ROLE OF THE IL-23/IL-17 PATHWAY IN CHRONIC IMMUNE-MEDIATED INFLAMMATORY DISEASES: MECHANISMS AND TARGETED THERAPIES

EDITED BY: Elisabetta Bianchi, Lars Rogge and Matteo Vecellio
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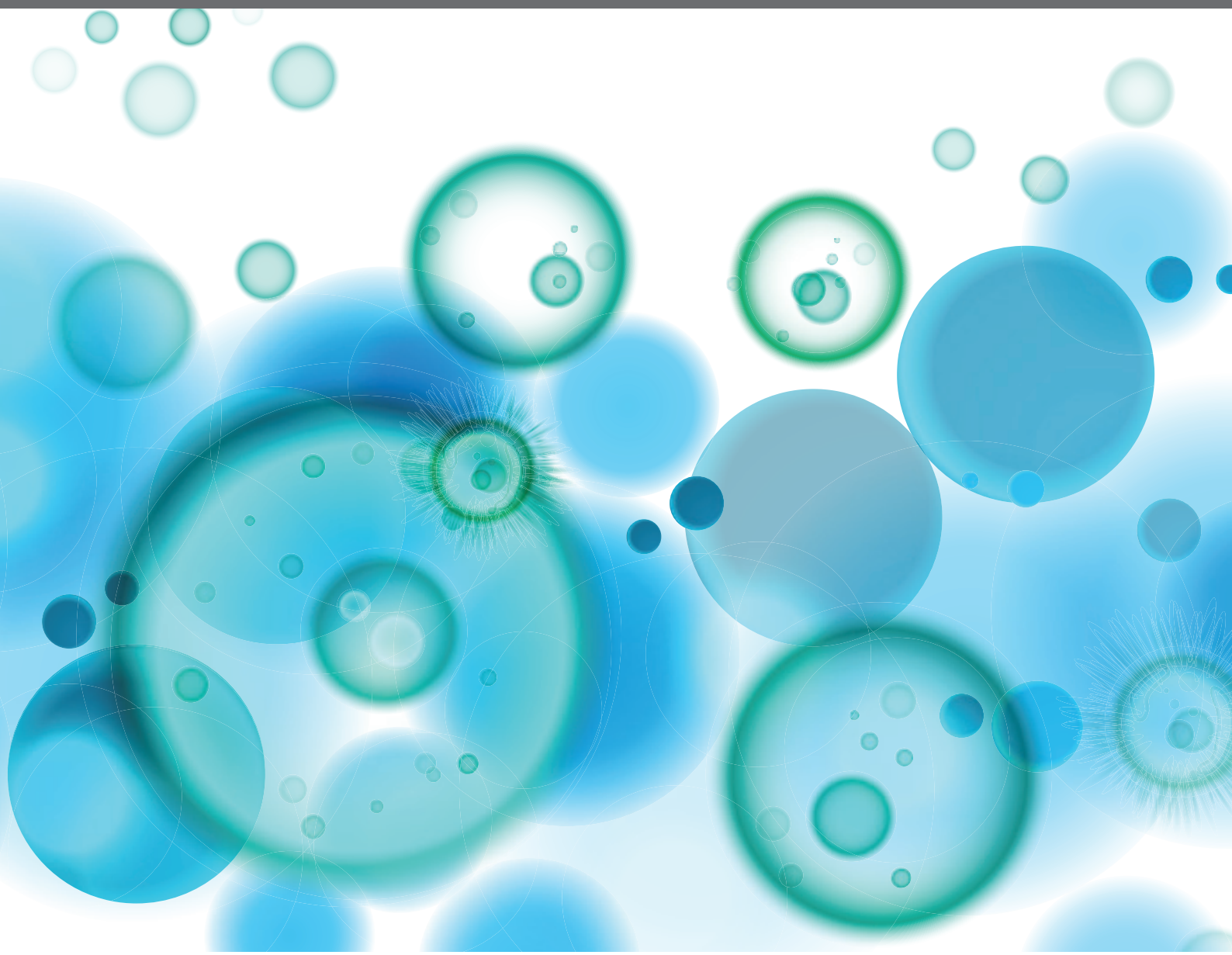

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ROLE OF THE IL-23/IL-17 PATHWAY IN CHRONIC IMMUNE-MEDIATED INFLAMMATORY DISEASES: MECHANISMS AND TARGETED THERAPIES

Topic Editors:
Elisabetta Bianchi, Institut Pasteur, France
Lars Rogge, Institut Pasteur, France
Matteo Vecellio, University of Oxford, United Kingdom

