DEFECTS IN REGULATION: HOW, WHERE AND WHEN THE IMMUNE SYSTEM CAN GO WRONG

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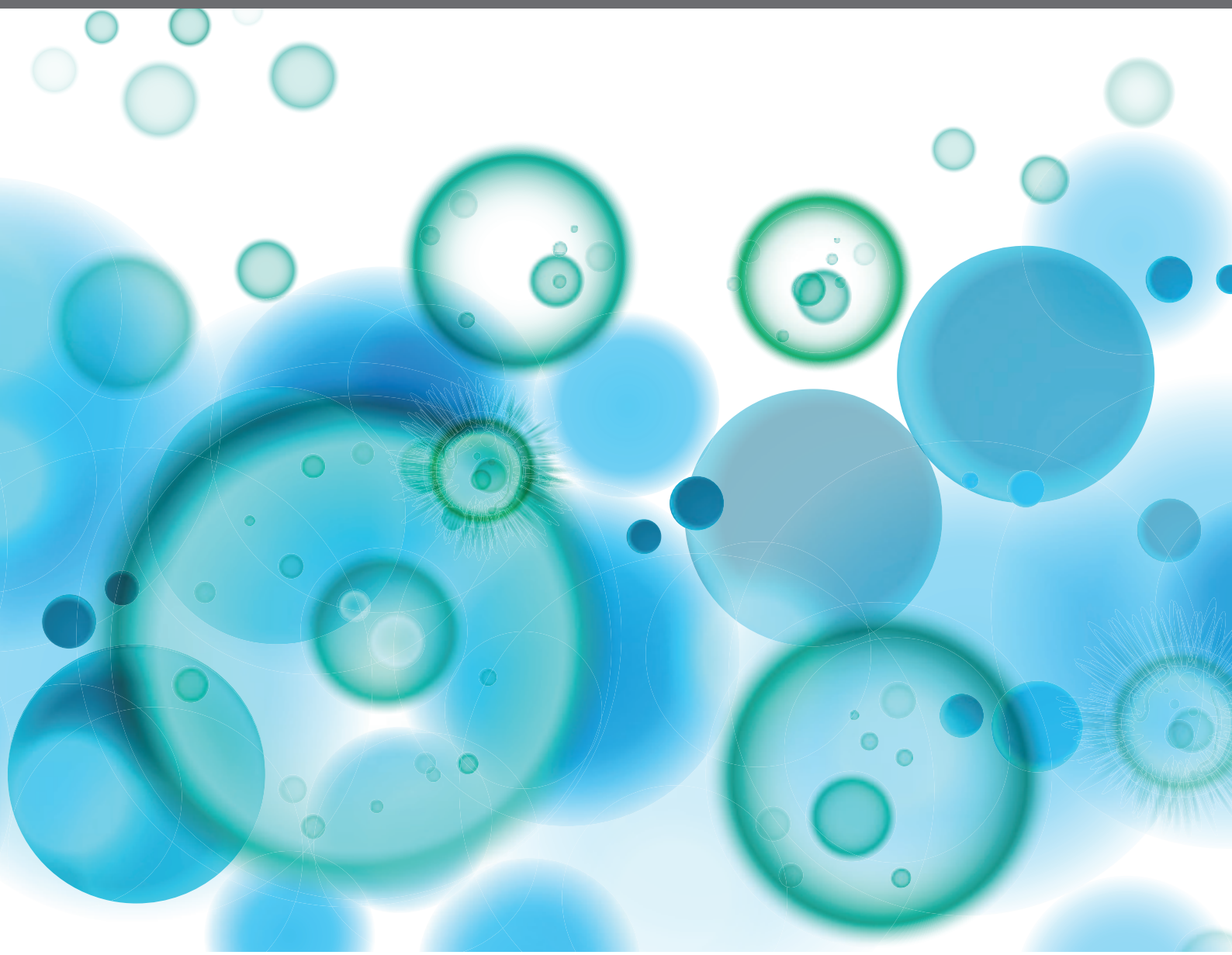

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DEFECTS IN REGULATION: HOW, WHERE AND WHEN THE IMMUNE SYSTEM CAN GO WRONG

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EDITORIAL
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Editorial: Defects in Regulation: How, Where and When the Immune System Can Go Wrong

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Keywords: immunoregulation, autoinflammation, autoimmunity, AIRE, glucocorticoids, TNF-blockade, addison, SLE

Editorial on the Research Topic

Defects in Regulation: How, Where and When the Immune System Can Go Wrong

The immune system is a highly complex cellular and molecular machinery conferring protection against infectious diseases and tumors. Like other complex machineries, the immune system requires strict regulation and guidance to function properly and to prevent it from going wrong. How, where and when can the immune system go wrong?

Crucial in keeping the level of immune- and inflammatory responses within the physiological range is the balance between different kinds of myeloid and lymphoid regulatory cells, including T regulatory cells, and antigen presenting cells. Moreover other cells, including for example dendritic cells, macrophages and neutrophils, also can contribute to the immune/inflammatory response and diseases.

Examples of where and when the immune system can go wrong are manifold. Defective expression of the autoimmune regulator (AIRE) gene, which prevents thymic transcription of tissue-specific self-antigens, disables the proper sensing of "self" and results in autoreactivity and self-destruction. Uncontrolled proliferation of immune cells may result in immunoproliferative diseases, such as leukemia. A compromised immune responses lead to immunodeficiencies including the autoinflammatory periodic fever syndromes. Finally, overactivation of the immune system may result in autoimmune diseases or allergies.

The aim of this Research Topic was to provide further insight into the relation between physiological functions of the immune system and the failure of regulatory mechanisms leading to immunodeficiency and autoimmunity with a focus on autoinflammation.

Discovery of a genetic defect in genes involved in immune responses may (as an "experiment of nature") lead to a better understanding of the physiology of the immune system. Thus, a defect in AIRE could explain how selection against recognition of self could be realized in the thymus. In addition, defects may also help to unravel the pathophysiology of immune mediated diseases. Thus, AIRE mutations link molecular defects to the pathophysiology of polyglandular autoimmune diseases such as Addison's disease. Perniola et al. review the history and current evidence of the role of AIRE in autoimmune Addison's disease as Part of the Autoimmune Polyglandular Syndrome Type 1.

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In other cases, a given genetic defect may not point directly to the pathophysiological mechanism of the disease. Martirosyan et al. from Yerevan in Armenia have studied the transmigration of neutrophils in patients with Familial Mediterranean Fever (FMF). FMF is caused by mutations in the pyrin encoding MEFV gene. Their data show that the mutated pyrin inflammasome is highly sensitive for slight changes in the cytoskeleton, even in the absence of pathogens. This could explain the apparent autoinflammatory nature of this disease.

Overexpression of interferon, based on gain-of-function mutations in *STING1* (Stimulator of interferon response cGAMP interactor), causes a rare autoinflammatory interferonopathy with systemic inflammation, vasculopathy and interstitial lung disease. Lin et al. from NIH in Bethesda with an international team of researchers now have found novel variants at several domains of the molecule, including the transmembrane domain, which also lead to STING autoactivation and vascular disease in the absence of ligand binding.

Epigenetic modifications also can be associated with autoimmune conditions. Kakan et al. have investigated miRNA signatures in the NOD mouse model of Sjögren's syndrome and suggest that upregulation of a set of five miRNAs dysregulates inflammation pathways, likely contributing to systemic inflammation and lymphocytic infiltration of the exocrine glands. Yu et al. from Jinan University in Shenzhen, China have set out to identify the transcription factors that contribute to the immune dysregulation in SLE by determining the chromatin accessibility landscape. By performing single cell analysis they show that 12 transcription factors, regulating 12 immune genes that characterize SLE. The complexity of this disease is illustrated by the fact that different profiles are found in T cells, B cells, monocytes and NK cells.

Glucocorticoids are important endocrine regulators of the immune system and as such used for a number of decades as pharmacological treatment of many autoimmune and autoinflammatory diseases. Ronchetti et al., from the group of Carlo Riccardi in Perugia, Italy, has reviewed the use of glucocorticoids (GC) for rare inflammatory and autoimmune diseases such as insulin autoimmune syndrome, relapsing polychondritis syndrome, dermatomyositis, and hemophagocytic lymphohistiocytosis. Prolonged use of GC comes with severe side-effects and therefore alternative, less toxic drugs would be needed. The potential for GC-induced proteins, such as glucocorticoid-induced leucine zipper (GILZ) for this purpose is discussed.

Relative little attention has been paid to the impact of GC, both physiologically as well as a drug, on regulation of tissue resident macrophages, a topic that is addressed by Diaz-Jimenez et al. from the NIH at Research Triangle Park. In heart tissue, in the central nervous system, in the gastrointestinal tract, and in the liver, glucocorticoids regulate immune surveillance by their effects on tissue resident macrophages. Glucocorticoids also exert their functions *via* GITR, the glucocorticoid-induced TNFR related protein as described by Tian et al. from Zhenjiang, China. As a consequence of GC, GITR is expressed on regulatory T cells and effector T cells as well as on NK cells and neutrophils. GITR is activated when interacting with its ligand, GIRTL, expressed constitutively on B cells and dendritic cells. Abnormalities in GITR/GITRL may contribute to the immune dysregulation in Sjögren's syndrome. The emerging data indicate that GITR blockade could be a potential treatment option for Sjögren's syndrome as well as other autoimmune diseases.

TNF plays a central role in many autoimmune and inflammatory diseases, and biological TNF blockade has revolutionized the therapy of rheumatoid arthritis, psoriasis, and Crohn's disease. In latter disease TNF blockade is effective in 60-70% of patients. Lykowska-Szuber et al. from Poznan University, Poland have studied expression of apoptosis genes in inflamed colon tissue of responder and non-responder Crohn's disease patients. They find reduced expression of the TNF receptor superfamily member 1B gene, *TNFRSF1B* in refractory patients which explains why these patients do not respond to this therapy.

Maintaining or restoring a balanced immune system is more complicated than keeping a buffered solution at neutral pH. Especially the autoimmune and autoinflammatory diseases are like a poke bowl mixture of genetic, epigenetic, hormonal and environmental ingredients. Detailed knowledge on how, where, and when these ingredients may go wrong will be needed to further our insight into the cellular and molecular mechanisms of immune (dys)regulation. And that will allow for the development and implementation of new immunotherapeutic interventions and further refinement of existing ones.

AUTHOR CONTRIBUTIONS

GR, CR, and FK were the guest editors of this Research Topic. GR wrote the first draft. CR and FK commented on and contributed to this editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Small RNA Deep Sequencing Identifies a Unique miRNA Signature Released in Serum Exosomes in a Mouse Model of Sjögren's Syndrome

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Sjögren's Syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration and loss of function of moisture-producing exocrine glands as well as systemic inflammation. SS diagnosis is cumbersome, subjective and complicated by manifestation of symptoms that overlap with those of other rheumatic and ocular diseases. Definitive diagnosis averages 4–5 years and this delay may lead to irreversible tissue damage. Thus, there is an urgent need for diagnostic biomarkers for earlier detection of SS. Extracellular vesicles called exosomes carry functional small non-coding RNAs which play a critical role in maintaining cellular homeostasis via transcriptional and translational regulation of mRNA. Alterations in levels of specific exosomal miRNAs may be predictive of disease status. Here, we have assessed serum exosomal RNA using next generation sequencing in a discovery cohort of the NOD mouse, a model of early-intermediate SS, to identify dysregulated miRNAs that may be indicative of SS. We found five miRNAs upregulated in serum exosomes of NOD mice with an adjusted $p < 0.05$—miRNA-127-3p, miRNA-409-3p, miRNA-410-3p, miRNA-541-5p, and miRNA-540-5p. miRNAs 127-3p and 541-5p were also statistically significantly upregulated in a validation cohort of NOD mice. Pathway analysis and existing literature indicates that differential expression of these miRNAs may dysregulate pathways involved in inflammation. Future studies will apply these findings in a human cohort to understand how they are correlated with manifestations of SS as well as understanding their functional role in systemic autoimmunity specific to SS.

Keywords: Sjögren's Syndrome, diagnostic miRNA biomarkers, extracellular vesicles, small-RNA sequencing, microRNA, piwi-RNA

INTRODUCTION

Sjögren's Syndrome (SS) is a chronic and systemic autoimmune disease marked by lymphocytic infiltration and loss of function of the body's moisture producing exocrine glands (e.g., lacrimal and salivary glands) as its defining manifestation. It is the second most common rheumatic autoimmune disease, affecting about 0.5–1% of the general population (1, 2). The progressive

inflammation of lacrimal and salivary exocrine glands is associated with their loss of function, leading to debilitating dry eye and dry mouth, respectively (3). SS is associated with increased inflammation of internal organs including brain, lung and liver (4) as well as a 44-fold increased risk of developing B-cell lymphoma (5, 6). SS can occur in the absence of another autoimmune disease (primary SS) or concurrently with another autoimmune disease such as rheumatoid arthritis or systemic lupus erythematosus (secondary SS).

Diagnosis of SS relies on the weighted score obtained from a series of criteria established in 2016 by the American College of Rheumatology (ACR) in collaboration with the European League Against Rheumatism (EULAR) (7). These criteria include: (1) labial salivary gland biopsy showing focal lymphocytic sialadenitis with a focus score $\geq$1; (2) anti-SSA (Ro) positivity (serum autoantibody); (3) ocular surface staining score $\geq$ 5 (or van Bijsterveld score $\geq$ 4) on at least one eye; (4) a Schirmer's value (tear flow) $\leq$ 5 mm/5 min on at least one eye; and (5) an unstimulated whole saliva flow rate $\leq$ 0.1 ml/min. Although, commonly used for inclusion in clinical trials, these criteria are not always practically applicable for clinical diagnosis. In particular, the labial salivary gland biopsy is painful, impractical and error-prone (8). Furthermore, these criteria have been developed primarily for patients with primary SS, and are not extensively validated in the far greater numbers of patients suffering from secondary SS. Therefore, many patients experience a substantial delay in diagnosis while some are never formally diagnosed. This delay in diagnosis may also delay treatment with anti-inflammatory agents to the point when irreversible damage to exocrine glands and other internal organs may already have occurred (9). Hence, there is an urgent need for an early, sensitive and non-invasive diagnostic test for SS.

In this study we utilized the Non Obese Diabetic mouse model of SS. The NOD/Shi strain originated from inbreeding of the Cataract Shionogi (CTS) strain, based on elevated fasting blood glucose level in cataract-free mice for the development of a model for insulin dependent diabetes mellitus. It was later also shown to develop features of exocrinopathy consistent with SS (10). Despite limitations to any animal model, this model exhibits several features of SS in humans including reduced tear and salivary secretion (11, 12), alterations in tear and salivary composition (13–15), lymphocytic infiltration of lacrimal and salivary glands (dacryoadenitis and sialadenitis, respectively) (11, 12), and the presence of many of the serum autoantibodies that are often present in human patients, such as autoantibodies to Ro/SSA and La/SSB (16), the M3 muscarinic acetylcholine receptor (17, 18), salivary gland protein 1 (19), carbonic anhydrase 6 (19) and parotid secretory protein (PSP) (19). Finally, the lacrimal glands (LG) of these mice show characteristic changes in specific proteins involved in the secretory process of exocrine glands typical of SS patients (20, 21). We have previously demonstrated that tear biomarkers identified in this murine model are also identified in SS patients (13–15).

Although, SS is more prevalent in women than in men at a 9:1 ratio (3), only male NOD mice were used in this study, because the male mice have been extensively characterized to exhibit the features of autoimmune dacryoadenitis and systemic disease prior to the development of diabetes. Females of this strain instead develop autoimmune sialadenitis concurrent with diabetes (22), complicating interpretation of any results. Thus, use of male NOD mice allows us to avoid confounding effects associated with the concurrent development of diabetes. Our mice were chosen at the age just after lymphocytic infiltration of the LG is typically established (14-weeks), representing an early-intermediate stage disease model of autoimmune dacryoadenitis in SS. As the disease development in this strain is polygenetic, and many of the diabetes resistant sub-strains that have been developed as controls for studying diabetes development still develop autoimmune exocrinopathy (11, 23, 24) there has been a lack of closely related healthy control strains for studies of SS disease development and treatment. With this said, the Balb/c strain has been the most commonly used for studies of SS exocrinopathy by multiple groups beyond our own (25–28). Therefore, we considered it the most prudent choice for use as a control strain.

MicroRNAs (miRNA) are evolutionarily conserved short non-coding RNA that function in gene silencing and post-transcriptional gene regulation (29), regulating nearly 60% of messenger RNA (mRNA) (30). A single miRNA can target several mRNA and any given mRNA may be targeted by more than one miRNA. Cooperativity between a group of dysregulated miRNAs targeting one or more mRNAs of a given signaling pathway or cellular process may substantially upregulate or downregulate that pathway and lead to development and progression of disease. Indeed, miRNA dysregulation is associated with cancer (31), obesity (32), heart disease (33), kidney disease (34), and diseases of the nervous system (35). Their diagnostic potential has been explored with high fidelity in various cancers (36), as well as neurodegenerative (37), autoimmune (38), and metabolic diseases (39). Thus, assessing the level of expression of a panel of functional mature miRNAs can be diagnostic of a given disease. Compared to proteins, miRNAs have lower inter-individual variation and less sequence heterogeneity, allowing for high specificity as biomarkers (40). Further, as miRNAs are highly evolutionarily conserved in mammals, results from an animal model are typically readily applicable to human subjects (41).

miRNAs circulate in a stable, cell-free form in all biofluids and are particularly enriched in serum (42). Relative to other biofluids such as saliva, urine, and cerebrospinal fluid (CSF), serum has a higher concentration of miRNA (42, 43). Most extracellular miRNAs as well as other non-coding RNAs (ncRNA) can be found in exosomes which are nano-sized extracellular vesicles, generated as intraluminal vesicles in multivesicular bodies (MVBs). Exosomes are actively shed from nearly every cell type and engage in intercellular signaling. Literature suggests that aberrant exosome-based intercellular communication plays a role in infectious and inflammatory diseases and various cancers. Exosomes are an extremely reliable source of miRNA as their contents are resistant to degradation by nucleases such as RNase (36, 44–47). Moreover, exosomal miRNAs are relatively stable following storage at -80°C, have a very low inter-individual variance, and also exhibit a low intra-individual variance over time (48).

The goal of this study was to understand how changes in regulatory small RNAs in serum, particularly miRNAs, might have utility as a source of diagnostic biomarkers for SS. Here we identify a subset of dysregulated miRNAs that may be specific to SS. We identified 5 exosomal miRNAs that showed significant changes in concert with establishment of SS-like symptoms in NOD mice, representing putative biomarkers for early disease diagnosis. Additionally, we have also assessed differential expression of other small non-coding RNA such as piRNA, that protect the genome by silencing transposons.

MATERIALS AND METHODS

Mice

Age-matched male NOD/ShiltJ (Stock No. 001976) and BALB/cJ (Stock No. 000651) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed with a 12 h light, 12 h dark cycle with *ad libitum* access to food and water until 14-weeks of age, when SS-like ocular symptoms are established in the NOD strain. All procedures performed on the mice were in accordance with protocols approved by the University of Southern California's Institutional Animal Care and Use Committee (IACUC) and the Guide for Care and Use of Laboratory Animals 8th edition (49).

LG Histology and Quantitative Analysis of Lymphocytic Infiltration

Lymphocytic infiltration in mouse lacrimal glands, indicative of autoimmune dacryoadenitis, was confirmed and quantified with hematoxylin & eosin staining as described (50). Briefly, lacrimal glands from NOD and BALB/c mice were fixed in 10% NBF (Richard-Allan Scientific, Kalamazoo, MI), fixed in paraffin, then cut into 5 μ horizontal sections and stained with hematoxylin and eosin. Sections were imaged using an Aperio Digital ScanScope (Leica Biosystems Inc., Buffalo Grove, IL) using the 40x objective lens. The percentage of lymphocytic infiltration in the tissue was determined by calculating the area of infiltrates manually using ImageJ (National Institutes of Health, http://imagej.nih.gov/ij). Data were analyzed by GraphPad Prism using one-way non-parametric ANOVA (Kruskal-Wallis).

Isolation of Serum Exosomes

Mice were anesthetized by intraperitoneal injection with ketamine/xylazine (60–70 mg + 5–10 mg/kg, respectively), and blood was collected by cardiac puncture using a 1 mL syringe (BD Biosciences, San Jose, CA) into MiniCollect 0.8 mL gold cap Z Serum Separator tubes (Greiner Bio-One, Kremsmünster, Austria). Thereafter the mice were euthanized by cervical dislocation. Blood was allowed to clot for 20 min at room temperature followed by centrifugation at 4°C, 2,000 × g for 15 min. Serum was collected and spun at 2,000 × g for 20 min at 4°C to pellet cellular debris. The supernatant was collected and spun at 12,000 × g for 45 min to remove microvesicles. Approximately 2000 μL of pooled supernatant from 5 mice was concentrated using 10 kDa Millipore Amicon Ultra concentrators (Burlington MA) to 500 μL and then loaded on an equilibrated iZON qEV original size exclusion column (Christchurch, New Zealand). Fractions 7–9 containing 1.5 mL of exosomes were collected and concentrated by 10-fold using 100 kDa concentrators (MilliporeSigma, Burlington, MA). Alternatively, exosomes were enriched from the supernatant obtained by centrifugation of serum as above and resolved by differential ultracentrifugation as previously described (51) with some modifications using a Beckman Coulter Optima LE-80k with a Beckman Coulter Type 50.2 Fixed Angle Rotor. Briefly, after the 12,000 × g spin of the clarified serum, the supernatant was centrifuged at 110,000 × g for 120 min. The pellet was resuspended in 2 mL of PBS containing 0.25 mM Trehalose (PBST) and centrifuged at 110, 000 × g for 70 min. The exosome pellet was resuspended in 200 μL of PBST. Purified exosomes from each protocol were used directly for RNA isolation, Western blotting or flash frozen and stored at −80°C for later analysis. A total of 5 groups per strain and 5 mice per group were utilized as biological replicates for the discovery as well as validation cohort.

Total RNA Isolation

RNA was isolated using the miRNeasy Serum/Plasma Mini Kit (Qiagen, Hilden, Germany). The manufacturer's protocols were followed as written, except for the final collection step which was performed sequentially in two steps. First, 25 μL of nuclease-free water was added to the spin column for 10 min before elution of sample. Then, this step was repeated using 15 μL of nuclease-free water to increase the recovery yield. Combination of both eluates yielded around 30 μL of total RNA collected per pooled exosome sample from five mice. The amount and quality of RNA was analyzed using a Nanodrop to assess initial concentration, and TapeStation (Agilent) to assess sample quality utilizing RNA integrity number (RIN).

Transmission Electron Microscopy

Exosomes stored at −80°C were thawed and fixed on 150 mesh copper carbon formvar grids (Electron Microscopy Sciences, Hatfield, PA). With high precision negative forceps (Electron Microscopy Sciences, Hatfield, PA), 10 μL of exosome samples were incubated with grids for 5 min. Excess liquid was absorbed using filter paper. The grid was incubated with 1% aqueous uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) for 5 min. After rinsing with 10 μL ultrapure water, the grid was air dried for 30 min before storage or immediate viewing in a JEM1400 transmission electron microscope operating at 100 keV.

Western Blotting

Equal volumes of exosome samples were heated for 5 min at 95°C under reducing conditions and resolved over 8–16% Novex WedgeWell Tris-Glycine Polyacrylamide Gels (ThermoFisher, Waltham, MA) for 90 min at 125 volts, under constant voltage. Proteins in gels were transferred to nitrocellulose membrane using an iBLOT 2 device and Invitrogen iBLOT 2 NC stacks (ThermoFisher, Waltham, MA). Membranes were rinsed in Phosphate Buffered Saline (PBS) and blocked in Rockland Blocking Buffer for Fluorescent Western Blotting (Pottstown, PA) for 1 h at room temperature. Membranes were incubated with rabbit primary polyclonal antibodies to TSG101 [Abcam—EPR7130(B), 1:250 dilution], and primary monoclonal antibody

to Cathepsin L (Abcam—EPR8011, 1:500 dilution) overnight at 4°C. After six 5 min washes in 1x PBS, membranes were incubated in goat-anti rabbit IR800 secondary antibody for 1 h at RT and rinsed again with 1x PBS, 6 times for 5 min each before imaging on a LI-COR Odyssey Fluorescent Imager. Images were analyzed using ImageStudio v5.2.5.

Particle Size Analysis

Size and concentration of exosomes was measured by Nanoparticle Tracking Analysis (NTA) using a ZetaView (Particle Metrix, Meerbusch, Germany). Some samples were also shipped to Alpha Nano Tech LLC (Chapel Hill, NC) for analysis by a ZetaView S/N 17-332 running the software ZetaView 8.04.02. After calibration with 100 nm standards (Applied Microspheres, The Netherlands), samples were diluted in varying amounts of PBS to reach the optimal concentration for analysis, then injected into the ZetaView cell for measurement. Eleven cell positions were sampled for two cycles each, with outliers automatically removed by the software. Measurements were taken at 22°C, using a sensitivity of 75, a frame rate of 30, and a shutter speed of 100. These measurements were analyzed using a minimum brightness of 20, a maximum size of 500 pixels, and a minimum size of 10 pixels. As it is the best determinant of particle size, the mode was selected as the main sizing parameter (52). Total particle count was calculated to account for varying resuspension volumes.

Particle size was also analyzed by Dynamic Light Scattering (DLS) using a Wyatt Dyna-Pro Plate reader II (Wyatt Technologies, Santa Barbara, CA). Briefly, 60 μL of exosome samples were run in triplicates at 25°C in a 384-well clear bottom plate (Greiner Bio One, Monroe, NC). The hydrodynamic radius of isolated exosomes was measured and presented as a normalized diameter. Data was analyzed using Dynamics V7 software (Wyatt, Santa Barbara, CA).

Small RNA Deep Sequencing

Library preparation and sequencing on exosome fractions were performed by GeneWiz (South Plainfield, NJ). Total RNA containing the small RNA fraction was converted into cDNA using the Illumina TruSeq Small RNA library prep kit according to the manufacturer's instructions. Briefly, 3' adapter "RA3" and 5' adapter "RA5," were ligated to total RNA which was then reverse transcribed. Adapter ligated cDNA library was enriched by PCR using primers that selectively anneal to the adapter sequence and then purified by gel electrophoresis. Quality of the cDNA library was assessed using a DNA chip on bioanalyzer. Barcodes were added to each sample and all 10 samples were sequenced on a single lane of an Illumina HiSeq system set to a 2 × 150 bp configuration. The output generated a total of ∼414 million reads which were then demultiplexed with the added barcode separating the files according to the samples into FASTQ files.

Bioinformatics

The raw FASTQ files obtained from Genewiz were assessed for their quality using FastQC v0.11.9. Adapter trimming was performed using Cutadapt v2.8 (https://github.com/marcelm/cutadapt). High quality reads of minimum length 15 nucleotides (nt) were mapped to whole genome (mm10 assembly GRCm38) using Bowtie v1.2.3 (53) and annotated using featureCounts v2.0.0 (54), using GENCODE (Release M24, GRCm38.p6) comprehensive gene annotation GTF file (PRI) to obtain distribution of reads over genome and raw counts for various non-coding RNA such as pre-miRNA, scRNA, scaRNA, snRNA, tRNA, rRNA, snoRNA, and lncRNA. Reads were then mapped to small RNA transcriptomes (miRbase v22, piRdbv2.0). The output files in the SAM file format were sorted to mapped reads that had an alignment CIGAR string of 18M or higher. Sam2counts, a python program (https://github.com/vsbuffalo/sam2counts), was used to acquire counts of reads aligned to transcriptomes (55).

Raw reads were also aligned to the piRNA and miRNA transcriptomes using an in-house aligner "miRGrep" (https://github.com/singhkakan/miRGrep) that applies brute force to count the number of reads containing a given miRNA or piRNA sequence. As miRNA are 19–26 bp long and piRNA are 24–30 bp long, the entirety of their sequence is read during sequencing. As a result, the reads (75 to 150 bp) are longer than the miRNA or piRNA of interest and contain their complete sequence. miRGrep yielded a final count table which was assembled in RStudio using the dplyr package for further processing. After the miRNA or piRNA counts table was generated, differential gene expression analysis was conducted using three statistical R packages DESEq2 (56), EdgeR (57), and LimmaVoom (58) in RStudio. Statistical significance was determined by adjusted $p < 0.05$ by DESEq2 or Limma or False Discovery Rate (FDR) < 0.1 by EdgeR. We have included miRNAs considered significant by at least 1 statistical package for downstream analyses. The experimental procedures and analysis pipeline are detailed in **Supplementary Figure 1**.

miRNA Validation Assays

In a separate validation cohort of 5 groups with five mice per group, we isolated serum exosomal RNA. The differential expression of miRNAs of interest was validated by qRT-PCR using individual Taqman Advanced miRNA Assays (Applied Biosystems). Briefly, poly-A tailing and adapter ligation was performed on 2 μL of total RNA isolated from serum exosomes of the validation cohort using the Taqman Advanced cDNA synthesis kit. Following this cDNA synthesis, miRNA amplification was conducted using the same kit. The amplified cDNA was diluted 1:10 and set up in triplicate qRT-PCRs with 1 μL of specific Taqman Advanced miRNA primer and run on a Quant-Studio Flex 6 (Applied Biosystems, Foster City, CA), using the assay's recommended cycling conditions. Results were analyzed by the ΔΔCt method with BALB/c serum exosomal small RNA as reference and miR-16-5p as housekeeping miRNA. 16-5p is identified in the literature as a suitable housekeeping miRNA (59–62) and was unchanged between serum exosomes in BALB/c and NOD mice in the sequencing data obtained in this study ($p = 0.995$, DESeq2).

Pathway and Functional Enrichment Analysis

Pathway analyses were conducted using miTALOS v2.0 (63) using StarBase2, a database of experimentally validated miRNA targets. Pathway data were extracted from KEGG, Reactome, and WikiPathways by miTALOS. Pathways with a corrected $p < 0.05$ and Enrichment score > 1 are expected to contain over-represented miRNA targets. Functional enrichment analysis was done using the custom heatmap calculator of miRPathDB v2.0 (64) using GeneOntology (Biological Enrichment) with at least two miRNA per pathway and two pathways per miRNA. The settings were chosen such that signaling pathways in which at least 2 of our miRNAs of interest have an mRNA target, would be identified. Additionally, the program was directed to identify at least two pathways targeted by each miRNA. With these constraints we may identify pathways that have a high probability of being dysregulated with the aberrant expression of the miRNA of interest. Pathway analysis was also conducted using Ingenuity Pathway Analysis (IPA) to visualize the interaction of miRNA "hits" with their targets in the signaling pathways relevant to autoimmunity, which were also identified by miTALOS and miRPathDB.

RESULTS

Characterization of Exosomes From Mouse Serum

We chose exosomes as the principal source of serum miRNAs based on findings that miRNAs are concentrated in these organelles in extracellular biofluids. Exosomes were isolated by differential ultracentrifugation (UC) for discovery experiments and by size exclusion chromatography (SEC) for validation experiments. Transmission electron microscopy (TEM) images showed ∼100 nm sized vesicles with the characteristic cup shaped morphology typical of exosomes (65) for both UC (**Figure 1A**) and SEC exosomes (**Figure 1C**). UC exosomes had a median diameter of 136 nm from male NOD mouse serum and 131 nm for BALB/c mouse serum, while SEC exosomes had a median diameter of 122 nm for NOD and BALB/c mouse serum (**Figures 1B,D**). There was no significant difference in the sizes of the exosomes isolated by the two methods, although exosomes isolated by UC were slightly larger in accord with previous reports (66). SEC has been reported to give a better yield than UC (67), consistent with our findings. There were no significant differences in the size or concentration of exosomes between the two strains of mice. Western blotting also showed the presence of the universal exosome membrane protein marker, TSG101, in exosomes isolated from both strains (**Figure 1E**). Various cathepsins (S, D, K and L) have been found in exosomes derived from plasma (68), macrophages (69), and microglia (70). Interestingly, we found enrichment of cathepsin L in exosomes isolated from both strains (**Figure 1E**). DLS found that SEC exosomes ranged from 60 to 130 nm with a mean diameter of 122 nm (**Figure 1F**). There was no strain specific difference in size, concentration or marker expression between exosomes isolated from the two strains. For study design, UC exosomes were used for miRNA "hit" identification and SEC exosomes were used for "hit" validation.

Mouse Serum Exosomes Contain Several Small RNA Biotypes

Since most extracellular ncRNA are associated with extracellular vesicles (71), UC serum exosomes were used as a source for small RNAs from NOD and BALB/c mice. Establishment of autoimmune dacryoadenitis, the most notable characteristic of SS in these mice, was confirmed in each NOD cohort relative to BALB/c by H & E staining of lacrimal gland sections and quantitation of lymphocytic infiltrates (**Supplementary Figure 2**). While we did not observe any lymphocytes infiltrating LG in the BALB/c, NOD mice had infiltration between 8 and 15% ($p < 0.0001$, one-way ANOVA). Using next-generation sequencing (NGS), we profiled small RNA in serum exosomes from each mouse strain to identify both novel and differentially expressed miRNA. A total of 417 million raw reads were generated which were mapped to the mouse genome and various small ncRNA transcriptomes. Of the reads that mapped to the mouse genome, roughly two-thirds of the reads mapped to intergenic or intronic regions while a third of the reads mapped to exons. Less than 10% of the reads mapped to transcription start or end sites (**Figure 2A**). Read distribution was fairly uniform across samples with no strain specific difference. More than 50% of mapped reads were comprised of miRNA (both mature and precursor) and piRNA. Nearly 27% of reads mapped to lncRNA, and 20% to rRNA, whereas <1% mapped to small nucleolar RNA (snoRNA) (**Figures 2B,C**) in serum exosomes of both NODs and BALB/c mice. Reads that mapped to miRNA, piRNA, lncRNA, snRNA, snoRNA, scaRNA and rRNA are provided in **Supplementary Table 1**. We did not observe any strain-specific differences in proportion of RNA sub-types within the small ncRNA libraries of NOD and BALB/c serum exosomes ($p = 0.925$, ordinary two-way ANOVA).

piRNA are involved in gene silencing of transposons by forming complexes with argonaute proteins (72), and appear to provide RNA-mediated adaptive immunity against transposons (73). In our analysis we found that 208 piRNA were expressed in NOD mouse UC exosome samples and 238 in BALB/c mouse samples. Of these, 171 were found in both but 37 were unique to NOD mouse samples, and 67 unique to BALB/c mouse samples (**Supplementary Figure 3B**). The 10 most highly expressed piRNA did not appear to be dysregulated in the NOD strain (**Supplementary Figure 3A**). There were no significant differences in the total number of distinct piRNA or the total number of reads aligning to piRdb between the two strains (**Supplementary Figure 3C**). We found 13 piRNA to be upregulated in NOD mouse samples with a $\log_2$ fold change >3, while 15 were downregulated with a $\log_2$ fold change < -3. Of these, only mmu-piR-58696 was significantly upregulated in the NODs as determined by LimmaVoom ($p_{adj} = 0.047$) (**Supplementary Figure 3D**).

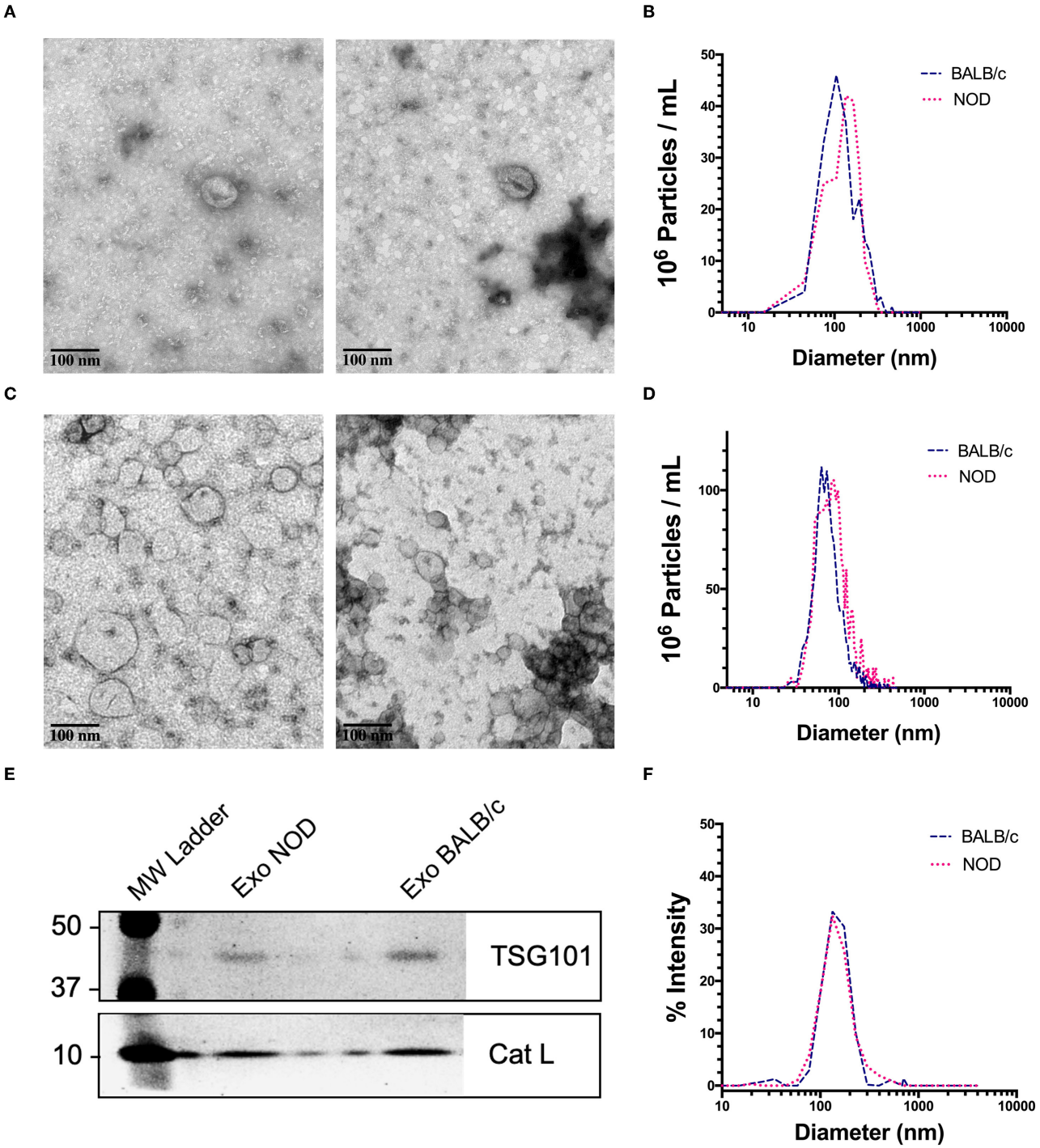

FIGURE 1 | Characterization of mouse serum-derived exosomes by differential Ultracentrifugation (UC) and Sice Exclusion Chromatography (SEC). **(A)** Transmission Electron Microscopy (TEM) images of exosomes isolated from NOD (left) and BALB/c (right) mouse serum using differential UC. The cup and saucer shaped morphology typical of exosomes is visible. **(B)** NTA showed median diameters of 136 and 131 nm for serum exosomes from NOD and BALB/c mice, respectively. The graph is representative of four separate experiments per mouse strain. No significant differences in exosome median diameter were observed between the two strains. **(C)** TEM images of SEC exosomes isolated from NOD (left) and BALB/c (right) mouse serum. **(D)** NTA of SEC exosomes. **(E)** Western blotting of NOD and BALB/c serum exosomes isolated by SEC show enrichment of TSG101 and Cathepsin L. **(F)** Exosome particle size of SEC exosomes analyzed by DLS showed a median diameter of 122 nm from both strains. The graph is representative of three separate experiments.

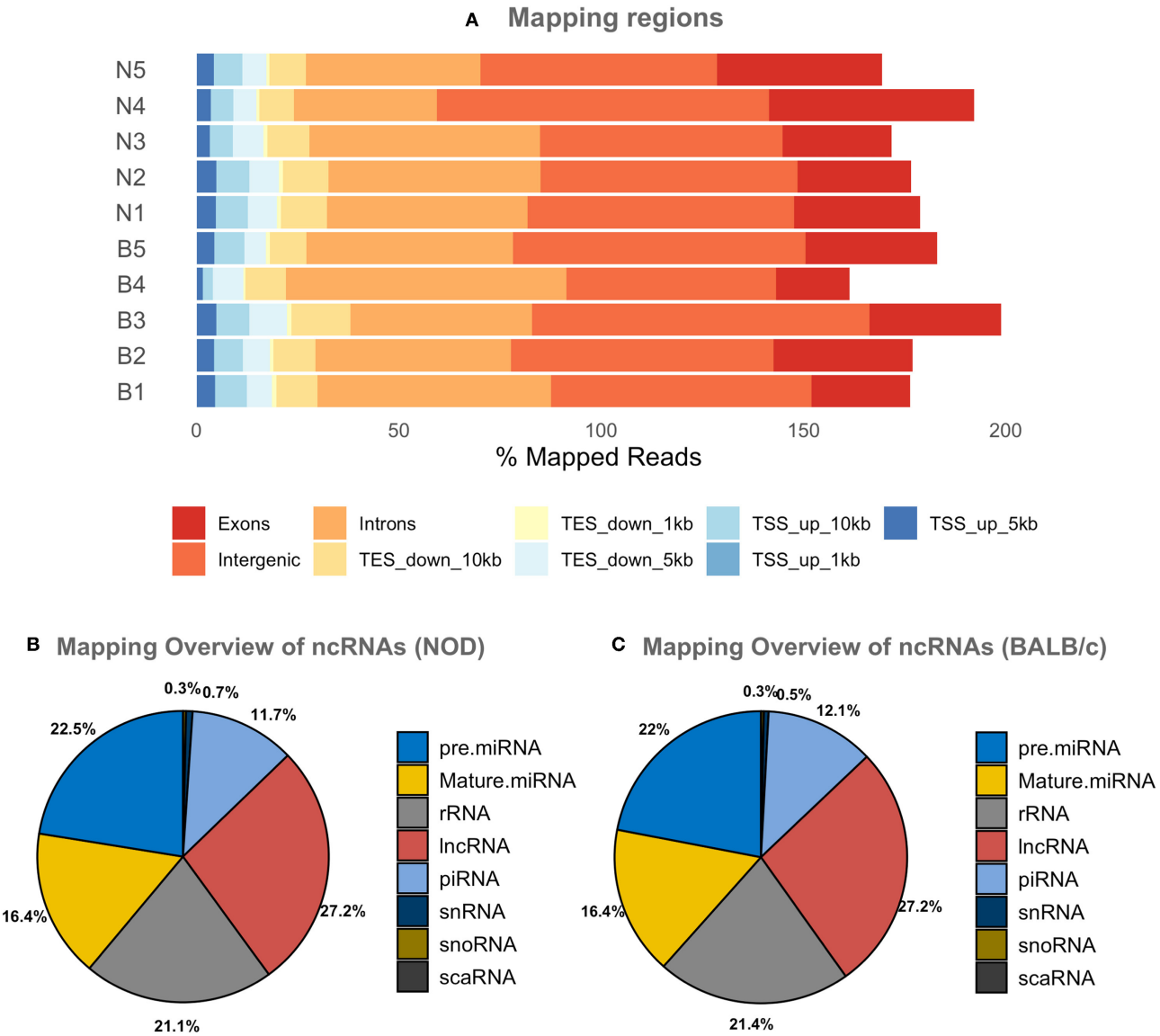

FIGURE 2 | Graphical presentations of the distribution of reads from NGS sequencing of RNAs. **(A)** Shows the regions of the mouse genome that reads of all samples were aligned to. The mapping percentage is >100 as multi-mapping reads were also counted. No significant sample to sample variation was observed. **(B,C)** Show the distribution of reads aligning to various ncRNAs in NOD & BALB/c serum UC exosomes. Results were obtained from $n = 5$ UC serum exosome samples per mouse strain, with each sample comprising pooled serum exosomes from five mice. TSS, Transcription Start Site; TES, Transcription End Site; kb, kilobases; pre miRNA, precursor microRNA; rRNA, ribosomal RNA; lnc RNA, long non-coding RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; scaRNA, small cajal body specific RNA; mature miRNA, mature microRNA; piRNA, piwi interacting RNA.

NOD Serum Exosomes Contain a Subset of Dysregulated miRNA

FASTQ reads were preprocessed using FASTqc which identified the presence of adapter in ∼90% of the reads. Cutadapt was used to remove adapter sequences and exclude reads of quality <20 and length <15. Pre-processed trimmed reads were then mapped to the miRNA transcriptome from miRbase v22.0 using Bowtie v1.2.3, as well as our in-house aligner miRGrep, which utilizes brute force and was written specifically for the alignment of RNA < 30 nt in length such as miRNA and piRNA. With Bowtie, we identified 550 distinct miRNAs in NODs and 255 in BALB/c. Using miRGrep, we identified 251 miRNAs in the NODs and 242 miRNAs in BALB/c. This is to be expected because miRGrep uses brute-force to align reads to miRNA, with no mismatch allowed in alignment and therefore, the miRNA identified by miRGrep are a subset of those identified by Bowtie (74). Of these, read counts for 38 miRNAs were found only in NOD serum exosomes while 29 miRNAs were found only in BALB/c serum exosomes (**Supplementary Figure 4A**) in at least 3 out of 5 sample groups per strain. The top 20 expressed

miRNA in NOD serum exosomes were overrepresented to the same extent in BALB/c (**Supplementary Figure 4B**) with no discernible strain specific differences. Of these miRNAs, miR-191-5p, miR-92a-3p, miR-22-3p, miR-16-5p, let-7f-5p, let-7i-5p, miR-26a-5p, miR-30e-5p, miR-186-5p, miR-30d-5p, miR-451a, miR-181a-5p, miR-148a-3p, miR-423-5p, let-7a-5p, and miR-25-3p have been previously reported to be abundant in serum exosomes (75). miR-486-5p (not shown) was the top over-represented miRNA as reported previously (75). It is possible that these miRNAs serve important regulatory functions that are evolutionarily conserved.

The volcano plot in **Figure 3A** shows the level of differential expression for all expressed miRNA identified in NOD mouse serum. miRNA that had an adjusted $p > 0.05$ and $\log_2$ fold change <3 or >-3 were not considered significant. DESeq2 and Limma determine significance when an adjusted $p < 0.05$ is reached whereas EdgeR considers a hit significant only when the FDR is $<$ 0.1 in RStudio (**Table 1**). We have reported and assessed miRNA that met our significance criteria by at least one of the statistical packages. Unsupervised hierarchical clustering analysis of top hits using the Euclidean method clustered NOD mouse miRNA samples in the same group, separate from BALB/c samples, as shown by the top tree (**Figure 3B**), suggesting that the differential expression observed may be attributed to dysregulation in the NOD strain. We performed an additional unsupervised technique to visualize the variability between the two groups. Principal component analysis (PCA) of the 10 samples with ∼500 expressed miRNAs revealed that 22% of the variance could be explained by the differences in strain (**Figure 3C**). All three packages determined that miR-127-3p, miR-409-3p, and miR-540-3p were significantly overexpressed in NOD mouse (**Figure 4A**). Limma identified miR-410-3p and miR-541-5p to be overexpressed in NOD serum exosomes (**Figure 4B**).

Furthermore, we found an additional 19 miRNAs that displayed an at least 3-fold higher expression in NOD serum exosomes than in Balb/c and 11 miRNAs that were under-expressed by at least 3-fold in NOD mice (**Supplementary Figure 4C**). Despite a meaningful fold change these did not reach statistical significance due to an outlier. Of these, miR-329-5p was found to be over 5-fold overexpressed in NOD serum exosomes (DESeq2, EdgeR). As NOD mice, even when age matched, show a variation in disease progression, it is possible that the outlier group may have progressed further in disease than other groups and vice versa. Thus, we included miR-329-5p in our downstream analyses as its differential expression may have biologically significance.

Validation of miRNA Differential Expression

To validate our deep sequencing findings of known miRNAs that were differentially expressed in NOD vs. BALB/c mouse serum exosomes, we purified serum exosomes from independent cohorts of mice, 5 samples per strain with each sample comprised of serum exosomes from each of five mice. For this round of exosome isolation, we used the SEC method (**Figures 1C–F**) which yielded exosomes of greater particle homogeneity. qRT-PCR was performed using Advanced Taqman miRNA Assays. In agreement with the sequencing data, miRNA miR-127-3p, miR-409-3p, miR-540-3p, miR-410-3p, miR-541-5p, and miR-329-5p were expressed at a higher level in NOD mouse serum exosomes (**Figure 5**). Five out of the six miRNAs were more than 25-fold over-expressed in the NODs on average. Of these, over-expression of mmu-miR-127-3p and mmu-miR-541-5p were found to be statistically significant ($p < 0.01$, Mann-Whitney U-test). miR-410-3p and miR-329-5p were upregulated in 4/5 groups whereas miR-409-3p was upregulated in 3/5 groups.

Pathway Analysis Identifies Several Pathways Involved in Lymphocyte Activation

To understand the signaling pathways the "hit" miRNA may be involved in, we used three methods (miTALOS, miRPathDB, and IPA) which utilize a range of databases of predicted and experimentally validated mRNA-miRNA interactions (such as Tarbase, TargetScan, miRanda, Starbase2) and databases of known pathways (KEGG, Reactome, WikiPathways) in their pathway analysis algorithm. These databases do not have information on all known miRNA for every species, and so we used multiple tools in our pathway analysis to be as comprehensive as possible. Functional over-representation analysis using Gene Ontology (Biological Functions) in miTALOS allows miRNA target prediction using the latest versions of TargetScan (6.2) and miRanda. Additionally, a database of experimentally validated targets—StarBase2- was also implemented in the pathway analysis and is available for both human and murine microRNAs. StarBase2 catalogs 3 miRNA hits identified in this study –mmu-miR-127-3p, mmu-miR-409-3p, mmu-miR-410-3p. As these have identical sequences for human and mice, pathway analysis for both species (**Supplementary Figures 5A,B**) was conducted using these three hits. Databases in miRPathDB catalog mmu-miR-541-5p in addition to the three miRNAs above. Using miRPathDB, both KEGG (not shown) and WikiPathways identified B cell receptor signaling (**Supplementary Figure 5C**) while Gene ontology—Biological functions identified lymphocyte activation (not shown). Other pathways identified are associated with cellular proliferation, pluripotency, apoptosis, and p53 signaling, all pathways that may be relevant to cancer. IPA identified several pathways involved in immune regulation (**Supplementary Figure 5D**). Pathways common to all three analyses include B-Cell Receptor (miR-targeted genes in lymphocyte in miTALOS), TGF-beta and IL-6 Signaling.

