IMMUNOPHYSIOLOGY OF PEDIATRIC RHEUMATIC DISEASES

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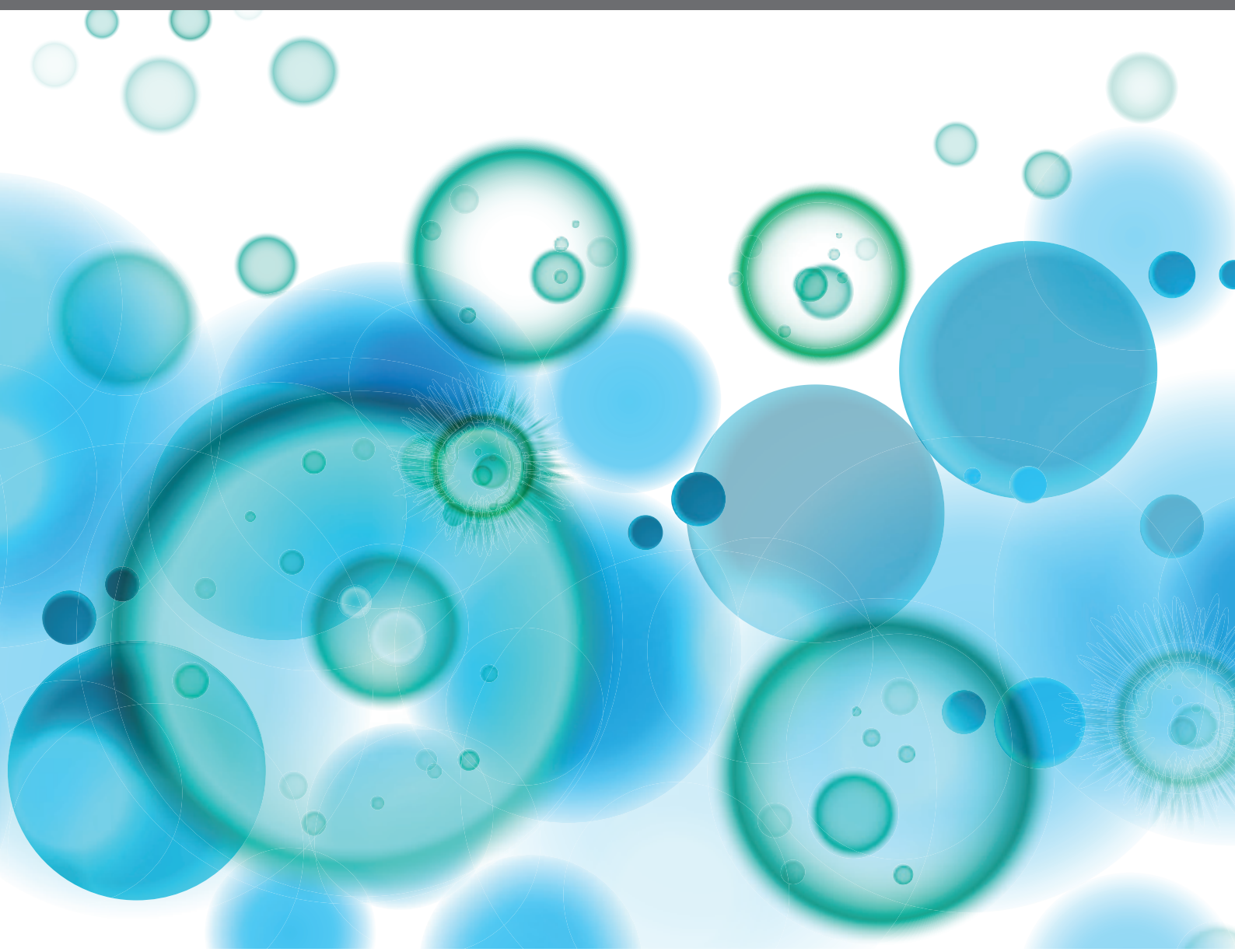

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IMMUNOPHYSIOLOGY OF PEDIATRIC RHEUMATIC DISEASES

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T Cell Receptor-Independent, CD31/IL-17A-Driven Inflammatory Axis Shapes Synovitis in Juvenile Idiopathic Arthritis

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T cells are considered autoimmune effectors in juvenile idiopathic arthritis (JIA), but the antigenic cause of arthritis remains elusive. Since T cells comprise a significant proportion of joint-infiltrating cells, we examined whether the environment in the joint could be shaped through the inflammatory activation by T cells that is independent of conventional TCR signaling. We focused on the analysis of synovial fluid (SF) collected from children with oligoarticular and rheumatoid factor-negative polyarticular JIA. Cytokine profiling of SF showed dominance of five molecules including IL-17A. Cytometric analysis of the same SF samples showed enrichment of αβT cells that lacked both CD4 and CD8 co-receptors [herein called double negative (DN) T cells] and also lacked the CD28 costimulatory receptor. However, these synovial αβT cells expressed high levels of CD31, an adhesion molecule that is normally employed by granulocytes when they transit to sites of injury. In receptor crosslinking assays, ligation of CD31 alone on synovial $CD28^{null}CD31^{+}$ DN αβT cells effectively and sufficiently induced phosphorylation of signaling substrates and increased intracytoplasmic stores of cytokines including IL-17A. CD31 ligation was also sufficient to induce RORγT expression and *trans*-activation of the *IL-17A* promoter. In addition to T cells, SF contained fibrocyte-like cells (FLC) expressing IL-17 receptor A (IL-17RA) and CD38, a known ligand for CD31. Stimulation of FLC with IL-17A led to CD38 upregulation, and to production of cytokines and tissue-destructive molecules. Addition of an oxidoreductase analog to the bioassays suppressed the CD31-driven IL-17A production by T cells. It also suppressed the downstream IL-17A-mediated production of effectors by FLC. The levels of suppression of FLC effector activities by the oxidoreductase analog were comparable to those seen with corticosteroid and/or biologic inhibitors to IL-6 and TNFα. Collectively, our data suggest that activation of a CD31-driven, αβTCR-independent, IL-17A-mediated T cell-FLC inflammatory circuit drives and/or perpetuates synovitis. With the notable finding that the oxidoreductase mimic suppresses

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the effector activities of synovial CD31^{+}CD28^{null} αβT cells and IL-17RA^{+}CD38^{+} FLC, this small molecule could be used to probe further the intricacies of this inflammatory circuit. Such bioactivities of this small molecule also provide rationale for new translational avenue(s) to potentially modulate JIA synovitis.

Keywords: CD31, double negative alpha beta T cells, fibrocyte-like cells, IL-17, juvenile idiopathic arthritis, oxidoreductase, synovial inflammation, TCR-independent

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a highly prevalent rheumatic disease of childhood before the age of 16 years. It is a genetic and clinical entity distinct from adult-onset rheumatoid arthritis (RA). The clinical subtypes of JIA have their own treatment patterns and prognostic courses (1). Thus, elucidating immunopathways unique to JIA may be informative for therapeutic innovations.

Multiple HLA associations of JIA implicate involvement of T cells (2). Oligoclonal expansions of these cells in blood and synovial fluid (SF) are well documented (3). However, the antigenic driver(s) of oligoclonality, or antigenic cause(s) of JIA disease remain elusive. Nevertheless, T cells play a role in JIA pathogenesis as they comprise a significant proportion of joint-infiltrating cells (4, 5).

Early studies on T cells in JIA showed many cells lacked expression of the costimulatory molecule CD28 (6). We have reported that there is a pervasive CD28^{null}CD8^{+} T cell population in oligoarticular and rheumatoid factor (RF)-negative polyarticular JIA (7), the two most prevalent clinical subtypes. CD28 is required to sustain T cell activation, but is irreversibly lost with chronologic aging (8). Aged human CD28^{null} CD4^{+} and CD8^{+} T cells are nonetheless functionally active (9), due in part to *de novo* expression of other molecules such as NK-related receptors CD56 and NKG2D that are capable of directly activating T cells (10). In JIA, we reported the *in vivo* accumulation of CD28^{null}CD8^{+} T cells disproportionately with age (7). This CD8 subset is prematurely senescent as indicated by their shortened telomeres, limited proliferative capacity, and expression of mitotic inhibitors. Furthermore, they express CD31, a receptor normally employed by granulocytes during their entry into sites of injury (11). In mice, *CD31*$^{-/-}$ granulocytes are unable to traverse endothelial barriers (12). Thus, accumulation of CD31^{+}CD28^{null}CD8^{+} T cells in JIA SF suggests their pathogenic role.

We sought to further evaluate the functional relevance of CD31 expression on joint-infiltrating T cells. We analyzed SF from another JIA cohort. Here, we report a subset of CD31^{+}CD28^{null} αβT cells that lack expression of both CD4 and CD8, referred to as double negative (DN) T cells. We hypothesized that CD31-driven T cell activation elaborates an inflammatory signature of SF. Of interest is whether synovial CD31^{+}CD28^{null} DN and CD8^{+} αβT cells respond similarly, or differently, to CD31 triggering. A corollary hypothesis is whether molecular effector(s) derived from CD31-activated synovial αβT cells leads to downstream activation of other SF mononuclear cells (SFMC) thereby compounding local inflammation.

Abbreviations (non-standard Immunology): ANA, anti-nuclear antibody; DN, double negative; FLC, fibrocyte-like cells; IL6i, clinically used biologic inhibitor of IL-6; JIA, juvenile idiopathic arthritis; MnT2E, Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin; RA, rheumatoid arthritis; RF, rheumatoid factor; SF, synovial fluid; SFMC, mononuclear cells in SF; TNFi, clinically used biologic inhibitor of TNF.

MATERIALS AND METHODS

Human Subjects and Biological Specimens

Institutional Review Boards of the University of Pittsburgh approved all research protocols. Written informed consent from parents/legal guardians, and assent of child subjects as appropriate, were obtained. Children with oligoarticular or RF^{-} polyarticular with JIA were recruited from the Rheumatology Clinic of Children's Hospital of Pittsburgh. RF^{+} polyarthritis, enthesitis-related arthritis, and psoriatic arthritis were excluded since these are considered separate genetic and clinical entities (13, 14). Patients undergoing arthrocentesis were targeted as donors of SF. Blood were also collected from our broader JIA patient base. Similar blood samples were collected from healthy controls, or those de-identified waste/residual clinical samples from our Clinical Laboratory Services.

As we have done in previous studies (7, 15–17), cell-free plasma was prepared by low speed centrifugation of blood. Similar centrifugation was carried out to isolate cell-free SF preparations from whole SF. Blood and SF samples with evidence of hemolysis were not used for these cell-free preparations. The centrifuged blood and SF samples were subsequently used to isolate peripheral blood mononuclear cells (PBMC) and SFMC, respectively, by standard isopycnic centrifugation. Cell contamination of plasma/SF preparations, and cell viability of PBMC/SFMC were verified by trypan blue staining, and/or by live cell counting using Countess II (Life Technologies). Aliquots of cell-free plasma/SF were stored in −80°C freezer until analysis. PBMC and SFMC aliquots were cryopreserved using a standardized protocol that we had validated previously to yield >90% recovery (7, 16).

Flow Cytometry

Multicolor cytometry was performed on SFMC and PBMC stained with fluorochrome-conjugated antibodies to cell surface and intracellular markers. All samples included a cell viability dye (ZombieUV™, BioLegend), which was used for the electronic gating of live cells. Raw cytometry data were acquired using a custom 5-laser Aria II cytometer (BD Biosciences). Off-life

analyses of cell populations were performed using FlowJo software (Tree Star). Immunostaining, instrument calibration, signal optimization, and off-line analyses employed our standardized procedures (7, 16).

We focused on conventional T cells defined as TCRαβ$^+$ (T10B9, BD), and were examined for expression of CD4 (OKT4, BD), CD8 (RPA-T8, BioLegend), CD28 (CD28.2, Novus Biologicals), and CD31 (2H8, Abcam). Discrimination of αβT cell subsets was achieved by exclusion staining for γδT cells (5A6.E91, ThermoFisher), monocytes (CD14: M5E2, BD), B cells (CD19: SJ25-C1, BD), NK cells (CD16/CD56: 3G8/NCAM16.2, BD), and plasma cells (CD138: 281-2, BD).

Non-lymphoid SFMC were also screened for co-expression of procollagen 1 (2Q576, Abcam) and proline-4-hydroxylase (EPR3661, Abcam), markers of mesenchymal fibrocytes reportedly found in circulation especially during injury (18). Since these markers are also characteristic of tissue fibroblasts (19, 20), we coined the term "fibrocyte-like cells (FLC)" reflecting their yet undetermined origin. We employed an immunostaining protocol for FLC by excluding the above mentioned hematopoietic markers and also tissue macrophages (CD68: Y1/82A, BD) in a single channel. For FLC, we screened for expression of IL-17 receptor A (IL-17RA) (0A01905, BD) and CD38 (HIT2, BioLegend).

Multiplex Analyses of Cell-Free SF and Plasma

A panel of 25 cytokines/chemokines was examined based on a broader global cytokine screening reported by de Jager et al. (21) and from our survey of current literature about their relevance to inflammation in general, and in disease settings of non-infectious arthritis. Using a customized kit (MILLIPLEX® MAP, Millipore Sigma) cytokine analyses were performed using previously standardized Luminex procedures (7). Raw data were acquired using MAGPIX or BioPlex200 (BioRad). Data quality was ascertained by a standard curve for each plate. We routinely set two overlapping standard curves, above and below the manufacturer's recommended setting. These plate curves were then used to determine confidence intervals in the construction a normalization curve. The latter was then used to adjust intra-plate variations and to calculate cytokine concentrations.

T Cell Bioassays

Preparation of cells for intracellular cytometry followed previous procedures (7, 16). CD31$^+$CD28^{null} DN and CD8 αβT cells were enriched from SFMC using EasySep (Stem Cell Technologies); preparations >85% enrichment used in experiments. Jurkat and JRT3 (both purchased from ATCC) were used as cell models for CD31$^+$CD3$^+$TCR$^+$, and CD31$^+$CD3$^-$TCR$^-$, respectively; these phenotypes verified by cytometry. Receptor crosslinking was performed according to previous procedures (7) using anti-CD31 (WM59, ab218, Abcam), anti-CD3 (OKT3, Centocor Ortho Biotech), anti-TCRαβ (1P26, BioLegend), or normal mouse Ig (BD) as stimulators. Crosslinking was achieved by excess amount of Cy5-conjugated anti-mouse Ig (BioLegend). Cytometry was performed by gating on cross-linked αβT cells, visualized as Cy5$^+$ cells that were also CD8$^+$CD4$^-$ or CD4$^-$CD8$^-$.

For some cultures, two small molecule inhibitors were added. One is Imatinib (Gleevec®, Selleck Chem), an inhibitor of catalytically active cAbl kinase that is currently used in the treatment of chronic myelogenous leukemia (CML) (22). It was added at 50 nM. The other is Mn(III) meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin (MnT2E), a Mn porphyrin mimic of superoxide dismutase that was originally developed to neutralize the tissue-destructive effects of superoxide (23). It has been shown experimentally to also inhibit the DNA-binding activity of NFκB (24). It was added at 34 μM (GMP grade, provided by Albany Molecular Research Inc., to JDP). The concentrations of Imatinib and MnT2E used were empirically determined as non-toxic (>95% cell viability).

For intracellular detection of IL-6 (AS12, BD), IL-17A (BL168, BioLegend), TNFα (Mab11, BD), and IFNγ (B27, BD), and RORγT (Q21-559, BD), a regulator *IL-17A* gene transcription (25), the crosslinked cells were cultured for 6 h in the presence of GolgiPlug™ reagent (BD) (7) in 7.5% CO_2 at 37°C. For signaling intermediates, the phosphorylated forms of ZAP70 (Y272; J34-602, BD), serine-threonine kinase Akt (S473; M89-61, BD), p16 subunit of NFκB referred to as RelA (S529; K10-895.12.50, BD), and Abelson kinase cAbl (Y245; ab62189, Abcam) were examined within 10 min of receptor crosslinking. These signaling phosphoproteins were identified from empirical proteomic screening (Hypromatrix). All intracellular cytometry procedures were performed according to our previous protocols (7).

Confocal Microscopy

Cells were incubated with anti-CD31 as described above. This was followed by crosslinking with anti-IgG immobilized onto microbeads labeled with Allophycocyanin (Spherotec). After 10 min, cells were fixed in paraformaldehyde, permeabilized with 0.1% Triton-PBS, washed, and blocked in 20% donkey serum. Cells were then incubated for 18 h with anti-phospho-Y245 cAbl (ab62189, Abcam) at 4°C, followed by anti-IgG conjugated with fluorescein isothiocyanate (Abcam) for 2 h at room temperature, counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen), and applied to a glass coverslip with Aqua PolyMount. Images were acquired on an Fluoview 1000 confocal microscope (Olympus).

FLC Bioassays

SFMC were first cultured overnight. The plastic-adherent cells were expanded to >70% confluence. Purity of the cultures determined cytometrically. FLC between second and fifth passages were incubated with or without non-toxic 20–2,000 ng/ml recombinant IL-17A (R&D Systems). In other experiments, FLC were cultured in 200 ng/ml IL-17A with the addition of 5 μM of a corticosteroid (Triamcinolone Acetonide, Aristospan®) or the biologic inhibitor of TNF (TNFi) Infliximab (Remicade®), or the biologic inhibitor of IL-6 (IL6i) Tocilizumab (Actemra®); or 34 μM MnT2E. After 24 h, CD38 expression was measured cytometrically, and the types and concentrations of soluble factors in the culture

supernatant were examined by Luminex using a kit (LXSAHM18, R&D Systems). This kit consists of 18 molecules based on the global SF screening of de Jager et al. (21) and reports about IL-17A-induced molecules in other experimental systems including adult arthritis (26–29).

Transient Transfection

With their homogeneous phenotype, Jurkat and JRT3 were used to test specifically the CD31-driven induction of IL-17A. Twenty μg luciferase plasmid reporter controlled by full-length *IL-17A* gene promoter (30), and 20 ng pRL *Renilla* luciferase plasmid (Promega) were co-transfected into 1×10^6 cells using Lipofectamine (ThermoFisher). Subsequently, receptor cross-linking was performed as described above. As system control, transfected cells were also stimulated with phorbol myristyl acetate (PMA) and ionomycin. Normalized luciferase reporter activity was determined as described previously (30).

Statistical Analysis

Data analyses were performed using SPSS software (V24, IBM). Due to intrinsic individual variations, data from T cell and FLC bioassays were normalized by expressing each response as stimulation index, or as percent (or fold) induction above or below the media or IgG controls as we have done previously (7). Stimulation indices were calculated from the difference of the experimental value and the media control divided by the appropriate IgG isotype control or solvent/carrier media as in the case of bioassays with Imatinib and MnT2E. We used this procedure reproducibly in a variety of experimental settings (7, 9, 16, 31–33). Kruskal–Wallis analysis of variance (ANOVA) was performed and *post hoc* pair-wise comparisons used the Tukey statistic. P-value <0.05 was considered significant.

RESULTS

Characteristics of the Study Cohort

Consistent with epidemiologic studies (34) JIA patients examined were predominantly female as shown in **Table 1**. This gender-bias was used as reference for the random subsampling of an equivalent female-biased healthy group. The entire cohort was predominantly Caucasian, representative of our patient population. Patients had long-standing oligoarticular or RF$^-$ polyarticular disease. They had varying age of disease onset and disease duration. There were no blood and SF samples from the same patient. Donors of SF were slightly older. There were multiple medications used, including topical steroids mirroring the patients with confirmed uveitis. None of the patients were newly diagnosed cases. Although anti-nuclear antibody (ANA) serology data were not available for all patients, about half of those tested were ANA$^+$. At the time of consent/assent, patients had 1–3 swollen large joints.

TABLE 1 | Characteristics of study cohort[a].

	Healthy	Oligoarticular juvenile idiopathic arthritis (JIA)		Rheumatoid factor$^-$ polyarticular JIA	
Specimen type	Blood	Blood	Synovial fluid (SF)	Blood	SF
Number of samples (# Black)	30 (4)	32	39 (2)	30	15
Sex, female/male	17/13	20/12	25/14	18/12	9/6
Mean age, years					
Boys					
At sampling	8.99	9.43	11.37	10.7	16.26
At disease onset	(n/a)	6.96	9.55	5.84	8.19
Girls					
At sampling	12.7	9.51	10.12	10.7	16.03
At disease onset	(n/a)	6.37	6.64	6.4	5.7
Mean of disease duration (years)	(n/a)	3.62	2.71	4.5	10.44
Medication[b]					
NSAID	(n/a)	25	25	16	9
Steroids, oral	(n/a)	2	1	2	1
Steroids, IA (total joints injected)	(n/a)	10 (20)	39 (62)	9 (22)	15 (18)
Steroids, IV	(n/a)	0	0	0	0
Steroids, topical	(n/a)	0	6	0	1
Methotrexate	(n/a)	12	7	19	5
Biologic agents	(n/a)	4	1	8	3
Other DMARD	(n/a)	2	2	2	3
Anti-nuclear antibody$^+$ (number subjects tested)	(n/a)	16 (30)	15 (38)	7 (19)	5 (15)
Number of involved joints at sampling	(n/a)	1.03	1.53	2.27	1.83
Uveitis history	(n/a)	0	6	0	1

[a]*Blood and SF aspirates were obtained at during routine medical visits of patients. There were no paired blood-SF samples.*

[b]*Medications were tallied at the time of consent/assent. Some patients were taking multiple medications. NSAID: meloxicam, ibuprofen, tolmentin, naproxen, diclofenac, and indomethacin. Biologic agents: etanercept, adalimumab, and infliximab. Other DMARD: sulfasalazine and hydroxychloroquine.*

(n/a), not applicable; DMARD, disease modifying anti-arthritic drug; IA, intraarticular injection; IV, intravenous injection; NSAID, non-steroidal anti-inflammatory drug.

Predominance of Five Cytokines in SF

Cytokines are recognized effectors of inflammation in JIA and have become therapeutic targets for neutralization using specific antibody blockers (35). Here, our primary goal was to examine the microenvironment of the JIA joint. Accordingly, experiments focused on SF, using PBMC and plasma from healthy controls and patients as internal references for SFMC phenotyping and cytokine profiling of cell-free SF, respectively.

Of 25 cytokines and chemokines examined, **Figure 1** shows IL-6, IL-10, IL-17A, IFNγ, and TNFα were found at significantly higher concentrations in SF compared to plasma. The cytokine SF/plasma levels of the two patient groups were equivalent. However, plasma levels of these five cytokines were not significantly different between patients and controls. Plasma cytokine levels were generally low. As depicted, IL-2 levels were not different between the subject groups thereby establishing confidence of the multiplex assay.

CD31$^+$CD28^{null} DN αβ T Cells Constitute a Major Cellular Component of SF

We reported previously that JIA carry CD31$^+$CD28^{null}CD8$^+$ T cells (7). **Figure 2A** illustrates the cytometry gating strategy for SFMC

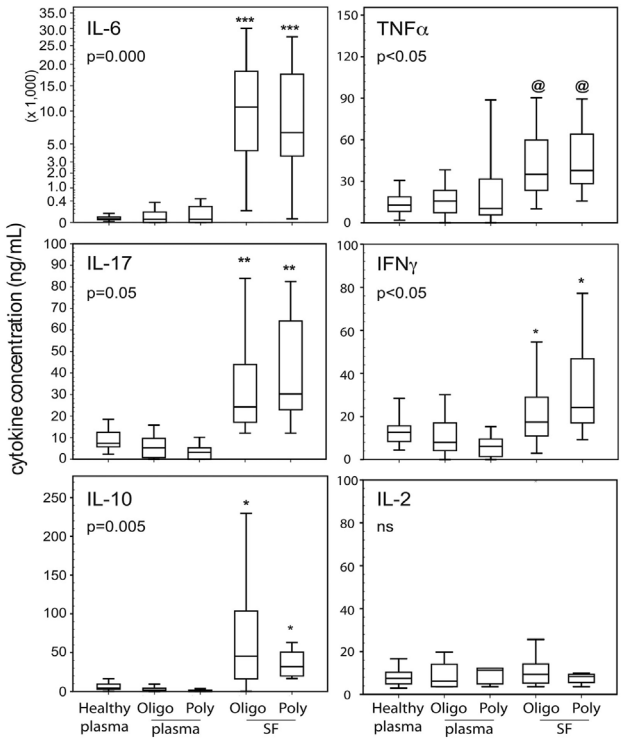

FIGURE 1 | Cytokine profiles in blood and cell-free SF of JIA patients. Data shown (n = 15–39 per group as in **Table 1**) are box-median-whisker plots of the five most dominant cytokines found in SF compared to plasma. The boxes represent the 25th and 75th percentile of values. The whiskers were the 5th and 95th percentile of values. There was not a global cytokine upregulation as shown by negligible detection of IL-2. The indicated P-values were determined by Kruskal–Wallis ANOVA. Between group comparisons were made using the *post hoc* Tukey statistic: [***], [**], and [*] $P < 0.005$, $P < 0.01$, and $P < 0.05$, respectively, indicate JIA SF is significantly different from healthy and JIA plasma; [@] indicates polyarticular [poly]/oligoarticular [oligo] SF were not significantly different from poly JIA plasma, but were significantly different ($P < 0.05$) from oligo JIA and healthy plasma. Abbreviation: NS, not significant.

and PBMC. Focusing on TCRαβ$^+$ gate, we found a new subset, namely, CD31$^+$CD28^{null}DN T cells. **Figure 2B** shows this subset comprised up to 80% (median ~48%) of the entire SF αβT cells in both oligoarticular and RF$^-$ polyarticular JIA. The frequency of this SFMC subset was equivalent between the two patient groups. Using the same gating strategy for PBMC, the data also show similar CD31$^+$CD28^{null}DN T cells that constituted up to 38% of the total circulating αβT cells in oligoarticular and polyarticular JIA (medians of 21 and 26%, respectively). This DN T cell subset was found at very low frequency (<8%) in healthy PBMC.

CD28 is lost progressively with chronologic aging (8). **Figure 2C** shows that age-adjustment of the frequency of CD31$^+$CD28^{null}DN T cells reveal their annual accumulations for up to 5.3% per age-year in blood. The yearly medians between the two patient groups were equivalent, but these medians were significantly higher than the <0.8% accumulation per age-year in blood of healthy children. In SF, there were significantly higher accumulations of CD31$^+$CD28^{null} DN T cells for up to 9.2% per age-year. The medians were 5 and 6.4% for oligoarticular and polyarticular JIA SFMC, respectively.

CD31-Driven, TCR-Independent Expression of Cytokines, and Phosphorylation of Signaling Intermediates in Synovial CD31$^+$CD28^{null} αβT Cells

In keeping with our primary goal to more closely examine the microenvironment of the inflamed joint, bioassays were performed to determine whether synovial CD31$^+$CD28^{null} subsets of DN and CD8$^+$ αβT cells were sources of the cytokines detected in SF. **Figure 3A** illustrates the strategy for receptor crosslinking using specific antibodies to either CD31 (WM59), αβTCR (1P26), or CD3 (OKT3, Orthoclone), followed by Cy5-conjugated anti-mouse Ig. Cy5$^+$ cells were then examined for the expression of CD8, CD4, CD31 (2H8), and intracellular cytokines. **Figure 3B** shows that within 6 h of CD31 ligation alone, there was induction of high levels of intracellular IL-6, IL-17A, IFNγ, and TNFα in primary CD31$^+$ DN and CD8$^+$ αβT cells. The levels of CD31-driven cytokine production were generally equivalent with those induced by CD3 or TCR ligation. For CD31$^+$ DN T cells, CD31-induced production levels of IFNγ, IL-6, and IL-17A were significantly higher than the other stimulation groups. Similar higher levels of CD31-driven production of IL-6 and IFNγ were observed for CD31$^+$ CD8$^+$ T cells. Due to high staining background, cytoplasmic IL-10 was not measured.

To verify these results in a more tractable model, we examined CD3$^+$TCR$^+$CD31$^+$ Jurkat. As depicted (**Figure 3B** third row), similar crosslinking of Jurkat recapitulated the cytokine production data from synovial CD31$^+$ DN and CD8$^+$ αβT cells. We also used JRT3, a somatic variant of Jurkat with mutated *CD3* and *TCRB* genes (ATCC) (36, 37), verified as CD3$^-$ and αβTCR$^-$, but CD31$^+$, by cytometry. Consistent with these phenotypic characteristics, JRT3 cells showed an exclusive CD31-driven cellular expression of the same four cytokines (**Figure 3B** fourth row).

We also examined whether ligation of CD31 alone is sufficient to induce phosphorylation signaling intermediates. Specifically, we focused on ZAP70, Akt, RelA, and cAbl, four molecules found during an empirical screening of phosphoprotein expression following CD31 ligation (see Materials and Methods). ZAP70 and Akt are components of conventional TCR-driven activation of T cells (38, 39). RelA is a known component of the NFkB pathway linked to many inflammatory cascades (40). cAbl is of interest since it is not a known component of classical CD31 signaling, which has been studied extensively in non-immune cells (41, 42). Proving its mobilization following CD31 ligation on αβT cells would validate it as component of CD31-driven TCR-independent T cell-mediated inflammation. **Figure 4** shows that within 10 min of CD31 ligation, there were highly significant phosphorylations of ZAP70, Akt, RelA, and cAbl compared to

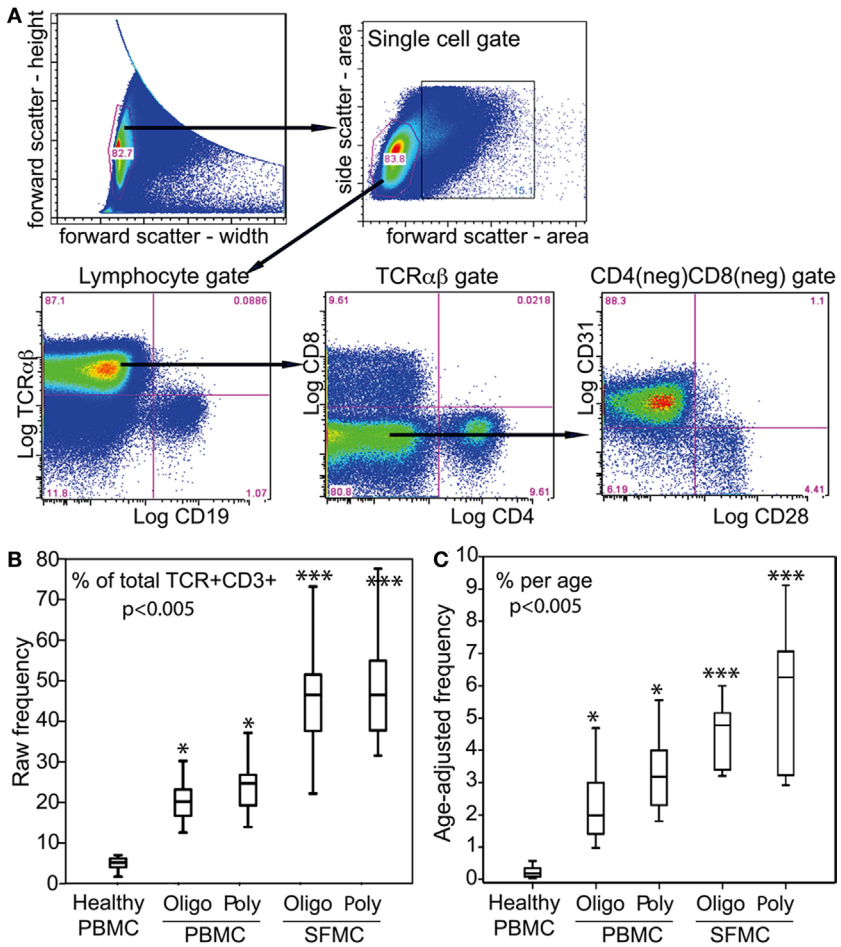

FIGURE 2 | αβT cell profiles in PBMC and SFMC of JIA patients. **(A)** Illustrative flow of the electronic gating strategy for cytometric determination of the expression of TCRαβ, CD4, CD8, CD28, and CD31. The gating profile shown was for an SFMC sample, which was very similar with a parallel PBMC gating profile. Following an initial live/dead electronic gate, the height and width of the forward scatter was used to set a single cell gate for lymphocytes that were sequentially examined for CD4, CD8, CD28, and CD31. **(B)** Box-median-whisker plots shown are the raw frequency and **(C)** the age-adjusted frequency of $CD31^{+}CD28^{null}DN$ αβT cells as a proportion of the total parent population of gated $αβTCR^{+}$ cells. The plots were constructed as in **Figure 1**. The indicated *P*-values were determined by Kruskal–Wallis analysis of variance. *Post hoc* group comparisons by Tukey: ****P* < 0.005 indicates SFMC of oligoarticular [oligo] and polyarticular [poly] JIA were significantly different from JIA/healthy PBMC; **P* < 0.05 indicates JIA PBMC was significantly different from healthy PBMC.

the IgG istotype controls in synovial DN and $CD8^{+}$ αβT cells. The phosphorylation levels were largely equivalent between CD31, TCR, and CD3 ligations. In Jurkat, these phosphorylation events were reproduced in similar crosslinking assays. In JRT3, the phosphorylations were seen only in response to CD31 ligation.

CD31-Driven cAbl Polarization, and Down Modulation of CD31-Driven Cytokine Production by an Inhibitor of cAbl and an Oxidoreductase Analog

Because classical CD31 signaling in non-immune cells (41, 42) has not been shown to involve cAbl, we analyzed whether cAbl was mobilized following TCR-independent CD31 ligation on T cells. **Figure 5A** shows representative imaging of phospho-cAbl polarization in five independent experiments. A Z-stack of confocal slices, and the single cell image (Inset, CD31-stimulated) showed phosphorylated cAbl localized at the point of contact between the T cell and the anti-CD31-bead. Polarized phospho-cAbl was observed at an average of 85% of cells per microscope field in 20 fields scanned in each of the five experiments. In contrast, cells incubated in IgG-bead had some random scattering of minutely speckled staining of cAbl, but there was no distinct polarization of the cAbl staining signal (Inset, Unstimulated). These observations indicated that CD31 signaling independent of TCR engagement in T cells involved cAbl.

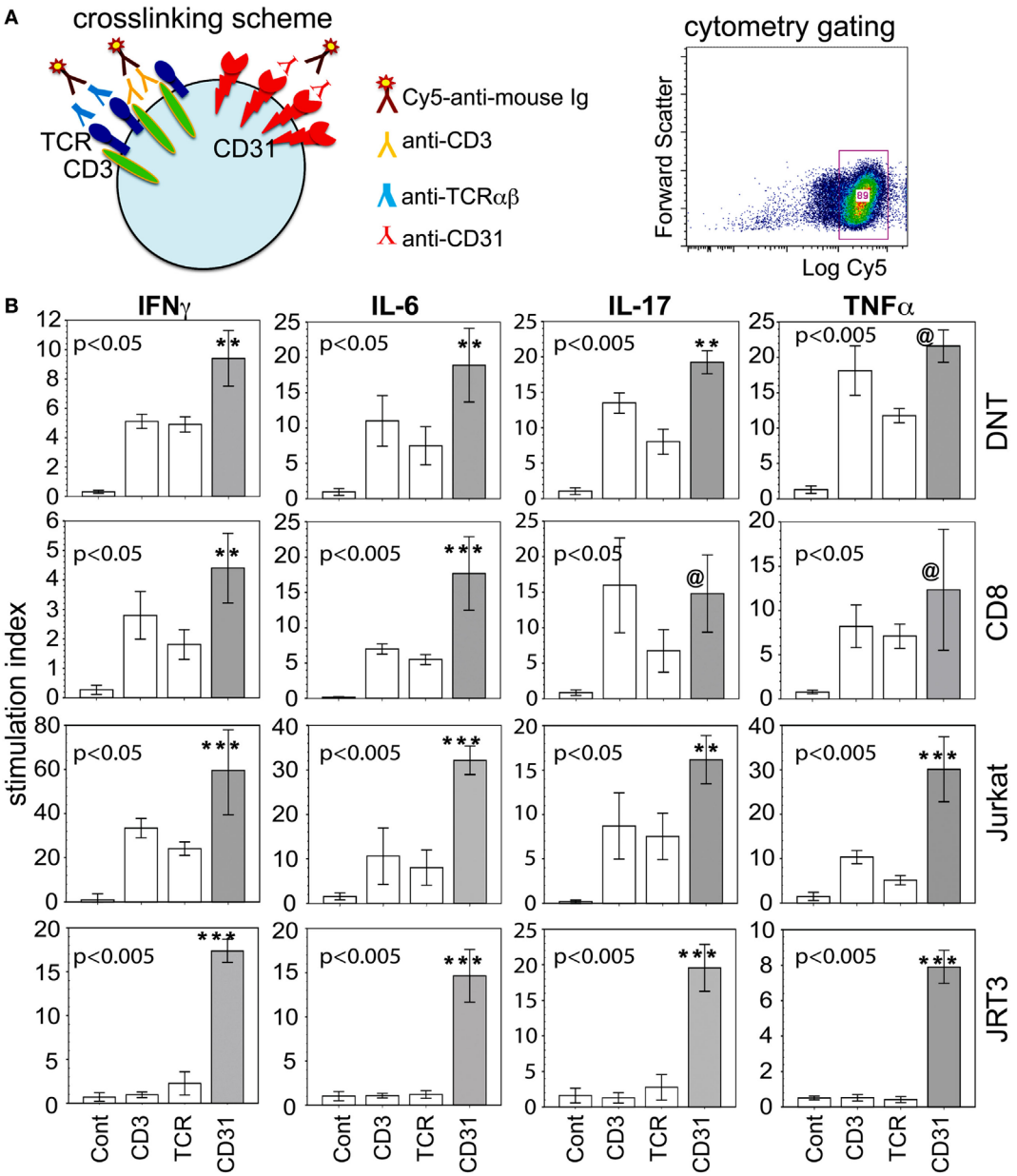

FIGURE 3 | CD31 ligation alone is sufficient to induce intracellular expression of cytokines. **(A)** Diagram of receptor crosslinking with specific antibody, and the relevant gate of crosslinked (Cy5$^+$) cells for subsequent cytometric analyses. **(B)** The data shown (bar means, SD whiskers; n = 5–11 per group) are intracellular levels of IFNγ, IL-6, IL-17, and TNFα in synovial CD31$^+$CD28^{null} double negative (DN) and CD8$^+$ αβT cells within 6 h of stimulation *via* CD3, TCRαβ, CD31, or IgG control. The data were expressed as stimulation index to normalize intrinsic variability between individual donors and culture batch differences of Jurkat or JRT3 cells. The plots were constructed as in **Figure 1**. The indicated P-values were determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: [***] and [**] $P < 0.005$ and $P < 0.05$, respectively, indicate CD31 crosslinking was significantly different from TCR or CD3 crosslinking and the IgG control [Cont] for synovial DN and CD8$^+$ T cells, Jurkat, and JRT3; [@] indicates CD31 crosslinking was not significantly different from TCR and CD3 crosslinking, but was significantly different ($P = 0.001$) from the IgG control.

The role of cAbl is further shown in **Figure 5B**. Imatinib, a known and clinically used inhibitor of constitutively active cAbl (22), consistently reduced the levels of CD31-driven phosphorylation of cAbl. In line with previous reports (43, 44), Imatinib also reduced the levels of RelA phosphorylation and intracellular expression of TNFα and IFNγ. Additionally, there was significant reduction of IL-17A expression. Similarly, MnT2E, a synthetic mimic of superoxide dismutase (23), significantly reduced CD31-driven RelA phosphorylation consistent with its reported inhibition of the DNA-binding activity of NFkB (24). MnT2E

also reduced cAbl phosphorylation and intracellular expression of IL-17A, IFNγ, and TNFα. Unexpectedly, the level of suppression of CD31-driven expression of IL-17A by both Imatinib and MnT2E was significantly greater than the levels of suppression of IFNγ and TNFα expression.

CD31-Driven, αβTCR-Independent Activation of *IL-17A* Gene Promoter

We further examined whether CD31 directly affected IL-17A production. **Figure 6A** shows CD31 ligation alone induced RORγT expression at significantly higher level than that induced conventionally *via* αβTCR or CD3 in synovial CD31$^+$ DN and CD8$^+$ αβT cells, and in Jurkat cells. For JRT3 cells, induction of RORγT expression was exclusive to CD31 ligation. Furthermore, **Figure 6B** shows CD31 ligation alone was sufficient to *trans*-activate the *IL-17A* gene promoter as assessed by luciferase reporter assays using Jurkat and JRT3. As expected, stimulation of Jurkat and JRT3 with the mitogen PMA/ionomycin, which bypasses TCR signaling, resulted in high luciferase activity compared to the IgG control albeit this JRT3 mitogenic response was higher than Jurkat.

FLC Are Downstream Targets of IL-17A and Are Sensitive to Suppression by MnT2E as Effective as TNFi and IL6i

In cytometric analyses of SFMC, we routinely noticed the presence of SFMC of larger size (forward scatter) and higher granularity (side scatter) compared to the electronically gated lymphocytes (depicted by bounding box in **Figure 2A**). **Figure 7A** illustrates individual variations in the proportions of these non-lymphoid SFMC, which were recognized from the exclusion staining of TCRαβ, TCRγδ, CD3, CD4, CD16, CD19, and CD56 by back-gating strategies. These non-lymphoid cells uniformly co-expressed procollagen 1 and proline-4-hydroxylase, the

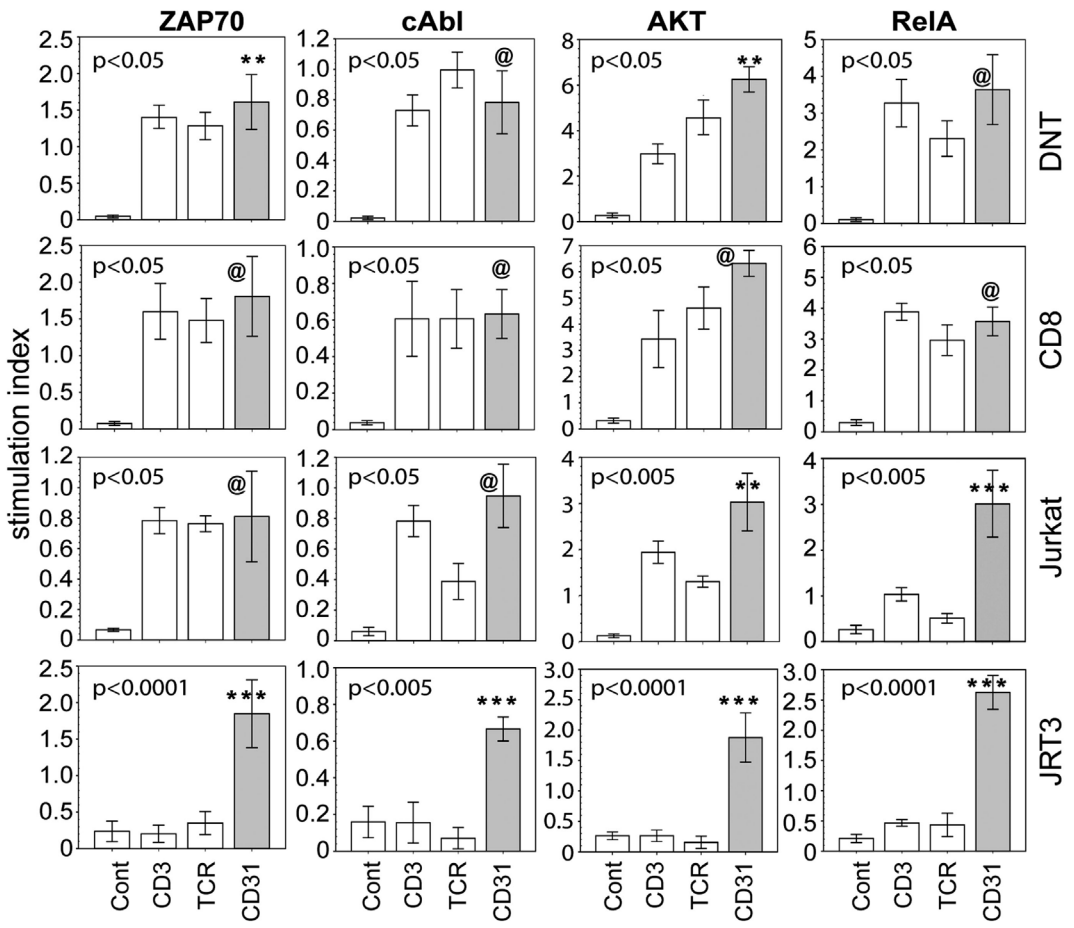

FIGURE 4 | CD31 ligation alone is sufficient to elicit phosphorylation of signaling intermediates. Using the same crosslinking bioassay in **Figure 3A**, the data shown (bar means, SD whiskers; $n = 5$–9 per group) are phosphorylation levels of ZAP70, cAbl, AKT, and RelA within 15 min of stimulation *via* CD31, TCR, CD3, or IgG control. The data are normalized stimulation indices as in **Figure 3B**. The indicated *P*-values were determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: [***] and [**] $P < 0.005$ and $P < 0.05$, respectively, indicate CD31 crosslinking was significantly different from TCR or CD3 crosslinking or the IgG control [Cont]; [@] indicates CD31 crosslinking was not significantly different from TCR or CD3 crosslinking, but was significantly different ($P = 0.0001$) from the IgG control.

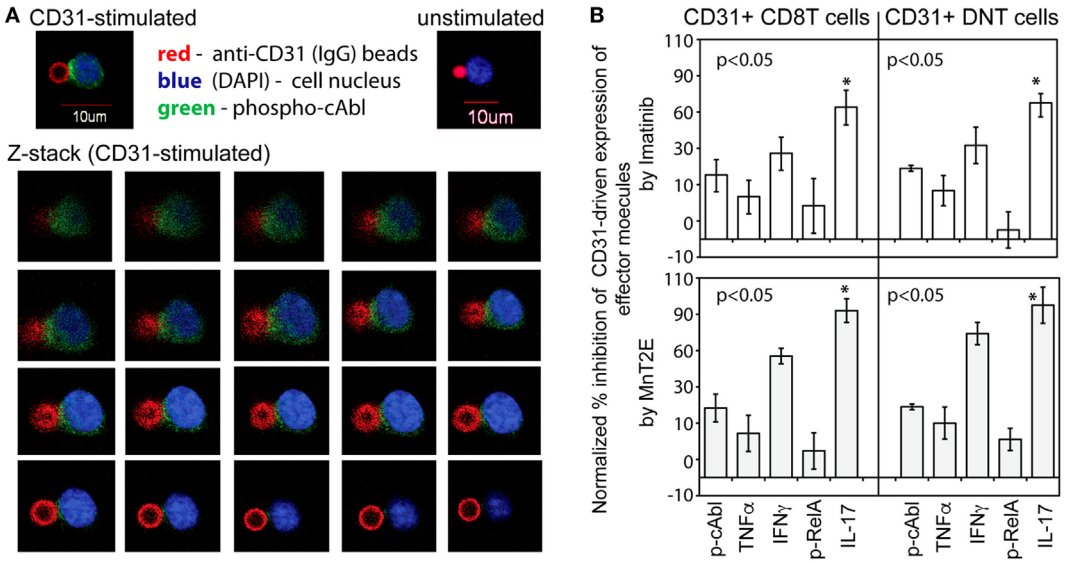

FIGURE 5 | cAbl is a signaling substrate of CD31-driven TCR-independent activation of synovial αβT cells. **(A)** Micrographs shown are representative confocal images of CD31-driven polarization of cAbl in αβT cells from five independent experiments. The Z-stack (left to right order) were sequential image slices from the top to the bottom of a cell (DAPI and Green staining) and a fluorescent bead (Red) with immobilized anti-CD31. **(B)** The data shown (bar means, SD whiskers; $n = 4–7$ per group) are percent inhibition of CD31-driven expression of phospho-cAbl and phospho-RelA, and intracellular expression of TNFα, IFNγ, and IL-17A by 50 nM Imatinib or 34 μM MnT2E in CD31-crosslinked synovial CD31^{+}CD28^{null} DN and CD8^{+} αβT cells CD31 crosslinking was performed as in **Figure 3A**. Data normalization was done as in **Figures 3B** and **4**. The indicated *P*-values were determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: $^{*}P < 0.05$ indicates MnT2E and Imatinib induced greater magnitudes of reduction of CD31-driven IL-17A expression than the inhibitor-induced reductions of cAbl, TNFα, IFNγ, and RelA expression.

typical markers of fibroblasts and circulating fibrocytes (18–20). They also expressed the receptor for IL-17 (IL-17RA) and CD38, a ligand for CD31 (45). Further, **Figure 7B** shows these non-lymphoid cells were adherent to plastic and were amenable for short-term propagation. They had varying stellate to ameboid morphology, and retained expression of procollagen 1, proline-4-hydroxylase, IL-17RA, and CD38. These morphological characteristics underscore their designation as "fibrocyte-like cells."

It is not yet known whether FLC in JIA SFMC derive from the same lineage as the hyperplastic fibroblast-like synoviocytes found in adult RA synovial tissue, which may be similarly positive for procollagen 1 and proline-4-hydroxylase (46). It is also unclear if FLC were fibrocytes, which could be found in small numbers in blood (18) and then infiltrated the joint. Neither is yet known if FLC were fibroblasts that detached from the pannus of the inflamed JIA joint. Irrespective of their origin, **Figure 7C** shows that consistent with their expression of IL-17RA, FLC exposed to recombinant IL-17A showed a dose-dependent increased expression of CD38. There were no differences in phenotype and responsiveness to IL-17A of FLC between oligoarticular and RF^{-} polyarticular JIA.

The responsiveness of FLC to IL-17A was further examined by the addition of MnT2E, the biologics of TNFi and IL6i, or corticosteroid to the bioassays. Here, we focused on IL-17A-induced molecular effectors from experimental systems including those implicated in juvenile and adult arthritis (21, 26–29). Out of 18 molecular effectors examined, 3 cytokines (TNFα, IL-6, and IL-1β) and 5 chemokines [CXCL1, CXCL8 (IL-8), CCL2 (MCP1), CCL3 (MIP1α), and CCL7 (MCP3)] were found to be significantly induced IL-17A (**Figure 8**). Additionally, IL-17A induced production of tissue-destructive proteins, namely, 6 metalloproteinases (MMP; MMP2, 3, 7, 8, 13, and 13) and vascular endothelial growth factor (VEGF) (**Figure 9**). There were no significant differences in MMP1, MMP9, and tissue inhibitor of metalloproteinase-1 levels between control FLC cultures and those incubated in IL-17A. Production of the 15 IL-17A-induced effectors by FLC was uniformly down-regulated by corticosteroid. Similarly, IL6i and TNFi down-regulated the expression of these effectors, albeit at lower magnitudes compared to those seen with corticosteroid.

The data also show that MnT2E was capable of inhibiting IL-17A-induced production of inflammatory effectors by FLC. Its inhibitory effects were not as high as those seen with corticosteroid. However, MnT2E was as effective as IL6i and TNFi in reducing IL-17A-mediated production of MMP3, MMP8, and IL8 (CXCL8). It elicited significantly higher degrees of inhibition of IL-17A-mediated production of TNFα, IL-6, MMP2, VEGF, and CCL2 (MCP1) than either IL6i or TNFi. MnT2E, IL6i, and TNFi did not affect the production of MMP7 and MMP12. MnT2E also did not affect production of IL-1β. Both TNFi and MnT2E had no effect on CCL7 (MCP3) production. Generally, there were no significant differences in FLC responses between oligoarticular and RF^{-} polyarticular JIA.

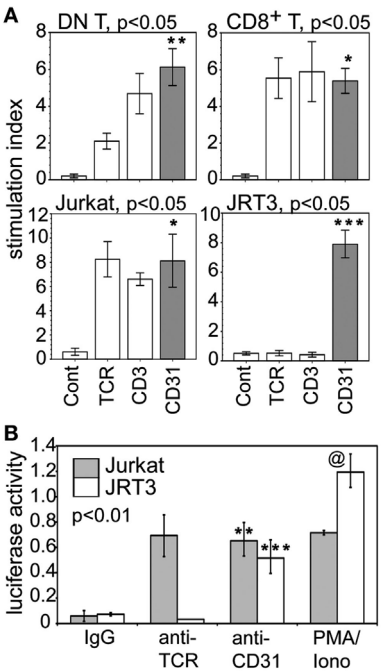

FIGURE 6 | CD31-driven expression of RORγT and *trans*-activation of *IL-17A* gene promoter. **(A)** Data shown (bar means, SD whiskers; $n = 5–7$ per group) are RORγT levels in synovial $CD31^{+}CD28^{null}$ DN and $CD8^{+}$ αβT cells, Jurkat, and JRT3 following CD31, TCR, CD3, or IgG stimulation. Crosslinking assays were performed as in **Figure 3A**. Data normalization done as in **Figures 3B** and **4**. The indicated *P*-values were determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: ***$P < 0.001$ indicates CD31 crosslinking in JRT3 were significantly different from TCR and CD3 crosslinking, and the IgG control; **$P < 0.05$ indicates CD31 crosslinking on DN T cells were significantly different from TCR or CD3 crosslinking, or IgG control; * indicates crosslinking of CD31, TCR, or CD3 on CD8 T cells and Jurkat were not significantly different but were significantly different ($P < 0.001$) from the IgG control. **(B)** Data shown (mean bars, SD whiskers; $n = 4–7$ per group) are *IL-17A* gene promoter-driven luciferase reporter activities of Jurkat and JRT3. The data were normalized for transfection efficiency by co-transfection of *Renilla* luciferase plasmid. Receptor crosslinking was performed as in **Figure 3A**. The indicated *P*-value was determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: [**] indicates crosslinking of CD31 and TCR on Jurkat was not significantly different, but either one was significantly different ($P < 0.005$) than the IgG control; [***] $P < 0.001$ indicates CD31crosslinking on JRT3 cells were significantly different than TCR crosslinking or the IgG control; [@] $P < 0.005$ indicates control luciferase activity of phorbol myristyl acetate/Iono-stimulated JRT3 without receptor crosslinking was higher than Jurkat.

DISCUSSION

Cytokines/chemokines are considered pathologic effectors of JIA. Their plasma/serum levels have been variably associated with disease activity, particular disease manifestations, or responses to biologics such as IL-6i and TNFi (47, 48). Regardless of treatment, many patients experience episodes of arthritic flares that are usually managed by systemic and/or by local therapy with arthrocentesis and corticosteroid injection. During such flares, there is exaggerated synovial inflammation compared to blood that is reflected by non-correspondence between plasma/serum and SF cytokine profiles (21). Thus, biological analysis of SF is a preferable approach to better understand the nature of synovitis in JIA (49).

The present study shows variable and low levels of plasma cytokines, some of which are not significantly different between JIA and healthy controls. In contrast, the SF cytokine profiles show dominance of IL-6, IL-10, IL-17A, IFNγ, and TNFα, which were among those identified by de Jager et al. (21). By comparison, our data show 4–5 orders of magnitudes for these five molecules (present data in **Figure 1** in ng/ml quantities versus data **Table 3** of de Jager et al. in pg/ml). Such quantitative differences could be related to intrinsic cohort differences. IL-6 and TNFα are two of the most consistently reported cytokines in JIA (21, 47). IL-10 has both pro- and anti-inflammatory effects in human disease and is among the upregulated cytokines in clinically active JIA (50). IFNγ has been associated with innate and adaptive responses in adult RA, with some reported association with systemic-onset JIA but not with oligoarticular or RF^{-} polyarticular JIA (51). IL-17A is a cytokine of interest in the biology of JIA (52). As shown by the present data and those reported by de Jager et al. (21), there are equivalent levels of these five cytokines in SF of oligoarticular and RF^{-} polyarticular JIA. Collectively, these findings support a growing opinion in Pediatric Rheumatology that these two clinical subtypes may represent a continuum of the same disease (14). Inasmuch as the present study is a cross section of patients with long-standing disease (disease duration up to 15 years) that have a treatment history with various medications, the dominance of IL-6, IL-10, IL-17A, IFNγ, and TNFα in SF suggests a common cytokine signature of JIA synovitis.

A key question is whether the SF cytokine milieu is linked to discrete subset(s) of joint-infiltrating cells. The present study provides evidence for the role of DN αβT cells. Whether these cells come from a distinct lineage or are derived from chronic inflammatory activation of single-positive CD4 and/or CD8 precursors remains to be examined. However, expression levels of CD4 and CD8 have been known to be transiently down-regulated during conventional TCR-crosslinking (53). In T cell cultures with highly mitogenic stimuli such as anti-CD3/CD28 or phorbol ester/ionomycin, extremely high rates of proliferation of CD8, but not CD4, cells were reportedly associated with the emergence of DN T cells (54). Such *in vitro* emergence of DN T cells has been linked to epigenetic modification of *CD8* that renders it inaccessible to transcription (55). Along these lines, DN T cells have also been reported to constitute ~10% of blood T cells in adults with systemic lupus erythematosus (56), a disease with known global and *CD8*-specific epigenetic modifications (57). Similar epigenetic modifications have been reported for $CD4^{+}$ T cells in JIA (58), but it is not yet clear if such changes lead to conversion of single-positive CD4 into DN T cells. Whether epigenetics regulate lineage decisions of joint-infiltrating T cells in manner differently from that of T cells residing/transiting in normal lymphoid tissue is also unknown. Regardless of the role of epigenetics, we report here that compared to adult lupus, oligoarticular and RF^{-} polyarticular JIA have greater than twice the frequency of DN T cells in blood, and up to sevenfold higher in SF.

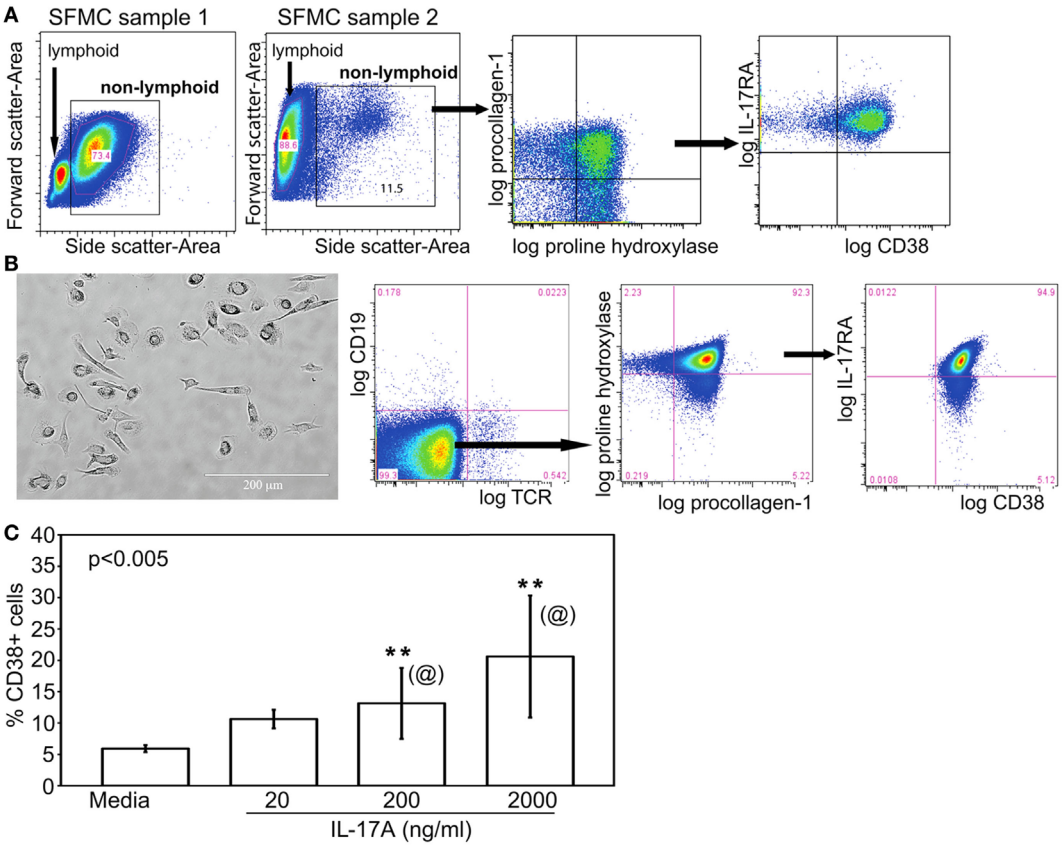

FIGURE 7 | Fibrocyte-like cells are non-lymphoid IL-17A-responsive components of SF. **(A)** The general electronic gating strategy to set a live single cell gate was done as in **Figure 2A**. The cytograms shown illustrate the gated larger sized cells in SFMC, i.e., boxed higher forward versus side scatter. As depicted, the proportions of these non-lymphoid SFMC varied widely among patients. These cells co-expressed procollagen 1 and proline hydroxylase, known markers of fibroblasts and mesenchymal fibrocytes. These cells, referred to as FLC, also expressed IL-17RA and CD38. **(B)** Representative micrograph of 10–15 day cultured, plate-adherent FLC showing stellate to ameboid morphology. Their typical cytogram profile showed negative staining for T and B cell markers, but positive staining of proline hydroxylase, procollagen 1, IL-17RA, and CD38. **(C)** CD38 expression on FLC (bar means, SD whiskers; $n = 5$ per group) incubated with three doses of recombinant IL-17A for 24 h. The indicated *P*-value was determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: [@] indicate no significant differences between 200 and 2,000 ng/ml doses of IL-17A; [**] indicates responses to 200 or 2,000 ng/ml was significantly different ($P < 0.05$) than those seen with 20 ng/ml IL-17A or media control.

Our new finding is that DN αβT cells in JIA are $CD31^{+}CD28^{null}$, a phenotype reminiscent of a $CD8^{+}$ subset we reported previously (7). This is unlike the situation in adult lupus where DN T cells are $CD28^{+}$ (56) and CD31 expression has not been examined. $CD28^{null}$ T cells are a biomarker of normal aging or premature aging in the human immune system (10). We have shown that the irreversible loss of CD28 can be accelerated by persistent TCR stimulation, and by inflammatory mediators (32, 59). As for CD31, it is a known marker for fresh naïve $CD28^{+}CD4^{+}$ T cells and is lost when they become $CD28^{+}$ activated or $CD28^{null}$ memory CD4 effectors (60). We have shown that CD31 is sporadically expressed on fresh naïve $CD8^{+}$ T cells, but is stably expressed on highly activated CD8 cells, and upon their conversion from $CD28^{+}$ to memory $CD28^{null}CD8^{+}$ effectors (7). Whether the losses of CD4, CD8, and CD28, and the corresponding gain of CD31 are independent, co-dependent, or sequential events remain to be examined.

The abundance of $CD31^{+}CD28^{null}$ DN and $CD8^{+}$ αβT cells in SF suggests their pathogenic role. Our data show >2.5-fold annual increase in $CD31^{+}CD28^{null}$DN αβT cell frequency in SF compared to that seen in blood. Such yearly accumulation is reminiscent of our original report for $CD31^{+}CD28^{null}CD8^{+}$ αβT cells (7). However, a longitudinal analysis of paired blood and SF samples is needed to ascertain whether there is concordance of their cell frequencies increases over time. Longitudinal studies could also inform whether arthritic flares may be due to, or predicted by cumulative increases in, $CD31^{+}CD28^{null}$ DN and/or $CD8^{+}$ αβT cells, or to particular effector subsets thereof.

An experimental support for a pathogenic role for $CD31^{+}CD28^{null}$ DN and $CD8^{+}$ αβT cells is their TCR-independent activation. Our data show CD31 ligation alone sufficiently increase intracellular IL-6, IL-17A, IFNγ, and TNFα, four of the five most upregulated cytokines we found from SF cytokine profiling. Furthermore, there is CD31-driven, TCR-independent phosphorylation of ZAP70, Akt, and RelA, three components of conventional TCR-driven CD28-dependent T cell signaling (38–40). Our data also show a primary role of cAbl in CD31-driven activation of synovial T cell activation; cAbl is not a known component of

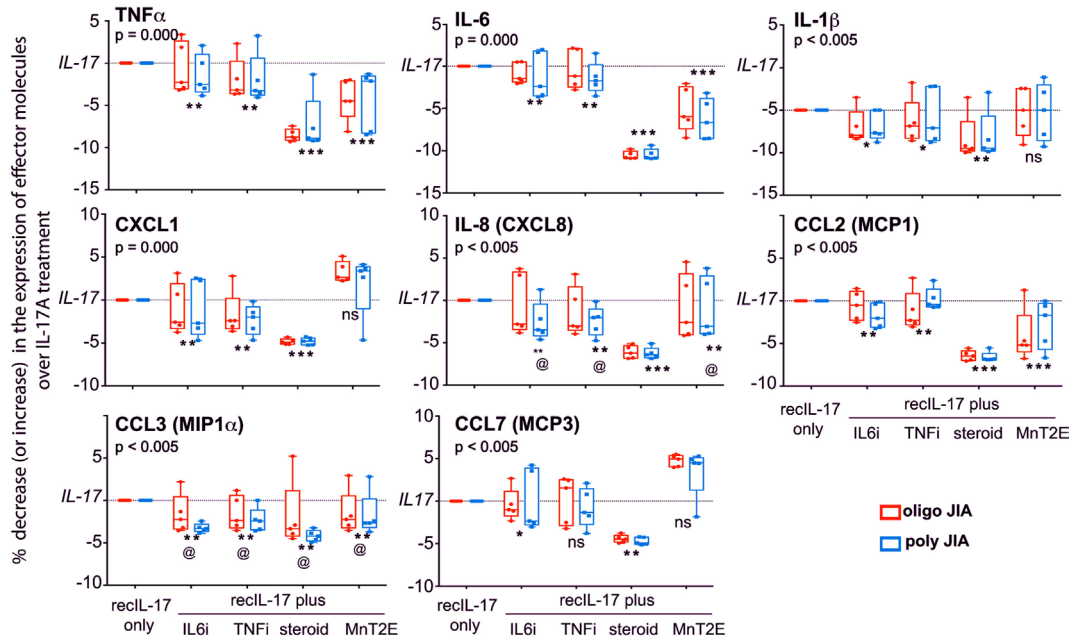

FIGURE 8 | IL-17A-mediated production of cytokines and chemokines by FLC, and their sensitivity to MnT2E. Data shown are box-median-whisker plots (n = 5–7 per group), which were constructed as in **Figure 1**. As depicted, the maximum levels of production of each of the indicated molecules by FLC cultures in 200 ng/ml recombinant IL-17A [recIL-17] were set as 100% response. Production levels of each indicated molecular effector in FLC cultures with recIL-17 combined with 5 μM corticosteroid or IL6i or TNFi, or 34 μM MnT2E were normalized as percent increase or decrease over the maximal response to recIL-17. The indicated P-values were determined by Kruskal–Wallis ANOVA. *Post hoc* group comparisons by Tukey: ***$P < 0.005$ indicate corticosteroid- and/or MnT2E-mediated suppression of IL-17A-mediated production of effectors was significantly greater than those elicited by TNFi or IL6i; [**] and [*]$P < 0.01$ and $P < 0.05$, respectively, indicate significant suppression of production of effector molecules compared recIL17 only group; [@] no significant differences among inhibitor-treated groups, but significantly different ($P < 0.05$) from the recIL-17 only group; ns, not significantly different between the inhibitor-treated group(s) and recIL-17 only group.

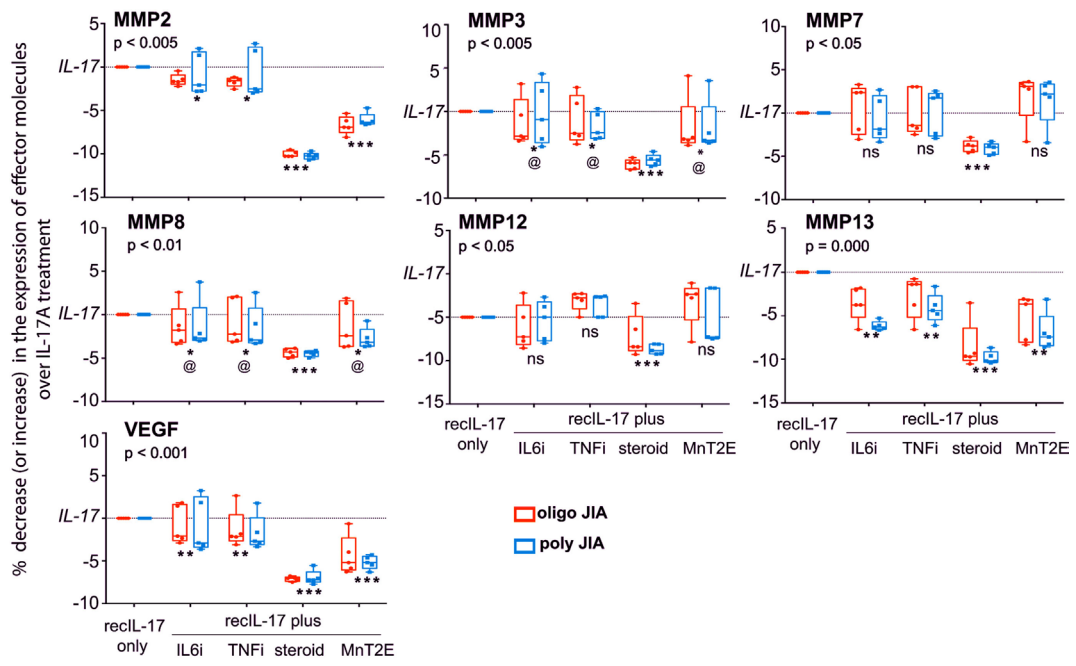

FIGURE 9 | Sensitivity of IL-17A-mediated production of tissue-destructive proteins by FLC to down modulation by MnT2E. Culture supernatants from the same FLC bioassays in **Figure 8** were also examined for MMPs and vascular endothelial growth factor (VEGF). Sample sizes, box-median-whisker plots, and statistical analyses were identical to that of **Figure 8**.

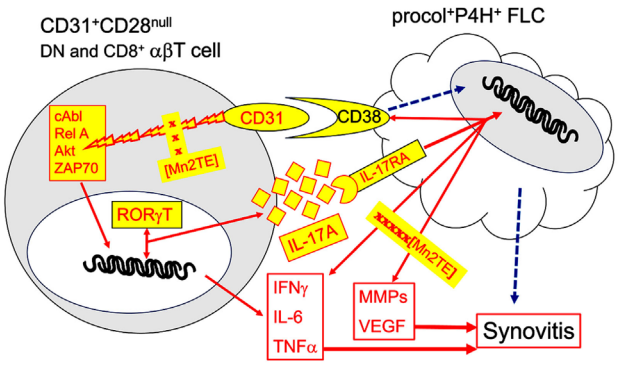

FIGURE 10 | Model for an inflammatory circuit between CD31^{+}CD28^{null} αβT cells and FLC in JIA synovitis. The present data (**Figures 2** and **7**) authenticate enrichment of CD31^{+}CD28^{null} DN αβT cells, as well as procollagen [procol]$^{+}$proline hydroxylase [P4H]$^{+}$ FLC in SF of oligoarticular and rheumatoid factor^{-} polyarticular JIA. In previous work, we have shown a similar CD31^{+}CD28^{null} subset in the CD8^{+} compartment (7). Ligation of CD31 with specific antibody on these two αβT cell subsets sufficiently and effectively signals phosphorylation of ZAP70, Akt, cAbl, and RelA (**Figure 4**). It is also sufficient to induce production of IL-17A, IL-6, IFNγ, and TNFα (**Figure 3**). These four cytokines are among the most dominant cytokines identified in SF (**Figure 1**). We have shown previously that similar cytokine production is achieved by CD31 ligation with CD38-Ig (7), indicating CD38 is a true ligand, CD31 ligand on synovial CD31^{+} αβT cells. Relevance of CD31 as a driver IL-17A is indicated by the specific *trans*-activation of *IL-17A* promoter and induction RORγT (**Figure 6**). CD31-driven production of cytokines and phosphorylation of cAbl and RelA are sensitive to down regulation by Imatinib, a known cAbl inhibitor, and by MnT2E, a superoxide mimic, and inhibitor of the DNA-binding activity of NFκB (**Figure 5**). IL-17RA^{+}CD38^{+} FLC is a likely target of IL-17A as indicated by the upregulation of CD38 (**Figure 7**). Futhermore, IL-17A induces FLC to produce additional inflammatory cytokines/chemokines and tissue-destructive effectors, which are sensitive to down modulation by MnT2E (**Figures 8** and **9**). Such inhibitory activity of MnT2E on FLC is comparable, and in some cases, better than the biologics IL6i and TNFi. Details of IL-17A and CD38 signaling in FLC [dashed arrows] remain to be examined.

CD31 signaling in non-immune cells (41, 42). For conventional αβT cells, cAbl has been implicated as a minor secondary participant of TCR/CD28 signaling (61). Our data indicate cAbl along with ZAP70, Akt, and RelA are proximal transducers of CD31 signaling. Whether these signaling molecules are independently or co-dependently required for the CD31-driven production of distal cytokine effectors, such as IL-6, IL-17A, IFNγ, and TNFα by αβT cells remains to be examined. Nonetheless, our data sets clearly show CD31 signaling in synovial αβT cells effectively suborn or co-opt the classical TCR signaling pathway in the absence of TCR engagement. This idea is supported by our data set with TCR^{+}CD3^{+}CD31^{+} Jurkat and TCR^{-}CD3^{-}CD31^{+} JRT3 that recapitulated the CD31-driven cytokine production and the phosphorylations of signaling intermediates. Given the basic tenet that antigen-specific TCR triggering confers protective immunity, CD31-driven TCR-independent activation of synovial CD31^{+} DN and CD8^{+} αβT cells may represent a form of immune dysregulation that contribute to JIA synovitis.

IL-17A is a cytokine of interest in many chronic inflammatory diseases (26, 52, 62, 63). We report here several novel findings. First, our data provide evidence for TCR-independent IL-17A production by synovial DN and CD8^{+} αβT cells. CD31 ligation alone is sufficient to induce expression of RORγT transcription factor and *trans*-activation of *IL-17A* gene promoter. Thus, in addition to IL-17A^{+} CD4^{+} αβT and γδT cells (52, 64, 65), CD31^{+}CD28^{null} DN and CD8^{+} αβT cells likely represent non-conventional Th17 subsets. Whether all these IL-17A-producing subsets of T cells work in concert or have differential roles during arthritic flares in JIA has yet to be examined.

Second, CD31-driven IL-17A production is suppressed by Imatinib, an anti-CML drug known to inhibit cAbl kinase activity (22). The signaling pathway linking CD31 ligation, cAbl phosphorylation, and IL-17A production needs further investigation.

Third, a target of IL-17A in the joint is IL-17RA^{+} FLC. Their exposure to IL-17A results in the production of several MMPs, chemokines, and VEGF, and additional TNFα and IL-6. FLC also express the CD31 ligand CD38 (45). We validated previously that recombinant CD38-Ig stimulates CD31^{+}CD28^{null}CD8^{+} αβT cells (7). The origin(s) of FLC has yet to be determined.

Fourth, CD31-driven production of IL-17A by CD31^{+}CD28^{null} DN and CD8^{+} αβT cells is sensitive to MnT2E, a mimic of superoxide dismutase (23). The suppressive activity of MnT2E on synovial αβT cell cytokine production is in line with its reported down modulatory effect on T cells in non-obese diabetic mice wherein oxyradicals play a significant role in insulitis and pancreas pathology (66). Our data showing MnT2E-mediated inhibition of RelA phosphorylation in synovial CD31^{+} DN and CD8^{+} T cells is also consistent with its reported blockade of the DNA-binding activity of NFκB in whole tissue explants (24). Furthermore, our data show MnT2E-dependent inhibition of cAbl phosphorylation, and a higher degree of MnT2E inhibition of CD31-driven production of IL-17A. These two particular suppressive effects MnT2E are equivalent to that seen with Imatinib. These suggest Imatinib and MnT2E may be useful tools to further probe CD31→cAbl→IL-17A directional signaling.

It remains to be investigated whether MnT2E directly affects structure or function of cAbl. However, c-Abl has been shown experimentally to be modulated to reactive oxygen species (67). This could explain our observation that c-Abl phosphorylation was indeed affected by MnT2E consistent with its known dismutase activity (23). It also remains to be examined whether MnT2E directly alters the transcriptional and/or translational machineries of IL-17A production. This is of interest since the *IL-17A* gene promoter includes an NFκB binding site (30). RelA complexes of NFκB are known targets of oxyradicals (68). IL-17A production itself appears to be sensitive to redox reactions (69).

Finally, MnT2E also affects IL-17A-mediated production of additional inflammatory effectors by FLC. MnT2E suppression of MMP3, MMP8, and IL-8 (CXCL8) expression is comparable to those seen with IL6i and TNFi. MnT2E produces greater degrees of inhibition TNFα, IL-6, MMP2, VEGF, and MCP1 (CCL2) production than either IL6i or TNFi. MnT2E, however,

has no effect on MMP7, MMP12, and IL-1β. The basis for these differential effects of MnT2E has to be examined.

In summary, the present work provides evidence for TCR-independent, CD31-driven activation of joint-infiltrating $CD31^{+}CD28^{null}$ DN and $CD8^{+}$ αβT cells in JIA. These cells are sources of IL-6, IL-17A, IFNγ, and TNFα, four abundant cytokines in SF. A downstream target of IL-17A is $IL17RA^{+}CD38^{+}$ FLC, which responds by further upregulating CD38, a ligand of CD31 (45), and the production of additional inflammatory and tissue-destructive effectors including TNFα and IL-6 that compounds the CD31-driven cytokine production by αβT cells. Collectively, our data suggest a synovial T cell-FLC inflammatory circuit illustrated in **Figure 10**. The working model highlights plausible CD31–CD38 interaction with an IL-17A-mediated feedback loop. We have shown previously (7) that CD38-Ig is potent inducer of cytokine production by $CD31^{+}CD28^{null}$ αβT cells. Specificity of a CD31–CD38 cognate interaction will have to be verified. Blocking CD31 and/or CD38 in usual T cell-FLC co-cultures yielded very variable results, which were likely due to a variety of compounding receptor-counter receptor interactions. Nuances of IL-17A-signaling and CD38-signaling cascades in FLC also remain to be examined. Admittedly, the model cannot exclude other possible feedback loops, since CD31-activated T cells and IL-17A-activated FLC produce multiple effectors. Nonetheless, a notable aspect of our model is the identification of MnT2E as an independent inhibitor of CD31-driven T cell activation and IL-17-A-mediated FLC activation. This points to its likely utility as a probe to further unravel intricacies of CD31 signaling in synovial αβT cells, as well as IL-17A and CD38-signaling cascades in FLC. The inhibitory activity of MnT2E also provides a translational rationale to test whether it is potentially disease-modifying.

ETHICS STATEMENT

Institutional Review Boards of the University of Pittsburgh approved all research protocols. Written informed consent from legal guardians, and assent of child subjects as appropriate, were obtained.

AUTHOR CONTRIBUTIONS

AV designed the study and secured funding. IF, PG, JM, and AD prepared the manuscript. AV, JM, and DK designed and managed the IRB protocol. IF, JM, JD, MR, and DK recruited/consented subjects and abstracted medical records. IF, PG, JM, RM, and JD collected, processed, and cataloged biological specimens. IF, PG, HY, RM, and JD performed experiments. SG and JP provided critical reagents and designed their use. IF, PG, JM, and AV performed statistical analysis. All authors reviewed/approved the manuscript.

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REFERENCES

1. Martini A. JIA in 2011: new takes on categorization and treatment. *Nat Rev Rheumatol* (2012) 8:67–8. doi:10.1038/nrrheum.2011.198
2. Macaubas C, Nguyen K, Milojevic D, Park JL, Mellins ED. Oligoarticular and polyarticular JIA: epidemiology and pathogenesis. *Nat Rev Rheumatol* (2009) 5:616–26. doi:10.1038/nrrheum.2009.209
3. Grom AA, Hirsch R. T-cell and T-cell receptor abnormalities in the immunopathogenesis of juvenile rheumatoid arthritis. *Curr Opin Rheumatol* (2000) 12:420–4. doi:10.1097/00002281-200009000-00012
4. Amariglio N, Klein A, Dagan L, Lev A, Padeh S, Rechavi G, et al. T-cell compartment in synovial fluid of pediatric patients with JIA correlates with disease phenotype. *J Clin Immunol* (2011) 31:1021–8. doi:10.1007/s10875-011-9580-0
5. Haufe S, Haug M, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, et al. Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells. *Arthritis Rheum* (2011) 63:3153–62. doi:10.1002/art.30503
6. Wedderburn LR, Maini MK, Patel A, Beverley PC, Woo P. Molecular fingerprinting reveals non-overlapping T cell oligoclonality between an inflamed site and peripheral blood. *Int Immunol* (1999) 11:535–43. doi:10.1093/intimm/11.4.535
7. Dvergsten JA, Mueller RG, Griffin P, Abedin S, Pishko A, Michel JJ, et al. Premature cell senescence and T cell receptor-independent activation of CD8+ T cells in juvenile idiopathic arthritis. *Arthritis Rheum* (2013) 65:2201–10. doi:10.1002/art.38015
8. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. *Immunol Rev* (2005) 205:158–69. doi:10.1111/j.0105-2896.2005.00256.x
9. Lemster BH, Michel JJ, Montag DT, Paat JJ, Studenski SA, Newman AB, et al. Induction of CD56 and TCR-independent activation of T cells with aging. *J Immunol* (2008) 180:1979–90. doi:10.4049/jimmunol.180.3.1979
10. Michel JJ, Griffin P, Vallejo AN. Functionally diverse NK-like T cells are effectors and predictors of successful aging. *Front Immunol* (2016) 7:530. doi:10.3389/fimmu.2016.00530
11. Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol* (2011) 6:323–44. doi:10.1146/annurev-pathol-011110-130224
12. Duncan GS, Andrew DP, Takimoto H, Kaufman SA, Yoshida H, Spellberg J, et al. Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J Immunol* (1999) 162:3022–30.
13. Prahalad S, Thompson SD, Conneely KN, Jiang Y, Leong T, Prozonic J, et al. Hierarchy of risk of childhood-onset rheumatoid arthritis conferred by HLA-DRB1 alleles encoding the shared epitope. *Arthritis Rheum* (2012) 64:925–30. doi:10.1002/art.33376
14. Martini A. It is time to rethink juvenile idiopathic arthritis classification and nomenclature. *Ann Rheum Dis* (2012) 71:1437–9. doi:10.1136/annrheumdis-2012-201388
15. Torok KS, Kurzinski K, Kelsey C, Yabes J, Magee K, Vallejo AN. Peripheral blood cytokine and chemokine profiles in juvenile localized scleroderma: T-helper cell-associated cytokine profiles. *Semin Arthritis Rheum* (2015) 45:284–93. doi:10.1016/j.semarthrit.2015.06.006
16. Vallejo AN, Hamel DL Jr, Mueller RG, Ives DG, Michel JJ, Boudreau RM. NK-like T cells and plasma cytokines, but not anti-viral serology, define immune fingerprints of resilience and mild disability in exceptional aging. *PLoS One* (2011) 6:e26558. doi:10.1371/journal.pone.0026558

17. Fitzpatrick ME, Singh V, Bertolet M, Lucht L, Kessinger C, Michel J, et al. Relationships of pulmonary function, inflammation, and T-cell activation and senescence in an HIV-infected cohort. *AIDS* (2014) 28:2505–15. doi:10.1097/QAD.0000000000000471
18. Blakaj A, Bucala R. Fibrocytes in health and disease. *Fibrogenesis Tissue Repair* (2012) 5:S6. doi:10.1186/1755-1536-5-S1-S6
19. Schwarz RI. Collagen I and the fibroblast: high protein expression requires a new paradigm of post-transcriptional, feedback regulation. *Biochem Biophys Rep* (2015) 3:38–44. doi:10.1016/j.bbrep.2015.07.007
20. Bonish BK, Foreman KE, Gutierrez-Steil C, Nickoloff BJ. Phenotype and proliferation characteristics of cultured spindle-shaped cells obtained from normal human skin and lesions of dermatofibroma, Kaposi's sarcoma, and dermatofibrosarcoma protuberans: a comparison with fibroblast and endothelial cells of the dermis. *J Dermatol Sci* (1997) 16:52–8.
21. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis* (2007) 66:589–98. doi:10.1136/ard.2006.061853
22. Ofran Y, Izraeli S. BCR-ABL (Ph)-like acute leukemia-pathogenesis, diagnosis and therapeutic options. *Blood Rev* (2017) 31:11–6. doi:10.1016/j.blre.2016.09.001
23. Batinic-Haberle I, Tovmasyan A, Spasojevic I. An educational overview of the chemistry, biochemistry and therapeutic aspects of Mn porphyrins – from superoxide dismutation to H2O2-driven pathways. *Redox Biol* (2015) 5:43–65. doi:10.1016/j.redox.2015.01.017
24. Bottino R, Balamurugan AN, Tse H, Thirunavukkarasu C, Ge X, Profozich J, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes* (2004) 53:2559–68. doi:10.2337/diabetes.53.10.2559
25. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* (2008) 28:29–39. doi:10.1016/j.immuni.2007.11.016
26. Lubberts E. The IL-23-IL-17 axis in inflammatory arthritis. *Nat Rev Rheumatol* (2015) 11:415–29. doi:10.1038/nrrheum.2015.53
27. Kirkham BW, Kavanaugh A, Reich K. Interleukin-17A: a unique pathway in immune-mediated diseases: psoriasis, psoriatic arthritis and rheumatoid arthritis. *Immunology* (2014) 141:133–42. doi:10.1111/imm.12142
28. Chiricozzi A, Nograles KE, Johnson-Huang LM, Fuentes-Duculan J, Cardinale I, Bonifacio KM, et al. IL-17 induces an expanded range of downstream genes in reconstituted human epidermis model. *PLoS One* (2014) 9:e90284. doi:10.1371/journal.pone.0090284
29. Brescia AC, Simonds MM, Sullivan KE, Rose CD. Secretion of pro-inflammatory cytokines and chemokines and loss of regulatory signals by fibroblast-like synoviocytes in juvenile idiopathic arthritis. *Proteomics Clin Appl* (2017) 11(5–6):1600088. doi:10.1002/prca.201600088
30. Liu XK, Lin X, Gaffen SL. Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17. *J Biol Chem* (2004) 279:52762–71. doi:10.1074/jbc.M405764200
31. Michel JJ, Turesson C, Lemster B, Atkins SR, Iclozan C, Bongartz T, et al. CD56-expressing T cells that have features of senescence are expanded in rheumatoid arthritis. *Arthritis Rheum* (2007) 56:43–57. doi:10.1002/art.22310
32. Lewis DE, Merched-Sauvage M, Goronzy JJ, Weyand CM, Vallejo AN. Tumor necrosis factor-alpha and CD80 modulate CD28 expression through a similar mechanism of T-cell receptor-independent inhibition of transcription. *J Biol Chem* (2004) 279:29130–8. doi:10.1074/jbc.M402194200
33. Vallejo AN, Yang H, Klimiuk PA, Weyand CM, Goronzy JJ. Synoviocyte-mediated expansion of inflammatory T cells in rheumatoid synovitis is dependent on CD47-thrombospondin 1 interaction. *J Immunol* (2003) 171:1732–40. doi:10.4049/jimmunol.171.4.1732
34. Harrold LR, Salman C, Shoor S, Curtis JR, Asgari MM, Gelfand JM, et al. Incidence and prevalence of juvenile idiopathic arthritis among children in a managed care population, 1996-2009. *J Rheumatol* (2013) 40:1218–25. doi:10.3899/jrheum.120661
35. Vanoni F, Minoia F, Malattia C. Biologics in juvenile idiopathic arthritis: a narrative review. *Eur J Pediatr* (2017) 176:1147–53. doi:10.1007/s00431-017-2960-6
36. Weiss A, Stobo JD. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med* (1984) 160:1284–99. doi:10.1084/jem.160.5.1284
37. Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, Calman AF, et al. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. *Nature* (1985) 316:606–9. doi:10.1038/316606a0
38. Katz ZB, Novotna L, Blount A, Lillemeier BF. A cycle of Zap70 kinase activation and release from the TCR amplifies and disperses antigenic stimuli. *Nat Immunol* (2017) 18:86–95. doi:10.1038/ni.3631
39. Cheng J, Phong B, Wilson DC, Hirsch R, Kane LP. Akt fine-tunes NF-kappaB-dependent gene expression during T cell activation. *J Biol Chem* (2011) 286:36076–85. doi:10.1074/jbc.M111.259549
40. Paul S, Schaefer BC. A new look at T cell receptor signaling to nuclear factor-kappaB. *Trends Immunol* (2013) 34:269–81. doi:10.1016/j.it.2013.02.002
41. Xu S, Ha CH, Wang W, Xu X, Yin M, Jin FQ, et al. PECAM1 regulates flow-mediated Gab1 tyrosine phosphorylation and signaling. *Cell Signal* (2016) 28:117–24. doi:10.1016/j.cellsig.2015.12.007
42. Lertkiatmongkol P, Liao D, Mei H, Hu Y, Newman PJ. Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). *Curr Opin Hematol* (2016) 23:253–9. doi:10.1097/MOH.0000000000000239
43. Gao H, Lee BN, Talpaz M, Donato NJ, Cortes JE, Kantarjian HM, et al. Imatinib mesylate suppresses cytokine synthesis by activated CD4 T cells of patients with chronic myelogenous leukemia. *Leukemia* (2005) 19:1905–11. doi:10.1038/sj.leu.2403933
44. Ciarcia R, Vitiello MT, Galdiero M, Pacilio C, Iovane V, d'Angelo D, et al. Imatinib treatment inhibit IL-6, IL-8, NF-KB and AP-1 production and modulate intracellular calcium in CML patients. *J Cell Physiol* (2012) 227:2798–803. doi:10.1002/jcp.23029
45. Deaglio S, Morra M, Mallone R, Ausiello CM, Prager E, Garbarino G, et al. Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J Immunol* (1998) 160:395–402.
46. Turner JD, Filer A. The role of the synovial fibroblast in rheumatoid arthritis pathogenesis. *Curr Opin Rheumatol* (2015) 27:175–82. doi:10.1097/BOR.0000000000000148
47. Spirchez M, Samasca G, Iancu M, Bolba C, Miu N. Relation of interleukin-6, TNF-alpha and interleukin-1alpha with disease activity and severity in juvenile idiopathic arthritis patients. *Clin Lab* (2012) 58:253–60.
48. Walters HM, Pan N, Lehman TJ, Adams A, Kalliolias GD, Zhu YS, et al. The impact of disease activity and tumour necrosis factor-alpha inhibitor therapy on cytokine levels in juvenile idiopathic arthritis. *Clin Exp Immunol* (2016) 184:308–17. doi:10.1111/cei.12782
49. Woo P. Cytokines and juvenile idiopathic arthritis. *Curr Rheumatol Rep* (2002) 4:452–7. doi:10.1007/s11926-002-0050-9
50. Barnes MG, Grom AA, Thompson SD, Griffin TA, Pavlidis P, Itert L, et al. Subtype-specific peripheral blood gene expression profiles in recent-onset juvenile idiopathic arthritis. *Arthritis Rheum* (2009) 60:2102–12. doi:10.1002/art.24601
51. Sikora KA, Fall N, Thornton S, Grom AA. The limited role of interferon-gamma in systemic juvenile idiopathic arthritis cannot be explained by cellular hyporesponsiveness. *Arthritis Rheum* (2012) 64:3799–808. doi:10.1002/art.34604
52. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum* (2008) 58:875–87. doi:10.1002/art.23291
53. Paillard F, Sterkers G, Vaquero C. Transcriptional and post-transcriptional regulation of TcR, CD4 and CD8 gene expression during activation of normal human T lymphocytes. *EMBO J* (1990) 9:1867–72.
54. Crispin JC, Tsokos GC. Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype. *J Immunol* (2009) 183:4675–81. doi:10.4049/jimmunol.0901533
55. Hedrich CM, Crispin JC, Rauen T, Ioannidis C, Koga T, Rodriguez Rodriguez N, et al. cAMP responsive element modulator (CREM) alpha mediates chromatin remodeling of CD8 during the generation of CD3+ CD4- CD8- T cells. *J Biol Chem* (2014) 289:2361–70. doi:10.1074/jbc.M113.523605
56. Anand A, Dean GS, Quereshi K, Isenberg DA, Lydyard PM. Characterization of CD3+ CD4- CD8- (double negative) T cells in patients with systemic lupus erythematosus: activation markers. *Lupus* (2002) 11:493–500. doi:10.1191/0961203302lu235oa
57. Patel DR, Richardson BC. Dissecting complex epigenetic alterations in human lupus. *Arthritis Res Ther* (2013) 15:201. doi:10.1186/ar4125

58. Spreafico R, Rossetti M, Whitaker JW, Wang W, Lovell DJ, Albani S. Epipolymorphisms associated with the clinical outcome of autoimmune arthritis affect CD4+ T cell activation pathways. *Proc Natl Acad Sci U S A* (2016) 113:13845–50. doi:10.1073/pnas.1524056113
59. Vallejo AN, Brandes JC, Weyand CM, Goronzy JJ. Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence. *J Immunol* (1999) 162:6572–9.
60. Kimmig S, Przybylski GK, Schmidt CA, Laurisch K, Mowes B, Radbruch A, et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med* (2002) 195:789–94. doi:10.1084/jem.20011756
61. Gu JJ, Ryu JR, Pendergast AM. Abl tyrosine kinases in T-cell signaling. *Immunol Rev* (2009) 228:170–83. doi:10.1111/j.1600-065X.2008.00751.x
62. Zhang Z, Kyttaris VC, Tsokos GC. The role of IL-23/IL-17 axis in lupus nephritis. *J Immunol* (2009) 183:3160–9. doi:10.4049/jimmunol.0900385
63. Crispin JC, Tsokos GC. IL-17 in systemic lupus erythematosus. *J Biomed Biotechnol* (2010) 2010:943254. doi:10.1155/2010/943254
64. Berkun Y, Bendersky A, Gerstein M, Goldstein I, Padeh S, Bank I. GammadeltaT cells in juvenile idiopathic arthritis: higher percentages of synovial Vdelta1+ and Vgamma9+ T cell subsets are associated with milder disease. *J Rheumatol* (2011) 38:1123–9. doi:10.3899/jrheum.100938
65. Bendersky A, Marcu-Malina V, Berkun Y, Gerstein M, Nagar M, Goldstein I, et al. Cellular interactions of synovial fluid gammadelta T cells in juvenile idiopathic arthritis. *J Immunol* (2012) 188:4349–59. doi:10.4049/jimmunol.1102403
66. Piganelli JD, Flores SC, Cruz C, Koepp J, Batinic-Haberle I, Crapo J, et al. A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes* (2002) 51:347–55. doi:10.2337/diabetes.51.2.347
67. El Jamali A, Valente AJ, Lechleiter JD, Gamez MJ, Pearson DW, Nauseef WM, et al. Novel redox-dependent regulation of NOX5 by the tyrosine kinase c-Abl. *Free Radic Biol Med* (2008) 44:868–81. doi:10.1016/j.freeradbiomed.2007.11.020
68. Batinic-Haberle I, Spasojevic I, Tse HM, Tovmasyan A, Rajic Z, St Clair DK, et al. Design of Mn porphyrins for treating oxidative stress injuries and their redox-based regulation of cellular transcriptional activities. *Amino Acids* (2012) 42:95–113. doi:10.1007/s00726-010-0603-6
69. Zhi L, Ustyugova IV, Chen X, Zhang Q, Wu MX. Enhanced Th17 differentiation and aggravated arthritis in IEX-1-deficient mice by mitochondrial reactive oxygen species-mediated signaling. *J Immunol* (2012) 189:1639–47. doi:10.4049/jimmunol.1200528

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MINI REVIEW
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Foxp3 Molecular Dynamics in Treg in Juvenile Idiopathic Arthritis

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Since the identification of the regulatory T-cell (Treg)-associated transcription factor Foxp3, there have been intensive research efforts to understand its biology and roles in maintaining immune homeostasis. It is well established that thymic selection of a repertoire of self-reactive Foxp3$^+$ T-cells provides an essential mechanism to minimize reactions to self-antigens in the periphery, and thus aid in the prevention of autoimmunity. It is clear from both genetic and immunological analyses of juvenile idiopathic arthritis (JIA) patients that T-cells have a strong role to play in both the initiation and propagation of disease. The current paradigm is to view autoimmunity as a consequence of an imbalance between inflammatory and immunoregulatory mechanisms. This view has led to the assigning of cells and inflammatory mediators to different classes based on their assumed pro- or anti-inflammatory roles. This is typically reported as ratios of effector T-cells to Treg cells. Problematically, many analyses are based on static "snapshots-in-time," even though both mouse models and human patient studies have highlighted the dynamic nature of Foxp3$^+$ T-cells *in vivo*, which can exhibit plasticity and time-dependent functional states. In this review, we discuss the role of Foxp3 dynamics in the control of T-cell responses in childhood arthritis, by reviewing evidence in humans and relevant mouse models of inflammatory disease. Whilst the cellular dynamics of Treg have been well evaluated—leading to standard data outputs such as frequency, quantity and quality (often assessed by *in vitro* suppressive capacity)—we discuss how recent insights into the molecular dynamics of *Foxp3* transcription and its post-translational control may open up tantalizing new avenues for immunotherapies to treat autoimmune arthritis.

Keywords: Foxp3, juvenile idiopathic arthritis (JIA), transcriptional dynamics, Tocky, Treg

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AIMS AND SCOPE

The aim of this article is to provide an overview of the literature reporting Foxp3$^+$ Regulatory T cell (Treg) cell biology in juvenile idiopathic arthritis (JIA) and place this in the context of recent advances in understanding basic Treg biology. For review of JIA Treg biology, a defined Pubmed search was performed with the following terms: "Treg" OR "Foxp3" OR "Regulatory T cell" AND "Juvenile Idiopathic Arthritis"[1]. What is clear from these papers is that there has been great industry in elucidating Treg and effector T-cell biology and relating this to disease mechanisms from cellular viewpoints. This article aims to build on this body of knowledge by detailing how recent new

[1]Search date July 10th 2018.

approaches are giving fresh insight into the molecular control of *Foxp3* and the dynamics of T-cell regulation. It is hoped that this approach may help human immunologists to disambiguate markers used to identify Treg [e.g., CD25 and CD127 expression (1)] and stimulate fresh thinking about Foxp3-mediated regulatory mechanisms in JIA.

INTRODUCTION

Foxp3 is essential for T-cell homeostasis and is considered one of the main drivers of Regulatory T-cell (Treg) differentiation (2). Mutations in the *FOXP3* gene in humans cause the complex multiorgan autoimmune disease, Immune Dysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX) (3). This condition parallels the Scurfy mouse (4), where a two base-pair insertion in the murine *Foxp3* gene results in a truncated form of Foxp3 protein (5) and loss of immune regulation. Scurfy mice typically die within 3 weeks, highlighting that loss of function of Foxp3 is not compatible with long-term survival. A Treg lineage (6) interpretation of these findings is that *Foxp3* mutations lead to loss of a dedicated line of suppressor T-cells, presumably because Foxp3 can no longer imprint the Treg suppressive phenotype (7). Treg are often divided into thymic and peripheral subsets, based on their sites of differentiation. Thymic Treg are self-reactive, and are important components of central tolerance. Peripheral Treg are thought to arise in response to innocuous antigens, and may be important for tolerance to dietary antigens or commensal bacteria. Together these subsets work to exert dominant tolerance to both self and foreign antigens (8).

Given the importance of Foxp3 to immune homeostasis and Treg biology, it has become a focal point of immunological research into diseases arising due to dysregulated T-cell responses, such as JIA. JIA is the most common form of autoimmune rheumatic disease with a prevalence in the region of one in a thousand in children under 16 years of age (9). JIA is a heterogeneous group of conditions, covering all forms of arthritis commencing in children under 16 years of age and lasting for at least 6 weeks duration (10). Although heterogeneity represents a challenge to translate basic immunological findings from animal models to JIA, access to the site of inflammation provides precious material for studying underlying immunological mechanisms.

GENETICS OF JIA POINT TO T-CELL REGULATION

Genetic association studies of JIA (incorporating the most common subtypes of disease) clearly highlight a key role for genes involved in the immune system, particularly those involved in the regulation of T-cell biology (11). Unsurprisingly, the strongest association is with human leukocyte antigen (*HLA*) alleles. This is a common occurrence in autoimmune diseases (12), which may be due to the finding that self-antigen-specific Treg selection is HLA allele-dependent and modulates susceptibility to autoimmunity (13). There also exists a striking association with genes involved in the molecular control of Treg biology. Although nine single nucleotide polymorphisms (SNPs) within the human *FOXP3* gene show no significant associations to JIA (14) (suggesting these do not impact on *FOXP3* in JIA), a key *FOXP3*-binding partner, Runt-related transcription factor 1 (*RUNX1),* is, however, significantly associated. In non-Foxp3 expressing T-cells, RUNX1 enhances interleukin (*IL)2* expression through direct binding of the *IL2* promoter in activated $CD4^+$ T-cells (7). However, in the context of Foxp3 expression, functional studies in mice have shown that Runx1 and Foxp3 form molecular complexes which lead to the repression of *Il2* and interferon gamma (*Ifng)*, and upregulation of Treg effector molecules, such as CD25 and cytotoxic lymphocyte antigen 4 (CTLA-4) (7). Thus, Foxp3 can re-direct the molecular machinery involved in T-cell activation in order to drive a T-cell-intrinsic suppressive programme. Whilst Foxp3 expression has been mainly studied in the context of Treg generation (15), the T-cell-intrinsic functions of FOXP3 expression during either T-cell activation (which is considered transient, but can have functional consequences for effector T-cells (16)) or peripheral (p)Treg generation in JIA remain unknown.

In addition to *RUNX1*, genes encoding proteins involved in cytokine signaling pathways that are critical to the development of $FOXP3^+$ T-cells are also significantly associated. *IL2RA* [gene encoding CD25, the original Treg marker (17)] and the *IL2/IL21* cytokine locus display significant disease associations (11). Furthermore, deficiency in the IL-2 signaling molecule signal transducer and activator of transcription (STAT)5b has been reported in patients with JIA (18). IL-2 signaling is critical for the survival and fitness of Treg in the periphery (19) and is sensed by the conserved non-coding sequence 2 (CNS2) of the *Foxp3* gene to maintain Treg cell identity (20). These findings suggest that molecular tuning of *Foxp3* transcription and function not only have genetic associations but may represent a new avenue through which to further our understanding of the pathogenesis of JIA.

$FOXP3^+$ TREG CELL BIOLOGY IN JIA

Analysis of Treg cells identified by CD25 expression has shown that such cells are enriched at the site of inflammation in JIA (21). The authors further dissected whether $CD25^+$ T-cells show any relationship to disease severity. Here analysis of the oligoarticular (O)-JIA subset of patients is very useful since the O-JIA patient group can have divergent clinical outcomes (22). The "persistent" O-JIA subtype presents with a mild, remitting form of disease, which can spontaneously resolve. However, there exists a subset of O-JIA patients in which the disease progresses and arthritis extends to include an increasing number of joints, which are referred to as extended O-JIA patients. These divergent forms of O-JIA have allowed translational immunologists to compare between different severities of disease, which has proven powerful for correlating the role of Treg and T-helper subsets in JIA with measurable disease outcomes. Interestingly, $CD25^+$ Treg cells are present in increased numbers within the joints of JIA patients with the milder persistent form of O-JIA compared to those

with the more severe form of extended O-JIA disease (21). These findings were further verified using the "gold standard" Treg marker, FOXP3, in a separate patient cohort (23). Interestingly, both of these studies highlighted that compared to Treg in blood, Treg at the inflamed site were not only increased in proportion but they also displayed increased FOXP3 protein expression at the single cell level (24). These seemingly paradoxical findings of a vastly increased Treg signature at inflamed sites suggest that Treg presence alone may not detail the whole picture. Indeed, many subsequent studies have highlighted that the balance of effector and regulatory mechanisms may be a major influencer of disease outcome. Evidence to support this in JIA comes from findings that Th17 cells, which drive chronic arthritis via a cellular cascade (25), are also increased in the synovial fluid (SF) of JIA patients and display a reciprocal relationship with Treg (23) or FOXP3 expression levels (24).

Alterations in Treg phenotypes are likely influenced by the joint environment (26, 27). Firstly, Treg from SF are susceptible to the downregulation of FOXP3 following removal from the inflamed environment (26), which can be prevented by addition of synovial fluid to cultures. Furthermore, analysis of thymic Treg output [which has been shown to be altered in adult rheumatic disease (28)] revealed that JIA patients are no different from controls (29), suggesting that the quantity of thymic Treg generation may be unaffected. Furthermore functional studies on Treg from JIA patients suggest no qualitative difference in their ability to suppress T-cell responses (30).

As to what may explain disease in the presence of increased Treg frequency, investigators have looked at the effector T-cells. *In vitro* suppression assays have shown that Treg from the blood and SF of JIA patients display similar abilities in regulating the proliferation of conventional T-cells (Tconv) from the peripheral blood environment (30, 31). However, Tconv from the inflamed site showed resistance to suppression by Treg. Neat follow up *in vitro* studies to this have gone on to show that this resistance to suppression could be overcome by the blocking of inflammatory cytokines such as tumor necrosis factor alpha (TNFα) (32, 33), which may provide a potential mechanism of action for biologics used to treat JIA. However, whilst these studies suggest that SF Tconv may be more resistant to regulation *in vitro*, it is unknown whether Tconv are resistant to regulation *in vivo*. In particular, analysis of Ki67$^+$ (marker of cell proliferation) T-cells in the joints of JIA patients suggests a more complex picture, since the majority of dividing cells in the joint are Treg or Treg-like [as evidenced by hypomethylation of the Foxp3 gene (27)]. Nonetheless a consensus has built based on the aforementioned work that site-specific Treg and Tconv interactions play central roles in the pathogenesis of JIA (**Figure 1**).

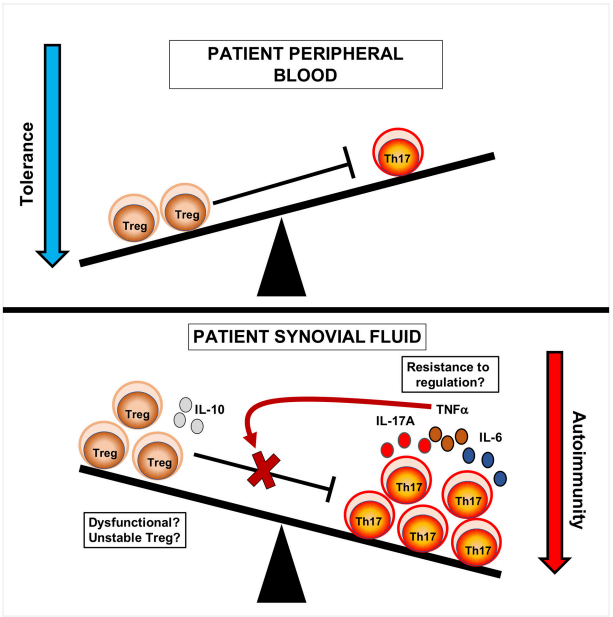

FIGURE 1 | Cellular seesaw view of Treg and Th17 cells in JIA pathogenesis. In blood, immune relationships are balanced and Treg can regulate effector T-cells. In synovial fluid, Treg are increased at the inflamed site but show a reciprocal relationship to Th17 cells. This suggests that the balance of Th17 to Treg cells may influence disease course, likely through increased production of inflammatory mediators. Treg may be dysfunctional, either through loss of Foxp3 and lineage stability, or are unable to regulate Tconv at the site of inflammation due to the release of inflammatory cytokines such as TNFα. Current strategies have aimed to tip the balance of this system in order to promote tolerance over autoimmunity.

DYNAMICS AND CLONAL RELATIONSHIPS BETWEEN FOXP3$^+$ AND FOXP3$^-$ T-CELLS IN JIA

Recent advances in sequencing technologies has allowed the profiling of the T-cell receptor (TCR) repertoires of Treg in JIA, which can be used to assess the relative clonal sharing between different CD4$^+$ T-cell subsets and provide great insight into cellular dynamics. Deep sequencing of TCRβ chains has highlighted more restricted and oligoclonal repertoires in SF T-cells compared to blood (26, 34). Comparison between controls and JIA peripheral blood Treg suggests that JIA patients have skewing of both blood and SF TCR repertoires (34). This skewing of the repertoire could arise from recirculation of T-cells between blood and SF (35), but potentially could be an important biomarker for treatment. Indeed, animal studies have shown that bone marrow transplantation following peptidoglycan-induced arthritis revealed that Treg from the graft repopulate the immune system and show improved repertoire diversity (36). In fact, transfer of additional Foxp3-GFP$^+$ T-cells together with the bone marrow transfer graft did not induce additional clinical improvement but moreover delayed TCR repertoire diversification, cautioning the use of Treg transfers in such settings. This is likely due to the fact that these additional Treg suppress T-cell proliferation, thus delaying expansion of donor-derived T-cells. In addition, the authors were able to show that JIA patients undergoing haematopoietic stem cell transfers also showed improved Treg diversity. These findings suggest that an immune reset provides an opportunity for graft-derived cells to regulate the autoimmune response, likely due to alterations in T-cell dynamics. Indeed, the SF environment

appears to drive dynamic changes in Treg expression of key molecules. TCR repertoire analysis of SF T-cells expressing different combinations of CD25 and FOXP3 have shown that there is remarkable clonal sharing between $CD25^{+}FOXP3^{-}$, $CD25^{+}FOXP3^{+}$, and $CD25^{-}FOXP3^{+}$ T-cells within the joint (26). These findings suggest that CD25 and FOXP3 expression within Treg-like populations may be dynamic within the joint; however, this does not preclude the possibility that CD25+FOXP3- T-cells also contain recently activated effector T-cells. Indeed, a thorough dissection of these populations would give useful insight to the dynamics of T-cell activation and FOXP3 expression in JIA. Given this observation, it will be intriguing for future studies to interrogate relationships between the TCR repertoires of Th17 and Treg cells within the joints of O-JIA patients, in order to establish whether the reciprocal relationship arises from discrete clones, or whether Th17/Treg plasticity is an important mechanism. For instance hybrid $IL\text{-}17A^{+}$ $FOXP3^{+}$ Treg, identified by expression of CD161, are significantly enriched at the site of inflammation (37, 38), and repertoire overlap between $CD161^{+}$ Treg and Tconv as a proportion of $CD161^{+}$ SF Treg was in the region of 20–30% (38), suggesting plasticity of T-cell responses within the joint. In summary, what these papers clearly show is that the joint environment is highly dynamic, and that snapshot in time analyses may obscure observations that may be accounted for by cellular and molecular dynamics.

MOLECULAR DYNAMICS REGULATE FOXP3+ T-CELL BIOLOGY

Whilst Foxp3 expression is used to define Treg subsets and is considered a stable marker of Treg cells, the clonal relationships between Treg and Tconv by TCR sequencing can also be explained by taking a molecular perspective (6). For instance, we have recently generated a new reporter system called Timer of cell kinetics and activity (Tocky) [(39); **Figure 2A**], which highlights a feedback control role for Foxp3 in regulating T-cell responses. This approach places the emphasis on the molecular dynamics of the system, with a reduced focus on cellular categorization, which can unnecessarily constrain analysis, particularly for high-dimensional data sets.

TIMER OF CELL KINETICS AND ACTIVITY (TOCKY) SYSTEM

Tocky reporter system uses a short-lived fluorescent Timer protein (40) to capture the activity of the *Foxp3* gene. Timer protein exhibits an initial blue fluorescent form, with an approximate half-life of 4 h in *Foxp3*-Tocky mice. Timer protein undergoes spontaneous and irreversible maturation into a red fluorescent form which has a half-life in the region of 5 days (41). *Foxp3*-Tocky therefore allows the detection of biologically important *Foxp3* gene settings. New *Foxp3* expressers are identified in *Foxp3*-Tocky mice by virtue of their pure blue fluorescence upon expression of the Timer gene, which is under the control of the *Foxp3* gene regulatory elements. This system captures rapid changes in *Foxp3* gene settings in response to immunological cues, giving insight to *Foxp3* gene regulation at the level of hours vs. days. In contrast, fate-mapping approaches, which have largely suggested at a cellular level Treg cells are stable (42, 43), do not capture the "real-time" changes in *Foxp3* gene activity which may greatly influence Treg function (41).

FOXP3-TOCKY REVEALS EFFECTOR TREG DIFFERENTIATION AND IDENTIFIES PTREG

We have revealed that mature Foxp3 expressers tune their *Foxp3* gene setting to a temporally persistent state to control the resolution of skin inflammation. Thus, our recent data suggest that studying Foxp3 as a binary marker (i.e., to identify Treg and non-Treg) can lose biological information. Upon immunization we observed dramatic changes in the activity of *Foxp3* transcription in various T-cell populations. At sites of inflammation (which are the relevant comparison for JIA) Treg increase transcription of *Foxp3* in a Foxp3-protein dependent fashion (41). This form of *Foxp3* autoregulation is key to driving what has been previously called the effector (e)Treg response, where cells display enhanced expression of immunoregulatory molecules, such as IL-10 and CTLA4. Thus, purely based on a molecular readout—the activity of the *Foxp3* gene—we could identify the major features of previously coined "quiescent" Treg and effector (e)Treg subsets (**Figure 2**).

Foxp3-Tocky mice allow visualization of the earliest stages of p Treg development during physiological T-cell responses. We reported an increased proportion of T-cells acquire *de novo* Foxp3 expression within inflamed skin compared to non-inflamed sites, during contact hypersensitivity. The biological significance of this is still to be fully determined, but we propose that induction of Foxp3 may be an important part of the resolution of T-cell responses through the intrinsic regulation of Tconv (16, 41).

POST-TRANSLATIONAL CONTROL OF FOXP3

Aside from the transcriptional control of *Foxp3*, insight into the post-translational control of Foxp3 protein has been revealed over the past few years (44). Foxp3 protein has been shown to be polyubiquitinated at multiple lysine residues, which can lead to its proteasome-mediated degradation (44, 45). This process appears to be regulated by the activity of two enzymes, the deubiquitinase USP7 (44) and E3 ubiquitin ligase STUB1 (45). Here the authors of the studies were able to show that inflammatory cytokines, such as IL-6 [which is elevated in JIA synovial fluid (46)] could repress USP7 expression, resulting in increased turnover of Foxp3 and loss of Treg control of inflammation. These findings have led to the suggestion that small molecular inhibitors of this process could improve the stabilization of Foxp3 protein in cells, and therefore their functions (47).

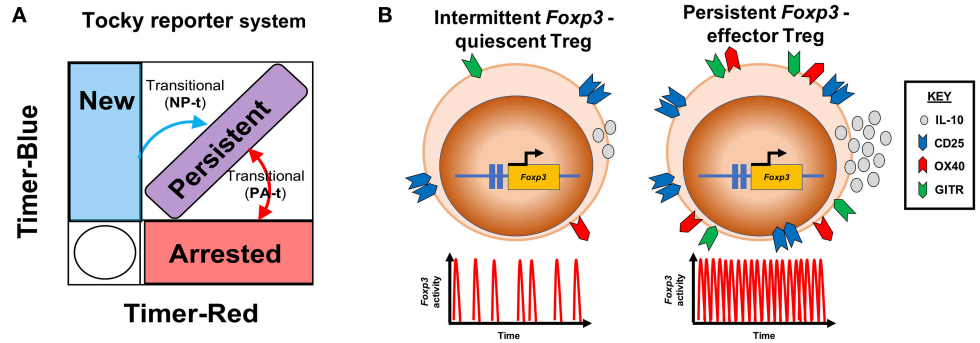

FIGURE 2 | Transcriptional dynamics of *Foxp3* define functional Treg profiles. **(A)** The Tocky system [adapted from (39)] identifies different temporal expression patterns of the *Foxp3* gene. Here a schematic is shown, illustrating how the Tocky system's timer locus approach can be used to identify different dynamics of *Foxp3* expression based on the position of cells within a theoretical flow cytometric Timer-blue and Timer-red two-dimensional space: New expressers are pure blue, gradually acquiring red fluorescence after 4–8 h, and moving to the New-Persistent transitional (NP-t) locus. Cells which continually transcribe *Foxp3*, accumulate in the blue$^+$ red$^+$ diagonal, called Persistent. And cells that have recently ceased *Foxp3* transcription are located in the pure red Arrested locus. As *Foxp3* gene activity changes, cells can move between Arrested and Persistent zones within the Persistent-Arrested transitional (PA-t) locus. **(B)** Summary of Treg *Foxp3* gene dynamics under different immunological contexts. In steady state, Treg exhibit intermittent *Foxp3* gene activity, and express lower levels of CD25, GITR, OX40, and IL-10. Upon immune challenge, responsive Treg increase their *Foxp3* transcriptional activity to a temporally persistent dynamic, which drives the Treg effector functions.

FOXP3 MOLECULAR "TUNING" FOR THERAPY

Foxp3$^+$ T-cells have a large number of molecules that have been proposed as surrogate markers, such as high CD25 expression and low CD127 (1) (the IL-7 receptor alpha chain). In addition, they have also been shown to express high levels of the Tumor necrosis factor receptor superfamily members, which are important for their thymic development (48). Indeed, it has been known for over 15 years that glucocorticoid-induced TNFR-related protein (GITR) is a marker of thymic CD4$^+$ CD25$^+$ subsets and targeting this membrane receptor with a monoclonal antibody could alter the course of autoimmunity (49). Using the *Foxp3*-Tocky tool, we have been able to give a dynamic perspective to common Treg cell surface markers (41). We were able to classify membrane receptors according to their relationship to *Foxp3* transcriptional activity, revealing that they can be classified into two main groups. Group I [containing amongst others TNFRII, C-C chemokine receptor type four (CCR4), CCR5] are high on activated T-cells and new *Foxp3* expressers and remain high whilst active *Foxp3* transcription occurs. We could show that the targeting of a marker within this group (TNFRII) was able to increase the proportion of T-cells acquiring new *Foxp3* expression. Group II (containing amongst others CD25, OX40, and GITR) membrane receptors appeared to parallel the activity of *Foxp3* transcription and increased as T-cells moved into persistent dynamics of *Foxp3* Transcription (i.e., eTreg phenotype). Very interestingly, expression levels of these molecules fell considerably in T-cells with low or arrested *Foxp3* transcriptional activity, implying they are selective to the eTreg-type programme. OX40 was one member of this group, and we showed that upon anti-OX40 treatment Foxp3$^+$ T-cells were shorter lived and "persistent" Foxp3 transcribers were reduced. This correlated with a delay in the resolution of allergic T-cell driven skin inflammation (41). Although these effects were modest, and the precise mechanisms of action remain to be fully elucidated, they show proof of concept that *Foxp3* transcriptional dynamics within T-cell populations can be modulated with measurable changes in disease outcomes.

CONCLUSIONS AND FUTURE DIRECTIONS

This review has highlighted the increases in our understanding of Foxp3$^+$ T-cell biology in JIA. Recent work revealing how molecular pathways regulate Foxp3 protein and *Foxp3* transcription should spur researchers to consider how these findings may be translated to human disease settings. For instance, it will be useful to determine whether the markers identified in basic animal models also hold equally true for humans. This could provide biomarkers to better understand the immunological effects of biologics for the treatment of autoimmune disorders such as JIA. In addition, given that Foxp3 protein can autoregulate its transcription, it will be very interesting to see whether post-translational modifications of Foxp3 can alter the Foxp3-driven autoregulatory loop (41, 50), and therefore enhance the Foxp3-driven T-cell programme. To take these ideas forward, however, the field may need to take a few steps back from the cell lineage paradigm and consider how the molecular dynamics of Foxp3-driven biology may provide new avenues for translational research in JIA.

AUTHOR CONTRIBUTIONS

DB conceived the review. AC and DB reviewed the literature and co-wrote the manuscript.

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REFERENCES

1. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med.* (2006) 203:1701–11. doi: 10.1084/jem.20060772
2. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* (2008) 133:775–87. doi: 10.1016/j.cell.2008.05.009
3. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* (2001) 27:20–1. doi: 10.1038/83713
4. Lyon MF, Peters J, Glenister PH, Ball S, Wright E. The scurfy mouse mutant has previously unrecognized hematological abnormalities and resembles Wiskott-Aldrich syndrome. *Proc Natl Acad Sci USA.* (1990) 87:2433–7.
5. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet.* (2001) 27:68–73. doi: 10.1038/83784
6. Ono M, Tanaka RJ. Controversies concerning thymus-derived regulatory T cells: fundamental issues and a new perspective. *Immunol Cell Biol.* (2016) 94:3–10. doi: 10.1038/icb.2015.65
7. Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, et al. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* (2007) 446:685–9. doi: 10.1038/nature05673
8. Lee HM, Bautista JL, Hsieh CS. Thymic and peripheral differentiation of regulatory T cells. *Adv Immunol.* (2011) 112:25–71. doi: 10.1016/B978-0-12-387827-4.00002-4
9. Manners PJ, Bower C. Worldwide prevalence of juvenile arthritis why does it vary so much? *J Rheumatol.* (2002) 29:1520–30.
10. Ravelli A, Martini A. Juvenile idiopathic arthritis. *Lancet* (2007) 369:767–78. doi: 10.1016/S0140-6736(07)60363-8
11. Hinks A, Cobb J, Marion MC, Prahalad S, Sudman M, Bowes J, et al. Dense genotyping of immune-related disease regions identifies 14 new susceptibility loci for juvenile idiopathic arthritis. *Nat Genet.* (2013) 45:664–9. doi: 10.1038/ng.2614
12. Gough SC, Simmonds MJ. The HLA region and autoimmune disease: associations and mechanisms of action. *Curr Genomics* (2007) 8:453–65. doi: 10.2174/138920207783591690
13. Ooi JD, Petersen J, Tan YH, Huynh M, Willett ZJ, Ramarathinam SH, et al. Dominant protection from HLA-linked autoimmunity by antigen-specific regulatory T cells. *Nature* (2017) 545:243–7. doi: 10.1038/nature22329
14. Eastell T, Group BS, Hinks A, Thomson W. SNPs in the FOXP3 gene region show no association with juvenile idiopathic arthritis in a UK Caucasian population. *Rheumatology* (2007) 46:1263–5. doi: 10.1093/rheumatology/kem129
15. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* (2007) 445:771–5. doi: 10.1038/nature05543
16. McMurchy AN, Gillies J, Gizzi MC, Riba M, Garcia-Manteiga JM, Cittaro D, et al. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood* (2013) 121:1265–75. doi: 10.1182/blood-2012-05-431023
17. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* (1995) 155:1151–64.
18. Kanai T, Jenks J, Nadeau KC The STAT5b pathway defect and autoimmunity. *Front Immunol.* (2012) 3:234. doi: 10.3389/fimmu.2012.00234
19. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol.* (2005) 6:1142–51. doi: 10.1038/ni1263
20. Feng Y, Arvey A, Chinen T, van der Veeken J, Gasteiger G, Rudensky AY, Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. *Cell* (2014) 158:749–63. doi: 10.1016/j.cell.2014.07.031
21. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol.* (2004) 172:6435–43. doi: 10.4049/jimmunol.172.10.6435
22. Macaubas C, Nguyen K, Milojevic D, Park JL, Mellins ED. Oligoarticular and polyarticular JIA: epidemiology and pathogenesis. *Nat Rev Rheumatol.* (2009) 5:616–26. doi: 10.1038/nrrheum.2009.209
23. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum.* (2008) 58:875–87. doi: 10.1002/art.23291
24. Olivito B, Simonini G, Ciullini S, Moriondo M, Betti L, Gambineri E, et al. Th17 transcription factor RORC2 is inversely correlated with FOXP3 expression in the joints of children with juvenile idiopathic arthritis. *J Rheumatol.* (2009) 36:2017–24. doi: 10.3899/jrheum.090066
25. Hirota K, Hashimoto M, Ito Y, Matsuura M, Ito H, Tanaka M, et al. Autoimmune Th17 cells induced synovial stromal and innate lymphoid cell secretion of the cytokine GM-CSF to initiate and augment autoimmune arthritis. *Immunity* (2018) 48:1220–32.e5. doi: 10.1016/j.immuni.2018.04.009
26. Bending D, Giannakopoulou E, Lom H, Wedderburn LR. Synovial regulatory T cells occupy a discrete TCR Niche in human arthritis and require local signals to stabilize FOXP3 protein expression. *J Immunol.* (2015) 195:5616–24. doi: 10.4049/jimmunol.1500391
27. Bending D, Pesenacker AM, Ursu S, Wu Q, Lom H, Thirugnanabalan B, et al. Hypomethylation at the regulatory T cell-specific demethylated region in CD25hi T cells is decoupled from FOXP3 expression at the inflamed site in childhood arthritis. *J Immunol.* (2014) 193:2699–708. doi: 10.4049/jimmunol.1400599
28. Koetz K, Bryl E, Spickschen K, O'Fallon WM, Goronzy JJ, Weyand CM. T cell homeostasis in patients with rheumatoid arthritis. *Proc Natl Acad Sci USA.* (2000) 97:9203–8. doi: 10.1073/pnas.97.16.9203
29. Lorenzi AR, Morgan TA, Anderson A, Catterall J, Patterson AM, Foster HE, et al. Thymic function in juvenile idiopathic arthritis. *Ann Rheum Dis.* (2009) 68:983–90. doi: 10.1136/ard.2008.088112
30. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood* (2011) 118:3538–48. doi: 10.1182/blood-2010-12-328187
31. Haufe S, Haug M, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, et al. Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells. *Arthritis Rheum.* (2011) 63:3153–62. doi: 10.1002/art.30503
32. Petrelli A, Wehrens EJ, Scholman RC, Prakken BJ, Vastert SJ, van Wijk F, Self-sustained resistance to suppression of CD8+ Teff cells at the site of autoimmune inflammation can be reversed by tumor necrosis factor and interferon-gamma blockade. *Arthritis Rheumatol.* (2016) 68:229–36. doi: 10.1002/art.39418
33. Wehrens EJ, Vastert SJ, Mijnheer G, Meerding J, Klein M, Wulffraat NM, et al. Anti-tumor necrosis factor alpha targets protein kinase B/c-Akt-induced resistance of effector cells to suppression in juvenile idiopathic arthritis. *Arthritis Rheum.* (2013) 65:3279–84. doi: 10.1002/art.38132
34. Henderson LA, Volpi S, Frugoni F, Janssen E, Kim S, Sundel RP, et al. Next-generation sequencing reveals restriction and clonotypic expansion of treg cells in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2016) 68:1758–68. doi: 10.1002/art.39606
35. Rossetti M, Spreafico R, Consolaro A, Leong JY, Chua C, Massa M, et al. TCR repertoire sequencing identifies synovial Treg cell clonotypes in the bloodstream during active inflammation in human arthritis. *Ann Rheum Dis.* (2017) 76:435–41. doi: 10.1136/annrheumdis-2015-208992
36. Delemarre EM, van den Broek T, Mijnheer G, Meerding J, Wehrens EJ, Olek S, et al. Autologous stem cell transplantation aids autoimmune patients by functional renewal and TCR diversification of regulatory T cells. *Blood* (2016) 127:91–101. doi: 10.1182/blood-2015-06-649145
37. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood* (2013) 121:2647–58. doi: 10.1182/blood-2012-08-443473
38. Duurland CL, Brown CC, O'Shaughnessy RF, Wedderburn LR. CD161(+) Tconv and CD161(+) Treg share a transcriptional and functional phenotype

despite limited overlap in TCRbeta repertoire. *Front Immunol.* (2017) 8:103. doi: 10.3389/fimmu.2017.00103
39. Bending D, Martin PP, Paduraru A, Ducker C, Marzaganov E, Laviron M, et al. A timer for analyzing temporally dynamic changes in transcription during differentiation *in vivo*. *J Cell Biol.* (2018) 217:2931–50. doi: 10.1083/jcb.201711048
40. Subach FV, Subach OM, Gundorov IS, Morozova KS, Piatkevich KD, Cuervo AM, et al. Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. *Nat Chem Biol.* (2009) 5:118–26. doi: 10.1038/nchembio.138
41. Bending D, Paduraru A, Ducker CB, Prieto Martin P, Crompton T, Ono M, A temporally dynamic Foxp3 autoregulatory transcriptional circuit controls the effector Treg programme. *EMBO J.* (2018) 37:e99013. doi: 10.15252/embj.201899013
42. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the regulatory T cell lineage *in vivo*. *Science* (2010) 329:1667–71. doi: 10.1126/science.1191996
43. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* (2012) 36:262–75. doi: 10.1016/j.immuni.2011.12.012
44. van Loosdregt J, Fleskens V, Fu J, Brenkman AB, Bekker CP, Pals CE, et al. Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. *Immunity* (2013) 39:259–71. doi: 10.1016/j.immuni.2013.05.018
45. Chen Z, Barbi J, Bu S, Yang HY, Li Z, Gao Y, et al. The ubiquitin ligase Stub1 negatively modulates regulatory T cell suppressive activity by promoting degradation of the transcription factor Foxp3. *Immunity* (2013) 39:272–85. doi: 10.1016/j.immuni.2013.08.006
46. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis.* (2007) 66:589–98. doi: 10.1136/ard.2006.061853
47. van Loosdregt J, Coffer PJ. Post-translational modification networks regulating FOXP3 function. *Trends Immunol.* (2014) 35:368–78. doi: 10.1016/j.it.2014.06.005
48. Mahmud SA, Manlove LS, Schmitz HM, Xing Y, Wang Y, Owen DL, et al. Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat Immunol.* (2014) 15:473–81. doi: 10.1038/ni.2849
49. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol.* (2002) 3:135–42. doi: 10.1038/ni759
50. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* (2010) 463:808–12. doi: 10.1038/nature08750

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REVIEW
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Using Chromatin Architecture to Understand the Genetics and Transcriptomics of Juvenile Idiopathic Arthritis

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The presence of abnormal gene expression signatures is a well-described feature of the oligoarticular and polyarticular forms of juvenile idiopathic arthritis. In this review, we discuss how new insights into genetic risk for JIA and the three dimensional architecture of the genome may be used to develop a better understanding of the mechanisms driving these gene expression patterns.

Keywords: juvenile idiopathic arthritis (JIA), genetics, enhancer, non-coding genome, topically associated domains

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The completion of the first assembly of the human genome was accompanied by considerable surprise with the discovery that only ∼2% of the genome contained protein-encoding genes (1). The lay press seized on these findings and disseminated the idea that 98% of human genomes contained so-called "junk DNA," on the assumption that the only things of interest in genomes were the protein-coding sequences. Subsequent genome annotation efforts, such as the Encyclopedia of DNA Elements (ENCODE) and Roadmap Epigenomics in the United States and Blueprint Epigenomics in Europe, demonstrated that much of this so-called "junk DNA" actually encoded RNA molecules (2, 3) or contained regulatory regions that played critical roles in fine-tuning transcription during development, cell differentiation, and in response to external stimuli (4).

The era of genome-wide association studies (GWAS), which were undertaken to better understand the genetic contribution to a broad range of complex traits, brought additional surprises. Investigators were perplexed to find that the strongest genetic associations occurred with single nucleotide polymorphisms (SNPs) that were located within non-coding regions of the genome. Indeed, taken together, GWAS SNPs are far more likely to occur in intronic or intergenic regions than in either exons or gene promoters (4).

These findings provide the basis for our inquiry into the causes of the well-described transcriptional abnormalities in JIA. In this paper, we will review the wealth of literature demonstrating abnormal patterns of gene expression in the peripheral blood cells of children with juvenile idiopathic arthritis (JIA). We will then discuss the role of non-coding genomic elements (especially enhancers) in regulating transcription and how an understanding of these mechanisms may allow a deeper understanding of the JIA-associated transcriptional patterns and/or the genetics of JIA.

In this review, we will focus on the oligoarticular and polyarticular, RF-negative subtypes of JIA, as these subtypes appear to represent a continuous spectrum of shared genetic risk (5). In contrast, systemic-onset (6) and RF+ polyarticular disease (7) show unique clinical and genetic features. We should note, however, that many of the broad ideas that we will present in this review are relevant to these other two JIA subtypes, as they are to most complex genetic traits.

TRANSCRIPTIONAL ABNORMALITIES IN JIA: LESSONS LEARNED FROM GENE EXPRESSION PROFILING

The emergence of technologies to assess transcription on a genome-wide basis was initially met with considerable optimism that these tools could be used to develop clinical biomarkers (8), to classify heterogeneous patient populations more accurately (9, 10), to develop a better understanding of the mechanisms of therapeutic response (11–13) and to better understand disease pathogenesis (14). Different groups have reported a broad range of transcriptional abnormalities in patients with polyarticular and oligoarticular JIA. These transcriptional abnormalities can be observed in whole blood (12), unsorted white blood cells (buffy coats) (15), peripheral blood mononuclear cells (PBMC) (9, 16), neutrophils (17), and CD4+ T cells (18). These studies have generally identified clusters of interferon gamma (IFNg) and tumor necrosis factor alpha (TNFa) regulated genes that display differential expression in children with active polyarticular JIA when they are compared to healthy control children. An IL8 "signature" can also be seen in both neutrophil (19) and whole blood (14) gene expression studies. In general, whole blood expression studies have suggested complex interactions between innate and adaptive immunity in JIA (14, 20). It is curious to note that, even though the oligoarticular and polyarticular rheumatoid factor (RF)-negative subtypes share common genetic risk loci, the largest published study comparing these two phenotypes found that each was distinguishable from the other at the gene expression level (21). Similarly, RF-negative and RF-positive subtypes, which are genetically distinct (7), have remarkably similar gene expression profiles on whole blood microarray analysis. Thus, it is clear that genetic factors are not the sole drivers of the gene expression abnormalities observed in oligoarticular and polyarticular JIA.

The question arises as to whether these transcriptional abnormalities reflect intrinsic defects in gene regulation in the cells of interest, or merely reflect the inflammatory milieu to which these cells are exposed. After all, elevated levels of a broad range of inflammatory mediators can be observed in the serum (22) or plasma (23) of children with JIA. On the other hand, our group has shown that the transcriptional abnormalities observed in JIA neutrophils are accompanied by aberrations in the metabolism of glucose via the hexose monophosphate shunt (19). Similarly, Throm and colleagues have shown distinct aberrations in interferon gamma-mediated (IFNg) signaling pathways in JIA T cells (24) studied *in vitro*. Finally, neither PBMC nor neutrophil signatures "normalize" after children have achieved clinical remission on medication (CRM), although it's curious to note that the neutrophil aberrations are more prominent (13). We should also note that the intrinsic defect vs. externally-driven hypotheses to explain the distinct transcriptional profiles of JIA peripheral blood cells are not mutually exclusive. Although we are coming to understand the strong effect that the environment (broadly considered) has on peripheral blood gene expression (25), underlying genetics and the immediate external milieu to which the cells are exposed may both play a role in the transcriptional patterns observed in peripheral blood leukocytes of children with JIA.

We were naturally led us to ask whether the emerging knowledge of the genetics of JIA might provide a useful framework from which to understand the mechanisms driving the transcriptional abnormalities in JIA peripheral blood cells. This necessarily leads to a brief discussion of the genetics on JIA. This discussion will not be comprehensive, and the reader wishing to have a deeper understanding is invited to read the recent reviews available on this subject (26, 27).

GWAS AND THE GENETICS OF JIA

Using candidate gene approaches, GWAS, and genetic fine mapping studies, investigators have identified >30 genetic loci associated with JIA (26–28). Each of these 3 approaches queries a single or small groups of SNPs (candidate gene approaches) or large numbers of SNPs (GWAS and genetic fine mapping studies) and asks whether specific alleles occur more frequently in individuals with a specific disease or phenotype than they do in controls (29) to identify alleles that have a strong *association* with the disease or trait of interest. It is important to note, however, that SNPs identified by such studies may not be the actual genetic variants that exert the biological effects that confer risk. This is because the so-called tag SNPs (or candidate SNPs used in candidate gene approaches) are in linkage disequilibrium (LD) with hundreds or thousands of other SNPs in the same region, any one of which may exert risk-enhancing biological effects. To use an analogy, GWAS can be understood as something like a crude global positioning satellite (GPS) that can tell you, say, that you are on the M1, somewhere between London and Sheffield, assuring you that you are not in Cornwall, but not providing any information as to whether you're actually closer to Leicester or Nottingham. Thus, GWAS have merely identified regions of the genome where genetic risk may be exerted. These regions can be referred to as "LD blocks" or, more commonly, risk haplotypes. It is common to refer to the risk haplotypes in JIA (and other complex traits) by the gene nearest to the tag SNP, and this has led to the common misunderstanding that the GWAS SNP: (1) is the one that actually exerts the relevant biological effects and (2) exerts those effects on the nearest gene. Neither is necessarily the case.

Figure 1 illustrates this point. *IL6R* is a haplotype that was identified on the genetic fine mapping study published by Hinks et al. (5). The haplotype spans the region marked by the genomic coordinates, chr1:154291718-154392674, a length of >100,000 bp. The reader will also note that there are functional elements *other than* the coding genes (*ATP8B2* and a portion of *IL6R*); this region is also characterized by prominent, overlapping H3K4me1/H3K27ac histone marks (for simplicity, only the H3K27 marks are shown in the figure), and dense transcription factor binding even in non-coding regions (i.e., within introns and the intergenic area). These chromatin features are commonly associated with enhancers, about which it is useful

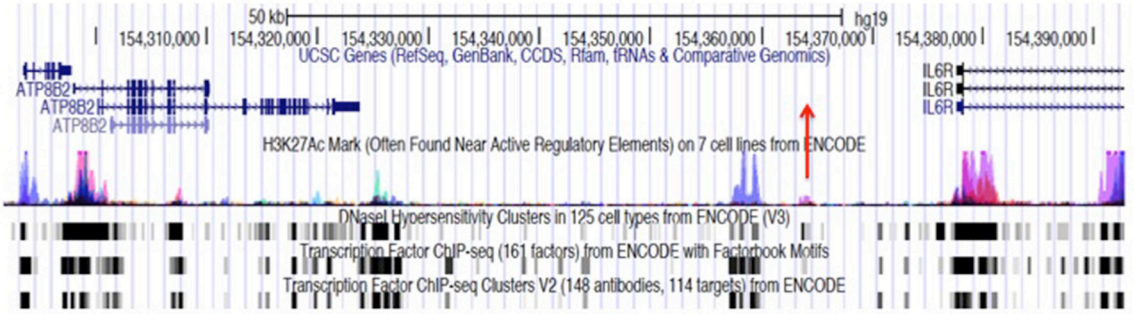

FIGURE 1 | Genome browser screen shot showing the JIA-associated *IL6R* locus, identified by the tag SNP, rs11265608, and encompassing the region chr1:154291718–154379369. Transcription is from left to right. This locus consists largely of an intergenic region between the *IL6R* and *ATP8B2* genes. The haplotype block also encompasses the first exon and intron of the *IL6R* gene and most of the coding sequence of the *ATP8B2* gene. The position of the tag SNP, rs11265608, is indicated by the red arrow. ChIPseq peaks for the histone mark H3K27ac from ENCODE and Roadmap Epigenomics data, are shown with the blue/magenta peaks. Transcription factor (TF) binding data and DNAse hypersensitivity data (also from ENCODE and Roadmap Epigenomics) are is represented black and gray boxes at the bottom of the figure. The regions where TF and H3K27ac marks overlap are putative enhancers. Almost identical chromatin architecture is seen in both CD4+ T cells and neutrophils within this haplotype. Note that neutrophils also express an intergenic RNA molecule at chr1:154350688-154350783 (not shown).

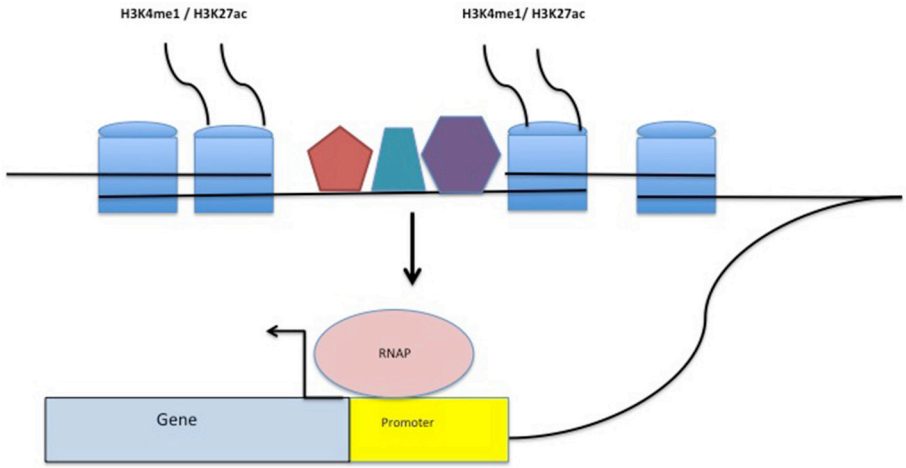

FIGURE 2 | Structure of a typical enhancer. H3K4me1/H3K27ac-marked histones flank a region densely bound by transcription factors (TFs-represented by the pentagon, hexagon, and trapezoid). The enhancer complex physically interacts with gene promoters, stabilizing TFs within the promoter as well as RNA polymerase (RNAP) binding, facilitating transcription.

to say more as we query the possible role of genetics in driving the transcriptional abnormalities observed in JIA.

JIA AND ENHANCERS

H3K4me1 and H3K27ac refer to covalent modifications to the tails of histones, epigenetic features that typically accompany enhancers, as noted above. One of the striking features that emerged from multiple GWAS studies was the high frequency with which the genetic "hits" occurred within H3K4me1/H3K27ac-marked regions of pathologically-relevant cells for the traits of interest (4). The reverse is also true: if one maps enhancer elements in specific cell types, those mapped regions are highly enriched in GWAS-identified SNPs (30) for diseases that affect those cells/tissues. We have reported that the risk loci for both JIA (31, 32) and systemic lupus (33), are highly enriched (compared to randomly-selected regions of functional chromatin) for H3K4me1/H3K27ac histone marks. These findings have led us to suggest that much of the genetic risk for JIA is exerted through altered function of these enhancers.

Enhancers are non-coding DNA elements that play an important role in regulating gene expression, serving as rheostats that adjust gene expression to fit finely-tuned physiologic contexts (34). Enhancers have a characteristic structure that includes the presence of open chromatin bound by multiple transcription factors (TFs) flanked by H3K4me1/H3K27ac-marked boundaries (**Figure 2**). These TFs form a complex that typically includes p300, mediator, and cohesin, which together facilitate looping and physical contact with the promoters of target genes. While many enhancers function constitutively, others remain latent, activated only by specific cellular or environmental triggers (35). It is important, from the standpoint

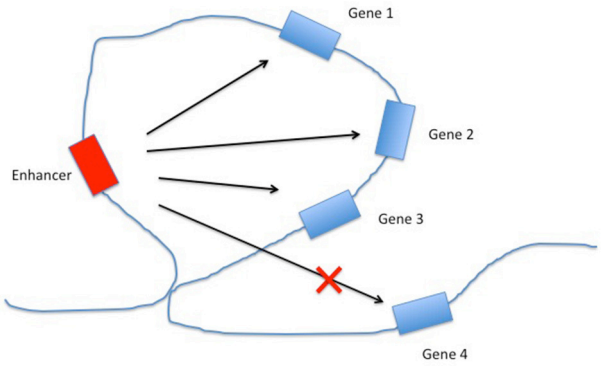

FIGURE 3 | Regulation of gene expression by enhancers within topologically associated domains (TADs). The chromatin loop structure facilitates interactions between the enhancer and some (or all) of the genes within the same TAD, while constricting the interactions between the enhancer and genes outside the TAD.

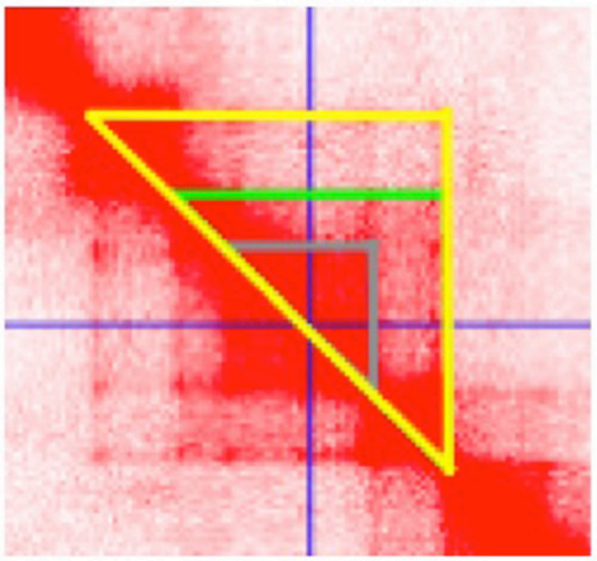

FIGURE 4 | Juicebox software visualization of the topologically associated domain (TAD) containing the JIA-associated *IL6R* haplotype. Data are from HiC analysis of K562 cells. The cursor (crossed blue lines) is set at the center of a putative enhancer in the region spanning chr1:154, 353, 745–154, 361, 534. Three distinct chromatin loops are identified, as indicted by the gray, green, and yellow triangles, suggesting complex chromatin architecture in this region.

of our understanding the genetics and pathobiology of JIA, to note that enhancers do not always regulate the nearest gene. Furthermore, a given enhancer may regulate more than one gene, and a given gene may be regulated by more than one enhancer. Although enhancers may not regulate the nearest gene, they typically regulate genes within the same chromatin loop or *topologically associated domain* (TAD). Our understanding of the three-dimensional structure of chromatin within the nucleus of eukaryotic cells has expanded significantly in the past 10 years, and readers interested in learning more on this fascinating topic and its relation to human disease may wish to consult some of the recent reviews (36–39). For the purposes of this review, TADs can be considered the basic chromatin loop structure that regulates enhancer-promoter interactions, as shown in **Figure 3**. TADs can be identified using non-targeted chromatin conformation techniques such as HiC (40), and visualized using publically available software such as JuiceBox (41). **Figure 4** shows how the TAD for the IL6R locus can be visualized. The larger loop contains multiple genes, including *IL6R*, *IL6R-AS1*, *ATP8B2*, *SHE*, *TDRD10*, *UBE2Q1*, and *UBE2Q1-AS1*. Any or all of these genes may be regulated by enhancer(s) within the *IL6R* locus, and thus *dys*regulated by genetic variants that disrupt or alter enhancer function within this locus.

QUERYING THE RISK HAPLOTYPES FOR DIFFERENTIALLY EXPRESSED GENES

Now we return to the question of the role of genes vs. environment (broadly considered) in driving the gene expression abnormalities in JIA. We recently reported on results of whole blood expression studies from children enrolled in the NIH-funded Trial of Early Aggressive Therapy in JIA (TREAT trial) (14). In that study, we identified 158 genes that showed differential expression when we compared children with new-onset polyarticular JIA with healthy controls. We then used conventional computational techniques to intersect the differentially expressed genes with the known JIA haplotypes. We were unable to identify a single differentially expressed gene within the known JIA haplotypes. We have subsequently repeated this technique using all publically available gene expression data from children with oligoarticular and polyarticular JIA and gotten the same result. This has led us to the conclusion that *if* genetic variants associated with JIA influence the observed transcriptional patterns, they must either do so within specific leukocyte subsets that are not detectable using whole blood, or they must act on longer-range chromatin interactions. Once again these are not mutually exclusive considerations. We anticipate that the emergence of single-cell technologies and perhaps the development of disease-specific three-dimensional chromatin maps will allow us to understand the genetic contribution (if any) to the observed peripheral blood expression abnormalities.

BRINGING IT ALL BACK HOME: A BROADER LOOK AT THE JIA RISK LOCI

Let us return to **Figure 1**, which shows the multiple features within the *IL6R* haplotype, which occupies the genomic coordinates chr1:154291718-154392674 and spans >100,000 bp. This risk locus is representative of the other JIA risk loci, which almost invariably show the same of similar chromatin features (31, 32).

The reader will note immediately that the haplotype contains only a portion of the *IL6R* gene as well as an additional gene, *ATP8B2*. The protein product of *ATP8B2* is an ATPase, and the

gene is expressed in both lymphoid cells as well as in myeloid cells such as macrophages (42); its exact role in these cells has not been investigated in any detail, although it is reasonable to speculate that it is involved in cellular energy production and utilization. Using RNA sequencing in human neutrophils, we have also shown that the haplotype also contains at least one non-coding intergenic RNA (ncRNA), a species of RNAs that are important in regulating both three dimensional chromatin architecture (43) and gene expression (44). We have previously shown that the presence of ncRNA molecules expressed in neutrophils is a common feature within the JIA risk loci (31, 32) and the presence of such RNA species in pathologically relevant cells is a characteristic that the JIA risk loci share with those of most other complex traits.