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Editorial: Role of the IL-23/IL-17 Pathway in Chronic Immune-Mediated Inflammatory Diseases: Mechanisms and Targeted Therapies

Elisabetta Bianchi[1,2]*, *Matteo Vecellio*[3,4] *and Lars Rogge*[1,2]

[1] Immunoregulation Unit, Department of Immunology, Institut Pasteur, Paris, France, [2] Unité Mixte de Recherche, Institut Pasteur/AP-HP Hôpital Cochin, Paris, France, [3] Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom, [4] Division of Rheumatology and Clinical Immunology, Humanitas Clinical and Research Center, IRCCS, Milan, Italy

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Editorial on the Research Topic

Role of the IL-23/IL-17 Pathway in Chronic Immune-Mediated Inflammatory Diseases: Mechanisms and Targeted Therapies

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***Correspondence:**
Elisabetta Bianchi
elisabetta.bianchi@pasteur.fr

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Chronic inflammatory diseases (CID) are clinically heterogeneous conditions that share common inflammatory pathways and derive from aberrant immune responses. The implication of the interleukin-23/interleukin-17 (IL-23/IL-17) axis in several CID is supported by studies in animal models of autoimmune disease (1, 2) and by the genome-wide association studies (GWAS) finding that several of the non-MHC loci genetically linked to Crohn's disease, psoriasis, and axial spondyloarthritis (axSpA), are associated with genes in this pathway (*IL23R*, *IL12B*, *IL6R*, *IL1R2*, *RORC*, *RUNX3*, *TYK2*, *JAK2*, *CARD9*) (3–5).

The clinical relevance of the IL-23/IL-17 axis has been validated by the successful treatment of psoriasis, psoriatic arthritis (PsA) and axSpA with IL-17A inhibitors (6–8). Furthermore, targeting IL-23 has proven highly effective for the treatment of psoriasis, and beneficial in PsA (9, 10), a disease belonging to the SpA spectrum. However, the clinical studies using these drugs have also given unexpected results, dissociating the effectiveness of IL-17 from IL-23 inhibitors in different diseases (11). A recent phase 2 study testing the IL-23 inhibitor risankizumab did not show any clinically improvement compared to placebo in patients with active axial SpA (12), despite the strong GWAS association of *IL23R* with SpA (4). Conversely, targeting IL-23 has proven effective for the treatment of Crohn's disease, while IL-17 inhibition induced worsening of symptoms in this disease (13).

These findings demonstrate our limited understanding of the pathogenic mechanisms of IL-17 and IL-23 in these CIDs, suggesting the need to reassess the link between IL-23 and IL-17 in these diseases.

This Research Topic offers an overview of the impact of the IL-23/IL-17 pathways in CIDs, in particular SpA, with a focus on the mechanisms driving pathogenesis and response to therapy.

IL-23 is important for the expansion and the functional activity of T helper 17 (Th17) cells, which secrete the pro-inflammatory cytokine IL-17 (14), but it may also act on several populations of innate immune cells that express the IL-23 receptor (IL-23R), including innate lymphoid cells (ILC), γδ T lymphocytes, iNKT cells, mucosal-associated invariant T cells (MAIT), and, neutrophils (15–20).

Some of these populations have been found to accumulate in the diseased tissues of patients or of model animals (21), suggesting that the inflammatory response in CID may be the result of a complex interplay of different immune cell types whose relative role in the pathogenesis of specific CIDs remains to be defined. Rosine and Miceli-Richard provide a comprehensive overview of IL-17 producing innate cell subsets in the context of SpA pathogenesis, while McGinty et al. propose that the immunoregulatory function of Tr1 cells may be impaired in SpA. IL-10 production by Tr1 was shown to prevent gut inflammation, and IL-23 downregulates IL-10 secretion in these cells (22). Given the therapeutic potential of these cells, the future challenge is the development of appropriate pre-clinical models to explore the role of Tr1 cells in CIDs.

Another cytokine regulated by IL-23 is IL-22, which is produced by Th17 cells, among other cell subsets (23). Lindhal and Olson explore in detail the role of IL-22 in Th1/Th17 cell polarization and in CIDs. Although the different studies are not always consistent, IL-22 seem in most models to reduce Th1 responses and may contribute to resolve inflammation by inducing IL-10 production.

Th17 differentiation is regulated at the transcriptional level by the IRF4 transcription factor (24). Using a T cell transfer model of colitis, Buchele et al. demonstrate that IRF4 also controls Th17 pathogenic function indirectly, by acting in a conventional Dendritic Cell 2 (cDC2) subset. This work highlights the role of IRF4 as a molecular switch that controls Th17 differentiation, as well as the importance of the cDC2 subset in the pathogenesis of colitis.