DISCUSSION

Here we report that an unbiased screen of exosomal serum miRNA in a murine model of early-intermediate stage autoimmune dacryoadenitis and SS, identified the significant upregulation of multiple miRNAs. qPCR analysis of serum exosomes from a separate set of disease-model vs. healthy control mice validated two significant hits, miR-127-3p and miR-541-3p, while confirming marked elevation with some variance across animal groups for three additional miRNAs, miR-409-3p, miR-410-3p, and miR-329-5p. SS is a complicated

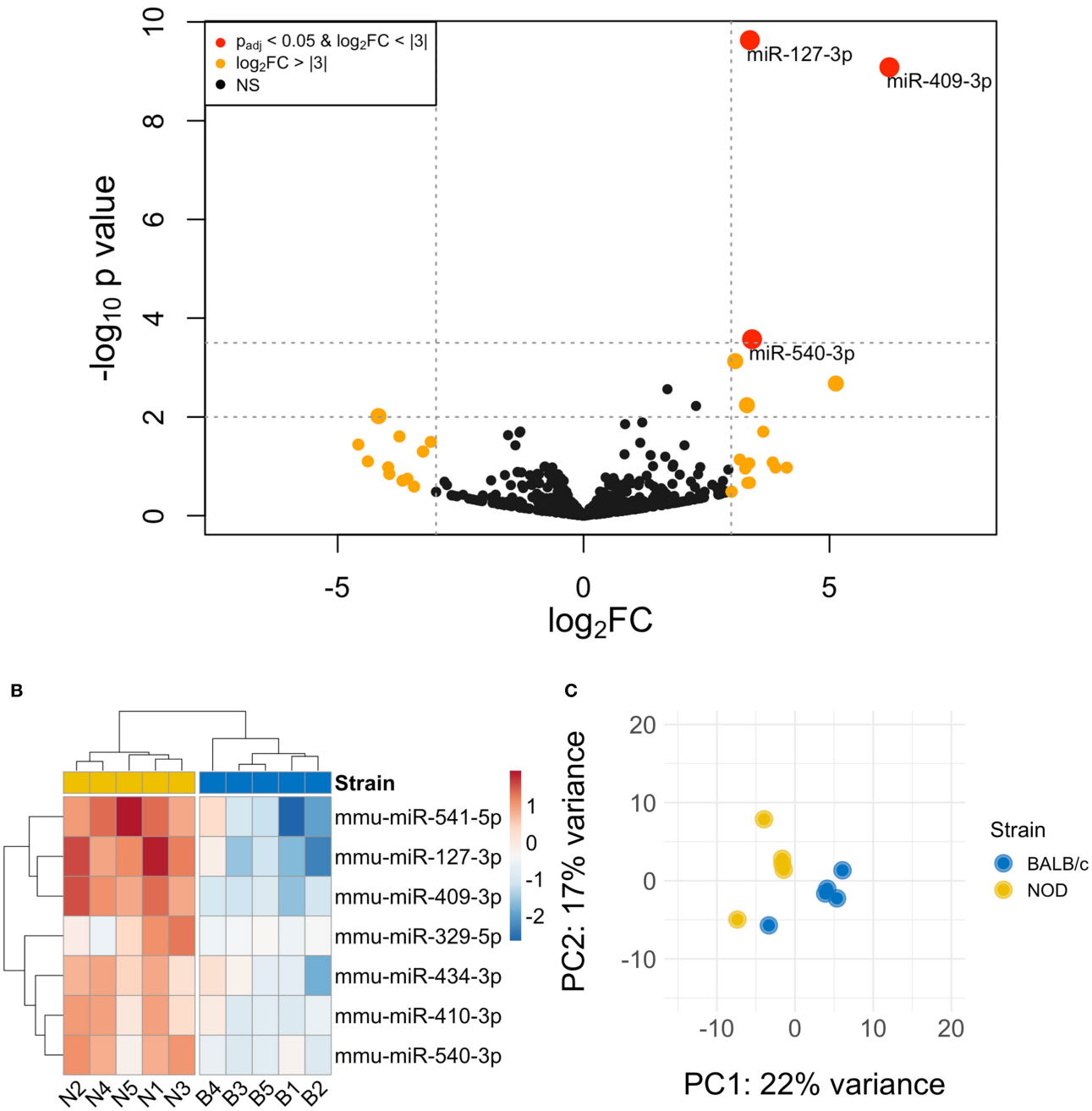

FIGURE 3 | Differential miRNA expression analysis. **(A)** The volcano plot highlights miRNAs that were identified in exosomes isolated by differential ultracentrifugation as upregulated or downregulated by more than 3-fold, shown in orange. Upregulated miRNAs that are statistically significantly different are shown in red. **(B)** Unsupervised hierarchically clustered heatmaps of the most differentially expressed miRNA (with highly expressed miRNAs in red and minimally expressed miRNAs in blue) in the 10 samples. Samples are labeled either N for NOD or B for BALB/c. Individual samples for the top differentially expressed miRNA cluster by strain. **(C)** PCA plot of all miRNAs shows that 22% of the variance was accounted for by strain.

autoimmune disease, with treatment hindered by a poorly understood pathogenesis and a lack of reliable diagnostics. Given that miRNAs are master regulators of gene expression with their dysregulation implicated in many diseases (76), identification of this group of dysregulated miRNAs in the male NOD mice at the initial stages of autoimmune dacryoadenitis and development of other indicators of established systemic disease in SS may be useful in establishment of future diagnostic biomarkers.

TABLE 1 | Summary of the 7 most differentially-expressed miRNAs detected in serum UC exosomes from 14-week male BALB/c and NOD mice using three statistical packages in RStudio.

	DESeq2		Limma		EdgeR	
miRNA	**Log_2 FC**	**p_{adj}**	**Log_2 FC**	**p_{adj}**	**Log_2 FC**	***p*-val**
miR-127-3p	3.38	**1.27×10^{-7}**	3.77	**6.08×10^{-6}**	3.40	**2.14×10^{-4}**
miR-409-3p	6.22	**2.25×10^{-7}**	5.41	**4.38×10^{-6}**	6.16	**5.06×10^{-5}**
miR-540-3p	3.42	**0.0485**	3.67	**5.0×10^{-3}**	3.37	**1.39×10^{-3}**
miR-410-3p	3.08	0.101	3.49	**8.29×10^{-3}**	3.02	2.49×10^{-3}
miR-541-5p	3.32	0.406	5.34	**2.3×10^{-4}**	3.24	8.88×10^{-3}
miR-329-5p	5.13	0.229	–	–	6.23	1.2×10^{-2}
miR-30d-3p	−4.17	0.580	−2.67	0.196	−4.4	5.79×10^{-3}

Values reaching statistical significance at $p_{adj} < 0.05$ are bolded, for DESeq2 and Limma Significance for EdgeR is determined by False Discovery Rate < 0.10.

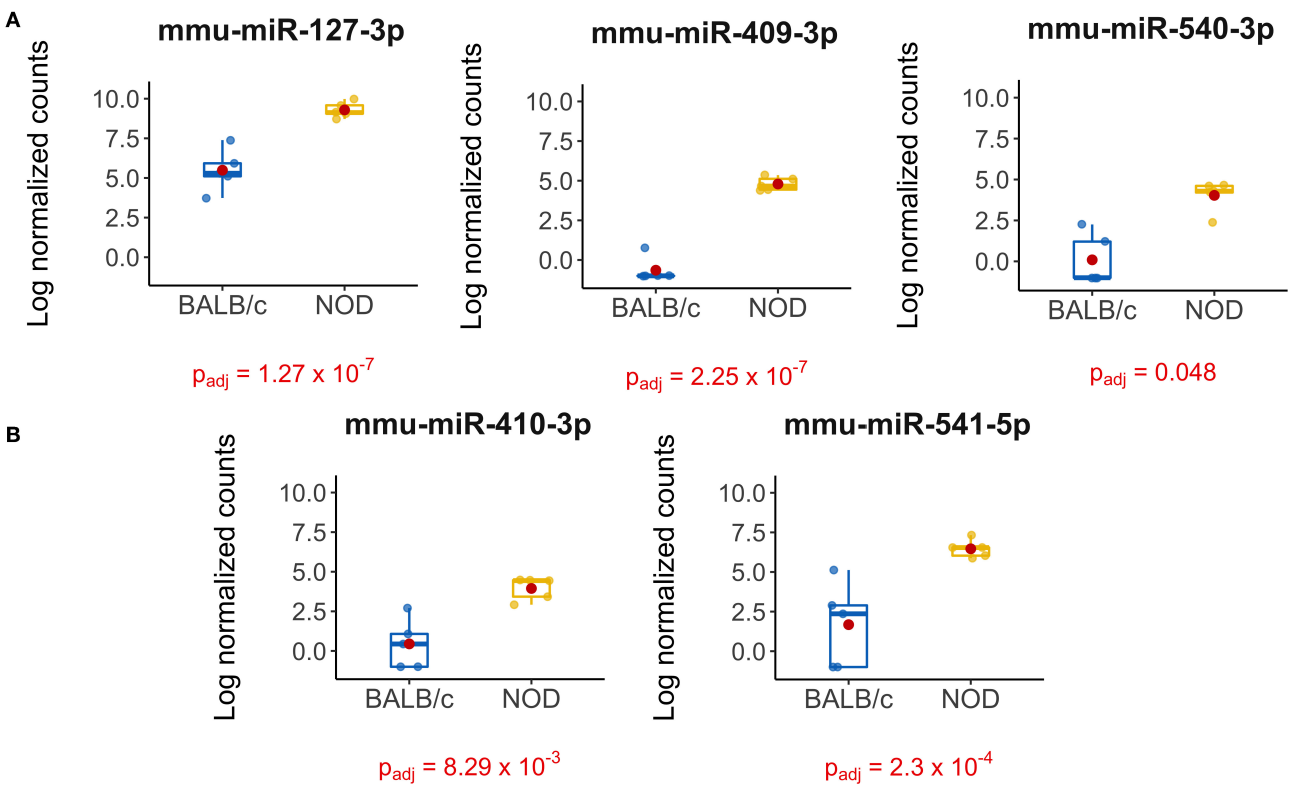

FIGURE 4 | Boxplots of differentially expressed miRNAs in exosomes isolated from 14-week male BALB/c and NOD mouse serum. Reads were aligned with Bowtie and an in-house brute force-based aligner, and differential gene expression analysis was conducted using DESEq2, EdgeR, and LimmaVoom in RStudio. **(A)** Both DESeq2 and LimmaVoom identified miR-127-3p, miR-409-3p, and miR-540-3p as "hits" with $p_{adj} < 0.05$. **(B)** Additionally, LimmaVoom identified miR-410-3p and miR-541-5p to be dysregulated with $p_{adj} < 0.05$. miR-329-5p failed to reach statistical significance due to one outlier but exhibited the highest fold change with $Log_2FC > 5$ in EdgeR and DESEq2. Results were obtained from data sets generated from small RNA sequencing of 5 sets of RNA from exosomes from each mouse cohort, each isolated from purified pooled serum exosomes from five groups of age-, gender-, and strain-matched mice, with 5 mice per group.

mmu-miR-127-3p, a significant and validated hit, has been shown to be necessary for the self-renewal and differentiation of hematopoietic stem cells (HSC) in a mouse model of HSC self-renewal defect (77). It has also been proposed to be a regulator of senescence as a tumor suppressor by directly targeting BCL6 (78, 79), a known protooncogene in human cell lines. As BCL6 is a transcriptional repressor and inhibits the production of IL10, its downregulation by miR-127-3p can lead to an increase in IL-10 (80). Increased levels of IL-10 are well-documented in SS patients (81, 82) and are also reported in the NOD mouse lacrimal gland in association with development of autoimmune dacryoadenitis (15). Our pathway analysis shows that mmu-miR-127-3p, is involved in the regulation of TGF-beta and B-Cell receptor

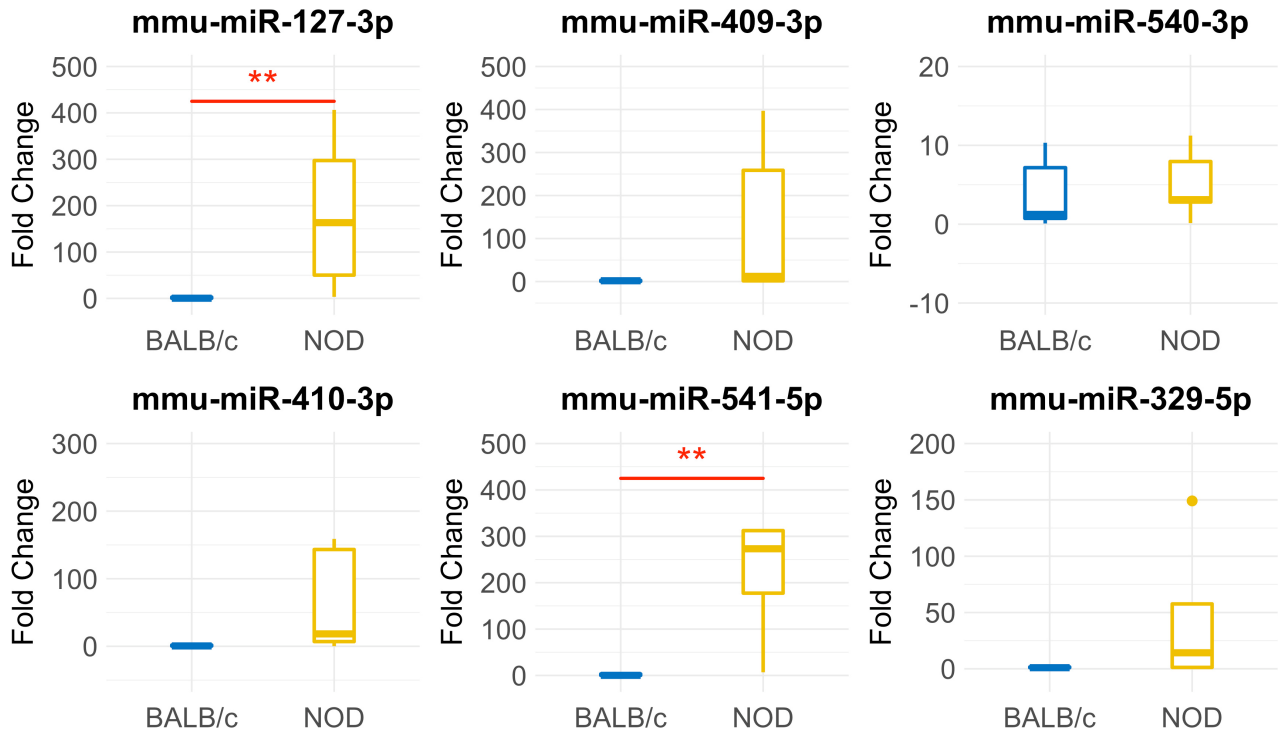

FIGURE 5 | Validation of miRNA 'hits' in Size Exclusion Chromatography (SEC)-purified serum exosomes from 14-week male BALB/c and NOD mice. Fold-change data represent mean ± SD normalized to miR-16-5p using the ΔΔCt method. Statistical significance was determined by the Mann-Whitney U-test; $^{**}p < 0.01$. Results were obtained from data sets generated from qRT-PCR from RNA isolated from SEC-purified pooled serum exosomes from five groups of mice per strain, with 5 mice per group. All mice were age matched.

Signaling (**Supplementary Figure 5C**) through its targeting of several MAP kinases and BCL6 (**Supplementary Figure 5D**). Appropriate regulation of BCL6 is also necessary for the development of germinal center B cell and follicular helper T cells (83). Upregulated levels of hsa-miR-127-3p are reported in testicular and nodal diffused large B-cell lymphoma, with an inverse correlation to BCL6 levels (84). Thus, regulated levels of miR-127-3p are necessary for appropriate control of lymphoproliferation and B cell homeostasis and elevated levels may be indicative of immune dysfunction/autoimmunity. This finding in the NOD mice is of particular relevance because a subset of SS patients develop B cell lymphoma (5) and there is great interest in biomarkers that may distinguish these patients from others with SS so that earlier interventions may be applied to suppress development of B cell lymphoma. It will be of great interest to study the potential dysregulation of miR-127-3p longitudinally in SS patients to explore its relationship to this debilitating and most destructive manifestation of SS.

mmu-miR-541-5p, a second significant and validated hit, may work in concert with mmu-miR-127-3p. In a mouse model of multiple sclerosis, miR-541-5p and miR-127-3p were upregulated in lymph nodes indicating that they may be involved in pathogenic neuro-inflammation (85). Interestingly, knockout of TNF-α in a mouse model led to downregulation of both miR-541-5p and miR-127-3p in epidermal skin, hinting at a close involvement of these miRNAs with pro-inflammatory cytokines (86).

Another hit, miR-409-3p, is broadly implicated in autoimmune disease in animal models and patients with chronic fatigue syndrome/myalgic encephalomyelitis (87), multiple sclerosis (88) and systemic lupus erythematosus (89). Our pathway analysis using miRPathDB and IPA indicates miR-409-3p's involvement in the B Cell Receptor, STAT3 and IL-6 signaling pathways (**Supplementary Figures 5C,D**). Studies have found that in mice with experimental autoimmune encephalomyelitis (EAE, a murine model of multiple sclerosis), mmu-miR-409-3p targets suppressor of cytokine signaling protein 3 (SOCS3). Upregulation of mmu-miR-409-3p in astrocytes of EAE mice silences SOCS3, leading to an increase in phosphorylation of STAT3 and increased production of inflammatory cytokines such as IL-1β, CXCL10, IL-6, MC-P1 (90). Another study found that in a co-culture of NOD mice salivary gland acinar cells (SGAC) and B-lymphocyte (an *in-vitro* model of salivary gland disease in SS), there was a significant increase in production of cytokines IL-6 and IL-1β by B-lymphocytes and increased phosphorylation of STAT3 in SGAC (91). IL-1β is upregulated in diseased NOD mouse LG, while its injection into murine LG further impairs tear production (92). Secretion of these cytokines by circulating lymphocytes is also increased in SS patients (93). Thus, mmu-miR-409-3p may be pro-inflammatory in nature and its upregulation

may increase cytokine production via the SOCS3/STAT3 signaling pathway.

According to TargetScan, mammals including mice and humans have an 8-mer conserved site on the Stat3 gene for miR-410 (30). hsa-miR-410-3p appears to directly target STAT3, leading to a reduction of IL-10 in T cells of patients with systemic lupus erythematosus (94), and was also shown to be elevated in the plasma of these patients (95). Increased expression of miR-410-3p was also observed in males with relapsing remitting multiple sclerosis, which is characterized by cycling of autoimmune inflammatory status (96). Expression of hsa-miR-410-3p was decreased in the synovial fluid and synoviocytes of rheumatoid arthritis (RA) patients. On the other hand, in an *in-vitro* model of RA, overexpression of hsa-miR-410-3p decreased the pro-inflammatory cytokines, TNF-α, IL-6, IL-1β, and MMP-9 (97) and was anti-proliferative and apoptotic in nature through targeting of transcription factor YY1 (98). Based on these results, upregulated miR-410-3p may have an immune-protective effect. If validated, use of this miRNA may have value as a potential therapeutic.

Of interest, five of the identified miRNAs (miR-127-3p, miR-329-5p, miR-409-3p, miR-410-3p and miR-541-5p) are encoded on the highly evolutionarily conserved Dlk1-Gtl2 locus on the maternally inherited allele of mouse chromosome 12, which is analogous to the locus Dlk1-Dio3 on the maternally inherited allele of human chromosome 14. The miRNAs from this locus seem to regulate ground state pluripotency in embryonic stem cells (99). Genes from this imprinted locus play a critical role in embryonic and fetal development and appear to be dysregulated in several diseases, including blood cancers such as lymphoma, acute myeloid and acute promyelocytic leukemia, as well as autoimmune diseases such as lupus nephritis (100) and multiple sclerosis (96). It is also interesting that the sequences of mature miRNA 127-3p, 409-3p, and 410-3p are identical in human and mouse. Sequences of miRNA-541-5p and miRNA-329-5p vary only by 3 nucleotides between human and mouse. This further highlights the applicability of miRNA-based biomarkers arising from murine model in this study to humans.

Studies of extracellular vesicles rely on particle size analysis which cannot discriminate between functional vesicles and lipid droplets of similar size, and the presence of these can be a confounding factor in our studies. Future studies will aim to validate these results utilizing different methods of exosome isolation. Although our choice of serum over plasma and the use of serum-separator tubes was aimed at reducing hemolysis, it may also be a confounding factor in our study. While miRNA stability studies in plasma have shown that levels of miR-127-3p are not altered by hemolysis (87), strengthening its potential as a biomarker candidate, similar investigations are needed for the other miRNAs identified in this study.

While we have identified the potential role of some miRNA in inflammatory pathways and SS, further study is required to better understand the relationships of these miRNA as well as the temporal changes that occur with disease progression. The panel of miRNA identified in this study reflect early changes in SS progression, while different patterns of dysregulation may be observed longitudinally as disease advances and/or as it impacts different organs. Since the secretory contents from both lacrimal and salivary glands are also influenced by the autoimmune inflammation characteristic of SS and have been used as sources of biomarkers to reflect both systemic and local inflammation, evaluating these biofluids in later studies as additional sources of potential biomarkers will be of importance. Future studies will also focus on assessing the utility of this panel of miRNA in identifying SS patients as well as exploring the utility of these miRNA in longitudinal disease progression in combination with existing blood-based biomarkers such as anti-La and anti-Ro antibodies and others.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The datasets can be found in the Sequence Research Archive under ID PRJNA622527 and are available for download here https://www.ncbi.nlm.nih.gov/bioproject/622527.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Southern California, Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

SK performed the bioinformatics, statistical analysis of next generation sequencing data, isolated and characterized mouse serum exosomes from the validation mouse cohort, isolated exosomal RNA, performed qPCR validation, and prepared the manuscript and supplementary files. SJ and BC isolated and characterized mice serum exosomes and exosomal RNA from the test cohort. SJ assisted with blood collection and performed qPCR validation of miRNA in the validation cohort. BC and SK optimized serum exosome isolation protocols. ME contributed to writing the manuscript and characterization of serum exosomes. DC contributed to data analysis and revised final manuscript. CO and SH-A secured research funding and contributed to experimental design and writing the manuscript. SH-A revised the final manuscript. All authors contributed to the article and approved the submitted version.

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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.2020.01475/full#supplementary-material

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The Role of GITR/GITRL Interaction in Autoimmune Diseases

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Glucocorticoid-induced TNFR-related protein (GITR) is a member of the TNFR superfamily which is expressed in various cells, including T cells, natural killer cells and some myeloid cells. GITR is activated by its ligand, GITRL, mainly expressed on antigen presenting cells and endothelial cells. It has been acknowledged that the engagement of GITR can modulate both innate and adaptive immune responses. Accumulated evidence suggests GITR/GITRL interaction is involved in the pathogenesis of tumor, inflammation and autoimmune diseases. In this review, we describe the effects of GITR/GITRL activation on effector T cells, regulatory T cells (Tregs) and myeloid cells; summarize its role and the underlying mechanisms in modulating autoimmune diseases.

Keywords: glucocorticoid-induced TNFR-related protein, GITRL, T cell, myeloid cells, autoimmune diseases

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INTRODUCTION

Glucocorticoid-induced TNFR related protein (GITR, also known as TNFRSF18) is a member of the TNFR superfamily, which is expressed on regulatory T cells (Tregs) and some activated immune cells, including effector T lymphocytes, nature killer (NK) cells, and neutrophils (1–4). Our previous data have shown that myeloid-derived suppressor cells (MDSCs) could also express GITR (5). GITR is triggered by its ligand, GITRL, which is mainly expressed on B cells, dendritic cells (DCs), macrophages and endothelial cells (6, 7). GITR engagement on effector T cells can generate a positive costimulatory signal and promote T cell activation and proliferation, whereas the activation of GITR on Tregs abrogates their suppressive function (6, 8). In addition, GITR triggering increases resistance to tumors and viral infections, and exacerbates autoimmune diseases and inflammation processes (9). This review mainly focuses on recent studies regarding the GITR/GITRL role in autoimmune diseases.

Abbreviations: Bcl-6, B-cell lymphoma; CIA, collagen-induced arthritis; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; EAT, experimental autoimmune thyroiditis; ESS, experimental SS; GC, germinal center; GITR, glucocorticoid-induced TNFR-related protein; GITRL, GITR ligand; GvHD, graft versus host disease; IDO, indoleamine-2,3-dioxygenase; MDSCs, myeloid-derived suppressor cells; MLR, mixed lymphocyte reaction; NF-κb, nuclear factor-κB; NK, nature killer; pSS, primary Sjögren's syndrome; pTreg, peripherally induced Treg; RA, rheumatoid arthritis; SF, synovial fluid; SLE, systemic lupus erythematosus; TCR, T-cell receptor; Treg, regulatory T cells; Tfh, T follicular helper; Th, T helper; Th17, IL-17-producing T helper; Th9, IL-9-producing T helper; Tfh, T follicular helper; TRAF2, tumor necrosis factor receptor-associated factor 2; Tresps, responder T lymphocytes; tTreg, thymus-derived Treg.

EFFECT OF GITR ON EFFECTOR T CELLS

GITR is expressed at low levels on resting $CD4^+$ and $CD8^+$T cells, and the level of GITR will be up-regulated following T-cell receptor (TCR) activation (8). GITR triggering exerts co-stimulatory effects on conventional T cells by increasing T cell survival, activation and proliferation (3). In fact, GITR stimulated by GITRL or anti-GITR Ab can increase TCR-induced T cell proliferation and cytokine production, and rescues T cells from anti-CD3-induced apoptosis (3, 10). Further investigations indicate a different role of GITR in $CD4^+$ and $CD8^+$ T cells. It has been acknowledged that the co-stimulatory capability of GITR is weaker and essentially different from that of CD28 (3, 11). Ronchetti et al. indicated that GITR could lower the threshold of CD28 co-stimulation in effector $CD8^+$ T cells, and GITR activity on $CD8^+$ T cells is considered to be independent of CD28 activation, which is different from $CD4^+$ T cells (10, 12). In effector $CD4^+$ T cells, the predominated conclusion is triggering GITR on $CD4^+CD25^-$ T cells can induce the survival, activation and proliferation of $CD4^+$T cells, and the effect is mainly dependent on TCR stimulation and CD28 co-triggering (13). However, several researches reported that GITR/GITRL interaction could also contribute to immune homeostasis by regulating effector $CD4^+$T cells (14, 15). GITR engagement can mediate cell apoptosis. By using a graft versus host disease (GvHD) model, Muriglan et al. showed GITR activation on $CD4^+$T cells reduced alloreactive proliferation in mixed lymphocyte reaction (MLR) experiments, and the reduction in expansion was due to the increased apoptosis *via* Fas-FasL pathway (16). Also, It has been found that co-stimulation of GITR showed a potent capacity to produce abundant IL-10, and caused the counter-regulation of increased proliferation responses (17).

Recent years, the regulation of GITR/GITRL was also investigated in several novel Th (T helper) cell subsets, including IL-17-producing T helper (Th17) cells, T follicular helper (Tfh) cells and IL-9-producing T helper (Th9) cells (18–22). Our previous study has found that recombinant GITRL can promote the differentiation and expansion of Th17 cells (18). Clouthier et al. reported that GITR expression was induced during the maturation phase of germinal center (GC) Tfh cells. Researches have implied that GITR was implicated in the differentiation and function of Tfh cells. GITR deficiency may result in impaired Tfh cell response, considering that Tfh cells in $GITR^{-/-}$ mice showed significant lower frequency and weaker capacity to provide help in antibody production than that in $GITR^{+/+}$ mice (23). Similarly, our previous study also showed that activation of GITR by GITRL could promote the differentiation of Tfh cells (19). Mechanically, GITR triggering initiated downstream canonical nuclear factor-κB (NF-κB) pathway through recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF5, which was responsible for B-cell lymphoma 6 (Bcl-6) transcription in Tfh cell program (24). Notably, a recent study identified the regulation of GITR signaling on Tfh cells in anti-tumor immune responses. They found that administration of anti-GITR agonistic antibody could induce IL-21-producing Tfh cells, and thus led to enhanced antitumor immune responses (22). Furthermore, GITR co-stimulation could also enhanced the differentiation of Th9 cells in a TRAF6- and NF-κB-dependent manner, and then promoted the tumor-specific cytotoxic T lymphocyte (CTL) responses (20). The effects of GITR triggering in different Th cell subsets are summarized in **Figure 1**.

EFFECT OF GITR ON TREG CELLS

GITR is constitutively expressed in Treg cells and is critical for the development and activity of Treg cells (2, 8, 25). The expression of GITR in Tregs can be up-regulated after activation or in tumor microenvironment, and the level of GITR usually positively correlates with the immunosuppressive function of Tregs (26, 27). GITR engagement in Treg cells can have multiple distinct effects. Both *in vitro* and *in vivo* data indicate that GITR activation abrogates the suppressive function of Tregs (2, 8). The initial study reported an agonist anti-GITR Ab inhibits the suppression of Tregs to break the immunological self-tolerance (8). By co-culture of Tregs from $GITR^{+/+}$ mice and $CD4^+$ effector cells from $GITR^{-/-}$ mice, Ronchetti et al. further confirmed the GITR activation on Tregs inhibited the suppressive effect of the cells (3). In fact, while GITR triggering inhibits the suppressive activity of Treg, several other studies identified it can induce Treg proliferation and expansion in vitro. The number of Treg cells was lower in $GITR^{-/-}$ mice, and the numbers of thymus-derived Treg (tTreg) and peripherally induced Treg (pTreg) cells were higher in GITRL transgenic mice (28–30). Nowakowska and Kissler have found that the number of Treg cells was increased in Ptpn22-deficient mice, but blockade of GITR ligand prevented Treg cell expansion caused by Ptpn22 knockdown, demonstrating the critical role of GITR signaling in regulating the size and composition of Tregs (31). tTreg cell progenitors express high levels of GITR, OX40 and TNFR2, agonists of these TNFRSF members enhance Treg differentiation whereas combined neutralization of their ligands diminishes the development of Treg cells (25). Additionally, GITRL was reported to be dispensable for the development of Treg cells in the thymus. Furthermore, DCs from $GITRL^{-/-}$ mice were less efficient in inducing proliferation of antigen-specific Treg cells in vitro than the cells from WT mice, indicating GITRL on antigen presenting cells is requisite for optimal Treg-mediated regulation of immune responses (32). Taken together, these results indicate that the effects of GITR binding in Tregs depend on the experimental model, the culture condition, the intensity of GITR activation and the pattern in which it is stimulated. The effects of GITR activation on Treg cells are summarized in **Figure 1**.

GITR/GITRL INTERACTION IN MYELOID CELLS

GITR can be detected on various myeloid cells, including monocytes, macrophages, DCs and MDSCs (5, 8, 33–35), and will be at high levels when activated. GITRL can also present on DCs and macrophages. It has been demonstrated that GITRL elicits a tolerogenic effect on plasmacytoid DCs. GITRL initiates the

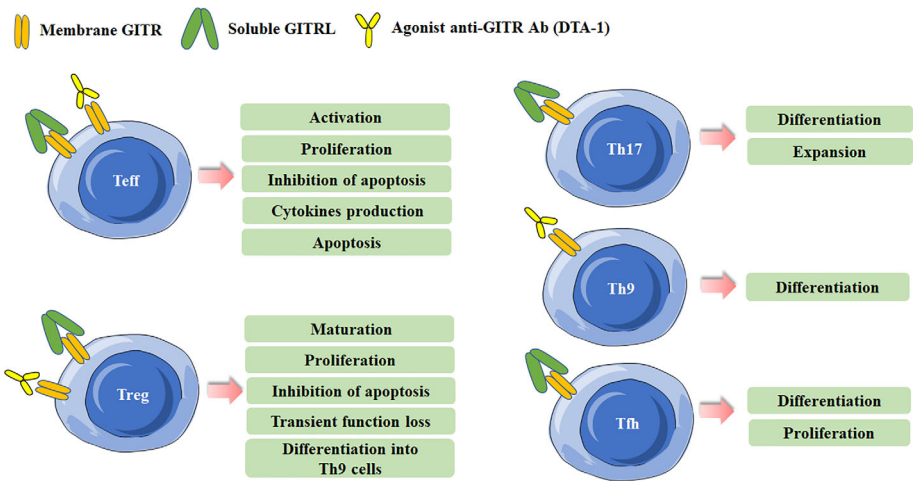

FIGURE 1 | Effects of Glucocorticoid-induced TNFR-related protein (GITR) triggering in effector T cells and Treg cells. Different effects are induced in effector T cells and Treg cells after GITR triggering by GITR ligand (GITRL) or agonist anti-GITR Ab (DTA-1). Triggering GITR can promote the differentiation and proliferation of Th17, Th9, and Tfh cells.

immunoregulatory pathway of tryptophan catabolism by inducing indoleamine-2,3-dioxygenase (IDO) after engagement by soluble GITR (36). In addition, by using the Candida albicans infection model, GITRL triggering downregulated IL-12 production in DCs when they were co-cultured with Treg cells, which acted as a modulator of DC activity (37). However, Ronchetti et al. reported that GITR is important for the activation of DCs and potentiates DC-induced activation of T lymphocytes (38). In macrophages, GITR/GITRL interaction can elicit a proinflammatory effect through both GITRL and GITR activation in macrophages (33). In rheumatoid arthritis (RA) patients, GITR stimulation by anti-GITR mAb increased the production of proinflammatory cytokines/chemokines and matrix metalloproteinase-9 in synovial macrophages (39). Additionally, GITRL triggering by anti-GITRL mAb or soluble GITR can also up-regulated production of proinflammatory and chemoattractant cytokines (33, 40). MDSCs, a population of immature myeloid cells with immunosuppressive function, has been demonstrated be involved in cancer, infectious diseases and autoimmune diseases (41–43). Our previous study has shown MDSCs from experimental Sjögren's syndrome (ESS) mice could express GITR, and triggering GITR on MDSCs by GITRL significantly reduced the suppressive function of MDSCs on $CD4^+$ T-cell proliferation, and the suppressive factors secreted by MDSCs, including arginase and NO, were also down-regulated. The effects of GITR and GITRL triggering in myeloid cells are summarized in **Figure 2**.

ROLE OF GITR/GITRL INTERACTION IN AUTOIMMUNE DISEASES

GITR/GITRL Interaction in RA

RA is a chronic autoimmune disease characterized by persistent synovitis and systemic inflammation, resulting in the cartilage damage and bone erosion (44). In the collagen-induced arthritis (CIA) mouse model, a lower incidence of CIA was induced in $GITR^{-/-}$mice than in $GITR^{+/+}$mice, and less neutrophil infiltration, joint injury, and bone erosion were observed in $GITR^{-/-}$mice. GITR triggering abrogated $GITR^{+/+}$ Treg suppressive effect and co-stimulated $GITR^{+/+}$ $CD4^+CD25^-$ effector T cells, indicating the reduced susceptibility to CIA was due to GITR modulation of effector and Treg cell function (45). Patel et al. demonstrated that activation of GITR by anti-GITR mAb significantly enhanced the production of Th1 and Th2-related cytokines and exacerbated the severity of CIA mice (46).

Of note, the regulation of GITR/GITRL interaction on Th17 and Tfh cells in RA has been explored in recent years. Th17 cells play an essential role in the pathogenesis of autoimmune arthritis (47). Our previous data have shown recombinant GITRL administration could cause an earlier onset of arthritis with markedly increased disease severity and joint damage in CIA mice, and an increasement of Th17 cells were observed in spleen and draining lymph nodes. Further in vitro data revealed that GITRL could efficiently promote naïve $CD4^+$T cells differentiate into Th17 cells. All these data identified the function of GITRL in enhancing Th17 differentiation and exacerbating arthritis progression in CIA mice (18). Further studies indicated that GITRL initiated p38 MAPK signal pathway and activated STAT3 signaling, which is responsible for the development of Th17 cells (48). Another novel Th cell subset, Tfh cells, have been reported to be involved in the development of multiple autoimmune diseases, including RA (49). Tfh cells facilitate humoral immunity through supporting GC generation, providing signals crucial for antibody class switching of B cells, generation of high affinity antibodies, and memory formation (50). Ma et al. have found that splenic Tfh cells from CIA mice expressed higher levels of GITR compared with non-Tfh cells, and the activation of GITR significantly enhanced the percentage

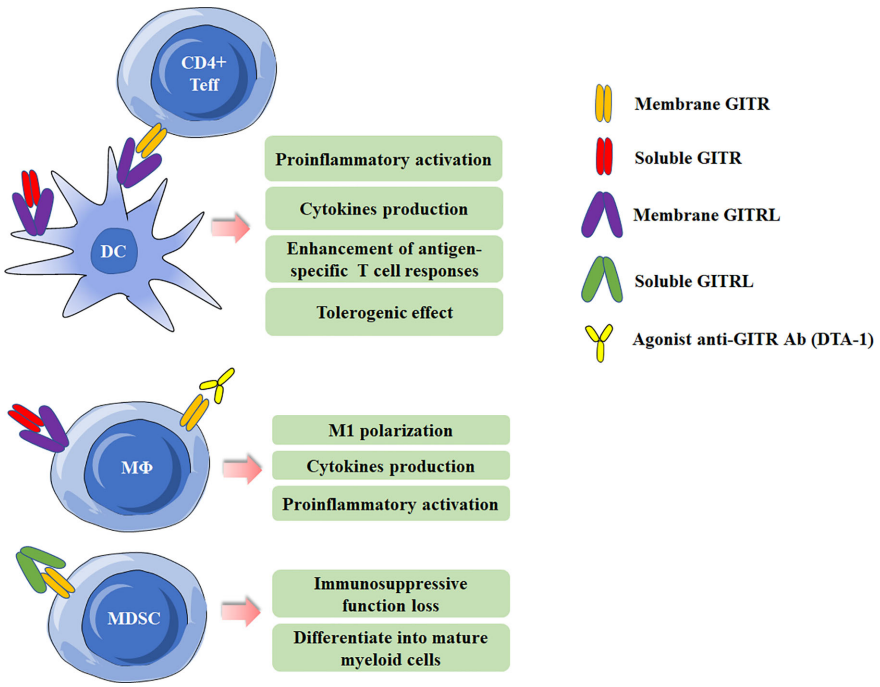

FIGURE 2 | Effects of Glucocorticoid-induced TNFR-related protein (GITR)/GITR ligand (GITRL) activation in myeloid cells. Multiple effects of GITR triggering by agonist anti-GITR Ab (DTA-1) or engagement by soluble/membrane GITR in DCs and macrophages. Activation of GITR by GITRL impairs the suppressive function of MDSCs.

and number of Tfh cells in vitro and in vivo. Furthermore, blocking the GITR/GITRL by GITR-Fc protein ameliorated the disease severity by suppressing the Tfh cell response (19). Together, all these studies indicate the critical role of GITR/GITRL signaling in CIA mouse model.

In RA patients, the GITRL level was significantly elevated in both serum and synovial fluid (SF). Positive correlations were found between serum GITRL levels and inflammation parameters or autoantibody production. indicating a role of GITRL in the development of RA (51). Additionally, GITR deficiency on RA synovial macrophages has been considered to inhibit the development of autoimmune arthritis via abatement of inflammatory response (39). Accordingly, macrophages function as a proinflammatory agent in the development of autoimmune diseases in a GITR-dependent manner.

GITR/GITRL Interaction in Primary Sjögren's Syndrome

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, such as salivary and lachrymal glands, leading to the loss of secretary function. The primary clinical symptoms are sicca syndrome, including dry eyes and mouth (52, 53). GITRL has been identified to be closely associated with the disease severity in MRL-Fas^{lpr} mice and pSS patients(54, 55). The expression of GITRL in salivary gland duct epithelial cells was evaluated to contribute greatly to the pathogenesis of Sjögren's syndrome-like autoimmune sialadenitis in MRL-Fas^{lpr} mice (54).The serum GITRL was demonstrated to positively correlate with the degree of lymphocytic infiltration in pSS patients (55). Our recent study further confirmed the role of GITRL in ESS mouse model and pSS patients, and explored the regulation of GITRL on MDSCs in pSS (5). We found that MDSCs gradually lost their suppressive function during the development of ESS, thus leading to progressive inflammation. Further exploration revealed that the increased GITRL in ESS mice could attenuate the suppressive function of MDSCs via activating GITR/GITRL pathway. Moreover, blocking GITR signal in MDSCs significantly restored their immunosuppressive function and ameliorated ESS progression in mice. The similar conclusion was also obtained in pSS patients. All these data identified a critical role of GITRL in modulating the suppressive function of MDSCs, which may facilitate the validation of GITRL as a therapeutic target for the treatment of pSS.

GITR/GITRL Interaction in Colitis

It has been studied the role of $CD4^+CD25^-GITR^+$ and $CD4^+CD25^+GITR^+$ T cells in a well-described $CD4^+CD45RB^{hi}$ T cell SCID-transferred colitis model. They demonstrated that both $CD4^+CD25^-GITR^+$ and $CD4^+CD25^+GITR^+$ T cells, regardless of the CD25 expression, could prevent the development of colitis, indicating $CD4^+CD25^-GITR^+$ and $CD4^+CD25^+GITR^+$ T cells can retain the regulatory function (56). Moreover, DTA-1 treatment significantly increased disease severity and death in TNBS-induced colitis (57). Soluble recombinant Fc-GITRL treatment have been reported to exacerbate IBD by inducing the proliferation of pathogenic IFN-γ producing T cells and reducing Treg cells in a mouse model (58). Additionally, GITR deficient mice protected against the colitis by

reducing innate immune responses and effector T cell activity. Effector T cells isolated from $GITR^{-/-}$ mice were less effective than T cells isolated from $GITR^{+/+}$ mice to transfer colitis in immunodeficient mice. Blocking the GITR/GITRL signal by soluble GITR prevented the colitis in normal $GITR^{+/+}$ and SCID mice (34). In contrast, Liao et al. evaluated the pathogenesis of colitis by using a $CD4^+$T cell transfer model of chronic enterocolitis. The results showed that the expression of GITR on the surface of regulatory and effector $CD4^+$T cells was dispensable for progression of the disease, but the presence of GITR on DCs and macrophages was requisite for controlling colitis (59).

GITR/GITRL Interaction in Other Autoimmune Diseases

Experimental autoimmune thyroiditis (EAT) is a murine model for Hashimoto's thyroiditis, which is characterized by mononuclear cell infiltration and destruction of the thyroid gland. Administration of anti-GITR mAb in EAT mice inhibits the $CD4^+CD25^+$ T cell mediated tolerance and aggravates the disease EAT, causing the increased autoantibody production and mononuclear infiltration in local tissues. Additionally, our group also found that the upregulated serum GITRL has a positive correlation with the percentage of Th17 cells in Hashimoto's thyroiditis patients, and the increased GITRL may impair the balance of Th17/Treg, thus contributing to the pathogenesis of Hashimoto's thyroiditis (60). In an experimental autoimmune encephalomyelitis (EAE) mouse model, It has been observed that anti-GITR Ab could significantly aggravate the disease severity and induce antigen-specific T cell proliferation and cytokine production (61). Also, the role of GITR in systemic lupus erythematosus (SLE) patients was explored. The expression of GITR on Tregs and $CD4^+CD25^-$ responder T lymphocytes (Tresps) were positively correlated with the severity of the disease. Glucocorticoid may achieve its therapeutic effect partly by inducing GITR expression on Tresps rather than Tregs, which initiates the apoptosis of Tresp cells in SLE patients (62).

MODULATION OF GITR/GITRL IN THE TREATMENT OF AUTOIMMUNE DISEASES

The involvement of GITR/GITRL in both innate and adaptive immunity may account for the inflammatory activation in autoimmune diseases (63). Thus, it is plausible that GITR blockade should be a potential treatment for autoimmune diseases, for inhibiting the activation of autoreactive T lymphocytes and inflammatory cells, and sustaining the immunocompetence of Tregs and MDSCs, which eventually corrects excessive autoimmunity. Although several researches have confirmed the therapeutic effects of GITR-Fc fusion protein or GITR gene knockout in murine autoimmune diseases models, the more explicit impact of GITR-Fc fusion protein on autoimmune microenvironment still needs future study prior to clinical trials (45).

CONCLUSION

Researches in recent years have described the roles of GITR/GITRL signaling on various immune cells involved in autoimmune diseases from different perspectives, including Th17 cells, Tfh cells, macrophages and MDSCs. In general, GITR triggering plays a proinflammatory role in the pathological mechanism of autoimmune diseases. Hence, GITR/GITRL system is a potential target for the immunotherapy of autoimmune diseases. Current studies have affirmed the therapeutic effects of GITR-Fc fusion protein in autoimmune diseases murine models, and future researches are expected to clarify the potential mechanism of this agent before application in clinical tests.

AUTHOR CONTRIBUTIONS

JT and BZ drafted the manuscript. KR discussed and revised the manuscript. SW designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Glance at the Use of Glucocorticoids in Rare Inflammatory and Autoimmune Diseases: Still an Indispensable Pharmacological Tool?

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Since their discovery, glucocorticoids (GCs) have been used to treat almost all autoimmune and chronic inflammatory diseases, as well as allergies and some forms of malignancies, because of their immunosuppressive and anti-inflammatory effects. Although GCs provide only symptomatic relief and do not eliminate the cause of the pathology, in the majority of treatments, GCs frequently cannot be replaced by other classes of drugs. Consequently, long-term treatments cause adverse effects that may, in turn, lead to new pathologies that sometimes require the withdrawal of GC therapy. Therefore, thus far, researchers have focused their efforts on molecules that have the same efficacy as that of GCs but cause fewer adverse effects. To this end, some GC-induced proteins, such as glucocorticoid-induced leucine zipper (GILZ), have been used as drugs in mouse models of inflammatory pathologies. In this review, we focus on some important but rare autoimmune and chronic inflammatory diseases for which the biomedical research investment in new therapies is less likely. Additionally, we critically evaluate the possibility of treating such diseases with other drugs, either GC-related or unrelated.

Keywords: glucocorticoids, rare disease, glucocorticoid-induced leucine zipper, inflammation, autoimmunity

INTRODUCTION

Reading a scientific article that dates back to the year 1900 definitely impresses on how pioneering in unknown medical and biological fields could have been more thrilling than a new discovery in current times, especially because empirical attempts based on experience and medical practice led to unprecedented successes in treating debilitating and life-threatening diseases. For example, the paroxysms of asthma were successfully treated by Doctor Solomon Solis-Cohen by using adrenal extracts administered as "grains of the desiccated gland substance." It not only provided the suffering patients relief from the recurrence of paroxysms but also helped them reach "a state of freedom from fear of their recurrence." Such a report clearly describes how debilitating, both in the body and mind, was a disease such as asthma or other chronic inflammatory or autoimmune diseases for which no specific treatment was available. At that time, scientists and physicians were not yet aware of the content of the adrenal extracts, whose benefits were clear, because adrenaline,

supposed to be the active compound, was not absorbed when given orally. It took another three decades for the isolation of cortisone and other steroids, which were the active compounds in the treatment of asthma (1). Starting from observations in 1929 and thereafter, Philip Hench, a clinician from the Mayo Clinic, successfully treated patients with rheumatoid arthritis (RA) by administering the so-called "compound E" in 1948. This compound was among the several steroids isolated from the adrenal cortex by the chemist Edward Kendall. Kendall, together with Philip Hench and the biochemist Tadeus Reichstein, was awarded the Nobel Prize in Physiology and Medicine in 1950 for "discoveries related to the hormones of the adrenal cortex, their structure and biological effects." Compound E was later identified as cortisone, the commercial production of which started in 1949 (2). Its undeniable usefulness in therapy has enabled the treatment of severe pathologies, ranging from autoimmune diseases to tumors, and driven the development of synthetically derived steroid compounds with stronger anti-inflammatory properties. Unfortunately, long-term treatment with glucocorticoids (GCs) is associated with many severe adverse effects, which counteract their beneficial effects (3, 4). To overcome these problems, several attempts have been made thus far to search for new drugs or modified molecules, such as the so-called "dissociated steroids" (5). However, these attempts have been unsuccessful, and seventy years after their first use are still not enough to get rid of these indispensable therapeutics, despite their unwanted adverse effects. Moreover, recently, GCs have been found to be efficacious as life-saving drugs in patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (6, 7).

PLEIOTROPIC ASPECTS OF GCS AS ANTI-INFLAMMATORY DRUGS

GCs are, by far, the most effective anti-inflammatory drugs for treating chronic inflammatory diseases, allergies, and autoimmune pathologies, such as RA, asthma, multiple sclerosis, and systemic lupus erythematosus (8, 9). Their mechanism of action has not yet been completely elucidated, owing to the different levels of tissue expression, several glucocorticoid receptor (GR) isoforms, and the complex gene regulation mediated by the activated monomeric or dimeric forms of GR. GC ligands, once bound to the GR, induce series of effects that depend either on the gene regulation activated by the interaction of ligand-bound GR with responsive DNA sequences (genomic actions) and/or molecular events that do not require gene modulation (non-genomic actions) (for a detailed description of the mechanism of action, please refer to references (10, 11)). As drugs, GCs are generally considered immunomodulatory rather than simply immunosuppressive, because of their complex effects on the cells of the immune system. Their anti-inflammatory action is mainly dependent on the suppression of pro-inflammatory cytokines and their transcription factors such as NF-κB and AP-1, and the activation of anti-inflammatory genes, such as *GILZ* (*TSC22D3*) and *DUSP-1* (12–15). Nevertheless, some pro-inflammatory effects of GCs have been reported, such as the induction of the expression of NLRP3, a central component of the inflammasome (16, 17). Furthermore, an extensive body of evidence suggests that GCs have different effects on the immune system depending on the duration of their administration. Prolonged exposure to GCs may cause immunosuppression, whereas acute exposure can activate the immune system (18).

In the case of chronic autoimmune or inflammatory diseases, long-term therapy with high-dose GCs elicits immunosuppressive and anti-inflammatory effects, which are necessary for symptomatic relief. Unfortunately, the consequent adverse effects are sometimes quite severe, requiring specific additional therapies or suspension of GC therapy. In some pathologies, such as asthma, the side effects of GCs have been partially resolved by topical administration (19). However, generally, adverse effects due to high doses cannot be fully avoided in systemic GC therapy. Attempts have been made to develop the so-called dissociated steroids, with the aim to favor the transrepression of activated monomeric GR over GR dimer transactivation, which is considered the cause of side effects. Studies in GR^{Dim} mutant mice, which harbor a mutation that causes impaired homodimerization of the ligand-bound GR, have initially shown a reduced functionality of the transcriptional activity (20). However, other recent studies have revealed that this mutant GR can still dimerize, although to a lower degree, making this model suboptimal for distinguishing differences between GR monomeric and homodimeric related effects (21). Furthermore, some side effects, but also some therapeutic effects, depend on both transactivation and transrepression. Thus, the concept of separating the beneficial anti-inflammatory effects from the adverse effects of GCs cannot be based on the simple separation of transrepression from transactivation activities (10). The dynamics of gene regulation by GR and its binding to the DNA remains a complex mechanism that needs to be more deeply studied. This is the reason why none of the selective glucocorticoid receptor agonist and modulators developed so far has still reached the market. Knowing the biology of the complex functions of these hormones will allow the development of pharmacological tools specifically targeting one of their sophisticated mechanisms (22, 23).

Another important aspect to consider in long-term treatments with GCs is that patients could develop adrenal insufficiency (e.g., 37% of RA patients), because GCs regulate their own secretion through a negative feedback loop, thereby inhibiting the hypothalamic–pituitary–adrenal (HPA) axis (24). It takes some time for the HPA axis to function properly after suppression. Recent studies have demonstrated that it takes as long as 1 year for a suppressed adrenal to again secrete these hormones. Conversely, if GC treatment lasts 1–2 weeks, it takes only 1 day to again secrete endogenous GCs (25). Interestingly, the suppression occurs also locally, at the level of adrenal steroidogenic activity (26). Furthermore, enzymes of the steroid biosynthesis are expressed not only by the adrenal cortex but also by other tissues, such as the lung, brain, spleen, skin and cells of the immune system. Interestingly, dysregulation of local steroidal activity has been found to be involved in the pathogenesis of some autoimmune or inflammatory diseases,

such as lupus erythematosus, multiple sclerosis, RA, and psoriasis (27). When local hormone production is altered, many GC-responsive genes are aberrantly expressed and may contribute to the pathogenesis of the above-mentioned diseases.