Finally, we note once again the rich in H3K4me1/H3K27ac histone marks, which overlap with abundant transcription factor binding sites, features that can be observed particularly in CD4+ T cells (ENCODE and Roadmap Epigenomics data) as well as our own neutrophil ChIP-seq data (31). Indeed the chromatin architecture at this locus suggests the presence of multiple intronic and intergenic enhancers in both cell types.

These observations raise multiple questions regarding the mechanism(s) through which genetic variants within this locus impinge on immune function. Do genetic variants alter the structure of the ncRNA or its regulation? Do they alter the expression of *ATP8B2 or IL6R* through alterations in their promoters? Do they alter the function of one or more of the enhancers? If so, what genes are dysregulated by altered enhancer function? If genetically-mediated dysregulation isn't exerted on peripheral blood cells, then where is it exerted?

These aren't either/or questions. That is, different genetic variants in different individuals might alter one or more of the genomic functions within the JIA haplotypes to different degrees. This might explain, for example, the considerable differences we see between individuals with JIA at both the phenotypic and gene expression levels. Indeed, we believe that one of the reasons why there is so much overlap in the genetic associations seen for a broad range of autoimmune/inflammatory diseases in the fact that these loci contain *multiple* important genomic elements which, if perturbed, could lead to an immune phenotype.

WHERE DO WE GO FROM HERE?

We are still rather in the dark as to the origin of the abnormal transcriptional signatures in JIA. It's clearly not as straightforward as we initially thought it might be: that polymorphisms in gene promoters, for example, would lead to alterations in gene expression that might be easily observed in cells or in serum protein levels. It should also be sufficiently clear to the reader that there is limited, if any, utility in trying to understand genetic mechanisms in JIA by focusing solely on the coding functions of the genes in close proximity to the SNPs identified on GWAS and genetic fine mapping studies. This "nearest gene" focus ignores the broader chromatin architecture in which the biologically relevant variants are likely to operate. We propose that future studies of individual JIA-associated loci consider not only the entire risk haplotype and the multiple genomic elements contained within it, but also broaden the inquiry to include three dimensional chromatin architecture and the genes included within the TADs that incorporate the risk haplotypes. This means that there is going to be a lot of work to do at each risk locus.

Enhancers seem to be the logical place to start, given their demonstrated importance in JIA (31, 32) and rheumatic diseases in general (45, 46). While the chromatin signatures within the JIA risk loci are strong indicators that these regions have enhancer function, the specific functional regions will need to be identified and verified empirically using reporter assays. Once the specific functional regions are identified, it will be a straightforward task to clarify the effects of genetic variants within these functional regions. Publically available data like the 1000 Genomes Project will give investigators a large but finite number of common genetic variants (allele frequencies >1%) to test within the defined regions. Our recent whole genome sequencing data from children with polyarticular, RF-negative JIA (47) have been made available to investigators through the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA343545) and will provide the scientific community with a rich list of rare variants to interrogate.

Once enhancer function is confirmed and the biologically-relevant genetic variants identified, we still have before us the task of identifying the genes (dys)regulated by the enhancers on which genetic variance operates. To accomplish this aim, our laboratory is taking advantage of the fact that most enhancers regulate genes within the same TAD. We are therefore using an epigenome editing approach (48) to attenuate enhancer function and identify genes whose expression is altered when specific enhancers are attenuated.

CONCLUSION

The identification of aberrant transcriptional patterns in the peripheral blood cells of children with JIA has opened the door to intriguing inquiries into the epigenetics and genetics of this family of diseases. With regard to the latter, our growing understanding of the structure and function of mammalian genomes makes it imperative that we broaden our investigations beyond the "nearest gene" to GWAS tag SNPs. Rather, developing a mechanistic understanding of how and where genetic variants alter transcription and immune function will require a complete understanding of the range of genomic functions altered by variants within the risk haplotypes. Furthermore, the field will require a detailed understanding of the larger chromatin "neighborhoods" within which each of the haplotypes resides. These are reachable goals that are most likely to be achieved by a focused and coordinated effort among the different pediatric rheumatology genetics research consortia to prioritize loci, share expertise and reagents, and develop plans for using genetic information to inform clinical care.

AUTHOR CONTRIBUTIONS

JJ developed the concept for the paper and assisted in the writing. HK assisted in the 3D chromatin analysis. KJ assisted in developing the concepts and generated data cited in this paper.

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REFERENCES

1. Stamatoyannopoulos J. What does our genome encode? *Genome Res.* (2012) 22:1602–11. doi: 10.1101/gr.146506.112
2. Jacquier A. The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs. *Nat Rev Genet*. (2009) 10:833–44. doi: 10.1038/nrg2683
3. Karpanov P, Willingham AT, Gingeras TR. Genome-wide transcription and implications for genomic organization. *Nat Rev Genet.* (2007) 8:413–23. doi: 10.1038/nrg2083
4. Maurano MT. Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, et al. Systematic localization of common disease association in regulatory DNA. *Science* (2013) 337:1190–5. doi: 10.1126/science.1222794
5. Hinks A, Cobb J, Marion MC, Prahalad S, Sudman M, Bowes JG, et al. Dense genotyping of immune-related disease regions identifies 14 new susceptibility loci for juvenile idiopathic arthritis. *Nat Genet.* (2013) 45:644–69. doi: 10.1038/ng.2614
6. Ombrello MJ. Arthur VL, Remmers EF, Hinks A, Tachmazidou I, Grom AA, et al. Genetic architecture distinguishes systemic juvenile idiopathic arthritis from other forms of juvenile idiopathic arthritis: clinical and therapeutic implications. *Ann Rheum Dis.* (2016) 76:906–13. doi: 10.1136/annrheumdis-2016-210324
7. Hinks A, Marion MC, Cobb J, Comeau ME, Sudman M, Ainsworth HC, et al. Brief report: the genetic profile of rheumatoid factor-positive polyarticular juvenile idiopathic arthritis resembles that of adult rheumatoid arthritis. *Arthritis Rheumatol.* (2018) 70:957–62. doi: 10.1002/art.40443
8. Jiang K, Sawle AD, Frank MB, Chen Y, Wallace CA, Jarvis JN. (2014). Whole blood gene expression profiling predicts therapeutic response in polyarticular juvenile idiopathic arthritis at 6 months. *Arthritis Rheumatol.* (2014) 66:1363–71. doi: 10.1002/art.38341
9. Griffin TA. Barnes MG, Ilowite NT, Olson JC, Sherry DD, Gottlieb BS, et al. Gene expression signatures in polyarticular juvenile idiopathic arthritis demonstrate disease heterogeneity and offer molecular classification of disease subsets. *Arthritis Rheumatol.* (2009) 60:2113–23. doi: 10.1002/art.24534
10. Eng SWM. Duong TT, Rosenberg AM, Morris Q, Yeung RSM. The biological basis of clinical heterogeneity in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2014) 66:3463–75. doi: 10.1002/art.38875
11. Du N, Jiang K, Sawle AD, Frank MB, Wallace CA, Zhang A, et al. Dynamic tracking of functional gene modules in treated juvenile idiopathic arthritis. *Genome Med.* (2015) 7:109. doi: 10.1186/s13073-015-0227-2
12. Hu Z, Jiang K, Frank MB, Chen Y, Jarvis JN. Transcriptional rewiring in neutrophils through the course of treated juvenile idiopathic arthritis. *Sci. Rep.* (2018) 8:7805. doi: 10.1038/s41598-018-26163-4
13. Jiang K, Frank MB, Chen Y, Osban J, Jarvis JN. Genomic characterization of remission in juvenile idiopathic arthritis. *Arthritis Res Ther.* (2013) 15:R100. doi: 10.1186/ar4280
14. Jiang K, Wong L, Sawle AD, Frank MB, Chen Y, Wallace CA, et al. Whole blood expression profiling from the TREAT trial: insights for the pathogenesis of polyarticular juvenile idiopathic arthritis. *Arthritis Res Ther.* (2016) 8:157. doi: 10.1186/s13075-016-1059-1
15. Jarvis JN, Dozmorov I, Jiang K, Frank MB, Szodoray P, Alex P, et al. Novel approaches to gene expression analysis of active polyarticular juvenile rheumatoid arthritis. *Arthritis Res Ther.* (2004) R15–32. doi: 10.1186/ar1018
16. Knowlton N, Jiang K, Frank MB, Aggarwal A, Wallace C, McKee R, et al. The meaning of clinical remission in polyarticular juvenile idiopathic arthritis: gene expression profiling in peripheral blood mononuclear cells identifies distinct disease states. *Arthritis Rheumatol.* (2009) 60:892–900. doi: 10.1002/art.24298
17. Jarvis JN. Jiang K, Frank MB, Knowlton N, Aggarwal A, Wallace CA, et al. Gene expression profiling in neutrophils of children with polyarticular juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2009) 60:1488–95. doi: 10.1002/art.24450
18. Wong L, Jiang K, Chen Y, Hennon T, Holmes L, Wallace CA, et al. Limits of peripheral blood mononuclear cells for gene expression-based biomarkers in juvenile idiopathic arthritis. *Sci Rep.* (2016) 6:29477 doi: 10.1038/srep29477
19. Jarvis JN, Petty HR, Tang Y, Frank MB, Tessier PA, Dozmorov I, et al. Evidence for chronic, peripheral activation of neutrophils in polyarticular juvenile rheumatoid arthritis. *Arthritis Res Ther.* (2006) 8: R154. doi: 10.1186/ar2048
20. Jarvis JN, Jiang K, Petty HR, Centola M. Neutrophils: the forgotten cell in JIA disease pathogenesis. *Pediatr Rheumatol.* (2007) 5:13. doi: 10.1186/1546-0096-5-13
21. Barnes MG, Grom AA, Thompson SD, Griffin TA, Pavlidis P, Itert L, et al. Subtype-specific gene expression profiles in recent-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2009) 60:2102–12. doi: 10.1002/art.24601
22. De Jaeger W, Hoppenreijs EP, Wulffrat, NM., Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis.* (2007) 66:589–98. doi: 10.1136/ard.2006.061853
23. Jarvis JN, Pousak T, Krenz M, Iodidze MV, Taylor H. Complement activation and immune complexes in juvenile rheumatoid arthritis. *J Rheumatol.* (1993) 20:114–7.
24. Thom AA, Moncrieffe H, Orandi AB, Pingel JT, Geurs TL, Miller HL, et al. Identification of enhanced IFN-γ signaling in polyarticular juvenile idiopathic arthritis with mass cytometry. *JCI Insight* (2018) 3:e121544. doi: 10.1172/jci.insight.121544
25. Favé MJ, Lamaze FC, Soave D, Hodgkinson A, Gauvin H, Bruat V, et al. Gene-by-environment interactions in urban populations modulate risk phenotypes. *Nat Comm.* (2018) 9:827. doi: 10.1038/s41467-018-03202-2
26. Hersh AO, Prahalad S. Immunogenetics of juvenile idiopathic arthritis: a comprehensive review. *J Autoimmun.* (2015) 64:113–24. doi: 10.1016/j.jaut.2015.08.002
27. Hersh AO, Prahalad S. Genetics of juvenile idiopathic arthritis. *Rheum Dis Clin N Am.* (2017) 43:435–48. doi: 10.1016/j.rdc.2017.04.007
28. McIntosh LA. Marion MC, Sudman M, Comeau ME, Becker ML, Bohnsack JF, et al. Genome-wide association meta-analysis reveals novel juvenile idiopathic arthritis susceptibility loci. *Arthritis Rheumatol.* (2017) 69:2222–32. doi: 10.1002/art.40216
29. McCarthy MI. Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JPA, et al. Genome-wide association studies for complex traits: consensus, uncertainty, challenges. *Nat Rev Genet.* (2008) 9:356–69. doi: 10.1038/nrg2344
30. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, Epstein CB, et al. Mapping and analysis of chromatin state dynamics in nine human cell lines. *Nature* (2011) 337:1190–5. doi: 10.1038/nature09906
31. Jiang K, Zhu L, Buck MJ, Chen Y, Carrier B, Liu T, et al. Disease-associated SNPs from non-coding regions in juvenile idiopathic arthritis are located within or adjacent to functional genomic elements of human neutrophils and CD4+ T cells. *Arthritis Rheumatol.* (2015) 67:1966–77. doi: 10.1002/art.39135
32. Zhu L, Jiang K, Webber K, Wong L, Liu T, Chen Y, et al. Chromatin landscapes and risk for juvenile idiopathic arthritis. *Arthritis Res Ther.* (2017) 19:57 doi: 10.1186/s13075-017-1260-x
33. Hui-Yuen JS, Zhu L, Wong L, Jiang K, Chen Y, Liu T, et al. Chromatin landscapes and genetic risk in systemic lupus. *Arthritis Res Ther.* (2016) 18:281. doi: 10.1186/s13075-016-1169-9
34. Shlyueva D, Stampfel G, Stark A. Transcriptional enhancers: from properties to genome-wide predictions. *Nat Rev Genet.* (2014) 15:272–86. doi: 10.1038/nrg3682

35. Ostuni R, Piccolo V, Barozzi I, Polletti S, Termanini A, Bonifacio S, et al. Latent enhancers activated by stimulation in differentiated cells. *Cell* (2013). 152:157–71. doi: 10.1016/j.cell.2012.12.018
36. Lupiáñez DG. Spielmann M, Mundlos S. Breaking TADs: how alterations of chromatin domains result in disease. *Trends Genet*. (2016) 32:225–37. doi: 10.1016/j.tig.2016.01.003
37. Hnisz D, Day DS, Young RA. Insulated neighborhoods: structural and functional units of mammalian gene control. *Cell* (2016) 167:1188–200. doi: 10.1016/j.cell.2016.10.024
38. Mishra A, Hawkins RD. Three-dimensional genome architecture and emerging technologies: looping in disease. *Genome Med*. (2017) 9:87. doi: 10.1186/s13073-017-0477-2
39. Roy SS, Mukherjee AK, Chowdhury S. Inisights about genome function from spatial organization of the genome. *Hum Genomics* (2018) 12:8. doi: 10.1186/s40246-018-0140-z
40. Belton JM, McCord RP, Gibcus JH, Naumova N, Zhan Y, Dekker. J. HiC: a comprehensive technique to capture the conformation of genomes. *Methods* (2012) 58:268–76. doi: 10.1016/j.ymeth.2012.05.001
41. Durand NC, Robinson JT, Shamim MS, Machol I, Mesirov JP, Lander ES, et al. Juicebox provides a visualization system for HiC contact maps with unlimited zoom. *Cell Systems* (2016) 3:99–101. doi: 10.1016/j.cels.2015.07.012
42. Poczobutt JM, De S, Yadav VK, Nguyen TT, Li H, Weiser-Evans MCM, et al. Expression profiling of macrophages reveals multiple populations with distinct biological roles in an immunocompetent orthotopic model of lung cancer. *J Immunol*. (2016) 196:2847–59. doi: 10.4049/jimmunol.1502364
43. Isoda T, Moore AJ, He Z, Chandra V, Aida M, Denholtz M, et al. Non-coding transcription instructs chromatin folding and compartmentalization to dictate enhancer-promoter communication and T cell fate. *Cell* (2017) 171:103–19.e18. doi: 10.1016/j.cell.2017.09.001
44. Chen YC, Satpathy AT, Chang HY. Gene regulation in the immune system by long non-coding RNAs. *Nat Immunol*. (2017) 18:962–72. doi: 10.1038/ni.3771
45. Fahr KK, Marson A, Kleinewietfeld M, Housely WJ, Beik S, Shoresh N, et al. Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* (2015) 518:337–43. doi: 10.1038/nature13835
46. Freudenberg J, Gregersen P, Li W. Enrichment of genetic variants for rheumatoid arthritis within T cell and NK cell enhancer regions. *Mol Med*. (2015) 21:180–4. doi: 10.2119/molmed.2014.00252
47. Wong L, Jiang K, Chen Y, Jarvis JN. Genetic insights into juvenile idiopathic arthritis derived from deep whole genome sequencing. *Sci Rep*. (2017) 7:2657. doi: 10.1038/s41598-017-02966-9
48. Klann TS, Black JB, Chellappan M, Safi A, Song L, Hilton IB, et al. CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome. *Nat Biotechnol*. (2017) 35:561–8. doi: 10.1038/nbt.3853

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REVIEW
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Systemic and Tissue Inflammation in Juvenile Dermatomyositis: From Pathogenesis to the Quest for Monitoring Tools

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Juvenile Dermatomyositis (JDM) is a systemic immune-mediated disease of childhood, characterized by muscle weakness, and a typical skin rash. Other organ systems and tissues such as the lungs, heart, and intestines can be involved, but may be under-evaluated. The inflammatory process in JDM is characterized by an interferon signature and infiltration of immune cells such as T cells and plasmacytoid dendritic cells into the affected tissues. Vasculopathy due to loss and dysfunction of endothelial cells as a result of the inflammation is thought to underlie the symptoms in most organs and tissues. JDM is a heterogeneous disease, and several disease phenotypes, each with a varying combination of affected tissues and organs, are linked to the presence of myositis autoantibodies. These autoantibodies have therefore been extensively studied as biomarkers for the disease phenotype and its associated prognosis. Next to identifying the JDM phenotype, monitoring of disease activity and disease-inflicted damage not only in muscle and skin, but also in other organs and tissues, is an important part of clinical follow-up, as these are key determinants for the long-term outcomes of patients. Various monitoring tools are currently available, among which clinical assessment, histopathological investigation of muscle and skin biopsies, and laboratory testing of blood for specific biomarkers. These investigations also give novel insights into the underlying immunological processes that drive inflammation in JDM and suggest a strong link between the interferon signature and vasculopathy. New tools are being developed in the quest for minimally invasive, but sensitive and specific diagnostic methods that correlate well with clinical symptoms or reflect local, low-grade inflammation. In this

review we will discuss the types of (extra)muscular tissue inflammation in JDM and their relation to vasculopathic changes, critically assess the available diagnostic methods including myositis autoantibodies and newly identified biomarkers, and reflect on the immunopathogenic implications of identified markers.

Keywords: juvenile dermatomyositis, tissue inflammation, vasculopathy, disease monitoring, biomarkers, interferon signature, autoantibodies, personalized medicine

INTRODUCTION

Juvenile Dermatomyositis (JDM) is a systemic immune-mediated disease of childhood. It is the most common idiopathic inflammatory myopathy in children, with an incidence of 2–4/million/year (1). Although the exact etiology is still elusive, both genetic and environmental factors are thought to play a role in the development of the disease (2–5). JDM is characterized by inflammation of skeletal muscles and skin, leading to muscle weakness and a typical skin rash of the face and hands (heliotrope rash and Gottron's papules, respectively), which are also used as classification criteria (6, 7). Next to the muscle and skin, other organs can be affected. Vital organ involvement, especially of the lungs, is still the major cause of death in JDM patients (8, 9). Although rare, cardiac involvement and microangiopathy of the intestine, brain and kidneys have been described (10). Thus, rather than being confined to specific tissues, JDM is a truly systemic disease, which can affect multiple organ systems.

Before the introduction of corticosteroids as a treatment option, mortality and morbidity among JDM patients were high, and long-term outcomes were not the primary focus. Since then, mortality rates have dropped from over 30% to 2–3% (11). With increasing survival, long-term outcomes become an important concern of patients and physicians, as patients' quality of life and societal participation depend on it. Long-term outcomes are likely dependent on various factors such as disease severity and activity, response to treatment and medication side effects which together determine the cumulative organ and tissue damage.

Especially low-grade inflammation and extramuscular manifestations of the disease are difficult to investigate in routine clinical care and may therefore be overlooked. Unrecognized, local inflammation leading to tissue damage and subsequent organ dysfunction may have serious consequences for short-term and long-term outcomes. So far, reliable assessment of disease activity and the type and extent of tissue involvement has been rather challenging. Current clinical tools for assessment of disease activity require active collaboration of patients, which can be difficult for young, unwell children. Detecting low-grade inflammation or differentiating clinically between various causes of muscle impairment is even more challenging. Hence, there is a great need for minimally invasive, objective and reliable diagnostic tools for the assessment and monitoring of (low-grade) disease activity and related organ involvement. Optimally, such tools could guide clinical decision making, facilitate individually tailored treatment regimens, and reduce the risk of over- and under-treatment.

In this review we will discuss the types of (extra)muscular tissue involvement that have been described in JDM and their relation to vasculopathic changes, critically assess the available diagnostic and monitoring tools and reflect on the immunopathogenic implications of identified markers.

SIGNS OF SYSTEMIC DISEASE ACTIVITY IN JDM BASED ON AFFECTED TISSUES AND ORGANS

JDM patients can present with a spectrum of symptoms. Most, but not all patients, have the classic combination of muscle involvement and typical skin rashes. Approximately 1–5% of JDM patients present with amyopathic JDM, but it was estimated that 26% of these patients will eventually progress to classical JDM, which can occur up to years after onset (12). This indicates that the phenotype can evolve over the course of the disease, possibly also dependent on treatment. True amyopathic JDM however is very rare and mild muscle involvement may be present but missed (13). Amyopathic JDM generally has a relatively mild disease course with fewer systemic manifestations, less required immunosuppressive treatment and a good prognosis (12, 14, 15).

(Sub)Cutaneous and Other Extramuscular Symptoms

Cutaneous symptoms can range from the pathognomonic heliotrope rash and Gottron's papules, to photosensitive rashes such as malar and truncal erythema, and severe complications such as skin ulceration and dystrophic calcinosis. Calcinosis occurs in 12–47% of patients and can occur in the skin and in subcutaneous, myofascial, or muscle tissue. Most often it is a long-term complication and its presence has been associated with delayed diagnosis and more severe disease with poorer functional outcomes. Effective treatment of calcinosis is still challenging, but aggressive high-dose immunosuppression or, in very severe cases, autologous stem cell transplantation have been shown to be able to reverse calcinosis, suggesting that chronic (low-grade) inflammation may be accountable for calcifications (16–20). Cutaneous and oral ulceration affects up to 30% of patients and is thought to result from occlusive endartheropathy of the small vessels (10, 21). Lipodystrophy affects 8–14% of JDM patients and is often associated with hormonal and metabolic changes (10, 22–24). We suspect that patients with lipodystrophy may therefore have an increased risk of cardiovascular events in the long-term. Limb edema and arthritis are also common, occurring in 11–32 and 23–58% of patients, respectively (10).

Next to the skin and musculoskeletal system, other organ systems can be involved, of which the lung is the most frequently affected. Up to 75% of children with JDM develop respiratory involvement, which may result from a complication of respiratory muscle weakness or immunosuppressive therapy, or from interstitial lung disease (ILD) (25, 26). ILD occurs in 8–19% of juvenile myositis patients and has been described as the major cause of death in JDM (27–30). Cardiac involvement may be present subclinically more often than recognized, as even in JDM patients without clinical cardiac dysfunction abnormal ECG and echocardiographic findings are relatively common (31–33). Conduction abnormalities and myocarditis have been reported, and systolic and diastolic dysfunction was found after long-term follow-up (34–37). Cardiac complications are thought to result from myocarditis and coronary artery disease as well as involvement of the small vessels of the myocardium (38). Involvement of the gut or neural system are rare complications of JDM and are also thought to result from an underlying small vessel angiopathy or vasculitis (39–41). Intestinal consequences of the small vessel angiopathy include ulceration, perforation, hemorrhage, pneumatosis intestinalis and malabsorption (42–44).

Vasculopathy

The pathologic changes underlying symptoms and tissue damage in the skin, muscles, and vital organs have a common factor: in all the affected tissues typical vasculopathic changes are observed, which include loss of capillaries (capillary dropout), perivascular inflammation, and (occlusive) small vessel angiopathy (21, 45). In a recently reported French JDM cohort of 116 patients, vasculopathy-related complications were the main cause of admission to the intensive care unit, illustrating the severity and relevance of vascular involvement in JDM (46). These complications include life-threatening disorders like systemic capillary leak syndrome, recently also described in 3 patients with JDM (47).

Deposition of complement, immune complexes and anti-endothelial antibodies is thought to play an important role in endothelial damage and subsequent capillary dropout (48–54). Clinically, the severity of vasculopathy and the disease phenotype have also been linked. The presence of prominent vascular injury in muscle biopsies identified a subgroup of patients with more severe clinical presentation and outcomes, including profound muscle weakness, limb edema and gastrointestinal involvement (55). This suggests that local vasculopathic changes can reflect systemic vasculopathy and the resulting clinical symptoms. Nailfold capillaroscopy, a commonly and easily used indicator of disease activity in clinical practice, is also based on this principle. The pathologic changes observed in nailfold capillaries, such as capillary dropout, branching and dilatation, likely reflect the systemic blood vessel abnormalities. Loss of end row nailfold capillaries is significantly associated with clinical disease activity scores for muscle and skin and can thus be used as a marker of skin and muscle activity. Nailfold capillaroscopy is especially suited as a non-invasive tool to follow up changes in disease activity over time in patients (56–59).

Taken together, JDM is a truly systemic disease in which not only the muscles and skin are affected, but also vital organs can be involved. The presence of typical vasculopathic changes in the various affected tissues points toward a central role for systemic endothelial dysfunction in the pathogenesis of JDM.

MONITORING OF DISEASE ACTIVITY AND TISSUE INVOLVEMENT

During clinical follow-up, monitoring of disease activity is crucial to determine the rate of medication tapering or to assess the requirement for intensification of immunosuppressive therapy. Next to clinical evaluation, various tools have been investigated for monitoring of disease activity, among which autoantibodies and other circulating biomarkers, and histopathologic evaluation of muscle biopsies, as well as several imaging techniques.

Clinical Assessment

The primary and most important evaluation of disease activity involves clinical assessment by experienced clinicians and health care professionals. Over the past years, several scoring tools have been devised for internationally standardized evaluation of disease activity (60). The most commonly used tools are now the childhood myositis assessment scale (CMAS), manual muscle testing of 8 muscle groups (MMT-8), physician's and patient's global assessment on a visual analog scale (PGA), cutaneous assessment tool (CAT), cutaneous dermatomyositis disease area and severity index (CDASI), disease activity score (DAS), myositis disease activity assessment tool (MDAAT) and childhood health assessment questionnaire (CHAQ) (61–69). Combined scoring systems are currently being developed (70). The Pediatric Rheumatology International Trials Organization (PRINTO) has composed criteria for defining clinically inactive disease (71). A recent re-evaluation of these PRINTO criteria showed that skin disease may be underestimated as a factor in the assessment of disease activity (72).

Clinical measures of disease activity, however, have limited capacity to detect low-grade inflammation in the tissues which does not cause overt symptoms, but may still contribute to tissue damage in the long term. Moreover, it is challenging to differentiate between various underlying causes of symptoms by clinical assessment. For example, muscle weakness may result from an ongoing inflammatory process, from medication side effects (e.g., steroid myopathy), muscle damage or effects of immobility. Biological assessment of the affected tissues and organs can therefore be helpful or even necessary to aid clinical decision-making concerning medication dose and additional interventions.

Biomarkers for Disease Course, Activity, and Tissue Involvement

Laboratory investigation of blood is a minimally invasive and time-efficient procedure, especially compared to muscle biopsy and some of the imaging methods. It is therefore particularly suited as a method for serial sampling during clinical follow-up. Laboratory investigation can be used for measurement of

autoantibodies and for biomarkers related to disease activity and specific (extra)muscular symptoms.

Autoantibodies

Antibodies found in myositis include myositis-specific autoantibodies (MSA), relatively specific to myositis, and myositis-associated antibodies (MAA), which are observed both in myositis and other connective tissue diseases (6). In the past years, different disease phenotypes have been linked to the presence of autoantibodies and particularly myositis-specific autoantibodies (16). The frequencies of autoantibodies in juvenile patients differ substantially from adult DM patients (73). Anti-TIF1 (p155/140) and anti-NXP2 (p140 or MJ) are the most commonly identified autoantibodies in Caucasian JDM patients (20–35 and 16–23%, respectively) (28, 73–76). Anti-TIF1 is associated with skin ulceration, photosensitive skin rashes, lipodystrophy, and edema (24, 75–78), whereas anti-NXP2 is associated with a severe disease course with more profound muscle involvement, calcinosis, gastrointestinal ulceration, joint contractures, and dysphonia (75, 77, 79, 80). A recently identified myositis specific autoantibody which is especially frequent in the Asian JDM population, is anti-MDA5 (CADM-140) (81). It is found in 33% of Asian JDM patients, compared to 7% of Caucasian patients (8, 82). Patients with anti-MDA5 have a higher risk of developing ILD than patients without these antibodies. This anti-MDA5 conferred risk is seen in both Asian and Caucasian JDM cohorts, although the risk difference appears to be more pronounced in Asian cohorts (8, 83). Common symptoms in Caucasian patients with anti-MDA5 antibodies include oral and cutaneous ulceration, arthritis, and milder muscle disease with fewer histologic abnormalities and a higher remission rate off medication after 2 years of follow-up (76, 82, 84, 85). Less frequently identified autoantibodies in the juvenile population include anti-Mi2 (4–10%) and anti-amino-acyl-tRNA synthetase antibodies such as anti-Jo-1 (1–3%) and anti-SAE (<1%). Anti-SRP and anti-HMG-CoA-reductase (Anti-HMGCR) autoantibodies, both accounting for <3% of juvenile myositis patients, are associated with a necrotizing type of myopathy with severe muscle weakness (73, 76, 86, 87).

It remains unclear whether each MSA reflects a distinct pathologic process, influencing the type and severity of disease phenotype and tissue involvement. Notably, autoantibodies against Jo-1, TIF1, SRP, and Mi-2 are not only informative at disease onset, but their levels have been found to correlate with disease activity during follow-up in the context of rituximab treatment (88). This highlights that perhaps autoantibodies should be measured during or soon after the first clinic visit as their levels may decline and become undetectable in remission.

A last and different (not myositis-specific) category of autoantibodies identified in JDM comprises autoantibodies against components of endothelial cells, which are thought to contribute to capillary loss. These anti-endothelial cell autoantibodies (AECA) were detected in 76% of JDM patients, as opposed to 30% of control patients (49). Twenty-two candidate target autoantigens for AECA were identified in JDM plasma, 17 of which were proteins associated with antigen processing and protein trafficking (50). Identification of autoantibody targets may provide novel insights into the auto-immune process and self-antigens involved in JDM.

Biomarkers for Systemic Inflammation and Muscle Disease Activity

Reliable assessment of disease activity during follow-up can be aided by laboratory markers that represent systemic and/or local inflammation. Especially for detection of low-grade inflammation and for differentiation between various causes of muscle weakness, laboratory investigation can be a helpful or even necessary tool.

So far, reliable and validated laboratory markers for disease activity and tissue involvement in JDM are still lacking. A large number of proteins in plasma, serum, and urine as well as circulating immune cell subsets have been investigated as potential biomarkers for (tissue-specific) disease activity in patients with JDM (**Tables 1**, **2**). In theory, every biological parameter that can be measured, could serve as a biomarker. To be suited for use in clinical practice however, a biomarker has to meet additional criteria, such as being reliable, robust, relatively stable and easy to measure. In the following paragraphs we highlight all biological markers that have been associated with disease activity in JDM, regardless of their suitability for use in clinical practice, as some of these identified markers may still contribute to the understanding of the immunopathogenesis of JDM. However, it is important to note that due to the rarity of the disease, many of these studies were carried out in small cohorts of <30 patients (as outlined in **Tables 1**, **2**). Insights based on such small numbers have limitations in a heterogeneous disease like JDM. Therefore, validation of identified markers in larger cohorts is crucial before implementation into clinical practice.

Currently used laboratory markers

The markers that are currently used in clinical practice, AST, ALT, LDH, aldolase and in particular creatine kinase activity (CK), do not correlate as well with disease activity in JDM as in DM (125–127). At diagnosis, any one muscle enzyme was only elevated in 80–86% of patients with JDM and CK was found to be elevated in only 61–64% of patients (125, 128). In almost 20% of patients the most abnormal measurement of CK was not elevated above normal values (28). Low muscle enzymes at first presentation may be associated with delayed diagnosis (129). During follow-up, CK may underestimate disease activity due to suppressed release by corticosteroids, circulating inhibitors of CK activity, or loss of muscle mass (127, 130–132). On the other hand, CK and aldolase can be elevated in steroid myopathy and are therefore not reliable as markers for disease activity requiring more potent immunosuppression (133). However, according to recent consensus guidelines, these muscle enzymes are still regarded as an important monitoring tool (134, 135).

Markers related to the interferon signature

An important group of investigated biomarkers is related to the type 1 interferon (IFN) signature, which has been demonstrated in the peripheral blood and muscle biopsies of JDM patients (136, 137). Activated plasmacytoid dendritic cells (pDC) are generally thought to be the main producers of the type 1 IFNs

TABLE 1 | Biomarkers for disease activity in JDM cohorts.

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients	Material & technique	Cohort	References
Interferon related biomarkers							
MxA		++++** (DAS, O), +++* (DAS, FU)	NS (skin DAS)	14 act JDM: 7 untreated, 7 treated	PBMC, qRT-PCR	USA	O'Connor (89)
IFNα activity	###* (DAS, off therapy at 36 months)	NS (DAS)	###** (skin DAS, off therapy at 36 months), ** (vsHC)	39 JDM and 19 ped HC	Serum, Functional reporter assay	USA	Niewold (90)
IFN gene score	r_p: NS (DAS)	r_p: NS (DAS)		27 JDM	Whole blood, qRT-PCR	USA	Baechler (91)
IFN chemokine score	r_p: ++** (DAS)	r_p: ++* (DAS)		29 JDM			
Eotaxin	+** (DAS)	NS (DAS)	+** (skin DAS), * (vsHC)	54 JDM	Serum, Luminex	Norway	Sanner (92)
MCP-1	+* (DAS)	NS (DAS)	NS (skin DAS), ** (vsHC)	54 age+sex matched controls			
IP-10	NS (DAS)	NS (DAS)	NS (skin DAS), * (vsHC)	Median time 16.8 yrs after onset			
IP-10	+++*** (PGA)	###*** (CMAS)		2014: 25 JDM (18 act, 19 rem), 14 ped HC, 8 NIMD	Plasma and serum, Luminex	NL	Enders (93, 94)
TNFR2	+++*** (PGA)	NS (CMAS)					
Galectin-9	+++*** (PGA)	##** (CMAS)		2015: 3 refractory JDM (pre and post aSCT)			
Soluble IL-2R	** (diagnosis vs. rem)			7 JDM: 7 at diagnosis, 7 in rem	Serum, ELISA & HPLC	Japan	Kobayashi (95)
Neopterin	Higher in act than rem						
Neopterin		+++*** (act), ++++* (FU 3 pts) (both with study-specific DAS)		15 JDM (21 samples: 12 act, 9 rem)	Serum, radioimmunoassay	Italy	De Benedetti (96)
Urine neopterin	++** (PGA)	###* (MMT), ##** (CMAS)	++** (skin VAS), ++** (CHAQ), ++** (MRI)	39 JDM, 3 JDM with overlap CTD, 3 JPM	Urine and plasma, ELISA, HPLC, gas chromatographic mass spectrometry	USA	Rider (97)
Urine quinolonic acid	++** (PGA)	###* (MMT), ##*** (CMAS)	++*** (CHAQ), ++** (MRI)				
Plasma neopterin/quinolonic acid	NS (PGA)	NS (CMAS, MMT)					
OTHER MARKERS OF INFLAMMATION							
MRP8/14	+++*** (PGA)	##** (CMAS)	NS (CHAQ)	56 JDM	Serum, ELISA	UK	Nistala (98)

(Continued)

TABLE 1 | Continued

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients	Material & technique	Cohort	References
CRP			Low during relapse in 4 patients	9 JDM: 4 during relapse and 3 rem	Serum	UK	Haas (99)
IMMUNE CELL SUBSETS							
Changes in %CD19+ cells	+ + * (DAS)				PBMC, flow cytometry	USA	Eisenstein (100)
T cell subsets	NS (DAS)						
T cell activation (CD25, HLA-DR)	NS (DAS)						
T cell recognition of human Hsp60	Higher in rem than act			22 JDM: 6 new-onset, 6 act, 10 rem	PBMC, ^{3}H-thymidine assay	NL	Elst (101)
Th1 within CXCR5+ CD4 T cells	Higher in rem than act***			45 JDM (52 samples): 26 act, 26 rem, 43 ped HC	PBMC, Flow cytometry	USA	Morita (102)
Ratio (Th2+Th17)/Th1 in CXCR5+ CD4 T cells	Higher in act than rem***						
% Plasmablasts (CD19+CD20–CD27+CD38++)	Higher in act than rem***						
Change in % CD3+CD69+ T cells	+ + *			24 JDM	PBMC, Flow cytometry	USA	Ernste (103)
Change in HLA-DR- CD11c+ mDC			+ + * (extra)				
Change in HLA-DR- CD123+ pDC		##*					
% FOXP3+ Tregs		NS (CMAS)		48 JDM: 21 act, 27 rem	Muscle biopsies, immunohistochemistry	NL	Vercoulen (104)
Defective suppressive function of Tregs	In 4/11 active pts vs. 0/9 in remission				Flow cytometry, ^{3}H-thymidine incorporation		
RORC	*	*	NS (extra)	26 JDM new-onset	Whole blood, qRT-PCR	USA	Lopez de Padilla (105)
IL-17F	NS	*	NS (extra)				
GATA3	NS	*	NS (extra)				
STAT4	***	***	NS (extra)				
Changes in STAT6	NS	NS	* (extra)				
Changes in IL-17D	NS	NS	** (extra)				
Changes in BCL6	NS	NS	** (extra)				
% Immature transitional B cells	+ + +*** (PGA)			68 JDM (113 samples): 20 pre-treatment, 93 on treatment	PBMC, Flow cytometry	UK	Piper (106)
Absolute number immature transitional B cells	+ + +*** (PGA)						

(Continued)

TABLE 1 | Continued

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients	Material & technique	Cohort	References
MARKERS RELATED TO ENDOTHELIAL ACTIVATION OR DYSFUNCTION							
vWF			Sens 0.85; Spec 0.45 (for flare)	16 JDM, prospective	Serum	CA	Guzman (107)
vWF	Sens 0.40 (6/15 act had high vWF)	NS (muscle strength)	NS (skin rash, calcinosis)	15 JDM	Serum	USA	Bloom (108)
C3d	Elevated in 6/7 pts with act			15 JDM: 7 act, 5 mild disease, 3 rem, 15 ped HC	Plasma, radioimmuno assay and rocket immuno-elektrophoresis	USA	Scott (109)
Fibrinopeptide A			* (vsHC)				
Factor VIII-related antigen			** (vsHC)				
MiRNA-10a	NS (DAS)	NS (DAS)	NS (skin DAS)	15 untreated JDM	Muscle boipsies, RT-PCR	USA	Xu (110)
EPC number		NS (DAS)	NS (skin DAS)	34 JDM: 6 untreated, 19 act on med, 9 rem	PBMC, Flow cytometry	USA	Xu (111)
LIPID METABOLISM							
HDL	NS (PGA)	++* (CMAS), NS (MMT)	NS (Skin, CHAQ)	16 JDM, 1 JPM	Serum	USA	Coyle (112)
LDL	r_p: ++* (DAS)	r_p: ##* (CMAS)	r_p: ++** (MYOACT)	25 JDM	Serum	Brazil	Kozu (113)
Triglycerides	All r_p: +++** (DAS), ++** (MITAX)	All r_p: ##** (CMAS), ##**(MMT)	r_p: +++** (MYOACT)				
IL-6	NS	#*	NS (extra)	26 JDM	Whole blood, qRT-PCR	USA	Olazagasti (114)
Resistin	++***	++***	+* (extra)				

*Spearman correlations (r_s) of biomarkers with global/muscle/skin/extraskeletal VAS are shown unless otherwise specified. +:$r_s \geq 0.2$, ++:$r_s \geq 0.4$, +++:$r_s \geq 0.6$, ++++:$r_s \geq 0.8$, #:$r_s \leq -0.2$, ##:$r_s \leq -0.4$, ###:$r_s \leq -0.6$, ####:$r_s \leq -0.8$. r_p: Pearson correlation. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant. Sens, sensitivity; Spec, specificity. Abbreviations biomarkers: IFN, interferon; MCP-1, CCL2; IP-10, CXCL10; TNFR2, Tumor necrosis factor receptor 2; IL-2R, Interleukin-2 receptor; MRP8/14, myeloid related protein 8/14 (S100A8/9); CRP, C-reactive protein; Hsp60, heat shock protein 60; Th, T helper; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; Treg, Regulatory T cell; IL, interleukin; vWF, von Willebrand factor; EPC, endothelial progenitor cell; HDL, high density lipoprotein; 25(OH)D, Vitamin D. Abbreviations disease activity: DAS, disease activity score; VAS, visual analog scale; PGA, physician's global activity VAS; MyoAct, Myositis disease activity assessment visual analog scales; MITAX, myositis intention to treat activity index; MMT, manual muscle testing; CMAS, childhood myositis assessment scale; vsHC, compared to healthy controls; CHAQ, childhood healthy assessment questionnaire; MRI, magnetic resonance imaging; ANA, anti-nuclear antibody; extra, extraskeletal/extramuscular symptoms. Abbreviations patients: JDM, juvenile dermatomyositis; JPM, juvenile polymyositis; HC, healthy control; ped, pediatric; act, active; rem, remission/asymptomatic/inactive disease; yrs, years; NIMD, non-inflammatory muscle disease; aSCT, autologous stem cell transplantation; CTD, connective tissue disease; O, onset of disease; FU, follow-up. Abbreviations material & technique: ELISA, enzyme-linked immuno sorbent assay; HPLC, high-performance liquid chromatography; PBMC, peripheral blood mononuclear cells; qRT-PCR, quantitative real time polymerase chain reaction.*

TABLE 2 | Biomarkers for disease activity in mixed cohorts containing patients with JDM.

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients (samples)	Material & technique	Cohort	References
Interferon related biomarkers							
Type I IFN signature	NS			2 JDM, 10 DM, 15 ad HC	Whole blood, microarray	USA	Baechler (115)
IP-10	NS				Serum, Luminex		
ITAC	NS						
MCP-1	++*						
MCP-2	+++**						
IFN gene score	++**	++** (VAS), ##** (MMT)	++** (skin), +* (extra)	19 JDM, 37 DM, 20 ad HC	Whole blood, qRT-PCR	USA	Bilgic (116)
IP-10	+++****				Serum, multiplexed sandwich immunoassay		
I-TAC	+++****						
MCP-1	++***						
MCP-2	++***						
MIP-1α	++***						
IL-6	++***	+** (VAS), ##** (MMT)	+* (skin), ++** (extra)				
IL-10	+*						
TNFα	+*						
TNFR1	+*						
MIG, MIP-1β, IL-8	NS						
Type I IFN chemokine score (Summarization of ITAC, IP-10, MCP-1, and MCP-2)	++* (JDM), +++**** (mixed)	++*** (VAS, mixed), ##** (MMT, mixed)	+* (skin), ++** (extra)				
Type I IFN gene score	+*	++**	NS (extra)	21 JDM (each active and remission sample), 30 DM (each active and remission sample)	Whole blood, qRT-PCR	USA	Reed (117)
Type I IFN chemokine score	++***	++***	++*** (extra)		Serum, multiplexed sandwich immunoassay		
IP-10	++***	++**	++*** (extra)				
ITAC	++**	++**	++** (extra)				
MCP-1	++**	++**	++** (extra)				
MCP-2	NS	++***	NS (extra)				
IL-6	++***	++***	+* (extra)				
IL-8	+*	+*	NS (extra)				
TNFα	+**	++***	+* (extra)				

(Continued)

TABLE 2 | Continued

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients (samples)	Material & technique	Cohort	References
OTHER MARKERS OF INFLAMMATION							
IL-1Ra	High in act than rem	++* (CK)	*** (vsHC)	2 JDM, 5 DM, 2 caDM, 4 PM, 2 OM, 12 HC	Serum, ELISA	Switzerland	Gabay (118)
sTNFR75 (sTNFR2)		++* (CK)					
BAFF		+*** (CK, mixed)		49 DM (of which ≥1 JDM), 44 PM, 6 IBM, 30 HC	Serum, ELISA	Sweden & Czech Republic	Krystufkova (119)
BAFF	+*** (mixed)	+* (mixed)	+*** (extra, mixed)	20 JDM, 45 DM, 26 PM, 7 IBM, 21 HC	PBMC, qRT-PCR	USA	Lopez de Padilla (120)
ΔBAFF (downregulates BAFF activity)	++*** (mixed)	+** (mixed)	++*** (extra, mixed)				
Anti-Jo1	LMM: ++***	LMM: ##*** (MMT), +++*** (ME)	LMM: +** (extra), ++*** (HAQ)	Refractory pts: 48 JDM, 76 DM, 76 PM (all analyses in mixed cohort)	Serum, ELISA & RNA and protein immunoprecipitation	USA	Aggarwal (88)
Anti-TIF1γ	LMM: ++**	LMM: ###*** (MMT), NS (ME)	LMM: +++*** (HAQ), NS (extra)				
Anti-SRP	LMM: NS	LMM: NS (MMT), +** (ME)	LMM: NS (extra, HAQ)				
Anti-Mi2	LMM: +++***	LMM: ##*** (MMT), ++** (ME)	LMM: +* (extra), NS (HAQ)				
IMMUNE CELL SUBSETS							
% CD3+ cells	Higher in rem than act* (DM, not JDM)			14 JDM, 24 DM, 17 ad HC, 9 ped HC	PBMC, Flow cytometry	Japan	Ishida (121)
% CD8+ cells	Higher in rem than act* (DM, not JDM)						
% CD20+ cells	Higher in act than rem* (DM, not JDM)						
% CD3+ cells	Higher in rem than act* (DM)			29 DM act (of which ≥1 JDM), 20 DM rem, 13 PM act, 37 PM rem, 32 ad HC	PBMC, Flow cytometry	Hungary	Aleksza (122)
% CD8+ cells	Higher in rem than act* (DM)						
% IFNγ+ of CD4 T cells	Higher in rem than act** (DM)						
% IFNγ+ of CD8 T cells	Higher in rem than act** (DM)						
% CD19+ cells	Higher in act than rem* (DM)						
% IL-4+ of CD4+ T cells	Higher in act than rem* (DM)						

(Continued)

TABLE 2 | Continued

Biomarker	Global disease activity	Muscle disease activity	Other activity measures	Patients (samples)	Material & technique	Cohort	References
MARKERS RELATED TO ENDOTHELIAL ACTIVATION OR DYSFUNCTION							
sVCAM-1		NS (CK, mixed)	NS (skin, mixed)	5 JDM, 27 DM, 4 PM, 25 HC	Serum, ELISA	Japan	Kubo (123)
sE-selectin		* (CK, mixed)					
sICAM	Higher in act than rem* (mixed)			4 JDM, 2 ped MCTD, 8 ped SLE, 4 ped Vasculitis	Serum, ELISA	USA	Bloom (124)
sICAM-3, sVCAM-1, sL-selectin, sE-selectin	NS (mixed)						

*Spearman correlations (r_S) of biomarkers with global/muscle/skin/extraskeletal VAS are shown unless otherwise specified. +-:$r_S \geq 0.2$, ++-:$r_S \geq 0.4$, +++-:$r_S \geq 0.6$, ++++-:$r_S \geq 0.8$, #:$r_S \leq -0.2$, ##:$r_S \leq -0.4$, ###:$r_S \leq -0.6$, ####:$r_S \leq -0.8$. r_p: Pearson correlation, LMM, linear mixed model. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, NS, not significant. Abbreviations biomarkers: IFN, interferon; MCP-1, CCL2; MCP-2, CCL8; IP-10, CXCL10; ITAC, CXCL11; MIP-1α, CCL3; IL, interleukin; TNF, Tumor necrosis factor; TNFR1/2= TNF receptor 1/2, MIG, CXCL9; MIP-1β, CCL4; IL-1Ra, IL-1 receptor alpha; BAFF, B cell activating factor. Abbreviations disease activity: CK, creatine kinase; ME, muscle enzymes; VAS, visual analog scale; MMT, manual muscle testing; HAQ, health assessment questionnaire; extra, extraskeletal/extramuscular symptoms. Abbreviations patients: JDM juvenile dermatomyositis; JPM, juvenile polymyositis; DM, adult dermatomyositis; caDM, cancer-associated DM; PM, adult polymyositis; OM, overlap myositis; IBM, inclusion body myositis; SPA, spondylartropathy; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; MCTD, mixed connective tissue disease; HC, healthy control; ped, pediatric; ad, adult; act, active; rem, remission/asymptomatic/inactive disease. Abbreviations material & technique: ELISA, enzyme-linked immuno sorbent assay; PBMC, peripheral blood mononuclear cells; qRT-PCR, quantitative real time polymerase chain reaction.*

(IFNα and IFNβ) in JDM. This notion may be challenged by a recent study measuring circulating IFNα with a highly sensitive assay and investigating the cellular source of IFNα in several systemic inflammatory diseases. JDM patients had higher levels of circulating IFNα than patients with systemic lupus erythematosus (SLE), but lower levels than patients with monogenic interferonopathies. However, neither isolated circulating pDC nor other circulating immune cell subsets from JDM patients expressed more IFNα than cells from healthy controls, suggesting that a non-circulating cellular source may be responsible for IFNα production in JDM (138).

Due to the lack of available methods to measure circulating IFNα and IFNβ until recently, the type 1 IFN signature, consisting of genes upregulated in response to IFNα or IFNβ stimulation, was used as a surrogate marker of type 1 IFN levels. The type 1 IFN signature in whole blood of three mixed DM and JDM cohorts correlated weakly to moderately with global disease activity [spearman r (r_s) = 0.33–0.44] and muscle activity (r_s = 0.44–0.47), while single IFN signature related serum chemokines MCP-1, IP-10 (CXCL10) and ITAC (CXCL11) had moderate to strong correlations with global (r_s = 0.42–0.66), muscle (r_s = 0.44–0.50), and extraskeletal disease activity (r_s = 0.42–0.55) (115–117). MxA expression in PBMC, also used as a surrogate for the IFN signature, had a very strong correlation with muscle disease activity of JDM patients (r_s = 0.80) at disease onset, but not with skin disease activity (89). IFNα activity measured by a functional reporter assay was also higher in JDM patients than controls (90).

Recently, IP-10, TNF receptor 2 (TNFR2) and galectin-9 were found to strongly correlate with global disease activity (r = 0.60–0.75) in two studies by Enders et al. (93, 94) IP-10, together with MCP-1 and eotaxin, was also higher in 54 JDM patients a median of 17 years after disease onset than matched healthy controls (92). TNFR2 correlated with CK in a mixed IIM cohort (r_s = 0.55) (118). Galectin-9 was recently identified as a biomarker for the IFN signature in SLE and anti-phospholipid syndrome (139). IP-10, TNFR2 and galectin-9 are promising biomarkers for disease activity, as they can potently discriminate between active disease and remission even during treatment (93, 94). After stem cell transplantation and concomitant eradication of circulating immune cells, their levels stayed high over several months, which suggests that these proteins are not primarily produced by circulating immune cells, but rather by non-circulating immune or tissue cells, just as IFNα (94, 138). IP-10 and galectin-9 are currently being validated as biomarkers for disease activity in two large international JDM cohorts.

One of the best investigated biomarkers so far in JDM is neopterin, a catabolic product of guanosine triphosphate, which was previously shown to be a marker of immune activation that can be induced by stimulation with IFNγ (140). In the first study identifying serum neopterin as a biomarker for JDM, neopterin levels correlated strongly with muscle strength impairment in 15 JDM patients (r_s = 0.68) (96). Elevated serum neopterin levels at diagnosis compared to remission were confirmed in an independent cohort (95). In a juvenile myositis validation cohort, plasma neopterin (n = 13), and quinolonic acid (n = 24), however, did not correlate with myositis disease activity measures

(97). Urine neopterin ($n = 45$) moderately correlated with global ($r_s = 0.42$), muscle ($r_s = 0.50–0.62$) and skin activity ($r_s = 0.49$), and edema on MRI ($r_s = 0.55$). Urine quinolonic acid also correlated with global and muscle activity and edema on MRI ($r_s = 0.45–0.61$) (97). Despite these efforts of validation, neopterin has not been widely implemented into clinical practice as a biomarker for disease activity in JDM.

Other inflammatory mediators

Next to type 1 IFN-related markers, other inflammatory mediators have been studied as biomarkers for JDM. The innate TLR4 ligand myeloid related protein 8/14 (MRP8/14 or S100A8/9), originally found to be elevated in patients with systemic-onset juvenile idiopathic arthritis (JIA), correlated moderately to strongly with global and muscle disease activity in a large cohort of 56 JDM patients ($r_s = 0.55–0.65$) (98, 141). Another marker adopted from studies in JIA, the soluble IL-2 receptor, was elevated at disease onset compared to remission (95, 142). Serum/plasma levels of the more conventional pro-inflammatory cytokines IL-6, IL-8, and TNFα also moderately correlated with global ($r_s = 0.19–0.46$) and muscle disease activity ($r_s = 0.35–0.52$) in three mixed JDM and DM cohorts (116, 117). Remarkably, CRP levels did not increase during disease flares (99). BAFF and especially its antagonistic non-cleavable form ΔBAFF, both important for survival and maturation of B cells, moderately correlated with global, muscle and extraskeletal VAS ($r_s = 0.27–0.54$), and CK ($r_s = 0.37$) in two mixed IIM cohorts (119, 120).

Markers related to vasculopathy and cardiovascular risk

Due to the vasculopathic component of JDM, markers related to endothelial activation and dysfunction were explored for their association with disease activity. Von Willebrand factor (vWF) was increased during most periods of active disease in a prospective cohort study, but did not reliably predict disease flares in another study (107, 108). sICAM-1, a marker of endothelial activation, was higher during active disease than remission in a combined cohort of juvenile patients with various systemic autoimmune diseases. VCAM-1, sICAM-3, and L-selectin did not correlate with disease activity, although expression of MiRNA-10a in JDM muscle, which is negatively associated with VCAM-1 expression, showed a correlative trend with muscle and global DAS [Pearson r (r_p) $= -0.45$] (110, 123, 124). C3d and fibrinopeptide A, which are related to vasculopathic changes, were higher in JDM patients with active disease than in remission (109). Endothelial progenitor cell numbers did not differ between JDM patients and controls and did not correlate with disease activity (111).

In view of the increased cardiovascular risk in JDM patients, the lipid profile has been investigated in relation to disease activity (41). Serum HDL negatively correlated with muscle activity ($r_s = -0.54$), but not global or skin activity (112). Triglyceride levels correlated strongly with global disease activity assessed by DAS ($r_s = 0.61$) and LDL was higher in patients with a higher disease activity (113). Gene expression of the adipokine resistin in PBMC was also upregulated in JDM patients compared to controls and moderately correlated with global and muscle disease activity ($r_s = 0.51$ and $r_s = 0.50$, respectively) (114). These results indicate that the cardiovascular risk profile is more pronounced in JDM patients with active disease.

Circulating immune cell subsets as biomarkers for disease activity

Among the circulating immune cell subsets, T cells and B cells have been studied most extensively in relation to disease activity in JDM. In two mixed cohorts of JDM and DM patients, the frequency of T cells, and especially CD8+ and IFNγ-producing T cells, was decreased during active disease, while the frequency of B cells and IL-4 producing CD4+ T cells was increased compared to remission (121, 122). This may suggest a shifted balance toward a T helper 2 (Th2) type immune response. In cohorts with only JDM patients, total B cell numbers were also increased compared to controls and changes in B cell frequencies accompanied changes in disease activity ($r_s = 0.47$) (100, 106). Within the B cell compartment, numbers and frequencies of circulating immature transitional B cells correlated strongly with global disease activity ($r_s = 0.69–0.71$). Compared to healthy pediatric controls, these specialized B cells were highly proliferative, had a prominent IFN signature and produced less of their regulatory signature cytokine IL-10 (106). Plasmablast frequencies were also increased during active disease compared to remission (102).

Several T cell subsets have been studied in JDM. In 26 new-onset JDM patients the blood gene expression of Th17-related genes, such as RORC and IL-17F, Th1-related genes, including STAT4, and Th2-related genes, including GATA3 and STAT6, was studied in relation to disease activity. RORC, IL-17F, STAT4, and GATA3 positively correlated with muscle activity and RORC and STAT4 correlated with global activity. This would suggest that the immune response is not specifically skewed toward a certain T helper response. However, at baseline, JDM patients had higher gene expression of Th17 related cytokines IL-23, IL-17F, IL-6, and IL-21 than DM patients, indicating that the Th17 pathway may play a more prominent role in the pathogenesis of JDM than DM. Changes in BCL6, a transcription factor for follicular helper T cells, correlated negatively with a change in extramuscular activity (105). Within CXCR5+ follicular helper T cells, the Th1 subset was decreased in active JDM compared to remission and controls, and Th2 and Th17 subsets were increased in JDM compared to controls (102). Regulatory T cell frequencies in muscle biopsies did not correlate with muscle activity, but suppressive activity of circulating Tregs may be impaired during active disease (104). Finally, global disease activity correlated moderately with the activation status of circulating T cells assessed by CD69 expression ($r_s = 0.43$), but not with CD25 and HLA-DR expression (100, 103). The expansion and functional alteration of particular B cell and CD4+ T cell subsets, coinciding with changes in disease activity, hints toward the involvement of these cell subsets in the pathogenesis of JDM.

In conclusion, many circulating, either soluble or cellular, markers have been studied for their relation with muscle and global disease activity. Correlations with disease activity were only moderate for most markers, and some of these molecules are relatively unstable in blood samples or complicated to

measure, rendering them unsuited for use in clinical practice. The highest correlations with disease activity were found for markers related to the IFN signature, the lipid profile, for MRP8/14, and immature transitional B cells. However, most of these biomarkers were identified in small patient cohorts and except for neopterin, so far none have been reproduced or thoroughly validated in independent and large JDM cohorts. Neopterin was investigated in a validation cohort, but its correlation with disease activity could only be confirmed in urine, not in plasma. Galectin-9 and IP-10 are currently being validated in two international cohorts and are promising biomarkers for implementation in clinical practice due to their high sensitivity and stability in serum.

Biomarkers for Extramuscular Disease Activity

Next to markers for global and muscle disease activity, biomarkers for involvement of specific tissues and organs have been investigated. Four studies by Kobayashi et al. have focused on biomarkers for ILD, and specifically the rapid progressive (RP-ILD) and chronic ILD type, in a Japanese JDM cohort. Not only the presence, but also the level of anti-MDA5 was a sensitive and specific marker for ILD, with the highest levels found in patients with RP-ILD (8, 143, 144). In addition, BAFF, APRIL, KL-6, and IL-18 levels were higher in patients with RP-ILD compared to chronic ILD and JDM patients without ILD (145). KL-6 was prognostic for ILD, as it stayed high in patients with persistent damage on HRCT (144). Biomarkers for cardiac involvement were tested in a Norwegian JDM cohort, a median of 17 years after diagnosis. Eotaxin and MCP-1 were elevated in patients with cardiac dysfunction and correlated moderately to strongly with systolic and diastolic dysfunction especially in patients with persistently active disease (r_s = 0.45–0.65) (146). In the same cohort, a reduced heart rate variability, which is an indicator of cardiac disease, correlated moderately with ESR, hsCRP, and also MCP-1 and eotaxin levels (r_s = 0.29–0.47) (147). Next to the autoantibody NXP2, which is prognostic for the development of calcinosis, phosphorylated matrix Gla protein was shown to be higher in patients with calcinosis than without calcinosis (79, 148). Reduced osteocalcin levels were found to be predictive of reduced bone mass, even before start of steroids (149). The presence of the TNFα-308A allele is associated with a more severe disease in JDM. However, apparent associations with this allele are likely to reflect the association with ancestral haplotype 8.1 due to linkage disequilibrium and should be interpreted with this in mind (150). Patients with this genotype are reported to show prolonged symptoms requiring ≥36 months of immunosuppressive therapy, a higher incidence of pathologic calcifications, increased production of TNFα by peripheral blood mononuclear cells *in vitro* and JDM muscle fibers *in vivo*, a higher IFNα activity and a higher rate of complications arising from occlusion of capillaries. Vascular occlusion has been linked to higher levels of the anti-angiogenic thrombospondin-1 (90, 151–154). In summary, a number of potential biomarkers for extramuscular disease activity has been identified, and especially for ILD and cardiac dysfunction the biomarkers seem promising. Validation in independent cohorts will have to confirm their potential as biomarkers for these extramuscular symptoms.

Histopathology of Muscle and Skin Biopsies

The diagnostic criteria for JDM by Peter and Bohan encompass histopathological findings consistent with DM: "necrosis of myofibers, phagocytosis, regeneration with basophils, large vesicular sarcolemmal nuclei, and prominent nucleoli, atrophy in a perifascicular distribution, variation in fiber size and an inflammatory exudate, often perivascular" (155, 156). For a long time, muscle biopsies were therefore taken as part of routine diagnostic workup. However, with evolving diagnostic options and more specialized trained pediatric rheumatologists muscle biopsies are currently not always considered a necessity for diagnosis (135).

One of the main problems hindering standardized evaluation of muscle biopsies was the lack of an internationally agreed upon scoring tool. An international consensus group of pediatric rheumatologists and pathologists developed such a tool, which encompasses 4 histopathological scoring domains: inflammatory, vascular, muscle fiber and connective tissue changes (157). The scoring tool has now been validated in an independent cohort consisting of 55 patients and was found to correlate with clinical measures of disease activity, including CMAS, PGA, and MMT-8 (r_s = 0.40–0.62) (45). Muscle biopsy scores may also have prognostic potential: in combination with MSA group, these scores were found to predict the risk of remaining on treatment over time, based on analysis of muscle biopsies from 101 JDM patients (158).

The most common findings in muscle biopsy specimens in JDM compared to healthy individuals or patients with non-inflammatory muscle diseases, are profound upregulation of MHC I expression on muscle fibers, increased expression of integrins and complement and membrane attack complex deposition on capillaries and perimysial large vessels, a type 1 IFN signature and immune cell infiltrates consisting mostly of mature pDC, memory CD4+ T cells, and B cells (48, 52, 159–169). (**Figure 1**) The IFN signature, measured by expression of MxA, correlated with muscle disease activity (166). In skin biopsies similar features are found, with the additional presence of diffuse mast cell infiltration (164).

Several studies have suggested associations between histopathological findings in muscle biopsies and disease duration before the biopsy or disease severity at a later time point. Biopsy specimens taken after a short duration of untreated disease (<2 months), showed higher expression of VCAM-1 (which correlated with higher serum soluble VCAM-1) and expression of genes involved in stress response and protein turnover, whereas biopsies taken after more than 2 months of untreated disease had more pDC infiltration, higher expression of genes involved in the immune response and vascular remodeling and more apoptosis-related markers (171–173). Thus, it should be taken into account that histological findings can depend on the disease duration before the biopsy. In addition, these findings may indicate that endothelial activation is an early feature of JDM, which precedes immune cell infiltration and vasculopathy.

The degree of vasculopathy and vascular injury (as defined by marked capillary dropout, increased direct immunofluorescent arterial staining and lymphocytic vasculitis, amongst others)

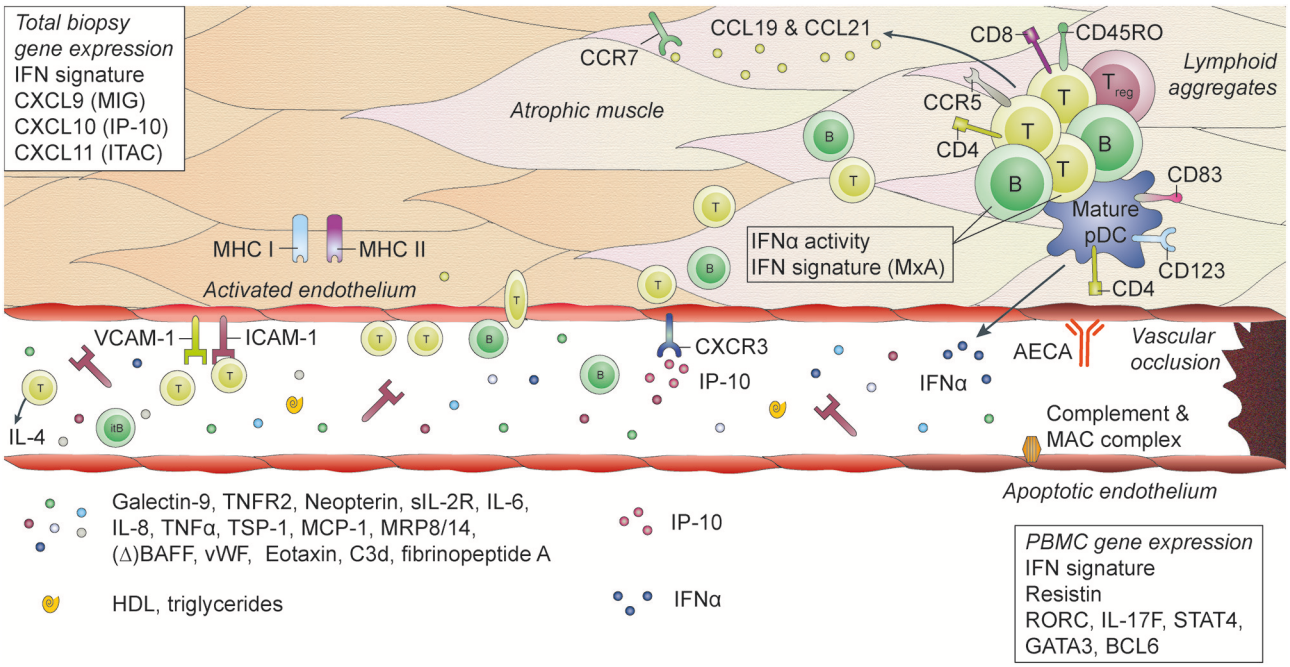

FIGURE 1 | Histopathological features and biomarkers in JDM. JDM is characterized by vasculopathic changes in the tissues, with loss and dysfunction of endothelial cells, leading to capillary dropout and subsequent atrophy of muscle fibers. The exact chain of events leading to loss of blood vessels and muscle fibers is not known, but it is thought that both overexpression of MHC-I (and MHC-II) on myocytes and endothelial damage are early events in the cascade (159, 170). They result in the first attraction of immune cells to the tissue, probably by a stress response of the myocytes and endothelial cells, leading to a first production of chemoattractants. The immune cell infiltrates, which can be organized in lymphoid structures, consist mostly of CD4+ and CD8+ memory T cells, B cells, mature plasmacytoid dendritic cells (pDC) and monocytes. CD4+ and CD8+ T cells are considered responsible for direct killing of muscle cells. pDC are considered the main producers of type I interferons (IFNs), which explains the IFN-I signature that is found in the muscles of JDM patients. Some typical IFN-inducible chemokines, CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (ITAC), are known for their angiostatic properties. The receptor for these cytokines, CXCR3, is upregulated on endothelial cells in JDM muscle, which may be one of the factors contributing to endothelial dysfunction (137). Other factors include anti-endothelial circulating antibodies (AECA), complement and membrane attack complex (MAC) deposition on endothelial cells. Endothelial cells in muscle also express high levels of ICAM-1 and VCAM-1, which further enables extravasation of immune cells into the tissues and promotes a positive feedback loop resulting in further tissue damage. Not only immune cells in the tissues, but also circulating immune cells show a type I IFN signature and increased IFNα activity. Various circulating markers reflecting immune activation and endothelial activation or distress are increased during active disease in JDM and can potentially be used as biomarkers for disease activity.

was associated with a more severe and chronic disease, with severe or persistent weakness, low remission rates at 12 months requiring additional treatment, subcutaneous edema, and chronic ulcerative disease of the skin and gastrointestinal tract (21, 55, 165). The degree of vasculopathy was also correlated with the expression of angiostatic chemokines MIG, IP-10 and ITAC (137). This indicates that the degree of vascular injury may be one of the most important factors determining long-term disease outcomes and that it is related to the IFN signature.

Not only the type of immune cell infiltration, but also the organization of immune cells in the muscle is of significance in JDM. Organization of immune infiltrates in lymphocytic aggregates or lymphoid follicle–like structures with dendritic cells and T cells, as compared to diffuse infiltrates, was associated with a more severe disease course and less response to treatment (174). MHC I expression, one of the most prominent and early histological features in JDM, did not correlate with clinical features of the disease (159, 160, 175).

The importance of thorough and standardized assessment of tissue involvement is underlined by the fact that even in cases with amyopathic DM, with normal EMG and MRI findings, the muscle biopsy can show focal endomysial lymphocyte and macrophage aggregates and 90% positivity for HLA class I in the sarcolemma (176). Unrecognized, low-grade muscle inflammation may be undertreated, resulting in a larger risk of long term damage. However, muscle biopsy is not routinely performed for children with JDM in all centers and therefore in future, biomarkers which are measureable in blood and correlate with biopsy features would represent a major advance.

IMMUNOPATHOGENIC IMPLICATIONS: INTERFERONS AND VASCULOPATHY

From the biological research conducted in JDM so far, it has become clear that IFNs and their signature play an important role in the immunopathogenesis of JDM (**Figure 1**). The IFN signature is detectable in muscle fibers, myogenic precursor cells, endothelial cells, skin and several circulating cell subsets of patients with JDM and could point toward a viral etiology (89, 106, 167). Although it has never been demonstrated definitively, several studies suggest that infections may be more common

before onset of JDM (177–180). Not only are IFNs potent drivers of (auto)inflammation, they may also be anti-angiogenic factors that could directly or indirectly contribute to endothelial damage and loss in JDM: directly by inhibiting angiogenesis and disrupting the vascular network organization and indirectly by inducing several other angiostatic factors such as galectin-9, IP-10, and ITAC (137, 181–186). In addition, type 1 IFNs inhibit the generation of myotubes and induce atrophy-associated genes in differentiated myotubes. Human skeletal muscle cells can also produce large quantities of IP-10 upon stimulation with IFNγ and TNFα (186, 187).

Rather than being produced by circulating immune cells, IFNs are probably mainly produced within inflamed tissues. Satellite cells, active myogenic cells and endothelial cells in JDM muscle strongly express IFNβ (167). The notion that non-circulating cells within tissues are responsible for IFN production also fits observations by Rodero et al. (138). In particular within muscle of JDM patients the dysbalance between angiogenic and angiostatic factors can contribute to endothelial loss (137, 188). Endothelial cells in JDM muscle downregulate genes related to vessel development, cell adhesion and migration, which are essential for angiogenesis (167). Downregulation of these genes is likely a key event in the development of vasculopathy. Next to being a target of the inflammation, the endothelium may also play an active role in the inflammatory process. In biopsies from JDM patients endothelial cells express inflammatory features, such as high levels of adhesion molecules ICAM-1 and VCAM-1, and produce cytokines and chemokines (161). These can facilitate the attraction and invasion of immune cells into tissues, thereby supporting the inflammatory process and subsequent damage. IP-10 and ITAC were the most highly upregulated genes in endothelial cells from JDM muscle and correlated with the degree of vasculopathy (137, 167). Endothelium-derived IP-10 can even stabilize the interaction between T cells and endothelial cells, thereby possibly contributing to the chronicity of T cell infiltration (189). Recently, a new function has been ascribed to endothelial cells as "semi-professional" antigen presenting cells, which act as sentinels for antigens, and possibly self-antigens, in tissues and facilitate T cell trafficking into these tissues (190, 191). The high expression of MHC molecules on endothelial cells in JDM muscle may support the notion that this process is involved in JDM (160, 175). Although the exact mechanisms of interaction between immune cells and endothelial cells in JDM are still elusive, they may be more elaborate than so far recognized.

CONCLUSIONS AND FUTURE PERSPECTIVES FOR BIOMARKER RESEARCH

JDM is a multisystem disease. Not only the skin and skeletal muscles are affected, but also other organ systems and tissues such as the lungs, heart and intestines are frequently (subclinically) involved and may be under-evaluated. Vasculopathy due to loss and dysfunction of endothelial cells as a result of the inflammatory process is thought to underlie the symptoms in most of these organs and tissues. Monitoring of disease activity and damage in all of these affected tissues is important during clinical follow-up, as these are key determinants for the long-term outcomes of patients. Tools for monitoring of tissue activity and damage include histopathological investigation of biopsies, and laboratory testing of blood for specific biomarkers as well as several imaging methods. Each of these methods has their strengths and weaknesses and can be of value for specific diagnostic questions at disease onset or during follow-up, as outlined in the consensus-based recommendations for the management of JDM (135, 192). There is still a need for minimally invasive, but at the same time sensitive and specific diagnostic methods that correlate well with clinical symptoms or reflect low-grade, local inflammation. Tissue-specific biomarkers can therefore be of great value as a monitoring tool.

To be able to identify sensitive, robust and reliable biomarkers or develop monitoring tools, it is of key importance to set up well-defined and large prospective patient cohorts, with a thorough longitudinal collection of a standardized clinical dataset assessing disease activity and organ involvement, paired with collection of patient material (193). Such a dataset is required to ensure a strict definition of active and inactive disease [e.g., as proposed by Almeida et al. (72)]. An important consideration for a successful biomarker study is the timing of data and sample collection: depending on the purpose of the biomarker, time points before start of immunosuppressive treatment, before each adjustment of medication, during flares, at paired time points during active and inactive disease or even at regular intervals of max 3–4 months may be crucial to reliably investigate the potency of a biomarker.

Next to the "classical" statistical approach, comparing patients with active disease and patients in remission (cross-sectionally or in paired samples), new computational approaches providing analysis methods that can integrate longitudinal data from multiple patients and multiple (bio)markers or scoring tools should be considered. These methods take into account the fluctuating nature of a relapsing-remitting disease such as JDM and are therefore better suited to test the reliability of a tool that will be used for longitudinal follow-up in clinical practice (194, 195).

To achieve implementation of a marker or tool into clinical practice, both clinical and technical validation in independent cohorts is of utmost importance. Only few markers prove to be stable, reliable and easy to measure, which are key features for a marker or tool to be suited for implementation into clinical practice. Also the invasiveness of the method should be taken into account. Ideally, a period of experimental implementation can demonstrate the added value and feasibility of a marker or tool in clinical practice. To achieve all this in a large group of JDM patients to ensure sufficient statistical power, international networks with well-established collaborations are fundamental.

Eventually, monitoring of disease activity with a reliable tool can be used to guide treatment and thereby facilitate precision medicine, with high dose therapy when indicated but also preventing overtreatment. This may reduce both the duration of active disease and thereby the disease-inflicted damage, and medication side effects, which will benefit the long-term outcomes on various domains, such as muscle

weakness, organ damage, cardiopulmonary fitness, and quality of life. Next to facilitating personalized treatment strategies, newly identified biomarkers may also provide insights into the immunopathogenesis of JDM and provide new treatment targets. For instance, new treatment strategies targeting the IFN signature, such as anti-IFN antibodies (sifalimumab) or JAK-inhibition (ruxolitinib) have been shown to reduce the IFN signature in blood and muscle of adult dermatomyositis patients, and may therefore be promising new strategies for patients with JDM (186, 196, 197). Several studies discussed in this review suggest a strong link between the IFN signature and vasculopathy; and vasculopathy has been related to disease severity. Targeting the IFN signature may thus benefit vascularization in JDM and thereby improve outcomes.

AUTHOR CONTRIBUTIONS

JW collected literature and wrote the manuscript draft. FvW and AvR-K supervised JW, outlined the manuscript focus and revised the manuscript. CD and LW critically revised the manuscript.

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REFERENCES

1. Meyer A, Meyer N, Schaeffer M, Gottenberg JE, Geny B, Sibilia J. Incidence and prevalence of inflammatory myopathies: a systematic review. *Rheumatology* (2015) 54:50–63. doi: 10.1093/rheumatology/keu289
2. Miller FW, Cooper RG, Vencovsky J, Rider LG, Danko K, Wedderburn LR, et al. Genome-wide association study of dermatomyositis reveals genetic overlap with other autoimmune disorders. *Arthritis Rheum.* (2013) 65:3239–47. doi: 10.1093/med/9780199732920.003.0012
3. Miller FW, Chen W, O'Hanlon TP, Cooper RG, Vencovsky J, Rider LG, et al. Genome-wide association study identifies HLA 8.1 ancestral haplotype alleles as major genetic risk factors for myositis phenotypes. *Genes Immun.* (2015) 16:470–80. doi: 10.1038/gene.2015.28
4. Rothwell S, Cooper RG, Lundberg IE, Miller FW, Gregersen PK, Bowes J, et al. Dense genotyping of immune-related loci in idiopathic inflammatory myopathies confirms HLA alleles as the strongest genetic risk factor and suggests different genetic background for major clinical subgroups. *Ann Rheum Dis.* (2016) 75:1558–66. doi: 10.1136/annrheumdis-2015-208119
5. Wedderburn LR, Rider LG. Juvenile dermatomyositis: new developments in pathogenesis, assessment and treatment. *Best Pract Res Clin Rheumatol.* (2009) 23:665–78. doi: 10.1016/j.berh.2009.07.007
6. Lundberg IE, Tjarnlund A, Bottai M, Werth VP, Pilkington C, de Visser M, et al. 2017 European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. *Ann Rheum Dis.* (2017) 76:1955–64. doi: 10.1136/annrheumdis-2017-211468
7. Lundberg IE, Tjarnlund A, Bottai M, Werth VP, Pilkington C, de Visser M, et al. 2017 European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. *Arthritis Rheumatol.* (2017) 69:2271–82. doi: 10.1002/art.40320
8. Kobayashi N, Takezaki S, Kobayashi I, Iwata N, Mori M, Nagai K, et al. Clinical and laboratory features of fatal rapidly progressive interstitial lung disease associated with juvenile dermatomyositis. *Rheumatology* (2015) 54:784–91. doi: 10.1093/rheumatology/keu385
9. Huber AM, Mamyrova G, Lachenbruch PA, Lee JA, Katz JD, Targoff IN, et al. Early illness features associated with mortality in the juvenile idiopathic inflammatory myopathies. *Arthritis Care Res.* (2014) 66:732–40. doi: 10.1002/acr.22212
10. Feldman BM, Rider LG, Reed AM, Pachman LM. Juvenile dermatomyositis and other idiopathic inflammatory myopathies of childhood. *Lancet* (2008) 371:2201–12. doi: 10.1016/S0140-6736(08)60955-1
11. Huber A, Feldman BM. Long-term outcomes in juvenile dermatomyositis: how did we get here and where are we going? *Curr Rheumatol Rep.* (2005) 7:441–6. doi: 10.1007/s11926-005-0048-1
12. Gerami P, Walling HW, Lewis J, Doughty L, Sontheimer RD. A systematic review of juvenile-onset clinically amyopathic dermatomyositis. *Br J Dermatol.* (2007) 157:637–44. doi: 10.1111/j.1365-2133.2007.08055.x
13. Plamondon S, Dent PB. Juvenile amyopathic dermatomyositis: results of a case finding descriptive survey. *J Rheumatol.* (2000) 27:2031–4.
14. Oberle EJ, Bayer ML, Chiu YE, Co DO. How often are pediatric patients with clinically amyopathic dermatomyositis truly amyopathic? *Pediatr Dermatol.* (2017) 34:50–7. doi: 10.1111/pde.13013
15. Mamyrova G, Kishi T, Targoff IN, Ehrlich A, Curiel RV, Rider LG. Features distinguishing clinically amyopathic juvenile dermatomyositis from juvenile dermatomyositis. *Rheumatology* (2018) 57:1956–1963. doi: 10.1093/rheumatology/key190
16. Rider LG, Nistala K. The juvenile idiopathic inflammatory myopathies: pathogenesis, clinical and autoantibody phenotypes, and outcomes. *J Intern Med.* (2016) 280:24–38. doi: 10.1111/joim.12444
17. Rider LG. The heterogeneity of juvenile myositis. *Autoimmun Rev.* (2007) 6:241–7. doi: 10.1016/j.autrev.2006.08.009
18. Huber AM, Lang B, LeBlanc CM, Birdi N, Bolaria RK, Malleson P, et al. Medium- and long-term functional outcomes in a multicenter cohort of children with juvenile dermatomyositis. *Arthritis Rheum.* (2000) 43:541–9. doi: 10.1002/1529-0131(200003)43:3<541::AID-ANR9>3.0.CO;2-T
19. Fisler RE, Liang MG, Fuhlbrigge RC, Yalcindag A, Sundel RP. Aggressive management of juvenile dermatomyositis results in improved outcome and decreased incidence of calcinosis. *J Am Acad Dermatol.* (2002) 47:505–11. doi: 10.1067/mjd.2002.122196
20. Holzer U, van Royen-Kerkhof A, van der Torre P, Kuemmerle-Deschner J, Well C, Handgretinger R, et al. Successful autologous stem cell transplantation in two patients with juvenile dermatomyositis. *Scand J Rheumatol.* (2010) 39:88–92. doi: 10.3109/03009740903096622
21. Crowe WE, Bove KE, Levinson JE, Hilton PK. Clinical and pathogenetic implications of histopathology in childhood polydermatomyositis. *Arthritis Rheum.* (1982) 25:126–39. doi: 10.1002/art.1780250203
22. Huemer C, Kitson H, Malleson PN, Sanderson S, Huemer M, Cabral DA, et al. Lipodystrophy in patients with juvenile dermatomyositis–evaluation of clinical and metabolic abnormalities. *J Rheumatol.* (2001) 28:610–5.
23. Verma S, Singh S, Bhalla AK, Khullar M. Study of subcutaneous fat in children with juvenile dermatomyositis. *Arthritis Rheum.* (2006) 55:564–8. doi: 10.1002/art.22108
24. Bingham A, Mamyrova G, Rother KI, Oral E, Cochran E, Premkumar A, et al. Predictors of acquired lipodystrophy in juvenile-onset dermatomyositis and a gradient of severity. *Medicine* (2008) 87:70–86. doi: 10.1097/MD.0b013e31816bc604
25. Pouessel G, Deschildre A, Le Bourgeois M, Cuisset J-M, Catteau B, Karila C, et al. The lung is involved in juvenile dermatomyositis. *Pediatr Pulmonol.* (2013) 48:1016–25. doi: 10.1002/ppul.22742

26. Richardson AE, Warrier K, Vyas H. Respiratory complications of the rheumatological diseases in childhood. *Arch Dis Child* (2016) 101:752–8. doi: 10.1136/archdischild-2014-306049
27. Kobayashi S, Higuchi K, Tamaki H, Wada Y, Wada N, Kubo M, et al. Characteristics of juvenile dermatomyositis in Japan. *Acta Paediatr Jpn Overseas Ed.* (1997) 39:257–62.
28. Shah M, Mamyrova G, Targoff IN, Huber AM, Malley JD, Rice MM, et al. The clinical phenotypes of the juvenile idiopathic inflammatory myopathies. *Medicine* (2013) 92:25–41. doi: 10.1097/MD.0b013e31827f264d
29. Sanner H, Aalokken TM, Gran JT, Sjaastad I, Johansen B, Flato B. Pulmonary outcome in juvenile dermatomyositis: a case-control study. *Ann Rheum Dis.* (2011) 70:86–91. doi: 10.1136/ard.2010.131433
30. Morinishi Y, Oh-Ishi T, Kabuki T, Joh K. Juvenile dermatomyositis: clinical characteristics and the relatively high risk of interstitial lung disease. *Mod Rheumatol.* (2007) 17:413–7. doi: 10.3109/s10165-007-0610-y
31. Cantez S, Gross GJ, MacLusky I, Feldman BM. Cardiac findings in children with juvenile Dermatomyositis at disease presentation. *Pediatr Rheumatol Online J.* (2017) 15:54. doi: 10.1186/s12969-017-0182-0
32. Pachman LM, Cooke N. Juvenile dermatomyositis: a clinical and immunologic study. *J Pediatr* (1980) 96:226–34. doi: 10.1016/S0022-3476(80)80807-9
33. Na S-J, Kim SM, Sunwoo IN, Choi Y-C. Clinical characteristics and outcomes of juvenile and adult dermatomyositis. *J Korean Med Sci.* (2009) 24:715–21. doi: 10.3346/jkms.2009.24.4.715
34. Constantin T, Ponyi A, Orban I, Molnar K, Derfalvi B, Dicso F, et al. National registry of patients with juvenile idiopathic inflammatory myopathies in Hungary–clinical characteristics and disease course of 44 patients with juvenile dermatomyositis. *Autoimmunity* (2006) 39:223–32. doi: 10.1080/08916930600622819
35. Shehata R, al-Mayouf S, al-Dalaan A, al-Mazaid A, al-Balaa S, Bahabri S. Juvenile dermatomyositis: clinical profile and disease course in 25 patients. *Clin Exp Rheumatol.* (1999) 17:115–118.
36. Schwartz T, Sanner H, Gjesdal O, Flato B, Sjaastad I. In juvenile dermatomyositis, cardiac systolic dysfunction is present after long-term follow-up and is predicted by sustained early skin activity. *Ann Rheum Dis.* (2014) 73:1805–10. doi: 10.1136/annrheumdis-2013-203279
37. Schwartz T, Sanner H, Husebye T, Flato B, Sjaastad I. Cardiac dysfunction in juvenile dermatomyositis: a case-control study. *Ann Rheum Dis.* (2011) 70:766–71. doi: 10.1136/ard.2010.137968
38. Schwartz T, Diederichsen LP, Lundberg IE, Sjaastad I, Sanner H. Cardiac involvement in adult and juvenile idiopathic inflammatory myopathies. *RMD open* (2006) 2:e000291. doi: 10.1136/rmdopen-2016-000291
39. Jimenez C, Rowe PC, Keene D. Cardiac and central nervous system vasculitis in a child with dermatomyositis. *J Child Neurol* (1994) 9:297–300. doi: 10.1177/088307389400900315
40. Ramanan A V, Sawhney S, Murray KJ. Central nervous system complications in two cases of juvenile onset dermatomyositis. *Rheumatology* (2001) 40:1293–8. doi: 10.1093/rheumatology/40.11.1293
41. Silverberg JI, Kwa L, Kwa MC, Laumann AE, Ardalan K. Cardiovascular and cerebrovascular comorbidities of juvenile dermatomyositis in US children: an analysis of the National Inpatient Sample. *Rheumatology* (2018) 57:694–702. doi: 10.1093/rheumatology/kex465
42. Robinson AB, Hoeltzel MF, Wahezi DM, Becker ML, Kessler EA, Schmeling H, et al. Clinical characteristics of children with juvenile dermatomyositis: the Childhood Arthritis and Rheumatology Research Alliance Registry. *Arthritis Care Res.* (2014) 66:404–10. doi: 10.1002/acr.22142
43. Laskin BL, Choyke P, Keenan GF, Miller FW, Rider LG. Novel gastrointestinal tract manifestations in juvenile dermatomyositis. *J Pediatr.* (1999) 135:371–4. doi: 10.1016/S0022-3476(99)70137-X
44. Mamyrova G, Kleiner DE, James-Newton L, Shaham B, Miller FW, Rider LG. Late-onset gastrointestinal pain in juvenile dermatomyositis as a manifestation of ischemic ulceration from chronic endarteropathy. *Arthritis Rheum.* (2007) 57:881–4. doi: 10.1002/art.22782
45. Varsani H, Charman SC, Li CK, Marie SKN, Amato AA, Banwell B, et al. Validation of a score tool for measurement of histological severity in juvenile dermatomyositis and association with clinical severity of disease. *Ann Rheum Dis.* (2015) 74:204–10. doi: 10.1136/annrheumdis-2013-203396
46. Besancon A, Brochard K, Dupic L, Gitiaux C, Delville M, Krid S, et al. Presentations and outcomes of juvenile dermatomyositis patients admitted to intensive care units. *Rheumatology* (2017) 56:1814–6. doi: 10.1093/rheumatology/kex267
47. Meneghel A, Martini G, Birolo C, Tosoni A, Pettenazzo A, Zulian F. Life-threatening systemic capillary leak syndrome in juvenile dermatomyositis. *Rheumatology* (2017) 56:1822–3. doi: 10.1093/rheumatology/kex262
48. Goncalves FGP, Chimelli L, Sallum AME, Marie SKN, Kiss MHB, Ferriani VPL. Immunohistological analysis of CD59 and membrane attack complex of complement in muscle in juvenile dermatomyositis. *J Rheumatol.* (2002) 29:1301–7.
49. Yu HH, Chang HM, Chiu CJ, Yang YH, Lee JH, Wang LC, et al. Detection of anti-p155/140, anti-p140, and antiendothelial cells autoantibodies in patients with juvenile dermatomyositis. *J Microbiol Immunol Infect.* (2016) 49:264–70. doi: 10.1016/j.jmii.2014.05.012
50. Karasawa R, Tamaki M, Sato T, Tanaka M, Nawa M, Yudoh K, et al. Multiple target autoantigens on endothelial cells identified in juvenile dermatomyositis using proteomics. *Rheumatology* (2018) 57:671–6. doi: 10.1093/rheumatology/kex468
51. Whitaker JN, Engel WK. Vascular deposits of immunoglobulin and complement in idiopathic inflammatory myopathy. *N Engl J Med.* (1972) 286:333–8. doi: 10.1056/NEJM197202172860701
52. Kissel JT, Mendell JR, Rammohan KW. Microvascular deposition of complement membrane attack complex in dermatomyositis. *N Engl J Med.* (1986) 314:329–34. doi: 10.1056/NEJM198602063140601
53. Kissel JT, Halterman RK, Rammohan KW, Mendell JR. The relationship of complement-mediated microvasculopathy to the histologic features and clinical duration of disease in dermatomyositis. *Arch Neurol.* (1991) 48:26–30. doi: 10.1001/archneur.1991.00530130034016
54. Emslie-Smith AM, Engel AG. Microvascular changes in early and advanced dermatomyositis: a quantitative study. *Ann Neurol.* (1990) 27:343–56. doi: 10.1002/ana.410270402
55. Gitiaux C, De Antonio M, Aouizerate J, Gherardi RK, Guilbert T, Barnerias C, et al. Vasculopathy-related clinical and pathological features are associated with severe juvenile dermatomyositis. *Rheumatology* (2016) 55:470–9. doi: 10.1093/rheumatology/kev359
56. Schmeling H, Stephens S, Goia C, Manlhiot C, Schneider R, Luthra S, et al. Nailfold capillary density is importantly associated over time with muscle and skin disease activity in juvenile dermatomyositis. *Rheumatology* (2011) 50:885–93. doi: 10.1093/rheumatology/keq407
57. Feldman BM, Rider LG, Dugan L, Miller FW, Schneider R. Nailfold capillaries as indicators of disease activity in juvenile idiopathic inflammatory myopathies (JIIM). *Arthritis Rheum.* (1999) 42:S181.
58. Smith RL, Sundberg J, Shamiyah E, Dyer A, Pachman LM. Skin involvement in juvenile dermatomyositis is associated with loss of end row nailfold capillary loops. *J Rheumatol.* (2004) 31:1644–9.
59. Bertolazzi C, Cutolo M, Smith V, Gutierrez M. State of the art on nailfold capillaroscopy in dermatomyositis and polymyositis. *Semin Arthritis Rheum.* (2017) 47:432–44. doi: 10.1016/j.semarthrit.2017.06.001
60. Rider LG, Werth VP, Huber AM, Alexanderson H, Rao AP, Ruperto N, et al. Measures of adult and juvenile dermatomyositis, polymyositis, and inclusion body myositis: physician and Patient/Parent Global Activity, Manual Muscle Testing (MMT), Health Assessment Questionnaire (HAQ)/Childhood Health Assessment Questionnaire (C-HAQ). *Arthritis Care Res.* (2011) 63(Suppl. 1):S118–57.
61. Lovell DJ, Lindsley CB, Rennebohm RM, Ballinger SH, Bowyer SL, Giannini EH, et al. Development of validated disease activity and damage indices for the juvenile idiopathic inflammatory myopathies. *II The Childhood Myositis Assessment Scale (CMAS): a quantitative tool for the evaluation of muscle function The Juvenile Dermatomyositis, D. Arthritis Rheum.* (1999) 42:2213–9.
62. Rider LG, Koziol D, Giannini EH, Jain MS, Smith MR, Whitney-Mahoney K, et al. Validation of manual muscle testing and a subset of eight muscles for adult and juvenile idiopathic inflammatory myopathies. *Arthritis Care Res.* (2010) 62:465–72. doi: 10.1002/acr.20035
63. Rider LG, Feldman BM, Perez MD, Rennebohm RM, Lindsley CB, Zemel LS, et al. Development of validated disease activity and damage indices for the juvenile idiopathic inflammatory myopathies: I. *Physician,*

parent, and patient global assessments Juvenile Dermatomyositis Disease Activity Collaborative Study Group. Arthritis Rheum. (1997) 40:1976–83. doi: 10.1002/art.1780401109
64. Huber AM, Dugan EM, Lachenbruch PA, Feldman BM, Perez MD, Zemel LS, et al. Preliminary validation and clinical meaning of the Cutaneous Assessment Tool in juvenile dermatomyositis. *Arthritis Rheum.* (2008) 59:214–21. doi: 10.1002/art.23340
65. Huber AM, Lachenbruch PA, Dugan EM, Miller FW, Rider LG. Alternative scoring of the Cutaneous Assessment Tool in juvenile dermatomyositis: results using abbreviated formats. *Arthritis Rheum.* (2008) 59:352–6. doi: 10.1002/art.23313
66. Bode RK, Klein-Gitelman MS, Miller ML, Lechman TS, Pachman LM. Disease activity score for children with juvenile dermatomyositis: reliability and validity evidence. *Arthritis Rheum.* (2003) 49:7–15. doi: 10.1002/art.10924
67. Huber AM, Hicks JE, Lachenbruch PA, Perez MD, Zemel LS, Rennebohm RM, et al. Validation of the Childhood Health Assessment Questionnaire in the juvenile idiopathic myopathies. *Juvenile Dermatomyositis Disease Activity Collaborative Study Group. J Rheumatol.* (2001) 28:1106–11.
68. Tiao J, Feng R, Berger EM, Brandsema JF, Coughlin CC, Khan N, et al. Evaluation of the reliability of the Cutaneous Dermatomyositis Disease Area and Severity Index and the Cutaneous Assessment Tool-Binary Method in juvenile dermatomyositis among paediatric dermatologists, rheumatologists and neurologists. *Br J Dermatol.* (2017) 177:1086–92. doi: 10.1111/bjd.15596
69. Sultan SM, Allen E, Cooper RG, Agarwal S, Kiely P, Oddis C V, et al. Interrater reliability and aspects of validity of the myositis damage index. *Ann Rheum Dis.* (2011) 70:1272–6. doi: 10.1136/ard.2010.142117
70. McCann LJ, Kirkham JJ, Wedderburn LR, Pilkington C, Huber AM, Ravelli A, et al. Development of an internationally agreed minimal dataset for juvenile dermatomyositis (JDM) for clinical and research use. *Trials* 16:268. doi: 10.1186/s13063-015-0784-0
71. Lazarevic D, Pistorio A, Palmisani E, Miettunen P, Ravelli A, Pilkington C, et al. The PRINTO criteria for clinically inactive disease in juvenile dermatomyositis. *Ann Rheum Dis.* (2013) 72:686–93. doi: 10.1136/annrheumdis-2012-201483
72. Almeida B, Campanilho-Marques R, Arnold K, Pilkington CA, Wedderburn LR, Nistala K. Analysis of published criteria for clinically inactive disease in a large juvenile dermatomyositis cohort shows that skin disease is underestimated. *Arthritis Rheumatol.* (2015) 67:2495–502. doi: 10.1002/art.39200
73. Betteridge ZE, Gunawardena H, McHugh NJ. Novel autoantibodies and clinical phenotypes in adult and juvenile myositis. *Arthritis Res Ther.* (2011) 13:209. doi: 10.1186/ar3275
74. Rider LG, Miller FW. Deciphering the clinical presentations, pathogenesis, and treatment of the idiopathic inflammatory myopathies. *JAMA* (2011) 305:183–90. doi: 10.1001/jama.2010.1977
75. Rider LG, Shah M, Mamyrova G, Huber AM, Rice MM, Targoff IN, et al. The myositis autoantibody phenotypes of the juvenile idiopathic inflammatory myopathies. *Medicine* (2013) 92:223–43. doi: 10.1097/MD.0b013e31829d08f9
76. Tansley SL, Simou S, Shaddick G, Betteridge ZE, Almeida B, Gunawardena H, et al. Autoantibodies in juvenile-onset myositis: their diagnostic value and associated clinical phenotype in a large UK cohort. *J Autoimmun.* (2017) 84:55–64. doi: 10.1016/j.jaut.2017.06.007
77. Gunawardena H, Wedderburn LR, North J, Betteridge Z, Dunphy J, Chinoy H, et al. Clinical associations of autoantibodies to a p155/140 kDa doublet protein in juvenile dermatomyositis. *Rheumatology* (2008) 47:324–8. doi: 10.1093/rheumatology/kem359
78. Habers GEA, Huber AM, Mamyrova G, Targoff IN, O'Hanlon TP, Adams S, et al. Brief Report: association of myositis autoantibodies, clinical features, and environmental exposures at illness onset with disease course in juvenile myositis. *Arthritis Rheumatol.* (2016) 68:761–8. doi: 10.1002/art.39466
79. Tansley SL, Betteridge ZE, Shaddick G, Gunawardena H, Arnold K, Wedderburn LR, et al. Calcinosis in juvenile dermatomyositis is influenced by both anti-NXP2 autoantibody status and age at disease onset. *Rheumatology* (2014) 53:2204–8. doi: 10.1093/rheumatology/keu259
80. Gunawardena H, Wedderburn LR, Chinoy H, Betteridge ZE, North J, Ollier WER, et al. Autoantibodies to a 140-kd protein in juvenile dermatomyositis are associated with calcinosis. *Arthritis Rheum.* (2009) 60:1807–14. doi: 10.1002/art.24547
81. Sato S, Hirakata M, Kuwana M, Suwa A, Inada S, Mimori T, et al. Autoantibodies to a 140-kd polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. *Arthritis Rheum.* (2005) 52:1571–6. doi: 10.1002/art.21023
82. Tansley SL, Betteridge ZE, Gunawardena H, Jacques TS, Owens CM, Pilkington C, et al. Anti-MDA5 autoantibodies in juvenile dermatomyositis identify a distinct clinical phenotype: a prospective cohort study. *Arthritis Res Ther.* (2016) 16:R138. doi: 10.1186/ar4600
83. Hoshino K, Muro Y, Sugiura K, Tomita Y, Nakashima R, Mimori T. Anti-MDA5 and anti-TIF1-γ antibodies have clinical significance for patients with dermatomyositis. *Rheumatology* (2010) 49:1726–33. doi: 10.1093/rheumatology/keq153
84. Hall JC, Casciola-Rosen L, Samedy L-A, Werner J, Owoyemi K, Danoff SK, et al. Anti-melanoma differentiation-associated protein 5-associated dermatomyositis: expanding the clinical spectrum. *Arthritis Care Res.* (2013) 65:1307–15. doi: 10.1002/acr.21992
85. Fiorentino D, Chung L, Zwerner J, Rosen A, Casciola-Rosen L. The mucocutaneous and systemic phenotype of dermatomyositis patients with antibodies to MDA5 (CADM-140): a retrospective study. *J Am Acad Dermatol.* (2011) 65:25–34. doi: 10.1016/j.jaad.2010.09.016
86. Tansley SL, Betteridge ZE, Simou S, Jacques TS, Pilkington C, Wood M, et al. Anti-HMGCR autoantibodies in juvenile idiopathic inflammatory myopathies identify a rare but clinically important subset of patients. *J Rheumatol.* (2017) 44:488–492. doi: 10.3899/jrheum.160871
87. Kishi T, Rider LG, Pak K, Barillas-Arias L, Henrickson M, McCarthy PL, et al. Association of anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase autoantibodies with DRB1*07:01 and severe myositis in juvenile myositis patients. *Arthritis Care Res.* (2017) 69:1088–94. doi: 10.1002/acr.23113
88. Aggarwal R, Oddis CV, Goudeau D, Koontz D, Qi Z, Reed AM, et al. Autoantibody levels in myositis patients correlate with clinical response during B cell depletion with rituximab. *Rheumatology* (2016) 55:991–9. doi: 10.1093/rheumatology/kev444
89. O'Connor KA, Abbott KA, Sabin B, Kuroda M, Pachman LM. MxA gene expression in juvenile dermatomyositis peripheral blood mononuclear cells: association with muscle involvement. *Clin Immunol.* (2006) 120:319–25.
90. Niewold TB, Kariuki SN, Morgan GA, Shrestha S, Pachman LM. Elevated serum interferon-alpha activity in juvenile dermatomyositis: associations with disease activity at diagnosis and after thirty-six months of therapy. *Arthritis Rheum.* (2009) 60:1815–24. doi: 10.1002/art.24555
91. Baechler EC, Bilgic H, Reed AM. Type I interferon pathway in adult and juvenile dermatomyositis. *Arthritis Res Ther.* (2011) 13:249. doi: 10.1186/ar3531
92. Sanner H, Schwartz T, Flato B, Vistnes M, Christensen G, Sjaastad I. Increased levels of eotaxin and MCP-1 in juvenile dermatomyositis median 16.8 years after disease onset; associations with disease activity, duration and organ damage. *PLoS ONE* (2014) 9:e92171. doi: 10.1371/journal.pone.0092171
93. Bellutti Enders F, van Wijk F, Scholman R, Hofer M, Prakken BJ, van Royen-Kerkhof A, et al. Correlation of CXCL10, tumor necrosis factor receptor type II, and galectin 9 with disease activity in juvenile dermatomyositis. *Arthritis Rheumatol.* (2014) 66:2281–9. doi: 10.1002/art.38676
94. Enders FB, Delemarre EM, Kuemmerle-Deschner J, van der Torre P, Wulffraat NM, Prakken BP, et al. Autologous stem cell transplantation leads to a change in proinflammatory plasma cytokine profile of patients with juvenile dermatomyositis correlating with disease activity. *Ann Rheum Dis.* (2015) 74:315–7. doi: 10.1136/annrheumdis-2014-206287
95. Kobayashi I, Ono S, Kawamura N, Okano M, Kobayashi K. Elevated serum levels of soluble interleukin-2 receptor in juvenile dermatomyositis. *Pediatr Int.* (2001) 43:109–11. doi: 10.1046/j.1442-200x.2001.01367.x
96. De Benedetti F, De Amici M, Aramini L, Ruperto N, Martini A. Correlation of serum neopterin concentrations with disease activity in juvenile dermatomyositis. *Arch Dis Child.* (1993) 69:232–5. doi: 10.1136/adc.69.2.232
97. Rider LG, Schiffenbauer AS, Zito M, Lim KL, Ahmed A, Zemel LS, et al. Neopterin and quinolinic acid are surrogate measures of disease activity

in the juvenile idiopathic inflammatory myopathies. *Clin Chem.* (2002) 48:1681–8.
98. Nistala K, Varsani H, Wittkowski H, Vogl T, Krol P, Shah V, et al. Myeloid related protein induces muscle derived inflammatory mediators in juvenile dermatomyositis. *Arthritis Res Ther.* (2013) 15:R131. doi: 10.1186/ar4311
99. Haas RH, Dyck RF, Dubowitz V, Pepys MB. C-reactive protein in childhood dermatomyositis. *Ann Rheum Dis.* (1982) 41:483–5. doi: 10.1136/ard.41.5.483
100. Eisenstein DM, O'Gorman MR, Pachman LM. Correlations between change in disease activity and changes in peripheral blood lymphocyte subsets in patients with juvenile dermatomyositis. *J Rheumatol.* (1997) 24:1830–2.
101. Elst EF, Klein M, de Jager W, Kamphuis S, Wedderburn LR, van der Zee R, et al. Hsp60 in inflamed muscle tissue is the target of regulatory autoreactive T cells in patients with juvenile dermatomyositis. *Arthritis Rheum.* (2008) 58:547–55. doi: 10.1002/art.23202
102. Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* (2011) 34:108–21. doi: 10.1016/j.immuni.2010.12.012
103. Ernste FC, Crowson CS, de Padilla CL, Hein MS, Reed AM. Longitudinal peripheral blood lymphocyte subsets correlate with decreased disease activity in juvenile dermatomyositis. *J Rheumatol.* (2013) 40:1200–11. doi: 10.3899/jrheum.121031
104. Vercoulen Y, Bellutti Enders F, Meerding J, Plantinga M, Elst EF, Varsani H, et al. Increased presence of FOXP3+ regulatory T cells in inflamed muscle of patients with active juvenile dermatomyositis compared to peripheral blood. *PLoS ONE* (2014) 9:e105353. doi: 10.1371/journal.pone.0105353
105. Lopez De Padilla CM, Crowson CS, Hein MS, Pendegraft RS, Strausbauch MA, Niewold TB, et al. Gene expression profiling in blood and affected muscle tissues reveals differential activation pathways in patients with new-onset juvenile and adult dermatomyositis. *J Rheumatol.* (2017) 44:117–24. doi: 10.3899/jrheum.160293
106. Piper CJM, Wilkinson MGL, Deakin CT, Otto GW, Dowle S, Duurland CL, et al. CD19(+)CD24(hi)CD38(hi) B cells are expanded in juvenile dermatomyositis and exhibit a pro-inflammatory phenotype after activation through toll-like receptor 7 and interferon-alpha. *Front Immunol.* (2018) 9:1372. doi: 10.3389/fimmu.2018.01372
107. Guzman J, Petty RE, Malleson PN. Monitoring disease activity in juvenile dermatomyositis: the role of von Willebrand factor and muscle enzymes. *J Rheumatol.* (1994) 21:739–43.
108. Bloom BJ, Tucker LB, Miller LC, Schaller JG. von Willebrand factor in juvenile dermatomyositis. *J Rheumatol.* (1995) 22:320–5.
109. Scott JP, Arroyave C. Activation of complement and coagulation in juvenile dermatomyositis. *Arthritis Rheum.* (1987) 30:572–6. doi: 10.1002/art.1780300513
110. Xu D, Huang C-C, Kachaochana A, Morgan GA, Bonaldo MF, Soares MB, et al. MicroRNA-10a regulation of proinflammatory mediators: an important component of untreated juvenile dermatomyositis. *J Rheumatol.* (2016) 43:161–8. doi: 10.3899/jrheum.141474
111. Xu D, Kacha-Ochana A, Morgan GA, Huang C-C, Pachman LM. Endothelial progenitor cell number is not decreased in 34 children with Juvenile Dermatomyositis: a pilot study. *Pediatr Rheumatol Online J.* (2017) 15:42. doi: 10.1186/s12969-017-0171-3
112. Coyle K, Rother KI, Weise M, Ahmed A, Miller FW, Rider LG. Metabolic abnormalities and cardiovascular risk factors in children with myositis. *J Pediatr.* (2009) 155:882–7. doi: 10.1016/j.jpeds.2009.06.009
113. Kozu KT, Silva CA, Bonfa E, Sallum AM, Pereira RMR, Viana VS, et al. Dyslipidaemia in juvenile dermatomyositis: the role of disease activity. *Clin Exp Rheumatol.* (2013) 31:638–44.
114. Olazagasti JM, Hein M, Crowson CS, de Padilla CL, Peterson E, Baechler EC, et al. Adipokine gene expression in peripheral blood of adult and juvenile dermatomyositis patients and their relation to clinical parameters and disease activity measures. *J Inflamm.* (2015) 12:29. doi: 10.1186/s12950-015-0075-2
115. Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. *Mol Med.* (2007) 13:59–68. doi: 10.2119/2006-00085.Baechler
116. Bilgic H, Ytterberg SR, Amin S, McNallan KT, Wilson JC, Koeuth T, et al. Interleukin-6 and type I interferon-regulated genes and chemokines mark disease activity in dermatomyositis. *Arthritis Rheum.* (2009) 60:3436–46. doi: 10.1002/art.24936
117. Reed AM, Peterson E, Bilgic H, Ytterberg SR, Amin S, Hein MS, et al. Changes in novel biomarkers of disease activity in juvenile and adult dermatomyositis are sensitive biomarkers of disease course. *Arthritis Rheum.* (2012) 64:4078–86. doi: 10.1002/art.34659
118. Gabay C, Gay-Croisier F, Roux-Lombard P, Meyer O, Maineti C, Guerne PA, et al. Elevated serum levels of interleukin-1 receptor antagonist in polymyositis/dermatomyositis. *A biologic marker of disease activity with a possible role in the lack of acute-phase protein response. Arthritis Rheum.* (1994) 37:1744–51. doi: 10.1002/art.1780371206
119. Krystufkova O, Vallerskog T, Helmers SB, Mann H, Putova I, Belacek J, et al. Increased serum levels of B cell activating factor (BAFF) in subsets of patients with idiopathic inflammatory myopathies. *Ann Rheum Dis.* (2009) 68:836–43. doi: 10.1136/ard.2008.091405
120. Lopez De Padilla CM, McNallan KT, Crowson CS, Bilgic H, Bram RJ, Hein MS, et al. BAFF expression correlates with idiopathic inflammatory myopathy disease activity measures and autoantibodies. *J Rheumatol.* (2013) 40:294–302. doi: 10.3899/jrheum.120555
121. Ishida T, Matsumoto Y, Ohashi M, Sasaki R. Analysis of lymphocyte subpopulations in peripheral blood in adult and juvenile cases of dermatomyositis. *J Dermatol.* (1993) 20:30–4. doi: 10.1111/j.1346-8138.1993.tb03825.x
122. Aleksza M, Szegedi A, Antal-Szalmas P, Irinyi B, Gergely L, Ponyi A, et al. Altered cytokine expression of peripheral blood lymphocytes in polymyositis and dermatomyositis. *Ann Rheum Dis.* (2005) 64:1485–9. doi: 10.1136/ard.2003.017715
123. Kubo M, Ihn H, Yamane K, Yazawa N, Kikuchi K, Soma Y, et al. Increased serum levels of soluble vascular cell adhesion molecule-1 and soluble E-selectin in patients with polymyositis/dermatomyositis. *Br J Dermatol.* (2000) 143:392–8. doi: 10.1046/j.1365-2133.2000.03668.x
124. Bloom BJ, Miller LC, Blier PR. Soluble adhesion molecules in pediatric rheumatic diseases. *J Rheumatol.* (2002) 29:832–6.
125. McCann LJ, Juggins AD, Maillard SM, Wedderburn LR, Davidson JE, Murray KJ, et al. The Juvenile Dermatomyositis National Registry and Repository (UK and Ireland)–clinical characteristics of children recruited within the first 5 yr. *Rheumatology* (2006) 45:1255–60. doi: 10.1093/rheumatology/kel099
126. Pachman LM, Hayford JR, Chung A, Daugherty CA, Pallansch MA, Fink CW, et al. Juvenile dermatomyositis at diagnosis: clinical characteristics of 79 children. *J Rheumatol.* (1998) 25:1198–204.
127. Targoff IN. Laboratory manifestations of polymyositis/dermatomyositis. *Clin Dermatol.* (1988) 6:76–92. doi: 10.1016/0738-081X(88)90051-X
128. Mathiesen PR, Zak M, Herlin T, Nielsen SM. Clinical features and outcome in a Danish cohort of juvenile dermatomyositis patients. *Clin Exp Rheumatol.* (2010) 28:782–9.
129. Ntusi NBA, Heckmann JM. Myopathy with a normal creatine kinase level in juvenile myopathic dermatomyositis. *S Afr Med J.* (2010) 100:24–5.
130. Hinderks GJ, Frolich J. Low serum creatine kinase values associated with administration of steroids. *Clin Chem.* (1979) 25:2050–1.
131. Kagen LJ, Aram S. Creatine kinase activity inhibitor in sera from patients with muscle disease. *Arthritis Rheum.* (1987) 30:213–7. doi: 10.1002/art.1780300212
132. Rider LG, Miller FW. Laboratory evaluation of the inflammatory myopathies. *Clin Diagn Lab Immunol.* (1995) 2:1–9.
133. Naim MY, Reed AM. Enzyme elevation in patients with juvenile dermatomyositis and steroid myopathy. *J Rheumatol.* (2006) 33:1392–4.
134. Hinze CH, Oommen PT, Dressler F, Urban A, Weller-Heinemann F, Speth F, et al. Development of practice and consensus-based strategies including a treat-to-target approach for the management of moderate and severe juvenile dermatomyositis in Germany and Austria. *Pediatr Rheumatol Online J* (2018) 16:40. doi: 10.1186/s12969-018-0257-6
135. Enders FB, Bader-Meunier B, Baildam E, Constantin T, Dolezalova P, Feldman BM, et al. Consensus-based recommendations for the

management of juvenile dermatomyositis. *Ann Rheum Dis.* (2017) 76:329–40. doi: 10.1136/annrheumdis-2016-209247
136. Rice GI, Melki I, Fremond M-L, Briggs TA, Rodero MP, Kitabayashi N, et al. Assessment of type I interferon signaling in pediatric inflammatory disease. *J Clin Immunol.* (2017) 37:123–32. doi: 10.1007/s10875-016-0359-1
137. Fall N, Bove KE, Stringer K, Lovell DJ, Brunner HI, Weiss J, et al. Association between lack of angiogenic response in muscle tissue and high expression of angiostatic ELR-negative CXC chemokines in patients with juvenile dermatomyositis: possible link to vasculopathy. *Arthritis Rheum.* (2005) 52:3175–80. doi: 10.1002/art.21303
138. Rodero MP, Decalf J, Bondet V, Hunt D, Rice GI, Werneke S, et al. Detection of interferon alpha protein reveals differential levels and cellular sources in disease. *J Exp Med.* (2017) 214:1547–55. doi: 10.1084/jem.20161451
139. van den Hoogen LL, van Roon JAG, Mertens JS, Wienke J, Lopes AP, de Jager W, et al. Galectin-9 is an easy to measure biomarker for the interferon signature in systemic lupus erythematosus and antiphospholipid syndrome. *Ann Rheum Dis.* (2018) 77:1810–14. doi: 10.1136/annrheumdis-2018-213497
140. Wachter H, Fuchs D, Hausen A, Reibnegger G, Werner ER. Neopterin as marker for activation of cellular immunity: immunologic basis and clinical application. *Adv Clin Chem.* (1989) 27:81–141. doi: 10.1016/S0065-2423(08)60182-1
141. Holzinger D, Frosch M, Kastrup A, Prince FHM, Otten MH, Van Suijlekom-Smit LWA, et al. The Toll-like receptor 4 agonist MRP8/14 protein complex is a sensitive indicator for disease activity and predicts relapses in systemic-onset juvenile idiopathic arthritis. *Ann Rheum Dis.* (2012) 71:974–80. doi: 10.1136/annrheumdis-2011-200598
142. Silverman ED, Laxer RM, Nelson DL, Rubin LA. Soluble interleukin-2 receptor in juvenile rheumatoid arthritis. *J Rheumatol.* (1991) 18:1398–402.
143. Kobayashi I, Okura Y, Yamada M, Kawamura N, Kuwana M, Ariga T. Anti-melanoma differentiation-associated gene 5 antibody is a diagnostic and predictive marker for interstitial lung diseases associated with juvenile dermatomyositis. *J Pediatr.* (2011) 158:675–7. doi: 10.1016/j.jpeds.2010.11.033
144. Kobayashi I, Ono S, Kawamura N, Okano M, Miyazawa K, Shibuya H, et al. KL-6 is a potential marker for interstitial lung disease associated with juvenile dermatomyositis. *J Pediatr.* (2001) 138:274–6. doi: 10.1067/mpd.2001.110324
145. Kobayashi N, Kobayashi I, Mori M, Sato S, Iwata N, Shigemura T, et al. Increased serum B cell activating factor and a proliferation-inducing ligand are associated with interstitial lung disease in patients with juvenile dermatomyositis. *J Rheumatol.* (2015) 42:2412–8. doi: 10.3899/jrheum.140977
146. Schwartz T, Sjaastad I, Flato B, Vistnes M, Christensen G, Sanner H. In active juvenile dermatomyositis, elevated eotaxin and MCP-1 and cholesterol levels in the upper normal range are associated with cardiac dysfunction. *Rheumatology* (2014) 53:2214–22. doi: 10.1093/rheumatology/keu256
147. Barth Z, Nomeland Witczak B, Schwartz T, Gjesdal K, Flato B, Koller A, et al. In juvenile dermatomyositis, heart rate variability is reduced, and associated with both cardiac dysfunction and markers of inflammation: a cross-sectional study median 13.5 years after symptom onset. *Rheumatology* (2016) 55:535–43. doi: 10.1093/rheumatology/kev376
148. van Summeren MJH, Spliet WGM, van Royen-Kerkhof A, Vermeer C, Lilien M, Kuis W, et al. Calcinosis in juvenile dermatomyositis: a possible role for the vitamin K-dependent protein matrix Gla protein. *Rheumatology* (2008) 47:267–71. doi: 10.1093/rheumatology/kem360
149. Reed A, Haugen M, Pachman LM, Langman CB. Abnormalities in serum osteocalcin values in children with chronic rheumatic diseases. *J Pediatr.* (1990) 116:574–80. doi: 10.1016/S0022-3476(05)81605-1
150. Chinoy H, Salway F, John S, Fertig N, Tait BD, Oddis C V, et al. Tumour necrosis factor-alpha single nucleotide polymorphisms are not independent of HLA class I in UK Caucasians with adult onset idiopathic inflammatory myopathies. *Rheumatology* (2007) 46:1411–6. doi: 10.1093/rheumatology/kem145y
151. Lutz J, Huwiler KG, Fedczyna T, Lechman TS, Crawford S, Kinsella TR, et al. Increased plasma thrombospondin-1 (TSP-1) levels are associated with the TNF alpha-308A allele in children with juvenile dermatomyositis. *Clin Immunol.* (2002) 103:260–3. doi: 10.1006/clim.2001.5212
152. Niewold TB, Kariuki SN, Morgan GA, Shrestha S, Pachman LM. Gene-gene-sex interaction in cytokine gene polymorphisms revealed by serum interferon alpha phenotype in juvenile dermatomyositis. *J Pediatr.* (2010) 157:653–7. doi: 10.1016/j.jpeds.2010.04.034
153. Pachman LM, Liotta-Davis MR, Hong DK, Kinsella TR, Mendez EP, Kinder JM, et al. TNFalpha-308A allele in juvenile dermatomyositis: association with increased production of tumor necrosis factor alpha, disease duration, and pathologic calcifications. *Arthritis Rheum.* (2000) 43:2368–77. doi: 10.1002/1529-0131(200010)43:10<2368::AID-ANR26>3.0.CO;2-8
154. Pachman LM, Fedczyna TO, Lechman TS, Lutz J. Juvenile dermatomyositis: the association of the TNF alpha-308A allele and disease chronicity. *Curr Rheumatol Rep.* (2001) 3:379–86. doi: 10.1007/s11926-996-0007-5
155. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). *N Engl J Med.* (1975) 292:344–7. doi: 10.1056/NEJM197502132920706
156. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). *N Engl J Med.* (1975) 292:403–7. doi: 10.1056/NEJM197502202920807
157. Wedderburn LR, Varsani H, Li CKC, Newton KR, Amato AA, Banwell B, et al. International consensus on a proposed score system for muscle biopsy evaluation in patients with juvenile dermatomyositis: a tool for potential use in clinical trials. *Arthritis Rheum.* (2007) 57:1192–201. doi: 10.1002/art.23012
158. Deakin CT, Yasin SA, Simou S, Arnold KA, Tansley SL, Betteridge ZE, et al. Muscle biopsy findings in combination with myositis-specific autoantibodies aid prediction of outcomes in juvenile dermatomyositis. *Arthritis Rheumatol.* (2016) 68:2806–16. doi: 10.1002/art.39753
159. Li CKC, Varsani H, Holton JL, Gao B, Woo P, Wedderburn LR. MHC Class I overexpression on muscles in early juvenile dermatomyositis. *J Rheumatol.* (2004) 31:605–9.
160. Sallum AME, Kiss MHB, Silva CAA, Wakamatsu A, Sachetti S, Lotufo S, et al. MHC class I and II expression in juvenile dermatomyositis skeletal muscle. *Clin Exp Rheumatol.* (2009) 27:519–26.
161. Sallum AME, Marie SKN, Wakamatsu A, Sachetti S, Vianna MAAG, Silva CAA, et al. Immunohistochemical analysis of adhesion molecule expression on muscle biopsy specimens from patients with juvenile dermatomyositis. *J Rheumatol.* (2004) 31:801–7.
162. Sakuta R, Murakami N, Jin Y, Nagai T, Nonaka I, Nishino I. Diagnostic significance of membrane attack complex and vitronectin in childhood dermatomyositis. *J Child Neurol.* (2005) 20:597–602. doi: 10.1177/08830738050200071201
163. Tezak Z, Hoffman EP, Lutz JL, Fedczyna TO, Stephan D, Bremer EG, et al. Gene expression profiling in DQA1*0501+ children with untreated dermatomyositis: a novel model of pathogenesis. *J Immunol.* (2002) 168:4154–63. doi: 10.4049/jimmunol.168.8.4154
164. Shrestha S, Wershil B, Sarwark JF, Niewold TB, Philipp T, Pachman LM. Lesional and nonlesional skin from patients with untreated juvenile dermatomyositis displays increased numbers of mast cells and mature plasmacytoid dendritic cells. *Arthritis Rheum.* (2010) 62:2813–22. doi: 10.1002/art.27529
165. Wargula JC, Lovell DJ, Passo MH, Bove KE, Santangelo JD, Levinson JE. What more can we learn from muscle histopathology in children with dermatomyositis/polymyositis? *Clin Exp Rheumatol.* (2006) 24:333–43.
166. Soponkanaporn S, Deakin CT, Schutz PW, Marshall LR, Yasin SA, Johnson CM, et al. Expression of myxovirus-resistance protein A: a possible marker of muscle disease activity and autoantibody specificities in juvenile dermatomyositis. *Neuropathol Appl Neurobiol.* (2018). [Epub ahead of print].
167. Gitiaux C, Latroche C, Weiss-Gayet M, Rodero MP, Duffy D, Bader-Meunier B, et al. Myogenic progenitor cells exhibit type i interferon-driven proangiogenic properties and molecular signature during juvenile dermatomyositis. *Arthritis Rheumatol.* (2018) 70:134–45. doi: 10.1002/art.40328
168. Minetti C, Gattorno M, Repetto S, Gregorio A, Pedemonte M, Assereto S, et al. Chemokine receptor CCR7 is expressed in muscle fibers in juvenile dermatomyositis. *Biochem Biophys Res Commun.* (2005) 333:540–543. doi: 10.1016/j.bbrc.2005.05.146
169. Lopez de Padilla CM, Vallejo AN, McNallan KT, Vehe R, Smith SA, Dietz AB, et al. *Plasmacytoid dendritic cells in inflamed muscle of patients with juvenile dermatomyositis. Arthritis Rheum.* (2007) 56:1658–68.
170. Englund P, Nennesmo I, Klareskog L, Lundberg IE. Interleukin-1alpha expression in capillaries and major histocompatibility complex class I expression in type II muscle fibers from polymyositis and dermatomyositis patients: important pathogenic features independent

of inflammatory cell clusters in muscle. *Arthritis Rheum.* (2002) 46:1044–55. doi: 10.1002/art.10140

171. Kim E, Cook-Mills J, Morgan G, Sredni ST, Pachman LM. Increased expression of vascular cell adhesion molecule 1 in muscle biopsy samples from juvenile dermatomyositis patients with short duration of untreated disease is regulated by miR-126. *Arthritis Rheum.* (2012) 64:3809–17. doi: 10.1002/art.34606
172. Chen YW, Shi R, Geraci N, Shrestha S, Gordish-Dressman H, Pachman LM. Duration of chronic inflammation alters gene expression in muscle from untreated girls with juvenile dermatomyositis. *BMC Immunol.* (2008) 9:43. doi: 10.1186/1471-2172-9-43
173. Zhao Y, Fedczyna TO, McVicker V, Caliendo J, Li H, Pachman LM. Apoptosis in the skeletal muscle of untreated children with juvenile dermatomyositis: impact of duration of untreated disease. *Clin Immunol.* (2007) 125:165–72. doi: 10.1016/j.clim.2007.06.011
174. Lopez De Padilla CM, Vallejo AN, Lacomis D, McNallan K, Reed AM. Extranodal lymphoid microstructures in inflamed muscle and disease severity of new-onset juvenile dermatomyositis. *Arthritis Rheum.* (2009) 60:1160–72. doi: 10.1002/art.24411
175. Shinjo SK, Sallum AME, Silva CA, Marie SKN. Skeletal muscle major histocompatibility complex class I and II expression differences in adult and juvenile dermatomyositis. *Clinics* (2012) 67:885–90. doi: 10.6061/clinics/2012(08)05
176. Iglesias E, Jou C, Bou R, Anton J. [Importance of muscle biopsy in the diagnosis of juvenile dermatomyositis]. *An Pediatr.* (2014) 80:e25–6. doi: 10.1016/j.anpedi.2013.04.017
177. Mamyrova G, Rider LG, Ehrlich A, Jones O, Pachman LM, Nickeson R, et al. (2017). Environmental factors associated with disease flare in juvenile and adult dermatomyositis. *Rheumatology* 56:1342–7. doi: 10.1093/rheumatology/kex162
178. Manlhiot C, Liang L, Tran D, Bitnun A, Tyrrell PN, Feldman BM. Assessment of an infectious disease history preceding juvenile dermatomyositis symptom onset. *Rheumatology* (2008) 47:526–9. doi: 10.1093/rheumatology/ken038
179. Pachman LM, Lipton R, Ramsey-Goldman R, Shamiyeh E, Abbott K, Mendez EP, et al. History of infection before the onset of juvenile dermatomyositis: results from the National Institute of Arthritis and Musculoskeletal and Skin Diseases Research Registry. *Arthritis Rheum.* (2005) 53:166–72. doi: 10.1002/art.21068
180. Pachman LM, Litt DL, Rowley AH, Hayford JR, Caliendo J, Heller S, et al. Lack of detection of enteroviral RNA or bacterial DNA in magnetic resonance imaging-directed muscle biopsies from twenty children with active untreated juvenile dermatomyositis. *Arthritis Rheum.* (1995) 38:1513–8. doi: 10.1002/art.1780381019
181. Jia H, Thelwell C, Dilger P, Bird C, Daniels S, Wadhwa M. Endothelial cell functions impaired by interferon *in vitro*: Insights into the molecular mechanism of thrombotic microangiopathy associated with interferon therapy. *Thromb Res.* (2018) 163:105–16. doi: 10.1016/j.thromres.2018.01.039
182. Aanhane E, Schulkens IA, Heusschen R, Castricum K, Leffler H, Griffioen AW, et al. Different angioregulatory activity of monovalent galectin-9 isoforms. *Angiogenesis* (2018) 21:545–55. doi: 10.1007/s10456-018-9607-8
183. O'Brien MJ, Shu Q, Stinson WA, Tsou P-S, Ruth JH, Isozaki T, et al. A unique role for galectin-9 in angiogenesis and inflammatory arthritis. *Arthritis Res Ther.* (2018) 20:31. doi: 10.1186/s13075-018-1519-x
184. Heusschen R, Schulkens IA, van Beijnum J, Griffioen AW, Thijssen VL. Endothelial LGALS9 splice variant expression in endothelial cell biology and angiogenesis. *Biochim Biophys Acta.* (2014) 1842:284–92. doi: 10.1016/j.bbadis.2013.12.003
185. Campanella GS V, Colvin RA, Luster AD. CXCL10 can inhibit endothelial cell proliferation independently of CXCR3. *PLoS ONE* (2017) 5:e12700. doi: 10.1371/journal.pone.0012700
186. Ladislau L, Suarez-Calvet X, Toquet S, Landon-Cardinal O, Amelin D, Depp M, et al. JAK inhibitor improves type I interferon induced damage: proof of concept in dermatomyositis. *Brain* (2018) 141:1609–21. doi: 10.1093/brain/awy105
187. Crescioli C, Sottili M, Bonini P, Cosmi L, Chiarugi P, Romagnani P, et al. Inflammatory response in human skeletal muscle cells: CXCL10 as a potential therapeutic target. *Eur J Cell Biol.* (2012) 91:139–49. doi: 10.1016/j.ejcb.2011.09.011
188. Nagaraju K, Rider LG, Fan C, Chen Y-W, Mitsak M, Rawat R, et al. Endothelial cell activation and neovascularization are prominent in dermatomyositis. *J Autoimmune Dis.* (2006) 3:2. doi: 10.1186/1740-2557-3-2
189. Sorensen EW, Lian J, Ozga AJ, Miyabe Y, Ji SW, Bromley SK, et al. CXCL10 stabilizes T cell-brain endothelial cell adhesion leading to the induction of cerebral malaria. *JCI Insight* (2018) 3:98911. doi: 10.1172/jci.insight.98911
190. Fu H, Kishore M, Gittens B, Wang G, Coe D, Komarowska I, et al. Self-recognition of the endothelium enables regulatory T-cell trafficking and defines the kinetics of immune regulation. *Nat Commun.* (2014) 5:3436. doi: 10.1038/ncomms4436
191. Pober JS, Merola J, Liu R, Manes TD. Antigen presentation by vascular cells. *Front Immunol.* (2017) 8:1907. doi: 10.3389/fimmu.2017.01907
192. Huber AM, Robinson AB, Reed AM, Abramson L, Bout-Tabaku S, Carrasco R, et al. Consensus treatments for moderate juvenile dermatomyositis: beyond the first two months. *Results of the second Childhood Arthritis and Rheumatology Research Alliance consensus conference. Arthritis Care Res.* (2012) 64:546–53. doi: 10.1002/acr.20695
193. McCann LJ, Pilkington CA, Huber AM, Ravelli A, Appelbe D, Kirkham JJ, et al. Development of a consensus core dataset in juvenile dermatomyositis for clinical use to inform research. *Ann Rheum Dis.* (2018) 77:241–50. doi: 10.1136/annrheumdis-2017-212141
194. van Dijkhuizen, E. P., Deakin, C. T., Wedderburn, L. R., and De Iorio, M. (2017). Modelling disease activity in juvenile dermatomyositis: a Bayesian approach. *Stat Methods Med Res. (2017)*. doi: 10.1177/0962280217713233
195. Lim LSH, Pullenayegum E, Moineddin R, Gladman DD, Silverman ED, Feldman BM. Methods for analyzing observational longitudinal prognosis studies for rheumatic diseases: a review and worked example using a clinic-based cohort of juvenile dermatomyositis patients. *Pediatr Rheumatol Online J.* (2017) 15:18. doi: 10.1186/s12969-017-0148-2
196. Higgs BW, Zhu W, Morehouse C, White WI, Brohawn P, Guo X, et al. A phase 1b clinical trial evaluating sifalimumab, an anti-IFN-alpha monoclonal antibody, shows target neutralisation of a type I IFN signature in blood of dermatomyositis and polymyositis patients. *Ann Rheum Dis.* (2014) 73:256–62. doi: 10.1136/annrheumdis-2012-202794
197. Guo X, Higgs BW, Rebelatto M, Zhu W, Greth W, Yao Y, et al. Suppression of soluble T cell-associated proteins by an anti-interferon-alpha monoclonal antibody in adult patients with dermatomyositis or polymyositis. *Rheumatology* (2014) 53:686–695. doi: 10.1093/rheumatology/ket413

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Neutrophils From Children With Systemic Juvenile Idiopathic Arthritis Exhibit Persistent Proinflammatory Activation Despite Long-Standing Clinically Inactive Disease

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Background: Systemic juvenile idiopathic arthritis (SJIA) is a chronic childhood arthropathy with features of autoinflammation. Early inflammatory SJIA is associated with expansion and activation of neutrophils with a sepsis-like phenotype, but neutrophil phenotypes present in longstanding and clinically inactive disease (CID) are unknown. The objective of this study was to examine activated neutrophil subsets, S100 alarmin release, and gene expression signatures in children with a spectrum of SJIA disease activity.

Methods: Highly-purified neutrophils were isolated using a two-step procedure of density-gradient centrifugation followed by magnetic-bead based negative selection prior to flow cytometry or cell culture to quantify S100 protein release. Whole transcriptome gene expression profiles were compared in neutrophils from children with both active SJIA and CID.

Results: Patients with SJIA and active systemic features demonstrated a higher proportion of $CD16^{+}CD62L^{lo}$ neutrophil population compared to controls. This neutrophil subset was not seen in patients with CID or patients with active arthritis not exhibiting systemic features. Using imaging flow cytometry, $CD16^{+}CD62L^{lo}$ neutrophils from patients with active SJIA and features of macrophage activation syndrome (MAS) had increased nuclear hypersegmentation compared to $CD16^{+}CD62L^{+}$ neutrophils. Serum levels of S100A8/A9 and S100A12 were strongly correlated with peripheral blood neutrophil counts. Neutrophils from active SJIA patients did not show enhanced resting S100 protein release; however, regardless of disease activity, neutrophils from SJIA patients did show enhanced S100A8/A9 release upon PMA stimulation compared to control neutrophils. Furthermore, whole transcriptome analysis of highly purified neutrophils from children with active SJIA identified 214 differentially expressed genes (DEG) compared to neutrophils from healthy controls. The most significantly upregulated gene pathway was Immune System Process, including *AIM2, IL18RAP,* and *NLRC4*. Interestingly, this gene set showed intermediate levels of expression in neutrophils

from patients with long-standing CID yet persistent serum IL-18 elevation. Indeed, all patient samples regardless of disease activity demonstrated elevated inflammatory gene expression, including inflammasome components and *S100A8*.

Conclusion: We identify features of neutrophil activation in SJIA patients with both active disease and CID, including a proinflammatory gene expression signature, reflecting persistent innate immune activation. Taken together, these studies expand understanding of neutrophil function in chronic autoinflammatory disorders such as SJIA.

Keywords: macrophage activation syndrome, S100, neutrophil subsets, gene expression, systemic JIA, autoinflammation

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA) is a severe and distinctive subtype of juvenile idiopathic arthritis (JIA). Along with arthropathy, SJIA is characterized by quotidian fevers, evanescent rash, adenopathy, hepatomegaly, and serositis (1). Children with SJIA are also at risk for life-threating complications including macrophage activation syndrome (MAS) and severe lung disease (2, 3). The pathogenesis of SJIA is incompletely understood; however, it has many shared features with the monogenic autoinflammatory disorders. In contrast to autoimmune diseases, autoinflammatory disorders typically lack autoreactive lymphocytes or high-titer autoantibodies, and are instead defined by excessive and uncontrolled activation of innate immunity (4). In support of this, SJIA is characterized by increased circulating innate immune effectors, upregulation of monocyte/macrophage differentiation genes, as well as high levels of monocyte-derived proinflammatory cytokines including IL-1, IL-6, and IL-18 (5–12). Children with SJIA also typically have excellent clinical response to biologic therapy targeting IL-1 and IL-6 (13).

Neutrophils are the most numerous innate immune effectors in the circulation, and have key roles in both host defense and autoinflammation. Neutrophils ingest particulate material through phagocytosis, and kill microbes through overlapping oxidative and non-oxidative mechanisms (14). Neutrophils are also key sources of proinflammatory mediators, including the alarmin proteins S100A8/A9 (calprotectin) and S100A12 (calgranulin C), which amplify innate immune signaling (15). During severe systemic inflammation, neutrophils become rapidly activated, leading to reactive oxygen species (ROS) production, release of neutrophil extracellular traps (NETs), and changes in gene expression profiles (16). While neutrophils have classically been considered terminally differentiated cells with homogenous functions, increasing evidence suggests that functional neutrophil subsets emerge during states of systemic inflammation. This includes both immature "banded" neutrophils as well as mature neutrophils with immunomodulatory properties (16, 17). These later cells, sometimes termed "suppressive neutrophils," are defined as $CD16^{+}CD62L^{dim}$, can display nuclear hypersegmentation, have distinct transcriptomes (18), and suppress T cell responses through ROS and direct cell contact (18–20).

Neutrophils also appear to have key roles in the pathogenesis of autoinflammatory disorders such as SJIA. Circulating neutrophils are markedly increased in active SJIA (21), and neutrophil numbers are associated with IL-1 related gene expression profiles in whole blood (22). Neutrophil-derived mediators such as S100 proteins are markedly elevated in active SJIA (23). A recent comprehensive analysis of neutrophil phenotypes in primarily new-onset disease significantly advanced the understanding of these cells in SJIA (24). This work found that neutrophil numbers strongly correlate with inflammatory disease parameters, have a primed and sepsis-like phenotype, and that circulating counts rapidly normalize with successful IL-1 blockade. In many children however, SJIA has a chronic and sustained disease course, associated with persistently elevated serum IL-18 levels and epigenetic changes in monocytes (12, 25). In contrast, little is known regarding whether neutrophil phenotypic and functional abnormalities persist in chronic SJIA and/or clinically inactive disease (CID).

In this study, we examined neutrophil subsets, functional properties, and gene expression signatures in large cohort of children with SJIA, including new-onset disease, active disease (both systemic and/or arthritic features), and CID. We hypothesized that active SJIA is associated with significant, proinflammatory changes in neutrophil phenotypes. We also hypothesized that these changes persist in patients with longstanding inactive disease but with signs of persistent immune activation.

MATERIALS AND METHODS

Patients

This study was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center (IRB 2016-2234), and written informed consent was obtained from all adult patients and from the parents or legal guardians of enrolled children. Systemic juvenile idiopathic arthritis (SJIA) was diagnosed based on the International League of Associations for Rheumatology diagnostic criteria (26), though for several patients, samples were obtained and treatment initiated with disease duration <6 weeks, in agreement with the operational definition of SJIA as described (27). Patients were considered as having active SJIA if they had presence of any active arthritis; any systemic features including rash, fever, adenopathy, or hepatosplenomegaly; or elevated ESR or CRP. Patients were

considered to have CID based on the Wallace criteria (28). MAS was diagnosed per the treating physician; however, all MAS episodes also satisfied the 2018 MAS Classification Criteria (29). Patients were enrolled and peripheral blood samples were collected during routine visits, and laboratory information was gathered from testing done during the routine clinical care. Serum was collected, alloquated and stored at −80°C until analyzed.

Control samples were recruited from children undergoing evaluation for joint pain at the pediatric rheumatology clinic at CCHMC but found to have non-inflammatory conditions, as well as healthy young adult donors (ages 18–30) through the Cell Processing and Manipulation Core at CCHMC.

Neutrophil Isolation and Purification

Neutrophils were isolated from fresh whole blood collected in ACD solution A vacutainer (Becton Dickinson, Franklin Lakes, NJ) tubes. Briefly, neutrophils were isolated through density gradient centrifugation as described (30). Cells were then further purified using magnetic-bead based negative selection with the MACSexpress Human Whole Blood Neutrophil Isolation kit (Miltenyi Biotec, Germany), followed by hypotonic lysis of remaining red blood cells. Neutrophil purity was assessed by flow cytometry (see below) and was typically >98%.

Antibodies and Flow Cytometry

All antibodies were from BD Biosciences. Antibodies used for these studies were Pacific Blue conjugated anti-human CD14 (clone M5E2), FITC conjugated anti-human CD15 (clone MMA), BV711 or PE conjugated anti-human CD16 (clone 3G5), APC, or AF647 conjugated anti-human CD62L (clone DREG56), BV421 conjugated anti-human CD193 (clone 5E8), and PE conjugated anti-human Siglec8 (clone 7C9). Approximately 1 million neutrophils were stained for surface markers for 30 min at 4°C. For imaging cytometry experiments, cells were then stained with 300nM DAPI (Thermofisher, Waltham, MA) for 10 min. The cells were then washed with FACS buffer (PBS supplemented with 1% fetal calf serum) prior to filtering and transferred to 12 × 75 mm polystyrene tubes. Cells were acquired using a BD LSR Fortessa analytical cytometer. Data was analyzed by FACSDiva and FlowJo software.

Imaging flow cytometry was performed on an ImageStreamX (EMD Millipore) two camera system equipped with 405, 488, 642, and 785 nm lasers. Cells were imaged using the 60 × objective with the 785 nm laser turned off. Laser powers were set to optimize fluorescence detection with 405 nm set to 20 mW, 288 nm set to 20 mW, and the 642 nm set to 50 mW. Classifiers were set on brightfield to eliminate debris and on fluorescence channels to eliminate saturated images. Focused images of single cells were analyzed using the lobe count feature on the morphology mask of the DAPI signal to compare nuclear segmentation of CD16+ cells with either high or low expression of CD62L.

Neutrophil Culture and S100 Alarmin Release

To quantify release of S100 alarmin proteins, 1×10^6 isolated purified neutrophils were incubated in RPMI and either left untreated or stimulated with phorbol myristate acetate (PMA) for 4 h. Subsequently, culture supernatants were collected, centrifuged to remove cells, and stored at −80°C. S100A8/A9 was determined using specific ELISA kit obtained from ALPCO (Salem, NH), and serum S100A12 levels using specific ELISA kits obtained from MBL (Woburn, MA).

Whole Transcriptome Analysis

Total RNA from purified neutrophils was extracted using the MagMax −96 Total RNA Isolation Kit (Life Technologies), and quantified via Qubit RNA HS Assay Kit (Life Technologies). Using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), 10 ng of RNA was reverse transcribed to make cDNA. The Ion AmpliSeq Library Kit Plus (Life Technologies) and the Ion AmpliSeq Transcriptome Human Gene Expression Core Panel was used to amplify target genes per manufacturer's directions. Each amplicon was then barcoded with the Ion Express Barcode Adapter (Life Technologies), and purified through Agencourt AMPure XP Beads (Beckman Coulter) and freshly prepared 70% ethanol. Finally, libraries were analyzed with High Sensitivity NGS Fragment Analysis Kit (AATI). All libraries were peaked around 200 bp.

Before sequencing, the concentration of the library was determined via Qubit dsDNA HS kit (Life Technologies) and diluted with nuclease-free water to 100 pM. Using the Ion 540 Kit-OT2 along with the Ion OneTouch 2 Instrument, the library was amplified on the Ion Spheres Particles (ISP) through emulsion PCR. Then, template-positive ISPs was recovered, and their quality was assessed through the Ion Sphere Quality Control Kit and the Qubit 2.0 Fluorometer. The ISPs were then enriched with Dynabead MyOne Streptavidin C1 Beads to select the clonally amplified DNA. Sequencing primers were annealed to the enriched ISPs and loaded on the Ion 540 Chip along with the Ion S5 Sequencing Polymerase. The loaded chip was run on the Ion S5 sequencer, and the amplicon regions were mapped with hg19_AmpliSeq_Transcriptome_ERCC_V1 reference from Ion Community.

After reads were mapped they were converted into reads per kilobase of transcript per million mapped reads (RPKM). Differentially expressed genes (DEG) (fold change >2, $p < 0.05$) were determined using AltAnalyze (31). This package was also utilized to perform principle component analysis, hierarchical clustering of DEGs, and pathway analysis for significantly enriched gene ontology pathways and transcription factor targets. The gene expression datasets for this study can be found in Gene Expression Omnibus (GSE122552).

RESULTS

Mature CD16$^+$CD62L^{dim} Neutrophils in Systemically Active SJIA

Circulating neutrophil counts are markedly elevated in patients with active SJIA, and recent work has described a sepsis-like phenotype during the early inflammatory phase at disease onset (24). To further characterize functional neutrophil properties throughout SJIA disease course, we utilized a two-step procedure to obtain highly purified (>98%) and untouched cell populations (**Figure 1A**). First, granulocytes were separated through density

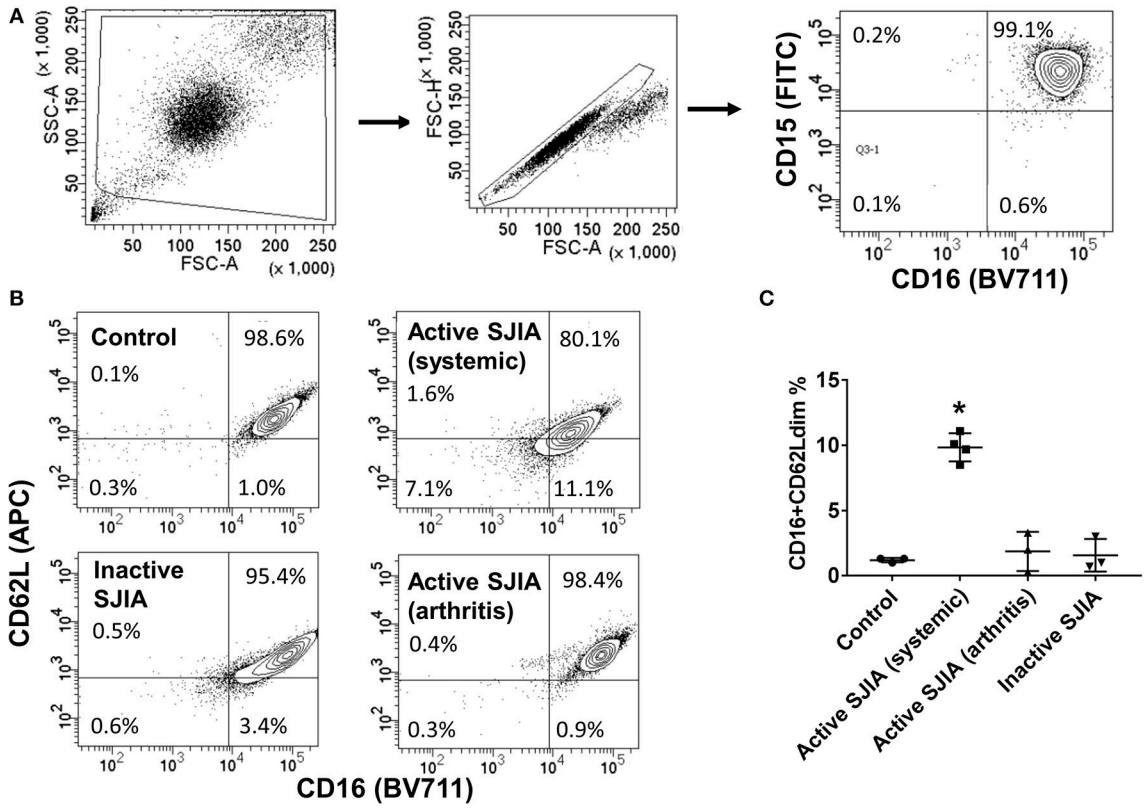

FIGURE 1 | Flow cytometry analysis of highly purified neutrophil populations. Neutrophils were stained with FITC-conjugated anti-CD15, BV711-conjugated anti-CD16, and APC-conjugated anti-CD62L before being analyzed. **(A)** Cells were gated for live cells by FSC/SSC, then for doublet discrimination, then to identify percentage of CD15+CD16+ neutrophils. **(B,C)** Neutrophils were analyzed for relative percentage of mature $CD16^{+}CD62L^{dim}$ cells. Gates were set using healthy controls to define CD62L thresholds. Data are representative of control neutrophils, neutrophils from patients with SJIA and active systemic features, active arthritis only, and inactive SJIA. Pooled data are shown in **(C)**. $^{*}p = 0.001$ vs. control as determined by ANOVA with Dunnett's multiple comparisons test.

gradient centrifugation (30). Following this, a magnetic-bead based negative selection step further purified the neutrophil suspension. Of note, this procedure produced populations with minimal (<1%) contamination with CD14+ monocytes, the higher RNA content of which can alter interpretation of transcriptional profiles (32), or from CD193+Siglec8+ eosinophils (data not shown).

Purified peripheral blood neutrophils were obtained from a large cohort of patients with SJIA including various states of disease activity including new-onset disease, active disease (both systemic and/or arthritic features) and CID, as well as both pediatric and adult healthy controls (**Table 1**). While circulating mature neutrophils had generally been considered to be a homogenous cell population, recent work has identified functional neutrophil subsets emerging in states of systemic inflammation (19). These include neutrophils described as $CD16^{+}CD62L^{dim}$, and capable of suppressing T cell proliferation through integrins and ROS production (17). We examined purified neutrophils from SJIA patients with both active and inactive disease for presence of this $CD16^{+}CD62L^{dim}$ subset. Approximately 10% of neutrophils from children with SJIA and active systemic features were $CD16^{+}CD62L^{dim}$, which was significantly higher than that seen with control neutrophils (**Figures 1B,C**). Patients with active SJIA also manifested lower neutrophil CD16 signal, possibly reflecting either an increase in both immature banded neutrophils ($CD16^{dim}$ $CD62L^{+}$) and apoptotic neutrophils ($CD16^{dim}CD62L^{dim}$) and/or shedding of CD16 by sustained neutrophil activation (33). In contrast, patients with either CID or active SJIA with only arthritis and no systemic features did not show increases in $CD16^{+}CD62L^{dim}$ (**Figures 1B,C**). These findings suggest that mature $CD16^{+}CD62L^{dim}$ neutrophils are specific to the systemic inflammatory phase of SJIA.