Adding complexity to the regulation of Th17 function, Peng et al. demonstrate a post-transcriptional mechanism that controls IL-17-mediated inflammation. The authors have shown that Tristetraprolin (TTP), an RNA-binding protein, inhibits IL-23 expression. In the present work, Peng et al. show that TTP conditional KO ($CD4CreTTP^{f/f}$) mice displayed increased systemic IL-17A and skin Th17 cells, and increased susceptibility to DSS-induced colitis. These data indicate that TTP is an important regulator of inflammation and a potential new therapeutic target.

Animal models of CID have been crucial to improve our understanding of the molecular processes that drive CIDs, as comprehensively illustrated by Mandour et al., in particular for diseases such as axSpA, for which access to human diseased tissue is difficult. Rodent models for SpA have been useful to study the molecular mechanisms of IL-23 induced pathogenesis, despite the fact that none reflects the whole range of pathologic findings of this disease. The study of these models has highlighted the possible role of IL-23-dependent gut and skin inflammation in triggering joint pathology. Another interesting finding of these studies is the importance of IL-23 in the early phases of SpA pathogenesis, demonstrated by the ability of IL-23 blockers to prevent disease onset when administered before the development of symptoms. The study of early events in these models may help develop predictive tools and identify targets for early therapeutic intervention. Whether IL-23 plays a similar role in the pre-clinical phase of the disease in humans remains to be established.

In human studies, GWAS have proven very useful to indicate potential pathogenic pathways in CIDs.

Disease-associated genetic variants may have the power to discriminate between similar conditions, such as psoriasis, PsA and ankylosing spondylitis (AS). In their article, Vecellio et al. highlighted the contribution of the IL-17/IL-23 axis to PsA, a disorder sharing most of the genetics and molecular mechanisms with other inflammatory diseases, like psoriasis, AS, inflammatory bowel disease and Behçet disease. The association of loci in the IL-17/IL-23 axis is the *usual suspect* that characterizes these disorders, together with the contribution of Th17 lymphocytes. The development of biologics blocking IL-17, such as Secukinumab in AS, or IL-23 such as Ustekinumab in psoriasis/PsA, demonstrates the value of a combination of genetic markers as an approach to identify credible targets for treatment. Wordsworth et al. elegantly summarize the progress made in the last years by the research community to identify candidate genes that contribute to increased AS susceptibility. More than 100 loci have been found to be associated with increased AS risk, but this could be an underestimate: it is crucial to have large cohorts and bigger sample size to increase the power of these studies. The authors point out that still no reliable genetic predictors of disease severity in AS or response to treatment are available, despite the efforts of the scientific community. The identification of credible therapeutic targets and the translation of genome wide association studies (GWAS) findings in AS, is the main massage from Zaroour et al.'s contribution. Despite recent progress, several challenges are still present in order to predict which are the causal genes regulated by disease-associated genetic variants and to define the relevant cell-type where these SNPs act. The success of biologics targeting the IL-17/IL-23 axis highlights the value of genetic studies for drug development. However, since it's very unlikely that two patients will have the same genetic makeup, stratification based on genetic predictors remains challenging.

Schinocca et al. summarize recent findings in human and animal models supporting the role of the IL-23/IL-17 pathway in SpA and other rheumatic diseases, including Rheumatoid Arthritis, Sjögren Syndrome and Systemic Lupus Erythematosus, linking molecular pathology to the development of biologic therapies. Several novel biologics targeting the IL-23/IL-17 pathway (including IL-17F and the Janus kinases (JAK) downstream of IL-23R) are being currently tested, as detailed by Ceribelli et al. in their comprehensive review of ongoing clinical trials for SpA treatment.

Hammitzsch et al. focus their attention on the role of JAKs in SpA pathogenesis, and the development of inhibitors for treatment. Given the pleiotropic role of a JAK in multiple signaling pathways, they argue for the use of selective inhibitors, to avoid undesired alterations of bone homeostasis.

In a bedside to bench approach, Fiechter et al. interrogate the effect of IL-23 inhibition on molecular pathways and cellular populations in the synovia of PsA patients. Major pathways modulated by treatment were MAPK/ERK, mTOR and Wnt signaling, while IL-17A production was not significantly affected, supporting a non-linearity in the IL-23/IL-17 pathway, and the

possibility of other pathogenic targets downstream of IL-23. Changes induced by treatment in the Wnt pathway also indicate the importance of better investigating the effects of IL-23 on bone metabolism.