Sometimes, GC treatments are not efficacious because of the development of resistance to GC effects. Resistance was described first in the 1970s in *in vitro* cell systems and has been largely studied in asthma and RA (28). The lack of a therapeutic response in 4%–10% and 30% of patients with asthma and RA, respectively, is attributable to treatment resistance. Some other inflammatory diseases, such as chronic obstructive pulmonary disease (COPD), are up to 100% resistant to GC treatments (29). There are multiple underlying mechanisms for GC resistance, from those genetic in origin to molecular alterations including the overexpression of the non-ligand–binding GRβ isoform, which functions as a decoy receptor (8, 29, 30). For comprehensive reviews on this subject, see references (31–33). Overcoming the problem of resistance by reactivating the sensitivity to GCs, when possible, is the only strategy for using GC treatment in pathologies that cannot be treated with alternative drugs.

GCs are released by the HPA axis under a specific rhythm, which is regulated by the circadian clock in anticipation of daily energy-demanding situations. In rodents, GC release peaks slightly before nighttime, whereas in humans, this happens before daybreak, just in the proximity of their respective active phase (34). Therefore, the time of administration of exogenous GCs in chronic autoimmune or inflammatory diseases is critical and should be carefully chosen because of the circadian clock. Furthermore, the existence of cross-talk between the HPA axis and the immune system further complicates the scenario in which GCs must act. It has been suggested that late-night administration is more effective than early morning administration, since the immune system starts to be activated between 1:00 and 2:00 a.m. and peaks early in the morning. This immune activation increases in some inflammatory conditions, such as RA, gout, or allergic asthma, and cannot be controlled by endogenous GCs, whose production is already inadequate due to chronic inflammation and consequent downregulation of the HPA axis. Therefore, administration of exogenous GCs during nighttime ameliorates morning symptoms in RA patients (35).

Considering the multiple aspects related to GCs as drugs, the need to optimize GC therapy is crucial, especially in the case of rare diseases where GCs are the only choice and often used in combination with other drugs (**Figure 1**).

RARE PATHOLOGIES THAT REQUIRE GC TREATMENT

Rare diseases are usually defined as those conditions that affect fewer than 200,000 people in the US and no more than 5 in 10,000 people within the general population in Europe. Independently of the numbers, a rare disease occurs in a small number of people compared to other diseases prevalent in the general population. Documented rare diseases range between 5,000 and 8,000, thus complicating the findings for appropriate pharmacological treatments, owing to the low number of cases for each pathology and its distribution across countries (36). The majority of rare diseases are life-threatening and genetic in origin, and most of them affect children, resulting in significant developmental problems or death. Therefore, treatments for rare

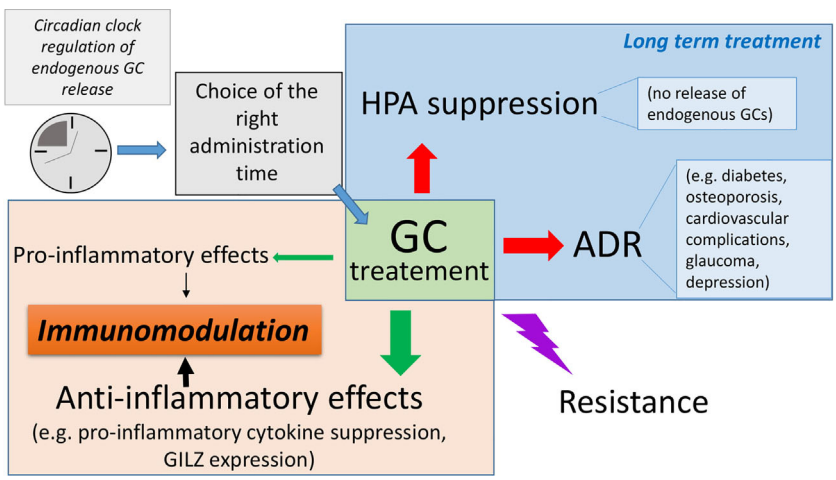

FIGURE 1 | Complex regulation of glucocorticoid (GC) activity during pharmacological administration: GCs exert immunomodulatory activities, both by transactivating anti-inflammatory proteins such as glucocorticoid-induced leucine zipper (GILZ) and transrepressing pro-inflammatory genes, such as those encoding for pro-inflammatory cytokines, and therefore exerting powerful anti-inflammatory and immunosuppressive effects. They can also favor inflammation, e.g., by induction of inflammasome. Importantly, long-term GC treatment causes undesired adverse drug reactions (ADR), which can often result in severe pathologies such as diabetes, osteoporosis, and metabolic disorders. The hypothalamic–pituitary–adrenal (HPA) axis is inevitably suppressed after long-term treatment, thus indicating the use of dose-escalating regimens to avoid life-threatening consequences. The occurrence of resistance to GC efficacy can further complicate the management of autoimmune and inflammatory diseases, especially for diseases whose treatments other than GCs are not available.

diseases remain an unmet medical need. Any drug developed for the treatment of a rare disease is called "orphan drug." Considerable costs associated with the development of these drugs often hinder the launch of a specific medical product in the market (37). Nevertheless, rare diseases whose pathogenesis has a proven inflammatory or autoimmune basis can be successfully treated with GCs, ensuring they are under control throughout patients' lifetimes, although they cannot be cured. Needless to say, the severe adverse effects of long-term and high-dose GC therapy warrant the search of new pharmacological tools that could help avoid GC use while affording the same anti-inflammatory and immunosuppressive effects. A brief description of examples of GC treatments for some rare diseases follows.

Insulin autoimmune syndrome, a rare and systemic disease, is characterized by spontaneous episodes of hyperinsulinemic hypoglycemia caused by high titers of serum insulin autoantibodies. Self-remission occurs often in the affected individual; however, high-dose GC therapy is required and has resulted in successful outcomes because of its autoimmune origin. Other therapies are available, from immunosuppressive agents, such as azathioprine, to monoclonal antibodies-based therapies, such as rituximab (an anti-CD20 antibody) which targets IgG producer B cells (38).

Another systemic inflammatory orphan disease is relapsing polychondritis, which can be considered a syndrome, because it is frequently associated with other autoimmune conditions, including systemic vasculitis, RA, and spondyloarthritis. It is characterized by an inflammatory infiltrate, IgG deposits, and islands of cartilage with fibrosis. High-dose steroid therapy represents the first line of treatment only in life-threatening cases, followed by a switch to less toxic drugs in the maintenance phase, such as azathioprine. Non-steroidal anti-inflammatory drugs (NSAIDs) are also used in cases involving the nose, external ear, or joints. Biologicals including tocilizumab (an anti-IL-6 antibody), abatacept (a CTLA4 fusion protein), and TNF inhibitors can be used in patients who do not respond to standard therapies (39, 40).

Some rare diseases affect the skin, and sometimes, also other organs. Autoimmune bullous disorders encompass autoimmune diseases with high morbidity and mortality. Aberrant IgG and IgA autoantibody production directed against adhesion molecules of the epidermis or the dermal-epidermal basement membrane zone leads to a loss of skin adhesion. In pemphigus, IgG autoantibodies react against epidermal adhesion complexes of keratinocytes, whereas in pemphigoid diseases, loss of adhesion is typical of the basement membrane zone. The result of the action of these autoantibodies is the occurrence of mucosal or cutaneous blisters and erosions. First-line treatment involves systemic corticosteroids that may be combined with immunosuppressive agents, such as azathioprine, which help reduce the risk of relapse (41). As in other autoimmune diseases that involve autoantibody production by B cells, the anti-CD20 antibody rituximab has been found to be efficacious to such an extent that it has been recently considered as a first-line treatment (42).

The skin can be affected by pyoderma gangrenosum, a rare neutrophilic dermatosis with an inflammatory basis that is often associated with comorbidities such as IBD, arthritis, or malignancies. The pathogenesis of this condition remains still poorly understood, although an imbalance in the ratio of Treg/Th17 cells has been recently identified (43). Because of the absence of controlled and randomized clinical trials and the unknown pathogenic mechanisms, topical or systemic steroids have been used as first-line therapeutic agents, either alone (for example prednisone) or in combination with cyclosporin. However, the severity of adverse effects of these drugs has necessitated the identification of alternative agents, including anti-TNFα biologics (infliximab, adalimumab, golimumab, and etanercept) and IL-1 or IL-6 antagonists (anakinra and tocilizumab, respectively), whose beneficial effects need to be confirmed because of the paucity of available studies. A better understanding of the underlying molecular pathogenic pathways will allow more targeted and efficacious therapy (44).

Another rare disease that requires treatment with GCs is dermatomyositis, which can affect the skin, muscles and, occasionally, the lungs. The pathogenesis of this disease is still largely unknown, but inappropriate inflammatory mechanisms were found to be the basis of injuries in the skin or the parenchyma of muscles. Mild disease can be treated with topical GCs, whereas more severe diseases are usually treated with antimalarials as first-line treatment. However, oral systemic GCs coupled with immunosuppressants are generally used as therapy for both skin and muscle diseases. Other drugs include biologicals such as rituximab, abatacept, and tocilizumab (45).

The combination of GCs and rituximab was highly efficacious in increasing the response rate and prolonging the relapse-free survival phase in patients with warm-antibody reactive autoimmune hemolytic anemia, in comparison with that in the patients treated with prednisolone. This form of acquired hemolytic anemia is caused by the formation of autoantibodies directed against antigens of the red blood cell membrane, for which the recommended first-line treatment is prednisolone (46).

For life-threatening immunological syndromes such as hemophagocytic lymphohistiocytosis, GCs remain the best choice as first-line treatment. The pathogenesis of this condition, although largely unknown, involves sustained activation of the immune system, especially CD8+ cells and macrophages, and uncontrolled cytokine release, notably large amounts of IFNγ. Immunosuppressive and anti-inflammatory drugs are the only effective therapies, although newer drugs are being tested both in preclinical and clinical studies. Among these newer drugs, small molecule inhibitors of Janus kinases (JAKs) have proven to be efficacious because of the inhibition of IFNγ and IL-6 signals (47).

GCs represent the only choice for the treatment of IgG4-related disease, which is an immunoinflammatory disorder characterized by lymphoplasmacytic infiltrates of plasma cells bearing IgG4 on their surface. The disease mostly involves the pancreas, but other organs can be affected by infiltrates consisting of CD4+ T cells, B cells, and IgG4-plasma cells. Fibrosis, phlebitis, and eosinophilia are other pathological features of this disease (48). Monotherapy with either prednisolone or prednisone can induce remission in 82%–100%

of patients when used as shortly as 3-4 weeks, followed by a tapering dosage. According to the Japanese guidelines (75% of patients are Japanese), a low-dose maintenance GC therapy is recommended for patients at high risk of relapse (49). The attempt to use biologics such as rituximab has proven unsuccessful because of the high percentages of relapse, but was efficacious in obtaining a good response in the totality of patients (50). IgG4-related hypophysitis is a recently identified subtype of hypophysitis that can be successfully treated with prednisone, which, occasionally, in case of relapse, can be continued at the maintenance dose for up to 3 years or even more (51). The same treatment schedule and dosage applies to other types of hypophysitis.

Although not in monotherapy, GCs are used in induction therapy for the treatment of anti-neutrophil circulating antibodies (ANCA)-associated vasculitis, a systemic, potentially life-threatening autoimmune disease that affects multiple organs and encompasses granulomatosis with polyangitis and microscopic polyangitis. Glomerulonephritis, which occurs frequently, can lead to renal failure; thus, prompt initiation of therapy is important to obtain rapid control of the disease. Cyclophosphamide is often used in combination with GCs, but the excessive immunosuppression and subsequent infections can result in high rates of mortality. Thus, there is a need for new therapies with both reduced toxicity and improved disease control (52–54).

Primary angiitis is a rare inflammatory disease of the central nervous system (CNS). Cells of the immune system infiltrate the CNS blood vessels, leading to thickening of the vessel walls and subsequent reduced circulation or, conversely, blood leakage and hemorrhage. Since the onset of this disease is sometimes insidious but progressive, prompt initiation of induction treatment with a combination of high-dose i.v. GCs and cyclophosphamide is important. This combination therapy can help avoid relapses, and this pharmacological approach is similar to the above-described orphan diseases. In addition, biological agents such as rituximab or infliximab have proven to be efficacious, but they represent an alternative option for patients who are intolerant to conventional therapies. After induction therapy, maintenance is achieved with corticosteroid-sparing low-risk immunosuppressants (azathioprine, mycophenolate mofetil, and metothrexate), with the aim of preventing severe side effects and relapses (55).

Although the majority of rare diseases do not show gender-specific patterns, some are more prevalent in females (in older patients). One such disease is juvenile localized scleroderma, a rare pediatric rheumatic condition associated with skin thickening and fibrosis that also occurs in adults. In this condition, inflammatory cells such as lymphocytes, eosinophils, and plasma cells infiltrate the reticular dermis, occasionally with the formation of edema. Anti-nuclear autoantibodies can be found in about 40% of patients. The recommended therapy includes GCs in combination with methotrexate, although there is no consensus on the dosage. Prednisone and prednisolone are used for oral therapy. The second-line therapy encompasses hydroxychloroquine, tocilizumab, infliximab, and abatacept (56).

For all the aforementioned diseases (**Figure 2**) and those not discussed in this review, when GC long-term therapy is needed, a

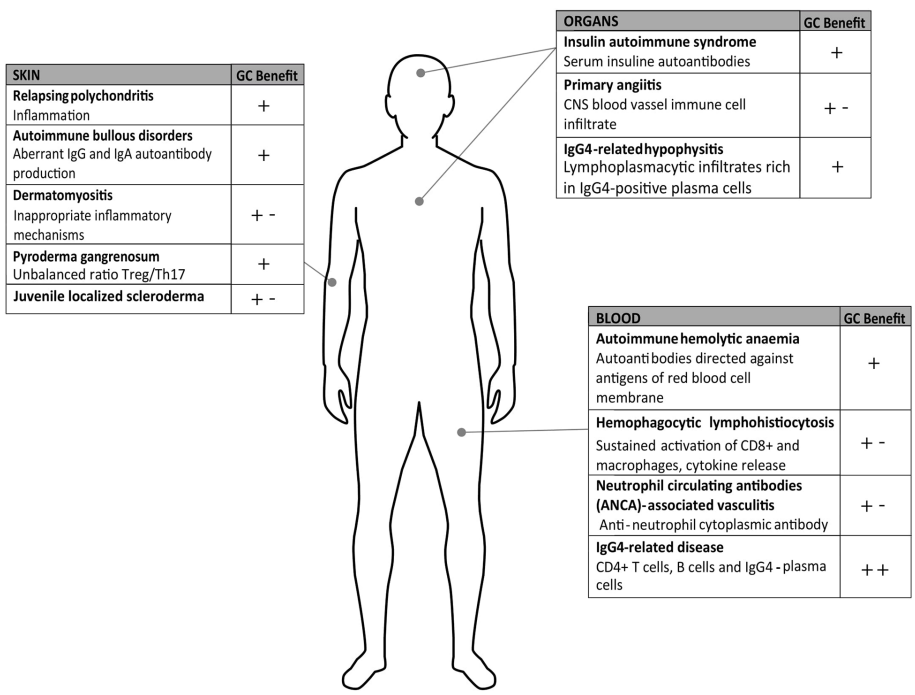

FIGURE 2 | Rare diseases on autoimmune or inflammatory basis. The figure summarizes the pathologies described in the text and the known pathogenesis and highlights the glucocorticoids (GC) benefits as monotherapy (+) or combination therapies (+-); (++) benefit represents remission close to 100%.

careful risk-benefit assessment must be considered because of the severe adverse effects of GCs that often turn into comorbidities, including diabetes, infections, osteoporosis or even cardiovascular pathologies. Therefore, new strategies must be pursued with the aim of identifying new effective drugs.

DISCUSSION: GILZ-BASED PROTEINS AS POTENTIAL PHARMACOLOGICAL TOOLS

A review of literature clearly highlights the difficulty in establishing an efficacious treatment protocol for rare diseases regardless of the use of GCs. Several problems are linked to this aspect, starting from the paucity or complete absence of randomized, placebo-controlled clinical trials and the limited literature highlighting comparisons across therapies. As a result, for some rare pathologies, there is no established dose regimen for the use of GCs, which is based on clinical practice and sporadic or retrospective studies on small groups of patients (**Table 1**). In such cases, the treatment is tailored according to each clinical case, the patient's response, and the time of the diagnosis in the progression of disease. Furthermore, the relative disinterest on the part of the biomedical industry to develop therapeutic agents for rare diseases is dictated by the small market potential, which, undoubtedly, leaves several clinical problems unsolved. Finally, from the economic point of view, care for a patient with a rare disease is much more expensive that for patients suffering from a common pathology. Rare diseases require a therapeutic challenge that can focus the efforts to optimize new pharmacological tools for their treatment. On the basis of what is known about the pathophysiology of autoimmune and inflammatory rare diseases, a field of research that is largely unexplored, new targets can be identified to allow the use of new drugs either in monotherapy or in combination. As an example, avacopan, a complement 5a receptor inhibitor, has proven to be effective in replacing high-dose GCs for the treatment of ANCA-associated vasculitis (77). Other experimental therapies include the use of monoclonal antibodies other than rituximab, but targeting the same B cells, such as ofatumumab, with optimized cytotoxicity, for phemphigus treatment. Since B cell intracellular signals activate p38MAPK, inhibitors of p38MAPK may represent an alternative strategy in pemphigus when applied topically, thereby avoiding the severe adverse events observed with oral administration (61). Other small-molecule inhibitors include tofacitinib, a Janus kinase (JAK)-1/3 inhibitor, and ruxolitinib, a selective JAK-1/2 inhibitor, which suppress interleukin and interferon signaling in dermatomyositis (45). Although they have been used in a small number of patients, they proved promising. Therefore, several efforts are needed to overcome the numerous problems linked to the treatment of rare diseases, even though these new drugs may occasionally show dose-limiting effects. We propose the potential use of a recombinant protein that was found to be therapeutic in some experimental models of autoimmune diseases in preclinical studies. The TAT-GILZ recombinant molecule is a fusion protein of full-length GILZ, an early GC-induced protein that mimics several beneficial effects of GCs without exerting GC-related adverse effects (13, 78). The first attempt in the use of TAT-GILZ was performed in a mouse model of DNBS-induced colitis, in which TAT-GILZ treatment successfully reduced the severity of spontaneously developed colitis in IL-10-knock-out mice. GILZ can inhibit NF-κB, a pivotal transcription factor in the regulation of pro-inflammatory cytokines, thereby contributing to the regulation of the CD4+ response in the gut (79). In the same model of colitis, but in a different strain of genetically modified mice, the *GILZ* B cell-conditional knock-out mice (GILZ B cKO), 3 day-treatment with the recombinant TAT-GILZ protein reversed the symptoms of DNBS-induced colitis, similar to wild-type mice (80, 81). In the context of a neutrophilic inflammation in a model of LPS-induced pleurisy in mice, TAT-GILZ was able to lead to inflammation resolution by decreasing cytokine production and promoting apoptosis in neutrophils (82). Another type of GILZ peptide was employed in the experimental encephalomyelitis, a mouse model of human multiple sclerosis. In this model, the proline-rich portion of the carboxyl terminus of GILZ protein (GILZ-P), which can bind the p65 subunit of NF-κB, was found to inhibit the transactivation of inflammatory cytokines, thus ameliorating the disease (83). Another interesting study demonstrated that TAT-GILZ can reduce Th-17 frequency and increase Treg cells in another inflammatory context, such as acute kidney injury (84). Furthermore, in human B lymphocytes, intracellular delivery of the HHph-GILZ peptide inhibited cell proliferation *in vitro*, explaining the observation of reduced GILZ in B cells of *systemic lupus erythematosus* patients and *lupus*-prone mice (85). Another proof of the efficacy of GILZ-based peptides is given by the use of a synthetic TAT-GILZ peptide (GILZ-p) in a model of ocular uveitis in rats. The inflammatory response was counteracted by the intravitreal injection of GILZ-p, which could reduce the expression of IL-1β and TNF-α (86).

Since GILZ is a pivotal intermediate of GC anti-inflammatory and immunoregulatory actions, the rationale for the use of GILZ protein-based pharmaceuticals is attractive in the rare diseases described above. Several autoimmune and inflammatory rare diseases share common altered immune responses, including uncontrolled B cell activation (see insulin autoimmune syndrome, autoimmune bullous disease, etc.). B cells are targets of GILZ effects, since GILZ is indispensable to control the over production of IFNγ in B cells, as demonstrated by the elevated levels of IFNγ in GILZ B cKO B lymphocytes. These mice are prone to develop a severe disease in the experimental model of colitis, in which IFNγ-secreting B cells have a pathogenic role (80). Moreover, GILZ-deficient mice develop a progressive B lymphocytosis, with expansion of B220+ cells in the bone marrow because of an increased survival of B cells (87). GILZ plays a role not only in B cells but also in T cell subtypes. A study demonstrated that GILZ is involved in the regulation of Th17 activity, in that it maintains a threshold for Th17 activation in an experimental model of psoriasis. Interestingly, GILZ expression inversely correlates with disease severity, suggesting that low amounts of GILZ may worsen this disease and/or may

TABLE 1 | Summary of the presented rare diseases, related treatments and references.

Disease	Treatment	References
Insulin autoimmune syndrome	Prednisone (first line), azathioprine, acarbose, thiamazole, biologics (anti-CD20 -*rituximab*-)	(38, 57)
Relapsing polychondritis	Steroids (first line), azathioprine, Non-steroidal anti-inflammatory drugs (NSAIDs), biologics (anti-TNFα -*infliximab, adalimumab, golimumab, certolizumab, etanercept*-, -anti-IL-6 -*tocilizumab*-, anti-IL-1 -*anakinra*-, CTLA-4 Ig -*abatacept*-, *rituximab*)	(58, 59)
Autoimmune bullous disorders, pemphigus, pemphingoid diseases	Prednisone (first or second line), clobetasol propionate, mycophenolate mofetil, methotrexate, azathioprine, combination of tetracyclines, doxycycline or minocycline and niacinamide, IVIG, biologics (*rituximab*, anti-IgE -*omalizumab*-).	(41, 60–62)
Pyoderma gangrenosum	Prednisone/prednisolone (first line), tacrolimus, sodium cromoglycate, cyclosporine, methotrexate, mycophenolate mofetil, azathioprine, intravenous immunoglobulins, (IVIG), biologics (*infliximab*, *adalimumab, golimumab, etanercept*, anti-IL-12 and anti-IL-23 –*ustekinumab*-, *anakinra*, anti-IL1β -*canakinumab*-, *tocilizumab*), JAK/STAT inhibitors (tofacitinb),	(44, 63)
Dermatomyositis	Steroids (first or second line), antimalarials, methotrexate, mycophenolate mofetil, IVIG, biologics (*rituximab, abatacept, tocilizumab*), tofacitinib	(45, 64)
Warm Antibody reactive autoimmune hemolytic anemia	Prednisolone (first line), azathioprine, biologics (*rituximab*)	(46, 65–67)
Haemophagocytic limphohistiocytosis	Methylprednisolone/dexamethasone/hydrocortisone (first line), biologics (*rituximab, anakinra*, anti-CD52 -*alemtuzumab*-), etoposide, cyclosporine A, methotrexate	(47, 68)
IgG4-related disease	Prednisolone/prednisone (first line), azathioprine, 6-mercaptopurine, methotrexate, mycophenolate mofetil, biologics (*rituximab*, *infliximab*), hydroxychloroquine	(48, 50, 69, 70)
IgG4-related hypophysitis	Prednisone (first line), methotrexate, biologics (*rituximab*)	(51, 71, 72)
Neutrophil circulating antibodies (ANCA)-associated vasculitis	Prednisone (first line), cyclophosphamide, methotrexate, mycophenolate mofetil, biologics (*tocilizumab*)	(52, 53, 73)
Primary angiitis	Prednisone (first line), cyclophosphamide, methotrexate, mycophenolate mofetil, azathioprine, IVIG, biologics (*rituximab, infliximab, etarnecept*)	(55, 74, 75)
Juvenile localized scleroderma	Prednisone/prednisolone (first line), methotrexate, hidroxychloroquine, imiquimod, cyclosporine A, biologics (*tocilizumab, infliximab, abatacept*), tofacitinib	(56, 76)

Management of the listed pathologies can be found in the indicated references.

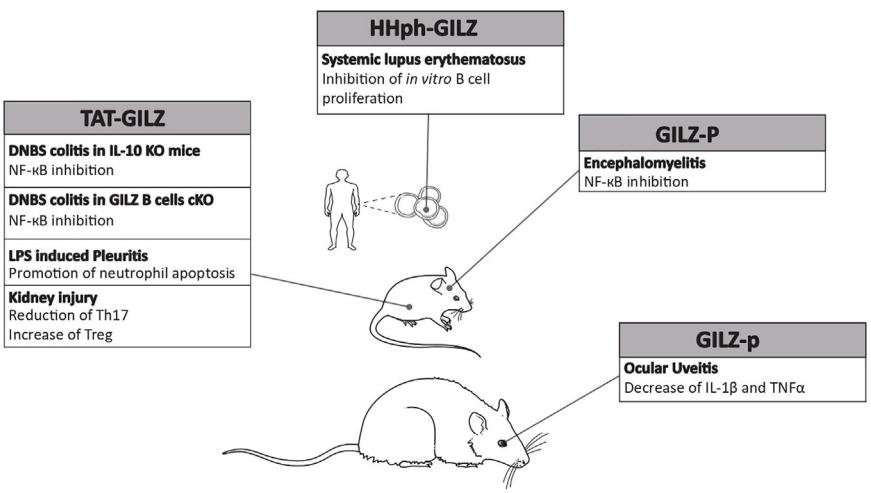

FIGURE 3 | Efficacy of glucocorticoid-induced leucine zipper (GILZ)-based proteins in experimental models. Summary of GILZ proteins/peptides that have been used in rodent models of autoimmune or inflammatory diseases and proven efficacious. The underlying molecular and cellular mechanisms are also indicated.

be part of the pathogenesis (88–90). Moreover, GILZ-deficient mice show spontaneous production of IL-17A and IL-22 in the imiquimod model of psoriasis, and their dendritic cells produce high amounts of IL-1, IL-23, and IL-6 (90). A recent study in pyoderma gangrenosum reported that, in the skin of patients suffering from this disease, Th17 cells are increased in number whereas Treg cells are reduced, with their balance shifted toward the Th17 pathogenic cells (43). The potential treatment with a GILZ protein would prove beneficial to restore the right balance of Th17/Treg cells, since GILZ is also involved in the production of Treg cells, as demonstrated by GILZ conditional knock-out mice (91). In addition, because pyoderma gangrenosum belongs to those rare inflammatory diseases referred to as neutrophilic dermatosis, GILZ treatment could reduce neutrophil activation, since we have previously demonstrated that GILZ is indispensable to restrain the activity of neutrophils in the site of inflammation (44, 92).

Finally, in all rare diseases characterized by an overproduction of pro-inflammatory cytokines, GILZ might be the protein that counteracts the increase. GILZ was found to inhibit the pro-inflammatory effects of TNFα in human adipocytes, to reduce macrophage inflammatory protein 1 (MIP-1) in macrophages, to inhibit the expression of the adhesion molecules like ICAM-1 in endothelial cells, and to interfere with other inflammatory mechanisms (93–96). More importantly, GILZ can heterodimerize with NF-κB, thus inhibiting the transactivation of downstream known pro-inflammatory genes. Therefore, GILZ, as a regulator of uncontrolled immune response, might be an ideal candidate for the development of new biological drugs (**Figures 2** and **3**). Up to date there are still no trials in humans, because further pre-clinical studies about the pharmacokinetics and pharmacodynamics of exogenous GILZ protein, as well as about its possible toxicity, are needed. It is reasonable to hypothesize a future use for the treatment of rare inflammatory and autoimmune diseases with a high benefit/cost ratio, since protein-based therapies are expensive but need low doses and less frequent dosing regimens. Furthermore, no comorbidities are expected to develop with no need of additional pharmacological treatments. Overall, GILZ-based proteins may represent an actual alternative to GCs.

CONCLUSIONS

Rare diseases with an inflammatory basis can be successfully treated with GCs, but the consequences of long-term or high-dose treatments can be detrimental. Thus, there is an urgent need to discover new drugs either to reduce the GC doses in co-treatments or hopefully replace them. Knowledge of the pathophysiology of these diseases is mandatory, so that new targets can be identified. On the other hand, proteins like GILZ, which mimic the GC beneficial effects, could be ideal candidates to reduce inflammation where its components are causative or contribute to the pathogenesis of the pathology. Furthermore, the ability of GILZ to change the balance between immune cells toward an anti-inflammatory or autoimmune phenotype can be exploited to restore a correct immune response. GILZ-based proteins may represent the next step in the treatment of rare inflammatory diseases, predicting their future hope and use in humans.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Autoimmune Addison's Disease as Part of the Autoimmune Polyglandular Syndrome Type 1: Historical Overview and Current Evidence

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The autoimmune polyglandular syndrome type 1 (APS1) is caused by pathogenic variants of the autoimmune regulator (*AIRE*) gene, located in the chromosomal region 21q22.3. The related protein, AIRE, enhances thymic self-representation and immune self-tolerance by localization to chromatin and anchorage to multimolecular complexes involved in the initiation and post-initiation events of tissue-specific antigen-encoding gene transcription. Once synthesized, the self-antigens are presented to, and cause deletion of, the self-reactive thymocyte clones. The clinical diagnosis of APS1 is based on the classic triad idiopathic hypoparathyroidism (HPT)—chronic mucocutaneous candidiasis—autoimmune Addison's disease (AAD), though new criteria based on early non-endocrine manifestations have been proposed. HPT is in most cases the first endocrine component of the syndrome; however, APS1-associated AAD has received the most accurate biochemical, clinical, and immunological characterization. Here is a comprehensive review of the studies on APS1-associated AAD from initial case reports to the most recent scientific findings.

Keywords: Addison's disease, autoimmune polyendocrinopathies, cytochrome P450 enzyme system, history, transcription factors

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INTRODUCTION

The term autoimmune polyglandular syndrome (APS) designates a heterogeneous group of diseases sharing a common fundamental characteristic—damage to more than one organ, essentially but not exclusively endocrine—caused by pathological processes identifiable as autoimmune (1, 2). APSs were classified in the early 1980s (3–5). APS type 1 (APS1), often referred to as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) to summarize clinical features, is a monogenic disease with an autosomal recessive inheritance (6, 7). Traditionally, at least two of the major components—chronic mucocutaneous candidiasis (CMC), idiopathic hypoparathyroidism (HPT), and autoimmune Addison's disease (AAD)—are required for clinical diagnosis (8–10). The diagnostic pathway of APS1 cases with uncommon presentation and course may take advantage of early non-triad components, such as urticaria-like eruptions, gastrointestinal dysfunction, and enamel hypoplasia, along with type-1 interferon (IFN) antibody assay, pending molecular confirmation (11–16).

In APS type 2 (APS2), or Schmidt's syndrome, AAD is associated with autoimmune thyroid disease (ATD), type-1 diabetes (T1D), or both (17–19). In contrast, ATD along with other autoimmune diseases, HPT and AAD excluded, identifies APS type 3 (APS3) (20). Worldwide medical literature is closely aligned with this classification (21–28). However, it has been suggested to merge non-monogenic APSs into a single disease entity (29), as they recognize a multifactorial genetic predisposition, mostly related to the major histocompatibility complex class-I and class-II (*MHCI* and *MHCII*, respectively) genes and their regulators (30–35).

Since AAD is the bridge between APS1 and APS2, the revision of its principles implies the treatment of these subjects (36–41). This work aims to review the characteristics of APS1-associated AAD, providing a historical overview and looking at the most recent results deriving from studies on the animal models of the disease. Due to the common embryogenesis of the adrenal cortex and gonads (42), AAD intersects the autoimmune events involving the gonads, especially in the form of autoimmune primary ovarian insufficiency (APOI) of the female sex.

AIRE, APS1, AND THE ANIMAL MODELS OF DISEASE

AIRE: Basic Properties and Functions

Autoimmune regulator (*AIRE*), the gene responsible for APS1, is found in the chromosomal region 21q22.3 (6, 7, 43–45). The murine homolog (*Aire*) lies on chromosome 10 (46–48). The pool of tissues in which the gene is transcribed must be fully delineated (49–51), but undoubtedly the highest degree of expression is in medullary thymic epithelial cells (mTECs); here, the protein (AIRE/Aire) forms distinct nuclear speckles and co-localizes with the microtubular cytoskeleton (52, 53).

Earlier studies proved that bipotent thymic epithelial progenitor cells (TEPCs) give rise to mTEC and cortical TEC (cTEC) compartments in the embryonic and early neonatal thymus (54–58). It was then shown that selected TEPC clusters differentiate into mTEC sublineage derailing from a predefined cTEC development program (59–63). In the post-natal thymus, bipotent TEPCs become progressively quiescent, and the replenishment of TEC compartments is supported by sublineage-restricted precursors (64–69).

Committed mTECs descend from the apical layer of the thymic anlage, marked by tight-junction claudins 3 and 4 (70), while the initial stages of maturation require lymphostromal "crosstalk" with early T-cell subsets (71–73). $MHC^{lo}CD80^{lo}AIRE^{-}$ mTECs ($mTECs^{lo}$) include not only precursors of the mature $MHC^{hi}CD80^{hi}AIRE^{+}$ mTECs ($mTECs^{hi}$), but also cortico-medullary junctional TECs (jTECs) that recruit positively selected thymocytes into the medulla (74–78). In turn, $mTECs^{hi}$ have a rapid turnover and give rise to various post-AIRE subsets (79–81); these include corneocyte-like mTECs and thymic tuft cells, which play a presumed role in addressing cytokine responses (82–87).

AIRE contains a caspase-activation and recruitment domain (CARD), a nuclear localization signal (NLS), a SAND (for Sp100-AIRE-NucP41/75-Deaf-1) domain, and two plant-homeodomain zinc fingers (PHD1 and PHD2, respectively) (88, 89). Amino-terminal and middle regions perform auxiliary functions, such as oligomerization, pro-apoptosis, nuclear shuttling, and DNA binding (90–93). At the carboxyl-terminal end, PHD1 binds to the tail of unmethylated histone H3 by electrostatic complementarity, and PHD2 activates gene transcription (94–97). To perform this function, AIRE interacts with enzymes, such as DNA-topoisomerases (DNA-TOPs) and DNA-activated protein kinase (DNA-PK), which belong to the multimolecular complex involved in DNA break and repair by non-homologous end joining (98–100).

As demonstrated in the murine thymus, Aire and co-actors localize to long stretches of chromatin known as super-enhancers, which enclose the transcription start sites of most Aire-dependent genes (101). Initiation of gene transcription is made effective by AIRE-induced recruitment of the positive transcriptional elongation factor b (P-TEFb), which enables elongation and pre-mRNA splicing into mature mRNA by phosphorylation and release of the stalled RNA-polymerase II (102, 103).

Due to the above properties, AIRE plays a crucial role in promiscuous gene expression within the thymus; in other words, AIRE drives the ectopic expression of genes that encode for enzymes, hormones, receptors, structural proteins and other molecules acting as self-antigens and normally synthesized in a few tissues (104–107). Their presentation to thymocytes induces apoptosis and deletion of self-reactive clones, which prevent their spreading as mature T cells (108–111). However, AIRE controls only a part of these genes; furthermore, the expression of any single AIRE-dependent gene affects a small percentage of mTECs and follows a stochastic pattern (112, 113), though co-expression pools of overlapping and complementary gene sets have been established (114–116).

AIRE also promotes the generation of regulatory T cells (111, 117, 118). The result of self-reactive thymocytes (i.e., negative selection or switch to tolerogenic function) depends on mTEC subsets and AIRE availability (119–122), division of labor and

Abbreviations: AAD, autoimmune Addison's disease; AC-Abs, adrenal-cortex antibodies; ACTH, adrenocorticotropic hormone; AIRE/Aire, autoimmune regulator; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; APOI, autoimmune primary ovarian insufficiency; APS, autoimmune polyglandular syndrome; ATD, autoimmune thyroid disease; CARD, caspase-activation and recruitment domain; CMC, chronic mucocutaneous candidiasis; CF, complement fixation; CYP, cytochrome P450; CYP17, CYP 17α-hydroxylase/17,20 lyase; CYP17-Abs, antibodies to CYP17; CYP21, CYP 21-hydroxylase; CYP21-Abs, antibodies to CYP21; CYP11A1/CYPscc, CYP cholesterol side-chain cleavage enzyme; CYPscc-Abs, antibodies to CYPscc; DNA-PK, DNA-activated protein kinase; DNA-TOP, DNA topoisomerase; HPT, hypoparathyroidism; HSD, hydroxysteroid dehydrogenase; IFN, interferon; IIF, indirect immunofluorescence; IP, immunoprecipitation; KHDC3L, KH-domain-containing 3-like protein; MAGEB2, melanoma-associated antigen B2; Mater, maternal antigen that embryo requires; MHC, major histocompatibility complex; NALP5, leucine-rich-repeat protein 5; NLS, nuclear localization signal; PDILT, disulfide isomerase-like protein of the testis; PHD, plant-homeodomain (zinc finger); P-TEFb, positive transcriptional elongation factor b; SAND, Sp100-AIRE-NucP41/75-Deaf-1; StC-Abs, steroid-cell antibodies; T1D, type-1 diabetes; TEC, thymic epithelial cell; TEPC, thymic epithelial progenitor cell; TSGA10, testis-specific protein 10; WB, Western blot.

interplay between antigen-presenting cells (123–127), and degree of affinity between self-antigens and T-cell receptors (128, 129).

For a careful dissection of the above-mentioned arguments, the authors suggest readers to refer recent reviews that have thoroughly analyzed TEC dynamics and functions (130, 131), the molecular properties of AIRE, and its role in self-tolerance (132–135).

Clinical Picture of APS1 and the Animal Models of Disease

The clinical picture of APS1 is quite repetitive, although, as indicated, minor or uncommon entities may precede the main triad in a number of cases (15, 16). In other patients, severe forms of autoimmune hepatitis (136–139), lung disease (140, 141), and oral/esophageal carcinoma (142, 143) worsen the disease and progress into a life-threatening condition.

In vitro mutagenesis suggests that specific *AIRE* genotypes, particularly that of Iranian-Jewish patients with APS1, give rise to distinctive clinical features (144–147). Other peculiarities concern patients carrying mutated AIRE chains that co-localize with the wild-type protein and undermine the oligomeric structure in a dominant way. Incomplete penetrance, late-onset autoimmunity, and a milder phenotype characterize the clinical picture (148–151).

Additional genetic traits, with particular reference to the MHC alleles, can influence the APS1 phenotype so that in large APS1 patient cohorts most of the disease components mirror the HLA associations established for the general population (152). As an example, Finnish patients with APS1 have a high prevalence of T1D, while ATD is surprisingly common in those of Southern Italy (8, 153); actually, both observations reflect the MHC predispositions of the respective populations (154, 155).

Regarding APS1-associated AAD, no susceptibility related to MHCI and MHCII was initially reported, while HLA-DR3 and -DR4 were recognized to confer a significant risk for sporadic and APS2-associated forms (5, 156, 157). Subsequent analysis of larger patient cohorts contradicted the previous claim and revealed that patients with APS1 carrying the DRB1*03 allele have a significantly higher prevalence of AAD, while DRB1*04 appears to be more closely associated with alopecia (152). A number of other genes have been linked to non-APS1-associated AAD, but none of these play a role in APS1 phenotype (35). On the contrary, *AIRE* sequencing allows a correct classification of some AAD cases, which were previously framed within sporadic forms and actually being part of APS1 (158, 159).

The animal models of APS1 provide a formidable support for delineating AIRE-related self-tolerance mechanisms, but provide scant information, if any, on the characteristics of the disease components (108–111). This is because *Aire*-deficient ($Aire^{-/-}$) mice exhibit different pictures than human APS1 (160, 161); typically, the exocrine glands are targets of autoimmunity, while the adrenal cortex, and other endocrine glands, shows little or no damage (108, 109, 111). Furthermore, the clinical picture of $Aire^{-/-}$ mice is modulated by the strain background, supporting the idea that gene modifiers control patterns of autoimmunity to each organ (162–164).

More recently, $Aire^{-/-}$ rats were engineered by zinc-finger nucleases; the animals exhibited various APS1-like ectodermal dystrophies, periportal lymphocyte infiltrates (with "piecemeal" necrosis) of the liver, and a broad spectrum of antibodies to self-antigens, type-1 IFNs included, but the endocrine glands were not significantly affected. In part contradicting the above findings, the reproductive capacity of the animals was impaired by the damage of testis Leydig cells (165).

Interestingly, the thymic expression of genes encoding for three enzymes of the cytochrome-P450 (CYP) family involved in the steroid pathway and electively targeted by adrenal autoimmunity in APS1 suggests differing and somewhat contrasting regulatory mechanisms between humans and mice. Murine mitochondrial cholesterol side-chain cleavage enzyme (CYP11A1, or CYPscc) and microsomal 21-hydroxylase (CYP21) have a strong and intermediate degree, respectively, of dependence on Aire, while the gene expression of another microsomal CYP enzyme, 17α-hydroxylase/17,20 lyase (CYP17), does not change significantly in murine $Aire^{-/-}$ mTECs (86, 109, 166–168). Unexpectedly, the expression of the human corresponding genes is unrelated to that of *AIRE* in thymoma and HEK293 cells, underlining that dependence on AIRE follows a species-specific pattern (169–171).

AAD AND APOI AS PARTS OF APS1

Prevalence of APS1-Associated AAD and APOI

The prevalence of AAD in APS2 and APS3 is, by definition, 100 and 0%, respectively. Regarding the prevalence of AAD in APS1, it should be noted that, prior to the APS classification (3–5), patients with APS1 are either described in case reports or grouped together with subjects suffering from other diseases. Consequently, a comprehensive overview of these APS1 cases is missing. Thanks to the collection of hundreds of scientific articles covering the whole relevant literature and considering the utmost care to avoid counting each patient more than once; the Authors reviewed 282 certain or highly probable APS1 cases reported till 1980. Personal data and clinical course of each patient and related references are listed in **Supplementary Material**. Any further information can be requested by e-mail to the corresponding author.

As shown in **Table 1**, AAD has a prevalence of ∼57%, with no gender preponderance. Moreover, the mean age of the patients at disease onset was comparable: just under 12 years in males, and just over 11 years in female patients. AAD, in the form of acute adrenal failure neither readily recognized nor effectively treated, was a significant cause of death, starting with the little girl whose clinical course and autopsy findings were detailed by Ostertag (172).

Among Finnish patients with APS1, who represent a reference cohort in terms of number, genetic homogeneity, data centralization, and serial updates (8, 173–180), AAD reaches an incidence of 84%. To retrieve the data, Finnish researchers estimate the onset of each APS1 component over fixed age ranges, assuming that all patients live up to 50 years old. As of the 2006

TABLE 1 | Summary of data from 282 confirmed or highly probable cases of autoimmune polyglandular syndrome type 1 (APS1) reported up until 1980.

	Whole casuistic	Male patients[a]	Female patients[a]
APS1 cases	282	126	153
Age at last observation/death	14.9 (0–56)	15.1 (0–38)	15.0 (0–56)
AAD patients	162 (57.4%)	72 (57.1%)	88 (57.5%)
Age at AAD onset	11.3 (0–34)	11.8 (0–33)	11.1 (0–34)
Patients deceased	64 (22.7%)	33 (26.2%)	30 (19.6%)
Age at decease	10.0 (0–33)	8.6 (0–24)	11.6 (0–33)
Patents deceased by acute adrenal failure	26 (9.2%)	12 (9.5%)	14 (9.2%)
APOI patients[b]	—	—	32/88 (36.4%)
Primary amenorrhea	—	—	21/88 (23.9%)
Secondary amenorrhea	—	—	11/88 (12.5%)

Prevalence of autoimmune Addison's disease (AAD) and autoimmune primary ovarian insufficiency (APOI) is specified. Personal data and medical history of each patient are detailed in ***Supplementary Material****.*

[a]*Gender was not specified in three (two Addisonian) patients.*

[b]*Calculated on female patients aged ≥13.*

update, no AAD cases were reported up to a patient age of 2 years, while AAD rate increased to 8, 40, and 65% in the age ranges 2–5, 5–10, and 10–15, respectively (180).

Ferre et al. calculated the incidence of APS1 components in American (mostly the US) patients in the same way and obtained consistent percentages for AAD (16). Again, AAD had a prevalence of 73 and 67% in two major reviews of 41 and 112 patients with APS1 from Northern Italy and Russia, respectively (9, 10).

In **Table 2**, the prevalence of AAD and APOI in various APS1 patient cohorts is reported, with the specification of their ethnic backgrounds (15, 144, 153, 181–197). AAD prevalence can sometimes be influenced by the mean patient age, for example, in Saudi patients with APS1 described by Bin-Abbas et al. (193). Conversely, the extent of follow-up suggests that the low prevalence of AAD in Iranian-Jewish patients with APS1 does not recognize an age-related factor (144).

Closely related to the mechanisms involved in AAD, a large percentage of patients with APS1 suffer from primary hypogonadism, with a privileged gender association. APS1 women have been included in APOI studies (198, 199), and APS1 has become a well-known condition causing this disease (200–218). Recently, the clinical and immunological features of APS1-associated APOI have been detailed in the Finnish cohort of APS1 women (219).

As shown in **Table 2**, APOI approaches or exceeds AAD prevalence in some APS1 patient cohorts. Surprisingly, Adamson et al. reported that 6 out of 19 UK APS1 male patients had gonadal insufficiency, but the criteria adopted to satisfy this diagnosis were not reported (186). Similar findings were outlined in Slovenian patients with APS1, but again the diagnostic criteria were not specified (195).

Clinical History and Particular Issues

Irvine and Barnes observed a bimodal age distribution at AAD onset; the first peak emerged at the end of the first decade and involved the majority of HPT patients. A later peak (fifth decade) mainly affected women with T1D, ATD, or both (220). In subsequent years, long-term observation of large AAD cohorts confirmed that the disease onset occurs at an early age only in patients with APS1 (221–227). Conversely, more recent nationwide AAD studies have purposely excluded patients with APS1, emphasizing the difference between monogenic and multifactorial pathogenesis (228, 229).

Furthermore, the prediction of AAD onset in patients with APS1 with related humoral autoimmunity has contributed to characterize the biochemical and clinical stages of the disease. Damage typically begins in the zona glomerulosa and causes impaired mineralocorticoid secretion and increased plasma renin activity. The subsequent involvement of the zona fasciculata has been divided into three stages of hypocortisolism: subnormal cortisol response after adrenocorticotropic-hormone (ACTH) stimulation test, persistent ACTH increase, and decrease in basal cortisol level, respectively (230–235).

Replacement therapy is unable to reproduce the natural hormone pulse, so the treatment of AAD is a challenge in itself and carries risks of suboptimal or excessive dosage (236). In APS1, the problem is accentuated by the coexistence of other hormonal deficits, with particular reference to HPT and ATD. Untreated AAD masks the early stages of HPT, as the hypercalciuric and hypocalcemic effects of glucocorticoids wear off. By the same principle, AAD replacement therapy can induce hypocalcemic seizures in patients with APS1 with subclinical HPT (237, 238). Fortunately, HPT treatment mitigates the negative impact of glucocorticoids on bone health (239).

Again, it is important to remember that, regardless of the underlying disease, untreated AAD can cause reversible thyroid dysfunction (240–242). Co-occurrence of AAD and ATD should caution when initiating thyroid replacement therapy due to the risk of raising the basal metabolic rate and precipitating an adrenal crisis.

Patients with APS1 have been included in AAD therapeutic trials (243–245), and special attention is paid to them in consensus statements on diagnosis, treatment, and follow-up (246).

TABLE 2 | Prevalence of autoimmune Addison's disease (AAD) and autoimmune primary ovarian insufficiency (APOI) in cohorts of autoimmune polyglandular syndrome type-1 (APS1) patients, with specification of their ethnic background.

	N	Mean age			AAD			Mean age at AAD onset			APOI[a]	Mean age at APOI onset
		Total	M	F	Total	M	F	Total	M	F	F	F
Wagman et al. (181) Canadian patients	16	20.6	22.4	19.7	9/16	3/5	6/11	nr	nr	nr	2/7	nr
Zlotogora and Shapiro (144) Iranian-Jewish patients	23	nr	nr	nr	5/23	2/11	3/12	21.8	21.0	22.3	1/9	32.0
Wang et al. (182) US patients	20	nr	nr	nr	19/20	8/8	11/12	nr	nr	nr	nr	—
Cihakova et al. (183) Central-Eastern European patients	27	nr	nr	nr	24/27	nr	nr	nr	nr	nr	nr	—
Stolarski et al. (184) Polish patients	16	21.4	19.6	22.3	8/16	3/5	5/11	8.9	9.3	8.6	4/10	16.0
Dominguez et al. (185) Irish patients	31	19.9	20.2	19.6	21/31	8/13	13/18	nr	nr	nr	11/16	nr
Adamson et al. (186) UK patients	33	24.2	24.9	23.1	24/33	19/24	5/9	nr	nr	nr	3/8	nr
Wolff et al. (187) Norwegian patients	34	33.8	35.2	32.0	23/34	13/19	10/15	12.3	11.8	12.9	7/13	17.0
Trebušak Podkrajšek et al. (188) Dutch, Eastern European patients	11	16.2	14.7	18.0	10/11	5/6	5/5	9.0	9.2	8.8	3/4	14.3
Perniola et al. (153) Southern Italian patients	12	26.7	28.7	24.7	9/12	5/6	4/6	8.3	10.8	5.2	5/6	19.6
Zaidi et al. (189) Indian patients	9	15.1	23.5	12.7	7/9	2/2	5/7	10.9	15.0	9.2	1/4	nr
Proust-Lemoine et al. (190) North-Western French patients	19	31.2	28.2	36.4	15/19	10/12	5/7	12.6	11.3	15.2	5/7	18.8
Tóth et al. (191) Hungarian patients	7	15.1	14.0	16.7	4/7	1/4	3/3	nr	nr	nr	1/2	nr
Orlova et al. (192) Russian patients	46	nr	nr	nr	30/46	nr	nr	9.9	nr	nr	nr	—
Bin-Abbas et al. (193) Saudi patients	20	10.9	9.4	12.2	8/20	3/9	5/11	7.5	6.7	8.0	1/4	15.0
Mazza et al. (15) Northern Italian patients	24	16.5	nr	nr	15/24	nr	nr	8.0	nr	nr	nr	—
Meloni et al. (194) Sardinian patients	22	25.8	28.1	24.3	15/22	5/9	10/13	10.0	10.3	9.8	8/10	24.5
Bratanic et al. (195) Slovenian patients	15	24.5	26.9	17.7	8/15	6/11	2/4	8.8	9.7	5.9	2/3	16.3
Fierabracci et al. (196) Turkish patients	23	17.5	18.3	17.0	14/23	4/7	7/12	15.0	16.2	14.2	2/8	15.0
Weiler et al. (197) Brazilian patients	14	18.7	15.5	19.5	10/14	3/4	7/10	nr	nr	nr	5/7	nr

[a] *Calculated on female patients aged ≥13.*

nr, not reported.

APS1-ASSOCIATED AAD AND APOI: HUMORAL IMMUNITY

Although in statistical terms AAD is the third component of APS1 triad, it has received the best immunological characterization; it is probably accepted that APS1 studies have made a substantial contribution to the delineation of the pathogenetic and immunological aspects of AAD.