$CD16^{+}CD62L^{dim}$ neutrophils have also been reported to have a hypersegmented nuclear appearance, both when associated with systemic LPS administration (19) and with *Helicobacter pylori* infection (34). To quantify this process, we utilized imaging cytometry to determine the number of nuclear lobes present in $CD16^{+}CD62L^{+}$ and $CD16^{+}CD62L^{dim}$ neutrophils. Using DAPI nuclear staining and spot counting, we could discriminate subpopulations of neutrophils with bi-lobed nuclei (**Figure 2A**) from those with hypersegmented nuclei (>4 nuclear lobes; **Figure 2B**). Through this imaging cytometry approach, we found that ∼10% of neutrophils in all samples had ≥4

TABLE 1 | Clinical and laboratory characteristics of patients enrolled for neutrophil collection.

	Active SJIA ($n = 23$)	Inactive SJIA ($n = 22$)
Age, median	8 (5–14)	11 (6.75–16)
Sex	11F, 12M	13F, 9M
Ferritin (ng/mL)	559.5 (65.6–5379)	23.5 (17.25–33.5)
CRP (mg/dL)	5.04 (0.22–9.88)	<0.29 (<0.29 to <0.29)
ESR (mm/hr)	59 (15–76)	5 (2–9)
IL-18 (pg/mL)	19852 (3632–111678)	956 (321–2697)
S100A8/A9 (ng/mL)	6869 (442.5–33116)	1615 (680–3431)
S100A12 (ng/mL)	220 (56–605.8)	76 (55–182)
Fever	34.8% (8/23)	N/A
Arthritis	70.0% (16/23)	N/A
Systemic features	34.8% (8/23)	N/A
Elevated ESR/CRP	82.6% (19/23)	N/A
New-onset SJIA	17.4% (4/23)	N/A
MAS or subclinical MAS	21.7% (5/23)	N/A
Time in CID, median	N/A	8 months (5.75–18.5)
History of MAS	60.9% (14/23)	31.8% (7/22)
History of chronic lung disease	17.4% (4/23)	4.5% (1/22)

IL-18 standard ranges 89–540 pg/mL. S100A8/A9 standard ranges 716–3004 ng/mL. S100A12 standard ranges 32–385 ng/mL.

nuclear lobes. However, in SJIA patients with systemically active disease and features of early/subclinical MAS, substantially more hypersegmented cells were visualized, particularly amongst the $CD16^{+}CD62L^{dim}$ population compared to $CD16^{+}CD62L^{+}$ cells (22.9% vs. 11.3%; **Figures 2C,D**). Together, this demonstrates that systemically active SJIA patients have circulating, mature $CD16^{+}CD62L^{dim}$ neutrophils.

Neutrophils From Both Active and Inactive SJIA Have Enhanced S100A8/A9 Release Capacity

S100 alarmin proteins, including S100A8/A9 (calprotectin) and S100A12 (calgranulin C), are host derived proinflammatory mediators that amplify innate immune responses by signaling through pattern recognition receptors (PRR) including TLR4 (15). These proteins are believed to be produced primarily by activated phagocytes, and present at very high levels in systemic inflammation as seen in SJIA (23). First, we confirmed that patients with active SJIA had significantly higher serum levels of S100A8/A9 and S100A12 than those with inactive disease (**Table 1**). We also confirmed prior work that peripheral neutrophil counts strongly and significantly correlate with serum S100A8/A9 ($R = 0.55$, $p < 0.001$) and S100A12 ($R = 0.64$, $p < 0.001$) levels (**Figure 3A**). In contrast, there was no significant correlation between the absolute lymphocyte count and S100A8/A9 levels ($R = -0.08$ and 0.07, respectively).

Recent work has shown that in the autoinflammatory disease familial Mediterranean fever (FMF), during active disease peripheral neutrophils constitutively and spontaneously release high levels of S100A12 (35). In order to quantify S100 protein release, 1×10^6 highly purified neutrophils were placed in tissue culture and incubated for 4 h. After incubation, cell-free supernatants were collected to determine S100A8/A9 and S100A12 release. Interestingly, there was no significant difference in levels of S100 alarmin proteins released by unstimulated neutrophils during either active or inactive SJIA (**Figure 3B**). We also determined the amount of S100 proteins that could be released by neutrophils upon stimulating with PMA to induce activation and degranulation. Compared to control neutrophils, cells from patient with both active and inactive SJIA released significantly more S100A8/A9 upon PMA stimulation (**Figure 3C**). There was no significant difference in amount of S100A12 released by neutrophils from either active or inactive disease. Taken together, these findings suggest that even during CID, neutrophils have increased capacity for S100A8/A9 alarmin release upon cell activation.

Proinflammatory Neutrophils Gene Expression Signatures in Both Active and Inactive SJIA

While previous work has described gene expression profiling of neutrophils from small cohorts of children with active SJIA (24, 36), little is known regarding these signatures in longstanding and inactive disease. We utilized the Ampliseq Transcriptome platform to determine gene expression profiles from highly purified neutrophils. Ampliseq Transcriptome is an amplicon-based gene expression system with near whole transcriptome coverage (>20,000 coding genes) and high correlation to microarray and RNA-sequencing (37). Gene expression profiling was performed on 14 neutrophil samples: (1) 4 from patients with systemically active SJIA but without MAS features, (2) 5 from patients with CID on medication, and (3) 5 healthy controls (**Supplemental Table 1**). Of note, the included patients with CID had longstanding remission on medication (6–48 months) but had persistently elevated serum IL-18 levels at least twice upper limit of normal (1163–8729 ng/ml). Principal component analysis showed clear distinction between transcriptomes of active SJIA neutrophils from control neutrophils, with inactive disease samples showing intermediate changes as compared to neutrophils from controls (**Figure 4A**). Using cut-offs of >2.0 fold change and $p < 0.05$, 139 genes were significantly upregulated and 75 genes significantly downregulated in neutrophils from active SJIA compared to controls. Hierarchical clustering based on these DEG is shown in **Figure 4B**; full list of DEG is shown in **Supplemental Table 2**. These genes included cell surface and PRR such as Fc-gamma receptor genes, *CR1, TLR2,* and *TLR5*; cytosolic PRR including *AIM2*, *NLRC4*, and *DDX58*; and *IL18RAP*.

To identify functional pathways of DEG in active SJIA neutrophils, Gene Ontology (GO) analysis was performed using AltAnalyze. The most significantly enriched GO pathways among upregulated DEG are shown in **Table 2**. The top GO pathway was Immune System Process (adjusted $p = 3.44 \times 10^{-16}$), with other immune/inflammatory pathways also highly enriched. The specific DEG contributing to this enriched pathway are shown in **Table 3**. Among downregulated DEG, no GO pathways had

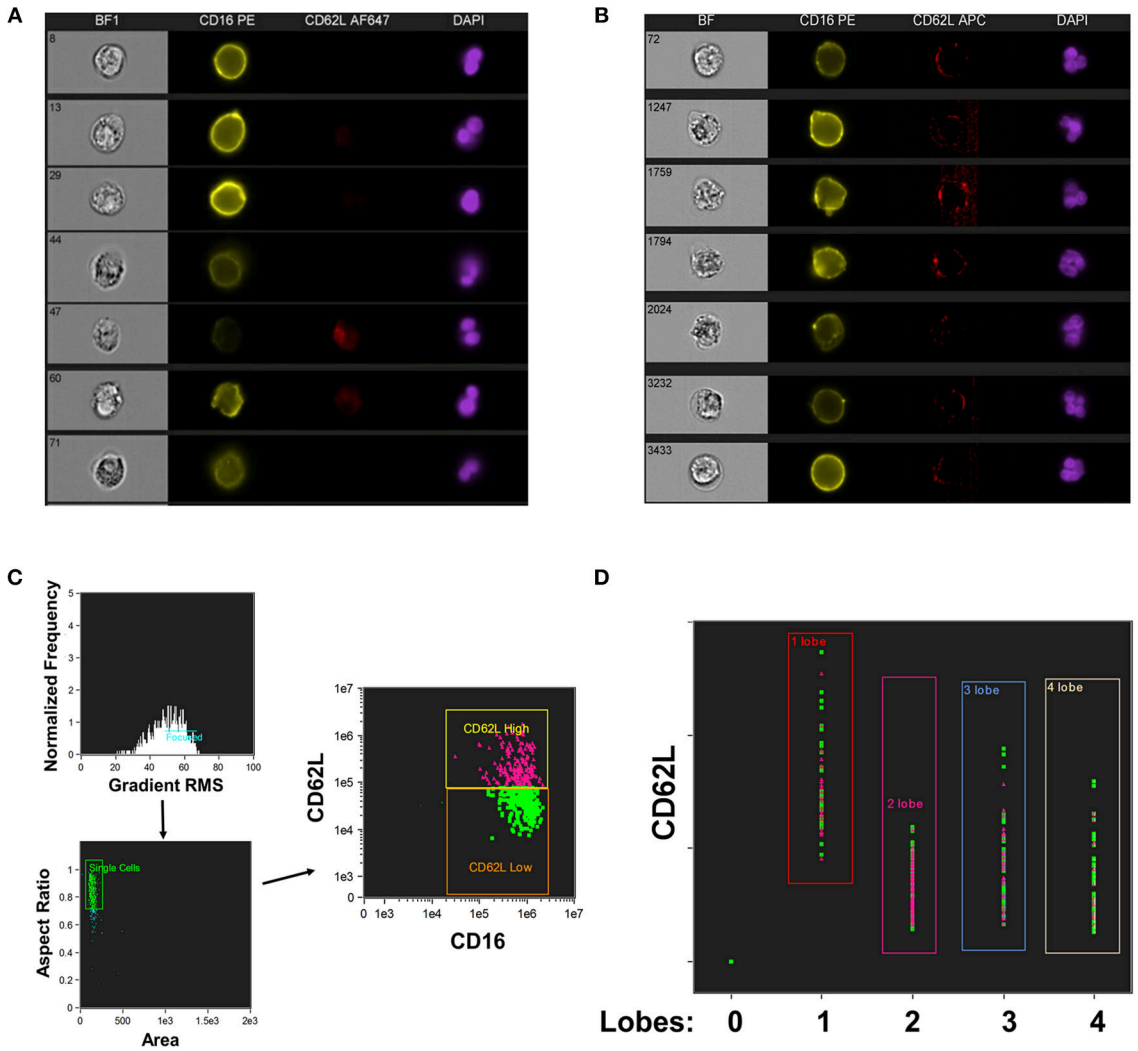

FIGURE 2 | Nuclear segmentation in neutrophils from active SJIA. Highly purified neutrophils were stained with PE-conjugated anti-CD16, APC- or AF647-conjugated anti-CD62L, and DAPI nuclear stain before analyzing with the Imagestream imaging cytometer. **(A,B)** representative images of neutrophils scored as having 2 lobes **(A)** and ≥4 nuclear lobes **(B)**. **(C,D)** Analyzed neutrophils from a patient with active SJIA and features of subclinical MAS. **(C)** Cells were gated for focused cells, single cells, and finally for $CD16^{+}CD62L^{+}$ (pink) and $CD16^{+}CD62L^{dim}$ (green) neutrophils. **(D)** Distribution of $CD16^{+}CD62L^{+}$ (pink) and $CD16^{+}CD62L^{dim}$ (green) neutrophils by number of identified nuclear lobes.

an adjusted $p < 0.05$; the most significantly enriched pathway was negative regulation of cellular metabolism (raw $p = 0.005$; z-score 3.11). When examining upregulated DEG for enriched transcription factor regulatory circuits, the most enriched circuit was immune response genes (**Figure 4C**). Specific transcription factor binding sites among upregulated genes included ISGF-3 ($p = 0.006$), STAT3 ($p = 0.02$), and Elf-1 ($p = 0.04$). Of note, ISGF-3 can be activated by type I interferon signaling (38, 39). Multiple serum cytokine studies have failed to identify type I or type II interferon signatures in the peripheral blood of SJIA patients with active disease (40, 41). Similarly, while we did find a significant enrichment in "regulation of type I interferon production" GO pathway ($p = 0.003$), this was driven by a small number of genes with pleotropic activation (*DDX58*, *IFI16*, *IFIH1*, *NFKBIA*, and *TLR2*), none of which were found to be highly specific for type I interferon responses (42).

Interestingly, hierarchical clustering based on DEG from active SJIA neutrophils also largely differentiated neutrophils from patients with CID from controls (**Figure 4B**). Indeed, 4/5 inactive disease samples clustered together, with intermediate expression of upregulated DEG, while 4/5 control samples clustered separately with the lowest expression of this signature. To further investigate neutrophil gene expression changes in CID, we determined DEG between all SJIA patients (active and inactive disease) and controls. This revealed 97 significantly upregulated genes and 81 downregulated genes (fold change >2.0, $p < 0.05$; **Supplemental Table 3**). When this signature was used for hierarchical clustering, clear distinction was shown

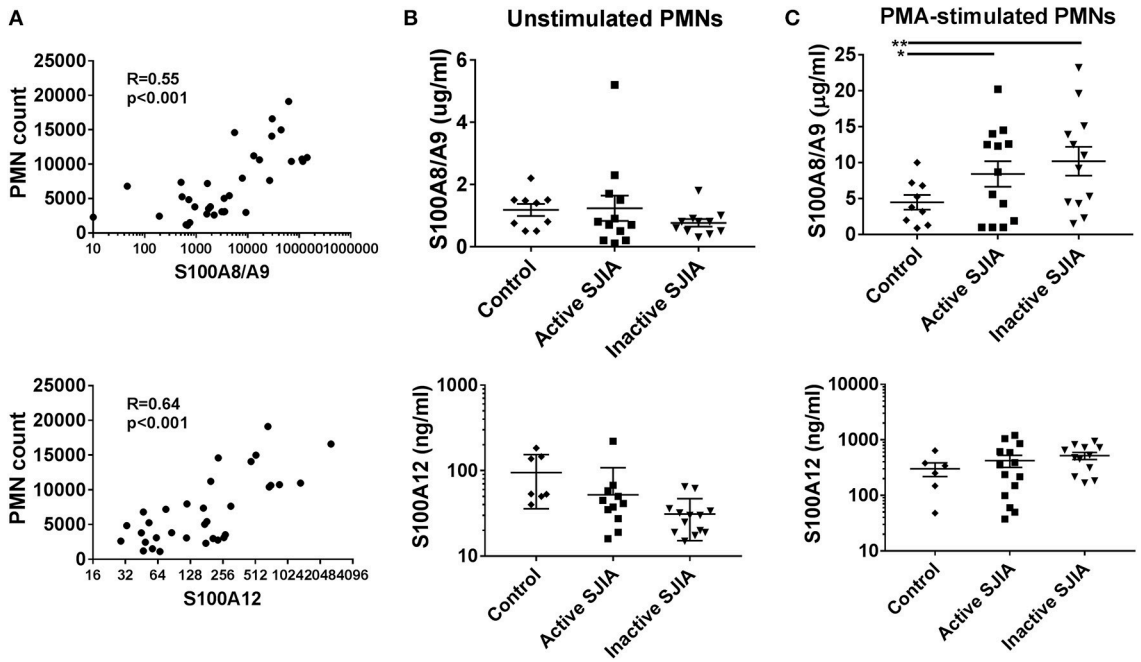

FIGURE 3 | S100 alarmin proteins release by neutrophils in SJIA. **(A)** Correlation between circulating neutrophil count and serum S100A8/A9 (top) and S100A12 (bottom) in patients with SJIA. **(B,C)** S100 alarmin protein release from cultured neutrophils either at rest **(B)** or after PMA stimulation **(C)**. Top panels represent S100A8/A9, bottom panels S100A12. Error bars represent mean ± SEM. *$p < 0.05$, **$P < 0.01$.

between patients and controls (**Figure 4D**). Neutrophils from SJIA patients clustered into two distinct groups; interestingly however, each contained samples from patients with both active and inactive disease. These DEG in all SJIA patient samples included *S100A8*, further supporting increased S100 release capacity as described above. GO analysis of upregulated DEG identified an overlapping network of pathways including immune system process ($p = 1.28 \times 10^{-11}$), vesicle-mediated transport (adjusted $p = 8.23 \times 10^{-4}$), protein complex ($p = 0.02$), and metabolic process ($p = 0.04$) (**Figure 4E**). Together, these findings suggest that in SJIA, neutrophils demonstrate a marked proinflammatory gene expression signature, and this can persist despite longstanding and clinically effective biologic treatment.

Proinflammatory Neutrophil Gene Expression Signature During Inactive Disease With High and Normal Serum IL-18

The above findings are particularly intriguing given that the CID patients, while in longstanding remission on medication, had persistently elevated serum IL-18 levels. To examine this further, we determined gene expression signatures from a second cohort of patients with SJIA. RNA extracted from highly enriched neutrophils from 10 patients with inactive SJIA was sequenced using Ampliseq Transcriptome. These patients had all been in longstanding remission (6–36 months); however, only three patients had serum IL-18 levels greater than twice normal (1188–4247 ng/ml; **Supplemental Table 4**). These gene expression profiles were then utilized for unsupervised clustering using the upregulated DEG signature found above in active SJIA neutrophils. As shown in **Figure 5**, these samples showed a broad range of expression levels of this gene set. However, samples clustered into two primary groups, with 2/4 samples in the highest expression group from "high IL-18" patients and 5/6 samples in the lowest expression group from "low IL-18" patients. Samples also largely clustered based on time in CID, with samples <12 months in CID showing higher expression of this signature (**Figure 5**), possibly relating to the gradual decline of serum IL-18 over many months of treatment reported in SJIA (43). Together, these findings first confirms the persistence of proinflammatory gene expression signatures in neutrophils from patients with inactive SJIA, and second suggest an association between signature persistence and elevated serum IL-18 levels.

DISCUSSION

Neutrophils serve as key innate immune effector cells in systemic inflammation. In the early inflammatory phase of SJIA, neutrophils are markedly expanded and activated with a sepsis-like phenotype (24); however, cell properties in long-standing and CID are less defined. Here, we examined neutrophil subsets, S100 alarmin release, and gene expression profiles from a large cohort of SJIA patients across disease states. Taken together, this work has several important and novel findings. First, we identified hypersegmented, $CD16^{+}CD62L^{dim}$ suppressor neutrophils only in SJIA patients with active systemic disease, including features of MAS. Second, in contrast to FMF, neutrophils from SJIA patients did not constitutively release high

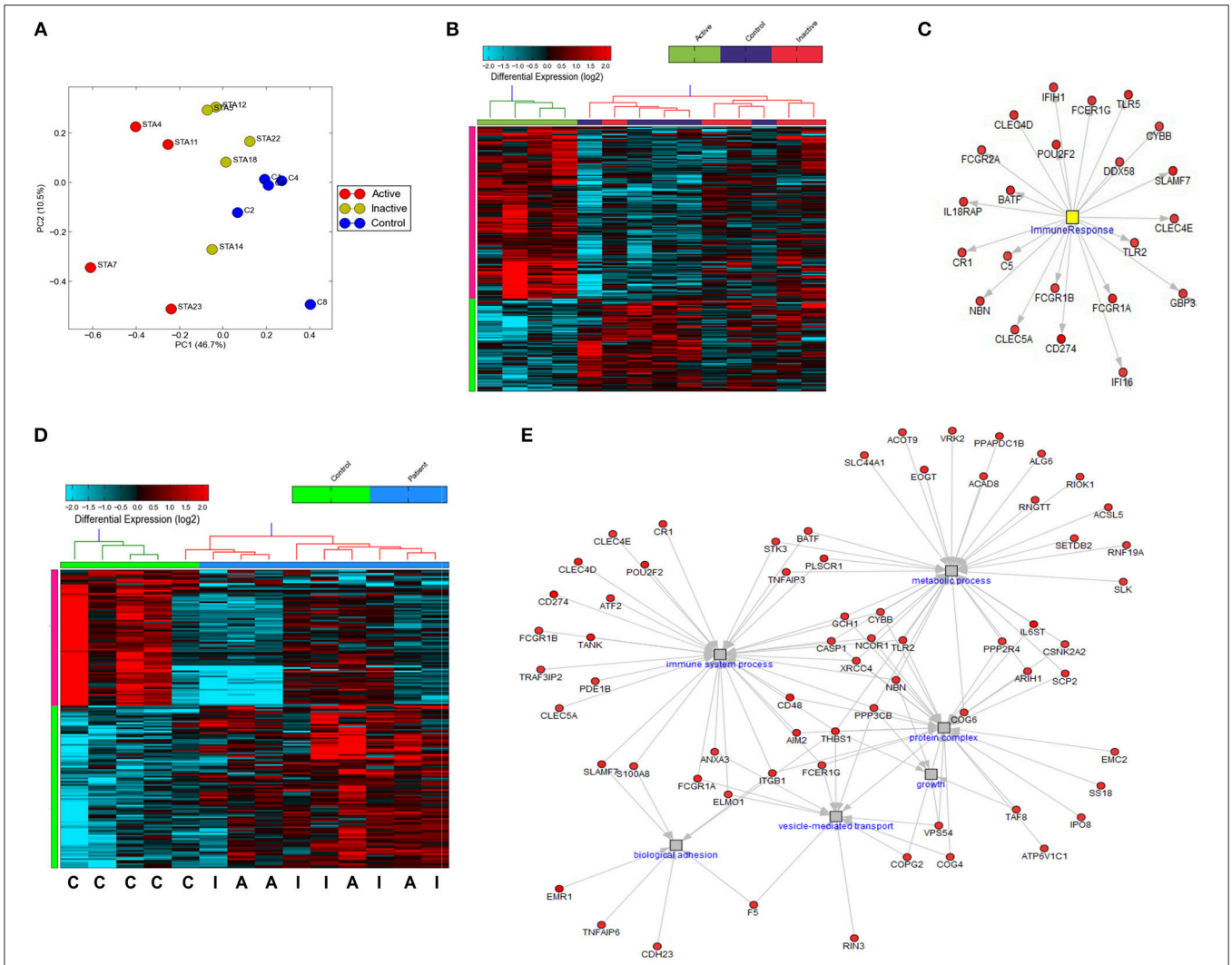

FIGURE 4 | Gene expression profiling of highly purified neutrophils from patients with SJIA. **(A)** Principle component analysis of differentially expressed genes (DEG) between active SJIA and control neutrophils. **(B)** Hierarchical clustering of DEGs between active SJIA and control neutrophils (fold change >2.0, $p < 0.05$). **(C)** Network analysis of immune response transcription factor cluster genes upregulated in active SJIA neutrophils. **(D)** Hierarchical clustering of DEGs between all SJIA patients and control neutrophils (fold change >2.0, $p < 0.05$). **(E)** Network analysis of genes in significantly enriched GO pathways upregulated in all SJIA patients.

levels of S100 proteins. Third, neutrophils from patients with both active and inactive SJIA had significantly increased capacity to release S100A8/A9 upon activation. Fourth, these neutrophils had a marked proinflammatory gene expression signature that is present in both active disease and longstanding CID on medication. Finally, proinflammatory gene expression profiles were associated with persistent serum IL-18 elevations. These findings expand our understanding of neutrophil phenotypes in SJIA, particularly during inactive disease.

The traditional view of neutrophils as short-lived cells with little capacity for phenotypic or transcriptional diversity has been challenged by the description of numerous functional neutrophil subsets (17). Among these are $CD16^{+}CD62L^{dim}$ cells which have been called "suppressor neutrophils" due to their capacity to inhibit T cell responses (19). $CD16^{+}CD62L^{dim}$ neutrophils have recently been shown to have distinct proteomic profiles, with an upregulation in pathways involved in adhesion and activation, response to stimuli, and immune processes, further supporting these cells representing a functional subset in systemic inflammation (44). Recent work has reported that ∼10–20% of circulating neutrophils in active SJIA are $CD16^{+}CD62L^{dim}$ (24), similar to that observed here. We have confirmed and extended those observations to note that this population was only increased in patients with active systemic features (fever, rash, liver enlargement), and not in children who had only active arthritis. $CD16^{+}CD62L^{dim}$ neutrophils seen after experimental LPS challenge in human volunteers (19) or after infection by *H. pylori* (34) are reported to exhibit nuclear hypersegmentation. Here, we utilized imaging cytometry to quantify nuclear segmentation, and

TABLE 2 | Gene ontology pathways of upregulated genes in neutrophils from children with active SJIA.

Gene ontology pathway	Number changed	Number measured	Z score	Fisher exact test *P*	Adjusted *P*
Immune system process (GO:0002376)	51	2091	10.88	3.78E-18	3.44E-16
Defense response (GO:0006952)	39	1284	11.13	8.98E-17	7.06E-13
Response to wounding (GO:0009611)	24	577	10.76	2.64E-13	1.04E-09
IgG binding (GO:0019864)	5	11	18.66	4.43E-09	5.80E-06
Response to other organism (GO:0051707)	16	460	7.73	4.21E-08	3.15E-05
Cell activation (GO:0001775)	18	594	7.42	4.62E-08	3.23E-05
Negative regulation of apoptosis (GO:0043066)	17	665	6.31	1.21E-06	0.000613
Response to lipopolysaccharide (GO:0032496)	10	229	7.12	2.22E-06	0.000965
External side of plasma membrane (GO:0009897)	9	196	6.98	4.84E-06	0.001901
Positive regulation of sequence-specific DNA binding transcription factor activity (GO:0051091)	9	199	6.91	5.47E-06	0.002048

found that in patients with systemically active disease, more $CD16^{+}CD62L^{dim}$ neutrophils had $\geq$4 visualized lobes than $CD16^{+}CD62L^{+}$ cells, particularly in patients with features of MAS. This is unsurprising, given the emerging view of both severe sepsis and MAS as "cytokine storm syndromes" with similar immune dysregulation (45). The significance of nuclear hypersegmentation, and of $CD16^{+}CD62L^{dim}$ neutrophils more broadly in early inflammatory SJIA, remains to be determined. Of note, there is no direct evidence regarding the functional roles of neutrophils in SJIA pathogenesis, including whether $CD16^{+}CD62L^{dim}$ neutrophils have suppressive properties *in vivo*.

Phagocytes including neutrophils are key sources for S100 alarmin production during inflammation (15). S100A8/A9 and S100A12 levels are markedly elevated in active SJIA, and may serve as useful biomarkers to distinguish SJIA from other disorders (46–49). These levels also strongly correlate with circulating neutrophil counts in SJIA (24). Recently, Gohar et al. reported that in the monogenic autoinflammatory disorder FMF, unstimulated neutrophils from patients with *MEFV* mutations spontaneously secreted S100A12 (35). In contrast, we report here that neutrophils from children with active or inactive SJIA do not show spontaneous S100A8/A9 or S100A12 release. This likely relates to the multifactorial nature of SJIA vs. the specific role of pyrin dysfunction in FMF. In contrast, we found that when activated by PMA, neutrophils from SJIA patients secreted significantly more S100A8/A9, regardless of disease activity. It is unknown why S100A8/A9 release was comparable between neutrophils from patients with active and inactive disease despite transcriptional profiles suggesting an intermediate phenotype, but this suggests the need for further linkage between gene expression and functional/protein data. The clinical implications of enhanced S100A8/A9 release capacity are unclear; however, it suggests that there are persistent changes in neutrophil phenotypes even in longstanding CID. In support of this, ter Haar et al. reported extensive *ex vivo* analysis of neutrophils from patients with active SJIA, demonstrating increased surface expression of degranulation markers and a primed phenotype for response to N-formyl peptides (24). While most of these changes reverted in neutrophils from patients with inactive disease, some, such as surface CD35 expression, had an intermediate phenotype reflecting increased degranulation.

Although neutrophils have been historically considered to have limited capacity for gene expression, recent findings instead suggest that they can undergo functional transcriptional alterations including epigenetic modifications (50). Complicating this analysis is that neutrophils generally contain 10–20 fold less mRNA than PBMC (32), and thus even in 95% pure cell populations other cell types may be large contributors to the gene expression signature. Here, we utilized a two-step purification to collect an "untouched" >98% pure neutrophil populations, with <1% contamination from blood monocytes or eosinophils (**Figure 1A**). Detection of IL6 mRNA, typically absent from neutrophils, has also been suggested as a marker for cell contamination (32); in all samples we detected <1 copy *IL6* per million reads.

Compared to control neutrophils, cells from children with active SJIA displayed a proinflammatory gene expression signature, including upregulation in PRR, inflammasome components, and the IL-18 receptor component *IL18RAP*. Although this sequencing was performed on highly purified but unsorted neutrophils, these changes are likely not solely due to the presence of $CD16^{+}CD62L^{dim}$ neutrophils in active disease. Gene expression studies on sorted $CD16^{+}CD62L^{dim}$ neutrophils during human experimental sepsis found the most upregulated genes compared to $CD16^{+}CD62L^{+}$ cells were in signal transduction and regulation of apoptosis pathways (18), which were not highly represented in the present study. In addition, we found that the proinflammatory signature identified here was also present in neutrophils from children with longstanding clinically-inactive SJIA. This neutrophil gene expression signature was broadly similar to that found in sorted neutrophils from patients with early inflammatory SJIA (24), although that study did not examine cells from patients with CID. Another small study examining gene expression in neutrophils from SJIA patients related to tocilizumab treatment found primarily changes related to mitochondrial and oxidative stress genes (36).

TABLE 3 | Immune System Process Gene Ontology pathway significantly upregulated genes in neutrophils from active SJIA.

Gene symbol	Description	Active vs. control (log-fold change)	Active vs. control (*P*-value)
NLRC4	NLR family, CARD domain containing 4	1.422914671	0.019850074
PLSCR1	Phospholipid scramblase 1	1.836792175	0.004685105
CR1	Complement component (3b/4b) receptor 1	1.465308433	0.036471508
CLEC5A	C-type lectin domain family 5, member A	1.306982102	0.008155234
POU2F2	POU class 2 homeobox 2	2.273993247	0.001974801
FCAR	Fc fragment of IgA, receptor for	1.039351747	0.042448537
THBS1	Thrombospondin 1	1.730758578	0.031004871
CLEC4E	C-type lectin domain family 4, member E	1.196901191	0.026681298
BATF	Basic leucine zipper transcription factor, ATF-like	2.098660806	0.001496821
TLR2	Toll-like receptor 2	1.310788972	0.047550579
SERPINB9	Serpin peptidase inhibitor, clade b (ovalbumin), member 9	2.279513514	0.014669102
CYBB	Cytochrome b-245, beta polypeptide	1.251099322	0.026243187
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	1.364191125	0.010664911
CTSH	cathepsin H	1.471367492	0.036661403
PDE1B	Phosphodiesterase 1B, calmodulin-dependent	1.059161606	0.018171438
CX3CR1	Chemokine (C-X3-C motif) receptor 1	1.137782624	0.007845546
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2.18776973	0.019294383
SLAMF7	SLAM family member 7	2.336817458	0.013387457
IFIH1	Interferon induced with helicase C domain 1	1.373101981	0.040081759
MEF2A	Myocyte enhancer factor 2A	1.328120472	0.019727678
LGALS8	Lectin, galactoside-binding, soluble, 8	1.043496856	0.043430423
CD274	CD274 molecule	3.231874521	0.000717605
ANXA1	Annexin A1	1.314782826	0.006487109
GBP3	Guanylate binding protein 3	1.634893254	0.038159331
VNN1	Vanin 1	2.111799896	0.006807828
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	1.159652732	0.015990704
NBN	Nibrin	1.760176462	0.041724081
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.293830277	0.006803898
AIM2	Absent in melanoma 2	2.083271913	0.003477437
FCAR	Fc fragment of IgA, receptor for	1.039351747	0.042448537
IFI16	Interferon, gamma-inducible protein 16	1.291412088	0.001107247
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	1.156808218	0.021307235
TANK	TRAF family member-associated NFKB activator	2.495474762	0.030111499
CLEC5A	C-type lectin domain family 5, member A	1.306982102	0.008155234
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	1.452965375	0.046330661
CLEC4D	C-type lectin domain family 4, member D	1.755561907	0.005050456
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	2.137983366	0.003672704
FCAR	Fc fragment of IgA, receptor for	1.039351747	0.042448537
CYBB	Cytochrome b-245, beta polypeptide	1.251099322	0.026243187
LNPEP	leucyl/cystinyl aminopeptidase	1.326215551	0.02050466
FCAR	Fc fragment of IgA, receptor for	1.039351747	0.042448537
C5	Complement component 5	1.180276747	0.019419926
EDN1	Endothelin 1	1.357923043	0.021389531
TLR5	Toll-like receptor 5	2.129276632	0.014710728
IL18RAP	Interleukin 18 receptor accessory protein	1.737091117	0.006320721
CD48	CD48 molecule	2.1469991	0.000478521
DUSP3	Dual specificity phosphatase 3	1.034227595	0.040091537
SELP	Selectin P (granule membrane protein 140kDa, antigen CD62)	1.175328323	0.040344487
ANXA3	Annexin A3	1.526571135	0.001808271

(Continued)

TABLE 3 | Continued

Gene symbol	Description	Active vs. control (log-fold change)	Active vs. control (*P*-value)
TRIM22	Tripartite motif containing 22	1.324747508	0.005971802
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2.18776973	0.019294383
SMAD3	SMAD family member 3	1.765847458	0.015380056
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	1.364191125	0.010664911
IRAK2	Interleukin-1 receptor-associated kinase 2	1.185197716	0.035499696

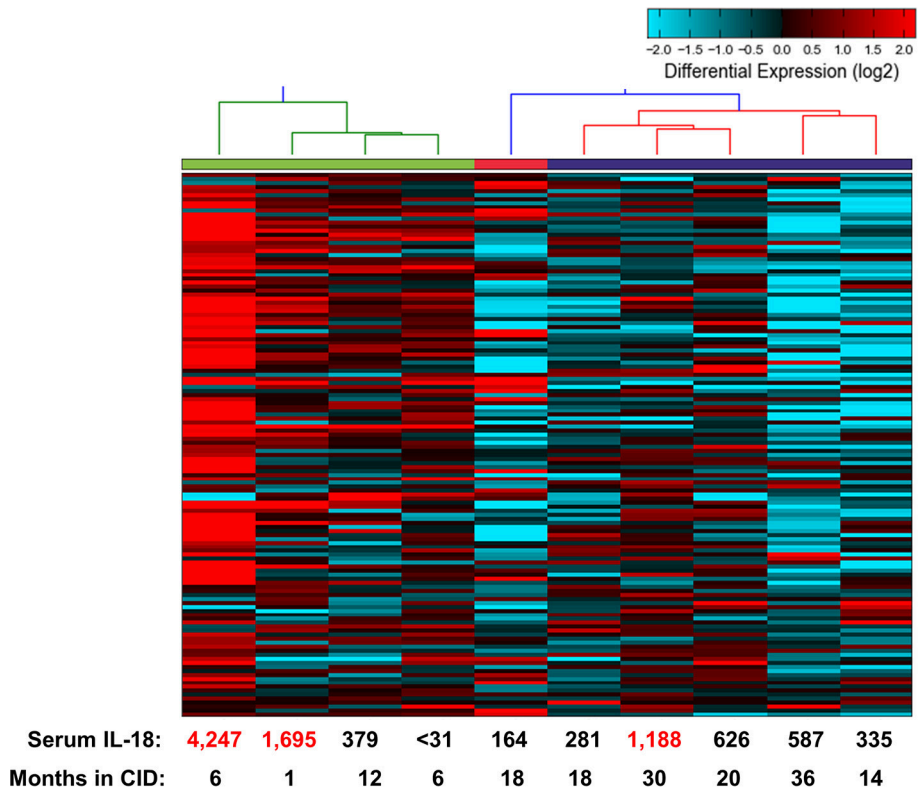

FIGURE 5 | Proinflammatory gene expression signature in neutrophils from patients with CID. Unsupervised clustering performed using 139 upregulated DEGs identified as upregulated in neutrophils from active SJIA patients. Serum IL-18 levels at time of sample collection indicated on bottom. Numbers in red indicate values more than twice upper limit of normal.

Interestingly this study did not find substantive changes in proinflammatory pathways pre- and post-treatment, supporting our findings of persistence of gene expression changes despite clinically effective treatment. Further work utilizing single-cell approaches for both gene expression and protein marker expression are needed to confirm and extend this work.

The changes in neutrophil gene expression profile reported here are particularly interesting in comparison to those reported in other inflammatory arthropathies affecting adults and children. Extensive work by Jarvis et al. has defined a proinflammatory phenotype of neutrophils from non-systemic, rheumatoid factor-negative polyarticular JIA (51–54). In this disorder however, neutrophils demonstrated upregulated gene clusters linked to IL-8 and IFNγ, which were not highly represented in the present study. Indeed, only 4/42 most highly upregulated genes in neutrophils from polyarticular JIA were identified in our patients with SJIA (54). On the other hand, Jarvis et al. did find that neutrophil alternations in polyarticular JIA persisted in disease remission, supporting a model of long-term cell alterations in JIA (53). Gene expression studies of neutrophils in adults with rheumatoid arthritis similarly identified IFN signaling as the most differentially regulated pathway, distinguishing patients with improved response to treatment (55). Findings in this variety of disorders also highlight the capacity of neutrophils for highly specific transcriptional responses in the setting of distinct pathological settings (51, 52).

Together, we report both functional and gene expression evidence of neutrophil alterations in SJIA patients with longstanding CID. This is in agreement with our previous data on monocytes, which found persistent epigenetic changes in children with inactive disease (25). Of note, more than half of patients with CID in the present cohort continued to have elevated serum IL-18 levels. There is significant evidence that IL-18 has pleotropic effects on neutrophil activation, including cytokine gene expression and release, degranulation, and priming of the oxidative burst (56–58). Indeed, we demonstrated that persistently high IL-18 levels were associated with proinflammatory neutrophil gene expression signatures in patients with CID. IL-18 is also reported to trigger further autocrine expression and secretion of this cytokine by neutrophils, potentially implicating neutrophils in a feed-forward process to perpetuate IL-18 production. As such, further studies are needed to define the role of neutrophil subpopulation activation in both early inflammatory SJIA and in persistence of chronic disease.

AUTHOR CONTRIBUTIONS

RB, AG, and GS designed the study. RB, MH, TD, SY, and GS collected samples, isolated cells, and performed flow cytometry and cell culture experiments. TD performed gene expression profiling experiments. MD and ST performed imaging cytometry. RB, ST, AG, and GS analyzed the data. All authors contributed to drafting of the work and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02995/full#supplementary-material

REFERENCES

1. Mellins ED, Macaubas C, Grom AA. Pathogenesis of systemic juvenile idiopathic arthritis: some answers, more questions. *Nat Rev Rheumatol.* (2011) 7:416–26. doi: 10.1038/nrrheum.2011.68
2. Kimura Y, Weiss JE, Haroldson KL, Lee T, Punaro M, Oliveira S, et al. Pulmonary hypertension and other potentially fatal pulmonary complications in systemic juvenile idiopathic arthritis. *Arthritis Care Res.* (2013) 65:745–52. doi: 10.1002/acr.21889
3. Grom AA, Horne A, De Benedetti F. Macrophage activation syndrome in the era of biologic therapy. *Nat Rev Rheumatol.* (2016) 12:259–68. doi: 10.1038/nrrheum.2015.179
4. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annu Rev Immunol.* (2009) 27:621–68. doi: 10.1146/annurev.immunol.25.022106.141627
5. de Benedetti F, Massa M, Robbioni P, Ravelli A, Burgio GR, Martini A. Correlation of serum interleukin-6 levels with joint involvement and thrombocytosis in systemic juvenile rheumatoid arthritis. *Arthritis Rheum.* (1991) 34:1158–63. doi: 10.1002/art.1780340912
6. Ling XB, Park JL, Carroll T, Nguyen KD, Lau K, Macaubas C, et al. Plasma profiles in active systemic juvenile idiopathic arthritis: Biomarkers and biological implications. *Proteomics* (2010) 10:4415–30. doi: 10.1002/pmic.201000298
7. Fall N, Barnes M, Thornton S, Luyrink L, Olson J, Ilowite NT, et al. Gene expression profiling of peripheral blood from patients with untreated new-onset systemic juvenile idiopathic arthritis reveals molecular heterogeneity that may predict macrophage activation syndrome. *Arthritis Rheum.* (2007) 56:3793–804. doi: 10.1002/art.22981
8. Allantaz F, Chaussabel D, Stichweh D, Bennett L, Allman W, Mejias A, et al. Blood leukocyte microarrays to diagnose systemic onset juvenile idiopathic arthritis and follow the response to IL-1 blockade. *J Exp Med.* (2007) 204:2131–44. doi: 10.1084/jem.20070070
9. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J Exp Med.* (2005) 201:1479–86. doi: 10.1084/jem.20050473
10. Macaubas C, Nguyen K, Deshpande C, Phillips C, Peck A, Lee T, et al. Distribution of circulating cells in systemic juvenile idiopathic arthritis across disease activity states. *Clin Immunol.* (2010) 134:206–16. doi: 10.1016/j.clim.2009.09.010
11. Shimizu M, Nakagishi Y, Yachie A. Distinct subsets of patients with systemic juvenile idiopathic arthritis based on their cytokine profiles. *Cytokine* (2013) 61:345–8. doi: 10.1016/j.cyto.2012.11.025
12. Weiss ES, Girard-Guyonvarc'h C, Holzinger D, de Jesus AA, Tariq Z, Picarsic J, et al. Interleukin-18 diagnostically distinguishes and pathogenically promotes human and murine macrophage activation syndrome. *Blood* (2018) 131:1442–55. doi: 10.1182/blood-2017-12-820852
13. Correll CK, Binstadt BA. Advances in the pathogenesis and treatment of systemic juvenile idiopathic arthritis. *Pediatr Res.* (2013) 75:176–83. doi: 10.1038/pr.2013.187
14. Nauseef WM, Borregaard N. Neutrophils at work. *Nat Immunol.* (2014) 15:602–11. doi: 10.1038/ni.2921
15. Bertheloot D, Latz E. HMGB1, IL-1α, IL-33 and S100 proteins: dual-function alarmins. *Cell Mol Immunol.* (2017) 14:43–64. doi: 10.1038/cmi.2016.34
16. Leliefeld PHC, Wessels CM, Leenen LPH, Koenderman L, Pillay J. The role of neutrophils in immune dysfunction during severe inflammation. *Crit Care* (2016) 20:73. doi: 10.1186/s13054-016-1250-4

17. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood* (2016) 127:2173–81. doi: 10.1182/blood-2016-01-688887
18. de Kleijn S, Langereis JD, Leentjens J, Kox M, Netea MG, Koenderman L, et al. IFN-γ-stimulated neutrophils suppress lymphocyte proliferation through expression of PD-L1. *PLoS ONE* (2013) 8:e72249. doi: 10.1371/journal.pone.0072249
19. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers J-W, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest.* (2012) 122:327–36. doi: 10.1172/JCI57990
20. Kamp VM, Pillay J, Lammers J-WJ, Pickkers P, Ulfman LH, Koenderman L. Human suppressive neutrophils CD16 ^{bright} /CD62L ^{dim} exhibit decreased adhesion. *J Leukoc Biol.* (2012) 92:1011–20. doi: 10.1189/jlb.0612273
21. Gattorno M, Piccini A, Lasiglie D, Tassi S, Brisca G, Carta S, et al. The pattern of response to anti-interleukin-1 treatment distinguishes two subsets of patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2008) 58:1505–15. doi: 10.1002/art.23437
22. Nirmala N, Brachat A, Feist E, Blank N, Specker C, Witt M, et al. Gene-expression analysis of adult-onset Still's disease and systemic juvenile idiopathic arthritis is consistent with a continuum of a single disease entity. *Pediatr Rheumatol Online J.* (2015) 13:50. doi: 10.1186/s12969-015-0047-3
23. Holzinger D, Kessel C, Omenetti A, Gattorno M. From bench to bedside and back again: translational research in autoinflammation. *Nat Rev Rheumatol.* (2015) 11:573–85. doi: 10.1038/nrrheum.2015.79
24. ter Haar NM, Tak T, Mokry M, Scholman RC, Meerding JM, de Jager W, et al. Reversal of sepsis-like features of neutrophils by Interleukin-1 blockade in patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2018) 70:943–56. doi: 10.1002/art.40442
25. Schulert GS, Fall N, Harley JB, Shen N, Lovell DJ, Thornton S, et al. Monocyte microRNA expression in active systemic juvenile idiopathic arthritis implicates miR-125a-5p in polarized monocyte phenotypes. *Arthritis Rheumatol.* (2016) 68:2300–13. doi: 10.1002/art.39694
26. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* (2004) 31:390–2.
27. DeWitt EM, Kimura Y, Beukelman T, Nigrovic PA, Onel K, Prahalad S, et al. Consensus treatment plans for new-onset systemic juvenile idiopathic arthritis. *Arthritis Care Res.* (2012) 64:1001–10. doi: 10.1002/acr.21625
28. Wallace CA, Giannini EH, Huang B, Itert L, Ruperto N. American College of Rheumatology provisional criteria for defining clinical inactive disease in select categories of juvenile idiopathic arthritis. *Arthritis Care Res.* (2011) 63:929–36. doi: 10.1002/acr.20497
29. Ravelli A, Minoia F, Davì S, Horne A, Bovis F, Pistorio A, et al. 2016 Classification Criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborat. *Arthritis Rheumatol.* (2016) 68:566–76. doi: 10.1002/art.39332
30. Nauseef WM. Isolation of human neutrophils from venous blood. *Methods Mol Biol.* 412:15–20. doi: 10.1007/978-1-59745-467-4_2
31. Emig D, Salomonis N, Baumbach J, Lengauer T, Conklin BR, Albrecht M. AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. *Nucleic Acids Res.* (2010) 38:W755–62. doi: 10.1093/nar/gkq405
32. Scapini P, Calzetti F, Cassatella MA. On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). *J Immunol Methods* (1999) 232:121–9. doi: 10.1016/S0022-1759(99)00170-2
33. Moldovan I, Galon J, Maridonneau-Parini I, Roman Roman S, Mathiot C, Fridman WH, et al. Regulation of production of soluble Fc gamma receptors type III in normal and pathological conditions. *Immunol Lett.* (1999) 68:125–34.
34. Whitmore LC, Weems MN, Allen L-AH. Cutting edge: *Helicobacter pylori* induces nuclear hypersegmentation and subtype differentiation of human neutrophils *in vitro*. *J Immunol.* (2017) 198:1793–7. doi: 10.4049/jimmunol.1601292
35. Gohar F, Orak B, Kallinich T, Jeske M, Lieber M, von Bernuth H, et al. Correlation of secretory activity of neutrophils with genotype in patients with familial mediterranean fever. *Arthritis Rheumatol.* (2016) 68:3010–22. doi: 10.1002/art.39784
36. Omoyinmi E, Hamaoui R, Bryant A, Jiang MC, Athigapanich T, Eleftheriou D, et al. Mitochondrial and oxidative stress genes are differentially expressed in neutrophils of sJIA patients treated with tocilizumab: a pilot microarray study. *Pediatr Rheumatol.* (2016) 14:7. doi: 10.1186/s12969-016-0067-7
37. Zhang JD, Schindler T, Küng E, Ebeling M, Certa U. Highly sensitive amplicon-based transcript quantification by semiconductor sequencing. *BMC Genomics* (2014) 15:565. doi: 10.1186/1471-2164-15-565
38. Park C, Li S, Cha E, Schindler C. Immune response in Stat2 knockout mice. *Immunity* (2000) 13:795–804. doi: 10.1016/S1074-7613(00)00077-7
39. Ho HH, Ivashkiv LB. Role of STAT3 in type I interferon responses. *J Biol Chem.* (2006) 281:14111–8. doi: 10.1074/jbc.M511797200
40. Bracaglia C, de Graaf K, Pires Marafon D, Guilhot F, Ferlin W, Prencipe G, et al. Elevated circulating levels of interferon-gamma and interferon-gamma induced chemokines characterize patients with macrophage activation syndrome complicating systemic juvenile idiopathic arthritis (in press). *Ann Rheum Dis.* (2016) 76:209020. doi: 10.1136/annrheumdis-2015-209020
41. van den Ham H-J, de Jager W, Bijlsma JWJ, Prakken BJ, de Boer RJ. Differential cytokine profiles in juvenile idiopathic arthritis subtypes revealed by cluster analysis. *Rheumatology* (2009) 48:899–905. doi: 10.1093/rheumatology/kep125
42. Kim H, de Jesus AA, Brooks SR, Liu Y, Huang Y, VanTries R, et al. Development of a validated interferon score using Nanostring technology. *J Interf Cytokine Res.* (2018) 38:171–85. doi: 10.1089/jir.2017.0127
43. Brachat AH, Grom AA, Wulffraat N, Brunner HI, Quartier P, Brik R, et al. Early changes in gene expression and inflammatory proteins in systemic juvenile idiopathic arthritis patients on canakinumab therapy. *Arthritis Res Ther.* (2017) 19:13. doi: 10.1186/s13075-016-1212-x
44. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJR, et al. Human CD62Ldim neutrophils identified as a separate subset by proteome profiling and *in vivo* pulse-chase labeling. *Blood* (2017) 129:3476–85. doi: 10.1182/blood-2016-07-727669
45. Canna SW, Behrens EM. Making sense of the cytokine storm: a conceptual framework for understanding, diagnosing, and treating hemophagocytic syndromes. *Pediatr Clin North Am.* (2012) 59:329–44. doi: 10.1016/j.pcl.2012.03.002
46. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, et al. Monitoring neutrophil activation in juvenile rheumatoid arthritis by S100A12 serum concentrations. *Arthritis Rheum.* (2004) 50:1286–95. doi: 10.1002/art.20125
47. Frosch M, Vogl T, Seeliger S, Wulffraat N, Kuis W, Viemann D, et al. Expression of myeloid-related proteins 8 and 14 in systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* (2003) 48:2622–6. doi: 10.1002/art.11177
48. Wittkowski H, Hirono K, Ichida F, Vogl T, Ye F, Yanlin X, et al. Acute Kawasaki disease is associated with reverse regulation of soluble receptor for advance glycation end products and its proinflammatory ligand S100A12. *Arthritis Rheum.* (2007) 56:4174–81. doi: 10.1002/art.23042
49. Wittkowski H, Frosch M, Wulffraat N, Goldbach-Mansky R, Kallinich T, Kuemmerle-Deschner J, et al. S100A12 is a novel molecular marker differentiating systemic-onset juvenile idiopathic arthritis from other causes of fever of unknown origin. *Arthritis Rheum.* (2008) 58:3924–31. doi: 10.1002/art.24137
50. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, et al. Chromatin remodelling and autocrine TNFα are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun.* (2015) 6:6061. doi: 10.1038/ncomms7061
51. Jiang K, Sun X, Chen Y, Shen Y, Jarvis JN. RNA sequencing from human neutrophils reveals distinct transcriptional differences associated with chronic inflammatory states. *BMC Med Genomics* (2015) 8:55. doi: 10.1186/s12920-015-0128-7
52. Hu Z, Jiang K, Frank MB, Chen Y, Jarvis JN. Complexity and specificity of the neutrophil transcriptomes in juvenile idiopathic arthritis. *Sci Rep.* (2016) 6:27453. doi: 10.1038/srep27453
53. Jarvis JN, Jiang K, Frank MB, Knowlton N, Aggarwal A, Wallace CA, et al. Gene expression profiling in neutrophils from children with

polyarticular juvenile idiopathic arthritis. *Arthritis Rheum.* (2009) 60:1488–95. doi: 10.1002/art.24450
54. Jarvis JN, Petty HR, Tang Y, Frank M, Tessier PA, Dozmorov I, et al. Evidence for chronic, peripheral activation of neutrophils in polyarticular juvenile rheumatoid arthritis. *Arthritis Res Ther.* (2006) 8:R154. doi: 10.1186/ar2048
55. Wright HL, Thomas HB, Moots RJ, Edwards SW. Interferon gene expression signature in rheumatoid arthritis neutrophils correlates with a good response to TNFi therapy. *Rheumatology* (2015) 54:188–93. doi: 10.1093/rheumatology/keu299
56. Leung BP, Culshaw S, Gracie JA, Hunter D, Canetti CA, Campbell C, et al. A role for IL-18 in neutrophil activation. *J Immunol.* (2001) 167:2879–86. doi: 10.4049/jimmunol.167.5.2879
57. Elbim C, Guichard C, Dang PMC, Fay M, Pedruzzi E, Demur H, et al. Interleukin-18 primes the oxidative burst of neutrophils in response to Formyl-Peptides: role of cytochrome b558 translocation and N-Formyl Peptide receptor endocytosis. *Clin Vaccine Immunol.* (2005) 12:436–46. doi: 10.1128/CDLI.12.3.436-446.2005
58. Fortin CF, Ear T, McDonald PP. Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation by human neutrophils. *FASEB J.* (2009) 23:194–203. doi: 10.1096/fj.08-110213

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REVIEW
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Autoantibodies in the Pathogenesis, Diagnosis, and Prognosis of Juvenile Idiopathic Arthritis

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Autoantibody production occurs in juvenile idiopathic arthritis (JIA) and numerous other autoimmune diseases. In some conditions, the autoantibodies are clearly pathogenic, whereas in others the roles are less defined. Here we review various autoantibodies associated with JIA, with a particular focus on antinuclear antibodies and antibodies recognizing citrullinated self-antigens. We explore potential mechanisms that lead to the development of autoantibodies and the use of autoantibody testing in diagnosis and prognosis. Finally, we compare and contrast JIA-associated autoantibodies with those found in adults with rheumatoid arthritis (RA).

Keywords: autoantibodies, juvenile idiopathic arthritis, antinuclear antibodies, rheumatoid factor, citrullinated self-antigens, anti-citrullinated protein antibodies, carbamylated self-antigens, anti-carbamylated protein antibodies

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INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory disease affecting hundreds of thousands of children worldwide (1). Advances in our understanding of the pathogenesis of JIA over the last two decades have revolutionized therapy, reduced morbidity, and improved quality of life for those affected.

Although often referred to as a single entity, JIA represents a heterogeneous group of inflammatory arthropathies. By definition, JIA is arthritis that begins in a child under the age of 16 years, lasts at least 6 weeks, and is not attributable to any other cause (e.g., Lyme disease, septic arthritis, or "reactive" forms of arthritis). The International League of Associations for Rheumatology (ILAR) has defined seven subtypes of JIA, which are summarized in **Table 1** (2). While there are shared genetic and immunologic features between JIA and rheumatoid arthritis (RA) in adults, only a small subset of JIA patients with polyarticular disease and a positive rheumatoid factor (RF) clinically resemble adult RA patients (3, 4).

As we explore in further detail below, although the subtypes of JIA likely differ in their specific pathophysiologic mechanisms, most forms of JIA appear to be rooted in the breakdown of immunologic self-tolerance. Some of the earliest and strongest genetic associations recognized involve the major histocompatibility complex (MHC) class II alleles (4–6), suggesting a critical role for CD4+ T helper (Th) cells. Synovial fluid from inflamed joints in children with oligoarticular, polyarticular, and systemic JIA (sJIA) show an abnormal ratio of Th17 to regulatory T cell subsets, and Th17 cell numbers correlate with arthritis severity (7). Systemic JIA is a distinct subtype driven largely by defects in innate immune mechanisms (8).

Abbreviations: JIA, juvenile idiopathic arthritis; ANA, antinuclear antibody; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MHC, major histocompatibility complex; NETs, neutrophil extracellular traps.

TABLE 1 | Second revision of the ILAR classification of juvenile idiopathic arthritis (2001).

Oligoarticular JIA	Involvement of 1–4 joints in the first 6 months of disease, further defined by the addition of additional involved joints over time (persistent vs. extended)
Polyarticular JIA, RF-negative	Involvement of 5 or more joints in the first 6 months, further defined by absence of rheumatoid factor
Polyarticular JIA, RF-positive	Involvement of 5 or more joints in the first 6 months, further defined by presence of rheumatoid factor
Enthesitis-related JIA	Defined by the presence of arthritis and enthesitis (inflammation of ligamentous and tendinous insertions). Associated with sacroiliitis leading to frequent low-back pain, HLA-B27 positivity, arthritis associated with inflammatory bowel syndrome, and "reactive" forms of arthritis
Psoriatic JIA	Arthritis and psoriasis. Associated with dactylitis, nail changes, and a family history of psoriasis.
Systemic JIA (sJIA)	Arthritis associated with fevers, rash, lymphadenopathy, hepatomegaly, splenomegaly, and/or serositis. Thought to be a systemic auto-inflammatory disease with a distinct pathophysiology as compared to other forms of JIA (10).
Undifferentiated JIA	Chronic idiopathic arthritis which does not fit with one category, or which fits with more than one category above (15–20% of patients)

Interestingly, however, recent work from Ombrello and colleagues highlights the numerous genetic differences between sJIA and the other forms of JIA, yet still identifies the strongest genetic linkage of sJIA as the MHC class II allele *DRB1*11* (9, 10). These findings support the notion that autoreactive CD4+ T cells are key contributors to the pathogenesis of each of the JIA subtypes.

One way CD4+ T cells contribute to JIA pathogenesis is by providing help to autoreactive B cells. In general, linked T- and B- cell recognition of self-antigens allows CD4+ T cells to promote affinity maturation of B cell clones. Analysis of JIA synovial fluid reveals changes consistent with B cell activation, including alterations in the immunoglobulin light chain repertoire suggestive of secondary V(D)J-recombination, and increased numbers of class-switched memory B cells and plasmablasts secreting IgG within the synovial fluid of affected joints (11–13).

Although autoreactive B cells also have other important pathogenic functions in JIA, such as functioning as antigen presenting cells within the synovium (14), this review focuses on autoantibody production as a consequence of autoreactive B cell activation. Various autoantibodies have been associated with JIA, including anti-nuclear antibodies (ANA), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), and others. In the following sections, we explore the role of autoantibodies in the pathogenesis, diagnosis, prognosis, and response to therapy in JIA.

ANTINUCLEAR ANTIBODIES (ANA): OVERVIEW AND USE IN DIAGNOSIS

The discovery of substances in sera of patients with autoimmunity that can bind to nuclear elements of healthy cells dates back over six decades (15–17). Ultimately these serum factors were shown to be IgG antibodies recognizing nuclear antigens and named antinuclear antibodies (ANA). Modern clinical laboratories detect ANA via an immunofluorescence based assay (FANA, or fluorescent antinuclear antibody test) or an enzyme-linked immunosorbent assay (ELISA). It is now known that several key autoantigens are identified by ANA, including nucleic acids, nucleosomes, phospholipids, and several nuclear and nucleolar proteins (18, 19). These autoantigens are theorized to normally be "hidden" but are exposed to antigen presenting cells during cell death, particularly during apoptosis—a process which has been shown to be abnormal in patients with SLE (20).

The ANA is a highly sensitive test (>95%) for SLE in both adults and children, but it is also commonly misunderstood by clinicians as a general test for autoimmune or rheumatic disease (21–24). Other diseases strongly associated with a positive ANA include mixed connective tissue disorder, juvenile dermatomyositis, Sjogren's syndrome, scleroderma, autoimmune hepatitis, primary biliary cirrhosis, ulcerative colitis, and autoimmune thyroiditis (25–31).

The ANA test is not used to diagnose JIA. However, it has important prognostic value with respect to the risk of uveitis (explored further below). The overall seroprevalence of a positive ANA among all subtypes of JIA combined is <50% (32). Simply stated, although a positive ANA is more common in children with JIA than among healthy children, the presence or absence of ANA does not change the likelihood that a given patient will have or will develop JIA. Furthermore, false positivity and transient positivity of the ANA (e.g., secondary to infections) are common occurrences (33).

ANA positivity amongst the JIA subtypes is highest in patients with oligoarticular JIA (up to 70%) and is particularly more prevalent in young, female patients (34). Similarly, among patients with psoriatic JIA, ANA positivity is associated with early-onset disease and female predominance (35). ANA positivity is less common in patients with undifferentiated JIA and systemic JIA (32), although a recent study showed patients with systemic JIA have rising ANA and rheumatoid factor titers over time (36).

ANA AND JIA PROGNOSIS

There is mixed evidence in the literature about a potential association between ANA status and arthritis prognosis in patients with JIA. In perhaps the largest study to date addressing this question, there were no significant differences found relating to ANA positivity in Danish children with JIA and the number of active joints at follow-up, remission rate on medication for more than 6 months, or remission rate off medication for more than 12 months. However, patients with RF, HLA-B27, and/or uveitis

(either past or present) tended to have lower rates of remission at follow-up, irrespective of ANA status (32). A separate study found that ANA status did not predict risk of relapse when withdrawing tumor necrosis factor inhibitors from patients in disease remission (37).

Another group used a microarray approach to test sera of patients with oligoarticular JIA for reactivity to over 100 autoantigens. A heat map analysis of these arrays was used to perform a cluster analysis and identified two primary groups of patients. Children in cluster 1 were more likely to have high titers of autoantibodies recognizing nuclear antigens such as histone and chromatin, while those in cluster 2 were more likely to have low levels of these autoantibodies, similar to healthy controls. ANA status was not significantly different between these clusters of patients; however, children in cluster 1 were more likely to have active arthritis at follow-up at 5 months (90 vs. 36%; $p < 0.024$) despite there being no significant differences in their treatment regimens (38). This study suggests that autoantibodies directed against more specific autoantigens may be better biomarkers than the ANA with respect to JIA prognosis.

Although the standard ANA test is not particularly helpful in predicting arthritis outcomes in JIA, its prognostic utility for the risk of developing JIA-associated uveitis is clear. Specifically, patients with oligo- or poly-articular JIA with ANA positivity tend to develop disease at a younger age, have asymmetric patterns of arthritis, and are at an increased risk of developing chronic anterior uveitis (39, 40). This form of uveitis is typically asymptomatic. If undetected and untreated, it can result in permanent vision loss. It is therefore recommended that all patients with certain subtypes of JIA and a positive ANA have more frequent screening eye exams (41). Of note, while a positive ANA indicates increased risk of uveitis, it does not seem to be helpful in predicting the timing or severity of this comorbidity (26).

RHEUMATOID FACTOR (RF): OVERVIEW AND ROLE IN DIAGNOSIS

Nearly 80 years ago, Eric Waller discovered a serum factor capable of agglutinating of sheep red blood cells, which others subsequently found to be more common among patients with RA (42, 43). This factor is now known as rheumatoid factor (RF) and refers to a group of antibodies of various classes whose antigen binding sites are specific to the Fc portion of IgG molecules. Not surprisingly, RF has a notable capacity to induce false positivity in laboratory assays such as those designed to detect antibody responses to vaccines or infectious pathogens or autoantibodies such as those seen in antiphospholipid syndrome (44).

RF is most commonly associated with RA in adults and is one of the two serologic tests (along with ACPA, discussed below) included in the current classification criteria for RA (45). A recent meta-analysis of adult RA showed pooled values of sensitivity and specificity of 69% (CI, 65–73%) and 85% (CI, 82–88%), respectively (46). RF can also be positive in other autoimmune disorders such as acute rheumatic fever, SLE, and Sjogren's syndrome. It can be seen non-specifically positive in infections such as tuberculosis and Lyme disease, as well as in otherwise healthy individuals (44, 47, 48).

RF was discovered in some patients with juvenile arthritis long ago (49). Although the overall seroprevalance of RF in patients with JIA is very low (<5%), it confers a worse prognosis (50). In particular, patients with RF+ polyarticular JIA are at higher risk of a more aggressive disease course and bone erosion than JIA patients without RF (51–54). RF+ polyarticular JIA has long been recognized to represent the true pediatric version of RA, and genetic analyses confirm this (4, 55). Consensus treatment plans developed by the Childhood Arthritis and Rheumatology Research Association (CARRA) recognize RF (and ACPA, discussed below) as poor prognostic risk factors among patients with polyarticular JIA, leading most pediatric rheumatologists to use more aggressive, early therapy (e.g., TNF inhibitors) for these patients than for patients without RF (56).

Interestingly, RFs play physiologic roles in the normal immune system. RF of the IgM isotype (IgM-RF), for example, promotes phagocytosis and the removal of antigen-antibody complexes in the course of infection, fixation of complement, and enhancing B cell antigen uptake and presentation to $CD4^+$ T cells (57). However, these naturally-occurring IgM-RFs are of low affinity and polyreactive, whereas pathogenic IgM-RFs tend to have undergone affinity maturation (58). Although IgM-, IgG-, and IgA-RFs are often elevated in RA, with IgM-RF being the most common, the isotype-switched IgG and IgA classes are felt to be more causally linked to immunopathology and bone erosion (47, 59). The mechanisms that go awry resulting in the production of RF are not entirely understood, but appear to depend on immune-complex recognition by B cell receptors in the context of toll-like receptor stimulation, as well as T cell help (60, 61). Additional roles for RF in arthritis pathogenesis are explored further below.

ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPA): EVIDENCE OF A BREACH IN SELF-TOLERANCE

In 1964, work by Nienhuis and Mandema led to the discovery of another class of autoantibodies that recognize post-translationally modified autoantigens, now called anti-citrullinated protein antibodies or ACPA (tested clinically as anti-cyclic citrullinated peptide or anti-CCP). Nienhuis and Mandema studied sera from patients with SLE, RA, and ankylosing spondylitis and found a factor more commonly present in RA patients that stained the cytoplasm surrounding the nucleus "like the rings of Saturn." They called this "the antiperinuclear factor" (62). Subsequent work showed that anti-keratin antibodies stained with a similar pattern as the antiperinuclear factors derived from patients with RA (63, 64). We now know that a variety of self-proteins including collagen, fibrinogen, vimentin, filaggrin, alpha-enolase, and others are bound by these antibodies (51, 65–73).

Citrullination is a form of post-translational modification in which arginine residues are changed to citrulline. This modification is mediated by a class of enzymes called

peptidylarginine deiminases, or PADs. Several isoforms of PADs exist, and PAD2 and PAD4 have been specifically implicated in inflammatory states. Expression of these enzymes, primarily derived from neutrophils, is increased in the synovial fluid of both mice and humans with inflammatory arthritis (74, 75).

PAD enzymes are required for the formation of neutrophil extracellular traps (NETs), a phenomenon in which neutrophils project DNA and histones into the extracellular environment to aid in phagocytosis. PAD4-activity is required for hypercitrullination of histones, which causes heterochromatin decondensation and the unfolding of chromatin, forming NETs (76). This natural innate immune mechanism sets the stage for modification of self-antigens in the context of inflammatory states and can lead to breakdown of immune self-tolerance (77).

Normally, thymocytes bearing T cell receptors with high affinity for self-antigens are either clonally deleted or are diverted into the regulatory T cell lineage, reducing the likelihood of autoimmunity—also known as central tolerance (78). However, citrullination of peptides generates neo-epitopes—peptides not present during thymic selection and therefore incapable of inducing central tolerance. Thymocytes specific for these citrullinated neo-epitopes can therefore escape central tolerance and go on to help drive autoantibody production (**Figure 1**).

Naïve T cells specific for citrullinated autoantigens have the potential to become activated, leading to pathogenic helper T cell responses including those that drive autoantibody formation. Indeed, sophisticated approaches using class II MHC tetramers loaded with citrullinated self-peptides have shown that self-reactive T cells recognizing citrullinated self-antigens exist more commonly among patients with adult RA. Relative to healthy control subjects, RA subjects have similar frequencies of influenza-specific CD4+ T cells, but have ∼5-fold increased frequencies of T cells specific for a panel of citrullinated self-peptides (derived from vimentin, fibrinogen, enolase, and cartilage intermediate-layer protein). These cells are predominately Th1 effector-memory cells expressing CD45RO and CXCR3. Treatment of RA patients with biologic therapies (TNF inhibitors, abatacept, or rituximab) restores the numbers of these cells to levels seen in healthy controls, whereas therapy with conventional disease modifying anti-rheumatic drugs such as methotrexate, hydroxychloroquine, or leflunamide) does not (79).

To our knowledge, additional approaches to characterize other effector and regulatory populations of citrullinated self-antigen-specific T cells have not yet been undertaken in either RA or JIA. For example, although the total numbers of follicular T helper cells (Tfh cells) are increased in the peripheral blood and synovial fluid of RA patients (80), their antigenic specificity is not known. Tfh cells drive germinal center B cell affinity maturation and are hypothesized to play a key role in autoantibody generation. One would predict that since the numbers of Tfh cells correlates with ACPA titer in RA patients (81), at least a fraction of these cells should be specific for citrullinated self-antigens. Moreover, a population of regulatory T cells co-expressing both FOXP3 and IL-17 has been observed to accumulate at the sites of inflamed joints in RA patients (82).

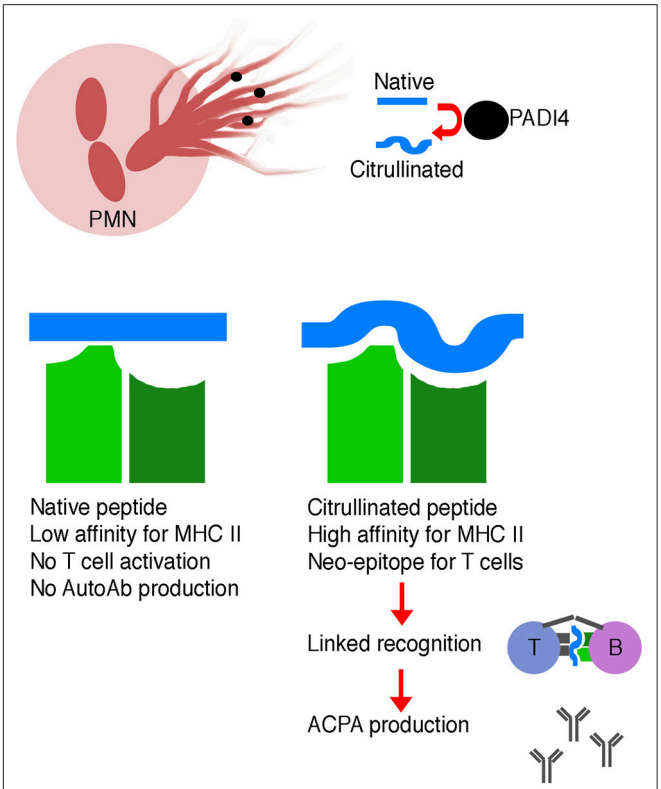

FIGURE 1 | Schematic illustrating the generation of ACPA. Neutrophils are a source of PADI4 enzyme, which leads to citrullination of self-peptides. Citrullinated self-peptides may have altered MHC class II binding affinity, which may result in more efficient presentation to T cells. These modified peptides are neo-epitopes, and T cells specific for these modified peptides may not have been deleted during negative selection in the thymus. Linked recognition of a citrullinated self-antigen and a corresponding citrullinated self-peptide by B and T cells, respectively, stimulates affinity maturation and production of high affinity ACPA.

Future studies should focus on defining the antigen specificity of these Tfh and Treg cells, focusing on citrullinated self-antigens.

ACPA: USE IN DIAGNOSIS AND PROGNOSIS OF JIA

Children with RF+ polyarticular JIA also commonly have positive ACPA tests (83, 84). This is particularly true amongst patients with the *HLA-DRB1*0401* (DR4) and *HLA-DRB1*0101* (DR1) haplotypes. As earlier studies have pointed to marked similarity between these patients and adult RA patients, it is not surprising that there is a high frequency of ACPA in adult RA patients that express the same MHC class II molecules (85–87). ACPA are highly specific for adult RA, and have been shown to predict future risk for developing RA in otherwise healthy individuals (88). As with RF, the sensitivity of ACPA for detecting JIA is very low, but in patients with RF+ polyarticular JIA, these autoantibodies are highly specific (68, 84, 89–95).

The presence of ACPA in polyarticular RF+ JIA has been shown by numerous studies to confer a greater risk of more aggressive and erosive disease (51, 84, 85, 90, 93, 95–99). This is mirrored in patients with ACPA-positive adult RA, who also have a more severe disease course (98, 100, 101). As with RF, ACPA positivity among children with polyarticular JIA typically leads pediatric rheumatologists to recommend earlier, more aggressive therapy (56).

RF, ACPA, AND CIRCULATING IMMUNE COMPLEXES: POTENTIAL ROLES IN THE PATHOGENESIS OF INFLAMMATORY ARTHRITIS

Whether or not ACPA have a direct role in the pathogenesis of inflammatory arthritis is unclear. ACPA- and fibrinogen-containing immune complexes have been shown *in vitro* to induce macrophage TNF production via binding to Fc-gamma receptor IIa (102, 103). ACPA have also been shown *in vitro* to activate complement and elicit macrophage activation by crosslinking TLR4 and Fc gamma receptors, and *in vivo,* to enhance tissue injury in murine models of inflammatory arthritis. Importantly, RF and ACPA may synergize to augment some of these functions (104–107).

Circulating immune complexes are elevated in RA and JIA. Immune complexes containing RF, complement factors C1q, C4, C3, C4, and components of the membrane attack complex (MAC) are detectable in synovial fluid of patients with polyarticular RF+ JIA (108). Binding of MAC to circulating immune complexes correlates with erythrocyte sedimentation rate, suggesting that in JIA, complement-mediated tissue damage is induced by the classical complement activation pathway (109).

Given the strong association between RF and ACPA positivity in RA patients, questions about a shared role in pathogenesis are being investigated. A large study of US veterans with RA demonstrated that patients with doubly positive ACPA and RF have higher clinical disease activity scores, serum CRP, and levels of TNF-alpha, IL-1 beta, IL-6, IL-12, and IL-17A. The same authors also performed additional *in vitro* experiments using peripheral blood mononuclear cells from healthy controls incubated with IgG-ACPA containing immune complexes derived from RA patients, and found that the addition of IgM-RF significantly increased TNF-alpha production in monocytes (110). Recently, immune complexes containing both IgG-ACPA and IgM-RF have also been identified in RA patients (111).

Interestingly, anti-PAD4 autoantibodies that increase PAD4 catalytic activity have been discovered in a subset of adult RA patients with particularly erosive disease (112). This indicates a possible feed-forward loop in driving additional self-reactivity against citrullinated autoantigens, and identifies a possible pathogenic role for anti-PAD4 autoantibodies.

Unfortunately, because there are far fewer children with JIA than adults with RA, much of our knowledge about the role of RF, ACPA, and other autoantibodies in inflammatory arthritis is derived from the adult literature. This is clearly a limitation, since these studies only pertain to a small subset of JIA patients overall, specifically children with RF+ polyarticular JIA.

AUTOANTIBODIES RECOGNIZING OTHER MODIFIED ANTIGENS

Another class of autoantibodies that have recently garnered attention are those that recognize carbamylated self-proteins. Like citrullination, carbamylation is another post-translational modification; in the case of carbamylation, lysine and taurine residues are replaced by isocyanic acid. The phagocyte peroxidase enzymes, myeloperoxidase, and eosinophil peroxidase oxidize thiocyanate, forming two compounds that can result in protein carbamylation: cyanate and hypothiocyanous acid (or HOSCN) (113, 114). Like PAD4, myeloperoxidase is also found in NETs and thus may be involved in promoting carbamylation of self-antigens at sites of neutrophilic inflammation (115).

Interestingly, smoking may be linked to carbamylation. Cigarette smoke contains cyanide, which can be oxidized by myeloperoxidase into cyanate. Cyanate can subsequently participate in non-enzymatic reactions that result in carbamylation under physiologic conditions (116). Exposing mice to cigarette smoke resulted in the generation of carbamylated vimentin, and the sera from exposed mice were broadly reactive against a variety of carbamylated antigens, unlike control mice (117). Smoking and non-smoking RA patients were also studied, and while anti-carbamylated protein antibodies were detectable in both, titers were significantly higher in smokers than in non-smokers (117).

A variety of autoantibodies have now been identified that recognize carbamylated proteins in patients with RA, SLE, and JIA (118–121). A recent study of pediatric rheumatology patients revealed detectable anti-carbamylated protein antibodies in 31% of patients with oligoarticular JIA, 21% of patients with polyarticular-RF positive JIA, in 13% of patients with polyarticular-RF negative JIA, and in 0% of healthy controls (119). Notably, a recent meta-analysis showed that triple positivity for RF, ACPA, and anti-carbamylated protein antibodies was highly specific to patients with active RA, and patients who would go on to develop RA (122).

Additional forms of post-translational modification are relevant to the JIA "peptidome." For instance, carbonylation is the modification of arginine into glutamic semialdehyde, and proline or methionine residues can undergo oxidation (123). Oxidation has been studied in the context of the molecular chaperone, transthyretin (TTR), which normally functions as a transporter of thyroxine, retinol, and other substances. Both TTR and anti-TTR antibodies are detected in the synovial fluid and plasma in greater concentrations in JIA patients than in controls. TTR can form aggregates when oxidized, and these aggregates were detected in JIA synovium. Interestingly, experiments in HLA-DR1 transgenic mice revealed that *in vivo* T cell proliferation was significantly higher when mice were immunized with

oxidized, rather than native TTR, suggesting that the aggregated forms of this autoantigen were more immunogenic (123). Finally, malondialdehyde-acetaldehyde (MAA) adducts are a consequence of oxidative stress; MAA adduction of proteins is increased among patients with RA, and the presence of anti-MAA antibodies correlates with ACPA positivity (124).

While knowledge regarding T and B cell responses to post-translationally modified autoantigens is burgeoning, it is becoming clear that these alterations represent distinct portals for breakdowns in immune self-tolerance, as these modified proteins can serve as neoantigens.

AUTOANTIBODIES AND POTENTIAL EXAMPLES OF MOLECULAR MIMICRY IN RA AND JIA

A significant body of research shows an association between periodontal disease and RA, and detectable titers of antibodies to the pathobiont, *Porphyromonas gingivalis*, are seen more commonly in RA patients than controls (125). Alpha-enolase is an established autoantigen in RA which undergoes citrullination and is present in the inflamed synovium (126). Immunization of human HLA-DR4-expressing transgenic mice with enolase derived from *P. gingivalis* induced arthritis and the development of anti-citrullinated enolase autoantibodies. Thus, molecular mimicry between exogenous enolase derived from *P. gingivalis* may be a potential mechanism driving autoantibody formation in RA, particularly in patients with periodontal disease and colonization with *P. gingivalis* (127).

Another example of molecular mimcry which could contribute to the pathogenesis of JIA involves binding immunoglobulin protein (BiP), a member of the heat shock protein (HSP) 70 family, and another established autoantigen in inflammatory arthritis. Approximately 60% of adult patients with RA and 37% of pediatric patients with polyarticular-RF+ JIA have anti-BiP antibodies (128, 129). BiP appears to have immunomodulatory effects, including inducing the production of IL-10, IL-1 receptor antagonist, soluble TNFRII, and downregulation of CD86 and HLA-DR (130). DBA/1J mice immunized with citrullinated-, but not native BiP, developed broadly reactive ACPAs. In a collagen-induced arthritis model, pre-immunizing mice with citrullinated BiP exacerbated the phenotype. The same authors also detected autoantibodies to both native and citrullinated BiP in adult RA patients (131).

Interestingly, a putative mimetope for $BiP_{336-355}$ has been discovered in bacterial HSPs. Immunization of HLA-DR4 transgenic mice with $HSP70_{287-306}$ derived from *Mycobacterium leporae* induced the development of BiP autoantibodies. In the collagen-induced arthritis model, oral administration of this mycobacterial peptide induced tolerance, blunting the development of arthritis and reducing anti-BiP autoantibody production (132). Thus, tolerance against BiP, a common autoantigen in RA and JIA, may be broken by immune responses directed against a mycobacterial pathogen.

NEW APPROACHES TO AUTOANTIBODY IDENTIFICATION

Admittedly, much of the knowledge of autoantibodies in JIA derives from experience and knowledge in adult RA. While this is not unreasonable, it has major shortcomings since the vast majority of patients with JIA do not fall into the RF+ polyarticular subtype, and are therefore clinically and likely pathophysiologically distinct.

In recognizing this shortcoming, one group applied a novel high-throughput nucleic acid programmable protein array in a small sample of JIA patients to screen for autoantibodies reactive against 768 proteins and identified 18 antibody specificities that could segregate two clusters of patients (133). To our knowledge, the autoantibodies discovered in this study have not been tested in control patients, nor has this approach been applied to a larger sample of patients. However, this or similar methods have the potential to detect additional autoantibody specificities that may promote new insight into the pathogenesis of JIA.

Another group recently looking to develop a novel diagnostic tool for JIA looked to overcome the diversity of autoantibodies in JIA and screened a random library of peptides displayed via Phage-ELISA for mimetopes with reactivity to large percentages of patients with JIA. This method successfully identified a mimetope named "PRF+1" which discriminated JIA patients from controls with a sensitivity of 61% and specificity of 91%. This peptide was subsequently applied to a differential pulse voltammetry system to generate an electro-biochemical sensor for rapid detection of anti-PRF+1 antibodies (134, 135).

SUMMARY

Autoantibody testing is commonly performed among children with suspected JIA. It is essential to recognize that JIA is much more heterogeneous than RA in adults, and that information regarding the value of particular autoantibodies in adult RA (e.g., RF and ACPA) apply to only a small subset of JIA patients, i.e., those with RF+ polyarticular JIA. We have provided an overview of commonly used autoantibody tests in JIA, seeking to explain their clinical utility as well as limitations and challenges to the field.

The ANA test is a non-specific test of autoantibody reactivity against nuclear antigens. Although highly sensitive for SLE and other related conditions, it is not a diagnostic test for JIA. ANA positivity is most commonly seen in young, female patients with oligoarticular disease. It does not clearly associate with differences in prognosis in any subtype of JIA. Positive ANA status does, however, increase the risk of uveitis and thus its use in clinical practice is primarily focused on predicting the ophthalmologic complications of JIA.

Patients with polyarticular JIA with RF and ACPA positivity have more aggressive and erosive disease. These markers are also not diagnostic tests for JIA, since most JIA patients do not have the seropositive polyarticular subset of disease. Although there are no studies proving roles for RF and ACPA in arthritis

pathogenesis, *in vitro* studies indicate that these two groups of antibodies may interact to drive immunopathology.

The multitude of targets recognized by all major classes of autoantibodies in JIA has made determining roles in pathogenesis more difficult. It is likely these roles will not become clear until advances in proteomics allow us to screen more patients for more specific autoantibodies, and by embracing the complexities of these autoimmune responses rather than attempting to simplify them.

AUTHOR CONTRIBUTIONS

SM wrote and edited the manuscript. BB helped conceive of and edit the manuscript.

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REFERENCES

1. Harrold LR, Salman C, Shoor S, Curtis JR, Asgari MM, Gelfand JM, et al. Incidence and prevalence of juvenile idiopathic arthritis among children in a managed care population, 1996-2009. *J Rheumatol.* (2013) 40:1218–25. doi: 10.3899/jrheum.120661
2. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of associations for rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* (2004) 31:390–2.
3. Nigrovic PA, Raychaudhuri S, Thompson SD. Review: genetics and the classification of arthritis in adults and children. *Arthritis Rheumatol.* (2018) 70:7–17. doi: 10.1002/art.40350
4. Vehe RK, Begovich AB, Nepom BS. HLA susceptibility genes in rheumatoid factor ositive juvenile rheumatoid arthritis. *J Rheumatol.* (1990) 26:11–5.
5. De Silvestri A, Capittini C, Poddighe D, Marseglia GL, Mascaretti L, Bevilacqua E, et al. HLA-DRB1 alleles and juvenile idiopathic arthritis: diagnostic clues emerging from a meta-analysis. *Autoimmun Rev.* (2017) 16:1230–6. doi: 10.1016/j.autrev.2017.10.007
6. Hersh AO, Prahalad S. Immunogenetics of juvenile idiopathic arthritis: a comprehensive review. *J Autoimmun.* (2015) 64:113–24. doi: 10.1016/j.jaut.2015.08.002
7. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum.* (2008) 58:875–87. doi: 10.1002/art.23291
8. Pardeo M, Bracaglia C, De Benedetti F. Systemic juvenile idiopathic arthritis: New insights into pathogenesis and cytokine directed therapies. *Best Pract Res Clin Rheumatol.* (2017) 31:505–16. doi: 10.1016/j.berh.2018.02.002
9. Ombrello MJ, Remmers EF, Tachmazidou I, Grom A, Foell D, Haas J-P, et al. HLA-DRB1*11 and variants of the MHC class II locus are strong risk factors for systemic juvenile idiopathic arthritis. *Proc Natl Acad Sci USA.* (2015) 112:15970–5. doi: 10.1073/pnas.1520779112
10. Ombrello MJ, Arthur VL, Remmers EF, Hinks A, Tachmazidou I, Grom AA, et al. Genetic architecture distinguishes systemic juvenile idiopathic arthritis from other forms of juvenile idiopathic arthritis: clinical and therapeutic implications. *Ann Rheum Dis.* (2017) 76:906–13. doi: 10.1136/annrheumdis-2016-210324
11. Corcione A, Ferlito F, Gattorno M, Gregorio A, Pistorio A, Gastaldi R, et al. Phenotypic and functional characterization of switch memory B cells from patients with oligoarticular juvenile idiopathic arthritis. *Arthritis Res Ther.* (2009) 11:R150. doi: 10.1186/ar2824
12. Low JM, Chauhan AK, Moore TL. Abnormal kappa:lambda light chain ratio in circulating immune complexes as a marker for B cell activity in juvenile idiopathic arthritis. *Scand J Immunol.* (2007) 65:76–83. doi: 10.1111/j.1365-3083.2006.01859.x
13. Morbach H, Richl P, Faber C, Singh SK, Girschick HJ. The kappa immunoglobulin light chain repertoire of peripheral blood B cells in patients with juvenile rheumatoid arthritis. *Mol Immunol.* (2008) 45:3840–6. doi: 10.1016/j.molimm.2008.05.011
14. Morbach H, Wiegering V, Richl P, Schwarz T, Suffa N, Eichhorn E-M, et al. Activated memory B cells may function as antigen-presenting cells in the joints of children with juvenile idiopathic arthritis. *Arthritis Rheum.* (2011) 63:3458–66. doi: 10.1002/art.30569
15. Hargraves MM, Richmond H, Morton R. Presentation of two bone marrow elements; the tart cell and the L.E. cell. *Proc Staff Meet Mayo Clin.* (1948) 23:25–8.
16. Haserick JR, Bortz DW. Normal bone marrow inclusion phenomena induced by lupus erythematosus plasma. *J Invest Dermatol.* (1949) 13:47–9.
17. Sundberg RD, Lick NB. L. E. cells in the blood in acute disseminated lupus erythematosus. *J Invest Dermatol.* (1949) 12:83.
18. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med.* (1994) 179:1317–30.
19. Rahman A, Isenberg DA. Systemic lupus erythematosus. *N Engl J Med.* (2008) 358:929–39. doi: 10.1056/NEJMra071297
20. Munoz LE, Gaipl US, Franz S, Sheriff A, Voll RE, Kalden JR, et al. SLE–a disease of clearance deficiency? *Rheumatology* (2005) 44:1101–7. doi: 10.1093/rheumatology/keh693
21. Correll CK, Spector LG, Zhang L, Binstadt BA, Vehe RK. Use of rheumatology laboratory studies among primary pediatricians. *Clin Pediatry* (2016) 55:1279–88. doi: 10.1177/0009922815624902
22. Malleson PN, Sailer M, Mackinnon MJ. Usefulness of antinuclear antibody testing to screen for rheumatic diseases. *Arch Dis Child.* (1997) 77:299–304.
23. Malleson PN, Mackinnon MJ, Sailer-Hoeck M, Spencer CH. Review for the generalist: the antinuclear antibody test in children - when to use it and what to do with a positive titer. *Pediatr Rheumatol Online J.* (2010) 8:27. doi: 10.1186/1546-0096-8-27
24. McGhee JL, Kickingbird LM, Jarvis JN. Clinical utility of antinuclear antibody tests in children. *BMC Pediatr.* (2004) 4:13. doi: 10.1186/1471-2431-4-13
25. Barahona-Garrido J, Camacho-Escobedo J, García-Martínez CI, Tocay H, Cabiedes J, Yamamoto-Furusho JK. Antinuclear antibodies: a marker associated with steroid dependence in patients with ulcerative colitis. *Inflamm Bowel Dis.* (2009) 15:1039–43. doi: 10.1002/ibd.20852
26. Campanilho-Marques R, Bogas M, Ramos F, Santos MJ, Fonseca JE. Prognostic value of antinuclear antibodies in juvenile idiopathic arthritis and anterior uveitis. Results from a systematic literature review. *Acta Reum Port* (2014) 39:116–22.
27. Radic M, Herrmann M, van der Vlag J, Rekvig OP. Regulatory and pathogenetic mechanisms of autoantibodies in SLE. *Autoimmunity* (2011) 44:349–56. doi: 10.3109/08916934.2010.536794
28. Segni M, Pucarelli I, Truglia S, Turriziani I, Serafinelli C, Conti F. High prevalence of antinuclear antibodies in children with thyroid autoimmunity. *J Immunol Res.* (2014) 2014:150239. doi: 10.1155/2014/150239
29. Thomson AM, West DC. Factors affecting slow regular firing in the suprachiasmatic nucleus in vitro. *J Biol Rhythms* (1990) 5:59–75. doi: 10.1177/074873049000500106
30. Weiss JE. Pediatric systemic lupus erythematosus: more than a positive antinuclear antibody. *Pediatr Rev.* (2012) 33:62–73; quiz 74. doi: 10.1542/pir.33-2-62
31. Yu C, Gershwin ME, Chang C. Diagnostic criteria for systemic lupus erythematosus: a critical review. *J Autoimmun.* (2014) 48–49:10–3. doi: 10.1016/j.jaut.2014.01.004

32. Glerup M, Herlin T, Twilt M. Remission rate is not dependent on the presence of antinuclear antibodies in juvenile idiopathic arthritis. *Clin Rheumatol.* (2017) 36:671–6. doi: 10.1007/s10067-017-3540-x
33. Litwin CM, Binder SR. ANA testing in the presence of acute and chronic infections. *J Immunoassay Immunochem.* (2016) 37:439–52. doi: 10.1080/15321819.2016.1174136
34. Guillaume S, Prieur AM, Coste J, Job-Deslandre C. Long-term outcome and prognosis in oligoarticular-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2000) 43:1858–65. doi: 10.1002/1529-0131(200008)43:8<1858::AID-ANR23>3.0.CO;2-A
35. Stoll ML, Punaro M. Psoriatic juvenile idiopathic arthritis: a tale of two subgroups. *Curr Opin Rheumatol.* (2011) 23:437–43. doi: 10.1097/BOR.0b013e328348b278
36. Hügle B, Hinze C, Lainka E, Fischer N, Haas J-P. Development of positive antinuclear antibodies and rheumatoid factor in systemic juvenile idiopathic arthritis points toward an autoimmune phenotype later in the disease course. *Pediatr Rheumatol Online J.* (2014) 12:28. doi: 10.1186/1546-0096-12-28
37. Iglesias E, Torrente-Segarra V, Bou R, Ricart S, González MI, Sánchez J, et al. Non-systemic juvenile idiopathic arthritis outcome after reaching clinical remission with anti-TNF-α therapy: a clinical practice observational study of patients who discontinued treatment. *Rheumatol Int.* (2014) 34:1053–7. doi: 10.1007/s00296-013-2884-z
38. Stoll ML, Li QZ, Zhou J, Punaro M, Olsen NJ. Elevated IgG autoantibody production in oligoarticular juvenile idiopathic arthritis may predict a refractory course. *Clin Exp Rheumatol.* (2011) 29:736–42.
39. Asproudis I, Katsanos A, Kozeis N, Tantou A, Konstas AG. Update on the treatment of uveitis in patients with Juvenile idiopathic arthritis: a review. *Adv Ther.* (2017) 34:2558–65. doi: 10.1007/s12325-017-0635-3
40. Oberle EJ, Harris JG, Verbsky JW. Polyarticular juvenile idiopathic arthritis - epidemiology and management approaches. *Clin Epidemiol.* (2014) 6:379–93. doi: 10.2147/CLEP.S53168
41. Heiligenhaus A, Minden K, Föll D, Pleyer U. Uveitis in juvenile idiopathic arthritis. *Dtsch Arztebl Int.* (2015) 112:92–100. doi: 10.3238/arztebl.2015.0092
42. Rose H, Ragan C. Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc Soc Exp Biol Med.* (1948) 68:1–6.
43. Waaler E. On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. *Acta Pathol Microbiol Immunol Scand.* (1940) 10:172–8. doi: 10.1111/j.1600-0463.2007.apm_682a.x
44. Ingegnoli F, Castelli R, Gualtierotti R. Rheumatoid factors: clinical applications. *Dis Markers* (2013) 35:727–34. doi: 10.1155/2013/726598
45. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid arthritis classification criteria: an American college of rheumatology/European league against rheumatism collaborative initiative. *Arthritis Rheum.* (2010) 62:2569–81. doi: 10.1002/art.27584
46. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Ann Intern Med.* (2007) 146:797–808. doi: 10.7326/0003-4819-146-11-200706050-00008
47. Soltys AI, Axford JS, Sutton BJ. Rheumatoid factors: where are we now? *Ann Rheum Dis.* (1997) 56:285–6.
48. Williams R, Kunkel HG. Rheumatoid factor, complement, and conglutinin aberrations in patients with subacute bacterial endocarditis. *J Clin Invest.* (1962) 41:666–75. doi: 10.1172/JCI104523
49. Toumbis A, Franklin EC, McEwen C, Kuttner AG. Clinical and serologic observations in patients with juvenile rheumatoid arthritis and their relatives. *J Pediatr.* (1963) 62:463–73.
50. Eichenfield AH, Athreya BH, Doughty RA, Cebul RD. Utility of rheumatoid factor in the diagnosis of juvenile rheumatoid arthritis. *Pediatrics* (1986) 78:480–4.
51. Berntson L, Nordal E, Fasth A, Aalto K, Herlin T, Nielsen S, et al. Anti-type II collagen antibodies, anti-CCP, IgA RF and IgM RF are associated with joint damage, assessed eight years after onset of juvenile idiopathic arthritis (JIA). *Pediatr Rheumatol Online J.* (2014) 12:22. doi: 10.1186/1546-0096-12-22
52. Fantini F, Gerloni V, Gattinara M, Cimaz R, Arnoldi C, Lupi E. Remission in juvenile chronic arthritis: a cohort study of 683 consecutive cases with a mean 10 year followup. *J Rheumatol.* (2003) 30:579–84.
53. Flatø B, Lien G, Smerdel A, Vinje O, Dale K, Johnston V, et al. Prognostic factors in juvenile rheumatoid arthritis: a case-control study revealing early predictors and outcome after 14.9 years. *J Rheumatol.* (2003) 30:386–93.
54. Gilliam BE, Chauhan AK, Low JM, Moore TL. Pediatric rheumatology Measurement of biomarkers in juvenile idiopathic arthritis patients and their significant association with disease severity : a comparative study. *Clin Exp Rheumatol.* (2013) 26:492–7.
55. Hinks A, Marion MC, Cobb J, Comeau ME, Sudman M, Ainsworth HC, et al. Brief report: the genetic profile of rheumatoid factor-positive polyarticular juvenile idiopathic arthritis resembles that of adult rheumatoid arthritis. *Arthritis Rheum.* (2018) 70:957–62. doi: 10.1002/art.40443
56. Ringold S, Weiss PF, Colbert RA, DeWitt EM, Lee T, Onel K, et al. Childhood arthritis and rheumatology research alliance consensus treatment plans for new-onset polyarticular juvenile idiopathic arthritis. *Arthritis Care Res (Hoboken).* (2014) 66:1063–72. doi: 10.1002/acr.22259
57. Carson DA, Chen PP, Kipps TJ. New roles for rheumatoid factor. *J Clin Invest.* (1991) 87:379–83. doi: 10.1172/JCI115007
58. Børretzen M, Chapman C, Natvig JB, Thompson KM. Differences in mutational patterns between rheumatoid factors in health and disease are related to variable heavy chain family and germ-line gene usage. *Eur J Immunol.* (1997) 27:735–41. doi: 10.1002/eji.1830270323
59. Päi S, Päi L, Birkenfeldt R. Correlation of serum IgA rheumatoid factor levels with disease severity in rheumatoid arthritis. *Scand J Rheumatol.* (1998) 27:252–6.
60. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* (2002) 416:603–7. doi: 10.1038/416603a
61. Song YW, Kang E. The pathogenic role of rheumatoid factor in rheumatoid arthritis. *Int J Clin Rheumtol.* (2010) 5:651–8. doi: 10.2217/ijr.10.62
62. Nienhuis RL, Mandema E. A new serum factor in patients with rheumatoid arthritis; the antiperinuclear factor. *Ann Rheum Dis.* (1964) 23:302–5.
63. Sebbag M, Simon M, Vincent C, Masson-Bessière C, Girbal E, Durieux JJ, et al. The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies. *J Clin Invest.* (1995) 95:2672–9. doi: 10.1172/JCI117969.
64. Young BJ, Mallya RK, Leslie RD, Clark CJ, Hamblin TJ. Anti-keratin antibodies in rheumatoid arthritis. *Br Med J.* (1979) 2:97–9.
65. Bang H, Egerer K, Gauliard A, Lüthke K, Rudolph PE, Fredenhagen G, et al. Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis. *Arthritis Rheum.* (2007) 56:2503–11. doi: 10.1002/art.22817
66. Gilliam BE, Reed MR, Chauhan AK, Dehlendorf AB, Moore TL. Evidence of fibrinogen as a target of citrullination in IgM rheumatoid factor-positive polyarticular juvenile idiopathic arthritis. *Pediatr Rheumatol Online J.* (2011) 9:8. doi: 10.1186/1546-0096-9-8
67. Gilliam BE, Chauhan AK, Moore TL. Evaluation of anti-citrullinated type II collagen and anti-citrullinated vimentin antibodies in patients with juvenile idiopathic arthritis. *Pediatr Rheumatol Online J.* (2013) 11:31. doi: 10.1186/1546-0096-11-31
68. Hromadnikova I, Stechova K, Pavla V, Hridelova D, Houbova B, Voslarova S, et al. Anti-cyclic citrullinated peptide antibodies in patients with juvenile idiopathic arthritis. *Autoimmunity* (2002) 35:397–401. doi: 10.1080/0891693021000014970
69. Kuna AT, Lamot L, Miler M, Harjacek M, Simundic A-M, Vrkic N. Antibodies to mutated citrullinated vimentin and antibodies to cyclic citrullinated peptides in juvenile idiopathic arthritis. *Clin Chem Lab Med.* (2009) 47:1525–30. doi: 10.1515/CCLM.2009.288
70. Moore TL, Gilliam TL, Crespo-Pagnussat S, Feller L, Chauhan AK. Measurement and evaluation of isotypes of anti-citrullinated fibrinogen and anti-citrullinated alpha-enolase antibodies in juvenile idiopathic arthritis. *Clin Exp Rheumatol.* (2014) 32:740–6.
71. Pratesi F, Tommasi C, Anzilotti C, Chimenti D, Migliorini P. Deiminated epstein-barr virus nuclear antigen 1 is a target of anti-citrullinated protein

antibodies in rheumatoid arthritis. *Arthritis Rheum.* (2006) 54:733–41. doi: 10.1002/art.21629
72. Vittecoq O, Incaurgarat B, Jouen-Beades F, Legoedec J, Letourneur O, Rolland D, et al.. Autoantibodies recognizing citrullinated rat filaggrin in an ELISA using citrullinated and non-citrullinated recombinant proteins as antigens are highly diagnostic for rheumatoid arthritis. *Clin Exp Immunol.* (2004) 135:173–80. doi: 10.1111/j.1365-2249.2004.02341.x
73. Vossenaar ER, Després N, Lapointe E, van der Heijden A, Lora M, Senshu T, et al. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther.* (2004) 6:R142–R150. doi: 10.1186/ar1149
74. Spengler J, Lugonja B, Ytterberg AJ, Zubarev RA, Creese AJ, Pearson MJ, et al. Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. *Arthritis Rheum.* (2015) 67:3135–45. doi: 10.1002/art.39313
75. Willis VC, Banda NK, Cordova KN, Chandra PE, Robinson WH, Cooper DC, et al. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. *Clin Exp Immunol.* (2017) 188:263–74. doi: 10.1111/cei.12932
76. Leshner M, Wang S, Lewis C, Zheng H, Chen XA, Santy L, et al. PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. *Front Immunol.* (2012) 3:307. doi: 10.3389/fimmu.2012.00307
77. Rohrbach AS, Slade DJ, Thompson PR, Mowen KA. Activation of PAD4 in NET formation. *Front Immunol.* (2012) 3:360. doi: 10.3389/fimmu.2012.00360.
78. Stritesky GL, Jameson SC, Hogquist KA. Selection of self-reactive T cells in the thymus. *Annu Rev Immunol.* (2012) 30:95–114. doi: 10.1146/annurev-immunol-020711-075035
79. James EA, Rieck M, Pieper J, Gebe JA, Yue BB, Tatum M, et al. Citrulline-specific Th1 cells are increased in rheumatoid arthritis and their frequency is influenced by disease duration and therapy. *Arthritis Rheum.* (2014) 66:1712–22. doi: 10.1002/art.38637
80. Liu R, Wu Q, Su D, Che N, Chen H, Geng L, et al. A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis. *Arthritis Res Ther.* (2012) 14:R255. doi: 10.1186/ar4100
81. Fukuyo S, Nakayamada S, Iwata S, Kubo S, Saito K, Tanaka Y. Abatacept therapy reduces CD28+CXCR5+follicular helper-like T cells in patients with rheumatoid arthritis. *Clin Exp Rheumatol.* (2017) 35:562–570.
82. Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C, et al. Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. *Ann Rheum Dis.* (2015) 74:1293–301. doi: 10.1136/annrheumdis-2013-204228
83. Spârchez M, Miu N, Bolba C, Iancu M, Spârchez Z, Rednic S. Evaluation of anti-cyclic citrullinated peptide antibodies may be beneficial in RF-negative juvenile idiopathic arthritis patients. *Clin Rheumatol.* (2016) 35:601–7. doi: 10.1007/s10067-015-2971-5
84. van Rossum M, van Soesbergen R, de Kort S, ten Cate R, Zwinderman AH, de Jong B, et al. Anti-cyclic citrullinated peptide (anti-CCP) antibodies in children with juvenile idiopathic arthritis. *J Rheumatol.* (2003) 30:825–8.
85. Ferucci ED, Majka DS, Parrish LA, Moroldo MB, Ryan M, Passo M, et al. Antibodies against cyclic citrullinated peptide are associated with HLA-DR4 in simplex and multiplex polyarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* (2005) 52:239–46. doi: 10.1002/art.20773
86. Senkpiehl I, Marget M, Wedler M, Jenisch S, Georgi J, Kabelitz D, et al. HLA-DRB1 and anti-cyclic citrullinated peptide antibody production in rheumatoid arthritis. *Int Arch Allergy Immunol.* (2005) 137:315–8. doi: 10.1159/000086463
87. Wagner CA, Sokolove J, Lahey LJ, Bengtsson C, Saevarsdottir S, Alfredsson L, et al. Identification of anticitrullinated protein antibody reactivities in a subset of anti-CCP-negative rheumatoid arthritis: association with cigarette smoking and HLA-DRB1 "shared epitope" alleles. *Ann Rheum Dis.* (2015) 74:579–86. doi: 10.1136/annrheumdis-2013-203915
88. van Venrooij WJ, Zendman AJW, Pruijn GJM. Autoantibodies to citrullinated antigens in (early) rheumatoid arthritis. *Autoimmun Rev.* (2006) 6:37–41. doi: 10.1016/j.autrev.2006.03.008
89. Avcin T, Cimaz R, Falcini F, Zulian F, Martini G, Simonini G, et al. Prevalence and clinical significance of anti-cyclic citrullinated peptide antibodies in juvenile idiopathic arthritis. *Ann Rheum Dis.* (2002) 61:608–11. doi: 10.1136/ard.61.7.608
90. Brunner J, Sitzmann FC. The diagnostic value of anti-cyclic citrullinated peptide (CCP) antibodies in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* (2006) 24:449–51.
91. Brunner JKH, Sitzmann FC. Anticyclic citrullinated peptide antibodies in juvenile idiopathic arthritis. *Mod Rheumatol.* (2006) 16:372–5. doi: 10.1007/s10165-006-0520-4
92. Kasapçopur O, Altun S, Aslan M, Karaarslan S, Kamburoglu-Göksel A, Saribas S, et al. Diagnostic accuracy of anti-cyclic citrullinated peptide antibodies in juvenile idiopathic arthritis. *Ann Rheum Dis.* (2004) 63:1687–9. doi: 10.1136/ard.2003.019331
93. Low JM, Chauhan AK, Kietz DA, Daud U, Pepmueller PH, Moore TL. Determination of anti-cyclic citrullinated peptide antibodies in the sera of patients with juvenile idiopathic arthritis. *J Rheumatol.* (2004) 31:1829–33.
94. Pang SY, Liu HY, Huang YJ, Liu YF, Dai YM, Zeng P, et al. Diagnostic performance of anti-citrullinated protein/peptide antibodies in juvenile idiopathic arthritis. *Genet Mol Res.* (2016) 15. doi: 10.4238/gmr.15028641
95. Tebo AE, Jaskowski T, Davis KW, Whiting A, Clifford B, Zeft A, et al. Profiling anti-cyclic citrullinated peptide antibodies in patients with juvenile idiopathic arthritis. *Pediatr Rheumatol Online J.* (2012) 10:29. doi: 10.1186/1546-0096-10-29
96. Habib HM, Mosaad YM, Youssef HM. Anti-cyclic citrullinated peptide antibodies in patients with juvenile idiopathic arthritis. *Immunol Invest.* (2008) 37:849–57. doi: 10.1080/08820130802438057
97. Omar A, Abo-Elyoun I, Hussein H, Nabih M, Atwa H, Gad S, et al. Anti-cyclic citrullinated peptide (anti-CCP) antibody in juvenile idiopathic arthritis (JIA): correlations with disease activity and severity of joint damage (a multicenter trial). *Joint Bone Spine* (2013) 80:38–43. doi: 10.1016/j.jbspin.2012.03.008
98. Skare TS, Nisihara RM, Silva RM, Munhoz da Silva DJ, Gameiro Silva MB, Utiyama SRR. Anti-cyclic citrullinated peptide antibodies in adult patients with juvenile idiopathic arthritis. *J Clin Rheumatol.* (2011) 17:421–3. doi: 10.1097/RHU.0b013e31823a4d0a
99. Syed RH, Gilliam BE, Moore TL. Rheumatoid factors and anticyclic citrullinated peptide antibodies in pediatric rheumatology. *Curr Rheumatol Rep.* (2008) 10:156–63. doi: 10.1007/s11926-008-0027-4
100. Peckham H, Cambridge G, Bourke L, Sen D, Radziszewska A, Leandro M, et al. Antibodies to cyclic citrullinated peptides in patients with juvenile idiopathic arthritis and patients with rheumatoid arthritis: shared expression of the inherently autoreactive 9G4 idiotype. *Arthritis Rheumatol.* (2017) 69:1387–95. doi: 10.1002/art.40117
101. Snir O, Widhe M, Hermansson M, von Spee C, Lindberg J, Hensen S, et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. *Arthritis Rheum.* (2010) 62:44–52. doi: 10.1002/art.25036
102. Clavel C, Nogueira L, Laurent L, Iobagiu C, Vincent C, Sebbag M, et al. Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. *Arthritis Rheum.* (2008) 58:678–88. doi: 10.1002/art.23284
103. Mathsson L, Lampa J, Mullazehi M, Rönnelid J. Immune complexes from rheumatoid arthritis synovial fluid induce FcgammaRIIa dependent and rheumatoid factor correlated production of tumour necrosis factor-alpha by peripheral blood mononuclear cells. *Arthritis Res Ther.* (2006) 8:R64. doi: 10.1186/ar1926
104. Anquetil F, Clavel C, Offer G, Serre G, Sebbag M. IgM and IgA rheumatoid factors purified from rheumatoid arthritis sera boost the Fc receptor– and complement-dependent effector functions of the disease-specific anti–citrullinated protein autoantibodies. *J Immunol.* (2015) 194:3664–74. doi: 10.4049/jimmunol.1402334
105. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, et al. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *J Clin Invest.* (2006) 116:961–73. doi: 10.1172/JCI25422

106. Sokolove J, Zhao X, Chandra PE, Robinson WH. Immune complexes containing citrullinated fibrinogen costimulate macrophages via toll-like receptor 4 and Fcγ receptor. *Arthritis Rheum.* (2011) 63:53–62. doi: 10.1002/art.30081
107. Trouw LA, Haisma EM, Levarht EWN, Van Der Woude D, Ioan-Facsinay A, Daha MR, et al. Anti-cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. *Arthritis Rheum.* (2009) 60:1923–31. doi: 10.1002/art.24622
108. Moore TL. Immune complexes in juvenile idiopathic arthritis. *Front Immunol.* (2016) 7:177. doi: 10.3389/fimmu.2016.00177
109. Gilliam BE, Reed MR, Chauhan AK, Dehlendorf AB, Moore TL. Significance of complement components C1q and C4 bound to circulating immune complexes in juvenile idiopathic arthritis: support for classical complement pathway activation. *Clin Exp Rheumatol.* (2011) 29:1049–56.
110. Sokolove J, Johnson DS, Lahey LJ, Wagner CA, Cheng D, Thiele GM, et al. Rheumatoid factor as a potentiator of anti-citrullinated protein antibody-mediated inflammation in rheumatoid arthritis. *Arthritis Rheumatol.* (2014) 66:813–21. doi: 10.1002/art.38307
111. Haraguchi A, Yamada H, Kondo M, Okazaki K, Fukushi J-I, Oyamada A, et al. Serum IgG ACPA-IgM RF immune complexes were detected in rheumatoid arthritis patients positive for IgM ACPA. *Clin Exp Rheumatol.* (2018) 36:612–8.
112. Darrah E, Giles JT, Ols ML, Bull HG, Andrade F, Rosen A. Erosive rheumatoid arthritis is associated with antibodies that activate PAD4 by increasing calcium sensitivity. *Sci Transl Med.* (2013) 5. doi: 10.1126/scitranslmed.3005370
113. Slungaard A, Mahoney JR. Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids: implications for cytotoxicity. *J Biol Chem.* (1991) 266:4903–10.
114. van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J.* (1997) 327(Pt 2):487–92. doi: 10.1042/bj3270487
115. Sur Chowdhury C, Giaglis S, Walker UA, Buser A, Hahn S, Hasler P. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther.* (2014) 16. doi: 10.1186/ar4579
116. Delporte C, Zouaoui Boudjeltia K, Furtmüller PG, Maki RA, Dieu M, Noyon C, et al. Myeloperoxidase-catalyzed oxidation of cyanide to cyanate: a potential carbamylation route involved in the formation of atherosclerotic plaques? *J Biol Chem.* (2018) 293:6374–86. doi: 10.1074/jbc.M117.801076
117. Ospelt C, Bang H, Feist E, Camici G, Keller S, Detert J, et al. Carbamylation of vimentin is inducible by smoking and represents an independent autoantigen in rheumatoid arthritis. *Ann Rheum Dis.* (2017) 76:1176–83. doi: 10.1136/annrheumdis-2016-210059
118. Ceccarelli F, Perricone C, Colasanti T, Massaro L, Cipriano E, Pendolino M, et al. Anti-carbamylated protein antibodies as a new biomarker of erosive joint damage in systemic lupus erythematosus. *Arthritis Res Ther.* (2018) 20:126. doi: 10.1186/s13075-018-1622-z
119. Moore TL, Bhardwaj PPP.Anti-carbamylated protein antibodies in juvenile idiopathic arthritis. *Autimmune Res.* (2017) 4:1022.
120. Muller PCEH, Anink J, Shi J, Levarht EWN, Reinards THCM, Otten MH, et al. Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients. *Ann Rheum Dis.* (2013) 72:2053–5. doi: 10.1136/annrheumdis-2013-203650
121. Regueiro C, Ortiz AM, Boveda MD, Castañeda S, Gonzalez-Alvaro I, Gonzalez A. Association of high titers of anti-carbamylated protein antibodies with decreased bone mineral density in early arthritis patients. *PLoS ONE* (2018) 13:e0202583. doi: 10.1371/journal.pone.0202583
122. Verheul MK, Böhringer S, van Delft MAM, Jones JD, Rigby WFC, Gan RW, et al. Triple positivity for anti-citrullinated protein autoantibodies, rheumatoid factor, and anti-carbamylated protein antibodies conferring high specificity for rheumatoid arthritis: implications for very early identification of at-risk individuals. *Arthritis Rheumatol.* (2018) 70:1721–31. doi: 10.1002/art.40562
123. Clement CC, Moncrieffe H, Lele A, Janow G, Becerra A, Bauli F, et al. Autoimmune response to transthyretin in juvenile idiopathic arthritis. *JCI insight* (2016) 1:e85633. doi: 10.1172/jci.insight.85633
124. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. *Arthritis Rheumatol.* (2015) 67:645–55. doi: 10.1002/art.38969
125. Bender P, Bürgin WB, Sculean A, Eick S. Serum antibody levels against *Porphyromonas gingivalis* in patients with and without rheumatoid arthritis - a systematic review and meta-analysis. *Clin Oral Investig.* (2017) 21:33–42. doi: 10.1007/s00784-016-1938-5
126. Kinloch A, Tatzer V, Wait R, Peston D, Lundberg K, Donatien P, et al. Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. *Arthritis Res Ther.* (2005) 7:R1421–9. doi: 10.1186/ar1845
127. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, et al. Immunization with *Porphyromonas gingivalis* enolase induces autoimmunity to mammalian α-enolase and arthritis in DR4-IE-transgenic mice. *Arthritis Rheum.* (2011) 63:3818–23. doi: 10.1002/art.30639
128. Bläss S, Union A, Raymackers J, Schumann F, Ungethüm U, Müller-Steinbach S, et al. The stress protein BiP is overexpressed and is a major B and T cell target in rheumatoid arthritis. *Arthritis Rheum.* (2001) 44:761–71. doi: 10.1002/1529-0131(200104)44:4<761::AID-ANR132>3.0.CO;2-S
129. Bodman-Smith MD, Fife MF, Wythe H, Corrigal VM, Panayi GS, Wedderburn LR, et al. Anti-BiP antibody levels in juvenile idiopathic arthritis (JIA). *Rheumatology (Oxford)* (2004) 43:1305–6. doi: 10.1093/rheumatology/keh186
130. Corrigall VM, Bodman-Smith MD, Brunst M, Cornell H, Panayi GS. Inhibition of antigen-presenting cell function and stimulation of human peripheral blood mononuclear cells to express an antiinflammatory cytokine profile by the stress protein BiP: relevance to the treatment of inflammatory arthritis. *Arthritis Rheum.* (2004) 50:1164–71. doi: 10.1002/art.20134
131. Shoda H, Fujio K, Shibuya M, Okamura T, Sumitomo S, Okamoto A, et al. Detection of autoantibodies to citrullinated BiP in rheumatoid arthritis patients and pro-inflammatory role of citrullinated BiP in collagen-induced arthritis. *Arthritis Res Ther.* (2011) 13:R191. doi: 10.1186/ar3520
132. Shoda H, Hanata N, Sumitomo S, Okamura T, Fujio K, Yamamoto K. Immune responses to Mycobacterial heat shock protein 70 accompany self-reactivity to human BiP in rheumatoid arthritis. *Sci Rep.* (2016) 6:22486. doi: 10.1038/srep22486
133. Gibson DS, Qiu J, Mendoza EA, Barker K, Rooney ME, LaBaer J. Circulating and synovial antibody profiling of juvenile arthritis patients by nucleic acid programmable protein arrays. *Arthritis Res Ther.* (2012) 14:R77. doi: 10.1186/ar3800
134. Araujo GR, Fujimura PT, Vaz ER, Silva TA, Rodovalho VR, Britto-Madurro AG, et al. A novel reactive epitope-based antigen targeted by serum autoantibodies in oligoarticular and polyarticular juvenile idiopathic arthritis and development of an electrochemical biosensor. *Immunobiology* (2016) 221:634–40. doi: 10.1016/j.imbio.2016.01.006
135. Rodovalho VR, Araujo GR, Vaz ER, Ueira-Vieira C, Goulart LR, Madurro JM, et al. Peptide-based electrochemical biosensor for juvenile idiopathic arthritis detection. *Biosens Bioelectron.* (2018) 100:577–82. doi: 10.1016/j.bios.2017.10.012

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Targeting Tregs in Juvenile Idiopathic Arthritis and Juvenile Dermatomyositis—Insights From Other Diseases

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Regulatory T cells (Tregs) are believed to be dysfunctional in autoimmunity. Juvenile idiopathic arthritis (JIA) and juvenile dermatomyositis (JDM) result from a loss of normal immune regulation in specific tissues such as joints or muscle and skin, respectively. Here, we discuss recent findings in regard to Treg biology in oligo-/polyarticular JIA and JDM, as well as what we can learn about Treg-related disease mechanism, treatment and biomarkers in JIA/JDM from studies of other diseases. We explore the potential use of Treg immunoregulatory markers and gene signatures as biomarkers for disease course and/or treatment success. Further, we discuss how Tregs are affected by several treatment strategies already employed in the therapy of JIA and JDM and by alternative immunotherapies such as anti-cytokine or co-receptor targeting. Finally, we review recent successes in using Tregs as a treatment target with low-dose IL-2 or cellular immunotherapy. Thus, this mini review will highlight our current understanding and identify open questions in regard to Treg biology, and how recent findings may advance biomarkers and new therapies for JIA and JDM.

Keywords: regulatory T cells, juvenile idiopathic arthritis, juvenile dermatomyositis, biomarker, therapy

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INTRODUCTION

$CD4^+FOXP3^+$ regulatory T cells (Tregs) are a subset of $CD4^+$ T helper cells present in lymphoid and non-lymphoid tissues, and are crucial for mediating tolerance to self, preventing allergies and controlling immune reactions after infections (1). They develop in the thymus or are induced in the periphery and exhibit contact-dependent and -independent mechanisms of action (1). Inactivating mutations in FOXP3 lead to multi-organ autoimmune disease [immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)], highlighting the importance of Tregs (2). Importantly, it is becoming clear that the local microenvironment affects the phenotype and function of tissue-localized Tregs, which also have additional roles in repair and regeneration (3).

Treg–tissue interaction might be particular important in autoimmunity with tissue-specific presentation, such as juvenile idiopathic arthritis (JIA) and juvenile dermatomyositis (JDM). While JIA is the most common inflammatory rheumatic disease in children, JDM is rare. JIA is characterized by persistent arthritis and subtype-dependent symptoms [reviewed in (4)]. Here, we focus on polyarticular and oligoarticular JIA, which present without involvement of systemic

organs or skin. JDM is characterized by inflammation of muscles and skin, resulting in muscle weakness and rashes [reviewed in (5)]. Interestingly, for both conditions researchers may take advantage of clinical sample collection from the site of inflammation: synovial fluid (SF) drained during therapeutic joint injection (JIA) and biopsies (mostly muscle, JDM). While some patients respond to therapy, others do not and studying the underlying differences may lead to better understanding and treatments.

Here, we discuss recent advances in the understanding of Treg biology in oligo-/ polyarticular JIA and JDM, and what we can learn about Treg-related disease mechanisms, treatments and biomarkers from other diseases.

ALTERED TREGS IN JIA AND JDM

The phenotype of $CD4^+FOXP3^+$ Tregs in JIA has been considerably characterized in the past (6) with the molecular roles of FOXP3 in JIA reviewed by Copland and Bending in this special collection (7). It is now clear that the Treg TCR (T cell receptor) repertoire is highly restricted in JIA, both at the site of inflammation (8–11) and in circulation (10, 12). Interestingly, in blood only Tregs but not conventional $CD4^+$ non-Treg cells (Tconv) are more clonal (10, 12). Some suggest that the TCR repertoires of Tregs from SF and peripheral blood (PB) significantly overlap (8), while others only found a very small overlap (9, 11). These differences might be explained by different sequencing depth and analysis strategies and/or by different Treg subsets studied: total (11) or effector Tregs defined by HLA-DR (8) or CD161 expression (9). Further, one study found that SF Tregs, but not Tconv, share specificity at an amino acid sequence level among different patients (10), suggesting disease-associated Treg clones might foster JIA.

Besides a restricted TCR repertoire, Tregs from the JIA inflammatory sites show unstable FOXP3 and CD25 (13), altered homing markers (9), cytokine production (6, 9), deficiency in specific chemokine production (14), and low responsiveness to IL-2 (13)—indicating impaired Treg function in JIA. Nevertheless, many reports found that JIA SF and PB Tregs are fully demethylated (8, 13), thus committed to the Treg-lineage, and suppressive *in vitro* (6, 8, 9, 13, 15). Hence, JIA Tregs are likely functioning inappropriately or insufficiently in the context of the inflammatory microenvironment. Interestingly, adding SF to *in vitro* cultures can both increase/stabilize Treg FOXP3 expression (11, 16) and *in situ* induce effector T cells to be resistant to Treg-mediated suppression *ex vivo* (17, 18). Thus, more research is needed to decipher the effects of the inflammatory microenvironment on Treg function.

In comparison, we know little about the contribution of Tregs to JDM pathogenesis. Similar to JIA, the Treg repertoire is restricted with a lack of diversity (12). $FOXP3^+$ Tregs were found to be enriched in JDM muscle compared to muscle tissue from patients with Duchenne muscular dystrophy (19). Since the latter is already enriched in Tregs compared to normal muscle (20), this suggests a hyper-enrichment in JDM in response to autoimmune inflammation. PB Tregs of active JDM also appear less suppressive *in vitro* with decreased expression of CTLA4 (19). Adult DM/ polymyositis muscle biopsies are also enriched with Tregs (21). Interestingly, both Treg and effector T cell numbers decreased post immunosuppressive therapy in adult myositis, suggesting that Treg enrichment is a response to inflammation. However, juvenile and adult DM have different clinical presentation (22) and JDM PB express more Th17-type and FOXP3 transcripts (23). JDM and other myopathies are characterized by a type 1 IFN signature (24–26) and interferons may be a potential therapeutic target (27), but their effects on Tregs remain to be investigated.

Tregs are crucial in resolving muscle injury in animal studies (28) and Treg-deficient mice develop more severe myopathies in response to antigen, while adoptive Treg transfer prevents inflammation (29, 30). Thorough immune-profiling recently revealed pan-tissue and tissue-specific signatures and enhancers of murine Tregs (31). The muscle Treg signature was highly enriched in cell cycle genes, showed a dynamic response to injury and was more similar to circulating Treg signatures than to other tissue Tregs (31), indicating that muscle Tregs might acutely infiltrate muscle and are not necessarily long-term resident cells. While myopathy is a defining characteristic of JDM, skin inflammation and rash are other symptoms (5). Skin-resident Tregs are crucial for immune homeostasis (3) and have been characterized in health and various disease settings (32). However, studies on JDM-affected skin are lacking, and more work is needed to characterize JDM skin-resident Tregs.

TREGS AS A BIOMARKER?

JIA and JDM can exhibit an unpredictable disease course. While mounting evidence indicates that an early aggressive treatment is best for severe disease (4, 27, 33, 34), the disease course is unpredictable at presentation. Additionally, due to potential short- and long-term side effects children should not be exposed to unnecessary medication. Unfortunately, once a patient appears to be in clinical remission (on or off medications), disease may flare without any notice or obvious trigger (**Figure 1A**). Indeed, among JIA patients who are in clinical remission, 30–50% experience flares (35, 36).

Hence, reliable biomarkers need to predict (i) the future disease course, (ii) treatment response, and (iii) the safety for medication withdrawal during clinical remission (**Figure 1B**).

Inflammation markers in the serum can indicate disease activity and potentially treatment response in JIA [reviewed in (36)]. In JDM, histology of biopsies and myositis-specific auto-antibodies can indicate future disease severity or complications [reviewed in (27)].

Only a few putative biomarkers probe the immunoregulatory balance in autoimmune arthritis and myopathies. The frequency of inflammation-associated Tregs ($HLA\text{-}DR^+$) in PB was proposed as a biomarker for disease activity in arthritis (8). TCR sequence overlap of these PB $HLA\text{-}DR^+$ Tregs with SF Tregs in JIA, and an increase of $HLA\text{-}DR^+$ Tregs in active rheumatoid arthritis (RA) were found. Low expression of the immunoregulatory receptor CD39 has been suggested as an

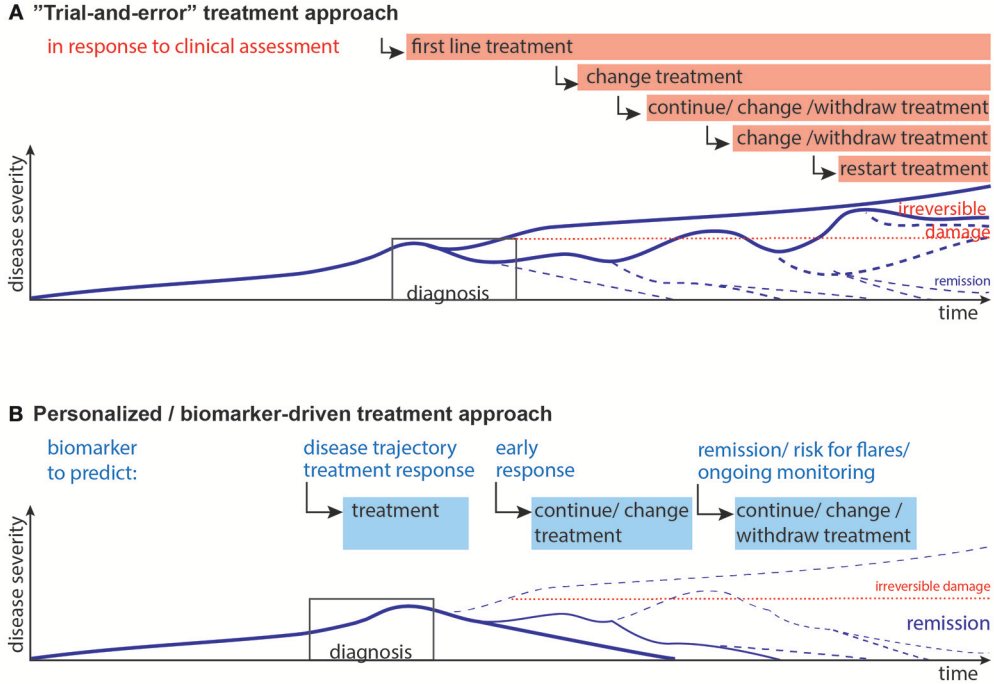

FIGURE 1 | Current and desired disease progression models. **(A)** Current "Trial-and-error" model: Upon diagnosis the first line of treatment is started, which may lead to remission, or partial remission. Often, a second, third or fourth treatment strategy needs to be implemented when the previous treatments are not effective. Choice of treatments is guided by previous experience, e.g., upon presentation non-steroidal anti-inflammatory drugs with glucocorticoids are used, after a couple of month many patients are switched to methotrexate as first disease modifying therapy, often followed by anti-TNF-α agents with/without methotrexate (4). This disease progression and subsequent staggering of therapy can result in irreversible damage and long-term therapy. **(B)** Desired personalized/biomarker-driven model: Biomarkers could be used for prediction and aid decisions at the following stages: disease trajectory, selection of treatment, early response to therapy, remission/minimal disease activity and the risk of flare upon withdrawal of therapy, ongoing monitoring of immune activity and risk of flares. Biomarkers might thus contribute to more efficient therapy, pre-empt flares, and minimize short- and long-term effects of flares and reduce long-term damage. Lines represent models of disease progression, with line thickness representing frequency estimates.

indicator of methotrexate resistance in RA (37). Also, response to the TNF-α blocker adalimumab could be predicted by a Treg increase in PBMCs from RA patients cultured with adalimumab prior to treatment (38). Finally, the soluble form of the high affinity IL-2 receptor α chain (CD25), crucial for Treg phenotype and function, might be a biomarker for adult myositis disease activity (39).

In the recent past, gene signatures have been defined as multi-parameter biomarkers. Thus, far, efforts to define JIA immune-based gene biomarkers have focused on whole genome expression profiling (40–42) and epigenomic signatures (43, 44). JIA displays an altered immune signature, which changes during remission, but does not return to a state comparable to healthy controls (41, 42). Myositis is characterized by type 1 IFN signatures (27). While interesting and highlighting potential disease mechanisms, whole-genome/exome expression profiling is not feasible for routine clinical practice due to cost, logistics and data interpretation. We have recently developed a Treg gene signature associated with Treg competency using the clinically-applicable multiplex platform nanoString (45). NanoString is fast and fewer than 10,000 lysed cells are sufficient without the need to purify RNA. Although the proportion of Tregs that express FOXP3 was similar between type 1 diabetes (T1D) and controls, there was a significant change in their Treg signature (45). Future work will elucidate whether the Treg gene signature may also be used as a biomarker in JIA and other autoimmune conditions.

In summary, some progress has been made, but more biomarkers are needed for biological disease activity, prognosis, treatment success, and risk of flares. Further, a consensus of criteria to describe active/inactive disease is needed to better estimate the currently widely variable incidence of clinically inactive JIA disease (46). For JDM, a comprehensive set of criteria to assess disease activity and damage has been proposed (47).

TREGS AS THERAPEUTIC TARGET/TOOL?

Convincing evidence demonstrates that functioning Tregs are crucial to prevent autoimmunity and our understanding of how different immunotherapies affect Tregs has improved.

(Unforeseen) Treg Effects of Immuno-Therapy

High levels of TNF-α in the inflamed JIA joint (32, 48) offer a clear rationale for anti-TNF therapy in JIA with marked success (4, 49). Anti-TNF therapy has also been used in refractory JDM (50), but with mixed evidence for its effectiveness (27, 51–53).

Blocking TNF-α can, however, also elicit further autoimmune responses, particular in the skin and muscle (54–57). TNF-α itself can have both positive and negative effects on Tregs (32, 58, 59). Interestingly, the negative effects are found especially in inflamed joints (32, 58, 60), whereas positive effects of TNF-α on Treg function were reported using healthy human cells or in mice (58, 61–64). TNF-α has two receptors CD120a (TNFR1) and CD120b (TNFR2) (58). CD120b may mediate the pro-Treg functions of TNF-α, including Treg proliferation, stabilizing Tregs, and preventing disease in mouse models (58, 62–64). Little is known about the effects of ligation of CD120a in Tregs, but some research suggests targeting CD120a while sparing CD120b-TNF-interaction can alleviate collagen-induced arthritis (65). In RA, adalimumab has been shown to enhance Treg frequency and potency via CD120b (38, 66, 67). Etanercept, a soluble CD120b as TNF-α blocker, instead might predominantly affect effector T cells, by reversing their resistance to suppression in JIA (68). Unfortunately, a considerable group of JIA/JDM patients do not respond to anti-TNF therapy (27, 49, 53) and anti-TNF agents are immunogenic (69), with 50% of patients developing anti-drug antibodies leading to resistance to therapy and disease progression.

Ustekinumab is another potentially attractive anti-cytokine therapy which targets the p40 subunit of IL-12 and IL-23, key cytokines driving Th1, Th17, and Th17.1, (ex-)Th17 cells with a Th1-like phenotype, function (70–74). Ustekinumab is well-tolerated in adult and pediatric patients for treating psoriasis, psoriatic arthritis, systemic lupus erythematosus (SLE), and Crohn's disease (70, 71, 75–77), and has shown lower immunogenicity compared to most anti-TNF agents (69). Th17.1 are enriched in JIA (72, 73), and ustekinumab therapy had some success in enthesitis-related JIA (78), psoriatic arthritis (69, 79) and is in trial for various rheumatological diseases (80). While no imbalance in IL-17 has been established in JDM (19), Th17.1 have not been investigated. Ustekinumab has been suggested as a potential therapy for JDM, and a case of JDM with psoriasis was treated successfully with ustekinumab (81). Due to the reciprocal relationship between Th17 and Tregs (82), Tregs might also be affected by ustekinumab therapy, and this was indeed suggested in a case report of giant cell arteritis (83) and in T1D treated with ustekinumab (NCT02117765; Pesenacker et al.).

IL-6 also drives inflammatory environments, including skewing the Treg/Th17 balance toward Th17 (71). Anti-IL-6 receptor therapy (tocilizumab) increases Treg frequency and numbers in RA (71). IL-6 has also been implicated in JIA and JDM (11, 17, 84, 85) and is used in polyarticular, extended oligoarticular, systemic JIA (49), and refractory JDM (50), but mechanistic studies in pediatric disease are lacking.

Whether drugs such as ustekinumab and tocilizumab act on Tregs directly or through changing the microenvironment is unclear. Human Tregs can express the receptors for IL-6 (86), IL-12 (87), and IL-23 (88), but evidence for direct drug action on Tregs is lacking.

Alternatively, co-receptors can be targeted to manipulate the immunoregulatory balance. Initially established for cancer therapy (checkpoint blockade), mimicking checkpoints such as CTLA4 (CTLA4-Ig, abatacept, belatacept) is used as treatment for autoimmunity. Abatacept has been shown to be safe and effective in oligo- and polyarticular JIA (49, 89–92), adult DM/polymyositis (34, 53), a case report of steroid-sparing abatacept in complex JDM (93) and a trial in JDM is underway (27). A reduction of the T cell activation state is the main reported effect of abatacept (90, 94–96). Surprisingly, the majority of studies found abatacept decreases the frequency of Tregs (90, 95, 97–99), with some studies showing an increase in function (99). On the other hand, increased Treg frequency, but decreased activity after abatacept therapy in RA was demonstrated (100). In muscle tissue of adult DM/polymyositis, more Tregs were found following abatacept (34), suggesting that abatacept treatment could change Treg localization. Other co-receptor targeting therapies are in use/development for malignancies (e.g., anti-PD1, anti-TIM3, anti-TIGIT, etc.) and these pathways might be useful targets in autoimmunity.

Treg (-Targeted) Therapy

Adoptive transfer of Tregs has been shown to be safe and possibly effective at reducing inflammation, inducing transplant tolerance, preventing graft-vs.-host disease (GVHD) and treating autoimmunity [reviewed in (101)].

Important considerations for Treg-therapy currently under investigation are the source of therapeutic cells, antigen-specificity and possibly tailoring homing characteristics for improved activity. Isolating and expanding sufficient numbers of Tregs from patients awaiting transplantation, under immunosuppression or with autoimmune disease is feasible and can restore their function (101–103), although achieving clinically relevant Treg numbers from pediatric JIA and JDM patients might prove challenging. Third-party Tregs from umbilical cord blood have been found safe and possibly effective as GVHD prophylaxis in adults (104, 105) and pediatric thymus—routinely removed during pediatric cardiac surgery—might be a plentiful source for highly functional therapeutic Tregs (106, 107). Antigen-specific Tregs are more effective than polyclonal Tregs for therapy and with recent successes of chimeric antigen receptor (CAR) T effector therapies for cancer, there has been a surge to adapt this technology to generate CAR-Tregs [reviewed in (108)]. While generation of antigen-specific Tregs recognizing allogeneic HLA-molecules is relatively straightforward in transplantation, generation of CAR-Tregs for autoimmunity without known antigen (i.e., JIA) might be difficult. Still, CAR-Tregs reacting with antigen found at the site of inflammation (i.e., JIA joints or JDM muscle) could activate Tregs locally. Alternatively, Tregs could be conditioned *in vitro* to home to specific sites (107) or Tregs could be injected locally, as shown with intra-dermal injection of Tregs to inhibit murine allograft skin inflammation (109).

Since Treg cell therapies are challenging and expensive, targeting Treg expansion *in vivo* might be more feasible for conditions such as JIA and JDM. The most promising advances of non-cellular therapies targeting Tregs have been low-dose IL-2, IL-2 complexes, or IL-2 bio-similars (110–112). While high doses of IL-2 stimulate mainly effector cells, low-dose IL-2 [0.3–3 $\times$ 10^6 units/day (112)] skews the response toward Tregs. Low-dose IL-2 increases the frequency of activated, functional

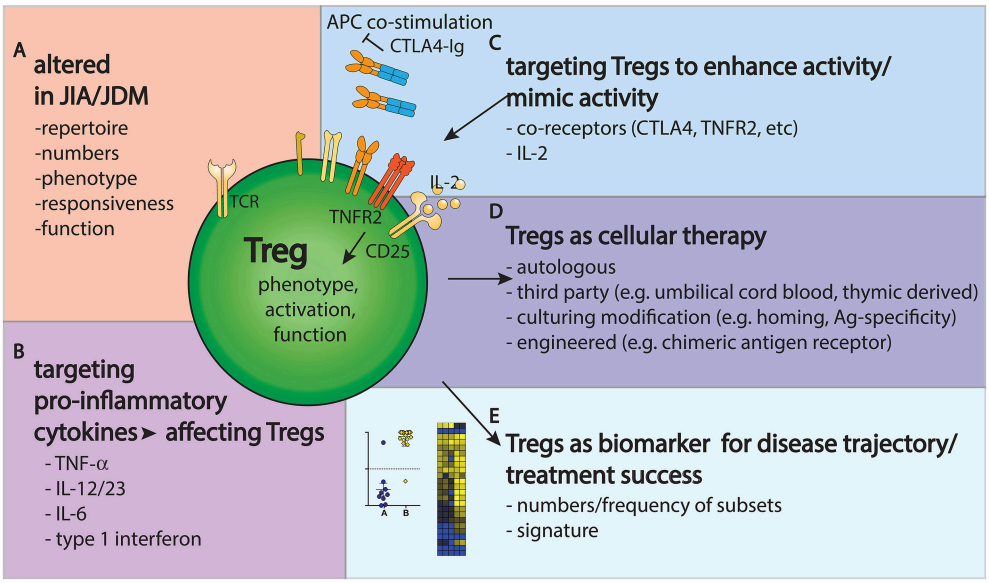

FIGURE 2 | Tregs provide challenges and opportunities in JIA/JDM pathogenesis, treatment and monitoring. **(A)** Tregs are altered in repertoire, phenotype and frequency in JIA/JDM, particularly at the site of inflammation. **(B–D)** Treatment options to restore the immunoregulatory balance include targeting pro-inflammatory cytokines **(B)**, targeting Tregs to enhance their activity **(C)** or using Tregs as a cellular therapy **(D)**. **(E)** Further, changes in Treg gene signatures could aid as biomarkers to measure or predict disease/treatment response.

and fully demethylated $CD25^{+}FOXP3^{+}$ Tregs (113–115) and induces STAT5 phosphorylation *in vivo* (114, 115). Low-dose IL-2 therapy has been deemed safe and successful in the treatment of T1D (112, 114, 115), GVHD (116, 117), and SLE (113). Indeed, low-dose IL-2 therapy rescued Tregs with low levels of CD25 in SLE (113), indicating that it might also rescue JIA Tregs with low CD25 expression (13). To further fine-tune specificity or increase the half-life of IL-2, IL-2 complexes, and bio-similars are in development (110, 111, 118); these expand Tregs and induce phosphorylated STAT5 *in vitro*, *in vivo*, and prevent disease in animal models (118–121), including resolution of muscular dystrophy (20). Covalently linking IL-2 to anti-IL-2 (122), to non-FcRγ-binding human IgG1 (123) or CD25 (124) may enhance potential clinical application by mitigating the risk of *in vivo* dissociation of complexes.

However, increasing Treg numbers alone might not be sufficient to overcome the highly inflammatory environment and effector cell resistance. Thus, to achieve sustained remission combination-therapy might be necessary to reduce the inflammatory milieu paralleled with boosting Tregs to maintain a renewed tolerance.

CONCLUDING REMARKS

Taken together, it is clear that Tregs present challenges and opportunities in JIA and JDM research and clinical management (**Figure 2**). Their phenotype and function are clearly altered in JIA and JDM, targeting them might improve disease outcome and Tregs could be used as biomarkers to gage the state and progress of disease.

The role of the microenvironment on Treg function and phenotype in JIA- and JDM-affected tissues remains to be explored further. Researchers should take advantage of biopsies taken for clinical diagnosis (JDM) and SF aspirated during therapeutic joint injections (JIA). Novel techniques, such as single cell sequencing, multidimensional mass/flow cytometry and microscopy, will aid using clinical samples to their full potential (125–127). Additionally, co-culture with SF or muscle-derived cells could highlight how the microenvironment affects Tregs. Since JDM in particular is a rare disease, collaborations between groups are crucial to increase sample size for fundamental research, biomarker-finding and -validation studies and controlled treatment trials. This could be achieved by consortiums similar to juvenile diabetes research foundation (JDRF) biomarker working group for T1D (114) and the immune tolerance network trials (128).

While there is progress toward unified measures of disease activity (46, 47), these will need to be tested and verified, followed by development of feasible, reliable and cost-effective biomarkers to predict disease activity, risk of flare and ideal treatment strategies. The ultimate goal, aided by biomarkers, is to go from a trial-and-error treatment approach toward a more efficient and personalized medicine approach with more patients achieving drug-free remission without major long-term disabilities (**Figure 1**).

Success of various agents affecting the immunoregulatory balance in other diseases point to potential uses in JIA and JDM. Any (new) therapy will need to be considered

in regards to both effector cells AND Tregs, since some therapies might have unexpected effects on Tregs. Thus, it is important to continuously build our understanding of how various agents affect the immunoregulatory balance.

In conclusion, important recent advances might lead to valid future contributions to the widened arsenal of treatment options available to restore the immunoregulatory balance in a heterogeneous disease spectrum.

AUTHOR CONTRIBUTIONS

AP conceived the review. RH and AP reviewed the literature and co-wrote the manuscript.