Liu et al. explore the role of IL-23 and the effects of its inhibition in a wide range of inflammatory skin diseases. Their analysis suggests that IL-23 may be important for the development of several skin diseases, including Hidradenitis Suppurativa or Pityriasis Rubra, which show clinical improvement upon IL-23 blockade. Bugaut and Aractingi focus on the pathogenesis and treatment of psoriasis, with an eye to new therapeutics inhibiting selective JAKs and the transcription factor RORγt, which is essential for the function of Th17 cells. The authors underline how improved understanding of IL-23/IL-17 biology, and of the many cell types involved, may lead to the identification of new therapeutic targets, necessary for severe and refractory cases.

The role of the IL-23/IL-17 axis in inflammatory bowel disease (IBD) is reviewed by Schmitt et al. and by Noviello et al. An interesting concept that emerges from this overview is the importance of considering T cell plasticity and changes in immune profiles during disease progression, which may explain the need for a different biological treatment at different stages of disease. Noviello et al. also suggest the possibility of stratifying patients for treatment according to baseline cytokine levels.

Finally, Baeten and Adamopoulos and McGonagle et al. discuss potentials reasons why IL-23-inhibition failed in AS. Revisiting the scientific rationale for conducting trials of IL-23-inhibitors in AS, Baeten and Adamopoulos caution that the evidence supporting a central role of IL-23 in the pathobiology of AxSpA was circumstantial, at best. In particular, they state that systemic IL-23 exposure induced chronic arthritis, severe bone loss and myelopoiesis in the bone marrow and spleen of mice (25), a phenotype which is not compatible with AxSpA. This report clearly contrasts the more publicized observation that IL-23 overexpression induces a SpA-like phenotype in mice (26). This perspective concludes with the notion that the IL-23/IL-17 axis is not a linear "cascade" and that genetic data are an excellent tool to generate hypotheses, but are not sufficient to prove or disprove them. McGonagle et al. argue that IL-23 blockade can prevent disease onset but not established disease in an experimental SpA model (27). Even if it is currently not clear if these observations can be translated to human disease, they point to a role of IL-23 in disease initiation, while persistent disease may be maintained by IL-23-independent IL-17 production by memory T cells. IL-23-independent IL-17 production by various lymphocyte populations is discussed in several reviews in this topic. McGonagle et al. point out that there is heterogeneity within human γδ T cell populations with respect to IL-23 receptor expression. Both δ1 and δ2 γδ T cell populations express IL-17A following stimulation, however only the δ2 population further upregulated IL-17A production when stimulated in the presence of IL-23 (16). An interesting point also discussed by McGonagle et al. is that patients with active PsA and imaging-confirmed sacroiliitis (axial PsA) benefit from treatment with the anti-IL-12/23 inhibitor ustekinumb (28) and the IL-23 inhibitor guselkumab (29). Thus, a subgroup of SpA patients with axial inflammation may actually benefit from IL-23-blockade, and McGonagle et al. propose that adequate IL-23 posology may be critical in this condition.

In conclusion, the articles included in this collection reached their "primary endpoint", that is raising more questions to guide future clinical and fundamental research.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Innate Cells: The Alternative Source of IL-17 in Axial and Peripheral Spondyloarthritis?

Nicolas Rosine[1] *and Corinne Miceli-Richard*[1,2]*

[1] Unité Mixte AP-HP/Institut Pasteur, Institut Pasteur, Immunoregulation Unit, Paris, France, [2] Paris University, Department of Rheumatology—Hôpital Cochin. Assistance Publique—Hôpitaux de Paris, EULAR Center of Excellence, Paris, France

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Remo Castro Russo,
Federal University of Minas Gerais, Brazil

***Correspondence:**
Corinne Miceli-Richard
corinne.miceli@aphp.fr

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Spondyloarthritis (SpA) is a chronic inflammatory rheumatism characterized by inflammation of sacroiliac joints, peripheral joints, and spine. The Assessment of SpondyloArthritis Society describes three disease forms: axial (axSpA), peripheral, and enthesitic SpA. Each may be associated with extra-articular manifestations: psoriasis, inflammatory bowel disease, and acute anterior uveitis. Genome-wide association studies performed in axSpA and psoriatic arthritis (PsA) have shown a shared genetic background, especially the interleukin 23 (IL-23)/IL-17 pathway, which suggests pathophysiological similarities. The convincing positive results of clinical trials assessing the effect of secukinumab and ixekizumab (anti-IL-17A monoclonal antibodies) in axSpA and PsA have reinforced the speculated crucial role of IL-17 in SpA. Nevertheless, and obviously unexpectedly, the differential efficacy of anti-IL-23–targeted treatments between axSpA (failure) and PsA (success) has profoundly disrupted our presumed knowledge of disease pathogeny. The cells able to secrete IL-17, their dependence on IL-23, and their respective role according to the clinical form of the disease is at the heart of the current debate to potentially explain these observed differences in efficacy of IL-23/IL-17–targeted therapy. In fact, IL-17 secretion is usually mainly related to T helper 17 lymphocytes. Nevertheless, several innate immune cells express IL-23 receptor and can produce IL-17. To what extent these alternative cell populations can produce IL-17 independent of IL-23 and their respective involvement in axSpA and PsA are the crucial scientific questions in SpA. From this viewpoint, this is a nice example of a reverse path from bedside to bench, in which the results of therapeutic trials allow for reflecting more in depth on the pathophysiology of a disease. Here we provide an overview of each innate immunity-producing IL-17 cell subset and their respective role in disease pathogeny at the current level of our knowledge.