The Founding Studies

Antibodies to adrenal cortex (AC-Abs) were first demonstrated by complement fixation (CF) in sera from patients with sporadic or APS2-associated AAD (247). Sera from unselected patients with APS1 were included in subsequent studies using both CF and indirect immunofluorescence (IIF) (248–255). The cell cytoplasm of the adrenal layers was stained in positive samples (248); the mitochondrial and microsomal fractions of the tissue extracts retained the antigenic properties, since pre-absorption with them inhibited the reaction (249, 253). In addition, AAD sera from the patients with associated HPT, CMC, or both contained other antibody specificities, such as those to thyroid (248, 249, 252, 254), salivary gland ducts (249), gastric parietal cells and intrinsic factor (250, 252, 254), parathyroid glands (251, 255), and liver (252).

In parallel studies, the same methods were used to test for antibodies to steroid-producing cells (StC-Abs). Five out of 77 AAD sera reacted against granulosa and theca interna cells of Graafian follicles, luteal cells, ovarian interstitial cells, Leydig cells, and placental syncytiotrophoblasts; three of these sera belonged to APS1 women with coexistent APOI (256, 257). A similar result was obtained with serum from a male patient with APS1 (258). Moreover, StC-Ab-positive sera from patients with APS1 showed *in vitro* cytotoxicity on granulosa cells of the Graafian follicle (259). The above-mentioned results were confirmed by subsequent studies (260–262).

Pre-absorption with adrenal and gonadal extracts reduced or abolished the antibody titer, leading researchers to infer that the self-antigens shared by the adrenal cortex and steroid-producing extra-adrenal tissues were related to steroid pathway enzymes (256–258, 262).

Antibodies to germline cells were also detected in some patients with APS1 (256, 257, 260, 263), following the studies of Vallotton and Forbes, who found them in patients with gonadal dysgenesis (264–266).

Unlike Anglo-American patients in the 1970s, Finnish patients with APS1 had already been grouped into a distinct disease entity called moniliasis-polyendocrinopathy syndrome; precipitating AC-Abs were detected in the serum of these patients by gel diffusion (267, 268). The association appeared

TABLE 3 | Fraction of sera positive for antibodies to adrenal cortex (AC-Abs), steroid-producing cells (StC-Abs), cytochrome-P450 21-hydroxylase (CYP21-Abs), cytochrome-P450 cholesterol side-chain cleavage enzyme (CYPscc-Abs), and cytochrome-P450 17α-hydroxylase/17,20 lyase (CYP17-Abs) in cohorts of autoimmune polyglandular syndrome type-1 (APS1) patients, with specification of their ethnic background.

	N	AC-Abs	StC-Abs	CYP21-Abs	CYPscc-Abs	CYP17-Abs
Uibo et al. (283) Finnish patients	50	nd	nd	16/50	22/50	16/50
Chen et al. (287) Northern Italian patients	11	8/11	5/11	7/11	5/11	6/11
Perniola et al. (296) Southern Italian patients	10	10/10	10/10	8/10	9/10	5/10
Myhre et al. (297) Norwegian patients	20	nd	nd	13/20	7/20	5/20
Cihakova et al. (183) Central-Eastern European patients	18	nd	nd	8/18	11/18	12/18
Halonen et al. (152) Finnish, Scandinavian patients	60	nd	nd	38/60	38/60	29/60
Söderbergh et al. (298) Finnish, Scandinavian patients	90	nd	nd	59/90	47/90	40/90
Wolff et al. (187) Norwegian patients	29	nd	nd	20/29	12/29	6/29
Meloni et al. (194) Sardinian patients	13	nd	nd	10/13	12/13	11/13
Ferre et al. (16) American patients	35	nd	nd	18/35	24/35	nd

nd, not determined.

to be restricted to APS1-associated AAD (269). Two specific adrenal antigens were targeted, one named P (particulate) and precipitating in the mitochondrial fraction, and the other named S (soluble) since it was present in all subcellular fractions; the latter contained a variety of determinants, partly common to human sera and sera from other species (270, 271).

Antibodies to CYP Enzymes

In the mid-1980s, genes encoding for the above-mentioned CYP enzymes were identified and cloned (272–274). CYP21 was soon identified as the major self-antigen target of adult-onset, either sporadic or APS2-associated AAD; generally, AC-Abs were searched for by IIF; the assay was followed by Western blot (WB) on tissue fractions separated by gel electrophoresis (275–277). Data were confirmed by immunoprecipitation (IP) of ^{35}S-labeled-cell lysates from human adrenal cells (275) and by the reaction of human antibodies to recombinant CYP21 (CYP21-Abs) expressed in *Saccharomyces cerevisiae* (276, 277).

In contrast, the definition of adrenal autoimmunity in APS1 was delayed by contradicting data. For the first time, CYP17 was identified as the self-antigen recognized by precipitating AC-Abs of APS1 sera (278). In another study, while CYP21-Abs were found in sera from non-APS1 AAD patients as indicated, the serum from the only patient with APS1 stained testis Leydig cells and targeted CYPscc (275). Further analysis of APS1 sera confirmed these results (279, 280). Thus, APS1 sera appeared to react against either CYP17 or CYPscc, while CYP21 was thought to represent an exclusive target of autoimmunity in sporadic and APS2-associated AAD (281, 282).

Finally, Uibo et al., using WB on *Escherichia coli* lysates, which expressed recombinant CYP fragments, stated that all three enzymes are self-antigens in APS1 (283). Furthermore, the absorption studies were congruent with tissue distribution: since CYP21 is restricted to the adrenal cortex, CYP17 is also represented in the gonads, and the ubiquitous CYPscc is also found in the placenta, only incubation with adrenal homogenates could completely abolish reactivity. Ovarian and testis homogenates absorbed reactivity against CYPScc and CYP17, while placental homogenates selectively absorbed that against CYPScc (281). Several subsequent studies have confirmed the strong association (60–100% of cases) of adrenal autoimmunity, any form of AAD included, with AC-Abs and CYP21-Abs (284–293).

In contrast, StC-Abs and antibodies to CYP17 and CYPscc (CYP17-Abs and CYPscc-Abs, respectively) can be found in a limited number (5–42%) of sporadic or APS2-associated AAD cases and mainly identified in APOI women (286, 287, 292–295). As shown in **Table 3**, their prevalence is significantly higher in patients with APS1 (16, 152, 183, 187, 194, 283, 287, 296–298).

The same results have been achieved starting from the opposite point of view, i.e., extrapolating from patients with various autoimmune diseases, precisely those with gonadal insufficiency. Furthermore, gonadal autoimmunity markers prevail in APS1 and APS2 APOI women, while the percentage of sera positive for StC-Abs and CYPscc-Abs/CYP17-Abs falls in women with non-AAD-associated APOI, and in patients with other autoimmune diseases (299–302).

Lastly, in a minority of APS1 sera, it is possible to find antibodies against members of other steroid pathways; this is the case of antibodies to an enzyme belonging to the hydroxysteroid dehydrogenase (HSD) family, namely 3βHSD (303).

Concordance Between IIF and Antibodies to CYP Enzymes

Comparing IIF, a tool normally available in laboratory medicine, and the assays used to detect antibodies to CYP enzymes, which are generally reserved for experimental settings, presumably contains the most useful information for clinical purposes. Due to the selective synthesis of CYP21 in the adrenal cortex, AC-Abs and CYP21-Abs have a close relationship that produces very high correlation coefficients, regardless of the analytical methods (284, 285, 289, 290, 292, 293).

Progressive depletion of self-antigen source causes antibody titers to show an inverse correlation with disease duration (285, 291). However, the sensitivity of the analytical methods makes CYP21-Abs more reliable than AC-Abs in long-lasting AAD (more than 15–20 years of duration) (288). In other studies, CYP21-Abs neither correlate with AAD duration (286) nor are they prevalent over AC-Abs (293).

In relation to the tissue distribution of CYPs, StC-Abs, routinely tested on ovary and testis specimens, are associated with positivity for CYPscc-Abs, CYP17-Abs, or both; again, this relationship does not differ among the subcohorts of AAD patients (287, 293).

Predictive Role of AC-Abs, StC-Abs, and Antibodies to CYP Enzymes

In most cases, AC-Abs and CYP21-Abs anticipate the onset of AAD, and their predictive role is higher in patients with APS1 (233, 304). The same is true for StC-Abs, though the overlap of AC-Abs and StC-Abs creates a phenomenon of statistical redundancy in univariate analyses (233).

The above-mentioned results are more relevant when adding patients with other diseases. An Italian research group followed adult and pediatric patients suffering from various autoimmune diseases and divided according to AC-Ab positivity: while only three patients with APS1 were included in the group of adult subjects (one only being AC-Ab-positive and acquiring overt AAD at follow-up), all but one AC-Ab-positive children enrolled in the latter group were patients with APS1 (305, 306).

In a reappraisal of these studies, the same research group observed that, among the parameters at highest risk of acquiring overt AAD in AC-Ab-positive patients, was an impaired adrenal function at enrollment, the simultaneous presence of idiopathic HPT, CMC, or both, pediatric age, and a high titer of AC-Abs and CYP21-Abs. In particular, the cumulative risk of developing AAD at 11 years of age was 100% in patients with APS1, a much higher percentage than in patients with other autoimmune and non-autoimmune diseases (307, 308).

On the other hand, StC-Abs play a prominent role in predicting APOI in APS1 women, but the statistical power is slightly lower than that achieved by AC-Abs (and StC-Abs themselves) in predicting AAD (231). Interestingly,

measurement of AC-Abs and CYP21-Abs in women with sporadic APOI may be important in identifying patients at risk of acquiring overt AAD (299).

Epitope Targeting

A first determination of the epitopes recognized by CYP21-Abs tested only sporadic and APS2-associated AAD sera (309). A subsequent study showed that the middle region of CYP21 ($CYP21_{164-356}$) retained antigenic sites, and sera from patients with APS1 reacted not unlike those from patients with other AAD forms (310). The use of CYP21 fragments and mouse monoclonal antibodies emphasized the antigenic relevance of the middle and carboxyl-terminal regions of CYP21 and resulted in the detection of two short amino-acid stretches ($CYP21_{335-339}$ and $CYP21_{406-411}$) as the main epitopes independently from patient disease (sporadic AAD, APS1, APS2, and isolated CYP21-Ab positivity) (311, 312). The same is true for CYPscc epitopes (313).

Due to the similarity between the antigenic targets, it was suggested that CYP17-Abs and CYP21-Abs have immunological cross-reactivity (314), but subsequent studies in sera from patients with APS1 disproved this hypothesis (315, 316).

Finally, it has been shown that antibodies to CYP enzymes belong mainly to the IgG1 subclass in patients with APS1, indicating a predominant Th1 response (317). However, IgG4 isotype specificity identifies a small subset of patients with Th2-oriented response (318).

Other Antibody Specificities and Proteomics

Adrenal antibody specificities other than those related to steroid pathways have been occasionally described in patients with APS1: Kendall-Taylor et al. found IgG-class antibodies that antagonized ACTH action in an APS1 girl with AAD (319).

The view is somewhat richer if we look at the gonadal self-antigens; testis-specific protein 10 (TSGA10) was identified by immunoscreening of testis and pituitary cDNA expression library with sera from patients with APS1, but no clinical phenotype correlated with antibody positivity (320, 321).

In turn, based on the observation that the maternal antigen that the embryo requires (Mater) acts as an ovarian target of autoimmunity in an early thymectomy mouse model (322, 323), Brozzetti et al. searched for antibodies to the leucine-rich repeat protein 5 (NALP5), which represents the corresponding human self-antigen. Interestingly, antibody positivity involved the majority of patients with APS1 and included a number of patients with non-APS1-associated AAD, APOI, or both (324).

Currently, proteomics appears to be capable of capturing the enormous biodiversity of potential targets and offers an undisputable advantage over previous candidate-based approaches (325). Landegren et al. used sera from patients with APS1 to probe proteome arrays containing thousands of full-length human proteins. Together with established autoimmunity targets, they detected melanoma-associated antigen B2 (MAGEB2) and disulfide isomerase-like protein of the testis (PDILT) as gonadal self-antigens (326).

More recently, Vazquez et al., employing a high-throughput, proteome-wide phage display method, identified some novel self-antigens targeted by APS1 sera. No adrenal targets were included but, in turn, antibodies to the ovarian KH-domain-containing 3-like protein (KHDC3L), which form an oocyte-specific critical complex with NALP5, were involved in APS1-associated APOI (327).

APS1-ASSOCIATED AAD: CELLULAR IMMUNITY

Although antibodies to CYP enzymes play a prominent role in indicating the nature, antigenic targets and, especially in the APS1-associated form, the time interval before the clinical onset of AAD, it is well-known that cellular immunity is responsible for organ damage (328). Even circulating T-lymphocytes show clear signs of activation in the early stage of the disease (16, 185, 329–333). Further evidence is provided by the (rare) animal models of autoimmune adrenalitis (334).

Findings from patients with APS1 strongly support this belief—starting from the cited study of Ostertag (172), autopsy samples of patients with Addisonian APS1 show massive infiltration of the adrenal cortex by mononuclear cells; with time, gross atrophy of the gland is evident, characterized by the replacement of the adrenal layers with connective tissue. The medulla is usually spared (335–340). As expected, a variable degree of adrenal inflammation is frequently found in patients with APS1 who had not yet reached a symptomatic stage, an uncommon feature of autopsy, if present, in the general population (341).

Interestingly, the delineation of CYP21 epitopes benefited from interferon-γ (IFNγ, a type-2 IFN) assay in cultures of peripheral blood mononuclear cells challenged with several CYP21 fragments: some patients with APS1 carrying the MHCI HLA-B35 allele were included in these studies. A $CYP21_{431-450}$ fragment stimulated lymphocytes from non-APS1 AAD patients, while those from two APS1 siblings reacted against $CYP_{131-150}$. Lymphocytes from the third patient with APS1 did not proliferate, presumably due to long-lasting AAD (342, 343).

CONCLUSIONS

Several key issues need to be clarified in the relationship between the loss of self-tolerance resulting from AIRE/Aire deficiency and related organ failures. In particular, the non-dependence on AIRE of the main adrenal self-antigens in the human thymus raises questions about the intrinsic mechanisms that trigger autoimmunity in APS1. Dissection of these aspects can help plan suitable strategies for causal therapy.

Meanwhile, a look at the history of APS1 and APS1-associated AAD confirms the challenge that such disease implies—interpolation between AAD and other endocrine failures, mainly HPT and ATD, amplifies the risks and benefits of replacement therapies and highlights the need for proper use of mineralo- and glucocorticoids.

Above all, correct management of specific laboratory tests can identify early markers of adrenal impairment and avoid pitfalls

and dangers deriving from the onset and stabilization of one of the most insidious APS1 components.

AUTHOR CONTRIBUTIONS

RP revised APS1 case reports and cohort studies, detailed AIRE properties and drew up the manuscript. AFi described APS1 clinical picture and examined the genotype/phenotype relationship. AFa dissected the immunological aspects of APS1. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.606860/full#supplementary-material

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Effect of Anti-TNF Therapy on Mucosal Apoptosis Genes Expression in Crohn's Disease

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Crohn's disease (CD) is a chronic immune-mediated disorder for which there is not a fully effective treatment. Moreover, biological therapy with anti-tumor necrosis factor-α (anti-TNF-α) monoclonal antibodies leads to an effective response in only 60–70% of patients. Our previous data suggested that specific *loci* polymorphism of the *TNFRSF1B*, *FCGR3A*, *IL1R*, *IL1B*, and *FAS* genes could be a predictor of the primary non-response to anti-TNF therapy in CD patients. In this work, we propose to explain this hypothesis by functional analysis in colon biopsies and in a cell culture model. Using the RT-qPCR analysis, we estimated the *FCGR3A*, *IL1R*, *TNFRSF1B*, *IL1B*, *FAS,* and *ADAM17* genes mRNA level in colon biopsies material from inflamed and non-inflamed tissue from 21 CD patients (14 responders and 7 non-responders to anti-TNF therapy) and 6 controls, as well as *in vitro* in a peripheral blood mononuclear cells (PBMCs) from 14 CD patients (seven responders and seven non-responders to anti-TNF therapy) and eight controls cultured for 72 h with 10 μg/ml of anti-TNF antibody. Our findings demonstrated a significant down-regulation of *TNFRSF1B* gene expression in non-responders both in inflamed and in non-inflamed colon tissue, while the expression of the *FCGR3A* and *IL1B* genes was significantly up-regulated in non-responders in the inflamed colon region. *In vitro* research results indicate that the anti-TNF drug induced a significant decrease in *TNFRSF1B*, *FCGR3A,* and *FAS* gene expression in non-responders. These results show that altered *TNFRSF1B*, *FCGR3A,* and *IL1B* genes expression can be a predictor of the primary non-response to anti-TNF therapy in CD patients.

Keywords: Crohn's disease, anti-TNF therapy, immunomodulation, genes expression, apoptosis

INTRODUCTION

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD), which generally affects the ileum and colon and is the result of an abnormal inflammatory response to antigens derived from the gastrointestinal tract (1). The main cytokine with a proven role in the pathogenesis of CD is the tumor necrosis factor TNF alpha (TNF-α). Its increased expression is found in both intestinal inflammatory infiltrates and in the serum of patients (2). In the last few years, the therapy that

involves the blockage of this cytokine has been increasingly used in the treatment of IBD (3). With the use of anti-TNF-α drugs, it is possible to achieve clinical and endoscopic remission of the disease. However, there is a group of about one-third of IBD patients who primarily do not respond to this type of therapy. Despite the anti-TNF-α treatment, no clinical improvement or inhibition of the inflammatory process in the endoscopic image is observed (4, 5).

While there are many pro-inflammatory cytokines in the inflamed mucosa, TNF-α is one of the main cytokines. CD is characterized by high activity of Th1/Th17 lymphocytes, which produce pro-inflammatory cytokines, such as interferon-gamma, interleukin 17A and interleukin 2. The increase in the expression of pro-inflammatory cytokine genes thus reflects the degree of activity of the immune system involved in the pathogenesis of the disease. Biological therapy induces endoscopic remission, which should be reflected by the reduction of inflammatory activity in the intestinal mucosa (6). On this basis, it can be hypothesized that a complete response to treatment is manifested by a reduction in the activity of pro-inflammatory cytokines in the intestinal mucosa and the associated decrease in the expression of genes encoding individual cytokines, as well as other proteins associated with the onset of and decrease in the inflammatory process. The exact mechanism of action of anti-TNF antibodies in IBD is still uncertain, but there is a general consensus that one of their main therapeutic properties is mediated by binding to membrane-bound TNF (mTNF)-bearing immune cells in intestine mucosa. These drugs bind to mTNF-expressing macrophages, thereby inducing apoptosis in TNF-RII-expressing mucosal T cells. The mTNF/TNF-RII signaling pathway is therefore a basis regulator in mediating resistance to intestinal T-cell apoptosis and may contribute to the perpetuation of mucosal inflammation (7–9). Moreover, our previous data suggested that specific *loci* of apoptosis genes including TNF receptor superfamily member 1B (*TNFRSF1B*, OMIM 191191), Fc fragment of IgG receptor IIIa (*FCGR3A,* OMIM 146740), interleukin 1 receptor type 1 (*IL1R*, OMIM 147810), interleukin 1 beta (*IL1B*, OMIM 147720) and Fas cell surface death receptor (*FAS*, OMIM 134637) genes could be predictors of the primary non-response to anti-TNF therapy in CD patients (10). In this work, we propose to explain this hypothesis by functional analysis based on the real-time quantitative PCR (RT-qPCR) in the colon biopsies of CD patients treated with anti-TNF and in cell culture model.

MATERIALS AND METHODS

Clinical Characterization

All patients enrolled in this study were hospitalized in the Department of Gastroenterology, Dietetics, and Internal Diseases of Poznan University of Medical Sciences in Poznan, Poland. They were assigned to two groups. The first group consisted of 21 individuals, for whom the research was carried out on tissue material collected during a routine colonoscopy. The second group included 13 patients, from whom peripheral blood samples for cell culture studies were collected. The detailed clinical characteristics of both study groups are presented in **Tables 1**, **2**. All patients had a confirmed diagnosis of CD. The subjects were treated with anti-TNF monoclonal antibodies (mAb') in the therapeutic program of the National Health Fund (which is an official reimbursement program for all biological therapies in Poland) in the Department of Gastroenterology, Dietetics, and Internal Diseases of Poznan University of Medical Sciences in Poznan between 2013 and 2019. The inclusion criteria for patients were that they had to be aged >18 years, have a diagnosis of active CD, be biologic-naïve, as well as having had treatment failure or intolerance to first-line therapies, such corticosteroids, and/or immunosuppressants. The exclusion criteria were the presence of an ileostomy or colostomy and infectious complications (including intraabdominal infections). The diagnosis was based on previously defined criteria (12). Clinical disease activity was assessed by using the Crohn's Disease Activity Index (CDAI) (13). Patients who had never smoked or had quit smoking at least 10 years prior to participating in the study were considered non-smokers. The patients were administered infusions of infliximab (IFX) at a dose of 5 mg/kg

TABLE 1 | Characteristics of the patients group in the mucosa studies.

Parameter	Total patients *n* = 21	Responders *n* = 14	Non-responders *n* = 7	*p*-value
Gender, (F/M), *n* (%)	10 (47.61)/11 (52.39)	7 (50.00)/7 (50.00)	3 (42.86)/4 (57.14)	0.7574
Age, (years) mean ± SD	33.81 ± 12.51	30.64 ± 8.21	40.14 ± 17.47	0.2858
Smoker, *n* (%)	1 (4.76)	1 (7.14)	0 (0.00)	0.4687
Previous surgeries, *n* (%)	8 (38.09)	2 (14.28)	6 (85.71)	0.0015
Disease duration, months, mean ± SD	59.62 ± 42.42	53.29 ± 35.51	72.29 ± 54.63	0.5190
Intestinal location,* *n* (%)				
Colonic (L2)	6 (28.57)	3 (21.43)	3 (42.86)	0.3055
Ileal (L1)	4 (19.05)	3 (21.43)	1 (14.28)	0.6944
Ileocolonic (L3)	11 (52.38)	8 (57.14)	3 (42.86)	0.5366
Behavior, *n* (%)				
Nonstricturing nonpenetrating (B1)	21 (100.00)	14 (100.00)	7 (100.00)	>0.9999
Stricturing (B2)	0 (0.00)	0 (0.00)	0 (0.00)	>0.9999
Penetrating (B3)	0 (0.00)	0 (0.00)	0 (0.00)	>0.9999
Histological score, median (range)	10 (6–14)	9 (6–14)	12 (8–14)	0.0744
Medication, *n* (%)				
Mesalamine	21 (100.00)	14 (100.00)	7 (100.00)	>0.9999
Corticosteroids	8 (38.90)	4 (28.57)	4 (57.14)	0.2037
Azathioprine	13 (61.90)	9 (63.28)	4 (57.14)	0.7507
Adalimumab	4 (19.05)	3 (21.43)	1 (14.28)	0.6944
Infliximab	17 (80.95)	11 (78.57)	6 (85.71)	0.6944

**Disease locations were classified according to the Montreal Classification (11).*

TABLE 2 | Characteristics of patients group in PBMC culture studies.

Parameter	Total patients *n* = 14	Responders *n* = 7	Non-responders *n* = 7	*p*-value
Gender, F/M, *n* (%)	4 (28.57)/10 (71.43)	2 (28.57)/5 (71.43) 33.33/66.67 (%)	2 (28.57)/5 (71.43)	>0.9999
Age, (years) mean ± SD	29.86 ± 7.29	28.71 ± 6.08	31 ± 8.68	0.7372
Smoker, *n* (%)	0 (0.00)	0 (0.00)	0 (0.00)	>0.9999
Previous operations, *n* (%)	6 (42.86)	2 (28.57)	4 (57.14)	0.2801
Disease duration, months, mean ± SD	61.71 ± 50.44	65.14 ± 47.93	58.29 ± 56.47	0.5233
Intestinal location,* *n* (%)				
Colonic (L2)	4 (28.57)	2 (28.57)	2 (28.57)	>0.9999
Ileal (L1)	2 (14.29)	2 (28.57)	0 (0.00)	0.1266
Ileocolonic (L3)	7 (50.00)	3 (42.86)	5 (71.43)	0.2801
Behavior, *n* (%)				
Non-stricturing, non-penetrating (B1)	10 (71.43)	6 (85.71)	4 (57.14)	0.2367
Stricturing (B2)	2 (14.29)	1 (14.28)	1 (14.28)	>0.9999
Penetrating (B3)	2 (14.29)	0	2 (28.57)	0.1266
Medication, *n* (%)				
Mesalamine	14 (100.00)	7 (100.00)	7 (100.00)	>0.9999
Corticosteroids	4 (28.57)	1 (14.28)	3 (42.86)	0.2367
Azathioprine	8 (57.14)	3 (42.86)	5 (71.43)	0.2801
Adalimumab	6 (42.86)	3 (42.86)	3 (42.86)	>0.9999
Infliximab	7 (50.00)	4 (57.14)	3 (42.86)	0.5930

**Disease locations were classified according to the Montreal Classification (11).*

body weight at Weeks 0, 2, 6 (the induction phase), and then every 8 weeks until 1 year (54 weeks-the maintenance phase). Adalimumab (ADA) was given subcutaneously at Week 0 at a dose of 160 mg, 80 mg was given at Week 2, and then every other week a 40-mg dose was given until 1 year (54 weeks).

The anti-TNF treatment response was assessed following 12 weeks of the therapy. The CDAI score was used to determine the clinical response. The clinical response to the therapy was defined as a CDAI reduction by ≥70 points. Moreover, each patient in the mucosal studies group, before qualifying for treatment and after 12 weeks of therapy, had a colonoscopy and/or, in the case of small intestine involvement, MR enterography. The activity of lesions in colonoscopy was assessed according to the Simple Endoscopic Score for Crohn's Disease (SES-CD) and the Simple Enterographic Activity Score for Crohn's Disease (SEAS-CD) in MR enterography (14, 15) (**Figure 1**).

The control groups consisted of 6 healthy (two female, four male) subjects with an average age of 46.7 years in mucosal studies and eight healthy subjects (four female, four male) with an average age 40.2 years in PBMCs culture studies.

All individuals gave their written consent to genetic testing and the evaluation of biochemical parameters in serum and colonoscopy examination. The research was approved by the Bioethical Committee of Poznan University of Medical Sciences, Poland, under Resolution No. 762/13 approved on 9 November 2013 and Resolution No. 1042/18 approved on 11 October 2018. All experiments were performed in accordance with the principles of the 1964 Declaration of Helsinki with its later amendments.

Biopsy Preparation

Approximately 1–2 mg of biopsies were obtained from sites of inflammation and non-inflamed regions from treatment-naïve patients with CD and from healthy controls during a colonoscopy. Next, the collected biopsies were suspended in 300 µl of RNALatter® reagent (Sigma) and were frozen at −80°C until RNA isolation begun started. In each case, material for microscopy analysis was also collected (H+E). We assessed the collected material according to the CD histopathological activity. The applied index was developed by D'Haens et al. and it consists of 8 histological parameters (variables) assessed individually (16). The results are presented in **Table 1**.

PBMCs Isolation, Culture, and Treatment With the Antibody

PBMCs were isolated from 9 ml samples of whole blood using LYMPHOSEP™ (MP Biomedicals LLC, Ohio, USA), according to the manufacturer's instructions. The obtained pellet was suspended in 4 ml of a Lymphogrow medium (Cytogen-Polska Sp. z o.o., Zgierz, Poland) containing phytohemagglutinin (PHA) and recombinant IL-2 (4 ng, 100 U, BioLegend, San Diego, CA, USA). The suspension was then transferred to a 25-ml vessel for adherent culture. Cells were grown under standard conditions at 37°C, 5% CO_2 with shaking for 24 h. Non-adherent cells were washed with PBS and transferred to a 25-ml vessel for suspension culture with fresh Lymphogrow medium supplemented with IL-2. After another 48 h, the cells were passaged and maintained in a culture using a standard RPMI-1630 medium supplemented with L-glutamine (2 mM), FBS (10%), penicillin (100 IU/ml), streptomycin (100 µg/ml), and with the addition of IL-2. Cell differentiation was measured by CD3, CD4, CD8, CD45, and HLA-DR by flow cytometry analysis. In the third passage, anti-TNF mAbs' (Sigma) was added (10 µg/ml). In parallel, a control culture without the addition of the antibody was carried out. After 72 h of culture, cells were suspended in 200 µl stayRNA solution (A&A Biotechnology, Gdansk, Poland) and frozen at −80°C for RNA isolation. Moreover, part of the cells was subjected to apoptosis analysis.

Apoptosis Assessment

Apoptosis was measured using a Muse® Annexin V and Dead Cell Assay Kit (Merck, Darmstadt, Germany) in a Muse Cell Analyzer (Luminex Corporation, Austion, USA), according to manufacturer's protocol. The cells were resuspended in a medium containing at least 1% FBS. Hundred microliter of Muse™ Annexin V & Dead Cell Reagent and 100 µL of cells were added to each tube. The cell suspension was incubated (RT, 20 min) and loaded onto a Muse® Cell Analyzer.

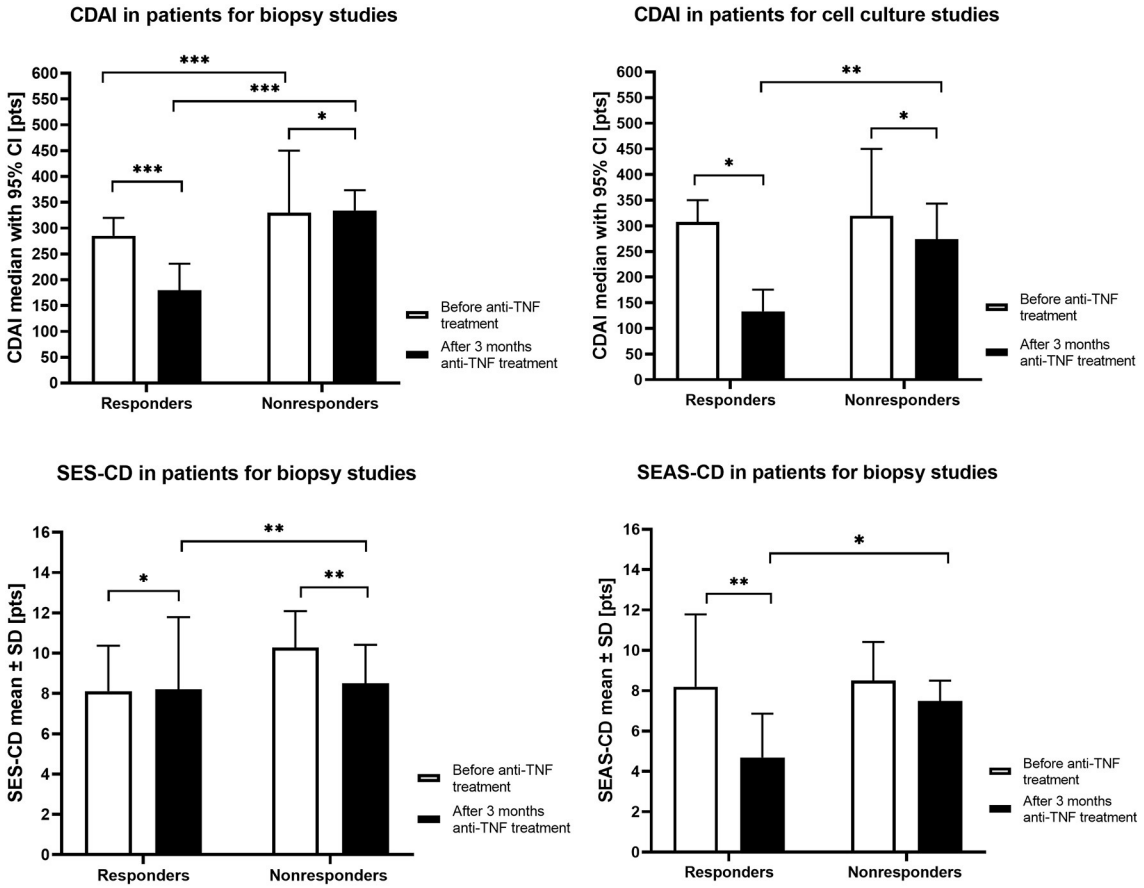

FIGURE 1 | The clinical parameters in responders and non-responders CD patients groups before anti-TNF treatment and after 3 months of therapy. CDAI, Crohn's Disease Activity Index; SES-CD, Simple Endoscopic Score for Crohn's Disease; SEAS-CD, Simple Enterographic Activity Score for Crohn's Disease; CI, confidence interval; SD, standard deviation; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RNA Isolation, cDNA Synthesis, and Quality Control

Tissue samples suspended in TRI Reagent® (Sigma-Aldrich, Saint Louis, MO, USA) were homogenized with mortar and pestle and subjected to RNA isolation with RNeasy Mini Kit (Qiagen, Hilden, Germany). The total RNA from cell culture containing up to 10^5-5×10^5 cells was extracted using Total RNA Mini Plus Concentrator (A&A BIOTECHNOLOGY, Gdansk, Poland), according to the manufacturer's procedure. For all the RNA samples obtained, a quantitative and qualitative evaluation was carried out using Agilent RNA 6000 Nano Kit and the Bioanalyzer 2.0 equipment (Agilent, Santa Clara, CA, USA). A 900 ng of total RNA with RIN $\geq$ 7 was converted to cDNA with an iScript Advanced Reverse Reaction kit (Bio-Rad) with the following conditions: 25°C for 5 min – annealing step, 42°C for 30 min – reverse transcription and 95°C for 1 min – inactivation.

Real-Time Quantitative PCR (RT-qPCR)

The mRNA level of selected genes: *ADAM17*, *FAS*, *FCGR3A*, *IL1B*, *IL1R*, and *TNFRSF1B* was measured by a real-time quantitative polymerase chain reaction on a BioRad CFX Connect 96-well Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California, United States) using the iTaq UniverSYBR Green assay (Bio-Rad Laboratories, Inc., Hercules, California, USA), according to the manufacturer's instructions. Primers for *ADAM17*, *FAS*, *FCGR3A*, *IL1B,* and *IL1R* genes analysis were designed using a Primer-BLAST tool, and their sequences are presented in **Table 3**. Primers for *TNFRSF1B* gene, as well as reference *PPIA* and *RPLP0* genes, were ordered as PrimePCR™ SYBR® Green Assay by Bio-Rad Laboratories, Inc. manufacturer. The results of qPCR reactions are presented as $dCt = (dCt_{referencegene} - dCt_{geneofinterest})$. Every reaction was performed in duplicate.

Statistical Analysis

Analysis of gene expression in intestinal biopsies was based on a multiple comparison non-parametric Kruskal-Wallis test (due to the failure to meet the assumptions for parametric tests) which compare every group in terms of the median value. *Post-hoc* analysis was performed based on Dunn's test, taking into account the Benjamin-Hochberg correction for multiple comparisons to specify which groups are significantly different.

TABLE 3 | Primers sequence and qPCR parameters.

Gene	Sequence 5′-3′	Annealing temperature	Amplicon (bp)	Reaction efficiency (%)
ADAM17	AGAATGTTTCACGTTTGCAGTCTC	55–65°C	117	97.95
	CTCGATGAACAAGCTCTTCAGGTG			
FAS	GTGAGGGAAGCGGTTTACGA	55–65°C	193	96.61
	AGATGCCCAGCATGGTTGTT			
FCGR3A	CACATATTTACAGAATGGCACAGG	55–65°C	173	95.31
	ACACTGCCAAACCTTGAGTGATGG			
IL1B	AAAGCTTGGTGATGTCTGGTC	55–65°C	89	91.62
	GGACATGGAGAACACCACTTG			
IL1R	TTGGGTTAAGAGGACAGGGA	55–65°C	105	90.00
	TGATTTCTTCTCTGGAGGCTG			

The Mann-Whitney non-parametric test was used to compare median value of the two groups in case of apoptosis and gene expression analysis in T lymphocytes cultures, as well as to compare clinical parameters between responders and non-responders. *P*-values < 0.05 were considered as statistically significant. All analyses were performed using R software (version 3.2.3) and R Studio.

RESULTS

Previously, in the research concerning the identification of molecular markers useful in predicting non-response to anti-TNF biological agents, we described that polymorphism in apoptosis and inflammatory pathways genes could be associated with this phenomenon in CD patients (10). These investigations were performed based on long-range PCR libraries and next-generation sequencing analysis of the selected genes panel (17). In the present study, we test whether changes in mRNA expression of these genes are associated with a primary non-response in our group of Polish patients. We also investigate the impact of anti-TNF mAbs on patients' T cells apoptosis and how the treatment affects the activity of selected targets.

Gene Expression in Intestinal Biopsy Samples

The mRNA expression level of *TNFRSF1B*, *FAS*, *FCGR3A*, *IL1B*, *IL1R,* and *ADAM17* genes was investigated to find potential differences between patients who did not respond to the therapy, patients who did respond, and healthy individuals. Additionally, we compared the expression in the inflamed and non-inflamed regions. In addition to the *FAS* gene, this analysis demonstrated significant differences in the mRNA level between the above-mentioned groups. For *TNFRSF1B*, in non-responders, both the inflamed and non-inflamed tissue presented a decreased level of the gene compared to biopsies from the responders and control group (**Figure 2**) (detailed values shown in **Supplementary Table 1**). *FAS* and *TNFRSF1B* genes are essential mediators in apoptosis pathways. Furthermore, other molecular factors involved in TNFα biology were investigated. For the *ADAM17* gene, a decreased level of transcripts was demonstrated in the material from non-responders to anti-TNF in inflamed and non-inflamed biopsies (**Figure 3**, **Supplementary Table 2**), similar to *TNFRSF1B*. On the contrary, the *FCGR3A* gene showed up-regulated levels in non-responding patients, though only in inflamed tissues when compared to the controls ($p = 0.0044$, **Figure 2**). Moreover, it should be emphasized that a relevant difference was found in non-responding patients between non-inflamed and inflamed colon tissue ($p = 0.0033$, **Figure 3**). Likewise, in the case of *IL1B* and its receptors, some significant differences were observed. The *IL1B* mRNA level was up-regulated in inflamed tissues on non-responders as compared to the control group ($p = 0.0002$, **Figure 3**). For the *IL1R* gene, there was only a significant difference between the control group and inflamed biopsy from responders ($p = 0.0047$, **Figure 3**).

Infliximab Did Not Induce Apoptosis in T Lymphocytes

PBMC fractions of patients and controls after stimulation of the cell culture with interleukin 2 in order to increase the fraction required for cell research and confirm the positive stimulation were subjected to a flow cytometry analysis to assess the differentiation level of the cells. On average $CD3^+$ lymphocytes represented 74.8% (SD ± 30.3%) of the whole fraction. $CD3^+$ $CD4^+$ - 31.3% (SD ± 16.6%), $CD3^+$ $CD8^+$ - 31.3% (SD ± 13.5%) and NK cells represented 9.4% (SD ± 4.5%). Based on the available data and the cytotoxicity test (data not shown), it should be assumed that 0–100 μg/ml of infliximab is the concentration *in vitro,* which reflects the concentration of the drug in plasma (18, 19).

The impact of anti-TNF mAbs on apoptosis of T lymphocytes was investigated by incubation with 10 μg/ml of infliximab for 72 h with cells obtained from responders, non-responders, and healthy controls. Simultaneously, control cultures were grown. Then, the overall apoptosis level was measured. However, no statistically significant differences between any of the three groups and parallel controls were observed. The cells viability was on average 74.9% (SD ± 8.8), 69.9% (SD ±12.1), and 72.7% (SD ± 7.5) for controls, responders and non-responders treated with anti-TNF vs. 75.4% (SD ± 10.4), 71.7% (SD ±10.5), and 73.1% (SD ±5.7) for untreated cells, respectively.

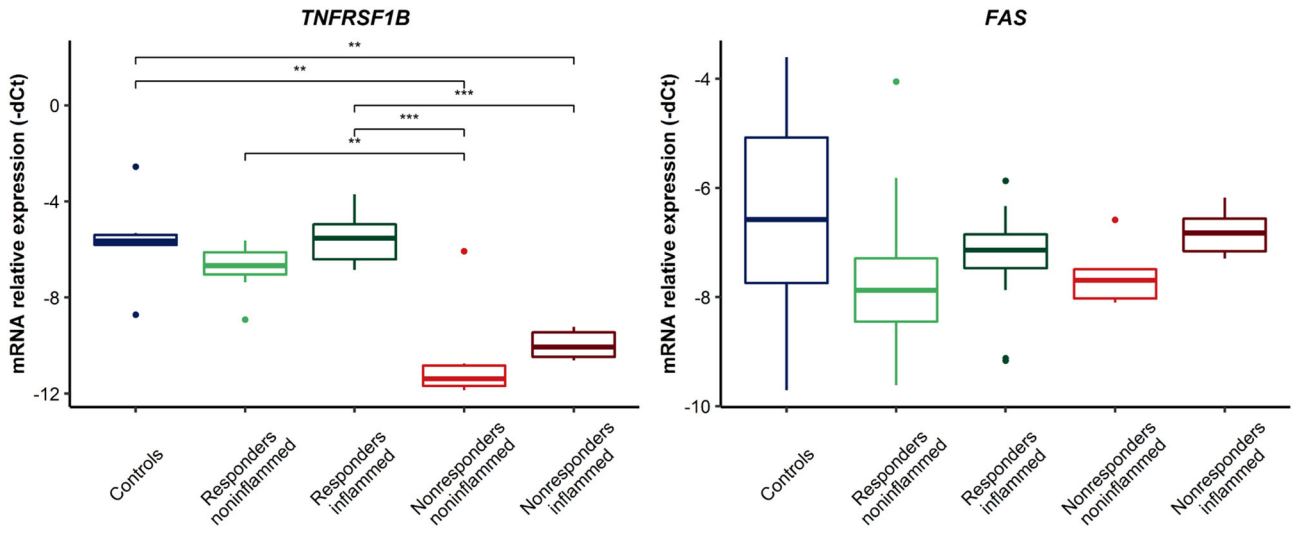

FIGURE 2 | Relative expression of genes involved in apoptosis in CD patients' colon biopsies. ***$p < 0.001$; **$p < 0.01$.

Infliximab Modified Expression Level of Apoptosis-Related Genes

However, no impact of infliximab on T cells apoptosis was observed. We wondered whether the treatment can modify the expression level of our genes of interest. Indeed, in the case of two apoptotic genes – *TNFRSF1B* and *FAS*, and interestingly also in *FCGR3A*, which is responsible for antibody-independent cellular cytotoxicity (AICC) mechanism, the same pattern of changes in expression was described. In healthy controls, the significant induction of mRNA expression was observed between treatment and control (*TNFRSF1B*, $p = 0.0007$; *FAS*, $p = 0.01998$; *FCGR3A*, $p = 0.0012$). In the case of responding patients, no significant changes were detected, and in non-responding patients, down-regulation of the three genes occurred (*TNFRSF1B*, $p = 0.0022$; *FAS*, $p = 0.0087$; *FCGR3A*, $p = 0.0087$; **Figures 4**, **5**).

For the remaining genes, only in *IL1B* mRNA in the control group was a significant increment observed ($p = 0.0217$, **Figure 5**).

DISCUSSION

The introduction of anti-TNF agents in CD therapy was an irrefutable breakthrough, although this form of treatment is not effective in almost 30–40% of patients. *In vivo*, inflammatory processes are complex and multifactorial, and their exact mechanism is still a matter of debate. In previous studies, based on a custom panel of genes and NGS technology, the genetic background of non-response was investigated in a selected group of genes which seem to play a key role in TNFα biology and anti-TNF action. Polymorphic variants of *TNFRSF1B*, *FAS*, *FCGR3A*, *IL1B,* and *IL1R,* genes were identified as potentially associated with a lack of response to mAbs' (10). However, mostly intronic, 3'-UTR location suggests their potential impact on gene expression regulation. Since genes that remain altered in non-responders constitute a potential new therapeutic target to induce remission in anti-TNF refractory patients, their activity was the subject of this work. Moreover, the *ADAM17* gene, which encodes the main enzyme responsible for converting transmembrane TNF-α into its soluble form, was included in our research. Thus, in intestinal biopsies, significant changes in mRNA expression levels were detected between responders, non-responders, and the control group. Down-regulation of *TNFRSF1B* and *ADAM17* mRNA was detected in inflamed and non-inflamed tissues of refractory patients. Additionally, in non-inflamed biopsies of non-responders, a significant increment of *FCGR3A* and *IL1B* was described. These expression changes seem to be important and could play a direct role in mAbs action. To provide the answer as to whether mAbs influences the mRNA activity of the genes in question, we performed an experiment on T lymphocytes treated with the anti-TNF drug. Overall, no influence of anti-TNF on cell apoptosis was observed, although somehow the treatment modified significantly the expression of *TNFRSF1B*, *FAS,* and *FCGR3A* in the same pattern, up-regulating mRNA level in the control group and down-regulating in non-responders, with no significant effect in patients who responded to anti-TNF mAbs. However, it should be noted that the lack of differences in cell viability measured by a flow cytometry does not exclude the initiation of apoptosis-related processes manifested by a change in concentration of membrane proteins such as TNFRI, TNRII, and FcγRIII receptors.

In our study group, apart from anti-TNF treatment, other drugs were also used (mesalamine, corticosteroids or azathioprine), whose influence on the apoptosis process cannot be excluded, and which may affect the expression of individual receptors. However, in subgroups, patients did not differ statistically in mesalamine, corticosteroids and azathioprine treatment. Therefore, we did not perform an analysis in this respect. From the studies conducted, it is known that the use of azathioprine and anti-TNF drugs may be more effective than the

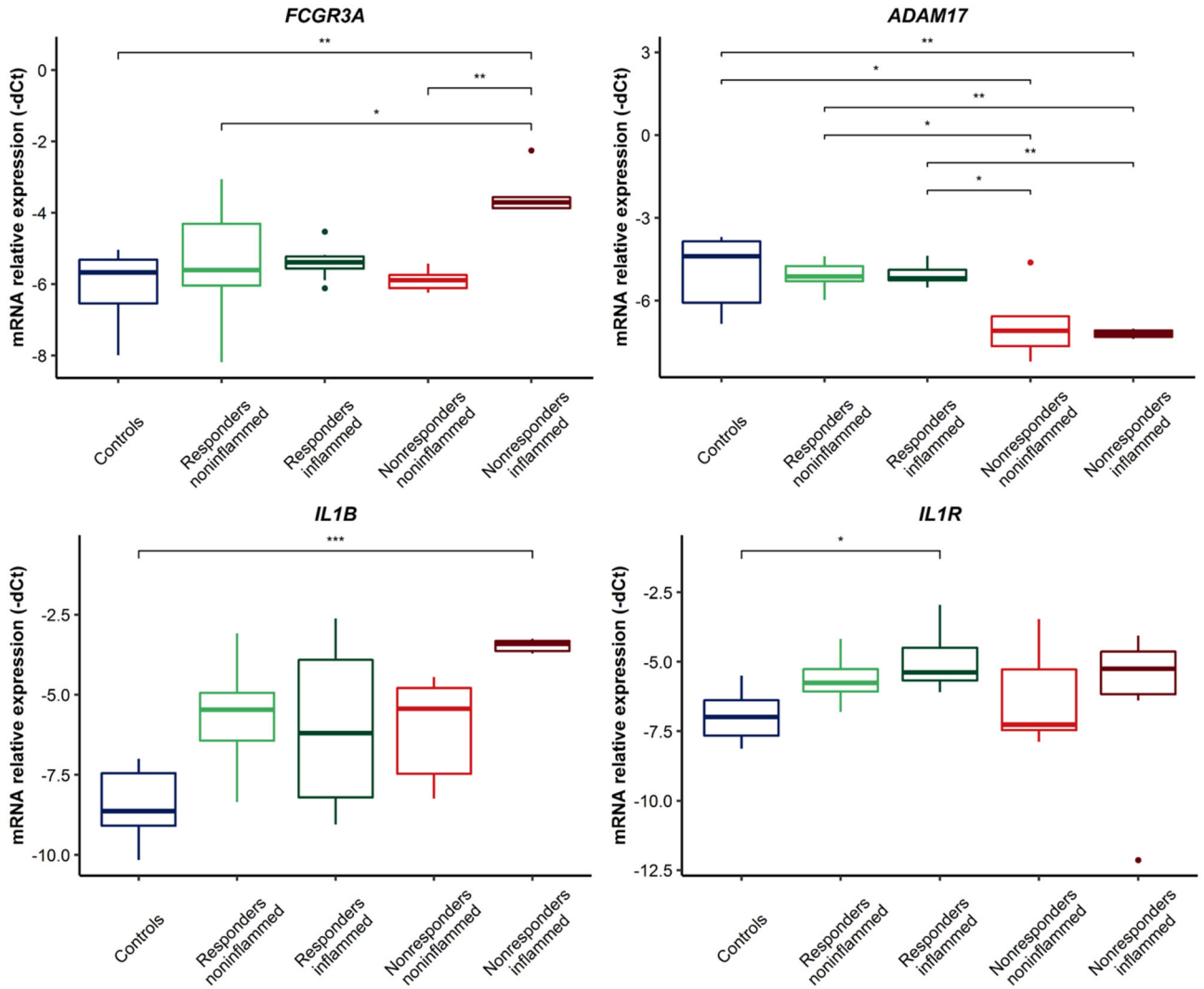

FIGURE 3 | Relative expression of genes involved in TNFα and anti-TNF monoclonal antibodies action in colon biopsies. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

use of biological drugs in monotherapy. In our case, however, trial treatment with azathioprine was used in every patient. Subject who did not continue thiopurine therapy showed intolerance to the drug. Azathioprine were applied for a period not shorter than 3 months before being included in biological treatment. All persons enrolled in the study were therefore on stable doses of the drugs. There are no reports in the literature on the influence of mesalamine, corticosteroids or azathioprine on the receptors which we studied (20). However, it can be speculated that due to the reduction in the inflammatory process, drugs such as corticosteroids and azathioprine may contribute to accelerating the elimination of inflammatory cells. This should have no effect on the expression of membrane proteins, disturbances to which result from genetic polymorphisms, and such a situation cannot be ruled out in our study group.