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REFERENCES

1. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol.* (2010) 10:490–500. doi: 10.1038/nri2785
2. Bacchetta R, Barzaghi F, Roncarolo MG. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann N Y Acad Sci.* (2016) 1417:5–22. doi: 10.1111/nyas.13011
3. Li J, Tan J, Martino MM, Lui KO. Regulatory T-Cells: potential regulator of tissue repair and regeneration. *Front Immunol.* (2018) 9:585. doi: 10.3389/fimmu.2018.00585
4. Crayne CB, Beukelman T. Juvenile idiopathic arthritis: oligoarthritis and polyarthritis. *Pediatr Clin North Am.* (2018) 65:657–74. doi: 10.1016/j.pcl.2018.03.005
5. Rider LG, Nistala K. The juvenile idiopathic inflammatory myopathies: pathogenesis, clinical and autoantibody phenotypes, and outcomes. *J Intern Med.* (2016) 280:24–38. doi: 10.1111/joim.12444
6. Pesenacker AM, Wedderburn LR. T regulatory cells in childhood arthritis–novel insights. *Expert Rev Mol Med.* (2013) 15:e13. doi: 10.1017/erm.2013.14
7. Copland A, Bending D. Foxp3 molecular dynamics in treg in juvenile idiopathic arthritis. *Front Immunol.* (2018) 9:2273. doi: 10.3389/fimmu.2018.02273
8. Rossetti M, Spreafico R, Consolaro A, Leong JY, Chua C, Massa M, et al. TCR repertoire sequencing identifies synovial Treg cell clonotypes in the bloodstream during active inflammation in human arthritis. *Ann Rheum Dis.* (2017) 76:435–41. doi: 10.1136/annrheumdis-2015-208992
9. Duurland CL, Brown CC, O'Shaughnessy RF, Wedderburn LR. CD161(+) Tconv and CD161(+) Treg share a transcriptional and functional phenotype despite limited overlap in TCRbeta repertoire. *Front Immunol.* (2017) 8:103. doi: 10.3389/fimmu.2017.00103
10. Henderson LA, Volpi S, Frugoni F, Janssen E, Kim S, Sundel RP, et al. Next-generation sequencing reveals restriction and clonotypic expansion of treg cells in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2016) 68:1758–68. doi: 10.1002/art.39606
11. Bending D, Giannakopoulou E, Lom H, Wedderburn LR. Synovial regulatory T cells occupy a discrete TCR niche in human arthritis and require local signals to stabilize FOXP3 protein expression. *J Immunol.* (2015) 195:5616–24. doi: 10.4049/jimmunol.1500391
12. Delemarre EM, van den Broek T, Mijnheer G, Meerding J, Wehrens EJ, Olek S, et al. Autologous stem cell transplantation aids autoimmune patients by functional renewal and TCR diversification of regulatory T cells. *Blood* (2016) 127:91–101. doi: 10.1182/blood-2015-06-649145
13. Bending D, Pesenacker AM, Ursu S, Wu Q, Lom H, Thirugnanabalan B, et al. Hypomethylation at the regulatory T cell-specific demethylated region in CD25hi T cells is decoupled from FOXP3 expression at the inflamed site in childhood arthritis. *J Immunol.* (2014) 193:2699–708. doi: 10.4049/jimmunol.1400599
14. Patterson SJ, Pesenacker AM, Wang AY, Gillies J, Mojibian M, Morishita K, et al. T regulatory cell chemokine production mediates pathogenic T cell attraction and suppression. *J Clin Invest.* (2016) 126:1039–51. doi: 10.1172/JCI83987
15. Bulatovic Calasan M, Vastert SJ, Scholman RC, Verweij F, Klein M, Wulffraat NM, et al. Methotrexate treatment affects effector but not regulatory T cells in juvenile idiopathic arthritis. *Rheumatology* (2015) 54:1724–34. doi: 10.1093/rheumatology/kev101
16. Ohl K, Nickel H, Moncrieffe H, Klemm P, Scheufen A, Foll D, et al. The transcription factor CREM drives an inflammatory phenotype of T cells in oligoarticular juvenile idiopathic arthritis. *Pediatr Rheumatol Online J.* (2018) 16:39. doi: 10.1186/s12969-018-0253-x
17. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood* (2011) 118:3538–48. doi: 10.1182/blood-2010-12-328187
18. Haufe S, Haug M, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, et al. Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells. *Arthritis Rheum.* (2011) 63:3153–62. doi: 10.1002/art.30503
19. Vercoulen Y, Bellutti Enders F, Meerding J, Plantinga M, Elst EF, Varsani H, et al. Increased presence of FOXP3+ regulatory T cells in inflamed muscle of patients with active juvenile dermatomyositis compared to peripheral blood. *PLoS ONE* (2014) 9:e105353. doi: 10.1371/journal.pone.0105353
20. Villalta SA, Rosenthal W, Martinez L, Kaur A, Sparwasser T, Tidball JG, et al. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med.* (2014) 6:258ra142. doi: 10.1126/scitranslmed.3009925
21. Pandya JM, Loell I, Hossain MS, Zong M, Alexanderson H, Raghavan S, et al. Effects of conventional immunosuppressive treatment on CD244+ (CD28null) and FOXP3+ T cells in the inflamed muscle of patients with polymyositis and dermatomyositis. *Arthritis Res Ther.* (2016) 18:80. doi: 10.1186/s13075-016-0974-5
22. Tansley SL, McHugh NJ, Wedderburn LR. Adult and juvenile dermatomyositis: are the distinct clinical features explained by our current understanding of serological subgroups and pathogenic mechanisms? *Arthritis Res Ther.* (2013) 15:211. doi: 10.1186/ar4198
23. Lopez De Padilla CM, Crowson CS, Hein MS, Pendegraft RS, Strausbauch MA, Niewold TB, et al. Gene expression profiling in blood and affected muscle tissues reveals differential activation pathways in patients with new-onset juvenile and adult dermatomyositis. *J Rheumatol.* (2017) 44:117–24. doi: 10.3899/jrheum.160293
24. Gitiaux C, Latroche C, Weiss-Gayet M, Rodero MP, Duffy D, Bader-Meunier B, et al. Myogenic progenitor cells exhibit type I interferon-driven proangiogenic properties and molecular signature during juvenile dermatomyositis. *Arthritis Rheumatol.* (2018) 70:134–45. doi: 10.1002/art.40328
25. Greenberg SA. A gene expression approach to study perturbed pathways in myositis. *Curr Opin Rheumatol.* (2007) 19:536–41. doi: 10.1097/BOR.0b013e3282efe261
26. Zhu W, Streicher K, Shen N, Higgs BW, Morehouse C, Greenlees L, et al. Genomic signatures characterize leukocyte infiltration in myositis muscles. *BMC Med Genomics* (2012) 5:53. doi: 10.1186/1755-8794-5-53
27. Wu Q, Wedderburn LR, McCann LJ. Juvenile dermatomyositis: latest advances. *Best Pract Res Clin Rheumatol.* (2017) 31:535–57. doi: 10.1016/j.berh.2017.12.003
28. Deyhle MR, Hyldahl RD. The role of T lymphocytes in skeletal muscle repair from traumatic and contraction-induced injury. *Front Physiol.* (2018) 9:768. doi: 10.3389/fphys.2018.00768

29. Young NA, Sharma R, Friedman AK, Kaffenberger BH, Bolon B, Jarjour WN. Aberrant muscle antigen exposure in mice is sufficient to cause myositis in a Treg cell-deficient milieu. *Arthritis Rheum.* (2013) 65:3259–70. doi: 10.1002/art.38184
30. Allenbach Y, Solly S, Gregoire S, Dubourg O, Salomon B, Butler-Browne G, et al. Role of regulatory T cells in a new mouse model of experimental autoimmune myositis. *Am J Pathol.* (2009) 174:989–98. doi: 10.2353/ajpath.2009.080422
31. DiSpirito JR, Zemmour D, Ramanan D, Cho J, Zilionis R, Klein AM, et al. Molecular diversification of regulatory T cells in nonlymphoid tissues. *Sci Immunol.* (2018) 3:eaat5861. doi: 10.1126/sciimmunol.aat5861
32. Pesenacker AM, Broady R, Levings MK. Control of tissue-localized immune responses by human regulatory T cells. *Eur J Immunol.* (2015) 45:333–43. doi: 10.1002/eji.201344205
33. Wallace CA, Ringold S, Bohnsack J, Spalding SJ, Brunner HI, Milojevic D, et al. Extension study of participants from the trial of early aggressive therapy in juvenile idiopathic arthritis. *J Rheumatol.* (2014) 41:2459–65. doi: 10.3899/jrheum.140347
34. Tjarnlund A, Tang Q, Wick C, Dastmalchi M, Mann H, Tomasova Studynkova J, et al. Abatacept in the treatment of adult dermatomyositis and polymyositis: a randomised, phase IIb treatment delayed-start trial. *Ann Rheum Dis.* (2018) 77:55–62. doi: 10.1136/annrheumdis-2017-211751
35. Wallace CA, Huang B, Bandeira M, Ravelli A, Giannini EH. Patterns of clinical remission in select categories of juvenile idiopathic arthritis. *Arthritis Rheum.* (2005) 52:3554–62. doi: 10.1002/art.21389
36. Duurland CL, Wedderburn LR. Current developments in the use of biomarkers for juvenile idiopathic arthritis. *Curr Rheumatol Rep.* (2014) 16:406. doi: 10.1007/s11926-013-0406-3
37. Peres RS, Liew FY, Talbot J, Carregaro V, Oliveira RD, Almeida SL, et al. Low expression of CD39 on regulatory T cells as a biomarker for resistance to methotrexate therapy in rheumatoid arthritis. *Proc Natl Acad Sci USA.* (2015) 112:2509–14. doi: 10.1073/pnas.1424792112
38. Nguyen DX, Cotton A, Attipoe L, Ciurtin C, Doré CJ, Ehrenstein MR. Regulatory T cells as a biomarker for response to adalimumab in rheumatoid arthritis. *J Allergy Clin Immunol.* (2018) 142:978–80.e979. doi: 10.1016/j.jaci.2018.04.026
39. Tournadre A, Dubost JJ, Soubrier M, Ruivard M, Souteyrand P, Schmidt J, et al. Soluble IL-2 receptor: a biomarker for assessing myositis activity. *Dis Markers* (2014) 2014:472624. doi: 10.1155/2014/472624
40. Cui A, Quon G, Rosenberg AM, Yeung RS, Morris Q, Consortium BS. Gene expression deconvolution for uncovering molecular signatures in response to therapy in juvenile idiopathic arthritis. *PLoS ONE* (2016) 11:e0156055. doi: 10.1371/journal.pone.0156055
41. Jiang K, Frank M, Chen Y, Osban J, Jarvis JN. Genomic characterization of remission in juvenile idiopathic arthritis. *Arthritis Res Ther.* (2013) 15:R100. doi: 10.1186/ar4280
42. Moncrieffe H, Bennett MF, Tsoras M, Luyrink LK, Johnson AL, Xu H, et al. Transcriptional profiles of JIA patient blood with subsequent poor response to methotrexate. *Rheumatology* (2017) 56:1542–51. doi: 10.1093/rheumatology/kex206
43. Peeters JG, Vervoort SJ, Tan SC, Mijnheer G, de Roock S, Vastert SJ, et al. Inhibition of super-enhancer activity in autoinflammatory site-derived T Cells reduces disease-associated gene expression. *Cell Rep.* (2015) 12:1986–96. doi: 10.1016/j.celrep.2015.08.046
44. Spreafico R, Rossetti M, Whitaker JW, Wang W, Lovell DJ, Albani S. Epipolymorphisms associated with the clinical outcome of autoimmune arthritis affect CD4+ T cell activation pathways. *Proc Natl Acad Sci USA.* (2016) 113:13845–50. doi: 10.1073/pnas.1524056113
45. Pesenacker AM, Wang AY, Singh A, Gillies J, Kim YW, Piccirillo CA, et al. A Treg gene signature is a specific and sensitive biomarker to identify children with new onset type 1 diabetes. *Diabetes* (2016) 65:1031–9. doi: 10.2337/db15-0572
46. Shoop-Worrall SJW, Verstappen SMM, Baildam E, Chieng A, Davidson J, Foster H, et al. How common is clinically inactive disease in a prospective cohort of patients with juvenile idiopathic arthritis? The importance of definition. *Ann Rheum Dis.* (2017) 76:1381–8. doi: 10.1136/annrheumdis-2016-210511
47. McCann LJ, Pilkington CA, Huber AM, Ravelli A, Appelbe D, Kirkham JJ, et al. Development of a consensus core dataset in juvenile dermatomyositis for clinical use to inform research. *Ann Rheum Dis.* (2018) 77:241–50. doi: 10.1136/annrheumdis-2017-212141
48. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis.* (2007) 66:589–98. doi: 10.1136/ard.2006.061853
49. Shepherd J, Cooper K, Harris P, Picot J, Rose M. The clinical effectiveness and cost-effectiveness of abatacept, adalimumab, etanercept and tocilizumab for treating juvenile idiopathic arthritis: a systematic review and economic evaluation. *Health Technol Assess.* (2016) 20:1–222. doi: 10.3310/hta20340
50. Spencer CH, Rouster-Stevens K, Gewanter H, Syverson G, Modica R, Schmidt K, et al. Biologic therapies for refractory juvenile dermatomyositis: five years of experience of the childhood arthritis and rheumatology research alliance in North America. *Pediatr Rheumatol Online J.* (2017) 15:50. doi: 10.1186/s12969-017-0174-0
51. Rouster-Stevens KA, Ferguson L, Morgan G, Huang CC, Pachman LM. Pilot study of etanercept in patients with refractory juvenile dermatomyositis. *Arthritis Care Res* (2014) 66:783–7. doi: 10.1002/acr.22198
52. Wang CR. Successful treatment of refractory juvenile dermatomyositis with adalimumab. *J Clin Rheumatol.* (2017) 23:174–5. doi: 10.1097/RHU.0000000000000514
53. Sasaki H, Kohsaka H. Current diagnosis and treatment of polymyositis and dermatomyositis. *Mod Rheumatol.* 28:913–21. doi: 10.1080/14397595.2018.1467257
54. Omran NE, Noorwali AA. Nephritis, cerebritis, and myositis after adalimumab therapy in a patient with rheumatoid arthritis: a case report. *Int J Gen Med.* (2018) 11:151–4. doi: 10.2147/IJGM.S154835
55. Brunasso AM, Aberer W, Massone C. New onset of dermatomyositis/polymyositis during anti-TNF-alpha therapies: a systematic literature review. *ScientificWorldJournal* (2014) 2014:179180. doi: 10.1155/2014/179180
56. Liu SW, Velez NF, Lam C, Femia A, Granter SR, Townsend HB, et al. Dermatomyositis induced by anti-tumor necrosis factor in a patient with juvenile idiopathic arthritis. *JAMA Dermatol.* (2013) 149:1204–8. doi: 10.1001/jamadermatol.2013.5220
57. Ko JM, Gottlieb AB, Kerbleski JF. Induction and exacerbation of psoriasis with TNF-blockade therapy: a review and analysis of 127 cases. *J Dermatolog Treat.* (2009) 20:100–8. doi: 10.1080/09546630802441234
58. Goldstein JD, Perol L, Zaragoza B, Baeyens A, Marodon G, Piaggio E. Role of cytokines in thymus- versus peripherally derived-regulatory T cell differentiation and function. *Front Immunol.* (2013) 4:155. doi: 10.3389/fimmu.2013.00155
59. Nie H, Zheng Y, Li R, Zhang J. Reply to suppressive activity of human regulatory T cells is maintained in the presence of TNF. *Nat Med.* (2016) 22:18–9. doi: 10.1038/nm.4018
60. Wang J, van Dongen H, Scherer HU, Huizinga TW, Toes RE. Suppressor activity among CD4+,CD25++ T cells is discriminated by membrane-bound tumor necrosis factor alpha. *Arthritis Rheum.* (2008) 58:1609–18. doi: 10.1002/art.23460
61. Zaragoza B, Chen X, Oppenheim JJ, Baeyens A, Gregoire S, Chader D, et al. Suppressive activity of human regulatory T cells is maintained in the presence of TNF. *Nat Med.* (2016) 22:16–7. doi: 10.1038/nm.4019
62. Wang J, Ferreira R, Lu W, Farrow S, Downes K, Jermutus L, et al. TNFR2 ligation in human T regulatory cells enhances IL2-induced cell proliferation through the non-canonical NF-kappaB pathway. *Sci Rep.* (2018) 8:12079. doi: 10.1038/s41598-018-30621-4
63. Fischer R, Proske M, Duffey M, Stangl H, Martinez GF, Peters N, et al. Selective activation of tumor necrosis factor receptor ii induces antiinflammatory responses and alleviates experimental arthritis. *Arthritis Rheumatol.* (2018) 70:722–35. doi: 10.1002/art.40413
64. Lamontain V, Schmid T, Weber-Steffens D, Zeller D, Jenei-Lanzl Z, Wajant H, et al. Stimulation of TNF receptor type 2 expands regulatory T cells and ameliorates established collagen-induced arthritis in mice. *Cell Mol Immunol.* (2018) 16:76–85. doi: 10.1038/cmi.2017.138
65. McCann FE, Perocheau DP, Ruspi G, Blazek K, Davies ML, Feldmann M, et al. Selective tumor necrosis factor receptor I blockade is antiinflammatory

and reveals immunoregulatory role of tumor necrosis factor receptor II in collagen-induced arthritis. *Arthritis Rheumatol.* (2014) 66:2728–38. doi: 10.1002/art.38755
66. McGovern JL, Nguyen DX, Notley CA, Mauri C, Isenberg DA, Ehrenstein MR. Th17 cells are restrained by Treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy. *Arthritis Rheum.* (2012) 64:3129–38. doi: 10.1002/art.34565
67. Nguyen DX, Ehrenstein MR. Anti-TNF drives regulatory T cell expansion by paradoxically promoting membrane TNF-TNF-RII bindin–g in rheumatoid arthritis. *J Exp Med.* (2016) 213:1241–53. doi: 10.1084/jem.20151255
68. Petrelli A, Wehrens EJ, Scholman RC, Prakken BJ, Vastert SJ, van Wijk F. Self-sustained resistance to suppression of CD8+ Teff cells at the site of autoimmune inflammation can be reversed by tumor necrosis factor and interferon-gamma blockade. *Arthritis Rheumatol.* (2016) 68:229–36. doi: 10.1002/art.39418
69. Strand V, Balsa A, Al-Saleh J, Barile-Fabris L, Horiuchi T, Takeuchi T, et al. Immunogenicity of biologics in chronic inflammatory diseases: a systematic review. *BioDrugs* (2017) 31:299–316. doi: 10.1007/s40259-017-0231-8
70. Elliott M, Benson J, Blank M, Brodmerkel C, Baker D, Sharples KR, et al. Ustekinumab: lessons learned from targeting interleukin-12/23p40 in immune-mediated diseases. *Ann N Y Acad Sci.* (2009) 1182:97–110. doi: 10.1111/j.1749-6632.2009.05070.x
71. Fasching P, Stradner M, Graninger W, Dejaco C, Fessler J. Therapeutic potential of targeting the Th17/Treg axis in autoimmune disorders. *Molecules* (2017) 22:E134. doi: 10.3390/molecules22010134
72. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci USA*. (2010) 107:14751–6. doi: 10.1073/pnas.1003852107
73. Cosmi L, Cimaz R, Maggi L, Santarlasci V, Capone M, Borriello F, et al. Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* (2011) 63:2504–15. doi: 10.1002/art.30332
74. Piper C, Pesenacker AM, Bending D, Thirugnanabalan B, Varsani H, Wedderburn LR, et al. T cell expression of granulocyte-macrophage colony-stimulating factor in juvenile arthritis is contingent upon Th17 plasticity. *Arthritis Rheumatol.* (2014) 66:1955–60. doi: 10.1002/art.38647
75. Papp KA, Griffiths CE, Gordon K, Lebwohl M, Szapary PO, Wasfi Y, et al. Long-term safety of ustekinumab in patients with moderate-to-severe psoriasis: final results from 5 years of follow-up. *Br J Dermatol.* (2013) 168:844–54. doi: 10.1111/bjd.12214
76. Kellen R, Silverberg NB, Lebwohl M. Efficacy and safety of ustekinumab in adolescents. *Pediatric Health Med Ther.* (2016) 7:109–20. doi: 10.2147/PHMT.S75836
77. van Vollenhoven RF, Hahn BH, Tsokos GC, Wagner CL, Lipsky P, Touma Z, et al. Efficacy and safety of ustekinumab, an IL-12 and IL-23 inhibitor, in patients with active systemic lupus erythematosus: results of a multicentre, double-blind, phase 2, randomised, controlled study. *Lancet* (2018) 392:1330–9. doi: 10.1016/S0140-6736(18)32167-6
78. Mannion ML, McAllister L, Cron RQ, Stoll ML. Ustekinumab as a therapeutic option for children with refractory enthesitis-related arthritis. *J Clin Rheumatol.* (2016) 22:282–4. doi: 10.1097/RHU.0000000000000408
79. Kavanaugh A, Puig L, Gottlieb AB, Ritchlin C, Li S, Wang Y, et al. Maintenance of clinical efficacy and radiographic benefit through two years of ustekinumab therapy in patients with active psoriatic arthritis: results from a randomized, placebo-controlled phase III trial. *Arthritis Care Res (Hoboken).* (2015) 67:1739–49. doi: 10.1002/acr.22645
80. Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med.* (2015) 21:719–29. doi: 10.1038/nm.3895
81. Montoya CL, Gonzalez ML, Ospina FE, Tobon GJ. A rare case of amyopathic juvenile dermatomyositis associated with psoriasis successfully treated with ustekinumab. *J Clin Rheumatol.* (2017) 23:129–30. doi: 10.1097/RHU.0000000000000430
82. Weaver CT, Hatton RD. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol.* (2009) 9:883–9. doi: 10.1038/nri2660
83. Samson M, Ghesquiere T, Berthier S, Bonnotte B. Ustekinumab inhibits Th1 and Th17 polarisation in a patient with giant cell arteritis. *Ann Rheum Dis.* (2018) 77:e6. doi: 10.1136/annrheumdis-2017-211622
84. Barnes MG, Grom AA, Thompson SD, Griffin TA, Pavlidis P, Itert L, et al. Subtype-specific peripheral blood gene expression profiles in recent-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2009) 60:2102–12. doi: 10.1002/art.24601
85. Nistala K, Varsani H, Wittkowski H, Vogl T, Krol P, Shah V, et al. Myeloid related protein induces muscle derived inflammatory mediators in juvenile dermatomyositis. *Arthritis Res Ther.* (2013) 15:R131. doi: 10.1186/ar4311
86. Ferreira RC, Rainbow DB, Rubio Garcia A, Pekalski ML, Porter L, Oliveira JJ, et al. In-depth immunophenotyping data of IL-6R on the human peripheral regulatory T cell (Treg) compartment. *Data Brief* (2017) 12:676–91. doi: 10.1016/j.dib.2017.04.043
87. Bhairavabhotla R, Kim YC, Glass DD, Escobar TM, Patel MC, Zahr R, et al. Transcriptome profiling of human FoxP3+ regulatory T cells. *Hum Immunol.* (2016) 77:201–13. doi: 10.1016/j.humimm.2015.12.004
88. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood* (2013) 121:2647–58. doi: 10.1182/blood-2012-08-443473
89. Brunner HI, Tzaribachev N, Vega-Cornejo G, Louw I, Berman A, Calvo Penades I, et al. Subcutaneous abatacept in patients with polyarticular-course juvenile idiopathic arthritis: results from a phase III open-label study. *Arthritis Rheumatol.* (2018) 70:1144–54. doi: 10.1002/art.40466
90. Maggi L, Cimaz R, Capone M, Santarlasci V, Rossi MC, Mazzoni A, et al. Immunosuppressive activity of abatacept on circulating T helper lymphocytes from juvenile idiopathic arthritis patients. *Int Arch Allergy Immunol.* (2016) 171:45–53. doi: 10.1159/000450948
91. Lovell DJ, Ruperto N, Mouy R, Paz E, Rubio-Perez N, Silva CA, et al. Long-term safety, efficacy, and quality of life in patients with juvenile idiopathic arthritis treated with intravenous abatacept for up to seven years. *Arthritis Rheumatol.* (2015) 67:2759–70. doi: 10.1002/art.39234
92. Ruperto N, Lovell DJ, Quartier P, Paz E, Rubio-Perez N, Silva CA, et al. Abatacept in children with juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled withdrawal trial. *Lancet* (2008) 372:383–91. doi: 10.1016/S0140-6736(08)60998-8
93. Arabshahi B, Silverman RA, Jones OY, Rider LG. Abatacept and sodium thiosulfate for treatment of recalcitrant juvenile dermatomyositis complicated by ulceration and calcinosis. *J Pediatr.* (2012) 160:520–2. doi: 10.1016/j.jpeds.2011.11.057
94. Sumitomo S, Nagafuchi Y, Tsuchida Y, Tsuchiya H, Ota M, Ishigaki K, et al. A gene module associated with dysregulated TCR signaling pathways in CD4(+) T cell subsets in rheumatoid arthritis. *J Autoimmun.* (2018) 89:21–9. doi: 10.1016/j.jaut.2017.11.001
95. Orban T, Beam CA, Xu P, Moore K, Jiang Q, Deng J, et al. Reduction in CD4 central memory T-cell subset in costimulation modulator abatacept-treated patients with recent-onset type 1 diabetes is associated with slower C-peptide decline. *Diabetes* (2014) 63:3449–57. doi: 10.2337/db14-0047
96. Rochman Y, Yukawa M, Kartashov AV, Barski A. Functional characterization of human T cell hyporesponsiveness induced by CTLA4-Ig. *PLoS ONE* (2015) 10:e0122198. doi: 10.1371/journal.pone.0122198
97. Szentpetery A, Heffernan E, Gogarty M, Mellerick L, McCormack J, Haroon M, et al. Abatacept reduces synovial regulatory T-cell expression in patients with psoriatic arthritis. *Arthritis Res Ther.* (2017) 19:158. doi: 10.1186/s13075-017-1364-3
98. Langdon K, Haleagrahara N. Regulatory T-cell dynamics with abatacept treatment in rheumatoid arthritis. *Int Rev Immunol.* 37:206–14. doi: 10.1080/08830185.2018.1465943
99. Alvarez-Quiroga C, Abud-Mendoza C, Doniz-Padilla L, Juarez-Reyes A, Monsivais-Urenda A, Baranda L, et al. CTLA-4-Ig therapy diminishes the frequency but enhances the function of Treg cells in patients with rheumatoid arthritis. *J Clin Immunol.* (2011) 31:588–95. doi: 10.1007/s10875-011-9527-5

100. Bonelli M, Goschl L, Blumel S, Karonitsch T, Hirahara K, Ferner E, et al. Abatacept (CTLA-4Ig) treatment reduces T cell apoptosis and regulatory T cell suppression in patients with rheumatoid arthritis. *Rheumatology (Oxford)* (2016) 55:710–20. doi: 10.1093/rheumatology/kev403
101. Duggleby R, Danby RD, Madrigal JA, Saudemont A. Clinical grade regulatory CD4(+) T cells (Tregs): moving toward cellular-based immunomodulatory therapies. *Front Immunol.* (2018) 9:252. doi: 10.3389/fimmu.2018.00252
102. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, et al. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Sci Transl Med.* (2015) 7:315ra189. doi: 10.1126/scitranslmed.aad4134
103. Tang Q, Vincenti F. Transplant trials with Tregs: perils and promises. *J Clin Invest.* (2017) 127:2505–12. doi: 10.1172/JCI90598
104. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, Sumstad D, et al. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood* (2016) 127:1044–51. doi: 10.1182/blood-2015-06-653667
105. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) 117:1061–70. doi: 10.1182/blood-2010-07-293795
106. Dijke IE, Hoeppli RE, Ellis T, Pearcey J, Huang Q, McMurchy AN, et al. Discarded human thymus is a novel source of stable and long-lived therapeutic regulatory T cells. *Am J Transplant.* (2015) 16:58–71. doi: 10.1111/ajt.13456
107. Hoeppli RE, MacDonald KN, Leclair P, Fung VCW, Mojibian M, Gillies J, et al. Tailoring the homing capacity of human Tregs for directed migration to sites of Th1-inflammation or intestinal regions. *Am J Transplant.* (2018) 19:62–76. doi: 10.1111/ajt.14936
108. Sicard A, Boardman DA, Levings MK. Taking regulatory T-cell therapy one step further. *Curr Opin Organ Transplant.* (2018) 23:509–15. doi: 10.1097/MOT.0000000000000566
109. Landman S, de Oliveira VL, van Erp PEJ, Fasse E, Bauland SCG, Joosten I, et al. Intradermal injection of low dose human regulatory T cells inhibits skin inflammation in a humanized mouse model. *Sci Rep.* (2018) 8:10044. doi: 10.1038/s41598-018-28346-5
110. Spolski R, Li P, Leonard WJ. Biology and regulation of IL-2: from molecular mechanisms to human therapy. *Nat Rev Immunol.* (2018) 18:648–59. doi: 10.1038/s41577-018-0046-y
111. Leon K, Garcia-Martinez K, Carmenate T, Rojas G. Combining computational and experimental biology to develop therapeutically valuable IL2 muteins. *Semin Oncol.* (2018) 45:95–104. doi: 10.1053/j.seminoncol.2018.04.001
112. Dwyer CJ, Ward NC, Pugliese A, Malek TR. Promoting immune regulation in type 1 diabetes using low-dose interleukin-2. *Curr Diab Rep.* (2016) 16:46. doi: 10.1007/s11892-016-0739-1
113. von Spee-Mayer C, Siegert E, Abdirama D, Rose A, Klaus A, Alexander T, et al. Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. *Ann Rheum Dis.* (2015) 75:1407–15. doi: 10.1136/annrheumdis-2015-207776
114. Todd JA, Evangelou M, Cutler AJ, Pekalski ML, Walker NM, Stevens HE, et al. Regulatory T cell responses in participants with type 1 diabetes after a single dose of interleukin-2: a non-randomised, open label, adaptive dose-finding trial. *PLoS Med.* (2016) 13:e1002139. doi: 10.1371/journal.pmed.1002139
115. Rosenzwajg M, Churlaud G, Mallone R, Six A, Derian N, Chaara W, et al. Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J Autoimmun.* (2015) 58:48–58. doi: 10.1016/j.jaut.2015.01.001
116. Matsuoka K, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, et al. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci Transl Med.* (2013) 5:179ra143. doi: 10.1126/scitranslmed.3005265
117. Koreth J, Kim HT, Jones KT, Lange PB, Reynolds CG, Chammas MJ, et al. Efficacy, durability, and response predictors of low-dose interleukin-2 therapy for chronic graft-versus-host disease. *Blood* (2016) 128:130–7. doi: 10.1182/blood-2016-02-702852
118. Trotta E, Bessette PH, Silveria SL, Ely LK, Jude KM, Le DT, et al. A human anti-IL-2 antibody that potentiates regulatory T cells by a structure-based mechanism. *Nat Med.* (2018) 24:1005–14. doi: 10.1038/s41591-018-0070-2
119. Lee SY, Cho ML, Oh HJ, Ryu JG, Park MJ, Jhun JY, et al. Interleukin-2/anti-interleukin-2 monoclonal antibody immune complex suppresses collagen-induced arthritis in mice by fortifying interleukin-2/STAT5 signalling pathways. *Immunology* (2012) 137:305–16. doi: 10.1111/imm.12008
120. Yokoyama Y, Iwasaki T, Kitano S, Satake A, Nomura S, Furukawa T, et al. IL-2-Anti-IL-2 monoclonal antibody immune complexes inhibit collagen-induced arthritis by augmenting regulatory T cell functions. *J Immunol.* (2018) 201:1899–906. doi: 10.4049/jimmunol.1701502
121. Zeng Z, Yu K, Chen L, Li W, Xiao H, Huang Z. Interleukin-2/Anti-Interleukin-2 immune complex attenuates cardiac remodeling after myocardial infarction through expansion of regulatory T cells. *J Immunol Res.* (2016) 2016:8493767. doi: 10.1155/2016/8493767
122. Spangler JB, Trotta E, Tomala J, Peck A, Young TA, Savvides CS, et al. Engineering a single-agent cytokine/antibody fusion that selectively expands regulatory T cells for autoimmune disease therapy. *J Immunol.* (2018) 201:2094–106. doi: 10.4049/jimmunol.1800578
123. Bell CJ, Sun Y, Nowak UM, Clark J, Howlett S, Pekalski ML, et al. Sustained in vivo signaling by long-lived IL-2 induces prolonged increases of regulatory T cells. *J Autoimmun.* (2015) 56:66–80. doi: 10.1016/j.jaut.2014.10.002
124. Ward NC, Yu A, Moro A, Ban Y, Chen X, Hsiung S, et al. IL-2/CD25: a long-acting fusion protein that promotes immune tolerance by selectively targeting the IL-2 receptor on regulatory T cells. *J Immunol.* (2018) 201:2579–92. doi: 10.4049/jimmunol.1800907
125. Kunz DJ, Gomes T, James KR. Immune cell dynamics unfolded by single-cell technologies. *Front Immunol.* (2018) 9:1435. doi: 10.3389/fimmu.2018.01435
126. Goltsev Y, Samusik N, Kennedy-Darling J, Bhate S, Hale M, Vazquez G, et al. Deep profiling of mouse splenic architecture with CODEX multiplexed imaging. *Cell* (2018) 174, 968–81 e915. doi: 10.1016/j.cell.2018.07.010
127. Dixon AR, Bathany C, Tsuei M, White J, Barald KF, Takayama S. Recent developments in multiplexing techniques for immunohistochemistry. *Expert Rev Mol Diagn.* (2015) 15:1171–86. doi: 10.1586/14737159.2015.1069182
128. Ehlers MR, Nepom GT. Immune-directed therapy for type 1 diabetes at the clinical level: the Immune Tolerance Network (ITN) experience. *Rev Diabet Stud.* (2012) 9:359–71. doi: 10.1900/RDS.2012.9.359

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REVIEW
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Biochemistry of Autoinflammatory Diseases: Catalyzing Monogenic Disease

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Monogenic autoinflammatory disorders are a group of conditions defined by systemic or localized inflammation without identifiable causes, such as infection. In contrast to classical primary immunodeficiencies that manifest with impaired immune responses, these disorders are due to defects in genes that regulate innate immunity leading to constitutive activation of pro-inflammatory signaling. Through studying patients with rare autoinflammatory conditions, novel mechanisms of inflammation have been identified that bare on our understanding not only of basic signaling in inflammatory cells, but also of the pathogenesis of more common inflammatory diseases and have guided treatment modalities. Autoinflammation has further been implicated as an important component of cardiovascular, neurodegenerative, and metabolic syndromes. In this review, we will focus on a subset of inherited enzymatic deficiencies that lead to constitutive inflammation, and how these rare diseases have provided insights into diverse areas of cell biology not restricted to immune cells. In this way, Mendelian disorders of the innate immune system, and in particular loss of catalytic activity of enzymes in distinct pathways, have expanded our understanding of the interplay between many seemingly disparate cellular processes. We also explore the overlap between autoinflammation, autoimmunity, and immunodeficiency, which has been increasingly recognized in patients with dysregulated immune responses.

Keywords: autoinflammation, innate immunity, mutations, enzyme deficiency, metabolic sensors, ubiquitination, protein homeostasis

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INTRODUCTION

Autoinflammatory disorders are characterized by recurrent or persistent systemic or organ specific inflammation without inciting event and classically present with elevated acute phase reactants (1, 2). Characterization of these disorders has primarily focused on familial forms of disease due to highly penetrant mutations as was the case with the identification of the *MEFV* gene responsible for Familial Mediterranean Fever (FMF) and mutations in the *TNFRSF1A* gene as the cause of dominantly inherited TRAPS (Tumor Necrosis Factor Receptor Associated Periodic Syndrome). As genetic sequencing technology and analysis have improved and cost has decreased, there have now been nearly 30 genes identified as causative for autoinflammatory disorders (3). Many of the earliest identified monogenic autoinflammatory diseases were directly related to constitutive inflammasome activation and include FMF and cryopyrinopathies, or loss of a critical inhibitory mechanism as in deficiency of IL-1 (DIRA) or IL-36 (DITRA) receptor antagonist leading to imbalanced cytokine receptor signaling (4–9). Examples such as these have

led to classification systems focused on the primary molecular pathways that are altered and thus diseases have been denoted as inflammasomopathies, interferonopathies, and NF-kB related autoinflammatory disorders (10–13). These classifications have helped identify shared mechanisms of disease pathogenesis and principles of treatment. Generally, autoinflammatory disorders are due to gene dysregulation restricted to hematopoietic lineages, whereas involvement of non-inflammatory cells is limited. Although most monogenic autoinflammatory disorders can be placed into this paradigm, many newly identified disorders seem to defy this classification and they have revealed a role for pathways not previously linked to immune function.

Here we will focus on classifying a subset of disorders by the specific biochemical deficiency as opposed to the clinical manifestations or immune mechanism that is disrupted (**Table 1**). These disorders will be organized by the affected cellular function to highlight the unexpected links between specific biochemical processes and immune dysregulation. We will review disorders that are due to loss of a enzymatic activity and how these diseases may reveal important aspects not only of immunology but of basic cellular signaling. Enzymatic deficiencies offer unique potential treatment strategies based on either accumulation of toxic substrates or loss of catalytic products and can theoretically be treated with enzyme replacement therapy.

DISORDERS DUE TO DISRUPTION OF PROTEIN TRANSLATION AND HOMEOSTASIS

tRNA Nucleotidyltransferase, CCA-Adding, 1 (TRNT1) Deficiency

The *TRNT1* gene encodes a ubiquitously expressed tRNA nucleotidyltransferase, CCA-Adding, 1 (TRNT1) that is essential for protein synthesis. TRNT1 adds and repairs the conserved CCA sequence at the 3′ end of all precursor cytosolic and mitochondrial transfer ribonucleic acids (tRNAs), a step necessary for the attachment of conjugate amino acids (14). TRNT1 also regulates RNA stability through tRNA decay mechanisms and may play an important role in reducing levels of non-coding RNAs (15). TRNT1 is localized to the mitochondria via a 41 amino acid transit peptide and is expressed in all tissues. The crystal structure of human TRNT1 (PDB ID:1Ou5) shows that the protein functions as a homodimer via intermolecular disulfide bond (16). Complete deficiency of *Trnt1* in mice is embryonic lethal further highlighting the essential function of this gene.

Bi-allelic loss of function mutations in *TRNT1* lead to a recessively inherited syndrome named SIFD for sideroblastic anemia, B-cell immunodeficiency, developmental delay, and periodic fevers (**Figure 1A**) (17, 18). Given the ubiquitous expression of TRNT1, it is not surprising that reduced function of the enzyme leads to a complex phenotype. To date, more than 30 patients have been reported with significant clinical and immunologic heterogeneity (17, 19–22). At the severe end of the spectrum are patients with neonatal-onset severe anemia and prominent extramedullary erythropoiesis, profound immunodeficiency, metabolic and neurological abnormalities (17). In this first published cohort of 12 patients, median survival was 48 months and seven patients died due to cardiac or multiorgan failure. Recurrent fever has been reported in most but not all patients with SIFD. Immunodeficiency in SIFD is primarily due to defects in B cells differentiation and can manifest early in life or can be progressive and present later (23). T and NK cell numbers are in the low-normal range and some patients also carry a diagnosis of combined variable immunodeficiency but without serious bacterial or viral infections. At the milder end of the spectrum are patients with non-syndromic retinitis pigmentosa and subtle hematological features (24, 25).

SIFD-associated variants are loss-of-function and they include missense, non-sense, frameshift, and splice site mutations. *TRNT1* pathogenic variants are either novel or have a low frequency in the general population. As is the case with many other recessively inherited diseases, a subset

TABLE 1 | Summary of diseases, genes, and inheritance for autoinflammatory disorders discussed.

Disease	Acronym	MIM disease	Inheritance	Gene/protein	Transcript ID
Sideroblastic anemia, B-cell immunodeficiency, developmental delay and periodic fevers	SIFD	616084	AR	*TRNT1*/TRNT1	NM_001302946
Majeed syndrome	–	609628	AR	*LPIN2*/LPIN2	NM_014646.2
PLCγ2-associated antibody deficiency, and immune dysregulation	PLAID	614468	AD	*PLCG2*/PLCγ2	NM_002661.3
Autoinflammation, PLCγ2-associated antibody deficiency, and immune dysregulation	APLAID	614878	AD	*PLCG2*/PLCγ2	NM_002661.3
Monogenic systemic JIA/IBD	–	–	AR	*C13ORF31*/LACC1/FAMIN	NM_153218
Mevalonate kinase deficiency	MKD/HIDS	251170/ 260920	AR	*MVK*/MVK	NM_000431.3
Haploinsufficiency A20	HA20	616744	AD/*De novo*	*TNFAIP3*/A20	NM_006290.3
Otulipenia/Otulin-related autoinflammtory syndrome	ORAS	615712	AR	*OTULIN*/OTULIN	NM_138348.5
RIPK1 deficiency	–	618108	AR	*RIPK1*/RIPK1	NM_003804
Deficiency of adenosine deaminase 2	DADA2	615688	AR	*CECR1*/*ADA2*/ADA2	NM_001282225.1

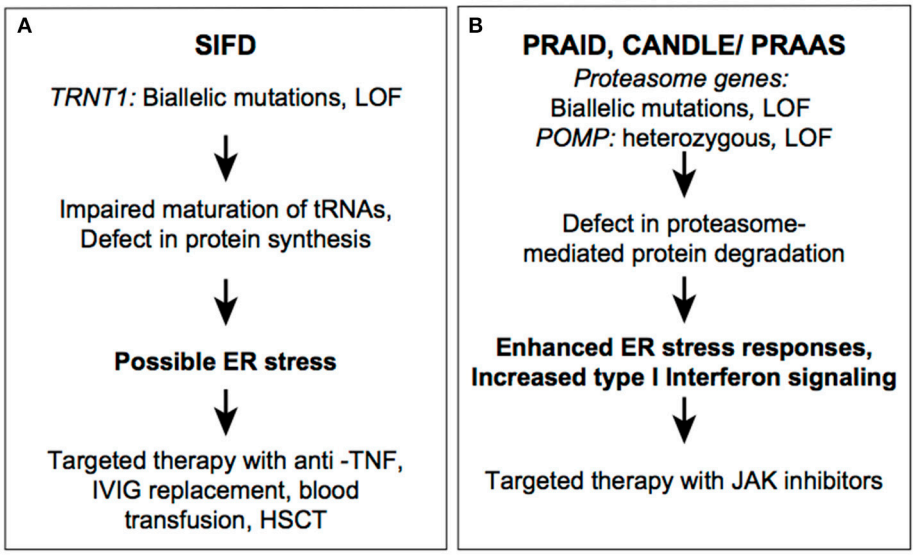

FIGURE 1 | Autoinflammatory diseases due to dysregulation in proteostasis. SIFD **(A)** and CANDLE **(B)** syndromes.

of patients were identified in founder populations with homozygous pathogenic variants. Hypomorphic mutations in TRNT1 reduce protein expression, affect protein stability, or alter its catalytic efficiency (18, 26). So far, no patients have been identified with biallelic non-sense or frameshift mutation, further emphasizing that residual TRNT1 protein is essential for development. In TRNT1-deficient cells, maturation of nuclear and mitochondrial tRNAs is impaired, which leads to a defect in global protein synthesis (21, 27). As result, these cells are unable to maintain protein homeostasis under stress conditions. Protein degradation pathways, which are essential for clearance of unprocessed/misfolded proteins, eventually become insufficient to remove an excessive load of misfolded proteins in mutant cells. Accumulation of misfolded proteins can then result in cell death and release of various proinflammatory cytokines through the unfolded protein response (UPR). Although ER stress and activation of the UPR has not been experimentally demonstrated in TRNT1-deficient cells, there are examples of other similar systemic inflammatory diseases of dysregulated protein homeostasis, such as Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) and POMP-related autoinflammation and immune dysregulation disease (PRAID). Both CANDLE and PRAID syndrome result from defects in proteasome assembly leading to the accumulation of ubiquinated proteins triggering cellular stress and the type 1 interferon (IFN) response (**Figure 1B**) (28, 29). In addition to the UPR stress-mediated inflammatory response, reactive oxygen species (ROS) accumulation has been observed in cultured SIFD patients' fibroblasts, and elevated ROS levels can activate other inflammatory pathways such as the NLRP3 inflammasome and type I interferon response (21, 30). Furthermore, some clinical manifestations of SIFD resemble patients with mitochondrial disorders and patient-derived fibroblasts showed decrease in cellular respiration and oxidative phosphorylation (31).

Thus, far, it has not been investigated why hypomorphic mutations in *TRNT1* cause severe defects in differentiation of hematopoietic cells, but it may have to do with tissue-specific regulatory functions or the specific metabolic demands of immune cells. Similar tissue specific clinical manifestations have been identified in genetic syndromes with loss of tRNA synthetases, enzymes responsible for tRNA conjugation to specific amino acids, despite their essential function across all tissues (32).

Most patients are treated symptomatically with blood transfusions, IgG replacement therapy, and corticosteroids, while the disease morbidity and mortality remains high. Necessity for blood transfusions for anemia is often increased during fevers. Anti-TNF therapy suppresses inflammation reducing the need for blood transfusions, and improving growth, although it is not clear if non-immune functions improve under these therapies (21). Molecular diagnosis in early life is crucial to prevent some of the severe disease consequences. Hematopoietic stem cell transplantation (HSCT) has been attempted and has helped with hematological features, however one patient died of transplant-related complications (17).

SECOND MESSENGER MEDIATED DISEASES

LPIN2-Deficiency

LPIN2 is a member of the lipin family of enzyme, which function in glycerolipid biosynthesis (33, 34). There are 2 other lipin family member enzymes, LPIN1, and LPIN3. LPIN2 is a phosphatidate phosphatase (PAP) that catalyzes the conversion of phosphatidic acid to diacylglycerol (DAG), a critical byproduct necessary for the production of triacylglycerol, phophatidylcholine, and phosphatidylethanolamine (35). DAG works as a secondary lipid messenger by activating protein kinases and RAS signaling

pathways among others (36). In addition to the PAP activity, LPIN1 and 2 may function as a transcriptional co-activator with peroxisome proliferator-activated receptors (PPARα) (37). LPIN1 and LPIN2 are expressed in macrophages and seem to have opposite roles in regulating immune responses (38, 39). While LPIN1 acts as a proinflammatory mediator during Toll-like receptor (TLR) signaling, LPIN2 downregulates proinflammatory signaling induced by saturated fatty acids (40). *In vitro* depletion of *LPIN2* causes increased expression of IL-6 and TNF in macrophages. *In vivo* studies of LPIN2 function have also demonstrated its role as a negative regulator of the NLRP3 inflammasome and TLR4 signaling (41). LPIN2-deficient mouse bone marrow derived macrophages (BMDM) display low cholesterol levels, an increase in ATP-promoted potassium flux, and increased activity of the PTX_7 receptor. Thus, reduced function of LPIN2 results in upregulation of the inflammatory pathways and leads to overproduction of IL-1β, IL-18, and TNF cytokines. Similar results were obtained in primary patient-derived macrophages. These studies established a critical link between lipid biosynthesis and inflammation.

LPIN2 loss of function in humans leads to Majeed Syndrome, a rare, recessively inherited disorder that is characterized by the triad of early-onset chronic recurrent multifocal osteomyelitis (CRMO), dyserythropoietic anemia (typically microcytic), and neutrophilic skin lesions (**Figure 2A**) (42, 43). While CRMO and skin inflammation can be explained by a loss of LPIN2 anti-inflammatory function, the molecular mechanism of anemia is less clear. Interestingly, LPIN1 is highly expressed in muscle, and loss of enzymatic activity leads to a recessively inherited muscle disease (44). Given the ubiquitous expression of *LPIN1* and *LPIN2* and the widespread importance of glycerolipids in diverse cellular functions, it is intriguing why deficiency of these enzymes would cause these very distinct, tissue-specific phenotypes. To date, 14 cases of Majeed syndrome have been reported, all in consanguineous families of Middle Eastern ancestry. As all patients carry a private homozygous mutation there is a limited number of identified pathogenic variants (*Infevers*). Causal variants include mostly non-sense and splice site mutations that lead to reduced protein expression. A single pathogenic missense variant, p.Ser734Leu, was shown *in vitro* to abolish the PAP activity of LPIN2, suggesting that this activity is important for the pathogenesis of the disease (45).

IL-1 blockade results in dramatic improvement in clinical and laboratory parameters of inflammation in Majeed syndrome, confirming that loss of LPIN2 in these patients leads to autoinflammation via enhanced IL-1β production (46).

PLCG2-Associated Diseases

Phospholipase C (PLC) enzymes have a key role in the regulation of variety of cellular functions (47). The PLC family of enzymes include PLCγ1 and PLCγ2, encoded by *PLCG1* and *PLCG2* genes, respectively. These enzymes catalyze the hydrolysis of phospholipids and generate secondary messengers that provide a signal between many cellular receptors with downstream intracellular pathways. While *PLCG1* is ubiquitously expressed, *PLCG2* is highly expressed in hematopoietic cells. PLCγ2 is activated by immune receptors such as B cell and Fc receptors and has an important role in mediating innate immune responses (48). In response to receptor stimulation, PLCγ2 catalyzes the formation of the second messengers inositol triphosphate (IP_3) and DAG from phosphatidylinositol 4,5-bisphosphate (PIP2). IP_3 binds to IP_3 receptors on the endoplasmic reticulum, resulting in calcium release and subsequent activation of various signaling pathways including mitogen activated protein kinases (MAPK). DAG remains bound to the cell membrane where it can activate protein kinase C (PKC) and other kinases. Pathogenic mutations in *PLCG2* lead to two distinct phenotypes: PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) and autoinflammation, antibody deficiency, and immune dysregulation (APLAID) (**Figures 2B,C**).

PLAID is a dominantly inherited disease characterized by cold-induced urticarial or blistering rash with onset in infancy, variable degrees of immunodeficiency and autoimmunity, and skin granuloma formation (49). In some patients, the skin lesions get worse over time leading to tissue destruction. APLAID is also dominantly inherited and presents early in life with fever, blistering, or erythematous relapsing skin lesions often triggered by heat and sweating, arthralgia, ocular inflammation, enterocolitis, and progressive interstitial lung disease (50). To date over thirty patients with PLAID and only two patients with APLAID have been reported (51). The main distinguishing features between PLAID and APLAID are that PLAID patients have cold-induced urticaria and prominent autoimmune features as compared to APLAID patients. Features of autoimmunity were found in about 25% of patients with PLAID. B cell immunodeficiency is in the spectrum of both diseases, and manifests with recurrent bacterial respiratory and GI infections. Common laboratory findings include antibody deficiency (low serum IgG and IgM) and decreased levels of circulating CD19+ and class-switched memory B cells.

The disease pathophysiology is due to a complex combination of temperature-sensitive, cell-specific gain and loss-of-function variants in the PLCγ2 signaling pathway (52). PLAID families were found to have in-frame genomic deletions spanning the cSH2 autoinhibitory domain of PLCγ2. This domain blocks the active site of the enzyme and deletion of this region results in diminished PLCγ2-mediated signaling at physiologic temperature but enhanced signaling at sub-physiologic temperatures. Primary B and NK cells from PLAID patients have an anergic phenotype while mast cells spontaneously activate when exposed to lower temperature due to loss of the autoinhibitory domain. On the other hand, APLAID patients, who carry a gain-of-function missense mutation instead of a deletion in the same cSH2 autoinhibitory domain of PLCγ2, have very different cellular and clinical phenotypes. The gain-of-function mutation identified in APLAID patients, p.Ser707Tyr, causes temperature-independent production of IP_3, intracellular Ca^{2+} release and ERK phosphorylation in patient-derived PBMCs (50). In addition, there is evidence for Ca-dependent activation of the NLRP3 inflammasome in primary cells (53). Collectively, these data suggest constitutive hyper activation of myeloid cells in APLAID.

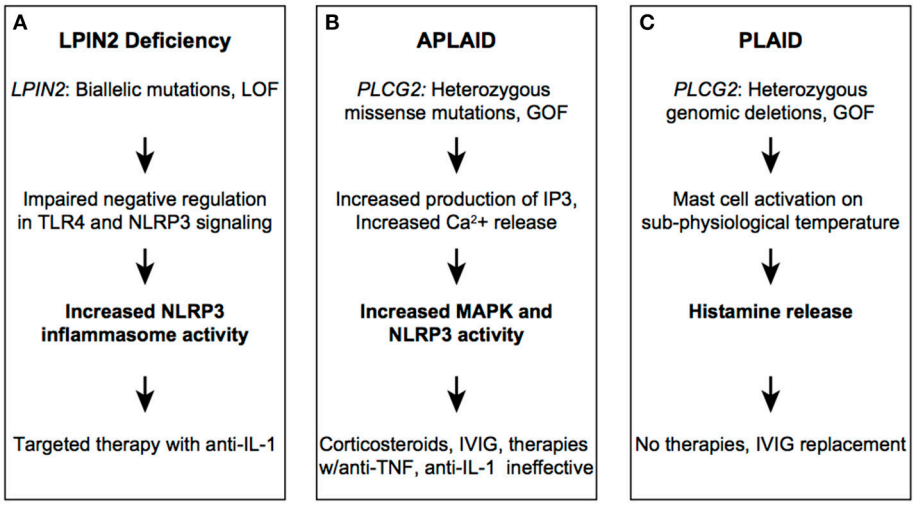

FIGURE 2 | Autoinflammatory diseases due to dysregulation in lipid-mediated signaling. LPIN2 **(A)**, PLAID **(B)**, and APLIAD **(C)** syndromes.

PLAID and APLAID patients with noticeable immunodeficiency require immunoglobulin replacement therapy, while patients with a severe inflammatory phenotype have proven difficult to treat. Although the NLRP3 inflammasome was shown to have a role in mediating inflammation in APLAID, IL-1 inhibitors have been mostly ineffective. Similarly, other cytokine inhibitors have been trialed but were ineffective in suppressing disease activity. This observation suggests function for other yet unknown pathways in the disease pathogenesis.

METABOLIC SENSORS IMPLICATED IN AUTOINFLAMMATORY DISEASES

LACC1 Deficiency

LACC1 encodes for the protein FAMIN (Fatty Acid Metabolism and Immunity Nexus), which has homology to bacterial multicopper oxidoreductase enzymes (54, 55). FAMIN interacts with fatty acid synthase (FASN) and localizes to the peroxisome. FAMIN is predominantly expressed in macrophages, where it regulates fatty acid oxidation and lipogenesis to maximize metabolism (55). The exact mechanism of how FAMIN controls lipid balance is not clear but patient monocytes with different *LACC1* alleles have altered laccase (phenol-oxidoreductase) suggesting loss of this enzymatic activity may be the mechanism of disease (56, 57).

The first link between *LACC1* and disease was identified through an association with leprosy and Crohn's disease (CD) by genome wide association studies (GWAS) (58–61). Interestingly leprosy and Crohn's disease are both granulomatous disorders, and share the same *LACC1* risk allele, the specific missense p.Ile254Val variant (rs3764147; MAF = 0.27). Later studies have demonstrated an association between a SNP (rs2121033; MAF = 0.27) downstream of *LACC1* with Behcet's Disease (**Figure 3A**) (62, 63). It is possible that these two susceptibility alleles are in linkage disequilibrium and therefore inherited on the same haplotype. *LACC1* was subsequently identified as a cause of recessively inherited monogenic CD, based upon a single consanguineous Saudi Arabian family with multiple members presenting with early-onset disease (64). The novel p.Cys284Arg variant segregated with the disease phenotype in this large family with four affected and seven unaffected members. This mutation is predicted to be detrimental for protein stability and activity, although this has not been confirmed experimentally. *LACC1* was further implicated in monogenic autoinflammatory disease through identification of families with recessively inherited systemic juvenile idiopathic arthritis (SoJIA) (65). The same mutation, p.Cys284Arg, identified in the family with CD, was identified in 13 children affected with juvenile idiopathic arthritis from five consanguineous Saudi Arabian families. All patients had symmetrical polyarthritis, the characteristic quotidian fevers, evanescent rash and were homozygous for the missense mutation p.Cys284Arg (65). Interestingly, none of the patients with inflammatory arthritis had CD or ulcerative colitis. Thus, the same population-specific variant has been linked to distinct inflammatory phenotypes. Two recent studies reported 19 additional patients with primarily juvenile idiopathic arthritis, all from Middle Eastern consanguineous families, with distinct genotypes including p.M1I, p.R414*, p.I330del, p.I254V, p.Cy370Tyrfs*6, and p. T276fs*2 in *LACC1* (66, 67). Some of these mutations have been shown to result in decreased protein expression, confirming that the disease is caused by loss of function of FAMIN.

Effective treatment modalities reported include TNF blockade with adalimumab or IL-6 inhibition with tocilizumab (66). Given the fact that *LACC1*/FAMIN controls reactive oxygen species production in macrophages, loss of this enzyme was predicted to result in decreased IL-1β production. However, knockout and knock-in disease mutation murine models show increased IL-1β, likely due to compromised pathogen clearance (55). Similar findings were made in patient-derived cells with a p.I254V

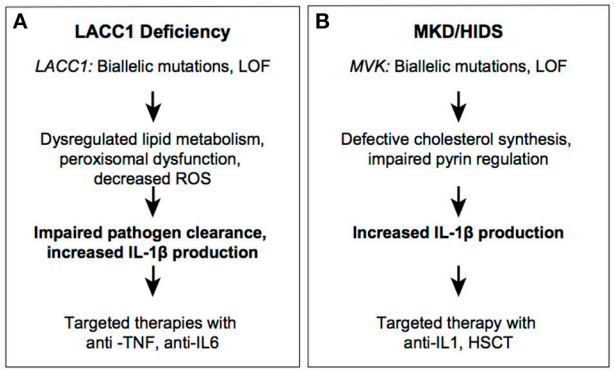

FIGURE 3 | Autoinflammatory diseases due to impaired metabolic sensors. LACC1 **(A)** and MVK **(B)** syndromes.

homozygous variant, which revealed decreased ROS production and decreased NOD2-dependent bacterial clearance (56). The central function of *LACC1* in granulomatous disease and pathogen clearance is similar to that of *NOD2*, a key pathogen recognition receptor mutated in a Mendelian granulomatous disease, Blau Syndrome, and polygenic CD. Because the same *LACC1* mutations, p.Ile254Val and p.Cys284Arg, have been associated with multiple phenotypes including, leprosy, BD, SoJIA and early-onset CD, it is likely that environmental factors, such as microbiota, play an important role in disease expressivity and manifestations. The study of phenotypes associated with mutations in *NOD2* and *LACC1* implies that environmental factors may be important in the pathogenesis of more common granulomatous diseases including sarcoidosis (68). The critical position of FAMIN in cellular energy homeostasis reveals unique regulatory functions that exist in phagocytes to ensure a balance of energy sources available for clearance of pathogens.

Mevalonate Kinase-Associated Diseases

Another example of dysregulated immunometabolism is mevalonate kinase deficiency/hyper IgD syndrome (MKD/HIDS). Mevalonate kinase (MVK) is a key enzyme in the biosynthesis of cholesterol and isoprenoids, catalyzing the conversion of mevalonic acid to mevalonate-5-phosphate. Through this role in cholesterol synthesis, MVK regulates levels of geranylgeranyl pyrophosphate, which is important for prenylation and regulation of the small GTPases. RhoGTPases inhibit the pyrin inflammasome by activating protein kinase N enzymes (PKNs), which suppress pyrin function (9). Protein geranylgeranylation is also critical for TLR-induced activation of phosphatidylinositol-3-OH kinase (PI(3)K) through the interaction between the small GTPase KRAS and the PI(3)K catalytic subunit p110delta. Compromised (PI(3)K) activity results in constitutive activation of pyrin (69). Thus, loss of MVK activity leads to reduced prenylation of GTPases, which in turn results in increased activity of the pyrin inflammasome and excessive production of IL-1β.

Recessive loss of function mutations in *MVK* can result in a spectrum of disease ranging from severe multisystem disease (mevalonic aciduria; MKD) to a milder autoinflammatory disease (HIDS) (**Figure 3B**) (70). Patients with MKD manifest dysmorphic features, psychomotor retardation, progressive cerebellar ataxia, and systemic inflammation. Patients with HIDS have episodes of high fever, rash, abdominal pain, aphthous ulcers, pharyngitis, swollen lymph nodes, and arthralgia/arthritis. These symptoms are often triggered by immunization or other stresses. Cells from patients with HIDS still have residual MVK enzymatic activity (about 1–10% of the activity found in healthy control cells), whereas patients with MKD have complete deficiency in enzyme activity (71, 72). Most severe MKD-associated mutations create truncated proteins, whereas HIDS-associated mutations are missense substitutions and are thought to impair MVK stability (73, 74). The inflammatory phenotype in patients with HIDS is ameliorated with anti-IL-1 therapy consistent with the finding that MVK-deficient PBMCs secrete higher levels of IL-1β (75–77).

POST-TRANSLATION MODIFYING ENZYMES IN AUTOINFLAMMATORY DISEASES

Several recently described autoinflammatory disorders highlight the role of post-translational modifications (PTMs), in the regulation of TNF and IL-1β signaling pathways and NF-κB activation (10, 78). Ubiquitination is the covalent attachment of an evolutionarily conserved 76-amino acid ubiquitin (Ub) protein to target substrates in the form of a monomer or polymers (ubiquitin chains; Ub chains). Ubiquitin chains can be conjugated at different lysine residues (K6, K11, K29, K33, K48, K63) along with the amino terminal methionine (M1), which can determine the fate of the modified protein. Proteins conjugated with Lys48 (K48) Ub chains are targeted for degradation via the ubiquitin-proteasome system (UPS), while Lys63 (K63) linked and linear Ub chains have essential roles in promoting signaling cascades. Ubiquitination can be reversed by a class of enzymes known as deubiqutylases or deubiquitinases (DUBs). There are more than 100 known DUBs expressed in various cells and with different degrees of specificity for Ub chains. Several DUBs are highly expressed in hematopoietic cells where they function as negative regulators of NF-kB signaling (A20, OTULIN, CYLD, and Cezanne). Mutations in genes encoding some of these DUB enzymes, along with other PTM enzymes such as kinases, have been recently implicated in autoinflammatory disorders.

Haploinsufficiency of *TNFAIP3*/A20 (HA20)

TNFAIP3/A20 has two enzymatic activities that synergize to restrict inflammatory responses: it has deubiquitinase activity by which it causes hydrolysis of K63 Ub linkages in receptor signaling complexes, and it has E3 Ub ligase activity through which substrates are modified with K48 Ub chains to target them for proteasomal degradation (79). TNFAIP3/A20 is a 790-residue protein that consists of an amino-terminal ovarian tumor domain (OTU) followed by 7 zinc finger domains (ZFs). The ZnF4 domain is essential for A20 E3 ligase activity and dimerization. The protein has a critical role as negative regulator of canonical NF-κB signaling. A20 null mice exhibit multi-organ

inflammation, cachexia, and early lethality, while conditional A20 knockout in B cells, T cells and/or epithelial cells alone do not lead to spontaneous inflammation suggesting that the inflammatory phenotype is specific to myeloid cells (80).

Heterozygous loss-of-function mutations in TNFAIP3/A20 are associated with an autoinflammatory disease named haploinsufficiency of A20 (HA20) (**Figure 4A**) (81). Most patients with HA20 present with an early-onset autoimmune/autoinflammatory phenotype, with a variety of organ-specific features that are analogous to diseases such as Behcet's disease, systemic lupus erythematosus, Hashimoto's thyroiditis, autoimmune lymphoproliferative syndrome, and Crohn's disease. Many patients present with oral and genital ulcers, which are uncommon symptoms in other monogenic autoinflammatory diseases (82–86). There is substantial variability in expressivity of disease even among patients with the same genotype. Recently, large deletions on chromosome 6 encompassing up to 55 genes including *TNFAIP3,* were identified in patients with systemic inflammation, psychomotor and growth delay, and *situs inversus* (87, 88). The heterotaxy (abnormal organ arrangement) was likely the consequence of the deleted *CITED2* gene (89, 90). Thus, anti-inflammatory therapy should be considered in patients with a complex phenotype and heterozygous genomic deletions on Chr. 6q23-q24.

HA20-associated mutations are mostly located within the OTU domain and lead to truncated proteins of different length, with some partial protein products stably expressed in patient cells (91). Whether these truncated protein affect signaling remains unclear, although a dominant negative mode of action for these byproducts could mechanistically explain the phenotype. In addition to germline mutations in *TNFAIP3*, a low-frequency somatic mutation was reported in two unrelated Japanese families, which is of particular concern for genetic counseling and disease reoccurrence risk stratification (84). Irrespective of mutation type and position, patients with active disease have high serum levels of many proinflammatory cytokines such as IL-1β, IL-6, IL-9, IL-17, IL-18, TNF, suggesting constitutive activation of canonical NF-κB and NLRP3 inflammasome pathways (81, 84). Therapies with cytokine inhibitors, such as anti-TNF, anti-IL-1, and anti-IL-6 have been successfully employed in controlling disease activity. HSCT was curative in one reported patient (92).

Otulipenia/Otulin-Related Autoinflammatory Syndrome (ORAS)

OTULIN is a highly conserved deubiquitinase that hydrolyzes Met1-linked (linear) Ub chains from conjugated substrates. OTULIN has a critical role in regulation of angiogenesis and is required for craniofacial and neuronal development by regulating canonical Wnt signaling (93). OTULIN also functions as a negative regulator of the canonical NF-kB pathway by deubiquitinating the linear ubiquitination chain assembly complex (LUBAC) (94). LUBAC, which consists of HOIP, HOIL1, and SHARPIN, catalyzes linear ubiquitination and is essential for NF-κB signaling (95). Conditional knockout of *Otulin* in immune lineages results in a viable but severe inflammatory phenotype in mice with a more severe phenotype in the KO myeloid lineage than lymphoid lineage (96).

Recessively inherited, loss of function mutations in the OTU domain of OTULIN are associated with the early-onset severe inflammatory disease (**Figure 4B**) (96, 97). Patients present with failure to thrive, recurrent fevers, rash, joint swelling, and gastrointestinal inflammation. The cutaneous manifestations include painful erythematous rashes, subcutaneous skin nodules, and lipodystrophy. In one highly inflamed patient, skin biopsy showed neutrophilic dermatitis and panniculitis. To date, three mutations (2 missense and 1 frameshift) in five individuals from Middle Eastern, consanguineous families, have been reported. The phenotypic characteristics of OTULIN deficiency will expand with increased awareness and identification of new mutations. Pathogenic mutations affect the catalytic activity of OTULIN thereby impairing removal of linear ubiquitination; mutant cells accumulate linear Ub chains on various substrates such as IKKγ/NEMO, RIPK1, and ASC (97). Increased linear ubiquitination of IKKγ and RIPK1 results in constitutive activation of the canonical NF-kB pathway. Patient-derived immune cells produce higher levels of many proinflammatory cytokines that are secreted both by myeloid cells and T cells, such as IL-17 and IFNγ. Therapy with TNF inhibitors has been very effective in suppressing systemic inflammation and in improving growth and development. Hematopoietic stem cell transplantation (HSCT) may also hypothetically rescue the hematological phenotype although it has not been attempted due to the favorable response to treatment with TNF-inhibitors.

Receptor Interacting Protein Kinase 1 (RIPK1)-Associated Immunodeficiency and Autoinflammation

Receptor Interacting Protein Kinase 1 (RIPK1) is a widely expressed serine/threonine kinase that regulates TNFR (TNF receptor) signaling and cell death pathways (98). RIPK1 plays an important role in the regulation of immune responses through its dual functions either in activating NF-κB signaling or initiating cell death programs. RIPK1 serves as a scaffold in plasma membrane-associated protein complexes that transmit signals from TNFR1, TLR2, and TLR4 cell surface receptors. Stimulation of these receptors activates the canonical NF-κB pathway, MAPK, and pro-survival genes. Cytoplasmic RIPK1 kinase activity is important for initiation of cell death signaling pathways, apoptosis and necroptosis (99, 100). RIPK1 kinase activity acts primarily in initiating necroptosis, a caspase-independent form of cell death, through auto-phosphorylation, which triggers a protein interaction cascade. During necroptosis, the plasma membrane permeabilizes, releasing damage associated molecular patterns (DAMPs) that can in turn lead to a prolonged immune response. RIPK1 has been extensively studied in murine models, with knockout mice displaying postnatal lethality due to systemic inflammation and increased cell death (101, 102). Inhibition of RIPK1 kinase activity using necroptosis inhibitors, necrostatin-1 and its analogs, can ameliorate a variety of mouse models of disease, including septic shock, myocardial infarction, and amyotrophic lateral sclerosis, along with inflammatory

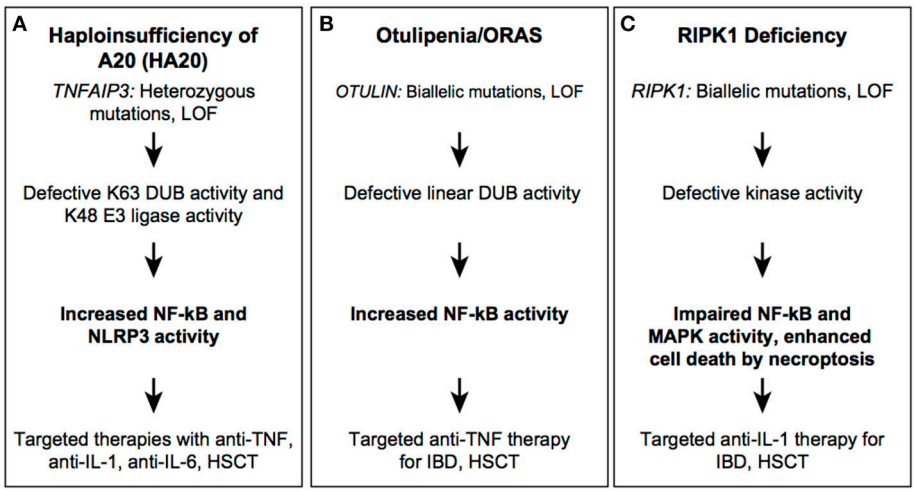

FIGURE 4 | Autoinflammatory diseases due to dysregulation in post-translational modification. HA20 **(A)**, Otulipenia **(B)**, and RIPK1 deficiency **(C)** syndromes.

phenotypes such as rheumatoid arthritis and ulcerative colitis implicating necroptosis as a major contributor in disease pathogenesis (103).

Recently, four patients with severe immunodeficiency, gut inflammation, and progressive polyarthritis were identified with *RIPK1* homozygous loss of function alleles (**Figure 4C**) (104). The disease causing variants are small nucleotide deletions in the N-terminal kinase domain that lead to frameshift and loss of a substantial portion of the protein. The autoinflammatory phenotypic features consists of a GI inflammation with variable onset and severity, which suggests that environmental factors may modulate the disease expressivity. The four described patients suffered from recurrent infections, mostly viral but some bacterial and fungal infection. Immunophenotyping revealed lymphopenia, most prominently of CD4+ T cells, with one patient demonstrating low antibody titers and another with low serum immunoglobulins (IgG, IgM, IgA). RIPK1-deficient patient fibroblasts stimulated with TNF exhibit impaired MAPK activation, reduced cytokine production and decreased cell viability consistent with the immunodeficient phenotype. Similar to fibroblasts, LPS-stimulated patient monocytes showed decreased production of proinflammatory cytokines including IL-6, IL-12, and TNF however, they secreted high levels of IL-1β. Cell death in cultured fibroblasts was predominantly mediated by necroptosis as apoptosis inhibitors were unable to reduce cell death. Although the mechanism of inflammation in humans, like mice, is due to increased cell death via necroptosis, the absence of immunodeficiency in *Ripk1* $^{-/-}$ mice and the viability of null alleles in humans, hints at a unique role for necroptosis specific to human immune cells.

This disease phenotype is fairly severe and with high mortality. As IL-1β likely contributes to both IBD and arthritis in these patients, anti-IL-1 therapy may be considered to suppress the inflammatory component of the disease although none of the reported patients received targeted IL-1 inhibitors. Three of the patients with RIPK1 deficiency underwent HSCT, two died shortly after transplant due to infectious causes.

NUCLEIC ACID REGULATION IN AUTOINFLAMMATORY DISEASE

Adenosine Deaminase 2 Gene (*ADA2*) Deficiency

Adenosine deaminase (ADA) is an aminohydrolase that regulates purine metabolism and adenosine homeostasis by catalyzing the deamination of adenosine (Ado) and 2′-deoxyadenosine (dAdo) into inosine and deoxyinosine, respectively. Extracellular levels of adenosine increase during hypoxia and tissue injury and can lead to persistent inflammation through activation of adenosine receptors (AdoR) (105). In humans, there are two isoenzymes encoded by different genes, *ADA1* (also known as *ADO*) and *ADA2* (also known as *CECR1*) that have different enzymatic properties. ADA2 has a 100-fold higher Michaelis-Menton constant for adenosine ($K_m = 2$ mM) than ADA1 and is not essential for intracellular deaminase activity. ADA2 is a secreted protein and under physiological conditions is present in low levels in plasma. In addition, ADA2 shares homology with adenosine deaminase growth factors (ADGFs) that have been shown to play a role in development (106). ADA1 and 2 are both highly expressed in immune cells and are critical for the development of the immune system. ADA1 is predominantly expressed in lymphocytes, while ADA2 is secreted by activated myeloid cells (107).

The first human disease linked to a defect in ADA1 function was severe combined immunodeficiency. A complete deficiency of ADA1 is fatal early in life, while patients with partial ADA1 deficiency present with milder clinical symptoms, later in childhood. In the absence of ADA1, deoxyadenosine nucleotides accumulate in lymphocytes and are toxic resulting in T-B-NK- SCID (108). Deficiency of adenosine deaminase type 2 (DADA2) is a recessively inherited disease manifesting with fevers, vasculitis, livedo racemosa, early-onset ischemic stroke, liver disease, and mild immunodeficiency (**Figure 5**) (109, 110). The vasculopathy/vasculitis associated with DADA2 predominantly affects small- and medium-sized arteries

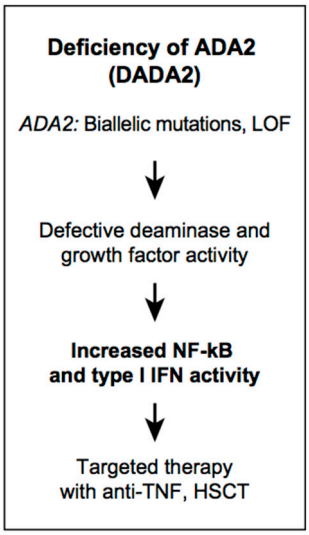

FIGURE 5 | Autoinflammatory diseases due to dysregulation in purine metabolism. DADA2.

with histologic findings consistent with necrotizing ANCA-negative vasculitis or polyarteritis nodosa (109, 111). Livedo racemosa is the most common skin manifestation, and it can be complicated by skin ulcerations, distal arterial occlusion, and sometimes digital necrosis (112). Neurological manifestations result from lacunar ischemic infarcts in the deep-brain nuclei, midbrain, and/or brainstem. Some patients were reported with intracerebral hemorrhage as the first disease manifestation (111, 113). Hematological manifestations were not appreciated initially, however it has become evident that some patients present with hypocellular bone marrow (114–116). Their clinical presentations include aplastic anemia, pure red cell aplasia, lymphopenia, neutropenia, thrombocytopenia, and hypogammaglobilinemia, in particular low IgG and IgM levels. Immunodeficiency is generally milder than in patients with ADA1 deficiency, and resembles common variable immunodeficiency (115). Lymphoproliferative features are also in the spectrum of DADA2 hematopoietic manifestations (117, 118). Autoimmune findings in DADA2 have been found in a small subset of patients, and manifest as autoimmune cytopenias, transiently positive lupus anticoagulant, and systemic lupus. This is consistent with increased type 1 interferon (IFN) gene expression signature in blood samples of patients with DADA2 (119, 120).

To date, there are more than 170 patients diagnosed with DADA2 described from all over the world (121–123). Many DADA2-associated mutations are present at a low frequency in the general population and it is possible that the disease is still underdiagnosed. In addition to standard genetic testing by sequencing, DADA2 can be diagnosed by measuring plasma or serum ADA2 activity. ADA2 activity testing is recommended to confirm the pathogenicity of novel variants identified by sequencing. The molecular mechanisms by which mutations in ADA2 lead to disease are still largely unknown. Proinflammatory cytokines have been identified in skin biopsies and blood samples of DADA2 patients however, the function of ADA2 in differentiation of hematopoietic and endothelial cells has not been comprehensively investigated. Deficiency of ADA2 is associated with monocyte-macrophage polarization toward the M1 subset, and M1 macrophages are known to promote inflammation, although it is not clear if this is directly dependent on adenosine, aminohydrolase catalytic activity on a novel substrate, growth factor function, or another as yet unidentified mechanism.

Primary treatment of DADA2 relies on anti-TNF agents in patients with a mostly inflammatory phenotype, while patients with bone marrow failure may require HSCT (124, 125). Identification of patients with ADA1-SCID and DADA2 has helped identify unexpected roles for adenosine as a key molecule in the regulation of immune physiology.

Interferonopathies

Interferonopathies are a group of systemic inflammatory diseases with continuum of features of autoinflammation and autoimmunity. The underlying mechanism of inflammation in monogenic interferonopathies is a defect in sensing and degradation of nucleic acids leading to constitutive activation of innate immune responses (126, 127). These disorders show a striking resemblance to congenital viral infections although occur in the absence of any identified pathogen.

Two of the best studied diseases are Aicardi-Goutières syndrome (AGS) and stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI). AGS is a heterogenous early-onset disorder manifesting with basal ganglia calcifications, encephalopathy, neurological impairments and other features of autoimmunity. Disease-associated mutations have been identified in genes that either impact nucleic acid degradation (*TREX1, SAMHD1, RNASEH2A, RNASEH2B, RNASEH2C, DNASE2),* or sensing of nucleic acids (*IFIH1*/MDA5) or RNA editing *(ADAR)* and result in activation of the type I interferon pathway. Patients with SAVI have gain-of-function mutations in *TMEM173,* which encodes the STING protein, and they present with prominent skin lesions, small vessel vasculitis, peripheral amputations, and interstitial lung disease. STING is an endoplasmic transmembrane protein that functions as an indirect sensor of endogenous or pathogen-derived cytosolic dsDNA (128). Upon binding to cGAMP, which is generated by the sensing receptor cGAS, STING activates IRF3, a transcription factor for type 1 interferon and related genes. Production of type 1 interferon cytokines results in an amplification loop that can be blocked by JAK inhibitors providing further insights into the mechanism of pathogenesis (129). Type I interferonopathies mechanisms, disease manifestations, and treatment have been reviewed in great detail elsewhere (127, 130).

CONCLUSIONS

The disorders discussed in this review exemplify how unbiased identification of mutated genes in patients with rare

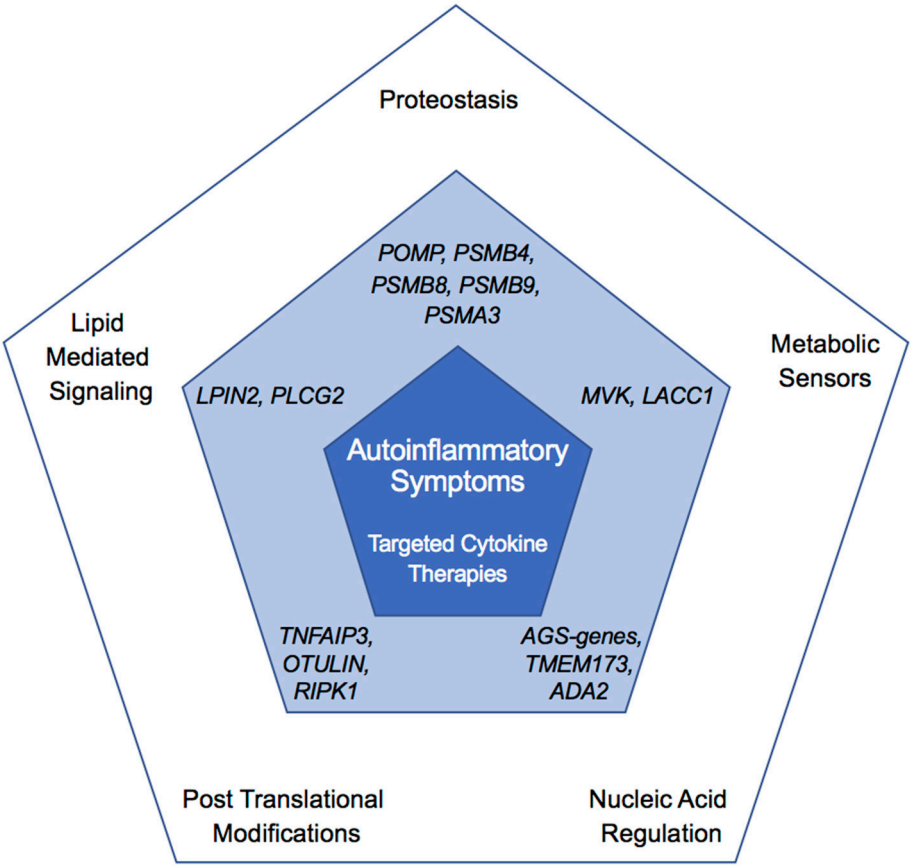

FIGURE 6 | Overlapping and unique features of autoinflammatory diseases. Outer pentagon shows pathways altered in autoinflammatory disease, central pentagon highlights genes in the corresponding node found mutated in monogenic disease (dark blue), and inner pentagon showing shared clinical features and treatment (light blue).

autoinflammatory diseases has uncovered novel mechanisms governing innate immune responses. Collectively, these findings point to previously unrecognized functions for these genes and their associated pathways. These atypical autoinflammatory genes not only help identify unique pathways regulating inflammatory pathways, but also illuminate shared features with other disorders based on treatment response and clinical manifestations (**Figure 6**). It is unclear whether these genes have a specific function in immune system regulation or if the relatively specific immune phenotypes are due to particular sensitivity of the immune system to disruption in basic cell machinery and metabolism. Furthermore, unlike disruption of canonical autoinflammatory pathways, mutations in many of these genes can result in concomitant overactivity of the innate immune system (autoinflammation) and of the adaptive immune system (autoimmunity) and be accompanied with ineffective immune responses (immunodeficiency). This provides evidence for cell-specific function of these enzymes in the regulation of metabolic and immune signaling pathways. Besides targeted anti-cytokine therapies, hematopoietic stem cell transplantation has been attempted in some patients refractory to conventional treatments, and can cure hematological and immunological manifestations. Like with other metabolic disorders, early diagnosis and treatment is critical for preventing disease complications. Unbiased genetic sequencing of patients with early-onset immune dysregulation disorders will continue to identify novel disease-causing genes and further delineate the molecular mechanisms governing inflammation in humans.

WEB LINK

https://infevers.umai-montpellier.fr/web.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. De Jesus AA, Canna SW, Liu Y, Goldbach-Mansky R. Molecular mechanisms in genetically defined autoinflammatory diseases: disorders of amplified danger signaling. *Annu Rev Immunol.* (2015) 33:823–74. doi: 10.1146/annurev-immunol-032414-112227
2. Manthiram K, Zhou Q, Aksentijevich I, Kastner DL. The monogenic autoinflammatory diseases define new pathways in human innate immunity and inflammation. *Nat Immunol.* (2017) 18:832–42. doi: 10.1038/ni.3777
3. Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. *J Clin Immunol.* (2018) 38:129–43. doi: 10.1007/s10875-017-0465-8
4. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, Van Royen-Kerkhoff A, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N Engl J Med.* (2009) 360:2426–37. doi: 10.1056/NEJMoa0807865
5. Marrakchi S, Guigue P, Renshaw BR, Puel A, Pei XY, Fraitag S, et al. Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N Engl J Med.* (2011) 365:620–8. doi: 10.1056/NEJMoa1013068
6. Cowen EW, Goldbach-Mansky R. DIRA, DITRA, and new insights into pathways of skin inflammation: what's in a name? *Arch Dermatol.* (2012) 148:381–4. doi: 10.1001/archdermatol.2011.3014
7. Hoffman HM, Broderick L. The role of the inflammasome in patients with autoinflammatory diseases. *J Allergy Clin Immunol.* (2016) 138:3–14. doi: 10.1016/j.jaci.2016.05.001
8. Masters SL, Lagou V, Jeru I, Baker PJ, Van Eyck L, Parry DA, et al. Familial autoinflammation with neutrophilic dermatosis reveals a regulatory mechanism of pyrin activation. *Sci Transl Med.* (2016) 8:332ra345. doi: 10.1126/scitranslmed.aaf1471
9. Park YH, Wood G, Kastner DL, Chae JJ. Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. *Nat Immunol.* (2016) 17:914–21. doi: 10.1038/ni.3457
10. Aksentijevich I, Zhou Q. NF-kappaB pathway in autoinflammatory diseases: dysregulation of protein modifications by ubiquitin defines a new category of autoinflammatory diseases. *Front Immunol.* (2017) 8:399. doi: 10.3389/fimmu.2017.00399
11. Oda H, Kastner DL. Genomics, biology, and human illness: advances in the monogenic autoinflammatory diseases. *Rheum Dis Clin North Am.* (2017) 43:327–45. doi: 10.1016/j.rdc.2017.04.011
12. Davidson S, Steiner A, Harapas CR, Masters SL. An update on autoinflammatory diseases: interferonopathies. *Curr Rheumatol Rep.* (2018) 20:38. doi: 10.1007/s11926-018-0748-y
13. Harapas CR, Steiner A, Davidson S, Masters SL. An update on autoinflammatory diseases: inflammasomopathies. *Curr Rheumatol Rep.* (2018) 20:40. doi: 10.1007/s11926-018-0750-4
14. Wellner K, Betat H, Morl M. A tRNA's fate is decided at its 3' end: collaborative actions of CCA-adding enzyme and RNases involved in tRNA processing and degradation. *Biochim Biophys Acta Gene Regul Mech.* (2018) 1861:433–41. doi: 10.1016/j.bbagrm.2018.01.012
15. Wilusz JE, Whipple JM, Phizicky EM, Sharp PA. tRNAs marked with CCACCA are targeted for degradation. *Science* (2011) 334:817–21. doi: 10.1126/science.1213671
16. Augustin MA, Reichert AS, Betat H, Huber R, Morl M, Steegborn C. Crystal structure of the human CCA-adding enzyme: insights into template-independent polymerization. *J Mol Biol.* (2003) 328:985–94. doi: 10.1016/S0022-2836(03)00381-4
17. Wiseman DH, May A, Jolles S, Connor P, Powell C, Heeney MM, et al. A novel syndrome of congenital sideroblastic anemia, B-cell immunodeficiency, periodic fevers, and developmental delay (SIFD). *Blood* (2013) 122:112–23. doi: 10.1182/blood-2012-08-439083
18. Chakraborty PK, Schmitz-Abe K, Kennedy EK, Mamady H, Naas T, Durie D, et al. Mutations in TRNT1 cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD). *Blood* (2014) 124:2867–71. doi: 10.1182/blood-2014-08-591370
19. Wedatilake Y, Niazi R, Fassone E, Powell CA, Pearce S, Plagnol V, et al. TRNT1 deficiency: clinical, biochemical and molecular genetic features. *Orphanet J Rare Dis.* (2016) 11:90. doi: 10.1186/s13023-016-0477-0
20. Barton C, Kausar S, Kerr D, Bitetti S, Wynn R. SIFD as a novel cause of severe fetal hydrops and neonatal anaemia with iron loading and marked extramedullary haemopoiesis. *J Clin Pathol.* (2018) 71:275–8. doi: 10.1136/jclinpath-2017-204698
21. Giannelou A, Wang H, Zhou Q, Park YH, Abu-Asab MS, Ylaya K, et al. Aberrant tRNA processing causes an autoinflammatory syndrome responsive to TNF inhibitors. *Ann Rheum Dis.* (2018) 77:612–9. doi: 10.1136/annrheumdis-2017-212401
22. Lougaris V, Chou J, Baronio M, Gazzurelli L, Lorenzini T, Soresina A, et al. Novel biallelic TRNT1 mutations resulting in sideroblastic anemia, combined B and T cell defects, hypogammaglobulinemia, recurrent infections, hypertrophic cardiomyopathy and developmental delay. *Clin Immunol.* (2018) 188:20–2. doi: 10.1016/j.clim.2017.11.008
23. Frans G, Moens L, Schaballie H, Wuyts G, Liston A, Poesen K, et al. Homozygous N-terminal missense mutation in TRNT1 leads to progressive B-cell immunodeficiency in adulthood. *J Allergy Clin Immunol.* (2017) 139:360–3 e366. doi: 10.1016/j.jaci.2016.06.050
24. Deluca AP, Whitmore SS, Barnes J, Sharma TP, Westfall TA, Scott CA, et al. Hypomorphic mutations in TRNT1 cause retinitis pigmentosa with erythrocytic microcytosis. *Hum Mol Genet.* (2016) 25:44–56. doi: 10.1093/hmg/ddv446
25. Hull S, Malik AN, Arno G, Mackay DS, Plagnol V, Michaelides M, et al. Expanding the phenotype of TRNT1-related immunodeficiency to include childhood cataract and inner retinal dysfunction. *JAMA Ophthalmol.* (2016) 134:1049–53. doi: 10.1001/jamaophthalmol.2015.5833
26. Leibovitch M, Hanic-Joyce PJ, Joyce PBM. *In vitro* studies of disease-linked variants of human tRNA nucleotidyltransferase reveal decreased thermal stability and altered catalytic activity. *Biochim Biophys Acta Proteins Proteom.* (2018) 1866:527–40. doi: 10.1016/j.bbapap.2018.02.002
27. Sasarman F, Thiffault I, Weraarpachai W, Salomon S, Maftei C, Gauthier J, et al. The 3' addition of CCA to mitochondrial tRNASer(AGY) is specifically impaired in patients with mutations in the tRNA nucleotidyl transferase TRNT1. *Hum Mol Genet.* (2015) 24:2841–7. doi: 10.1093/hmg/ddv044
28. Torrelo A. CANDLE syndrome as a paradigm of proteasome-related autoinflammation. *Front Immunol.* (2017) 8:927. doi: 10.3389/fimmu.2017.00927
29. Poli MC, Ebstein F, Nicholas SK, De Guzman MM, Forbes LR, Chinn IK, et al. Heterozygous truncating variants in POMP escape nonsense-mediated decay and cause a unique immune dysregulatory syndrome. *Am J Hum Genet.* (2018) 102:1126–42. doi: 10.1016/j.ajhg.2018.04.010
30. Fremond ML, Melki I, Kracker S, Bondet V, Duffy D, Rice GI, et al. Comment on: 'Aberrant tRNA processing causes an autoinflammatory syndrome responsive to TNF inhibitors' by Giannelou et al: mutations in TRNT1 result in a constitutive activation of type I interferon signalling. *Ann Rheum Dis.* (2018). doi: 10.1136/annrheumdis-2018-213745. [Epub ahead of print].
31. Liwak-Muir U, Mamady H, Naas T, Wylie Q, Mcbride S, Lines M, et al. Impaired activity of CCA-adding enzyme TRNT1 impacts OXPHOS complexes and cellular respiration in SIFD patient-derived fibroblasts. *Orphanet J Rare Dis.* (2016) 11:79. doi: 10.1186/s13023-016-0466-3
32. Meyer-Schuman R, Antonellis A. Emerging mechanisms of aminoacyl-tRNA synthetase mutations in recessive and dominant human disease. *Hum Mol Genet.* (2017) 26:R114–27. doi: 10.1093/hmg/ddx231
33. Peterfy M, Phan J, Xu P, Reue K. Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat Genet.* (2001) 27:121–4. doi: 10.1038/83685
34. Reue K. The lipin family: mutations and metabolism. *Curr Opin Lipidol.* (2009) 20:165–70. doi: 10.1097/MOL.0b013e32832adee5
35. Donkor J, Sariahmetoglu M, Dewald J, Brindley DN, Reue K. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J Biol Chem.* (2007) 282:3450–7. doi: 10.1074/jbc.M610745200
36. Carrasco S, Merida I. Diacylglycerol, when simplicity becomes complex. *Trends Biochem Sci.* (2007) 32:27–36. doi: 10.1016/j.tibs.2006.11.004
37. Zhang P, Reue K. Lipin proteins and glycerolipid metabolism: roles at the ER membrane and beyond. *Biochim Biophys Acta Biomembr.* (2017) 1859(9 Pt B):1583–95. doi: 10.1016/j.bbamem.2017.04.007

38. Valdearcos M, Esquinas E, Meana C, Gil-De-Gomez L, Guijas C, Balsinde J, et al. Subcellular localization and role of lipin-1 in human macrophages. *J Immunol.* (2011) 186:6004–13. doi: 10.4049/jimmunol.1003279
39. Meana C, Pena L, Lorden G, Esquinas E, Guijas C, Valdearcos M, et al. Lipin-1 integrates lipid synthesis with proinflammatory responses during TLR activation in macrophages. *J Immunol.* (2014) 193:4614–22. doi: 10.4049/jimmunol.1400238
40. Valdearcos M, Esquinas E, Meana C, Pena L, Gil-De-Gomez L, Balsinde J, et al. Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *J Biol Chem.* (2012) 287:10894–904. doi: 10.1074/jbc.M112.342915
41. Lorden G, Sanjuan-Garcia I, De Pablo N, Meana C, Alvarez-Miguel I, Perez-Garcia MT, et al. Lipin-2 regulates NLRP3 inflammasome by affecting P2X7 receptor activation. *J Exp Med.* (2017) 214:511–28. doi: 10.1084/jem.20161452
42. Ferguson PJ, Chen S, Tayeh MK, Ochoa L, Leal SM, Pelet A, et al. Homozygous mutations in LPIN2 are responsible for the syndrome of chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anaemia (Majeed syndrome). *J Med Genet.* (2005) 42:551–7. doi: 10.1136/jmg.2005.030759
43. Cox AJ, Ferguson PJ. Update on the genetics of nonbacterial osteomyelitis in humans. *Curr Opin Rheumatol.* (2018) 30:521–5. doi: 10.1097/BOR.0000000000000530
44. Zeharia A, Shaag A, Houtkooper RH, Hindi T, De Lonlay P, Erez G, et al. Mutations in LPIN1 cause recurrent acute myoglobinuria in childhood. *Am J Hum Genet.* (2008) 83:489–94. doi: 10.1016/j.ajhg.2008.09.002
45. Donkor J, Zhang P, Wong S, O'loughlin L, Dewald J, Kok BP, et al. A conserved serine residue is required for the phosphatidate phosphatase activity but not the transcriptional coactivator functions of lipin-1 and lipin-2. *J Biol Chem.* (2009) 284:29968–78. doi: 10.1074/jbc.M109.023663
46. Herlin T, Fiirgaard B, Bjerre M, Kerndrup G, Hasle H, Bing X, et al. Efficacy of anti-IL-1 treatment in Majeed syndrome. *Ann Rheum Dis.* (2013) 72:410–3. doi: 10.1136/annrheumdis-2012-201818
47. Koss H, Bunney TD, Behjati S, Katan M. Dysfunction of phospholipase Cgamma in immune disorders and cancer. *Trends Biochem Sci.* (2014) 39:603–11. doi: 10.1016/j.tibs.2014.09.004
48. Gorjestani S, Yu M, Tang B, Zhang D, Wang D, Lin X. Phospholipase Cgamma2 (PLCgamma2) is key component in Dectin-2 signaling pathway, mediating anti-fungal innate immune responses. *J Biol Chem.* (2011) 286:43651–9. doi: 10.1074/jbc.M111.307389
49. Ombrello MJ, Remmers EF, Sun G, Freeman AF, Datta S, Torabi-Parizi P, et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. *N Engl J Med.* (2012) 366:330–8. doi: 10.1056/NEJMoa1102140
50. Zhou Q, Lee GS, Brady J, Datta S, Katan M, Sheikh A, et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cgamma2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. *Am J Hum Genet.* (2012) 91:713–20. doi: 10.1016/j.ajhg.2012.08.006
51. Aderibigbe OM, Priel DL, Lee CC, Ombrello MJ, Prajapati VH, Liang MG, et al. Distinct cutaneous manifestations and cold-induced leukocyte activation associated with PLCG2 mutations. *JAMA Dermatol.* (2015) 151:627–34. doi: 10.1001/jamadermatol.2014.5641
52. Milner JD. PLAID: a syndrome of complex patterns of disease and unique phenotypes. *J Clin Immunol.* (2015) 35:527–30. doi: 10.1007/s10875-015-0177-x
53. Chae JJ, Park YH, Park C, Hwang IY, Hoffmann P, Kehrl JH, et al. Connecting two pathways through Ca 2+ signaling: NLRP3 inflammasome activation induced by a hypermorphic PLCG2 mutation. *Arthritis Rheumatol.* (2015) 67:563–7. doi: 10.1002/art.38961
54. Lisova ZA, Lisov AV, Leontievsky AA. Two laccase isoforms of the basidiomycete Cerrena unicolor VKMF-3196. Induction, isolation and properties. *J Basic Microbiol.* (2010) 50:72–82. doi: 10.1002/jobm.200900382
55. Cader MZ, Boroviak K, Zhang Q, Assadi G, Kempster SL, Sewell GW, et al. C13orf31 (FAMIN) is a central regulator of immunometabolic function. *Nat Immunol.* (2016) 17:1046–56. doi: 10.1038/ni.3532
56. Lahiri A, Hedl M, Yan J, Abraham C. Human LACC1 increases innate receptor-induced responses and a LACC1 disease-risk variant modulates these outcomes. *Nat Commun.* (2017) 8:15614. doi: 10.1038/ncomms15614
57. Assadi G, Vesterlund L, Bonfiglio F, Mazzurana L, Cordeddu L, Schepis D, et al. Functional analyses of the Crohn's disease risk gene LACC1. *PLoS ONE* (2016) 11:e0168276. doi: 10.1371/journal.pone.0168276
58. Jostins L, Ripke S, Weersma RK, Duerr RH, Mcgovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) 491:119–24. doi: 10.1038/nature11582
59. Liu H, Irwanto A, Fu X, Yu G, Yu Y, Sun Y, et al. Discovery of six new susceptibility loci and analysis of pleiotropic effects in leprosy. *Nat Genet.* (2015) 47:267–71. doi: 10.1038/ng.3212
60. Assadi G, Saleh R, Hadizadeh F, Vesterlund L, Bonfiglio F, Halfvarson J, et al. LACC1 polymorphisms in inflammatory bowel disease and juvenile idiopathic arthritis. *Genes Immun.* (2016) 17:261–4. doi: 10.1038/gene.2016.17
61. Wang D, Fan Y, Malhi M, Bi R, Wu Y, Xu M, et al. Missense variants in HIF1A and LACC1 contribute to leprosy risk in Han Chinese. *Am J Hum Genet.* (2018) 102:794–805. doi: 10.1016/j.ajhg.2018.03.006
62. Takeuchi M, Mizuki N, Meguro A, Ombrello MJ, Kirino Y, Satorius C, et al. Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behcet's disease susceptibility. *Nat Genet.* (2017) 49:438–43. doi: 10.1038/ng.3786
63. Wu P, Du L, Hou S, Su G, Yang L, Hu J, et al. Association of LACC1, CEBPB-PTPN1, RIPK2 and ADO-EGR2 with ocular Behcet's disease in a Chinese Han population. *Br J Ophthalmol.* (2018) 102:1308–14. doi: 10.1136/bjophthalmol-2017-311753
64. Patel N, El Mouzan MI, Al-Mayouf SM, Adly N, Mohamed JY, Al Mofarreh MA, et al. Study of Mendelian forms of Crohn's disease in Saudi Arabia reveals novel risk loci and alleles. *Gut* (2014) 63:1831–2. doi: 10.1136/gutjnl-2014-307859
65. Wakil SM, Monies DM, Abouelhoda M, Al-Tassan N, Al-Dusery H, Naim EA, et al. Association of a mutation in LACC1 with a monogenic form of systemic juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2015) 67:288–95. doi: 10.1002/art.38877
66. Kallinich T, Thorwarth A, Von Stuckrad SL, Rosen-Wolff A, Luksch H, Hundsdoerfer P, et al. Juvenile arthritis caused by a novel FAMIN (LACC1) mutation in two children with systemic and extended oligoarticular course. *Pediatr Rheumatol Online J.* (2016) 14:63. doi: 10.1186/s12969-016-0124-2
67. Karacan I, Ugurlu S, Sahin S, Everest E, Kasapcopur O, Tolun A, et al. LACC1 gene defects in familial form of juvenile arthritis. *J Rheumatol.* (2018) 45:726–8. doi: 10.3899/jrheum.170834
68. Szymanski AM, Ombrello MJ. Using genes to triangulate the pathophysiology of granulomatous autoinflammatory disease: NOD2, PLCG2 and LACC1. *Int Immunol.* (2018) 30:205–13. doi: 10.1093/intimm/dxy021
69. Akula MK, Shi M, Jiang Z, Foster CE, Miao D, Li AS, et al. Control of the innate immune response by the mevalonate pathway. *Nat Immunol.* (2016) 17:922–9. doi: 10.1038/ni.3487
70. Van Der Burgh R, Ter Haar NM, Boes ML, Frenkel J. Mevalonate kinase deficiency, a metabolic autoinflammatory disease. *Clin Immunol.* (2013) 147:197–206. doi: 10.1016/j.clim.2012.09.011
71. Stoffels M, Simon A. Hyper-IgD syndrome or mevalonate kinase deficiency. *Curr Opin Rheumatol.* (2011) 23:419–23. doi: 10.1097/BOR.0b013e328349c3b1
72. Ter Haar NM, Jeyaratnam J, Lachmann HJ, Simon A, Brogan PA, Doglio M, et al. The phenotype and genotype of mevalonate kinase deficiency: a series of 114 cases from the eurofever registry. *Arthritis Rheumatol.* (2016) 68:2795–805. doi: 10.1002/art.39763
73. Houten SM, Frenkel J, Rijkers GT, Wanders RJ, Kuis W, Waterham HR. Temperature dependence of mutant mevalonate kinase activity as a pathogenic factor in hyper-IgD and periodic fever syndrome. *Hum Mol Genet.* (2002) 11:3115–24. doi: 10.1093/hmg/11.25.3115
74. Mandey SH, Schneiders MS, Koster J, Waterham HR. Mutational spectrum and genotype-phenotype correlations in mevalonate kinase deficiency. *Hum Mutat.* (2006) 27:796–802. doi: 10.1002/humu.20361
75. Frenkel J, Rijkers GT, Mandey SH, Buurman SW, Houten SM, Wanders RJ, et al. Lack of isoprenoid products raises *ex vivo* interleukin-1beta secretion in hyperimmunoglobulinemia D and periodic fever syndrome. *Arthritis Rheum.* (2002) 46:2794–803. doi: 10.1002/art.10550

76. Galeotti C, Meinzer U, Quartier P, Rossi-Semerano L, Bader-Meunier B, Pillet P, et al. Efficacy of interleukin-1-targeting drugs in mevalonate kinase deficiency. *Rheumatology* (2012) 51:1855–9. doi: 10.1093/rheumatology/kes097
77. De Benedetti F, Gattorno M, Anton J, Ben-Chetrit E, Frenkel J, Hoffman HM, et al. Canakinumab for the treatment of autoinflammatory recurrent fever syndromes. *N Engl J Med.* (2018) 378:1908–19. doi: 10.1056/NEJMoa1706314
78. Steiner A, Harapas CR, Masters SL, Davidson S. An update on autoinflammatory diseases: relopathies. *Curr Rheumatol Rep.* (2018) 20:39. doi: 10.1007/s11926-018-0749-x
79. Wertz IE, O'rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* (2004) 430:694–9. doi: 10.1038/nature02794
80. Das T, Chen Z, Hendriks RW, Kool M. A20/tumor necrosis factor alpha-induced protein 3 in immune cells controls development of autoinflammation and autoimmunity: lessons from mouse models. *Front Immunol.* (2018) 9:104. doi: 10.3389/fimmu.2018.00104
81. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. *Nat Genet.* (2016) 48:67–73. doi: 10.1038/ng.3459
82. Aeschlimann FA, Batu ED, Canna SW, Go E, Gul A, Hoffmann P, et al. A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease. *Ann Rheum Dis.* (2018) 77:728–35. doi: 10.1136/annrheumdis-2017-212403
83. Berteau F, Rouviere B, Nau A, Le Berre R, Sarrabay G, Touitou I, et al. Response to: 'A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease'. *Ann Rheum Dis.* (2018). doi: 10.1136/annrheumdis-2018-213347. [Epub ahead of print].
84. Kadowaki T, Ohnishi H, Kawamoto N, Hori T, Nishimura K, Kobayashi C, et al. Haploinsufficiency of A20 causes autoinflammatory and autoimmune disorders. *J Allergy Clin Immunol.* (2018) 141:1485–8 e1411. doi: 10.1016/j.jaci.2017.10.039
85. Lawless D, Pathak S, Scambler TE, Ouboussad L, Anwar R, Savic S. A case of adult-onset still's disease caused by a novel splicing mutation in TNFAIP3 successfully treated with tocilizumab. *Front Immunol.* (2018) 9:1527. doi: 10.3389/fimmu.2018.01527
86. Zheng C, Huang Y, Ye Z, Wang Y, Tang Z, Lu J, et al. Infantile Onset Intractable Inflammatory Bowel Disease Due to Novel Heterozygous Mutations in TNFAIP3 (A20). *Inflamm Bowel Dis.* (2018) 24:2613–20. doi: 10.1093/ibd/izy165
87. Franco-Jarava C, Wang H, Martin-Nalda A, Alvarez SD, Garcia-Prat M, Bodet D, et al. TNFAIP3 haploinsufficiency is the cause of autoinflammatory manifestations in a patient with a deletion of 13Mb on chromosome 6. *Clin Immunol.* (2018) 191:44–51. doi: 10.1016/j.clim.2018.03.009
88. Viel S, Cheyssac E, Pescarmona R, Besson L, Till M, Viremouneix L, et al. Large deletion in 6q associated to A20 haploinsufficiency and thoracoabdominal heterotaxy. *Ann Rheum Dis.* (2018) 77:1697–8. doi: 10.1136/annrheumdis-2018-213300
89. Sperling S, Grimm CH, Dunkel I, Mebus S, Sperling HP, Ebner A, et al. Identification and functional analysis of CITED2 mutations in patients with congenital heart defects. *Hum Mutat.* (2005) 26:575–82. doi: 10.1002/humu.20262
90. Lopes Floro K, Artap ST, Preis JI, Fatkin D, Chapman G, Furtado MB, et al. Loss of Cited2 causes congenital heart disease by perturbing left-right patterning of the body axis. *Hum Mol Genet.* (2011) 20:1097–110. doi: 10.1093/hmg/ddq554
91. Takagi M, Ogata S, Ueno H, Yoshida K, Yeh T, Hoshino A, et al. Haploinsufficiency of TNFAIP3 (A20) by germline mutation is involved in autoimmune lymphoproliferative syndrome. *J Allergy Clin Immunol.* (2017) 139:1914–22. doi: 10.1016/j.jaci.2016.09.038
92. Duncan CJA, Dinnigan E, Theobald R, Grainger A, Skelton AJ, Hussain R, et al. Early-onset autoimmune disease due to a heterozygous loss-of-function mutation in TNFAIP3 (A20). *Ann Rheum Dis.* (2018) 77:783–6. doi: 10.1136/annrheumdis-2016-210944
93. Rivkin E, Almeida SM, Ceccarelli DF, Juang YC, Maclean TA, Srikumar T, et al. The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. *Nature* (2013) 498:318–24. doi: 10.1038/nature12296
94. Heger K, Wickliffe KE, Ndoja A, Zhang J, Murthy A, Dugger DL, et al. OTULIN limits cell death and inflammation by deubiquitinating LUBAC. *Nature* (2018) 559:120–4. doi: 10.1038/s41586-018-0256-2
95. Shimizu Y, Taraborrelli L, Walczak H. Linear ubiquitination in immunity. *Immunol Rev.* (2015) 266:190–207. doi: 10.1111/imr.12309
96. Damgaard RB, Walker JA, Marco-Casanova P, Morgan NV, Titheradge HL, Elliott PR, et al. The deubiquitinase OTULIN is an essential negative regulator of inflammation and autoimmunity. *Cell* (2016) 166:1215–30 e1220. doi: 10.1016/j.cell.2016.07.019
97. Zhou Q, Yu X, Demirkaya E, Deuitch N, Stone D, Tsai WL, et al. Biallelic hypomorphic mutations in a linear deubiquitinase define otulipenia, an early-onset autoinflammatory disease. *Proc Natl Acad Sci USA.* (2016) 113:10127–32. doi: 10.1073/pnas.1612594113
98. He S, Wang X. RIP kinases as modulators of inflammation and immunity. *Nat Immunol.* (2018) 19:912–22. doi: 10.1038/s41590-018-0188-x
99. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature* (2015) 517:311–20. doi: 10.1038/nature14191
100. Weinlich R, Oberst A, Beere HM, Green DR. Necroptosis in development, inflammation and disease. *Nat Rev Mol Cell Biol.* (2017) 18:127–36. doi: 10.1038/nrm.2016.149
101. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, Wachsmuth L, et al. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. *Nature* (2014) 513:90–4. doi: 10.1038/nature13608
102. Rickard JA, O'donnell JA, Evans JM, Lalaoui N, Poh AR, Rogers T, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell* (2014) 157:1175–88. doi: 10.1016/j.cell.2014.04.019
103. Newton K, Manning G. Necroptosis and inflammation. *Annu Rev Biochem.* (2016) 85:743–63. doi: 10.1146/annurev-biochem-060815-014830
104. Cuchet-Lourenco D, Eletto D, Wu C, Plagnol V, Papapietro O, Curtis J, et al. Biallelic RIPK1 mutations in humans cause severe immunodeficiency, arthritis, and intestinal inflammation. *Science* (2018) 361:810–3. doi: 10.1126/science.aar2641
105. Hershfield MS. New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. *Eur J Immunol.* (2005) 35:25–30. doi: 10.1002/eji.200425738
106. Zavialov AV, Yu X, Spillmann D, Lauvau G, Zavialov AV. Structural basis for the growth factor activity of human adenosine deaminase ADA2. *J Biol Chem.* (2010) 285:12367–77. doi: 10.1074/jbc.M109.083527
107. Meyts I, Aksentijevich I. Deficiency of adenosine deaminase 2 (DADA2): updates on the phenotype, genetics, pathogenesis, and treatment. *J Clin Immunol.* (2018) 38:569–78. doi: 10.1007/s10875-018-0525-8
108. Mccusker C, Upton J, Warrington R. Primary immunodeficiency. *Allergy Asthma Clin Immunol.* (2018) 14:61. doi: 10.1186/s13223-018-0290-5
109. Navon Elkan P, Pierce SB, Segel R, Walsh T, Barash J, Padeh S, et al. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. *N Engl J Med.* (2014) 370:921–31. doi: 10.1056/NEJMoa1307362
110. Zhou Q, Yang D, Ombrello AK, Zavialov AV, Toro C, Zavialov AV, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. *N Engl J Med.* (2014) 370:911–20. doi: 10.1056/NEJMoa1307361
111. Garg N, Kasapcopur O, Foster J II, Barut K, Tekin A, Kizilkilic O, et al. Novel adenosine deaminase 2 mutations in a child with a fatal vasculopathy. *Eur J Pediatr.* (2014) 173:827–30. doi: 10.1007/s00431-014-2320-8
112. Gonzalez Santiago TM, Zavialov A, Saarela J, Seppanen M, Reed AM, Abraham RS, et al. Dermatologic features of ADA2 deficiency in cutaneous polyarteritis nodosa. *JAMA Dermatol.* (2015) 151:1230–4. doi: 10.1001/jamadermatol.2015.1635
113. Lee PY, Huang Y, Zhou Q, Schnappauf O, Hershfield MS, Li Y, et al. Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2. *J Allergy Clin Immunol.* (2018) 142:1363–5.e8. doi: 10.1016/j.jaci.2018.05.038

114. Ben-Ami T, Revel-Vilk S, Brooks R, Shaag A, Hershfield MS, Kelly SJ, et al. Extending the clinical phenotype of adenosine deaminase 2 deficiency. *J Pediatr.* (2016) 177:316–20. doi: 10.1016/j.jpeds.2016.06.058
115. Schepp J, Proietti M, Frede N, Buchta M, Hubscher K, Rojas Restrepo J, et al. Screening of 181 patients with antibody deficiency for deficiency of adenosine deaminase 2 sheds new light on the disease in adulthood. *Arthritis Rheumatol.* (2017) 69:1689–700. doi: 10.1002/art.40147
116. Cipe FE, Aydogmus C, Serwas NK, Keskindemirci G, Boztug K. Novel mutation in CECR1 leads to deficiency of ADA2 with associated neutropenia. *J Clin Immunol.* (2018) 38:273–7. doi: 10.1007/s10875-018-0487-x
117. Alsultan A, Basher E, Alqanatish J, Mohammed R, Alfadhel M. Deficiency of ADA2 mimicking autoimmune lymphoproliferative syndrome in the absence of livedo reticularis and vasculitis. *Pediatr Blood Cancer* (2018) 65:e26912. doi: 10.1002/pbc.26912
118. Trotta L, Martelius T, Siitonen T, Hautala T, Hamalainen S, Juntti H, et al. ADA2 deficiency: clonal lymphoproliferation in a subset of patients. *J Allergy Clin Immunol.* (2018) 141:1534–7 e1538. doi: 10.1016/j.jaci.2018.01.012
119. Uettwiller F, Sarrabay G, Rodero MP, Rice GI, Lagrue E, Marot Y, et al. ADA2 deficiency: case report of a new phenotype and novel mutation in two sisters. *RMD Open* (2016) 2:e000236. doi: 10.1136/rmdopen-2015-000236
120. Skrabl-Baumgartner A, Plecko B, Schmidt WM, Konig N, Hershfield M, Gruber-Sedlmayr U, et al. Autoimmune phenotype with type I interferon signature in two brothers with ADA2 deficiency carrying a novel CECR1 mutation. *Pediatr Rheumatol Online J.* (2017) 15:67. doi: 10.1186/s12969-017-0193-x
121. Van Montfrans JM, Hartman EA, Braun KP, Hennekam EA, Hak EA, Nederkoorn PJ, et al. Phenotypic variability in patients with ADA2 deficiency due to identical homozygous R169Q mutations. *Rheumatology* (2016) 55:902–10. doi: 10.1093/rheumatology/kev439
122. Caorsi R, Penco F, Grossi A, Insalaco A, Omenetti A, Alessio M, et al. ADA2 deficiency (DADA2) as an unrecognised cause of early onset polyarteritis nodosa and stroke: a multicentre national study. *Ann Rheum Dis.* (2017) 76:1648–56. doi: 10.1136/annrheumdis-2016-210802
123. Rama M, Duflos C, Melki I, Bessis D, Bonhomme A, Martin H, et al. A decision tree for the genetic diagnosis of deficiency of adenosine deaminase 2 (DADA2): a French reference centres experience. *Eur J Hum Genet.* (2018) 26:960–71. doi: 10.1038/s41431-018-0130-6
124. Van Eyck L, Liston A, Meyts I. Mutant ADA2 in vasculopathies. *N Engl J Med.* (2014) 371:478–9. doi: 10.1056/NEJMc1405506
125. Hashem H, Kumar AR, Muller I, Babor F, Bredius R, Dalal J, et al. Hematopoietic stem cell transplantation rescues the hematological, immunological, and vascular phenotype in DADA2. *Blood* (2017) 130:2682–8. doi: 10.1182/blood-2017-07-798660
126. Crow YJ, Manel N. Aicardi-Goutieres syndrome and the type I interferonopathies. *Nat Rev Immunol.* (2015) 15:429–40. doi: 10.1038/nri3850
127. Rodero MP, Crow YJ. Type I interferon-mediated monogenic autoinflammation: the type I interferonopathies, a conceptual overview. *J Exp Med.* (2016) 213:2527–38. doi: 10.1084/jem.20161596
128. Liu Y, Jesus AA, Marrero B, Yang D, Ramsey SE, Sanchez GAM, et al. Activated STING in a vascular and pulmonary syndrome. *N Engl J Med.* (2014) 371:507–18. doi: 10.1056/NEJMoa1312625
129. Sanchez GAM, Reinhardt A, Ramsey S, Wittkowski H, Hashkes PJ, Berkun Y, et al. JAK1/2 inhibition with baricitinib in the treatment of autoinflammatory interferonopathies. *J Clin Invest*. (2018) 128:3041–52. doi: 10.1172/JCI98814
130. Martinez-Quiles N, Goldbach-Mansky R. Updates on autoinflammatory diseases. *Curr Opin Immunol.* (2018) 55:97–105. doi: 10.1016/j.coi.2018.09.014

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REVIEW
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The Immunology of Macrophage Activation Syndrome

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Synonymous with secondary hemophagocytic lymphohistiocytosis, macrophage activation syndrome (MAS) is a term used by rheumatologists to describe a potentially life-threatening complication of systemic inflammatory disorders, most commonly systemic juvenile idiopathic arthritis (sJIA) and systemic lupus erythematosus (SLE). Clinical and laboratory features of MAS include sustained fever, hyperferritinemia, pancytopenia, fibrinolytic coagulopathy, and liver dysfunction. Soluble interleukin-2 receptor alpha chain (sCD25) and sCD163 may be elevated, and histopathology often reveals characteristic increased hemophagocytic activity in the bone marrow (and other tissues), with positive CD163 (histiocyte) staining. A common hypothesis as to the pathophysiology of many cases of MAS proposes a defect in lymphocyte cytolytic activity. Specific heterozygous gene mutations in familial HLH-associated cytolytic pathway genes (e.g., *PRF1*, *UNC13D*) have been linked to a substantial subset of MAS patients. In addition, the pro-inflammatory cytokine environment, particularly IL-6, has been shown to decrease NK cell cytolytic function. The inability of NK cells and cytolytic CD8 T cells to lyse infected and otherwise activated antigen presenting cells results in prolonged cell-to-cell (innate and adaptive immune cells) interactions and amplification of a pro-inflammatory cytokine cascade. The cytokine storm results in activation of macrophages, causing hemophagocytosis, as well as contributing to multi-organ dysfunction. In addition to macrophages, dendritic cells likely play a critical role in antigen presentation to cytolytic lymphocytes, as well as contributing to cytokine expression. Several cytokines, including tumor necrosis factor, interferon-gamma, and numerous interleukins (i.e., IL-1, IL-6, IL-18, IL-33), have been implicated in the cytokine cascade. In addition to broadly immunosuppressive therapies, novel cytokine targeted treatments are being explored to dampen the overly active immune response that is responsible for much of the pathology seen in MAS.

Keywords: macrophage activation syndrome, hemophagocytic lymphohistiocytosis, cytokine storm, IL-1, IL-6, IL-18, NK cell, anakinra

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INTRODUCTION

Synonymous with secondary hemophagocytic lymphohistiocytosis (HLH), macrophage activation syndrome (MAS) is a term used by rheumatologists to describe a potentially life-threatening complication of systemic inflammatory disorders, most commonly systemic juvenile idiopathic arthritis (sJIA) and its adult equivalent, adult onset Still disease. This syndrome was first reported in

juvenile rheumatoid arthritis (JRA) patients [now termed juvenile idiopathic arthritis (JIA)] with enlarged Kupffer cells (i.e., stellate macrophages in the liver) who concomitantly suffered from strikingly low counts of white blood cells and unusually low erythrocyte sedimentation rates (ESR) (1). Subsequent literature described the presence of activated macrophages and hemophagocytic histiocytes in patients with rheumatic disease, termed reactive hemophagocytic syndrome and now known as MAS (2–4).

A majority of clinical data available involves MAS as a complication of sJIA. The prevalence of fulminant MAS in patients with sJIA is reported to be about 10%; however, subclinical MAS may be present in as many as 30% of children with known or suspected sJIA (5–8). As MAS becomes more clinically recognized, an increasing frequency of occurrence in other systemic inflammatory disorders [i.e., systemic lupus erythematosus (SLE), Kawasaki disease, and periodic fever syndromes] has been reported (9–11). While MAS is known to complicate a variety of inflammatory conditions, including but not limited to malignancy, infection (i.e., Epstein-Barr virus), and primary immunodeficiencies, it is most commonly reported as a well-recognized complication of sJIA, and therefore, much of the understanding of the genetics, pathology, and subsequently immunology is derived from this specific cohort (12).

Early recognition of MAS remains diagnostically challenging as there is no diagnostic test or even a set of disease uniform diagnostic criteria to differentiate MAS from the underlying systemic inflammatory condition. Clinical and laboratory features of MAS include sustained fever, hyperferritinemia, pancytopenia, fibrinolytic consumptive coagulopathy, and liver dysfunction. In 2016, an expert consensus panel published a set of validated diagnostic criteria to help distinguish a sJIA flare from MAS. The final MAS criteria for children with sJIA proved to be both sensitive (0.73) and specific (0.99). The diagnosis of MAS can be made in a febrile patient with sJIA, or suspected sJIA, who has a serum ferritin level $>$ 684 ng/ml plus any 2 of the following: platelet count $\leq$ 181 $\times$ 109/l, aspartate aminotransferase $>$ 48 units/l, triglyceride concentration $>$ 156 mg/dl, or fibrinogen $\leq$ 360 mg/dl (5, 6). These relatively few total criteria are routinely readily available and timely. To date, these criteria have yet to prove diagnostic in other autoimmune diseases and remain limited to children with known or suspected sJIA, with the possible exception of adult onset Still disease (13).

The clinical similarity of MAS and secondary HLH has led some clinicians to use the longer-standing HLH-2004 diagnostic guidelines, which require five of the following eight criteria to be met for diagnosis: fever, splenomegaly, cytopenias (affecting $\geq$ 2 of 3: hemoglobin $<$ 90 g/l, platelets $<$ 100 $\times$ 109/l, neutrophils $<$ 1.0 $\times$ 109/l), hypertriglyceridemia ($\geq$265 mg/dl) and/or hypofibrinogenemia ($\leq$1.5 g/l), hemophagocytosis in bone marrow or spleen or lymph nodes, low or absent natural killer (NK) cell activity, ferritin $\geq$ 500 μg/l, and sCD25 $\geq$ 2,400 units/ml (14). Using this strict set of criteria may delay diagnosis in patients with a less severe initial presentation.

Hemophagocytosis is defined as the engulfment of blood cells, including red blood cells (RBC), white blood cells, or platelets by phagocytic cells (**Figure 1**). Hemophagocytosis by macrophages has been widely associated with the development of MAS in patients with sJIA and other rheumatologic diseases (15–17). Histopathology often reveals characteristic increased hemophagocytic activity in the bone marrow, liver, and spleen with positive CD163 (histiocyte) staining, although hemophagocytosis may not be present in initial stages and is neither sensitive nor specific for MAS (18–20). Detection of hemophagocytosis using serum laboratory tests includes soluble interleukin 2 receptor alpha chain (sCD25) and soluble CD163 (sCD163), a high affinity scavenger receptor for hemoglobin-haptoglobin complexes (**Figure 1**), both of which may be elevated, thereby suggesting sCD25 and sCD163 to be more sensitive in detection of MAS. These tests are only performed at select sites, making them costly with a long turnaround time for results thus leading to a delay in diagnosis and ultimately treatment (18). If inadequately treated, MAS can result in multi-organ failure and death. In the absence of universal diagnostic criteria or a gold-standard laboratory test, understanding the immune mechanisms of MAS may lead to more prompt recognition and target-specific therapies.

CYTOLYTIC CELL DYSFUNCTION IN MAS

MAS shares many etiologic similarities with familial HLH (fHLH), also referred to as primary HLH, not the least of which is the increased prevalence of heterozygous mutations in known fHLH genes that are now being increasingly recognized in MAS patients. fHLH is a severe form of cytokine storm syndrome occurring in infancy, typically within the first few days to months of life. fHLH is a result of homozygous, or compound heterozygous, mutations in genes involved in the perforin-mediated pathway of cytolysis shared by NK cells (innate immunity) and cytotoxic CD8 T cells (adaptive immunity) (21).

The first gene recognized to contribute to fHLH was *PRF1* which gives rise to perforin (22). Homozygous defects in *PRF1* were identified in several families with fHLH (23). Normally, perforin is packaged into cytolytic granules and upon NK cell or CD8 T cell activation is trafficked along the actin cytoskeleton to the immunologic synapse between the cytolytic lymphocyte and the antigen presenting cell (APC) or target cell (24). A variety of fHLH genes are involved in trafficking and docking of the cytolytic granules, including *LYST, RAB27A, UNC13D, STXBP2, STX11*, and others, to the cell membrane (**Table 1**). The polarized granules then allow release of perforin into the synapse to form a pore between the lytic cell and the target cell. Granzyme B, which is co-packaged with perforin, is then delivered to the target cell, resulting in apoptotic cell death. Homozygous disruption of any of the critical genes involved in this process of perforin-mediated cytolysis (**Table 1**) results in fHLH, occurring in about 1 in 50,000 live births and often associated with an infectious trigger. The inability to lyse the infected APC results in a prolonged interaction between the cytolytic lymphocyte and the APC yielding a pro-inflammatory cytokine storm believed to be responsible for the clinical features

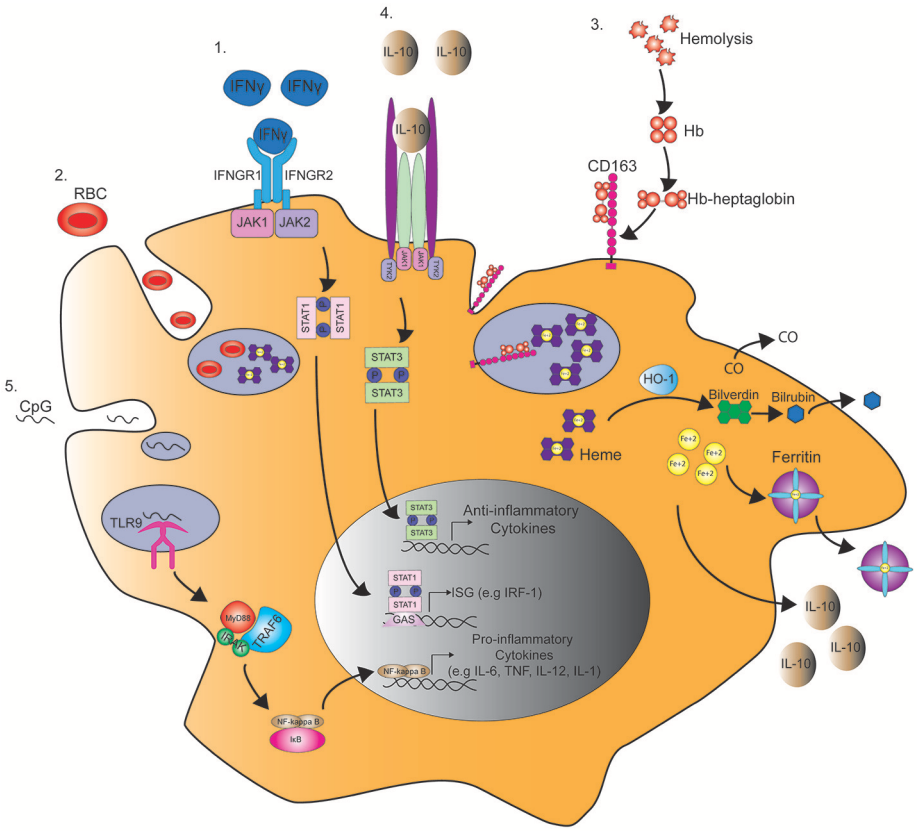

FIGURE 1 | Pathways regulating macrophage function in MAS. 1. IFNγ binds the IFNγ receptor (IFNGR) and subsequently induces the phosphorylation of STAT1 by JAK1/2 in the cytoplasm. STAT1 dimer then binds to γ-interferon activation site (GAS) and enhances the transcription of interferon-stimulated genes (ISG), such as interferon regulatory factor 1 (IRF1). 2. STAT1 activation by IFNγ also induces macropinocytosis leading to the engulfment and degradation of red blood cells (RBC) in a process known as hemophagocytosis. 3. Hemophagocytosis is also mediated by the uptake of hemoglobin (Hb)-heptaglobin complex by CD163. The Hb-heptaglobin complex is degraded in the lysosome followed by catalysis of heme by heme oxygenase-1 (HO-1) to carbon dioxide (CO), bilverdin, and iron (Fe^{2+}). Bilverdin is then converted to bilirubin by bilverdin reductase, and iron is bound to ferritin. 4. This process also leads to the production of IL-10 that through binding to IL-10 receptor induces STAT3 phosphorylation and the production of anti-inflammatory cytokines that counteract IFNγ signaling. 5. In a mouse model of MAS, serial injections of CpG induce the activation of toll-like receptor 9 (TLR9) in the macrophage endosome leading to the production of pro-inflammatory cytokines in a MyD88 and NFκB dependent manner.

of fHLH (31). Human fHLH has been modeled in *PRF1* deficient mice infected with LCMV, and both CD8 T cells and interferon-gamma (IFNγ), a cytokine known to be the main driver of anemia in models of fHLH and fulminant MAS (32, 33), were found to be critically important mediators of mouse mortality (34). IFNγ and its downstream JAK pathways are both considered as possible targets for therapy in man (**Table 2**). IL-33, a member of the IL-1 family of cytokines, may also play a role in T cell hyperactivation during HLH (**Table 2**) (42).

MAS or secondary HLH is much more common than fHLH and occurs in children and adults (44). Interestingly, heterozygous mutations in fHLH genes may be found in upwards of 40% of individuals with secondary HLH and MAS (45, 46). Some of these mutations are hypomorphic in nature, even those identified in genetic regulatory regions (47, 48), and others have dominant-negative effects (49, 50). Like in fHLH, these heterozygous gene mutations alter cytolytic function in NK cells, and presumably CD8 T cells as well.

TABLE 1 | Cytolytic pathway genes associated with HLH and MAS.

Gene	Protein	Function
PRF1	Perforin	Pore formation (23)
UNC13D	Munc13-4	Vesicle priming (25)
STX11	Syntaxin 11	Vesicle docking (26)
STXBP2	Munc18-2	Vesicle membrane fusing (27)
LYST	Lysosomal trafficking regulator	Vesicle sorting (28)
RAB27A	Rab27a	Vesicle fusing (29)
AP3B1	AP-3	Vesicle trafficking (30)

A combination of a chronic inflammatory state, such as in sJIA or SLE, with a genetic predisposition, and/or a triggering infection may result in fatal MAS or sHLH. Examples of this include identification of heterozygous fHLH gene mutations in patients with fatal influenza (H1N1) infections and associated hemophagocytosis (51), and increased percentages of *PRF1* and

TABLE 2 | MAS therapies directed at cytokine blockade and disruption of cell–cell interactions.

Reported cytokine target	Therapeutic mechanism	Example
IL-1	IL-1 receptor antagonist	Anakinra, canakinumab (35)
IL-6	Anti-IL-6R monoclonal Ab	Tocilizumab (36, 37)
IL-18	IL-18 binding protein	Not commercially available (38)
CD28	CTLA4-Ig	Abatacept (39)
JAK1/2	JAK inhibitor	Tofacitinib (40)
Theoretical cytokine target	**Proposed mechanism**	**Example**
IL-10	Recombinant IL-10 protein	None available (41)
IL-33	Anti-IL-33R monoclonal Ab	None available (42)
IFNγ	Anti-IFNγ monoclonal Ab	None available (34, 43)

TNF, tumor necrosis factor; Ab, antibody; IL, interleukin; R, receptor; CTLA, cytotoxic T-lymphocyte-associated protein 4; Ig, immunoglobulin; JAK, Janus kinase; IFNγ, interferon-gamma.

UNC13D heterozygous mutations in cohorts of sJIA patients who develop MAS (52, 53). This has led investigators to propose a threshold model of MAS, in which combinations of genetic predisposition, an underlying inflammatory state, and triggering infectious agents, results in a clinically relevant cytokine storm syndrome (54). Thus, genetic defects in cytolytic lymphocytes of the innate (NK cells) and adaptive (CD8 T cells) immune system can contribute to MAS. Moreover, there are other mechanisms by which MAS can be triggered by genetic mutations that directly affect cells (e.g., macrophages and dendritic cells) of the innate immune system through altering cytokine production via the inflammasome complex (55).

MACROPHAGES IN MAS

As the name implies, macrophage activation is a definitive characteristic of MAS (**Figure 1**). The role of macrophages in MAS has been largely established through their mediation of hemophagocytosis and hypercytokinemia. However, their potential role in dampening an overly exuberant immune response has also been suggested (56).

Hemophagocytosis

Despite the reported increase in hemophagocytic macrophages in the bone marrow and liver of sJIA and MAS patients, there are conflicting reports on the role of hemophagocytic macrophages in disease pathology induction. Several studies have shown that hemophagocytic macrophages induce pathogenesis. The cause of red blood cell (RBC) destruction in hemophagocytic syndromes is largely attributed to activated macrophages. In a model of autoimmune hemolytic anemia, treatment with liposomal chlodronate increased RBC counts by blocking the ability of macrophages to phagocytose RBC (57). Interestingly, hemophagocytosis was induced in macrophages treated with IFNγ (58). In addition, hemophagocytosis did not develop in two HLH patients with IFNγ receptor deficiency (59). Hemophagocytic macrophages were also found to produce the pro-inflammatory cytokine tumor necrosis factor (TNF) in the liver biopsy of MAS patients (60). Since both IFNγ and TNF are key cytokines for the polarization of classically activated or pro-inflammatory M1 macrophages (61, 62), these findings suggest that hemophagocytic macrophages in MAS could have an M1 phenotype.

The identification of hemophagocytic macrophages in bone-marrow aspirates and liver biopsies of MAS patients largely relies on histochemical analysis of CD163 staining. CD163 is an exclusive marker of cells of the monocyte/macrophage lineage. It is often expressed in activated macrophages but is not restricted to hemophagocytic macrophages (63). As previously mentioned, CD163 is a hemoglobin scavenger receptor that mediates the endocytosis of haptoglobin-hemoglobin complexes (64). Avcin et al. reported the increased frequency of $CD163^+$ hemophagocytic macrophages in three MAS patients who developed SLE, sJIA, and Kawasaki disease (65), suggesting that CD163 could be a diagnostic marker in MAS. In contrast, Behrens et al. demonstrated that CD163 expression was increased in the bone-marrow aspirates of 15 sJIA patients, of which two patients were diagnosed clinically with overt MAS, thereby suggesting that this increase is not exclusive to MAS patients. Interestingly, activated or hemophagocytic $CD163^+$ macrophages within the bone-marrow aspirates preceded the development of full-blown MAS, thus supporting the hypothesis that occult MAS could precede clinical MAS in sJIA patients (8). These findings further suggest that MAS and sJIA disease flare may be two ends of the same spectrum with MAS at the most extreme (66).

Since CD163 expression is increased during active sJIA, the ability of activated macrophages to shed this protein (67, 68) led to further speculations on the use of soluble CD163 (sCD163) as a diagnostic marker of macrophage activation. Several studies have reported that sCD163 is increased in the serum of sJIA patients and correlates with an increase in sCD25 and ferritin and with low platelet counts at disease peak (18, 69). Sakumura et al. reported increased levels of serum sCD163 in patients diagnosed with confirmed sJIA and MAS compared to patients with acute sJIA in the absence of MAS, suggesting a correlation between sCD163 levels and clinical MAS (70). Serum sCD163 shows promise as a diagnostic biomarker for MAS, although additional studies are needed to determine clinical significance.

In contrast to these findings, other studies have suggested that hemophagocytic macrophages have an M2 phenotype. Infusion of the M2-driving cytokine IL-4 with a micro-pump induced hemophagocytosis by macrophages. Surprisingly, hemophagocytosis was not inhibited by IFNγ blockade, and macrophages in IL-4 infused mice expressed arginase-1, a classical marker of M2 macrophages (71). Similarly, other reports have shown that $CD163^+$ macrophages have anti-inflammatory M2 properties. The anti-inflammatory cytokine IL-10 was found to upregulate the expression of CD163 expression on macrophages (72). In addition, $CD163^+$ macrophages are thought to play a protective role during inflammation due to their ability to clear free-hemoglobin (Hgb). Free Hgb binds to haptoglobin, which is then engulfed by macrophages through CD163-mediated endocytosis. This subsequently leads

to the production of the anti-inflammatory agents, interleukin-10 (IL-10) and heme oxygenase (HO-1), by macrophages (73, 74) (**Figure 1**). HO-1 may also have anti-inflammatory effects by mediating the catabolism of heme to carbon monoxide (CO) and free iron (Fe^{2+}) (**Figure 1**). Interestingly, in *in vitro* studies macrophages exposed to CO prior to lipopolysaccharide (LPS) stimulation have enhanced production of IL-10 and inhibited production of TNF (75) (**Figure 1**).

Similarly, ferritin is also considered to be cytoprotective through its ability to sequester free Fe^{2+}, therefore decreasing endothelial apoptosis mediated by increased oxidative stress (76) (**Figure 1**). Moreover, studies in animal models of MAS favor the anti-inflammatory role of IL-10, since blockade of IL-10 in mice treated with serial injections of CpG worsen disease and induce symptoms of fulminant MAS (33, 41). Overall, these findings suggest that the increased numbers of $CD163^+$ hemophagocytic macrophages and ferritin in MAS may be a compensatory mechanism rather than a cause of disease pathology in MAS.

In summary, the macrophage phenotype resides along a spectrum, due in part to the plasticity of macrophages. There are constant functional changes that occur in macrophages in response to changing stimuli during the progression of inflammation (77). In this line, the pro-inflammatory M1 and anti-inflammatory M2 phenotypes are considered to be two extremes of a continuous spectrum of various phenotypes that are finely tuned in response to external stimuli (78, 79). The degree of macrophage activation in MAS may therefore be reflective of the heterogeneity of macrophages within the inflammatory environment. Hemophagocytosis occurs in later stages of MAS and is only found in about 60% of HLH and MAS patient biopsies (80). This suggests that as the disease progresses, macrophages may switch from a pro-inflammatory to an anti-inflammatory phenotype, thereby balancing the extremely hyperactive inflammatory environment in patients with fulminant disease. Further investigations are needed to determine the role of hemophagocytic macrophages in the setting of MAS.

Hypercytokinemia

The acute phase of MAS is often associated with markedly elevated levels of pro-inflammatory cytokines. This cytokine storm triggers a cascade of inflammatory pathways that, if untreated, leads to tissue damage and death (81). The working hypothesis suggests macrophages/monocytes produce a cocktail of cytokines, notably TNF and various interleukins (i.e., IL-6, IL-1β, and IL-18), which triggers a cascade of inflammatory pathways and ultimately creating a cytokine storm (**Figure 1**). TNF is a pro-inflammatory cytokine that drives macrophage polarization toward the M1-end of the spectrum. This cytokine has been described as being an anti-M2 factor due to its ability to inhibit STAT6-dependent M2 gene expression in tumor models (62, 82), therefore, favoring macrophage polarization of the M1 phenotype. Macrophages are also thought to be the main source of TNF in MAS.

In situ expression of TNF by hemophagocytic macrophages was reported in the liver of MAS patients (60). Elevated levels of TNF have been found in patients with other rheumatic diseases [i.e., rheumatoid arthritis (RA)], making it a prime target for treatment. Anti-TNF biologics are a class of medications that target TNF directly as monoclonal antibodies or the TNF receptor to block the cytokine cascade and successfully modify disease activity in a milieu of rheumatic diseases (e.g., RA, JIA, uveitis) (83, 84). While successful treatment of MAS with etanercept, a TNF receptor antagonist, has been reported (85) (**Table 2**) other studies have shown that it may trigger or worsen disease progression (86, 87). Thus, the role of TNF and its blockade in MAS remains unclear.

Like TNF, IL-6 producing macrophages have been found in the liver of MAS patients (60). Increased levels of IL-6 have also been reported in the serum of sJIA and in sepsis patients (88–90). Despite the association of IL-6 levels and MAS, the role of IL-6 in the pathogenesis of disease is not well-understood. It remains unknown whether macrophages are the main cellular sources of IL-6 in MAS patients. A recent study by Norelli et al. demonstrated that human monocytes are the primary producers of IL-1β and IL-6 in cytokine release syndrome and that ablation of monocytes could be protective (91) (**Table 2**). In contrast, IL-6 in combination with GM-CSF drives the differentiation of suppressive monocytic myeloid-derived suppressor cells (M-MDSC) in bone marrow (92).

Tocilizumab is a monoclonal antibody targeting the IL-6 receptor and is approved for use in RA, giant cell arteritis, polyarticular JIA, and sJIA (93). Despite its success in treating acute sJIA, patients with sJIA treated with tocilizumab remain at risk for MAS, arguing that IL-6 blockade alone is insufficient to control the inflammatory cascade (36, 94, 95). These patients tended to be afebrile and had lower cell counts and ferritin levels with higher liver enzymes (94, 96). The mechanism of IL-6 in the pathogenesis of MAS remains controversial. IL-6 likely contributes to the cytokine storm, but its role in clinical disease manifestations of MAS is limited, thus making it a questionable target for therapy.

As members of the IL-1 family of cytokines, IL-1β and IL-18 are potent inducers of IL-6 production in monocytes and macrophages (97, 98). Levels of IL-1β and IL-18 are markedly increased in patients with active sJIA and MAS (99–103). Anakinra is a recombinant IL-1 receptor antagonist used off-label in patients with sJIA and less commonly in patients with MAS, either in combination with sJIA or secondary other etiology (35, 104, 105). Efficacy data in the treatment of MAS with anakinra is limited to case reports and series, but many patients achieve disease remission with normalization of lab abnormalities and fever despite prior poor response to more traditional therapies (**Table 2**) (105, 106).

Canakinumab is a monoclonal antibody that specifically targets the IL-1β cytokine and a common treatment target in patients with sJIA. Patients with sJIA treated with canakinumab also remain at risk for MAS, suggesting that IL-1β is not the sole contributor to the pathogenesis of MAS (96). In comparison, IL-1α also signals via the IL-1 receptor (107). By blocking the receptor with anakinra, both IL-1α and IL-1β signals are dampened. While the importance of IL-1β in sJIA is widely accepted, IL-1α may be more important in stimulating the

cytokine cascade in patients with MAS. Further research is needed to determine the efficacy of IL-1 blockade in treating MAS in non-sJIA patients.

Like many cytokines, the source(s) of IL-1 during MAS is unclear. Gene expression analysis of immune cells and murine tissues suggest that neutrophils may be better producers of IL-1β than monocytes, while an IL-1 family member, IL-18, may be largely produced by epithelial cells (108). Free IL-18 was shown to be highly elevated in the serum of MAS patients compared to patients with sJIA flare without MAS or familial HLH. In agreement with these findings, blockade of IL-18 receptor reduced inflammation in a murine model of MAS induced by repeated CpG injections (109). In addition, IL-18 inhibition with recombinant human IL-18 binding protein (IL-18BP) in combination with anakinra successfully improved life-threatening hyperinflammation in a patient with a dominant heterozygous mutation in NLRC4 (**Table 2**) (38). NLRC4 triggers the inflammasome, an innate immune complex that responds via caspase-1 activation and IL-1β and IL-18 secretion. Gain of function mutations, as seen in Familial Mediterranean Fever (FMF), result in hyperactivation of the NLRC4 inflammasone which can in turn result in MAS (102, 110). Adjunct therapy with mTOR inhibition (i.e., rapamycin) was reported in an infant with MAS refractory to anakinra and corticosteroids found to have an NLRC4 mutation (111).

Elevated free IL-18 may aid in the diagnosis of MAS, and as such, IL-18 blockade may be an effective cytokine-directed therapy in some forms of MAS. Of note, IL-18BP is not commercially available in the United States but has been used compassionately (38).

Hypercytokinemia correlates with a worse prognosis and is considered by many to be the main driver of disease pathology and subsequently the morbidity and mortality associated with MAS (112). Since macrophage activation appears to trigger the cytokine cascade in MAS (8), a solid understanding of the immunology and pathogenesis is critical to target-specific therapy. Known inducers of macrophage activation include toll-like receptor (TLR) ligands and cytokines (62, 77, 113) (**Figure 1**). The type of TLR stimuli and cytokines in the inflammatory milieu define the genetic programs, either pro- or anti-inflammatory adopted by macrophages in response to inflammatory stimuli (79).

Emerging studies in sJIA patients and in animal models of cytokine storm syndromes suggest TLR stimulation regulates cytokine activity via monocyte response. Gene expression analysis of Peripheral blood mononuclear cells (PBMC) from sJIA patients revealed an increased TLR/IL-1R signature and TLR2 expression (99, 114). Ablation of the TLR/IL-1R adaptor molecule Myd88 (115–117) reversed disease pathophysiology in models of fHLH (42, 118). Unlike fHLH, which typically presents in infancy due to one of many autosomal recessive gene mutations, MAS occurs across all ages and may present in the absence of a known pathogen or trigger. Two murine models were developed to better understand the role of TLR stimulation in MAS. Murine models show that repeated stimulation of TLR9 with CpG results in clinical MAS (41). In this model, monocytes were the main cells responsive to TLR9 stimulation which induced production of IL-12 (33). Further, IL-10 proved to be protective since blockade of IL-10R lead to fulminant MAS (**Table 2**) (33, 41). In a second model of MAS, TLR4 stimulation with LPS was shown to induce clinical symptoms consistent with MAS in IL-6 transgenic mice (119). These findings shed light on the combinatorial effect of TLR ligands and cytokines in the induction of pathogenesis in MAS.

DENDRITIC CELLS IN MAS

The role of dendritic cells (DC) in disease pathogenesis is largely mediated by the ability of these cells to present antigen to T cells (120). Most of our knowledge of the role of DC in MAS originates from studies in murine models of fHLH. Similar to patients with fHLH, impaired NK cell degranulation resulting from mutations in *PRF1, UNC13D, STXBP2*, and *RAB27A* has been reported in patients with MAS (45, 48, 121–123).

The current view on the contribution of DC to disease pathogenesis HLH, arises from studies in perforin-deficient ($Prf^{-/-}$) mice. Symptoms of fHLH can be reproduced by the infection of $Prf^{-/-}$ mice with LCMV, resulting in a fatal hyperinflammatory response characterized by hyperproliferation of IFNγ-producing $CD8^+$ T cells, which are central to disease pathogenesis (32, 34). Since T cell proliferation requires antigen presentation by DC, investigative studies focus on the mechanisms by which perforin regulates DC function.

Hermans et al. demonstrated that cytolytic T lymphocytes (CTL) regulate DC function by eliminating antigen-loaded DC and preventing their access to the lymph nodes, therefore acting as gate-keepers (124). Yang et al. later showed that this elimination was dependent on perforin, since in $Prf^{-/-}$ mice activated CTL failed to eliminate antigen-loaded DC (125). This suggests that in LCMV-infected $Prf^{-/-}$ mice, the extensive proliferation of CD8 T cells can result from continuous activation by antigen-presenting DC that cannot be eliminated by defective CTL. This hypothesis was further supported by Terrell et al. who showed that the antigen-presenting capacity of DC is increased in LCMV-infected $Prf^{-/-}$ mice, along with the numbers of DC containing viral antigen. Additionally, transfer of $Prf^{+/+}$ CD8 T cells to LCMV-infected $Prf^{-/-}$ mice reduced IFNγ production by CD8 T cells suggesting that CTL limit T cell activation likely by eliminating virus-infected DC (126). Furthermore, Lykens et al. demonstrated that the increased activation of cytotoxic CD8 T cells was not due to an intrinsic defect of activation threshold, but rather an enhanced presentation of antigen by DC (127). In line with these findings, persistence of antigen was found to be correlated with disease pathogenesis. In IFNγ knockout BALB/c mice infected with MCM virus, the severity of HLH-like symptoms was reduced in mice administrated with the antiviral cidofovir, further supporting the notion that antigen persistence drives constant antigen-presentation by DC (128). Collectively, these studies strongly propose that DC mediate disease pathogenesis in hosts with cytotoxic dysfunction. In cases where there is an infectious trigger, such as a viral infection, cytotoxic CTL fail to clear virus-infected DC. This leads to constant DC activation and antigen-presentation of viral antigens

to T cells (31), which in turn respond by hyperproliferation and production of pro-inflammatory cytokines responsible for multi-organ failure seen in MAS.

CONCLUSION

MAS is a potentially fatal inflammatory condition that can lead to multiorgan failure if inadequately treated. In the absence of validated diagnostic criteria, recognition is often delayed. A firm understanding of the pathogenesis of MAS can guide diagnosis and direct therapy toward target-specific treatment. A common hypothesis as to the pathophysiology of MAS proposes a defect in lymphocyte cytolytic activity. Normally, cytolytic cells induce cell apoptosis in abnormal cells. In the setting of an infection or inflammatory state, cytolytic cells may induce apoptosis in activated macrophages and T cells and serve to control the inflammatory response. A defect in cytolytic function may result in overstimulation of the immune system leading to the multi-organ failure seen in MAS.

The pro-inflammatory cytokine environment, particularly IL-6, has been shown to decrease NK cell cytolytic function. The inability of NK cells and cytolytic CD8 T cells to lyse infected and otherwise activated antigen presenting cells (APCs) results in prolonged cell-to-cell interactions and amplification of a pro-inflammatory cytokine cascade. The cytokine storm results in activation of macrophages, causing hemophagocytosis, as well as contributing to multi-organ dysfunction (**Figure 1**). Several cytokines, including TNF, IFNγ, and numerous interleukins (i.e., IL-1, IL-6, IL-18), have been implicated in the cytokine cascade. Specific heterozygous gene mutations in fHLH-associated cytolytic pathway genes (e.g., *PRF1*, *UNC13D*) have been linked to a substantial subset of MAS patients. These mutations cause defects in various proteins responsible for the production and transport of granules leading to apoptosis of target cells.

Historically, treatment of MAS focuses on controlling the underlying trigger, such as infection or sJIA treatment. However, not all cases present with a known pathogen or with a known etiology, making treatment of the underlying trigger impossible. It is important to understand the mechanism behind the uncontrolled cytokine storm seen in MAS to target specific cytokines upstream and prevent further stimulation of the activated macrophages. In addition to broadly immunosuppressive medications, such as corticosteroids and cyclosporine, cytokine specific therapy (e.g., IL-1 pathway blockade) may prove more effective in dampening the overly active immune system. Further studies and clinical trials are needed to better assess the role of pro-inflammatory cytokines in the pathogenesis of MAS and determine their clinical relevance.

AUTHOR CONTRIBUTIONS

CC: writing of the clinical and therapeutic sections, introduction and conclusion, organization of manuscript, primary editor; SA: writing of the basic science immunology and design of the figure; KN: writing of the basic science immunology, supervisor; RC: writing of the genetics sections, primary supervisor.