Keywords: spondyloarthritis, psoriatic arthritis, IL-17A, innate cells, IL-23, IL-17

INTRODUCTION

Spondyloarthritis (SpA) is a chronic inflammatory disease characterized by inflammation of the sacroiliac joints, peripheral joints, and spine. The Assessment of SpondyloArthritis Society describes three disease forms: axial (axSpA), peripheral, and enthesitic. Each of these forms can be associated with extra-articular manifestations: psoriasis, inflammatory bowel disease, and acute anterior uveitis. Genome-wide association studies performed in the axial and more severe form (i.e., ankylosing spondylitis [AS]) have revealed significant genetic associations with several polymorphisms involved in the T helper 17 cell (Th17) pathway: IL-23 receptor (IL-23R), IL-12B, IL-1R2, IL-6R, and RUNX3. These results, among others, have shed light on the role of the Th17 pathway in axSpA. Of note, GWAS performed in psoriasis and psoriatic arthritis (PsA) have shown similar genetic associations regarding Th17 pathway components, which suggests a common genetic background and potential shared pathogenic mechanisms for this spectrum of diseases.

Th17 cells are the third subset of effector $CD4^+$ Th cells characterized by the expression of IL-17. IL-17 contributes to the clearance of a range of pathogens (e.g., *Candida albicans*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*)—the Yin and beneficial face of IL-17. Nevertheless, they have also been associated with the pathogenesis of several immune-mediated inflammatory diseases—the Yang and deleterious face of Th17 cells.

In recent years, the involvement of the IL-23/IL-17 axis in the pathophysiology of SpA has been established both in peripheral blood and affected tissues. Several mouse models have shown the major role of Th17 cells in triggering autoimmunity and autoimmune diseases (1). Synovial fluid and/or serum from patients with active AS, undifferentiated SpA, and PsA show increased expression of IL-17A (2, 3). Consistent with the increased expression of serum IL-17, the increased absolute number and/or frequency of circulating $CD4^+$ Th17 cells have been reported in AS, PsA, and reactive arthritis (4–6), but these observations have been controversial (7, 8). Regardless, IL-17 production was found not restricted to $CD4^+$ Th17 cells. Other innate immune cells can produce IL-17, and even if some represent a quantitatively minor cell subset, their potential role in SpA pathophysiology should not be underestimated.

TH17 CELLS: THE "CLASSICAL" IL-17–SECRETING CELLS

Th17 cells are involved in the defense against certain bacterial and fungal infections, participate in the stimulation and recruitment of polynuclear neutrophil cells, and stimulate the production of antimicrobial peptides and pro-inflammatory cytokines by polynuclear neutrophil cells. IL-17A, IL-17F, IL-22, and IL-21 are the effector cytokines produced by Th17 cells; IL-23 is mainly produced by myeloid cells (macrophages), dendritic cells, and keratinocytes and favor expansion and stabilization of Th17 responses.

Biologically active IL-23 consists of IL-23p19 linked *via* a disulfide bond to IL-12p40 and signals through the IL-23R in complex with IL-12Rβ1 (9, 10). The co-localization of IL-23R and IL-12Rβ1 enables the complex to activate Janus kinase 2 (JAK2) and tyrosine kinase 2 (10), which subsequently phosphorylates signal transducer and activator of transcription 3 (STAT3) (10, 11). The phosphorylation of STAT3 leads to its translocation into the nucleus and further activates the transcription factor retinoic acid-related orphan receptor gamma t (RORγt). RORγt expression induces the transcription of downstream cytokines IL-17A, IL-17F, and IL-22 (12). RORγt also induces the expression of the chemokine receptor CCR6, which allows for the migration of Th17 in inflamed tissues. The binding of CCL20 on CCR6 allows for the chemoattraction of dendritic cells, effector and memory T cells and B cells, especially on the mucosal surface in homeostatic and pathogenic conditions (13). The IL-23 pathway induces a positive feedback loop able to maintain the pathogenic activity of this pathway (14).