Regardless of the unexplained mechanism of anti-TNF action, in current clinical practice, there is a necessity to identify molecular mediators of inflammation, primarily in order to describe molecular markers of non-response and perhaps in the future, implement it in everyday clinical practice. A great scientific effort has been taken to identify useful and reliable indicators of non-response rate, but to date, not one of the genes analyzed is supported by strong evidence. *TNFRSF1B* appears to be an obvious candidate, considering that it codes for one of two TNFα receptors, namely TNF-RI. This gene is expressed on the surface of lymphocytes, epithelial cells, and macrophages (3). According to the literature, TNF-RI and TNF-RII receptors are identified as responsible for activating the apoptosis cascade. To some extent, functions of receptors may overlap, depending on the participation of different factors in signal transducing, the degree of cell activation and the environment. Moreover, TNF-RII activation might contribute to stimulate apoptosis, limiting immune response in autoreactive T cells (21, 22). Therefore, it seems that the decreased expression level of *TNFRSF1B* may

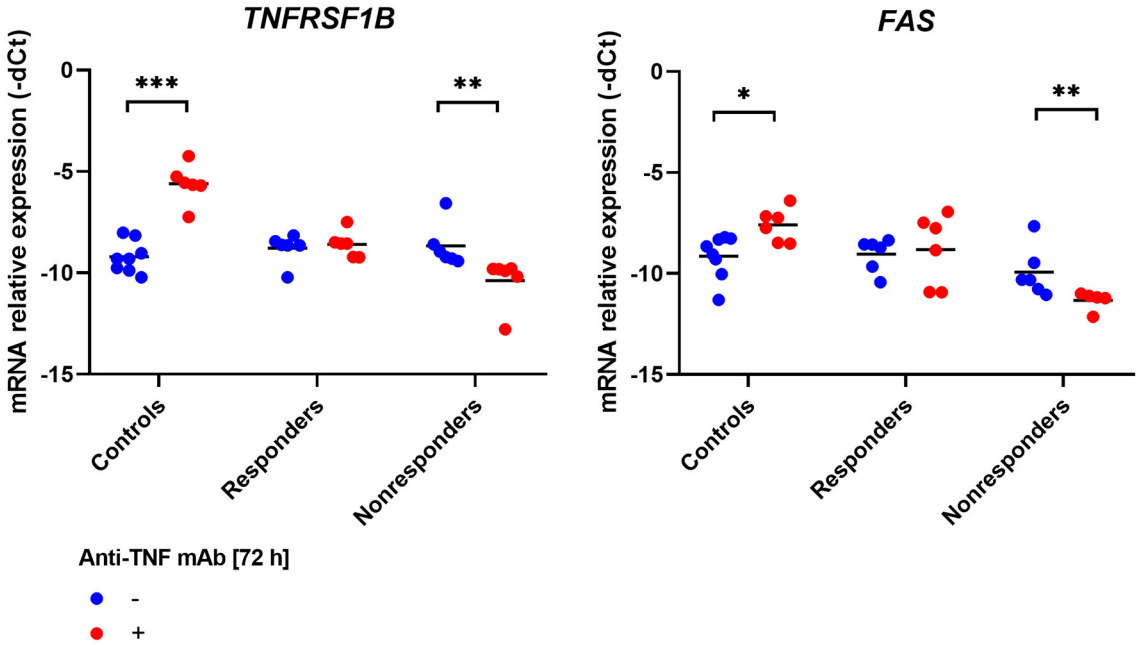

FIGURE 4 | Relative expression of receptor genes involved in apoptosis in PBMCs culture treated with anti-TNF monoclonal antibodies. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

be responsible for reduced patients' response by decreasing the ability of T cells elimination.

FCGR3A is a key player in the antibody-dependent cell cytotoxicity (ADCC) mechanism – one of the possible ways anti-TNF acts. The increased mRNA level in non-responding patients described in this paper may disturb ADCC activity and, as a consequence, eliminate cells expressing tmTNFα on their surface. Also, it is noteworthy that previously we found SNP in 3'-UTR of the gene which occurred with increased frequency in refractory patients. Additionally, this variant is placed in a potential seed region for miRNA particles. This suggests a possible regulation mechanism of the *FCGR3A* expression level. However, to confirm this, further functional studies should be performed (10). Moreover, the participation of anti-TNF mAbs in ADCC was indicated in research based on modified cell cultures, stably expressing tmTNFα (23, 24). *In vivo*, the impact of anti-TNF on T cells cytotoxicity remains uncertain (25, 26).

In the context of *FCGR3A*, the activity of *ADAM17* seems to be of value. A significant decrement of the mRNA level was observed in our group of non-responding patients compared to responders and controls. Other than controlling the balance between sTNFα and tmTNFα, this metalloproteinase is important for the activity of FcγRIII. ADAM 17 is a potential enzyme which inactivates the FcγRIII receptor (also called CD16) by a cleavage after cytotoxicity induction. It has been shown that after inhibition of the enzyme, FcγRIII activity was not decreased. ADAM 17 is also constitutively expressed on a surface of natural killer (NK) cells and other leucocytes (27–30). Additionally, inhibitors of this metalloproteinase are expected to be an effective treatment for oncological patients with decreased activity of FcγRIII, which is a potential cause of anti-TNF lack of activity (31). However, there is no data referring to CD in this matter. Based on the results presented in this study, we may hypothesize that down-regulated activity of *ADAM17* might influence the activity of *FCGR3A* but direct evidence was not provided and this requires further investigation.

Another candidate gene which may function as a molecular predictor of non-response is *IL1B*. Increased levels of this cytokine were described in the plasma of CD patients and other diseases triggered by autoimmunity. Also, up-regulated levels of the *IL1B* gene mRNA in the biopsies of refractory patients were described by transcriptomic studies (32, 33). We have confirmed these results in our study. Likewise, in the *FCGR3A* gene, we described polymorphisms in 3'-UTR, potentially affecting the miRNA binding site and additionally SNP in exonic sequence (10). If these variants affect the expression level of the gene, they should be investigated experimentally.

In IBD, the search for an ideal predictive marker of non-response in biological treatment has been ongoing for many years. A large number of different factors were identified as associated with resistance in patients, although the lack of reproducibility and predefined protocols for sample collection, small analyzed groups, and often contradictory results prevent the scientific community from drawing strong conclusions. According to West et al., oncostatin M seems to be a strong candidate for molecular marker, since the high pretreatment expression of *OSM* was associated with the failure of anti-TNF treatment (34). In pediatric patients, down-regulated *SMAD7* expression in whole blood cells prior and after 2 weeks of anti-TNF treatment was identified as associated with a lack of response. It is of utmost interest that the authors did not find any differences in the case of *TNFRSF1B*, and *OSM* (35). As

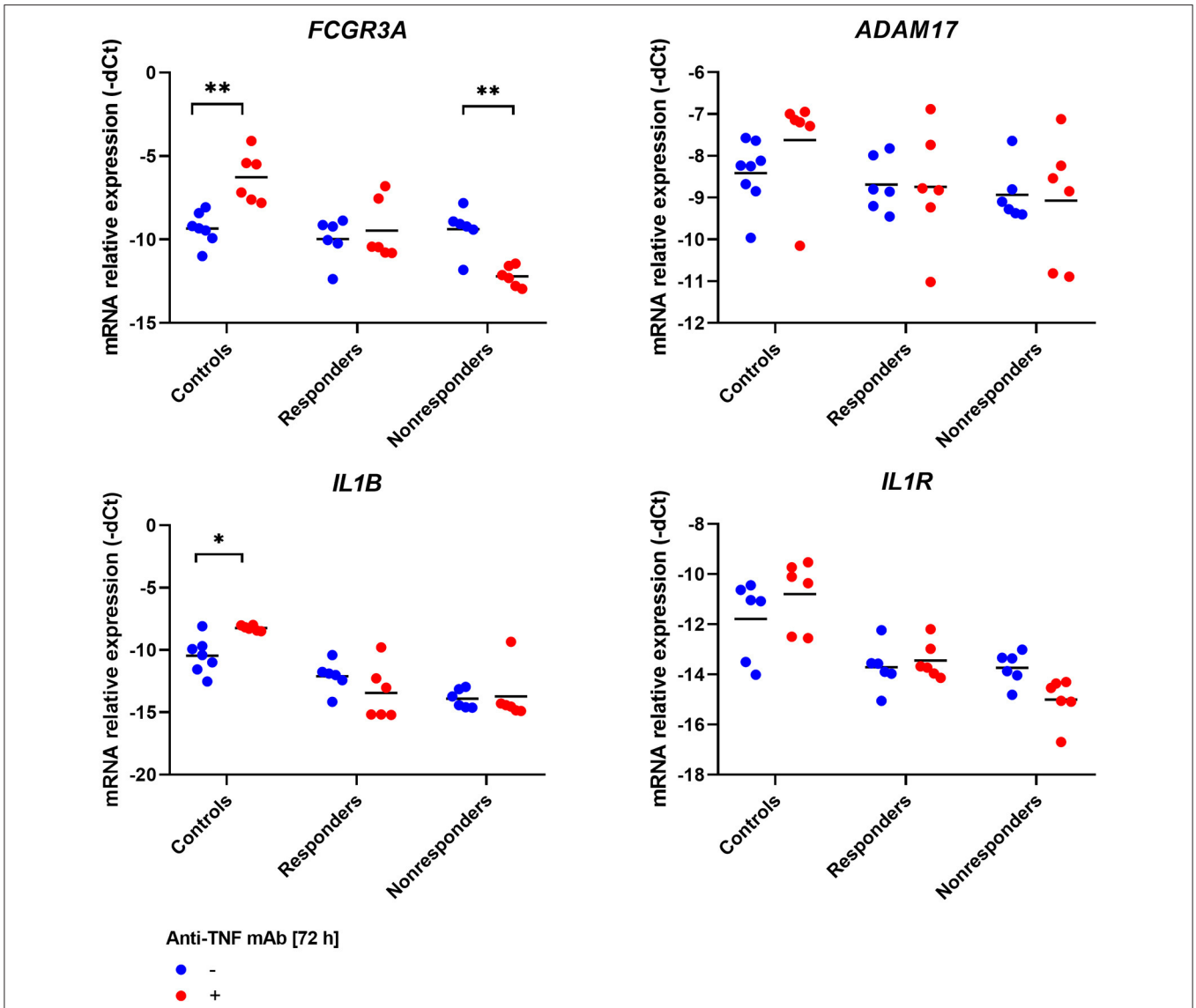

FIGURE 5 | Relative expression of receptor genes involved in the action of TNFα and anti-TNF monoclonal antibodies in PBMCs culture treated with anti-TNF drug. $^{**}p < 0.01$; $^{*}p < 0.05$.

mentioned, mostly this type of study is limited by the sample size. This is also the most restrictive factor. Another problem is the lack of standardized lab protocols, which might strongly affect RT-qPCR results and it should be equalized to improve the quality of results obtained in different laboratories.

In the set of genes analyzed, we revealed differences in expression between responders and non-responders, although biopsies were collected prior to anti-TNF treatment, and the ability to respond was assessed after 3 months of biological treatment. To investigate the impact of anti-TNF mAbs on the expression of our targets' level we decided to use cell cultures. We studied two aspects – whether the anti-TNF treatment would modify the expression level of genes of interest and whether mAbs are able to induce apoptosis in cells collected from patients.

Additionally, *TNFRSF1B*, *IL1B*, *FAS*, *FCGR3A*, and *ADAM17* are genes that are possibly involved in the multifactorial mechanism of anti-TNF action. One probable explanation of biological treatment efficacy is the induction of apoptosis of T lymphocytes, whose activity might be dysfunctional in refractory patients. Changes in the expression of a set of genes included in biopsies, characterized for non-responders, indicate their possible impact on the lack of response. However, in the conditions tested, we did not find significant differences in any of the three groups investigated between T cells population which were treated with anti-TNF mABs and the control culture. The results concerning the occurrence of apoptosis after anti-TNF treatment are also contradictory. Researchers from the Netherlands showed that infliximab induced apoptosis *in vitro* on lymphocytes isolated from *lamina propria*, as

well as from PBMC by activation of caspase-3 in patients who responded to the treatment. However, it was not clear if this result was a consequence of direct activation through tmTNFα or a simple block of contact between tmTNFα and TNF receptors bearing cells (36, 37). Similar results were obtained in monocytes cultures from healthy individuals and responding patients (38). Other studies did not confirm these observations in monocytes (39) and lymphocytes (8) cultures. Although we did not show apoptosis induction after infliximab treatment, our results are strictly limited. First, we measured the overall level of apoptosis, not direct pathways or caspases activation. Secondly, the conditions used in the experiment are narrow, and possibly in wider circumstances, results might be different.

The impact of infliximab on apoptosis and its influence on the expression level of selected genes was measured. The mRNA level was compared with the culture treated with infliximab and parallel control in responders, non-responders, and healthy individuals. Three genes, *TNFRSF1B* and *FAS,* both of which are involved in apoptosis and *FCGR3A*, which is essential for the AICC mechanism, showed the same tendency. In the group of healthy individuals, infliximab induced significant up-regulation of genes, in responding patients no change was observed, and in the case of non-responders, the expression was down-regulated. *FAS* is coding for a key AICD death receptor and is involved in the process of autoreactive T cells elimination as well as *TNFRSF1A*. Meng et al. showed that *TNFRSF1B* might also participate in apoptosis control. In the activated T lymphocytes, isolated from PBMC of healthy individuals, apoptosis was induced. The silencing of *TNFRSF1B*, in contrast to *TNFRSF1A*, inhibited cell death. Similar results were obtained in Jurkat cells treated with an anti-TNF drug. What is of value is that stimulation of healthy T cells induced up-regulation of *FAS,* and *TNFRSF1B* expression. This is possibly one of the AICD mechanisms in which the antibody sensitizes cells to the influence of apoptotic signals by induction of death receptors expression on the cell surface (40). These results are in line with previous observations concerning the impact of TNF-RII stimulation on inhibition of T lymphocyte proliferation and cytokine expression (41). Schmitt et al. also identified that increased expression of *TNFRSF1B* in T cells from *lamina propria* prior to the treatment is associated with an increased response (9). The amount of evidence confirming the contribution of *TNFRSF1B* in apoptosis of T lymphocytes is increasing, which goes beyond its predominantly described function of proliferation stimulation. Here, we confirmed the observation that anti-TNF in healthy cells stimulates the up-regulation of *FAS* and *TNFRSF1B.* However, we additionally determined that the opposed effect occurred in cells obtained from non-responders. Presumably, due to molecular abnormalities, instead of activation of both genes, down-regulation is presented, which finally results as insensitivity to apoptotic signals. This is an interesting hypothesis, which should be verified by the scientific community.

In summary, our research contributes to understanding the mechanisms behind the lack of a primary response in CD patients. We have observed that in naïve non-responding patients, there is a changed expression of proteins involved in the process of apoptosis and elimination of pro-inflammatory cells at the mucosal tissue level. Perhaps the assessment of an appropriate panel of apoptotic genes will be a valuable future marker for the first therapy in IBD patients. However, more detailed research is needed in this area.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethical Committee of the University of Medical Sciences in Poznan, Poland, under Resolution No. 762/13 approved on 9 November 2013 and Resolution No. 1042/18 approved on 11 October 2018. All experiments were performed in accordance with the principles of the 1964 Declaration of Helsinki with its later amendments. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LL-S and MS-Z: conceptualization. MW and LL-S: methodology and validation. MW, LL-S, JS-Z, KS-E, KW, PE, AW, and IK-K: investigation. MW: formal analysis. MW, LL-S, and MS-Z: writing-original draft preparation. PE and JS-Z: critical revision of the manuscript. KS-E, MS-Z, and RS: funding acquisition. RS and AD: supervision. All authors contributed to the article and approved the submitted version.

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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.2021.615539/full#supplementary-material

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CASE REPORT
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Case Report: Novel SAVI-Causing Variants in *STING1* Expand the Clinical Disease Spectrum and Suggest a Refined Model of STING Activation

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Gain-of-function mutations in *STING1* cause the monogenic interferonopathy, SAVI, which presents with early-onset systemic inflammation, cold-induced vasculopathy and/or interstitial lung disease. We identified 5 patients (3 kindreds) with predominantly peripheral vascular disease who harbor 3 novel *STING1* variants, p.H72N, p.F153V, and p.G158A. The latter two were predicted by a previous cryo-EM structure model to cause STING autoactivation. The p.H72N variant in exon 3, however, is the first SAVI-causing variant in the transmembrane linker region. Mutations of p.H72 into either charged residues or hydrophobic residues all led to dramatic loss of cGAMP response, while amino acid changes to residues with polar side chains were able to maintain the wild type status. Structural modeling of these novel mutations suggests a reconciled model of STING activation, which indicates that STING dimers can oligomerize in both open and closed states which would obliviate a high-energy 180° rotation of the ligand-binding head for STING activation, thus refining existing models of STING activation. Quantitative comparison showed that an overall lower autoactivating potential of the disease-causing mutations was associated with less severe lung disease, more severe peripheral vascular disease and the absence of a robust interferon signature in whole blood. Our findings are important in understanding genotype-phenotype correlation, designing targeted STING inhibitors and in dissecting differentially activated pathways downstream of different STING mutations.

Keywords: interferonopathy, autoinflammatory disease, type I interferon, SAVI, STING, whole exome sequencing, pediatrics

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INTRODUCTION

Autoactivating variants in Stimulator of interferon response cGAMP interactor 1 (*STING1*, also known as *TMEM173*), the gene that encodes STING (Stimulator of IFN genes) (1–3) cause a rare autoinflammatory interferonopathy, STING-associated vasculopathy with onset in infancy (SAVI, OMIM # 615934) (4–6). SAVI-causing variants in exons 5, 6 and 7 were so far found in 8 different amino acid residues that lead to STING autoactivation in the absence of ligand-binding (5–11). Here we report 3 novel SAVI-causing mutations in 3 unrelated kindreds, including variant p.H72N, which was disease-causing in a mother and 2 children, and is the first disease-causing gain-of-function (GOF) mutation in the transmembrane linker region of STING (12). Modeling of previously reported and these novel SAVI mutations extend our current understanding of STING activation involving critical residues that are mutated in the connector helix loop encoded by exon 5, the polymer interface encoded by exon 6 and 7, and the transmembrane linker region encoded by exon 3.

MATERIALS AND METHODS

IFNB1 Luciferase Reporter Assay

The assay was performed as previously described in detail (13). Briefly, HEK293T cells were co-transfected with STING constructs and *IFNB1* firefly luciferase reporter construct with Lipofectamine 3000 reagent (ThermoFisher Scientific, cat# L3000015) in 96-well plates (black wall with clear bottom, BD Falcon, cat# 353219) with a reverse transfection protocol. 50 pg of STING construct and 50 ng of *IFNB1* firefly luciferase reporter construct were mixed with 5 μL of Opti-MEM and 0.2 μL of P300 reagent. The vector dilutions were then mixed with the Lipofectamine 3000 dilutions containing 5 μL of Opti-MEM and 0.3 μL of Lipofectamine 3000, and then incubated at room temperature for 10-30 min. 30,000 HEK293T cells in 75 μL of complete DMEM were applied to each well and mixed by gently tapping, and then rocked back and forth to allow even distribution of the cells, followed by 10 min incubation at room temperature to allow the cells to settle. Plates were then returned to 37°C in a humidified atmosphere with 5% CO2 for 24 hr. Luciferase assay was carried out using ONE-Glo™ EX Luciferase Assay System (Promega, cat# E8120), with 50 μL luciferase reagent per well.

cGAMP Stimulation

1.8 μL of 2'3'-cGAMP (1 μg/μL, Invivogene, cat# tlrl-nacga23) were diluted with 3.2 μL of complete DMEM, and applied to one well (96-well plate) with 85 μL of culture at 6 hr post transfection for a final concentration of 20 μg/mL (13). For the 4 μg/mL dose, 0.36 μL of 2'3'-cGAMP were diluted with 4.64 μL of complete DMEM.

Abbreviations: *STING1*, Stimulator of interferon response cGAMP interactor 1; SAVI, STING-associated vasculopathy with onset in infancy; IFN, Interferon; cGAMP, 2'3'-cyclic GMP–AMP; WES, whole exome sequencing.

Western Blot

Transfections were carried out as described above, except 24-well plates were used (Corning Costar, cat# 3524) and reagents and cells were added in quantities 4-times as in the 96-well plate. Briefly, 800 pg of STING construct and 200 ng of *IFNB1* firefly luciferase reporter construct were co-transfected into 120,000 HEK293T cells in each well.

Western blot was carried out as previously described (13). Briefly, 12 μg of protein were loaded for each sample and transferred to PVDF membranes (Trans-Blot® Turbo™ Midi PVDF Transfer Packs, BIO-RAD, cat# 1704157) with Trans-Blot® Turbo™ Transfer System (BIO-RAD, cat#1704150). STING and ACTB were detected simultaneously by 0.2 μg/mL of anti-STING antibody (R&D Systems, cat# MAB7169-SP) and hFAB™ Rhodamine Anti-Actin Primary Antibody (BIO-RAD, cat# 12004163, 1:1,000 dilution) under chemiluminescent and rhodamine channels, respectively.

Modeling

The side chain of p.H72 residue in the original 4.1 Å resolution of full-length human STING cryo-electron microscopy (Cryo-EM) structure (pdb 6NT5) was flipped and the energy of the structure was minimized by MOE (Molecular Operating Environment) software, resulting in a stereochemically more favorable conformation of p.H72.

RESULTS

Clinical Presentations

All patients presented with peripheral vasculopathy (**Figures 1A–G**), while lung involvement and features of systemic inflammation were more variable between patients. Not all of the patients received immunomodulatory treatment. Patients' characteristics are summarized in **Table 1** and **Supplemental Table 1** and described below; family trees are shown in **Figure 1H**; exon locations of the mutations are shown in **Figure 1I**.

Patient 1 is a 7-year-old girl of French-Canadian origin, who presented at 12 months of age with chronic rhinorrhea and recurrent fever. At 17 months, due to neutropenia, a bone marrow biopsy was performed, showing a hypocellular marrow (20%) with markedly decreased granulopoiesis, mildly decreased megakaryocytes, and mildly increased plasma cells. At the age of 22 months she developed persistent erythematous rashes involving hands, feet, buttocks, and cheeks that were aggravated by cold temperatures. At the age of 2 years she presented with chronic cough, growth retardation and speech delay. Due to persistent neutropenia, she was treated with G-CSF between the age of 27 months and 4 years. Whole Exome Sequencing (WES) at the age of 5 revealed a *de novo* heterozygous *STING1* variant, p.G158A, which is not observed in healthy populations from gnomAD database (https://gnomad.broadinstitute.org), and she was diagnosed with SAVI. She had red-purple lesions on hands and feet, tapered fingers without ulcers. Other features included livedo reticularis, telangiectasias on cold exposed areas, nasal septum perforation and a flat nasal bridge consistent with a saddle nose deformity (**Figures 1A, B, E**).

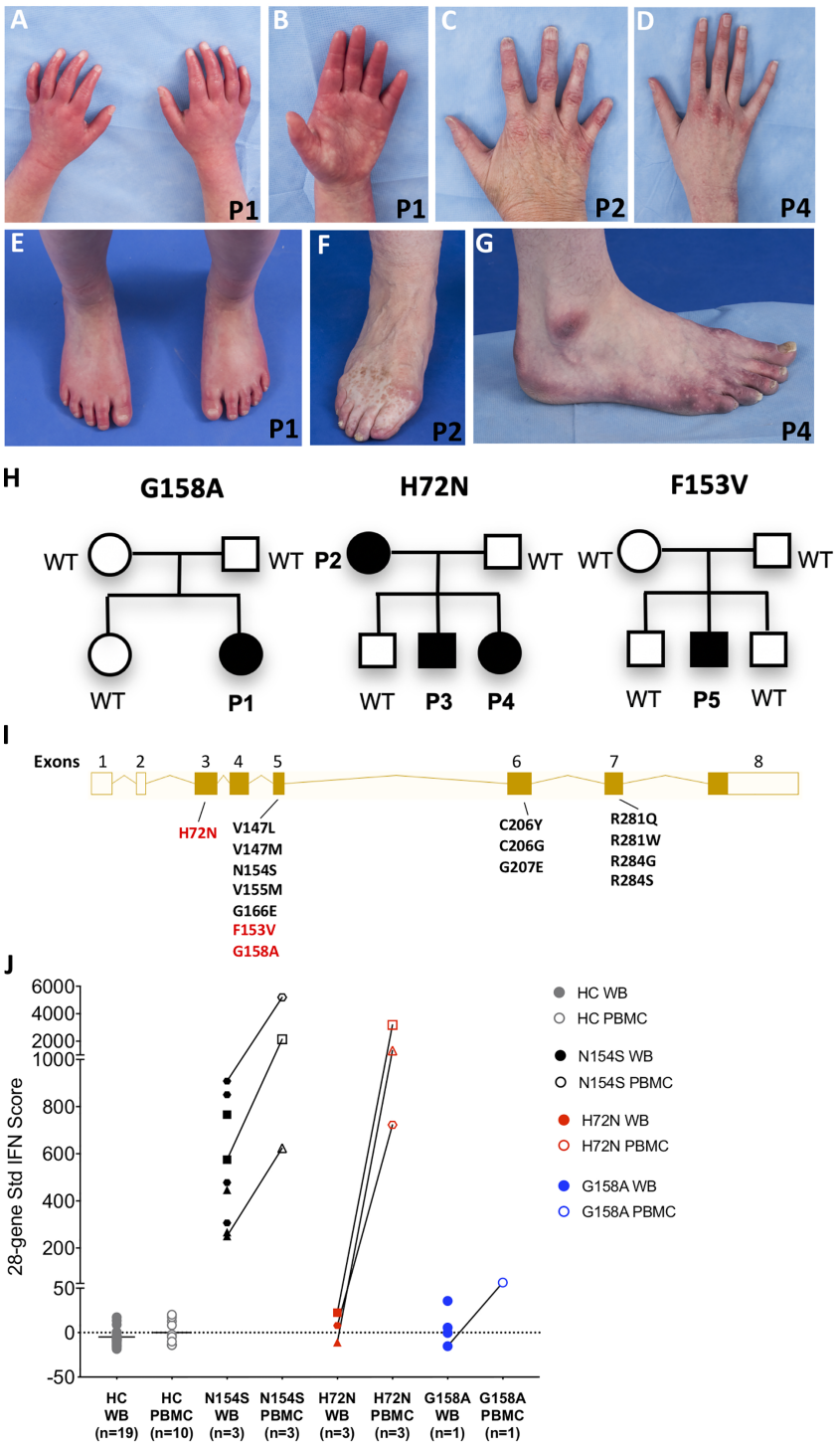

FIGURE 1 | Three novel variants in STING1 cause SAVI. **(A–D)** Erythematous lesions of the hands and tapered fingers in Patient 1 **(A, B)**, Patient 2 **(C)** and Patient 4 **(D)**. **(E–G)** Erythematous rash on the feet and tapered toes in Patient 1 **(E)**, with similar lesions and tissue loss of the feet in Patient 2 **(F)** and Patient 4 **(G)**. Patient number is depicted on the bottom right corner of each picture. **(H)** Pedigree charts. Solid symbols represent affected individuals; open symbols, unaffected individuals. P1-P5: patients, WT: wild type. **(I)** Exon locations of all SAVI-causing STING1 variants. Open boxes represent non-coding exons, solid boxes represent coding exons, while lines represent introns. Previously reported variants are in black and the three novel variants in red. Transcript diagram was obtained from ENSEMBL (www.ensembl.org). **(J)** 28-gene Interferon (IFN) scores from whole blood (WB) and peripheral blood mononuclear cells (PBMCs). Solid symbols indicate WB and open symbols indicate PBMCs. Healthy control (HC) samples included 19 WB and 10 PBMCs. SAVI patients with N154S mutation (n=3) in black, family members with H72N mutation (n=3) in red, and patient with G158A mutation (n=1) in blue. Paired PBMC and WB IFN scores from the same time point are connected by a line. Each patient with SAVI is represented by a different symbol.

TABLE 1 | Patient characteristics.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Gender	F	F	M	F	M
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
***STING1* mutation**	c.473G>C, p.G158A	c.214 C>A, p.H72N	c.214 C>A, p.H72N	c.214 C>A, p.H72N	c.457T>G, p.F153V
	De novo	-	Inherited	Inherited	De novo
Age at onset	12 months	4 years	3 months	2 months	1 year
Age at last evaluation	6 years and 7 months	54 years	21 years	19 years	28 years
Recurrent fever	+	–	–	–	+
Failure to thrive	+	–	–	–	–
Increased inflammatory markers	+	–	–	–	–
Hematologic manifestations	Neutropenia, leukopenia, anemia, thrombocytopenia	–	–	–	Lymphopenia
Positive autoantibodies	ANA, anticardiolipin IgG, anti-thyroid peroxidase	–	–	ANA	–
Increased IFN score (whole blood)	–	–	–	–	Not available
Lung involvement	Mild/Moderate	Mild	–	–	–
Peripheral vasculopathy	+	+	+	+	+
Tissue loss	Nasal septum perforation	–	–	–	–
Arthritis	–	–	–	–	–
Basal ganglia calcifications	Not available	–	Not available	–	Not available
Previous treatment	G-CSF, antibiotics	Sympathectomy	–	–	Diltiazem, nifedipine
Current treatment	Baricitinib	Amlodipine	Amlodipine	Amlodipine, Pentoxifylline	–

ANA, antinuclear antibodies; G-CSF, Granulocyte-colony stimulating factor.

Her weight was on the 8th percentile, height below the 1st percentile with delayed bone age. A chest computed tomography (CT) showed ground glass opacities in the left lower lobe. Her pulmonary function tests (PFTs) showed mildly reduced spirometric lung volumes. She had an increased erythrocyte sedimentation rate (ESR; 44 mm/h), autoimmune hypothyroidism and strongly positive antinuclear antibodies (ANA; 4 EU, normal range 0-0.9 EU); anticardiolipin IgG was equivocal. Baricitinib was started at the age of 5 years and 10 months. Her peripheral vasculopathy remained stable, and on PFTs the forced vital capacity improved from 86% to 95% of the predicted value on baricitinib treatment. She developed mild macrocytic anemia (Hb >10 g/dl), thrombocytopenia (>100.000/µl), and neutropenia (>1.000/µl). At the last evaluation, after 9 months of baricitinib treatment, her ESR was still elevated (37 mm/h), anticardiolipin IgG was negative, while ANA remained positive albeit at lower titers (2.7 EU). Weight had reached the 39th percentile, height the 3rd percentile.

Patient 2 is a 54-year-old Caucasian woman, with a history of chilblains and erythematous skin lesions over ears, nose, and cheeks since the age of 4. At the age of 6, she underwent sympathectomy and was started on calcium channel blockers which improved her symptoms. She also had a history of chronic upper respiratory congestion. Her right fifth finger was amputated after suspected post-traumatic osteomyelitis. Peripheral vasculopathy led to finger and toe tapering and dyschromic skin changes (**Figures 1C, F**). She never recalled fever, lymphadenopathy, hepatosplenomegaly, or respiratory symptoms. WES showed a heterozygous *STING1* variant, p.H72N, which is not observed in healthy populations from gnomAD database. Samples from her parents, who were reported to be healthy, were not available. A chest CT performed at the age of 54 showed reticular ground-glass opacities in the lower lobes, potentially representing scarring due to previous inflammation. Six-minute walking test (6MWT) and PFTs including diffusing capacity for carbon monoxide (DLCO) were normal. Inflammatory markers, complete blood count (CBC), lymphocyte subsets, and serum immunoglobulins were normal. Autoantibodies were negative. Height was normal, weight was above the 97th percentile. She has two sons and one daughter, of whom two harbor the same *STING1* variant (Patient 3 and 4) and also developed SAVI as described below; her son without the mutation is healthy.

Patient 3 is a 21-year-old Caucasian man, who presented during infancy with cold induced skin color changes, and hypothermic fingers and toes with dry skin. He was started on amlodipine with good response. Patient 4 is a 19-year-old Caucasian woman, whose perinatal history was significant for cyanosis with normal oxygenation. At two months of age she presented with chilblains involving feet, hands, nose and ears (**Figures 1D, G**). Cold would also cause numbness in feet, while hot temperatures would worsen rash and pain. She was started on amlodipine with good response and pentoxyphylline was later added. She had a history of a phonological speech disorder and chronic upper respiratory congestion. Both siblings did not develop ulcerations or tissue loss, fever, arthritis, lymphadenopathy, hepatosplenomegaly, or respiratory symptoms. Chest CT, 6MWT and PFTs including DLCO performed at the ages of 21 and 19 respectively were normal. Inflammatory markers, CBC, lymphocyte subsets, and serum immunoglobulins were normal. Autoantibodies including ANA were negative for the brother, but ANA was positive in the sister (2.2 EU). Both siblings had normal height and weight.

Patient 5 is a 28-year-old Caucasian man, who presented from the age of one year with telangiectasias and chilblains lesions of nose, cheeks, hands and feet during winter months.

Capillaroscopy revealed capillary dilation and tortuosity. Episodes of recurrent fevers started at age 26 without apparent triggers. His neurodevelopment was normal. He never developed failure to thrive, and denied arthritis, lymphadenopathy, hepatosplenomegaly, or respiratory symptoms. A chest CT performed at age 27 was normal, a CBC revealed lymphopenia. Inflammatory markers were normal and autoantibodies (including ANA, anti-dsDNA, antibodies to extractable nuclear antigens and antineutrophil cytoplasmic antibodies) were negative, serum immunoglobulins were normal. Genetic testing showed a heterozygous *STING1* variant. p.F153V, which is not observed in healthy populations from gnomAD database. He is treated with calcium channel blockers.

Interferon Signature in Mononuclear Cells but Not Whole Blood

Most SAVI patients present with an elevated interferon response gene signature recognized as elevated interferon scores (14). As expected, interferon scores from peripheral blood mononuclear cell (PBMC) samples of patient 1-4 are much higher than those of healthy controls (**Figure 1J**); samples from patient 5 were not available. Notably, interferon scores from the whole blood samples were negative, which is different from previously reported SAVI mutants including p.N154S (**Figure 1J**). The mechanism remains unclear. Further investigation is needed to assess the interferon scores in various cell populations of the blood, and in patients with the same mutation from different kindreds that may become available in the future.

p.H72N, p.G158A, p.F153V Variants Lead to STING Autoactivation

To assess the autoactivating potential of the 3 novel variants, constructs expressing mutant or wild type (WT) STING were transfected into HEK293T cells and *IFNB1* firefly luciferase reporter activation was measured (**Figure 2A**). All 3 mutant constructs showed autoactivation in the absence of ligand (**Figure 2A**). The autoactivation was not due to higher expression of the mutant proteins compared to the WT (**Supplementary Figure S1**). The p.F153V and p.H72N mutations remained responsive to ligand stimulation with cGAMP, while the p.G158A mutant was maximally activated at baseline and was not further enhanced by cGAMP stimulation. The constitutive auto-activation of p.G158A was even higher than WT construct activated with 20 μg/mL of cGAMP ligand (**Figure 2A**).

The p.G158A mutation in the connector helix loop increased STING activation more than 13-fold, which is comparable to two other "highly-autoactivating" mutations, p.R284S and p.R284G, in the polymer interface (**Figure 2A**, see also **Figure 2B** for the residue location). Their activation mechanism differs and was previously described by different models (12, 15). The p.G158A mutation is predicted by a rotation model to highly mimic the ligand-bound form of STING thus conferring autoactivation (12). The model suggests that cGAMP binding on wildtype STING triggers a 180° rotation of the ligand binding "head" of the STING dimer, which allows a closed dimer conformation, "flattening" of the polymer interface and STING polymerization *via* side-by-side packing (12). The STING oligomers recruit TBK1 and IRF3 which activate downstream signaling (16). Consistent with this model, the p.G158 residue that is mutated in patient 1 is situated in a closely packed location of the apo STING dimer, where only Glycine, the smallest residue, can be accommodated (12) (**Figure 2B**). Mutation to the larger Alanine results in "space limitation" that is predicted by the model to "force" a 180° rotation of the ligand-binding head, which mimics the ligand-bound STING conformation thus conferring autoactivation.

The p.R284S and p.R284G highly-autoactivating mutations, however, are not likely to trigger a rotation and are better explained by a polymer interface blocking model (15). This model suggests that STING is kept inactive *via* C-terminal tail binding to the polymer interface, which blocks polymerization and prevents autoactivation. As a result, mutating the polymer interface residues to various residues lead to broad autoactivation (13, 15). However, neither model explains STING autoactivation caused by the p.H72N and p.F153V mutations; both are unlikely to "push" a 180° rotation and are not located in the polymer interface.

The Variant p.H72N Identifies a Novel Class of SAVI-Causing Mutations That Are Located in the Transmembrane Linker Domain

While the previously reported SAVI-causing mutations are distributed around the dimer crossing-over region, the disease-causing p.H72N variant identified in patients 2-4 is the first SAVI-causing mutation in the transmembrane linker region of STING (**Figure 2B**) that, together with the N-terminal tail, forms a supporting arm which restrains the ligand-binding domain (12) (**Figure 2B**). However, neighboring mutations in the transmembrane linker region, p.E68A and p.E69A, resulted in loss-of-function (12) which raised the question how the p.H72N mutation would confer a gain-of-function.

To interrogate how residue p.H72 might control STING activation, we generated various mutant constructs at the p.H72 position and assessed their autoactivating potential and their response to cGAMP stimulation in activating interferon activity (**Figure 2A**). Mutations to charged residues, including Lysine (K), Arginine (R), or Aspartic acid (D), led to a complete loss-of-function with no autoactivation and loss of a cGAMP response (**Figure 2A**). Mutations to the hydrophobic residue Leucine (L) resulted in a dramatic reduction in the cGAMP response. In contrast, mutations to residues with uncharged polar side chains, including Glutamine (Q) or Phenylalanine (F), resulted in responses similar to wildtype construct, with a maintained dose dependent cGAMP response. These data suggest that the charged and hydrophobic residues may result in improper STING dimer formation, while residues with uncharged polar side chains may maintain the wildtype configuration of the dimer.

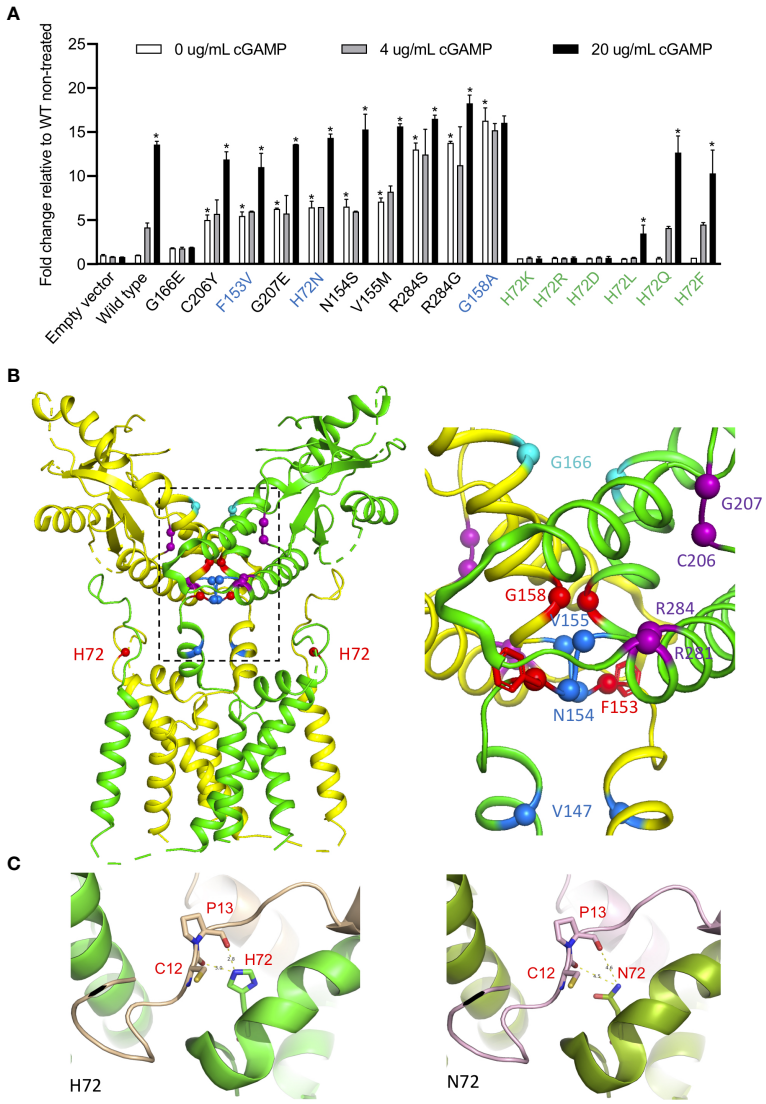

FIGURE 2 | H72N, G158A, F153V variants lead to autoactivation. **(A)** Novel STING variants lead to strong STING autoactivation as measured by *IFNB1* reporter activation in 293T cells. 50 pg of various STING constructs were transfected. The three novel SAVI-causing variants are labeled in blue, and other H72 variants are labeled in green. The SAVI-causing variants are shown in the order of autoactivating potential. *An *asterisk* above the *white bar* designating 0 μg/ml cGAMP indicates significant change compared ($p<0.0001$) to wildtype (WT) non-treated, by ordinary one-way ANOVA Dunnett's multiple comparisons tests. An *asterisk* above the *black bar* designating 20 μg/ml cGAMP indicates significant change ($p<0.003$) of 20 μg/ml cGAMP treated cells compared to the respective non-treated cells; by two-way ANOVA, Bonferroni's multiple comparisons test. Data are presented as mean±SD of duplicates. Similar results were obtained in independent experiments. **(B)** Ribbon structural model of apo STING dimer, with the two subunits colored in yellow and green. Location of SAVI-causing variants are highlighted with spheres, with the three novel variants in red, variants in the connector helix loop region in blue, p.G166E variants in the ligand-binding pocket in cyan, and variants in the polymer interface in purple. The major mutation region is enlarged on the right, showing how the F153 phenyl ring buttress the polymer interface. **(C)** Structure model of H72 compared to N72 indicates increased dimer flexibility of p.H72N mutation, which confers gain-of-function. The two STING monomers forming a dimer are in green and gold/purple respectively.

Structural Modeling of p.H72N Suggests a Refined Model of STING Activation

We hypothesized that residues with uncharged polar side chains at position p.H72 may maintain the "supporting arm" structure to restrain the ligand-binding head, and we generated structural models to investigate the effect of the mutation on its interactions with neighboring residues (**Figure 2C**). Structural modeling of the domain in fact confirms that residue p.H72 is essential in forming hydrogen bonds with residues 12 and 13 on the N-terminal tail of the second STING monomer within the STING dimer (**Figure 2C**, left), which stabilizes a "supporting arm". If mutated to Asparagine (N), these hydrogen bonds cannot form firmly (**Figure 2C**, right), which would result in a "looser support" of the ligand binding head and increased head

flexibility. The polymer interface, which is on the outer surface of the ligand binding head, would also become more flexible, which attenuates its binding to the C-terminal tail (15) thus conferring the autoactivation.

These findings highlight an essential role of hydrogen bonds at the p.H72 location: consistent with our observations, only polar residues can stabilize the supporting arm *via* hydrogen bonds, while both charged and hydrophobic residues fail to form hydrogen bonds and results in loss-of-function (**Figure 2A**). The autoactivation *via* a 180° rotation is energy consuming and although likely favored by the p.G158A mutation that would "force" the rotation due to space limitations, it is not a likely mechanism in patients with the p.H72N mutation, which may only allow random rotations that are inefficient in causing autoactivation.

In fact, rotation of the ligand-binding head is also an unlikely consequence of the highly-autoactivating polymer interface mutations (13) (**Figure 2A**), as these mutations are located in the dimer surface and should not cause a rotation of the ligand-biding head (**Figures 2A, B**, purple labeled). These findings are in concordance with a structural study of bacterial cyclic-di-GMP (CDG)-bound STING, which revealed that CDG can activate STING without a rotation and a STING dimer closing (15). Furthermore, STING binding to TBK1 is quite flexible through its unordered C-terminal tail, which was predicted to occur even in an open dimer conformation (16).

A presumed "small protrusion" in the polymer interface of unrotated STING was thought to be sufficient to keep STING inactive (12). However, the aforementioned examples of STING activation in an "unrotated state" (with the protrusion still present) raised questions about the exclusive validity of that model and led to the proposal of an inhibitor that blocked STING polymerization (13). In fact, the C-terminal tail binding can serve that function (15) and no other inhibitor protein is needed, as autoinhibitory STING dimers can be formed in a cryo-EM structure where only STING is present (12).

These observations led to the development of an adjusted model of STING activation that accommodates all currently reported STING-causing mutations in exons 3, 5, 6 and 7 (13). We hypothesize that STING is kept inactive by the C-terminal tail blocking of the polymer interface, which prevents polymerization *via* side-by-side packing (**Figure 3A**). Ligand binding induces 180° rotation of the ligand binding domain and leads to conformational change in the polymer interface, which results in loss of C-terminal tail binding. This clears the polymer interface and allows polymer formation *via* side-by-side packing (**Figure 3A**).

In this reconciled model, SAVI-causing mutations can be categorized into 4 classes. The p.G158A variant and possibly the p.N154S and p.V155M variants, which are all located in the tightly packed connector region, mimic the ligand-bound STING dimer by favoring a "rotated" conformation (**Figure 3B**). Mutations in the polymer interface at residues R281, R284, C206, and G207, constitute the second class, which directly abolishes binding to the autoinhibitory C-terminal tail and allows side-by-side packing without a 180° rotation (**Figure 3C**); p.H72N, p.F153V, p.V147L, and p.V147M constitute a third class which relieve the constraint on the polymer interface and autoactivate without a rotation. Residues H72 and V147 are part of the supporting arm which stabilizes the ligand-binding head and preserves the C-terminal tail binding to the polymer interface. Residue p.F153 lies right beneath the polymer interface to constrain it and prevents STING polymerization (12) (**Figure 2B**). A residue with reduced size such as the phenylalanine (F) to Valine (V) (p.F153V) mutation would be predicted to increase the flexibility of the polymer interface, thus relieving the autoinhibition by loosening the C-terminal tail binding. The p.G166E located in the ligand binding pocket constitutes class 4. This mutation confers a very low level of autoactivation and also abolishes cGAMP responsiveness (**Figure 2A**). Its autoactivating potential is not explained by current models, and it is not clear whether this low autoactivation causes SAVI. It is possible that binding of a non-cGAMP ligand is needed to activate STING and cause the disease.

Novel Mutations Are Associated With Milder Disease

Patients with mutations that were of lower auto-activating potential p.G166E, p.F153V, p.H72N (**Figure 2A**), showed milder disease with less severe or no lung manifestations, which is in contrast to the p.N154S, p.V155M mutations. However, the patient with the highly-autoactivating mutation, p.G158A, showed an intermediate phenotype, less severe than that of patients who develop interstitial lung disease and peripheral tissue loss including autoamputations. Her clinical picture was characterized by peripheral vasculopathy with nasal septal perforation, mild to moderate lung involvement, and hematologic manifestations (neutropenia). These findings indicate that disease severity in SAVI is influenced by additional factors that include family genetic backgrounds, treatment histories and yet unknown environmental factors such as infections which all may contribute to the variable disease severity which is even seen within the same family (6). Additional studies of patients from different families with the same mutations would help to address this question.

DISCUSSION

We have identified three novel SAVI-causing mutations in critical locations of STING. All patients had more prominent peripheral disease with mild or no lung disease. The p.H72N mutation is the first SAVI-causing mutation discovered in the transmembrane linker region, and the other two mutations, p.F153V and p.G158A, had been predicted to be autoactivating by Cryo-EM structures (12). The typical clinical phenotype and the autoactivation of these mutations in the *IFNB1* luciferase reporter assay coupled with the prediction of the autoactivating potential of these mutations in Cryo-EM structural studies and their absence in publicly available databases including gnomAD database confirms that they are disease-causing. Future identification of more patients from different families would help better address the variable disease severity caused by the respective mutations. Future studies of interferon-dependent and

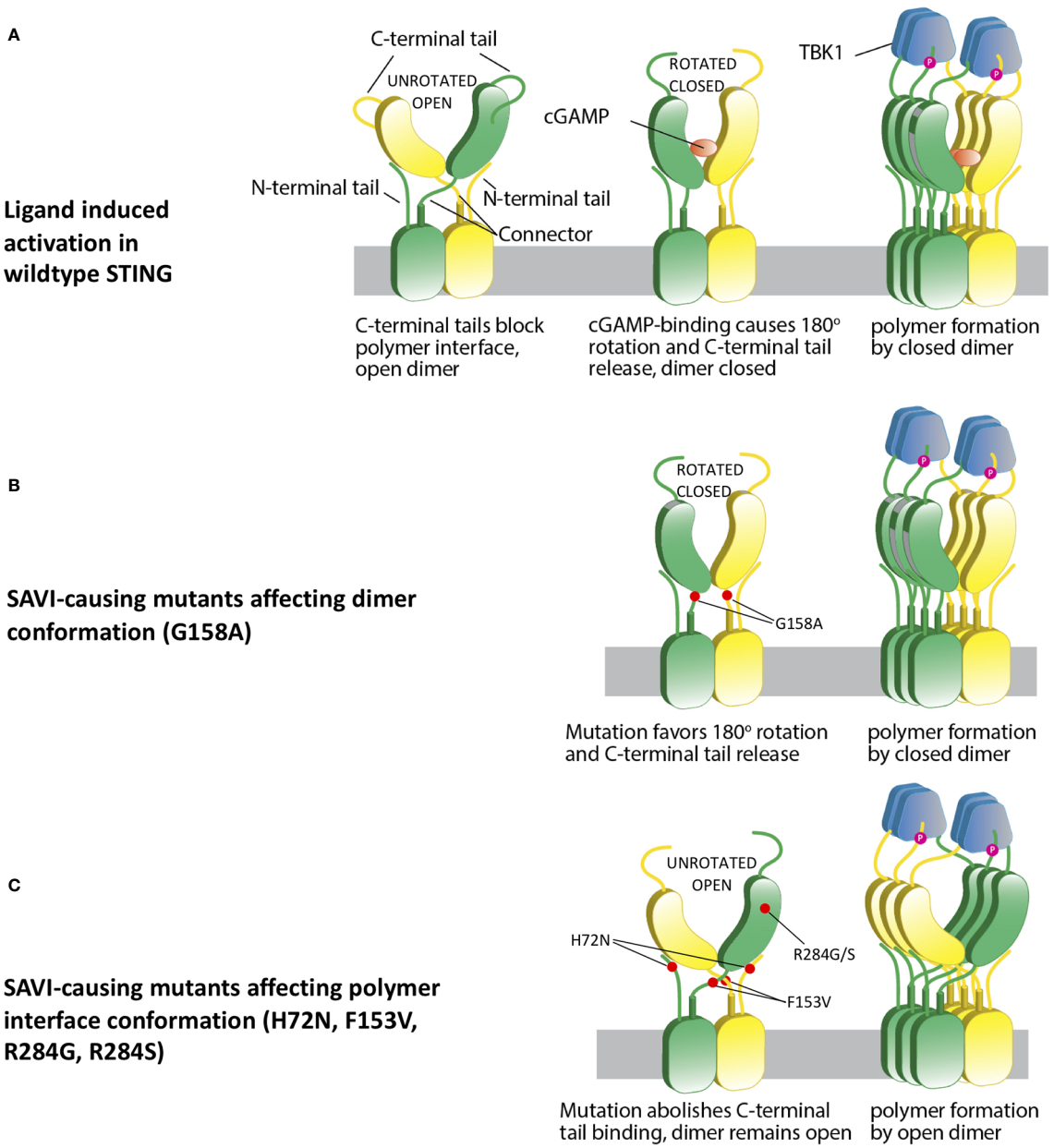

FIGURE 3 | A hypothetical STING activation model reconciles current structural models. **(A)** Wild type apo STING forms a dimer in open conformation, with C-terminal tail blocking the polymer interface and preventing polymerization. cGAMP binding leads to a 180° rotation and a closed dimer, which also changes the polymer interface conformation and subsequently loses C-terminal tail blocking. This leads to STING polymerization *via* side-by-side packing and TBK1 recruitment. **(B)** SAVI mutant G158A "pushes" the formation of a closed dimer, which mimics ligand-bound STING and confers autoactivation in the absence of ligand binding. **(C)** Some other SAVI mutants like R284G, R284S, H72N, F153V directly affect the polymer interface conformation, which leads to release of C-terminal tail binding. The STING dimer remains in the open state, but the polymer interfaces are free of C-terminal tail blocking, which allows polymerization and autoactivation.

-independent functions of STING that may contribute to the disease phenotype are also planned (17–21).