REFERENCES

1. Hadchouel M, Prieur AM, Griscelli C. Acute hemorrhagic, hepatic, and neurologic manifestations in juvenile rheumatoid arthritis: possible relationship to drugs or infection. *J Pediatr.* (1985) 106:561–6. doi: 10.1016/S0022-3476(85)80072-X
2. Stephan JL, Kone-Paut I, Galambrun C, Mouy R, Bader-Meunier B, Prieur AM. Reactive haemophagocytic syndrome in children with inflammatory disorders. A retrospective study of 24 patients. *Rheumatology (Oxford)* (2001) 40:1285–92. doi: 10.1093/rheumatology/40.11.1285
3. Athreya BH. Is macrophage activation syndrome a new entity? *Clin Exp Rheumatol.* (2002) 20:121–3.
4. Mouy R, Stephan JL, Pillet P, Haddad E, Hubert P, Prieur AM. Efficacy of cyclosporine A in the treatment of macrophage activation syndrome in juvenile arthritis: report of five cases. *J Pediatr.* (1996) 129:750–4. doi: 10.1016/S0022-3476(96)70160-9
5. Ravelli A, Minoia F, Davi S, Horne A, Bovis F, Pistorio A, et al. 2016 Classification criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. *Ann Rheum Dis.* (2016) 75:481–9. doi: 10.1136/annrheumdis-2015-208982
6. Ravelli A, Minoia F, Davi S, Horne A, Bovis F, Pistorio A, et al. 2016 Classification criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. *Arthritis Rheumatol.* (2016) 68:566–76. doi: 10.1002/art.39332
7. Minoia F, Davi S, Horne A, Bovis F, Demirkaya E, Akikusa J, et al. Dissecting the heterogeneity of macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. *J Rheumatol.* (2015) 42:994–1001. doi: 10.3899/jrheum.141261
8. Behrens EM, Beukelman T, Paessler M, Cron RQ. Occult macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis. *J Rheumatol.* (2007) 34:1133–8.
9. Garcia-Pavon S, Yamazaki-Nakashimada MA, Baez M, Borjas-Aguilar KL, Murata C. Kawasaki disease complicated with macrophage activation syndrome: a systematic review. *J Pediatr Hematol Oncol.* (2017) 39:445–51. doi: 10.1097/MPH.0000000000000872
10. Borgia RE, Gerstein M, Levy DM, Silverman ED, Hiraki LT. Features, treatment, and outcomes of macrophage activation syndrome in childhood-onset systemic lupus erythematosus. *Arthritis Rheumatol.* (2018) 70:616–24. doi: 10.1002/art.40417
11. Rigante D, Emmi G, Fastiggi M, Silvestri E, Cantarini L. Macrophage activation syndrome in the course of monogenic autoinflammatory disorders. *Clin Rheumatol.* (2015) 34:1333–9. doi: 10.1007/s10067-015-2923-0
12. Sepulveda FE, de Saint Basile G. Hemophagocytic syndrome: primary forms and predisposing conditions. *Curr Opin Immunol.* (2017) 49:20–6. doi: 10.1016/j.coi.2017.08.004
13. Tada Y, Inokuchi S, Maruyama A, Suematsu R, Sakai M, Sadanaga Y, et al. Are the 2016 EULAR/ACR/PRINTO classification criteria for macrophage activation syndrome applicable to patients with adult-onset Still's disease? *Rheumatol Int.* (2019) 39:97–104. doi: 10.1007/s00296-018-4114-1
14. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* (2007) 48:124–31. doi: 10.1002/pbc.21039
15. Ravelli A, De Benedetti F, Viola S, Martini A. Macrophage activation syndrome in systemic juvenile rheumatoid arthritis

successfully treated with cyclosporine. *J Pediatr.* (1996) 128:275–8. doi: 10.1016/S0022-3476(96)70408-0
16. Ravelli A, Magni-Manzoni S, Pistorio A, Besana C, Foti T, Ruperto N, et al. Preliminary diagnostic guidelines for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. *J Pediatr.* (2005) 146:598–604. doi: 10.1016/j.jpeds.2004.12.016
17. Alkoht A, Hanafi I, Khalil B. Macrophage activation syndrome: a report of two cases and a literature review. *Case Rep Rheumatol.* (2017) 2017:5304180. doi: 10.1155/2017/5304180
18. Bleesing J, Prada A, Siegel DM, Villanueva J, Olson J, Ilowite NT, et al. The diagnostic significance of soluble CD163 and soluble interleukin-2 receptor alpha-chain in macrophage activation syndrome and untreated new-onset systemic juvenile idiopathic arthritis. *Arthritis Rheum.* (2007) 56:965–71. doi: 10.1002/art.22416
19. Goel S, Polski JM, Imran H. Sensitivity and specificity of bone marrow hemophagocytosis in hemophagocytic lymphohistiocytosis. *Ann Clin Lab Sci.* (2012) 42:21–5.
20. Ho C, Yao X, Tian L, Li FY, Podoltsev N, Xu ML. Marrow assessment for hemophagocytic lymphohistiocytosis demonstrates poor correlation with disease probability. *Am J Clin Pathol.* (2014) 141:62–71. doi: 10.1309/AJCPMD5TJEFOOVBW
21. Filipovich AH, Chandrakasan S. Pathogenesis of hemophagocytic lymphohistiocytosis. *Hematol Oncol Clin North Am.* (2015) 29:895–902. doi: 10.1016/j.hoc.2015.06.007
22. Behrens EM, Cron RQ. Kill or be killed. *J Immunol.* (2015) 194:5041–3. doi: 10.4049/jimmunol.1500774
23. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* (1999) 286:1957–9. doi: 10.1126/science.286.5446.1957
24. Orange JS. The lytic NK cell immunological synapse and sequential steps in its formation. *Adv Exp Med Biol.* (2007) 601:225–33. doi: 10.1007/978-0-387-72005-0_23
25. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* (2003) 115:461–73. doi: 10.1016/S0092-8674(03)00855-9
26. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, et al. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet.* (2005) 14:827–34. doi: 10.1093/hmg/ddi076
27. Cote M, Menager MM, Burgess A, Mahlaoui N, Picard C, Schaffner C, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest.* (2009) 119:3765–73. doi: 10.1172/JCI40732
28. Rubin CM, Burke BA, McKenna RW, McClain KL, White JG, Nesbit ME, Jr., et al. The accelerated phase of Chediak-Higashi syndrome. An expression of the virus-associated hemophagocytic syndrome? *Cancer* (1985) 56:524–30.
29. Menasche G, Pastural E, Feldmann J, Certain S, Ersoy F, Dupuis S, et al. Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. *Nat Genet.* (2000) 25:173–6. doi: 10.1038/76024
30. Enders A, Zieger B, Schwarz K, Yoshimi A, Speckmann C, Knoepfle EM, et al. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. *Blood* (2006) 108:81–7. doi: 10.1182/blood-2005-11-4413
31. Jenkins MR, Rudd-Schmidt JA, Lopez JA, Ramsbottom KM, Mannering SI, Andrews DM, et al. Failed CTL/NK cell killing and cytokine hypersecretion are directly linked through prolonged synapse time. *J Exp Med.* (2015) 212:307–17. doi: 10.1084/jem.20140964
32. Binder D, van den Broek MF, Kägi D, Bluethmann H, Fehr J, Hengartner H, et al. Aplastic anemia rescued by exhaustion of cytokine-secreting $CD8^+$ T cells in persistent infection with lymphocytic choriomeningitis virus. *J Exp Med.* (1998) 187:1903–20.
33. Canna SW, Wrobel J, Chu N, Kreiger PA, Paessler M, Behrens EM. Interferon-γ mediates anemia but is dispensable for Fulminant Toll-Like receptor 9-induced macrophage activation syndrome and hemophagocytosis. *Arthritis Rheum.* (2013) 65:1764–75. doi: 10.1002/art.37958
34. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): $CD8^+$ T cells and interferon gamma are essential for the disorder. *Blood* (2004) 104:735–43. doi: 10.1182/blood-2003-10-3413
35. Miettunen PM, Narendran A, Jayanthan A, Behrens EM, Cron RQ. Successful treatment of severe paediatric rheumatic disease-associated macrophage activation syndrome with interleukin-1 inhibition following conventional immunosuppressive therapy: case series with 12 patients. *Rheumatology (Oxford)* (2011) 50:417–9. doi: 10.1093/rheumatology/keq218
36. De Benedetti F, Brunner HI, Ruperto N, Kenwright A, Wright S, Calvo I, et al. Randomized trial of tocilizumab in systemic juvenile idiopathic arthritis. *N Engl J Med.* (2012) 367:2385–95. doi: 10.1056/NEJMoa1112802
37. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med.* (2014) 371:1507–17. doi: 10.1056/NEJMoa1407222
38. Canna SW, Girard C, Malle L, de Jesus A, Romberg N, Kelsen J, et al. Life-threatening NLRC4-associated hyperinflammation successfully treated with IL-18 inhibition. *J Allergy Clin Immunol.* (2017) 139:1698–701. doi: 10.1016/j.jaci.2016.10.022
39. Record JL, Beukelman T, Cron RQ. Combination therapy of abatacept and anakinra in children with refractory systemic juvenile idiopathic arthritis: a retrospective case series. *J Rheumatol.* (2011) 38:180–1. doi: 10.3899/jrheum.100726
40. Das R, Guan P, Sprague L, Verbist K, Tedrick P, An QA, et al. Janus kinase inhibition lessens inflammation and ameliorates disease in murine models of hemophagocytic lymphohistiocytosis. *Blood* (2016) 127:1666–75. doi: 10.1182/blood-2015-12-684399
41. Behrens EM, Canna SW, Slade K, Rao S, Kreiger PA, Paessler M, et al. Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice. *J Clin Invest.* (2011) 121:2264–77. doi: 10.1172/JCI43157
42. Rood JE, Rao S, Paessler M, Kreiger PA, Chu N, Stelekati E, et al. ST2 contributes to T-cell hyperactivation and fatal hemophagocytic lymphohistiocytosis in mice. *Blood* (2016) 127:426–35. doi: 10.1182/blood-2015-07-659813
43. Prencipe G, Caiello I, Pascarella A, Grom AA, Bracaglia C, Chatel L, et al. Neutralization of IFN-gamma reverts clinical and laboratory features in a mouse model of macrophage activation syndrome. *J Allergy Clin Immunol.* (2018) 141:1439–49. doi: 10.1016/j.jaci.2017.07.021
44. Crayne CB, Cron RQ. Weathering a macrophage storm. *J Rheumatol.* (2017) 44:970–2. doi: 10.3899/jrheum.170370
45. Zhang M, Behrens EM, Atkinson TP, Shakoory B, Grom AA, Cron RQ. Genetic defects in cytolysis in macrophage activation syndrome. *Curr Rheumatol Rep.* (2014) 16:439. doi: 10.1007/s11926-014-0439-2
46. Kaufman KM, Linghu B, Szustakowski JD, Husami A, Yang F, Zhang K, et al. Whole-exome sequencing reveals overlap between macrophage activation syndrome in systemic juvenile idiopathic arthritis and familial hemophagocytic lymphohistiocytosis. *Arthritis Rheumatol.* (2014) 66:3486–95. doi: 10.1002/art.38793
47. Cichocki F, Schlums H, Li H, Stache V, Holmes T, Lenvik TR, et al. Transcriptional regulation of Munc13-4 expression in cytotoxic lymphocytes is disrupted by an intronic mutation associated with a primary immunodeficiency. *J Exp Med.* (2014) 211:1079–91. doi: 10.1084/jem.20131131
48. Schulert GS, Zhang M, Husami A, Fall N, Brunner H, Zhang K, et al. Brief report: novel UNC13D intronic variant disrupting an NF-kappaB enhancer in a patient with recurrent macrophage activation syndrome and systemic juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2018) 70:963–70. doi: 10.1002/art.40438
49. Spessott WA, Sanmillan ML, McCormick ME, Patel N, Villanueva J, Zhang K, et al. Hemophagocytic lymphohistiocytosis caused by dominant-negative mutations in STXBP2 that inhibit SNARE-mediated membrane fusion. *Blood* (2015) 125:1566–77. doi: 10.1182/blood-2014-11-610816
50. Zhang M, Bracaglia C, Prencipe G, Bemrich-Stolz CJ, Beukelman T, Dimmitt RA, et al. A heterozygous RAB27A mutation associated with delayed cytolytic granule polarization and hemophagocytic lymphohistiocytosis. *J Immunol.* (2016) 196:2492–503. doi: 10.4049/jimmunol.1501284

51. Schulert GS, Zhang M, Fall N, Husami A, Kissell D, Hanosh A, et al. Whole-exome sequencing reveals mutations in genes linked to hemophagocytic lymphohistiocytosis and macrophage activation syndrome in fatal cases of H1N1 influenza. *J Infect Dis.* (2016) 213:1180–8. doi: 10.1093/infdis/jiv550
52. Vastert SJ, van Wijk R, D'Urbano LE, de Vooght KM, de Jager W, Ravelli A, et al. Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis. *Rheumatology (Oxford)* (2010) 49:441–9. doi: 10.1093/rheumatology/kep418
53. Zhang K, Biroschak J, Glass DN, Thompson SD, Finkel T, Passo MH, et al. Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis is associated with MUNC13-4 polymorphisms. *Arthritis Rheum.* (2008) 58:2892–6. doi: 10.1002/art.23734
54. Strippoli R, Caiello I, De Benedetti F. Reaching the threshold: a multilayer pathogenesis of macrophage activation syndrome. *J Rheumatol.* (2013) 40:761–7. doi: 10.3899/jrheum.121233
55. Schulert GS, Canna SW. Convergent pathways of the hyperferritinemic syndromes. *Int Immunol.* (2018) 30:195–203. doi: 10.1093/intimm/dxy012
56. Behrens EM. Macrophage activation syndrome in rheumatic disease: what is the role of the antigen presenting cell? *Autoimmun Rev.* (2008) 7:305–8. doi: 10.1016/j.autrev.2007.11.025
57. Jordan MB, van Rooijen N, Izui S, Kappler J, Marrack P. Liposomal clodronate as a novel agent for treating autoimmune hemolytic anemia in a mouse model. *Blood* (2003) 101:594–601. doi: 10.1182/blood-2001-11-0061
58. Zoller EE, Lykens JE, Terrell CE, Aliberti J, Filipovich AH, Henson PM, et al. Hemophagocytosis causes a consumptive anemia of inflammation. *J Exp Med.* (2011) 208:1203–14. doi: 10.1084/jem.20102538
59. Tesi B, Sieni E, Neves C, Romano F, Cetica V, Cordeiro AI, et al. Hemophagocytic lymphohistiocytosis in 2 patients with underlying IFN-gamma receptor deficiency. *J Allergy Clin Immunol.* (2015) 135:1638–41. doi: 10.1016/j.jaci.2014.11.030
60. Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-gamma-producing lymphocytes and IL-6- and TNF-alpha-producing macrophages. *Blood* (2005) 105:1648–51. doi: 10.1182/blood-2004-08-2997
61. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med.* (1983) 158:670–89. doi: 10.1084/jem.158.3.670
62. Murray PJ. Macrophage polarization. *Annu Rev Physiol.* (2017) 79:541–66. doi: 10.1146/annurev-physiol-022516-034339
63. Lau SK, Chu PG, Weiss LM. CD163: a specific marker of macrophages in paraffin-embedded tissue samples. *Am J Clin Pathol.* (2004) 122:794–801. doi: 10.1309/QHD6YFN81KQXUUH6
64. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, et al. Identification of the haemoglobin scavenger receptor. *Nature* (2001) 409:198–201. doi: 10.1038/35051594
65. Avcin T, Tse SM, Schneider R, Ngan B, Silverman ED. Macrophage activation syndrome as the presenting manifestation of rheumatic diseases in childhood. *J Pediatr.* (2006) 148:683–6. doi: 10.1016/j.jpeds.2005.12.070
66. Ramanan AV, Grom AA. Does systemic-onset juvenile idiopathic arthritis belong under juvenile idiopathic arthritis? *Rheumatology (Oxford)* (2005) 44:1350–3. doi: 10.1093/rheumatology/keh710
67. Moller HJ, Peterslund NA, Graversen JH, Moestrup SK. Identification of the hemoglobin scavenger receptor/CD163 as a natural soluble protein in plasma. *Blood* (2002) 99:378–80. doi: 10.1182/blood.V99.1.378
68. Droste A, Sorg C, Hogger P. Shedding of CD163, a novel regulatory mechanism for a member of the scavenger receptor cysteine-rich family. *Biochem Biophys Res Commun.* (1999) 256:110–3. doi: 10.1006/bbrc.1999.0294
69. Schaer DJ, Schleiffenbaum B, Kurrer M, Imhof A, Bachli E, Fehr J, et al. Soluble hemoglobin-haptoglobin scavenger receptor CD163 as a lineage-specific marker in the reactive hemophagocytic syndrome. *Eur J Haematol.* (2005) 74:6–10. doi: 10.1111/j.1600-0609.2004.00318.x
70. Sakumura N, Shimizu M, Mizuta M, Inoue N, Nakagishi Y, Yachie A. Soluble CD163, a unique biomarker to evaluate the disease activity, exhibits macrophage activation in systemic juvenile idiopathic arthritis. *Cytokine* (2018) 110:459–65. doi: 10.1016/j.cyto.2018.05.017
71. Milner JD, Orekov T, Ward JM, Cheng L, Torres-Velez F, Junttila I, et al. Sustained IL-4 exposure leads to a novel pathway for hemophagocytosis, inflammation, and tissue macrophage accumulation. *Blood* (2010) 116:2476–83. doi: 10.1182/blood-2009-11-255174
72. Do T, Tan R, Bennett M, Medvedovic M, Grom AA, Shen N, et al. MicroRNA networks associated with active systemic juvenile idiopathic arthritis regulate CD163 expression and anti-inflammatory functions in macrophages through two distinct mechanisms. *J Leuk Biol.* (2018) 103:71–85. doi: 10.1002/JLB.2A0317-107R
73. Schaer CA, Schoedon G, Imhof A, Kurrer MO, Schaer DJ. Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ Res.* (2006) 99:943–50. doi: 10.1161/01.RES.0000247067.34173.1b
74. Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol.* (2003) 24:449–55. doi: 10.1016/S1471-4906(03)00181-9
75. Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, et al. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci USA.* (2001) 98:8798–803. doi: 10.1073/pnas.161272598
76. Vercellotti GM, Khan FB, Nguyen J, Chen C, Bruzzone CM, Bechtel H, et al. H-ferritin ferroxidase induces cytoprotective pathways and inhibits microvascular stasis in transgenic sickle mice. *Front Pharmacol.* (2014) 5:79. doi: 10.3389/fphar.2014.00079
77. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leuk Biol.* (2004) 76:509–13. doi: 10.1189/jlb.0504272
78. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* (2014) 41:14–20. doi: 10.1016/j.immuni.2014.06.008
79. Xue J, Schmidt S, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* (2014) 40:274–88. doi: 10.1016/j.immuni.2014.01.006
80. Cron RQ, Davi S, Minoia F, Ravelli A. Clinical features and correct diagnosis of macrophage activation syndrome. *Expert Rev Clin Immunol.* (2015) 11:1043–53. doi: 10.1586/1744666X.2015.1058159
81. Canna SW, Behrens EM. Making Sense of the cytokine storm: a conceptual framework for understanding, diagnosing and treating hemophagocytic syndromes. *Pediatr Clin North Am.* (2012) 59:329–44. doi: 10.1016/j.pcl.2012.03.002
82. Kratochvill F, Neale G, Haverkamp JM, Van de Velde LA, Smith AM, Kawauchi D, et al. TNF Counterbalances the emergence of M2 tumor macrophages. *Cell Rep.* (2015) 12:1902–14. doi: 10.1016/j.celrep.2015.08.033
83. Harris JG, Kessler EA, Verbsky JW. Update on the treatment of juvenile idiopathic arthritis. *Curr Allergy Asthma Rep.* (2013) 13:337–46. doi: 10.1007/s11882-013-0351-2
84. Stoll ML, Cron RQ. Treatment of juvenile idiopathic arthritis: a revolution in care. *Pediatr Rheumatol Online J.* (2014) 12:13. doi: 10.1186/1546-0096-12-13
85. Flammiger A, Fiedler W, Bacher U, Bokemeyer C, Schneider M, Binder M. Critical imbalance of TNF-alpha and soluble TNF receptor 1 in a patient with macrophage activation syndrome: potential implications for diagnostics and treatment. *Acta Haematol.* (2012) 128:69–72. doi: 10.1159/000338179
86. Sterba G, Sterba Y, Stempel C, Blank J, Azor E, Gomez L. Macrophage activation syndrome induced by etanercept in a patient with systemic sclerosis. *Israel Med Assoc J.* (2010) 12:443–5.
87. Buonuomo PS, Campana A, Insalaco A, Bracaglia C, Pardeo M, Cortis E. Necrotizing fasciitis in a pediatric patient treated with etanercept and cyclosporine for macrophage activation syndrome. *Rheumatol Int.* (2013) 33:1097–8. doi: 10.1007/s00296-011-2319-7
88. de Benedetti F, Massa M, Robbioni P, Ravelli A, Burgio GR, Martini A. Correlation of serum interleukin-6 levels with joint involvement and thrombocytosis in systemic juvenile rheumatoid arthritis. *Arthritis Rheum.* (1991) 34:1158–63. doi: 10.1002/art.1780340912
89. Lasiglie D, Traggiai E, Federici S, Alessio M, Buoncompagni A, Accogli A, et al. Role of IL-1 beta in the development of human T(H)17

cells: lesson from NLPR3 mutated patients. *PLoS ONE* (2011) 6:e20014. doi: 10.1371/journal.pone.0020014
90. Xu XJ, Tang YM, Song H, Yang SL, Xu WQ, Zhao N, et al. Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children. *J Pediatr.* (2012) 160:984–90.e1. doi: 10.1016/j.jpeds.2011.11.046
91. Norelli M, Camisa B, Barbiera G, Falcone L, Purevdorj A, Genua M, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med.* (2018) 24:739–48. doi: 10.1038/s41591-018-0036-4
92. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity* (2010) 32:790–802. doi: 10.1016/j.immuni.2010.05.010
93. Sterba Y, Ilowite N. Biologics in pediatric rheumatology: Quo Vadis? *Curr Rheumatol Rep.* (2016) 18:45. doi: 10.1007/s11926-016-0593-9
94. Grom AA, Horne A, De Benedetti F. Macrophage activation syndrome in the era of biologic therapy. *Nat Rev Rheumatol.* (2016) 12:259–68. doi: 10.1038/nrrheum.2015.179
95. Yokota S, Itoh Y, Morio T, Sumitomo N, Daimaru K, Minota S. Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis under treatment with tocilizumab. *J Rheumatol.* (2015) 42:712–22. doi: 10.3899/jrheum.140288
96. Schulert GS, Minoia F, Bohnsack J, Cron RQ, Hashad S, Kon EPI, et al. Effect of biologic therapy on clinical and laboratory features of macrophage activation syndrome associated with systemic juvenile idiopathic arthritis. *Arthritis Care Res (Hoboken)* (2018) 70:409–19. doi: 10.1002/acr.23277
97. Musso T, Espinoza-Delgado I, Pulkki K, Gusella GL, Longo DL, Varesio L. Transforming growth factor beta downregulates interleukin-1 (IL-1)-induced IL-6 production by human monocytes. *Blood* (1990) 76:2466–9.
98. Netea MG, Kullberg BJ, Verschueren I, Van Der Meer JW. Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1beta. *Eur J Immunol.* (2000) 30:3057–60. doi: 10.1002/1521-4141(200010)30:10<3057::AID-IMMU3057>3.0.CO;2-P
99. Fall N, Barnes M, Thornton S, Luyrink L, Olson J, Ilowite NT, et al. Gene expression profiling of peripheral blood from patients with untreated new-onset systemic juvenile idiopathic arthritis reveals molecular heterogeneity that may predict macrophage activation syndrome. *Arthritis Rheum.* (2007) 56:3793–804. doi: 10.1002/art.22981
100. Ling XB, Park JL, Carroll T, Nguyen KD, Lau K, Macaubas C, et al. Plasma profiles in active systemic juvenile idiopathic arthritis: biomarkers and biological implications. *Proteomics* (2010) 10:4415–30. doi: 10.1002/pmic.201000298
101. Shimizu M, Yokoyama T, Yamada K, Kaneda H, Wada H, Wada T, et al. Distinct cytokine profiles of systemic-onset juvenile idiopathic arthritis-associated macrophage activation syndrome with particular emphasis on the role of interleukin-18 in its pathogenesis. *Rheumatology (Oxford)* (2010) 49:1645–53. doi: 10.1093/rheumatology/keq133
102. Kaplanski G. Interleukin-18: Biological properties and role in disease pathogenesis. *Immunol Rev.* (2018) 281:138–53. doi: 10.1111/imr.12616
103. Shimizu M, Inoue N, Mizuta M, Nakagishi Y, Yachie A. Characteristic elevation of soluble TNF receptor II: I ratio in macrophage activation syndrome with systemic juvenile idiopathic arthritis. *Clin Exp Immunol.* (2018) 191:349–55. doi: 10.1111/cei.13026
104. Nigrovic PA, Mannion M, Prince FH, Zeft A, Rabinovich CE, van Rossum MA, et al. Anakinra as first-line disease-modifying therapy in systemic juvenile idiopathic arthritis: report of forty-six patients from an international multicenter series. *Arthritis Rheum.* (2011) 63:545–55. doi: 10.1002/art.30128
105. Sonmez HE, Demir S, Bilginer Y, Ozen S. Anakinra treatment in macrophage activation syndrome: a single center experience and systemic review of literature. *Clin Rheumatol.* (2018) 37:3329–35. doi: 10.1007/s10067-018-4095-1
106. Rajasekaran S, Kruse K, Kovey K, Davis AT, Hassan NE, Ndika AN, et al. Therapeutic role of anakinra, an interleukin-1 receptor antagonist, in the management of secondary hemophagocytic lymphohistiocytosis/sepsis/multiple organ dysfunction/macrophage activating syndrome in critically ill children. *Pediatr Crit Care Med.* (2014) 15:401–8. doi: 10.1097/PCC.0000000000000078
107. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev.* (2018) 281:8–27. doi: 10.1111/imr.12621
108. Weiss ES, Girard-Guyonvarc'h C, Holzinger D, Jesus AAd, Tariq Z, Picarsic J, et al. Interleukin-18 diagnostically distinguishes and pathogenically promotes human and murine macrophage activation syndrome. *Blood* (2018) 131:1442–55. doi: 10.1182/blood-2017-12-820852
109. Girard-Guyonvarc'h C, Palomo J, Martin P, Rodriguez E, Troccaz S, Palmer G, et al. Unopposed IL-18 signaling leads to severe TLR9-induced macrophage activation syndrome in mice. *Blood* (2018) 131:1430–41. doi: 10.1182/blood-2017-06-789552
110. Duncan JA, Canna SW. The NLRC4 inflammasome. *Immunol Rev.* (2018) 281:115–23. doi: 10.1111/imr.12607
111. Barsalou J, Blincoe A, Fernandez I, Dal-Soglio D, Marchitto L, Selleri S, et al. Rapamycin as an adjunctive therapy for NLRC4 associated macrophage activation syndrome. *Front Immunol.* (2018) 9:2162. doi: 10.3389/fimmu.2018.02162
112. Weaver LK, Behrens EM. Hyperinflammation, rather than hemophagocytosis, is the common link between macrophage activation syndrome and hemophagocytic lymphohistiocytosis. *Curr Opin Rheumatol.* (2014) 26:562–9. doi: 10.1097/BOR.0000000000000093
113. Gordon S, Martinez-Pomares L. Physiological roles of macrophages. *Pflugers Archiv Eur J Physiol.* (2017) 469:365–74. doi: 10.1007/s00424-017-1945-7
114. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J Exp Med.* (2005) 201:1479–86. doi: 10.1084/jem.20050473
115. Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* (1997) 7:837–47. doi: 10.1016/S1074-7613(00)80402-1
116. Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* (1997) 278:1612–5. doi: 10.1126/science.278.5343.1612
117. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* (1998) 2:253–8. doi: 10.1016/S1097-2765(00)80136-7
118. Krebs P, Crozat K, Popkin D, Oldstone MB, Beutler B. Disruption of MyD88 signaling suppresses hemophagocytic lymphohistiocytosis in mice. *Blood* (2011) 117:6582–8. doi: 10.1182/blood-2011-01-329607
119. Strippoli R, Carvello F, Scianaro R, De Pasquale L, Vivarelli M, Petrini S, et al. Amplification of the response to Toll-like receptor ligands by prolonged exposure to interleukin-6 in mice: implication for the pathogenesis of macrophage activation syndrome. *Arthritis Rheum.* (2012) 64:1680–8. doi: 10.1002/art.33496
120. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol.* (2012) 30:1–22. doi: 10.1146/annurev-immunol-100311-102839
121. Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. *J Pediatr.* (2003) 142:292–6. doi: 10.1067/mpd.2003.110
122. Hazen MM, Woodward AL, Hofmann I, Degar BA, Grom A, Filipovich AH, et al. Mutations of the hemophagocytic lymphohistiocytosis-associated gene UNC13D in a patient with systemic juvenile idiopathic arthritis. *Arthritis Rheum.* (2008) 58:567–70. doi: 10.1002/art.23199
123. Villanueva J, Lee S, Giannini EH, Graham TB, Passo MH, Filipovich A, et al. Natural killer cell dysfunction is a distinguishing feature of systemic onset juvenile rheumatoid arthritis and macrophage activation syndrome. *Arthritis Res Ther.* (2005) 7:R30–7. doi: 10.1186/ar1453

124. Hermans IF, Ritchie DS, Yang J, Roberts JM, Ronchese F. $CD8^+$ T cell-dependent elimination of dendritic cells *in vivo* limits the induction of antitumor immunity. *J Immunol.* (2000) 164:3095–101. doi: 10.4049/jimmunol.164.6.3095
125. Yang J, Huck SP, McHugh RS, Hermans IF, Ronchese F. Perforin-dependent elimination of dendritic cells regulates the expansion of antigen-specific $CD8^+$ T cells *in vivo*. *Proc Natl Acad Sci USA*. (2006) 103:147–52. doi: 10.1073/pnas.0509054103
126. Terrell CE, Jordan MB. Perforin deficiency impairs a critical immunoregulatory loop involving murine $CD8^+$ T cells and dendritic cells. *Blood*. (2013) 121:5184–91. doi: 10.1182/blood-2013-04-495309
127. Lykens JE, Terrell CE, Zoller EE, Risma K, Jordan MB. Perforin is a critical physiologic regulator of T-cell activation. *Blood* (2011) 118:618–26. doi: 10.1182/blood-2010-12-324533
128. Brisse E, Imbrechts M, Mitera T, Vandenhaute J, Wouters CH, Snoeck R, et al. Lytic viral replication and immunopathology in a cytomegalovirus-induced mouse model of secondary hemophagocytic lymphohistiocytosis. *Virol J.* (2017) 14:240. doi: 10.1186/s12985-017-0908-0

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REVIEW
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Restoring T Cell Tolerance, Exploring the Potential of Histone Deacetylase Inhibitors for the Treatment of Juvenile Idiopathic Arthritis

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Juvenile Idiopathic Arthritis (JIA) is characterized by a loss of immune tolerance. Here, the balance between the activity of effector T (Teff) cells and regulatory T (Treg) cells is disturbed resulting in chronic inflammation in the joints. Presently, therapeutic strategies are predominantly aimed at suppressing immune activation and pro-inflammatory effector mechanisms, ignoring the opportunity to also promote tolerance by boosting the regulatory side of the immune balance. Histone deacetylases (HDACs) can deacetylate both histone and non-histone proteins and have been demonstrated to modulate epigenetic regulation as well as cellular signaling in various cell types. Importantly, HDACs are potent regulators of both Teff cell and Treg cell function and can thus be regarded as attractive therapeutic targets in chronic inflammatory arthritis. HDAC inhibitors (HDACi) have proven therapeutic potential in the cancer field, and are presently being explored for their potential in the treatment of autoimmune diseases. Specific HDACi have already been demonstrated to reduce the secretion of pro-inflammatory cytokines by Teff cells, and promote Treg numbers and suppressive capacity *in vitro* and *in vivo*. In this review, we outline the role of the different classes of HDACs in both Teff cell and Treg cell function. Furthermore, we will review the effect of different HDACi on T cell tolerance and explore their potential as a therapeutic strategy for the treatment of oligoarticular and polyarticular JIA.

Keywords: juvenile idiopathic arthritis (JIA), acetylation, HDAC inhibitor (histone deacetylase inhibitor), T cells, tolerance

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INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is the most common rheumatic disease in children and an important cause of short- and long-term disability (1, 2). It includes several different entities and has an intriguing heterogeneity in disease course and outcome. Oligo-articular JIA (oJIA) has a relatively mild course, with lasting medication-free remission in approximately half of the children, while poly-articular JIA (pJIA) more often is non-remitting and can lead to severe disability (2, 3). The main pathophysiological concept of JIA is that the immunological balance is disturbed resulting in loss of immune tolerance (1). A unique subtype of JIA is systemic-onset JIA (sJIA), involving 10% of all JIA patients, and in contrast to oJIA and pJIA, sJIA is mainly characterized as

an autoinflammatory disease instead of an autoimmune disease. In the pathogenesis of sJIA there is a key role for cells of the innate immune system including monocytes and neutrophils (4, 5). This is illustrated by the high incidence of macrophage activation syndrome (MAS) in patients with sJIA (6, 7). Although cells from the innate immune system play an essential role in the pathogenesis of oJIA and pJIA as well, it is generally thought that activation of autoreactive $CD4^+$ T cells, leading to a T cell-driven immune response is a key manifestation in the pathogenesis of oJIA and pJIA. This subsequently results in recruitment of other immune cells, including innate immune cells and the production of several pro-inflammatory cytokines including tumor necrosis factor (TNF)α, interleukin (IL)-6, IL-17, and interferon (IFN) γ which collectively leads to joint inflammation. Innate immune cells, such as neutrophils and monocytes/macrophages to the site of inflammation (8). The pathogenic T cells present within the synovial compartment are predominantly Thelper (Th) 1 and Th17 cells (9, 10).

Regulatory T (Treg) cells are key players in maintaining immunological balance and tolerance (11, 12). The transcription factor forkhead box P3 (FOXP3) is crucial for Treg cell development and function and mutations in the *FOXP3* gene can result in severe dysregulation of the immune system due to a Treg cell deficiency (13, 14). Treg cell numbers and function have also been implicated in complex autoimmune diseases including rheumatoid arthritis (RA) and JIA, and in fact the first data on $CD4^+$ Treg cells in human chronic arthritis comes from JIA patients (15, 16). Treg cells can be identified by the high expression of several markers, such as (but not limited to) FOXP3, $CD25^{high}$, cytotoxic T lymphocyte associated protein (CTLA)-4 and low expression of CD127. Treg cells can adapt to local environment (tissues) and acquire additional characteristics in inflammatory conditions (12, 17). They seem to exert their regulatory or suppressive actions both cell-contact dependent and independent via the secretion of anti-inflammatory cytokines such as Transforming Growth Factor beta (TGF)β and IL-10 (18). In JIA, the balance between pro-inflammatory Teff cells and anti-inflammatory Treg cells can be associated with the course of the disease (16, 19–22). For instance, higher numbers of Treg and lower numbers of Teff cells (Th17 and Th1) at the site of inflammation have been correlated to a more favorable course and outcome in JIA (16, 20–22). These observations support the concept that treatment may be aimed to restore the immunological imbalance between effector mechanisms and regulatory mechanism in children with JIA.

Current treatment of JIA, consisting of intra-articular corticosteroids, disease modifying anti-rheumatic drugs (DMARDs) and biologicals, such as anti-TNFα, seem primarily directed at the effector side of the immunological imbalance (23–26). In the past two decades, biologicals are increasingly being used in JIA. They certainly have been a major- breakthrough in the treatment of JIA, but even today, a significant percentage of patients do not respond to therapy or only show partial response. Furthermore, after achieving clinical inactive disease on therapy, many patients suffer from relapse when treatment is discontinued (27, 28). Therefore, there is still a need for improved treatment strategies in chronic inflammatory diseases such as JIA. Restoring tolerance, either by; decreasing Teff cell function, increasing Treg cell function or preferentially both, might be a promising therapeutic strategy.

Histone deacetylases (HDACs) are a novel class of therapeutic targets that are being explored for the treatment of autoimmune disease. These enzymes can modulate epigenetic regulation and important cellular functions in many different cell types, including T cells by the deacetylation of both histone and non-histone proteins. In other diseases and research fields, mainly cancer research, HDAC inhibitors (HDACi) have already demonstrated therapeutic potential (29). Interestingly, in the context of autoimmune disease, HDAC inhibition proved to influence both the innate immune system and Teff cell and Treg cell function, potentially restoring immunological tolerance. We here provide an overview and focus on the role of the different types of HDACs in $CD4^+$ Teff cells and Treg cells, and explore the potential of specific HDACi as a therapeutic strategy for the treatment of autoimmune diseases, in specific oJIA and pJIA.

HISTONE ACETYLATION AS REGULATORY MECHANISM OF IMMUNE ACTIVATION

The function of many intracellular proteins, particularly transcription factors, and histones, can be altered by post-translational modifications. Here, one or more amino acids are covalently modified, often modulating subcellular localization, activation state, interaction with other proteins or protein turnover/degradation. Acetylation is one of the most prominent post-translational modifications. The majority of literature on acetylation is directed at its role in epigenetic regulation, which refers to changes in gene expression without altering the genetic code. In the nucleus, DNA is tightly wrapped around histones to form a nucleosome (30) which controls the accessibility of DNA binding sequence to their transcription factors (31). An important epigenetic mechanism that affects this accessibility is the post-translational modification of histones by acetylation (32), a process which is reciprocally regulated by lysine acetyl transferases (HATs) and lysine deacetylases (HDACs) (33–35) (**Figure 1**). In general, histone acetylation is associated with transcriptional activation by rendering the DNA more accessible to transcription factors (32, 36). The reverse process, deacetylation by HDACs, can therefore lead to condensation of chromatin structure and inhibition of gene transcription. However, deacetylation is also associated with activation of genes, and the inhibition of HDACs in fact results in both upregulation and downregulation of genes in equivalent percentages (37–41).

There are 18 different HDAC enzymes, which can be divided into 4 classes based upon homology to yeast HDACs and their function. Class I (HDAC1, 2, 3, and 8), Class II divided into class IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6, 10) and class IV (HDAC11) are all Zinc-dependent and are considered classical HDACs. Class III consist of the Sirtuin family (Sirtuin 1–7) and are NAD^+ dependent (42).

Histone modifications are widely associated with human disease, including malignancies and autoimmune disease such as RA, systemic lupus erythematosus (SLE) and JIA (35, 43–46).

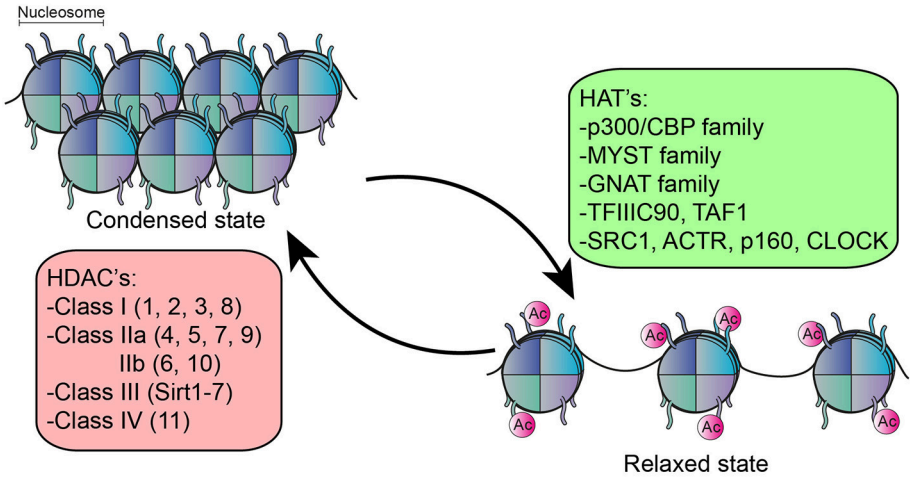

FIGURE 1 | Function of HDACs an HATs. HATs acetylate the lysine residue on histones resulting in relaxation of chromatin structure, rendering the DNA more accessible for transcription factors. The reverse process, deacetylation by HDACs results in condensation of chromatin structure. 5 families of HATs have been described and 18 different HDACs, divided into 4 classes.

For instance, autoimmune disease associated single nucleotide polymorphisms (SNP's) are significantly enriched in regions with high acetylation of lysine 27 on histone 3 (H3K27) (46–48). Accordingly, increased regions of H3K27 acetylation in $CD4^+$ T cells of JIA patients corresponded with increased expression of pro-inflammatory genes in these patients. Furthermore, in SLE, global acetylation of histone H3 and H4 in $CD4^+$ T cells was reduced in patients compared to healthy controls and the degree of histone H3 acetylation negatively correlated with diseases activity (43). This demonstrates an important role for histone acetylation in autoimmune disease.

Next to histones, HDACs can also deacetylate non-histone proteins, hereby affecting their localization in the cell, stability and function (49, 50). For example, the activity of the transcription factors FOXP3 and Rar-related orphan receptor gamma (RORγt), key regulators of T cell function, is directly regulated by acetylation. Numerous studies have directly assessed the role of acetylation in modulating immune responses. Although there is a clear role for HDACs in the regulation of innate immune responses, reviewed by others (51, 52), we will focus on the role of the different HDACs in T cells and discuss their potential implications in oJIA and pJIA.

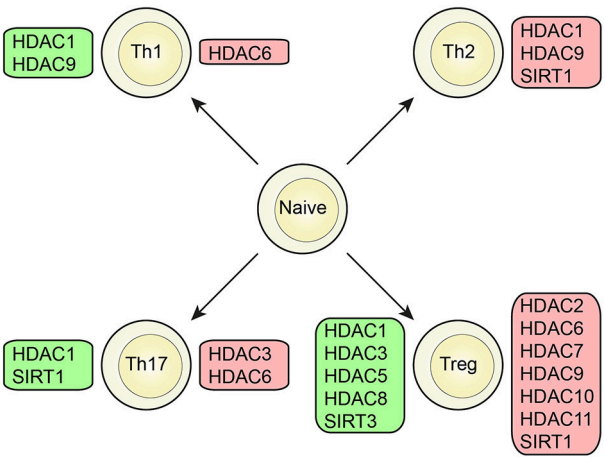

FIGURE 2 | Role of HDAC members in different T cell subsets. Specific HDAC members which was demonstrated to either promote (green box) or inhibit (red box) function or differentiation of $CD4^+$ T cells or the $CD4^+$ T cell subsets; Th1, Th2, Th17, and Treg cells.

HDAC FUNCTION IN $CD4^+$ T CELLS

Several classes of HDACs were demonstrated to have an important function in $CD4^+$T cell development and function (**Figures 2**, **3**).

Class I HDACs; HDAC 1, 2, 3, and 8

Several members of Class I HDACs are essential for T cell development and differentiation in mice. In mice, T cell-specific knock-out of *Hdac1* resulted in normal T cell numbers of both $CD4^+$ and $CD8^+$ T cells. However, these mice displayed enhanced Th2 responses, characterized by increased production of Th2 associated cytokines such as IL-4 and IL-5 combined with airway inflammation (53). In addition, *Hdac1* knock-out mice were resistant to the induction of experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis animal model which is associated with Th1 and Th17 responses (54). These data suggest that HDAC1 can skew T cell responses by impairing Th2 function and potentiating Th1 and Th17 activity. Furthermore, HDAC1 can also modulate Treg function. In a mouse cardiac transplant model, the deletion of HDAC1 in $FOXP3^+$ Treg cells resulted in an impaired function of these cells, combined with increased secretion of the pro-inflammatory cytokines IL-2, IL-17, IFNγ, and decreased cardiac allograft survival (55). This demonstrates that HDAC1 contributes to Treg cell suppressive

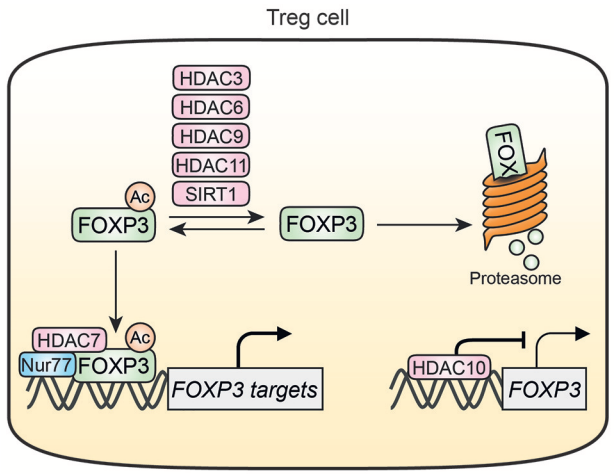

FIGURE 3 | HDAC-mediated regulation of FOXP3 in Treg cells. HDAC3, HDAC6, HDAC9, HDAC11, and SIRT1 can deacetylate FOXP3 thereby increasing FOXP3 susceptibility for degradation. Acetylation of FOXP3 also promotes associations with DNA where it can form a transcriptional complex with HDAC7 and Nur77. HDAC10 can bind to and inhibit the FOXP3 promotor resulting in decreased FOXP3 expression. Nur77 is inhibited by HDAC7 and interacts with the HDAC7/FOXP3 transcriptional complex.

capacity. While also T cell-specific deletion of HDAC2 did not affect T cell numbers, combined deletion of both HDAC1 and HDAC2 resulted in severe defects in mature T cell development, especially in the $CD4^+$ T cell lineage. This indicates that HDAC1 and HDAC2 have overlapping functions in T cell development. In a combined *Hdac1/2* T cell knock-out mouse there was a decreased differentiation from double negative to double positive T cells ($CD4^+/CD8^+$) accompanied with a 5-fold decrease in thymocyte cellularity indicating a block in T cell development (56). *Hdac1/2* knock-out was proposed to result in defective propagation of T cell receptor (TCR) signaling. In a later stage of T cell development, the combined knock-out of *Hdac1/2* in $CD4^+$ T cells specifically resulted in an increased CD8 surface expression by these $CD4^+$ T cells and a decrease in peripheral T cell numbers (55). It was demonstrated that HDAC1 and HDAC2 maintain CD4 T cell integrity via repressing Runx-CBFß, a heterodimeric transcription factor required for the appropriate expression of CD4 and CD8. Furthermore, in T cells a dose dependent tumor suppressor function of HDAC1/2 was observed (56, 57). In Treg cells, the inhibition or deletion of HDAC2 specifically resulted in an increased Treg function, promoting cardiac allograft survival. This indicates that selective inhibition of HDAC2 in contrast to HDAC1 could serve a protective role against graft rejection (55). Combined targeting of both HDAC1 and 2 could result in severe defects in T cell development and therefore seems not suited for the treatment of JIA based upon data from mouse knock-out studies.

Comparable to the developmental defects observed in *Hdac1/2* knock-outs, HDAC3 also was shown to be essential for T cell development and maturation (58–60). Knock-out of *Hdac3* in mice resulted in a block in T cell development during positive selection in the thymus resulting in a strong reduction in $CD4^+$ and $CD8^+$ peripheral T cells. Within the $CD4^+$ T cells present in the peripheral blood there was an increase in RORyt and IL-17 producing cells, indicating a skewing toward the Th17 phenotype (58). Furthermore, it was demonstrated that HDAC3 is an important mediator of the development and function of both induced and natural Treg cells. Interestingly, HDAC3 can directly associate with FOXP3, resulting in reduced IL-2 production by Treg cells. Moreover, mice with a FOXP3 cell-specific deletion of HDAC3 died within weeks from severe autoimmunity (61). For HDAC8, ongoing studies indicate that deletion of HDAC8 in Treg cells results in impaired Treg cell function (62). Together these data indicate that next to combined HDAC1/2 targeting, the selective inhibition of HDAC3 or HDAC8 could either result in T cell development defects or impaired Treg cell function and therefore does not seem to be of any therapeutic potential in a chronic autoimmune disease such as JIA.

Class IIa HDACs; HDAC4, 5, 7, and 9

Most members from Class IIa HDACs are protective for the development of autoimmunity in mice. HDAC4 has been mainly assessed in the nervous system due to its high expression in brain and skeletal tissue and little is known about its function in T cells. Although HDAC4 is expressed in all T cell subsets, $CD4^+$ T cells specific *Hdac4* knock-out mice display normal T cell numbers and function (63). A role for HDAC4 in $CD4^+$ T cells was suggested based upon hypermethylation of the *HDAC4* region of the DNA of $CD4^+$ T cells from RA patients. These data indicate that HDAC4 expression would be decreased in these patients, but gene expression or function of HDAC4 was not assessed in this study (64). Therefore, the exact role of HDAC4 in T cell and in autoimmune diseases is currently unknown.

Similarly, *Hdac5* knock-out mice display a normal $CD4^+$ T cell development and function. In these mice however, Treg cell displayed impaired suppressive capacity (65). In addition, IFNγ production was decreased in $CD8^+$ T cells, indicating that HDAC5 perhaps has a more profound role in $CD8^+$ T cells and Treg cells compared to $CD4^+$ Teff cells (65). In contrast to HDAC4 and HDAC5, HDAC7 is an important regulator of T cell development by regulating both positive and negative selection in the thymus (66–68). Knock down of *Hdac7* in mice T cells results in a defect in positive selection in the thymus with a decrease in T cell survival and TCR repertoire (66). The effect of HDAC7 on negative thymocyte selection was shown to be via inhibition of the expression of the transcription factor; Nur77 (69). Nur77 promotes thymocyte apoptosis during negative selection of autoreactive thymocytes (69) and the overexpression of Nur77 significantly decreases numbers of peripheral $CD4^+$ and $CD8^+$ T cells in mice (70). It was demonstrated that HDAC7 is recruited to the *Nur77* promotor via interaction with the transcription factor MEF2D resulting in decreased Nur77 expression. HDAC7 is exported out of the nucleus during T cell activation resulting in increased Nur77 expression. Blocking the nuclear export of HDAC7 in T cells in mice resulted in a block in negative selection in the thymus, promoted survival of auto-reactive T cells and was accompanied with the development of autoimmunity and

a decreased life span in mice (67). The exact mechanism of inhibition of expression of Nur77 by HDAC7 remains unknown, but it is demonstrated to be dependent on its deacetylase activity (69). A profound role for HDAC7 in Treg function was also shown. Overexpression of Nur77 in mice T cells was associated with an increase in Treg cell percentages and cardiac allograft survival. It was demonstrated that Nur77 can interact with a HDAC7/FOXP3 transcriptional complex in Treg cells and that Nur77 overexpression resulted in increased expression of Treg associated genes, including Foxp3, Foxp1, TIP60 (70). Implicating, that Nur77 affects the balance between Teff and Treg cells, favoring Treg cell survival. Taken together, HDAC7 plays an essential role in negative T cell selection in the thymus as an inhibitor of Nur77. Both in Teff and Treg cells a protective role of Nur77 with respect to development of autoimmunity is suggested making Nur77 an interesting treatment target. However, the importance of HDAC7 in the positive selection of T cells in the thymus and TCR repertoire formation and therefore T cell development makes HDAC7 an undesirable target for the treatment of autoimmune diseases.

A role for HDAC9 has been implicated in several T cell subsets, including Treg, Th1, and Th2 cells both in mice and humans. HDAC9 expression was found to be increased in different subsets of $CD4^+$ T cells of SLE patients and the autoimmune prone MLR/lpr mice. In addition, HDAC9 deficiency was associated with hyperacetylation of several lysine residues of histone H3 in mice (71). In the MLR/lpr mouse, knock-out of *Hdac9* resulted in a prolonged survival and decrease in autoimmune disease progression. This was determined by smaller lymph nodes and spleen and a decreased percentage of activated $CD4^+$ T cells and double negative T cells. Furthermore, knock-out of *Hdac9* resulted in an inhibition of T cell activation *in vitro* and a decrease in Th1 and increase in Th2 cytokine production *in vivo* (71). Therefore, HDAC9 appears to promote skewing toward Th1 subsets. In addition, there is a differential expression of HDAC9 in different T cell subsets. HDAC9 is higher expressed in Treg cells compared to non-Treg cells and HDAC9 expression is markedly decreased in non-Treg T cells after stimulation in contrast to Treg cells (72). This could implicate an important role for HDAC9 in Treg function or development. Indeed, knock-out of HDAC9 in mice resulted in increased Treg numbers with enhanced suppressive function (73) and predisposition to iTreg development (74). This was confirmed by *in vitro* knock-down of *Hdac9* in mice Treg cells which resulted in increased FOXP3 expression and increased suppressive function (73). Furthermore, the induction of colitis in mice was associated with increased HDAC9 expression, while the *Hdac9* knock-out mice were resistant to development of colitis (73). Taken together, HDAC9 showed to promote the development of autoimmune disease via its function in both Teff and Treg cells.

In summary class IIa HDACs in $CD4^+$ T cells seem to be protective against the development of autoimmunity or essential for T cell development, with the exception of HDAC9. The inhibition of HDAC9 could therefore be of therapeutic interest in the context of autoimmune diseases such as JIA, however it's specific role in arthritis had not been investigated yet.

Class IIb; HDAC6, 10

The two members of class IIb HDACs; HDAC6, and HDAC10 have both been assessed for their therapeutic potential as a new treatment of autoimmune disease. Importantly, in $CD4^+$ specific *Hdac6* knock-out mice, $CD4^+$ and $CD8^+$ T-cell development and function was described to be normal (74–76). However, in these mice the population of IL-17 producing gamma delta (γδ) T cells was increased which was accompanied by a decreased expression of the transcription factor SOX4. This was confirmed *in vitro,* by treating a murine lymphoma cell line (EL4) with the two different HDAC6 inhibitors; tubacin, and tubastatin. HDAC6 inhibition in these cells resulted in a concentration-dependent increase of IL-17 expression (75). Since IL-17 production is associated with autoimmunity, these observations implicate a protective role for HDAC6 in the development of autoimmune disease via inhibition of Th17 cell differentiation. However, in Treg cells, it has been shown that HDAC6 is involved in deacetylation of FOXP3 and selective inhibition of HDAC6 enhanced the suppressive function of Treg cells (76–78). This was confirmed in an arthritis mouse model were HDAC6 inhibition resulted in decreased arthritis scores in mice (79). Furthermore, in human peripheral blood mononuclear cells (PBMCs) from RA patients, *in vitro* HDAC6 inhibition resulted in a decreased expression of the pro-inflammatory cytokines TNFα and IL-1β and increased the anti-inflammatory cytokine IL-10 (79). These observations indicate that HDAC6 can exert different effects in different T cell subsets, both pro-inflammatory and anti-inflammatory. Due to the observation that HDAC6 inhibition in Treg cells resulted in enhanced suppressive function and a decrease in arthritis development in a mouse model, there is great interest in the potential application of specific HDAC6i immune suppression therapy in for example transplant recipients. (62) However, more research is needed to determine the potential risk of HDAC6i by promoting autoimmune disease by increasing IL-17 production.

For HDAC10, it was reported very recently that it can bind to the *FOXP3* promotor and inhibit its transcriptional activity (80). Treg cells from HDAC10 knock-out mice showed increased expression of FOXP3 accompanied by increased Treg suppressive function. Furthermore, transfer of Treg cells from HDAC10 knock-out mice in a colitis mouse model resulted in a reduced induction of colitis compared to transfer of wild-type (WT) Treg cells (80).

Altogether, both HDAC6 and HDAC10, as class IIb HDACs, seem to exert negative effects on Treg cells and the inhibition of class IIb HDACs could therefore be beneficial in inducing Treg cell function. However, for HDAC6 there is a differential effect on different Th subsets and a pro-inflammatory effect has also been demonstrated via the induction of IL-17 production.

Class IV; HDAC11

HDAC11, the only member of class IV HDACs is suggested to have an inhibitory role in maintaining immune tolerance. For Treg cells it was demonstrated in a human T cell line that HDAC11 can associate with and deacetylate FOXP3 (81). Knock-out of *Hdac11* in Treg cells resulted in an increased expression of FOXP3 and TGF-β, and an increased suppressive capacity

in vitro. This was confirmed *in vivo* by an increased cardiac allograft survival in *Hdac11* knock-out mice (81). In mice, *in vitro* activation of Teff cells resulted in a downregulation of HDAC11 expression. In addition, *Hdac11* knock-out in $CD4^+$ and $CD8^+$ T cells resulted in an increased proliferation and pro-inflammatory cytokine production after activation indicating an inhibitory role for HDAC11 in Teff cell activation. Interestingly, the *Hdac11* knock-out $CD4^+$ T cells were resistant to the *in vivo* induction of tolerance via the injection of a tolerogenic dose of ovalbumin (82). In summary, *Hdac*11 knock-out has a differential effect on the different T cell subsets with an increased Teff function and decreased tolerance induction after HDAC11 knock-out in all T cells. In contrast, Treg cell-specific knock-out of HDAC11 results in the opposite effect with an increased suppressive function of Treg cells and improved immune tolerance. The effect of HDAC11 inhibition *in vivo* therefore seems to depend on which cell type is affected most. To our knowledge, no specific inhibitor for HDAC11 is available yet and therefore the effect of HDAC11 inhibition *in vivo* remains incompletely understood.

Class III; Sirtuins1-7

The most extensively studied member of the sirtuin family, SIRT1, is an important player in chronic inflammation. The exact function of SIRT1 is under debate and both a pro-inflammatory and an anti-inflammatory role of SIRT1 have been described in human disease. It was demonstrated that SIRT1 is upregulated in the synovial tissue and PBMC from patients with RA compared to patients with osteoarthritis. In this study, a pro-inflammatory role for SIRT1 was implied in monocytes with a reduction in lipopolysaccharide (LPS) induced TNFα production *in vitro* after inhibition of SIRT1, either by incubation with SIRT1 inhibitors or siRNA targeting (83). However, the opposing effect has also been demonstrated where a protective role for SIRT1 against inflammation and tissue destruction is suggested in chondrocytes and osteoblasts via the deacetylation and therefore inactivation of Nuclear factor-kappa B (NF-κB) (84, 85). Part of this controversy may be explained by differences in experimental setup, cell type and readout.

In mice T cells, the germline knock-out of *Sirt1* resulted in an increased T cell proliferation and expression of IL-2, IFNγ, and IL-5 compared to WT upon *in vitro* activation. In these mice, this decreased T cell tolerance resulted in increased development of EAE (86). In contrast to what is found in the germline knock-out mice, T cell development, cytokine expression and proliferation upon *in vitro* activation is normal in $CD4^+$ T cell specific *Sirt1* knock-out mice (87). These observations indicate that the role of SIRT1 varies within different T cell subsets and different stages of T cell development. This is in line with the finding that basal sirtuin/SIRT1 levels differ between different T cell subsets. For instance, in mice, all sirtuin members are expressed in higher levels in Treg cells compared to Teff cells. Interestingly, upon *in vitro* activation via the T cell receptor there is a markedly increased expression of all sirtuin members in mouse Teff cells, but a downregulation of some sirtuins, including SIRT1, in Treg cells (76). Furthermore, SIRT1 plays an important role in the function of Treg cells via its effect on FOXP3. Both FOXP3 activity and stability is dependent on its acetylation status and it was demonstrated that SIRT1 associates with and deacetylates FOXP3, resulting in its degradation (55, 88–90). This was confirmed *in vivo* by Treg-specific *Sirt1* knock-out in mice where there was increased expression of FOXP3 (87). In addition, the increase in FOXP3 expression was accompanied by an increased suppressive capacity of Treg cells. Transfer of Treg cells from both $Cd4^+$ and $Foxp3^+$ specific knock-out mice into immune-deficient mice showed a more potent suppressive capacity compared to WT Treg cells (87). Moreover, in a colitis mouse model, the adoptive transfer of Teff cells isolated from $CD4^+$ specific *Sirt1* knock-out mice resulted in a nearly 3-fold increase in iTreg formation compared with mice receiving WT Teff cells. This correlated with reduced weight loss and reduced development of colitis (91).

In other T cell subsets, treatment with an siRNA for *Sirt1*, resulted in increased of IL-9 production *in vitro* by both mouse and human $CD4^+$ T cells which was accompanied with an increase in allergic airway inflammation in mice (89). In Th17 cells, *Sirt1* knock-out was demonstrated to inhibit Th17 differentiation via RORyt hyperacetylation and showed to be protective in an EAE mouse model (92). IL-9 producing Th9 cells are associated with a Th2 type response while Th17 cells are important pro-inflammatory players in autoimmune disease.

Altogether, the function of SIRT1 can exert varying effects on different T cell subsets and has been linked to both protective and aggravating effects in disease models. Overall, most studies suggest a pro-inflammatory role for SIRT1 in $CD4^+$ T cells, and especially Treg cells, in the setting of autoimmune diseases.

The role of SIRT2, SIRT4, SIRT5, SIRT6, and SIRT7 in $CD4^+$ T cells has not been studied to our knowledge. Germline knock-out of SIRT3 resulted in a normal T cell development and response to bacterial and fungal infections in mice (93). Treg cells from FOXP3 cell-specific *Sirt3* knock-out in mice had impaired suppressive function *in vitro* which resulted in an increased cardiac allograft rejection and chronic graft injury *in vivo*. These findings suggest a protective role for SIRT3 in the function of Treg cells (61). Collectively, these data demonstrate that, although opposite effects, both SIRT1 and 3 can be regarded as a potential therapeutic target in autoimmune disease.

HDAC INHIBITION BASED THERAPY FOR CHRONIC INFLAMMATORY DISEASES

Acetylation has directly been implicated in the control of cell cycle arrest and apoptosis, making modulators of acetylation such as HATs and HDACs interesting targets for treatment of various diseases, especially cancer. Presently, the therapeutic potential of various HDACi is being tested in clinical trials involving several different malignancies (29, 94–97). For example, the tumor suppressor gene p53, a master coordinator of crucial cellular functions such as apoptosis and genomic stability, is deacetylated by several members of class I and class III HDACs, thereby decreasing its activity (98–102). This underlies the therapeutic potential of HDACi in cancer treatment. HDACi are generally well-tolerated compared to other drugs used in

cancer therapy and the most commonly described side effects are gastro-intestinal complaints and fatigue (97, 103). Serious adverse events described include bone marrow depression, liver toxicity, electrolyte disturbances and electrocardiogram (ECG) changes. However, bone marrow depression was shown to be reversible after cessation of the therapeutic agent (97, 104) and intensive monitoring of ECG changes in clinical trials did not show an increase in cardiac adverse events, but long-term follow up is needed (97, 103). Furthermore, of the many HDACi tested in phase I/II clinical trials just a few have been approved for clinical use, which could be caused by absence of selectivity and unclear mechanism of action of many HDACi (97). The broad impact of HDACs on major cell functions, including on $CD4^+$ T cell function (both Teff and Treg), suggests that HDACs could be a potential therapeutic target as well for non-malignant diseases like chronic inflammatory diseases. However, especially for non-lethal chronic diseases such as JIA, potential side effects should be carefully studied, monitored and balanced against the possible or expected benefits.

Although on a different scale compared to cancer research, the role of HDACi in the context of autoimmune diseases has also been investigated (**Table 1**). HDACi were demonstrated to suppress key players of the innate immune system (115–121). For example, Trichostatin A (TSA) and nicotinamide, HDACi inhibiting class I/II or class III HDACs, respectively, decreased the *in vitro* production of IL-6 and TNFα by macrophages from healthy donors and patients with RA after stimulation with TNFα or LPS (115). In mice, oral treatment with the pan-HDACi; suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat, reduced the circulating levels of the pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IFNγ after LPS stimulation (116). Moreover, in a murine lethal LPS-induced septic shock model, treatment with SAHA improved survival by attenuation of several inflammatory markers including neutrophil infiltration in the lungs (117). Furthermore, in LPS stimulated cultured human PBMCs, the class I/II HDACi: ITF2357 (Givinostat) reduced the production of the pro-inflammatory cytokines TNFα, IL-1α, IL-1β, and IFNγ. This *in vitro* data was confirmed in an *in vivo* mouse model where oral treatment with Givinostat reduced LPS-induced serum TNFα and IFNγ by more than 50% (118). Next, HDAC inhibition in RA fibroblast like synoviocytes (FLS) suppressed inflammatory gene expression, including type I IFNγ, IL-6, IL-8 (120, 121) via regulation of cytokine mRNA stability (121). Accordingly, Etinostat (MS275), a class I HDAC inhibitor, selectively affecting HDAC1-3, showed to decrease cell proliferation and secretion of the pro-inflammatory cytokines IL-6 and IL-18 and nitric oxide in cultured human fibroblastic cells from RA patients (119). The nuclear accumulation of NFκ-B was decreased in this model indicating that the anti-inflammatory effect could be mediated via increased acetylation of NFκ-B. A more detailed overview of HDAC inhibition in innate immune cells can be found in literature (51, 52) and is beyond the scope of this review. These data demonstrate that HDAC inhibition of various classes can result in reduced pro-inflammatory cytokine production by innate immune cells, both *in vitro* and *in vivo*.

HDACi Affecting Class I and II HDAC

Next to suppressive effects on innate inflammation, HDACi could be of great interest as therapeutic strategy by suppressing key players of the adaptive immune system. Of special interest for this review is the effect of various HDACi on FOXP3 expression and Treg cell mediated suppression. Treatment of WT mice with the pan-HDACi TSA resulted in an increase of absolute numbers and percentages of $CD4^+FOXP3^+$ cells in lymphoid tissue by increased production in the thymus (72). This was accompanied by an increase in FOXP3 acetylation and expression of Treg associated genes, including *Foxp3*, *Ctla-4*, and *Il-10* (72). In addition, in a mouse colitis model, the pan HDACi TSA and SAHA inhibited the development of colitis, defined by weight loss, diarrhea, bleeding, and histological findings. In line with previous results, this was accompanied by an increase in $CD4^+FOXP3^+$ cells in both absolute numbers and percentages in the lymphoid tissue of these mice (73). Furthermore, in a mouse cardiac transplant model, SAHA treatment prolonged cardiac allograft survival which was associated with an increased percentage of $FOXP3^+$ cells in the thymus, lymph nodes and spleen (105). This was combined with an improved suppressive capacity of the Treg cells. However, depending on the dose used, different effects of SAHA on Treg cells were observed. A low dose of SAHA selectively promoted Teff cell apoptosis and hereby increased the relative percentage of Treg cells, while a high dose suppressed the generation of $FOXP3^+$ cells (105). Perhaps, this could be explained by differential effects of SAHA concentrations on the various types of HDACs (122). In an arthritis mouse model, Valproic acid (VPA), a strong class I but also class II HDACi, decreased the incidence and disease activity of collagen induced arthritis. In addition, there was an increase in the suppressive capacity and numbers of Treg cells (112). In contrast to TSA and SAHA, Etinostat, a selective inhibitor of HDAC1-3 from class I, had no effect on the development of colitis or on the percentage $CD4^+FOXP3^+$ cells in the colitis mouse model (73). Since TSA is an inhibitor of Class I and II and SAHA of class I, II, and IV HDACs this implies that the inhibition of one or more HDACs from class II are responsible for the increase in Treg cell numbers in this study. As we described above, an important role for HDAC9 in the development of autoimmune disease was demonstrated in several studies. Therefore, HDAC9 inhibition could potentially be responsible for the increased Treg cell numbers and the protective effect in colitis development in these mice treated with TSA and SAHA. HDAC9 inhibition could therefore be of important therapeutic potential in the treatment of autoimmune diseases such as JIA.

In humans, a phase I/II clinical trial aiming to reduce the incidence of graft vs. host disease in patients receiving allogeneic hematopoietic cell transplantation, add-on treatment with Vorinostat (SAHA) reduced pro-inflammatory cytokine levels in plasma and increased Treg cell numbers and suppressive capacity (106, 107).

Although non-specific, a compound known to exert an effect on T cells via HDAC8 inhibition is butyrate (113). Butyrate treatment resulted in a reduction in disease severity in a collagen induced arthritis mouse model. This was associated

TABLE 1 | Effect of HDAC inhibition on T cell subsets.

HDAC inhibitor	Targeted HDAC member	Effect on T-cell subsets
Trichostatin A (TSA)	Class I and II	- Increase of absolute numbers and percentages of FOXP3$^+$ cells in mice with increased FOXP3 acetylation and *Foxp3*, *Ctla-4* and *Il-10* expression (72). - Increase of FOXP3$^+$ cell numbers and percentages in mice. Accompanied with decreased colitis development in mice (73).
Suberoylanilide hydroxamic acid (SAHA)/Vorinostat	Class I, II, and IV	- Increase of FOXP3$^+$ cell numbers and percentages and decreased colitis development in mice (73). - Improved cardiac allograft survival in mice, with increased percentage of FOXP3$^+$ cells and increased suppressive capacity of these cells. However, different effects with different dosages (105). - Reduced incidence of graft versus host disease in allogenic hematopoietic stem cell transplantation patients in a phase I/II clinical trial. Accompanied by reduced pro-inflammatory cytokines and increased Treg cell numbers and suppressive capacity (106, 107).
ITF2357/Givinostat	Class I and II	- Decreased disease activity and increased survival in SLE prone mice (NZB/W). Increased percentage of Treg cells and decrease in IL-17 producing cells (108). - Decreased joint swelling and cell influx into joint cavity in arthritis mouse model. Reduction in pro-inflammatory cytokines; TNFα, IL-1β (109). - Reduced pro-inflammatory cytokine production after *ex vivo* stimulation of PBMC from healthy volunteers with LPS in a phase I clinical trial (110). - Decreased disease activity scores in 5/9 patients at 12 weeks in a phase II human clinical trial involving children with sJIA (111).
MS275/Etinostat	Class I members HDAC1-3	- No effect on percentage of FOXP3$^+$ cells percentage or development of colitis in mice (73).
Valproic acid (VPA)	Class I (primarily) and II	- Increased Treg cell number and suppressive capacity accompanied with a decreased incidence of collagen induced arthritis in mice (112).
Butyrate	Class I, IIa, and IV	- Decreased Th17 cell numbers and increased Treg cell numbers in mice via inhibition of HDAC8. Decreased expression of pro-inflammatory cytokines and reduction in inflammation, bone damage and cartilage damage (113).
EX-527	Class III member; SIRT1	- Increased suppressive capacity of mouse Treg cells *in vivo* (87). - Increased allograft survival and kidney function in a mouse kidney transplant model (114). - Reduction of weight loss and induction of iTreg development in mouse colitis model. Decreased production of IL-17 during *ex vivo* induction of mouse IL-17 cells (92)
Nicotinamide (NAM)/Vitamin B3	Class III member; SIRT1	- Increased percentage of FOXP3$^+$cell's *in vitro* in primary human cells (88). - Decreased production of IL-17 during *ex vivo* induction of mouse IL-17 cells (92).

with decreased expression levels of pro-inflammatory cytokines and a reduction in inflammation scores, bone damage and cartilage damage scores (113). Inhibition of HDAC8 in T cells by butyrate resulted in decreased Th17 cell number and increased number of Treg cells in these mice. However, the effect of butyrate on the immune system seems to be very broad, with the potential to inhibit Class I, IIa, and IV HDACs affecting several cell types, and being not restricted to HDAC inhibition (123–125).

Givinostat, another class I and II HDACi, is together with SAHA one of the few HDACi that has been investigated for its potential therapeutic effect in autoimmune disease in a clinical trial. Both *in vitro* (human primary cells) and in *in vivo* mouse models, Givinostat was demonstrated to have a strong anti-inflammatory effect by affecting key players of the innate and the adaptive immune system (108, 109, 118). The SLE prone NZB/W mice treated with Givinostat showed decreased SLE disease activity and increased survival (108). This was defined by a decrease in anti-nuclear antibodies and immune complex deposition, improvement of renal histopathology, decrease of the pro-inflammatory cytokine IL-1β and increase in the anti-inflammatory cytokine tumor growth factor (TGF)-β. In addition, an increased percentage of Treg cells and a decreased number of IL-17 producing cells was observed in the spleen (108). Furthermore, in an arthritis mouse model, Givinostat treatment showed to decrease joint swelling and cell influx into the joint cavity (109). A reduced production of the pro-inflammatory cytokines TNFα and IL-1β by synovial tissue was demonstrated which resulted in strong inhibition of bone resorption (109). These promising results from pre-clinical (animal) studies resulted in further exploring Givinostat as a potential therapeutic treatment in human autoimmune disease. First, oral treatment with Givinostat was proven to be safe in a phase I trial involving healthy volunteers (110). In addition, the *ex vivo* stimulation of the peripheral blood from these volunteers with LPS showed a reduction in pro-inflammatory cytokine production. Next, in a small phase II trial involving 17 children with systemic onset JIA (sJIA), oral treatment with Givinostat during 12 weeks proved to be relatively safe, with only mild adverse events. Although 4/17 patients discontinued treatment for safety reasons this was reported to be non-drug related. Interestingly, although not set-up and powered to demonstrate efficacy, possible therapeutic effects of treatment were suggested. In the per-protocol treated group, 5/9 patients showed a relevant decrease in disease activity scores (ACRPed50%) at 12 weeks. Moreover, some patients showed a decrease in neutrophil count and a decrease in pro-inflammatory cytokines such as CD40L, IL-1α, and IFNγ in whole blood lysates (111). As this was only a small phase II trial, the therapeutic potential of Givinostat in the

treatment of sJIA or other forms of JIA needs to be explored further.

HDACi Affecting Class III HDAC

The pro-inflammatory role of SIRT1, at least partially caused by an inhibitory effect on FOXP3 stability and function, makes SIRT1 a potentially interesting target for the treatment of autoimmune disease. In line with what was demonstrated in *Sirt1* knock-out mice, selective inhibition of SIRT1 with small-molecule inhibitors promoted Treg cell numbers and function in *in vitro* studies using primary human cells and in *in vivo* animal models for autoimmune diseases (87, 88, 91). Mouse Treg cells treated *in vitro* with the selective SIRT1 inhibitor EX-527 showed a more potent suppressive capacity (87). This was confirmed *in vivo* in a kidney transplant model where mice treated with EX-527 showed increased survival and improved kidney function (114). In addition, in a mouse colitis model, EX-527 treatment resulted in reduced weight loss and promoted the development of induced Treg cells (91). These studies outline the therapeutic potential of SIRT1 inhibition with EX-527 in autoimmune diseases although its safety and efficacy needs to be further investigated in humans.

Even more interesting for potential future use in patients is the relatively specific SIRT1 inhibitor nicotinamide, also known as Vitamin B3 and well-known for many years as a food additive. SIRT1 inhibition via nicotinamide proved to increase percentages of $FOXP3^+$ cells *in vitro* in primary human cells (88). Furthermore, both nicotinamide and EX-527 dose dependently decreased the production of IL-17 during *ex vivo* induction of mouse Th17 cells, indicating an important role for SIRT1 in Th17 differentiation (92). Therefore, SIRT1 inhibition, via nicotinamide or EX-527, has the potential to both induce Treg cells and inhibit Th17 cell differentiation, affecting both sides of the disturbed immune balance in autoimmune diseases such as JIA. Importantly, the potential therapeutic effects of nicotinamide in autoimmune diseases is not a new concept, as it has been studied in humans in a variety of diseases for over more than 50 years (126–134). In both adults and children, maintenance therapy with nicotinamide has been associated with improvement of beta-cell function and reduction in pancreatic inflammation in Type 1 diabetes (128, 131, 132, 134, 135). However, results proved variable in other studies and the effects on either Treg or Teff cells in these studies has not been investigated (127, 129, 130, 136). Importantly, in particular for intended future development of nicotinamide maintenance therapy in JIA, the long term use of high dose nicotinamide treatment proved to be safe in multiple clinical trials involving large numbers of adults and children from the age of 5 (130, 132, 136, 137).

DISCUSSION

In JIA, the distorted immunological balance results in chronic, sometimes lifelong inflammatory arthritis and imposes a significant risk for restricted mobility and even disability in children. Although therapy with DMARDs and biologicals has proven to be very successful in inducing remission in JIA, there is a high percentage of patients that relapse after tapering and stop of maintenance immunosuppressive treatment. Therefore, there is still a medical need for novel treatment strategies that focus on restoring immune tolerance and the prevention of relapses in these children. As increased insight of the mechanisms underlying JIA has revealed that the balance between anti-inflammatory mechanisms such as Treg cells and pro-inflammatory mechanisms such as Teff cells can determine the course of the disease, this balance represents therefore a promising therapeutic target.

HDACs have been demonstrated to directly regulate the differentiation, proliferation and function of $CD4^+$ Teff and Treg cells. Therefore, HDACi harbor therapeutic potential to restore immune tolerance and inhibit activation in autoimmune diseases such as JIA. Currently however, literature on HDAC function in T cells and autoimmune disease is mainly focused on mice. HDAC function in humans and autoimmune disease in specific, needs to be further investigated before conclusions about the therapeutic potential of HDACi can be drawn. These mice knock-out studies however help us to better understand the function of specific HDACs in the different T cell subtypes and indicate which HDACs have the best potential as a therapeutic target in autoimmune disease. As HDACs, specifically from class I, have shown to be crucial for the development, differentiation and function of $CD4^+$ T cells, the inhibition of some of these HDACs could potentially lead to severe dysregulation of the immune response. This indicates that, in order to use HDACs in the treatment of human autoimmune disease, it is extremely important that HDACi will selectively target specific HDACs. Several pan HDACi, which have a broad effect and target different HDACs and different HDAC classes, have shown a great potential for the treatment of numerous malignancies and pronounced anti-inflammatory effects by affecting both the innate and adaptive immune system both *in vitro* and *in vivo* without affecting general T cell differentiation or development. However, the broad and pronounced anti-inflammatory effect of these pan HDACi certainly hinders the applicability in chronic autoimmune diseases due to the potential side effects. Some of the HDACi however, may show the required selectivity that is needed for treating chronic inflammatory diseases. For example, the class I/II HDACi; Givinostat, was demonstrated to be safe in a small first clinical trial and data from this phase II trial suggested anti-inflammatory effects in sJIA patients, a disease characterized by hyperactivated (innate) inflammatory pathways. In this study, Givinostat seemed to mainly act on effector mechanisms, comparable to the current available treatment options for sJIA. Although promising in this small initial prove of concept trial, the effect of Givinostat treatment in other forms of JIA and the long-term side effects needs to be explored in more extent before it could be considered as part of a potential treatment strategy.

Other HDACi, targeting HDAC6, HDAC9, and SIRT1 not only show suppressive effects on effector mechanisms in inflammation, but may have promising anti-inflammatory effects

via improving Treg cell numbers and function as well. The selective and specific inhibition of these HDACs is therefore currently being explored as potential treatment for use in transplant patients suffering GVHD and could be attractive candidates as part of the treatment regimens in chronic autoimmune diseases such as JIA. Although selective deletion of HDAC9 showed promising anti-inflammatory effects, to our knowledge no specific inhibitor of HDAC9 is available yet. In contrast, specific inhibition of HDAC6 is possible and clinical trials involving specific HDAC6 inhibition to prevent allograft rejection in transplant patients are expected in the near future. Since HDAC6i mainly modulate Treg cell numbers and function, the expansion of treatment with specific HDAC6i to other autoimmune diseases seems a logical next step provided that these HDACi have been demonstrated to be safe and in these patients.

Another promising candidate for the treatment of chronic inflammatory diseases such as JIA is the specific inhibition of SIRT1, which showed an anti-inflammatory effect in several models both *in vitro* and *in vivo*. The beneficial effect of SIRT1 inhibition was shown to be the result of induction of Treg cells (numbers) and function as well as inhibition of Th17 function. This means that SIRT1 inhibition positively affects the disturbed immune balance in diseases like JIA in both ways. As nicotinamide is a selective inhibitor of SIRT1, and has already proven to be safe in multiple trials for a range of chronic (inflammatory) diseases, nicotinamide could therefore be considered an attractive compound, even in long term use for human adults and children. This is specifically of interest when used as part of tapering and stop regimens for already proven and effective therapies with either DMARDS and/or biologicals. Long term treatment with both DMARDS and biologicals have potential side effects and can be a burden for both patients (as weekly or bi-weekly injections and for example intolerance complaints for MTX) and society (high costs). Development of novel stop-strategies, directed to decrease the chance of relapse of disease once DMARD and/or biologicals are stopped, by introducing maintenance therapy with nicotinamide as HDACi, are therefore both interesting and attractive in chronic disease such as JIA.

In conclusion, there seems to be potential for HDACi, in particularly for specific HDACi, in restoring immunological tolerance in JIA and other autoimmune diseases. However, the data on HDACi in arthritis and specifically JIA is still very limited and needs to be further explored. When considered (nicotinamide) or proven (other HDACi) safe, these agents could first be potentially used as adjuvant agents in stopping and tapering strategies for conventional immunosuppressive treatment with DMARDS/biologicals. First, the effectivity of HDACi in such strategies need to be tested in double blind randomized controlled clinical trial.

AUTHOR CONTRIBUTIONS

LN, JP, SV, and JvL wrote the manuscript. LN, JP, and JvL made figures and table. SV and JvL edited the manuscript.

REFERENCES

1. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. *Lancet* (2011) 377:2138–49. doi: 10.1016/S0140-6736(11)60244-4
2. Lovell DJ. Update on treatment of arthritis in children: New treatments, new goals. *Bull NYU Hosp Jt Dis.* (2006) 64:72–6.
3. Griffin TA, Barnes MG, Ilowite NT, Olson JC, Sherry DD, Gottlieb BS, et al. Gene expression signatures in polyarticular juvenile idiopathic arthritis demonstrate disease heterogeneity and offer a molecular classification of disease subsets. *Arthritis Rheum.* (2009) 60:2113–23. doi: 10.1002/art.24534
4. Mellins ED, Macaubas C, Grom AA. Pathogenesis of systemic juvenile idiopathic arthritis: some answers, more questions. *Nat Rev Rheumatol.* (2011) 7:416–26. doi: 10.1038/nrrheum.2011.68
5. Schneider R, Laxer RM. Systemic onset juvenile rheumatoid arthritis. *Baillieres Clin Rheumatol.* (1998) 12:245–71.
6. Behrens EM, Beukelman T, Paessler M, Cron RQ. Occult macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis. *J Rheumatol.* (2007) 34:1133–8.
7. Minoia F, Davi S, Horne A, Demirkaya E, Bovis F, Li C, et al. Clinical features, treatment, and outcome of macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a multinational, multicenter study of 362 patients. *Arthritis Rheumatol.* (2014) 66:3160–9. doi: 10.1002/art.38802
8. Lin Y, Wang C, Gershwin ME, Chiang B. Autoimmunity reviews the pathogenesis of oligoarticular/polyarticular vs systemic juvenile idiopathic arthritis. *Autoimmun Rev.* (2011) 10:482–9. doi: 10.1016/j.autrev.2011.02.001
9. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17–producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum.* (2008) 58:875–87. doi: 10.1002/art.23291
10. Wedderburn LR, Robinson N, Patel A, Varsani H, Woo P. Selective recruitment of polarized T cells expressing CCR5 and CXCR3 to the inflamed joints of children with juvenile idiopathic arthritis. *Arthritis Rheum.* (2000) 43:765. doi: 10.1002/1529-0131(200004)43:4<765::AID-ANR7>3.0.CO;2-B
11. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* (2000) 101:455–8. doi: 10.1016/S0092-8674(00)80856-9
12. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* (1995) 155:1151–64.
13. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* (2001) 27:20–1. doi: 10.1038/83713
14. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet.* (2001) 27:18–20. doi: 10.1038/83707
15. de Kleer I. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the CD4+CD25+ immune regulatory network. *Blood* (2006) 107:1696–702. doi: 10.1182/blood-2005-07-2800
16. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4+CD25bright Regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol.* (2004) 172:6435–43. doi: 10.4049/jimmunol.172.10.6435
17. Kitagawa Y, Sakaguchi S. Molecular control of regulatory T cell development and function. *Curr Opin Immunol.* (2017) 49:64–70. doi: 10.1016/j.coi.2017.10.002
18. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, et al. Foxp3+CD25+CD4+ natural regulatory T cells in dominant

self-tolerance and autoimmune disease. *Immunol Rev.* (2006) 212:8–27. doi: 10.1111/j.0105-2896.2006.00427.x
19. Wehrens EJ, Prakken BJ, Van Wijk F. T cells out of control-impaired immune regulation in the inflamed joint. *Nat Rev Rheumatol.* (2013) 9:34–42. doi: 10.1038/nrrheum.2012.149
20. Nistala K, Wedderburn LR. Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. *Rheumatology* (2009) 48:602–6. doi: 10.1093/rheumatology/kep028
21. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, Van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood* (2011) 118:3538–48. doi: 10.1182/blood-2010-12-328187
22. Prakken B, Ellen Wehrens, Van Wijk F. Editorial: Quality or quantity? unraveling the role of treg cells in rheumatoid arthritis. *Arthritis Rheum.* (2013) 65:552–4. doi: 10.1002/art.37831
23. Bulatović Calasan M, Vastert SJ, Scholman RC, Verweij F, Klein M, Wulffraat NM, et al. Methotrexate treatment affects effector but not regulatory T cells in juvenile idiopathic arthritis. *Rheumatology* (2015) 54: 1724–34. doi: 10.1093/rheumatology/kev101
24. Petrelli A, Wehrens EJ, Scholman RC, Prakken BJ, Vastert SJ, van Wijk F. Self-Sustained resistance to suppression of CD8+ Teff Cells at the site of autoimmune inflammation can be reversed by tumor necrosis factor and interferon-gamma blockade. *Arthritis Rheumatol.* (2016) 68:229–36. doi: 10.1002/art.39418
25. van Loosdregt J, van Wijk F, Prakken B, Vastert B. Update on research and clinical translation on specific clinical areas from biology to bedside: unpacking the mysteries of juvenile idiopathic arthritis pathogenesis. *Best Pract Res Clin Rheumatol.* (2017) 31:460–75. doi: 10.1016/j.berh.2018.02.003
26. Wehrens EJ, Vastert SJ, Mijnheer G, Meerding J, Klein M, Wulffraat NM, et al. Brief report: anti-tumor necrosis factor α targets protein kinase B/c-akt-induced resistance of effector cells to suppression in juvenile idiopathic arthritis. *Arthritis Rheum.* (2013) 65:3279–84 doi: 10.1002/art.38132
27. Foell D, Wulffraat N, Wedderburn LR, Wittkowski H, Frosch M, Gerss J, et al. Methotrexate withdrawal at 6 vs 12 months in juvenile idiopathic arthritis in remission: a randomized clinical trial. *JAMA* (2010) 303:1266–73. doi: 10.1001/jama.2010.375
28. Guzman J, Oen K, Huber AM, Watanabe Duffy K, Boire G, Shiff N, et al. The risk and nature of flares in juvenile idiopathic arthritis: results from the ReACCh-Out cohort. *Ann Rheum Dis.* (2016) 75:1092–8. doi: 10.1136/annrheumdis-2014-207164
29. Lin H-Y, Chen C-S, Lin S-P, Weng J-R, Chen C-S. Targeting histone deacetylase in cancer therapy. *Med Res Rev.* (2006) 26:397–413. doi: 10.1002/med.20056
30. Kornberg RD. Structure of chromatin. *Annu Rev Biochem.* (1977) 46:931–54. doi: 10.1146/annurev.bi.46.070177.004435
31. Zlatanova J. Histone H1 and the regulation of transcription of eukaryotic genes. *Trends Biochem Sci.* (1990) 15:273–276.
32. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Molecular Biology of the Cell.* New York, NY: Garland Science (2002).
33. Clayton AL, Hazzalin CA, Mahadevan LC. enhanced histone acetylation and transcription: a dynamic perspective. *Mol Cell.* (2006) 23:289–96. doi: 10.1016/j.molcel.2006.06.017
34. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem.* (2007) 76:75–100. doi: 10.1146/annurev.biochem.76.052705.162114
35. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet.* (2009) 10:32–42. doi: 10.1038/nrg2485
36. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* (2004) 429:457–463. doi: 10.1038/nature02625
37. Nusinzon I, Horvath CM. Positive and negative regulation of the innate antiviral response and beta interferon gene expression by deacetylation. *Mol Cell Biol.* (2006) 26:3106–13. doi: 10.1128/MCB.26.8.3106-3113.2006
38. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol Cancer Ther.* (2003) 2:151–163.
39. Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, et al. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med.* (2001) 7:437–43. doi: 10.1038/86507
40. Nair AR, Boersma LJ, Schiltz L, Chaudhry MA, Muschel RJ. Paradoxical effects of trichostatin a: inhibition of NF-Y-associated histone acetyltransferase activity, phosphorylation of hGCN5 and downregulation of cyclin A and B1 mRNA. *Cancer Lett.* (2001) 166:55–64. doi: 10.1016/S0304-3835(01)00418-9
41. Tong X, Yin L, Giardina C. Butyrate suppresses Cox-2 activation in colon cancer cells through HDAC inhibition. *Biochem Biophys Res Commun.* (2004) 317:463–71. doi: 10.1016/j.bbrc.2004.03.066
42. de Ruijter AJM, van Gennip AH, Caron HN, Kemp S, van Kuilenburg ABP. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J.* (2003) 370:737–49. doi: 10.1042/BJ20021321
43. Hu N, Qiu X, Luo Y, Yuan J, Li Y, Lei W, et al. Abnormal histone modification patterns in lupus CD4+ T cells. *J Rheumatol.* (2008) 35:804–10.
44. Horiuchi M, Morinobu A, Chin T, Sakai Y, Kurosaka M, Kumagai S. Expression and function of histone deacetylases in rheumatoid arthritis synovial fibroblasts. *J Rheumatol.* (2009) 36:1580–9. doi: 10.3899/jrheum.081115
45. Gillespie J, Savic S, Wong C, Hempshall A, Inman M, Emery P, et al. Histone deacetylases are dysregulated in rheumatoid arthritis and a novel histone deacetylase 3-selective inhibitor reduces interleukin-6 production by peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Rheum.* (2012) 64:418–22. doi: 10.1002/art.33382
46. Peeters JGC, Vervoort SJ, Tan SC, Mijnheer G, de Roock S, Vastert SJ, et al. Inhibition of super-enhancer activity in autoinflammatory site-derived t cells reduces disease-associated gene expression. *Cell Rep.* (2015) 12:1986–96. doi: 10.1016/j.celrep.2015.08.046
47. Farh KK-H, Marson A, Zhu J, Kleinewietfeld M, Housley WJ, Beik S, et al. Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* (2015) 518:337–43. doi: 10.1038/nature13835
48. Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, et al. Systematic localization of common disease-associated variation in regulatory DNA. *Science* (2012) 337:1190–5. doi: 10.1126/science.1222794
49. Spange S, Wagner T, Heinzel T, Kramer OH. Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int J Biochem Cell Biol.* (2009) 41:185–98. doi: 10.1016/j.biocel.2008.08.027
50. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* (2009) 325:834–40. doi: 10.1126/science.1175371
51. Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol.* (2011) 32:335–43. doi: 10.1016/j.it.2011.04.001
52. Grabiec AM, Tak PP, Reedquist KA. Function of histone deacetylase inhibitors in inflammation. *Crit Rev Immunol.* (2011) 31:233–263.
53. Grausenburger R, Bilic I, Boucheron N, Zupkovitz G, El-Housseiny L, Tschismarov R, et al. Conditional deletion of histone deacetylase 1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production. *J Immunol.* (2010) 185:3489–97. doi: 10.4049/jimmunol.0903610
54. Goschl L, Preglej T, Hamminger P, Bonelli M, Andersen L, Boucheron N, et al. A T cell-specific deletion of HDAC1 protects against experimental autoimmune encephalomyelitis. *J Autoimmun.* (2018) 86:51–61. doi: 10.1016/j.jaut.2017.09.008
55. Wang L, Samanta A, Levine M, Beier U, Han R, Kalin J, et al. Vital Role of CoREST Complex as a master regulator of Foxp3+ T-regulatory cell gene expression and suppressive function. *Am J Transpl.* (2017) 17(Suppl. 3).
56. Dovey OM, Foster CT, Conte N, Edwards SA, Edwards JM, Singh R, et al. Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic stability in mice. *Blood* (2013) 121:1335–44. doi: 10.1182/blood-2012-07-441949
57. Heideman MR, Wilting RH, Yanover E, Velds A, de Jong J, Kerkhoven RM, et al. Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function. *Blood* (2013) 121:2038–50. doi: 10.1182/blood-2012-08-450916
58. Philips RL, Chen MW, McWilliams DC, Belmonte PJ, Constans MM, Shapiro VS. HDAC3 is required for the downregulation of RORgammat

during thymocyte positive selection. *J Immunol.* (2016) 197:541–54. doi: 10.4049/jimmunol.1502529
59. Stengel KR, Zhao Y, Klus NJ, Kaiser JF, Gordy LE, Joyce S, et al. Histone deacetylase 3 is required for efficient T cell development. *Mol Cell Biol.* (2015) 35:3854–65. doi: 10.1128/MCB.00706-15
60. Hsu F-C, Belmonte PJ, Constans MM, Chen MW, McWilliams DC, Hiebert SW, et al. Histone deacetylase 3 is required for T cell maturation. *J Immunol.* (2015) 195:1578–90. doi: 10.4049/jimmunol.1500435
61. Wang L, Liu Y, Han R, Beier UH, Bhatti TR, Akimova T, et al. FOXP3+ regulatory T cell development and function require histone/protein deacetylase 3. *J Clin Invest.* (2015) 125:1111–23. doi: 10.1172/JCI77088
62. Wang L, Beier UH, Akimova T, Dahiya S, Han R, Samanta A, et al. Histone/protein deacetylase inhibitor therapy for enhancement of Foxp3+ T-regulatory cell function posttransplantation. *Am J Transl.* (2018) 18:1596–603. doi: 10.1111/ajt.14749
63. Liu Q, Zhang X, Yin C, Chen X, Zhang Z, Brown S, et al. HDAC4 is expressed on multiple T cell lineages but dispensable for their development and function. *Oncotarget* (2017) 8:17562–72. doi: 10.18632/oncotarget.15077
64. Guo S, Zhu Q, Jiang T, Wang R, Shen Y, Zhu X, et al. Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis. *Mod Rheumatol.* (2017) 27:441–7. doi: 10.1080/14397595.2016.1218595
65. Xiao H, Jiao J, Wang L, O'Brien S, Newick K, Wang L-CS, et al. HDAC5 controls the functions of Foxp3(+) T-regulatory and CD8(+) T cells. *Int J Cancer* (2016) 138:2477–86. doi: 10.1002/ijc.29979
66. Kasler HG, Young BD, Mottet D, Lim HW, Collins AM, Olson EN, et al. Histone deacetylase 7 regulates cell survival and TCR signaling in CD4/CD8 double-positive thymocytes. *J Immunol.* (2011) 186:4782–93. doi: 10.4049/jimmunol.1001179
67. Kasler HG, Lim HW, Mottet D, Collins AM, Lee IS, Verdin E. Nuclear export of histone deacetylase 7 during thymic selection is required for immune self-tolerance. *EMBO J.* (2012) 31:4453–65. doi: 10.1038/emboj.2012.295
68. Kasler HG, Verdin E. Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. *Mol Cell Biol.* (2007) 27:5184–200. doi: 10.1128/MCB.02091-06
69. Dequiedt F, Kasler H, Fischle W, Kiermer V, Weinstein M, Herndier BG, et al. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. *Immunity* (2003) 18:687–698. doi: 10.1016/S1074-7613(03)00109-2
70. Tao R, Hancock WW. Resistance of Foxp3+ regulatory T cells to Nur77-induced apoptosis promotes allograft survival. *PLoS ONE* (2008) 3:e2321. doi: 10.1371/journal.pone.0002321
71. Yan K, Cao Q, Reilly CM, Young NL, Garcia BA, Mishra N. Histone deacetylase 9 deficiency protects against effector T cell-mediated systemic autoimmunity. *J Biol Chem.* (2011) 286:28833–43. doi: 10.1074/jbc.M111.233932
72. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med.* (2007) 13:1299–307. doi: 10.1038/nm1652
73. de Zoeten EF, Wang L, Sai H, Dillmann WH, Hancock WW. Inhibition of HDAC9 Increases T regulatory cell function and prevents colitis in Mice. *Gastroenterology* (2010) 138:583–94 doi: 10.1053/j.gastro.2009.10.037
74. Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Sci Signal* (2012) 5:ra45. doi: 10.1126/scisignal.2002873
75. Yan B, Liu Y, Bai H, Chen M, Xie S, Li D, et al. HDAC6 regulates IL-17 expression in T lymphocytes: implications for HDAC6-targeted therapies. *Theranostics* (2017) 7:1002–9. doi: 10.7150/thno.17615
76. de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H, et al. Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. *Mol Cell Biol.* (2011) 31:2066–78. doi: 10.1128/MCB.05155-11
77. Kalin JH, Butler KV, Akimova T, Hancock WW, Kozikowski AP. Second-generation histone deacetylase 6 inhibitors enhance the immunosuppressive effects of Foxp3+ T-regulatory cells. *J Med Chem.* (2012) 55:639–51. doi: 10.1021/jm200773h
78. Segretti MCF, Vallerini GP, Brochier C, Langley B, Wang L, Hancock WW, et al. Thiol-based potent and selective HDAC6 inhibitors promote tubulin acetylation and t-regulatory cell suppressive function. *ACS Med Chem Lett.* (2015) 6:1156–61. doi: 10.1021/acsmedchemlett.5b00303
79. Oh BR, Suh D, Bae D, Ha N, Choi Y Il, Yoo HJ, et al. Therapeutic effect of a novel histone deacetylase 6 inhibitor, CKD-L, on collagen-induced arthritis *in vivo* and regulatory T cells in rheumatoid arthritis *in vitro*. *Arthritis Res Ther.* (2017) 19:154. doi: 10.1186/s13075-017-1357-2
80. Dahiya S, Wang L, Beier ulf H, Han R, Hancock WW. HDAC10 Targeting Regulates Foxp3 Promoter, enhances T-regulatory (Treg) function and suppresses autoimmune colitis. *J.Immunol.* (2018) 200 (Suppl. 1) 54.11.
81. Huang J, Wang L, Dahiya S, Beier UH, Han R, Samanta A, et al. Histone/protein deacetylase 11 targeting promotes Foxp3+ Treg function. *Sci Rep.* (2017) 7:8626. doi: 10.1038/s41598-017-09211-3
82. Woods DM, Woan KV, Cheng F, Sodre AL, Wang D, Wu Y, et al. T cells lacking HDAC11 have increased effector functions and mediate enhanced alloreactivity in a murine model. *Blood* (2017) 130:146–55. doi: 10.1182/blood-2016-08-731505
83. Niederer F, Ospelt C, Brentano F, Hottiger MO, Gay RE, Gay S, et al. SIRT1 overexpression in the rheumatoid arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance. *Ann Rheum Dis.* (2011) 70:1866–73. doi: 10.1136/ard.2010.148957
84. Moon M-H, Jeong J-K, Lee Y-J, Seol J-W, Jackson CJ, Park S-Y. SIRT1, a class III histone deacetylase, regulates TNF-alpha-induced inflammation in human chondrocytes. *Osteoarthr Cartil.* (2013) 21:470–80. doi: 10.1016/j.joca.2012.11.017
85. Huang W, Shang W, Wang H, Wu W, Hou S. Sirt1 overexpression protects murine osteoblasts against TNF-alpha-induced injury *in vitro* by suppressing the NF-kappaB signaling pathway. *Acta Pharmacol Sin.* (2012) 33:668–74. doi: 10.1038/aps.2011.189
86. Zhang J, Lee S-M, Shannon S, Gao B, Chen W, Chen A, et al. The type III histone deacetylase Sirt1 is essential for maintenance of T cell tolerance in mice. *J Clin Invest.* (2009) 119:3048–58. doi: 10.1172/JCI38902
87. Beier UH, Wang L, Bhatti TR, Liu Y, Han R, Ge G, Hancock WW. Sirtuin-1 targeting promotes Foxp3+ T-regulatory cell function and prolongs allograft survival. *Mol Cell Biol.* (2011) 31:1022–9. doi: 10.1128/MCB.01206-10
88. Van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YYJ, Beekman JM, Van Beekum O, et al. Regulation of treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* (2010) 115:965–74. doi: 10.1182/blood-2009-02-207118
89. Wang Y, Bi Y, Chen X, Li C, Li Y, Zhang Z, et al. Histone deacetylase SIRT1 negatively regulates the differentiation of interleukin-9-producing CD4(+) T Cells. *Immunity* (2016) 44:1337–49. doi: 10.1016/j.immuni.2016.05.009
90. Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc Natl Acad Sci USA.* (2007) 104:4571–6. doi: 10.1073/pnas.0700298104
91. Akimova T, Xiao H, Liu Y, Bhatti TR, Jiao J, Eruslanov E, et al. Targeting sirtuin-1 alleviates experimental autoimmune colitis by induction of Foxp3+ T-regulatory cells. *Mucosal Immunol.* (2014) 7:1209–20. doi: 10.1038/mi.2014.10
92. Lim HW, Kang SG, Ryu JK, Schilling B, Fei M, Lee IS, et al. SIRT1 deacetylates RORgammat and enhances Th17 cell generation. *J Exp Med.* (2015) 212:607–17. doi: 10.1084/jem.20132378
93. Ciarlo E, Heinonen T, Lugrin J, Acha-Orbea H, Le Roy D, Auwerx J, et al. Sirtuin 3 deficiency does not alter host defenses against bacterial and fungal infections. *Sci Rep.* (2017) 7:3853. doi: 10.1038/s41598-017-04263-x
94. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* (2006) 5:769–84. doi: 10.1038/nrd2133
95. Weidle UH, Grossmann A. Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. *Anticancer Res.* (2000) 20:1471–85.
96. McGovern JL, Nguyen DX, Notley CA, Mauri C, Isenberg DA, Ehrenstein MR. Th17 cells are restrained by treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy. *Arthritis Rheum* (2012) doi: 10.1002/art.34565

97. Subramanian S, Bates SE, Wright JJ, Espinoza-delgado I, Piekarz RL. Clinical toxicities of histone deacetylase inhibitors. *Pharmaceuticals* (2010) 3:2751–67. doi: 10.3390/ph3092751
98. Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, et al. Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem.* (2000) 275:20436–43. doi: 10.1074/jbc.M000202200
99. Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* (2000) 408:377–81. doi: 10.1038/35042612
100. Ito A, Kawaguchi Y, Lai C-H, Kovacs JJ, Higashimoto Y, Appella E, et al. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *EMBO J.* (2002) 21:6236–45. doi: 10.1093/emboj/cdf616
101. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* (2001) 107:149–59.10. doi: 10.1016/S0092-8674(01)00527-X
102. Kuo Y-H, Qi J, Cook GJ. Regain control of p53: Targeting leukemia stem cells by isoform-specific HDAC inhibition. *Exp Hematol.* (2016) 44:315–21. doi: 10.1016/j.exphem.2016.02.007
103. Kim M, Thompson LA, Wenger SD, O'Bryant CL. Romidepsin: a histone deacetylase inhibitor for refractory cutaneous T-cell lymphoma. *Ann Pharmacother.* (2012) 46:1340–8. doi: 10.1345/aph.1R036
104. Bishton MJ, Harrison SJ, Martin BP, McLaughlin N, James C, Josefsson EC, et al. Deciphering the molecular and biologic processes that mediate histone deacetylase inhibitor-induced thrombocytopenia. *Blood* (2011) 117:3658–68. doi: 10.1182/blood-2010-11-318055
105. Zhang X, Han S, Kang Y, Guo M, Hong S, Liu F, et al. SAHA, an HDAC inhibitor, synergizes with tacrolimus to prevent murine cardiac allograft rejection. *Cell Mol Immunol.* (2012) 9:390–8. doi: 10.1038/cmi.2012.28
106. Choi SW, Gatza E, Hou G, Sun Y, Whitfield J, Song Y, et al. Histone deacetylase inhibition regulates inflammation and enhances Tregs after allogeneic hematopoietic cell transplantation in humans. *Blood* (2015) 125:815–9. doi: 10.1182/blood-2014-10-605238
107. Choi SW, Braun T, Chang L, Ferrara JLM, Pawarode A, Magenau JM, et al. Vorinostat plus tacrolimus and mycophenolate to prevent graft-versus-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: a phase 1/2 trial. *Lancet Oncol.* (2014) 15:87–95. doi: 10.1016/S1470-2045(13)70512-6
108. Regna NL, Chafin CB, Hammond SE, Puthiyaveetil AG, Caudell DL, Reilly CM. Class I and II histone deacetylase inhibition by ITF2357 reduces SLE pathogenesis *in vivo*. *Clin Immunol.* (2014) 151:29–42. doi: 10.1016/j.clim.2014.01.002
109. Joosten LAB, Leoni F, Meghji S, Mascagni P. Inhibition of HDAC activity by ITF2357 ameliorates joint inflammation and prevents cartilage and bone destruction in experimental arthritis. *Mol Med.* (2011) 17:391–6. doi: 10.2119/molmed.2011.00058
110. Furlan A, Monzani V, Reznikov LL, Leoni F, Fossati G, Modena D, et al. Pharmacokinetics, safety and inducible cytokine responses during a phase 1 trial of the oral histone deacetylase inhibitor ITF2357 (givinostat). *Mol Med.* (2011) 17:353–62. doi: 10.2119/molmed.2011.00020
111. Vojinovic J, Damjanov N. HDAC inhibition in rheumatoid arthritis and juvenile idiopathic arthritis. *Mol Med* (2011) 17:397–403. doi: 10.2119/molmed.2011.00030
112. Saouaf SJ, Li B, Zhang G, Shen Y, Furuuchi N, Hancock WW, et al. Deacetylase inhibition increases regulatory T cell function and decreases incidence and severity of collagen-induced arthritis. *Exp Mol Pathol.* (2009) 87:99–104. doi: 10.1016/j.yexmp.2009.06.003
113. Kim DS, Kwon J-E, Lee SH, Kim EK, Ryu J-G, Jung K-A, et al. Attenuation of Rheumatoid Inflammation by Sodium Butyrate Through Reciprocal Targeting of HDAC2 in Osteoclasts and HDAC8 in T Cells. *Front Immunol.* (2018) 9:1525. doi: 10.3389/fimmu.2018.01525
114. Levine MH, Wang Z, Xiao H, Jiao J, Wang L, Bhatti TR, et al. Targeting Sirtuin-1 prolongs murine renal allograft survival and function. *Kidney Int.* (2016) 89:1016–26. doi: 10.1016/j.kint.2015.12.051
115. Grabiec AM, Krausz S, de Jager W, Burakowski T, Groot D, Sanders ME, et al. Histone deacetylase inhibitors suppress inflammatory activation of rheumatoid arthritis patient synovial macrophages and tissue. *J Immunol.* (2010) 184:2718–28. doi: 10.4049/jimmunol.0901467
116. Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, et al. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proc Natl Acad Sci USA.* (2002) 99:2995–3000. doi: 10.1073/pnas.052702999
117. Li Y, Liu B, Zhao H, Sailhamer EA, Fukudome EY, Zhang X, et al. Protective effect of suberoylanilide hydroxamic acid against LPS-induced septic shock in rodents. *Shock* (2009) 32:517–23. doi: 10.1097/SHK.0b013e3181a44c79
118. Leoni F, Fossati G, Lewis EC, Lee J-K, Porro G, Pagani P, et al. The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines *in vitro* and systemic inflammation *in vivo*. *Mol Med.* (2005) 11:1–15. doi: 10.2119/2006-00005.Dinarello
119. Choo Q-Y, Ho PC, Tanaka Y, Lin H-S. Histone deacetylase inhibitors MS-275 and SAHA induced growth arrest and suppressed lipopolysaccharide-stimulated NF-kappaB p65 nuclear accumulation in human rheumatoid arthritis synovial fibroblastic E11 cells. *Rheumatology* (2010) 49:1447–60. doi: 10.1093/rheumatology/keq108
120. Angiolilli C, Kabala PA, Grabiec AM, Van Baarsen IM, Ferguson BS, Garcia S, et al. Histone deacetylase 3 regulates the inflammatory gene expression programme of rheumatoid arthritis fibroblast-like synoviocytes. *Ann Rheum Dis.* (2017) 76:277–85. doi: 10.1136/annrheumdis-2015-209064
121. Angiolilli C, Kabala PA, Grabiec AM, Rossato M, Lai WS, Fossati G, et al. Control of cytokine mRNA degradation by the histone deacetylase inhibitor ITF2357 in rheumatoid arthritis fibroblast-like synoviocytes: beyond transcriptional regulation. *Arthritis Res Ther.* (2018) 20:148. doi: 10.1186/s13075-018-1638-4
122. Huber K, Doyon G, Plaks J, Fyne E, Mellors JW, Sluis-Cremer N. Inhibitors of histone deacetylases: correlation between isoform specificity and reactivation of HIV type 1 (HIV-1) from latently infected cells. *J Biol Chem.* (2011) 286:22211–8. doi: 10.1074/jbc.M110.180224
123. Berni Canani R, Di Costanzo M, Leone L. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. *Clin Epigenet.* (2012) 4:4. doi: 10.1186/1868-7083-4-4
124. Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr.* (2003) 133:2485S–93S. doi: 10.1093/jn/133.7.2485S
125. Meijer K, de Vos P, Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? *Curr Opin Clin Nutr Metab Care* (2010) 13:715–21. doi: 10.1097/MCO.0b013e32833eebe5
126. Carlson LA. Nicotinic acid: the broad-spectrum lipid drug. a 50th anniversary review. *J Intern Med.* (2005) 258:94–114. doi: 10.1111/j.1365-2796.2005.01528.x
127. Visalli N, Cavallo MG, Signore A, Baroni MG, Buzzetti R, Fioriti E, et al. A multi-centre randomized trial of two different doses of nicotinamide in patients with recent-onset type 1 diabetes (the IMDIAB VI). *Diabetes Metab Res Rev.* (1999) 15:181–5.
128. Chianelli M, Parisella MG, Visalli N, Mather SJ, D'Alessandria C, Pozzilli P, et al. Pancreatic scintigraphy with 99mTc-interleukin-2 at diagnosis of type 1 diabetes and after 1 year of nicotinamide therapy. *Diabetes Metab Res Rev.* (2008) 24:115–22. doi: 10.1002/dmrr.767
129. Lampeter EF, Klinghammer A, Scherbaum WA, Heinze E, Haastert B, Giani G, et al. The Deutsche Nicotinamide Intervention Study: an attempt to prevent type 1 diabetes. DENIS Group. *Diabetes* (1998) 47:980–4.
130. Gale EAM, Bingley PJ, Emmett CL, Collier T. European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. *Lancet* (2004) 363:925–31. doi: 10.1016/S0140-6736(04)15786-3
131. Pozzilli P, Browne PD, Kolb H. Meta-analysis of nicotinamide treatment in patients with recent-onset IDDM. The Nicotinamide Trialists. *Diabetes Care* (1996) 19:1357–63.
132. Crino A, Schiaffini R, Manfrini S, Mesturino C, Visalli N, Beretta Anguissola G, et al. A randomized trial of nicotinamide and vitamin E in children with recent onset type 1 diabetes (IMDIAB IX). *Eur J Endocrinol.* (2004) 150:719–24.
133. HOFFER A. Treatment of arthritis by nicotinic acid and nicotinamide. *Can Med Assoc J.* (1959) 81:235–238.
134. Kamal M, Abbasy A-J Al, Muslemani A Al, Bener A. Effect of nicotinamide on newly diagnosed type 1 diabetic children. *Acta Pharmacol Sin.* (2006) 27:724–27. doi: 10.1111/j.1745-7254.2006.00313.x

135. Olmos PR, Hodgson MI, Maiz A, Manrique M, De Valdes MD, Foncea R, et al. Nicotinamide protected first-phase insulin response (FPIR) and prevented clinical disease in first-degree relatives of type-1 diabetics. *Diabetes Res Clin Pract.* (2006) 71:320–33. doi: 10.1016/j.diabres.2005.07.009
136. Pitocco D, Crino A, Di Stasio E, Manfrini S, Guglielmi C, Spera S, et al. The effects of calcitriol and nicotinamide on residual pancreatic beta-cell function in patients with recent-onset Type 1 diabetes (IMDIAB XI). *Diabet Med.* (2006) 23:920–3. doi: 10.1111/j.1464-5491.2006.01921.x
137. Knip M, Douek IF, Moore WP, Gillmor HA, McLean AE, Bingley PJ, et al. Safety of high-dose nicotinamide: a review. *Diabetologia* (2000) 43:1337–45. doi: 10.1007/s001250051536

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REVIEW
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B Cells as a Therapeutic Target in Paediatric Rheumatic Disease

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B cells carry out a central role in the pathogenesis of autoimmune disease. In addition to the production of autoantibodies, B cells can contribute to disease development by presenting autoantigens to autoreactive T cells and by secreting pro-inflammatory cytokines and chemokines which leads to the amplification of the inflammatory response. Targeting both the antibody-dependent and antibody-independent function of B cells in adult rheumatic disease has led to the advent of B cell targeted therapies in clinical practice. To date, whether B cell depletion could also be utilized for the treatment of pediatric disease is relatively under explored. In this review, we will discuss the role of B cells in the pathogenesis of the pediatric rheumatic diseases Juvenile Idiopathic Arthritis (JIA), Juvenile Systemic Lupus Erythematosus (JSLE) and Juvenile Dermatomyositis (JDM). We will also explore the rationale behind the use of B cell-targeted therapies in pediatric rheumatic disease by highlighting new case studies that points to their efficacy in JIA, JSLE, and JDM.

Keywords: B cells, autoimmunity, pediatric autoimmune diseases, inflammation, biologics

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INTRODUCTION—B CELLS

The most well-defined function of B cells is their ability to produce antibodies as part of the humoral immune response. However, other described roles of B cells in the immune system have recently emerged including the ability to present both peptide and lipid antigens and to produce an array of pro and anti-inflammatory cytokines (1–3). Following development in the bone marrow where they undergo processes that aim to ensure tolerance, B cells are released into the periphery as antigen-inexperienced cells. Upon activation and exposure to antigen, B cells proliferate and differentiate into antibody-producing plasma cells or memory B cells, or, depending upon the cytokine environment in which they find themselves, into cytokine-producing B effector (Be) cells (For detailed information see **Box 1**). Naïve B cells differentiate into Be-1, which produce type 1 cytokines, or Be-2 cells, which produce type 2 cytokines, when co-cultured with polarized T helper (Th)-1 or Th-2 cells, respectively (4). B cells can also produce immunoregulatory cytokines such as IL-10, IL-35, and TGFβ (5, 6). Production of immunoregulatory cytokines by B cells is usually attributed to a specialized subset of B cells known as regulatory B cells which directly influence T cell function in humans by suppressing the differentiation of Th-1/Th-17 cells and inducing the differentiation of T regulatory cells (Tregs) (5). B cell activation also leads to the upregulation of molecules that mediate antigen-presentation, such as MHC class II (MHC class II), or co-stimulatory molecules such as CD86/CD80. In some B cells, activation induces class-switching, the process by which B cells change their immunoglobulin class from one to another (i.e., IgM to IgG/IgA/IgE). All together, these diverse functions within the adaptive immune response make B cells an important target for investigation in rheumatic disease.

Murine models have now established that both antibody-dependent and antibody-independent functions of B cells contribute to development of experimental models of autoimmune disease. Seminal examples include the observation that B cell-deficient mice do not develop collagen-induced arthritis (7), that CD80/CD86 expression on B cells is required for autoreactive T cell activation and the development of arthritis (8), and that chimeric mice lacking IL-10 expressing B cells develop an exacerbated arthritis that is driven by an expanded Th17 compartment (9). These data have now been translated into human disease (10, 11). However, to date, the majority of studies have focused on the role of B cell pathology in adult rheumatic disease. Although these data have underpinned the rationale for the advent of B cell depletion therapy in adult-onset disease, there is comparatively a scarcity of studies investigating whether a similar rationale could be applied to pediatric disease. In this review, we will provide a broad overview of the current evidence that B cell abnormalities drive aspects of pathology in the most common pediatric inflammatory rheumatic diseases. We will also summarize current case studies and clinical trials demonstrating that targeting B cells in these diseases could be invaluable, especially in patients whose disease is refractory to first-line therapies.

JUVENILE IDIOPATHIC ARTHRITIS

Juvenile Idiopathic Arthritis (JIA) is an umbrella term that encompasses a heterogenous group of conditions with a wide spectrum of outcomes. The most common rheumatic disease in childhood, JIA is characterized by joint swelling that lasts for longer than 6 weeks and that develops before the age of 16, and can cause significant disability and loss of quality of life if not controlled (21). Within this group several subtypes of JIA exist, including oligoarticular (oligo-JIA), polyarticular (poly-JIA), enthesitis-related arthritis (ERA), psoriatic arthritis (PsA), and systemic JIA (21), as defined by the International League of Associations for Rheumatology (ILAR). At present there is no cure for JIA, which means that current clinical strategy focuses on achieving clinical remission (defined as no joint inflammation and no secondary characteristics of disease such as rash, fever, serositis and uveitis (22, 23). In the absence of clinical remission, treatment is quickly escalated from the use of non-steroidal anti-inflammatory drugs to include intra-articular steroids, disease modifying anti-rheumatic drugs such as methotrexate, and in the case of methotrexate failure to expensive biologic therapies (24). The first-line biologic used in JIA treatment is TNF inhibition, and despite being well-tolerated in many children, there remains a subset of children whose disease remain uncontrolled (25, 26). At present, therapeutic strategy is defined by failure of medication in controlling disease severity as there are no clinical or biological tools that allow stratification of patients before treatment is started. Greater understanding concerning the biological pathways underlying disease development will improve stratification of patients and increase early-remission rates.

Apart from systemic JIA, which is considered to be an autoinflammatory disorder whose pathogenesis is thought to be associated with IL-6 dysregulation and macrophage activation (and will therefore not be discussed further in this review) (21), the other subtypes of JIA are classically thought of as autoimmune, T cell driven diseases. A particular role for IL-17 producing T helper cells (Th17) has been postulated, which may be particularly dominant in the extended oligo-JIA and ERA forms of JIA (27, 28). However, subtypes of JIA can also be characterized by different patterns of auto-antibody production, which implicates a central role for B cells in JIA pathogenesis. Further to autoantibody production such as anti-nuclear antibodies (ANAs), B-cells may contribute to disease pathogenesis by producing pro-inflammatory cytokines and by presenting auto-antigens to T-cells (29).

Autoantibodies

Anti-nuclear antibodies (ANA) are most commonly detected in both oligo-JIA and rheumatoid factor (RF) negative poly-JIA suggesting a B cell involvement in both patient groups. Although the exact targets of these ANA are yet to be defined, the presence of ANA provides strong evidence that B cell tolerance is altered in both oligo-JIA and RF- poly-JIA. Indeed, it is thought that ANA+ oligo-JIA and RF- poly-JIA patients would be better considered as one subtype of disease based on similarities in pathogenesis, risk for uveitis and early disease-onset (30). ANA+ oligo-JIA and RF- poly-JIA are confined to childhood and there is thought to be no equivalent in post-pubertal patients (31, 32). While the contribution of ANA to disease pathology remains unclear, recent research has suggested that ANA positivity is associated with the development of ectopic lymphoid tissue in certain JIA patients, which by facilitating interactions between autoreactive T and B cells could directly support the production of these autoantibodies (33). In contrast to RF- poly-JIA, RF+ poly-JIA has a later onset, and many clinical and genetic features (34) that are analogous with adult-onset rheumatoid arthritis (RA), including the development of anti-citrullinated (anti-CCP) and RF autoantibodies (35). Furthermore, the multinational JIA consortium for Immunochip (JACI) recently established that RF+ poly-JIA is more genetically similar to adult-onset RA than to oligo-JIA and RF- poly-JIA (34). This provides further evidence that these diseases are likely to have similar underlying pathological mechanisms and that current pharmacological strategies employed in RA are directly relevant to RF+ poly-JIA. Although the detection of RF is used diagnostically to subcategorize poly-JIA in RF- and RF+ patients, anti-CCP antibodies are not standardly measured in clinical practice in pediatric patients despite evidence from RA patients suggesting that they may be useful in defining patients with more severe clinical disease (36, 37). An autoantibody involvement, and therefore B cell component, in the development of ERA and PsA is much less well-defined, similarly to their adult-onset counterparts Ankylosing Spondylitis and Psoriatic Arthritis, respectively. Therefore, whether B cell depletion therapy would convey much in the way of benefit for these diseases is yet to be elucidated and as such are not routinely tested.

Box 1 | Life Cycle of a B Cell

The earliest stages of B cell development occur in the bone marrow, which provides a specialized environment containing non-lymphoid cells such as osteolineage cells and stromal cells. These cells support haematopoiesis and B cell development by secreting specific cytokines and growth factors such as IL-7, FLT3 ligand, stem cell factor (SCF), RANKL and CXCL12 (12). In this specialized environment, repression of FLT3 in haematopoietic stem cells and induction of the master transcription factor PAX5 commits these pluripotent cells to the B cell lineage as they differentiate into as pre-pro B cells. Movement through the early stages of B cell development are defined by the re-arrangement of immunoglobulin (Ig) genes, which ultimately leads to the expression of a functional antigen-specific B cell receptor (BCR). Briefly, Ig re-arrangement is initiated at the pro-B cell stage where the RAG-1/RAG-2 complex mediates recombination by inducing double strand breaks at recombination signal sequences flanking the variable (V), diversity (D), and joining (J) regions of the heavy chain locus. RAG enzymes and accessory molecules then coordinate the rejoining of V, D, and J segments, excising intervening DNA to give a single coding sequence that is ligated to the μ heavy chain locus. In frame rearrangements that allow surface expression of a functional heavy chain in association with λ5 and V preB (the pre-B cell receptor) result in cessation of heavy chain rearrangement and initiation of analogous Igκ rearrangement (13). Failure to produce a functional Igκ light chain following re-arrangement of the two Igκ light chain alleles leads to re-arrangement of the alleles within the Igλ light chain locus giving a B cell four attempts to produce a functional BCR (14). It is at this stage that the BCR is tested for autoreactivity, immature B cell undergo processes that ensue central tolerance by one of three processes: 1, receptor editing—immature B cells that react with low to high avidity self-antigens can undergo receptor editing by a secondary rearrangement at the Igκ or rearrangement of the Igλ allele; 2, Clonal anergy—immature B cells that react with low avidity self-antigen can migrate to the spleen as anergic B cells; 3, clonal deletion—immature B cells are deleted by apoptosis, which occurs at a low rate for those cells that fail receptor editing (15). It is important to note that much of what we know about the early life cycle of a B cell is derived from mouse studies and although informative there may be some differences, as yet undiscovered, in humans.

Once released from the bone marrow in humans, B cell subsets found in human peripheral blood can be broadly defined based on the expression of CD19, CD20, CD24, CD38, IgD, and CD27. These peripheral subsets include antigen-inexperienced immature B cells ($CD20^{+}CD19^{+}CD24^{+}CD38^{+}$ IgD^{+}) and mature naïve B cells ($CD20^{+}CD19^{+}CD24^{int}CD38^{int}$ IgD^{+}) as well as antigen-experienced memory B cells ($CD20^{+}CD19^{+}CD24^{+}CD38^{-}$ IgD^{-}), plasmablasts ($CD20^{+}CD19^{+}CD24^{-}CD38^{+}IgD^{-}$) and antibody-producing plasma cells ($CD20^{-}CD19^{-}CD24^{-}CD38^{+}IgD^{-}$) (16). Following activation with TLR-agonists, anti-CD40 (which models T-B cell interactions *in vitro*) or pro-inflammatory cytokines (e.g., IFNα), antigen-inexperienced B cells can differentiate into IL-10 producing regulatory B cells or depending upon the cytokine-polarizing environment differentiate into cytokine producing B effector cells (5, 17). Following activation and encounter with antigen, activated B cells can also seed germinal centers (GC). Within a GC, the introduction of point mutations into the V regions B cell receptor genes by activation-induced cytidine deaminase (AICD), a process called somatic hypermutation, and subsequent competition for antigen and survival signals from T-follicular helper cells (T_{FH}) by daughter B cells, a process called affinity maturation, promotes the expansion of B cells with high affinity ag-specific BCRs. Class switching of BCRs to different isotypes, guided by the nature of the immune response etc can also occur in GCs (18). Following maturation in a GC, terminally differentiated plasma cells can migrate back to the bone marrow where they can reside for up to the lifetime of the host (19, 20).The life cycle of a B cell and the markers used to identify the various stages of B cell development by flow cytometry are summarized in **Figure 1**.

The underlying mechanisms that lead to auto-antibody production in JIA are yet to be elucidated. Nevertheless, current data suggests that altered peripheral B cell homeostasis could be one contributing factor. CD5+ B cells, a potential human equivalent of murine B-1a cells, are expanded in the peripheral blood of patients with oligo-JIA and poly-JIA (38); B-1 cells have polyreactive B cell receptors (BCR) that recognize conserved sequences on bacterial pathogens and are therefore enriched for autoreactive epitopes. CD5+ B cells in humans are not as well-defined as there murine counterparts and may also represent a population of pre-naïve B cells (39) or activated B cells (40). There is also a reported expansion in $CD24^{hi}CD38^{hi}$ transitional B cells (41), a high proportion of which still express polyreactive BCRs, as they are still undergoing negative selection (42). These data suggest that B cell central tolerance is abnormal in JIA, leading to autoreactive B cells escaping negative selection to join the mature B cell pool. This hypothesis is supported by data that critical checkpoints in B cell tolerance are altered in JIA. For example, B cells from JIA patients are still able to undergo receptor revision in the periphery (43), a process usually restricted to the bone marrow, which is accompanied by skewed lamba:kappa light chain usage (44). Taken together, these data demonstrate that changes in the frequency of potentially autoreactive B cell subsets is accompanied with changes in the molecular events that control B cell tolerance in JIA. Future studies are needed to understand whether this directly contributes to the production of autoantibodies and how this effects JIA pathology in general.

Cytokine Production and Antigen-Presentation by B Cells

As described above, B cells can also act as effectors of immune response by presenting antigens and producing cytokines and significantly, the $CD24^{hi}CD38^{hi}$ transitional B cell compartment which is altered in JIA also contains regulatory B cells; $CD24^{hi}CD38^{hi}$ transitional B cells produce the highest amount of IL-10 following culture with agonistic anti-CD40 or CpG (10, 45). A recent study has reported that IL-10 production by $CD19^{+}CD24^{hi}CD38^{hi}$ B cells is reduced in a small cohort of JIA patients (46). There was a drastic reduction in IL-$10^{+}CD19^{+}CD24^{hi}CD38^{hi}$ in peripheral blood compared to controls, with an even greater reduction in synovial fluid (46). Interestingly, an initial analysis of the difference between RF+ and RF- JIA patients revealed that frequency of IL-10+$CD19^{+}CD24^{hi}CD38^{hi}$ was lower in RF+ patients. It has been previously published that $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs are drastically reduced in RA patients, and can no longer suppress Th17 induction (11). These results strongly suggest that similarly to their adult counterparts, that in RF+ JIA patients T cell abnormalities may be at least in part driven by a dysfunctional regulatory B cell compartment. Future functional studies are needed to confirm this hypothesis.

In conjunction with a paucity of $CD19^{+}CD24^{hi}CD38^{hi}$ in the synovial fluid of JIA patients (41), there is an expansion of switched memory B cells in the synovial fluid B cell compartment compared to the periphery. These switched memory B cells are phenotypically defined as $CD19^{+}CD27^{+}IgD^{-}$ (41) and express high levels of the transcript for IL-12p40. Importantly,

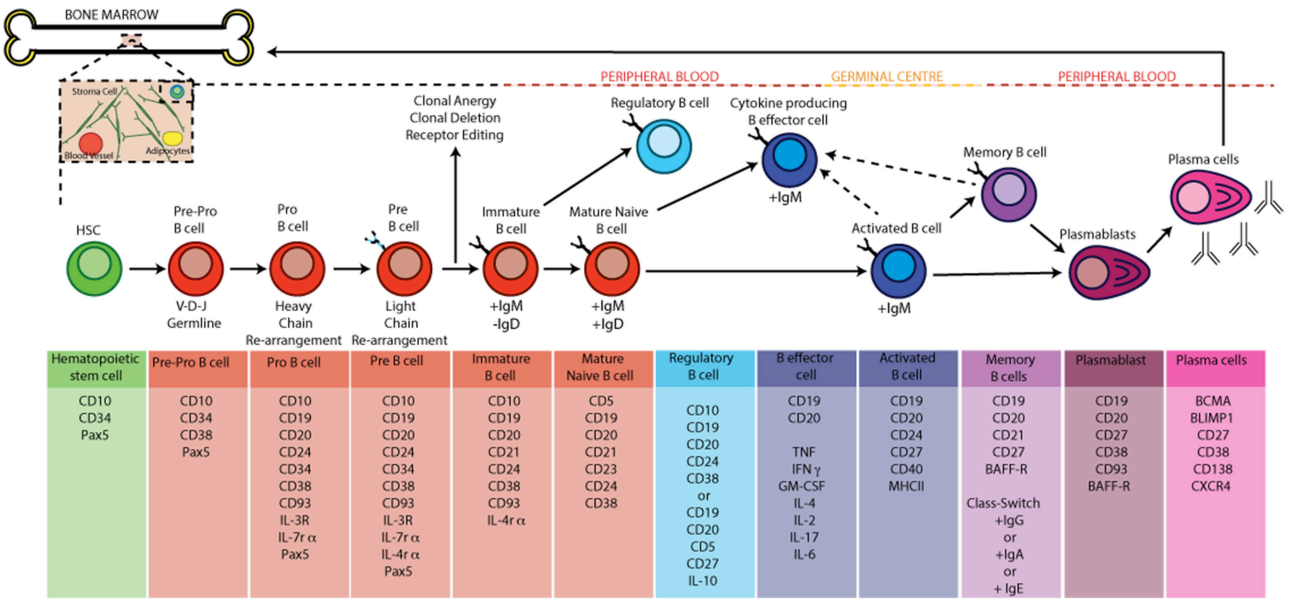

FIGURE 1 | B cell development and activation in humans**.** B cells develop from hematopoietic stem cells generated in the bone marrow. Pro-B cells express a unique antibody, the immunoglobulin (Ig) variable domain (Fv). They undergo re-arrangement of the heavy and light chains of this immunoglobulin (V-D-J and V-J recombination) leading to the development of pre-B cells. A pre-B cell acquires antigen specificity by the expression of a unique BCR and surface IgM as it matures into an immature B cell; at the this point the B cell under goes central tolerance by one of three processes: 1, receptor editing—immature B cells that react with low to high avidity self-antigens undergo receptor editing by a secondary rearrangement at the Igκ or rearrangement of the Igλ allele; 2, Clonal anergy—immature B cells that react with low avidity self-antigen can migrate to the spleen as anergic B cells; 3—clonal deletion: occurs at a low rate for those cells that fail receptor editing. If the immature B cell survives central tolerance they develop into mature antigen-inexperienced naïve B cells. In the germinal centre B cells mature into memory and plasma cells after antigen engagement. The GC produces memory and high affinity antibody-producing plasma B cells as a result of somatic hyper mutation, clonal expansion and class switch recombination (CSR). Terminally differentiated plasma cells migrate back to the bone marrow and memory B cells produce antibody when they encounter a secondary antigen.

switched memory B cells in the SF express higher levels of the co-stimulatory molecules such as CD86 indicating an important role in auto-antigen presentation by these cells at the inflamed site (47). Indeed, B cells isolated from the synovial fluid of patients with JIA are more efficacious at activating isolated allogenic T cells *in vitro* compared to B cells isolated from the peripheral blood (47). To date, whether switched memory B cells differentiate in the joint or are recruited from the blood is currently not known. A recent study has shown that switched memory B cells expand at an increased rate in patients with oligo-JIA and poly-JIA and that this expansion is inhibited by anti-TNFα therapy (48). Based on these data, it could be postulated that these cells are then recruited to the joint. Collectively, evidence demonstrating that B cell abnormalities in JIA can be found both in the periphery and at the inflamed site make B cells an interesting target for therapy, particularly those patients whose disease is refractory to current treatment protocols namely non-responders to methotrexate and anti-TNFα therapy.

JUVENILE SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematous (SLE) is an autoimmune disease characterized by the generation of auto-antibodies directed against nuclear components. It can present with a wide variety of symptoms including renal, musculoskeletal and neuropsychiatric manifestations. The disease has a prevalence of 50–100/100,000 people in the USA and Europe (49). Patients who are diagnosed in childhood and adolescence make up 10–15% of this population with highest rates of diagnosis in female patients between 12 and 16 years (50). The juvenile-onset form of disease has many similarities with adult-onset SLE but there are some noteworthy differences in clinical manifestation. Juvenile SLE (JSLE) has a more severe disease course with higher rates of aggressive renal disease, increased mortality rates when adjusted for age and need a higher dose of glucocorticoids such as prednisolone (49, 51). Glucocorticoids are the backbone of JSLE therapy, with other DMARDs including hydroxychloroquine, aziothioprine, sulfasalazine, mycophenolate mofetil, and cyclophosphamide. For many young women, whose are diagnosed pre or peri-pubertal, these drugs have life-changing side-effects such as increasing the risk of osteoporosis, increasing the risk in infertility problems and changes in weight gain (52, 53). These side effects, coupled with the increased in mortality rates and severity of disease, demonstrate a clinically unmet need for therapeutics that substantially improve both quality of life and reduce mortality in pediatric patients.

Autoantibodies

In the context of JSLE it is traditionally believed that autoantibodies are pathogenic through the deposition of immune complexes in the skin, renal glomerulus and sites of tissue injury, in addition to targeting specific localized antigens. More recently evidence suggests that autoantibodies act as immune modulators through the recognition of nucleic acid containing immune complexes that can directly induce cell signaling and new gene transcription through endosomal toll-like receptors (TLRs) (54). Thus, ANA positivity is a critical characteristic used to define the development of SLE and is observed in over 95% of cases. The importance of ANAs in adult SLE has been extensively reviewed elsewhere (55, 56) and due to the overlapping clinical spectra between pediatric and adult onset disease these studies are extremely informative. Both forms of the disease display positivity for a variety of ANAs including those directed against double stranded DNA (dsDNA) and extractable nuclear antigens (ENA) of which examples include anti-Sm/RNP and anti-SSA/SSB (also known as anti-Ro and anti-La autoantibodies) (55). There are however some observed differences in autoantibody profiles between the two diseases. It has been reported that there is a higher prevalence of anti-dsDNA, anti-Sm and anti-RNP antibodies in juvenile compared to adult SLE populations (57, 58), but that significantly less JSLE patients present with anti-SSA and anti-SSB antibodies (59). Whether these changes are caused by differences in the severity of pathology between SLE and JSLE remains unexplored.

Evidence on what causes the production of ANA in JSLE and SLE can be garnered from genome-wide association scanning (GWAS) studies. These studies have demonstrated that gene susceptibility loci identified in lupus patients, which include *PTPN22, BTK*, and *LYN,* are associated with the strength of BCR signaling (60). Importantly, transgenic, congenic, or knockout mice have demonstrated that modulation or deficiency in molecules that control the strength of BCR signaling leads to the productions of ANA and, in some cases, the development of lupus-like disease. Polymorphisms in *PTPN22,* whose role in BCR signaling is not well-defined, leads to hypo-responsiveness following BCR activation, impairing B cell central tolerance by diminishing both deletion and editing of autoreactive B cells (61). Conversely, gain of function mutations in *BTK* (bruton's tyrosine kinase), a major adaptor of the BCR signaling cascade, in transgenic mice leads to hyper-responsiveness of the BCR. This reduces the activation threshold of the BCR leading to spontaneous germinal center (GC) formation, a hallmark of lupus-like disease in mice, and ANA production due to ineffective deletion of autoreactive B cells during central tolerance. Similarly, mice with B cell specific deletion in *Lyn,* which is a negative regulator of BCR activation, also develop anti-dsDNA and anti-Sm antibodies, nephritis and spontaneous GC formation. These examples demonstrate that changes to the strength of B cell signaling impact activation, proliferation and both negative and positive selection of B cells, all which could lead to systemic autoimmunity in JSLE.

Autoimmunity in many mouse models of lupus is reversed by deletion of the toll-like receptors (TLRs) or the TLR adaptor protein *Myd88.* For example, in mice where there is a B cell specific deletion of Lyn, they no longer develop nephritis if *Myd88* is also deleted. Other significant examples include the observation that autoantibody profiles are altered in MRL/lpr mice based on the deletion of either TLR7 and TLR9. In humans, the TLR7 locus is found on the X chromosome suggesting that changes to X chromosome inactivation in immune cells may alter autoantibody profiles (62, 63). This is of interest as the predilection of lupus to develop in females over males is extremely high, over 90% of reported cases in adults are in females, and is especially high in individuals that develop JSLE after puberty. Future studies that stratify by sex and age are needed to address the role of X chromosome inactivation in autoantibody production and the onset of lupus nephritis. It is important to note that TLR-activation can drive both the terminal differentiation of plasma cells and memory cells and that many SLE autoantigens can be detected by the endosomal TLR compartment (64). Thus, a positive feedback loop may exist whereby activation of TLRs/BCR in lupus by nuclear antigens leads to the terminal differentiation of antinuclear B cells, which in turn produce ANA and cause immune complex deposition and antigenic spreading. Targeting these pathways are critical when considering the development of therapeutics in both SLE and JSLE.

Cytokine Production and Antigen-Presentation by B Cells

The data summarized above demonstrate that B cell hyper-reactivity in JSLE may be, in part, driven by endosomal TLR-activation by nuclear-antigens. Of note, TLR-activation can drive both the terminal differentiation of B cells and induce cytokine production by B cells. In patients with active SLE, TLR9 expression is increased on total B cells *ex vivo* (65). Moreover, TLR9-activation *in vitro* leads to an altered cytokine profile compared to healthy B cells which is characterized by a reduction in TNFα, IL-10, and IL-6. This suggests that B cells may be chronically activated *in vivo* (66). Studies have also demonstrated that the regulatory B cell compartment is abnormal is SLE, with a seminal study demonstrating that there is an inability of $CD24^{hi}CD38^{hi}$ B cells to produce IL-10 in response to anti-CD40 stimulation and an inability to suppress inflammatory T cell differentiation *in vitro* (10). More recently, it has been established that exogenous cytokine production by other cells of the immune system also impacts B cell cytokine-production in lupus. Plasmacytoid dendritic cells (pDCs), which are the highest producers of IFNα in the immune system, are chronically activated during lupus leading to over-production of IFNα *in vivo*, which skews the differentiation of B cells away from regulatory B cells toward plasmablasts (45). These studies have focused on total lupus cohorts, encapsulating both pediatric and adult-onset patients. Future studies should stratify how B cell cytokine production is affected by SLE age of onset. In one study that did focus specifically on JSLE patients, it was shown that there is increased mRNA and protein expression of TLR3, TLR7, TLR9 in total peripheral blood mononuclear cells (PBMCs), mirroring effects seen on isolated B cells from adult-onset lupus

patients. In this study, it was proposed that B cells sense apoptotic neutrophils via TLR-activation as a source of nuclear antigens in JSLE, as blocking Myd88-dependent signaling suppress IFNα production (67).

It is known that there are high serum levels of the B cell activating cytokine Blys in JSLE patients (68). Although, the cellular source of this Blys is unknown, it correlates with disease severity marking a subset of childhood-onset patients that develop a particularly severe form of disease (69). Studies in mice have shown that high-levels of Blys allow autoreactive B cells to escape deletion in the spleen and induce class switching of antibodies without the need for T cell help. To date, there is little research investigating the role of autoreactive T cells in the development of lupus. However, in transgenic mice, B cell specific deletion of MHCII, which prevents antigen-presentation by B cells, reduces glomerular nephritis and reduces IFNγ production by T cells (70). In this study, cognate TCR-Ag-MHCII interactions between B cells and T cells drives both T cell activation and further promotes B cell proliferation and differentiation. Although the importance of antigen-presentation by B cells is under-explored in SLE in humans, it has been demonstrated that pro-inflammatory cytokine productions by T cell is higher in patients with JSLE compared to age-matched controls (71). It is therefore tempting to postulate that altered T cell cytokine production is due to aberrant antigen-presentation by B cells.

JUVENILE DERMATOMYOSITIS

Juvenile dermatomyositis (JDM) is a rare disease, yet the most common form of childhood autoimmune myositis that presents with proximal muscle weakness and associated skin rash.

The mainstay treatments for JDM are prednisolone and methotrexate. Other immunosuppressive treatments currently used include mycophenolate mofetil, cyclophosphamide (72) and azathioprine. At present, there is limited evidence for biologic therapy and its efficacy in the treatment of JDM due to limited understanding of the mechanisms underlying disease pathology. In general adult and juvenile onset DM share a similar clinical and pathological phenotype. However, adult onset myositis patients have much higher associated risk of malignancy (73), a more chronic disease course and higher mortality rate. Key pathological differences between adult and juvenile onset DM are that JDM patients have increased risk of neovascularisation of capillaries, up-regulation of MHC class I on myofibers and type I interferon response (74). Investigating the functions of B cells in JDM may provide an insight into the mechanisms of the disease and lead to novel therapeutic pathways.

Autoantibodies

In approximately a third of JDM cases B-lymphocytes have been detected in inflamed muscle, and almost 50% of patients have detectable myositis-specific (MSA) or myositis-associated autoantibodies (MAA) (75, 76). MSA and MAA can be identified in the serum of up to 70% of JDM patients and closely correlate to specific homogenous clinical phenotypes (**Figure 2**). In adult myositis, the most frequent group of myositis autoantibodies detected are the anti-synthetase enzymes. The anti-synthetases are present in 25–40% of adult patients compared to 5% of juvenile patients (75). Longitudinal studies have also demonstrated that MSA phenotype and muscle biopsy score at time of diagnosis can predict the risk of staying on treatment (77). Thus, growing empirical evidence suggests that JDM patients should be stratified based on autoantibody subtype to predict both disease features, such as lung involvement or calcinosis, and to inform treatment strategies employed by clinicians (78). MSA and MAA also act as important diagnostic tools to discriminate JDM from other rheumatic diseases such as JSLE and JIA that can share some similar features of JDM, such as Raynaud's phenomenon and muscle weakness, and may lead to incorrect diagnoses (78). These antibodies are also useful to differentiate JDM from other rare immune-mediated myopathies such as those associated with anti-signal recognition particle (SRP).

The underlying cause of MSA and MAA production in JDM is yet to be elucidated. Although it has suggested that there is aberrant expression of some MAA and MSA targets, namely Jo-1 and Mi-2, on regenerating muscle fiber in myositis patients when compared to controls (79). Given the presence of these autoantigens, and that B cells have long been detected in the inflamed muscle of myositis patients (80), it may be that autoantigen specific B cell differentiation occurs in the muscle in myositis. Prospective studies that include the phenotyping of B cells recovered from inflammatory infiltrates in JDM muscle should determine whether this hypothesis is correct. Other targets of MSA and MAA antibodies include members of the nucleic acid sensing pathway (81). The connection between auto-antibodies against nucleic acid sensing molecules and the IFN signature, which is a hallmark of myositis immunopathology, is yet to be ascertained. However, taken together these data strongly suggest that aberrant sensing of nucleic acids is central in disease pathogenesis by driving both IFN production and production of autoantibodies against these molecules (82–84). Future work will need to establish what causes auto-antibody production against these molecules and what is the effect on B cell differentiation/activation.

Cytokine Production and Antigen-Presentation by B Cells

Due to the rarity of JDM cases, it is extremely hard to carry out functional assays to understand what underlies B cell dysfunction in JDM. Nevertheless, large cohorts of JDM patients such as Juvenile Dermatomyositis Cohort Biomarker Study and Repository (UK and Ireland) (JDCBS) and the Myositis Genetics Consortium (MyoGen) are now being used to address how B cell function is altered in children with JDM (85, 86). For example, using patients recruited to the JDCBS, it was recently reported that $CD19^{+}CD24^{hi}CD38^{hi}$ B cells are expanded in the peripheral blood of JDM patients that are naïve of immunosuppressive treatment, demonstrating that similarly to other rheumatic disorders normal B cell development is affected. In this study, it was also demonstrated that cytokine-production by immature B cells is altered in JDM, exhibiting a pro-inflammatory phenotype

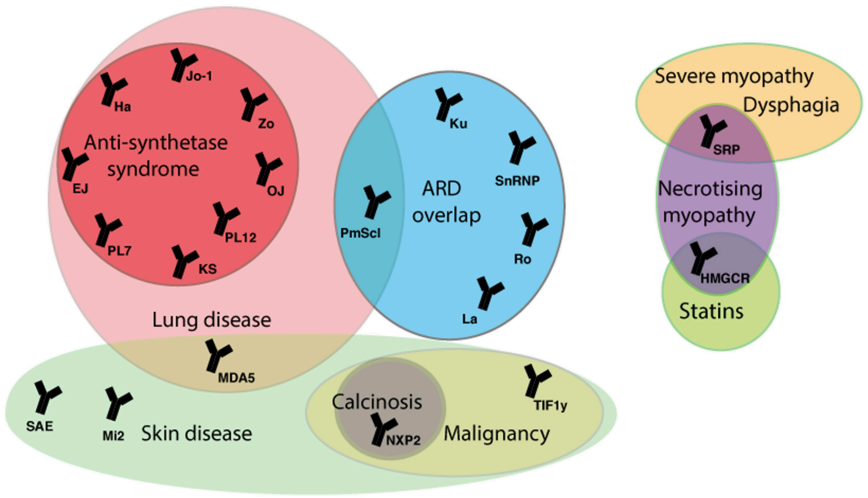

FIGURE 2 | Myositis autoantibodies and their key clinical associations**.** JDM patients can be stratified by myositis specific and associated auto-antibodies (MSA/MAA) to group with clinical phenotype. The anti-TIF1-γ and anti-NXP-2 autoantibodies are associated with calcinosis in JDM and malignancy in adults. The MDA5 autoantibody in juvenile cases is associated with mild muscle and skin disease, but strongly associated with interstitial lung disease (ILD). In adult IIM anti-Jo-1 is the most common of the anti-synthetase autoantibodies and is associated with ILD, arthritis, fevers, Raynaud's phenomenon and mechanic's hand. The majority of mortality in adult and children IIM patients is due to ILD. In rare cases treatment with statins can trigger an immune-mediated necrotizing myopathy that can be characterized by the presence of an autoantibody against HMGCR, the pharmacological target of statins. ARD, autoimmune rheumatic disease; SRP, signal recognition particle; HMGCR, 3-hydroxy-3methylglutaryl-conenzyme A reductase; TIF1, transcription intermediary factor 1; NXP2, nuclear matrix protein 2; MDA5, melanoma differentiation-association gene 5; SAE, small ubiquitin-like modifier activating enzyme; 5NT1A, cytosolic 5′nucleotidase 1A; Mi-2, nucleosome-remodeling deacetylase complex, Jo-1, histidyl tRNA synthetase; PL7, threonyl tRNA synthetase; PL12, ananyl tRNA synthetase; OJ, isoleucyl tRNA synthetases; EJ, glycyl tRNA synthetase; KS, asparanginyl tRNA synthetase; Zo, phenylalanyl tRNA synthetase, Ha; tyrosyl tRNA synthetase; snRNP, small nuclear ribonucleic protein.

after activation through TLR7 and IFN-α (84). To our knowledge, there is only one other report showing that cytokine production is affected in JDM B cells. The data was presented as part of a larger study comparing IL-10 production by B cells from children with autoimmune disease with healthy controls. In this study, as part of a collection with JSLE and undifferentiated or overlap (mixed) connective tissue disease patients, JDM patients were shown to have a reduced percentage of B10 cells; a population of regulatory B cells that have been identified in human and mouse (87).

To date, there are no studies published that directly investigate whether antigen presentation by B cells is altered in patients with JDM. However, a GWAS study carried out by MyoGen demonstrated that susceptibility of disease was associated with SNPs within the MHC locus (88). Miller et al. have also reported that risk of JDM development was particularly associated with the HLA haplotype *HLA-DRB1*0301* (89). Future studies are needed to directly understand how this affects auto-reactive T cell activation by B cells in JDM and whether this is altered in different autoantibody subtypes. Greater understanding of the B cell component that underlies JDM pathogenesis will provide evidence for the efficacy of B cell-targeted-therapies for this disease.

B CELL TARGETED-THERAPIES IN PEDIATRIC RHEUMATIC DISEASE

B Cell Depletion Therapy

The most well-known and widely used B cell depletion therapy for the treatment of rheumatic disease is Rituximab. This chimeric monoclonal antibody targets the surface protein CD20 expressed mainly by memory and naïve B cells and results in depletion by antibody-dependent cytotoxicity, complement-mediated lysis or apoptosis (90). **Table 1** summarizes the B cell depletion therapeutic agents that have been trialed in pediatric rheumatological diseases. The original rationale for treatment of adult patients with rheumatoid arthritis with rituximab, circa 1998, was that depletion of the memory B cell compartment would influence immune cell interactions that could potentially "re-set" immunological tolerance as crucially auto-antibody producing plasma cells no longer express CD20 (94). Although the actual picture is more complicated, numerus randomized trials have now confirmed that B cell depletion therapy with rituximab has some beneficial effects in the treatment of adult disease.

Despite failing to meet primary endpoints in two large double-blind randomized, placebo-controlled trials investigating both renal [LUNAR (91)] and non-renal [EXPLORER (95)] manifestations of SLE in adults, rituximab has been subsequently demonstrated as a potentially effective treatment in both adults and children with refractory disease (96). Although not currently approved by the UK national institute of clinical excellence (NICE), rituximab can be prescribed at the discretion of the treating physician on the basis of a specialist NHS England interim commissioning policy. It has been suggested that both LUNAR and EXPLORER trials may have failed to demonstrate efficacy for a number of reasons including problems with the study design, which is frequently quoted in the cases of clinical trials investigating the efficacy of new therapeutics

TABLE 1 | Summary of clinical trials investigating efficacy of B cell targetted therapies in pediatric rheumatic disease.

Authors	Title of trial	Drug mechanism	Kind of trial Patient	Patient group	Outcome Measures	NICE approved	Summary
Rovin et al. (91)	Lupus Nephritis Assessment with Rituximab Study (LUNAR)	Anti-lymphocyte monoclonal antibody leading to lysis of B lymphocytes.	Double-blind randomized, placebo controlled trial, Phase III	SLE ($n = 144$)	Assessed for renal response based on serum creatinine levels, urinary sediment and urine protein to creatinine ratio (UPC).	No	Despite rituximab leading to high response rate within patient cohort after the trial finished, no long-term outcomes were observed after 1 year of treatment
Merill et al., (95)	Exploratory Phase II/III SLE Evaluation of Rituximab (EXPLORER)	Anti-lymphocyte monoclonal antibody leading to lysis of B lymphocytes.	Double-blind randomized, placebo controlled trial, Phase II/III	SLE ($n = 257$)	Monthly assessments with the British Isles Lupus Assessment Group (BILAG) index and the Lupus Quality of Life (LupuQol) index, including pain and fatigue outcomes.	No	No significant differences were observed between the placebo and treatment groups
Oddis et al. (92)	Rituximab in Myositis Study (RIM Study)	Anti-lymphocyte monoclonal antibody leading to lysis of B lymphocytes.	Double-blind randomized controlled, placebo phase trial	PM ($n = 76$), DM ($n = 76$), JDM ($n = 48$)	Definition of improvement (DOI) based on International Myositis Assessment and Clinical Studies Group (IMACS). Improvement was classified as a =>20% increase in any 3 of 6 IMACS items and no more than 2 worsening items by >=25% compared to baseline.	No	No significant differences between treatment pathways however 83% of refractory myositis patients met DOI.
Hui-Yuen et al. (93)	Pediatric Lupus Trial Of Belimumab (PLUTO)	Binds to human B lymphocyte stimulator protein (BLyS) to prevent binding on B cell receptors, interfering with B cell survival.	Observational Stud	SLE ($n = 157$), JSLE ($n = 38$)	Comparison of overall physician assessment including clinical symptoms at baseline vs. endpoint.	Yes	71% of pediatric SLE patients presented a clinical improvement within 6 months and over two thirds were able to reduce steroid use.
Curiel et al., ongoing.	Abatacept in Juvenil Dermatomyositis (AID); Assessing the safety and efficacy of subcutaneous Abatacept in refractory JDM patients.	Soluble fusion protein that inhibits T lymphocyte activity	Single group clinical trial, Phase IV	JDM ($n = 10$)	Definition of improvement (DOI) based on International Myositis Assessment and Clinical Studies Group (IMACS). Improvement was classified as a =>20% increase in any 3 of 6 IMACS items and no more than 2 worsening items by >= 25% compared to baseline.	No	Results not yet released.

in SLE. Factors including high background steroid doses in the control group and issues with end-point measurements are likely to have contributed to this. It is important to note that evidence from animal models suggests that lupus-prone MRL mice are surprisingly refractory to B cell depletion due to high levels of serum IgG inhibiting FcγR-dependent phagocytosis by macrophages and neutrophils (97). Further studies are needed to understand whether lupus patients that do not respond to B cell depletion therapy have a similar acquired deficiency in their myeloid compartment caused by the burden of endogenous auto-antibody associated with disease pathology.