IL-17A was cloned in 1993 and was considered the IL-17 family leader, but other proteins structurally related to IL-17A were further identified in the 2000s. Thus, the IL-17 family consists of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A is mainly produced by Th17 cells. IL-6 and transforming growth factor β (TGFβ) promote the initial differentiation of Th0 to Th17 cells, whereas IL-23 stabilizes and expands Th17 cells in mice (15). The activity of IL-17A is mediated *via* a heterodimeric receptor consisting of IL-17RA and IL-17RC. This complex recruits the nuclear factor κB (NF-κB) activator 1 (ACT1) adaptor protein to activate several pathways such as mitogen-activated protein kinases (MAPKs) including p38 MAK, c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), JAK, STAT, and phosphoinositol 3 kinase (PI3K). It also induces several pro-inflammatory cytokines (IL-1β, IL-6, tumor necrosis factor α [TNFα], C-C motif chemokine ligand 2 [CCL2]), antimicrobial peptides (β-defensin), and matrix metalloproteinases [reviewed in (16)].

IL-21 and IL-22 are two other key cytokines secreted by Th17. IL-22 has a protective effect on the cutaneous, digestive, and respiratory-tract barriers *via* the production of anti-bacterial proteins and chemokines, the increase in cellular mobility, and the expression of molecules amplifying its action. IL-22 can act synergistically with TNF and appears to enhance the effect of IL-17A and IL-17F in some *in vitro* models [reviewed in (17)]. The other sources of IL-22 are somewhat like those of IL-17A (type 3 innate lymphoid cells [ILCs] mainly and invariant natural killer T [iNKT] cells) *via* RORγt. However, Th1 lymphocytes produce IL-22, with level correlated with interferon γ (IFNγ) and T-bet levels. Some authors have even described an independent population named Th22. The production of IL-22 goes through the transcription factors aryl hydrocarbon receptor (AhR) and RORγt as for Th17 (but with induced IL-22 mRNA expression less important for the latter). These results suggest that differentiation to either of these two cell types relies on RAR

Related Orphan Receptor C (RORC) expression [reviewed in (17) and (18)]. IL-21 is also produced by Th17 and has an autocrine action. Even if not mandatory for Th17 differentiation, IL-21 allows for the stabilization of the Th17 phenotype and proliferation capacities. IL-21 increases the expression of IL-23R and induces the expression of RORγt [reviewed in (19) and (20)] (**Figures 1** and **2A**).

ALTERNATIVE SOURCES OF IL-17: INNATE IMMUNE CELL SUBSETS

Several groups are currently investigating whether these innate immune subsets are dysregulated in SpA and to what extent they might contribute to disease pathogeny.

Mucosal-Associated Invariant T (MAIT) Cells

Various cytokines are expressed by MAIT cells, such as IFNγ, IL-17, or cytotoxicity granules after a large pattern of stimuli (21, 22). The cells display an effector phenotype with chemokine receptor expression suggesting their ability to migrate in tissues. MAIT cells express an invariant T-cell receptor (TCR; Vα7.2) activated by the major histocompatibility complex class I-related protein 1 (MR1) (**Figure 2B**). MR1 is ubiquitously expressed by many cell types, especially hematopoietic and epithelial cells, so they can act as antigen-presenting cells (23). At the exit of the thymus, MAIT cells are still naive cells but very quickly become memory cells after interacting with B cells and the commensal flora (24). Indeed, B cell-deficient patients lack MAIT cells. The mechanism of interaction and the stage of maturation at which this interaction occurs is not known, but the ability of B cells incubated with bacteria to induce MAIT cells suggests an interaction with MR1 (25, 26). The transcription factors promyelocytic leukaemia zinc finger protein, RORcT, and CD161 are also rapidly expressed so that once in the periphery, MAIT cells acquire a memory phenotype (26).

These cells are related to the CD8 cell subset. In fact, they express CD8αα homodimers or intermediate levels of CD8αβ or are double negative for CD8 α and β chains (27). MAIT cells are mainly present in the liver, where they represent up to 20% to 50% of T cells (28, 29), but they are also found in blood, mesenteric lymph nodes, lamina propria, and especially inflammatory tissues during different diseases. Indeed, the presence of chemokine receptors (CCR5, CCR6, CXCR6) suggests a tissue tropism, and the presence of cytokine receptors such as IL-23R and IL-18R is linked to their ability to secrete pro-inflammatory cytokines, mostly TNFα, IFNγ, and IL-17, after strong stimulation (28–30). However, stimulation with CD3/CD28 or antigen-presenting cell infection is sufficient to induce IFNγ, whereas PMA/ionomycin is necessary to induce IL-17A (21, 22, 31–34).