The p.H72N and p.F153V mutations, however, are not fully explained by a model that requires rotation of the ligand-binding head for downstream activation (12), as both are unlikely to force a rotation and form a closed dimer. This is similar to the highly-autoactivating mutations in the polymer interface, which were explained by another model (15). Our data suggest a reconciled model that allows both open and closed STING dimers to form polymers and lead to STING activation, as long as the polymer interface is cleared of the C-terminal tail binding which blocks oligomerization of the STING dimers.

Although our findings are limited by our inability to generate a cryo-EM model, which was outside the scope of our work, these

findings have important implications for the use and design of drugs that target STING for the treatment of SAVI patients and likely other diseases that may activate the cGAS-STING pathway. As described in **Figure 3**, successful polymerization is the most critical step for autoactivation and is required by all SAVI-causing mutations, suggesting that the polymer interface should be the preferred drug target site to attenuate STING autoactivation.

We have recommended JAK inhibitors for SAVI patients and have reported on their partial responses, particularly on the stabilization of the lung disease (22). Interestingly, Patients 1 and 4 were treated with baricitinib for 2-3 months with only marginal benefit to their peripheral vascular disease, and both discontinued treatments. Thus, the risks and benefits in patients with mild disease have to be considered carefully.

In summary, the novel mutations expand the phenotypic presentation of SAVI by including clinical presentations with mild or no lung involvement and propose novel mechanisms of STING activation. Our findings led to a refined model of STING activation, which will inform the development of drugs that target STING activation in patients with SAVI and other STING-dependent inflammatory diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of National Institutes of Health. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization: BL, AJ, and RG-M. Experiments: DK, BL, JM, AJ, and AA. Patient data collection: ST, DR, BW, MM-C, LS, SA, AJ, and RG-M. Structural modeling: ZJ and TJ. Writing of initial draft: BL, ST, and AJ. Writing – review and editing: BL and RG-M. All authors contributed to the article and approved the submitted version.

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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.2021.636225/full#supplementary-material

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Glucocorticoids as Regulators of Macrophage-Mediated Tissue Homeostasis

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Our immune system has evolved as a complex network of cells and tissues tasked with maintaining host homeostasis. This is evident during the inflammatory responses elicited during a microbial infection or traumatic tissue damage. These responses seek to eliminate foreign material or restore tissue integrity. Even during periods without explicit disturbances, the immune system plays prominent roles in tissue homeostasis. Perhaps one of the most studied cells in this regard is the macrophage. Tissue-resident macrophages are a heterogenous group of sensory cells that respond to a variety of environmental cues and are essential for organ function. Endogenously produced glucocorticoid hormones connect external environmental stress signals with the function of many cell types, producing profound changes in immune cells, including macrophages. Here, we review the current literature which demonstrates specific effects of glucocorticoids in several organ systems. We propose that tissue-resident macrophages, through glucocorticoid signaling, may play an underappreciated role as regulators of organ homeostasis.

Keywords: glucocorticoids, macrophages, homeostasis, glucocorticoid receptor, inflammation

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INTRODUCTION

For over 70 years, synthetic glucocorticoids have been used to treat numerous inflammatory conditions, including allergies, asthma, autoimmune diseases, sepsis, and cancer. This is partially due to their profound ability to modulate the immune response through anti-inflammatory and immunosuppressive mechanisms. Endogenous glucocorticoids (such as cortisol in humans and corticosterone in mice) are a class of adrenal cortex steroid hormones regulated through the hypothalamic-pituitary-adrenal axis. They are produced in response to stresses such as infection, but are also naturally secreted in circadian and ultradian cycles. Cortisol acts as a biochemical signaling molecule and is involved in numerous metabolic processes in the body. However, cortisol deficiency in the body leads to an exacerbated inflammatory response. Furthermore it is well recognized that the serum level of cortisol in the body is decreased in the elderly.

Both endogenous and synthetic glucocorticoids (dexamethasone and prednisone, among others) exert their therapeutic effects primarily through the glucocorticoid receptor (encoded by NR3C1, hereafter GR), and their efficacy in controlling inflammatory conditions results from the pleiotropic effects of the GR signaling pathways (1). GR is a member of the nuclear receptor superfamily and is a

ligand-dependent transcription factor. It is expressed ubiquitously in almost every human cell, including all immune cells (2). When bound by its ligands, GR translocates to the nucleus and occupies specific palindromic DNA sequences within the open chromatin, called glucocorticoid response elements (GREs), to activate or repress gene expression (3–8). GR activates gene expression through different mechanisms that involve direct binding of dimers to GREs within GR-binding sites or composite binding in which GR and another transcription factor interact with distinct response elements within the same genome location. For example, glucocorticoids enhance phosphoenolpyruvate carboxykinase gene expression through GR and CREB binding to GREs and a cyclic AMP response element, respectively, within close proximity (9).

Glucocorticoid-mediated gene repression or GR transrepression occurs through direct binding of GR to repressive DNA motifs (negative glucocorticoid response elements or nGREs) (10, 11) or tethered recruitment of ligand-bound GR to another transcription factor without DNA interaction. Tethering is likely the most studied mechanism for immune regulation by glucocorticoids. Many studies have linked this mechanism to the beneficial anti-inflammatory actions of glucocorticoids (12, 13). Tethering occurs when GR binds to another transcription factor without interacting with DNA. GR has been shown to tether key pro-inflammatory transcription factors, including nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), which antagonizes their interaction with chromatin, influences the recruitment of co-regulators, and results in gene expression inhibition. Remarkably, using genome-wide profiling in LPS activated macrophages upon Dex treatment, Uhlenhaut et al. found that 20% of GR-dependent repression is related to nGREs and tethered sites, suggesting that the positive and negative GR cistromes are predominantly composed of classical GREs in close proximity to NF-kB and AP-1 binding sites (6).

Interestingly, GR^{dim} mice carrying an amino acid substitution (A465T) in the D-loop of the DNA-binding domain of GR showed reduced, but not completely absent, transactivation ability in response to glucocorticoids (14), suggesting that the GR dimerization-dependent gene regulation was not essential for the effects of GCs. Direct binding of GR as monomers also has been described (5, 15). Initially, the mechanism of transrepression proposed that the monomeric state of GR repress the transcription by tethering to DNA-bound TFs (10, 11). Using mouse liver from WT and GR^{dim} under endogenous corticosterone exposure and chromatin immunoprecipitation with lambda exonuclease digestion and sequencing (ChIPexo), Lim HW et al., reported that monomeric GR interaction with a half-site motif is more prevalent than homodimer binding (5). This monomeric GR interaction with a half-site motif display greater cell-type specificity and enrichment for lineage-determining TFs relative to dimer sites. These data arguing in favor of a model termed half-site-facilitated tethering, where sequence-specific interaction of GR monomers to different motifs promotes transient contacts between monomers and nearby TFs (5).

The GR has been previously reported to modify chromatin structure as well (16–18). New evidence establish that glucocorticoids exert primary repressive effects on transcription through altering chromatin structure (18). For example, using a global run-on sequencing or GRO-seq, Sasse et al., demonstrated that the repression of many TNF-regulated genes and enhancers by dex treatment rapidly changes the chromatin structure in a process that does not required GR occupancy (18). This evidence suggest that either a transrepressive or nGRE mechanisms on the NF-kB signaling are not implicated. Moreover, the high resolution given by GRO-seq also allowed to discover a secondary anti-inflammatory effects resulting from transcriptional cooperation between GR and NF-kB at a subset of regulatory regions (18). This cooperative glucocorticoid-TNF crosstalk in the repression of inflammatory processes previously was observed by Vettorazzi et al. in a model of acute lung inflammation (7), where Dex and pro-inflammatory stimuli in macrophages, synergistically *via* GR increased sphingosine1-phosphate (SphK1) expression and the levels of S1P circulating that play a role in attenuating lung inflammation. These data provide evidence that reducing the expression of pro-inflammatory cytokines, a classic feature of glucocorticoids treatment, is not sufficient to resolve the inflammation.

Glucocorticoids do not only antagonize proinflammatory gene expression. They have recently been shown to induce proinflammatory gene expression in several cell types, including macrophages (19–21). For example, dexamethasone upregulated expression of the NLRP3 inflammasome in human THP-1 macrophages, causing them to be more responsive to the NLRP3 agonist ATP (19). In addition, co-regulation of genes by glucocorticoids and cytokines has been demonstrated in which glucocorticoids and cytokines synergize to enhance proinflammatory mediator production (20). Finally, GR-mediated induction of exopeptidase DPP4 contributed to the increased mobility of macrophages in response to dexamethasone (21). However, the extent of this co-regulation and its mechanism in immune cells is poorly understood.

Macrophages are innate immune system effector cells which, upon inflammation, phagocytose apoptotic and necrotic cells. They are involved in tissue repair and modulate inflammation by balancing pro- and anti-inflammatory responses. Interestingly, glucocorticoids seem to have limited efficacy in the control of inflammation in diseases related to macrophage activity, such as, atherosclerosis, ulcerative colitis and respiratory tract diseases (22, 23). While it is true that many of the diseases mentioned above are quite successfully controlled by corticosteroids treatment, this has been associated to early stages of the diseases because they are able to inhibit many components of the inflammatory response. Even in the clinical management of some of them, the use of corticoisteroids has been recommended as adjunct treatment at the lowest dose possible and for the shortest time possible. Although glucocorticoids induce cell death and reduce cell survival in immune cells such as T and B cells, macrophages are relatively resistant to glucocorticoid-induced apoptosis (2). These observations support the idea that the pro-inflammatory versus the anti-inflammatory regulatory actions of glucocorticoids may be predominant in macrophages.

The macrophage ontogeny has been challenged during the last two decades. The paradigm that tissue-resident macrophages

are continuously replenished by blood-circulating monocytes, which arose from bone marrow (BM)-derived precursors was updated since Merad et al, showed that Langerhans cells, a kind of macrophages in the skin, were resistant to the irradiation and were not derived from donor after congenic BM transplanstation (24). The current models of macrophage ontogeny have been established through genetic fate-mapping techniques. For example, now is well-known that major tissue-resident macrophage populations, including microglia, liver Kupffer cells, lung alveolar macrophages, epidermal Langerhans cells and splenic macrophages, are established during the embryogenesis from the yolk sac (YS) anf fetal liver and subsequently maintain themselves independently of replenishment by blood monocytes during adulthood (25, 26). Contrary, macrophages population from the gut and heart are constantly replenished by blood monocytes postnatal (27, 28). In the new era of "omics" techniques, single-cell RNA-sequencing have revealed a next level of complexity to the functional heterogeneity of the embryonic origin of key tissue-resident macrophage populations. For example, depth analysis of arterial macrophages at single-cell resolution in steady state and in response to angiotensin-II (AngII)-induced arterial inflammation revealed dual origin of arterial macrophages from both YS and BM-hematopoiesis, a process that is stable in adult mice, but declines in numbers during ageing and is not replenished by bone marrow (BM)-derived macrophages (29). In AngII inflammation, BM-derived macrophages invade the inflamed adventitial tissue, while resident -YS erythromyeloid progenitors (EMP)-derived macrophages- were self-renewal and proliferate locally providing a distinct transcriptional profile linked to tissue regeneration (29). Despite the fact that our understanding of ontogeny of macrophages is increasing, the precise developmental trajectories of tissue-resident macrophages remain undetermined.

Another level of complexity into the macrophages biology is given by the activation or polarization processes. Macrophages are polarized according to changes in their environment and are classically divided in two main categories, M1 macrophages and M2 macrophages (30). M1 macrophages are mainly involved in pro-inflammatory responses, classically generated upon induction by microbial products, such as LPS and pectidoglycan and pro-inflammatory cytokines such as interferon-gamma. M2 macrophages are mainly involved in anti-inflammatory responses, ultimately associated with promoting wound healing, tissue repair and for resolving inflammation (31, 32). Glucocorticoids have been related to a M2-like phenotype, where the capacity to promote tissue repair and wound healing has been demonstrated (33–35). However, the direct participation of GR in the polarization still are not as well-understood.

Macrophages play a critical role in determining the extent of our body's inflammatory response. However, macrophage function becomes impaired with increasing age and this could be linked to an imbalance between the amount of cortisol generated and the increase in the quantities of pro-inflammatory molecules produced in the body. Recently, has been proposed that low levels of the stress hormone cortisol and loss of the glucocorticoid-induced leucine zipper (GILZ) expression in macrophages can trigger chronic inflammatory responses in the body, contributing to the aging process (36).

Here, we review mechanisms whereby glucocorticoids can regulate physiological tissue homeostasis through the macrophage as a sensor, with emphasis on tissues where glucocorticoid signaling has been ablated using specific GR knockout mouse models. We propose that the pro-inflammatory or positive gene regulatory actions of glucocorticoids on macrophages may be a way in which macrophages shape the physiology of tissues.

GLUCOCORTICOIDS IN THE IMMUNE-SURVEILLANCE OF THE HEART

Glucocorticoid signaling has direct effects during cardiac development and in both physiological and pathological conditions of the cardiovascular system. Multiple studies have revealed an important role for circulating glucocorticoids in the regulation of heart function and in impaired infarct healing, but they have not discriminated between direct and systemic actions of these hormones (37). By generating mice lacking GR expression solely in heart tissue (the cardiomyocyte-specific GR knockout or cardioGRKO), our group found that mice died prematurely from pathological cardiac hypertrophy that progressed to dilated cardiomyopathy and heart failure (38). It is established that endogenous glucocorticoids can also signal through the closely related mineralocorticoid receptor (encoded by Nr3c2, hereafter MR). For example, Oakley et al. generated mice lacking both GR and MR in cardiomyocytes which were resistant to cardiac disease in comparison to cardioGRKOs (39). Interestingly, these findings suggest that an appropriate amount of glucocorticoid signaling through both GR and MR in cardiomyocytes is critical for maintaining a healthy heart.

Heart failure is one of the leading causes of morbidity and mortality. It is recognized that innate immune cell activation occurs in patients with heart failure. This activation is associated with adverse clinical outcomes for disease progression. While it is accepted that neutrophils produce robust inflammatory responses and contribute to heart damage after acute ischemic injury, macrophages improve healing and cardiac remodeling after injury by promoting neutrophil efferocytosis, suppressing free radical formation, and modulating fibroblast activation state; however, the exact roles played by macrophages continue to be explored and defined (40–42). Paradoxically, macrophages can also trigger a damaging inflammatory response, which was shown in a zebrafish model where macrophages directly contributed to fibrosis during heart repair (43).

It has been suggested that distinct macrophage populations, such as resident or recruited subsets, may favor healing of injured areas or promote inflammatory and reparative functions (44). In the heart, tissue-resident macrophages populate different regions, including the ventricular myocardium, where they are found throughout myocardial interstitial spaces and interact directly with capillary endothelial cells and cardiomyocytes (45). They are also found in the atrioventricular node, where they facilitate electrical conduction by coupling to cardiomyocytes through

connexin 43-containing gap junctions (46). Recently, Nicolas-Avila et al, demonstrated that macrophages can clean up dysfunctional mitochondria from cardiomyocytes, helping to maintain cardiac health and homeostasis (47). These data suggest broader homeostatic functions for heart resident macrophages; therefore, macrophages are an emerging target for therapeutic strategies aimed at minimizing cardiomyocyte death, ameliorating pathological cardiac remodeling, and treating heart failure after myocardial infarction.

Glucocorticoids play key roles in the regulation of macrophage homeostatic functions and in their functional properties to resolve inflammation and tissue damage (22). The loss of glucocorticoid-mediated regulation of macrophage function in the heart could result in the dysregulation of factors that control inflammation, neovascularization, collagen degradation, and scar tissue formation. In a model of myocardial infarction, mice lacking GR in myeloid cells under control of lysozyme M locus (LysM) promoter die earlier after infarction than wild type controls. GR-deficient macrophages were shown to exacerbate cardiac remodeling and to cause impairment of collagen scar formation and angiogenic response to ischemic injury, resulting in dysregulation of the resolution of inflammation and defects in wound healing (34).

Finally, the newly discovered macrophage function related to the active elimination of cardiomyocyte-derived mitochondria through the phagocytic receptor Mer tyrosine kinase (Mertk) (47) reinforces the idea that glucocorticoids contribute to cardiac tissue homeostasis (**Figure 1**). It's established that glucocorticoids upregulate Mertk expression in macrophages (48) and promote the phagocytosis of apoptotic neutrophils (49). The clearance of apoptotic cells and dysfunctional mitochondria by macrophages ensures mitochondrial and cardiomyocyte fitness, tissue proteostasis, and cardiac function. Therefore, glucocorticoids acting through macrophages could determine the balance between cardiac immunity and tolerance. Failure of this mechanism caused by defects in cardiac macrophage sensing of glucocorticoids, rather than from age related impairment of cardiomyocytes, could compromise cardiac homeostasis and promote heart disease.

GLUCOCORTICOIDS IN THE IMMUNE-SURVEILLANCE OF THE CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) is a prominent target of glucocorticoids because GR is ubiquitously expressed in neurons, glial cells (such as astrocytes, oligodendrocytes and microglia) (50–53) in addition to brain vasculature (54). Moreover, differential expression of the glucocorticoid receptor has been described in specific subregions of the human cortex such as the basolateral amygdala, CA1 hippocampus and dentate gyrus. Glucocorticoid effects on the brain are related to the adaptation to stress. They primarily depend on GR distribution and functional pattern, and vary with gender, age, hormone concentrations, timing, and duration of exposure (55). There is compelling evidence for direct GC effects on behavior, cognition and mood (56, 57). At the cellular level, glucocorticoids are necessary for neuronal growth and differentiation. They also have an impact on several neuronal functions, including cell survival, integrity, and synaptic plasticity (58, 59). In humans and rodents, it has been described that glucocorticoids play a role in both embryonic and adult neurogenesis (60). Similar to embryonic development, neurogenesis in the adult happens in the hippocampus and involves a multi-step process starting with the division of neural stem cells and subsequent maturation into neural progenitor cells, proliferation of progenitor cells, maturation, morphological changes, migration, physiological adaptation, and functional integration into the hippocampal network (61). Newly generated neurons in the hippocampus contribute to learning and memory (62), forgetting (63) and cognitive flexibility (64). Interestingly, chronically elevated glucocorticoid levels under prolonged exposure to stress has been related to changes in the hippocampal cytoarchitecture, such as atrophy of dendritic processes and inhibition of neurogenesis (65, 66). Hippocampal neurons also play an essential role in the negative feedback regulation of the HPA-axis (67). Consequently, impaired hippocampal neurogenesis is closely associated with brain disorders and neurodegeneration by disrupted hypothalamic-pituitary axis functions. Interestingly, Quarta C., et al. (68), developed a tissue-specific anti-inflammatory drug that conjugate glucagon-like peptide-1 (GLP-1) to dexamethasone (GLP-1/Dexa) to selectively delivers dexamethasone to GLP-1 receptor (GLP-1R)-expressing cells (68). They showed that GLP-1/Dexa ameliorates the diet-induced systemic inflammation and does not induce negative effects on HPA-axis activity however, they do not deepen into the identity of the GLP-1R-expressing cells (68). Some studies have shown that macrophages could be one of the cells responding to the drug because they also express GLP-1R (69, 70). In addition, we recently published that dexamethasone induced the expression of the exopeptidase DPP4 that is recognized as one of the most important inactivator of GLP1 (21).

Glucocorticoids also play a crucial role in regulation of the immune system and intermediate metabolism within the brain. By inhibiting the immune system, glucocorticoids prevent overproduction of inflammatory molecules that can be harmful to neurons. The most notable CNS immune cells affected by glucocorticoids are the microglia. As resident macrophages of the nervous system, microglia are the brain's professional phagocytes that sense and coordinate the brain inflammatory response. Microglia are the predominant immune cells of the CNS, comprising approximately 10–12% of the cells in the brain, with higher numbers within the hippocampus (71). Microglia normally exist in a quiescent or "resting" state in the healthy adult brain and, in response to tissue injury or disease, can transform rapidly from a quiescent state to different activation states (72). They are highly motile cells that survey the local environment and release cytokines that coordinate the response of both innate and adaptive immunity to control infection, remove cell debris and promote tissue repair (73). Upon activation, microglia upregulate cell surface molecules

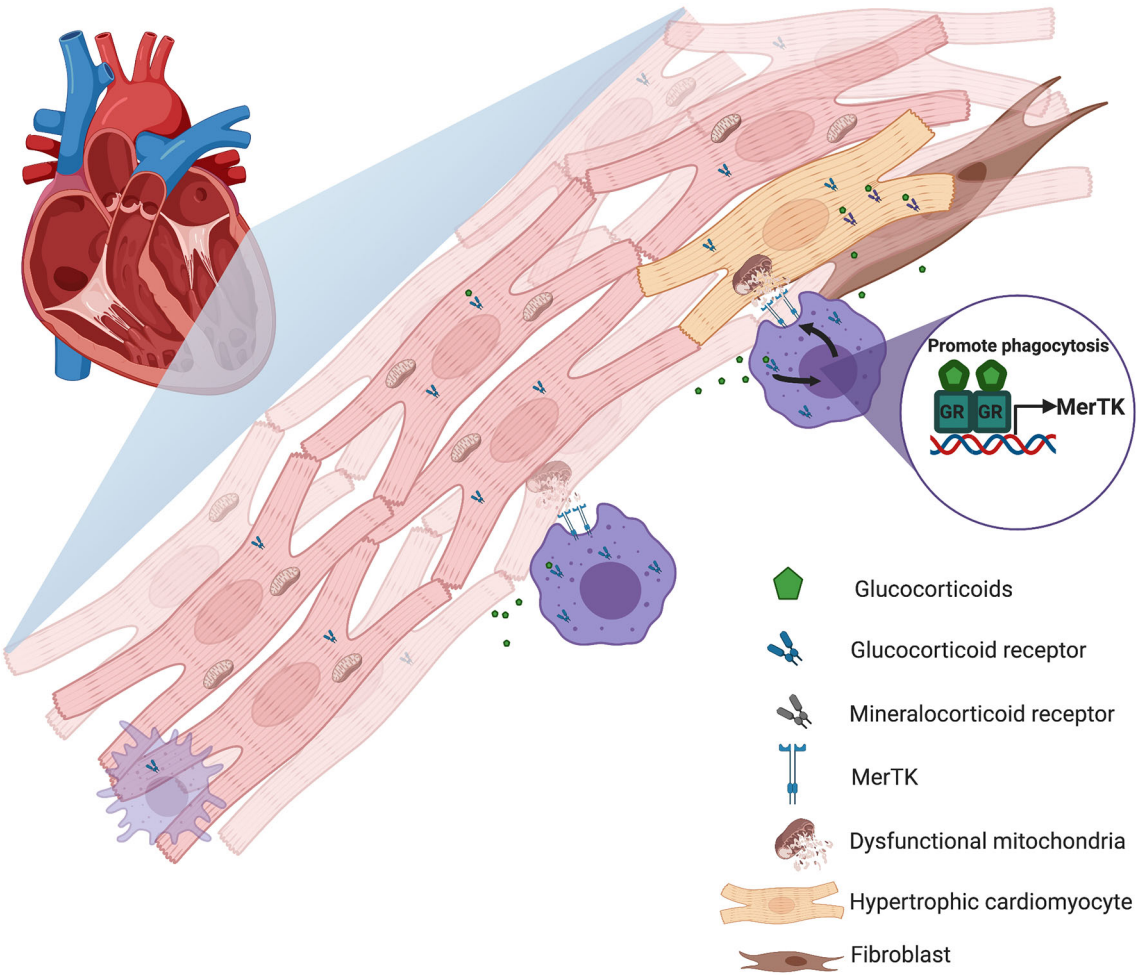

FIGURE 1 | Schematic representation of how glucocorticoids could contribute to cardiac tissue homeostasis. Upon damage or stress activation, glucocorticoid-activated cardiac macrophages promote the active elimination of hypertrophic cardiomyocyte-derived mitochondria and help maintain cardiac health and homeostasis through the induction of the phagocytic receptor Mer tyrosine kinase (Mertk).

including major histocompatibility complex class I and II, receptors for cytokines and chemokines, such as CD200R (74) and CX3CR1 (75), and several other cellular markers indicative of increased reactivity (76). The constitutive expression of HLA-DR in human microglia has been related to their immune-surveillance of the brain (77). Recently, it has been described that microglial mTOR-dependent metabolic flexibility and glutaminolysis support their effector functions within the brain parenchyma (78).

In addition to their roles as immune sentinel cells, microglia also play a direct role in the regulation of neuron networks and physiology. Microglia can produce factors that modulate proliferation or survival of neurons (79, 80). Consistent with the well-known microglia functions as a sensors and phagocyte cells, Wang et al. (81), demonstrated that microglia eliminate synaptic components in the adult hippocampus, leading to dissociation of engram cells and the forgetting of previously learned contextual fear memory in a complement- and activity-dependent manner.

Conditions that are commonly associated with microglial activation and inflammation in the brain, such as aging, chronic stress, and neurodegenerative diseases also affect adult hippocampal neurogenesis (82). Mechanisms of immune regulation in the CNS are largely dependent on neuronal viability and activity, so the interactions between neurons and microglia are essential in maintaining brain homeostasis (83). Recently, Diaz-Aparicio et al. (84) showed that microglia also modulate adult hippocampal neurogenesis through the secretome associated with phagocytosis of apoptotic newborn cells *via* purinergic P2Y12 receptor and MerTK. As we discussed above for heart immunosurveillance, glucocorticoids could also regulate the expression of MerTK on microglia and promote the long-term homeostasis of adult hippocampal neurogenesis (**Figure 2**).

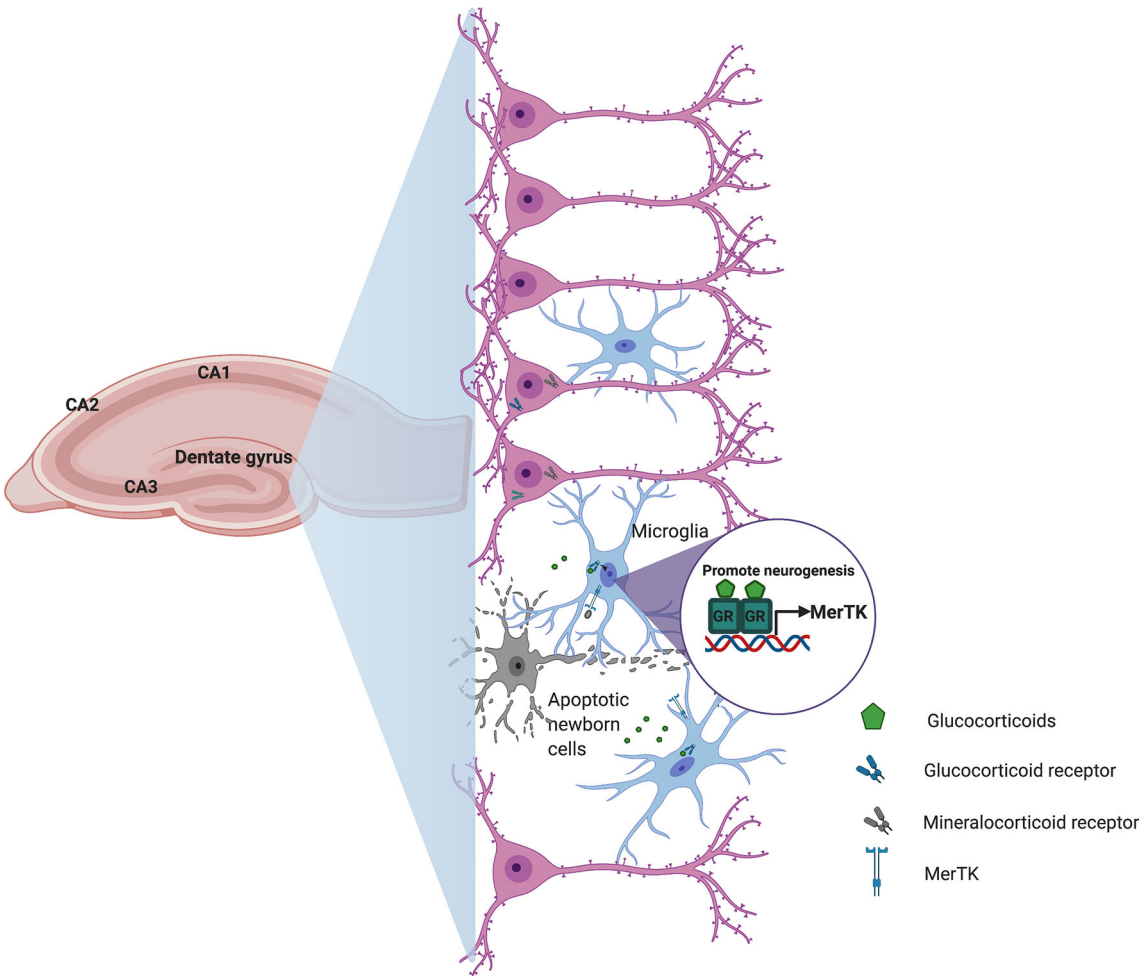

FIGURE 2 | Schematic representation of how glucocorticoids could contribute to central nervous system homeostasis. During hippocampal neurogenesis, cell debris derived from apoptotic newborn cells and stress-induced glucocorticoid secretion promote microglia activation and transcriptional induction of the phagocytic receptor Mertk to regulate the production of new neurons in order to maintain homeostasis in the adult hippocampal neurogenic niche.

The CNS is highly sensitive to damage and any inflammatory response occurring within this organ system must be regulated. Microglial activation, and subsequent suppression, is necessary for host defense and neuroprotection following insult. However, these microglial neuromodulatory mechanisms may become deficient and/or dysregulated under excessive or prolonged inflammatory stimulation induced by stress, disease, and injury (71). One proposed mechanism for maintaining control of microglial activation is through their interaction with neuronal signaling molecules. Healthy neurons maintain microglia in their resting state *via* secreted and membrane bound signals, including CD200, CX3CL1 (fractalkine), neurotransmitters and neurotrophins (83, 85). A reduction in these regulatory factors can lead to microglia hyperactivation, suggesting an important role for communication between neurons and microglia in regulating neuroinflammation.

Although it has been known that glucocorticoid signaling is required for proliferation, differentiation, and survival of neurons, it appears to also be a critical regulator of microglia immunosuppression. By inhibiting microglial activation, glucocorticoids may have opposite effects in changing the immune status of the brain and may make neurons more susceptible to damage. In contrast, studies have suggested that GR activation in microglia promotes their neuroprotective function (86, 87). For example, GR-deficiency in microglia exacerbated neuronal and axon damage caused by intraparenchymal injection of LPS, and GR signaling in microglia suppresses stress-induced neuronal death (86). Interestingly, Maatouk et al. demonstrated that the number of microglia-expressing GR was significantly reduced in the brain of post-mortem Parkinson's disease subjects compared to control tissue and also observed a significant upregulation of TLR9 protein (87). Moreover, in two mice model lacking GR in microglia/macrophages ($GRLysM^{cre}$ and $GRCX3CR1^{CreER2}$ GR mutant mice), intranigral injection of CpG-ODN (TLR9 ligand), resulted in significant loss of dopamine neurons in the brain (87). Although is well known that glucocorticoids are key regulators of TLRs activation upon inflammation, these data suggesting that the loss

of GR in microglia also could contribute to dopamine neurodegenerative process. While both increased microglia activation and neuronal injury can be the result of an exaggerated neuroimmune response, it is unknown if microglial overactivation precedes and causes neuronal damage, or if activation occurs in response to loss of normal neuronal integrity. The differential and separate effects of glucocroticoids on neurons and microglia might depend on the machinery each type of cell possesses, the timing of exposure (before, during, or after activation) and the way they ultimately integrate permissive, preparative, suppressive, and stimulatory effects.

GLUCOCORTICOIDS IN THE IMMUNE-SURVEILLANCE OF THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract represents the largest interface between the organism and the external environment. The GI tract is persistently exposed to a high antigenic load derived from the dense, but largely harmless, commensal microbiota. Because of the mutualistic relationship between microbiome and host, the GI tract establishes a delicate coupling of immune resistance to pathogens and tolerance to tissue damage and inflammation. An important player in this process is the tissue macrophage. Intestinal macrophages, which function as phagocytes, are crucial to maintain the homeostasis of normal healthy GI tract tissues, but are also important for protection against pathogens through the secretion of pro-inflammatory mediators. Intestinal macrophages are also involved in the repair of damaged tissue through the production of proteins that drive epithelial cell renewal (88–90). Tissue-resident macrophages in the steady state are strongly influenced by the microbiota, and major populations are distributed in the stomach as well as along the length of the small and large intestines (91, 92). Unlike many other tissue macrophages, those in the mucosa of the GI tract are derived by continuous but distinct replenishment rates from circulating monocytes (27, 93). Interestingly, a new population of self-maintaining macrophages that are closely positioned in the intestinal submucosa and muscularis externa and arise from both embryonic precursors and adult bone marrow-derived monocytes, persists throughout adulthood and promotes intestinal homeostasis (94). At the functional level, these self-maintaining macrophages control intestinal physiology by supporting the vascular architecture, the permeability, and the intestinal motility that regulates neuronal function in the myenteric plexus (94).

The important role of resident gastrointestinal macrophages in maintaining local homeostasis was discovered through a study by Zigmond et al. (95), where mice harboring IL-10 receptor alpha subunit (IL10RA) deficiency failed to sense interleukin-10 (IL-10; a pleiotropic and anti-inflammatory cytokine produced by T cells, B cells, and macrophages upon inflammation), resulting in spontaneous development of severe colitis. In a mouse model of inflammatory bowel disease (IBD), a chronic inflammatory disorder of the GI tract, mice lacking GR in myeloid cells (GRlysM) displayed impaired disease resolution to dextran sulfate sodium (DSS)-induced colitis and a diminished expression of IL-10 (35). The defect in the acquisition of an anti-inflammatory status and the lack of tissue repair caused by GR ablation in myeloid cells was characterized by persisting clinical symptoms and tissue damage, demonstrating an essential role for GR in macrophages for the induction of tissue repair mechanisms after intestinal tissue damage (**Figure 3**).

Glucocorticoid signaling in the GI tract plays a role in both regulation of the intestinal stress response and intestinal tissue homeostasis. This is suggested by the fact that synthetic glucocorticoid therapy is effective in inducing remission in IBD patients (96). Elevated GC levels as a result of stress or treatment also enhance the nutrient absorption by enterocytes (97, 98). Moreover, an increase in gastric acid secretion, induction of gastroparesis or gastric emptying, and the possible formation of gastric ulcers, in addition to enhanced intestinal glucose transport, have been observed after GC treatment (99). In a study using GRvillinCre mice, Reichardt et al. (100) demonstrated that the lack of GR in enterocytes did not protect mice from glucocorticoid-induced gastroparesis, suggesting that this pathology could be mediated directly by GR in the stomach. Moreover, Cipriani et al. demonstrated that proinflammatory macrophages were necessary for the development of gastroparesis in diabetic mice (101), suggesting that glucocorticoid signaling in the epithelium is needed to control macrophages activation.

Therefore, it is conceivable that GC effects on enterocytes might also contribute to the homeostasis of the entire GI tract. The role of glucocorticoid signaling in the stomach was recently investigated by our group through the depletion of circulating glucocorticoids in mice by adrenalectomy (ADX) (102). The lack of systemic endogenous glucocorticoids in mice resulted in the rapid onset of spontaneous gastric inflammation and the appearance of a clinical phenotype of spasmolytic polypeptide expressing metaplasia (SPEM), a precursor of gastric cancer (102). Moreover, the SPEM which developed in ADX mice was prevented by clodronate treatment and within the Cx3cr1 knockout mouse model, indicating that CX3CR1+ macrophages derived from monocytes are critical mediators of gastric inflammation (102). Intriguingly, the adrenalectomy does not trigger inflammation within another section of the stomach (gastric corpus greater curvature) and neither in other sections of the GI tract, such as ileum and colon (102). Interestingly, both small and large intestinal mucosa have been recognized as site of extra-adrenal glucocorticoid synthesis (103–105). One of the plausible reasons why ADX mice would not develop spontaneous inflammation in the intestine could be the local production of GCs. In order to cope the local stress, GCs would regulate the immune homeostasis, however this hypothesis have not been addressed yet. The nuclear receptor liver receptor homologue-1 (LRH-1, NR5A2) would be essential to regulate the intestinal glucocorticoid synthesis *in vivo* (104). Moreover, thought 3 different models, human intestinal organoids, humanized murine intestinal organoids, and a humanized murine IBD model Bayrer al. showed that LRH-1 promotes normal intestinal epithelial homeostasis suggesting that this NR can be an important regulator of intestinal tissue integrity

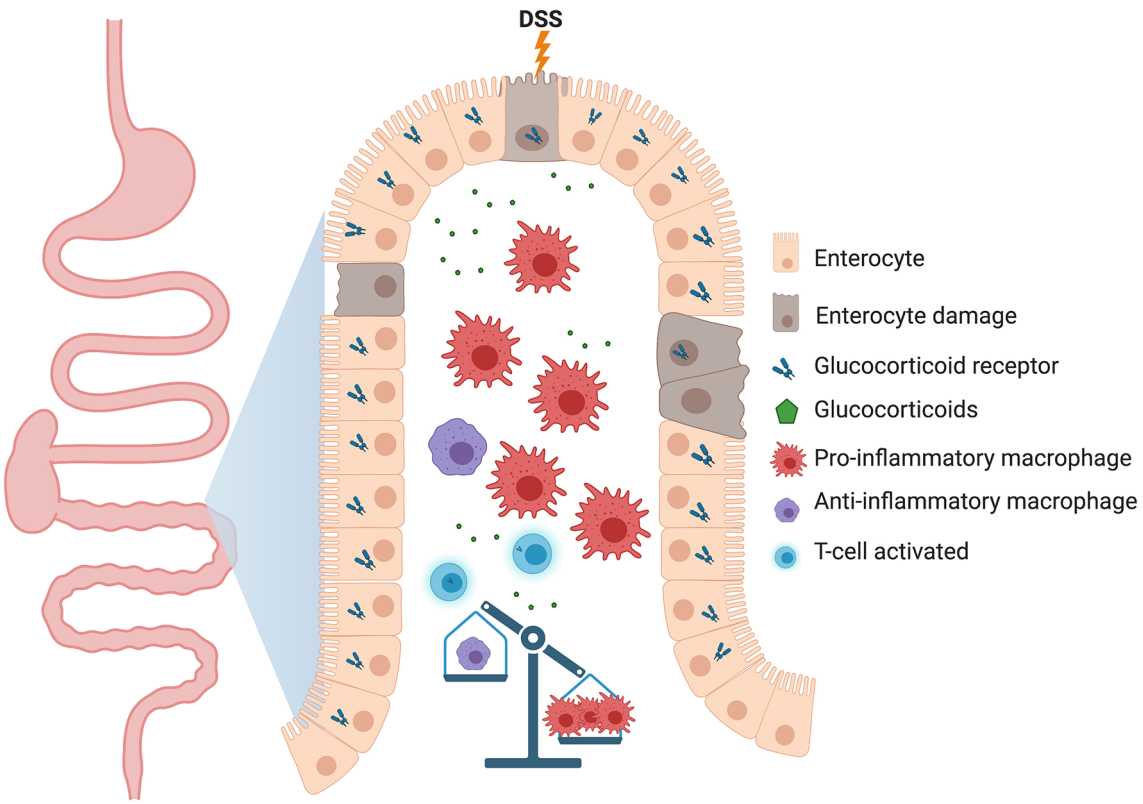

FIGURE 3 | Proposed model of how the lack of glucocorticoid receptor (GR) in macrophages could be detrimental to gastrointestinal tissue homeostasis. In the dextran sodium sulfate (DSS)-induced colitis model, deletion of GR in myeloid cells delays the resolution of inflammation through an increase in the number of pro-inflammatory macrophages which perpetuates tissue damage. Other immune cells, such as B cells, dendritic cells and ILCs relevant to the intestinal physiology have been deliberately neglected to highlight the function of macrophages.

(105). Summarizing, all these findings indicate that glucocorticoid signaling could participate in the immune-surveillance of the gastrointestinal tract and is a critical mediator of both gastric and intestinal homeostasis.

GLUCOCORTICOIDS IN THE IMMUNE-SURVEILLANCE OF THE LIVER

As their name suggests, glucocorticoids are profound regulators of glucose metabolism. It is no surprise then that the liver, the organ responsible for controlling glucose levels, is a major target of glucocorticoid action. Glucocorticoids exert permissive effects on glycogen metabolism and stimulate gluconeogenesis through direct regulation of rate limiting enzymes involved in this process, such as PEPCK and G6Pase (106, 107).

Studies using liver-specific GR knockout (L-GRKO) mice have demonstrated the many roles played by GR signaling in hepatocytes. The daily rhythmic production of endogenous glucocorticoids coordinate glucose, lipid, and fatty acid metabolism with periods of feeding and fasting (108, 109). This coordination is lost in L-GRKO mice, which exhibit fasting hypoglycemia and reduced body weight, which may involve impaired growth hormone signaling (110, 111). Our group evaluated the actions of glucocorticoids on hepatic expression of inflammatory genes in male and female L-GRKO mice. Interestingly, in response to pro-inflammatory LPS challenge, deletion of GR reduced the number of inflammatory genes in a sex-specific manner, with female mice exhibiting regulation of more genes than males (112). Therefore, GR signaling influences diverse gene expression programs in hepatocytes, some of which are sexually dimorphic.

In addition to its roles in regulating metabolism, the liver is also an important part of the immune system. It serves as a major filter for the blood coming from the digestive tract and can respond to potential threats such as bacterial toxins and cellular debris.

Hepatocytes release large amounts of immunoregulatory proteins into the circulation that function to eliminate pathogens and fine-tune innate immunity. These include members of the complement system, acute-phase proteins, LPS signaling regulators, and several iron-metabolism-related proteins (reviewed in (113)). The liver also contains the single largest reticuloendothelial cell network in the body, which is composed of tissue-resident macrophages called Kupffer cells. Kupffer cells are intimately involved in the hepatic response to various toxic insults. They constitute a primary line of defense against invading microorganisms, function as sensors for

altered tissue integrity, and control immunological tolerance in the liver by providing an anti-inflammatory microenvironment during homeostasis (114). Kupffer cells are largely stationary and adhere to the liver sinusoidal endothelial cells where they are exposed to the contents of the blood. During times of inflammation, the hepatic macrophage pool is expanded by circulating blood monocytes that give rise to monocyte-derived macrophages (115), which have been shown to resemble the transcriptional phenotype of Kupffer cells after lymphocyte choriomeningitis virus infection (116) or after acetaminophen-induced hepatotoxicity (117).

Relatively few studies have explored the role of GR signaling in Kupffer cells and monocyte-derived liver macrophages. Nevertheless, there is good evidence that glucocorticoid regulation of these immune cells may be essential for liver homeostasis. Kupffer cells respond to glucocorticoids by upregulating the anti-inflammatory gene Gilz, and mice with macrophage-specific deficiency in GR exhibit more severe obesity-induced liver inflammation (118). In human and mouse, Kupffer cells secrete the immunosuppressive cytokine IL-10 (119). IL-10 deficiency or depletion exacerbates hepatic immune-mediated liver damage and abrogates tolerance induction (120). For example, in the Concanavalin A hepatitis model, which is used to study tolerance induction and immune-mediated hepatitis, Kupffer cell-derived IL-10 exerts hepatoprotective and tolerogenic effects through Treg activation (121). Similarly, Kupffer cells interact directly with T cells in response to administration of particulate antigens, causing the expansion of IL-10-expressing Tregs (122). It is well known that IL-10 is a glucocorticoid-induced gene (123, 124); therefore, glucocorticoid levels may directly influence the inflammatory environment in the liver by modulating Kupffer cell IL-10 expression (**Figure 4**).

The liver's response to injury is complex and depends upon the interaction of multiple cell types, both parenchymal and non-parenchymal. Paradoxically, Kupffer cells have been implicated in both liver regeneration and fibrosis. They secrete pro-inflammatory mediators, such as reactive oxygen species, eicosanoids, prostaglandins, and cytokines that induce recruitment of additional inflammatory cells to the liver (125).

Glucocorticoid receptor ligands have been shown to suppress hepatic expression of pro-fibrotic genes, leading to decreased extracellular matrix deposition (126). In addition, GR ligands inhibit immune cell infiltration to the damaged liver, which can exacerbate injury in certain cases. Using models of tissue-specific deletion of GR, Kim et al. (126) demonstrated that GR signaling controls pro-fibrotic gene expression and immune cell infiltration *via* two cell types. Specifically, GR deletion in Kupffer cells (via LysM-cre) reversed inhibition of immune cell infiltration in response to dexamethasone. GR deletion in hepatic stellate cells (via hGFAP-cre) reversed downregulation of fibrotic gene expression in response to dexamethasone. These studies suggest that GR signaling in Kupffer cells modulates factors involved in cell recruitment to the liver, while GR signaling in hepatic stellate cells modulates fibrosis in response to injury. Other studies have also supported the idea that GR signaling in Kupffer cells promotes liver homeostasis. Direct targeting of dexamethasone to Kupffer cells promoted replenishment of glycogen stores lost during hepatic fibrosis caused by bile duct ligation (127). Interestingly, Rose et al. (128) demonstrated that the use of glucocorticoids completely sustained hepatocyte longevity and improved hepatocyte functionality during the establishment of co-culture conditions between hepatocytes and Kupffer cells. Additional studies are needed to pinpoint the effects of both endogenous and synthetic glucocorticoids on specific cells within the liver and to understand the interaction between these cell types during times of injury and homeostasis.

CONCLUSIONS AND PERSPECTIVES

Glucocorticoids mediate physiological processes in different tissues and cell types with high specificity to systematically influence behavior and cognition, metabolism, cardiovascular function, and the immune system. Synthetic glucocorticoids are administrated as drugs to treat several inflammatory conditions because of their ability to induce potent anti-inflammatory and immunosuppressive effects that occur due to the repression of pro-inflammatory genes and the activation of anti-inflammatory pathways in immune cells. Whereas restricted inflammation is beneficial, excessive or persistent inflammation could be associated with chronic diseases. The immune regulation process and anti-inflammatory homeostatic mechanisms mediated by glucocorticoids are essential in limiting and resolving the inflammatory process. The balance of pro- and anti-inflammatory pathways plays an important role in maintaining immune homeostasis. In addition to its immunosuppressive functions, GR signaling may regulate cellular metabolism and survival.

The main mechanism of action of GCs on immune cells has been linked to their ability to induce cell death and reduce cell survival through direct genomic effects. GR-regulated genes that are required to drive apoptosis include pro-apoptotic mediators, such as the BH3-protein BIM (BCL2LII), which is activated (129) and the anti-apoptotic BCL2, which is downregulated (130). Non-genomic effects of GR have also been proposed. Interestingly, a second mechanism that could explain the effectiveness of GC regulation of the immune response is the promotion of Treg proliferation directly or indirectly through macrophage activation. Bereshchenko et al. (131) reported that glucocorticoid-induced leucine zipper (GILZ) promotes Treg production and enhance Treg signaling. Macrophages have been shown to possess the potential to induce Treg function to maintain tissue homeostasis, while Tregs can enhance the ability of macrophages to engulf apoptotic cells which promotes resolution of inflammation (132). Disruption of the crosstalk between macrophages and Tregs leads to severe autoimmune disease and chronic inflammation.

The glucocorticoid regulation of local homeostatic mechanisms has been exemplified through the study of tissue-specific GR knockout mice. We still have limited knowledge of how GR-dependent gene expression contributes to the phenotypes of tissue-specific GR knockout mice. Future studies are needed to fully understand how GR signaling is acting in specific tissues and in different disease states.

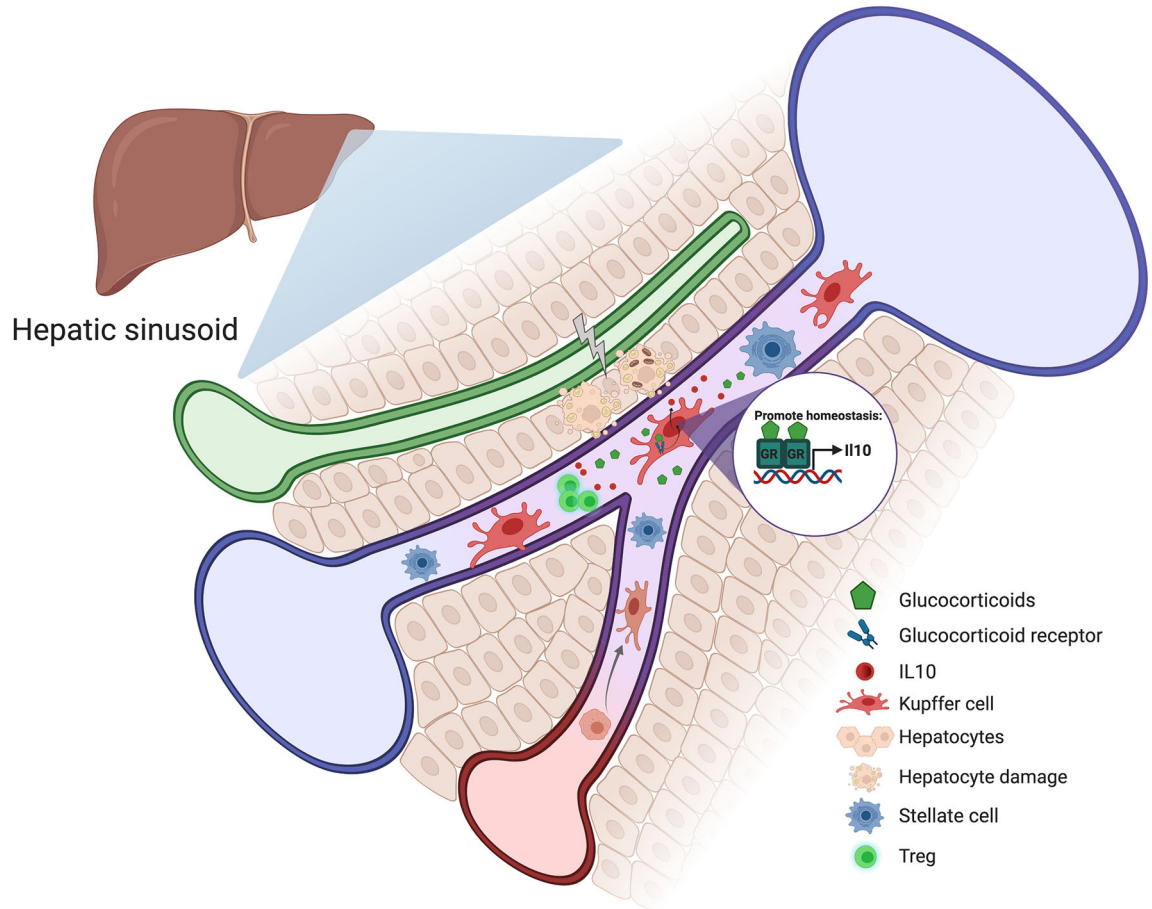

FIGURE 4 | Schematic representation of how glucocorticoids could contribute to liver homeostasis. Stress and hepatocyte injury around the hepatic sinusoid cause an increase in glucocorticoid levels which induces Kupffer cell activation and IL-10 production. Kupffer cell-derived IL-10 exerts hepatoprotective and tolerogenic effects through Treg activation.