For pediatric rheumatic disease, very few formal trials exist that have investigated the efficiency of B cell depletion therapy in suppressing disease symptoms. In spite of this, Rituximab is increasingly being used as an adjunctive therapy to treat children with JIA, JDM and JSLE. Importantly, despite the well-established use of Rituximab in RA, it is currently infrequently used as a biologic in RF+ poly-JIA, which, as discussed above, is thought of as the early-onset equivalent of RA. Future clinical trials are essential to establish its place in the clinician's therapeutic arsenal in the treatment of RF+ poly-JIA. In terms of oligo-JIA and RF- poly-JIA, especially those that have ANA, there is considerable evidence that rituximab treatment may be a worthwhile treatment option. Several case studies have demonstrated that rituximab therapy can produce sustained clinical improvement in patients with refractory disease (98–100). Currently, rituximab is often only considered following the failure of first-line treatments such as TNF inhibitors and methotrexate in both pediatric and adult disease. Taking-into-account the strong B cell signature observed in JIA patients with early-onset disease, rituximab treatment could potentially benefit these patients if used prior to failure to first-line therapies and could therefore prevent sustained disability as a result of established joint damage in some of these patients.

In JSLE, a retrospective cohort study recently reported that the use of rituximab for the treatment of active or refractory disease is increasing. There is increasing evidence to support that this treatment is effective in reducing specific disease activity biomarkers in SLE. Rituximab has been shown to reduce steroid use (and therefore importantly decrease the incidence of side-effects associated with long term corticosteroid use) in those not responding to standard therapies (101). It has been suggested that adverse reactions to rituximab are increased in JSLE patients compared to adult-onset SLE patients' (102). For example, half the children recruited to a small study in France reported by Willems et al. developed thrombocytopenia and neutropenia following treatment. However, it is often difficult to distinguish what findings are a result of side effects to treatment with rituximab and what may be attributed to an underlying disease flare (102). In comparison, another small study by Marks et al. (103) reported no side-effects or adverse reactions. This highlights the need for larger studies to prevent sampling bias and to establish the safety profile and efficacy of rituximab in childhood onset disease. Problems with study design in adult SLE, which include the treatment of rituximab alongside many other medications and lack of an appropriate control group, are also present in JSLE. Appropriate trials in adults and children are now required as the subtle immunological differences observed between SLE and JSLE means that rituximab treatment may have a different efficacy in disease depending on age of onset.

One of the few pediatric diseases where rituximab has been trialed is in JDM, in a study by Oddis et al. where efficacy in adult DM and JDM were compared (92). Although the primary end point of the randomized control trial was not met, Oddis et al. showed that a higher proportion of JDM patients (87%) treated with rituximab met the definition of improvement more quickly than adult dermatomyositis (ADM) patients (78%). These results might imply that B cells in JDM are either more pathogenic than in ADM, or perhaps B cells have a greater regulatory role in ADM compared to JDM. Future studies are needed to address these differences.

It is important to note that some patients with pediatric rheumatic disease are refractory to first-line therapies and thus may require monoclonal antibody therapy, such as rituximab. Clinical trials that directly compare rituximab therapy with methotrexate alone, TNF inhibiting drugs or, where appropriate, anti-IL-6 therapy would be extremely informative to help stratify treatment efficacy when compared to other current treatment strategies. Development of other CD20-targeting monoclonal antibodies is ongoing, recent additions include ofatumumab (104) and veltuzumab, which have a similar mechanism of action to rituximab. Notably, ofatumumab is a fully humanized form of CD20, which has been demonstrated to be effective in adults and children with SLE who have had adverse reactions to rituximab (104).

Other B Cell-Targeting Therapies

Further to B cell depletion therapy, there are other biologics in clinical practice with a method of action that targets B cell activation/function (**Figure 3**). The most well-known of which are Belimumab (a monoclonal antibody that inhibits BAFF/Blys) and Abatacept (which binds CD28 on T cells thus preventing its interaction with CD80/CD86 on antigen-presenting cells such as B cells). Data demonstrating that Blys levels in the sera differ between JIA and JSLE patients suggests that Belimumab treatment may be more efficacious in diseases that have higher levels of BAFF such as JSLE. Belimumab is currently FDA approved for the treatment of adult-onset SLE and there is currently an ongoing trial in pediatric lupus (PLUTO – NCT01649765) to investigate the safety and pharmacokinetics of this treatment in disease. Although PLUTO is yet to report, there is an abstract reporting that background steroid treatment was tapered in 63% of JSLE patients treated with Belimumab suggesting a favorable effect on disease (93). To our knowledge, similar studies do not yet exist in JIA or JDM.

Abatacept is primarily thought of as a drug that targets T cell function by preventing its interaction with antigen-presenting cells, which include both B cells and dendritic cells (DCs). Importance of B cells in the efficacy of abatacept-treatment is demonstrated by studies showing that in RA patients

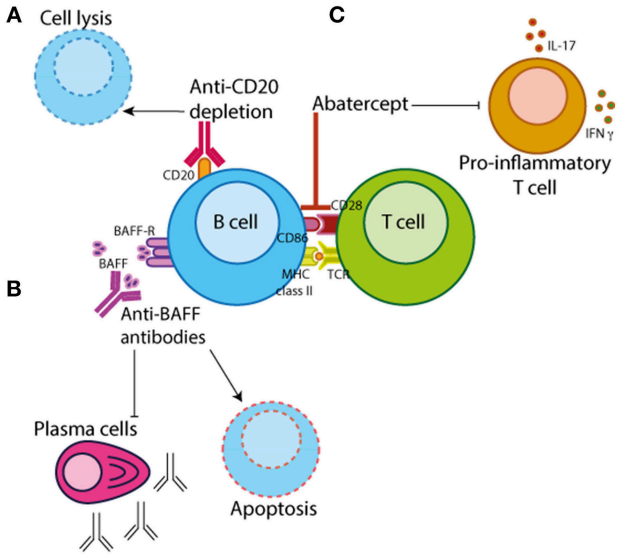

FIGURE 3 | B cell-targeted therapeutics. There are three main categories of B cell targeting therapeutics. **(A)** Chimeric monoclonal antibodies against CD20 (e.g., rituximab) which target B cells for depletion by antibody-dependent cytotoxicity, complement-mediated lysis or apoptosis. **(B)** Monoclonal antibodies that bind B cell activating factor/B-lymphocyte stimulator (BAFF/BLyS) (e.g., Belimumab) preventing B cell proliferation and survival. **(C)** Abatercept, a fusion protein of CTLA-4 and a fragment of the modified Fc domain of human IgG1, that binds to CD28 on T cells preventing the interaction with CD80/CD86 on antigen-presenting cells inhibiting T cell activation and differentiation.

abatacept-treatment is dependent upon the baseline levels of memory B cells (which express higher levels of CD80/CD86) (105, 106). There is also some evidence that abatacept inhibits phosphorylation of Syk in PB B cells from RA patients, potentially directly altering intracellular signaling cascades which affect B cell activation and proliferation (107). Although, abatacept treatment is likely to affect multiple immune cell subsets, these data demonstrate a decisive effect of this drug on the B cell compartment.

At present, in the UK, NICE only recommends Abatacept treatment for poly-JIA patients who are over 6 years of age, whose disease has not responded to treatment with a disease-modifying anti-rheumatic drug (DMARD) or at least one TNF inhibitor. This means to date that no studies have directly compared the efficacy on abatacept to drugs such as adalimumab and etanercept as clinical trials have focused on patients that have failed TNF therapy (108). The safety profile of Abatacept is thought to be generally good and studies have demonstrated that there is a favorable response in 70% of patients. Studies have also suggested a possible efficacy in oligo-JIA patients with Uveitis who have also failed TNF inhibition therapy (109). In the future, it should be possible to target patients that have mutations in PTPN22, a JIA susceptibility locus, which encodes a tyrosine phosphatase that downregulates CD28 activation (110). These patients are likely to fall across all JIA subtypes.

The efficacy of Abatacept treatment for JSLE and JDM is much less well-defined. Indeed, all trials investigating its efficacy of Abatacept in adult-onset SLE have not reached their primary end-point, although this is likely to be to problems in clinical trial design in lupus, similarly to those conducted with Rituximab, as there is some evidence it suppresses the severity of nephritis (101). In JDM, there is a case study suggesting that Abatacept-treatment suppresses recalcitrant disease that is complicated by calcinosis formation and there is a phase 4 interventional clinical trial (NCT02594735) underway to assess the safety and efficacy of subcutaneous abatacept in 10 patients 7 years of age and older with refractory JDM (111). Future work is necessary to establish its efficacy in both juvenile SLE and DM patients.

CONCLUDING REMARKS

B cell depletion therapy has improved the lives of many patients with adult-onset autoimmune rheumatic disease. The data summarized above demonstrate that B cell dysfunction is also central to the pathology of many patients with pediatric-onset rheumatic disease. However, due to inherent problems in the design and approval of clinical trials focused on pediatric-onset disease, the understanding of the efficacy of B cell depletion/targeting therapies is years behind that of adult rheumatic disease. The future of clinical practice will be centered around the stratification of patients and the increased cost-efficiency of multi-omics platforms means the age of personalized medicine is fast approaching. We propose that future stratification of JSLE, JDM, and JIA will identify a subset of patients whose disease is driven by a B cell component, which makes them ideal candidates for B cell depletion/targeting therapies, and that treatment protocols will be applied which define patients by immune-phenotype rather than by clinical manifestation. For these changes in clinical practice to take place, correctly controlled clinical trials must be performed that assess the safety, efficiency and pharmacokinetics of B cell targeting biologics in children, which is likely to be drastically different to adults. In particular, the effect of these drugs on the immature immune system should be addressed. This will provide information on particular drug profiles, but will also provide information on the processes underlying B cell development in early-life. Notably, there are relatively few studies that have carried out consensus B cell phenotyping in pre and peri-pubertal individuals. Moreover, to date, there are no studies that stratify B cell responses in children and young people by sex, despite an appreciation that there is a sex-biased development of rheumatic disease in women. Future studies are needed to improve the characterization of basic B cell biology in healthy children and those with autoimmune disease, which will aid the understanding of the usefulness of B cell depletion therapy in pediatric rheumatic disease. We believe this will allow current treatment protocols to be updated so that they more effectively target the "window-of-opportunity" based on immune-phenotype, hopefully preventing long-term disability in children with musculoskeletal disease.

AUTHOR CONTRIBUTIONS

ER and MW wrote the manuscript. All authors gave approval before submission.

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REFERENCES

1. Vazquez MI, Catalan-Dibene J, Zlotnik A. B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine* (2015) 74:318–26. doi: 10.1016/j.cyto.2015.02.00
2. Harwood NE, Batista FD. Visualizing the molecular and cellular events underlying the initiation of B-cell activation. *Curr Top Microbiol Immunol*. (2009) 334:153–77. doi: 10.1007/978-3-540-93864-4_7
3. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol.* (2010) 10:236–47. doi: 10.1038/nri2729
4. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol.* (2000) 1:475–82. doi: 10.1038/82717
5. Mauri C, Bosma A. Immune regulatory function of B cells. *Ann Rev Immunol.* (2012) 30:221–41. doi: 10.1146/annurev-immunol-020711-074934
6. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity* (2015) 42:607–12. doi: 10.1016/j.immuni.2015.04.005
7. Svensson L, Jirholt J, Holmdahl R, Jansson L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). *Clin Exp Immunol.* (1998) 111:521–6.
8. O'Neill SK, Cao Y, Hamel KM, Doodes PD, Hutas G, Finnegan A. Expression of CD80/86 on B cells is essential for autoreactive T cell activation and the development of arthritis. *J Immunol.* (2007) 179:5109–16 doi: 10.4049/jimmunol.179.8.5109
9. Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. *Arthr Res Therapy* (2012) 14:R32. doi: 10.1186/ar3736
10. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* (2010) 32:129–40. doi: 10.1016/j.immuni.2009.11.009
11. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med.* (2013) 5:173ra23. doi: 10.1126/scitranslmed.3005407
12. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol.* (2006) 6:107–16. doi: 10.1038/nri1780
13. Schlissel MS. Regulating antigen-receptor gene assembly. *Nat Rev Immunol.* (2003) 3:890–9. doi: 10.1038/nri1225
14. Luning Prak ET, Monestier M, Eisenberg RA. B cell receptor editing in tolerance and autoimmunity. *Ann NY Acad Sci.* (2011) 1217:96–121. doi: 10.1111/j.1749-6632.2010.05877.x
15. Pelanda R, Torres RM. Central B-cell tolerance: where selection begins. *Cold Spring Harb Perspect Biol.* (2012) 4:a007146. doi: 10.1101/cshperspect.a007146
16. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev.* (2004) 197:179–91. doi: 10.1111/j.0105-2896.2004.0109.x
17. Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. *Curr Opin Immunol.* (2008) 20:332–8. doi: 10.1016/j.coi.2008.03.003
18. De Silva NS, Klein U. Dynamics of B cells in germinal centres. *Nat Rev Immunol.* (2015) 15:137–48. doi: 10.1038/nri3804
19. Andraud M, Lejeune O, Musoro JZ, Ogunjimi B, Beutels P, Hens N. Living on three time scales: the dynamics of plasma cell and antibody populations illustrated for hepatitis a virus. *PLoS Comput Biol.* (2012) 8:e1002418. doi: 10.1371/journal.pcbi.1002418
20. Hammarlund E, Thomas A, Amanna IJ, Holden LA, Slayden OD, Park B, et al. Plasma cell survival in the absence of B cell memory. *Nat Commun.* (2017) 8:1781. doi: 10.1038/s41467-017-01901-w
21. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. *Lancet* (2011) 377:2138–49. doi: 10.1016/S0140-6736(11)60244-4
22. Wallace CA, Ravelli A, Huang B, Giannini EH. Preliminary validation of clinical remission criteria using the OMERACT filter for select categories of juvenile idiopathic arthritis. *J Rheumatol.* (2006) 33:789–95.
23. Ravelli A, Martini A. Remission in juvenile idiopathic arthritis. *Clin Exp Rheumatol.* (2006) 24(6 Suppl. 43):S105–10.
24. Ruth NM, Passo MH. Juvenile idiopathic arthritis: management and therapeutic options. *Ther Adv Musculoskelet Dis.* (2012) 4:99–110. doi: 10.1177/1759720X11413630
25. Dekker L, Armbrust W, Rademaker CM, Prakken B, Kuis W, Wulffraat NM. Safety of anti-TNFalpha therapy in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* (2004) 22:252–8.
26. Zannin ME, Birolo C, Gerloni VM, Miserocchi E, Pontikaki I, Paroli MP, et al. Safety and efficacy of infliximab and adalimumab for refractory uveitis in juvenile idiopathic arthritis: 1-year followup data from the Italian Registry. *J Rheumatol.* (2013) 40:74–9. doi: 10.3899/jrheum.120583
27. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheumat.* (2008) 58:875–87. doi: 10.1002/art.23291
28. Rosser EC, Lom H, Bending D, Duurland CL, Bajaj-Elliott M, Lucy R, Wedderburn MD. Innate Lymphoid Cells and T Cells Contribute to the Interleukin-17A Signature Detected in the Synovial Fluid of Patients With Juvenile Idiopathic Arthritis. *Arthritis & Rheumatology*. (2018) doi: 10.1002/art.40731. [Epub ahead of print].
29. Wiegering V, Girschick HJ, Morbach H. B-cell pathology in juvenile idiopathic arthritis. *Arthritis* (2010) 2010:759868. doi: 10.1155/2010/759868
30. Giancane G, Consolaro A, Lanni S, Davì S, Schiappapietra B, Ravelli A. Juvenile idiopathic arthritis: diagnosis and treatment. *Rheumatol Ther*. (2016) 3:187–207. doi: 10.1007/s40744-016-0040-4

31. Macaubas C, Nguyen K, Milojevic D, Park JL, Mellins ED. Oligoarticular and polyarticular JIA: epidemiology and pathogenesis. *Nat Rev Rheumatol.* (2009) 5:616–26. doi: 10.1038/nrrheum.2009.209
32. Guillaume-Czitrom S, Sibilia J, Nordal E. Growing up with chronic arthritis: the confusing matter of classification. *RMD Open* (2017) 3:e000417. doi: 10.1136/rmdopen-2016-000417
33. Gregorio A, Gambini C, Gerloni V, Parafioriti A, Sormani MP, Gregorio S., et al. Lymphoid neogenesis in juvenile idiopathic arthritis correlates with ANA positivity and plasma cells infiltration. *Rheumatology* (2007) 46:308–13. doi: 10.1093/rheumatology/kel225
34. Hinks A, Marion MC, Cobb J, Comeau ME, Sudman M, Ainsworth HC., et al. Brief report: the genetic profile of rheumatoid factor-positive polyarticular juvenile idiopathic arthritis resembles that of adult rheumatoid arthritis. *Arthritis Rheumatol.* (2018) 70:957–62. doi: 10.1002/art.40443
35. Peckham H, Cambridge G, Bourke L, Sen D, Radziszewska A, Leandro M., et al. Antibodies to cyclic citrullinated peptides in patients with juvenile idiopathic arthritis and patients with rheumatoid arthritis: shared expression of the inherently autoreactive 9G4 idiotype. *Arthritis Rheumatol.* (2017) 69:1387–95. doi: 10.1002/art.40117
36. Miriovsky BJ, Michaud K, Thiele GM, O'Dell JR, Cannon GW, Kerr G., et al. Anti-CCP antibody and rheumatoid factor concentrations predict greater disease activity in men with rheumatoid arthritis. *Ann Rheumat Dis.* (2010) 69:1292–7. doi: 10.1136/ard.2009.122739
37. Pruijn GJ, Wiik A, van Venrooij WJ. The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis. *Arthritis Res Ther.* (2010) 12:203. doi: 10.1186/ar2903
38. Wouters CH, Ceuppens JL, Stevens EA. Different circulating lymphocyte profiles in patients with different subtypes of juvenile idiopathic arthritis. *Clin Exp Rheumatol.* (2002) 20:239–48.
39. Lee J, Kuchen S, Fischer R, Chang S, Lipsky PE. Identification and characterization of a human CD5+ pre-naive B cell population. *J Immunol.* (2009) 182:4116–26. doi: 10.4049/jimmunol.0803391
40. Vernino LA, Pisetsky DS, Lipsky PE. Analysis of the expression of CD5 by human B cells and correlation with functional activity. *Cell Immunol.* (1992) 139:185–97
41. Corcione A, Ferlito F, Gattorno M, Gregorio A, Pistorio A, Gastaldi R., et al. Phenotypic and functional characterization of switch memory B cells from patients with oligoarticular juvenile idiopathic arthritis. *Arthritis Res Ther.* (2009) 11:R150. doi: 10.1186/ar2824
42. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood* (2005) 105:4390–8. doi: 10.1182/blood-2004-11-4284
43. Faber C, Morbach H, Singh SK, Girschick HJ. Differential expression patterns of recombination-activating genes in individual mature B cells in juvenile idiopathic arthritis. *Ann Rheumat Dis.* (2006) 65:1351–6. doi: 10.1136/ard.2005.047878
44. Low JM, Chauhan AK, Moore TL. Abnormal kappa:lambda light chain ratio in circulating immune complexes as a marker for B cell activity in juvenile idiopathic arthritis. *Scand J Immunol.* (2007) 65:76–83. doi: 10.1111/j.1365-3083.2006.01859.x
45. Menon M, Blair PA, Isenberg DA, Mauri C. A regulatory feedback between plasmacytoid dendritic cells and regulatory B cells is aberrant in systemic lupus erythematosus. *Immunity* (2016) 44:683–97. doi: 10.1016/j.immuni.2016.02.012
46. Zhao Q, Jung LK. Frequency of CD19(+)CD24(hi)CD38(hi) regulatory B cells is decreased in peripheral blood and synovial fluid of patients with juvenile idiopathic arthritis: a preliminary study. *Pediatric Rheumatol Online J.* (2018) 16:44. doi: 10.1186/s12969-018-0262-9
47. Morbach H, Wiegering V, Richl P, Schwarz T, Suffa N, Eichhorn EM., et al. Activated memory B cells may function as antigen-presenting cells in the joints of children with juvenile idiopathic arthritis. *Arthritis Rheumat.* (2011) 63:3458–66. doi: 10.1002/art.30569
48. Marasco E, Aquilani A, Cascioli S, Moneta GM, Caiello I, Farroni C., et al. Switched memory B cells are increased in oligoarticular and polyarticular juvenile idiopathic arthritis and their change over time is related to response to tumor necrosis factor inhibitors. *Arthritis Rheumatol.* (2018) 70:606–15. doi: 10.1002/art.40410
49. Houghton KM, Page J, Cabral DA, Petty RE, Tucker LB. Systemic lupus erythematosus in the pediatric North American native population of British Columbia. *J Rheumatol.* (2006) 33:161–3.
50. Kone-Paut I, Piram M, Guillaume S, Tran TA et al. Lupus in adolescence. *Lupus* (2007) 16:606–12. doi: 10.1177/0961203307079562
51. Carreño L, López-Longo FJ, Monteagudo I, Rodríguez-Mahou M, Bascones M, González CM, et al. Immunological and clinical differences between juvenile and adult onset of systemic lupus erythematosus. *Lupus* (1999) 8:287–92. doi: 10.1191/096120399678847786
52. McGill G, Ambrose N. The management of lupus in young people. *Br J Gen Pract.* (2018) 68:96–97. doi: 10.3399/bjgp18X694805
53. Levy DM, Kamphuis S. Systemic lupus erythematosus in children and adolescents. *Pediatr Clin North Am.* (2012) 59:345–64. doi: 10.1016/j.pcl.2012.03.007
54. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* (2010) 11:373–84. doi: 10.1038/ni.1863
55. Olsen NJ, Karp DR. Autoantibodies and SLE: the threshold for disease. *Nat Rev Rheumatol.* (2014) 10:181–6. doi: 10.1038/nrrheum.2013.184
56. Han S, Zhuang H, Shumyak S, Yang L, Reeves WH. Mechanisms of autoantibody production in systemic lupus erythematosus. *Front Immunol.* (2015) 6:228. doi: 10.3389/fimmu.2015.00228
57. Papadimitraki ED, Isenberg DA. Childhood- and adult-onset lupus: an update of similarities and differences. *Expert Rev Clin Immunol.* (2009) 5:391-403. doi: 10.1586/eci.09.29
58. Ambrose N, Morgan TA, Galloway J, Ionnoau Y, Beresford MW, Isenberg DA., et al. Differences in disease phenotype and severity in SLE across age groups. *Lupus* (2016) 25:1542–50. doi: 10.1177/0961203316644333
59. Tarr T, Dérfalvi B, Gyori N, Szántó A, Siminszky Z, Malik A., et al. Similarities and differences between pediatric and adult patients with systemic lupus erythematosus. *Lupus* (2015) 24:796–803. doi: 10.1177/0961203314563817
60. International Consortium for Systemic Lupus Erythematosus G, Harley JB, Alarcón-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, et al. International Consortium for Systemic Lupus Erythematosus G, Harley JB, Alarcon-Riquelme ME, et al. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat Genet.* (2008) 40:204–10. doi: 10.1038/ng.81
61. Chung SA, Criswell LA. PTPN22: its role in SLE and autoimmunity. *Autoimmunity* (2007) 40:582–90. doi: 10.1080/08916930701510848
62. Souyris M, Cenac C, Azar P, Daviaud D, Canivet A, Grunenwald S., et al. TLR7 escapes X chromosome inactivation in immune cells. *Sci Immunol.* (2018) 3:eaap8855. doi: 10.1126/sciimmunol.aap8855
63. Rubtsov AV, Rubtsova K, Kappler JW, Marrack P. TLR7 drives accumulation of ABCs and autoantibody production in autoimmune-prone mice. *Immunol Res.* (2013) 55:210–6. doi: 10.1007/s12026-012-8365-8
64. Wu YW, Tang W, Zuo JP. Toll-like receptors: potential targets for lupus treatment. *Acta Pharmacol Sin.* (2015) 36:1395–407. doi: 10.1038/aps.2015.91
65. Papadimitraki ED, Choulaki C, Koutala E, Bertsias G, Tsatsanis C, Gergianaki I., et al. Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: implications for the induction and maintenance of the autoimmune process. *Arthritis Rheumat.* (2006) 54:3601–11. doi: 10.1002/art.22197
66. Gies V, Schickel JN, Jung S, Joublin A, Glauzy S, Knapp AM, et al. Impaired TLR9 responses in B cells from patients with systemic lupus erythematosus. *JCI Insight* (2018) 3:96795. doi: 10.1172/jci.insight.96795
67. Midgley A, Thorbinson C, Beresford MW. Expression of Toll-like receptors and their detection of nuclear self-antigen leading to immune activation in JSLE. *Rheumatology* (2012) 51:824–32. doi: 10.1093/rheumatology/ker400
68. Hong SD, Reiff A, Yang HT, Migone TS, Ward CD, Marzan K., et al. B lymphocyte stimulator expression in pediatric systemic lupus erythematosus and juvenile idiopathic arthritis patients. *Arthritis Rheumat.* (2009) 60:3400–9. doi: 10.1002/art.24902
69. Kanakoudi-Tsakalidou F, Tzimouli V, Evangelia F, Maria T, Anna T, Panagiota N et al. B lymphocyte stimulator, interferon-α and HMGB 1 interrelation

in childhood onset systemic lupus erythematosus: associations with disease activity and severity. *Pediatr Rheumatol Online J.* (2014) 12 (Suppl. 1):321. doi: 10.1186/1546-0096-12-S1-P321
70. Giles JR, Kashgarian M, Koni PA, Shlomchik MJ. B Cell-Specific MHC Class II Deletion reveals multiple nonredundant roles for B cell antigen presentation in murine lupus. *J Immunol.* (2015) 195:2571–9. doi: 10.4049/jimmunol.1500792
71. Ballantine LE, Ong J, Midgley A, Watson L, Flanagan BF, Beresford MW. The pro-inflammatory potential of T cells in juvenile-onset systemic lupus erythematosus. *Pediatr Rheumatol Online J.* (2014) 12:4. doi: 10.1186/1546-0096-12-4
72. Deakin CT, Campanilho-Marques R, Simou S, Moraitis E, Wedderburn LR, Pullenayegum E., et al. Efficacy and safety of cyclophosphamide treatment in severe juvenile dermatomyositis shown by marginal structural modeling. *Arthritis Rheumatol.* (2018) 70:785–93. doi: 10.1002/art.40418
73. Chen YJ, Wu CY, Huang YL, Wang CB, Shen JL, Chang YT. Cancer risks of dermatomyositis and polymyositis: a nationwide cohort study in Taiwan. *Arthritis Res Therapy* (2010) 12:R70. doi: 10.1186/ar2987
74. Rider LG, Lindsley CB, Miller FW. Juvenile dermatomyositis. In: Petty RE, Laxer RM, Lindsley CB, et al., editors. *Textbook of Pediatric Rheumatology. 7th ed*. Philadelphia: Elsevier (2016). p. 351–405.
75. Shah M, Mamyrova G, Targoff IN, Huber AM, Malley JD, Rice MM., et al. The clinical phenotypes of the juvenile idiopathic inflammatory myopathies. *Medicine* (2013) 92:25–41. doi: 10.1097/MD.0b013e31827f264d
76. Nistala K, Wedderburn LR. Update in juvenile myositis. *Curr Opin Rheumatol.* (2013) 25:742–6. doi: 10.1097/01.bor.0000434674.62644.02
77. Deakin CT, Yasin SA, Simou S, Arnold KA, Tansley SL, Betteridge ZE, et al. Muscle biopsy in combination with myositis-specific autoantibodies aids prediction of outcomes in juvenile dermatomyositis. *Arthritis Rheumatol.* (2016) 11:2806–16. doi: 10.1002/art.39753
78. Tansley SL, Simou S, Shaddick G, Betteridge ZE, Almeida B, Gunawardena H, et al. Autoantibodies in juvenile-onset myositis: Their diagnostic value and associated clinical phenotype in a large UK cohort. *J Autoimmun.* (2017) 84:55–64. doi: 10.1016/j.jaut.2017.06.007
79. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, et al. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy. *J Exp Med.* (2005) 201:591–601. doi: 10.1084/jem.20041367
80. Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies. I: quantitation of subsets according to diagnosis and sites of accumulation and demonstration and counts of muscle fibers invaded by T cells. *Ann Neurol.* (1984) 16:193–208. doi: 10.1002/ana.410160206
81. Theofilopoulos AN, Kono DH, Beutler B, Baccala R. Intracellular nucleic acid sensors and autoimmunity. *J Interferon Cytokine Res.* (2011) 31:867–86. doi: 10.1089/jir.2011.0092
82. Walsh RJ, Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL., et al. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. *Arthritis Rheum.* (2007) 56:3784–92. doi: 10.1002/art.22928
83. Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R, et al. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. *Ann Neurol.* (2005) 57:664–78. doi: 10.1002/ana.20464
84. Piper CJM, Wilkinson MGL, Deakin CT, Otto GW, Dowle S, Duurand CL, et al. CD19(+)CD24(hi)CD38(hi) B cells are expanded in juvenile dermatomyositis and exhibit a pro-inflammatory phenotype after activation through toll-like receptor 7 and Interferon-alpha. *Front Immunol.* (2018) 9:1372. doi: 10.3389/fimmu.2018.01372
85. Martin N, Krol P, Smith S, Murray K, Pilkington CA, Davidson JE., et al. A national registry for juvenile dermatomyositis and other pediatric idiopathic inflammatory myopathies: 10 years' experience; the Juvenile Dermatomyositis National (UK and Ireland) Cohort Biomarker Study and Repository for Idiopathic Inflammatory Myopathies. *Rheumatology* (2011) 50:137–45. doi: 10.1093/rheumatology/keq261
86. Rider LG, Dankó K, Miller FW. Myositis registries and biorepositories: powerful tools to advance clinical, epidemiologic and pathogenic research. *Curr Opin Rheumatol.* (2014) 26:724–41. doi: 10.1097/BOR.0000000000000119
87. Kalampokis I, Venturi GM, Poe JC, Dvergsten JA, Sleasman JW, Tedder TF. The regulatory B cell compartment expands transiently during childhood and is contracted in children with autoimmunity. *Arthritis Rheumatol.* (2017) 69:225–38. doi: 10.1002/art.39820
88. Miller FW, Chen W, O'Hanlon TP, Cooper RG, Vencovsky J, Rider LG, et al. Genome-wide association study identifies HLA 8.1 ancestral haplotype alleles as major genetic risk factors for myositis phenotypes. *Genes Immun.* (2015) 16:470–80. doi: 10.1038/gene.2015.28
89. Rothwell S, Cooper RG, Lundberg IE, Miller FW, Gregersen PK, Bowes J, et al. Dense genotyping of immune-related loci in idiopathic inflammatory myopathies confirms HLA alleles as the strongest genetic risk factor and suggests different genetic background for major clinical subgroups. *Ann Rheumatic Dis.* (2016) 75:1558–66. doi: 10.1136/annrheumdis-2015-208119
90. Gürcan HM, Keskin DB, Stern JN, Nitzberg MA, Shekhani H, Ahmed AR. A review of the current use of rituximab in autoimmune diseases. *Int Immunopharmacol.* (2009) 9:10–25. doi: 10.1016/j.intimp.2008.10.004
91. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis assessment with rituximab study. *Arthritis Rheumatism* (2012) 64:1215–26. doi: 10.1002/art.34359
92. Oddis CV, Reed AM, Aggarwal R, Rider LG, Ascherman DP, Levesque MC, et al. Rituximab in the treatment of refractory adult and juvenile dermatomyositis and adult polymyositis: a randomized, placebo-phase trial. *Arthritis Rheumat.* (2013) 65:314–24. doi: 10.1002/art.37754
93. Hui-Yuen J, Taylor J, Qing Li X, Mariel Bermudez L, Isgro J, Eichenfield AH, et al. Favorable response to belimumab in pediatric-onset systemic lupus erythematosus. *Arthritis & Rheumatology* (2014). 66: S37 doi: 10.1002/art.38439
94. Bryant A, Moore J. Rituximab and its potential for the treatment of rheumatoid arthritis. *Ther Clin Risk Manag.* (2006) 2:207–12.
95. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheumat.* (2010) 62:222–33. doi: 10.1002/art.27233
96. Reddy V, Jayne D, Close D, Isenberg D. B-cell depletion in SLE: clinical and trial experience with rituximab and ocrelizumab and implications for study design. *Arthritis Res. Ther.* (2013) 15 Suppl 1:S2. doi: 10.1186/ar3910
97. Ahuja A, Teichmann LL, Wang H, Dunn R, Kehry MR, Shlomchik MJ. An acquired defect in IgG-dependent phagocytosis explains the impairment in antibody-mediated cellular depletion in Lupus. *J Immunol.* (2011) 187:3888–94. doi: 10.4049/jimmunol.1101629
98. Stoll ML, Cron RQ. Treatment of juvenile idiopathic arthritis: a revolution in care. *Pediatric Rheumatol Online J.* (2014) 12:13. doi: 10.1186/1546-0096-12-13
99. Sakamoto AP, Pinheiro MM, Barbosa CM, Fraga MM, Len CA, Terreri MT. Rituximab use in young adults diagnosed with juvenile idiopathic arthritis unresponsive to conventional treatment: report of 6 cases. *Rev Bras Reumatol.* (2015) 55:536–41. doi: 10.1016/j.rbr.2014.12.015
100. Berrada K, Abourazzak FE, El Mezouar I, Lazrak F, Aradoini N, Tahiri L, et al. A successful treatment of juvenile idiopathic arthritis with rituximab: A report of two cases. *Eur J Rheumatol.* (2014) 1:164–66. doi: 10.5152/eurjrheumatol.2014.140049
101. Watson L, Beresford MW, Maynes C, Pilkington C, Marks SD, Glackin Y, et al. The indications, efficacy and adverse events of rituximab in a large cohort of patients with juvenile-onset SLE. *Lupus* (2015) 24:10–7. doi: 10.1177/0961203314547793
102. Willems M, Haddad E, Niaudet P, Koné-Paut I, Bensman A, Cochat P., et al. Rituximab therapy for childhood-onset systemic lupus erythematosus. *J Pediatrics* (2006) 148:623–27. doi: 10.1016/j.jpeds.2006.01.041
103. Marks SD, Patey S, Brogan PA, Hasson N, Pilkington C, Woo P., et al. B lymphocyte depletion therapy in children with refractory systemic lupus erythematosus. *Arthritis Rheumat.* (2005) 52:3168–74. doi: 10.1002/art.21351
104. Lei L, Muhammad S, Al-Obaidi M, Sebire N, Cheng IL, Eleftheriou D, et al. Successful use of ofatumumab in two cases of early-onset juvenile SLE with thrombocytopenia caused by a mutation in protein kinase C delta. *Pediatric Rheumatol. Online J.* (2018) 16:61. doi: 10.1186/s12969-018-0278-1

105. Gazeau P, Devauchelle-Pensec V, Pochard P, Pers JO, Saraux A, Renaudineau Y, et al. Abatacept efficacy in rheumatoid arthritis is dependent upon baseline blood B-cell levels. *Rheumatology* (2016) 55:1138–40. doi: 10.1093/rheumatology/kev417
106. Gazeau P, Alegria GC, Devauchelle-Pensec V, Jamin C, Lemerle J, Bendaoud B, et al. Memory B Cells and response to abatacept in rheumatoid arthritis. *Clin Rev Allergy Immunol*. (2017) 53:166–76. doi: 10.1007/s12016-017-8603-x
107. Iwata S, Nakayamada S, Fukuyo S, Kubo S, Yunoue N, Wang SP, et al. Activation of Syk in peripheral blood B cells in patients with rheumatoid arthritis: a potential target for abatacept therapy. *Arthritis Rheumatol.* (2015) 67:63–73. doi: 10.1002/art.38895
108. Ruperto N, Lovell DJ, Quartier P, Paz E, Rubio-Pérez N, Silva CA, et al. Abatacept in children with juvenile idiopathic arthritis: a randomised, double-blind, placebo-controlled withdrawal trial. *Lancet* (2008) 372:383–91. doi: 10.1016/S0140-6736(08)60998-8
109. Birolo C, Zannin ME, Arsenyeva S, Cimaz R, Miserocchi E, Dubko M, et al. Comparable efficacy of abatacept used as first-line or second-line biological agent for severe juvenile idiopathic arthritis-related uveitis. *J Rheumatol.* (2016) 43:2068–73. doi: 10.3899/jrheum.151389
110. Burn GL, Svensson L, Sanchez-Blanco C, Saini M, Cope AP. Why is PTPN22 a good candidate susceptibility gene for autoimmune disease? *FEBS Lett*. (2011) 585:3689–98. doi: 10.1016/j.febslet.2011.04.032
111. Arabshahi B, Silverman RA, Jones OY, Rider LG. Abatacept and sodium thiosulfate for treatment of recalcitrant juvenile dermatomyositis complicated by ulceration and calcinosis. *J Pediat*. (2012) 160:520–2. doi: 10.1016/j.jpeds.2011.11.057

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REVIEW
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Alarmins of the S100-Family in Juvenile Autoimmune and Auto-Inflammatory Diseases

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Autoimmune and auto-inflammatory diseases in children are causing chronic inflammation, organ damage, and pain. Although several options for treatment are nowadays available a significant number of patients does not respond sufficiently to current therapies. In these diseases inflammatory processes are triggered by numerous exogenous and endogenous factors. There is now increasing evidence that especially a novel family of pro-inflammatory molecules, named alarmins, play a significant role in inflammatory processes underlying these diseases. Alarmins are endogenous proteins released during stress reactions that confer inflammatory signaling via Pattern Recognition Receptors (PRRs), like the Toll-like receptor 4 (TLR4). The most abundant alarmins in juvenile rheumatic diseases belong to the family of pro-inflammatory calcium-binding S100-proteins. In this review we will give a general introduction in S100-biology. We will demonstrate the functional relevance of these proteins in animal models of autoimmune and auto-inflammatory diseases. We will show the expression patterns of S100-alarmins and correlation to disease activity in different forms of juvenile idiopathic arthritis, auto-inflammatory diseases, and systemic autoimmune disorders. Finally, we will discuss the clinical use of S100-alarmins as biomarkers for diagnosis and monitoring of rheumatic diseases in children and will point out potential future therapeutic approaches targeting inflammatory effects mediated by S100-alarmins.

Keywords: S100, alarmins, autoinflammation, biomarker, DAMP, rheumatic diseases

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DANGER SIGNALS, ALARMINS AND PATTERN RECOGNITION RECEPTORS IN INFLAMMATION

It is now widely accepted that most pediatric rheumatic diseases are driven by mechanisms of both autoimmunity and auto-inflammation (1). It is believed that aberrant activation of dendritic cells (DC) due to presentation of autoantigens to T-cells in the context of pathological co-stimulation results in development of immune reactivity toward native antigens driven by autoreactive T- and B-cells. However, in many classical autoimmune diseases no dominant autoantigen has been identified so far and there is now increasing evidence that antigen-independent but self-directed inflammation, driven by local factors released during tissue damage or cellular stress, leads to activation of innate immune cells (2, 3). Primarily, inflammation is a protective response of the organism to infections or tissue damage eventually resulting in the elimination of the harmful trigger and tissue repair (4). However, rare inborn diseases of innate immunity, so-called auto-inflammatory diseases, demonstrated that uncontrolled activation of cytokine cascades,

mutations leading to recurrent tissue or cell damage or aberrant receptors for detection of microbes may result in tissue specific recurrent inflammation (3). Cells of the innate immune system, first of all neutrophils and monocytes, recognize invading pathogens by so called pathogen associated molecular patterns (PAMPS), conserved structures specific for a distinct group of microorganisms, like LPS of gram-negative bacteria or single stranded RNA of viruses. These hallmarks of invading pathogens are recognized by conserved receptors on immune and non-immune cells called pattern recognition receptors (PRRs). PRRs include the toll like receptors (TLRs), which are highly conserved in different species and were first identified in drosophila, a species that does not possess an adaptive immune system (5). Upon binding of these PAMPS to specific receptors a transcriptional response is initiated resulting in the production of inflammatory cytokines like interleukin-1ß (IL-1ß), IL-6, or tumor necrosis factor (TNF) as well as of chemokines to promote the recruitment and activation of inflammatory cells at the site of infection/injury in order to combat pathogens and tissue damage (5). It is now clear that some PRRs, in addition to PAMPS, can also recognize own cellular molecules that are released during cell stress and tissue injury. In analog to PAMPs they are called DAMPs (damage or danger associated molecular patterns) or alarmins (**Figure 1**). Most alarmins are primarily intracellular molecules involved in different cellular processes. After release during cell damage or secretion by activated cells they act as extracellular danger signals. Like PAMPs, alarmins are recognized by PRRs (6). Interestingly, some receptors such as the TLR4 seem to be able to recognize PAMPs as well as alarmins (2, 7) Like PAMPS the recognition of alarmins induces a transcriptional response resulting in strong local sterile inflammation (8). Under physiological conditions the function of this inflammatory program is the induction of a tissue repair/remodeling situation resulting in reconstitution of the integrity of the organism. Alarmins include amongst others heat shock proteins (HSP60, 70, Gp96), high mobility group box 1 protein (HMGB1) but also S100-proteins (S100A8/S100A9 and S100A12,) which are the main topic of this review (8). In addition to proteins also lipoproteins and fatty acids, proteoglycans as well as nucleic acids can serve as alarmins.

Characteristic for all alarmins is the recognition by conserved receptors. S100-proteins for example are recognized by the TLR4/MD2 complex, while heat shock proteins are recognized by TLR2. As a functional consequence the expression of the receptors on different cell types also restrict their biological activity. Both TLR4 and TLR2 are expressed extracellularly, while dsDNA, ssRNA or DNA-immune complexes are sensed by intracellular receptors like TLR7 and TLR9 (9). Depending on the structure of the alarmin the binding to the receptor results in a downstream response similar to the binding of a pathogen. Alarmins are important signals to induce tissue repair mechanisms. However, there is current evidence, that excessive or inappropriate amounts and locations of alarmins can induce harm to tissue as well. High amounts of HMGB1 and HSP 70 for example have been identified in the synovia of rheumatoid arthritis patients (10, 11), dsDNA complexes induce interferon signaling in dendritic cells (12) and even serve as diagnostic criteria of systemic lupus erythematosus (SLE). In addition, incubation of healthy tissue or cells with alarmins can induce a sterile inflammation (13), while specific deletion of alarmins in genetically targeted mice results in amelioration of experimental disease like arthritis or sepsis. Thus, the whole system of alarmins as an inducer of cell injury on the one side and of tissue repair on the other side needs to be tightly controlled.

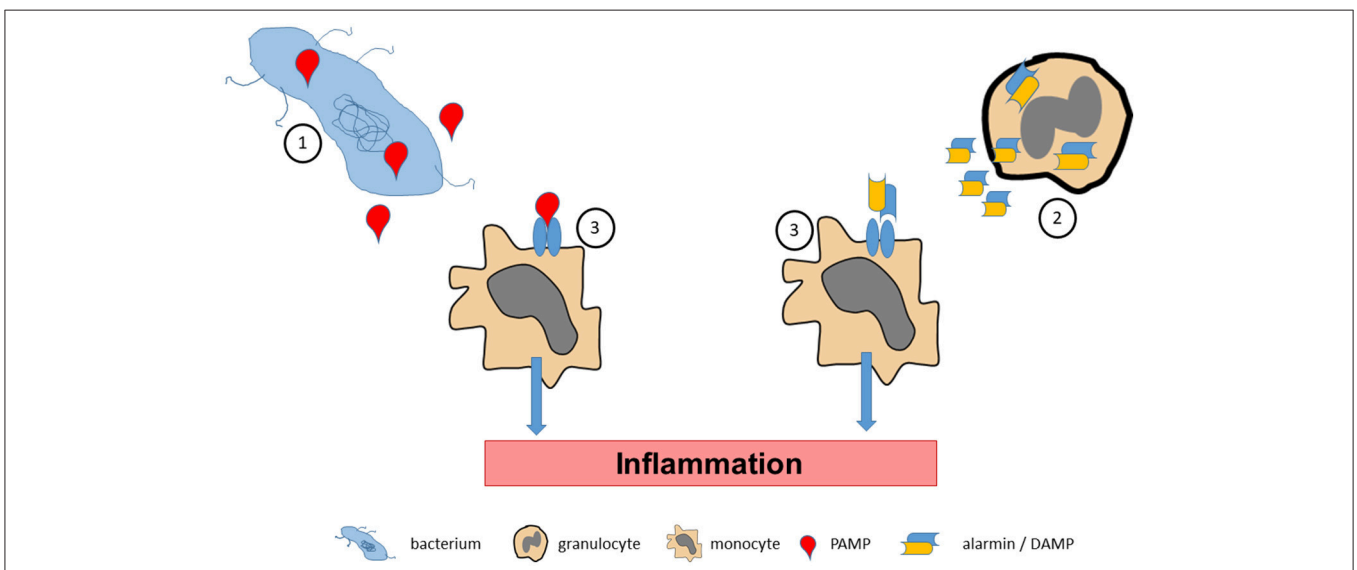

FIGURE 1 | Pathogen associated molecular pattern molecules (PAMPs) are released by bacterial pathogens during infections (1). Accordingly, tissue stress or cellular activation may lead to release of endogenous alarmins or danger associated molecular pattern molecules (DAMPs) under sterile conditions (2). PAMPs and alarmins/DAMPs bind to and activate specific pattern recognition receptors on effector cells (3), which promote inflammatory processes.

This suggests that alarmins as well as their binding partners (TLRs) can serve as therapeutic targets in certain disease settings. An example of this is again SLE, in which the drug chloroquine alters the pH of lysosomes, where TLR7 and 9 are located and thereby reduces the binding affinity of ds-DNA immune complexes to TLR9 and downregulates interferon production (9). The use of chloroquine has now come to a renaissance and is recommended for the basic treatment of SLE and used in nearly every patient (14).

THE FAMILY OF S100-PROTEINS

The most abundant alarmins in many inflammatory disorders, S100A8 and S100A9, belong to the group of S100-proteins defining a family of small (molecular weight of about 10–12 kDa) calcium-binding molecules. Members of this S100-protein family are characterized by a tissue or cell type-specific expression pattern. All S100-proteins have two calcium-binding sites of the so-called EF-hand type (15–17). Binding of calcium to these calcium binding sites induces conformational changes resulting in interaction of S100-proteins with different ligands or binding to specific receptors. A typical characteristic of most S100-proteins is the formation of homodimers, heterodimers and/or higher oligomers. Intracellularly S100-proteins have been described to be involved in many processes including cell cycle control, proliferation, differentiation, migration, metabolism, cellular dynamics, signaling, and cell death. A close correlation between high expression and release of different S100 proteins with disease activity has been shown in many inflammatory diseases, e.g., rheumatoid arthritis (RA), inflammatory bowel or lung disease, but also in Alzheimer's disease, cardiovascular disease and cancer (2, 15).

S100A8 AND S100A9: MAJOR CALCIUM-BINDING PROTEINS IN MONOCYTES AND GRANULOCYTES

As mentioned above the most abundant alarmins in many clinically relevant diseases are S100A8 and S100A9. Both proteins have been initially described as myeloid-related protein 8 (MRP8) and MRP14 due to the fact that both molecules are expressed in high amounts in neutrophilic granulocytes and inflammatory monocytes/macrophages whereas they cannot be found in lymphocytes or resting tissue macrophages. In granulocytes S100A8/S100A9 represent more than 40% of the detergent soluble protein amount, in monocytes up to 5% (18). Synonyms of S100A8 and S100A9 are calgranulin A and calgranulin B, respectively, and complexes of both molecules have been also described as calprotectin. In addition, expression of both proteins is induced in some epithelial cells of gut and skin, osteoclasts and synoviocytes during inflammatory processes (7, 19).

Like many alarmins S100A8 and S100A9 exhibit primarily intracellular functions. Critical for biological functions of these proteins is formation of non-covalently associated hetero-complexes. S100A8/S100A9 heterodimers represent the structural basis of these proteins, monomers are not stable and homodimers seem not to play a relevant role in humans. With increasing calcium concentrations two S100A8/S100A9 dimers associate to $(S100A8/S100A9)_2$ hetero-tetramers (20). Complexes of S100A8/S100A9 have been described to modulate cytoskeleton-membrane interactions in a calcium-dependent manner, which seems to be of relevance for cellular dynamics and migration of phagocytes. The latter effect seems to be mediated by regulating activity of small GTPases and polymerization of microtubules. The effect on tubulin polymerization is controlled by phosphorylation of S100A9 on threonine 113 by p38 mitogen-activated protein kinase (MAPK) (21). However, the intracellular functions of S100A8 and S100A9 are not well-defined.

SECRETION AND EXTRACELLULAR EFFECTS OF S100A8/S100A9

Beside the intracellular effects described above, S100A8 and S100A9 are secreted during many inflammatory diseases triggering inflammatory functions in many cell types, for example, endothelial cells, phagocytes, lymphocytes, or osteoclasts (22). S100A8 and S100A9 have been ascribed several extracellular functions, but the mode of secretion is still not completely clear since both proteins lack the necessary leader sequences for transport and release of the classical pathway via endoplasmic reticulum and Golgi complex. One potential mechanism is the passive release of both proteins due to necrosis of neutrophils and monocytes during inflammatory processes or during the formation of neutrophil extracellular traps (22). However, concentrations of these S100-proteins in sera do not correlate well with parameters of cell death. There is also a specific and energy-dependent release of S100A8/S100A9 by human monocytes which is induced within minutes after activation of these cells and which depends on activation of protein kinase C. Inhibitors of vesicular traffic through the endoplasmic reticulum and Golgi complex do not block release of S100A8/S100A9. The same is true for inhibitors of protein translation indicating that preformed S100-proteins are released during phagocyte activation. However, secretion of S100A8/S100A9 is an energy-dependent process and is significantly reduced by inhibitors of cellular energy metabolism and oxidative phosphorylation confirming an active and specific release pathway for these proteins (23). After induction of secretion S100A8/S100A9 complexes co-localize with microtubules and colchicine inhibits release of these molecules. Thus, the process of release is clearly distinct from classical secretion (eg., TNF) but shows also differences to the alternative pathway of release of IL-1ß (23). *In vivo* interaction of E-selectin with P-selectin glycoprotein ligand 1 (PSGL-1) triggers release of S100A8/S100A9 during rolling of neutrophils on TNF activated endothelial cells. Subsequently, S100A8/S100A9 act as an autocrine player promoting leukocyte adhesion to endothelium and transmigration. This process involves rapid ß2 integrin activation in a GTPase-dependent manner which results in reduced leukocyte rolling velocity and increased adhesion (24). Additional inflammatory stimuli of S100A8/S100A9 release in neutrophils include

C5a, N-Formylmethionyl-leucyl-phenylalanine (fMLP), or monosodium urate crystals in a tyrosine kinase (Src)/spleen tyrosine kinase (Syk)- and tubulin-dependent manner (25).

S100A8/S100A9 acts on different cell types and induces several molecular pathways highly relevant in the pathology of arthritis. On endothelial cells S100A8/S100A9 induce an inflammatory response resulting in induction of cytokines, loss of cell-cell contacts, and increasing permeability of endothelial monolayers (26). Recently, we performed a genome-wide expression analysis with S100A8-stimulated monocytes. This analysis identified around 500 up- and 1,000 downregulated genes that were overrepresented in specific functional clusters, such as immune cell activation, cell migration, leukocyte activation, and signal transduction (NF-κB signaling) (27). S100A8/S100A9 induces expression of cytokines like TNF and IL-6, chemokines like CXCL-10 as well as matrix metallo-proteinases MMP3, MMP9, and particularly MMP13 which is involved in cartilage and bone metabolism (27, 28).

However, continuous stimulation of TLR4 with S100A8/S100A9 may induce a status of "tolerance" in phagocytes as described for PAMPS like LPS and alarmins like heat shock proteins or HMGB1 (29–31). In addition, prolonged exposure of myeloid progenitor cells modulates development of dendritic cells and so called myeloid derived suppressor cells depending on time and dose of S100-stimulus (32).

S100A8/ S100A9 has an anti-apoptotic effect on neutrophils and increases cell survival, a pathway involving TLR4, CD11b/CD18, and mitogen activated protein kinase signaling (33).

RECEPTORS FOR S100A8/S100A9

After release into the extracellular compartment S100A8/S100A9 molecules are enriched at sites of inflammation by binding glycosaminoglycans. In addition, S100A8/S100A9 may interact with specific receptors, TLR4 and the receptor for advanced glycation end products (RAGE) (34). Another receptor, EMMPRIN (synonyms BASIGIN and CD149), binds S100A9 and has been reported to trigger monocyte/macrophage migration. However, the physiological relevance of this receptor for S100-biology is yet not clear (35).

Although S100A8/S100A9 bind to RAGE and especially carboxylated N-glycans expressed on this receptor knock-out of RAGE in myeloid cells does not interfere with the inflammatory response induced by S100A8/S100A9. In contrast, knock-out of TLR4 in murine phagocytes completely abolishes the response of these cells toward S100A8/S100A9 stimulation (36). The relevance of S100A8/S100A9-mediated TLR4 signaling was confirmed in human monocytes demonstrating an almost identical expression pattern induced by the classical TLR4-ligand LPS and S100A8 (27). Accordingly, transfection of HEK cells with TLR4 induces S100-sensitivity of these cells whereas RAGE transfection has no effect. S100A8/S100A9-binding to TLR4 activates MyD88 and TRIF-dependent signaling and results in activation of NF-kB and induction of inflammatory gene expression (27, 36).

Since TLR4 is also the LPS receptor possible endotoxin contamination of purified S100A8/S100A9 could be a major bias regarding inflammatory effects of these proteins. However, this possibility was excluded in several independent approaches. First of all, knock-out of S100A9 in mice has an anti-inflammatory effect in many murine models even under sterile conditions in the absence of any microbial stimulus (7, 22, 28). Furthermore, LPS contaminations of S100 charges were excluded by Limulus assay. Blocking LPS by the endotoxin antagonist polymyxin B has no effect on S100A8/S100A9 activities. Heating of S100A8/S100A9 samples on the other hand completely abolished inflammatory activities of these proteins under conditions which have no influence on LPS activity in the same experiments (27, 36). Last but not least, we have recently identified the specific TLR4-binding site within the S100A8 and S100A9 molecules which were confirmed by targeted mutagenesis, peptide binding and structural analysis. Point mutations in the TLR4-binding site of S100A8 or S100A9 abolished inflammatory activity which is the final proof to exclude any effect by LPS contaminations (37).

Recently we unraveled a novel regulatory mechanism which restricts the inflammatory effects of S100A8/S100A9 to the local process of inflammation. Interaction of S100A8/S100A9 with TLR4/MD2 is mediated by peptide sequences of about 10–15 amino acids within the second calcium-binding EF-hands of both S100-subunits. These TLR4 binding structures are freely accessible in heterodimers of S100A8/S100A9 which are released during inflammation by monocytes, macrophages and granulocytes. In the presence of high extracellular calcium concentrations S100A8/S100A9 dimers associate to $(S100A8/S100A9)_2$ tetramers which hide the specific TLR4/MD2-binding peptides within the tetramer interphase, thus representing an auto-inhibitory process limiting S100-effects to local sites of inflammation and avoiding undesirable systemic effects (**Figure 2**). Loss of this auto-inhibitory mechanism results in fatal inflammation in an animal model of TNF-driven arthritis and psoriasis (37).

S100A8/A9 IN ARTHRITIS AND AUTOIMMUNITY

S100A8 and S100A9 were initially identified as the two major calcium-binding proteins highly expressed in inflammatory granulocytes and macrophages during RA (38). Synovitis during RA is characterized by a high abundance of neutrophils and macrophages expressing S100A8 and S100A9 especially at the cartilage–pannus junctions indicating that S100-expression is closely associated with cartilage destruction and bone erosion in arthritis. Concentrations of S100A8/S100A9 are very high at the side of inflammation, ie., the synovial fluid, and correlate well with serum concentrations and disease severity in patients with RA (39). Also, in psoriasis arthritis patients, expression of S100A8 andS100A9 is very high in the synovial sub-lining layer and particularly in perivascular areas (40).

Functional relevance of S100A8/S100A9 expression was further confirmed by analysis of experimental models of arthritis and synovial inflammation in $S100A9^{-/-}$ mice.

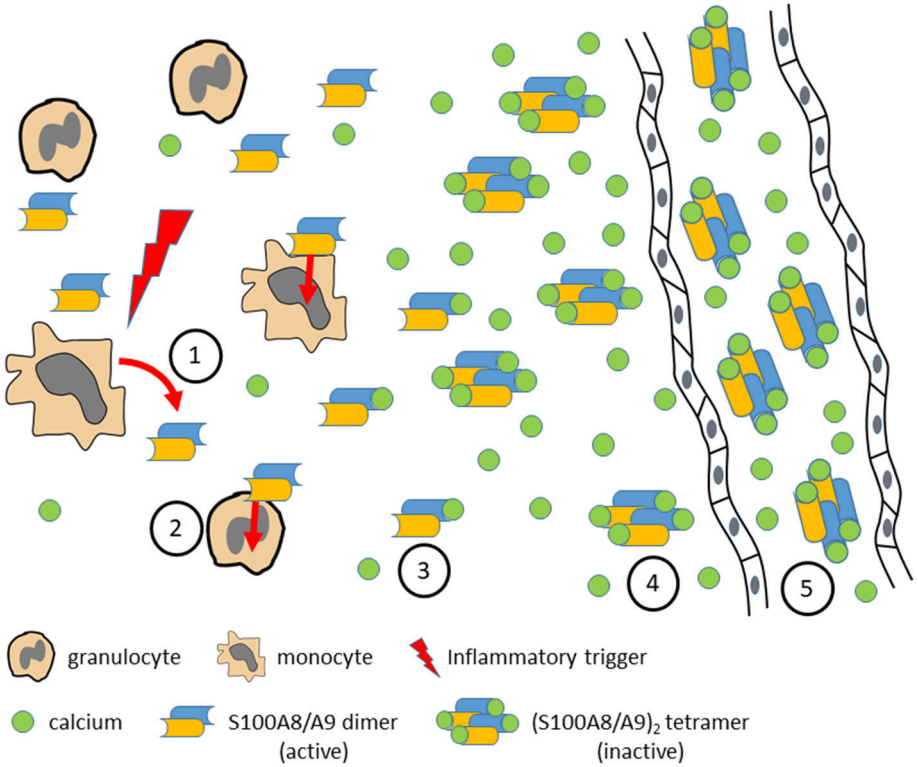

FIGURE 2 | Various inflammatory triggers lead to local release of S100A8/S100A9 dimers by activated monocytes or granulocytes (1). S100A8/S100A9 may bind to TLR4 on different target cells and amplify and perpetuate the inflammatory response (2). With increasing calcium concentrations toward systemic circulation S100A8/S100A9 dimers bind calcium ions (3) and form $(S100A8/S100A9)_2$ tetramers (4) which show no inflammatory activity any more due to the fact that the TLR4 binding site is hidden in the tetramer interface. In systemic circulation high calcium concentrations prevent systemic inflammatory side effects by stabilization of inactive $(S100A8/S100A9)_2$ tetramers (5) which, however, are useful biomarkers for monitoring local disease activity.

Lack of S100A8/A9 expression decreases joint inflammation, protease expression and cartilage destruction during antigen-induced arthritis (28). In this context S100A8 induces activating Fcγ receptors I and IV in macrophages in inflamed synovium in a TLR-4 dependent manner which eventually results in bone erosion (41, 42). There is a synergetic effect between TNF, IL-1ß, IL-17, and S100A8 leading to induction of erosive MMPs and exaggeration of cartilage erosion during arthritis (43). In addition, S100A8 and S100A9 have been shown to promote the development of functional autoreactive CD8+ T-cells in a mouse model of CD40L-induced lupus-like disease (44).

EXPRESSION OF S100A12 IN INFLAMMATORY PROCESSES

The third S100-protein expressed in granulocytes and monocytes is S100A12. Expression of S100A12 correlates closely with S100A8/S100A9, however, in at least 10-fold lower quantities. S100A12 resulted from a duplication of the S100A9 gene during evolution (15, 45). However, expression levels obviously decreased significantly compared to S100A9 during evolution and the gene is disrupted and no S100A12 protein is found in rodents including mice. Biological activity of S100A12 is low compared to S100A8/S100A9. There is no clear evidence *in vivo* whether S100A12 has a relevant function in inflammatory processes. However, expression of this protein correlates very well with disease activity in many pathological conditions and S100A12 is a useful biomarker for monitoring disease activity in many clinically relevant disorders like rheumatoid arthritis, inflammatory bowel disease, vasculitis, or psoriasis (46–48).

S100 PROTEINS AS BIOMARKERS OF INFLAMMATION

Since alarmins and cytokines are essential part of the pathophysiology of pediatric rheumatic diseases they are candidates for biomarkers of inflammatory processes. However, to be useful biomarkers should be able to support initial diagnosis, reflect disease activity, and predict the further outcome of inflammatory diseases with high diagnostic accuracy. Feasible biomarkers should be obtained through non-invasive, easily performed, reproducible, and cost-effective procedures.

S100 proteins are part of the local inflammatory process and reflect the disease activity when measured in the serum. Phagocyte-specific S100-proteins have been established as useful markers of both local and systemic inflammation.

They correlate with disease activity in rheumatic diseases, vasculitis, inflammatory bowel disease, pulmonary diseases, and infections (46–58). Their stability makes these proteins useful as biomarkers for the monitoring of pediatric rheumatic diseases in clinical practice. S100 proteins are stable at room temperature for several days in separated serum, so serum samples can be sent at room temperature (59). Additionally, commercial assays are available especially for the detection of S100A8/S100A9 but only some of these assays are certified for use in clinical diagnostics. However, these assays are not strictly comparable and especially the concentrations vary among different providers. Therefore, a strict evaluation including validation and standardization of these assays is mandatory before introducing commercial assays in clinical practice. For some of these assays validation studies have been already performed and some assays can be recommended for use to confirm inactive disease in clinical practice (60). However, all available assays have a limited linearity in serum compared to standard buffer at higher concentrations often requiring serial dilutions of individual samples. Serum concentrations of S100-proteins are independent of age and gender (59). Normalization of S100 levels can take 8 [in CAPS with effective canakinumab treatment (61)] to 30 days [in SJIA with effective anakinra treatment (62)].

S100 PROTEINS IN PEDIATRIC RHEUMATIC DISEASES

Juvenile Idiopathic Arthritis (JIA)

Pathophysiology and Marker of Disease Activity

Over the last 20 years the importance of S100-proteins as biomarkers of inflammatory activity and the understanding of its pathogenetic role in JIA has evolved rapidly. In 2000, Frosch et al. could demonstrate that S100A8 and S100A9 are specifically released during interaction of activated monocytes with TNF-stimulated endothelial cells. In JIA patients, S100A8, and S100A9 were strongly expressed in infiltrating neutrophils and monocytes within the inflamed joints and could be found in significantly higher concentrations in synovial fluid compared with serum. After intraarticular triamcinolone therapy, the serum concentrations of S100A8/S100A9 decreased significantly in the serum of therapy responders, whereas no differences were found in patients who showed no clinical benefit (63).

Comparable results could be obtained for S100A12. S100A12 serum concentrations were determined in 124 patients with chronic active polyarticular, oligoarticular, or systemic-onset JIA (SJIA). The mean serum level of S100A12 was 395 ng/ml in patients with active polyarticular JRA and 325 ng/ml in patients with active oligoarticular JIA (normal <120 ng/ml). The level of S100A12 was ∼ 10-fold higher in synovial fluid than in serum, indicating release at sites of local inflammation. Notably, in patients with SJIA, the mean level of S100A12 was 3700 ng/ml. Moreover, serum levels decreased in response to different anti-inflammatory therapies (i.e., intraarticular injections of corticosteroids, methotrexate (MTX), or etanercept). Moreover, S100A12 levels were elevated in 20 patients who experienced disease flares after the initial induction of remission, even weeks before the relapses became clinically apparent. This finding demonstrated that S100-proteins might indicate synovial inflammation even when other signs of arthritis are absent (52).

Marker of Subclinical Inflammation

Accordingly, the potential of S100-proteins to indicate subclinical inflammation was evaluated in a large prospective, open, multicenter, medication-withdrawal randomized clinical trial including 364 patients. This study aimed to analyze whether longer MTX treatment during remission of JIA prevents flares after withdrawal of medication and whether specific biomarkers identify patients at risk for flares. Primary outcome was relapse rate in the 2 treatment groups (withdrawal after 6 or 12 months); secondary outcome was time to relapse. Besides the finding that in patients with JIA in remission, a 12- vs. 6-month withdrawal of MTX did not reduce the relapse rate, it could be demonstrated that higher S100A8/S100A9 concentrations at time of MTX withdrawal were associated with risk of relapse after discontinuing MTX (59).

Clinical inactive disease with elevated inflammatory markers can be defined as subclinical disease activity, which may result in unstable remission (i.e., a status of clinical but not immunological remission). Therefore, in a sub-analysis of this study S100A12, S100A8/S100A9 as well as the acute phase reactant high-sensitivity C reactive protein (hsCRP) were compared as predictive biomarkers for the risk of a flare within a time frame of 6 months. Clinical or standard laboratory parameters could not differentiate between patients at risk of relapse and those not at risk. On the other hand S100A12 and S100A8/S100A9 levels were significantly higher in patients who subsequently developed flares than in patients with stable remission (64).

To implement these biomarkers for further studies and use in clinical practice the performance of different enzyme-linked immunosorbent assays (ELISAs) in order to validate systems available for routine use were tested. The tested commercial S100A8/S100A9 and S100A12 ELISAs showed a performance comparable to well-established experimental ELISA protocols when assay-specific cutoffs for the indication of relapse prediction were thoroughly applied (60). In another study S100A8/S100A9 levels before discontinuation of anti-tumor necrosis factor (TNF)-inhibitors were analyzed retrospectively. Patients who flared within 6 months after treatment discontinuation had higher S100A8/S100A9 levels compared to patients with stable remission. Results were confirmed by a commercial ELISA assay with high reproducibility but different overall levels (65). As mentioned above analysis of higher serum concentrations may require serial dilutions of individual samples to obtain reliable results. A recent study analyzed the relationship between serum S100A8/S100A9 and S100A12 and the maintenance of clinical inactive disease (CID) in patients with polyarticular forms of juvenile idiopathic arthritis (PF-JIA) while on anti-TNF- therapy and disease flare following withdrawal of treatment. Here, serum S100 levels did not predict maintenance of CID or disease flare, with S100A12 levels only moderately correlating inversely with time to disease flare (66). Further studies are needed to evaluate the clinical use of S100-proteins for stopping treatment.

Marker of Response to Therapy

Besides supporting tools for stopping treatment in remission there is an unmet need for biomarkers with which to identify patients who will respond well to anti-inflammatory therapy. Around one-third of patients with juvenile idiopathic arthritis (JIA) fail to respond to first-line MTX or TNF therapy, with even fewer achieving ≥ American College of Rheumatology Pediatric 70% criteria for response (ACRpedi70). Within the Childhood Arthritis Response to Medication Study (CHARMS) the prognostic value of baseline serum proteins (S100A8/S100A9, inflammatory cytokines, CRP), ESR and clinical variables in response to MTX was analyzed to identify whether the patient is likely to respond well to MTX. High disease activity (high serum S100A8/S100A9, active joint count, or physician's score) pre-MTX was observed in a subgroup of patients with a better response to therapy. In a multivariable analysis, after accounting for S100A8/S100A9 at baseline, no other factors were independently significantly associated with outcome (67). High levels of baseline S100A8/S100A9 are associated with good response to anti-TNF treatment. Baseline S100A8/S100A9 levels in patients before treatment with TNF-inhibitors were higher in responders compared to non-responders. Levels decreased after start of treatment only in responders. Change in JADAS-10 was correlated with baseline S100A8/S100A9 levels and documented the correlation of S100A8/S100A9 with disease activity (65). These results could also be confirmed for S100A12. Responders to MTX or anti-TNF treatment can be identified by higher pretreatment S100A12 serum concentration levels and baseline serum S100A12 in both univariate and multivariate regression models was significantly associated with change in JADAS-10 (68).

Juvenile Dermatomyositis (JDM)

The aetiopathogenesis of JDM and in particular the contribution of monocytes or macrophages, which are frequently observed to infiltrate muscle tissue very early in the disease process, remains poorly understood. Early results indicated a clear association of expression of S100A8 and S100A9 by infiltrating macrophages with degeneration of myofibers in muscle biopsies of patients with dermatomyositis, polymyositis, and inclusion body myositis. Furthermore, S100A8/S100A9 complex inhibited proliferation and differentiation of myoblasts and induced apoptosis via activation of caspase-3. In the course of inflammatory myopathies, activated macrophages seem to promote destruction and impair regeneration of myocytes via secretion of S100A8/S100A9 (69). These findings could be confirmed in a follow-up study. Here, S100A8/S100A9 levels of 56 JDM patients were compared with clinical measures of disease activity. S100A8/S100A9 serum levels correlated with physician's global assessment of disease activity in JDM and muscle strength/endurance, childhood myositis assessment score. S100A8/S100A9 was widely expressed by CD68+ macrophages in JDM muscle tissue. When cultured with human myoblasts, S100A8 led to the secretion of MCP-1 and IL-6, which was enhanced by ER stress. However, the usefulness of serum S100A8/S100A9 as a potential biomarker for disease activity in JDM has to be confirmed in further studies (70).

Autoinflammatory Diseases

Markedly elevated S100 levels are a hallmark of SJIA, Familial Mediterranean Fever (FMF) and PSTPIP1 associated inflammatory diseases (PAID) such as pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome or PSTPIP1-associated myeloid-related proteinaemia inflammatory (PAMI) syndrome (71). Here, S100 levels can differentiate these conditions from other infectious or auto-inflammatory conditions (**Table 1**). Hypersecretion of S100 proteins in these diseases can result in a sterile inflammatory environment, which triggers pro-inflammatory cytokine as well as further S100A8/S100A9 and S100A12 expression and thus can perpetuate disease activity (72, 73). In contrast, in the cryopyrin associated periodic syndromes (CAPS) or periodic fever, aphthous stomatitis, pharyngitis, adenitis (PFAPA) syndrome S100 levels are lower and within the range of other inflammatory diseases and cannot be used to differentiate from infectious diseases; however, they do correlate with disease activity.

MONOGENIC AUTO-INFLAMMATORY SYNDROMES

Familial Mediterranean Fever (FMF)

Pathophysiology

FMF is an auto-inflammatory syndrome associated with the activation of phagocytic cells and an over-secretion of IL-1β. The discovery of pyrin mutations as the genetic basis of this auto-inflammatory disorder identified the dysfunction of intracellular processes, e.g., alternative secretory pathways, and immune dysregulation involving inflammasome-dependent recruitment and processing of IL-1β as causes of FMF (74). During inflammatory attacks of FMF serum levels of S100A8/S100A9 and S100A12 are massively elevated and significantly higher than in patients with CAPS (75). Both S100A8/S100A9 and S100A12 exhibit pro-inflammatory effects *in vitro* at concentration found in FMF patients *in vivo* during active disease (**Table 1**). This hypothesis is further supported by the observation that S100A8/S100A9 co-localizes with the cytoskeleton and a Golgi-independent but tubulin-dependent release has been shown (21, 23). Pyrin is likewise associated with these structures while colchicine blocks tubulin-dependent processes at the molecular level and is therefore a possible inhibitor of alternative secretion of S100 proteins (80).

Marker of Disease Activity

During acute attacks serum levels of S100A8/S100A9 and S100A12 are massively elevated (71, 72). Moreover, patients with FMF well-controlled with anti-inflammatory treatment have significantly decreased serum levels (72). S100A12 may also allow stratification of FMF patients according to disease severity (72). Overall, S100A12 levels show an excellent correlation to disease activity (56, 75). S100A12 serum levels in patients with unstable disease under colchicine treatment were significantly higher than those without inflammatory attacks, supposed as stable disease. Moreover, homozygous MEFV mutation carriers exhibited clearly increased S100A12 serum levels despite of no clinical disease activity while classical inflammatory markers

TABLE 1 | Serum concentration of phagocyte-specific S100 proteins in inflammatory diseases [adapted and updated from Kessel et al. (73)].

	S100A8/A9 levels (ng/ml)	N†	References	S100A12 levels (ng/ml)	N†	References
POLYGENIC AUTOINFLAMMATORY DISEASES						
Systemic-onset JIA	14,920 ± 4,030	60	(76)	7,190 ± 2,690	60	(75)
	24,750 ± 11,410	20	(77)	3,700 (1,080)**	33	(78)
Polyarthritis JIA	2,380 ± 530	89	(52, 63)	395 (45)**	89	(78)
PFAPA	3846 ± 1197	15	(79)	685 ± 210	15	(79)
MONOGENIC AUTOINFLAMMATORY DISEASES						
FMF	110,000 ± 82,000	20	(71)	6,720 ± 4,960	17	(75)
				33,500 (22,200)**	7	(56)
PAPA	116,000 ± 74,000	11	(71)	–		
PAMI	2,045,000 ± 1,300,000	13	(71)	–		
NOMID	2,830 ± 580	18	(76)	720 ± 450	18	(75)
MWS	4,390 (2535)*	12	(61)	150 ± 60	17	(75)
FCAS	3,600 (4610)*	5	(61)	–	–	–
INFECTIONS						
Severe febrile infections	3,720 ± 870	66	(76)	470 ± 160	83	(75)
Healthy controls	340 ± 70	50	(76)	50 ± 10	45	(75)
				50 (5)**	74	(50)

FCAS, Familial cold autoinflammatory syndrome, FMF, familial Mediterranean fever; JIA, juvenile idiopathic arthritis; MWS, Muckle Wells syndrome; NOMID, Neonatal onset multisystem inflammatory disorder; PAMI, PSTPIP1-associated myeloid-related proteinemia inflammatory; PAPA, pyogenic sterile arthritis, pyoderma gangrenosum, and acne syndrome; PFAPA, periodic fever, aphthous stomatitis, pharyngitis, adenitis syndrome [mean (standard deviation), ** mean (standard error of the mean), all other data are mean ± 95% confidence interval, †N, number of patients studied].*

were in the range of normal controls. Also, heterozygous MEFV mutation carriers have significantly elevated S100A12 serum levels while classical inflammation markers were not increased (56). These findings indicate ongoing subclinical inflammatory activity of the innate immune system in otherwise clinically stable individuals.

PSTPIP1 Associated Inflammatory Diseases (PAID)

PAPA syndrome seems to be only one clinical entity within the expanding spectrum of PAID caused by mutations in PSTPIP1 (81). PAMI syndrome (PSTPIP1 E250K mutation) is a PAID presenting with clinical and biochemical features not found in patients with classical PAPA syndrome (71). Mutated PSTPIP1 markedly increases pyrin binding and IL-1ß production by peripheral blood leukocytes from patients with PAPA and in cell lines transfected with both PAPA associated mutants (82). Moreover, PAPA-associated PSTPIP1 mutants activate pyrin, thereby allowing it to interact with ASC and facilitate ASC oligomerization into an active ASC pyroptosome (83). A hallmark of PAID are very high (PAPA: 116 ± 74 μg/ml) or massively elevated S100A8/S100A9 serum concentrations (PAMI: 2,070 ± 1,190 μg/ml vs. 0.48±0.1 μg/ml in healthy controls) (71). Although the exact role of S100A8/S100A9 in the pathogenesis of PAID is not yet clear, there are important links between S100A8/S100A9, pyrin and PSTPIP1. S100A8/S100A9 serum levels are also highly elevated in FMF (110 ± 82 μg/ml) (71). Like PSTPIP1 and pyrin, S100A8, and S100A9 are highly expressed in phagocytes. Both proteins bind to both the subcellular actin network and microtubules in a calcium dependent manner (21). Interestingly, IL-1ß secretion is only apparent in monocytes of PAPA patients after stimulation with the exogenous TLR-4 ligand LPS (84), which points to a putative role of endogenous TLR-4 ligands S100A8 and S100A9 for the release of IL-1ß from PAPA monocytes.

Cryopyrin-Associated Periodic Syndromes (CAPS)

Pathophysiology

Cryopyrin-associated periodic syndromes (CAPS) comprise a group of rare auto-inflammatory diseases, which include the Familial Cold Auto-inflammatory Syndrome (FCAS), the Muckle-Wells Syndrome (MWS), and the Neonatal-Onset Multiorgan Inflammatory Disease (NOMID) and are caused by mutations in the *NLRP3* (*CIAS1/NALP3/PYPAF1)* gene, encoding for cryopyrin/NALP3 protein. Cryopyrin controls the assembly of proteins into the inflammasome complex, which regulates caspase-1 activity that induces the conversion of pro-IL-1β to biologically active IL-1β (85–88). Uncontrolled pro-IL-1β processing results in a constitutive excess of IL-1β release from phagocytic cells of CAPS patients (89–91). IL-1 hypersecretion is not easy to determine *in vivo* and is only one factor among others involved in a complex immune dysregulation including phagocyte activation during auto-inflammation (76). Although the exact role of the S100-proteins in CAPS has not yet been fully understood, they seem to reflect IL-1ß-driven inflammation in CAPS.

Marker of Disease Activity

Various states of subclinical disease activity were demonstrated in all categories of CAPS, depending on the type of anti-IL-1 therapy. Here, S100 levels were compared with CRP and ESR

and seemed to have a higher sensitivity to detect subclinical inflammation. In this context, S100A8/S100A9 proved to be a sensitive biomarker for monitoring disease activity, and response to IL-1 blockade in patients with CAPS and also indicated subclinical inflammation when CRP and ESR were already normal (57, 61). S100A12 has been shown to be elevated in patients with active NOMID and MWS (75). In patients with CAPS treated with IL-1-blockers, S100A12 and S100A8/S100A9 correlate with inflammatory activity and decline rapidly along with a normalization of neutrophil counts (92).

POLYGENIC AUTO-INFLAMMATORY DISEASES

Systemic Juvenile Idiopathic Arthritis (SJIA)

Pathophysiology

SJIA is a severe systemic inflammatory disease in childhood with significant morbidity and serious complications, especially in those children with a therapy-resistant course. Although defined as a subtype of JIA, the disease nowadays is attributed to the auto-inflammatory syndromes with a significant role of IL-1. Thus, clinical symptoms can be assigned to dysregulated innate immune mechanisms with only little involvement of adaptive immunity. Serum of SJIA patients induces the transcription of genes of the innate immune system including IL-1 in peripheral blood mononuclear cells (PBMCs) and activated monocytes from patients with SJIA secrete significantly higher amounts of IL-1β in comparison with monocytes of healthy controls (93) The predominant role of the innate immune system is furthermore underscored by very high S100A8/S100A9 and S100A12 serum levels (75, 76). The hypersecretion of IL-1, IL-18, S100A8/S100A9, and S100A12 indicates an important aspect regarding the pathogenesis of SJIA since they are all released by the alternative secretory pathway. In contrast to IL-1 and IL-18, S100-proteins are not processed by caspase 1 prior to release (23). Thus, a loss of control of the alternative secretory pathway downstream of caspase 1 has been proposed to be involved in release of pro-inflammatory proteins leading to the inflammatory process of SJIA (94). However, it cannot be determined whether secretion of IL-1β, IL-6, or S100-proteins is a primary or secondary step in the cause-and-effect chain of SJIA (95).

Detection Marker in Fever of Unknown Origin

At initial presentation, SJIA is difficult to differentiate from severe systemic infections. S100A8/S100A9 serum levels are closely correlated to disease activity in SJIA and these high concentrations can be found neither in other forms of inflammatory arthritis, nor in other autoimmune or infectious diseases (52, 78)- in contrast to markers like CRP, which are not able to differentiate SJIA from other causes of FUO (76, 96, 97). The same applies for S100A12 (75, 98). Although SJIA cannot be differentiated from FMF or PAID in this context, these autoinflammatory diseases may at least clinically be differentiated from other causes of FUO.

Marker of Disease Activity

S100A8/S100A9 serum levels correlate closely with response to drug treatment and disease activity and therefore might be an additional measurement for monitoring anti-inflammatory treatment of individual patients with SJIA (62, 77). S100A8/S100A9 serum concentrations are the first predictive biomarker in SJIA indicating subclinical disease activity and stratifying patients at risk of relapse during times of clinically inactive disease (77) and might be able to predict response to treatment with anakinra (62).

PERIODIC FEVER, APHTHOUS STOMATITIS, PHARYNGITIS, CERVICAL ADENITIS (PFAPA) SYNDROME

PFAPA syndrome is characterized by fever flares accompanied by pharyngitis, adenitis, and/or aphthous stomatitis without evidence of infection, asymptomatic intervals between the flares, and onset before the age of 5 years (99). The pathogenic mechanism of this syndrome is not known, but it has been shown that IL-1ß production by monocytes is dysregulated in patients with PFAPA syndrome. Twenty percentage of enrolled patients were found to have NLRP3 variants, suggesting that inflammasome-related genes might be involved in this auto-inflammatory syndrome. S100A8/S100A9 and S100A12 are upregulated in flares but within the range of healthy control in symptom-free intervals. The levels of active patients are within those of systemic infections and have no additional diagnostic value in PFAPA (79).

OUTLOOK

There is now increasing evidence that innate immune mechanisms triggered by local tissue signals, so called alarmins or DAMPs, are crucial factors in the pathogenesis of many pediatric rheumatic diseases. S100-alarmins, especially S100A8/S100A9, are highly upregulated in different forms of arthritis and autoimmune diseases in children. Specific secretion of S100-alarmins at the local site of inflammation by activated phagocytes makes these molecules useful markers for monitoring disease activity. For early diagnosis of SJIA S100A8/S100A9 is currently the most specific biomarker and is used in several specialized centers. In addition, it is used in clinical studies for prediction of disease flares in patients with JIA or RA in clinical remission on medication after stopping or reduction of therapy. However, use in clinical routine is limited by the fact that no commercial assay is available which guaranties reliable data in the whole range of serum levels found in different inflammatory disorders. Novel approaches to follow expression of these molecules *in vivo* by molecular imaging techniques, which already work very well in preclinical models, may even improve the diagnostic value of these molecules. There is already a small molecular compound described which can be used for monitoring S100A8/S100A9 expression in preclinical models of inflammation *in vivo*. Since the structure of this tracer is based on Q-compounds already used in clinical trials with very low

toxicity, transfer of such a tracer into clinical practice may be feasible within the next years (100, 101). Preclinical models in mice have also confirmed a functional role of S100A8/S100A9 in the process of arthritis and autoimmunity. Especially in some auto-inflammatory diseases like SJIA, FMF, and PAID S100-alarmins seem to play a dominant role. The Q-compounds mentioned above have been shown to specifically inhibit binding of S100A9 to TLR4 and RAGE (34). These drugs block the infiltration and activation of phagocytes in experimental models of inflammation (24) In addition, laquinimod, a member of the Q-compound family, showed significant effects in a randomized clinical trial (phase III) for treatment of relapsing–remitting multiple sclerosis (102) and in patients with Crohn's disease (phase II) (103). Treatment with laquinimod was well-tolerated and not associated with major side effects. There are no published data showing therapeutic effects of laquinimod or any other Q-compounds in pediatric rheumatic diseases so far. The recent identification of the TLR4-binding site in S100A8 and S100A9 offers novel molecular structures for targeted inhibition of these interaction. Since the active form of S100A8/S100A9 is restricted to local sites of inflammation such an approach may have very limited systemic side effects. However, data regarding blocking antibodies directed against these specific binding structures in preclinical mouse models are missing so far and would be a prerequisite for transfer of this novel therapeutic approach into clinical trials. In addition, pharmacological inhibition of the non-classical secretory pathway of S100A8/S100A9 may be an alternative strategy to specifically address this inflammatory mechanism. Interestingly, colchicine, which is already used for the treatment of FMF, has been shown to inhibit secretion of S100A8/S100A9 by activated phagocytes. Taken together there are now several lines of future research in the field of S100-biology which may offer innovative diagnostic or even therapeutic approaches for pediatric rheumatic diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. McGonagle D, Aziz A, Dickie LJ, McDermott MF. An integrated classification of pediatric inflammatory diseases, based on the concepts of autoinflammation and the immunological disease continuum. *Pediatr Res.* (2009) 65:38R−45R. doi: 10.1203/PDR.0b013e31819dbd0a
2. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, et al. Alarmins: awaiting a clinical response. *J Clin Invest.* (2012) 122:2711–9. doi: 10.1172/JCI62423
3. McGonagle D, McDermott MF. A proposed classification of the immunological diseases. *PLoS Med.* (2006) 3:e297. doi: 10.1371/journal.pmed.0030297
4. Netea MG, Balkwill F, Chonchol M, Cominelli F, Donath MY, Giamarellos-Bourboulis EJ, et al. A guiding map for inflammation. *Nat Immunol.* (2017) 18:826–31. doi: 10.1038/ni.3790
5. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol.* (2003) 21:335–76. doi: 10.1146/annurev.immunol.21.120601.141126
6. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol.* (2010) 28:367–88. doi: 10.1146/annurev.immunol.021908.132603
7. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol.* (2009) 86:557–66. doi: 10.1189/jlb.1008647
8. Nefla M, Holzinger D, Berenbaum F, Jacques C. The danger from within: alarmins in arthritis. *Nat Rev Rheumatol.* (2016) 12:669–83. doi: 10.1038/nrrheum.2016.162
9. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci USA* (2004) 101:5598–603. doi: 10.1073/pnas.0400937101
10. Kokkola R, Sundberg E, Ulfgren AK, Palmblad K, Li J, Wang H, et al. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis Rheumatism* (2002) 46:2598–603. doi: 10.1002/art.10540
11. Schett G, Redlich K, Xu Q, Bizan P, Groger M, Tohidast-Akrad M, et al. Enhanced expression of heat shock protein 70 (hsp70) and heat shock factor 1 (HSF1) activation in rheumatoid arthritis synovial tissue. differential regulation of hsp70 expression and hsf1 activation in synovial fibroblasts by proinflammatory cytokines, shear stress, and antiinflammatory drugs. *J Clin Invest.* (1998) 102:302–11. doi: 10.1172/JCI2465
12. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* (2001) 294:1540–3. doi: 10.1126/science.1064890
13. Caielli S, Athale S, Domic B, Murat E, Chandra M, Banchereau R, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med.* (2016) 213:697–713. doi: 10.1084/jem.20151876
14. Groot N, de Graeff N, Marks SD, Brogan P, Avcin T, Bader-Meunier B, et al. European evidence-based recommendations for the diagnosis and treatment of childhood-onset lupus nephritis: the SHARE initiative. *Ann Rheumatic Dis.* (2017) 76:1965–73. doi: 10.1136/annrheumdis-2017-211898
15. Marenholz I, Heizmann CW, Fritz G. S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem Biophys Res Commun.* (2004) 322:1111–22. doi: 10.1016/j.bbrc.2004.07.096
16. Moore BW. A soluble protein characteristic of the nervous system. *Biochem Biophys Res Commun.* (1965) 19:739–44. doi: 10.1016/0006-291X(65)90320-7
17. Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS. Calcium-dependent and -independent interactions of the S100 protein family. *Biochem J.* (2006) 396:201–14. doi: 10.1042/BJ20060195
18. Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem.* (1991) 266:7706–13.
19. Roth J, Vogl T, Sorg C, Sunderkotter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol.* (2003) 24:155–8. doi: 10.1016/S1471-4906(03)00062-0
20. Leukert N, Sorg C, Roth J. Molecular basis of the complex formation between the two calcium-binding proteins S100A8 (MRP8) and S100A9 (MRP14). *Biol Chem.* (2005) 386:429–34. doi: 10.1515/BC.2005.051
21. Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, et al. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. *Blood* (2004) 104:4260–8. doi: 10.1182/blood-2004-02-0446

22. Austermann J, Spiekermann C, Roth J. S100 proteins in rheumatic diseases. *Nat Rev Rheumatol*. (2018) 14:528–41. doi: 10.1038/s41584-018-0058-9
23. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem*. (1997) 272:9496–502. doi: 10.1074/jbc.272.14.9496
24. Pruenster M, Kurz AR, Chung KJ, Cao-Ehlker X, Bieber S, Nussbaum CF, et al. Extracellular MRP8/14 is a regulator of beta2 integrin-dependent neutrophil slow rolling and adhesion. *Nat Commun*. (2015) 6:6915. doi: 10.1038/ncomms7915
25. Tardif MR, Chapeton-Montes JA, Posvandzic A, Page N, Gilbert C, Tessier PA. Secretion of S100A8, S100A9, and S100A12 by neutrophils involves reactive oxygen species and potassium efflux. *J Immunol Res*. (2015) 2015:296149. doi: 10.1155/2015/296149
26. Viemann D, Strey A, Janning A, Jurk K, Klimmek K, Vogl T, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood* (2005) 105:2955–62. doi: 10.1182/blood-2004-07-2520
27. Fassl SK, Austermann J, Papantonopoulou O, Riemenschneider M, Xue J, Bertheloot D, et al. Transcriptome assessment reveals a dominant role for TLR4 in the activation of human monocytes by the alarmin MRP8. *J Immunol*. (2015) 194:575–83. doi: 10.4049/jimmunol.1401085
28. van Lent PL, Grevers L, Blom AB, Sloetjes A, Mort JS, Vogl T, et al. Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. *Ann Rheumatic Dis*. (2008) 67:1750–8. doi: 10.1136/ard.2007.077800
29. Ulas T, Pirr S, Fehlhaber B, Bickes MS, Loof TG, Vogl T, et al. S100-alarmin-induced innate immune programming protects newborn infants from sepsis. *Nat Immunol*. (2017) 18:622–32. doi: 10.1038/ni.3745
30. Austermann J, Friesenhagen J, Fassl SK, Petersen B, Ortkras T, Burgmann J, et al. Alarmins MRP8 and MRP14 induce stress tolerance in phagocytes under sterile inflammatory conditions. *Cell Rep*. (2014) 9:2112–23. doi: 10.1016/j.celrep.2014.11.020
31. Aneja R, Odoms K, Dunsmore K, Shanley TP, Wong HR. Extracellular heat shock protein-70 induces endotoxin tolerance in THP-1 cells. *J Immunol*. (2006) 177:7184–92. doi: 10.4049/jimmunol.177.10.7184
32. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. (2009) 9:162–74. doi: 10.1038/nri2506
33. Atallah M, Krispin A, Trahtemberg U, Ben-Hamron S, Grau A, Verbovetski I, et al. Constitutive neutrophil apoptosis: regulation by cell concentration via S100 A8/9 and the MEK-ERK pathway. *PLoS ONE* (2012) 7:e29333. doi: 10.1371/journal.pone.0029333
34. Bjork P, Bjork A, Vogl T, Stenstrom M, Liberg D, Olsson A, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. *PLoS Biol*. (2009) 7:e97. doi: 10.1371/journal.pbio.1000097
35. Alexaki VI, May AE, Fujii C, SN VU-S, Mund C, Gawaz M, et al. S100A9 induces monocyte/ macrophage migration via EMMPRIN. *Thromb Haemost*. (2017) 117:636–9. doi: 10.1160/TH16-06-0434
36. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med*. (2007) 13:1042–9. doi: 10.1038/nm1638
37. Vogl T, Stratis A, Wixler V, Voller T, Thurainayagam S, Jorch SK, et al. Autoinhibitory regulation of S100A8/S100A9 alarmin activity locally restricts sterile inflammation. *J Clin Invest*. (2018) 128:1852–66. doi: 10.1172/JCI89867
38. Odink K, Cerletti N, Bruggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature* (1987) 330:80–2. doi: 10.1038/330080a0
39. Youssef P, Roth J, Frosch M, Costello P, Fitzgerald O, Sorg C, et al. Expression of myeloid related proteins (MRP) 8 and 14 and the MRP8/14 heterodimer in rheumatoid arthritis synovial membrane. *J Rheumatol*. (1999) 26:2523–8.
40. Kane D, Roth J, Frosch M, Vogl T, Bresnihan B, FitzGerald O. Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. *Arthritis Rheumatism* (2003) 48:1676–85. doi: 10.1002/art.10988
41. Di Ceglie I, Ascone G, Cremers NAJ, Sloetjes AW, Walgreen B, Vogl T, et al. Fcgamma receptor-mediated influx of S100A8/A9-producing neutrophils as inducer of bone erosion during antigen-induced arthritis. *Arthritis Res Ther*. (2018) 20:80. doi: 10.1186/s13075-018-1584-1
42. van Lent PL, Grevers LC, Schelbergen R, Blom A, Geurts J, Sloetjes A, et al. S100A8 causes a shift toward expression of activatory Fcgamma receptors on macrophages via toll-like receptor 4 and regulates Fcgamma receptor expression in synovium during chronic experimental arthritis. *Arthritis Rheumatism* (2010) 62:3353–64. doi: 10.1002/art.27654
43. Koenders MI, Marijnissen RJ, Devesa I, Lubberts E, Joosten LA, Roth J, et al. Tumor necrosis factor-interleukin-17 interplay induces S100A8, interleukin-1beta, and matrix metalloproteinases, and drives irreversible cartilage destruction in murine arthritis: rationale for combination treatment during arthritis. *Arthritis Rheumatism* (2011) 63:2329–39. doi: 10.1002/art.30418
44. Loser K, Vogl T, Voskort M, Lueken A, Kupas V, Nacken W, et al. The Toll-like receptor 4 ligands Mrp8 and Mrp14 are crucial in the development of autoreactive CD8+ T cells. *Nat Med*. (2010) 16:713–7. doi: 10.1038/nm.2150
45. Fuellen G, Foell D, Nacken W, Sorg C, Kerkhoff C. Absence of S100A12 in mouse: implications for RAGE-S100A12 interaction. *Trends Immunol*. (2003) 24:622–4. doi: 10.1016/j.it.2003.10.004
46. Foell D, Ichida F, Vogl T, Yu X, Chen R, Miyawaki T, et al. S100A12 (EN-RAGE) in monitoring Kawasaki disease. *Lancet* (2003) 361:1270–2. doi: 10.1016/S0140-6736(03)12986-8
47. Foell D, Kane D, Bresnihan B, Vogl T, Nacken W, Sorg C, et al. Expression of the pro-inflammatory protein S100A12 (EN-RAGE) in rheumatoid and psoriatic arthritis. *Rheumatology* (2003) 42:1383–9. doi: 10.1093/rheumatology/keg385
48. Foell D, Kucharzik T, Kraft M, Vogl T, Sorg C, Domschke W, et al. Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut* (2003) 52:847–53. doi: 10.1136/gut.52.6.847
49. Foell D, Frosch M, Schulze zur Wiesch A, Vogl T, Sorg C, Roth J. Methotrexate treatment in juvenile idiopathic arthritis: when is the right time to stop? *Ann Rheumatic Dis*. (2004) 63:206–8. doi: 10.1136/ard.2003.005686
50. Foell D, Hernandez-Rodriguez J, Sanchez M, Vogl T, Cid MC, Roth J. Early recruitment of phagocytes contributes to the vascular inflammation of giant cell arteritis. *J Pathol*. (2004) 204:311–6. doi: 10.1002/path.1660
51. Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, et al. Expression of S100A12 (EN-RAGE) in cystic fibrosis. *Thorax* (2003) 58:613–7. doi: 10.1136/thorax.58.7.613
52. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, et al. Monitoring neutrophil activation in juvenile rheumatoid arthritis by S100A12 serum concentrations. *Arthritis Rheumatism* (2004) 50:1286–95. doi: 10.1002/art.20125
53. Foell D WH, Luken A, Dabritz J, Viemann D, Heidemann J, Vogl T, Roth J. The Mediator S100a12 is critically involved in early inflammatory events of inflammatory bowel disease. *Gastroenterology* (2009) 136(Suppl. 1):A254–A. doi: 10.1016/S0016-5085(09)61153-7
54. Frosch M, Metze D, Foell D, Vogl T, Sorg C, Sunderkotter C, et al. Early activation of cutaneous vessels and epithelial cells is characteristic of acute systemic onset juvenile idiopathic arthritis. *Exp Dermatol*. (2005) 14:259–65. doi: 10.1111/j.0906-6705.2005.00271.x
55. Frosch M, Vogl T, Seeliger S, Wulffraat N, Kuis W, Viemann D, et al. Expression of myeloid-related proteins 8 and 14 in systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheumatism* (2003) 48:2622–6. doi: 10.1002/art.11177
56. Kallinich T, Wittkowski H, Keitzer R, Roth J, Foell D. Neutrophil-derived S100A12 as novel biomarker of inflammation in familial Mediterranean fever. *Ann Rheumatic Dis*. (2010) 69:677–82. doi: 10.1136/ard.2009.114363
57. Kuemmerle-Deschner JB, Tyrrell PN, Koetter I, Wittkowski H, Bialkowski A, Tzaribachev N, et al. Efficacy and safety of anakinra therapy in pediatric and adult patients with the autoinflammatory Muckle-Wells syndrome. *Arthritis Rheumatism* (2011) 63:840–9. doi: 10.1002/art.30149
58. Kuemmerle-Deschner JB, Wittkowski H, Tyrrell PN, Koetter I, Lohse P, Ummenhofer K, et al. Treatment of Muckle-Wells syndrome: analysis of two IL-1-blocking regimens. *Arthritis Res Ther*. (2013) 15:R64. doi: 10.1186/ar4237

59. Foell D, Wulffraat N, Wedderburn LR, Wittkowski H, Frosch M, Gerss J, et al. Methotrexate withdrawal at 6 vs 12 months in juvenile idiopathic arthritis in remission: a randomized clinical trial. *JAMA* (2010) 303:1266–73. doi: 10.1001/jama.2010.375
60. Rothmund F, Gerss J, Ruperto N, Dabritz J, Wittkowski H, Frosch M, et al. Validation of relapse risk biomarkers for routine use in patients with juvenile idiopathic arthritis. *Arthritis Care Res*. (2014) 66:949–55. doi: 10.1002/acr.22248
61. Wittkowski H, Kuemmerle-Deschner JB, Austermann J, Holzinger D, Goldbach-Mansky R, Gramlich K, et al. MRP8 and MRP14, phagocyte-specific danger signals, are sensitive biomarkers of disease activity in cryopyrin-associated periodic syndromes. *Ann Rheumatic Dis.* (2011) 70:2075–81. doi: 10.1136/ard.2011.152496
62. Vastert SJ, de Jager W, Noordman BJ, Holzinger D, Kuis W, Prakken BJ, et al. Effectiveness of first-line treatment with recombinant interleukin-1 receptor antagonist in steroid-naive patients with new-onset systemic juvenile idiopathic arthritis: results of a prospective cohort study. *Arthritis Rheumatol.* (2014) 66:1034–43. doi: 10.1002/art.38296
63. Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkotter C, et al. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheumatism* (2000) 43:628–37. doi: 10.1002/1529-0131(200003)43:3<628::AID-ANR20>3.0.CO;2-X
64. Gerss J, Roth J, Holzinger D, Ruperto N, Wittkowski H, Frosch M, et al. Phagocyte-specific S100 proteins and high-sensitivity C reactive protein as biomarkers for a risk-adapted treatment to maintain remission in juvenile idiopathic arthritis: a comparative study. *Ann Rheumatic Dis.* (2012) 71:1991–7. doi: 10.1136/annrheumdis-2012-201329
65. Anink J, Van Suijlekom-Smit LW, Otten MH, Prince FH, van Rossum MA, Dolman KM, et al. MRP8/14 serum levels as a predictor of response to starting and stopping anti-TNF treatment in juvenile idiopathic arthritis. *Arthritis Res Ther.* (2015) 17:200. doi: 10.1186/s13075-015-0723-1
66. Hinze CH, Foell D, Johnson AL, Spalding SJ, Gottlieb BS, Morris PW, et al. Serum S100A8/A9 and S100A12 Levels in children with polyarticular forms of juvenile idiopathic arthritis: relationship to maintenance of clinical inactive disease during and flare after discontinuation of anti-TNF therapy. *Arthritis Rheumatol*. (2018). doi: 10.1002/art.40727. [Epub ahead of print].
67. Moncrieffe H, Ursu S, Holzinger D, Patrick F, Kassoumeri L, Wade A, et al. A subgroup of juvenile idiopathic arthritis patients who respond well to methotrexate are identified by the serum biomarker MRP8/14 protein. *Rheumatology* (2013) 52:1467–76. doi: 10.1093/rheumatology/ket152
68. Gohar F, Anink J, Moncrieffe H, Van Suijlekom-Smit LWA, Prince FHM, van Rossum MAJ, et al. S100A12 is associated with response to therapy in juvenile idiopathic arthritis. *J Rheumatol.* (2018) 45:547–54. doi: 10.3899/jrheum.170438
69. Seeliger S, Vogl T, Engels IH, Schroder JM, Sorg C, Sunderkotter C, et al. Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases. *Am J Pathol.* (2003) 163:947–56. doi: 10.1016/S0002-9440(10)63454-2
70. Nistala K, Varsani H, Wittkowski H, Vogl T, Krol P, Shah V, et al. Myeloid related protein induces muscle derived inflammatory mediators in juvenile dermatomyositis. *Arthritis Res Ther.* (2013) 15:R131. doi: 10.1186/ar4311
71. Holzinger D, Fassl SK, de Jager W, Lohse P, Rohrig UF, Gattorno M, et al. Single amino acid charge switch defines clinically distinct proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1)-associated inflammatory diseases. *J Allergy Clin Immunol.* (2015) 136:1337–45. doi: 10.1016/j.jaci.2015.04.016
72. Gohar F, Orak B, Kallinich T, Jeske M, Lieber M, von Bernuth H, et al. Correlation of secretory activity of neutrophils with genotype in patients with familial mediterranean fever. *Arthritis Rheumatol.* (2016) 68:3010–22. doi: 10.1002/art.39784
73. Kessel C, Holzinger D, Foell D. Phagocyte-derived S100 proteins in autoinflammation: putative role in pathogenesis and usefulness as biomarkers. *Clin Immunol.* (2013) 147:229–41. doi: 10.1016/j.clim.2012.11.008
74. Chae JJ, Komarow HD, Cheng J, Wood G, Raben N, Liu PP, et al. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. *Mol Cell* (2003) 11:591–604. doi: 10.1016/S1097-2765(03)00056-X
75. Wittkowski H, Frosch M, Wulffraat N, Goldbach-Mansky R, Kallinich T, Kuemmerle-Deschner J, et al. S100A12 is a novel molecular marker differentiating systemic-onset juvenile idiopathic arthritis from other causes of fever of unknown origin. *Arthritis Rheumatism* (2008) 58:3924–31. doi: 10.1002/art.24137
76. Frosch M, Ahlmann M, Vogl T, Wittkowski H, Wulffraat N, Foell D, et al. The myeloid-related proteins 8 and 14 complex, a novel ligand of toll-like receptor 4, and interleukin-1beta form a positive feedback mechanism in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheumatism* (2009) 60:883–91. doi: 10.1002/art.24349
77. Holzinger D, Frosch M, Kastrup A, Prince FH, Otten MH, Van Suijlekom-Smit LW, et al. The Toll-like receptor 4 agonist MRP8/14 protein complex is a sensitive indicator for disease activity and predicts relapses in systemic-onset juvenile idiopathic arthritis. *Ann Rheumatic Dis*. (2012) 71:974–80. doi: 10.1136/annrheumdis-2011-200598
78. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheumatism* (2004) 50:3762–71. doi: 10.1002/art.20631
79. Kolly L, Busso N, von Scheven-Gete A, Bagnoud N, Moix I, Holzinger D, et al. Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis syndrome is linked to dysregulated monocyte IL-1beta production. *J Allergy Clin Immunol.* (2013) 131:1635–43. doi: 10.1016/j.jaci.2012.07.043
80. Mansfield E, Chae JJ, Komarow HD, Brotz TM, Frucht DM, Aksentijevich I, et al. The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments. *Blood* (2001) 98:851–9. doi: 10.1182/blood.V98.3.851
81. Holzinger D, Roth J. Alarming consequences - autoinflammatory disease spectrum due to mutations in proline-serine-threonine phosphatase-interacting protein 1. *Curr Opin Rheumatol.* (2016) 28:550–9. doi: 10.1097/BOR.0000000000000314
82. Shoham NG, Centola M, Mansfield E, Hull KM, Wood G, Wise CA, et al. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proc Natl Acad Sci USA* (2003) 100:13501–6. doi: 10.1073/pnas.2135380100
83. Yu JW, Fernandes-Alnemri T, Datta P, Wu J, Juliana C, Solorzano L, et al. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell* (2007) 28:214–27. doi: 10.1016/j.molcel.2007.08.029
84. Omenetti A, Carta S, Caorsi R, Finetti M, Marotto D, Lattanzi B, et al. Disease activity accounts for long-term efficacy of IL-1 blockers in pyogenic sterile arthritis pyoderma gangrenosum and severe acne syndrome. *Rheumatology* (2016) 55:1325–35. doi: 10.1093/rheumatology/kew031
85. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, et al. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem*. (2006) 281:36560–8. doi: 10.1074/jbc.M607594200
86. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* (2006) 440:228–32. doi: 10.1038/nature04515
87. Martinon F, Glimcher LH. Gout: new insights into an old disease. *J Clin Invest.* (2006) 116:2073–5. doi: 10.1172/JCI29404
88. Sutterwala FS, Ogura Y, Zamboni DS, Roy CR, Flavell RA. NALP3: a key player in caspase-1 activation. *J Endotoxin Res.* (2006) 12:251–6. doi: 10.1177/09680519060120040701
89. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* (2004) 20:319–25. doi: 10.1016/S1074-7613(04)00046-9
90. Aksentijevich I, Nowak M, Mallah M, Chae JJ, Watford WT, Hofmann SR, et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheumatism* (2002) 46:3340–8. doi: 10.1002/art.10688
91. Janssen R, Verhard E, Lankester A, Ten Cate R, van Dissel JT. Enhanced interleukin-1beta and interleukin-18 release in a patient with chronic

infantile neurologic, cutaneous, articular syndrome. *Arthritis Rheumatism* (2004) 50:3329–33. doi: 10.1002/art.20494
92. Lachmann HJ, Lowe P, Felix SD, Rordorf C, Leslie K, Madhoo S, et al. *In vivo* regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med.* (2009) 206:1029–36. doi: 10.1084/jem.20082481
93. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J Exp Med.* (2005) 201:1479–86. doi: 10.1084/jem.20050473
94. Frosch M, Roth J. New insights in systemic juvenile idiopathic arthritis–from pathophysiology to treatment. *Rheumatology* (2008) 47:121–5. doi: 10.1093/rheumatology/kem271
95. Mellins ED, Macaubas C, Grom AA. Pathogenesis of systemic juvenile idiopathic arthritis: some answers, more questions. *Nat Rev Rheumatol.* (2011) 7:416–26. doi: 10.1038/nrrheum.2011.68
96. Gohar F, Kessel C, Lavric M, Holzinger D, Foell D. Review of biomarkers in systemic juvenile idiopathic arthritis: helpful tools or just playing tricks? *Arthritis Res Ther.* (2016) 18:163. doi: 10.1186/s13075-016-1069-z
97. Shenoi S, Ou JN, Ni C, Macaubas C, Gersuk VH, Wallace CA, et al. Comparison of biomarkers for systemic juvenile idiopathic arthritis. *Pediatr Res.* (2015) 78:554–9. doi: 10.1038/pr.2015.144
98. Foell D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Practice Rheumatol.* (2007) 3:382–90. doi: 10.1038/ncprheum0531
99. Tasher D, Somekh E, Dalal I. PFAPA syndrome: new clinical aspects disclosed. *Archi Dis Childhood* (2006) 91:981–4. doi: 10.1136/adc.2005.084731
100. Faust A, Voller T, Busch F, Schafers M, Roth J, Hermann S, et al. Development and evaluation of a non-peptidic ligand for the molecular imaging of inflammatory processes using S100A9 (MRP14) as a novel target. *Chem Commun.* (2015) 51:15637–40. doi: 10.1039/C5CC07019H
101. Voller T, Faust A, Roth J, Schafers M, Vogl T, Hermann S. A Non-Peptidic S100A9 specific ligand for optical imaging of phagocyte activity *in vivo*. *Mol Imaging Biol.* (2018) 20:407–16. doi: 10.1007/s11307-017-1148-9
102. Comi G, Jeffery D, Kappos L, Montalban X, Boyko A, Rocca MA, et al. Placebo-controlled trial of oral laquinimod for multiple sclerosis. *N Engl J Med.* (2012) 366:1000–9. doi: 10.1056/NEJMoa1104318
103. D'Haens G, Sandborn WJ, Colombel JF, Rutgeerts P, Brown K, Barkay H, et al. A phase II study of laquinimod in Crohn's disease. *Gut* (2015) 64:1227–35. doi: 10.1136/gutjnl-2014-307118

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REVIEW
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Neutrophil Heterogeneity as Therapeutic Opportunity in Immune-Mediated Disease

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Neutrophils are versatile innate effector cells essential for immune defense but also responsible for pathologic inflammation. This dual role complicates therapeutic targeting. However, neither neutrophils themselves nor the mechanisms they employ in different forms of immune responses are homogeneous, offering possibilities for selective intervention. Here we review heterogeneity within the neutrophil population as well as in the pathways mediating neutrophil recruitment to inflamed tissues with a view to outlining opportunities for therapeutic manipulation in inflammatory disease.

Keywords: neutrophil, immune mediated disease, therapeutic opportunities, granulopoiesis, neutrophil migration, CD177, autoimmune, therapeutic targeting

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INTRODUCTION

Circulating leukocytes have long been categorized by microscopic appearance as lymphocytes, monocytes, and granulocytes. In the late 1870s, Paul Ehrlich distinguished neutrophils from eosinophils and basophils using aniline stains (1). Neutrophils are diverse in phenotype, although the understanding of this heterogeneity remains relatively basic. Related challenges include the relative homogeneity of neutrophils on microscopic examination, a paucity of surface markers defining clear-cut subgroups, short *in vitro* lifespan, and susceptibility to activation with manipulation. Experiments using newer techniques such as mass cytometry and RNAseq often exclude neutrophils by restricting analysis to cryopreserved peripheral blood mononuclear cells (PBMC) or to cells with high mRNA content. The definition of subpopulations within neutrophils has thus lagged behind work in other lineages.

This gap does not reflect doubt about the immune importance of neutrophils. Quantitative and qualitative neutrophil defects expose patients to a high risk of infection, amply displayed in both congenital and acquired neutrophil disorders (2, 3). In mice, neutropenia resulting, for example, from congenital deficiency of the transcription factor *Gfi1* translates into high mortality from bacterial pathogens (4). Safety concerns translate into an understandable reluctance to target neutrophils therapeutically.

The failure to develop such strategies passes up potential opportunities to intervene in human disease. Neutrophils feature prominently in pathogenic sterile inflammation. For example, neutrophils are ubiquitous in the inflamed joint in rheumatoid arthritis (RA), in peritonitis associated with familial Mediterranean fever, and in the neutrophilic dermatoses (5–7). Among the pediatric rheumatic diseases, neutrophils are uniformly present in inflamed juvenile idiopathic arthritis (JIA) synovial fluid and have been implicated in the pathogenesis of the childhood-restricted vasculitis Kawasaki disease (8–11) While presence alone does not establish causation, evidence for a pathogenic role is frequently compelling. For example, experimental arthritis is

abrogated in mice that lack neutrophils or with impaired neutrophil migration or function (12–15). Analogous studies implicate neutrophils as key effectors in a myriad of immune mediated diseases, including neuroinflammation, colitis, and bullous pemphigoid (16, 17). Neutrophils therefore remain an interesting drug target.

The therapeutic challenge is to develop strategies that preserve the defensive contribution of neutrophils while hindering their capacity to mediate sterile inflammation. Selectivity may be achieved by leveraging differences within the neutrophil population, in the way that cancer chemotherapy for targets cells that undergo frequent mitosis or bear specific mutations. Opportunities to drive a "wedge" between protective and pathogenic functions could also arise through differences in effector pathways that neutrophils engage in responding to sterile and septic triggers. This review will explore these possibilities with a view to highlighting potential treatment targets in neutrophils.

NEUTROPHIL BIOLOGY: ONTOGENY AND LIFECYCLE

Neutrophils arise from hematopoietic stem cells (HSCs) in bone marrow, spleen, and probably lung (**Figure 1**) (24, 25) HSCs give rise to multipotent progenitors (MPP), which yield common myeloid progenitors (CMP) and then granulocyte monocyte progenitors (GMP). The latter commit to a program to become monocyte/dendritic cells, mast cells, basophils, or neutrophil/monocytes (26). A proliferation-competent committed progenitor termed a preNeu develops into post-mitotic immature neutrophils (myelocytes, metamyelocytes, band cells) and finally segmented mature neutrophils (18). Immature neutrophils are also be found in peripheral blood in time of immunologic stress. Granulopoiesis is stimulated predominantly through the IL-23/IL-17/G-CSF axis and to a lesser extent by GM-CSF and M-CSF, although mice lacking all three colony stimulating factors still have ∼10% of normal circulating neutrophils (19, 27). Other cytokines have also been implicated, for example IL-6, which has a special importance in emergency granulopoiesis in response to systemic infection (24, 28).

Studies in mice suggested a circulating neutrophil half-life of 1.5 h by exogenous labeling followed by transfer and 8–10 h after *in vivo* labeling (29, 30). In humans, endogenous labeling raised the possibility that the neutrophil lifespan may be as long as 5.4 days (half-life 3.7 days) (20). This surprising result reflects assumptions about the relationship between marrow and circulation that have been disputed, and more recent studies suggest instead a half-life of 19 h, conforming more closely to murine data and to conventional expectations (31, 32). *In vitro*, human neutrophils typically undergo apoptosis within 24 h, but >90% viability even after 9 days can be achieved in the presence of GM-CSF (33). Thus, whatever the basal half-life of circulating neutrophils, it is likely that some neutrophils live for a prolonged period *in vivo*, especially in an inflamed context.

Neutrophils released into circulation can follow several paths (**Figure 1**) (34). The simplest is uneventful aging followed by return to the bone marrow, a process influenced by the microbiome and mediated through progressive expression of the SDF-1 (CXCL12) receptor CXCR4 in older neutrophils (35–38). Production of mediators by marrow macrophages phagocytizing aged neutrophils in turn regulates granulopoiesis, forming a "neutrostat" that contributes to circadian release of fresh neutrophils into blood (36). Some circulating neutrophils marginate in lung, also under the influence of CXCR4, where they can combat infection locally or be released at need into the circulation (34, 39). A marginated neutrophil population is also observed in liver and bone marrow (40).

Neutrophils recruited to inflamed tissues undergo several distinct fates. Some die locally through apoptosis or other forms of cell death, including ejection of their DNA as neutrophil extracellular traps (NETs) (41). Neutrophils can egress from tissues via lymphatics, appearing in lymph nodes loaded with antigen (42). Neutrophils from inflammatory infiltrates can return to the circulation, a possibility originally visualized in zebrafish and subsequently observed in mice (38, 43, 44). More recently, it has been recognized that neutrophils enter some tissues even without an exogenous trigger. For example, neutrophils promote angiogenesis in the regenerating uterine lining and modulate metabolism in adipose tissue, particularly in obesity (34). In healthy mice, neutrophils have also been observed in liver, skin, intestine, skeletal muscle, kidneys, and heart, although not in brain or gonads (45). While the role of these patrolling neutrophils remains to be established, one function may be to modulate the activity of local macrophages by which they are phagocytosed at the end of their lifespan (45, 46).

NEUTROPHIL HETEROGENEITY AS A FUNCTION OF MATURITY

The neutrophil lifecycle accounts for substantial phenotypic heterogeneity (**Figure 1**). Mature human neutrophils exhibit a characteristic multi-lobular nucleus and high surface expression of CD16 (FcγRIII), CD62L (L-selectin), and CD10 (neutral endopeptidase), along with neutrophil lineage markers CD15 and CD66b (21, 22). By contrast, immature neutrophils released from marrow after immune stress are $CD16^{lo}$ and $CD10^{lo}$, often but not invariably together with band nuclear morphology (22, 47, 48) Neutrophils less mature than band cells exhibit elevated CD33 (Siglec-3) and lower CXCR2, the receptor that enables mobilization out of the bone marrow niche (18, 21, 49). Immature neutrophils express more CXCR4 than mature neutrophils, likely promoting their retention in the bone marrow (18). As neutrophils age, expression of CXCR4 again increases, licensing return to the bone marrow for clearance (35). Aging is accompanied by other changes, including elevated expression of the integrins CD11b and CD11c, lower CD62L, and lower CD47, an inhibitor of phagocytosis (23). This maturation-related variation corresponds to changes in effector function. Aged neutrophils can be particularly effective in migration to sites of inflammation for immune defense, although immature neutrophils display superior bactericidal function against certain pathogens (50, 51). Murine spleen contains a mature $Ly6G^{hi}$

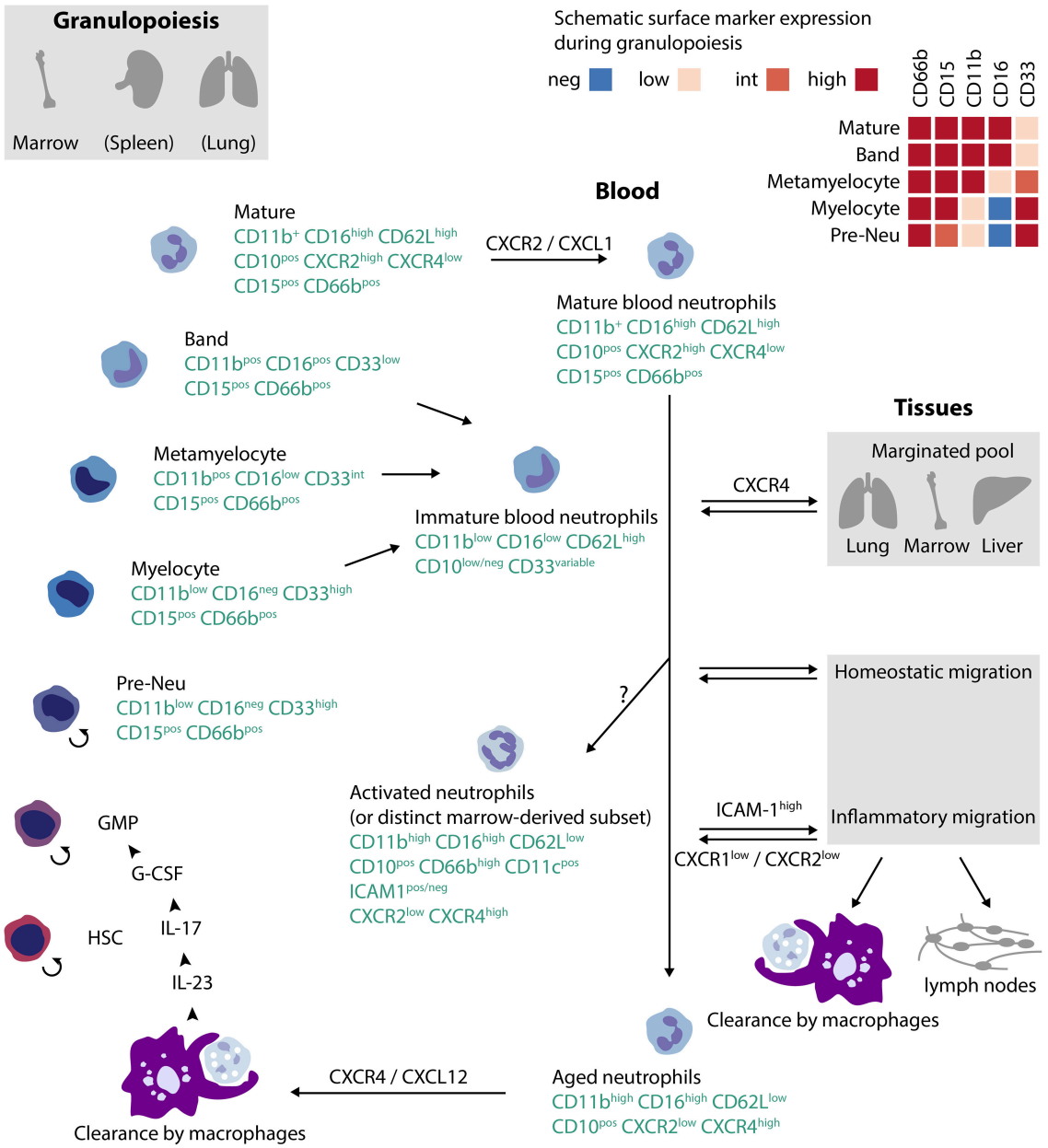

FIGURE 1 | Lifecycle of human neutrophils. Neutrophils arise in bone marrow, spleen and (at least in mice) in lung from hematopoietic stem cells (HSC), progressing to committed granulocyte-monocyte progenitors (GMP), and then through a set of intermediate stages to mature neutrophils. Neutrophils exit to blood under the control of CXCR2, usually as mature cells but under conditions of stress also as immature cells. Over time, neutrophils age, expressing CXCR4 that mediates return to marrow. Alternate pathways for blood neutrophils include intravascular activation, intravascular margination, homeostatic migration into tissues, or migration into inflamed tissues. Clearance occurs via macrophages either in tissues or in bone marrow. The localization of the recently-defined preNeu in the previously-accepted neutrophil ontology (GMP → myeloblast → promyelocyte → myelocyte) remains uncertain; one plausible configuration is shown. The small circular arrow ↻ reflects replication competence. References:(18–23).

population of motile, highly phagocytic neutrophils as well as immature Ly6G^{lo} neutrophils with a preserved capacity for mitosis, limited mobility, and low phagocytic capacity (49). In humans receiving G-CSF, immature CD10^{neg} neutrophils stimulate T cells while mature CD10^{pos} neutrophils are suppressive (22).

NEUTROPHIL HETEROGENEITY AS A FUNCTION OF ACTIVATION STATE

Beyond maturational changes, neutrophils shift phenotype with activation. Mobilization of intracellular granules brings not only soluble mediators but also pre-formed membrane proteins to

the neutrophil surface. These include CD66b, the α and β chains of the β2 integrin CD11b/CD18 (Mac-1), and in some individuals CD177 with its associated protease proteinase 3 (PR3) (21, 52, 53). Other surface markers are lost, including CD62L, which is rapidly shed in activated neutrophils. Activated neutrophils exhibit multiple changes in function compared with resting neutrophils. Inside-out signaling and clustering enhance integrin binding, and activated neutrophils thus exhibit enhanced mobility as well as production of lipid mediators, cytokines, chemokines, and reactive oxygen species (54).

Neutrophils acquire additional surface markers that reflect their migratory history (**Figure 1**). Many neutrophils that transmigrate into inflamed tissue do not die by apoptosis, as was previously assumed, but rather migrate back into the circulation (38, 43, 44). In human neutrophils, *in vitro* reverse transendothelial migration correlates with the appearance of surface ICAM-1 (CD54), elevation of CD18, and lower CD62L, CXCR1 and CXCR2 (55). In mice, reverse-migrated neutrophils are characterized by ICAM-1 and upregulation of CXCR4 through which they can "transplant" inflammation from the periphery to the lung before returning to the bone marrow for final clearance (38, 44, 56). ICAM-1 elevation has also been reported in human neutrophils after prolonged *in vitro* stimulation and in a $CD16^{hi}CD62L^{lo}CD11b^{hi}CD11c^{hi}$ peripheral blood neutrophil population induced in normal donors treated with i.v. LPS, and hence is not restricted to reverse-migrated neutrophils (20). Thus healthy donors exhibit an almost uniform signature of mature neutrophils in blood, $CD11b+CD16^{hi}CD62L^{hi}CD10^{hi}$. After administration of LPS or G-CSF, additional populations appear, including immature cells ($CD11b^{lo}CD16^{lo}CD62L^{hi}CD10^{lo}$, banded nuclear morphology) and cells with a phenotype suggestive of activated mature cells ($CD11b^{hi}CD16^{hi}CD62L^{lo}CD10+$, increased nuclear lobulation) (20, 22) Intriguingly, this latter subgroup can be found even in normal marrow; labeling studies suggest a similar age to mature neutrophils, raising the possibility that some $CD62L^{lo}$ cells may not be mature neutrophils activated intravascularly but rather a distinct type of neutrophil released directly from marrow under stress (57).

NEUTROPHIL HETEROGENEITY BEYOND AGING AND ACTIVATION

The broad phenotypic variability associated with maturation and activation complicates the task of discerning additional axes of heterogeneity in the form of discrete neutrophil subsets. This topic has been expertly reviewed (40, 58, 59). We will focus on targetable neutrophil heterogeneity by limiting consideration here to three areas: low-density neutrophils, immunomodulatory neutrophils, and neutrophil subgroups defined by the surface marker CD177.

Low-Density Neutrophils

The average density of neutrophils from healthy subjects is >1.080 g/ml and therefore higher than lymphocytes (1.073–1.077 g/ml) and monocytes (1.067–1.077 g/ml) (60). Density gradient centrifugation leverages these differences to separate PBMC from granulocytes. Low-density neutrophils (LDN, also termed low-density granulocytes) are neutrophils found in the PBMC layer rather than the granulocyte pellet (61, 62). On microscopic examination, many display an immature nuclear morphology, and gene expression studies suggest immaturity of the population as a whole, although expression of CD10 and other markers of maturity (e.g., $CD16^{hi}$) suggest that not all LDN are neutrophils released prematurely from the marrow (22, 62, 63). Importantly, normal-density neutrophils (NDN) exposed to sera containing complement or immune complexes can also segregate with PBMC, highlighting the dynamic nature of density as a physical property of neutrophils that reflects factors such as granule content and cytoplasmic volume (22, 61). Elevated CD66b and CD11b further suggest that some LDN represent activated mature cells (62). In mice, interconversion between LDN and NDN occurs in neutrophils adoptively transferred into live animals, while *ex vivo* TGF-β treatment induces LDN-like features in NDN (64). Thus, LDN likely represent a diverse population of immature and activated mature neutrophils.

Elevation in peripheral blood LDN has been observed in many states of immune stress, including acute rheumatic fever, JIA, RA, systemic lupus erythematosus (SLE), autoinflammatory diseases, G-CSF administration, cancer, and sepsis (61–67). Their characteristics vary widely with context, and can for example include enhanced production of pro-inflammatory cytokines such as TNF and type I interferons, spontaneous NET generation (discussed further below), and immunosuppressive capacity (40). LDN thus reflect the phenotypic and functional plasticity of the neutrophil lineage. Whether some LDN also represent a discrete, stable neutrophil subset remains to be determined.

Immunomodulatory Neutrophils

The view of neutrophils as simple foot-soldiers of immunity has given way to a more nuanced understanding of these cells as full participants in the immune network. Examples of the reciprocal interchange between neutrophils and adaptive immunity are abundant. Neutrophils home to lymph nodes in response to CCL19 and CCL21, carrying antigen for presentation to T cells in the context of MHC II and the canonical costimulatory molecules CD80 and CD86 (68–74). Neutrophils can differentiate into cells with surface and functional similarity to dendritic cells (75, 76). $CD15^{int/lo}CD16^{int/low}CD11b^{high}$ "B helper" (N_{BH}) neutrophils have been reported in the marginal zone between lymphoid white pulp and non-lymphoid red pulp of human spleen than interact with B cells to promote IgM production and Ig class switching (77). The N_{BH} phenotype develops under the influence of IL-10 from local cells, including splenic endothelial cells, and confers the capacity to produce mediators including APRIL, BAFF, and IL-21. Of note, not all investigators have observed these cells in human spleen, such that further exploration of N_{BH} cells is required (78).

Neutrophils can also suppress adaptive immunity. This capacity has gained particular attention in cancer biology, where neutrophils can promote tumor growth by inhibiting responding lymphocytes (64, 79, 80). Mechanisms include arginase-1 to deplete extracellular arginine required for T

cell function, reactive oxygen and nitrogen species to impair effector T cells in favor of regulatory T cells, IL-10, and TGF-β as immunosuppressive mediators, and pathways mediated through direct cell-cell contact (20, 34, 81). Myeloid cells with the capacity to block T cell activation (and under some conditions B cells and NK cells) have been termed myeloid-derived suppressor cells (MDSC), a loosely-defined category now recognized to include both neutrophil-like and monocyte-like cells (82, 83). Neutrophilic MDSC (so-called PMN-MDSC) are typically considered relatively immature, but in G-CSF-treated donors suppressive capacity in fact resides within the mature (CD10+) fraction, both LDN and NDN (22, 83). Suppressive capacity can also be elicited *ex vivo* in healthy-donor neutrophils exposed to TLR ligands, consistent with *in vivo* LPS challenge data (20, 81). Immunosuppressive capacity is thus available to neutrophils at a range of maturational states with appropriate stimulation. It remains to be established whether this capacity represents part of a broader differentiation program in a limited group of neutrophils—i.e., whether PMN-MDSC represent one or more distinct neutrophil subsets.

CD177

Another protein expressed dichotomously in human neutrophils is CD177, originally known as NB1 (84). A glycoprotein of ∼60 kD attached to the neutrophil surface via a GPI linker, CD177 is present on 40–60% of neutrophils in most donors, with a range extending from 0 to 100%; some individuals manifest a $CD177^{int}$ population as well (52, 85, 86). Like another dichotomously-expressed neutrophil protein olfactomedin 4 (OLFM4), CD177 is localized to the specific granules, residing in the granule membrane for rapid mobilization to the surface with cell activation; however, $CD177^{pos}$ and $OLFM4^{hi}$ subsets otherwise exhibit no interdependence (52, 87).

The function of CD177 is incompletely understood. Lacking a transmembrane domain, CD177 cannot itself transmit a signal intracellularly, but antibody ligation studies show that CD177 can signal through the β2 integrins with which it associates in *cis* at the neutrophil surface (88, 89). Resulting enhancement in integrin expression and affinity translate CD177 ligation into neutrophil arrest, blocking transmigration (89). CD177 thus functionally echoes murine Ly6G, a neutrophil-restricted GPI-linked protein from the same Ly6/UPAR protein family that also interacts with β2 integrins and can modulate neutrophil migration, although Ly6G ligation appears to impair rather than enhance integrin binding (15, 90, 91). The endogenous receptor for CD177 remains uncertain. *In vitro* data implicate the endothelial adhesion molecule PECAM-1; however $CD177^{pos}$ neutrophils display no particular affinity for PECAM-1-expressing platelets or *in vivo* migratory advantage, rendering the physiological significance of the *in vitro* observations uncertain (89, 92–94) Interestingly, CD177 specifically binds the neutrophil protease PR3, which is stored primarily in azurophilic and specific granules in resting neutrophils and mobilized to the membrane during activation, such that $CD177^{pos}$ cells are identical to $PR3^{pos}$ cells among activated neutrophils (95–97) Some data suggest that PR3 may promote the migration of $CD177^{pos}$ neutrophils, but more recent data indicate that CD177 binding impairs PR3 function, leaving the functional implications of the CD177-PR3 interaction uncertain (92, 98).

The basis for the expression of CD177 in some neutrophils but not others is partially understood. *CD177* resides adjacent to a related pseudogene *CD177P1* that is characterized by a stop codon in the region corresponding to *CD177* exon 7. Through a process of homologous recombination (gene conversion), approximately 12% of *CD177* alleles feature the *CD177P1* stop codon and thus represent null variants. Accordingly, the observed allelic distribution matches that expected by Hardy-Weinberg equilibrium, with 78% WT/WT, 19% WT/null, and 3% null/null (85, 99). In subjects with 2 intact copies of *CD177*, the $CD177^{pos}$ fraction is typically 50–98%; in WT/null, 10–60%; and in null/null 0%. Why some neutrophils from WT/WT donors lack CD177 expression remains undefined, but presumably reflects epigenetic regulation (100). Interestingly, in individuals with 2 intact copies of *CD177*, epigenetic control enforces expression of single parental allele in all CD177-expressing neutrophils, although the purpose of such tight control is unknown (101).

Distinct immunological roles of $CD177^{pos}$ and $CD177^{neg}$ neutrophils have not yet been established. Individuals with nearly 100% $CD177^{pos}$ neutrophils or lacking CD177 altogether appear healthy. The proportion of neutrophils expressing CD177 in an individual typically remains stable over time but rises in pregnancy, sepsis, and pathologic conditions including polycythemia vera, vasculitis, and SLE (86, 102). Studies of circulating $CD177^{pos}$ and $CD177^{neg}$ neutrophils reveal similar expression of integrins and Fc receptors, fibronectin adhesion, *in vitro* migration, and reactive oxygen species production (89, 103). Gene expression profiling using microarrays identified minor differences, principally in genes encoding granule proteins, although protein levels remained similar (104). Surface PR3 has been proposed as a potential modulator of T cell proliferation (105). A recent study suggested that $CD177^{pos}$ cells express enhanced bactericidal capacity as well as IL-22 production, reflecting a potentially protective role in inflammatory bowel disease, including through use of a murine model (106). However, the use of an antibody clone that is known to activate neutrophils (MEM-166) to sort CD177 populations complicate the interpretation of these data, as functional measures may become confounded by the activation-mediated effect of the antibody itself (89). CD177 may be of interest in vasculitis, as it can mediate the tethering of PR3, the target of c-ANCA autoantibodies to the neutrophil surface.

MIGRATORY PATHWAYS AS A WEDGE BETWEEN PROTECTIVE AND PATHOLOGIC FUNCTIONS IN NEUTROPHILS

Beyond population heterogeneity, opportunities for intervention in neutrophil biology could emerge through effector pathways that are employed differentially in pathogenic and defensive functions. Given the myriad effector pathways employed by neutrophils, it is likely that there are multiple such opportunities.

For example, in zebrafish, H_2O_2 is required for initiation of neutrophil recruitment to wounding but dispensable for migration toward injected bacteria (107). The zebrafish IL-1β ortholog and its downstream signaling partner MyD88 are similarly required for neutrophil recruitment triggered by wounding but not bacteria (108). We will focus here on another intriguing discrepancy between sterile and septic neutrophil migration related to the role of neutrophil β2 integrins.

The leukocyte recruitment cascade is well-established (54, 109). Circulating neutrophils roll across the endothelial surface under the influence of adhesive interactions between endothelial P- and E-selectins and neutrophil ligands including PSGL-1, further slowed by weak, transient interactions between other receptor-ligand pairs such as endothelial ICAM-1 and low-affinity neutrophil β2 integrins. With activation, endothelial cells upregulate these adhesion molecules and neutrophils augment the quantity and affinity of surface integrins, resulting in neutrophil arrest. Further neutrophil activation via chemokines presented on the endothelial glycocalyx and/or transported by endothelial cells to the luminal surface solidifies the attachment through post-adhesion strengthening (54, 110). Adherent neutrophils crawl in an integrin-dependent manner to sites suitable for transmigration between or through endothelial cells and then along sub-endothelial pericytes to sites of eventual egress into tissue (111, 112). While much of this cascade has been defined in mice, human relevance is supported by the susceptibility to infection in patients lacking the β2 chain CD18 (113).

Yet this selectin-integrin paradigm is not the whole story. Neutrophils lacking all integrins can migrate through the 3-dimensional matrix of tissue interstitium via amoeboid motion ("flowing and squeezing") (114) Mice lacking β2 integrins or subject to integrin blockade still mount neutrophilic infiltrates. In particular, neutrophils can enter the airway without β2 integrins, although entry into the pulmonary parenchyma exhibits partial integrin dependence, as shown in studies employing adoptive transfer of mixed wild-type and CD18–/– neutrophils (90, 115–120) Indeed, under certain circumstances integrins slow neutrophil migration into lung, such that impairing integrins actually promotes neutrophil entry (116, 120). Consistent with these murine findings, neutrophilic pneumonia is observed in humans and cows lacking CD18, although recurrent pulmonary infections remain a clinical feature of this immunodeficiency in both species (113, 121, 122). In peritoneum, migratory impairment resulting from integrin deficiency or blockade is partial, with marked variability among experimental systems (90, 115, 116, 123–125). In the liver, integrins mediate neutrophil accumulation after thermal injury but are dispensable for migration induced by live bacteria in favor of CD44-hyaluronan adhesion (126). In other sites, β2 integrins play a more clear-cut role, including skin and in joints inflamed through immune complex deposition, although at least in joints the initial dependence on integrins may become less prominent as inflammation proceeds (13, 110, 114, 116, 127–129). Thus, mechanisms employed by neutrophils to enter tissues vary with site and also with stimulus, via pathways not limited to the classic leukocyte adhesion cascade.

Could this variability be exploited therapeutically? In liver, sterile infiltration is dependent on β2 integrins, while these integrins appear dispensable for septic infiltration (126). The generalizability of this principle was tested in murine peritoneum. Co-transfer of WT and CD18–/– neutrophils identified a markedly greater role for β2 integrins in sterile than septic peritonitis (i.p. IL-1β vs. live *E. coli*) (90). Correspondingly, targeting integrin-mediated neutrophil recruitment via an antibody directed against the integrin modulator Ly6G attenuated only sterile neutrophil infiltration, and then only in neutrophils expressing CD18. Consistent with the known variation in integrin dependence, ligation of Ly6G attenuated integrin-dependent arthritis and integrin-mediated post-adhesion strengthening on inflamed cremaster muscle but had no effect on integrin-independent neutrophil infiltration into lung (15, 90). These findings suggest that neutrophil migration in sterile disease could potentially be targeted without impairing antimicrobial defense. To the extent that integrin compromise can be rendered neutrophil-selective, as with Ly6G ligation, blockade is unlikely to phenocopy human CD18 deficiency, which impacts not only neutrophils but also monocytes, macrophages, and T cells. Humans do not express *Ly6G*, but *CD177* could potentially fulfill a similar role, given its similar structure, selective expression in neutrophils, spatial association with β2 integrins, and capacity to block neutrophil migration upon ligation (88, 89, 91, 93). Anti-CD177 (clone MEM166) arrests migration by enhancing integrin-mediated adhesion via mechanisms including inside-out signaling and impaired integrin recycling (89). Since the relevant endogenous counterligands of both Ly6G and CD177 are unknown, it remains unclear if this difference in effect reflects intrinsic differences between these proteins or variability among the available targeting antibodies.

Importantly, not only integrin binding but also timely integrin release is required for successful transmigration. Interference with this step through interventions that prevent integrin affinity modulation represent a further opportunity for intervention in migration, a "leukadherin"-type mechanism (120, 130). To date, however, this approach lacks specificity for neutrophils.

NEUTROPHIL TARGETING IN INFLAMMATORY DISEASES

Heterogeneity within the neutrophil population and in the pathways that neutrophils employ to access inflamed tissues represent opportunities for intervention in neutrophil-mediated disease. None of these have yet been explored definitively, so this discussion remains necessarily speculative. We will focus on three diseases: inflammatory arthritis, SLE, and vasculitis.

Inflammatory Arthritis

This disease family encompasses conditions including JIA, adult RA, and crystalline arthropathies such as gout (131). The presence of neutrophils in the joint fluid is the *sine qua non* of active inflammation across this spectrum. Experimental

data across species implicate neutrophils in both initiation and perpetuation of disease (13–15, 132–134). Neutrophils stimulated via C5a arrest at the synovial endothelium in a β2 integrin-dependent manner and transmigrate under the influence of leukotriene B_4 (LTB_4) and other chemokines (110, 135, 136). Within the joint, activated neutrophils provide LTB_4 and IL-1β that amplify the inflammatory process (13, 136, 137). Abundant in synovial fluid, neutrophils remain sparse in synovial tissues, although they can be observed in the inflamed pannus early in disease and at the cartilage-pannus junction (138, 139). Their proteases can injure cartilage, including through "frustrated phagocytosis" of embedded immune complexes (140). More recently, neutrophils have been recognized as a source of citrullinated autoantigens in seropositive RA at sites including joint, oropharynx and lung (141–147). Interestingly, not all neutrophil activity in arthritis is pathogenic. Neutrophil microvesicles can protect cartilage by promoting local production of TGF-β (148). In gout, aggregated NETs can help resolve flares through protease-mediated clearance of pro-inflammatory mediators, though the practical contribution of these mechanisms remains unclear (149, 150).

Neutrophils likely play roles in arthritis beyond their immediate impact within the joint environment. Their capacity for regulation of B cells and T cells, and for transport and presentation of antigen, has been noted above. Neutrophils provide mediators that contribute to systemic inflammation. For example, they are a major source of the pro-inflammatory calgranulins S100A8/A9 and S100A12, danger-associated molecular pattern (DAMP) proteins that can activate other cells via pathways including TLR4 ligation (151–153). In systemic JIA, a form of childhood arthritis characterized by fever and rash, concentrations of these mediators in blood are highly elevated, correlating with circulating neutrophil counts and potentially contributing to IL-1β release by monocytes and other cells (48, 154). Recent studies have identified a specific expansion of hypersegmented $CD16^{pos}$ $CD62L^{dim}$ neutrophils in patients with systemic JIA with active, systemic symptoms compared to patients with active arthritis or inactive disease (155). These changes in phenotype and count are accompanied by a sepsis-like transcriptomic pattern in systemic JIA circulating neutrophils (67).

Points of intervention in neutrophil biology in arthritis range across a broad spectrum, including recruitment, effector pathways, and antigen generation. Mechanisms of recruitment blockade in mice include neutrophil-specific integrin blockade and chemokine antagonism (15, 90, 156). Targeting toxins and other compounds to neutrophils, e.g., via scavenger receptors, could potentially hasten resolution of inflammation (157). A similar strategy could alter the ability of neutrophils to generate citrullinated autoantigens, for example by introducing inhibitors of peptidylarginine deiminase enzymes (158). Of note, RA was one of the first diseases to be associated with LDN (61). Induction of the LDN phenotype by RA plasma, complement, or aggregated IgG suggests that LDN could represent an activated and/or degranulated cell population (61, 159). Direct *ex vivo* analysis of RA LDN has found markers of immaturity but failed to identify the enhanced capacity for NET formation observed in SLE LDN, such that their role in antigen generation remains uncertain (63).

Systemic Lupus Erythematosus

The evidence for a role for neutrophils in SLE is now compelling, as has been reviewed in depth (158, 160, 161) Most of the attention has focused on LDN and NETs. The presence of neutrophils in PBMC preparations was described originally in a cohort of diseases including SLE, and it was in SLE that the term "low density granulocytes" was first applied (61, 62). LDN within SLE have several features that suggest a role in disease pathogenesis. They can elaborate type I interferons and generate NETs *in vitro* without exogenous stimuli, expose SLE-associated autoantigens and stimulate interferon production by plasmacytoid dendritic cells. (62, 162–164). Free DNA has been observed in lupus nephritis kidneys, potentially reflecting an impaired ability to clear NETs (165). Indeed, some SLE patients exhibit autoantibodies against DNase I, the enzyme primarily responsible for NET clearance, or against the NET themselves that block enzymatic attack (165). NETs can be elicited by anti-phospholipid antibodies, potentially contributing to elevated thrombosis risk in SLE (166). Finally, neutrophils may contribute to aberrant B cell development in SLE bone marrow through production of IFNα and B cell growth factors (167).

This substantial evidence base renders neutrophils, and particular NETosis, an intriguing therapeutic target in SLE (158). Interference with NETosis can ameliorate manifestations of experimental SLE (168, 169). Importantly, however, genetic deficiency of PAD4 and other pathways required for NETosis can have no effect or even worsen SLE-like disease in mice (170–172). Which murine studies replicate the human potential of NET blockade remains to be determined. Depleting or blocking LDN could represent an avenue forward, but will require further understanding of their origin and role in immune defense as well as in pathogenic inflammation.

Vasculitis

Inflammatory disease of blood vessels can assume many forms, with a severity ranging from trivial to catastrophic. Neutrophilic infiltration into blood vessels is a common feature of vasculitis. For example, neutrophils are the dominant tissue leukocyte in immune complex-mediated leukocytoclastic vasculitis and in inflamed coronary arteries in Kawasaki disease (173–175). Neutrophils may mediate Henoch-Schönlein purpura (HSP), the most common childhood vasculitis, through their ability to recognize the Fc portion of IgA molecules (176). In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis and related *in vivo* disease models, the neutrophil enzymes PR3, and myeloperoxidase are targeted by autoantibodies and play a potential pathogenic role, mediating neutrophil activation and β2 integrin-dependent adhesion to endothelium (177–182). For PR3, the subset of neutrophils expressing CD177 may play a particularly important role, because CD177 binds PR3 to enable surface expression at high level (92, 96). This binding enables anti-PR3 antibodies to activate neutrophils by signaling via CD177-associated integrins, potentially similar to the activation of neutrophils via anti-CD177 antibodies (88, 89, 103). Of

note, PR3 may be expressed via CD177-independent pathways, and CD177 expression is not an invariable requirement for neutrophil-mediated inflammation induced via anti-PR3 antibodies (102, 183). ANCAs bound to the neutrophil surface also activate neutrophils via their surface Fc receptors and can trigger NETs that contribute to tissue injury (184–186). Detailed mechanistic understanding of the role of neutrophils in non-ANCA vasculitis remains more limited, but it is plausible to suspect that their presence in these sterile inflammatory infiltrates reflects a pathogenic role (187).

These considerations render neutrophils an interesting target population in vasculitis. In some patients, there is an emergent need to shut down inflammation to protect affected tissues including lung, kidney, brain, nerve, and heart. In such cases, short-term infectious risk might be a tolerable exchange for rapid cessation of disease activity. In other cases, vasculitis is chronic and indolent, and a more careful balancing act is required. The neutrophil populations and pathways to be targeted vary with the disease. These include $CD177^{pos}$ neutrophils in anti-PR3 ANCA-associated vasculitis, which could be depleted or treated to interfere with the ability of CD177 to bind PR3, although non-CD177-mediated PR3 expression may limit the effectiveness of such a strategy (102). Blockade of neutrophil β2 integrins could attenuate vasculitis mediated through firm adhesion between neutrophil and endothelium. Intracellular activation pathways and NETs have also been proposed as targets (188, 189).

CONCLUSIONS

Neutrophils exhibit a broad range of phenotypes. Much of this variability reflects developmental stage and activation status, integrating both stimulatory exposures and migratory history. As a result, neutrophils diverge from one another in nuclear morphology, buoyancy, surface markers, migratory, and phagocytic capacity, NET generation, and immunomodulatory function, among other characteristics. There remains intense interest in the possibility that this diversity manifests specific developmental programs to which individual neutrophils become committed, reflecting thereby true neutrophil subsets. However, to date evidence in favor of discrete subsets is insufficient to reject the alternative hypothesis that phenotypic variation reflects the impact of diverse environments on neutrophils within a single developmental continuum. The growing capacity for single-cell analysis of immune populations will likely provide important insights into this biology in coming years.

For the purposes of therapeutic targeting, the ontogeny of neutrophils is less important than the fact of their phenotypic diversity, now well-established if still incompletely delineated. This diversity opens the possibility of targeting neutrophils engaged in disease pathogenesis without similarly perturbing neutrophils engaged in antimicrobial defense. Such "wedge opportunities" arise not only with respect to heterogeneity within neutrophil population but also, somewhat less appreciated, in the pathways employed by neutrophils to respond to different stimuli. We reviewed here the evidence in favor of a greater role for neutrophil β2 integrins in neutrophil migration toward sterile than septic triggers, at least in some sites, and the potential role for neutrophil-specific integrin modulators (Ly6G in mice, potentially CD177 in humans) to enable lineage-specific integrin targeting. Better understanding of neutrophil biology will open further possibilities for the selective manipulation of this lineage in human therapeutics.

AUTHOR CONTRIBUTIONS

RG-B and PAN conceptualized and wrote the review and created the figure.