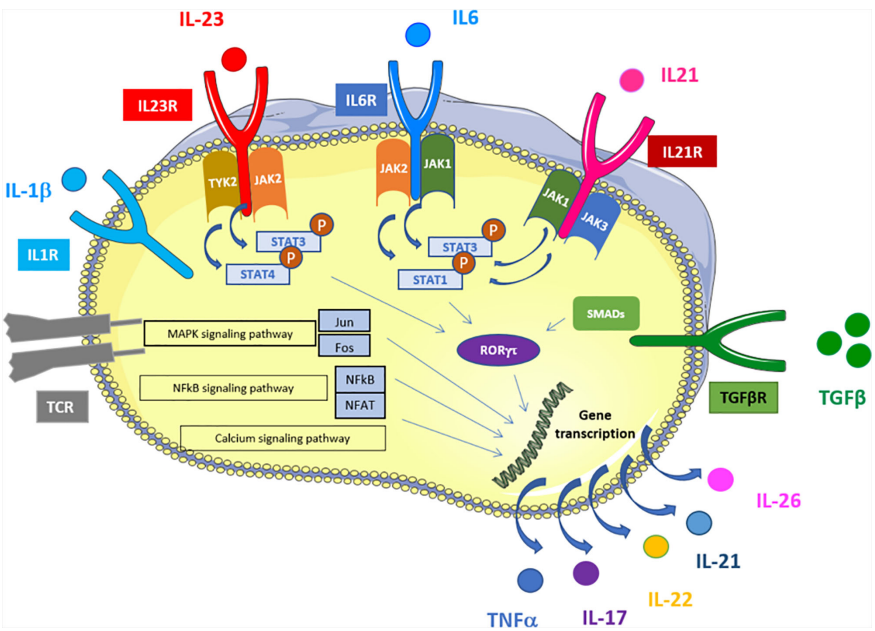

FIGURE 1 | Schematic representation of signaling and transcriptional regulation of Th17 polarization. Th17 cells are induced upon TCR activation in the presence of TGFβ, IL-1β, IL-6, IL-21, and IL-23. IL-6, IL-21 and IL-23 activate several STATs that bind to the promoter regions and activates transcription of RORγt. In addition, IL-1 induces RORgt via P38/mTOR and IRF4. TGFβ stimulation induces RORγt activation via SMADs. RORgt leads to IL-17 and other Th17-related cytokines expression. TCR activation activates MAPK, NFkB and calcium signaling pathways that also induce Th17-related cytokines expression via alternative transcription factors (NFAT, NFkB, Jun and Fos). Adapted from KEGG pathways https://www.genome.jp/kegg-bin/show_pathway?hsa04659.