Its well-known that glucocorticoids can regulate different stages of macrophage biology, including differentiation, survival, movement, activation and polarization. While it has been long believed that tissue macrophages were originated from myeloid cells and circulating adult blood monocytes, it is now clear that many resident tissue macrophages are established during embryonic development and persist by self-renewal. Even, we now know that multiple populations of macrophage-like cells co-exist in both, steady-state and inflammation. Moreover, under inflammatory conditions, the macrophage pool is expanded by pro-inflammatory infiltrating blood monocytes that may or may not acquire the phenotype of the resident macrophages in a given tissue. Whether glucocorticoids can regulate the gene expression profile of macrophages independently of their ontogeny, activation or polarization states is one of the most important questions that must be addressed. Uncovering distinct glucocorticoid-mediated gene expression networks in macrophages may aid in the production of targeted therapies for diseases characterized by dysregulation of homeostasis.

AUTHOR CONTRIBUTIONS

DD-J and JK wrote the manuscript with supervision from JC. All authors contributed to the article and approved the submitted version.

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Transmigration of Neutrophils From Patients With Familial Mediterranean Fever Causes Increased Cell Activation

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Familial Mediterranean fever (FMF) is caused by pyrin-encoding *MEFV* gene mutations and characterized by the self-limiting periods of intense inflammation, which are mainly mediated by a massive influx of polymorphonuclear neutrophils (PMNs) into the inflamed sites. Perturbation of actin polymerization by different pathogens was shown to activate the pyrin inflammasome. Our aim was to test whether cytoskeletal dynamics in the absence of pathogens may cause abnormal activation of PMNs from FMF patients. We also aimed to characterize immunophenotypes of circulating neutrophils and their functional activity. Circulating PMNs displayed heterogeneity in terms of cell size, granularity and immunophenotypes. Particularly, PMNs from the patients in acute flares (FMF-A) exhibited a characteristic of aged/activated cells (small cell size and granularity, up-regulated CXCR4), while PMNs form the patients in remission period (FMF-R) displayed mixed fresh/aged cell characteristics (normal cell size and granularity, up-regulated CD11b, CD49d, CXCR4, and CD62L). The findings may suggest that sterile tissue-infiltrated PMNs undergo reverse migration back to bone marrow and may explain why these PMNs do not cause immune-mediated tissue damage. A multidirectional expression of FcγRs on neutrophils during acute flares was also noteworthy: up-regulation of FcγRI and down-regulation of FcγRII/FcγRIII. We also observed spontaneous and fMPL-induced activation of PMNs from the patients after transmigration through inserts as seen by the increased expression of CD11b and intracellular expression of IL-1β. Our study suggests heightened sensitivity of mutated pyrin inflammasome towards cytoskeletal modifications in the absence of pathogens.

Keywords: Familial Mediterranean fever, neutrophils, fresh/aged neutrophils, transmigration, IL-1β, immunophenotype, FcγRs

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INTRODUCTION

Familial Mediterranean fever [FMF, MIM249100] is an autoinflammatory syndrome characterized by the recurrent episodes of fever and aseptic polyserositis. Gain-of-function mutations in the MEFV gene which encodes pyrin are the cause for FMF (1, 2). Interaction of the N-terminal part of pyrin with microtubules and co-localization of pyrin with actin (3, 4) suggest that pyrin might be a sensor for

actin homeostasis (5). Later on, pyrin was shown to recognize downstream Rho modifications, most likely involving the actin cytoskeleton modifications (6, 7). Pyrin has been shown to activate caspase-1 and mature IL-1β and IL-18 release in response to Rho-inactivating toxins from a number of pathogenic bacteria (5, 6, 8). The involvement of cytoskeleton might be confirmed by the therapeutic efficiency of colchicine, antimitotic drug, which causes microtubule disruption/depolymerization as well as re-organization of actin cytoskeleton (9, 10).

The pyrin is primarily expressed in neutrophils (polymorphonuclear cells, PMNs), eosinophils, and cytokine-activated monocytes (11). Despite the main players of self-limited inflammatory attacks are neutrophils, remarkably little is known about their phenotypic and functional characteristics. PMNs carrying *MEFV* mutations display characteristics of activation status (12, 13). Increased spontaneous release of IL-18, S100A12, myeloperoxidase (MPO), caspase-1, and proteinase 3 have been shown by neutrophils from FMF patients. Moreover, the highest levels of IL-18 were released by the cells derived from the patients with homozygous M694V mutations (14). In our previous experiments, we have observed a phenomenon of heightened sensitivity of neutrophils from FMF patients towards *in vitro* conditions in the inductor-free media (13, 15). Similar results with the use of monocytes have been obtained later by another group (16). *In vitro* experiments unavoidably impose mechanical forces on cells that may affect cytoskeletal structure and modulate cellular behaviour (17). It is already known that mechanical deformation has the ability to increase expression of adhesion molecules, reorganize cell cytoskeleton, increase free intracellular Ca2+ concentration, and induce cell activation (18, 19). The routine experimental procedures involving external mechanical forces applied to the cells may lead to the generation of danger signals that are sensed by the pyrin inflammasome. Recent investigations showing the pyrin inflammasome activation in response to actin modifications (6, 7), together with our results suggesting the excessive activation of neutrophils from FMF patients in *ex vivo* experiments (13, 15), warrants further studies of pyrin-cytoskeleton interactions. We hypothesize that mechanical forces induced by the transmigration of the cells may enhance pyrin inflammasome priming. To address our hypothesis and evaluate the role of cytoskeletal dynamics in increased activation of the cells, we performed a series of *in vitro* assays where PMNs were influenced by different stressful signals such as transmigration through Transwell insert, stress hormone epinephrine, and bacterial ligands. In parallel, immunophenotypes of circulating neutrophils and their functional activity were analysed.

MATERIALS AND METHODS

Patients

A total of 35 patients with fulfilled Tel Hashomer criteria for FMF diagnosis and at least one mutation in the *MEFV* gene were enrolled in the study (20). All FMF patients were recruited at the"Arabkir" Medical Center – Institute of child and adolescent health (Arabkir MC-ICAH, Yerevan, Armenia). Fourteen of the 35 patients expressed a typical FMF attack and were identified as FMF-A group, and the remaining 21 clinically asymptomatic FMF patients in attack-free period were identified as FMF-R group. All patients within FMF-R groups were receiving colchicine, while the patients from FMF-A group were newly diagnosed colchicine-naive patients. Determination of the acute phase was based on clinical and laboratory findings (fever, FMF-related symptoms such as abdominal and/or thoracic pain, arthritis, C-reactive protein, white blood cell counts, erythrocyte sedimentation rate, etc.). The remission phase was defined as being free of attacks for at least 3 months. The control group consisted of 20 healthy, age and gender-matched volunteers without any concomitant chronic disorders. The potential carriage of *MEFV* mutations within the control group was not assessed, however, healthy individuals with family history of FMF in three successive generations were excluded from the study. The clinical and demographic characteristics of the participants involved in the study are summarized in **Table 1**. The study was approved by the Ethical Committee of the Institute of Molecular Biology NAS RA (IRB IORG0003427). Written informed consent was obtained from all adult participants or from parents/legal guardians of participants under 18.

Cell Surface Staining

Peripheral venous blood samples were drawn from patients with FMF and healthy donors (HD) into EDTA- and Heparin-containing tubes and processed within 2h. To investigate the expression of cell surface markers in PMNs, whole blood was aliquoted (50 µl per tube) and stained with optimal concentrations of fluorochrome-conjugated monoclonal antibody combinations directed against the following antigens: CD11b, CD15, CD16, CD32, CD49d, CD62L, CD64, and CD184 (CXCR4) for 20 minutes at room temperature, in the dark. Isotype matched FITC, PE, PE/Cy7, APC-conjugated irrelevant antibodies were used to establish background staining. PMNs population was identified through sequential gating strategy: based on forward scatter (FSC), side scatter (SSC), and CD15 expression. Data was acquired on a FACSCalibur flow cytometer (BD) equipped with BD CellQuest™ Pro acquisition software and analysed using the FlowJo vX0.7 software (Tree Star, Inc, San Carlos, CA). At least 10,000 total events were collected per sample. Cell viability was verified with PI staining. Results are expressed as the percentage and median fluorescence intensity (MFI) of the cells for each examined marker.

Oxidative Burst Assay

To determine PMNs oxidative burst capacity, dihydrorhodamine 123 (DHR-123) (Sigma-Aldrich) conversion into the fluorophore rhodamine was evaluated. Briefly, the aliquots of heparinized whole blood were incubated for 20 min with DHR-123 at final concentration 10 µM in the dark at 37°C. fMLP (100 ng/ml) and as a positive control PMA (50 ng/ml) were added to the corresponding tubes and incubated for further 30 min in the dark at 37°C. As negative control cells were left in the RPMI medium alone. Reaction was stopped by placing tubes on ice for 10 min. Following erythrocytes lysis with cold hypotonic solution and a single washing step, fluorescence intensity of the cells was immediately analysed by flow cytometry.

TABLE 1 | Demographic and clinical characteristics of FMF patients and healthy donors.

	HD	FMF-R	FMF-A
№ of participants	20	21	14
Age	21 (16–20)	16 (10–18)	15 (7–18)
Leucocytes [10*9/L]	7.36 ± 1.95	7.57 ± 1.91	9.51 ± 2.61
PMNs [10*9/L]	3.95 ± 2.18	4.73 ± 1.54	6.58 ± 2.88
PMNs [%]	49.2 ± 6.48	56.4 ± 7.36	65.15 ± 11.97
CRP [mg/dL]	0.9 ± 1.31	3.26 ± 4.65	101.08 ± 89.16
ESR [mm/hr]	7.3 ± 4.62	9.71 ± 5.67	15.42 ± 11.02
Colchicine treatment	0/20	21/21	0/14
MEFV mutations	no family history of FMF	2 homozygous/ 19 heterozygous	4 homozygous/ 10 heterozygous

Phagocytosis

PMNs phagocytosis capacity was assessed in aliquots (50 µl per tube) of heparinized whole blood, stimulated with following inducers: LPS (100 ng/ml), PGN (1 µg/ml) in the dark at 37°C for 60 min. Afterward, cells were incubated with 0,5 µl (density: $4.55x10^{10}$ particles/ml) microbeads (Polysciences, Inc., Fluoresbrite® YG Microspheres 1.00 µm size) for 30 min at 37°C. For negative control, cells were incubated with microbeads at 4°C for 30 min, without prior stimulation. Following the incubation, cells were washed with ice-cold PBS to remove any free (non-ingested) particles and analysed by flow cytometry.

Neutrophil Transmigration Assay

To analyse whether mechanical stress abnormally affects cell activation, we performed a transmigration assay. Briefly, PMNs were isolated with Histopaque-1077 gradient (Sigma, St. Louis, MO) and subjected to transmigration using 24-well plate and 6.5-mm Transwell Inserts with 3-µm pore Polyester Membrane (Corning, NY, USA). Inserts (upper compartment) were placed into the wells containing 600 µl RPMI-1640 supplemented with 0.5% bovine serum albumin (BSA) in the absence or presence of fMLP (100 ng/ml). PMNs were suspended at final concentration $1x10^6$/100 µl, loaded into the upper compartment and allowed to transmigrate for 2h, at 37°C under a humidified atmosphere of 5% CO_2. In parallel, PMNs were cultured at the same conditions in a 24-well plate without inserts as a non-migrated control. Following cultivation period, migrated and non-migrated cells were surface-labelled with CD11b, fixed/permeabilized with Fixation Buffer/Permeabilization Wash buffer (BioLegend), blocked to avoid non-specific binding, stained intracellularly with IL-1β for 20 min, and analysed with flow cytometry. Supernatants of migrated and non-migrated cells were collected and stored at -80°C for further IL-1β quantification.

Actin Polymerization Assay

Isolated PMNs (500.000 cells per measurement) were stimulated with fMLP (100 ng/ml) or left untreated for 5, 15, 30, 60, 120 and 180 sec. For every time point, regardless of stimulation, reaction was stopped by adding a Fixation Buffer (BioLegend) for 20 min at room temperature. Thereafter, cells were permeabilized and blocked to avoid non-specific binding. Following 20 min incubation, cells were intracellularly stained with FITC-conjugated Phalloidin (Abcam, 1:1000). As negative control, PMNs were left either unstimulated or unstained. Following 30 min incubation in the dark at 4°C, actin polymerization was determined by the level of Phalloidin expression, as measured by flow cytometry.

Kinetic of Cultured PMNs

Time-dependent dynamics of neutrophils activation in the absence of any external forces were analysed using isolated PMNs ($1x10^6$) cultured in a complete RPMI medium separately for 1h, 2h, 3h and 4h. After each hour, cell culture supernatants were aspirated and stored for further IL-1β analysis. The cells were then surface-labeled with CD11b, fixed/permeabilized and stained intracellularly with IL-1β for 20 min. Samples were acquired on the flow cytometer.

Whole Blood Cultivation With Epinephrine

Aliquots of 50 µl whole blood were cultured in the absence or presence of Epi (10, 100, 1000 µM), LPS (100 ng/ml) or LPS (100 ng/ml) + Epi (1000 µM) in complete RPMI, supplemented with 10% FBS and 2 mmol/L glutamine, at 37°C under a humidified atmosphere of 5% CO_2 for 4h. After the cultivation period, the whole blood cells were labeled for surface markers and analysed with flow cytometry. Cell culture supernatants were collected and stored for IL-1β measurements.

IL-1β Quantification by ELISA

Duplicate samples of the cell culture supernatants were analysed using Human IL-1β ELISA MAX Deluxe Set kit (Biolegend, UK) commercial assay, according to the manufacturer's instructions. The samples were read at 450 nm on a microplate photometer HiPo MPP-96 (BioSan, Riga, Latvia). Results were calibrated with serial dilutions of known quantities of recombinant cytokines. The minimum detectable concentration for IL-1β was 0.5 pg/mL.

Statistical Analyses

Data analysis was performed with GraphPad Prism 5.01 software (GraphPad Software, USA). All values are given as mean ± standard deviation (SD). Normal distribution was checked with Shapiro–Wilk's W test. One-way ANOVA and Wilcoxon signed-rank tests as appropriate were used to estimate the effect of inducers within investigated groups. The Mann–Whitney test was used for the comparisons between studied groups. Principal component analysis (PCA) was performed using R software (version 4.0.5). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Spontaneous Expression of Surface Markers

Peripheral blood PMNs were identified and described according to their FSC, SSC characteristics and CD15 expression. PMNs from the FMF-A group displayed low FSC and SSC parameters as measured by flow cytometry (**Figure 1**). Particularly, FMF-A PMNs showed lower size compared with both HD and FMF-R groups ($P = 0.054$ and $P < 0.01$, respectively), and tendency to the lower granularity compared with the cells from the FMF-R group (P=0.058). The cells from the FMF-A group also exhibited a higher percentage of SSC^{hi} cells compared to both HD and FMF-R ($P < 0.05$) (**Figures S1A, B**).

Next, we examined the surface expression of CD11b, CD16, CD32, CD64, CD49d, CD62L, and CD184 (CXCR4) which is shown in **Figure 2**. Both FMF-R and FMF-A neutrophils displayed increased expression of CXCR4 ($P < 0.05$) compared to the HD group, while expression of CD11b ($P < 0.01$), CD62L ($P < 0.05$) and a percentage of integrin alpha subunit CD49-positive cells ($P < 0.05$) were increased only in the FMF-R group. A positive correlation between surface expression of CD11b and neutrophil percentage in patients from FMF-A group ($R = 0.729$, $P = 0.01$) was observed.

Subsets of circulating neutrophils with pro-inflammatory and anti-inflammatory features, were identified by the co-expression of CD11b and CD49d (**Figures S2A, B**), which are the markers able to characterize N1 ($CD11b^{lo}/CD49d^{+}$) and N2 ($CD11b^{hi}/CD49d^{-}$), respectively (21). There was an increase in $CD11b^{lo}/CD49d^{+}$ subset of PMNs with pro-inflammatory features in the FMF-R group compared to HD ($P < 0.05$) (**Figure S2C**).

Interestingly, while analysing expression of FcγRs on circulating PMNs, we observed multidirectional surface expression of these receptors. Particularly, a significant downregulation of low-affinity FcγRII (CD32) and FcγRIII(CD16) and upregulation of high-affinity FcγRI (CD64) expression in the cells from the FMF-A group were observed (**Figure 3**).

To determine if circulating neutrophils from all studied groups are distinct in expression profiles of surface markers and visualize the structure within the data, we used PCA. The analyses revealed clustering of three subpopulations (HD, FMF-R, FMF-A) with several dispersed cases, and few cases displayed in-between characteristics (2 HD, 3 FMF-R, and 2 FMF-A cases). In total, 56% of the cases were classified correctly. It is worth mentioning that overlapping of expression profiles was observed only between patients and controls not between FMF-R and FMF-A groups. Within studied markers, CD11b, CD16, CD32, CD64, and CXCR4 markers had the most consistent ability to correctly classify the cells from studied groups (**Figure 4**).

Functional Activity of Circulating PMNs

Next, we addressed the question whether the patterns of FcRs expressed in the studied groups are associated with the

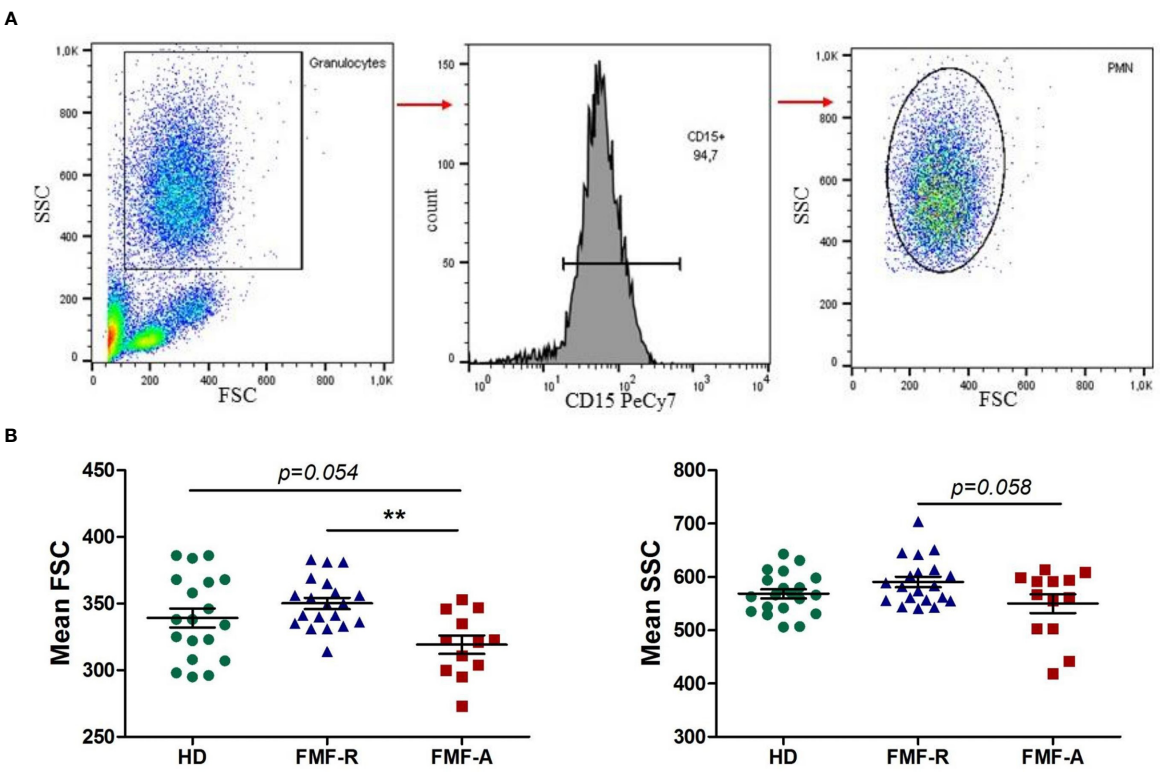

FIGURE 1 | Analysis of morphological parameters of PMN. **(A)** Backgating strategy for PMN identification and FSC/SSC values. **(B)** Mean FSC and SSC parameters values of circulating PMNs in HD, FMF-R and FMF-A groups. $^{**}p < 0.01$.

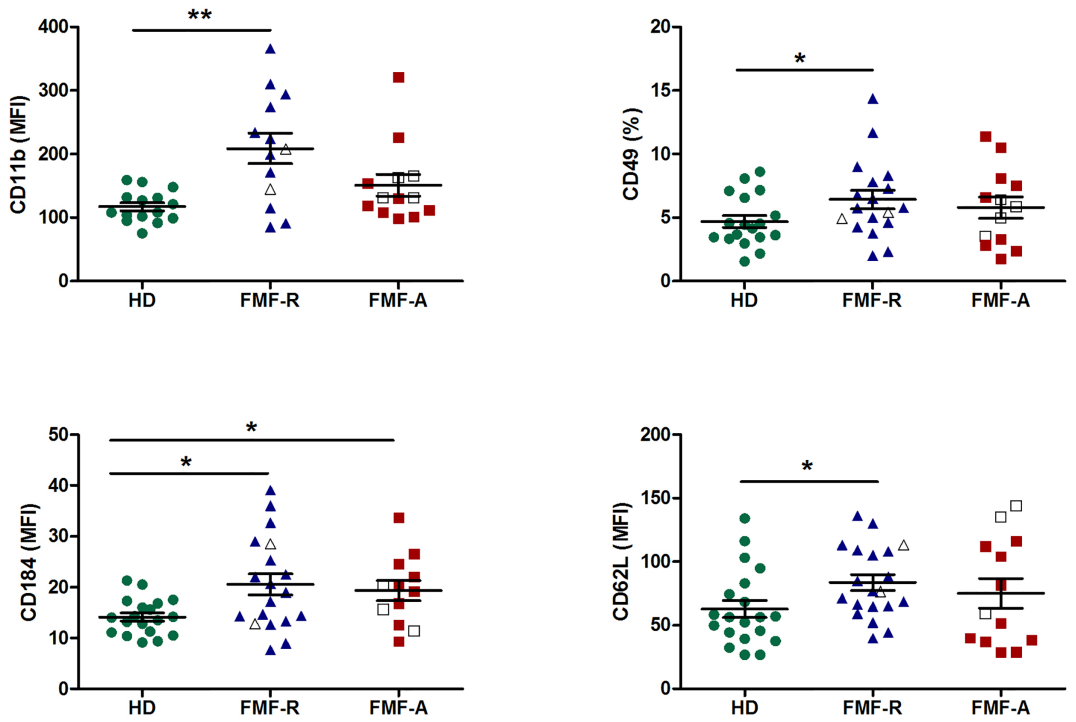

FIGURE 2 | Surface expression of CD11b, CD49d, CD184 (CXCR4), and CD62L on circulating PMNs from HD, FMF-R and FMF-A patients. *$p < 0.05$; **$p < 0.01$.

phagocytosis. As appeared, phagocytic activity of PMNs was not different in both unstimulated and stimulated cells from all studied groups. Notably, PMNs from the FMF-R group failed to increase phagocytic activity in response to both fMLP and PGN which might be explained by the colchicine therapy received by the patients (**Figure S3A**).

As reactive oxygen species (ROS) can be found in higher amounts when neutrophils are activated, we used ROS as a parameter for neutrophil activity. Spontaneous oxidative burst of neutrophils in the FMF-A group was decreased compared to HD one ($P < 0.05$). A significant increase in ROS activity was detected upon fMLP stimulation in HD and FMF-R groups compared with unstimulated samples ($P < 0.05$ and $P < 0.01$, respectively). In the FMF-A group, fMLP-stimulated and unstimulated oxidative activity was reduced compared to the HD group ($P < 0.05$) and fMLP-stimulated compared to the FMF-R group ($P < 0.05$) (**Figure S3B**).

Transmigration of Neutrophils From FMF Patients Activates the Cells

To determine whether mechanical forces induce increased activation of neutrophils, we mimicked conditions distinctive for the transendothelial migration. For this, we used 3μm pore Transwell inserts to allow neutrophils to migrate towards fMLP. In parallel, we cultured the cells in the absence or presence of fMLP in 24-well plates as a control (non-migrated) for the migrated cells. After 2h, we analysed the number of migrated cells, surface expression of CD11b, intracellular expression of

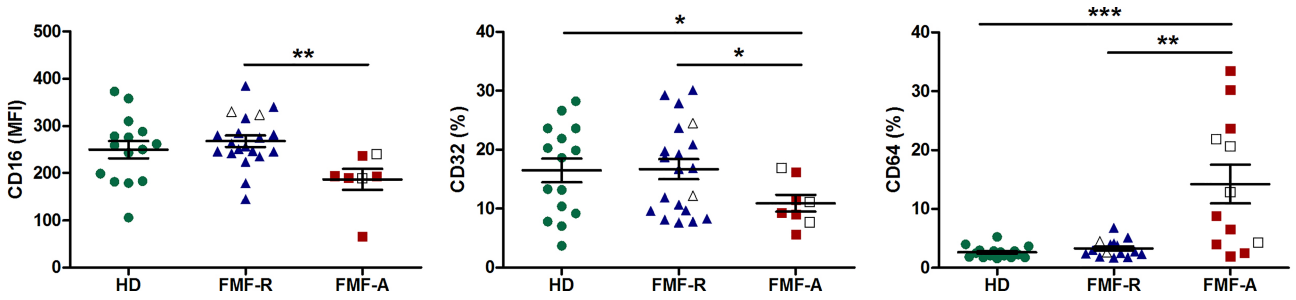

FIGURE 3 | Surface expression of FcγRIII(CD16), FcγRII (CD32), FcγRI (CD64) on circulating PMNs from HD, FMF-R and FMF-A patients. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

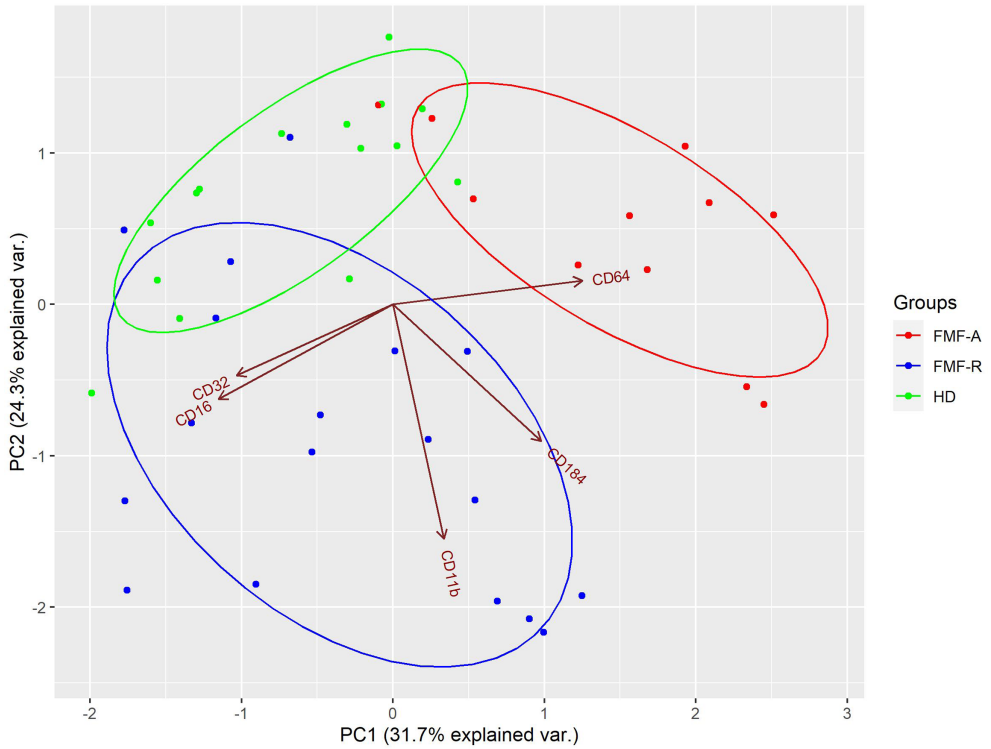

FIGURE 4 | Principal component analysis (PCA) of PMN from HD (green), FMF-R (blue), and FMF-A (red), characterized by combination of CD11b, CD16, CD32, CD64, and CXCR4 expression. Each dot on bi-plot graph represents a single patient/control. The model loadings are represented by vectors and indicate how each surface marker contributes to the cell variability in a specific direction. Percentages represent variance captured by PC 1 and 2.

IL-1β and production of IL-1β by migrated and non-migrated neutrophils. PMNs from FMF-A group displayed a tendency to a higher rate of transmigration compared to both HD and FMF-R cells (**Figure S4**).

When analysing the effect of transmigration on cell activation, an increase in activation markers in the cells from the FMF-R group passing through the inserts was detected. When passing inserts, fMLP-induced cells from the FMF-R group revealed higher expression of IL-1β compared to those from the HD group ($P < 0.05$), except the non-migrated fMLP-stimulated cells. More notably, IL-1β expression within the FMF-R group was higher in migrated fMLP-stimulated PMNs compared to non-migrated ones ($P < 0.05$). When analysing surface CD11b expression, differences between studied groups were even more marked and exciting. Within the FMF-R and FMF-A groups, the migrated cells had a higher CD11b expression compared to the non-migrated cells ($P < 0.05$). FMF-R cells also responded to the fMLP stimulation more intensively as seen by up-regulated surface expression of CD11b on fMLP-stimulated cells compared to the unstimulated ones both after migration and without. Worth to mention that even without migration, cultured not-stimulated FMF-R cells exhibited up-regulation of IL-1β in comparison to those from the HD groups ($P < 0.05$) (**Figures 5A, B**).

Similarly to intracellular expression, the levels of released IL-1β were higher in the supernatants from the migrated fMLP-induced FMF-R cells compared with healthy ones ($P < 0.05$). However, the highest release of IL-1β was observed in the supernatants from the non-migrated FMF-R cells stimulated with fMLP which significantly higher compared to the FMF-A group ($P < 0.05$). Worth to mention that in the HD group, production of IL-1β by fMLP-stimulated PMNs in non-migrated was found to be higher than in migrated (**Figure S5**).

Actin Dysfunctions in Neutrophils From FMF Patients

To analyse the kinetic of actin polymerization, we have measured the amounts of F-actin in neutrophils induced by fMLP at 5s, 15s, 30s, 60s, 120s, and 180s time points *in vitro*. PMNs from FMF-R group displayed the lowest actin polymerization activity at all-time points both in the absence or presence of fMLP, which might be explained by the received colchicine therapy by the patients. The cells from the FMF-A group stimulated with fMLP displayed a maximal F-actin polymerization earlier (at 5s) than in HD and FMF-R groups (at 30s) ($P < 0.05$ and $P < 0.01$, respectively). In contrast, plateau levels of polymerized F-actin content were reached earlier in HD and FMF-A neutrophils (after 1 min), while plateau levels in FMF-R were reached later (after 2 min) (**Figure S6**). The faster polymerization activity of the cells in acute flares is confirmed by a higher rate of transmigration observed in our study (albeit not significant).

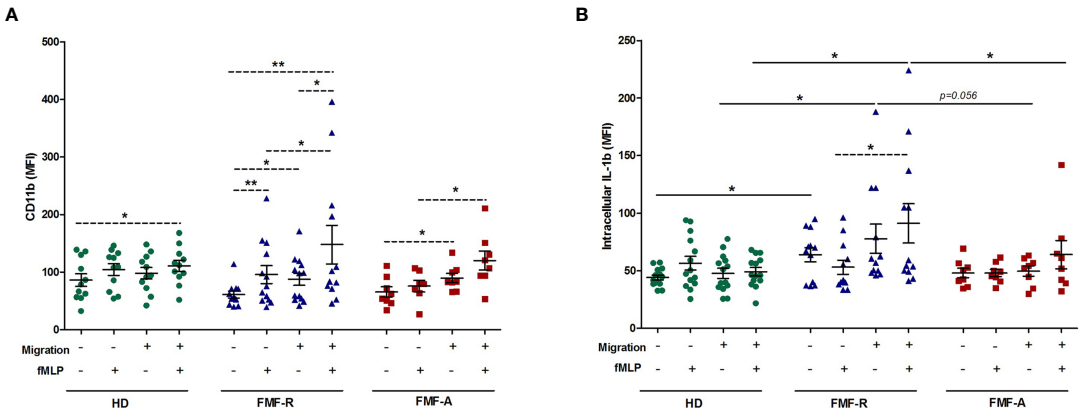

FIGURE 5 | Activation patterns of migrated and non-migrated PMNs. PMNs were allowed to transmigrate through 3-μm pore Polyester Membrane Inserts or cultured in 24-well plates (non-migrated control cells) for 2h in absence or presence of fMLP. **(A)** Surface expression of CD11b (MFI), and **(B)** Intracellular expression of IL-1β (MFI), analysed by flow cytometry. *$p < 0.05$; **$p < 0.01$.

Production of IL-1β by Neutrophils From FMF-R Group Is Increased in a Time-Dependent Manner

As was seen by migratory study, IL-1β expression in FMF-R cells was increased in induction-free media. To analyse time-dependent changes, we cultured PMNs continuously during 4h in RPMI media. The cells and supernatants were collected at 1, 2, 3, and 4h and analysed for IL-1β production and surface expression of CD11b. As shown in **Figure 6A**, cultivation of FMF-R and FMF-A cells has led to the increased production of IL-1β compared to the HD cells at different time points. Within the FMF-R group, an increase in IL-1β production was significant with the highest levels at 4h cultivation. In contrast, CD11b surface expression was gradually decreasing during the cultivation hours (**Figure 6B**). At 4h cultivation CD11b expression was higher on the cells from FMF-R and FMF-A groups compared to the HD group ($P = 0.064$ and $P < 0.01$, respectively).

Epinephrine Is Not Significant Inducer of PMNs Activation in FMF

Since stress has been linked to the FMF flares, next we addressed the question whether stress hormone epinephrine (Epi) might be implicated in abnormal activation of FMF PMNs. For this, different concentrations of Epi were used to induce neutrophil's response. As the analyses showed, there was no difference in expression of surface markers CD62L, CD11b on neutrophils exposed to different concentrations of epinephrine in all studied groups. In contrast, production of IL-1β was significantly increased in the supernatants from the cells exposed to LPS and LPS+Epi in FMF-R and FMF-A compared to those in the HD group (**Figure 7**). However, the differences in IL-1β concentrations between LPS-stimulated and LPS+Epi were not found which suggest that the observed effects of IL-1β were caused by LPS.

DISCUSSION

Despite pyrin function has been shown is an innate sensor for a number of pathogenic bacteria (5, 6, 8), it appeared that pyrin senses an event downstream of Rho modification, most likely involving the actin cytoskeleton modifications and do not directly detecting a microbial product (6, 7). Recently, Liston and Masters introduced a term named 'homeostasis-altering molecular processes' (HAMPs), which describes the ability of innate immunity to recognize novel infections and trigger sterile inflammation (22). Pyrin inflammasome can sense cellular imbalance rather than a distinct pathogen enabling it to provide a defence to a large number of infections even novel (22, 23). The hypothesis that pyrin senses the changes in the cytoskeleton organization (6, 7, 23) is further confirmed by our study showing spontaneous and fMPL-induced activation of PMNs from FMF patients after transmigration as seen by the increased expression of CD11b and IL-1β. Transendothelial migration is a crucial step in the inflammatory response allowing PMNs exit circulation and enter a tissue. PMN migration is mediated by polarized shape changes and mechanical interactions with the extracellular tissues, driven by the cytoskeleton and associated proteins (24, 25). The selective activation of small Rho GTPases, including Rho, Rac, and Cdc42 results in the regulation of actin networks required to form varying structures involved in cell motility (26). Whether cytoskeletal activation caused by cell transmigration in the absence of virulent agents is able to assemble pyrin inflammasome or increased activation of FMF PMNs after transmigration was caused by another unidentified mechanism are unknown. The fact that colchicine blocks activation of the pyrin inflammasome after RhoA inactivation, favour that microtubule/actin dynamics might control or regulate pyrin activation (27).

In parallel, we analysed the population of circulating PMNs in terms of their immunophenotypes and functionality. As it turned out, circulating PMNs displayed heterogeneity as seen by the diverse phenotypes and FSC/SSC in the diseased groups. PMNs

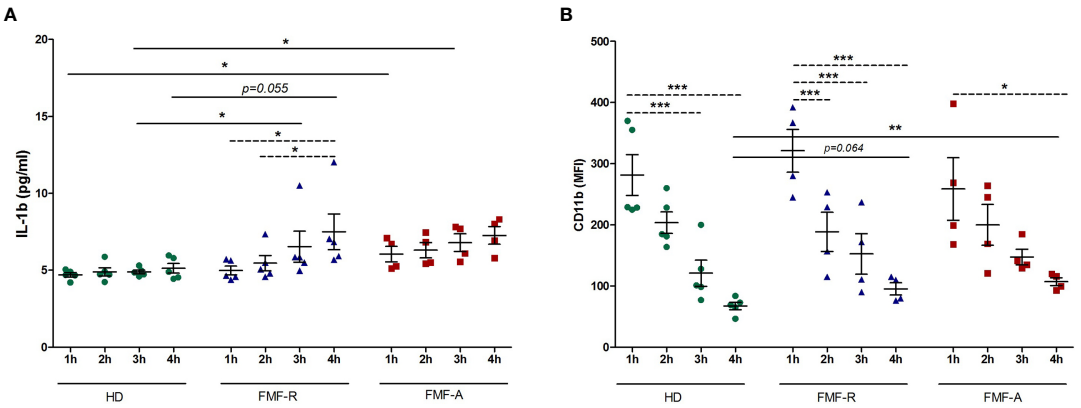

FIGURE 6 | Analysis of time-dependent kinetics of PMNs activation. The cells were isolated from HD, FMF-R and FMF-A patients and cultured for 1h, 2h, 3h and 4h in induction-free media. After each cultivation period, PMNs and culture supernatants were analysed. **(A)** Production of IL-1β in supernatants of cultured PMNs as assessed by ELISA; **(B)** Surface expression of CD11b (MFI) analysed by flow cytometry. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

rapidly change their characteristics and behaviour as they get activated, aged, or primed by different mediators, and are able to polarize and produce alternative effector or immune-regulatory molecules (28). In norm, neutrophils display different phenotypes based on the time they egress bone marrow and enter the circulation (fresh neutrophils) to the time they leave the circulation (aged neutrophils). Fresh neutrophils are characterized by $CD62L^{hi}CXCR4^{lo}$ phenotype, while aged neutrophils are $CD62L^{lo}CXCR4^{hi}$ (29, 30). CXCR4 is a chemokine receptor regulating the distribution and trafficking of neutrophils. Up-regulation of CXCR4 on aged PMNs was shown to promote their re-entering vasculature and migrating selectively back to the bone marrow in response to CXCL12 (31). Additionally, aged neutrophils are smaller, contain fewer granules and upregulate CD11b and CD49d, which promote their migration and adherence to inflamed tissues (29). In our study, FMF-A cells were characterized by the small cell size and granularity, increased number of the cells with SSC^{hi} parameters, and up-regulated CXCR4 receptor which reflects the presence of aged PMNs in the circulation of the patients in acute period. The aged PMNs with up-regulated CD11b and CXCR4 observed in the acute period of the disease may reflect their activated state and the direction of migration to the bone marrow resulting in the clearance of these leukocytes by resident macrophages (30). Here these aged neutrophils might be involved in the mobilization of fresh neutrophils from the bone marrow to replenish the number of circulating PMNs (32). FMF-R cells were of normal size and granularity and also characterized by the up-regulated CD11b, CD49d, CXCR4, and CD62L which suggests the mixed pool of the cells containing both fresh and aged fraction of the cells in the circulation. Heterogeneous population of FMF-R cells might also reflect a high turnover of

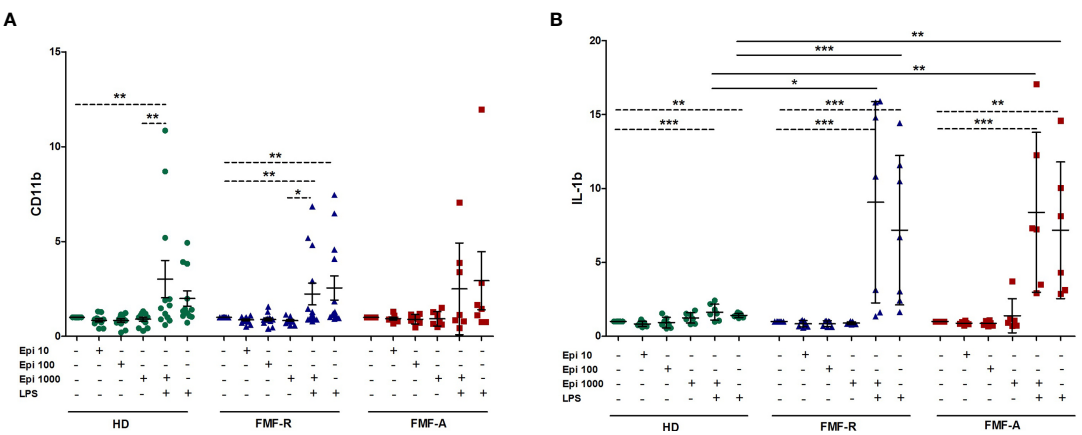

FIGURE 7 | Effect of stress-related hormone Epinephrine (Epi) on PMNs activation. Whole blood cells were cultured in the absence or presence of Epi (10, 100, 1000 μM), LPS (100 ng/ml) or LPS (100 ng/ml) + Epi (1000 μM) for 4h. **(A)** Surface expression of CD11b (MFI) analysed by flow cytometry; **(B)** IL-1β production by whole blood cells was assessed in culture supernatants using ELISA. Data presented as fold change for stimulated CD11b and IL-1β production versus unstimulated ones. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

circulating PMNs and their activated status even in the absence of acute flares. Results of the current study may explain why tissue-infiltrated PMNs do not cause immune-mediated tissue damage. Rather, sterile tissue-infiltrated aged PMNs undergo reverse transmigration migrating back into the circulation and then to the bone marrow *via* CXCR4 (29, 33, 34). This phenomenon is a form of biologic recycling, an event that is unlikely to occur in infections (34).

In addition to the changes in the heterogeneity of neutrophils, a down-regulation of CD16 (FcγRIII) and CD32 (FcγRIIA) in PMNs from FMF-A group were observed, which also confirms aged/activation status of the cells during acute flares. Modulation of FcγRII- and FcγRIII-induced cell activation might be achieved by receptor internalization or shedding of the extracellular portion of the FcγRs. The cleavage of FcRs from the cell surface has been shown after neutrophil stimulation and during neutrophil apoptosis (35). It was shown that cross-linking of FcγRIIA (spontaneously expressed on neutrophils) leads to the activation, degranulation, and production of inflammatory mediators and ROS (35). A decrease in expression of FcγRIII was shown in heat-stressed neutrophils which was suggested to contribute to the anti-inflammatory signalling at inflamed sites and preceded the development of spontaneous apoptosis (36). In contrast to FcγRII and FcγRIII, FcγRI was significantly high in the FMF-A group which has been shown to be strongly upregulated in the presence of inflammatory cytokines and reflects disease activity in numerous inflammatory conditions (37, 38). The reason for multidirectional expression FcγRs is unknown. A possible explanation might reside in differential differences in monomeric/multimeric IgGs or other factors in the blood of FMF patients during acute flares, however experimental data to support this hypothesis are missing.

In conclusion, the current study raised the possibility of heightened sensitivity of mutated pyrin inflammasome towards cytoskeletal modifications in the absence of pathogens. Whether pyrin in turn modulates biomechanics of the cells is unknown. Further studies should be directed towards understanding whether cytoskeleton perturbations alone are able to influence pyrin phosphorylation state.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Institute of Molecular Biology NAS RA (IRB IORG0003427). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

GM conceived the study and planned the experiments, interpreted the data, and wrote the manuscript. NM and GA collected the patient samples and clinical data. AM, DP, and SG performed the analysis. AM and DP performed the statistical, data mining analysis and designed the figures. AM, DP, and GA revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.672728/full#supplementary-material

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The Chromatin Accessibility Landscape of Peripheral Blood Mononuclear Cells in Patients With Systemic Lupus Erythematosus at Single-Cell Resolution

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Objective: Systemic lupus erythematosus (SLE) is a complex autoimmune disease, and various immune cells are involved in the initiation, progression, and regulation of SLE. Our goal was to reveal the chromatin accessibility landscape of peripheral blood mononuclear cells (PBMCs) in SLE patients at single-cell resolution and identify the transcription factors (TFs) that may drive abnormal immune responses.

Methods: The assay for transposase accessible chromatin in single-cell sequencing (scATAC-seq) method was applied to map the landscape of active regulatory DNA in immune cells from SLE patients at single-cell resolution, followed by clustering, peak annotation and motif analysis of PBMCs in SLE.

Results: Peripheral blood mononuclear cells were robustly clustered based on their types without using antibodies. We identified twenty patterns of TF activation that drive abnormal immune responses in SLE patients. Then, we observed ten genes that were highly associated with SLE pathogenesis by altering T cell activity. Finally, we found 12 key TFs regulating the above six genes (*CD83*, *ELF4*, *ITPKB*, *RAB27A*, *RUNX3*, and *ZMIZ1*) that may be related to SLE disease pathogenesis and were significantly enriched in SLE patients ($p < 0.05$, FC >2). With qPCR experiments on *CD83*, *ELF4*, *RUNX3*, and *ZMIZ1* in B cells, we observed a significant difference in the expression of genes (*ELF4*, *RUNX3*, and *ZMIZ1*), which were regulated by seven TFs (EWSR1-FLI1, MAF, MAFA, NFIB, NR2C2 (var. 2), TBX4, and TBX5). Meanwhile, the seven TFs showed highly accessible binding sites in SLE patients.

Conclusions: These results confirm the importance of using single-cell sequencing to uncover the real features of immune cells in SLE patients, reveal key TFs in SLE-PBMCs, and provide foundational insights relevant for epigenetic therapy.

Keywords: systemic lupus erythematosus, single-cell chromatin accessible assay, peripheral blood mononuclear cells, transcription factor, marker

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that affects every organ and system in the body. The loss of tolerance in the immune system results in autoantibody production, immune complex deposition, and complement activation, which lead to systemic inflammation and target tissue damage (1). Genetic factors are essential in SLE susceptibility, according to family studies and the concordance rate between twins (2). Nevertheless, gene sequences alone explain only a minority of SLE heritability. Epigenetic marks have emerged as keys to understanding a portion of the missing heritability (3), and noncoding elements within cell-type-specific genomes are vital to understanding SLE pathogenesis (4). However, little is known about the related pathogenesis.

Genetic and transcriptomic analyses have unveiled some genes and noncoding loci associated with SLE. As a result, over 80 SLE risk loci are known to influence SLE predisposition, and the majority of risk variants alter regulatory elements that govern gene expression (5). Since chromatin accessibility plays a vital role in gene regulation and genome stability, and since changes in chromatin accessibility patterns may alter the accessibility of the genome's regulatory regions to critical proteins, chromatin accessibility patterns are emerging as an essential component of human diseases (6). Notably, the assessment of chromatin accessibility in immune cells from SLE patients lags behind that of other genome-wide measurements, such as DNA modifications or transcription (7).

The assay for transposase-accessible chromatin using sequencing (ATAC-seq) (8) technique is widely used to profile genome-wide chromatin accessibility patterns at base-pair resolution due to its simplicity and sensitivity. A plate-based ATAC-seq method for single-cell analysis (scATAC-seq) was recently developed to map open chromatin regions and identify regulatory regions (9). This method has the advantages of (i) recognizing different cell types, including subtle and rare cell subtypes, (ii) revealing cell type-specific transcription factor (TF) motifs, and (iii) allowing the exploration of cell type-specific gene regulatory networks. Using scATAC-seq to study blood-derived cells in SLE patients helps to understand the role of involved cell subsets without bias and discloses the mechanism of cell type-specific gene regulation.

Therefore, we used the scATAC-seq method to analyze the open chromatin regions of peripheral blood mononuclear cells (PBMCs) from seven SLE patients and 12 healthy controls. We identified the cell types and investigated novel and rare cell populations in SLE patients after performing cell clustering. We also parse cell-type-specific regulatory patterns and summarize TF motifs with significant differences between SLE patients and healthy controls. With Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, we explored SLE-related target genes and critical signaling pathways to provide mechanistic insights into the pathogenesis of SLE.

MATERIAL AND METHODS

Human PBMC Collection

The classification of SLE patients was based on the 2012 guidelines (10). All SLE subjects (n = 7, female, mean age 33 ± 13 years, SLE disease activity (SLEDAI) >10) and healthy controls (n = 12, male/female = 6/6, mean age 34 ± 9 years) were recruited from outpatient clinics or from among the medical staff in Shenzhen People's Hospital (Shenzhen, China). No patient had been treated with an immune suppressant within the previous three months. The human sample studies and procedures were approved by the ethics committees of both Shenzhen People's Hospital and Guangzhou Institutes of Biomedicine and Health (Guangzhou, China) (LL-KY-2019590) with informed written consent.

Eight milliliters of peripheral venous blood was drawn from both SLE patients and control subjects, followed by the addition of Ficoll–Hypaque solution (GE Healthcare, Switzerland) and density-gradient centrifugation. Red blood cell (RBC) lysis buffer was added to eliminate the remaining RBCs, and chilled PBS was used to wash the PBMCs. After quantification with a cell counting plate, PBMCs were stored on ice for further analysis.

scATAC-seq Library Construction and Sequencing

As described previously (11) and as described on the manufacturer's website https://support.10xgenomics.com/single-cell-atac, scATAC-seq libraries were generated according to the Chromium Single Cell ATAC protocol (10x GENOMICS, CG000168). In brief, the isolated nuclei were incubated with the Transposition Mix. Then, transposed nuclei, barcoded gel beads, partitioning oil, and a Master Mix were loaded on a Chromium Chip E. Next, silane magnetic beads and solid phase reversible immobilization (SPRI) beads were used. After adding a sample index (P7) and Read 2 (Read 2N) sequence, the final libraries containing the P5 and P7 primers were constructed *via* PCR with Illumina® bridge amplification. Finally, Illumina® sequencer compatibility, sequencing depth and run parameters, sample indices, library loading, and pooling were summarized.

Raw scATAC-seq Data Processing

All protocols for data processing are available on the following website: https://support.10xgenomics.com/single-cell-atac/software/pipelines/latest/algorithms/overview. The main steps are as follows:

Barcode Processing

The 16 bp barcode sequence was obtained from the "I2" index read. Each barcode sequence was checked against a 'whitelist' of correct barcode sequences, and the frequency of each whitelisted barcode was counted. All observed barcodes with two or fewer differences (Hamming distance ≤2) from the whitelisted sequence were scored based on the abundance of the incorrect bases' read data and quality values. Consequently, if the probability of being the real barcode based on this model was more than 90%, the observed barcode outside the whitelist was corrected to a whitelisted barcode.

Genome Alignment

The reference-based analysis was performed through the Cell Ranger ATAC pipeline (https://support.10xgenomics.com/single-cell-atac/software/overview/welcome). First, the adapter and primer oligo sequences were trimmed. Then, the cutadapt (12) tool was used to identify and trim the reverse complement sequence. Next, BWA-MEM (13) with default parameters was applied to align the trimmed read pairs of more than 25 bp to GRCh38.

Duplicate Marking

Groups of read pairs across all barcodes were identified to find duplicate reads, where the 5' ends of both R1 and R2 had identical mapping positions on the reference. Thus, the unique read pair was reported as a fragment in the file.

Peak Calling

The combined signal from each fragment's ends was analyzed to identify regions of the genome enriched for open chromatin. First, the number of transposition events at each base pair along the genome was counted. A smoothed profile of these events with a 401 bp moving window around each base pair and fitting a ZINBA-like mixture model was generated. The model consisted of a geometric distribution to model the zero-inflated count, a negative binomial distribution to model noise, and another negative binomial distribution to model the signal. Meanwhile, a signal threshold that determined whether a region was a peak signal (enriched for open chromatin) or noise was set based on an odds ratio of 1/5. Next, peaks within 500 bp of each other were merged to produce a position-sorted BED file.