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REFERENCES

1. Ehrlich P. *Beiträge für Theorie und Praxis der Histologischen Färbung*. Leipzig (1878). Available online at: https://www.worldcat.org/title/beitrage-fur-theorie-und-praxis-der-histologischen-farbung/oclc/63372150
2. Bodey GP, Buckley M, Sathe YS, Freireich EJ. Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. *Ann Intern Med.* (1966) 64:328–40. doi: 10.7326/0003-4819-64-2-328
3. Andrews T, Sullivan KE. Infections in patients with inherited defects in phagocytic function. *Clin Microbiol Rev.* (2003) 16:597–621. doi: 10.1128/CMR.16.4.597-621.2003
4. Hock H, Hamblen MJ, Rooke HM, Traver D, Bronson RT, Cameron S, et al. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity.* (2003) 18:109–20. doi: 10.1016/S1074-7613(02)00501-0
5. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat Rev Rheumatol.* (2014) 10:593–601. doi: 10.1038/nrrheum.2014.80
6. Ben-Chetrit E, Levy M. Familial Mediterranean fever. *Lancet.* (1998) 351:659–64. doi: 10.1016/S0140-6736(97)09408-7
7. Callen JP. Neutrophilic dermatoses. *Dermatol Clin.* (2002) 20:409–19. doi: 10.1016/S0733-8635(02)00006-2
8. Biezeveld MH, van Mierlo G, Lutter R, Kuipers IM, Dekker T, Hack CE, et al. Sustained activation of neutrophils in the course of Kawasaki disease: an association with matrix metalloproteinases. *Clin Exp Immunol.* (2005) 141:183–8. doi: 10.1111/j.1365-2249.2005.02829.x
9. Punzi L, Ramonda R, Glorioso S, Schiavon F, Mariuz S, Gambari PF. Predictive value of synovial fluid analysis in juvenile chronic arthritis. *Ann Rheum Dis.* (1992) 51:522–4. doi: 10.1136/ard.51.4.522
10. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, et al. Monitoring neutrophil activation in juvenile rheumatoid arthritis

by S100A12 serum concentrations. *Arthritis Rheum.* (2004) 50:1286–95. doi: 10.1002/art.20125
11. Deanehan JK, Nigrovic PA, Milewski MD, Tan Tanny SP, Kimia AA, Smith BG, et al. Synovial fluid findings in children with knee monoarthritis in lyme disease endemic areas. *Pediatr Emerg Care.* (2014) 30:16–9. doi: 10.1097/PEC.0000000000000028
12. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J Immunol.* (2001) 167:1601–8. doi: 10.4049/jimmunol.167.3.1601
13. Monach PA, Nigrovic PA, Chen M, Hock H, Lee DM, Benoist C, et al. Neutrophils in a mouse model of autoantibody-mediated arthritis: critical producers of Fc receptor gamma, the receptor for C5a, and lymphocyte function-associated antigen 1. *Arthritis Rheum.* (2010) 62:753–64. doi: 10.1002/art.27238
14. Elliott ER, Van Ziffle JA, Scapini P, Sullivan BM, Locksley RM, Lowell CA. Deletion of Syk in neutrophils prevents immune complex arthritis. *J Immunol.* (2011) 187:4319–30. doi: 10.4049/jimmunol.1100341
15. Wang JX, Bair AM, King SL, Shnayder R, Huang YF, Shieh CC, et al. Ly6G ligation blocks recruitment of neutrophils via a beta2-integrin-dependent mechanism. *Blood.* (2012) 120:1489–98. doi: 10.1182/blood-2012-01-404046
16. Németh T, Mócsai A, Lowell CA. Neutrophils in animal models of autoimmune disease. *Semin Immunol.* (2016) 28:174–86. doi: 10.1016/j.smim.2016.04.001
17. Liu Z, Giudice GJ, Zhou X, Swartz SJ, Troy JL, Fairley JA, et al. A major role for neutrophils in experimental bullous pemphigoid. *J Clin Invest.* (1997) 100:1256–63. doi: 10.1172/JCI119639
18. Evrard M, Kwok IWH, Chong SZ, Teng KWW, Becht E, Chen J, et al. Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions. *Immunity.* (2018) 48:364–79. e368. doi: 10.1016/j.immuni.2018.02.002
19. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity.* (2005) 22:285–94. doi: 10.1016/j.immuni.2005.01.011
20. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest.* (2012) 122:327–36. doi: 10.1172/JCI57990
21. Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol Dis.* (2002) 28:260–74. doi: 10.1006/bcmd.2002.0513
22. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, et al. Mature CD10(+) and immature CD10(-) neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood.* (2017) 129:1343–56. doi: 10.1182/blood-2016-04-713206
23. Adrover JM, Nicolás-Ávila JA, Hidalgo A. Aging: a temporal dimension for neutrophils. *Trends Immunol.* (2016) 37:334–45. doi: 10.1016/j.it.2016.03.005
24. Manz MG, Boettcher S. Emergency granulopoiesis. *Nat Rev Immunol.* (2014) 14:302–14. doi: 10.1038/nri3660
25. Lefrançais E, Ortiz-Muñoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature.* (2017) 544:105–9. doi: 10.1038/nature21706
26. Iwasaki H, Akashi K. Myeloid lineage commitment from the hematopoietic stem cell. *Immunity.* (2007) 26:726–40. doi: 10.1016/j.immuni.2007.06.004
27. Hibbs ML, Quilici C, Kountouri N, Seymour JF, Armes JE, Burgess AW, et al. Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. *J Immunol.* (2007) 178:6435–43. doi: 10.4049/jimmunol.178.10.6435
28. King CL, Xianli J, Malhotra I, Liu S, Mahmoud AA, Oettgen HC. Mice with a targeted deletion of the IgE gene have increased worm burdens and reduced granulomatous inflammation following primary infection with Schistosoma mansoni. *J Immunol.* (1997) 158:294–300.
29. Basu S, Hodgson G, Katz M, Dunn AR. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood.* (2002) 100:854–61. doi: 10.1182/blood.V100.3.854
30. Suratt BT, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS. Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol Lung Cell Mol Physiol.* (2001) 281:L913–21. doi: 10.1152/ajplung.2001.281.4.L913
31. Tofts PS, Chevassut T, Cutajar M, Dowell NG, Peters AM. Doubts concerning the recently reported human neutrophil lifespan of 5.4 days. *Blood.* (2011) 117:6053–4. doi: 10.1182/blood-2010-10-310532
32. Lahoz-Beneytez J, Elemans M, Zhang Y, Ahmed R, Salam A, Block M, et al. Human neutrophil kinetics: modeling of stable isotope labeling data supports short blood neutrophil half-lives. *Blood.* (2016) 127:3431–8. doi: 10.1182/blood-2016-03-700336
33. Brach MA, deVos S, Gruss HJ, Herrmann F. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood.* (1992) 80:2920–4.
34. Nicolás-Ávila JÁ, Adrover JM, Hidalgo A. Neutrophils in homeostasis, immunity, and cancer. *Immunity.* (2017) 46:15–28. doi: 10.1016/j.immuni.2016.12.012
35. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity.* (2003) 19:583–93. doi: 10.1016/S1074-7613(03)00263-2
36. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chèvre R, A-González N, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell.* (2013) 153:1025–35. doi: 10.1016/j.cell.2013.04.040
37. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature.* (2015) 525:528–32. doi: 10.1038/nature15367
38. Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. *Science.* (2017) 358:111–6. doi: 10.1126/science.aam9690
39. Devi S, Wang Y, Chew WK, Lima R, González AN, Mattar CN, et al. Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. *J Exp Med.* (2013) 210:2321–36. doi: 10.1084/jem.20130056
40. Deniset JF, Kubes P. Neutrophil heterogeneity: Bona fide subsets or polarization states? *J Leukoc Biol.* (2018) 103:829–38. doi: 10.1002/JLB.3RI0917-361R
41. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
42. Hampton HR, Chtanova T. The lymph node neutrophil. *Semin Immunol.* (2016) 28:129–36. doi: 10.1016/j.smim.2016.03.008
43. Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *J Leukoc Biol.* (2006) 80:1281–8. doi: 10.1189/jlb.0506346
44. Colom B, Bodkin JV, Beyrau M, Woodfin A, Ody C, Rourke C, et al. Leukotriene B4-neutrophil elastase axis drives neutrophil reverse transendothelial cell migration *in vivo*. *Immunity.* (2015) 42:1075–86. doi: 10.1016/j.immuni.2015.05.010
45. Casanova-Acebes M, Nicolás-Ávila JA, Li JL, García-Silva S, Balachander A, Rubio-Ponce A, et al. Neutrophils instruct homeostatic and pathological states in naive tissues. *J Exp Med.* (2018) 215:2778–95. doi: 10.1084/jem.20181468
46. A-Gonzalez N, Quintana JA, García-Silva S, Mazariegos M, González de la Aleja A, Nicolás-Ávila JA, et al. Phagocytosis imprints heterogeneity in tissue-resident macrophages. *J Exp Med.* (2017) 214:1281–96. doi: 10.1084/jem.20161375
47. Pillay J, Ramakers BP, Kamp VM, Loi AL, Lam SW, Hietbrink F, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J Leukoc Biol.* (2010) 88:211–20. doi: 10.1189/jlb.1209793
48. Ter Haar NM, Tak T, Mokry M, Scholman RC, Meerding JM, de Jager W, et al. Reversal of sepsis-like features of neutrophils by interleukin-1 blockade in patients with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2018) 70:943–56. doi: 10.1002/art.40442

49. Deniset JF, Surewaard BG, Lee WY, Kubes P. Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of *S. pneumoniae*. *J Exp Med.* (2017) 214:1333–50. doi: 10.1084/jem.20161621
50. Uhl B, Vadlau Y, Zuchtriegel G, Nekolla K, Sharaf K, Gaertner F, et al. Aged neutrophils contribute to the first line of defense in the acute inflammatory response. *Blood*. (2016) 128:2327–37. doi: 10.1182/blood-2016-05-718999
51. Leliefeld PHC, Pillay J, Vrisekoop N, Heeres M, Tak T, Kox M, et al. Differential antibacterial control by neutrophil subsets. *Blood Adv*. (2018) 2:1344–55. doi: 10.1182/bloodadvances.2017015578
52. Goldschmeding R, van Dalen CM, Faber N, Calafat J, Huizinga TW, van der Schoot CE, et al. Further characterization of the NB 1 antigen as a variably expressed 56-62 kD GPI-linked glycoprotein of plasma membranes and specific granules of neutrophils. *Br J Haematol.* (1992) 81:336–45. doi: 10.1111/j.1365-2141.1992.tb08237.x
53. Halbwachs-Mecarelli L, Bessou G, Lesavre P, Lopez S, Witko-Sarsat V. Bimodal distribution of proteinase 3 (PR3) surface expression reflects a constitutive heterogeneity in the polymorphonuclear neutrophil pool. *FEBS Lett.* (1995) 374:29–33. doi: 10.1016/0014-5793(95)01073-N
54. Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. *Immunity*. (2014) 41:694–707. doi: 10.1016/j.immuni.2014.10.008
55. Buckley CD, Ross EA, McGettrick HM, Osborne CE, Haworth O, Schmutz C, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol.* (2006) 79:303–11. doi: 10.1189/jlb.0905496
56. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils *in vivo*. *Nat Immunol.* (2011) 12:761–9. doi: 10.1038/ni.2062
57. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJR, et al. Human CD62L(dim) neutrophils identified as a separate subset by proteome profiling and *in vivo* pulse-chase labeling. *Blood*. (2017) 129:3476–85. doi: 10.1182/blood-2016-07-727669
58. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. *Blood.* (2016) 127:2173–81. doi: 10.1182/blood-2016-01-688887
59. Scapini P, Marini O, Tecchio C, Cassatella MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. *Immunol Rev*. (2016) 273:48–60. doi: 10.1111/imr.12448
60. Zipursky A, Bow E, Seshadri RS, Brown EJ. Leukocyte density and volume in normal subjects and in patients with acute lymphoblastic leukemia. *Blood*. (1976) 48:361–71.
61. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. *Arthr Rheum.* (1986) 29:1334–42. doi: 10.1002/art.1780291105
62. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol.* (2010) 184:3284–97. doi: 10.4049/jimmunol.0902199
63. Wright HL, Makki FA, Moots RJ, Edwards SW. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. *J Leukoc Biol.* (2017) 101:599–611. doi: 10.1189/jlb.5A0116-022R
64. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep.* (2015) 10:562–73. doi: 10.1016/j.celrep.2014.12.039
65. Mistry P, Carmona-Rivera C, Ombrello AK, Hoffmann P, Seto NL, Jones A, et al. Dysregulated neutrophil responses and neutrophil extracellular trap formation and degradation in PAPA syndrome. *Ann Rheum Dis.* (2018) 77:1825–33. doi: 10.1136/annrheumdis-2018-213746
66. Morisaki T, Goya T, Ishimitsu T, Torisu M. The increase of low density subpopulations and CD10 (CALLA) negative neutrophils in severely infected patients. *Surg Today*. (1992) 22:322–7. doi: 10.1007/BF00308740
67. Ramanathan K, Glaser A, Lythgoe H, Ong J, Beresford MW, Midgley A, et al. Neutrophil activation signature in juvenile idiopathic arthritis indicates the presence of low-density granulocytes. *Rheumatology (Oxford, England).* (2018) 57:488–98. doi: 10.1093/rheumatology/kex441
68. Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood.* (2011) 117:1196–204. doi: 10.1182/blood-2009-11-254490
69. Chtanova T, Schaeffer M, Han SJ, van Dooren GG, Nollmann M, Herzmark P, et al. Dynamics of neutrophil migration in lymph nodes during infection. *Immunity.* (2008) 29:487–96. doi: 10.1016/j.immuni.2008.07.012
70. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, et al. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood.* (2005) 106:1843–50. doi: 10.1182/blood-2005-03-1281
71. Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, Guo TL, et al. Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. *Blood.* (1997) 89:4128–35.
72. Mudzinski SP, Christian TP, Guo TL, Cirenza E, Hazlett KR, Gosselin EJ. Expression of HLA-DR (major histocompatibility complex class II) on neutrophils from patients treated with granulocyte-macrophage colony-stimulating factor for mobilization of stem cells. *Blood.* (1995) 86:2452–3.
73. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3. *J Immunol.* (1993) 151:1482–90.
74. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells *in vitro* and *ex vivo*. *Blood.* (2017) 129:1991–2001. doi: 10.1182/blood-2016-10-744441
75. Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, et al. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. *Blood.* (2013) 121:1677–89. doi: 10.1182/blood-2012-07-445189
76. Fites JS, Gui M, Kernien JF, Negoro P, Dagher Z, Sykes DB, et al. An unappreciated role for neutrophil-DC hybrids in immunity to invasive fungal infections. *PLoS Pathog.* (2018) 14:e1007073. doi: 10.1371/journal.ppat.1007073
77. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol.* (2011) 13:170–80. doi: 10.1038/ni.2194
78. Nagelkerke SQ, aan de Kerk DJ, Jansen MH, van den Berg TK, Kuijpers TW. Failure to detect functional neutrophil B helper cells in the human spleen. *PLoS ONE.* (2014) 9:e88377. doi: 10.1371/journal.pone.0088377
79. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer cell.* (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
80. Shaul ME, Fridlender ZG. Cancer related circulating and tumor-associated neutrophils - subtypes, sources and function. *FEBS J.* (2018) 285:4316–42. doi: 10.1111/febs.14524
81. Aarts CEM, Kuijpers TW. Neutrophils as myeloid-derived suppressor cells. *Eur J Clin Investig.* (2018) 48 (Suppl. 2):e12989. doi: 10.1111/eci.12989
82. Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S, et al. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.* (2007) 67:426. doi: 10.1158/0008-5472.CAN-06-3037
83. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun.* (2016) 7:12150. doi: 10.1038/ncomms12150
84. Lalezari P, Murphy GB, Allen FH Jr. NB1, a new neutrophil-specific antigen involved in the pathogenesis of neonatal neutropenia. *J Clin Investig.* (1971) 50:1108–15. doi: 10.1172/JCI106582
85. Wu Z, Liang R, Ohnesorg T, Cho V, Lam W, Abhayaratna WP, et al. Heterogeneity of human neutrophil CD177 expression results from CD177P1 pseudogene conversion. *PLoS Genet.* (2016) 12:e1006067. doi: 10.1371/journal.pgen.1006067
86. Stroncek DF, Caruccio L, Bettinotti M. CD177: a member of the Ly-6 gene superfamily involved with neutrophil proliferation and polycythemia vera. *J Transl Med.* (2004) 2:8. doi: 10.1186/1479-5876-2-8
87. Welin A, Amirbeagi F, Christenson K, Björkman L, Björnsdottir H, Forsman H, et al. The human neutrophil subsets defined by the presence or absence of

OLFM4 both transmigrate into tissue *in vivo* and give rise to distinct NETs *in vitro*. *PLoS ONE.* (2013) 8:e69575. doi: 10.1371/journal.pone.0069575
88. Jerke U, Rolle S, Dittmar G, Bayat B, Santoso S, Sporbert A, et al. Complement receptor Mac-1 is an adaptor for NB1 (CD177)-mediated PR3-ANCA neutrophil activation. *J Biol Chem.* (2011) 286:7070–81. doi: 10.1074/jbc.M110.171256
89. Bai M, Grieshaber-Bouyer R, Wang J, Schmider AB, Wilson ZS, Zeng L, et al. CD177 modulates human neutrophil migration through activation-mediated integrin and chemoreceptor regulation. *Blood.* (2017) 130:2092–100. doi: 10.1182/blood-2017-03-768507
90. Cunin P, Lee PY, Kim E, Schmider AB, Cloutier N, Pare A, et al. Differential attenuation of β2 integrin-dependent and -independent neutrophil migration by Ly6G ligation. *Blood Adv.* (2019) 3:256–67. doi: 10.1182/bloodadvances.2018026732
91. Lee PY, Wang JX, Parisini E, Dascher CC, Nigrovic PA. Ly6 family proteins in neutrophil biology. *J Leukoc Biol.* (2013) 94:585–94. doi: 10.1189/jlb.0113014
92. Jerke U, Marino SF, Daumke O, Kettritz R. Characterization of the CD177 interaction with the ANCA antigen proteinase 3. *Sci Rep.* (2017) 7:43328. doi: 10.1038/srep43328
93. Bayat B, Werth S, Sachs UJ, Newman DK, Newman PJ, Santoso S. Neutrophil transmigration mediated by the neutrophil-specific antigen CD177 is influenced by the endothelial S536N dimorphism of platelet endothelial cell adhesion molecule-1. *J Immunol.* (2010) 184:3889–96. doi: 10.4049/jimmunol.0903136
94. Wang L, Ge S, Agustian A, Hiss M, Haller H, von Vietinghoff S. Surface receptor CD177/NB1 does not confer a recruitment advantage to neutrophilic granulocytes during human peritonitis. *Eur J Haematol.* (2013) 90:436–7. doi: 10.1111/ejh.12095
95. Bauer S, Abdgawad M, Gunnarsson L, Segelmark M, Tapper H, Hellmark T. Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils. *J Leukoc Biol.* (2007) 81:458–64. doi: 10.1189/jlb.0806514
96. von Vietinghoff S, Tunnemann G, Eulenberg C, Wellner M, Cristina Cardoso M, Luft FC, et al. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. *Blood.* (2007) 109:4487–93. doi: 10.1182/blood-2006-10-055327
97. Witko-Sarsat V, Cramer EM, Hieblot C, Guichard J, Nusbaum P, Lopez S, et al. Presence of proteinase 3 in secretory vesicles: evidence of a novel, highly mobilizable intracellular pool distinct from azurophil granules. *Blood.* (1999) 94:2487–96.
98. Kuckleburg CJ, Tilkens SB, Santoso S, Newman PJ. Proteinase 3 contributes to transendothelial migration of NB1-positive neutrophils. *J Immunol.* (2012) 188:2419–26. doi: 10.4049/jimmunol.1102540
99. Li Y, Mair DC, Schuller RM, Li L, Wu J. Genetic mechanism of human neutrophil antigen 2 deficiency and expression variations. *PLoS Genet.* (2015) 11:e1005255. doi: 10.1371/journal.pgen.1005255
100. Jelinek J, Li J, Mnjoyan Z, Issa JP, Prchal JT, Afshar-Kharghan V. Epigenetic control of PRV-1 expression on neutrophils. *Exp Hematol.* (2007) 35:1677–83. doi: 10.1016/j.exphem.2007.09.008
101. Eulenberg-Gustavus C, Bähring S, Maass PG, Luft FC, Kettritz R. Gene silencing and a novel monoallelic expression pattern in distinct CD177 neutrophil subsets. *J Exp Med.* (2017) 214:2089–101. doi: 10.1084/jem.20161093
102. Hu N, Westra J, Huitema MG, Bijl M, Brouwer E, Stegeman CA, et al. Coexpression of CD177 and membrane proteinase 3 on neutrophils in antineutrophil cytoplasmic autoantibody-associated systemic vasculitis: anti-proteinase 3-mediated neutrophil activation is independent of the role of CD177-expressing neutrophils. *Arthr Rheum.* (2009) 60:1548–57. doi: 10.1002/art.24442
103. Schreiber A, Luft FC, Kettritz R. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. *Kidney Int.* (2004) 65:2172–83. doi: 10.1111/j.1523-1755.2004.00640.x
104. Hu N, Mora-Jensen H, Theilgaard-Mönch K, Doornbos-van der Meer B, Huitema MG, Stegeman CA, et al. Differential expression of granulopoiesis related genes in neutrophil subsets distinguished by membrane expression of CD177. *PLoS ONE.* (2014) 9:e99671. doi: 10.1371/journal.pone.0099671
105. Yang TH, St John LS, Garber HR, Kerros C, Ruisaard KE, Clise-Dwyer K, et al. Membrane-associated proteinase 3 on granulocytes and acute myeloid leukemia inhibits T cell proliferation. *J Immunol.* (2018) 201:1389–99. doi: 10.4049/jimmunol.1800324
106. Zhou G, Yu L, Fang L, Yang W, Yu T, Miao Y, et al. CD177+ neutrophils as functionally activated neutrophils negatively regulate IBD. *Gut.* (2017) 67:1052–63. doi: 10.1136/gutjnl-2016-313535
107. Deng Q, Harvie EA, Huttenlocher A. Distinct signalling mechanisms mediate neutrophil attraction to bacterial infection and tissue injury. *Cell Microbiol.* (2012) 14:517–28. doi: 10.1111/j.1462-5822.2011.01738.x
108. Yan B, Han P, Pan L, Lu W, Xiong J, Zhang M, et al.IL-1beta and reactive oxygen species differentially regulate neutrophil directional migration and Basal random motility in a zebrafish injury-induced inflammation model. *J Immunol.* (2014) 192:5998–6008. doi: 10.4049/jimmunol.1301645
109. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol.* (2007) 7:678–89. doi: 10.1038/nri2156
110. Miyabe Y, Miyabe C, Murooka TT, Kim EY, Newton GA, Kim ND, et al. Complement C5a receptor is the key initiator of neutrophil adhesion igniting immune complex-induced arthritis. *Sci Immunol.* (2017) 2:eaaj2195. doi: 10.1126/sciimmunol.aaj2195
111. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med.* (2006) 203:2569–75. doi: 10.1084/jem.20060925
112. Proebstl D, Voisin MB, Woodfin A, Whiteford J, D'Acquisto F, Jones GE, et al. Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls *in vivo*. *J Exp Med.* (2012) 209:1219–34. doi: 10.1084/jem.20111622
113. Almarza Novoa E, Kasbekar S, Thrasher AJ, Kohn DB, Sevilla J, Nguyen T, et al. Leukocyte adhesion deficiency-I: a comprehensive review of all published cases. *J Allergy Clin Immunol Pract.* (2018) 6:1418–20 e1410. doi: 10.1016/j.jaip.2017.12.008
114. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R, Hirsch K, et al. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature.* (2008) 453:51–5. doi: 10.1038/nature06887
115. Doerschuk CM, Winn RK, Coxson HO, Harlan JM. CD18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J Immunol.* (1990) 144:2327–33.
116. Mizgerd JP, Kubo H, Kutkoski GJ, Bhagwan SD, Scharffetter-Kochanek K, Beaudet AL, et al. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J Exp Med.* (1997) 186:1357–64. doi: 10.1084/jem.186.8.1357
117. Mizgerd JP, Horwitz BH, Quillen HC, Scott ML, Doerschuk CM. Effects of CD18 deficiency on the emigration of murine neutrophils during pneumonia. *J Immunol.* (1999) 163:995–9.
118. Basit A, Reutershan J, Morris MA, Solga M, Rose CE Jr, Ley K. ICAM-1 and LFA-1 play critical roles in LPS-induced neutrophil recruitment into the alveolar space. *Am J Physiol Lung Cell Mol Physiol.* (2006) 291:L200–7. doi: 10.1152/ajplung.00346.2005
119. Kadioglu A, De Filippo K, Bangert M, Fernandes VE, Richards L, Jones K, et al. The integrins Mac-1 and alpha4beta1 perform crucial roles in neutrophil and T cell recruitment to lungs during *Streptococcus pneumoniae* infection. *J Immunol.* (2011) 186:5907–15. doi: 10.4049/jimmunol.1001533
120. Wilson ZS, Ahn LB, Serratelli WS, Belley MD, Lomas-Neira J, Sen M, et al. Activated beta2 integrins restrict neutrophil recruitment during murine acute pseudomonal pneumonia. *Am J Respir Cell Mol Biol.* (2017) 56:620–7. doi: 10.1165/rcmb.2016-0215OC
121. Hawkins HK, Heffelfinger SC, Anderson DC. Leukocyte adhesion deficiency: clinical and postmortem observations. *Pediatr Pathol.* (1992) 12:119–30. doi: 10.3109/15513819209023288
122. Nagahata H, Masuyama A, Masue M, Yuki M, Higuchi H, Ohtsuka H, et al. Leukocyte emigration in normal calves and calves with leukocyte adhesion deficiency. *J Vet Med Sci.* (1997) 59:1143–7. doi: 10.1292/jvms.59.1143
123. Schmits R, Kündig TM, Baker DM, Shumaker G, Simard JJ, Duncan G, et al. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. *J Exp Med.* (1996) 183:1415–26. doi: 10.1084/jem.183.4.1415

124. Lu H, Smith CW, Perrard J, Bullard D, Tang L, Shappell SB, et al. LFA-1 is sufficient in mediating neutrophil emigration in Mac-1-deficient mice. *J Clin Investig.* (1997) 99:1340–50. doi: 10.1172/JCI119293
125. Mócsai A, Zhou M, Meng F, Tybulewicz VL, Lowell CA. Syk is required for integrin signaling in neutrophils. *Immunity.* (2002) 16:547–58. doi: 10.1016/S1074-7613(02)00303-5
126. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science.* (2010) 330:362–6. doi: 10.1126/science.1195491
127. Watts GM, Beurskens FJ, Martin-Padura I, Ballantyne CM, Klickstein LB, Brenner MB, et al. Manifestations of inflammatory arthritis are critically dependent on LFA-1. *J Immunol.* (2005) 174:3668–75. doi: 10.4049/jimmunol.174.6.3668
128. Ding ZM, Babensee JE, Simon SI, Lu H, Perrard JL, Bullard DC, et al. Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol.* (1999) 163:5029–38.
129. Harding MG, Zhang K, Conly J, Kubes P. Neutrophil crawling in capillaries; a novel immune response to *Staphylococcus aureus*. *PLoS Pathog.* (2014) 10:e1004379. doi: 10.1371/journal.ppat.1004379
130. Maiguel D, Faridi MH, Wei C, Kuwano Y, Balla KM, Hernandez D, et al. Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease. *Sci Signal.* (2011) 4:ra57. doi: 10.1126/scisignal.2001811
131. Nigrovic PA, Raychaudhuri S, Thompson SD. Genetics and the classification of arthritis in adults and children. *Arthritis Rheumatol.* (2018) 70:7–17. doi: 10.1002/art.40350
132. Nandakumar KS, Svensson L, Holmdahl R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol.* (2003) 163:1827–37. doi: 10.1016/S0002-9440(10)63542-0
133. Phelps P, McCarty DJ Jr. Crystal-induced inflammation in canine joints. II. Importance of polymorphonuclear leukocytes. *J Exp Med.* (1966) 124:115–26. doi: 10.1084/jem.124.1.115
134. Podolin PL, Bolognese BJ, Foley JJ, Schmidt DB, Buckley PT, Widdowson KL, et al. A potent and selective nonpeptide antagonist of CXCR2 inhibits acute and chronic models of arthritis in the rabbit. *J Immunol.* (2002) 169:6435–44. doi: 10.4049/jimmunol.169.11.6435
135. Kim ND, Chou RC, Seung E, Tager AM, Luster AD. A unique requirement for the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis. *J Exp Med.* (2006) 203:829–35. doi: 10.1084/jem.20052349
136. Chou RC, Kim ND, Sadik CD, Seung E, Lan Y, Byrne MH, et al. Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis. *Immunity.* (2010) 33:266–78. doi: 10.1016/j.immuni.2010.07.018
137. Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, et al. Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. *J Exp Med.* (2006) 203:837–42. doi: 10.1084/jem.20052371
138. Mohr W, Westerhellweg H, Wessinghage D. Polymorphonuclear granulocytes in rheumatic tissue destruction. III. an electron microscopic study of PMNs at the pannus-cartilage junction in rheumatoid arthritis. *Ann Rheum Dis.* (1981) 40:396–9. doi: 10.1136/ard.40.4.396
139. Bromley M, Woolley DE. Histopathology of the rheumatoid lesion. Identification of cell types at sites of cartilage erosion. *Arthritis Rheum.* (1984) 27:857–63. doi: 10.1002/art.1780270804
140. Henson PM. The immunologic release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces. *J Immunol.* (1971) 107:1547–57.
141. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med.* (2013) 5:178ra140. doi: 10.1126/scitranslmed.3005580
142. Carmona-Rivera C, Carlucci PM, Moore E, Lingampalli N, Uchtenhagen H, James E, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci Immunol.* (2017) 2:eaag3358. doi: 10.1126/sciimmunol.aag3358
143. Demoruelle MK, Harrall KK, Ho L, Purmalek MM, Seto NL, Rothfuss HM, et al. Anti-citrullinated protein antibodies are associated with neutrophil extracellular traps in the sputum in relatives of rheumatoid arthritis patients. *Arthritis Rheumatol.* (2017) 69:1165–75. doi: 10.1002/art.40066
144. Demoruelle MK, Bowers E, Lahey LJ, Sokolove J, Purmalek M, Seto NL, et al. Antibody responses to citrullinated and noncitrullinated antigens in the sputum of subjects with rheumatoid arthritis and subjects at risk for development of rheumatoid arthritis. *Arthritis Rheumatol.* (2018) 70:516–27. doi: 10.1002/art.40401
145. Corsiero E, Bombardieri M, Carlotti E, Pratesi F, Robinson W, Migliorini P, et al. Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs. *Ann Rheum Dis.* (2016) 75:1866–75. doi: 10.1136/annrheumdis-2015-208356
146. Romero V, Fert-Bober J, Nigrovic PA, Darrah E, Haque UJ, Lee DM, et al. Immune-mediated pore-forming pathways induce cellular hypercitrullination and generate citrullinated autoantigens in rheumatoid arthritis. *Sci Transl Med.* (2013) 5:209ra150. doi: 10.1126/scitranslmed.3006869
147. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. *Sci Transl Med.* (2016) 8:369ra176. doi: 10.1126/scitranslmed.aaj1921
148. Headland SE, Jones HR, Norling LV, Kim A, Souza PR, Corsiero E, et al. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. *Sci Transl Med.* (2015) 7:315ra190. doi: 10.1126/scitranslmed.aac5608
149. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med.* (2014) 20:511–7. doi: 10.1038/nm.3547
150. Reber LL, Gaudenzio N, Starkl P, Galli SJ. Neutrophils are not required for resolution of acute gouty arthritis in mice. *Nat Med.* (2016) 22:1382–4. doi: 10.1038/nm.4216
151. Vogl T, Pröpper C, Hartmann M, Strey A, Strupat K, van den Bos C, et al. S100A12 is expressed exclusively by granulocytes and acts independently from MRP8 and MRP14. *J Biol Chem.* (1999) 274:25291–6. doi: 10.1074/jbc.274.36.25291
152. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med.* (2007) 13:1042–9. doi: 10.1038/nm1638
153. Foell D, Wittkowski H, Kessel C, Lüken A, Weinhage T, Varga G, et al. Proinflammatory S100A12 can activate human monocytes via Toll-like receptor 4. *Am J Respir Crit Care Med.* (2013) 187:1324–34. doi: 10.1164/rccm.201209-1602OC
154. Frosch M, Ahlmann M, Vogl T, Wittkowski H, Wulffraat N, Foell D, et al. The myeloid-related proteins 8 and 14 complex, a novel ligand of toll-like receptor 4, and interleukin-1beta form a positive feedback mechanism in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum.* (2009) 60:883–91. doi: 10.1002/art.24349
155. Brown RA, Henderlight M, Do T, Yasin S, Grom AA, DeLay M, et al. Neutrophils from children with systemic juvenile idiopathic arthritis exhibit persistent proinflammatory activation despite long-standing clinically inactive disease. *Front Immunol.* (2018) 9:2995. doi: 10.3389/fimmu.2018.02995
156. Angelini A, Miyabe Y, Newsted D, Kwan BH, Miyabe C, Kelly RL, et al. Directed evolution of broadly crossreactive chemokine-blocking antibodies efficacious in arthritis. *Nat Commun.* (2018) 9:1461. doi: 10.1038/s41467-018-03687-x
157. Robertson JD, Ward JR, Avila-Olias M, Battaglia G, Renshaw SA. Targeting neutrophilic inflammation using polymersome-mediated cellular delivery. *J Immunol.* (2017) 198:3596–604. doi: 10.4049/jimmunol.1601901
158. Gupta S, Kaplan MJ. The role of neutrophils and NETosis in autoimmune and renal diseases. *Nat Rev Nephrol.* (2016) 12:402–13. doi: 10.1038/nrneph.2016.71
159. Chang M, Nigrovic PA. Mechanisms of antibody-dependent and -independent arthritis. *JCI insight.* (in press).
160. Smith CK, Kaplan MJ. The role of neutrophils in the pathogenesis of systemic lupus erythematosus. *Curr Opin Rheumatol.* (2015) 27:448–53. doi: 10.1097/BOR.0000000000000197

161. Apel F, Zychlinsky A, Kenny EF.Apel F, Zychlinsky A, Kenny EF. The role of neutrophil extracellular traps in rheumatic diseases. *Nat Rev Rheumatol.* (2018) 14:467–75. doi: 10.1038/s41584-018-0039-z
162. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol.* (2011) 187:538–52. doi: 10.4049/jimmunol.1100450
163. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med.* (2011) 3:73ra19. doi: 10.1126/scitranslmed.3001180
164. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med.* (2011) 3:73ra20. doi: 10.1126/scitranslmed.3001201
165. Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci USA.* (2010) 107:9813–8. doi: 10.1073/pnas.0909927107
166. Yalavarthi S, Gould TJ, Rao AN, Mazza LF, Morris AE, Núñez-Álvarez C, et al. Release of neutrophil extracellular traps by neutrophils stimulated with antiphospholipid antibodies: a newly identified mechanism of thrombosis in the antiphospholipid syndrome. *Arthritis Rheumatol.* (2015) 67:2990–3003. doi: 10.1002/art.39247
167. Palanichamy A, Bauer JW, Yalavarthi S, Meednu N, Barnard J, Owen T, et al. Neutrophil-mediated IFN activation in the bone marrow alters B cell development in human and murine systemic lupus erythematosus. *J Immunol.* (2014) 192:906–18. doi: 10.4049/jimmunol.1302112
168. Knight JS, Zhao W, Luo W, Subramanian V, O'Dell AA, Yalavarthi S, et al. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Investig.* (2013) 123:2981–93. doi: 10.1172/JCI67390
169. Knight JS, Subramanian V, O'Dell AA, Yalavarthi S, Zhao W, Smith CK, et al. Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Ann Rheum Dis.* (2015) 74:2199–206. doi: 10.1136/annrheumdis-2014-205365
170. Gordon RA, Herter JM, Rosetti F, Campbell AM, Nishi H, Kashgarian M, et al. Lupus and proliferative nephritis are PAD4 independent in murine models. *JCI Insight.* (2017) 2:92926. doi: 10.1172/jci.insight.92926
171. Kienhöfer D, Hahn J, Stoof J, Csepregi JZ, Reinwald C, Urbonaviciute V, et al. Experimental lupus is aggravated in mouse strains with impaired induction of neutrophil extracellular traps. *JCI Insight.* (2017) 2:92920. doi: 10.1172/jci.insight.92920
172. Campbell AM, Kashgarian M, Shlomchik MJ. NADPH oxidase inhibits the pathogenesis of systemic lupus erythematosus. *Sci Transl Med.* (2012) 4:157ra141. doi: 10.1126/scitranslmed.3004801
173. Carlson JA. The histological assessment of cutaneous vasculitis. *Histopathology.* (2010) 56:3–23. doi: 10.1111/j.1365-2559.2009.03443.x
174. Fujiwara H, Hamashima Y. Pathology of the heart in Kawasaki disease. *Pediatrics.* (1978) 61:100–7.
175. Takahashi K, Oharaseki T, Naoe S, Wakayama M, Yokouchi Y. Neutrophilic involvement in the damage to coronary arteries in acute stage of Kawasaki disease. *Pediatr Int.* (2005) 47:305–10. doi: 10.1111/j.1442-200x.2005.02049.x
176. Heineke MH, Ballering AV, Jamin A, Ben Mkaddem S, Monteiro RC, Van Egmond M. New insights in the pathogenesis of immunoglobulin A vasculitis (Henoch-Schonlein purpura). *Autoimmun Rev.* (2017) 16:1246–53. doi: 10.1016/j.autrev.2017.10.009
177. Savage CO. Pathogenesis of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis. *Clin Exp Immunol.* (2011) 164 (Suppl. 1):23–6. doi: 10.1111/j.1365-2249.2011.04362.x
178. Radford DJ, Savage CO, Nash GB. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. *Arthritis Rheum.* (2000) 43:1337–45. doi: 10.1002/1529-0131(200006)43:6<1337::AID-ANR16>3.0.CO;2-M
179. Radford DJ, Luu NT, Hewins P, Nash GB, Savage CO. Antineutrophil cytoplasmic antibodies stabilize adhesion and promote migration of flowing neutrophils on endothelial cells. *Arthritis Rheumat.* (2001) 44:2851–61. doi: 10.1002/1529-0131(200112)44:12<2851::AID-ART473>3.0.CO;2-2
180. Dick J, Gan PY, Ford SL, Odobasic D, Alikhan MA, Loosen SH, et al. C5a receptor 1 promotes autoimmunity, neutrophil dysfunction and injury in experimental anti-myeloperoxidase glomerulonephritis. *Kidney Int.* (2018) 93:615–25. doi: 10.1016/j.kint.2017.09.018
181. Kuligowski MP, Kwan RY, Lo C, Wong C, James WG, Bourges D, et al. Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood.* (2009) 113:6485–94. doi: 10.1182/blood-2008-12-192617
182. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, et al. Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. *Blood.* (2005) 106:2050–8. doi: 10.1182/blood-2005-03-0921
183. Millet A, Martin KR, Bonnefoy F, Saas P, Mocek J, Alkan M, et al. Proteinase 3 on apoptotic cells disrupts immune silencing in autoimmune vasculitis. *J Clin Investig.* (2015) 125:4107–21. doi: 10.1172/JCI78182
184. Porges AJ, Redecha PB, Kimberly WT, Csernok E, Gross WL, Kimberly RP. Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol.* (1994) 153:1271–80.
185. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. *Nature medicine.* (2009) 15:623-625. doi: 10.1038/nm.1959
186. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *J Am Soc Nephrol.* (2014) 25:990–7. doi: 10.1681/ASN.2013060606
187. Stock AT, Hansen JA, Sleeman MA, McKenzie BS, Wicks IP. GM-CSF primes cardiac inflammation in a mouse model of Kawasaki disease. *J Exp Med.* (2016) 213:1983–98. doi: 10.1084/jem.20151853
188. Schreiber A, Rolle S, Peripelittchenko L, Rademann J, Schneider W, Luft FC, et al. Phosphoinositol 3-kinase-gamma mediates antineutrophil cytoplasmic autoantibody-induced glomerulonephritis. *Kidney Int.* (2010) 77:118–28. doi: 10.1038/ki.2009.420
189. Schreiber A, Rousselle A, Becker JU, von Mässenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proc Natl Acad Sci USA.* (2017) 114:E9618–25. doi: 10.1073/pnas.1708247114

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Th17 and Th1 Lymphocytes in Oligoarticular Juvenile Idiopathic Arthritis

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In the last years much attention has focused on the Th17 and Th1 phenotypes and on their pathogenic role in juvenile idiopathic arthritis, investigating how the cytokines produced by T helper cells act on resident cells on the synovia and which signal transduction pathways regulate Th17 cells proliferation and plasticity. In this context, an important milestone was represented by the identification of the non-classic Th1 phenotype, developed from the shift of Th17 cells. The cytokine TNF-α, beyond its well-known proinflammatory activity is involved in this process and this is one of the reasons why the TNF-α inhibitors are widely used in the treatment of juvenile idiopathic arthritis patients.

Keywords: Th17, Th1, CD161+ T cells, TNF-α, synoviocytes

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INTRODUCTION

Juvenile idiopathic arthritis (JIA) is one of the most common chronic conditions of childhood, comprising several forms of arthritis characterized by persistent joint inflammation for at least 6 weeks, with an onset before the age of 16 years and with unknown cause (1, 2). The term JIA covers seven pathologic conditions that differ for clinical presentation, disease course and treatment response; in particular it includes systemic arthritis (sJIA), oligoarthritis, polyarthritis (both rheumatoid factor positive or negative), psoriatic arthritis (JPsA), enthesitis-related arthritis (ERA), and undifferentiated arthritis (1, 2). Although the cause of disease is unknown, immune cells, including T and B lymphocytes, infiltrate the synovial membrane of inflamed joints, suggesting that the adaptive immune system is involved in the pathogenesis of JIA (3). Human effector CD4+ T lymphocytes can be classified in three main subsets based mainly on their immunological functions, their cytokines production profile and their typical transcription factor expression (4). Th1 lymphocytes express the transcription factor T-bet, produce interferon (IFN)-γ, and defend the body from intracellular infections. Th2 cells express the transcription factor GATA-3, produce type 2 cytokines (interleukin (IL)-4, IL-5, IL-9, and IL-13) and are important to protect against helminths (5, 6). Finally, the Th17 subset produce IL-17A, IL-17F and IL-22 (7–10), express the transcription factor ROR-γT (11–13) and the lectin receptor CD161 (14), as typical surface marker. Beyond their protective role against extracellular bacterial and fungal infections, Th17 cells have been demonstrated to be important in the pathogenesis of several autoimmune and inflammatory diseases, including multiple sclerosis, inflammatory bowel disease (IBD), psoriasis, rheumatoid arthritis (RA), and JIA (15, 16). In humans, Th17 lymphocytes are included within the CD161+ cell fraction of circulating and tissue-infiltrating CD4+ T cells, and they develop from a CD161+ T cell precursor found in umbilical cord blood and neonatal thymus (14, 17, 18).

Since JIA can be considered as an immune-mediated disorder, the pharmacologic therapy is essentially based on immunosuppressive drugs, at least when the usage of non-steroidal anti-inflammatory drugs (NSAIDs) does not control symptoms. Among these disease-modifying antirheumatic drugs (DMARDs) methotrexate (MTX), is considered the first line treatment, since its positive clinical effects are associated with low toxic effects (19). For those patients with a suboptimal response to non-biologic DMARDs, in particular those with polyarthritis, the usage of biologic drugs such as tumor necrosis factor-α (TNF-α) inhibitors (etanercept, infliximab and adalimumab), IL-1 inhibitors (anakinra, canakinumab, and rilonacept), IL-6 inhibitor (tocilizumab), CD20/B-cell targeted (rituximab) and T-cell co-stimulatory signal blocker (abatacept), has been proven to be effective (19–22).

T HELPER EFFECTOR CELLS IN OLIGOARTICULAR JIA

The synovial membrane of JIA inflamed joints shows high degree of infiltrating mononuclear cells, including T and B lymphocytes, dendritic cells and macrophages (3, 18, 23). Among T cells, Th1 are the most represented since these cells can migrate in the synovia in response to the chemokine CXCL10 (24). For this reason, these cells were thought to play a key role in the pathogenesis of oligoarticular JIA (25, 26), at least until 10 years ago. More recently, after the identification of the Th17 subset, many experimental data suggested their potential pathogenic role both in adult and childhood arthritis as well as in other inflammatory and autoimmune diseases (27, 28). Indeed, increased levels of IL-17A and of the transcription factor ROR-γT, as well as of Th17 cells were reported in the synovial fluid (SF) of oligoarticular JIA patients (26, 28–31).

Moreover, Th17 cells have been demonstrated to be pathogenic in several murine models of chronic inflammatory disorders (16), such as experimental autoimmune encephalomyelitis (7), collagen-induced arthritis (32), and IBD (10–12).

However, despite their supposed pathogenicity, Th17 are very rare at inflammatory sites if compared to Th1 cells (28, 33). A first explanation for this rarity is a self-regulatory mechanism that controls Th17 cells clonal expansion. In particular, ROR-γT favors the up-regulation of the interleukin (IL)-4 induced gene 1 (IL4I1), which encodes an l-phenylalanine oxidase that down-regulates CD3ε expression on T cells via the production of H_2O_2 (28, 34). By this way, Th17 cells display an impaired signaling pathway downstream of the T-cell receptor (TCR), leading to inappropriate proliferation and reduced IL-2 production upon TCR triggering (28, 34). In addition, high IL4I1 expression in Th17 cells induces up-regulation of Tob1, a member of the Tob/BTG anti-proliferative protein family, involved in the negative control of the cell cycle (35).

It has also been recently described that Th17 cells show reduced IL-2 responsiveness since they express Musculin (MSC), a member of the basic helix-loop-helix transcription factors, dependent by ROR-γT, which negatively regulates the phosphorylation level of STAT5B upon IL-2 signaling (36). In agreement with these findings, both IL4I1 and MSC were found to be selectively expressed by CD161+ T cells obtained from SF of oligoarticular JIA inflamed joints (34, 36).

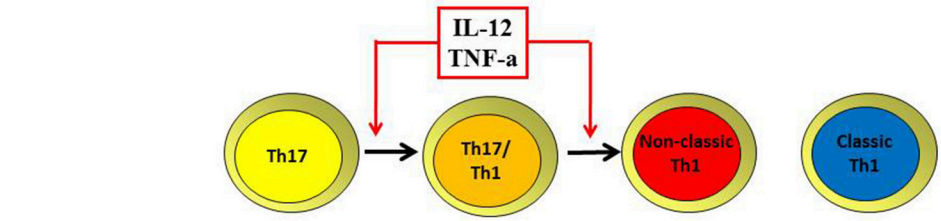
It has also been demonstrated that the development of JIA and of other autoimmune diseases depends not only on the amount and the phenotype of Th effector cells, but also on their balance with Treg cells (37). Indeed, several papers demonstrated an accumulation of Th17 and Treg cells in SF and PB of JIA patients (31, 38, 39), in particular in active versus inactive JIA (31), hypothesizing that the joint inflammatory status persists despite the high frequency of Treg cells because Th17 cells show a reduced susceptibility to their regulatory function. This could be also related to the low proliferation rate of Th17 cells (33–36).

ROLE OF Th17 PLASTICITY IN THE PATHOGENESIS OF OLIGOARTICULAR JIA

A second explanation for human Th17 cells rarity at inflamed tissues is their phenotype plasticity (28). Indeed, in presence of local inflammatory cytokines such as IL-12 and TNF-α, Th17 cells acquire the ability to produce IFN-γ. At a first stage, cells display an intermediate phenotype known as Th17/Th1 and produce both IFN-γ and IL-17, but they can also rapidly loose IL-17 secretion and become IFN-γ single producers (12, 18, 40). These Th17-derived Th1 cells are defined as non-classic Th1 cells because, differently from classic Th1 cells, they maintain the expression of ROR-γT, CD161, and CCR6 (17, 18, 28, 40), typical molecules of the Th17 subset (**Figure 1**).

Th17/Th1 and non-classic Th1 cells were found to be enriched in the SF of oligoarticular JIA children compared to their peripheral blood (18, 40). Moreover, a positive correlation between the frequencies of CD4+CD161+ Th17/Th1 cells in the SF of inflamed joints and disease activity parameters was described (18, 28). The shift of Th17 cells toward the non-classic Th1 phenotype is driven by IL-12, that has been found to be elevated in the SF of oligoarticular JIA patients (18, 40). Moreover, the finding that SF-derived Th17 clones share similar TCR Vβ spectra with Th1 CD161+ clones but not with Th1 CD161- ones (18, 40), strongly supported the data of the shift of Th17 toward non-classic Th1 cells. These findings are in agreement with several studies describing an accumulation of Th1 cells in the SF of JIA patients (25, 26), characterized as Th cells producing IFN-γ but without any distinction between the classic and non-classic phenotypes Additional data at epigenetic level confirm that non-classic Th1 cells originate from Th17 (41); indeed it was observed that non-classic Th1 cells exhibit demethylation of RORC2 and IL17A genes, as reported for Th17 cells, whereas classic Th1 cells are completely methylated at these loci (41).

Th17 plasticity consists not only in the acquisition of the ability to produce IFN-γ, but also GM-CSF (42). In fact, it has been described an enrichment of GM-CSF producing T cells with a non-classic Th1 phenotype in the SF of oligoarticular JIA patients (42) and induced *in vitro* by IL-12. This data suggests a possible involvement not only of IFN-γ but also



ROR-γt	+++	++	+	-
Tbet	+	+	+++	+++
Eomes	-	-	++	+++
CD161	+++	+++	+++	-
CXCR3	-	+	+++	+++
CCR6	+++	+++	+++	-
IL-17A	+	+	-	-
IFN-γ	-	+	+	+

FIGURE 1 | Similar and distinct features of human Th1 and Th17 cells. Both classic and non-classic Th1 cells produce IFN-γ, express the transcription factor T-bet and Eomes and the surface marker CXCR3. Differently from classic, non-classic Th1 cells maintain also the expression of ROR-γT, CD161, and CCR6 as typical transcription factor and surface markers of the Th17 phenotype. In fact, non-classic Th1 cells originate from the shift of Th17 toward the Th17/Th1 phenotype when in the presence of IL-12 and/or TNF-α.

of GM-CSF in JIA pathogenesis, and, accordingly, a positive correlation between GM-CSF protein levels in the SF and the serum parameters of disease activity was described (42).

Moreover it has been recently described that human non-classic Th1 cells development is promoted by the transcription factors Eomes (43), which induces and reinforces IFN-γ production, maintains the Th1 phenotype stability by inhibiting and preventing the re-expression of ROR-γT and IL-17A and promotes GM-CSF secretion (43). Finally, it was shown that Eomes induces, *in vitro*, the production of IFN-γ and GM-CSF by human CD4+ T cells and that cells with this cytokines profile were enriched in the SF of inflamed joints of children with oligoarticular JIA (43). All these data further support the pathogenic role of Th17-derived non-classic Th1 cells.

Additional evidence of the pathogenic features of CD4+CD161+ T cells, including pure Th17 cells and their derivative phenotypes Th17/Th1 and non-classic Th1, derived from a recent paper (44), describing that all these subsets express CHI3L1. This chitinase-like protein without enzymatic activity is defined in the literature as a well-known marker of disease activity and inflammation in several immune-mediated disorders (44–47): its levels are elevated in SF of children affected by oligoarticular JIA and positively correlated to inflammatory parameters (44).

These data of Th17 plasticity, partially solve the literature debate on the pathogenic or protective role of Th17 cells in immunomediated disorders (48, 49), supporting the hypothesis that, at least in JIA, the Th1 subset, both classic and non-classic, is directly involved in the active phase of the disease when the clinical manifestations are evident, and that the pure Th17 cells acquire a pathogenic feature when they start to produce IFN-γ shifting toward the Th17/Th1 and the non-classic Th1 phenotype (18, 20, 40). Anyway, it cannot be excluded that even pure Th17 cells may play a key role in the pathogenesis of JIA during the onset and/or the early-phase of the disease.

CROSS TALK BETWEEN T HELPER CELLS AND RESIDENT SYNOVIAL CELLS

CD4+ T helper lymphocytes orchestrate both RA and JIA chronic inflammation producing cytokines that initiate and maintain the process of synovial proteolysis and proliferation as well as the angiogenesis related to the inflammatory status (50, 51). Synovial fibroblasts (SFbs) are the main tissue resident cell population in the synovia and it has been demonstrated that in adult RA SFbs produce cytokines and matrix-degrading enzymes, crucial to promote cartilage destruction and to mediate inflammation (28, 52). In particular, it has been reported (51) that SFbs derived from SF of oligoarticular JIA patients express high levels of CD106 (VCAM), a sialoglycoprotein which mediates leukocyte-endothelial cell adhesion and signal transduction (51), and whose upregulation is critical to favor leukocytes retention in the inflamed synovia (51). SFbs also showed a peculiar morphology consisting in polygonal cell body, large, and oval-shaped nucleus, many slender protrusions and branches extended out of the cell body (51, 53). This peculiar phenotype of oligoarticular JIA-derived SFbs, was resembled *in vitro* stimulating healthy-derived SFbs with culture supernatants from activated classic and non-classic Th1, but not from Th17, lymphocytes. Indeed, also in these experimental conditions SFbs upregulated CD106 expression and underwent morphological

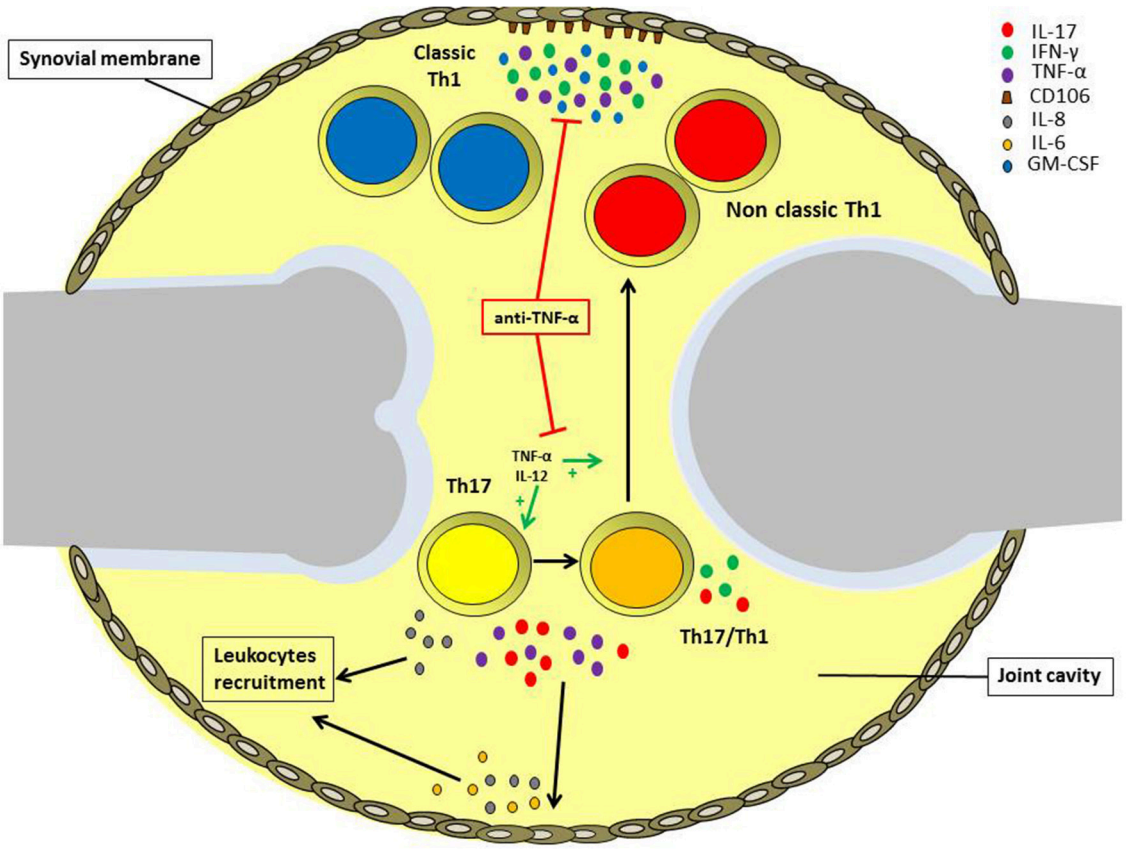


FIGURE 2 | Role of different T helper cell subsets in the pathogenesis of oligoarticular juvenile idiopathic arthritis. IL-17A and TNF-α produced by Th17 and Th17/Th1 cells infiltrating the inflamed joints of oligoarticular juvenile idiopathic arthritis patients, promotes IL-6 and IL-8 release by endothelial cells and synoviocytes in the synovial membrane. These cytokines, in turn, maintain joint inflammation and induce leukocytes recruitment. Inflamed joints are also characterized by high IL-12 and TNF-α levels, which promote Th17 cells phenotypic shift toward Th17/Th1 and non-classic Th1 cells. Non-classic Th1 cells proliferate at higher rate than Th17, and together with classic Th1 cells secrete IFN-γ and TNF-α, favoring the expression of the adhesion molecule CD106 by synoviocytes. Anti-TNF-α treatment can reduce inflammation via both impairing Th17 cell phenotypic shift and also inhibiting CD106 upregulation on the synovial membrane.

changes (50). It has been demonstrated that TNF-α is the main cytokine involved in this process and that IFN-γ exerts a synergic effect (51, 54). The concept that cytokines produced by T cells play an important role on the activation of SFbs has been confirmed also by the paper of Lavocat et al. (55). It demonstrates with *in vitro* experiments that IL-17A and TNF-α alone are able to induce the expression of IL-6 and IL-8 (55) by both endothelial cells and synoviocytes (even if with different kinetics on each cell type), and that a synergistic effect can be achieved from the use of both cytokines (55). Similar results were obtained also by stimulating endothelial cells and synoviocytes in the presence of culture supernatants from activated T cell clones or recombinant cytokines. Indeed, the main increase in IL-6 and IL-8 production was observed when cells were cultured in presence of supernatants from Th17/Th1 T cell clones that contained both IL-17A and TNF-α (55). The early expression of IL-8 in inflamed joints, directly produced also by Th17 cells itself (9), might explain the massive neutrophil recruitment in the acute phase (56). On the other hand, IL-6 production might be important to sustain the pro-inflammatory process since it is involved in the differentiation and expansion of Th17 cell (57), in VEGF production [thus mediating angiogenesis (58)], as well as in antibody production (59) and in osteoclast activation (55).

The IL-17 signature, which is typical of JIA, is important also for bone and cartilage erosion. In fact, it has been demonstrated that IL-17A acts on SFbs increasing the expression of different types of matrix metalloproteinases, MMP-1, MMP-3 (60). Finally, it is important to note that IL-17A production is not strictly associated to Th17 cells, since it is produced also by additional cells of the immune system enriched in SF of JIA patients, such as CD3+CD8+ and CD3+CD4-CD8- T cells (17, 61, 62) and innate lymphoid cells (62). Collectively, these data suggest that mechanisms actively contributing to joint inflammation in the synovia of JIA patients depend on the final balance and cross-talk between tissue resident cells and immune cells from both the adaptive and innate immune systems.

EFFECTS OF BIOLOGICAL DRUGS IN THE TREATMENT OF JIA: *EX-VIVO* AND *IN-VITRO* OBSERVATIONS

Cytokines produced by immune cells (in particular T cells and monocytes) and by tissue resident cells in the synovia contribute to the development of JIA and are responsible for most of the clinical manifestations of the disease. In this view, pro-inflammatory cytokines represent a key therapeutic target for biological treatment. The drugs mainly used and effective in JIA inhibit the activity of TNF-α, IL-1, or IL-6. TNF-α has pleiotropic effects in the inflamed environment of affected joints, acting on different cell populations (51): TNF-α mediates monocyte, macrophage and SFb activation, and it is also responsible for inflammation induction, cartilage degradation, bone erosion and tissue damage (51). Moreover, as previously stated, TNF-α acts on SFbs inducing the upregulation of CD106, thus favoring leukocytes retention within the synovia and increasing joint inflammatory status (51). TNF-α is also involved in the neovascularization process, leading to synovial membrane growth, and in the process of osteoclast-containing 'pannus' formation (51). Additionally, TNF-α interferes with T helper cells phenotype plasticity, mediating the shifting of Th17 lymphocytes toward non-classic Th1 cells (20, 51). Nowadays, JIA patients are treated with non-steroidal antiinflammatory drugs, corticosteroids, and disease modifying antirheumatic drugs including TNF-α antagonists (28). Among these antagonists etanercept is a soluble dimeric fusion protein binding soluble TNF-α. Etanercept has been reported to induce improvement of clinical symptoms (as measured by radiological progression and laboratory parameters of disease activity) in patients affected by immune-mediated arthritis, including RA, JIA, and psoriatic arthritis (28, 63). Moreover, etanercept efficacy in JIA treatment has been demonstrated in randomized clinical trials, as well as in long-term observational registries (28, 64). These clinical effects were sustained by its well-known anti-inflammatory properties on the innate and adaptive immune responses. Moreover, it has been reported a new mechanism of action of etanercept, defining its inhibitory role in the plasticity of Th17 cells toward the non-classic Th1 phenotype mediated by TNF-α (18, 20, 28). In fact, etanercept reduces the proportion of circulating non-classic Th1 cells (20), supposed to play a key role in the pathogenesis of oligoarticular JIA and leads to an increased frequency of Th17 cells (20). Similar evidence was observed also during the treatment of RA patients with adalimumab, a fully humanized monoclonal IgG1 antibody against TNF-α (65), supporting again the important role of this cytokine in the pathogenesis of such diseases. In fact, after 12 weeks of treatment an increase was found in the frequency of IL-17A producing cells that significantly correlated with a reduction in joint inflammation (65, 66). These data support again the important role of this cytokine in the pathogenesis of such diseases. Moreover, it was demonstrated that etanercept acts also on the regulation of CD106 expression on SFbs, in fact *in vitro* administration of this drug negatively interferes with the ability of both classic and non-classic Th1cells supernatants to significantly induce CD106 expression on SFbs (51). Taking into account the important role of CD106 expression on SFbs to mediate leukocytes adhesion, these recent data define also the role of etanercept in interfering with the adhesion of immune cells on SFbs. Even if these data were obtained with *in-vitro* experimental models (51), they may suggest that the reduction of inflammatory cells in the synovia, occurring during etanercept treatment of oligoarticular JIA, may be driven by the reduced retention of immune cells within inflamed joints.

All these data define the immunomodulatory properties of TNF-α inhibitors, especially of etanercept and adalimumab, which could further explain its disease-modifying effect in JIA (20, 51, 66). Regarding infliximab (a human–mouse chimeric anti-TNF-α antibody) and golimumab (a fully humanized monoclonal anti-TNF-α antibody), their use is related mainly to polyarticular JIA and reported in case reports and open-label trials (22).

Among additional drugs inhibiting inflammatory cytokines, the IL-6 receptor antagonist (tocilizumab) and the IL-1 antagonists (anakinra, canakinumab and rilonacept), are currently used in systemic JIA. Anyway these drugs were also tested in clinical practice for oligoarticular or polyarticular JIA (22, 67), and their efficacy may be due, at least in part, to the interference with Th17 expansion and differentiation in the synovia mediated by IL-1 and IL-6.

Moreover, the use of anti-IL-12/IL-23 p40 inhibitors could improve the course of JIA, since both cytokines are involved in Th1 and Th17 differentiation and are important regulators of Th17 plasticity. Ustekinumab, the human monoclonal antibody anti- p40 subunit, is often used in the treatment of psoriatic arthritis and ankylosing spondylarthritis in adults and children (68) and its use in oligoarticular JIA is poor. These data suggest that although these cytokines contribute to joint inflammation, they may not be the principal factors responsible and it is likely that other key mediators are involved (i.e., TNF-α).

Similarly, the use of anti-IL-17A in the treatment of JIA has been explored. In fact data from clinical trials show that secukinumab, the high-affinity fully human monoclonal antibody neutralizing the activity of IL-17A, can be effective in the treatment of JPsA and ERA, suggesting that IL-17 cytokine and Th17 cells play a key role in the pathogenesis of these subtypes of JIA and their role is instead marginal in oligoarticular JIA (18, 20, 40). These data suggest that the use of the appropriate treatment and its effectiveness are related to the different biological conditions found in the different subtypes of JIA (69).

Finally, since oligoarticular JIA is mainly considered to arise due to a dysregulated adaptive immunity, involving Th1 and Th17 effector cells and Treg cells, abatacept was used for its treatment. Abatacept is a chimeric CTLA4 and IgG Fc fusion protein, that, binding to CD80/86 molecules instead of CD28, reduces T helper cells activation. In the treatment of oligoarticolar JIA, it has been demonstrated that abatacept reduced proliferation of CD4+ T cells and their cytokines production (mainly IFN-γ and TNF-α) (21) and reduced the induction of Ig production by B cells (70).

CONCLUSION

In this review, we analyzed the role of different types of T helper cell subsets in the pathogenesis of JIA with particular attention to Th17 and Th1 phenotype (**Figure 2**). Both Th1 and Th17 cells are critical for the pathogenensis of the disease: Th1 lymphocytes through the production of pro-inflammatory cytokines IFN-γ and TNF-α; Th17 lymphocytes thanks to the shift toward the non-classic Th1 phenotype. Th1 cells subsets exert their function through inducing the expression of CD106 on SFbs, which is crucial in mediating immune cells retention in inflamed synovia. Intriguingly the TNF-α inhibitors are the main biological drugs used in JIA and interfere both with the shift of Th17 to Th1 cells and the TNF-α mediated CD106 upregulation on SFbs. All these data give an explanation at both cellular and molecular level for the efficacy of etanercept treatment in JIA and represent the beginning for further investigation with the aim to identify more specific therapeutic targets. In this view, it is important to underline that different subtypes of JIA are characterized by different inflammatory conditions, whose characterization is crucial for the choice of the efficacious biological treatment.

AUTHOR CONTRIBUTIONS

LM and LC wrote the paper. LM and AM prepared the figure. RC, FL, FA, and LC revised the manuscript.

REFERENCES

1. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International league of associations for rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* (2004) 31:390–2.
2. Ferrara G, Mastrangelo G, Barone P, La Torre F, Martino S, Pappagallo G, et al. Methotrexate in juvenile idiopathic arthritis: advice and recommendations from the MARAJIA expert consensus meeting. *Pediatr Rheumatol Online J.* (2018) 16:46. doi: 10.1186/s12969-018-0255-8
3. Murray KJ, Luyrink L, Grom AA, Passo MH, Emery H, Witte D, et al. Immunohistological characteristics of T cell infiltrates in different forms of childhood onset chronic arthritis. *J Rheumatol.* (1996) 23:2116–24.
4. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Defining the human T helper 17 cell phenotype. *Trends Immunol.* (2012) 33:505–12. doi: 10.1016/j.it.2012.05.004
5. Mosmann TR, Coffman RL. TH1 And TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* (1989) 7:145–73. doi: 10.1146/annurev.iy.07.040189.001045
6. Romagnani S. Lymphokine production by human T cells in disease states. *Annu Rev Immunol.* (1994) 12:227–57. doi: 10.1146/annurev.iy.12.040194.001303
7. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature.* (2003) 421:744–8. doi: 10.1038/nature01355
8. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* (2000) 13:715–25. doi: 10.1016/S1074-7613(00)00070-4
9. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood.* (2010) 115:335–43. doi: 10.1182/blood-2009-04-216085
10. Ouyang W, Koli JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity.* (2008) 28:454–67. doi: 10.1016/j.immuni.2008.03.004
11. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgt directs the differentiation program of proinflammatory IL-17+T helper cells. *Cell.* (2006) 126:1121–31. doi: 10.1016/j.cell.2006.07.035
12. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
13. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol.* (2007) 8:639–46. doi: 10.1038/ni1467
14. Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, et al. Human interleukin- 17-producing cells originate from a CD161+ CD4+ T-cell precursor. *J Exp Med.* (2008) 205:1903–16. doi: 10.1084/jem.20080397
15. Fouser LA, Wright JF, Dunussi-Joannopoulos K, Collins M. Th17 cytokines and their emerging roles in inflammation and autoimmunity. *Immunol Rev.* (2008) 226:87–102. doi: 10.1111/j.1600-065X.2008.00712.x
16. Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 and non-classic Th1 cells in chronic inflammatory disorders: two sides of the same coin. *Int Arch Allergy Immunol.* (2014)164:171–7 doi: 10.1159/000363502
17. Maggi L, Santarlasci V, Capone M, Rossi MC, Querci V, Mazzoni A, et al. Distinctive features of classic and nonclassic (Th17 derived) human Th1 cells. *Eur J Immunol.* (2012) 42:3180–8. doi: 10.1002/eji.201242648
18. Cosmi L, Cimaz R, Maggi L, Santarlasci V, Capone M, Borriello F, et al. Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* (2011) 63:2504–15. doi: 10.1002/art.30332
19. Ungar WJ, Costa V, Burnett HF, Feldman BM, Laxer RM. The use of biologic response modifiers in polyarticular-course juvenile idiopathic arthritis: a systematic review. *Semin Arthritis Rheum.* (2013) 42:597–618. doi: 10.1016/j.semarthrit.2012.10.006
20. Maggi L, Cimaz R, Capone M, Santarlasci V, Querci V, Simonini G, et al. Brief report: etanercept inhibits the tumor necrosis factor α-driven shift of Th17 lymphocytes toward a nonclassic Th1 phenotype in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2014) 66:1372–7. doi: 10.1002/art.38355
21. Maggi L, Cimaz R, Capone M, Santarlasci V, Rossi MC, Mazzoni A, et al. Immunosuppressive activity of abatacept on circulating T helper lymphocytes from juvenile idiopathic arthritis patients. *Int Arch Allergy Immunol.* (2016) 171:45–53 doi: 10.1159/000450948
22. Vanoni F, Minoia F, Malattia C. Biologics in juvenile idiopathic arthritis: a narrative review. *Eur J Pediatr.* (2017) 176:1147–53. doi: 10.1007/s00431-017-2960-6
23. Gregorio A, Gambini C, Gerloni V, Parafioriti A, Sormani MP, Gregorio S, et al. Lymphoid neogenesis in juvenile idiopathic arthritis correlates with ANA positivity and plasma cells infiltration. *Rheumatology.* (2007) 46:308–13. doi: 10.1093/rheumatology/kel225
24. Aggarwal A, Agarwal S, Misra R. Chemokine and chemokine receptor analysis reveals elevated interferon-inducible protein-10 (IP)-10/CXCL10 levels and increased number of CCR5+ and CXCR3+ CD4 T cells in synovial fluid of patients with enthesitis-related arthritis. *Clin Exp Immunol.* (2007) 148:515–9. doi: 10.1111/j.1365-2249.2007.03377.x
25. De Jager W, Hoppenreijs EP, Wulffraat NM. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. *Ann Rheum Dis.* (2007) 66:589–98. doi: 10.1136/ard.2006.061853
26. Mahendra A, Misra R, Aggarwal A. Th1 and Th17 Predominance in the enthesitis-related arthritis form of juvenile idiopathic arthritis. *J Rheumatol.* (2009) 36:1730–6. doi: 10.3899/jrheum.081179

27. Singh RP, Hasan S, Sharma S, Nagra S, Yamaguchi DT, Wong DT, et al. Th17 cells in inflammation and autoimmunity. *Autoimmun Rev*. (2014)13:1174–81 doi: 10.1016/j.autrev.2014.08.019
28. Maggi L, Cosmi L, Simonini G, Annunziato F, Cimaz R. T cell subpopulations in juvenile idiopathic arthritis and their modifications after biotherapies. *Autoimmun Rev*. (2016) 15:1141–4. doi: 10.1016/j.autrev.2016.09.012
29. Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum*. (2008) 58:875–87. doi: 10.1002/art.23291
30. Olivito B, Simonini G, Ciullini S, Moriondo M, Betti L, Gambineri E, et al. Th17 transcription factor RORC2 is inversely correlated with FOXP3 expression in the joints of children with juvenile idiopathic arthritis. *J Rheumatol*. (2009) 36:2017–24. doi: 10.3899/jrheum.090066
31. Wu SA, Yeh KW, Lee WI, Yao TC, Huang JL. Persistent improper upregulation of Th17 and TReg cells in patients with juvenile idiopathic arthritis. *J Microbiol Immunol Infect*. (2016) 49:402–8. doi: 10.1016/j.jmii.2014.07.002
32. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med*. (2003)198:1951–8. doi: 10.1084/jem.20030896
33. Annunziato F, Santarlasci V, Maggi L, Cosmi L, Liotta F, Romagnani S. Reasons for rarity of Th17 cells in inflammatory sites of human disorders. *Semin Immunol*. (2013) 25:299–304. doi: 10.1016/j.smim.2013.10.011
34. Santarlasci V, Maggi L, Capone M, Querci V, Beltrame L, Cavalieri D, et al. Rarity of human T helper 17 cells is due to retinoic acid orphan receptor-dependent mechanisms that limit their expansion. *Immunity*. (2012)36:201–14. doi: 10.1016/j.immuni.2011.12.013
35. Santarlasci V, Maggi L, Mazzoni A, Capone M, Querci V, Rossi MC, et al. IL-4-induced gene 1 maintains high Tob1 expression that contributes to TCR unresponsiveness in human T helper 17 cells. *Eur J Immunol*. (2014) 44:654–61. doi: 10.1002/eji.201344047
36. Santarlasci V, Mazzoni A, Capone M, Rossi MC, Maggi L, Montaini G, et al. Musculin inhibits human T helper 17 cells response to interleukin 2 by controlling STAT5B activity. *Eur J Immunol*. (2017) 47:1427–42. doi: 10.1002/eji.201746996
37. Nguyen LT, Jacobs J, Mathis D, Benoist C. Where FoxP3- dependent regulatory T cells impinge on the development of inflammatory arthritis. *Arthritis Rheum*. (2007)56:509–20. doi: 10.1002/art.22272
38. De Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4þCD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol*. (2004) 172:6435–43. doi: 10.4049/jimmunol.172.10.6435
39. Grose R, Millard D, Mavrangelos C, Barry S, Zola H, Nicholson IC, et al. Comparison of blood and synovial fluid Th17 and novel peptidase inhibitor 16 treg cell subsets in juvenile idiopathic arthritis. *J Rheumatol*. (2012)39:2021–31. doi: 10.3899/jrheum.111421
40. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci USA*. (2010) 107:14751–6. doi: 10.1073/pnas.1003852107
41. Mazzoni A, Santarlasci V, Maggi L, Capone M, Rossi MC, Querci V, et al. Demethylation of the RORC2 and IL17A in Human CD4+ T lymphocytes defines Th17 origin of nonclassic Th1 cells. *J Immunol*. (2015) 194:3116–26. doi: 10.4049/jimmunol.1401303
42. Piper C, Pesenacker AM, Bending D, Thirugnanabalan B, Varsani H, Wedderburn LR, et al. T Cell expression of granulocyte–macrophage colony-stimulating factor in juvenile arthritis is contingent upon Th17 plasticity. *Arthritis Rheumatol*. (2014) 66:1955–60. doi: 10.1002/art.38647
43. Mazzoni A, Maggi L, Siracusa F, Ramazzotti M, Rossi MC, Santarlasci V, et al. Eomes controls the development of Th17-derived (non-classic) Th1 cells during chronic inflammation. *Eur J Immunol*. (2019) 49:79–95. doi: 10.1002/eji.201847677
44. Capone M, Maggi L, Santarlasci V, Rossi MC, Mazzoni A, Montaini G, et al. Chitinase 3-like-1 is produced by human Th17 cells and correlates with the level of inflammation in juvenile idiopathic arthritis patients. *Clin Mol Allergy*. (2016) 14:16. doi: 10.1186/s12948-016-0053-0
45. Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, et al. A chitinase-like protein in the lung and circulation of patients with severe asthma. *N Engl J Med*. (2007) 357:2016–27. doi: 10.1056/NEJMoa073600
46. Vos K, Steenbakkers P, Miltenburg AM, Bos E, van Den Heuvel MW, van Hogezand RA, et al. Raised human cartilage glycoprotein-39 plasma levels in patients with rheumatoid arthritis and other inflammatory conditions. *Ann Rheum Dis*. (2000) 59:544–8. doi: 10.1136/ard.59.7.544
47. Baeten D, Steenbakkers PG, Rijnders AM, Boots AM, Veys EM, De Keyser F. Detection of major histocompatibility complex/human cartilage of MHC/HC gp-39 complexes in rheumatoid arthritis synovitis as a specific and independent histological marker. *Arthritis Rheum*. (2004)50:444–51. doi: 10.1002/art.20012
48. Marwaha AK, Leung NJ, McMurchy AN, Levings MK. TH17 cells in autoimmunity and immunodeficiency: protective or pathogenic? *Front Immunol*. (2012) 3:129. doi: 10.3389/fimmu.2012.00129
49. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature*. (2012) 484:514–8. doi: 10.1038/nature10957
50. Guiducci S, Del Rosso A, Cinelli M, Margheri F, D'Alessio S, Fibbi G, et al. Rheumatoid synovial fibroblasts constitutively express the fibrinolytic pattern of invasive tumor-like cells. *Clin Exp Rheumatol*. (2005)23:364–72.
51. Maggi L, Margheri F, Luciani C, Capone M, Rossi MC, Chillà A, et al. Th1-Induced CD106 expression mediates leukocytes adhesion on synovial fibroblasts from juvenile idiopathic arthritis patients. *PLoS ONE*. (2016) 11:e0154422. doi: 10.1371/journal.pone.0154422
52. Brescia AC, Simonds MM, McCahan SM, Fawcett PT, Rose CD. The role of transforming growth factor β signaling in fibroblast-like synoviocytes from patients with oligoarticular juvenile idiopathic arthritis: dysregulation of transforming growth factor β signaling, including overexpression of bone morphogenetic protein 4, may lead to a chondrocyte phenotype and may contribute to bony hypertrophy. *Arthritis Rheumatol*. (2014) 66:1352–62. doi: 10.1002/art.38336
53. Casnici C, Lattuada D, Tonna N, Crotta K, Storini C, Bianco F, et al. Optimized "*in vitro*" culture conditions for human rheumatoid arthritis synovial fibroblasts. *Mediators Inflamm*. (2014) 2014:702057. doi: 10.1155/2014/702057
54. Lombardi A, Cantini G, Mello T, Francalanci M, Gelmini S, Cosmi L, et al. Molecular mechanisms underlying the pro-inflammatory synergistic effect of tumor necrosis factor alpha and interferon gamma in human microvascular endothelium. *Eur J Cell Biol*. (2009) 88:731–42. doi: 10.1016/j.ejcb.2009.07.004
55. Lavocat F, Maggi L, Annunziato F, Miossec P. T-cell clones from Th1, Th17 or Th1/17 lineages and their signature cytokines have different capacity to activate endothelial cells or synoviocytes. *Cytokine*. (2016) 88:241–50. doi: 10.1016/j.cyto.2016.09.019
56. Griffin GK, Newton G, Tarrio ML, Bu DX, Maganto-Garcia E, Azcutia V, et al. IL-17 and TNFalpha sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation. *J. Immunol*. (2012) 188:6287–99. doi: 10.4049/jimmunol.1200385
57. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature*. (2008) 454:350–2. doi: 10.1038/nature07021
58. Pickens SR, Volin MV, Mandelin AM II, Kolls JK, Pope RM, Shahrara S. IL-17 contributes to angiogenesis in rheumatoid arthritis. *J Immunol*. (2010) 184:3233–41. doi: 10.4049/jimmunol.0903271
59. Gottenberg JE, Dayer JM, Lukas C, Ducot B, Chiocchia G, Cantagrel A, et al. Serum IL-6 and IL-21 are associated with markers of B cell activation and structural progression in early rheumatoid arthritis: results from the ESPOIR cohort. *Ann Rheum Dis*. (2012) 71:1243–8. doi: 10.1136/annrheumdis-2011-200975
60. Agarwal S, Misra R, Aggarwal A. Interleukin 17 levels are increased in juvenile idiopathic arthritis synovial fluid and induce synovial fibroblasts to produce proinflammatory cytokines and matrix metalloproteinases. *J Rheumatol*. (2008) 35:515–9.
61. Omoyinmi E, Hamaoui R, Pesenacker A, Nistala K, Moncrieffe H, Ursu S, et al. Th1 and Th17 cell subpopulations are enriched in the peripheral blood of patients with systemic juvenile idiopathic arthritis. *Rheumatology*. (2012) 51:1881–6. doi: 10.1093/rheumatology/kes162

62. Rosser EC, Lom H, Bending D, Duurland CL, Bajaj-Elliott M, Wedderburn LR. Brief report: innate lymphoid cells and T-cells contribute to the IL-17A signature detected in the synovial fluid of patients with Juvenile Idiopathic Arthritis. *Arthritis Rheumatol*. (2019) 71:460–7. doi: 10.1002/art.40731
63. Marotte H, Cimaz R. Etanercept - TNF receptor and IgG1 Fc fusion protein: is it different from other TNF blockers? *Expert Opin Biol Ther*. (2014) 14:569–72. doi: 10.1517/14712598.2014.896334
64. Lovell DJ, Giannini EH, Reiff A, Cawkwell GD, Silverman ED, Nocton JJ, et al. Etanercept in children with polyarticular juvenile rheumatoid arthritis. *N Engl J Med*. (2000) 342:763–9. doi: 10.1056/NEJM200003163421103
65. Hull DN, Cooksley H, Chokshi S, Williams RO, Abraham S, Taylor PC. Increase in circulating Th17 cells during anti-TNF therapy is associated with ultrasonographic improvement of synovitis in rheumatoid arthritis. *Arthritis Res Ther*. (2016) 18:303. doi: 10.1186/s13075-016-1197-5
66. Hull DN, Williams RO, Pathan E, Alzabin S, Abraham S, Taylor PC. Anti-tumour necrosis factor treatment increases circulating T helper type 17 cells similarly in different types of inflammatory arthritis. *Clin Exp Immunol*. (2015) 181:401–6. doi: 10.1111/cei.12626
67. Poddighe D, Romano M, Gattinara M, Gerloni V. Biologics for the treatment of juvenile idiopathic arthritis. *Curr Med Chem*. (2018) 25:5860–93. doi: 10.2174/0929867325666180522085716
68. Webb K, Wedderburn LR. Advances in the treatment of polyarticular juvenile idiopathic arthritis. *Curr Opin Rheumatol*. (2015) 27:505–10 doi: 10.1097/BOR.0000000000000206
69. Davies R, Gaynor D, Hyrich KL, Pain CE. Efficacy of biologic therapy across individual juvenile idiopathic arthritis subtypes: a systematic review. *Semin Arthritis Rheum*. (2017) 46:584–93. doi: 10.1016/j.semarthrit.2016.10.008
70. Scarsi M, Paolini L, Ricotta D, Pedrini A, Piantoni S, Caimi L, et al. Abatacept reduces levels of switched memory B cells, autoantibodies, and immunoglobulins in patients with rheumatoid arthritis. *J Rheumatol*. (2014) 41:666–72. doi: 10.3899/jrheum.130905

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A Path to Prediction of Outcomes in Juvenile Idiopathic Inflammatory Myopathy

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Humans have an innate desire to observe and subsequently dissect an event into component pieces in an effort to better characterize the event. We then examine these pieces individually and in combinations using this information to determine the outcome of future similar events and the likelihood of their recurrence. Practically, this attempt to foretell an occurrence and predict its outcomes is evident in multiple disciplines ranging from meteorology to sociologic studies. In this manuscript we share the historical and present-day tools to predict course and outcome in juvenile idiopathic inflammatory myopathy including clinical features, testing, and biomarkers. Further we discuss considerations for building more complex predictive models of outcome especially in diseases such as juvenile idiopathic inflammatory myopathy where patients numbers are low. Many of the barriers to developing risk prediction models for juvenile idiopathic inflammatory myopathy outcomes have improved with many remaining challenges being addressed.

Keywords: juvenile myositis/deratomyositis, outcomes, myositis, predictive model, biomarkers

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Humans have an innate desire to observe and subsequently dissect an event into component pieces in an effort to better characterize the event. We then examine these pieces individually and in combinations using this information to determine the outcome of future similar events and the likelihood of their recurrence. Practically, this attempt to foretell an occurrence and predict its outcomes is evident in multiple disciplines ranging from meteorology to sociologic studies. In medicine, the ancient Greek physicians, most notably Hippocrates and Asclepius, relied on examination and observation of patients to develop the art and science of diagnostic and prognostic medicine. The word prognosis (Greek: πρόγνωσις) translates into "knowledge beforehand," how an event is likely to conclude (1). The ability to make a prognosis is a tenet and legacy of Hippocratic medicine. The study of biological systems evolved from defining the essential character of an observation or occurrence, natural philosophy, to using empiric methods for descriptions of how they occurred, the scientific method. The evolution of scientific capabilities led to the ability to look at these systems on a microscopic and molecular level, to attempt to understand the larger entity by breaking it into the smallest component pieces, known as reductionism. Advances in medical research have paralleled the advances in biologic research. Currently, we are in an era of system level observation of cellular networks where high-throughput technologies allow further resolution of these systems and generate complex data. Multivariate statistical methods are integral to the analysis of these biologic networks and are vital tools in the effort to discover biomarkers that are predictive of disease activity, severity, and response to therapeutic interventions.

Predictive and prognostic models are statistical tools that predict a clinical outcome determined by at least 2 points of patient data and ideally more with novel statistical models which take into account change over time (2, 3). Adequate prediction of prognostic endpoints generally requires multiple prognostic factors (variables, predictors, or markers) (2). Therefore, the original dataset may contain numerous covariates including clinical and biological markers identified as potential predictors of disease characteristics (phenotype/severity, or outcomes). Development is a process that includes identification of a relevant pool of predictors, formulation of a statistical model that may employ techniques such as linear regression, logistic regression, or Bayesian models, among others. Once a model is developed, it undergoes internal and external validation (2, 3). In the context of patient care, the goal is to develop a model that predicts an accurate diagnosis based on entered data. Treatment tailored to that disease has the potential to limit morbidity associated with inappropriate therapy (4). This is important in a disease with numerous clinical phenotypes such as the Juvenile Idiopathic Inflammatory Myopathies (JIIMs). A second combination of data utilizing a different statistical model may predict disease course or prognosis. Prognostic models have advanced from basic decision rules (prediction rules) used at the bedside to aid diagnostic and clinical decision making into complex mathematical formulas developed based on large population databases (3, 5).

The JIIMs represent a rare heterogeneous group of systemic autoimmune vasculopathies characterized by variable involvement of the skin and muscle primarily, but with the potential to affect multiple organs. The most common JIIM is juvenile dermatomyositis (JDM). Other JIIMs include juvenile polymyositis (JPM), immune-mediated necrotizing myositis, and myositis associated with connective tissue disease (6). Prediction of course and prognosis in JIIM has been difficult. In 1983, Bowyer et al. stated, "It has been impossible to predict at the onset of juvenile dermatomyositis whether a child will have complete recovery..." (7) More recently, van Dijkhuizen reporting for the Juvenile Dermatomyositis Research Group (JDRG) stated, "It is currently impossible to predict the prognosis of patients with JDM" (4). Bowyer's group sought to identify factors present early in the course of disease that might determine significant morbidity looking at clinical and treatment variables. Van Dijkhuizen's group, 35 years later, reports employing a Bayesian model of disease activity utilizing four continuous outcome variables to stratify patients by disease activity and allow for more sign/symptom-specific treatment based on these variables. Logically, predictors of disease course and prognosis have historically paralleled development of capabilities to characterize and examine disease manifestations, first by description of signs and symptoms, and later by immunohistochemistry and molecular techniques (8–16).

The years just prior to and since the new millennium have seen development, validation, and revision of measures of disease activity, severity, and outcome in the JIIMs, particularly JDM. Organizations instrumental in development of these measures include the International Myositis Assessment and Clinical Studies Group (IMACS) and the pediatric Rheumatology International Trials Organization (PRINTO). These measures assess various domains of the JIIMs including global disease activity, muscle strength, physical function, and quality of life (**Table 1**) (14). Core set measures (CSMs) have been developed and validated for assessment of disease and treatment variables in JDM (16, 18, 19). CSMs are the minimum set of measures, in aggregate, that allow for adequate assessment of the disease within the various domains studied, and are required for implementation in all clinical and therapeutic trials (20, 21). In order for measures to be useful in clinical care and research, definitions of disease improvement, severity, and response to therapy need to be available. For example, response to therapy being defined as at least a 20% improvement in three of six CSMs with no more than one or two worsening (which cannot be muscle strength) had been established as preliminary response criteria employed by both PRINTO and IMACS (20).

Predictive modeling utilizing clinical and laboratory data has been employed in JDM. Van Dijkhuizen's et al. utilized data from the UK Juvenile dermatomyositis cohort and biomarker study (JDCBS) in which data were analyzed using a Bayesian model to develop a model of disease activity (21). They identified signs and symptoms that associated with four outcome parameters. These parameters measured longitudinally included creatinine kinase

TABLE 1 | Disease related measures used in predicting disease severity and outcome (17).

Domain	Measure	Grading
Disease Activity—Includes extramuscular	Physician global activity Patient/parent global Disease Activity Score (DAS); developed for JDM Myositis Disease Assessment Tool (MDAAT) –Combined tool that includes the Myositis Disease Assessment VAS (MYOACT) and Myositis Intent to Treat Activities Index (MITAX)	Visual Analog Scale (VAS) or Likert scale Visual Analog Scale (VAS) or Likert scale 10 items scored dichotomously, 3 polychotomously; also DAS skin (range 0–9) and muscle (range 0–11) scores Combined tool: VAS for each organ (MYOACT) and polychotomous response (MITAX)
Overall Health Status	Child Health Questionnaire (CHQ)	Consists of 14 health concepts
Physical Function	Childhood Health Assessment Questionnaire (CHAQ) Childhood Myositis Assessment Scale (CMAS); physical function, muscle strength, and endurance in JIIM	Questionnaire measuring degree of difficulty performing activities of daily living (ADLs); VAS for pain assessment and overall well being Observational, performance-based grading
Muscle Strength	Manual Muscle Testing 8 (MMT8)	10-point scale; 8 muscle groups
Cutaneous Involvement	Cutaneous Assessment Tool (CAT)	Scoring based on lesion characteristics: 0–2 or 0–7 depending on item
Global Damage	Physician Global Damage Myositis Damage Index (MDI)	VAS or Likert scale 11 separate VAS ratings
Laboratory Assessment	Muscle enzymes (creatinine kinase, aldolase, LDH, AST, ALT)	

(CK), childhood myositis assessment scale (CMAS), manual muscle testing 8 (MMT8), and physician global assessment (PGA). Among other associations, they discuss the association of periorbital rash with lower CMAS and higher CK values concluding this may support the opinion that ongoing skin disease reflects ongoing systemic disease activity (21). Deakin reporting for the Juvenile Dermatomyositis Research Group (JDRG) describes the use of marginal structural modeling (MSM) in determining the efficacy and safety of cyclophosphamide (CYC) treatment in severe JDM (22).

Marginal systems modeling (MSM), a statistical strategy utilizing multi-step estimation was employed by Lam et al. to adjust for baseline confounding bias to establish causal relationships using observational data between control and treatment groups in a cohort of JDM patients receiving IVIG (23). This study was significant in its application of bias-reduction methods to demonstrate the efficacy of IVIG in controlling JDM, most notably in corticosteroid-resistant patients. Deakin et al. also used MSM to determine the efficacy and safety of cyclophosphamide (CYC) in the treatment of severe JDM. The retrospective study consisted of 200 cases, 56 patients receiving, and 144 not receiving CYC (22). Descriptive analysis as well as MSM revealed improvements in three domains of disease activity (skin, muscle, and overall) in CYC-treated patients vs. those not treated. In the MSM analysis, the improvement was greatest at 12 months after the start of CYC in skin disease and global disease activity. Only minor adverse events were noted in three patients within 1 year of stopping CYC. In addition to efficacy and safety, the relative cost was lower and course of treatment was shorter using CYC as compared to biologic therapies.

Key factors for validation across universal cohorts is the ability to have comparable measures and outcomes. Work by CARRA, IMACS, PRINTO, cure JM, and JDRG all have worked to identify an international set of evaluations, measures for disease change and treatment protocols and place them into routine care of patients and not only in clinical trials (14, 16, 24–29).

In addition to clinical and laboratory measures, additional biomarkers are now being assessed as variables utilizing statistical methods to develop predictive and prognostic models.

HISTORICAL BIOMARKERS

Our ability to predict outcomes in JIIM continues to be limited by our subjective clinical assessments and our minimal and insensitive laboratory data. Exploration into biomarkers has been ongoing and continues to include cytokines, dysregulated inflammatory markers, autoantibodies, and muscle tissue markers. Individual biomarkers hold promise to be informative. However, it is more likely that these measures in concert hold a stronger association than they do individually, as demonstrated with disease modeling.

Over the past few decades, the development of new technologies, specifically high-throughput systems, has allowed identification of markers such as individual proteins, RNA immune related elements, and autoantibodies as markers of disease. Many of these same markers have been studied longitudinally and related to disease activity markers.

TRANSCRIPTIONAL ANALYSIS

RNA profiling of peripheral blood, muscle and skin biopsies in JIIM demonstrated similar patterns of the activation of the innate immune system with type 1 interferon (IFN1) induced, and the adaptive immune system with IL-17 and IL-6 pathway involvement, along with Th1 and Th2 related transcripts dysregulation (**Figure 1**) (30–33). The earliest differentially expressed transcripts reported in JIIM muscle tissue included an increase in IFN1 and Human Leucocyte Antigen (HLA) class I and II. The upregulation of HLA class I is now used in the disease diagnosis (31, 33–37). Chemokines related to both monocyte and lymphocyte immune function, such as CXCL9 [Monokine induced by gamma interferon (MIG)], CXCL10 (also known as IP-10, interferon gamma-induced proteins), and CXCL11 [Interferon-inducible T-cell alpha chemoattractant (I-TAC)], along with Calcium Binding Proteins such as S100A10, TNFSF13B (BAFF), ISG15 an ubiquitin-like modifier, are all reported dysregulated in JIIM (30, 38–40). An early study demonstrated that a cluster of IFN-regulated transcripts are upregulated in most JIIM patients and consist of classical IFN-induced genes including STAT1, SOCS1, the myxovirus resistance genes MX1 and MX2, the oligoadenylate synthetase transcripts OAS1, OAS2, and OAS3, Fcγ receptor FCGR1A, the interleukin 1 receptor antagonist (IL1RN), the pro-apoptotic TNFSF1 0/TRAIL, and another inflammatory chemokine CXCL10/IP-10 (30, 37).

Non-immune genes are also differentially expressed and include transcripts related to the oxidative pathways and mitochondrial function such as cytochrome C oxidase and NADH dehydrogenase (30, 33).

Not only have these transcripts been reported as dysregulated in JIIM, but they have also been reported to correlate with disease activity measures. Many individual transcripts were statistically significant. However, as with other disorders such as Systemic Lupus and JIIM, Baechler et al found combinations of interferon related transcripts specifically built as interferon scores appeared to be more reliable and withstand longitudinal disease variability including treatment (30, 41, 42). Further refinement of the IFN gene signature in JIIM was determined using the expression levels of 3 IFN-regulated genes (IFIT1, G1P2, and IRF7) using quantitative real-time reverse transcription-polymerase chain reaction and normalization to obtain an IFN score (41). This was later verified and used in adult myositis (40). This also led to the development of an IFN protein score with proteins found to be associated with disease activity. (MCP-2, CXCL10, and CXCL11) (25, 36).

PROTEIN MARKERS

Just as microarray technologies have allowed identification of new biomarkers, advances in proteomics have allowed identification of protein changes with disease states and

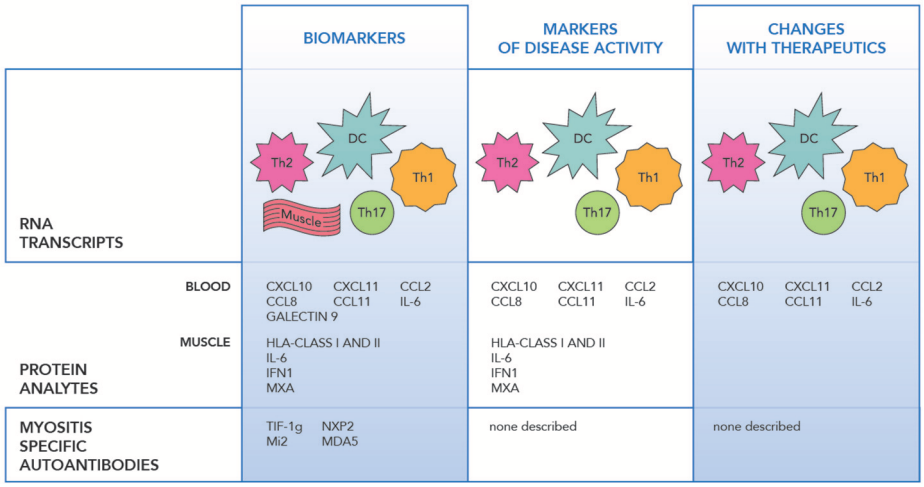

FIGURE 1 | The figure illustrates the transcript, protein and antibody biomarkers in juvenile idiopathic myopathy. RNA Transcripts include DC dendritic cells (IFNα/β, OAS1, 2), Th1 (IL-1, CXCR3, FCGR1A), Th2 (IL-4, IL-13, and GATA3), Th17 (IL-6, IL17D, IL-17F, IL-21, IL-23A, IL-27, RORC/RORγt, and IRF4), Monocytes (CXCL9, CXCL10, CXCL11), Muscle (HLA class I and II, MX1 and MX2, MxA, SGF-15, RORc, STAT3, cytochrome C oxidase, and NADH dehydrogenase), IFNβ, IRF7 (24–34).

interventions. A variety of proteins, including levels of IFN regulated proteins included anti-viral proteins, humoral and adaptive immune proteins, and chemokines are seen in the peripheral blood, in specific cell subsets and in the muscle tissue in JIIM. Specific analytes include IFN alpha and beta, IL-6, IL-17, chemokines including MCP-1/CCL2, MCP-2/CCL8, IP-10/CXCL10, I-TAC/CXCL11, IFNγ, Galectin 9, IL-1Ra, GM-CSF, and Eotaxin (**Figure 1**) (41, 43–46). Markers including those related to IFN1 upregulation along with IL-6, CCL11, MCP-1, CXCL11, and CXCL10 appear to hold the strongest correlation with disease activity in JIIM (37, 41, 43, 45, 47, 48).

Many of these proinflammatory cytokines induce or enhance the metabolic effects on muscle tissue, especially during the regeneration process (49). Cytokines such as type-1 interferon, IL-1, and TNFa are upregulated in myocytes along with the increased expression of HLA-class I. In addition, with myocyte regeneration, seen in IIM, there also is upregulation of HLA-class I along with type-1 IFN and IL-6, which could lead to further inflammation (44, 50–53).

Tissue-specific markers, such as those related to atrophic myofibers, include the ISG15-conjugation pathway proteins such as MxA, which are upregulated in active disease. Several IFN-regulated chemokines showed significant positive correlations with muscle enzymes, including MCP-1, MCP-2, and CXCL10. Baechler (30) and Bilgic (41) demonstrated that JIIM with the highest degree of disease activity had elevation of IFN-regulated proteins CXCL10, MCP-1, and MCP-2. Similar to RNA transcripts, scores using multiple transcripts and multiple protein analytes combined together created a stronger association with disease activity measures (41). The set of JIIM markers indicate the intricate matrix and interconnectedness of the innate, humoral and adaptive immune systems in autoimmunity.

AUTOANTIBODIES

Identification of a myositis specific autoantibody (MSAs) is highly suggestive of an inflammatory myositis and more recently recognized in JIIM. This has led to autoantibody relationships with clinical disease phenotypes, and possibly antibody levels themselves fluctuate with disease activity. Those more commonly seen in JIIM include anti-TIF 1(Transcriptional intermediary factor 1), NXP2 (Nuclear Matric protein 2), MDA5 (Melanoma differentiation-associated gene 5), Mi2 (Nucleosome-remodeling deacetylase complex), and less commonly anti-SAE, ASA (synthetase), SRP (Signal recognition particle and HMGCR (3-hydroy-3-methlglutaryl coenzyme A reductase (54–56).

Myositis-specific antibodies have an increasing utility as both diagnostic and prognostic biomarkers with multiple publications suggesting clinical features related to antibody specificity (54–56) (**Figure 1**). Not only are MSAs markers of the clinical phenotype, but they have also been investigated as disease activity markers. Myositis-associated antibody levels for anti-Jo-1 TIF1-γ, SRP, and -Mi-2 were investigated after B cell depletion in adult and pediatric DM and adult PM and correlated with disease activity. Anti-Jo-1 serum levels correlated with clinical improvement specifically MMT and muscle enzymes ($p = 0.007$) (17). In DM patients who had anti-CADM-140/MDA5 autoantibodies and rapidly progressive interstitial lung disease, the mean titer of anti-CADM-140/MDA5 of anti-CADM-140/MDA5 significantly decreased in the responder group compared to non-responders ($P = 0.033$) (57).

In the future, predicting disease response to treatment as well as disease- and treatment-related outcomes will require classification of myositis patients in more homologous groups than the traditional PM and DM subtypes.

Predicting Disease Outcomes in JIIM

While risk prediction models have recently become commonplace in the medical literature, there have been few attempts to develop risk prediction models in patients with JIIM. This is likely due to the rarity of the disease, as a minimum sample size of 200 patients is preferred for risk prediction models. In addition, the heterogeneity of disease activity and therapeutic strategies in patients with JIIM has made risk prediction difficult in this patient population. For example, a small observational study of 39 patients with JIIM, among whom six achieved clinical remission, found that female sex, negative Gower's sign and photosensitivity were associated with achievement of complete remission, but the small sample size precluded development of a multivariable model that could be used for prediction purposes (58).

Cooperative efforts including registries and biobanks are now making this task more feasible (59). Challa et al. recently published a model to predict changes in disease activity among children with JIIM using the Childhood Arthritis and Rheumatology Research Alliance (CARRA) Legacy Registry (60). They found that anti-nuclear antibody (ANA) positivity and use of hydroxychloroquine predicted improvement in patient/parent global health score over 6 months, and ANA positivity along with V/shawl sign predicted improvement in patient pain.

The Rituximab in Myositis (RIM) trial also provided opportunities to develop risk prediction models in patients with refractory disease. Aggarwal et al. (45) found that the presence of anti-synthetase and anti-Mi-2 autoantibodies and lower disease damage strongly predicted clinical improvement in these patients, and that the juvenile patients had better prognosis than the adults (61). Reed et al. found that biomarker signatures involving type-1 interferon regulated and other proinflammatory chemokines and cytokines in conjunction with autoantibodies predicted response to rituximab in patients with refractory myositis (45). Furthermore, Olazagasti et al. found that adding gene expression, cytokine and chemokine data to clinical and standard laboratory assessments improved prediction of response to rituximab in patients with JIIM (62).

Practical Considerations for Building Predictive Models in JIIM

In addition to the importance of a sufficient sample size, there are several other important considerations for building risk prediction models. First, the study design and population of interest should be considered. As mentioned above, registries are making risk prediction in JIIM more feasible. While registries are observational studies, which may suffer from confounding, they can be used to predict outcomes from baseline characteristics. However, confounding by indication is common, as patients with more severe disease often require more therapies and interventions. This confounding can make it difficult to assess the impact of treatment on the outcome of interest. Alternatives to address confounding my indication are causal inference analysis methods (e.g., MSM) or randomized clinical trials. By randomizing patients to receive a treatment, confounding by indication is eliminated. However, clinical trials also have limitations because they often have strict inclusion criteria. Thus, patients enrolled in clinical trials may not represent the population of interest, as some of the patients for whom the risk prediction model could be useful were excluded from the trial.

Risk factor selection is another important consideration when developing a risk prediction model. The existence and assessment of relevant disease activity measures and biomarkers, such as those mentioned previously in this review, is critical. Without reliable disease activity measures, it is not possible to build models that will predict disease-related outcomes. In addition, causality is important, because when factors included in a risk prediction model improve, it is assumed that the risk of the outcome will be reduced. However, proving causality is difficult and requires causal inference analysis methods or a randomized clinical trial. Finally, the practicality and cost of each potential risk factor needs to be considered. Measurements that are financially costly or those that require a great deal of effort to assess, such as cumulative measures, provide challenges for implementing a risk prediction model in clinical practice.

The choice of model is also an important consideration. Historically, logistic regression models for cross-sectional data and Cox regression models for longitudinal time-to-event data were commonly used for risk prediction. These models had the advantage of providing an easily understandable equation for predicting the risk of an outcome. However, they were often overly simplistic and only considered linear effects of the continuous risk factors. More recent advances in statistical methods, which evolved from computer science methodology, including machine-learning techniques, have demonstrated improved performance for risk prediction models resulting from complex algorithms that do not provide simple risk calculation formulas. Computing advances have made it much easier to implement these complex algorithms into clinical practice using web-based tools, so simple formulas are no longer required and has opened the possibility of novel algorithms to apply to complex biomedical datasets.

Finally, once the risk prediction model has been developed, it is important to objectively assess its performance. Model performance assessment should include both discrimination and calibration. Discrimination is the ability to correctly rank patients from low to high risk. Calibration is the ability to accurately predict the absolute risk level. There is a wealth of literature on how to assess model performance for different types of models, which is beyond the scope of this review.

Risk prediction models should be validated prior to use in clinical decision-making. External validation in a separate dataset is preferred. However, internal validation based on subdividing the study patients into training and test dataset is also common. Szodoray et al. randomly subdivided their patients into 50 cases and 50 controls for training and 29 cases and 20 controls for testing (44). Analyzing multiplex cytokine assays using principal components, hierarchical clustering and discriminant function analyses, they identified unique immune profiles that seem to perpetuate autoimmune processed in patients with JIIM and may be able to identify disease subsets. Many of the modern modeling techniques use cross-validation methods to avoid

over-optimism or over-fitting of a risk prediction model. None of the previously mentioned risk prediction models for JIIM have been validated due to study sample sizes that were too small to subdivide and a lack of available external data sources for validation.

In conclusion, while many of the barriers to developing risk prediction models for JIIM outcomes have improved, there are still many remaining challenges. The availability of registries to provide larger sample sizes and newer biomarkers that can predict outcomes has reduced the challenges, but careful planning will still be required to navigate these challenges and ultimately develop useful risk prediction models for patients with JIIM. However, there continues to be a major need to compare across data sets and cohorts that require continued standardization and validation across cohorts and centers.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Kleisiaris CF, Sfakianakis C, Papathanasiou IV. Health care practices in ancient Greece: the hippocratic ideal. *J Med Ethics Hist Med*. (2014) 7:6.
2. Steyerberg EW, Moons KG, van der Windt DA, Hayden JA, Perel P, Schroter S, et al. Prognosis Research Strategy (PROGRESS) 3: prognostic model research. *PLoS Med*. (2009) 10:e1001381. doi: 10.1371/journal.pmed.1001381
3. Vogenberg FR. Predictive and prognostic models: implications for healthcare decision-making in a modern recession. *Am Health Drug Benefits*. (2009) 2:218–22.
4. van Dijkhuizen EP, Deakin CT, Wedderburn LR, De Iorio M. Modelling disease activity in juvenile dermatomyositis: a Bayesian approach. *Stat Methods Med Res*. (2019) 28:35–49. doi: 10.1177/0962280217713233
5. Stiell IG, Wells GA. Methodologic standards for the development of clinical decision rules in emergency medicine. *Ann Emerg Med*. (1998) 5:437–47.
6. Huber AM. Juvenile idiopathic inflammatory myopathies. *Pediatr Clin North Am*. (2018) 65:739–56. doi: 10.1016/j.pcl.2018.04.006
7. Bowyer SL, Blane CE, Sullivan DB, Cassidy JT. Childhood dermatomyositis: factors predicting functional outcome and development of dystrophic calcification. *J Pediatrics*. (1983) 103:882–8. doi: 10.1016/S0022-3476(83)80706-9
8. Rider LG, Gurley RC, Pandey JP, Garcia de la Torre I, Kalovidouris AE, O'Hanlon TP, et al. Clinical, serologic, and immunogenetic features of familial idiopathic inflammatory myopathy. *Arthritis Rheum*. (1998) 41:710–9. doi: 10.1002/1529-0131(199804)41:4<710::AID-ART19>3.0.CO;2-K
9. Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies. I: quantitation of subsets according to diagnosis and sites of accumulation and demonstration and counts of muscle fibers invaded by T cells. *Ann Neurol*. (1984) 16:193–208. doi: 10.1002/ana.410160206
10. Lovell DJ, Lindsley CB, Rennebohm RM, Ballinger SH, Bowyer SL, Giannini EH, et al. Development of validated disease activity and damage indices for the juvenile idiopathic inflammatory myopathies. II. The Childhood Myositis Assessment Scale (CMAS): a quantitative tool for the evaluation of muscle function. The Juvenile Dermatomyositis Disease Activity Collaborative Study Group. *Arthritis Rheum*. (1999) 42:2213–9. doi: 10.1002/1529-0131(199910)42:10<2213::AID-ANR25>3.0.CO;2-8
11. Huber AM, Hicks JE, Lachenbruch PA, Perez MD, Zemel LS, Rennebohm RM, et al. Validation of the Childhood Health Assessment Questionnaire in the juvenile idiopathic myopathies. Juvenile Dermatomyositis Disease Activity Collaborative Study Group. *J Rheumatol*. (2001) 28:1106–11.
12. Rider LG, Feldman BM, Perez MD, Rennebohm RM, Lindsley CB, Zemel LS, et al. Development of validated disease activity and damage indices for the juvenile idiopathic inflammatory myopathies: I. Physician, parent, and patient global assessments. Juvenile Dermatomyositis Disease Activity Collaborative Study Group. *Arthritis Rheum*. (1997) 40:1976–83. doi: 10.1002/art.1780401109
13. Rider LG, Koziol D, Giannini EH, Jain MS, Smith MR, Whitney-Mahoney K, et al. Validation of manual muscle testing and a subset of eight muscles for adult and juvenile idiopathic inflammatory myopathies. *Arthritis Care Res*. (2010) 62:465–72. doi: 10.1002/acr.20035
14. Rider LG, Werth VP, Huber AM, Alexanderson H, Rao AP, Ruperto N, et al. Measures of adult and juvenile dermatomyositis, polymyositis, and inclusion body myositis. *Arthritis Care Res*. (2011) 63(S11):S118–57. doi: 10.1002/acr.20532
15. Varnier GC, Rosina S, Ferrari C, Pistorio A, Consolaro A, Bovis F, et al. Development and testing of a hybrid measure of muscle strength in juvenile dermatomyositis in routine care. *Arthritis Care Res*. (2018) 70:1312–9. doi: 10.1002/acr.23491
16. McCann LJ, Pilkington CA, Huber AM, Ravelli A, Appelbe D, Kirkham JJ, et al. Development of a consensus core dataset in juvenile dermatomyositis for clinical use to inform research. *Ann Rheum Dis*. (2018) 77:241–50. doi: 10.1136/annrheumdis-2017-212141
17. Aggarwal R, Oddis CV, Goudeau D, Koontz D, Qi Z, Reed AM, et al. Autoantibody levels in myositis patients correlate with clinical response during B cell depletion with rituximab. *Rheumatology*. (2016) 55:1710. doi: 10.1093/rheumatology/kew275
18. McCann LJ, Kirkham JJ, Wedderburn LR, Pilkington C, Huber AM, Ravelli A, et al. Development of an internationally agreed minimal dataset for juvenile dermatomyositis (JDM) for clinical and research use. *Trials*. (2015) 16:268. doi: 10.1186/s13063-015-0784-0
19. Ruperto N, Ravelli A, Murray KJ, Lovell DJ, Andersson-Gare B, Feldman BM, et al. Preliminary core sets of measures for disease activity and damage assessment in juvenile systemic lupus erythematosus and juvenile dermatomyositis. *Rheumatology*. (2003) 42:1452–9. doi: 10.1093/rheumatology/keg403
20. Rider LG, Aggarwal R, Pistorio A, Bayat N, Erman B, Feldman BM, et al. 2016 American College of Rheumatology/European League against rheumatism criteria for minimal, moderate, and major clinical response in juvenile dermatomyositis: an International Myositis Assessment and Clinical Studies Group/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. *Arthritis Rheumatol*. (2017) 69:911–23. doi: 10.1002/art.40060
21. van Dijkhuizen EHP, De Iorio M, Wedderburn LR, Deakin CT, JDRG. Clinical signs and symptoms in a joint model of four disease activity parameters in juvenile dermatomyositis: a prospective, longitudinal, multicenter cohort study. *Arthritis Res Ther*. (2018) 20:180. doi: 10.1186/s13075-018-1687-8
22. Deakin CT, Campanilho-Marques R, Simou S, Moraitis E, Wedderburn LR, Pullenayegum E, et al. Efficacy and safety of cyclophosphamide treatment in severe juvenile dermatomyositis shown by marginal structural modeling. *Arthritis Rheumatol*. (2018) 70:785–93. doi: 10.1002/art.40418
23. Lam CG, Manlhiot C, Pullenayegum EM, Feldman BM. Eficacy of Ig therapy in juvenile dermatomyositis. *Ann Rheum Dis*. (2011) 70:2089–94. doi: 10.1136/ard.2011.153718
24. Stringer E, Bohnsack J, Bowyer SL, Griffin TA, Huber AM, Lang B, et al. Treatment approaches to juvenile dermatomyositis (JDM) across North America: the Childhood Arthritis and Rheumatology Research Alliance (CARRA) JDM Treatment Survey. *J Rheumatol*. (2010) 37:1953–61. doi: 10.3899/jrheum.090953
25. Huber AM, Lang B, LeBlanc CM, Birdi N, Bolaria RK, Malleson P, et al. Medium- and long-term functional outcomes in a multicenter cohort of children with juvenile dermatomyositis. *Arthritis Rheum*. (2000) 43:541–9. doi: 10.1002/1529-0131(200003)43:3<541::AID-ANR9>3.0.CO;2-T
26. Huber AM, Robinson AB, Reed AM, Abramson L, Bout-Tabaku S, Carrasco R, et al. Consensus treatments for moderate juvenile dermatomyositis:

beyond the first two months. Results of the second Childhood Arthritis and Rheumatology Research Alliance consensus conference. *Arthritis Care Res.* (2012) 64:546–53. doi: 10.1002/acr.20695
27. Huber AM, Kim S, Reed AM, Carrasco R, Feldman BM, Hong SD, et al. Childhood Arthritis and Rheumatology Research Alliance Consensus Clinical Treatment Plans for Juvenile Dermatomyositis with Persistent Skin Rash. *J Rheumatol.* (2017) 44:110–6. doi: 10.3899/jrheum.160688
28. Kim S, Kahn P, Robinson AB, Lang B, Shulman A, Oberle EJ, et al. Childhood Arthritis and Rheumatology Research Alliance consensus clinical treatment plans for juvenile dermatomyositis with skin predominant disease. *Pediatr Rheumatol Online J.* (2017) 15:1. doi: 10.1186/s12969-016-0134-0
29. Isenberg DA, Allen E, Farewell V, Ehrenstein MR, Hanna MG, Lundberg IE, et al. International consensus outcome measures for patients with idiopathic inflammatory myopathies. Development and initial validation of myositis activity and damage indices in patients with adult onset disease. *Rheumatology.* (2004) 43:49–54. doi: 10.1093/rheumatology/keg427
30. Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. *Mol Med.* (2007) 13:59–68. doi: 10.2119/2006-00085.Baechler
31. Liao AP, Salajegheh M, Nazareno R, Kagan JC, Jubin RG, Greenberg SA. Interferon beta is associated with type 1 interferon-inducible gene expression in dermatomyositis. *Ann Rheum Dis.* (2011) 70:831–6. doi: 10.1136/ard.2010.139949
32. López De Padilla CM, Crowson CS, Hein MS, Pendegraft RS, Strausbauch MA, Niewold TB, et al. Gene expression profiling in blood and affected muscle tissues reveals differential activation pathways in patients with new-onset juvenile and adult dermatomyositis. *J Rheumatol.* (2017) 44:117–24. doi: 10.3899/jrheum.160293
33. Tezak Z, Hoffman EP, Lutz JL, Fedczyna TO, Stephan D, Bremer EG, et al. Gene expression profiling in DQA1*0501+ children with untreated dermatomyositis: a novel model of pathogenesis. *J Immunol.* (2002) 168:4154–63. doi: 10.4049/jimmunol.168.8.4154
34. Walsh RJ, Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL, et al. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. *Arthritis Rheum.* (2007) 56:3784–92. doi: 10.1002/art.22928
35. Chen YW, Shi R, Geraci N, Shrestha S, Gordish-Dressman H, Pachman LM. Duration of chronic inflammation alters gene expression in muscle from untreated girls with juvenile dermatomyositis. *BMC Immunol.* (2008) 9:43. doi: 10.1186/1471-2172-9-43
36. Greenberg SA, Bradshaw EM, Pinkus JL, Pinkus GS, Burleson T, Due B, et al. Plasma cells in muscle in inclusion body myositis and polymyositis. *Neurology.* (2005) 65:1782–7. doi: 10.1212/01.wnl.0000187124.92826.20
37. Greenberg SA, Higgs BW, Morehouse C, Walsh RJ, Kong SW, Brohawn P, et al. Relationship between disease activity and type 1 interferon- and other cytokine-inducible gene expression in blood in dermatomyositis and polymyositis. *Genes Immun.* (2012) 13:207–13. doi: 10.1038/gene.2011.61
38. Suárez-Calvet X, Gallardo E, Nogales-Gadea G, Querol L, Navas M, Díaz-Manera J, et al. Altered RIG-I/DDX58-mediated innate immunity in dermatomyositis. *J Pathol.* (2014) 233:258–68. doi: 10.1002/path.4346
39. Zhou X, Dimachkie MM, Xiong M, Tan FK, Arnett FC. cDNA microarrays reveal distinct gene expression clusters in idiopathic inflammatory myopathies. *Med Sci Monit.* (2004) 10:BR191–7.
40. Salajegheh M, Kong SW, Pinkus JL, Walsh RJ, Liao A, Nazareno R, et al. Interferon-stimulated gene 15 (ISG15) conjugates proteins in dermatomyositis muscle with perifascicular atrophy. *Ann Neurol.* (2010) 67:53–63. doi: 10.1002/ana.21805
41. Bilgic H, Ytterberg SR, Amin S, McNallan KT, Wilson JC, Koeuth T, et al. Interleukin-6 and type I interferon-regulated genes and chemokines mark disease activity in dermatomyositis. *Arthritis Rheum.* (2009) 60:3436–46. doi: 10.1002/art.24936
42. Petri M, Singh S, Tesfasyone H, Dedrick R, Fry K, Lal P, et al. Longitudinal expression of type I interferon responsive genes in systemic lupus erythematosus. *Lupus.* (2009) 18:980–9. doi: 10.1177/0961203309105529
43. Sanner H, Schwartz T, Flatø B, Vistnes M, Christensen G, Sjaastad I. Increased levels of eotaxin and MCP-1 in juvenile dermatomyositis median 16.8 years after disease onset; associations with disease activity, duration and organ damage. *PLoS ONE.* (2014) 9:e92171. doi: 10.1371/journal.pone.0092171
44. Szodoray P, Alex P, Knowlton N, Centola M, Dozmorov I, Csipo I, et al. Idiopathic inflammatory myopathies, signified by distinctive peripheral cytokines, chemokines and the TNF family members B-cell activating factor and a proliferation inducing ligand. *Rheumatology.* (2010) 49:1867–77. doi: 10.1093/rheumatology/keq151
45. Reed AM, Crowson CS, Hein M, de Padilla CL, Olazagasti JM, Aggarwal R, et al. Biologic predictors of clinical improvement in rituximab-treated refractory myositis. *BMC Musculoskelet Disord.* (2015) 16:257. doi: 10.1186/s12891-015-0710-3
46. Bellutti Enders F, van Wijk F, Scholman R, Hofer M, Prakken BJ, van Royen-Kerkhof A, et al. Correlation of CXCL10, tumor necrosis factor receptor type II, and galectin 9 with disease activity in juvenile dermatomyositis. *Arthritis Rheumatol.* (2014) 66:2281–9. doi: 10.1002/art.38676
47. Gao S, Luo H, Zhang H, Zuo X, Wang L, Zhu H. Using multi-omics methods to understand dermatomyositis/polymyositis. *Autoimmun Rev.* (2017) 16:1044–8. doi: 10.1016/j.autrev.2017.07.021
48. López De Padilla CM, Crowson CS, Hein MS, Strausbauch MA, Aggarwal R, Levesque MC, et al. Interferon-regulated chemokine score associated with improvement in disease activity in refractory myositis patients treated with rituximab. *Clin Exp Rheumatol.* (2015) 33:655–63.
49. Nagaraju K. Role of major histocompatibility complex class I molecules in autoimmune myositis. *Curr Opin Rheumatol.* (2005) 17:725–30. doi: 10.1097/01.bor.0000179947.58271.9a
50. Malm C, Nyberg P, Engstrom M, Sjodin B, Lenkei R, Ekblom B, et al. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *J Physiol.* (2000) 529(Pt 1):243–62. doi: 10.1111/j.1469-7793.2000.00243.x
51. Tournadre A, Miossec P. A critical role for immature muscle precursors in myositis. *Nat Rev Rheumatol.* (2013) 9:438–42. doi: 10.1038/nrrheum.2013.26
52. Kuru S, Inukai A, Kato T, Liang Y, Kimura S, Sobue G. Expression of tumor necrosis factor-alpha in regenerating muscle fibers in inflammatory and non-inflammatory myopathies. *Acta Neuropathol.* (2003) 105:217–24. doi: 10.1007/s00401-002-0635-4
53. Reed A, Hein MS, Colbert RA. UPR activation and IFNb production in myocytes in adult and juvenile myositis. *Arthritis Rheum.* (2010) 62(S10):1376.
54. Betteridge ZE, Gunawardena H, McHugh NJ. Novel autoantibodies and clinical phenotypes in adult and juvenile myositis. *Arthritis Res Ther.* (2011) 13:209. doi: 10.1186/ar3275
55. Betteridge Z, McHugh N. Myositis-specific autoantibodies: an important tool to support diagnosis of myositis. *J Intern Med.* (2016) 280:8–23. doi: 10.1111/joim.12451
56. Tansley SL, Simou S, Shaddick G, Betteridge ZE, Almeida B, Gunawardena H, et al. Autoantibodies in juvenile-onset myositis: their diagnostic value and associated clinical phenotype in a large UK cohort. *J Autoimmun.* (2017) 84:55–64. doi: 10.1016/j.jaut.2017.06.007
57. Sato S, Kuwana M, Fujita T, Suzuki Y. Anti-CADM-140/MDA5 autoantibody titer correlates with disease activity and predicts disease outcome in patients with dermatomyositis and rapidly progressive interstitial lung disease. *Mod Rheumatol.* (2013) 23:496–502. doi: 10.3109/s10165-012-0663-4
58. Sun C, Lee JH, Yang YH, Yu HH, Wang LC, Lin YT, et al. Juvenile dermatomyositis: a 20-year retrospective analysis of treatment and clinical outcomes. *Pediatr Neonatol.* (2015) 56:31–9. doi: 10.1016/j.pedneo.2014.02.006
59. Wu Q, Wedderburn LR, McCann LJ. Juvenile dermatomyositis: latest advances. *Best Pract Res Clin Rheumatol.* (2017) 31:535–57. doi: 10.1016/j.berh.2017.12.003
60. Challa D, Crowson CS, Niewold TB, Reed AM, CARRA Legacy Registry Investigators. Predictors of changes in disease activity among children with juvenile dermatomyositis enrolled in the Childhood Arthritis and Rheumatology Research Alliance (CARRA) Legacy Registry. *Clin Rheumatol.* (2018) 37:1011–5. doi: 10.1007/s10067-017-3901-5

61. Aggarwal R, Bandos A, Reed AM, Ascherman DP, Barohn RJ, Feldman BM, et al. Predictors of clinical improvement in rituximab-treated refractory adult and juvenile dermatomyositis and adult polymyositis. *Arthritis Rheumatol.* (2014) 66:740–9. doi: 10.1002/art.38270
62. Olazagasti J, Crowson C, Hein M, de Padilla C, Aggarwal R, Oddis C, et al. Predictive models of clinical improvement in rituximab-treated myositis patients using clinical features, autoantibodies, and biomarkers. predictive models of clinical improvement in rituximab-treated myositis patients using clinical features, autoantibodies, and biomarkers. *Open J Rheumatol Autoimmune Dis*. (2015) 5:68–80. doi: 10.4236/ojra.2015.53012

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Immunopathogenesis of Pediatric Localized Scleroderma

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Localized scleroderma (LS) is a complex disease characterized by a mixture of inflammation and fibrosis of the skin that, especially in the pediatric population, also affects extracutaneous tissues ranging from muscle to the central nervous system. Although developmental origins have been hypothesized, evidence points to LS as a systemic autoimmune disorder, as there is a strong correlation to family history of autoimmune disease, the presence of shared HLA types with rheumatoid arthritis, high frequency of auto-antibodies, and elevated circulating chemokines and cytokines associated with T-helper cell, IFNγ, and other inflammatory pathways. This inflammatory phenotype of the peripheral blood is reflected in the skin via microarray, RNA Sequencing and tissue staining. Research is underway to identify the key players in the pathogenesis of LS, but close approximation of inflammatory lymphocytic and macrophage infiltrate with collagen and fibroblasts deposition supports the notion that LS is a disease of inflammatory driven fibrosis. The immune system is dynamic and undergoes changes during childhood, and we speculate on how the unique features of the immune system in childhood could potentially contribute to some of the differences in LS between children and adults. Interestingly, the immune phenotype in pediatric LS resembles to some extent the healthy adult cellular phenotype, possibly supporting accelerated maturation of the immune system in LS. We discuss future directions in better understanding the pathophysiology of and how to better treat pediatric LS.

Keywords: localized scleroderma, morphea, pediatric rheumatology, immunophenotype, disease etiology, autoimmune disease, skin, fibrosis

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DISEASE MANIFESTATIONS AND UNIQUE FEATURES OF LOCALIZED SCLERODERMA (LS) IN CHILDHOOD

Localized scleroderma (LS) is the most common form of pediatric scleroderma, a disease whose histologic pathology involves inflammation and fibrosis, similar to that of systemic sclerosis (SSc), although the clinical phenotypes are markedly different. Overall, the annual incidence in the US of LS collectively in adults and children is slighter higher than for systemic sclerosis (SSc) (2.7 vs. 1.9

per 100,000, respectively) (1, 2). A much larger difference is found for childhood onset of these diseases, however, with 34% of LS beginning in childhood (by age 18 years), compared to <<5% of SSc (by age 16 years) (2, 3).

The mean age of pediatric LS disease onset is 6.4–8.7 years, with the disease more prevalent in Caucasians (4). As is true for other autoimmune disease, females are more commonly affected than males (2.3 4:1 ratio) (4). This female preponderance is consistent over different ethnicities, as clinical centers with more homogeneous racial populations, such as Mexico (5), have reported similar ratios of female:male patients.

LS can present in several different patterns (subtypes) depending on depth and distribution of lesions, including circumscribed morphea (plaque lesions), linear scleroderma of the trunk/limb or head (band-like lesion), generalized morphea (multiple plaque lesions), pansclerotic morphea, or a combination of two or more of these subtypes (mixed morphea) (6). Besides the obvious difference in age of disease onset, there are several major differences between pediatric and adult onset disease. These include a different subtype predominance, higher frequency of deep tissue and extracutaneous involvement in pediatric disease, and longer disease duration in pediatric disease [reviewed in (4)]. These differences contribute to the higher frequency of serious morbidity in patients with pediatric compared to adult onset disease (4, 7), with morbidity including arthropathy, uveitis, facial hemiatrophy, seizures, and neuropathy (7, 8). Functional impairment has been reported in 30–38% of juvenile LS patients (9–11).

Twenty to 70% of juvenile LS patients have been reported to have extracutaneous involvement, with higher frequencies reported in prospective studies (7, 8, 11–15). The most common type of extracutaneous involvement is musculoskeletal, which includes joint, tendon, muscle, and bone issues. Joint and tendon issues include arthralgia, arthritis, joint contractures, and angulation defects, some of which require corrective surgeries (16). Muscle involvement includes myalgia, myositis, and muscle atrophy (8). Because the disease commonly begins before most children have undergone their major growth spurt, children are at risk for undergrowth of the affected side, which can lead to limited physical function, pain, and major disfigurement. Growth impairment is common, with studies reporting facial hemiatrophy in half the patients with linear scleroderma of the head, deformity from tissue atrophy and/or muscle bulk reduction in half, and a bone length difference in 15–18% of patients (15–17).

Central nervous system (CNS) involvement is a less common but notable extracutaneous manifestation. The overall frequency of CNS involvement in juvenile LS is ∼5% but in patients with linear scleroderma of the head (LSh), it ranges between 44% (18) and 50% (19). Seizures, in particular, partial complex seizures, are the most common neurological symptom, followed by headache, hemiparesis, cranial nerve palsy, optic neuritis; and less commonly, neuropsychiatric disorders, deterioration of intelligence, and/or ischemic stroke (8). Temporal relationship between onset of the neurological symptoms and skin lesions is variable, with the majority of the patients having preceding scalp and facial lesions before CNS presentation, though approximately one-quarter of the cases can present with neurological manifestations (20) (21). Radiological and cerebrospinal fluid (CSF) laboratory findings in LSh patients further support that the disease affects the CNS, likely in an autoimmune manner. When brain imaging is performed in symptomatic patients, abnormalities, such as cortical and subcortical white matter lesions, atrophy, and calcinosis are common, with 34 of the 54 reported patients in one review (63%) found to have multiple or diffuse brain lesions on magnetic resonance imaging (MRI) (20). These brain lesions seem to be more epileptogenic than other autoimmune diseases, such as multiple sclerosis (20). Furthermore, analysis of CSF obtained via lumbar puncture reveals findings consistent with an inflammatory process in some LSh patients demonstrating oligoclonal bands, elevated IgG levels, and autoantibodies (22–24). Further evidence supporting CNS inflammation includes histologic findings of LSh brain biopsies, which demonstrate the same changes as seen in skin: chronic perivascular lymphocytic inflammation with some vessels showing intimal thickening and hyalinization (25).

POTENTIAL PATHOGENIC ETIOLOGIES

Genetics

Familial history of disease, either immediate or remote, is common for those with autoimmune diseases. For LS patients, 10–30% of patients reported having a family history of autoimmune disease, such as lupus and arthritis (26–30). Ten percent of LS patients have concurrent autoimmune diseases; in children, the most commonly identified diseases are vitiligo, alopecia areata, and juvenile rheumatoid arthritis (26).

Few studies have examined HLA associations in LS. The largest to date was performed using participants from the Morphea in Adults and Children Cohort, which includes about 1/3 childhood-onset LS. In this case control study, HLA Class II genotyping and SSCP typing of HLA A, B, and C alleles was performed and associations between HLA-Class I and II alleles and LS as well as its subphenotypes was determined. Notably, there was only one common allele with adult SSc, DRB*04:04, implying LS and SSc are immunogenetically distinct. In contrast, the strongest associations were with DRB1*04:04 and HLA-B*37 (31). DRB1*04:04 is also strongly associated with risk for rheumatoid arthritis. Interestingly, population based studies examining the autoimmune profile of RA have identified increased risk (SIR) of LS in patients with RA. Conversely, studies have indicated there may be increased risk of RA in LS patients. Taken together, this implies that there may be common genetic susceptibility in LS and RA as well as other autoimmune disorders (27, 32). The strong association of LS with specific class-I HLA alleles supports the role of CD8 or natural killer cell associated immune responses in the pathogenesis of LS and implicate loss of tolerance to an unknown self-antigen (31).

Developmental Etiology

Several studies have confirmed that localized scleroderma follows the distribution pattern of Blaschko lines, invisible patterns in the skin, distinct from dermatomes, which is a

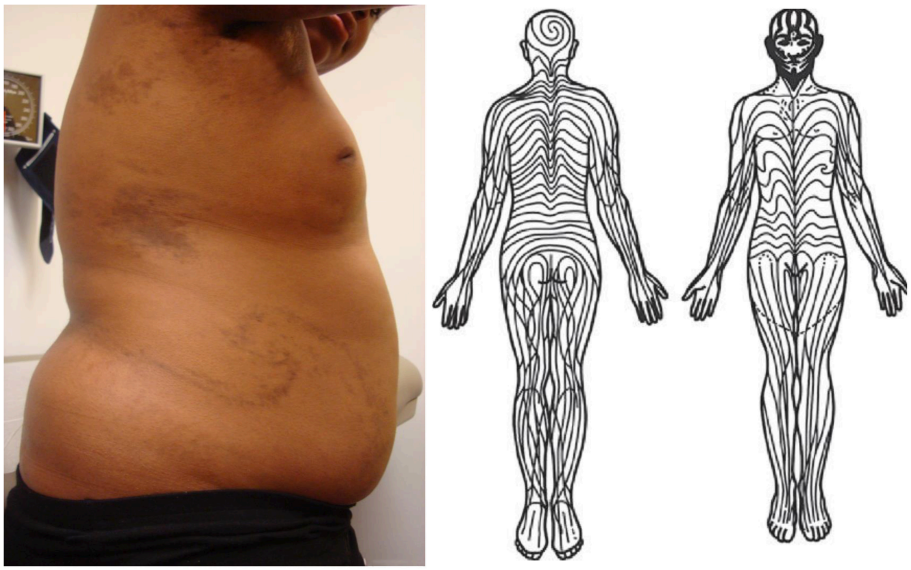

FIGURE 1 | (Left) Swirling lines of Blaschko on patient's right trunk with pediatric-onset localized scleroderma. Note several other patches of morphea on trunk and axilla. She also had linear bands of fibrosis traveling down posterior aspects of bilateral arms and right greater than left leg following lines of Blaschko. Written informed consent was obtained for the clinical photograph. (Right) Diagram of lines of Blaschko. Without modification from Tenea (33).

normal embryonic pattern (**Figure 1**) (34, 35). This includes a retrospective chart review of 65 children with linear scleroderma, plotting skin lesions on standardized head and body charts and then comparing these clinical diagrams to a computer-assisted comparison, which demonstrated excellent correlation (35). Some dermatologic diseases which distribute along lines are believed to be evidence of genomic mosaicism, caused by a somatic mutation during embryogenesis that gives rise to an individual with two genetically distinct populations of cells (36). Whole exome sequencing has been used to prove that affected tissue from other conditions that follow Blaschko lines, including some types of epidermal nevi, contains somatic mutations not present in germline tissue from the same patient (37). Though these findings suggest that localized scleroderma is a form of cutaneous mosaicism, this theory has not yet been confirmed on a molecular basis.

A neuroectodermal origin has been postulated for CNS disease in juvenile LS. Given that face and brain parenchyma tissues derive from a common progenitor in the ectodermal site of the neural tube, an early mutation in the rostral area might cause both cerebral dysgenesis and progressive facial hemiatrophy (38). In support of this hypothesis are the coexistence of ipsilateral cutaneous and neurological lesions and reports of Sturg-Weber syndrome-like intracranial lesions and multiple hamartomas in patients with linear scleroderma of the face (39, 40).

Immune Etiology

LS as an Autoimmune Disease

Takehara and colleagues were among the first to recognize and publish the summary of immunologic findings to support localized scleroderma as an autoimmune disorder with serological supportive evidence being the presence of autoantibodies (autoAb), elevated circulating cytokines, their soluble receptors and soluble cell adhesion molecules (41). Further, examination of the histopathology of LS lesions shows close approximation of immune cell infiltrates with sclerosis in inflammatory lesions, further supporting the interplay between immune dysregulation and increased extracellular matrix components including type I collagen in LS (42). Specifically in regards to LSh, oligoclonal bands and elevated IgG in the CSF have been documented, further supporting an autoimmune phenomenon. Additionally, clinical response of skin, musculoskeletal, and neurological manifestation of LS to immunosuppressive agents, such as corticosteroids and methotrexate, further provides support for the autoimmune hypothesis (43–46). Here, we provide an updated summary of the literature that builds upon this foundation, summarize immune signature grouping or speculations of findings, as well as future directions to give a better understanding of the LS immunophenotype.

Histopathologic Evidence for Immune Involvement

In a cross-sectional study of 83 patients with localized scleroderma in which 101 biopsy specimens were examined the authors found that the microanatomical location and degree of inflammation and sclerosis were associated with the stage of evolution of the lesion as well as clinical disease manifestations. The authors categorized sclerosis patterns as top heavy (confined to the papillary dermis), bottom heavy (confined to the reticular dermis and beyond), and throughout (extending from the papillary dermis to subcutis and beyond). They also quantified the degree and location of inflammation and cell types present. Important observations included that all patterns of sclerosis (top, bottom, and throughout) were present in circumscribed, generalized, and linear subtypes. Interestingly, regardless of subtype the bottom heavy or throughout pattern of sclerosis

TABLE 1 | Peripheral Blood Cytokine Profiles in LS: Elevated cytokines associate with T helper cell linages and correlate with other inflammatory disease indicators, such as activity scores and clinical laboratory tests.

		Patient (n) and LS, SSc	Subtype	Ages median (IQR) mean ± SD	Sex (% female)	Clinical and serological correlations
CCL2/MCP-1	Torok et al. (66)†	69 Ped LS	8 generalized 41 linear 8 plaque morphea	13.0 (10.0–16.0)	67%	–
CCL3/MIP-1a	O'Brien et al. (67)†	87 LS	49 generalized 28 linear 8 plaque morphea	50 ± 20 years (89% adult)	69%	–
CXCL8/IL−8	Ihn et al. (68)	48 LS 20 SSc	16 generalized 22 linear 10 plaque morphea	–	–	–
IL-2	Ihn et al. (69)	48 LS 20 SSc	16 generalized 22 linear 10 plaque morphea	–	–	RF
IL−2R	O'Brien et al. (67)†	87 LS	49 generalized 28 linear 8 plaque morphea	50 ± 20 years (89% adult)	69%	LoSDI
IL−4	Ihn et al. (69)	48 LS 20 SSc	16 generalized 22 linear 10 plaque morphea	–	–	AHA
IL−6	Ihn et al. (69)	48 LS 20 SSc	16 generalized 22 linear 10 plaque morphea	–	–	AHA
IL−13	Hasegawa et al. (70)†	45 LS	12 generalized 22 linear 11 plaque morphea	27 (range 5–67 years old)	59%	Number of plaque lesions Number of lesions
IL−12	Torok et al. (66)†	69 Ped LS	8 generalized 41 linear 8 plaque morphea	13.0 (10.0–16.0)	67%	–
	O'Brien et al. (67)†	87 LS	49 generalized 28 linear 8 plaque morphea	50 ± 20 years (89% adult)	69%	LoSDI
TNFα	Hasegawa et al. (70)†	45 LS	12 generalized 22 linear 11 plaque morphea	27 (range 5–67 years old)	59%	IgM AHA IgG ssDNA Muscle involvement
TGFβ1	Uziel et al. (71)†	55 Ped LS	16 generalized 28 linear 10 plaque morphea	9.2 ± 3.6 years	–	–
TGFβ2	Budzynska-Włodarczyk et al. (72)	17 LS	9 generalized 6 other subtypes	45.3 ± 14.6 years	100%	–
sIL−2r	Uziel et al. (73)	17 Ped LS	–	8.1 years	–	–
sIL−6r	Nagaoka et al. (74)	45 LS 20 SSc	12 generalized 22 linear 11 plaque	–	–	IgM RF Number of lesions Number of body areas
	Budzynska-Włodarczyk et al. (72)	17 LS	9 generalized 6 other subtypes	45.3 ± 14.6 years	100%	ESR
	Nagaoka et al, (74)	45 LS 20 SSc	–	–	–	IgM AHA RF Number of lesions Number of body areas

(Continued)

TABLE 1 | Continued

		Patient (n) and LS, SSc	Subtype	Ages median (IQR) mean ± SD	Sex (% female)	Clinical and serological correlations
IL−23	Danczak-Pazdrowska et al. (75)*	41 LS	14 generalized 7 linear 20 plaque	43.7 ± 17.5 years	53%	mLoSSI in plaque patients Disease duration in all subtypes
IL−17A	Danczak-Pazdrowska et al. (75)*	41 LS	14 generalized 7 linear 20 plaque	43.7 ± 17.5 years	53%	mLoSSI in plaque patients Disease duration in all subtypes
	Torok et al. (66)†	69 Ped LS	8 generalized 41 linear 8 plaque morphea	13.0 (10.0–16.0)	67%	–
IL−1	Danczak-Pazdrowska et al. (75)	41 LS	14 generalized 7 linear 20 plaque	44 ± 18 years	50%	–
CXCL9./MIG	O'Brien et al. (67)*†	87 LS	49 generalized 28 linear 8 plaque morphea	50 ± 20 years (89% adult)	69%	mLoSSI LoSDI
	Mertens et al. (76)	80 LS	16 generalized 30 linear 8 plaque morphea	−80% adult	59%	mLoSSI
CXCL10./IP-10	O'Brien et al. (67)	87	49 generalized 28 linear 8 plaque morphea	50 ± 20 years (89% adult)	69%	LoSDI
	Mertens et al. (76)	80 LS	16 generalized 30 linear 8 plaque morphea	−80% adult	59%	mLoSSI
	Magee et al. (77)*†	69 Ped LS	8 generalized 40 linear 5 plaque morphea	12.5 (10.0–16.0)	46%	PGA-A mLoSSI
sgp130	Nagaoka et al, (74)	45 LS 20 SSc	–	–	–	IgG Number of lesions Number of body areas

*Also demonstrated in skin
†Includes pediatric patients
CCL2/MCP-1, Monocyte chemoattractant protein-1;
CCL3/MIP-1a, macrophage inflammatory protein 1 alpha;
CXCL8/IL-8, interleukin 8;
IL-2, interleukin 2;
IL-2R, interleukin 2 receptor;
IL-4, interleukin 4;
IL-6, interleukin 6;
IL-13, interleukin 13;
IL-12, interleukin 12;
TNFα, Tumor necrosis factor alpha;
TGFβ1, Transforming growth factor beta 1;
TGFβ2, Transforming growth factor beta 2;
sIL-2r, soluble interleukin 2 receptor;
sIL-6r, soluble interleukin 6 receptor;
IL-23, interleukin 23;
IL-17A, interleukin 17A;
IL-1, interleukin 1;
CXCL9/MIG, Chemokine (C-X-C motif) ligand 9/Monokine induced by gamma interferon;
CXCL10/IP-10, C-X-C motif chemokine 10/Interferon gamma-induced protein 10;
Sgp130, soluble gp130;
AHA, anti-histone antibody;
ANA, anti-nuclear antibody;
ESR, Erythrocyte sedimentation rate (sed rate);
IgG, Immunoglobulin G;
IgM, Immunoglobulin M;
LoSDI, Localized Scleroderma Damage Index;
mLoSSI, modified LS Skin Severity Index;
RF, Rheumatoid factor;
ssDNA, single stranded DNA antibody.

was associated with increased risk of pain and functional limitation, implying that both the location of a lesion on the skin as well as the microanatomical location of the pathology are important in determining those at risk for more serious sequelae. In terms of inflammatory cell types, lymphocytes predominated with plasma cells as second most common. Surprisingly eosinophils were present in 21% of specimens. The microanatomical location of inflammation closely mirrored that of the pattern of sclerosis present in an individual specimen. In other words, sclerosis occurred in areas that were enriched in inflammatory cell infiltrate. This implies a link between inflammation and activation of fibroblasts in the surrounding dermis and subcutis. Taken together, these results support the use of microanatomical location of inflammation and sclerosis in assessing risk of pain and functional limitations in localized scleroderma as well as further linking immune dysregulation as a driver of sclerosis (42).

In addition to fibroblasts, the immune cells could potentially activate skin structures. The location of the inflammatory cell infiltrate in skin is typically in a perivascular, peri-eccrine and peri-neural distribution, with the interplay with the neurovascular bundles sometimes termed "lymphocytic neurovasculitis" (47). In CNS disease, chronic perivascular lymphocytic infiltrate with intimal thickening and hyalinization of the vessels are also found when brain biopsies are obtained (23–25). T lymphocytes were also identified in a brain biopsy (48). Together, the histopathology in multiple organs in LS further suggest an autoimmune phenomenon.

Autoantibodies and Clinical Associations in LS

Autoantibodies (autoAb) are commonly observed in individuals with LS which reflects activation of the immune system and auto-reactivity to self-antigens. Although not as specific with regard to organ manifestation or scleroderma subtype as seen in systemic sclerosis (SSc), autoAb may be helpful in associating to disease severity and/or depth of LS. When more classic SSc-associated autoAb, such as anti-centromere and anti-topoisomerase, are tested in LS patients, they occur in 3–18% of the LS subjects (4, 8, 26, 49, 50), none of which had or developed SSc in addition to their LS diagnosis. A recent study of the SSc line immunoassay (LIA) (Euroimmun, Germany) in pediatric LS subjects found similar percentages (centromere 14%, topoisomerase 10%, RNA Polymerase III 12%) and when compared to clinical parameters, correlated to deep tissue involvement, signified by joint contractures, muscle involvement and nerve entrapment (51). Interestingly, none of the SSc-antibodies associated with LS subtype designation (51).

A high proportion of pediatric LS patients are Anti-Nuclear Antibody (ANA) positive, ranging from 30 to 70% when tested by indirect immunofluorescence (4, 11, 26–29, 41, 52, 53). The frequency and clinical utility of autoantibodies in LS has been the subject of numerous studies, with varied results. The presence ANA in several studies corresponded to deeper disease involvement (beyond the subcutis) by associating with features, such as joint contractures, muscle atrophy and extremity shortening, but not disease subtype or age of onset (4, 26–29, 53, 54). A recent longitudinal cohort study supports a positive ANA at LS diagnosis to be predictive of likelihood for recurrence; therefore, ANA is likely promoting autoimmunity in some fashion or reflecting a more auto-reactive state (55). Other auto-antibodies which may be reflecting positive ANA in LS have been reported, most commonly, anti-histone antibody (AHA) and anti–single-stranded DNA antibody (ssDNA Ab). In those tested, a range of 10–50% of LS patients are positive for ssDNA and/or AHA (56–58) with both correlating to severity features, such as deep muscle involvement, joint contractures, and increased number of lesions (41, 57, 59), and is able to track with disease activity status in a subset of patients (60).

When evaluated, rheumatoid factor was present in 16- 29% of patients and associated with arthritis (29, 50). Other markers of immune activation, such as IgG, IgA, and IgM were found to be increased in patients with linear scleroderma, and deep and pansclerotic morphea (29). Elevation of other more typical laboratory parameters tested in connective tissue diseases, such as muscle enzymes, corresponded to deeper tissue involvement. Elevated creatine phosphokinase (CPK) and aldolase were associated with disease parameters including muscle atrophy and extremity shortening in a North American pediatric LS cohort, indicating muscle involvement (11).

Cytokine and Cellular Signatures in LS

The exact cellular signature of LS is still being investigated. In both adult and pediatric LS analyses, lymphocytes and their associated cytokine and chemokine populations are observed in both the blood and skin. Flow cytometry studies of the circulating cellular phenotype of LS (pediatric and adult) have shown a predominance of $CD4^+$T helper cells along with decreased functional T regulatory cells (61–63). This decrease in T regulatory cells, possibly reflecting a more "permissive state," was also seen in pediatric SSc without increases in other T cell populations (64). Furthermore, when comparing paired active to inactive PBMC phenotypes in LS, those with active disease states demonstrated much higher populations of IFNγ-expressing T cells (reflecting T_H1 cells; $CD4^+$ IFNγ + T cells) (63). An expanded study utilizing multiparameter mass cytometry by time-of-flight spectrometry (CyTOF) also supported increased IFNγ expression from $CD4^+$T cells, as well as NK cell populations, in active LS PBMC samples (65).

T helper cells consist of 3 main types including T_H1, T_H2, and T_H17. These cell types produce distinct interactive cytokine profiles. T_H1 cells secrete IFN-γ and IL-2 and are stimulated by IL-12, whereas T_H2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and are activated by IL-4. T_H17 cells produce IL-17 A/F, IL-21, IL-22 and are propagated by IL-23, IL-6, and IL-1. While elevation of cytokines associated with all three T_H lineages have been observed in LS, the pattern of expression is consistent with that of a T_H1 predominance (**Table 1**). In peripheral blood, elevation of T_H1 related cytokines, chemokines, and their receptors include: IL-2 (67, 69, 73), IL-12 (66, 67), TNFα (70), TGFβ (66, 67, 71, 72), MCP-1 (66), IFNγ related proteins, CXCL9 (monokine induced by gamma interferon [MIG]) (67, 76, 77), CXCL10 (Interferon gamma-induced protein 10 [IP-10]) (67, 76) and CXCL11 (Interferon-inducible T-cell alpha chemoattractant [I-TAC]), and IFNγ chemokine receptor (CXCR3). T_H2 related

cytokines IL-4 (69), IL-6 (69, 72), IL-13 (70), and T_H17 related cytokines, IL-17A (66, 75), and IL-23 (75), were also reported as significantly elevated in peripheral blood of LS patients compared to controls. Peripheral blood levels of the IFNγ-related chemokines CXCL9 and CXCL10 also correlated to disease activity measures, such as clinical scores, the modified Localized scleroderma skin severity index (mLoSSI), and the Physician Global Assessment of disease activity (66, 67, 78) (**Table 1**), underscoring their potential as serological biomarkers of disease activity.