Cell Calling

For each barcode, the mapped high-quality fragments that passed all filters were recorded, and the number of fragments that overlapped any peak regions was determined to separate the signal from the noise. Briefly, a depth-dependent fixed count was first subtracted from all barcode counts to model whitelist contamination. Notably, this fixed count was the estimated number of fragments per barcode that originated from a different GEM, assuming a contamination rate of 0.02. A mixture model of two negative binomial distributions to capture the signal and noise was set, and barcodes that corresponded to real cells were separated from the non-cell barcodes by setting an odds ratio of 1,000. Next, a count matrix consisting of the counts of fragment ends within each peak region for each barcode was produced. The matrix was filtered to include only cell barcodes and then used in subsequent analysis.

Clustering and t-SNE Projection

Based on Cell Ranger ATAC, dimensionality reduction was first performed *via* latent semantic analysis (LSA) (14). Then, the data were normalized *via* the inverse-document frequency (IDF) transform. Singular value decomposition (SVD) was performed on this normalized matrix using IRLBA. Before clustering, depth normalization was carried out by scaling each barcode data point to the unit L2-norm in the lower dimensional space. Next, k-medoids clustering that produced three to six clusters and graph-based clustering and visualization *via* t-SNE were provided (15), and the data were normalized to the unit norm before performing graph-based clustering and t-SNE projection.

Peak Annotation

BEDtools was used to associate each peak with a gene based on the closest TSS (16). Peak-related GO enrichment analysis was performed. First, all peak-related genes were mapped to GO terms in the Gene Ontology database (http://www.geneontology.org/). Then, gene numbers were calculated for each term, and GO terms significantly enriched in peak-related genes compared to the genome background were defined by the hypergeometric test. The p-value was calculated using the following formula:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

N is the number of all genes with GO annotation; n is the number of peak-related genes in N; M is the number of all genes that are annotated with the specific GO terms; and m is the number of peak-related genes in M. The calculated p-value was adjusted by the FDR, and an FDR-corrected p-value of less than 0.05 was used as the threshold. GO terms meeting this condition were significantly enriched in peak-related genes. This analysis identifies the main biological functions of related genes.

Similar to GO analysis, KEGG enrichment analysis was also performed to identify significantly enriched metabolic pathways and signal transduction pathways in peak-related genes compared to the whole-genome background. The p-value was calculated using the same equation as that in GO analysis. Notably, N is the number of all transcripts with KEGG annotation, and M is the number of transcripts annotated to specific pathways.

TF Motif Analysis

To identify TF binding sites, the position weight matrix (PWM) of the TF motifs was obtained from the JASPAR database (17),

and each peak was scanned using MOODS (https://github.com/jhkorhonen/MOODS). The threshold p-value was 1E−7, and the background nucleotide frequencies were set to be those observed in the peak regions of each GC bucket. The list of motif-peak matches was unified across these buckets to avoid GC bias in the scan. To analyze motif enrichment, the reads for a TF motif within a barcode were first counted. Then, the ratio of this number to the total read number for that barcode was calculated. Next, the value was normalized to read depth. TF motif enrichment was detected by z-scoring the distribution over barcodes of these proportion values. A modified z-score calculation using the median and the scaled median absolute deviation from the median (MAD) was performed to ensure that the analysis was robust.

Differentially Accessible Peak Analysis

Cell Ranger ATAC was used to perform the fast asymptotic beta test used in edgeR to find differentially accessible motifs between groups of cells. For each cluster, the algorithm was run on that cluster versus all other cells, yielding a list of motifs that were differentially expressed in that cluster relative to the rest of the sample. Finally, the relative library size was computed as the total cut site count for each cell divided by the median number.

RNA Extraction and qPCR

Three SLE patients (female, mean age 30 ± 8 years, SLEDAI >10) and three healthy controls (female, mean age 32 ± 2 years) were recruited, 8 ml of Peripheral blood was collected from each sample, and PBMCs were isolated using density-gradient centrifugation with Ficoll–Hypaque. Then B cells were isolated from PBMCs by CD19 positive selection using MACS magnetic beads (Miltenyi). The RNA was extracted from B cells using RNAiso Plus (TAKARA, 9109), chloroform, and isopropanol. Total RNA was reversed transcribed into cDNA with PrimeScript RT Master Mix (TAKARA, RR036A). The cDNA was then used for quantitative real-time PCR (RT-qPCR) analysis with SYBR Premix Ex Taq II (TAKARA, RR820A) and PCR primers. The relative expression of genes in B cells was calculated by Student's t-test, and the differences were considered significant if the P-value was less than 0.05.

The primer sequences for genes were as follows: CD83 forward, GGTGGCTTGCTCCGAAGATG, CD83 reverse, TGACCCAGGAGACCGTGTAG; ELF4 forward, GACTG GAGTTGGACGACGTTC, ELF4 reverse, GGTGGCCTCAT TGTCATCTGTC; RNUX3 forward, CGAGCATCAGCAGCC TCAG, RNUX3 reverse, TGTCCCGTAGTAGAGGTGGTAG; ZMIZ1 forward, GCAGCAGAACACCAACCAG; ZMIZ1 reverse, GTTGCCGCCTGGATTCATG; Actin forward, CATGTACGTTGCTATCCAGGC, Actin reverse, CTCCTTAATGTCACGCACGAT.

Statistical Analysis

For data analysis, Cell Ranger ATAC software was used to perform the initial data processing and downstream analysis. As described above, Loupe Cell Browser interactive visualization software was used to generate scATAC-seq peak profiles for cell clusters. The p-values in this manuscript were calculated with Loupe Cell Browser 3.1.1 through the difference analysis feature and adjusted using the Benjamini–Hochberg correction for multiple tests.

RESULTS

Cell Type Identification and Cell Type-Specific TF Motif Exploration

To construct the landscape of cell type-specific open chromatin features in SLE patients, we used the scATAC-seq method to analyze PBMCs in both SLE patients (PBMC_SLE) and healthy controls (PBMC_NC) (**Figure 1A**). It is desirable to have the same sex ratio for the SLE patients and HCs (18). Since we used a fresh sample for higher data quality, our study focuses on the general landscapes of immune cells instead of cell heterogeneity in SLE patients. In addition, the results of scATAC-seq from six healthy male controls plus six healthy female controls were similar to those from seven healthy female controls. Thus, it is acceptable to further analyze PBMCs from seven SLE patients and 12 healthy controls (**Table 1**). As a result, we observed periodic peaks as nucleosome-bound fragments (**Figure 1B**) and enrichment around transcription start sites (TSSs) for both the PBMC_SLE and PBMC_NC groups (**Figure 1C**). Meanwhile, we obtained 4,993 cells from the PBMC_SLE group and 8,393 cells from the PBMC_NC group.

Among the 13,386 cells, we captured a median of 5,344 unique fragments per cell. The fraction of fragments overlapping the targeted region, including TSSs, enhancer regions, promoter regions, and nucleosome-free regions, was 49.6%. The fraction of transposition events in peaks in cell barcodes was 18.3%. The total number of mapped read pairs and unique fragments in the PBMC_SLE library were 174,494,332 and 158,667,523, respectively, and in the PBMC_NC library, 193,333,851 and 165,563,843, respectively. After peak analysis, we used Cell Ranger ATAC for dimension reduction and t-SNE projection. As a result, we divided 13,386 cells into six clusters.

Based on the activity of cell marker genes, we identified and annotated five clusters (19, 20). They were T cells (cluster 1), natural killer (NK) cells (cluster 2), monocytes (cluster 3), B cells (cluster 5), and dendritic cells (DCs) (cluster 6). Interestingly, we did not observe any enriched markers in cluster 4 (**Figures 1D, E**). Since we purified PBMCs to analyze lymphocytes and all data passed quality control, cluster 4 should be an immune cell subtype. Furthermore, cluster 4 seems to be a part of cluster 1 (T cells), and our UMAP results confirmed this hypothesis. Thus, cluster 4 was regarded as a T cell subtype. Taking genes based on the enriched peaks in cluster 4 ($p < 0.05$, $|FC| > 1.2$) into consideration, this T cell subtype was involved in the metaphase/anaphase transition of the cell cycle (**Figure 3A**). Our results indicated that cluster 4 represented proliferative T cells (**Table 2** and **Supplementary Figure S1**).

To explore cell type-specific TF motifs, we summarized the motifs that were significantly enriched in each cluster of PBMC_SLEs ($p < 0.05$ and fold change (FC) >1.2). Notably,

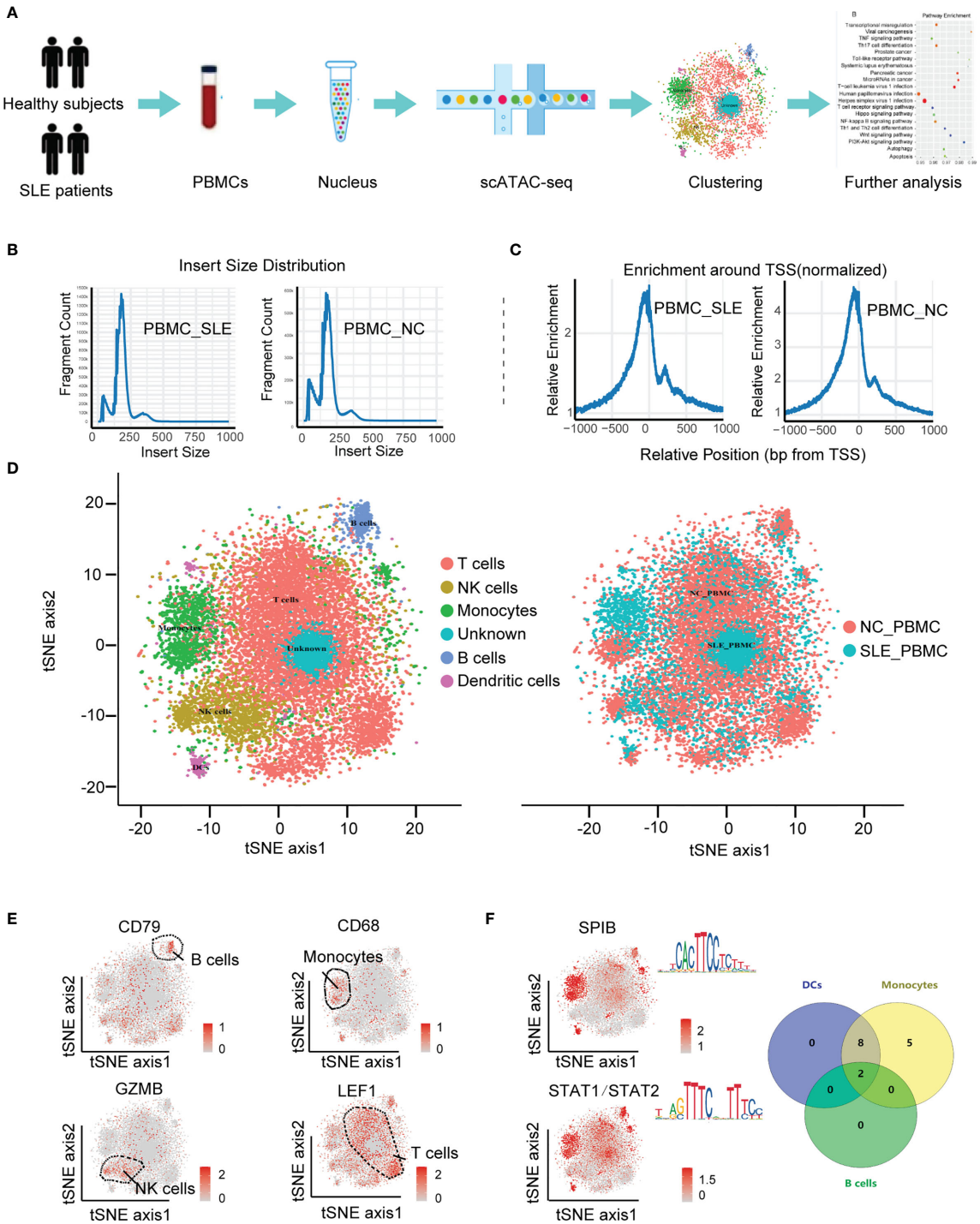

FIGURE 1 | Cell-type-specific clustering of human PBMCs according to scATAC-seq. **(A)** Schematic showing the process of isolating PBMCs for scATAC-seq; **(B)** Histogram of the distribution of fragment lengths in reads from the SLE_PBMC and NC_PBMC groups; **(C)** Histograms showing enrichment of fragments at TSSs; **(D)** tSNE plot of cellular populations in the SLE_PBMC and NC_PBMC groups; **(E)** tSNE plot of canonical cell markers used to label clusters, color-coded for expression levels (gray to red); **(F)** tSNE plot of cell type-specific TF motifs (left), color-coded for expression levels (gray to red), and Venn diagram showing the distribution of 15 TF motifs with significantly differences ($p < 0.05$) between different clusters (right). PBMCs, peripheral blood mononuclear cells; scATAC-seq, assaying transposase-accessible chromatin in single-cell sequencing; SLE_PBMC, PBMCs from patients with systemic lupus erythematosus (SLE); NC_PBMC, PBMCs from healthy controls; NK cells, natural killer cells; DCs, dendritic cells; TF, transcription factor; TSSs, transcription start sites. The p-values were calculated with Loupe Cell Browser 3.1.1 through the difference analysis feature and adjusted using the Benjamini–Hochberg correction for multiple tests.

TABLE 1 | Clinical features of patients with SLE and NC studied for scATAC-seq experiments.

Clinical characteristic (number of samples)	SLE (n = 7)	HC (n = 12)
Age (years)	33 ± 13	34 ± 9
Sex, Female/Male	7/0	6/6
Joint injury	7/7	NA
Skin lesions	3/7	NA
Hematologic abnormalities	2/7	NA
dsDNA (IU/ml)	710 ± 474	<24
ANA (AU/ml)	3,462 ± 1,824	<32
SLEDAI	>10	NA

SLE, systemic lupus erythematosus; NC, healthy controls; NA, not applicable; SLE disease activity (SLEDAI) >10.

these TF motifs were not significantly different between the PBMC_SLE and PBMC_NC libraries ($p > 0.05$). In total, we found two TF motifs in B cells, 15 TF motifs in monocytes, and 10 TF motifs in DCs (**Figure 1F**). Moreover, the 15 TF motifs in monocytes included the 10 TF motifs in DCs, and the ten motifs in DCs included the two motifs in B cells. Regarding the remaining two clusters (T cells and NK cells), there was no TF motif with an FC value of more than 1.2 (**Table 2**).

Comparison of Open Chromatin Patterns Between PBMC_SLE and PBMC_NC

When calculating the cell ratios in both the PBMC_SLE and PBMC_NC groups, we found a significant difference in T cells and B cells (Student's t-test, $p < 0.05$, FDR <0.05). Notably, proliferative T cells (cluster 4) existed only in PBMCs from SLE patients (**Figure 2A**). Then, we counted the number of significantly different loci in each cluster (Student's t-test, $p < 0.05$, $|FC| > 1.2$). We observed 2,092 significantly different peaks between the PBMC_SLE and PBMC_NC·libraries. In detail, there were 447 significantly different peaks in B cells, 544 in DCs, 680 in monocytes, 347 in NK cells, 58 in T cells, and 16 in the unknown group (**Figure 2B**). In addition, we calculated the number of significantly enriched motifs in each cluster ($p < 0.05$, FC >1.2). We obtained 7, 68, 20, 102, 45, and 21 significantly enriched motifs in T cells, NK cells, monocytes, B cells, DCs, and the unknown cluster, respectively (**Figure 2C**). Our results indicated that both proliferative T cells and non-T cells were active in SLE patients.

We then reclustered T cells, NK cells, monocytes, B cells, and DCs separately for further analysis. In addition, we obtained 20 subclusters (**Figure 2D**). The cell number ratios of subcluster 0 in monocytes (Monocyte-0) and subcluster 1 in NK cells (NK-1) increased by more than three times in the PBMC_SLE group compared with the PBMC_NC group. In contrast, the cell number ratios of subcluster 1 (B-1) and subcluster 3 (B-3) in B cells, subcluster 2 in monocytes (Monocyte-2), and subcluster 1 in T cells (T-1) decreased by more than three times (**Figure 2E**). Interestingly, the number of significantly different peaks in each subcluster of NK cells, monocytes, B cells, and DCs was much larger than that in the T cell subsets ($p < 0.05$, $|FC| > 1.2$, 748 ± 426 *vs.* 53 ± 47) (**Figure 2F**). Similarly, the number of significantly enriched motifs in each subgroup of NK cells, monocytes, B cells, and DCs was much larger than that in the T cell subgroups ($p < 0.05$, FC >1.2, 72 ± 46 *vs.* 7 ± 8), except for subgroup 3 in monocytes (Monocyte-3), which showed 12 significantly enriched motifs. Meanwhile, seven subclusters showed enriched motifs with a FC greater than 2 (**Figure 2G** and **Table 3**).

Functional Analysis of Significantly Changed Peaks in PBMC_SLE

In B cells, DCs, monocytes, NK cells, and T cells, we chose peaks with absolute FC values greater than 1.2 for GO and KEGG analysis. The observed abnormal genes in different cells were found to be involved in various pathways. For example, B cell genes that differed between the PBMC_SLE and PBMC_NC groups were critical in neural tube formation and closure (**Figure 3B**). In contrast, the abnormal genes in DCs may play a vital role in neutrophil-mediated immunity, according to GO analysis. In monocytes and NK cells, the abnormal genes showed a high likelihood ($p < 0.001$) of being involved in antigen processing and presentation and ubiquitin-like protein ligase binding. Meanwhile, abnormal T cell genes may be related to kinase activity and MHC class II protein complex binding.

With further GO and KEGG analysis of genes with differential expression between the PBMC_SLE and PBMC_NC groups ($p < 0.05$, $|FC| > 1.2$) in each subcluster of T cells, NK cells, monocytes, B cells, and DCs, we obtained more detailed information. In detail, the abnormal genes in B-0, B-1, B-2, and B-3 may be involved in axon part, the regulation of RNA splicing and apoptotic signaling pathway, transcription initiation from RNA polymerase II promoter, and T cell activation, respectively. The differentially expressed genes in DC-0 and DC-2 participate in endocytosis and T cell activation in DC subclusters, respectively. Meanwhile, the differentially expressed genes in Monocyte-0, Monocyte-1, Monocyte-2, and Monocyte-3 were active in neutrophil-mediated immunity, Toll-like receptor signaling pathways, MAPK signaling pathway, and T cell activation, respectively. In NK cell subclusters, the differentially expressed genes in NK-0, NK-1, NK-2, NK-3, and NK-4 were critical in autophagy, kinase regulator activity, negative regulation of phosphorylation, transferase activity, and negative regulation of dephosphorylation, respectively (**Figure 3C**). In T cell subclusters, the abnormal genes in T-0 and T-2 were related to histone binding and Lys63-specific deubiquitinase activity, respectively (**Figure 3D**). Notably, we did not find an available record with significantly enriched peaks in DC-1, T-0, and T-2.

Unexpectedly, in SLE patients, B-3, DC-2, and Monocyte-3 not only showed a decrease in cell number ratio but also displayed obvious abnormal signals related to T cell activation, with 59, 75, and 55 differentially expressed genes between the PBMC_SLE and PBMC_NC groups ($p < 0.05$, $|FC| > 1.2$), respectively (**Figures 3E–G**). As mentioned above, proliferative T cells (cluster 4) were found only in PBMCs from SLE patients. Thus, genes contributing to T cell activity may be important factors that drive T cell proliferation and lead to an abnormal immune response in SLE patients. After deduplication, there were 137 genes in B-3, DC-2, and Monocyte-3, which were involved in T cell activity (**Figure 3H**). Meanwhile, ten genes (*BCL11B*, *CCR7*, *CD83*, *ELF4*, *ITPKB*, *NCK2*,

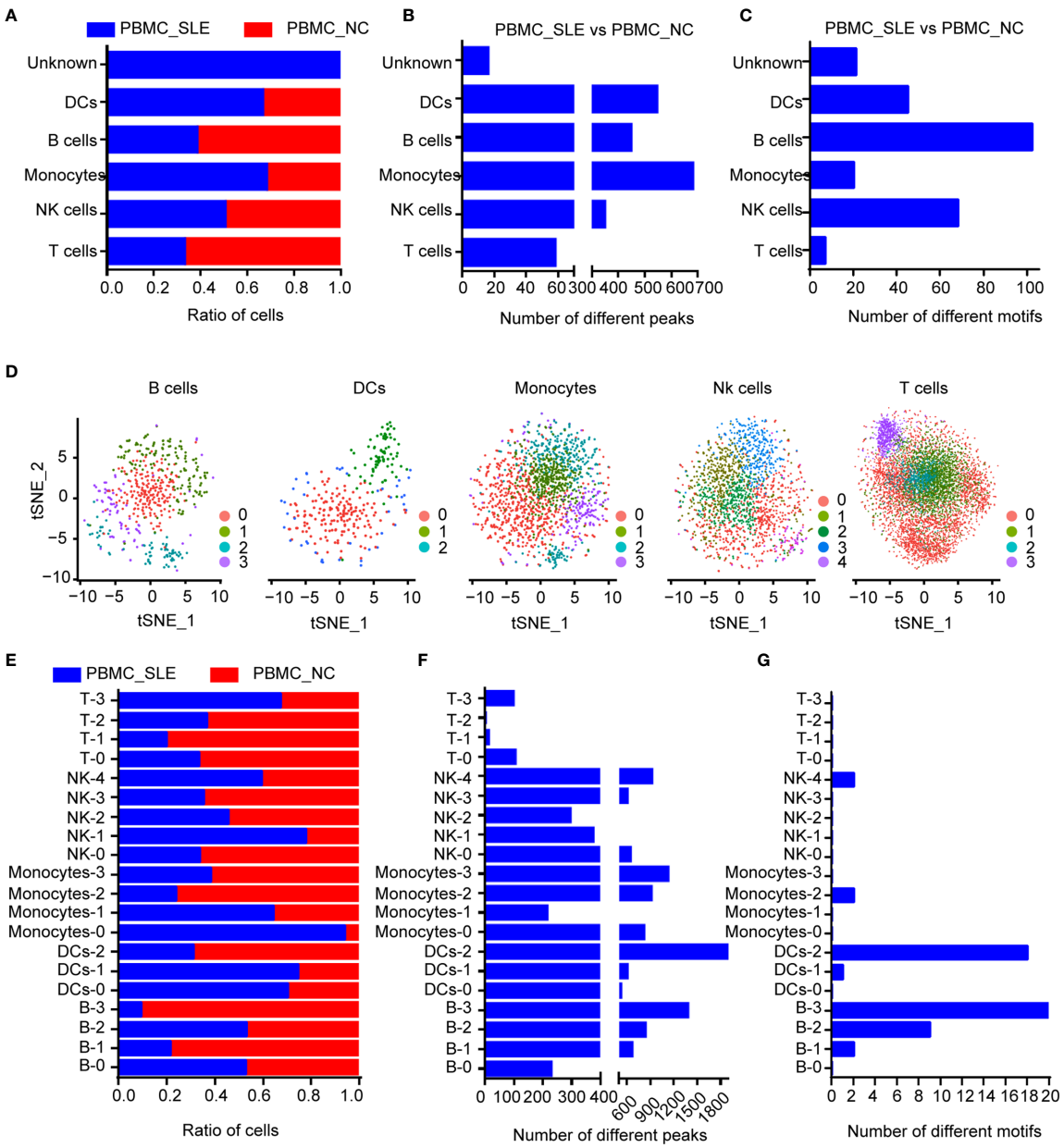

FIGURE 2 | Epigenomic analysis of human PBMCs. **(A)** Cell ratios in each cell type for comparison between the SLE_PBMC and NC_PBMC libraries; **(B)** Number of different peaks in each cell type for comparison between the SLE_PBMC and NC_PBMC libraries ($p < 0.05$); **(C)** Number of different TF motifs in each cell type for comparison between the SLE_PBMC and NC_PBMC libraries ($p < 0.05$); **(D)** tSNE plot of B cells, DCs, monocytes, NK cells and T cells, color-coded by their associated subcluster; **(E)** Cell ratios in each subcluster for comparison between the SLE_PBMC and NC_PBMC libraries; **(F)** Number of different peaks in each subcluster for comparison between the SLE_PBMC and NC_PBMC libraries ($p < 0.05$); **(G)** Number of different TF motifs in each subcluster for comparison between the SLE_PBMC and NC_PBMC libraries ($p < 0.05$); PBMCs, peripheral blood mononuclear cells; SLE_PBMC, PBMCs from patients with systemic lupus erythematosus (SLE); NC_PBMC, PBMCs from healthy controls; NK cells, natural killer cells. The p-values were calculated with Loupe Cell Browser 3.1.1 through the difference analysis feature and adjusted using the Benjamini–Hochberg correction for multiple tests.

NKAP, *RAB27A*, *RUNX3*, *ZMIZ1*) were found in all three subgroups, which suggests that these genes should be prioritized as targets for therapy (**Figure 3H**). Further analysis indicated that *BCL11B* was highly enriched in both B-3 and Monocyte-3 compared with other B cell subclusters and monocyte subclusters, respectively (**Figure 3I**). This result indicates that *BCL11B* may be used as a marker to purify B-3 and Monocyte-3 cells from B cells and monocytes, respectively.

Exploration of Key TFs in PBMC_SLE

Since we found ten genes that may be important for understanding SLE pathogenesis and further targeted therapy,

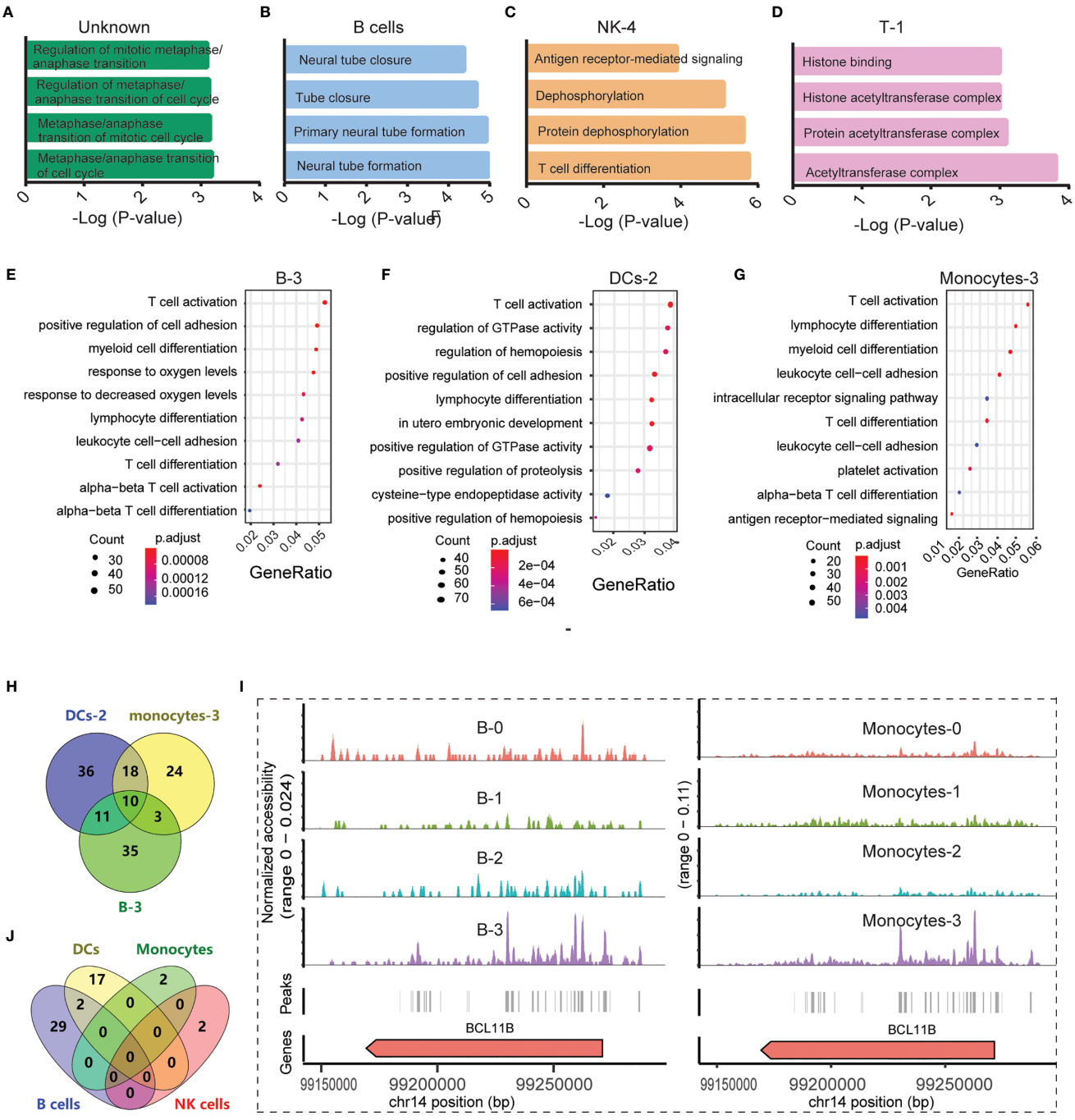

FIGURE 3 | Functional analysis of significantly differential peaks between the SLE_PBMC and NC_PBMC libraries (p <0.05). **(A)** GO analysis of 16 differential genes between the SLE_PBMC and NC_PBMC libraries in the unknown group; **(B)** GO analysis of 447 differential genes between the SLE_PBMC and NC_PBMC libraries in B cells; **(C)** GO analysis of 917 differential genes between the SLE_PBMC and NC_PBMC libraries in subcluster 4 of NK cells (NK-4); **(D)** GO analysis of 11 differential genes between the SLE_PBMC and NC_PBMC libraries in subcluster 1 of T cells (T-1); **(E)** GO analysis revealing the 10 most significant pathways in subcluster 3 of B cells (B-3); **(F)** GO analysis revealing the 10 most significant pathways in subcluster 2 of DCs (DC-2); **(G)** GO analysis revealing the 10 most significant pathways in subcluster 3 of monocytes (Monocyte-3); **(H)** Venn-diagram showing distribution of genes corresponding to T cell activation in **(E–G)**; **(I)** Example locus near BCL11B with differential accessibility across B cell subclusters and monocyte subpopulations; **(J)** Venn diagram showing the distribution of 52 observed enriched TF motifs between the SLE_PBMC and NC_PBMC libraries. GO, Gene Ontology; SLE_PBMC, PBMCs from patients with systemic lupus erythematosus (SLE); NC_PBMC, PBMCs from healthy controls; NK cells, natural killer cells; DCs, dendritic cells; TF, transcription factor. The p-values were calculated with Loupe Cell Browser 3.1.1 through the difference analysis feature and adjusted using the Benjamini–Hochberg correction for multiple tests.

TABLE 2 | Identified markers and transcription factors in each cluster for scATAC-seq experiments.

Clusters	Cell types	Markers	Transcription factors
1	T cells	*CD3D*, *CD3G*, *CD8A*, *IL2RA*	None
2	Natural killer cells	*GZMB*, *KLRD1*, *NKG7*	None
3	Monocytes	*CD14*, *CD68*, *ITGAM*	EHF, ELF1, ELF3, ETV4, EWSR1-FLI1, GABPA, IKZF1, IRF1, KLF5, MAZ, SPIB, SPI1, STAT1::STAT2, ZKSCAN5, ZNF263,
4	Unknown (Proliferative T cells)	None	None
5	B cells	*CD79A*, *CD79B*, *MS4A1*	IRF1, STAT1::STAT2
6	Dendritic cells	*CST3*	EHF, ELF1, ELF3, EWSR1-FLI1, IKZF1, IRF1, SPIB, SPI1, STAT1::STAT2, ZKSCAN5

the TFs regulating these genes were explored. In total, 157 TFs were found to be involved in regulating these nine genes (*BCL11B*, *CCR7*, *CD83*, *ELF4*, *ITPKB*, *NKAP*, *RAB27A*, *RUNX3*, and *ZMIZ1*) ($p < 0.05$) according to the database, and there was no record of a TF regulating *NCK2*. We further identified the significantly enriched motifs in each subcluster of SLE patients compared to healthy controls ($p < 0.05$, FC >1.2) (**Table 3**). In summary, there were 31 enriched motifs in B cells, including two motifs in B-1, nine motifs in B-2, and 20 motifs in B-3. In DCs, there was 1 enriched motif in DC-1 and 18 enriched motifs in DC-2. In both monocytes and NK cells, there were only two enriched motifs, one each in Monocyte-2 and NK-4. Since both B-3 and DC-1 showed the MLX motif and both B-2 and DC-2 showed the TFAP2A motif, we observed 52 enriched TF motifs in PBMC_SLE in total (**Figure 3J**). Notably, the TFs PRDM1 and IRF8, which are well-known to be associated with SLE pathogenesis, were found to be enriched in B-3 and DC-2, respectively, based on scATAC-seq analysis. When overlapping the TFs enriched in B-3, DC-2, and Monocyte-3 of SLE patients with the 157 TFs that could regulate genes to trigger T cell activity, we found 12 TFs with enriched binding sites in PBMC_SLE (FC >2), including four TFs in DC-2 (HNF1B, POU3F2, TFAP2A, and ZNF740) and eight TFs in B-3 (EWSR1-FLI1, MAF, MAFA, NFIB, NR2C2 (var. 2), REL, TBX4, and TBX5) (**Figures 4A, B**). Thus, the 12 TFs may be key elements for SLE pathogenesis by regulating their target genes *CD83*, *ELF4*, *ITPKB*, *RAB27A*, *RUNX3*, and *ZMIZ1* and thereby promoting abnormal T cell activation (**Figures 4B, C**).

Taking the blood cells we could obtain from both the SLE patients and healthy controls and the cell number we need for the qPCR experiments into consideration, we have only checked the expression of four genes in B cells (*CD83*, *ELF4*, *RUNX3*, *ZMIZ1*). As a result, we observed significant difference in 3 gene expression in SLE patients compared to healthy controls (*ELF4*, 0.326 ± 0.097 *vs.* 1.162 ± 0.596, $p = 0.001$; *RUNX3*, 0.280 ± 0.044 *vs.* 1.048 ± 0.344, $p < 0.001$; *ZMIZ1*, 0.334 ± 0.154 *vs.* 1.119 ± 0.587, $p = 0.001$) (**Supplementary Figure S2**).

DISCUSSION AND CONCLUSIONS

Since SLE is highly correlated with epigenetic modification (21), we used scATAC-seq to analyze the genome-wide chromatin accessibility of PBMC_SLE and PBMC_NC. We identified the main five cell types, namely, T cells, NK cells, monocytes, B cells, and DCs, without the use of antibodies. We also summarized the significantly enriched motifs in each cluster, which can be used as an option for cell identification. Unexpectedly, we observed a

TABLE 3 | Subclusters with enriched motifs in PBMC_SLE for scATAC-seq experiments.

Subclusters	Top 3 enriched motifs in each subcluster ($p < 0.05$, FC >1.2)	Enriched motifs in PBMC_SLE ($p < 0.05$, FC >2)	
		ID	Transcription factors
B-1	MA1125.1, MA0684.2, MA0025.2	MA0057.1, MA1100.2	MZF1(var.2), ASCL1
B-2	MA0500.2, MA0080.5, MA1635.1	MA0089.2, MA0501.1, MA0659.2, MA1640.1, MA0495.3, MA0846.1, MA0003.4, MA0032.2, MA0808.1	NFE2L1, MAF::NFE2, MAFG, MEIS2(var.2), MAFF, FOXC2, TFAP2A, FOXC1, TEAD3
B-3	MA0080.5, MA0687.1, MA0081.2	MA1535.1, MA0663.1, MA0649.1, MA0101.1, MA0829.2, MA0806.1, MA0807.1, MA0664.1, MA0508.3, MA1520.1, MA1464.1, MA1643.1, MA0812.1, MA0592.3, MA1527.1, MA0149.1, MA1536.1, MA1151.1, MA1581.1, MA1521.1	NR2C1, MLX, HEY2, REL, SREBF1(var.2), TBX4, TBX5, MLXIPL, PRDM1, MAF, ARNT2, NFIB, TFAP2B (var.2), ESRRA, NFIC(var.2), EWSR1-FLI1, NR2C2 (var.2), RORC, ZBTB6, MAFA
DCs-1	MA0901.2, MA1104.2, MA0076.2	MA0663.1	MLX
DCs-2	MA1528.1, MA1142.1, MA0489.1	MA0046.2, MA0725.1, MA0524.2, MA0814.2, MA0153.2, MA0811.1, MA1496.1, MA0754.1, MA0694.1, MA0787.1, MA1651.1, MA0753.2, MA0652.1, MA0671.1, MA0789.1, MA0810.1, MA0037.3, MA0003.4	HNF1A, VSX1, TFAP2C, TFAP2C(var.2), HNF1B, TFAP2B, HOXA4, CUX1, ZBTB7B, POU3F2, ZFP42, ZNF740, IRF8, NFIX, POU3F4, TFAP2A(var.2), GATA3, TFAP2A
Monocytes-2	MA0748.2, MA0506.1, MA0765.2	MA0863.1, MA0682.2	MTF1, PITX1
NK-4	MA0687.1, MA0081.2, MA0080.5	MA1529.1, MA1544.1	NHLH2, OVOL1

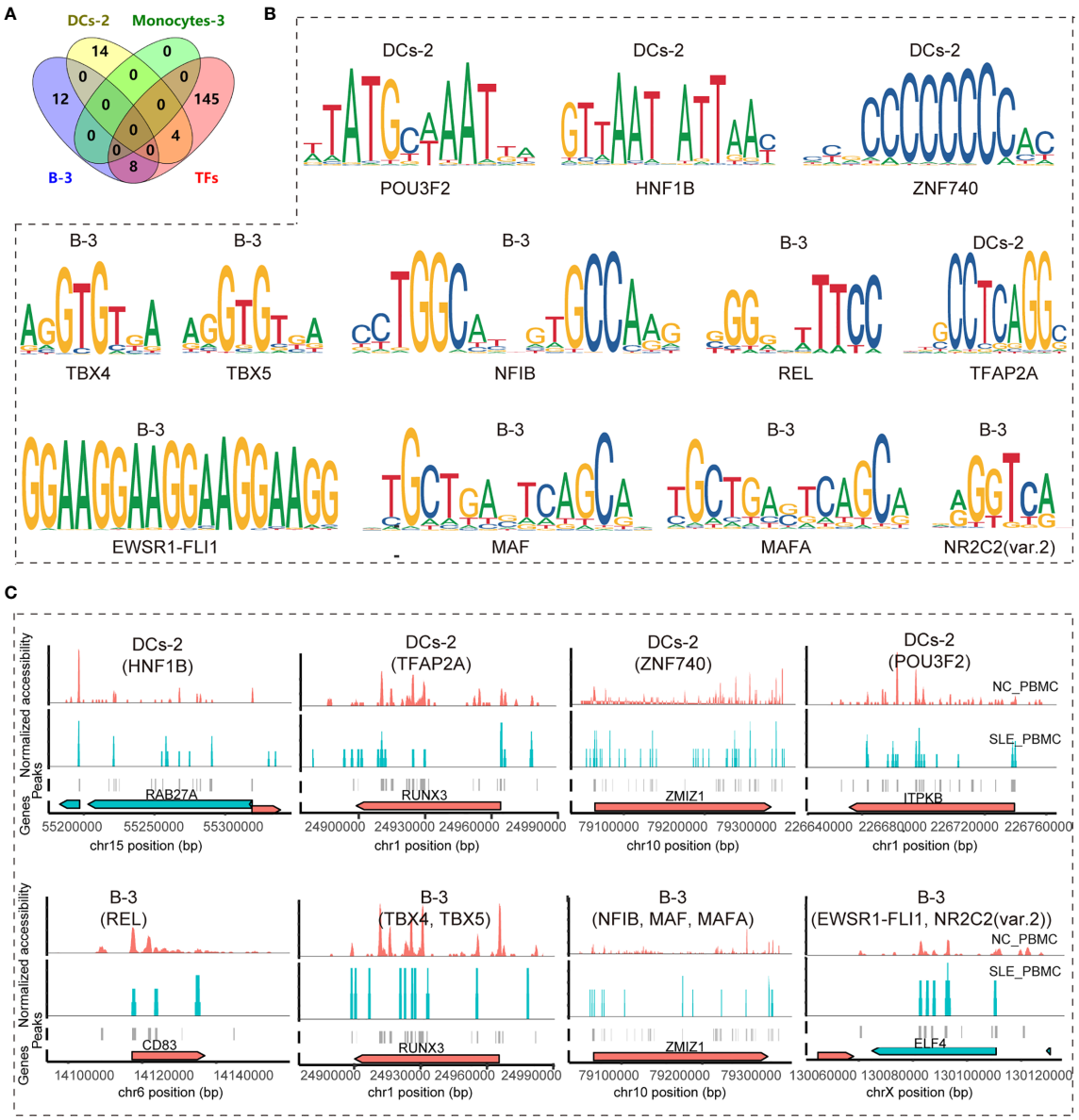

FIGURE 4 | Identifying key TF regulators and their regulatory networks involved in the T cell activation pathway. **(A)** Venn diagram showing the distribution of enriched TF regulators in B-3, Monocyte-3, and DC-2 between the SLE_PBMC and NC_PBMC libraries ($p < 0.05$, FC >2) and the 157 TF regulators involved in regulating the target genes (BCL11B, CCR7, CD83, ELF4, ITPKB, NCK2, NKAP, RAB27A, RUNX3, and ZMIZ1) to activate T cells; **(B)** Position weight matrices (PWMs) for the shared TFs in **(A)**; **(C)** Example locus near RAB27A, RUNX3, ZMIZ1, ITPKB, CD83 and ELF4 with differential accessibility between the SLE_PBMC and NC_PBMC libraries across DC-2 and B-3. SLE_PBMC, PBMCs from patients with systemic lupus erythematosus (SLE); NC_PBMC, PBMCs from healthy controls; NK cells, natural killer cells; DCs, dendritic cells. The p-values in this manuscript were calculated with Loupe Cell Browser 3.1.1 through the difference analysis feature and adjusted using the Benjamini–Hochberg correction for multiple tests.

disease-specific cluster that did not express any classic markers. Through further functional analysis of the peaks enriched in PBMC_SLE, we found that this cluster was involved in the metaphase/anaphase transition of the cell cycle based on GO analysis. This result explains why we observed few markers and indicates that a state of high proliferation activity and differentiation occurred in SLE. This unknown cluster seems to be a part of cluster 1 (T cells), and our UMAP results confirmed this hypothesis. In addition, T cell activation and differentiation are typical processes in SLE patients (22). Thus, the SLE-specific cluster 4 was presumed to be proliferating T cells. Our results revealed that T cells in SLE patients experienced activation and proliferation from the perspective of open chromatin.

Based on cell reclustering, we obtained 20 accessible chromatin patterns in five main cell types. Moreover, we calculated the significantly enriched peaks and motifs in the 20

subclusters. Interestingly, the subgroups revealed detailed information different from that obtained from the five main cell types described above. For example, the abnormal genes in B cells showed functions related to neural tube formation and closure according to the GO analysis of significantly different peaks ($p < 0.05$, $|FC| > 1.2$). The subclusters of B cells displayed abnormal signals in the axon part (B-0), T cell differentiation, and B cell receptor signaling pathways (B-3). Similarly, we observed only seven significantly enriched motifs ($p < 0.05$, $FC > 1.2$) in T cells, but we found 1, 5, 0 and 20 enriched motifs ($p < 0.05$, $FC > 1.2$) in T-0, T-1, T-2 and T-3, respectively. This result confirms the cell heterogeneity and highlights the importance of single-cell sequencing.

B-1 showed a reduced cell number ratio, and the genes that were changed in B-1 played a role in regulating the apoptotic signaling pathway and RNA splicing, with 31 and 19 genes, respectively. In the literature, there was an increase in apoptotic debris in SLE patients, and B cells played a role in apoptotic cell clearance (23). In addition, defects in highly active B cell clearance lead to B cell tolerance (23). Our results indicate that decreased B-1 may lead to insufficient clearance of apoptotic cells and B cell tolerance loss, thereby promoting B cell activity (23). The abnormal genes in B-2 showed transcription initiation from the RNA polymerase II promoter, which was consistent with the literature reporting that the transcriptional regulation associated with the less accessible *RXRA* locus in PBMC_SLE might lead to the production of more antibodies to nuclear antigens (24). B-3 also showed a reduction in the cell number ratio and was active in the B cell and T cell receptor signaling pathways together with T helper (Th) cell differentiation (**Figure 4G**). In addition, we found that the TF motif of PRDM1 associated with B cell differentiation was significantly enriched in B-3 cells ($FC > 2$). B cells that produce immunoglobulins are typical clinical findings in SLE patients. Meanwhile, Th cells could promote autoreactive B cells and induce immunoglobulins through cytokines and receptor binding (25). Our results suggest that B-3 is an essential subgroup in SLE pathogenesis and is highly correlated with B cell activation and antibody production. Additionally, reduced B-3 may help increase immunoglobulin production. Notably, B-0 explains why SLE often exhibits abnormal performance of the central nervous system (CNS) (26), because some B cells express genes associated with abnormal axon function.

Among the DCs, only DC-0 and DC-2 played a role in SLE pathogenesis based on functional analysis of significantly different peaks ($p < 0.05$, $|FC| > 1.2$). According to the literature, DCs can recognize antigens, produce chemokines, present antigens to T and B cells, absorb complex antigens and induce T and B cell immunity (21). Moreover, IRF8 was a potent repressor of BAFF, reducing autoantibodies and autoreactive B cell clones (27). Our results are consistent with the literature and show that we may find effective SLE targets by further studying DC-0 and DC-2, especially DC-2.

Similar to DCs, monocytes are antigen-presenting cells (APCs) that can present self-antigens to autoreactive cells, thereby inducing the inflammatory response of SLE patients. In addition, monocytes can secrete chemokines such as IL-1β in a TLR signal-dependent manner or produce IFNα through TLR7 signals, which indicates that monocytes and TLR-mediated pathways are essential in SLE (28). In addition, monocytes from SLE patients showed downregulation of the MAP kinase and NF-κB pathways (21). Monocyte-0 showed an increased cell number ratio and was active in neutrophil-mediated immunity. Monocyte-1 and Monocyte-3 showed abnormal Toll-like receptor signaling pathways and T cell activation, respectively. In contrast, Monocyte-2 cells showed decreases in the cell number ratio and in the effects of the MAPK signaling pathway and NF-κB signaling pathway. These results are closely related to the findings in the literature and explain the different roles of monocyte subpopulations in SLE pathology.

In NK cells, NK-0 was active in autophagy. Since both B and T cells show abnormal autophagic processes in SLE (29), NK-0 may play a role in this process. Based on the functional analysis of different peaks between PBMC_SLE and PBMC_NC, NK-1 cells showed an increased cell number ratio and abnormal signals related to kinase regulatory activity. This result was related to protein kinase activation-induced inflammation, as reported in the literature, in which NFκB-mediated and mitogen-activated kinase participate in the NK cell response (30). Binding antigens and inducing cytotoxicity through chemical modification of the substance and stimulating other immune cells (such as T cells) and secreting cytokines are typical characteristics of NK cells (31). NK-2 and NK-3 showed negatively regulated phosphorylation and transfer of the acyl group, respectively. At the same time, NK-4 showed protein dephosphorylation and T cell differentiation. This result indicates that NK-4 may be an important subgroup of NK cells in SLE due to dephosphorylation-induced cytokine secretion and the response of other immune cells.

In T cells, we did not find a functional record of T-0, nor did we observe different peaks in T-2 ($p < 0.05$, $|FC| > 1.2$). Since Lys63-specific deubiquitinase activity could be involved in innate immune signaling (32), T-3 may be the population that triggers this pathway. T-1 showed a decreased cell number ratio and abnormal signals related to histone binding and the histone acetyltransferase complex. This result indicates that T-1 may consist of CD4+ T cells and therefore experience a differentiating response to inflammation, as histone acetylation contributes to the expression of genes involved in regulatory T cell differentiation and function, including increased *Foxp3* stability (33).

As described above, B-3, DC-2, and Monocyte-3 cells showed obvious abnormal signals related to T cell activation. We observed T cell activation and proliferation in SLE patients. Therefore, ten genes shared in the three subgroups (*BCL11B*, *CCR7*, *CD83*, *ELF4*, *ITPKB*, *NCK2*, *NKAP*, *RAB27A*, *RUNX3*, and *ZMIZ1*) may be critical markers related to SLE disease. Based on the scATAC-seq analysis, 12 TFs with highly accessible binding sites in SLE patients were involved in regulating these genes, indicating that they may be potential targets for SLE diagnosis and treatment. Consistent with the literature, we observed that the expression of the TFs PRDM1 and IRF8 correlated with SLE pathogenesis and that these

genes showed an FC value greater than 2 in PBMC_SLE. This result confirmed the accuracy of our findings. With qPCR experiments, we observe a significant difference in the expression of *ELF4*, *RUNX3*, and *ZMIZ1*. This result indicates that at least EWSR1-FLI1, MAF, MAFA, NFIB, NR2C2 (var. 2), TBX4, and TBX5 may play a key role in SLE disease pathogenesis, through regulating *ELF4*, *RUNX3*, *ZMIZ1* and mediating abnormal T cell activity.

In conclusion, we obtained 20 chromatin accessibility patterns in T cells, NK cells, monocytes, B cells, and DCs, mapping the landscape of active regulatory DNA in PBMC_SLE. Our results showed the necessity of using the single-cell sequencing method to reveal cell heterogeneity and real features. In addition, we identified ten crucial genes associated with T cell activity from B-3, DC-2, and Monocyte-3 cells and revealed their regulatory network. The 12 TFs with highly accessible binding sites regulating these genes may be critical targets for SLE diagnosis and treatments. Meanwhile, three out of four target genes in B cells have been validated to show significantly different expression between SLE patients and healthy controls. Thus, at least the seven TFs (EWSR1-FLI1, MAF, MAFA, NFIB, NR2C2 (var. 2), TBX4, and TBX5) regulating the three genes (*ELF4*, *RUNX3*, and *ZMIZ1*) may be key targets for SLE diagnosis and treatments. In the future, scRNA-seq or bulk RNA-seq coupled with cell sorting can be conducted to confirm these candidate markers, and ChIP-seq is a choice to verify the key TFs. Our results reveal candidate markers in SLE-PBMCs, demonstrate the feasibility of epigenetic therapy in patient samples, and provide foundational insights relevant to precision medicine.

DATA AVAILABILITY STATEMENT

The raw and processed data presented in the study are deposited in the Gene Expression Omnibus under accession number GSE158263.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shenzhen People's Hospital (affiliation: Shenzhen People's Hospital). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HY: Investigation, writing—original draft, formal analysis, and visualization. HW: Investigation, formal analysis, and visualization. FZ and ZZ: Resources. WD: Investigation. LY and XH: Writing—review and editing. DL: Conceptualization, supervision, and funding acquisition. DT: Writing—review and editing and project administration. YD: Conceptualization, writing—review and editing, and supervision. All authors contributed to the article and approved the submitted version.

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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.2021.641886/full#supplementary-material

Supplementary Figure 1 | UMAP visualization of cellular populations in the SLE_PBMC and NC_PBMC groups.

Supplementary Figure 2 | Relative expression of genes in B cells involved in T cell activity.

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