In LS skin, T_H1/IFNγ related chemokines CXCL9 (67) and CXCL10 (77) were increased, while T_H17 related cytokines IL-23 (75) and IL-17A (75) were decreased compared to healthy control skin. Both CXCL9 and CXCL10 were found to be present in the perivascular lymphocytic infiltrate of the papillary and reticular dermis (67, 77). Additionally, CXCL9 was found to stain in close approximation to both $CD4^+T_H$ cells and macrophages (67), suggesting potential interaction between lymphocytes and macrophages utilizing IFNγ chemokine signaling (**Figure 2**). Overall, this may synergistically promote fibroblasts to increase collagen expression in LS, eventually causing increased collagen deposition, fibrosis, and a shift toward a later T_H2 profile.

In summary, in LS there is a T_H1/IFNγ signature prevalent in the active or initial inflammatory stage of the disease and likely a more fibrotic T_H2 signature follows in the collagenous stage of LS, which more closely resembles long term SSc disease profiles. These two states of inflammatory and fibrotic disease have unique profiles that reflect the clinical perception of disease. In pediatric patients, CXCL9, CXCL10, CXCL11, MIP-3β, IL-9, IL-2, and CCL-1 were elevated in the active state compared to the inactive state of disease (77, 78) as indicated by these cytokine profiles of the blood and skin. The exact relationship of the circulating cellular and cytokine/chemokine players to skin pathophysiology is still under study. One study focused on skin expression of select transcripts, and found increased mRNA levels of CCR7 and CCL5/RANTES in LS lesions (79). This supported the idea that lymphocytes are recruited from the blood to lesional skin. Investigation into skin-homing T cell profiles are underway in LS, with preliminary data showing that both skin homing $CD8^+$(Tc)$CCR10^+$ and $CD4^+(T_H)CCR10^+$ T cells subsets produce inflammatory cytokine populations, including IFNγ, that were significantly increased in the active disease state compared to the inactive disease state (80). In adult SSc, IL-13^+, and not IFNγ^+, $CD8^+CCR10^+$ cells appear to be the skin homing cell type that propagates disease (81), emphasizing possible biological differences in scleroderma phenotypes: a more inflammatory, T_H1/T_C1 phenotype in LS and a more fibrotic, T_H2/T_C2 phenotype in SSc.

The LS Transcriptome

Investigation into the transcriptome of scleroderma, specifically SSc, has been successful at classifying samples based on genetic differences that could indicate or predict disease progression and potential future treatment plans. Adult SSc subjects were classified into 4 distinct genetic signatures based on overall microarray expression of the skin (82). These classifications include a *diffuse-proliferation* group composed of diffuse SSc patients, the *inflammatory* group containing diffuse SSc, limited SSc and LS (4 morphea subjects were included in the analyses), the *limited* group composed of limited SSc, and the *normal-like* group, which includes healthy controls along with a few SSc patients (82). All of the LS subjects included fell nicely into the inflammatory group, expressing primarily T-lymphocyte and IFNγ related genes (82), which corresponds to the peripheral blood and protein skin expression discussed above. Recent transcriptional analysis using RNA bulk sequencing of pediatric LS skin showed a distinct subset of patients expressing similar inflammatory genes including interferon-inducible chemokines, such as CXCL9, CXCL10, CXCL11, and IFNγ itself (83). Similar to SSc, subgroupings of LS patients are expected to be present as these inflammatory genes were more highly expressed in LS patients with more inflammatory or active lesions, with associated higher clinical activity scores, mLoSSI and PGA-A (83). This transcriptome classification system in SSc has been used more recently to predict patient response to therapy, such as the inflammatory subset showing better response to mycophenolate mofetil and the fibroproliferative group showing better response to stem cell transplant (84, 85). A methodologically similar classification of LS using immunophenotyping of the transcriptome could help to delineate immunological subtypes and determine therapeutic responses to disease.

Speculation: Differences in Immune System Function in Children and Adults

The greater prevalence of extracutaneous manifestations and autoimmunity in children than in adults suggests the possibility of some age-related differences in disease mechanisms that may potentially reflect differences in immune system function between children and adults. The immune system is relatively immature at birth, and many changes occur within the first year of life. Newborn dependency on antibodies from the maternal circulation and immune cells undergo phenotypic changes over the first few months of life including a robust thymic output of T cells that subsides over the first few years (86, 87). However, there are also changes from early childhood to adulthood. For example, in the peripheral blood, CD4 and CD8 T cell counts increase from childhood to adulthood while B cell counts drop (88). At the same time, regulatory T cell (87), monocyte (89) and NK cell (90) counts are higher in infancy or early childhood than in adulthood. Whether these differences in cell population reflect differential generation, trafficking, or survival is currently unclear. T and B cells are continuously trafficking between blood and secondary lymphoid organs, such as spleen and lymph nodes (91), and an increased proportion of B cells in the blood circulation at a young age could reflect relative reductions in B cell entry into lymph nodes, and potentially less exposure to an environment that supports the generation of a robust but also a well-regulated response. Further understanding how differences in immune cell numbers reflect differential immune system function could help to better understand disease.

In addition to differences in cell numbers, immune cells also differ in function in children and adults. Generally, early life is dominated by a more regulatory state that favors healing

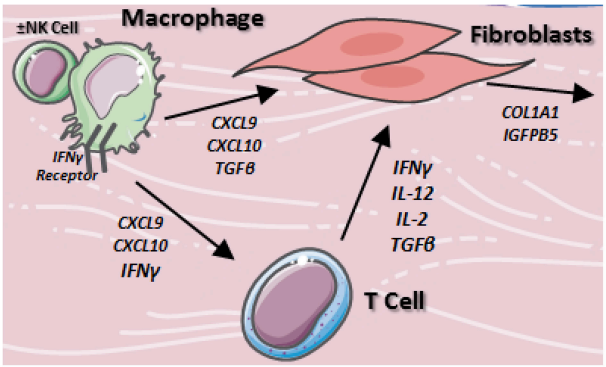

FIGURE 2 | Proposed cellular interactions of macrophages, fibroblasts, and T cells in localized scleroderma. Resident macrophages stimulate T cells and fibroblasts via TH1/IFNγ associated cytokine network to produce inflammation and collagen accumulation in the skin.

and repair (92). For innate immunity, NK cell phenotype is more consistent with cytokine producing rather than cytotoxic function early in post-natal life, with more mature phenotype during the first years of life (90). Responses to toll like receptor (TLR) stimulation are reduced during early months to years in Western countries (90, 92). At the same time, production of IL-10, a regulatory cytokine decreases during infancy to below adult levels, and then increases again until adult levels are reached (90). The functional implications of these alterations for different manifestations of scleroderma in childhood and adulthood are unclear, but these differences drive home the point that immunopathogenesis at different times of life may be different.

Childhood is also a time of immune repertoire development for lymphocytes, whereby lymphocytes that are naïve will be exposed to antigen, and antigen- experienced lymphocytes will constitute a larger proportion of the body's lymphocytes (87). Exposure over infancy and childhood through the gut, skin, and respiratory tract to different microbes, food, and the environment is thought to contribute to shaping and increasing the memory T cell compartment and protective antibodies. While the immune repertoire is modulated with intercurrent infections, even in the absence of frank infections, these exposures help to generate memory cells and antibodies that can potentially cross react enough with potential pathogens to protect the child later on (87). In addition to the antigenic exposure, the diversity of an individual's immune repertoire is also shaped by stochastic events. The selection of the exact T and B cells that will dominate in the response to antigen exposure has a large element of randomness, as reflected in the different immune repertoires in twins (93). During this period of high expansion of the immune repertoire, T and B cells that are cross-reactive to self and pathogenic could incidentally be generated.

Given these differences in immune function in children and adults, it is interesting to consider that the biomarkers of disease seen in pediatric LS compared to healthy pediatric controls—the reduced regulatory T cell, increased T_H1, T_H2, and T_H17 cells, and increased innate cell activation—resemble the pattern seen in a healthy adult immune system. We speculate that, while autoreactivity could contribute to pathogenesis, the cell distribution and activity in pediatric LS may actually signal an accelerated development of the immune system. This would be complementary but distinct from "aging" of the immune system and generation of senescent pro-inflammatory cells (94), but an acceleration of normal transition to a more permissive immune system that, perhaps in addition to the increased antigenic exposure during childhood, contributes to pathology in children.

FUTURE DIRECTIONS FOR RESEARCH

While treatment strategies effective for most patients have been identified, there is a major need for additional treatment options and strategies. Thirty percent of jLS patients may fail to respond to initial standard immunosuppressive treatment (95), and 15–53% of patients can relapse following treatment (15, 28, 95). Active disease can persist for decades (53, 96). Failure to achieve remission and relapsing disease are both associated with poorer outcome (97).

Identifying optimal treatment strategies for jLS will require comparative effectiveness studies; the feasibility of this approach was demonstrated by a recent pilot study of three standardized methotrexate based regimens (13). Because treatment is focused on controlling inflammation, sensitive monitoring of disease activity is essential for conducting such trials. A recent study identified specific lesion features for tracking disease activity that are likely to improve the sensitivity and specificity of existing clinical measures (14). Future work may lead to development of a weighted clinical activity measure to further improve our ability to identify relative differences in treatment efficacies. The identification of biomarkers that facilitate monitoring activity level and/or help identify response to specific treatments will enable us to work toward personalized medicine for these patients.

Further understanding disease pathophysiology will aid in the development of new therapeutic approaches. Insight into the exact nature of the immune dysfunction and how it contributes to skin fibrosis and the extracutaneous manifestations may help to better target the immune system to treat the disease. Understanding how the unique aspects of the immune system of childhood intersect with the disease process can further provide insight into disease etiology and perhaps teach us how to best reduce the immune dysfunction, or at the very least, repair the damage.

AUTHOR CONTRIBUTIONS

KT, SL, HJ, ST, AS, FZ, and TL conceived of and wrote the manuscript.

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REFERENCES

1. Mayes MD, Lacey JVJr, Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum*. (2003) 48:2246–55. doi: 10.1002/art.11073
2. Peterson L, Nelson A, Su W, Mason T, O'Fallon W, Gabriel S. The epidemiology of morphea (localized scleroderma) in Olmstead County 1960–1993. *J Rheumatol*. (1997) 24:73–80.
3. Scalapino K, Arkaschaisri T, Lucas M, Fertig N, Helfrich D, Londino AJ, et al. Childhood onset systemic sclerosis: classification, clinical and serologic features, and survival in comparison with adult onset disease. *J Rheumatol*. (2006) 33:1004–13.
4. Li SC. Scleroderma in children and adolescents: localized scleroderma and systemic sclerosis. *Pediatr Clin North Am*. (2018) 65:757–81. doi: 10.1016/j.pcl.2018.04.002
5. Guevara-Gutierrez E, Yinh-Lao J, Garcia-Gutierrez P, Tlacuilo-Parra A. Frequency of antinuclear antibodies in mestizo Mexican children with morphea. *Clin. Rheumatol*. (2010) 29:1055–9. doi: 10.1007/s10067-010-1515-2
6. Laxer R, Zulian F. Localized scleroderma. *Curr Opin Rheumatol*. (2006) 18:606–13. doi: 10.1097/01.bor.0000245727.40630.c3
7. Ardalan K, Zigler CK, Torok KS. Predictors of longitudinal quality of life in juvenile localized scleroderma. *Arthritis Care Res*. (2017) 69:1082–7. doi: 10.1002/acr.23101
8. Zulian F, Vallongo C, Woo P, Russo R, Ruperto N, Harper J, et al. Localized scleroderma in childhood is not just a skin disease. *Arthritis Rheum*. (2005) 52:2873–81. doi: 10.1002/art.21264
9. Piram M, McCuaig CC, Saint-Cyr C, Marcoux D, Hatami A, Haddad E, et al. Short- and long-term outcome of linear morphoea in children. *Br J Dermatol*. (2013) 169:1265–71. doi: 10.1111/bjd.12606
10. Weibel L, Laguda B, Atherton D, Harper JI. Misdiagnosis and delay in referral of children with localized scleroderma. *Br J Dermatol*. (2011) 165:1308–13. doi: 10.1111/j.1365-2133.2011.10600.x
11. Wu EY, Li SC, Torok KS, Virkud Y, Fuhlbrigge R, Rabinovich CE. Baseline description of the juvenile localized scleroderma subgroup from the childhood arthritis and rheumatology research alliance legacy registry. *ACR Open Rheumatol In press*. (2019) 1:119–124. doi: 10.1002/acr2.1019
12. Condie D, Grabell D, Jacobe H. Comparison of outcomes in adults with pediatric-onset morphea and those with adult-onset morphea: a cross-sectional study from the morphea in adults and children cohort. *Arthritis Rheumatol*. (2014) 66:3496–504. doi: 10.1002/art.38853
13. Li S, Torok K, Hong S, Ferguson P, Rabinovich C, Becker M, et al. (2018a). Working Towards Comparative Effectiveness Studies for Moderate to Severe Juvenile Localized Scleroderma: Results of a 1-Year Pilot Study of Three Methotrexate-Based Consensus Treatment Plans. *Pediatric Rheumatol Online J*. 16(Suppl 1):42. doi: 10.1186/s12969-018-0252-y
14. Li SC, Li X, Pope E, Stewart K, Higgins GC, Rabinovich CE, et al. New Features for Measuring Disease Activity in Pediatric Localized Scleroderma. *J Rheumatol in press*. (2018b) 45:1680–8: doi: 10.3899/jrheum.171381
15. Weibel L, Sampaio MC, Visentin MT, Howell KJ, Woo P, Harper JI. Evaluation of methotrexate and corticosteroids for the treatment of localized scleroderma (morphoea) in children. *Br J Dermatol*. (2006) 155:1013–20. doi: 10.1111/j.1365-2133.2006.07497.x
16. Schoch J, Schoch B, Davis D. Orthopedic complications of linear morphea: Implications for early interdisciplinary care. *Pediatric Dermatol*. (2018) 35:43–6. doi: 10.1111/pde.13336
17. Tollefson M, Witman P. En coup de sabre morphea and parry-romberg syndrome: a retrospective review of 54 patients. *J Am Acad Dermatol*. (2007) 56:257–63. doi: 10.1016/j.jaad.2006.10.959
18. Blaszczyk M, Krolicki L, Krasu M, Glinska O, Jablonska S. Progressive facial hemiatrophy: central nervous system involvement and relationship with scleroderma en coup de sabre. *J Rheumatol*. (2003) 30:1997–2004.
19. Moko SB, Mistry Y, Blandin de Chalain TM. Parry-Romberg syndrome: intracranial MRI appearances. *J Cranio Maxillo Facial Surgery*. (2003) 31:321–4. doi: 10.1016/S1010-5182(03)00028-3
20. Kister I, Inglese M, Laxer RM, Herbert J. Neurologic manifestations of localized scleroderma: a case report and literature review. *Neurology*. (2008) 71:1538–45. doi: 10.1212/01.wnl.0000334474.88923.e3
21. Sartori S, Martini G, Calderone M, Patrizi A, Gobbi G, Zulian F. Severe epilepsy preceding by four months the onset of scleroderma en coup de sabre. *Clin Exp Rheumatol*. (2009) 27:64–7.
22. De Somer L, Morren MA, Muller PC, Despontin K, Jansen K, Lagae L, et al. Overlap between linear scleroderma, progressive facial hemiatrophy and immune-inflammatory encephalitis in a paediatric cohort. *Eur J Pediatr*. (2015) 174:1247–54. doi: 10.1007/s00431-015-2532-6
23. Luer W, Jockel D, Henze T, Schipper HI. Progressive inflammatory lesions of the brain parenchyma in localized scleroderma of the head. *J Neurol*. (1990) 237:379–81. doi: 10.1007/BF00315664
24. Sathornsumetee S, Schanberg L, Rabinovich E, Lewis D Jr, Weisleder P. Parry-Romberg syndrome with fatal brain stem involvement. *J Pediatr*. (2005) 146:429–31. doi: 10.1016/j.jpeds.2004.10.026
25. Stone J, Franks AJ, Guthrie JA, Johnson MH. Scleroderma "en coup de sabre": pathological evidence of intracerebral inflammation. *J Neurol Neurosurg Psychiatr*. (2001) 70:382–5. doi: 10.1136/jnnp.70.3.382
26. Christen-Zaech S, Hakim MD, Afsar FS, Paller AS. Pediatric morphea (localized scleroderma): review of 136 patients. *J Am Acad Dermatol*. (2008) 59:385–96. doi: 10.1016/j.jaad.2008.05.005
27. Leitenberger JJ, Cayce RL, Haley RW, Adams-Huet B, Bergstresser PR, Jacobe HT. Distinct autoimmune syndromes in morphea: a review of 245 adult and pediatric cases. *Arch Dermatol*. (2009) 145:545–50. doi: 10.1001/archdermatol.2009.79
28. Pequet MS, Holland KE, Zhao S, Drolet BA, Galbraith SS, Siegel DH, et al. Risk factors for morphoea disease severity: a retrospective review of 114 paediatric patients. *Br J Dermatol*. (2014) 170:895–900. doi: 10.1111/bjd.12758
29. Zulian F, Athreya BH, Laxer R, Nelson AM, Feitosa de Oliveira SK, Punaro MG, et al. Juvenile localized scleroderma: clinical and epidemiological features in 750 children. An international study. *Rheumatology*. (2006) 45:614–20. doi: 10.1093/rheumatology/kei251
30. el-Azhary RA, Aponte CC, Nelson AM, Weaver AL, Homburger HA. Antihistone antibodies in linear scleroderma variants. *Int. J. Dermatol*. (2006) 45:1296–9. doi: 10.1111/j.1365-4632.2006.02891.x
31. Jacobe H, Ahn C, Arnett FC, Reveille JD. Major histocompatibility complex class I and class II alleles may confer susceptibility to or protection against morphea: findings from the morphea in adults and children cohort. *Arthritis Rheumatol*. (2014) 66:3170–7. doi: 10.1002/art.38814
32. Hemminki K, Li X, Sundquist J, Sundquist K. Familial associations of rheumatoid arthritis with autoimmune diseases and related conditions. *Arthritis Rheum*. (2009) 60:661–8. doi: 10.1002/art.24328
33. Tenea D. The puzzle of the skin patterns. *Integr Med Int*. (2017) 4:1–12. doi: 10.1159/000452949
34. Jue MS, Kim MH, Ko JY, Lee CW. Digital image processing for the acquisition of graphic similarity of the distributional patterns between cutaneous lesions of linear scleroderma and Blaschko's lines. *J Dermatol*. (2011) 38:778–83. doi: 10.1111/j.1346-8138.2010.01162.x
35. Weibel L, Harper JI. Linear morphoea follows blaschko's lines. *Br J Dermatol*. (2008) 159:175–81. doi: 10.1111/j.1365-2133.2008.08647.x
36. Molho-Pessach V, Schaffer JV. Blaschko lines and other patterns of cutaneous mosaicism. *Clin Dermatol*. (2011) 29:205–25. doi: 10.1016/j.clindermatol.2010.09.012
37. Levinsohn JL, Tian LC, Boyden LM, McNiff JM, Narayan D, Loring ES, et al. Whole-exome sequencing reveals somatic mutations in HRAS and KRAS, which cause nevus sebaceus. *J Invest Dermatol*. (2013) 133:827–30. doi: 10.1038/jid.2012.379
38. Dupont S, Catala M, Hasboun D, Semah F, Baulac M. Progressive facial hemiatrophy and epilepsy: a common underlying dysgenetic mechanism. *Neurology*. (1997) 48:1013–8. doi: 10.1212/WNL.48.4.1013
39. Chung MH, Sum J, Morrell MJ, Horoupian DS. Intracerebral involvement in scleroderma en coup de sabre: report of a case with neuropathologic findings. *Ann Neurol*. (1995) 37:679–81. doi: 10.1002/ana.410370519
40. Derex L, Isnard H, Revol M. Progressive facial hemiatrophy with multiple benign tumors and hamartomas. *Neuropediatrics*. (1995) 26:306–9. doi: 10.1055/s-2007-979779
41. Takehara K, Sato S. Localized scleroderma is an autoimmune disorder. *Rheumatology*. (2005) 44:274–9. doi: 10.1093/rheumatology/keh487
42. Walker D, Susa JS, Currimbhoy S, Jacobe H. Histopathological changes in morphea and their clinical correlates: results from the morphea in

adults and children cohort V. *J Am Acad Dermatol*. (2017) 76:1124–30. doi: 10.1016/j.jaad.2016.12.020
43. Goldberg-Stern HT, deGrauw Passo M, Ball WS, Jr. Parry-Romberg syndrome: follow-up imaging during suppressive therapy. *Neuroradiology*. (1997) 39:873–6. doi: 10.1007/s002340050525
44. Holland KE, Steffes B, Nocton JJ, Schwabe MJ, Jacobson RD, Drolet BA. Linear scleroderma en coup de sabre with associated neurologic abnormalities. *Pediatrics*. (2006) 117:e132–6. doi: 10.1542/peds.2005-0470
45. Torok KS, Arkachaisri T. Methotrexate and corticosteroids in the treatment of localized scleroderma: a standardized prospective longitudinal single-center study. *J Rheumatol*. (2012) 39:286–94. doi: 10.3899/jrheum.110210
46. Zulian F, Martini G, Vallongo C, Vittadello F, Falcini F, Patrizi A, et al. Methotrexate treatment in juvenile localized scleroderma: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum*. (2011) 63:1998–2006. doi: 10.1002/art.30264
47. Pensler JM, Murphy GF, Mulliken JB. Clinical and ultrastructural studies of Romberg's hemifacial atrophy. *Plast Reconstr Surg*. (1990) 85:669–74; discussion 675–66. doi: 10.1097/00006534-199005000-00001
48. Doolittle DA, Lehman VT, Schwartz KM, Wong-Kisiel LC, Lehman JS, Tollefson MM. CNS imaging findings associated with Parry-Romberg syndrome and en coup de sabre: correlation to dermatologic and neurologic abnormalities. *Neuroradiology*. (2015) 57:21–34. doi: 10.1007/s00234-014-1448-6
49. Kreuter A, Wischnewski J, Terras S, Altmeyer P, Stucker M, Gambichler T. Coexistence of lichen sclerosus and morphea: a retrospective analysis of 472 patients with localized scleroderma from a German tertiary referral center. *J Am Acad Dermatol*. (2012) 67:1157–62. doi: 10.1016/j.jaad.2012.04.003
50. Vancheeswaran R, Black CM, David J, Hasson N, Harper J, Atherton D, et al. Childhood-onset scleroderma: is it different from adult-onset disease. *Arthritis Rheum*. (1996) 39:1041–9. doi: 10.1002/art.1780390624
51. Porter A, Mirizio E, Fritzler MJ, Brown R, Choi M, Schollaert-Fitch K, et al. Autoantibody testing in pediatric localized scleroderma (LS). *Arthritis Rheumatol*. (2018) 70(suppl 10). Available online at: https://acrabstracts.org/abstract/autoantibody-testing-in-pediatric-localized-scleroderma-ls/
52. Kreuter A, Tigges C, Gaifullina R, Kirschke J, Altmeyer P, Gambichler T. Pulsed high-dose corticosteroids combined with low-dose methotrexate treatment in patients with refractory generalized extragenital lichen sclerosus. *Arch Dermatol*. (2009) 145:1303–8. doi: 10.1001/archdermatol.2009.235
53. Marzano AV, Menni S, Parodi A, Borghi A, Fuligni A, Fabbri P, et al. Localized scleroderma in adults and children. clinical and laboratory investigations on 239 cases. *Eur J Dermatol*. (2003) 13:171–6.
54. Kreuter A. Localized scleroderma. *Dermatol Ther*. (2012) 25:135–47. doi: 10.1111/j.1529-8019.2012.01479.x
55. Kurzinski K, Zigler CK, Torok KS. Prediction of disease relapse in a cohort of juvenile localized scleroderma patients. *Br J Dermatol*. (2018) doi: 10.1111/bjd.17312
56. Dharamsi JW, Victor S, Aguwa N, Ahn C, Arnett F, Mayes MD, et al. Morphea in adults and children cohort III: nested case-control study–the clinical significance of autoantibodies in morphea. *JAMA Dermatol*. (2013) 149:1159–65. doi: 10.1001/jamadermatol.2013.4207
57. Falanga V, Medsger TAJr, Reichlin M, Rodnan GP. Linear scleroderma. clinical spectrum, prognosis, and laboratory abnormalities. *Ann Intern Med*. (1986) 104:849–57. doi: 10.7326/0003-4819-104-6-849
58. Sato S, Ihn H, Kikuchi K, Takehara K. Antihistone antibodies in systemic sclerosis. *Assoc Pulmonary Fibrosis Arthritis Rheum*. (1994) 37:391–4. doi: 10.1002/art.1780370313
59. Arkachaisri T, Fertig N, Pino S, Medsger TAJr. Serum autoantibodies and their clinical associations in patients with childhood- and adult-onset linear scleroderma. a single-center study. *J Rheumatol*. (2008) 35:2439–44. doi: 10.3899/jrheum.080098
60. Arkachaisri T, Pino S. Localized scleroderma severity index and global assessments: a pilot study of outcome instruments. *J Rheumatol*. (2008) 35:650–7.
61. Inoshita T, Whiteside TL, Rodnan GP, Taylor FH. Abnormalities of T lymphocyte subsets in patients with progressive systemic sclerosis (PSS, scleroderma). *J Lab Clin Med*. (1981) 97:264–77.
62. Keystone EC, Lau C, Gladman DD, Wilkinson S, Lee P, Shore A. Immunoregulatory T cell subpopulations in patients with scleroderma using monoclonal antibodies. *Clin Exp Immunol*. (1982) 48:443–8.
63. Mirizio E, Marathi A, Hershey N, Ross C, Schollaert K, Salgadgo CM, et al. Identifying the signature immune phenotypes present in pediatric localized scleroderma. *J Invest Dermatol*. (2018) 139:715–8. doi: 10.1016/j.jid.2018.09.025
64. Reiff A, Weinberg KI, Triche T, Masinsin B, Mahadeo KM, Lin CH, et al. T lymphocyte abnormalities in juvenile systemic sclerosis patients. *Clin Immunol*. (2013) 149:146–55. doi: 10.1016/j.clim.2013.07.005
65. Mirizio E, Mellins ED, Macaubas C, Maecker HT, Gartner F, Konnikova L, et al. Multiparameter mass cytometry by time-of-flight spectrometry (CyTOF) phenotyping in pediatric localized scleroderma [abstract]. *Arthritis Rheumatol*. (2018a) 70(suppl 10). Available online at: https://acrabstracts.org/abstract/multiparameter-mass-cytometry-by-time-of-flight-spectrometry-cytof-phenotyping-in-pediatric-localized-scleroderma/
66. Torok KS, Kurzinski K, Kelsey C, Yabes J, Magee K, Vallejo AN, et al. Peripheral blood cytokine and chemokine profiles in juvenile localized scleroderma: T-helper cell-associated cytokine profiles. *Semin Arthritis Rheum*. (2015) 45:284–93. doi: 10.1016/j.semarthrit.2015.06.006
67. O'Brien JC, Rainwater YB, Malviya N, Cyrus N, Auer-Hackenberg L, Hynan LS, et al. Transcriptional and cytokine profiles identify CXCL9 as a biomarker of disease activity in morphea. *J Invest Dermatol*. (2017) 137:1663–70. doi: 10.1016/j.jid.2017.04.008
68. Ihn H, Sato S, Fujimoto M, Kikuchi K, Takehara K. Demonstration of interleukin 8 in serum samples of patients with localized scleroderma. *Arch Dermatol*. (1994) 130:1327–8.
69. Ihn H, Sato S, Fujimoto M, Kikuchi K, Takehara K. Demonstration of interleukin-2, interleukin-4 and interleukin-6 in sera from patients with localized scleroderma. *Arch Dermatol Res*. (1995) 287:193–7. doi: 10.1007/BF01262331
70. Hasegawa M, Sato S, Nagaoka T, Fujimoto M, Takehara K. Serum levels of tumor necrosis factor and interleukin-13 are elevated in patients with localized scleroderma. *Dermatology*. (2003) 207:141–7. doi: 10.1159/000071783
71. Uziel Y, Feldman BM, Krafchik BR, Laxer RM, Yeung RS. Increased serum levels of TGFbeta1 in children with localized scleroderma. *Pediatr Rheumatol Online J*. (2007) 5:22. doi: 10.1186/1546-0096-5-22
72. Budzynska-Wlodarczyk J, Michalska-Jakubus MM, Kowal M, Krasowska D. Evaluation of serum concentrations of the selected cytokines in patients with localized scleroderma. *Postepy Dermatol Alergol*. (2016) 33:47–51. doi: 10.5114/pdia.2015.48044
73. Uziel Y, Krafchik BR, Feldman B, Silverman ED, Rubin LA, Laxer RM. Serum levels of soluble interleukin-2 receptor. a marker of disease activity in localized scleroderma. *Arthritis Rheum*. (1994) 37:898–901. doi: 10.1002/art.1780370618
74. Nagaoka T, Sato S, Hasegawa M, Ihn H, Takehara K. Serum levels of soluble interleukin 6 receptor and soluble gp130 are elevated in patients with localized scleroderma. *J Rheumatol*. (2000) 27:1917–21.
75. Danczak-Pazdrowska A, Kowalczyk M, Szramka-Pawlak B, Gornowicz-Porowska J, Szewczyk A, Silny W, et al. Interleukin-17A and interleukin-23 in morphea. *Arch Med Sci*. (2012) 8:1089–95. doi: 10.5114/aoms.2012.32421
76. Mertens JSEM, de Jong GJ, Pandit AM, Seyger MB. Regarding "transcriptional and cytokine profiles identify CXCL9 as a biomarker of disease activity in morphea". *J Invest Dermatol*. (2018) 138:1212–5. doi: 10.1016/j.jid.2017.11.032
77. Magee KE, Kelsey CE, Kurzinski KL, Ho J, Mlakar LR, Feghali-Bostwick CA, et al. Interferon-gamma inducible protein-10 as a potential biomarker in localized scleroderma. *Arthritis Res Ther*. (2013) 15:R188. doi: 10.1186/ar4378
78. Torok KS, Mi Q, Mirizio E, Schollaert-Fitch K, Fritzler M, Fritzler MJ. Chemokine ligand 9 (CXCL9) [monokine induced by gamma interferon (MIG)] as a predictor of active disease status in localized scleroderma [abstract]. *Arthritis Rheumatol*. (2017) 69(suppl 10). Available online at: https://acrabstracts.org/abstract/chemokine-ligand-9-cxcl9-monokine-induced-by-gamma-interferon-mig-as-a-predictor-of-active-disease-status-in-localized-scleroderma/
79. Gambichler T, Skrygan M, Labanski AA, Kolios AG, Altmeyer P, Kreuter A. Significantly increased CCL5/RANTES and CCR7 mRNA levels in localized scleroderma. *Regul Pept*. (2011) 170:4–6. doi: 10.1016/j.regpep.2011.05.003

80. Macaubas C, Mirizio E, Schollaert-Fitch K, Mellins ED, Torok KS. (2017). Nterferon gamma (IFN-γ) Subpopulations in skin homing T cells of localized scleroderma [abstract]. *Arthritis Rheumatol*. 69(suppl 10). Available online at: https://acrabstracts.org/abstract/interferon-gamma-ifn-%ce%b3-subpopulations-in-skin-homing-t-cells-of-localized-scleroderma/
81. Li G, Larregina AT, Domsic RT, Stolz DB, Medsger TAJr, Lafyatis R, et al. Skin-resident effector memory CD8(+)CD28(-) T cells exhibit a profibrotic phenotype in patients with systemic sclerosis. *J Invest Dermatol*. (2017) 137:1042–50. doi: 10.1016/j.jid.2016.11.037
82. Milano A, Pendergrass SA, Sargent JL, George LK, McCalmont TH, Connolly MK, et al. Molecular subsets in the gene expression signatures of scleroderma skin. *PLoS ONE*. (2008) 3:e2696. doi: 10.1371/journal.pone.0002696
83. Mirizio E, Mandal R, Yan Q, Horne W, Schollaert-Fitch K, Torok KS. Genetic signatures from RNA sequencing of pediatric localized scleroderma (LS) skin. *Arthritis Rheumatol*. (2018b) 70 (suppl 10). Available online at:https://acrabstracts.org/abstract/genetic-signatures-from-rna-sequencing-of-pediatric-localized-scleroderma-ls-skin/
84. Franks J, Martyanov V, Wood TA, Croord L, Keyes-Elstein L, Furst DE, et al. Machine learning classification of peripheral blood gene expression identifies a subset of patients with systemic sclerosis most likely to show clinical improvement in response to hematopoietic stem cell transplant. *Arthritis Rheumatol*. (2018) 90(Suppl. 10). Available online at: https://acrabstracts.org/abstract/machine-learning-classification-of-peripheral-blood-gene-expression-identifies-a-subset-of-patients-with-systemic-sclerosis-most-likely-to-show-clinical-improvement-in-response-to-hematopoietic-stem-c/
85. Hinchcliff M, Huang CC, Wood TA, Matthew Mahoney J, Martyanov V, Bhattacharyya S, et al. Molecular signatures in skin associated with clinical improvement during mycophenolate treatment in systemic sclerosis. *J Invest Dermatol*. (2013) 133:1979–89. doi: 10.1038/jid.2013.130
86. Olin A, Henckel E, Chen Y, Lakshmikanth T, Pou C, Mikes J, et al. Stereotypic immune system development in newborn children. *Cell*. (2018) 174:1277–92.e1214. doi: 10.1016/j.cell.2018.06.045
87. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. *Proc Biol Sci*. (2015) 282:20143085. doi: 10.1098/rspb.2014.3085
88. Valiathan R, Ashman M, Asthana D. Effects of ageing on the immune system: infants to elderly. *Scand J Immunol*. (2016) 83:255–66. doi: 10.1111/sji.12413
89. Adeli K, Raizman JE, Chen Y, Higgins V, Nieuwesteeg M, Abdelhaleem M, et al. Complex biological profile of hematologic markers across pediatric, adult, and geriatric ages: establishment of robust pediatric and adult reference intervals on the basis of the Canadian health measures survey. *Clin Chem*. (2015) 61:1075–86. doi: 10.1373/clinchem.2015.240531
90. Georgountzou A, Papadopoulos NG. Postnatal innate immune development: from birth to adulthood. *Front Immunol*. (2017) 8:957. doi: 10.3389/fimmu.2017.00957
91. Young AJ. The physiology of lymphocyte migration through the single lymph node *in vivo*. *Semin Immunol*. (1999) 11:73–83. doi: 10.1006/smim.1999.0163
92. Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O. Protecting the newborn and young infant from infectious diseases: lessons from immune ontogeny. *Immunity*. (2017) 46:350–63. doi: 10.1016/j.immuni.2017.03.009
93. Hawes GE, Struyk L, van den Elsen PJ. Differential usage of T cell receptor V gene segments in $CD4^+$ and $CD8^+$ subsets of T lymphocytes in monozygotic twins. *J Immunol*. (1993) 150:2033–45.
94. Vallejo AN. Immune remodeling: lessons from repertoire alterations during chronological aging and in immune-mediated disease. *Trends Mol Med*. (2007) 13:94–102. doi: 10.1016/j.molmed.2007.01.005
95. Zulian F, Vallongo C, Patrizi A, Belloni-Fortina A, Cutrone M, Alessio M, et al. A long-term follow-up study of methotrexate in juvenile localized scleroderma (morphea). *J Am Acad Dermatol*. (2012) 67:1151–6. doi: 10.1016/j.jaad.2012.03.036
96. Saxton-Daniels S, Jacobe HT. An evaluation of long-term outcomes in adults with pediatric-onset morphea. *Arch Dermatol*. (2010) 146:1044–5. doi: 10.1001/archdermatol.2010.239
97. Martini G, Fadanelli G, Agazzi A, Vittadello F, Meneghel A, Zulian F. Disease course and long-term outcome of juvenile localized scleroderma: experience from a single pediatric rheumatology centre and literature review. *Autoimmun Rev*. (2018) 17:727–34. doi: 10.1016/j.autrev.2018.02.004

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REVIEW
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T-Cell Compartmentalization and Functional Adaptation in Autoimmune Inflammation: Lessons From Pediatric Rheumatic Diseases

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Chronic inflammatory diseases are characterized by a disturbed immune balance leading to recurring episodes of inflammation in specific target tissues, such as the joints in juvenile idiopathic arthritis. The tissue becomes infiltrated by multiple types of immune cell, including high numbers of CD4 and CD8 T-cells, which are mostly effector memory cells. Locally, these T-cells display an environment-adapted phenotype, induced by inflammation- and tissue-specific instructions. Some of the infiltrated T-cells may become tissue resident and play a role in relapses of inflammation. Adaptation to the environment may lead to functional (re)programming of cells and altered cellular interactions and responses. For example, specifically at the site of inflammation both CD4 and CD8 T-cells can become resistant to regulatory T-cell-mediated regulation. In addition, CD8 and CD4 T-cells show a unique profile with pro- and anti-inflammatory features coexisting in the same compartment. Also regulatory T-cells are neither homogeneous nor static in nature and show features of functional differentiation, and plasticity in inflammatory environments. Here we will discuss the recent insights in T-cell functional specialization, regulation, and clonal expansion in local (tissue) inflammation.

Keywords: T-cell adaptation, autoimmune-inflammation, regulatory T-cell, CD8 T-cell, tissue-resident memory T-cell, clonal expansion, JIA, SLE

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INTRODUCTION

A typical hallmark of immune-mediated inflammatory diseases (IMIDs) is the intermittent presence of inflammation that manifest in specific target tissues. In affected tissues, activated antigen-specific T-cells with a memory phenotype are present. The combination of memory T-cell infiltrated at affected sites and genetic associations have pointed to T-cells as key players in the pathophysiology of chronic inflammatory diseases. Due to the ongoing inflammation in target tissues there is an increasing risk for collateral tissue damage. Especially in children this can have long-term effects and consequences. Understanding the biology of immune cells that are actively involved in the local inflammatory process is crucial for the development of therapeutic approaches. However, because of technical challenges, the vast majority of data in human studies comes from peripheral blood cells. With the emergence of novel techniques

including (single-cell) RNA-sequencing and mass cytometry it is now possible to unravel T-cell signatures in a local (tissue) setting. Here, we will discuss the most recent findings on human T-cell functional programming and adaptation at the site of chronic inflammation with a focus on pediatric rheumatologic disease.

T-CELLS AT THE SITE OF INFLAMMATION

Inflammation is a process aimed at eliminating the triggering agent, but in chronic inflammation the (auto)antigens persist, inducing a sustained inflammation. When inflammation does resolve, a population of antigen-specific T-cells may remain in the tissue as memory cells and become tissue resident (1), the so called tissue-resident memory T-cells (Trm). This makes them unique compared to central memory, effector memory and naïve T-cells that recirculate within lymphoid organs and blood and/or lymphatics. A hallmark of Trm cells is their ability to respond quickly and robustly after re-encountering the antigen as well as the expression of inhibitory molecules to keep them in check. In steady-state, Trm play an important role in tissue homeostasis and protection. After immune re-activation of Trm, circulating immune cells are attracted to the site of inflammation as well, resulting in local accumulation of antigen-specific memory T-cells. So both Trm and infiltrating T-cells that might eventually become Trm themselves, may actively participate during the inflammatory response. Because of their vigilance and specific localization in tissues, Trm are suspected to play a dominant role in the typical relapsing remitting course of chronic inflammatory diseases. Not much is known about the dynamics and interaction between both memory subsets. An accumulation of both $CD4^+$ and $CD8^+$ T-cells are found at sites of chronic inflammation. However, research on the involvement of T-cells in disease pathogenesis has mainly focused on $CD4^+$ T-cells, whereas $CD8^+$ T-cells have been the focus of the Trm research field. In this review we summarize the findings on $CD8^+$, $CD4^+$, and $FOXP3^+$ regulatory T-cell (Treg) subsets in the contexts of local (tissue) adaptation and functional differentiation. In line with the divarication of T helper cells, with each subset having a specified function in the immune system, it is now becoming apparent that specialization is also true for T-cells present in different tissues and types of inflammation. The local acquisition of additional or adjusted functions and phenotypes will be referred to as adaptation.

$CD4^+$ T Helper Responses in Local Pathogenesis

For a long time, $CD4^+$ T have been recognized as central players in the immune-pathogenesis of autoimmune diseases, which is supported by strong associations of rheumatic diseases with MHC class II alleles (2). The $CD4^+$ T-cell population is comprised of several T helper subsets that develop after the T-cell receptor (TCR) on naïve $CD4^+$ T-cells interacts with activated antigen presenting cells (APCs). Next to this TCR stimulation, co-stimulation, subsequent signaling and cytokines in the micro-environment are important in determining the fate of a specific subset, by activating signaling molecules that establish a lineage-specific enhancer landscape and lead to the expression of master transcription factors. Those, together with a complex network of accessory transcription factors, can coordinate cellular differentiation programs committed toward a lineage, while simultaneously repressing the developmental programs of opposing Th lineages (3). For many years the only identified lineages were Th1 and Th2 subsets, with IFNγ producing Th1 cells being specialized in cell-mediated immune responses against intracellular bacteria and Th2 cells producing IL-4 and IL-13 targeting helminths. The discovery of other subsets, such as Th17, Th9, and T follicular helper (Tfh) cells has shifted the paradigm of two opposing lineages (4–6). Th17 cells, producing various cytokines including IL-17, IL-22, and GM-CSF, induce defense against fungi and extracellular bacteria and are crucial for the maintenance of mucosal homeostasis (7). Despite their relatively recent discovery, Th17 cells are implicated in the pathogenesis of many human (autoimmune-) diseases. This also accounts for Tfh cells that provide help to cognate B cells to produce high affinity antibodies and memory B cells and as such control humoral immunity (8). Recent reports indicate that Th9 cells, mainly producing IL-9, may also be involved in the pathogenesis of auto-immune diseases, possibly by promoting Th17 differentiation (4).

Many immune-mediated diseases are associated with aberrant Th responses. In the lamina propria of Crohn's disease, the synovial fluid (SF) of Juvenile Idiopathic Arthritis (JIA) and Rheumatoid Arthritis (RA) patients and renal tissue of Systemic Lupus Erythematosus (SLE) patients for example, Th1 cells are implicated in disease pathogenesis (9–12). In JIA, a mixed Th17/Th1 phenotype is also found in inflamed joints, capable of producing both IL-17 and IFNγ. The presence of this subset correlates with disease activity (13, 14) and its IL-17-producing capacity is associated with CD161 expression (15). Interestingly, this subset seems to be present specifically in inflammatory environments, and can be a transiently induced from Th17 cells upon exposure to IL-12 and/or TNFα (13, 14, 16). Whereas, the Th1 lineage is shown to be fairly stable, the Th17 lineage is known for its instability and is severely impacted by the environment (17). In line with this, the mixed Th1/Th17 phenotype likely derives from Th17 cells instead of Th1 cells (18). Next to pathogenic Th17 cells, non-pathogenic Th17 cells have been described in autoimmune diseases as well [reviewed in (19)]. A recent paper found delayed IL-10 production in about 25% of activated human Th17 clones in culture (20). This indicates that IL-10 production is an intrinsic property of a subset of Th17 cells after antigenic stimulation, perhaps to regulate and balance the immune response. Transcriptional analysis of the $IL\text{-}10^+$ and $IL\text{-}10^-$ Th17 clones demonstrated immune-regulatory and tissue-resident properties of IL-10 producing Th17 cells, and a pro-inflammatory profile of $IL\text{-}10^-$ Th17 cells with high CCR7 expression, which may indicate circulatory properties (20). Local pathogenic Th17 cells have been described in multiple autoimmune disease [reviewed in (17)]. In muscle of JDM patients and affected kidneys of SLE patients, IL-17 producing cells are increased (21–23). One mechanism that explains the elevated production of IL-17 in JIA and

SLE is the increased expression of the transcription factor cAMP-responsive element modulator (CREM)α. This induces repression of IL-2 transcription but also epigenetic changes of the IL-17A locus, resulting in enhanced IL-17A promoter activity (24, 25). Although evidence for involvement of the IL-17 signaling pathway in SLE pathogenesis is expanding, direct interference with IL-17 or it's receptor does not seem to be effective, at least in mouse models (26, 27). Also in JIA, IL-17 blockade is not part of standard treatment. This might be related to the pathogenic role of another $CD4^+$ subset, Tfh cells that is increased in inflamed tissues of RA and SLE patients and located near B cells in affected kidneys in SLE patients (28, 29). Interestingly, STAT-3 and IL-21, signature molecules shared by Th17 and Tfh cells, are heavily implicated in SLE pathology and are capable of inducing autoreactive B cells (30, 31). Alternatively, the lack of IL-17 blockade efficacy in JIA might be explained by Th17 cells that can be polarized in the inflamed joints to shift toward the so called non-classic Th1 subset. These cells have been shown to lose the ability to produce IL-17 while maintaining both RORc and CD161 expression and produce high levels of IFNγ, in line with the mixed Th1/Th17 cells described above (18). Altogether, overactive $CD4^+$ T-cells are present at the site of human inflammation and represent a mixed population of which especially Th17 cells show plasticity.

A Continuum of Th Cell Fates

The presence of mixed $CD4^+$ phenotypes found in human sites of autoimmune inflammation is of great interest, but might not be inflammation-specific. Recent studies using novel technologies have revealed a continuum of cell fates rather than limited and distinct Th subsets in healthy tissues. Mass cytometry measuring T-cell trafficking receptor and cytokine expression in eight different human tissues revealed tissue-specific and unique T-cell phenotypes (32). This indicates that T-cells cannot be easily classified into separate lineages across human tissues. Furthermore, multi-color cytometry of peripheral blood cells of a healthy human cohort showed substantial subject-to-subject differences in T-cell populations that yet remained relatively stable for months within individuals (33). Age and disease associated genetic polymorphisms were identified as important factors in the identified variation. These publications highlight the importance of age and tissue influences on T cells [reviewed in (34)]. On top of this homeostatic variety, inflammation will probably add another layer of complexity by driving local tissue cells into an adapted phenotype.

$CD8^+$ T Helper Responses in Local Pathogenesis

Although long neglected in autoimmune diseases compared to $CD4^+$ T-cells, $CD8^+$ T-cells are equipped with different capacities by which they can contribute to the inflammatory process. For example, $CD8^+$ T-cells have cytolytic activity, produce pro-inflammatory cytokines and can react to self-antigens upon cross-presentation (35). In several chronic inflammatory diseases $CD8^+$ T-cells are described to be present in the inflamed tissues and increased $CD8^+$ T-cell numbers are associated poor prognosis in several rheumatic diseases including JIA, RA and SLE (36–39). $CD8^+$ T-cells form a diverse group of cells but in contrast to $CD4^+$ T-cells subsets are less well-defined. The phenotype of $CD8^+$ T-cells at the inflamed site of human autoimmune arthritis is heterogeneous, with both pro- and anti-inflammatory features [reviewed in (35)]. In SF of RA patients for example, $CD8^+$ T-cells are characterized by increased expression of activation markers (CD80, CD86, CD25), pro-inflammatory cytokines like IL-6 and TNFα, and with a proliferative signature, but also by elevated levels of negative co-stimulatory markers, such as TIM-3 and PD-1 (36, 40, 41). In affected kidneys of SLE patients, the majority of $CD8^+$ T-cells are located in the periglomerular regions where tissue damage occurs, and this infiltrate is correlated with renal injury (42). Despite multiple studies reporting $CD8^+$ T-cells accumulation in SLE affected tissues, the phenotype of local $CD8^+$ T-cells remains largely unexplored. One study reports a differentiated effector memory phenotype with loss of CD28 on infiltrating $CD8^+$ T-cells, indicating active involvement of these cells in disease pathology (43). However, although counter-intuitive for effector cells in an autoimmune environment, a recent study shows that kidney-infiltrating T-cells are metabolically and functionally "exhausted" in three mouse models of lupus nephritis (44). The term "exhausted" stems from numerous studies on $CD8^+$ T-cells in chronic viral infections and, to a lesser extent, in cancers. Chronic antigen exposure in these settings goes along with the upregulation of negative co-stimulatory markers, such as PD-1 in combination with reduced secretion of effector cytokines and proliferation. This has led to the hypothesis that these cells are terminally differentiated and severely functionally impaired (45, 46). However, recently this concept was challenged by the discovery that PD-1^+ $CD8^+$ T-cells are functionally adapted cells able to control the viral load or tumor cells without causing excessive immune pathology, and can be therapeutically reinvigorated by blocking PD-1/PD-L1 interaction (47, 48).

Chronic Stimulation in Auto-Immune Inflammation: From an Exhausted to a Trm Phenotype

Not much is known about the functional profile of tissue-specific memory $CD8^+$ T-cell in human chronic autoimmune diseases. Like $CD8^+$ T-cells in infectious diseases and tumors, these cells are chronically activated. However, instead of resulting in strengthened regulation as is described for the aforementioned conditions, chronic stimulation in autoimmune diseases seems to lead toward overzealous and pathogenic effector functions. Previous observations from peripheral blood derived $CD8^+$ T-cells show that the transcriptional signature reflecting exhaustion is associated with poor clearance of chronic viral infection, but conversely predicts better prognosis in multiple auto-immune diseases, including SLE (49). A recent paper addressing the enriched PD-1^+ $CD8^+$ T-cell population in SF of JIA patients is the first to study this cell subset locally, derived from the site of inflammation in humans (50). In this setting, PD-1 expressing $CD8^+$ T-cells maintain their effector function, such as pro-inflammatory cytokine production, cytotoxic profile,

and use of glycolysis as a metabolic pathway and thereby are suspected to have a detrimental role in autoimmune tissue damage. Strong inflammatory signals, and high levels of soluble PD-1 that block interaction with APCs (51), may overrule or hamper PD-1-signaling in $CD8^+$ T-cells in SF of JIA patients. In line with this, Odorizzi et al. have shown that PD-1 expression is not a prerequisite for exhaustion to occur, by using a mouse model of chronic viral infection with genetic absence of PD-1. In this model, PD-1 prevented $CD8^+$ T-cells overstimulation and apoptosis, as the absence of PD-1 led to more cytotoxic but terminally differentiated $CD8^+$ T-cells (47). It is tempting to speculate that this mechanism may also play a role in local auto-immune inflammation, leading to survival of auto-antigen induced clonally expanding effector cells.

In line with this, other T-cell subsets have been described that may be induced by chronic stimulation and acquire a pathogenic role. The latter is reflected by the secretion of inflammatory cytokines and expression of granzymes and perforin. These include $CD8^+$ T-cells expressing CD57 (52, 53) and $CD4^+/CD8^+$ T-cells that have downregulated CD28, so called $CD28^{null}$ cells (54, 55). Some reports have demonstrated that these cells are also present in SF of RA patients, whereas others failed to confirm this (56, 57). There are indications that these chronically activated $CD4^+$ and $CD8^+$ T-cells cells are induced by CMV infection in RA patients (58), although a causative linkage has not been established thus far (54, 59).

Interestingly, the combined cytotoxic and regulatory profile of $CD8^+$ T-cells, defined by increased expression of effector molecules, such as Granzyme B on the one hand, and of negative co-stimulatory molecules on the other hand, is also typical for Trm cells (60). In line with this, the $PD\text{-}1^+$ $CD8^+$ cells in SF were shown to be enriched for a Trm transcriptional profile compared to the $PD\text{-}1^-$ $CD8^+$ T-cells from the same environment (50). Furthermore, Trm are defined by CD69 expression and downregulation of S1PR1, which is also found on $PD\text{-}1^+$ $CD8^+$ cells derived from SF of JIA patients. So local $CD8^+$ T-cells from the site of autoimmune inflammation in JIA cannot be classified as exhausted, despite some overlapping features, but share much of their profile with Trm cells. Interestingly, tumor-infiltrating CD8+ T-cells in human cancers also display a Trm profile (61). Moreover, a recent paper describing the transcriptional, metabolic, and functional signatures of intra-tumoral $PD\text{-}1^+$ $CD8^+$ T-cells has revealed that, next to many commonalities, such as impaired cytokine production, these cells also differ from exhausted cells as described in chronic infections (62). The intra-tumoral $CD8^+$ T-cells showed increased proliferation and glycolysis, and lack of enrichment of the exhausted T-cell gene signature (62), as was observed in SF of JIA patients. The interpretation now arises that $CD8^+$ "exhausted" cells are a heterogeneous group of memory cells with diverse differentiation states but all driven by persistence of antigen that induces upregulation of inhibitory receptors, and with functional properties that are influenced by the environment (63, 64). This concept shares many features with the current view on Trm cells as they are also regarded as highly specialized cells with a tissue adapted profile, tightly regulated to prevent excessive tissue damage. The commonalities between $CD8^+$ T-cells at inflammatory sites and the Trm profile suggest interplay between inflammation and tissue residency. Indeed, several studies indicate that inflammation is the trigger for initial homing of Trm cells, by providing the migratory signals needed to direct them to tissues (61). PD-1 expressing $CD8^+$T-cells in an inflammatory exudate, such as SF of JIA could be tissue derived, but how they developmentally relate to Trm cells remains unknown. All in all, local $CD8^+$ T-cells situated in the affected tissue of chronic inflammation are heterogeneous with both effector and regulatory responses that are highly influenced by chronic inflammation, possibly with disease specific profiles.

FOXP3 Regulatory T-Cells Responses in Local Pathogenesis

Overzealous $CD4^+$ Th responses carry the risk of initiating detrimental pro-inflammatory responses that can result in collateral tissue damage. Thus, the maintenance of immune homeostasis and prevention of immunopathology requires tight regulatory mechanisms. Regulatory T-cells (Treg) are a subset of $CD4^+$ T-cells with unique homeostatic functions. Absence of the Treg master transcription FOXP3 leads to fatal multi-organ autoimmunity in mice and men (65, 66). The capacity of Treg to dampen immune responses have made them attractive therapeutic targets in diverse settings, such as in autoimmune diseases, transplantation, and cancer. The best discriminative surface markers for Treg are high expression of CD25 (IL-2 receptor α) in combination with low expression of CD127 (IL-7 receptor α). Early studies often used solely high CD25 expression to identify or purify Treg, resulting in contamination with activated conventional CD4+ T-cells, and contradicting data.

Effector and Polarized Treg

Traditionally, the Treg lineage was considered as a homogeneous group of committed cells with suppressive capacities toward other immune cells. As it increasingly becoming apparent that most, if not all, immune cells have the capacity to adapt to their environment, data from the last decade demonstrated that Treg are perhaps the most heterogeneous in phenotype and function (67). Their high turnover and sensitivity to environmental signals leads to a large degree of adaptation that allows Treg to control diverse immune responses and even to exert tissue-specific functions. In that respect novel Treg phenotypes have been identified that differ markedly from their naïve recirculating counterparts (central Treg). Liston and Gray have proposed the model of environment-instructed effector Treg and polarized Treg differentiation (68). Effector Treg, or eTreg, are characterized in humans by $Foxp3^{high}CD25^{high}CD45RA^{low}$ expression representing a small fraction of circulating Treg (69). They have signs of recent antigen encounter, have an heightened activation status and migratory potential, and express markers similar to activated conventional $CD4^+$ T-cells while maintaining Treg functions (68). For example, increased expression of Foxp3, as well as typical functional markers, such as ICOS and CTLA4 is observed in eTreg (69, 70). Polarized (tissue-resident)

Treg are present in non-lymphoid tissues, express specific homing receptors and exert tissue-specific functions and immune regulation. Treg do so by utilizing the transcription factor program of the population they are suppressing, or tissue specific transcription factors, respectively (67, 68, 71). For example, Treg that co-express T-BET next to FOXP3 can efficiently suppress Th1 responses (72), whereas the expression of adipose tissue-specific peroxisome proliferator-activated receptor gamma (PPARγ) is needed for Treg to control insulin sensitivity (73). In the latter case, Treg are tissue-resident in a physiological condition and there is an increasing list of tissue specific phenotypes of tissue-resident Treg exerting non-immunological but tissue-homeostatic functions [reviewed in (67)]. To what extend the profile of conventional Trm is applicable to tissue-resident Treg remains to be explored.

Using the proper gating strategy for Treg markers, the frequency of Treg in rheumatic diseases is increased at the site of inflammation in JIA and JDM (74–76). Whether Treg function is impaired in these diseases is still under debate, partly related to differences in phenotyping markers and conditions of *in vitro* assays used to test Treg functionality. Multiple studies have however shown that Treg derived from SF of JIA patients maintain their suppressive function and upregulate Treg functional markers, such as CD25, CTLA4, and GITR, rather pointing toward an eTreg profile (77–79) (**Figure 1**).

Treg Stability

Instability of Treg has long been suspected to play a role in disease pathology. Instability is defined by loss of FOXP3 expression and suppressive function, with a concomitant acquisition of an effector phenotype. The stability of Treg is a contentious issue, with contradicting data from several studies (80). Multiple mouse models, including genetic fate-mapping models that allow tracking Foxp3 expressing cells, revealed that Treg are fairly stable *in vivo* with a small proportion of cells that lose Foxp3 expression (81–84).

At the site of autoimmune inflammation in humans, FOXP3-expressing Treg that produce pro-inflammatory cytokines have been described (85, 86). In specific tissues however, it is unknown if aberrant adapted Treg add to disease pathogenesis. In this regard, it is important to distinguish between functional plasticity/adaptability and lineage instability. In JIA, a small fraction of SF Treg expresses CD161 and is capable of producing pro-inflammatory cytokines. At the same time, FOXP3 expression remains high and suppressive capacity is also maintained (87, 88). Another paper studying Treg stability in SF of JIA patients, demonstrated that the T-cell receptor (TCR) repertoires of Treg is very distinct from conventional T-cells in SF, indicating a different origin and thus excluding a large degree of instability of Treg (89). On top of that, the same paper showed that Treg need inflammatory signals present in SF to maintain their FOXP3 expression, supporting the idea that local signals in an inflammatory environment can stabilize or even enhance the Treg phenotype.

Systemic administration of IL-2 as a therapy to maintain and possibly expand Treg is currently being tested for SLE patients. A recent paper reports on reduced CD25 expression on peripheral blood Treg of SLE patients, that correlates to the reduced production of IL-2 from circulating memory T-cells (90). Since the increased expression of CREMα leads to reduced IL-2 production of effector T-cells, and IL-2 receptor (CD25) signaling via STAT5 is pivotal for maintained Foxp3 expression in Treg, impaired Treg function could be a consequence (91). This provides a rationale for Treg targeted therapy by low dose IL-2 administration (92). However, it is not known whether the reduced CD25 expression on Treg also occurs at the site of inflammation. Moreover, in peripheral blood of active SLE patients enhanced levels of functional, non-cytokine producing Helios$^+$ Treg have been identified that positively correlate with disease activity (93, 94). In addition, these cells were shown to express CXCR3 and CCR4, allowing them to migrate to inflammatory sites (93).

The stability of Treg is regulated on multiple levels. The acquisition of a specific epigenetic landscape is a strong determinant (95), as well as transcriptional and post-transcriptional regulation of FOXP3. *In vitro* studies have shown that environmental cues can modulate these processes. For example ubiquitination of FOXP3, that targets its proteosomal degradation, is highly regulated by the ubiquitin ligase Stub1 and the deubiquitinating enzyme USP7. *In vitro*, inflammatory stimuli allow Stub1 to bind FOXP3 and promote its degradation, which is further facilitated by the downregulation of USP7 (96, 97). However, *ex vivo* gene expression analysis of SF Treg from JIA patients shows stable expression of both proteins (Mijnheer et al., unpublished data). This illustrates that *in vivo* regulation of FOXP3 and Treg function is a highly complex organized process in which multiple proteins are involved. Also, depending on the environmental conditions, different proteins can take part in this network, allowing fine-tuning of the cells to a specific environment and further polarization (81). Altogether, there are no indications for Treg instability on a large scale *in vivo*, but inflammation does seem to impact Treg by differentiation toward an eTreg phenotype.

Resistance of CD4$^+$ and CD8$^+$ Cells to Suppression

Unresponsiveness of T-cells to Treg suppression is most likely a normal transient phenomenon during the assembly of an immune response to clear an infectious threat. However, in auto-immune diseases this resistance of local effector T-cells to suppression contributes to a sustained inflammatory response and subsequent disease pathogenesis (98). In JIA, both CD4$^+$ and CD8$^+$ T-cells from the synovial fluid of affected joints have been found to be intrinsically resistant to suppression (78, 79, 99). The same has been described for CD4$^+$ T-cells from SLE patients (100). The resistance to suppression is at least partly mediated by protein kinase B (PKB)/c-akt hyper-activation, induced by local cytokines. TNFα and IL-6 were found to induce resistance in CD4$^+$ T-cells, whereas TNFα and autocrine release of IFNγ were responsible for the intrinsic resistance in CD8$^+$ T-cells (79, 99). The hyperphosphorylation of PKB/c-akt is interesting in the light of PD-1 signaling, since PD-1 is a strong negative regulator of PKB/c-akt (101). Apparently PD-1 signaling in SF T-cells is

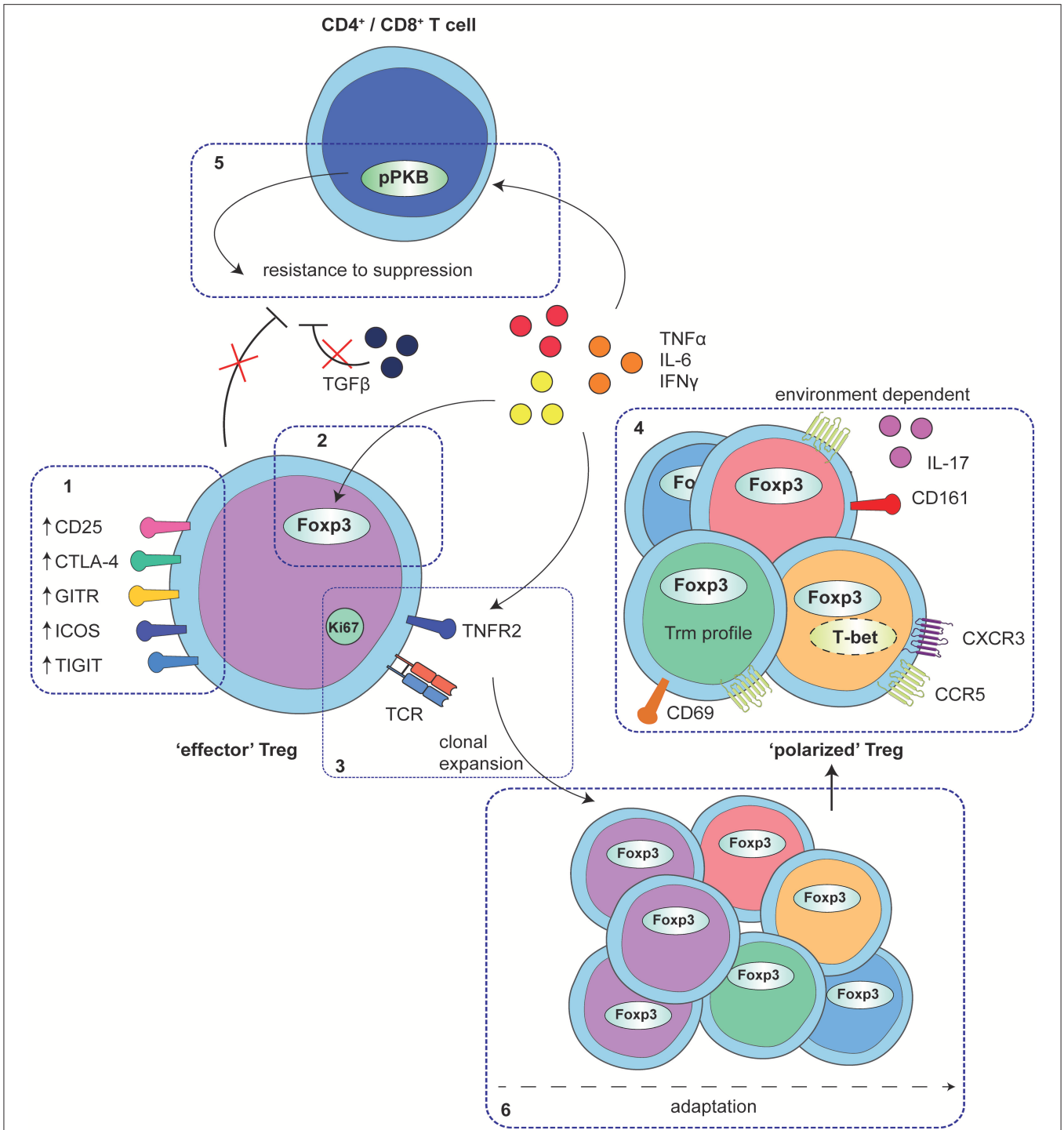

FIGURE 1 | Adaptation of Treg to local auto-immune inflammation. At the site of human autoimmune-inflammation functional Treg are present that display an effector phenotype. This phenotype is characterized by increased expression of functional Treg markers, including CD25, CTLA4, GITR, ICOS, and TIGIT (1) and stable increased expression of Foxp3, and at least in part, instructed by local inflammatory signals (2). Furthermore, Treg are clonally expanding as is reflected by increased Ki67 expression and a local clonal TCR repertoire, possibly mediated by TNFα signaling via TNFR2 on Treg (3). In addition to the effector profile, Treg may also display a specific environment instructed profile, including e.g., expression of CD161 and IL-17 production, upregulation of Th1- and inflammation-associated markers and chemokine receptors and/or characteristics of a Trm profile (4). These polarization profiles are not exclusive but rather are overlapping, depending on the specific local conditions. Whereas, adapted Treg are functional, local cytokines produced by monocytes and fibroblasts also affect $CD4^+$ and $CD8^+$ T-cells, in part by hyper-phosphorylation of PKB/c-AKT, conferring resistance of local $CD4^+$ and $CD8^+$ T-cells to Treg suppression (5). Overall, the process of Treg adaptation in inflammatory settings is highly influenced by the local environment, most likely starting with an expanding effector population that can be further fine-tuned with environmental adaptations (6).

not sufficient enough to downregulate this signaling pathway. Blockade of TNFα can restore the susceptibility of CD4 and CD8 T-cells to suppression, which is in line with the effectiveness of therapies targeting TNFα (99, 102). In IBD, resistance of lamina propria T-cells to Treg-mediated suppression has been described as well. In this setting high levels of Smad7, causing insensitivity to TGFβ, have been related to the resistance of effector cells (103). Thus, impaired regulation of the local immune response involves resistance of effector cells, possibly despite functional Tregs.

Local T-Cell Clonal Expansion

When activated T-cells encounter their cognate antigen in the context of co-stimulation and cytokines, specific clones will expand to initiate a robust adaptive immune response. A diverse repertoire of T-cell receptors (TCRs) of conventional T-cells allows a response to a multitude of possible pathogens. Also thymic derived Treg need a diverse TCR repertoire to regulate auto-immune responses. The TCR repertoire of Treg, representing only a small fraction of the total T-cell pool, is equally diverse as the larger effector pool (104). The need for a diverse (auto-antigen specific) TCR repertoire of Treg has been illustrated by studies using transgenic mice with a restricted TCR repertoire of Treg. In these models, a loss of tolerance toward commensal bacteria and develop autoimmune diseases has been observed (105–108).

At the site of human autoimmune inflammation in both JIA and SLE, clonal T-cell expansions of $CD4^+$ T-cell, $CD8^+$ T-cell and $FOXP3^+$ Treg populations are found (43, 50, 89, 109, 110). Remarkably, especially Treg were found to be hyper-expanded, and to express high levels of Ki67. It is possible that dominant auto-antigens present at the affected sites induce this proliferation (79, 89). Local TNFα can also act as a contributing factor to Treg expansion, as TNFα induces Treg proliferation and effector Treg differentiation via TNFR2 signaling [(111–114); Mijnheer et al., unpublished data]. Since hyper-expanded Treg clones were demonstrated to display a very distinct repertoire compared to conventional $CD4^+$ T-cells, local induction of Treg from conventional T-cells is unlikely (89). Tissue resident Treg also show a considerable oligoclonality regarding their TCR repertoire, supporting the notion that tissue Treg and Treg from the site of autoimmune inflammation share typical features (115, 116) (**Figure 1**). Interestingly, in refractory JIA and JDM patients undergoing hematopoietic stem cell transplantation the TCR repertoire of circulating Treg prior to transplantation was also found to be highly clonal. After transplantation, the Treg TCR repertoire became more diverse over time, except for one patient that experienced a relapse (117). These dates suggest that Treg TCR repertoire abnormalities may contribute to disease pathogenesis, possibly by limiting the chance of antigen encounter, thereby being outcompeted by the less restricted Teff.

Recent data from multiple affected joints in RA show that total TCR repertoires are substantially overlapping (118). In JIA patients, especially $PD\text{-}1^+$ $CD8^+$ T-cells were shown to have a clonal repertoire, with a high overlap in dominant clones between different affected joints (35, 62). The overlap may be explained by the presence of common antigens that drive the disease at multiple sites, and/or (re)circulation of dominant T-cell clones. In affected kidneys of SLE patients expanded $CD8^+$ T-cells clones were found to be present for years in sequential biopsies (43), suggesting long-term persistence of dominant T-cell clones. It is tempting to speculate that these dominant T-cell clones play a role in disease relapses, but further studies are need to conclude this.

CONCLUSION AND FUTURE PERSPECTIVES

The expanding field of T-cells, including the discovery of multiple Th subsets as well as observations of mixed phenotypes in inflamed tissues, has made the classification of subsets increasingly complex (5). This complexity, however, likely represents human immunity that is continuously exposed to multiple microorganisms and environmental conditions, in contrast to highly controlled mouse models that have contributed to most of the current knowledge (32, 119). A more nuanced view on T-cells in tissues now arises, with fine-tuning of immune cells to the local environment allowing tailored responses explaining the observed diversity in phenotypes. When inflammation becomes chronic this fine-tuning of T-cells might be unrestrained or insufficient, and as a result cause or contribute to pathogenesis. Potential determinants in this process could be the strength and frequency of TCR stimulation, as well as the presence of absence of CD4 help or co-stimulation, whereas local metabolites also seem to be important. How this is regulated exactly, and what the importance is of different factors remains to be answered, as well as the question what is different in affected human tissue in disease vs. healthy human tissue. The possibilities of gaining new insight are enormous, with newly developed high-throughput technologies that require only small numbers of cells and that allow for analysis of rare T-cell populations from small tissue samples. Single cell sequencing combined with TCR sequencing and mass cytometry on whole tissues will give a deeper understanding about the heterogeneity of T-cells present in human autoimmunity. This, together with smart use of patients samples, such as sequential sampling and sampling from multiple affected sites, will provide novel insights and undoubtedly improve the therapeutic options for patients with rheumatic diseases.

AUTHOR CONTRIBUTIONS

GM and FvW have designed the structure and discussed the content of the manuscript. GM has searched the literature and has drafted the text and figure. FvW has critically edited the manuscript.

REFERENCES

1. Moro-García MA, Mayo JC, Sainz RM, Alonso-Arias R. Influence of inflammation in the process of T lymphocyte differentiation: proliferative, metabolic, and oxidative changes. *Front Immunol.* (2018) 9:339. doi: 10.3389/fimmu.2018.00339
2. Okada Y, Kishikawa T, Sakaue S, Hirata J. Future directions of genomics research in rheumatic diseases. *Rheum Dis Clin N Am.* (2017) 43:481–7. doi: 10.1016/j.rdc.2017.04.009
3. Loo TT, Gao Y, Lazarevic V. Transcriptional regulation of $CD4^+$ TH cells that mediate tissue inflammation. *J. Leukoc. Biol.* (2018) 104:1069–85. doi: 10.1002/JLB.1RI0418-152RR
4. Deng Y, Wang Z, Chang C, Lu L, Lau CS, Lu Q. Th9 cells and IL-9 in autoimmune disorders: Pathogenesis and therapeutic potentials. *Hum Immunol.* (2017) 78:120–8. doi: 10.1016/j.humimm.2016.12.010
5. Palmer MT, Weaver CT. Autoimmunity: increasing suspects in the $CD4^+$ T cell lineup. *Nat Immunol.* (2010) 11:36–40. doi: 10.1038/ni.1802
6. Skapenko A, Leipe J, Lipsky PE, Schulze-Koops H. The role of the T cell in autoimmune inflammation. *Arthritis Res Ther.* (2005) 7(Suppl. 2):S4. doi: 10.1186/ar1703
7. Okada S, Markle JG, Deenick EK, Mele F, Averbuch D, Lagos M, et al. Immunodeficiencies. Impairment of immunity to Candida and Mycobacterium in humans with bi-allelic RORC mutations. *Science.* (2015) 349:606–13. doi: 10.1126/science.aaa4282
8. Gensous N, Charrier M, Duluc D, Contin-Bordes C, Truchetet M-E, Lazaro E, et al. T follicular helper cells in autoimmune disorders. *Front Immunol.* (2018) 9:1637. doi: 10.3389/fimmu.2018.01637
9. Masutani K, Akahoshi M, Tsuruya K, Tokumoto M, Ninomiya T, Kohsaka T, et al. Predominance of Th1 immune response in diffuse proliferative lupus nephritis. *Arthritis Rheum.* (2001) 44:2097–106. doi: 10.1002/1529-0131(200109)44:9<2097::AID-ART360>3.0.CO;2-6
10. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med.* (2002) 195:1129–43. doi: 10.1084/jem.20011956
11. Wedderburn LR, Robinson N, Patel A, Varsani H, Woo P. Selective recruitment of polarized T cells expressing CCR5 and CXCR3 to the inflamed joints of children with juvenile idiopathic arthritis. *Arthritis Rheum.* (2000) 43:765–74. doi: 10.1002/1529-0131(200004)43:4<765::AID-ANR7>3.0.CO;2-B
12. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi J, Oyamada A, et al. Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints. *J Rheumatol.* (2011) 38:1569–75. doi: 10.3899/jrheum.101355
13. Cosmi L, Cimaz R, Maggi L, Santarlasci V, Capone M, Borriello F, et al. Evidence of the transient nature of the Th17 phenotype of $CD4^+CD161^+$ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* (2011) 63:2504–15. doi: 10.1002/art.30332
14. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci USA.* (2010) 107:14751–6. doi: 10.1073/pnas.1003852107
15. Maggi L, Santarlasci V, Capone M, Peired A, Frosali F, Crome SQ, et al. CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *Eur J Immunol.* (2010) 40:2174–81. doi: 10.1002/eji.200940257
16. Maggi L, Cimaz R, Capone M, Santarlasci V, Querci V, Simonini G, et al. Brief report: etanercept inhibits the tumor necrosis factor α-driven shift of Th17 lymphocytes toward a nonclassic Th1 phenotype in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2014) 66:1372–7. doi: 10.1002/art.38355
17. Stadhouders R, Lubberts E, Hendriks RW. A cellular and molecular view of T helper 17 cell plasticity in autoimmunity. *J Autoimmun.* (2018) 87:1–15. doi: 10.1016/j.jaut.2017.12.007
18. Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 and non-classic Th1 cells in chronic inflammatory disorders: two sides of the same coin. *Int Arch Allergy Immunol.* (2014) 164:171–7. doi: 10.1159/000363502
19. Wu X, Tian J, Wang S. Insight into non-pathogenic Th17 cells in autoimmune diseases. *Front Immunol.* (2018) 9:1112. doi: 10.3389/fimmu.2018.01112
20. Aschenbrenner D, Foglierini M, Jarrossay D, Hu D, Weiner HL, Kuchroo VK, et al. An immunoregulatory and tissue-residency program modulated by c-MAF in human TH17 cells. *Nat. Immunol.* (2018) 19:1126–36. doi: 10.1038/s41590-018-0200-5
21. Crispín JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol.* (2008) 181:8761–6. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/19050297
22. De Pasquale L, D'Amico A, Verardo M, Petrini S, Bertini E, De Benedetti F. Increased muscle expression of interleukin-17 in Duchenne muscular dystrophy. *Neurology.* (2012) 78:1309–14. doi: 10.1212/WNL.0b013e3182518302
23. Zickert A, Amoudruz P, Sundström Y, Rönnelid J, Malmström V, Gunnarsson I. IL-17 and IL-23 in lupus nephritis–association to histopathology and response to treatment. *BMC Immunol.* (2015) 16:7. doi: 10.1186/s12865-015-0070-7
24. Ohl K, Nickel H, Moncrieffe H, Klemm P, Scheufen A, Föll D, et al. The transcription factor CREM drives an inflammatory phenotype of T cells in oligoarticular juvenile idiopathic arthritis. *Pediatr Rheumatol.* (2018) 16:39. doi: 10.1186/s12969-018-0253-x
25. Rauen T, Hedrich CM, Juang Y-T, Tenbrock K, Tsokos GC. cAMP-responsive element modulator (CREM)α protein induces interleukin 17A expression and mediates epigenetic alterations at the interleukin-17A gene locus in patients with systemic lupus erythematosus. *J Biol Chem.* (2011) 286:43437–46. doi: 10.1074/jbc.M111.299313
26. Katsuyama T, Tsokos GC, Moulton VR. Aberrant T cell signaling and subsets in systemic lupus erythematosus. *Front Immunol.* (2018) 9:1088. doi: 10.3389/fimmu.2018.01088
27. Schmidt T, Paust H-J, Krebs CF, Turner J-E, Kaffke A, Bennstein SB, et al. Function of the Th17/interleukin-17A immune response in murine lupus nephritis. *Arthritis Rheumatol.* (2015) 67:475–87. doi: 10.1002/art.38955
28. Liarski VM, Kaverina N, Chang A, Brandt D, Yanez D, Talasnik L, et al. Cell distance mapping identifies functional T follicular helper cells in inflamed human renal tissue. *Sci Transl Med.* (2014) 6:230ra46. doi: 10.1126/scitranslmed.3008146
29. Penatti A, Facciotti F, De Matteis R, Larghi P, Paroni M, Murgo A, et al. Differences in serum and synovial $CD4^+$ T cells and cytokine profiles to stratify patients with inflammatory osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther.* (2017) 19:103. doi: 10.1186/s13075-017-1305-1
30. Wang S, Wang J, Kumar V, Karnell JL, Naiman B, Gross PS, et al. IL-21 drives expansion and plasma cell differentiation of autoreactive $CD11chiT\text{-}bet^+$ B cells in SLE. *Nat Commun.* (2018) 9:1758. doi: 10.1038/s41467-018-03750-7
31. Zhang C-J, Wang C, Jiang M, Gu C, Xiao J, Chen X, et al. Act1 is a negative regulator in T and B cells via direct inhibition of STAT3. *Nat Commun.* (2018) 9:2745. doi: 10.1038/s41467-018-04974-3
32. Wong MT, Ong DEH, Lim FSH, Teng KWW, McGovern N, Narayanan S, et al. A high-dimensional atlas of human T cell diversity reveals tissue-specific trafficking and cytokine signatures. *Immunity.* (2016) 45:442–56. doi: 10.1016/j.immuni.2016.07.007
33. Lu Y, Biancotto A, Cheung F, Remmers E, Shah N, McCoy JP, et al. Systematic analysis of cell-to-cell expression variation of T lymphocytes in a human cohort identifies aging and genetic associations. *Immunity.* (2016) 45:1162–75. doi: 10.1016/j.immuni.2016.10.025
34. Kumar BV, Connors TJ, Farber DL. Human T cell development, localization, and function throughout life. *Immunity.* (2018) 48:202–13. doi: 10.1016/j.immuni.2018.01.007
35. Petrelli A, van Wijk F. $CD8^+$ T cells in human autoimmune arthritis: the unusual suspects. *Nat Rev Rheumatol.* (2016) 12:421–8. doi: 10.1038/nrrheum.2016.74
36. Cho B-A, Sim JH, Park JA, Kim HW, Yoo W-H, Lee S-H, et al. Characterization of effector memory $CD8^+$ T cells in the synovial fluid of rheumatoid arthritis. *J Clin Immunol.* (2012) 32:709–20. doi: 10.1007/s10875-012-9674-3

37. Costello P, Bresnihan B, O'Farrelly C, FitzGerald O. Predominance of $CD8^+$ T lymphocytes in psoriatic arthritis. *J Rheumatol.* (1999) 26:1117–24. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/10332977
38. Hunter PJ, Nistala K, Jina N, Eddaoudi A, Thomson W, Hubank M, et al. Biologic predictors of extension of oligoarticular juvenile idiopathic arthritis as determined from synovial fluid cellular composition and gene expression. *Arthritis Rheum.* (2010) 62:896–907. doi: 10.1002/art.27284
39. McKinney EF, Lyons PA, Carr EJ, Hollis JL, Jayne DRW, Willcocks LC, et al. A $CD8^+$ T cell transcription signature predicts prognosis in autoimmune disease. *Nat Med.* (2010) 16:586–91, 1p following 591. doi: 10.1038/nm.2130
40. Carvalheiro H, Duarte C, Silva-Cardoso S, da Silva JAP, Souto-Carneiro MM. $CD8^+$ T cell profiles in patients with rheumatoid arthritis and their relationship to disease activity. *Arthritis Rheum.* (2015) 67:363–71. doi: 10.1002/art.38941
41. Li S, Peng D, He Y, Zhang H, Sun H, Shan S, et al. Expression of TIM-3 on $CD4^+$ and $CD8^+$ T cells in the peripheral blood and synovial fluid of rheumatoid arthritis. *APMIS.* (2014) 122:899–904. doi: 10.1111/apm.12228
42. Couzi L, Merville P, Deminière C, Moreau J-F, Combe C, Pellegrin J-L, et al. Predominance of $CD8^+$ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis. *Arthritis Rheum.* (2007) 56:2362–70. doi: 10.1002/art.22654
43. Winchester R, Wiesendanger M, Zhang H-Z, Steshenko V, Peterson K, Geraldino-Pardilla L, et al. Immunologic characteristics of intrarenal T cells: trafficking of expanded $CD8^+$ T cell β-chain clonotypes in progressive lupus nephritis. *Arthritis Rheum.* (2012) 64:1589–600. doi: 10.1002/art.33488
44. Tilstra JS, Avery L, Menk AV, Gordon RA, Smita S, Kane LP, et al. Kidney–infiltrating T cells in murine lupus nephritis are metabolically and functionally exhausted. *J Clin Invest.* (2018) 128:4884–97. doi: 10.1172/JCI120859
45. Gallimore A, Glithero A, Godkin A, Tissot AC, Plückthun A, Elliott T, et al. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med.* (1998) 187:1383–93. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/9565631
46. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature.* (1993) 362:758–61. doi: 10.1038/362758a0
47. Odorizzi PM, Pauken KE, Paley MA, Sharpe A, Wherry EJ. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted $CD8^+$ T cells. *J Exp Med.* (2015) 212:1125–37. doi: 10.1084/jem.20142237
48. Speiser DE, Utzschneider DT, Oberle SG, Münz C, Romero P, Zehn D. T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion? *Nat Rev Immunol.* (2014) 14:768–74. doi: 10.1038/nri3740
49. McKinney EF, Lee JC, Jayne DRW, Lyons PA, Smith KGC. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature.* (2015) 523:612–6. doi: 10.1038/nature14468
50. Petrelli A, Mijnheer G, van Konijnenburg DPH, van der Wal MM, Giovannone B, Mocholi E, et al. PD-$1^+$$CD8^+$ T cells are clonally expanding effectors in human chronic inflammation. *J Clin Invest.* (2018) 128:4669–81. doi: 10.1172/JCI96107
51. Bommarito D, Hall C, Taams LS, Corrigall VM. Inflammatory cytokines compromise programmed cell death-1 (PD-1)-mediated T cell suppression in inflammatory arthritis through up-regulation of soluble PD-1. *Clin Exp Immunol.* (2017) 188:455–66. doi: 10.1111/cei.12949
52. Chiang SCC, Theorell J, Entesarian M, Meeths M, Mastafa M, Al-Herz W, et al. Comparison of primary human cytotoxic T-cell and natural killer cell responses reveal similar molecular requirements for lytic granule exocytosis but differences in cytokine production. *Blood.* (2013) 121:1345–56. doi: 10.1182/BLOOD-2012-07-442558
53. Le Priol Y, Puthier D, Lécureuil C, Combadière C, Debré P, Nguyen C, et al. High cytotoxic and specific migratory potencies of senescent $CD8^+$ $CD57^+$ cells in HIV-infected and uninfected individuals. *J Immunol.* (2006) 177:5145–54. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/17015699
54. Dumitriu IE. The life (and death) of $CD4^+$ CD28 null T cells in inflammatory diseases. *Immunology.* (2015) 146:185–93. doi: 10.1111/imm.12506
55. Kared H, Martelli S, Ng TP, Pender SLF, Larbi A. CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunol Immunother.* (2016) 65:441–52. doi: 10.1007/s00262-016-1803-z
56. Fasth AER, Snir O, Johansson AAT, Nordmark B, Rahbar A, Af Klint E, et al. Skewed distribution of proinflammatory $CD4^+$CD28null T cells in rheumatoid arthritis. *Arthritis Res Ther.* (2007) 9:R87. doi: 10.1186/ar2286
57. Schmidt D, Martens PB, Weyand CM, Goronzy JJ. The repertoire of $CD4^+$ $CD28^-$ T cells in rheumatoid arthritis. *Mol Med.* (1996) 2:608–18. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/8898376
58. Hooper M, Kallas EG, Coffin D, Campbell D, Evans TG, Looney RJ. Cytomegalovirus seropositivity is associated with the expansion of $CD4^+CD28^-$ and $CD8^+CD28^-$ T cells in rheumatoid arthritis. *J Rheumatol.* (1999) 26:1452–57. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/10405929
59. Halenius A, Hengel H. Human cytomegalovirus and autoimmune disease. *Biomed Res Int.* (2014) 2014:1–15. doi: 10.1155/2014/472978
60. Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human tissue-resident memory T cells are defined by core transcriptional and functional signatures in lymphoid and mucosal sites. *Cell Rep.* (2017) 20:2921–34. doi: 10.1016/j.celrep.2017.08.078
61. Amsen D, van Gisbergen KPJM, Hombrink P, van Lier RAW. Tissue-resident memory T cells at the center of immunity to solid tumors. *Nat Immunol.* (2018) 19:538–46. doi: 10.1038/s41590-018-0114-2
62. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A transcriptionally and functionally distinct PD-1^+ $CD8^+$ T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. *Nat Med.* (2018) 24:994–1004. doi: 10.1038/s41591-018-0057-z
63. Bengsch B, Ohtani T, Khan O, Setty M, Manne S, O'Brien S, et al. Epigenomic-guided mass cytometry profiling reveals disease-specific features of exhausted CD8 T cells. *Immunity.* (2018) 48:1029–45.e5. doi: 10.1016/j.immuni.2018.04.026
64. Utzschneider DT, Alfei F, Roelli P, Barras D, Chennupati V, Darbre S, et al. High antigen levels induce an exhausted phenotype in a chronic infection without impairing T cell expansion and survival. *J Exp Med.* (2016) 213:1819–34. doi: 10.1084/jem.20150598
65. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet.* (2001) 27:68–73. doi: 10.1038/83784
66. Ochs HD, Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* (2001) 27:20–1. doi: 10.1038/83713
67. Sharma A, Rudra D. Emerging functions of regulatory T cells in tissue homeostasis. *Front Immunol.* (2018) 9:883. doi: 10.3389/fimmu.2018.00883
68. Liston A, Gray DHD. Homeostatic control of regulatory T cell diversity. *Nat Rev Immunol.* (2014) 14:154–65. doi: 10.1038/nri3605
69. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. *Immunity.* (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
70. Cretney E, Kallies A, Nutt SL. Differentiation and function of Foxp3+ effector regulatory T cells. *Trends Immunol.* (2013) 34:74–80. doi: 10.1016/j.it.2012.11.002
71. Panduro M, Benoist C, Mathis D. Tissue Tregs. *Annu Rev Immunol.* (2016) 34:609–33. doi: 10.1146/annurev-immunol-032712-095948
72. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol.* (2009) 10:595–602. doi: 10.1038/ni.1731
73. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, et al. PPAR-? Is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature.* (2012) 486:549. doi: 10.1038/nature11132
74. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. $CD4^+$CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol.* (2004) 172:6435–43. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/15128835

75. Ohl K, Tenbrock K. Regulatory T cells in systemic lupus erythematosus. *Eur J Immunol.* (2015) 45:344–55. doi: 10.1002/eji.201344280
76. Vercoulen Y, Bellutti Enders F, Meerding J, Plantinga M, Elst EF, Varsani H, et al. Increased presence of FOXP3$^+$ regulatory T cells in inflamed muscle of patients with active juvenile dermatomyositis compared to peripheral blood. *PLoS ONE.* (2014) 9:e105353. doi: 10.1371/journal.pone.0105353
77. Chandrasekaran U, Yi W, Gupta S, Weng C-H, Giannopoulou E, Chinenov Y, et al. Regulation of effector Treg cells in murine lupus. *Arthritis Rheum.* (2016) 68:1454–66. doi: 10.1002/art.39599
78. Haufe S, Haug M, Schepp C, Kuemmerle-Deschner J, Hansmann S, Rieber N, et al. Impaired suppression of synovial fluid CD4$^+$CD25$^-$ T cells from patients with juvenile idiopathic arthritis by CD4$^+$CD25$^+$ Treg cells. *Arthritis Rheum.* (2011) 63:3153–62. doi: 10.1002/art.30503
79. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood.* (2011) 118:3538–48. doi: 10.1182/blood-2010-12-328187
80. Lee W, Lee GR. Transcriptional regulation and development of regulatory T cells. *Exp Mol Med.* (2018) 50:e456. doi: 10.1038/emm.2017.313
81. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune disease. *Nat Immunol.* (2018) 19:665–73. doi: 10.1038/s41590-018-0120-4
82. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3$^+$ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci USA.* (2009) 106:1903–8. doi: 10.1073/pnas.0811556106
83. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the regulatory T cell lineage *in vivo*. *Science.* (2010) 329:1667–71. doi: 10.1126/science.1191996
84. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martínez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat Immunol.* (2009) 10:1000–7. doi: 10.1038/ni.1774
85. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic conversion of Foxp3$^+$ T cells into TH17 cells in autoimmune arthritis. *Nat Med.* (2014) 20:62–8. doi: 10.1038/nm.3432
86. MacDonald KG, Dawson NAJ, Huang Q, Dunne JV, Levings MK, Broady R. Regulatory T cells produce profibrotic cytokines in the skin of patients with systemic sclerosis. *J Allergy Clin Immunol.* (2015) 135:946–55.e9. doi: 10.1016/j.jaci.2014.12.1932
87. Duurland CL, Brown CC, O'Shaughnessy RFL, Wedderburn LR. CD161$^+$ Tconv and CD161$^+$ Treg share a transcriptional and functional phenotype despite limited overlap in TCRβ repertoire. *Front Immunol.* (2017) 8:103. doi: 10.3389/fimmu.2017.00103
88. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood.* (2013) 121:2647–58. doi: 10.1182/blood-2012-08-443473
89. Bending D, Giannakopoulou E, Lom H, Wedderburn LR. Synovial regulatory T cells occupy a discrete TCR niche in human arthritis and require local signals to stabilize FOXP3 protein expression. *J Immunol.* (2015) 195:5616–24. doi: 10.4049/jimmunol.1500391
90. Costa N, Marques O, Godinho SI, Carvalho C, Leal B, Figueiredo AM, et al. Two separate effects contribute to regulatory T cell defect in systemic lupus erythematosus patients and their unaffected relatives. *Clin Exp Immunol.* (2017) 189:318–30. doi: 10.1111/cei.12991
91. Solomou EE, Juang YT, Gourley MF, Kammer GM, Tsokos GC. Molecular basis of deficient IL-2 production in T cells from patients with systemic lupus erythematosus. *J Immunol.* (2001) 166:4216–22. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/11238674
92. Mizui M, Tsokos GC. Targeting regulatory T cells to treat patients with systemic lupus erythematosus. *Front Immunol.* (2018) 9:786. doi: 10.3389/fimmu.2018.00786
93. Alexander T, Sattler A, Templin L, Kohler S, Groß C, Meisel A, et al. Foxp3$^+$ Helios$^+$ regulatory T cells are expanded in active systemic lupus erythematosus. *Ann Rheum Dis.* (2013) 72:1549–58. doi: 10.1136/annrheumdis-2012-202216
94. Golding A, Hasni S, Illei G, Shevach EM. The percentage of FoxP3$^+$Helios$^+$ Treg cells correlates positively with disease activity in systemic lupus erythematosus. *Arthritis Rheum.* (2013) 65:2898–906. doi: 10.1002/art.38119
95. Morikawa H, Sakaguchi S. Genetic and epigenetic basis of Treg cell development and function: from a FoxP3-centered view to an epigenome-defined view of natural Treg cells. *Immunol Rev.* (2014) 259:192–205. doi: 10.1111/imr.12174
96. Chen Z, Barbi J, Bu S, Yang H-Y, Li Z, Gao Y, et al. The ubiquitin ligase Stub1 negatively modulates regulatory T cell suppressive activity by promoting degradation of the transcription factor Foxp3. *Immunity.* (2013) 39:272–85. doi: 10.1016/j.immuni.2013.08.006
97. van Loosdregt J, Fleskens V, Fu J, Brenkman AB, Bekker CPJ, Pals CEGM, et al. Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. *Immunity.* (2013) 39:259–71. doi: 10.1016/j.immuni.2013.05.018
98. Wehrens EJ, Prakken BJ, van Wijk F. T cells out of control—impaired immune regulation in the inflamed joint. *Nat Rev Rheumatol.* (2012) 9:34–42. doi: 10.1038/nrrheum.2012.149
99. Petrelli A, Wehrens EJ, Scholman RC, Prakken BJ, Vastert SJ, van Wijk F. Self-sustained resistance to suppression of CD8$^+$ Teff cells at the site of autoimmune inflammation can be reversed by tumor necrosis factor and interferon-γ blockade. *Arthritis Rheumatol.* (2016) 68:229–36. doi: 10.1002/art.39418
100. Venigalla RKC, Tretter T, Krienke S, Max R, Eckstein V, Blank N, et al. Reduced CD4$^+$, CD25$^-$ T cell sensitivity to the suppressive function of CD4$^+$, CD25^{high}, CD127$^-$/low regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum.* (2008) 58:2120–30. doi: 10.1002/art.23556
101. Riley JL. PD-1 signaling in primary T cells. *Immunol Rev.* (2009) 229:114–25. doi: 10.1111/j.1600-065X.2009.00767.x
102. Wehrens EJ, Vastert SJ, Mijnheer G, Meerding J, Klein M, Wulffraat NM, et al. Anti-tumor necrosis factor α targets protein kinase B/c-Akt-induced resistance of effector cells to suppression in juvenile idiopathic arthritis. *Arthritis Rheum.* (2013) 65:3279–84. doi: 10.1002/art.38132
103. Fantini MC, Rizzo A, Fina D, Caruso R, Sarra M, Stolfi C, et al. Smad7 controls resistance of colitogenic T cells to regulatory T cell-mediated suppression. *Gastroenterology.* (2009) 136:1308–16.e1–3. doi: 10.1053/j.gastro.2008.12.053
104. Wing JB, Sakaguchi S. TCR diversity and Treg cells, sometimes more is more. *Eur J Immunol.* (2011) 41:3097–100. doi: 10.1002/eji.201142115
105. Adeegbe D, Matsutani T, Yang J, Altman NH, Malek TR. CD4(+) CD25(+) Foxp3(+) T regulatory cells with limited TCR diversity in control of autoimmunity. *J Immunol.* (2010) 184:56–66. doi: 10.4049/jimmunol.0902379
106. Föhse L, Suffner J, Suhre K, Wahl B, Lindner C, Lee C-W, et al. High TCR diversity ensures optimal function and homeostasis of Foxp3$^+$ regulatory T cells. *Eur J Immunol.* (2011) 41:3101–13. doi: 10.1002/eji.201141986
107. Nishio J, Baba M, Atarashi K, Tanoue T, Negishi H, Yanai H, et al. Requirement of full TCR repertoire for regulatory T cells to maintain intestinal homeostasis. *Proc Natl Acad Sci USA.* (2015) 112:12270–5. doi: 10.1073/pnas.1516617112
108. Yu H, Paiva R, Flavell RA. Harnessing the power of regulatory T cells to control autoimmune diabetes: overview and perspective. *Immunology.* (2017) 153:161–70. doi: 10.1111/imm.12867
109. Henderson LA, Volpi S, Frugoni F, Janssen E, Kim S, Sundel RP, et al. Next-generation sequencing reveals restriction and clonotypic expansion of Treg cells in juvenile idiopathic arthritis. *Arthritis Rheumatol.* (2016) 68:1758–68. doi: 10.1002/art.39606
110. Rossetti M, Spreafico R, Consolaro A, Leong JY, Chua C, Massa M, et al. TCR repertoire sequencing identifies synovial Treg cell clonotypes in the bloodstream during active inflammation in human arthritis. *Ann Rheum Dis.* (2016) 76:135–41. doi: 10.1136/annrheumdis-2015-208992
111. Okubo Y, Mera T, Wang L, Faustman DL. Homogeneous expansion of human T-regulatory cells via tumor necrosis factor receptor 2. *Sci Rep.* (2013) 3:3153. doi: 10.1038/srep03153
112. Vasanthakumar A, Liao Y, Teh P, Pascutti MF, Oja AE, Garnham AL, et al. The TNF receptor superfamily-NF-κB axis is critical to maintain effector

regulatory T cells in lymphoid and non-lymphoid tissues. *Cell Rep*. (2017) 20:2906–20. doi: 10.1016/j.celrep.2017.08.068

113. Wang J, Ferreira R, Lu W, Farrow S, Downes K, Jermutus L, et al. TNFR2 ligation in human T regulatory cells enhances IL2-induced cell proliferation through the non-canonical NF-κB pathway. *Sci Rep*. (2018) 8:12079. doi: 10.1038/s41598-018-30621-4
114. Zaragoza B, Chen X, Oppenheim JJ, Baeyens A, Gregoire S, Chader D, et al. Suppressive activity of human regulatory T cells is maintained in the presence of TNF. *Nat Med*. (2016) 22:16–7. doi: 10.1038/nm.4019
115. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, et al. A special population of regulatory T cells potentiates muscle repair. *Cell*. (2013) 155:1282–95. doi: 10.1016/j.cell.2013.10.054
116. Sanchez Rodriguez R, Pauli ML, Neuhaus IM, Yu SS, Arron ST, Harris HW, et al. Memory regulatory T cells reside in human skin. *J Clin Invest*. (2014) 124:1027–36. doi: 10.1172/JCI72932
117. Delemarre EM, van den Broek T, Mijnheer G, Meerding J, Wehrens EJ, Olek S, et al. Autologous stem cell transplantation benefits autoimmune patients through functional renewal and TCR diversification of the regulatory T cell compartment. *Blood*. (2015) 127:91–101. doi: 10.1182/blood-2015-06-649145
118. Musters A, Klarenbeek PL, Doorenspleet ME, Balzaretti G, Esveldt REE, van Schaik BDC, et al. In rheumatoid arthritis, synovitis at different inflammatory sites is dominated by shared but patient-specific T cell clones. *J Immunol*. (2018) 201:417–22. doi: 10.4049/jimmunol.1800421
119. Eizenberg-Magar I, Rimer J, Zaretsky I, Lara-Astiaso D, Reich-Zeliger S, Friedman N. Diverse continuum of CD4+ T-cell states is determined by hierarchical additive integration of cytokine signals. *Proc Natl Acad Sci USA*. (2017) 114:E6447–56. doi: 10.1073/pnas.1615590114

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REVIEW
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Immunopathogenesis of Juvenile Systemic Sclerosis

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Juvenile-onset systemic sclerosis (jSSc) is a rare and severe autoimmune disease with associated life-threatening organ inflammation and evidence of fibrosis. The organ manifestations of jSSc resemble adult SSc, but with better outcomes and survival. The etiology of jSSc appears to reflect adult-onset SSc, with similar inflammatory mediators and autoantibodies, but with a significant population of children with uncharacterized anti-nuclear antibodies. The genetics of patients with jSSc differ from women with SSc, resembling instead the genes of adult males with SSc, with additional HLA genes uniquely associated with childhood-onset disease. Current treatments are aimed at inhibiting the inflammatory aspect of disease, but important mechanisms of fibrosis regulated by dermal white adipose tissue dendritic cells may provide an avenue for targeting and potentially reversing the fibrotic stage.

Keywords: systemic sclerosis, Pediatric Rheumatology, genetics, disease etiology, autoimmune disease, skin, fibrosis

CLINICAL ASPECTS

General Demographics and Clinical Characteristics

Juvenile systemic sclerosis (jSSc) is a multisystem connective tissue disease characterized by skin induration and widespread fibrosis of internal organs. The incidence has been reported at 0.27–0.50 per million children per year in the UK and Finland (1, 2), with a prevalence of 3 per million (3–5). Less than 5% of SSc has an onset in childhood (6–8). As in adults, there are three main subtypes of jSSc: diffuse cutaneous, limited cutaneous, and overlap (6, 9–12).

The onset of jSSc is usually insidious. Isolated Raynaud phenomenon is often the presenting symptom along with positive antinuclear antibodies (ANA) and nailfold capillary changes. Months or years later, tightening of the skin, especially of fingers and face, cutaneous telangiectasias and symptoms related to internal organ involvement gradually develop [**Table 1**, (6, 9–13)].

Cutaneous changes characteristically evolve in a sequence beginning with oedema, followed by induration and eventually atrophy. Nailfold capillary abnormalities are common, and include capillary dropout, tortuous dilated loops, and occasionally distorted capillary architecture (14, 15).

TABLE 1 | Clinical features of juvenile systemic sclerosis in comparison with adult-onset systemic sclerosis*.

	Juvenile SSc %**	Adult-onset SSc %**
Cutaneous	**66–86**	**64–96**
Sclerodactyly	46–84	52–90
Skin induration	64–86	77–96
Peripheral vascular	**84–100**	**91–100**
Raynaud's phenomenon	84–100	91–100
Digital ulcers	29–50	22–41
Respiratory	**36–55**	**44–64**
Pulmonary arterial hypertension	2–13	14
Pulmonary fibrosis	9–26	22–36
Cardiovascular	**5–18**	**11–25**
Hypertension	3–8	11–17
Cardiac abnormalities	2–17	3–20
Musculoskeletal	**31–42**	**48–71**
Muscle weakness	20–32	15–27
Arthritis	10–35	15–17
Joint contracture	30–45	32–42
Tendon friction rubs	8–11	12–23
Gastrointestinal	**42–74**	**56–78**
Esophageal	24–60	65
Gastric	16–30	26
Ileum-colon	10–15	10–22
Renal	**3–5**	**2–13**
Proteinuria	3–5	6
Renal crisis	0–4	2–13

Cumulative data from references: (6, 9–13).
**During the overall disease course.*

Musculoskeletal symptoms are common in jSSc and characteristically occur at or near onset of the disease in one-third of children (6, 13).

Gastrointestinal involvement affects 42–74% of patients, and has been associated with interstitial lung disease, malnutrition, and poor quality of life (6, 11, 13, 16). Although the esophagus is often involved quite early, many patients are asymptomatic and rarely present heartburn or dysphagia. Malabsorptive diarrhea and delayed colonic transit, when present, reflect long-standing disease.

Cardiopulmonary involvement is the leading cause of morbidity and mortality in jSSc (17, 18). Cardiac inflammation and fibrosis may lead to conduction defects, arrhythmias, and impaired ventricular function. In the largest published study on jSSc, cardiac involvement was found in 8.4% of cases at onset, and in 24% during the overall course of disease (13). Cardiac involvement, especially pericarditis, is a major prognostic factor in jSSc, with a cardiac cause of death in 50% of deceased patients in a large international case series (odds ratio 41.3) (17, 18).

Pulmonary artery hypertension occurs in a minority of jSSc patients and, as in adults, may be either an isolated vascular complication or a consequence of pulmonary fibrosis (6, 13). Interstitial pulmonary fibrosis is reported in approximately one of four children (11, 13). The kidney is rarely affected (1).

Morbidity and Survival

As compared with adults, children with jSSc have overall better outcomes, related to a lower frequency of major visceral organ involvement and lower mortality (6, 13, 17). During follow-up, interstitial lung involvement, gastroesophageal dysmotility, and renal involvement are significantly more common in adults, while arthritis and muscle inflammation are more common in children, because of the higher prevalence of the overlap form of jSSc (3, 4, 6).

Morbidity is a major issue for jSSc, with most patients having multi-organ manifestations. In a recent North American study ($n = 64$ jSSc), 38% were found to have >4 organ systems involved, likely associating with the findings that 36% of the jSSc patients were found to have impaired function (ACR functional scores >1), and 64% reported having pain in the prior week (11). Poorer QOL scores were found to be associated with gastrointestinal symptoms, arthritis and pulmonary diseases (19).

Data from the jSSc Pediatric Rheumatology European Society registry reported a 5, 10, 15, and 20 years survival of 89, 87.4, 87.4, and 82.5%, respectively—significantly better than in adult-onset disease (17). Death in jSSc is usually related to the involvement of cardiac, renal, and pulmonary systems (9, 17, 18). A small subset of jSSc patients can have a rapidly progressive disease course that leads to early death as 60% of deaths are within 5 years (13). A raised creatinine level, pericarditis, and signs of fibrosis on chest X ray at diagnosis are potential risk factors for early mortality, similar to adult-onset SSc (17). In contrast to adult disease, however, malignancy is not an additional risk factor in jSSc for mortality as it has not been associated with jSSc (9, 13, 20, 21).

Autoantibodies and Clinical Associations

Autoimmunity plays an important role in juvenile and adult SSc, with a high prevalence of positive ANA and scleroderma-related autoantibodies, which can further assist clinical characterization and organ risk assessment as an adjunct to skin thickening distribution assignment of limited or diffuse cutaneous (6, 11, 13, 22–24). For example, Anti-topoisomerase antibodies (ATA; Scl-70) would be expected in a patient with diffuse cutaneous SSc, and would be worrisome for rapid skin progression and development of interstitial lung disease (ILD), prompting more aggressive pulmonary monitoring (6, 13, 23).

ANA positivity is reported in 78–97% of patients in the jSSc cohorts (6, 9–13), with scleroderma-associated antibodies reflecting the majority of extractable nuclear antigens causing ANA reactivity, such as ATA (Scl-70), centromere, and U1-RNP as reported in adult SSc, but in divergent frequencies, mirroring the slightly different clinical phenotype in children with SSc (**Table 2**). Another observation is that a significant proportion of jSSc patients, up to 23%, have a positive ANA without a specific extractable nuclear antigen identified, which is rarely observed in adults (6, 11, 13).

TABLE 2 | Comparison between pediatric and adult-onset systemic sclerosis (SSc) subtype and autoantibody distribution.

	Pediatric-Onset SSc	Adult-Onset SSc
Clinical Subsets (%)		
Diffuse cutaneous SSc	30–65	35–45
Limited cutaneous SSc	30–50	40–55
Overlap	**10–39***	**9–18**
Antibody Positivity (%)		
Antinuclear antibody	78–97	90–99
Anti-topoisomerase (ATA, Scl-70)	20–46	20–40
Anti-centromere (ACA)	**2–15†**	**20–30**
Anti-U1 ribonucleoprotein (U1-RNP)	**15–20***	**5**
Anti-polymyositis-scleroderma (PM-Scl)	**15***	**5**
RNA polymerase III (POL3)	**2–4†**	**10–30**

**Significantly higher in pediatric onset SSc compared to adult-onset SSc group.*
† Significantly lower in pediatric onset SSc compared to adult-onset SSc group.
Boldface text highlights a difference between pediatric and adult SSc.
Pediatric Onset data from: juvenile SSc cohorts (6, 9–13).

Table 2 shows the general distribution of SSc clinical subtypes and the SSc-associated auto-antibodies in pediatric-onset compared to adult SSc. The diffuse cutaneous SSc subtype and associated ATA frequencies are similar between pediatric and adult SSc. A higher proportion of overlap patients and associated antibodies U1-RNP and PM-Scl was observed across several jSSc cohorts (6, 9–12) and accounts for the relatively common disease presentation in children of the combination of arthritis, myositis, Raynaud phenomenon and digital ulcers (6). The jSSc patients with overlap syndrome and associated antibodies (U1-RNP, Pm-Scl, U3-RNP) that have myositis may be of the most concern, as myopathy of the peripheral skeletal muscle is likely related to cardiac skeletal myopathy, and these patients have been demonstrated to have more conduction defects and other cardiac manifestations, a major contributor to mortality (6, 12, 13, 18).

Another main difference from adults with SSc is the lack of anti-centromere antibody–positive patients with jSSc (*leq*5% in most cohorts) (6, 9–13, 25), especially with pre-pubertal patients (<10 years at onset) (10). This is true despite ∼30% to 50% of patients with jSSc being clinically classified as limited cutaneous SSc (**Table 2**). The relatively low rate of centromere antibodies found in jSSc does not seem to correlate with pulmonary arterial hypertension (PAH), as PAH is found with a similar frequency in juvenile and adult SSc (**Table 1**) (6, 9–13, 23). Specifically, in the Scalapino jSSc cohort manuscript (n =111, 4 with intrinsic PAH) it was noted there was no association of ACA positivity with manifestation of PAH (6).

There are several other SSc-related autoantibodies that associate with different organ manifestations in adult SSc; however, they are much less common in childhood onset SSc. One of these is RNA polymerase III (POL3) antibody, which relates to severe renal disease in the form of scleroderma renal crisis in adult SSc. POL3 is rarely observed in jSSc (<5% in all cohorts; 4 or fewer patients per cohort), but can be found in up to 30% of adult onset SSc patients and reflects the clinically significant higher proportion of renal disease in adult SSc (6, 23). POL3 antibodies have also been associated with malignancy in SSc, a clinical outcome not reported in jSSc (21). Some of this data in jSSc is limited by the incomplete serological testing of scleroderma-associated antibodies in children. A more comprehensive evaluation for less common autoantibodies, such as POL3 and Th/To, to complete the full scleroderma-associated auto-antibody profile in jSSc cohorts may allow for better disease characterization and allow for more significant clinical associations.

GENETIC BASIS OF JUVENILE SYSTEMIC SCLEROSIS

Evidence for a Genetic Contribution to jSSc

Although the etiology of SSc is undoubtedly multifactorial, association studies have revealed immunoregulatory genes that most likely contribute to the pathogenesis of disease. The genetic risk factors appear to be lower for adult-onset SSc than for other autoimmune diseases [reviewed recently in (26)]. Only one study has examined history of autoimmunity in family members of SSc patients, and found a rate of 11% for a first or second degree relative of jSSc patients, suggesting that the disease is not monogenic (13).

The concordance rate for adult-onset SSc in twins is low (5.6% for dizygotic (DZ) twins and 4.2% for monozygotic (MZ) twins) (27). However, the genetic predisposition for loss of immune self-tolerance is high in families with SSc, with greatly increased concordance for ANA in MZ twins (90%) compared with DZ twins (40%) (27). Some of the discordance in disease penetrance may be attributed to differential methylation of X-encoded genes (28). A genetic contribution has also been suggested by reports of familial clustering in three cohorts in which first degree relatives of patients with SSc carried a 10–16-fold relative risk for disease and siblings a 10–27-fold risk (29).

In families with more than one case of SSc, affected individuals shared cutaneous subsets of disease severity and SSc-associated autoantibodies, with similar ages of onset (30). Finally, gene expression studies in cultured dermal fibroblasts suggest a heritable profibrotic program. In this study, healthy adult-onset MZ twins shared fibroblast gene expression patterns with their twins with SSc, whereas gene expression in unaffected adult-onset DZ twins resembled that of healthy controls (31). Moreover, serum from SSc patients or MZ twins could induce a SSc gene expression pattern in fibroblasts from healthy controls (31).

Peculiar Genetic Aspects of Juvenile SSc

Although numerous studies have investigated HLA associations in adult SSc, age of onset has not generally been considered. In pediatric studies, some HLA alleles found associated with adult SSc have also been associated with jSSc (32). An initial study in the UK examined HLA alleles in 27 patients with jSSc-dermatomyositis overlap, demonstrated an increase of DQA1*05 and DRB1*03 and a trend toward decreased DRB1*15. This finding was confirmed in a larger study of 76 Caucasian patients with a mean onset of SSc onset at 10 years of age

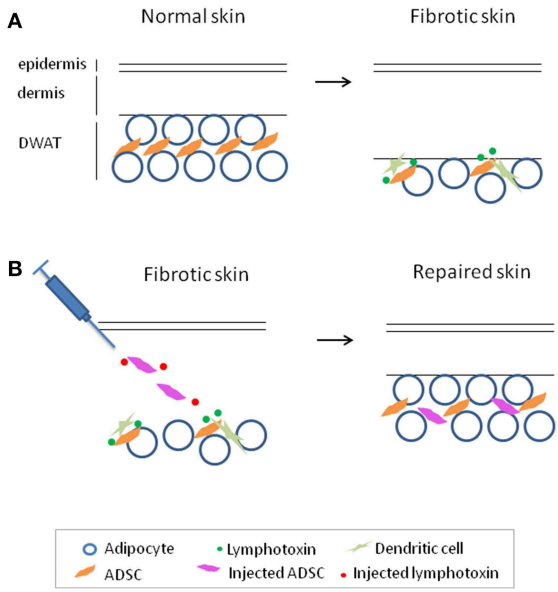

FIGURE 1 | Adipose-derived stromal cells (ADSC) maintenance by dendritic cell-derived lymphotoxin in fibrotic skin and enhancing ADSC therapy with lymphotoxin stimulation to treat fibrosis. **(A)** Reparative and regenerative ADSCs are lost with skin fibrosis and are maintained in fibrotic skin by dendritic cell-derived lymphotoxin. **(B)** Adding lymphotoxin stimulation to therapeutic ADSC injection enhances survival and effectiveness of the injected ADSCs in skin repair in a model of skin fibrosis. This model may be helpful to investigate its potential application for the treatment of fibrosis.

in the US (25). Children with SSc did not have an increased frequency of DRB1*01, which has been associated with adult limited cutaneous SSc and ACA or HLA-DRB1*11 alleles, the group most consistently described in association with SSc in Caucasian adults.

One novel HLA association was discovered in jSSc patients: DRB1*10. DRB1*10 appeared to be a risk factor specific for jSSc, present in 10.5% of jSSc compared to 1.5% of controls, similar to one report of adult Han Chinese, but not otherwise described in adult SSc (33).

Protective HLA alleles in adult-onset SSc (DRB1*07, DQB1*02:02) were not found to be protective for jSSc. Instead, DQB1*06 was protective for jSSc (25).

Age and Sex Matter

The frequency of the adult limited cutaneous SSc-associated allele DRB1*01 differed in children with age of disease onset and autoantibody status (25). DRB1*01 was less prevalent in patients who were <6 years of age compared to patients 11–16 years at disease onset. DRB1*01 was under-represented among anti-topoisomerase 1 (ATA) -positive jSSc patients, consistent with a similar observation reported in adult SSc (34). In this study, just as the ATA-associated alleles decreased with age, the prevalence of ATA significantly decreased with increasing age (25).

Thus, children with SSc genetically resemble adult males with SSc, rather than the more prevalent women with SSc, with an increase frequency of DQA1*05 but not DRB1*11. This phenomenon could be explained by pregnancy immunopathology. It has been hypothesized that fetal microchimerism could trigger or perpetuate a graft-vs. -host type chronic inflammatory response (35). Women with SSc more often have been pregnant, and carry high levels of fetal microchimerism, whereas males and children do not. About 1 in 10 children with SSc carry an HLA allele not found in adults with SSc (DRB1*10), suggesting a unique childhood antigen trigger.

PATHOLOGY OF SYSTEMIC SCLEROSIS

Histology of the Skin in SSc

The histology of the skin reflects the main three pathways affected in SSc, endothelial/vascular, immune/inflammatory, and fibrosis. The epidermis is relatively spared, though may be thinned, and rete ridges reduced. The dermis is thickened from the fibrosis and the connective tissue appears homogenous from collagen deposition. There is a loss of adnexal structures such as the pilosebaceous and eccrine glands (36). There are also vascular changes characterized by capillary rarefaction in the papillary dermis, thickened arteriolar walls and intimal thickening (37, 38). There can be inflammatory infiltrates of lymphocytes and macrophages, similar to that found in localized scleroderma (37), though may limited to early disease in SSc.

The Role of the Adipocytes

The dermal alterations that are most often discussed are the features that are apparent on a full thickness skin biopsy. However, intrinsic to the dermis but sitting mainly under the connective tissue-rich portions of the dermis and immediately adjacent to subcutaneous adipose tissue in humans is the dermal white adipose tissue (DWAT) (39). Fleishmajer et al. performed excisional biopsies in SSc patients, sampling skin down to the fascia, and thereby were able to characterize the DWAT (36). They reported that DWAT was replaced by connective tissue comprised of immature disorganized collagen fibrils and massive ground substance. Interestingly, this loss of DWAT is also observed in multiple murine models of scleroderma skin fibrosis (40–42), and reflects at least in part transdifferentiation of adipocytes into myofibroblasts (40, 41). Furthermore, this DWAT loss can further compromise skin function, as adipocytes can express anti-fibrotic molecules such as adiponectin and are involved in hair regeneration. DWAT is a niche for regenerative and reparative adipose-derived stromal cells (ADSCs) that participate in wound healing (41, 43, 44).

Upon loss of DWAT in murine skin fibrosis models, there is an 80% loss of ADSCs, likely due to cell death, which could potentially contribute to the inability to reverse and heal the fibrosis (42). Interestingly, dendritic cells helped to maintain the survival of the remaining ADSCs in fibrotic skin, and stimulating with the dendritic cell-derived signal lymphotoxin can enhance the survival and therapeutic effectiveness of intradermally injected ADSCs in a murine skin fibrosis model. ADSCs or a related type of mesenchymal stromal cells (MSCs) given either locally or systemically are being tested in human SSc (45);

if clinical trials show efficacy of these approaches, it may be helpful to investigate whether adding lymphotoxin beta receptor stimulation can enhance these approaches (**Figure 1**).

CYTOKINE AND CELLULAR SIGNATURES

There have been multiple studies in adult SSc evaluating the cellular and cytokine profiles of tissue and peripheral blood, with only a few studies in jSSc owing to the rarity of disease in children. Skin lesions in jSSc show lymphocytes as the predominant cell type within the dermal and subcutaneous infiltrate (46, 47).

The lymphocytes have been further characterized, with T lymphocytes (CD4 and CD8) as the predominant cell type in the dermis of SSc patients (48, 49). T cell activation and associated cytokine release are thought to play a pivotal role in SSc pathogenesis through stimulating fibroblasts to promote fibrosis (50–52). CD4+ T-helper (T_H) cell lineage and its effector cytokines have been identified in biopsies of skin lesions (48, 53, 54), peripheral circulation (adult and pediatric) (55–58) and culture in supernatants of peripheral blood mononuclear cells (PBMC) of SSc patients (59).

T_H cells consist of three main effector cell types including T_H1, T_H2, and T_H17; each are differentiation states characterized by the predominant cytokines they produce, namely interferon-γ (IFN-γ), interleukin-4 (IL-4)/IL-13, and IL-17, respectively. Many autoimmune diseases, including scleroderma, are thought to be propagated by an imbalance of T_H cell subsets and their associated cytokines (48, 53–57, 59–61).

There is an extensive amount of literature (mainly adult studies) supporting a T_H2 cellular and associated cytokine predominance in SSc, with peripheral blood and tissue derived IL-4 and IL-13, and more recently identified T_H2 associated cytokines, IL-33, IL-34, and IL-35 (62, 63), correlating with the degree of skin and lung fibrosis and disease burden (50, 51, 57, 64–67). Both IL-4 and IL-13 are effector cytokines of the T_H2 lineage characterized as pro-fibrotic and anti-inflammatory due to their respective actions as initiators of extracellular matrix production and inhibitors of T_H1 function (68).

Therefore, it is suspected that the T_H2 cytokine signature in SSc supports the fibrotic component of the disease, with peripheral cytokines serving as prognostic markers in SSc patients (50, 51). A recent study analyzing 14 pediatric SSc subjects compared to 24 healthy pediatric controls found a significantly elevated proportion of circulating CD4+IL-4+ (T_H2) cells in pediatric SSc, and notably 10 of the 14 subjects had later stage disease (>2 years from onset) (58). Conversely, although higher than pediatric LS, the T_H17 (CD4+IL-17+) profile of these pediatric SSc patients was significantly lower than pediatric healthy controls, perhaps reflecting their later stage of disease (58).

Cytokines associated with a T_H1 profile (IL-1, TNF-α, and IFN-γ) have been identified as elevated in the peripheral blood of adult SSc patients compared to healthy controls, and their levels decrease overtime signifying their elevation during the earlier, active phase of the disease (69, 70). Specifically, the IFN-γ associated chemokines, IFN-inducible protein 10 (IP-10/CXCL10) and monocyte chemoattractant protein 1 (MCP-1/CCL2), have been demonstrated in more early, inflammatory adult SSc (71) as well as preliminary data in jSSc (72). The sera levels of jSSc are even more elevated than adult SSc and reflect those of juvenile LS (73), which in general, is considered the more inflammatory scleroderma subset. The "hallmark" effector cytokine of T_H17 cells, IL-17a, has been demonstrated in significant amounts in the skin, lungs, and sera of adult SSc patients during the early, more active, stages of the disease (55). These findings suggest that T_H17 and T_H1 cells may contribute to cellular inflammation in SSc via production of associated pro-inflammatory cytokines (51) in the earlier stage of disease, as hypothesized in the pediatric SSc findings (58, 72).

Disease stage	Pre-disease: genetics	Early disease: inflammation	Late disease: fibrosis
Mechanisms	HLA DQA1*05 HLA DRB1*10	? Macrophages, other innate cells, early vascular damage ↑ Th1, Th17 cells and cytokines Loss of DWAT and ADSCs	↑ Th2→TGFb, CTGF→ fibrosis, further endothelial damage ↓ Regulatory T cell function Limitation of skin damage by DC-ADSC axis
Opportunities for new treatment approaches		Targeting macrophages, Th1 and Th17 inflammation, limiting DWAT loss	Targeting Th2 axis, improving Treg function, enhancing ADSC function and reparative processes

FIGURE 2 | Schematic representation of proposed disease pathogenesis of pediatric SSc.

It is thought that these T cell–associated chemokines and cytokines then stimulate fibroblasts and endothelial cells to produce TGF-β and connective tissue growth factor (CTGF) to stimulate tissue fibrosis (via increased collagen production) and endothelial cell damage (via influence of adhesion molecules ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1), with elevation of these adhesion molecules demonstrated in the peripheral blood of SSc patients (74–78).

The counterbalance to the inflammatory T_H cell phenotype, the regulatory T cell (Treg) immunophenotype, has been recently reported by Torok and Reiff in jSSc patients (58, 79), and collectively demonstrate decrease in functional Tregs compared to healthy controls, as well as association to more severe clinical phenotype and longer disease duration. This could, in part, be due to the differentiation of Tregs in the blood and skin to proinflammatory (T_H17) and fibrotic (T_H2) cells, therefore decreasing their frequency and effectiveness, as reported in adult SSc (80, 81).

Although the focus has been more on lymphocytes, innate cells such as macrophages and plasmacytoid dendritic cells are becoming increasingly recognized as potential contributors to systemic sclerosis, especially during early disease (42, 82–85). In addition to directly stimulating fibroblasts, innate cells can contribute to activating endothelial cells, which then further recruits circulating cells into the tissue, fueling the inflammation and further injury of the endothelium (86–88). While most patient data is gathered from adult SSc patients, a recent gene expression and expression quantitative trait loci analysis of monocyte-derived macrophages generated from pediatric and adult SSc patients suggested that changes in macrophage gene expression is an important contributor to disease and that upregulation of GSMDA, a regulator of pyroptosis in macrophages but not other cell types, contributes to disease risk (89). Further delineation of innate cell phenotype and function in pediatric SSc awaits.

Figure 2 summarizes, in a schematic way, the main immunologic/genetic pathways involved in the pathogenesis of the disease, in its different stages.

FUTURE DIRECTIONS FOR RESEARCH

In summary, early-onset SSc seems to be a distinct entity, whereas genetically-programmed manifestations in older children resemble adult disease. Disease manifestations and histopathology of jSSc resemble adult SSc, albeit with less organ involvement and superior outcomes.

Although genome-wide association studies cannot be performed on a disease as rare as jSSc, a genetic basis is likely in early-onset disease, and HLA class II associations with jSSc resemble adult males rather than adult females with SSc.

A pediatric-specific association of DRB1*10 suggests a unique trigger leading to younger onset disease. Younger children with SSc tended to carry genes associated with ATA, diffuse cutaneous SSc type, and not DRB1*01. More extensive genetic studies in families of children with early-onset (<6 years old) disease, with special focus on those with rapidly progressive organ fibrosis (90) are needed.

Novel therapeutic approaches could target specific HLA molecules, inflammatory chemokine, and cytokine genes over-expressed in jSSc. Recent reports of promising results of hematopoietic stem cell transplantation in adult-onset SSc encourage optimism for this therapeutic approach in children, who may have less organ damage when disease is recognized (91) Anti-fibrotic cellular therapies with adipose-derived stromal cells are being investigated, and recent findings in the laboratory suggest the potential power of stromal cell survival factors to enhance cellular therapy of the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Herrick AL, Ennis H, Bhushan M, Silman AJ, Baildam EM. Incidence of childhood linear scleroderma and systemic sclerosis in the UK and Ireland. *Arthritis Care Res.* (2010) 62:213–8. doi: 10.1002/acr.20070
2. Pelkonen PM, Jalanko HJ, Lantto RK, Mäkelä AL, Pietikäinen MA, Savolainen HA, et al. Incidence of systemic connective tissue diseases in children: a nationwide prospective study in Finland. *J Rheumatol.* (1994) 21:2143–6.
3. Royle JG, Lanyon PC, Grainge MJ, Abhishek A, Pearce FA. Correction to: the incidence, prevalence, and survival of systemic sclerosis in the UK Clinical Practice Research Datalink. *Clin Rheumatol.* (2018) 37:2601. doi: 10.1007/s10067-018-4219-7
4. Royle JG, Lanyon PC, Grainge MJ, Abhishek A, Pearce FA. The incidence, prevalence, and survival of systemic sclerosis in the UK Clinical Practice Research Datalink. *Clin Rheumatol.* (2018) 37:2103–11. doi: 10.1007/s10067-018-4182-3
5. Beukelman T, Xie F, Foeldvari I. Assessing the prevalence of juvenile systemic sclerosis in childhood using administrative claims data from the United States. *J Scler Relat Disord.* (2018) 3:189–90. doi: 10.1177/2397198318763701
6. Scalapino K, Arkachaisri T, Lucas M, Fertig N, Helfrich DJ, Londino AV, et al. Childhood onset systemic sclerosis: classification, clinical and serologic features, and survival in comparison with adult onset disease. *J Rheumatol.* (2006) 33:1004–13.
7. Misra R, Singh G, Aggarwal P, Aggarwal A. Juvenile onset systemic sclerosis: a single center experience of 23 cases from Asia. *Clin Rheumatol.* (2007) 2007:1259–62. doi: 10.1007/s10067-006-0483-z
8. Li SC. Scleroderma in children and adolescents: localized scleroderma and systemic sclerosis. *Pediatr Clin North Am.* (2018) 65:757–81. doi: 10.1016/j.pcl.2018.04.002
9. Foeldvari I, Nihtyanova SI, Wierk A, Denton CP. Characteristics of patients with juvenile onset systemic sclerosis in an adult single-center cohort. *J Rheumatol.* (2010) 37:2422–6. doi: 10.3899/jrheum.100001
10. Foeldvari I, Tyndall A, Zulian F, Müller-Ladner U, Czirjak L, Denton C, et al. Juvenile and young adult-onset systemic sclerosis share the same organ involvement in adulthood: data from the EUSTAR

database. *Rheumatology*. (2012) 51:1832–7. doi: 10.1093/rheumatology/kes144
11. Stevens BE, Torok KS, Li SC, Hershey N, Curran M, Higgins GC, et al. Clinical characteristics and factors associated with disability and impaired quality of life in children with juvenile systemic sclerosis. *Arthritis Care Res*. (2018) 70:1806–13. doi: 10.1002/acr.23547
12. Foeldvari I, Klotsche J, Torok KS, et al. Are diffuse and limited juvenile systemic sclerosis different in clinical presentation? Clinical characteristics of a juvenile systemic sclerosis cohort. *J Scler Relat Disord*. (2018) 4:49–61. doi: 10.1177/2397198318790494
13. Martini G, Foeldvari I, Russo R, Cuttica R, Eberhard A, Ravelli A, et al. Systemic sclerosis in childhood: clinical and immunologic features of 153 patients in an international database. *Arthritis Rheum*. (2006) 54:3971–8. doi: 10.1002/art.22207
14. Ingegnoli F, Boracchi P, Gualtierotti R, Smith V, Cutolo M, Foeldvari I, et al. A comparison between nailfold capillaroscopy patterns in adulthood in juvenile and adult-onset systemic sclerosis: a EUSTAR exploratory study. *Microvasc Res*. (2015) 102:19–24. doi: 10.1016/j.mvr.2015.07.007
15. Dolezalova P, Young SP, Bacon PA, Southwood TR. Nailfold capillary microscopy in healthy children and in childhood rheumatic diseases: a prospective single blind observational study. *Ann Rheum Dis*. (2003) 62:444–9. doi: 10.1136/ard.62.5.444
16. Ambartsumyan L, Zheng HB, Iyer RS, Soares J, Henstorf G, Stevens AM. Relationship between esophageal abnormalities on fluoroscopic esophagram and pulmonary function testing in juvenile systemic sclerosis. *Arthritis Care Res*. (2018). doi: 10.1002/acr.23778. [Epub ahead of print].
17. Martini G, Vittadello F, Kasapçopur O, Magni Manzoni S, Corona F, Duarte-Salazar C, et al. Factors affecting survival in juvenile systemic sclerosis. *Rheumatology*. (2009) 48:119–22. doi: 10.1093/rheumatology/ken388
18. Quartier P, Bonnet D, Fournet JC, Bodemer C, Acar P, Ouachée-Chardin M, et al. Severe cardiac involvement in children with systemic sclerosis and myositis. *J Rheumatol*. (2002) 29:1767–73.
19. Baildam EM, Ennis H, Foster HE, Shaw L, Chieng AS, Kelly J, et al. Influence of childhood scleroderma on physical function and quality of life. *J Rheumatol*. (2011) 38:167–73. doi: 10.3899/jrheum.100447
20. Hatta Y, Hasegawa M, Matsushita T, Hamaguchi Y, Fujimoto M, Takehara K. The clinical characteristics of juvenile-onset systemic sclerosis in Japanese patients. *Mod Rheumatol*. (2013). doi: 10.1007/s10165-013-0884-1
21. Maria ATJ, Partouche L, Goulabchand R, Rivière S, Rozier P, Bourgier C, et al. Intriguing relationships between cancer and systemic sclerosis: role of the immune system and other contributors. *Front Immunol*. (2018) 9:3112. doi: 10.3389/fimmu.2018.03112
22. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol*. (1988) 15:202–5.
23. Foeldvari I, Zhavania M, Birdi N, Cuttica RJ, de Oliveira SH, Dent PB, et al. Favourable outcome in 135 children with juvenile systemic sclerosis: results of a multi-national survey. *Rheumatology*. (2000) 39:556–9. doi: 10.1093/rheumatology/39.5.556
24. Steen VD. The many faces of scleroderma. *Rheum Dis Clin North Am*. (2008) 34:1–15; v. doi: 10.1016/j.rdc.2007.12.001
25. Stevens AM, Kanaan SB, Torok KS, Medsger TA, Mayes MD, Reveille JD, et al. Brief report: HLA-DRB1, DQA1, and DQB1 in juvenile-onset systemic sclerosis. *Arthritis Rheumatol*. (2016) 68:2772–7. doi: 10.1002/art.39765
26. Angiolilli C, Marut W, van der Kroef M, Chouri E, Reedquist KA, Radstake TRDJ. New insights into the genetics and epigenetics of systemic sclerosis. *Nat Rev Rheumatol*. (2018) 14:657–73. doi: 10.1038/s41584-018-0099-0
27. Feghali-Bostwick C, Medsger TA Jr., Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis Rheum*. (2003) 48:1956–63. doi: 10.1002/art.11173
28. Selmi C, Feghali-Bostwick CA, Lleo A, Lombardi SA, De Santis M, Cavaciocchi F, et al. X chromosome gene methylation in peripheral lymphocytes from monozygotic twins discordant for scleroderma. *Clin Exp Immunol*. (2012) 169:253–62. doi: 10.1111/j.1365-2249.2012.04621.x
29. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. *Arthritis Rheum*. (2001) 44:1359–62. doi: 10.1002/1529-0131(200106)44:6<1359::AID-ART228>3.0.CO;2-S
30. Stephens CO, Briggs DC, Whyte J, Artlett CM, Scherbakov AB, Olsen N, et al. Familial scleroderma–evidence for environmental versus genetic trigger. *Br J Rheumatol*. (1994) 33:1131–5. doi: 10.1093/rheumatology/33.12.1131
31. Zhou X, Tan FK, Xiong M, Arnett FC, Feghali-Bostwick CA. Monozygotic twins clinically discordant for scleroderma show concordance for fibroblast gene expression profiles. *Arthritis Rheum*. (2005) 52:3305–14. doi: 10.1002/art.21355
32. Wedderburn LR, McHugh NJ, Chinoy H, Cooper RG, Salway F, Ollier WE, et al. HLA class II haplotype and autoantibody associations in children with juvenile dermatomyositis and juvenile dermatomyositis-scleroderma overlap. *Rheumatology (Oxford)*. (2007) 46:1786–91. doi: 10.1093/rheumatology/kem265
33. He D, Wang J, Yi L, Guo X, Guo S, Guo G, et al. Association of the HLA-DRB1 with scleroderma in Chinese population. *PLoS ONE*. (2014) 9:e106939. doi: 10.1371/journal.pone.0106939
34. Beretta L, Rueda B, Marchini M, Santaniello A, Simeón CP, Fonollosa V, et al. Analysis of class II human leucocyte antigens in Italian and Spanish systemic sclerosis. *Rheumatology*. (2012) 51:52–9. doi: 10.1093/rheumatology/ker335
35. Nelson JL. The otherness of self: microchimerism in health and disease. *Trends Immunol*. (2012) 33:421–7. doi: 10.1016/j.it.2012.03.002
36. Fleischmajer R, Damiano V, Nedwich A. Scleroderma and the subcutaneous tissue. *Science*. (1971) 171:1019–21. doi: 10.1126/science.171.3975.1019
37. Fleischmajer R, Perlish JS, Reeves JR. Cellular infiltrates in scleroderma skin. *Arthritis Rheum*. (1977) 20:975–84. doi: 10.1002/art.1780200410
38. Fleming JN, Nash RA, McLeod DO, Fiorentino DF, Shulman HM, Connolly MK,et al. Capillary regeneration in scleroderma: stem cell therapy reverses phenotype? *PLoS ONE*. (2008) 3:e1452. doi: 10.1371/annotation/6b021f46-17bd-4ffe-a378-a1b8d24a1398
39. Driskell RR, Jahoda CA, Chuong CM, Watt FM, Horsley V. Defining dermal adipose tissue. *Exp Dermatol*. (2014) 23:629–31. doi: 10.1111/exd.12450
40. Marangoni RG, Korman BD, Wei J, Wood TA, Graham LV, Whitfield ML, et al. Myofibroblasts in murine cutaneous fibrosis originate from adiponectin-positive intradermal progenitors. *Arthritis Rheumatol*. (2015) 67:1062–73. doi: 10.1002/art.38990
41. Marangoni RG, Lu TT. The roles of dermal white adipose tissue loss in scleroderma skin fibrosis. *Curr Opin Rheumatol*. (2017) 29:585–90. doi: 10.1097/BOR.0000000000000437
42. Chia JJ, Zhu T, Chyou S, Dasoveanu DC, Carballo C, Tian S, et al. Dendritic cells maintain dermal adipose-derived stromal cells in skin fibrosis. *J Clin Invest*. (2016) 126:4331–45. doi: 10.1172/JCI85740
43. Festa E, Fretz J, Berry R, Schmidt B, Rodeheffer M, Horowitz M, et al. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. *Cell*. (2011) 146:761–71. doi: 10.1016/j.cell.2011.07.019
44. Driskell RR, Lichtenberger BM, Hoste E, Kretzschmar K, Simons BD, Charalambous M, et al. Distinct fibroblast lineages determine dermal architecture in skin development and repair. *Nature*. (2013) 504:277–81. doi: 10.1038/nature12783
45. Peltzer J, Aletti M, Frescaline N, Busson E, Lataillade JJ, Martinaud C. Mesenchymal stromal cells based therapy in systemic sclerosis: rational and challenges. *Front Immunol*. (2018) 9:2013. doi: 10.3389/fimmu.2018.02013
46. Succaria F, Kurban M, Kibbi AG, Abbas O. Clinicopathological study of 81 cases of localized and systemic scleroderma. *J Eur Acad Dermatol Venereol*. (2013) 27:e191–6. doi: 10.1111/j.1468-3083.2012.04581.x
47. Torres JE, Sánchez JL. Histopathologic differentiation between localized and systemic scleroderma. *Am J Dermatopathol*. (1998) 20:242–5. doi: 10.1097/00000372-199806000-00003
48. Roumm AD, Whiteside TL, Medsger TA Jr., Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. *Arthritis Rheum*. (1984) 27:645–53. doi: 10.1002/art.1780270607
49. Kalogerou A, Gelou E, Mountantonakis S, Settas L, Zafiriou E, Sakkas L. Early T cell activation in the skin from patients with systemic sclerosis. *Ann Rheum Dis*. (2005) 64:1233–5. doi: 10.1136/ard.2004.027094
50. Baraut J, Farge D, Jean-Louis F, Kesmandt H, Durant C, Verrecchia F, et al. [Cytokines in systemic sclerosis]. *Pathol Biol*. (2012) 60:127–39. doi: 10.1016/j.patbio.2009.11.003

51. Hasegawa M, Takehara K. Potential immunologic targets for treating fibrosis in systemic sclerosis: a review focused on leukocytes and cytokines. *Semin Arthritis Rheum*. (2012) 42:281–96. doi: 10.1016/j.semarthrit.2012.03.014
52. Fuschiotti P. T cells and cytokines in systemic sclerosis. *Curr Opin Rheumatol*. (2018) 30:594–9. doi: 10.1097/BOR.0000000000000553
53. Keystone EC, Lau C, Gladman DD, Wilkinson S, Lee P, Shore A. Immunoregulatory T cell subpopulations in patients with scleroderma using monoclonal antibodies. *Clin Exp Immunol*. (1982) 48:443–8.
54. Inoshita T, Whiteside TL, Rodnan GP, Taylor FH. Abnormalities of T lymphocyte subsets in patients with progressive systemic sclerosis (PSS, scleroderma). *J Lab Clin Med*. (1981) 97:264–77.
55. Kurasawa K, Hirose K, Sano H, Endo H, Shinkai H, Nawata Y, et al. Increased interleukin-17 production in patients with systemic sclerosis. *Arthritis Rheum*. (2000) 43:2455–63. doi: 10.1002/1529-0131(200011)43:11<2455::AID-ANR12>3.0.CO;2-K
56. Matsushita T, Hasegawa M, Hamaguchi Y, Takehara K, Sato S. Longitudinal analysis of serum cytokine concentrations in systemic sclerosis: association of interleukin 12 elevation with spontaneous regression of skin sclerosis. *J Rheumatol*. (2006) 33:275–84.
57. Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. *Arthritis Rheum*. (1992) 35:67–72. doi: 10.1002/art.1780350111
58. Mirizio E, Marathi A, Hershey N, Ross C, Schollaert K, Salgado C, et al. Identifying the signature immune phenotypes present in pediatric localized scleroderma. *J Invest Dermatol*. (2018) 139:715–8. doi: 10.1016/j.jid.2018.09.025
59. Valentini G, Baroni A, Esposito K, Naclerio C, Buommino E, Farzati A, et al. Peripheral blood T lymphocytes from systemic sclerosis patients show both Th1 and Th2 activation. *J Clin Immunol*. (2001) 21:210–7. doi: 10.1023/A:1011024313525
60. Ihn H, Sato S, Fujimoto M, Kikuchi K, Takehara K. Demonstration of interleukin-2, interleukin-4 and interleukin-6 in sera from patients with localized scleroderma. *Arch Dermatol Res*. (1995) 287:193–7. doi: 10.1007/BF01262331
61. Hasegawa M, Sato S, Nagaoka T, Fujimoto M, Takehara K. Serum levels of tumor necrosis factor and interleukin-13 are elevated in patients with localized scleroderma. *Dermatology*. (2003) 207:141–7. doi: 10.1159/000071783
62. Zhang YJ, Zhang Q, Yang GJ, Tao JH, Wu GC, Huang XL, et al. Elevated serum levels of interleukin-1beta and interleukin-33 in patients with systemic sclerosis in Chinese population. *Z Rheumatol*. (2018) 77:151–9. doi: 10.1007/s00393-016-0202-3
63. Kuzumi A, Yoshizaki A, Toyama S, Fukasawa T, Ebata S, Nakamura K, et al. Serum interleukin-34 levels in patients with systemic sclerosis: Clinical association with interstitial lung disease. *J Dermatol*. (2018) 45:1216–20. doi: 10.1111/1346-8138.14538
64. Atamas SP, Yurovsky VV, Wise R, Wigley FM, Goter Robinson CJ, Henry P, et al. Production of type 2 cytokines by CD8+ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis. *Arthritis Rheum*. (1999) 42:1168–78. doi: 10.1002/1529-0131(199906)42:6<1168::AID-ANR13>3.0.CO;2-L
65. Parel Y, Aurrand-Lions M, Scheja A, Dayer JM, Roosnek E, Chizzolini C. Presence of CD4+CD8+ double-positive T cells with very high interleukin-4 production potential in lesional skin of patients with systemic sclerosis. *Arthritis Rheum*. (2007) 56:3459–67. doi: 10.1002/art.22927
66. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. *J Rheumatol*. (1997) 24:328–32.
67. Fuschiotti P, Medsger TA Jr., Morel PA. Effector CD8+ T cells in systemic sclerosis patients produce abnormally high levels of interleukin-13 associated with increased skin fibrosis. *Arthritis Rheum*. (2009) 60:1119–28. doi: 10.1002/art.24432
68. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol*. (2004) 4:583–94. doi: 10.1038/nri1412
69. Gourh P, Arnett FC, Assassi S, Tan FK, Huang M, Diekman L, et al. Plasma cytokine profiles in systemic sclerosis: associations with autoantibody subsets and clinical manifestations. *Arthritis Res Ther*. (2009) 11:R147. doi: 10.1186/ar2821
70. Dantas AT, Almeida AR, Sampaio MCPD, Cordeiro MF, Oliveira PSS, Mariz HA, et al. Different profile of cytokine production in patients with systemic sclerosis and association with clinical manifestations. *Immunol Lett*. (2018) 198:12–6. doi: 10.1016/j.imlet.2018.03.011
71. Liu X, Mayes MD, Tan FK, Wu M, Reveille JD, Harper BE, et al. Correlation of interferon-inducible chemokine plasma levels with disease severity in systemic sclerosis. *Arthritis Rheum*. (2013) 65:226–35. doi: 10.1002/art.37742
72. Kurzinski KKC, Arkachaisri T, Feghali-Bostwick C, Torok KS. Circulating IP-10 and MCP-1 levels in active localized scleroderma. *Arthritis Rheum*. (2011) 63:S115.
73. Magee KE, Kelsey CE, Kurzinski KL, Ho J, Mlakar LR, Feghali-Bostwick CA, et al. Interferon-gamma inducible protein-10 as a potential biomarker in localized scleroderma. *Arthritis Res Ther*. (2013) 15:R188. doi: 10.1186/ar4378
74. Varga J, Rosenbloom J, Jimenez SA. Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. *Biochem J*. (1987) 247:597–604. doi: 10.1042/bj2470597
75. Gore-Hyer E, Pannu J, Smith EA, Grotendorst G, Trojanowska M. Selective stimulation of collagen synthesis in the presence of costimulatory insulin signaling by connective tissue growth factor in scleroderma fibroblasts. *Arthritis Rheum*. (2003) 48:798–806. doi: 10.1002/art.10953
76. Uziel Y, Feldman BM, Krafchik BR, Laxer RM, Yeung RS. Increased serum levels of TGFbeta1 in children with localized scleroderma. *Pediatr Rheumatol Online J*. (2007) 5:22. doi: 10.1186/1546-0096-5-22
77. Higley H, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black C. Immunocytochemical localization and serologic detection of transforming growth factor beta 1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum*. (1994) 37:278–88. doi: 10.1002/art.1780370218
78. Thakkar V, Patterson KA, Stevens W, Wilson M, Roddy J, Sahhar J, et al. Increased serum levels of adhesion molecules ICAM-1 and VCAM-1 in systemic sclerosis are not specific for pulmonary manifestations. *Clin Rheumatol*. (2018) 37:1563–71. doi: 10.1007/s10067-018-4081-7
79. Reiff A, Weinberg KI, Triche T, Masinsin B, Mahadeo KM, Lin CH, et al. T lymphocyte abnormalities in juvenile systemic sclerosis patients. *Clin Immunol*. (2013) 149:146–55. doi: 10.1016/j.clim.2013.07.005
80. Slobodin G, Rimar D. Regulatory T cells in systemic sclerosis: a comprehensive review. *Clin Rev Allergy Immunol*. (2017) 52:194–201. doi: 10.1007/s12016-016-8563-6
81. MacDonald KG, Dawson NA, Huang Q, Dunne JV, Levings MK, Broady R. Regulatory T cells produce profibrotic cytokines in the skin of patients with systemic sclerosis. *J Allergy Clin Immunol*. (2015) 135:946–.e9. doi: 10.1016/j.jaci.2014.12.1932
82. Ah Kioon MD, Tripodo C, Fernandez D, Kirou KA, Spiera RF, Crow MK, et al. Plasmacytoid dendritic cells promote systemic sclerosis with a key role for TLR8. *Sci Transl Med*. (2018) 10:eaam8458. doi: 10.1126/scitranslmed.aam8458
83. Raker V, Haub J, Stojanovic A, Cerwenka A, Schuppan D, Steinbrink K. Early inflammatory players in cutanous fibrosis. *J Dermatol Sci*. (2017) 87:228–35. doi: 10.1016/j.jdermsci.2017.06.009
84. Stifano G, Sornasse T, Rice LM, Na L, Chen-Harris H, Khanna D, et al. Skin gene expression is prognostic for the trajectory of skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheumatol*. (2018) 70:912–9. doi: 10.1002/art.40455
85. Asano Y. Systemic sclerosis. *J Dermatol*. (2018) 45:128–38. doi: 10.1111/1346-8138.14153
86. Volkmann ER, Varga J. Emerging targets of disease-modifying therapy for systemic sclerosis. *Nat Rev Rheumatol*. (2019) 15:208–24. doi: 10.1038/s41584-019-0184-z
87. Matucci-Cerinic M, Kahaleh B, Wigley FM. Review: evidence that systemic sclerosis is a vascular disease. *Arthritis Rheumatism*. (2013) 65:1953–62. doi: 10.1002/art.37988

88. Sierra-Sepúlveda A, Esquinca-González A, Benavides-Suárez SA, Sordo-Lima DE, Caballero-Islas AE, Cabral-Castañeda AR, et al. Systemic sclerosis pathogenesis and emerging therapies, beyond the fibroblast. *BioMed Res Int.* (2019) 2019:4569826. doi: 10.1155/2019/4569826
89. Moreno-Moral A, Bagnati M, Koturan S, Ko JH, Fonseca C, Harmston N, et al. Changes in macrophage transcriptome associate with systemic sclerosis and mediate GSDMA contribution to disease risk. *Ann Rheum Dis.* (2018) 77:596–601. doi: 10.1136/annrheumdis-2017-212454
90. Stock CJW, Renzoni EA. Genetic predictors of systemic sclerosis-associated interstitial lung disease: a review of recent literature. *Eur J Hum Genet.* (2018) 26:765–77. doi: 10.1038/s41431-018-0104-8
91. Sullivan KM, Majhail NS, Bredeson C, Carpenter PA, Chatterjee S, Crofford LJ, et al. Systemic sclerosis as an indication for autologous hematopoietic cell transplantation: position statement from the American society for blood and marrow transplantation. *Biol Blood Marrow Transplant.* (2018) 24:1961–4. doi: 10.1016/j.bbmt.2018.06.025

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