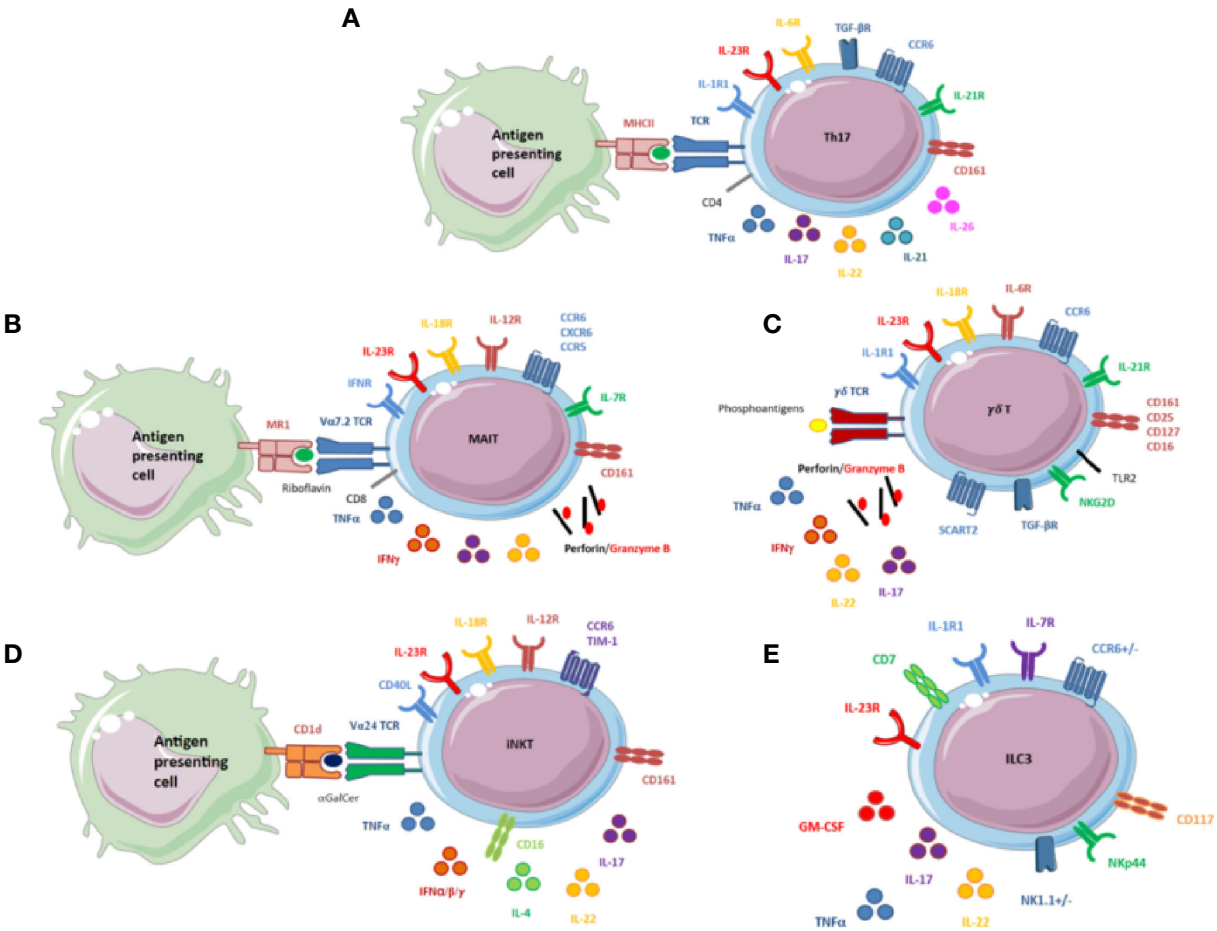

FIGURE 2 | **(A)** Schematic overview of the main receptors and secreted cytokines of Th17 cells. CD4+Th17 cells are induced on T-cell receptor (TCR) activation in the presence of TGFβ, IL-1β, IL-6, IL-21, and IL-23. IL-6, IL-21, and IL-23 activate STAT3, which binds to the promoter regions and activates transcription of RORγt and IL-17. Th17 cells also produce other cytokines, mainly pro-inflammatory cytokines: TNFα, IL-21, IL-22, and IL-26. Th17 cells possess 2 markers specific to IL-17 producing cells: CD161, a marker of activation belonging to the C-lectin family, and CCR6, a chemokine receptor that binds only one chemokine, CCL20. It may regulate the migration and recruitment of dendritic cells and T cells during inflammatory and immunological responses. **(B)** Schematic overview of the main receptors and secreted cytokines of mucosal-associated invariant T (MAIT) cells. MAIT cells express an invariant TCR, Vα7.2. They recognize the conserved major histocompatibility complex-like protein 1 (MR1), which presents a bacterial-derived ligand, riboflavin. MAIT cells express various cytokines and receptors. In the cytokine environment, MAIT cells can release cytotoxicity granules, perforin and granzyme **(B)** They also display specific receptors to induce a type 1 immune response (IFN, IL-12, and IL-18 receptors to produce TNFα and IFNγ) and a type 3 immune response (IL-23R to produce IL-17 and IL-22). IL-7 receptor (IL-7R) is also displayed on the surface of MAIT cells. IL-7R plays a critical role in the development of immune cells and could be of particular interest for these cells. MAIT cells possess the 2 markers specific to IL-17–producing cells, CD161 and CCR6. CXCR6 and CCR5 are also expressed, but their roles are not fully understood. **(C)** Schematic overview of the main receptors and secreted cytokines of γδ T cells. γδ T cells express a TCR that does not engage major histocompatibility complex antigen complexes but rather conserved phosphoantigens of bacterial metabolic pathways. Self-induced proteins overexpressed by infected cells or tumor cells are detected by NKG2D expressed at the surface of γδ T cells. IL-1, IL6, IL-18, IL-23R, and TGFβ induce the production of IL-17. TLR2 is expressed at the surface of these cells and could also be involved in the production of IL-17 by γδ T cells. γδ T cells can also release cytotoxicity granules: perforin and granzyme B and other pro-inflammatory cytokines, IL-22, TNFα, and IFNγ. IL-7 receptor (IL-7R) is on the surface of γδ T cells and participates with CD25 and SCART2 in maintenance of the phenotype. γδ T cells possess the 2 markers specific to IL-17–producing cells, CD161 and CCR6. **(D)** Schematic overview of the main receptors and secreted cytokines of invariant natural killer T (iNKT) cells. iNKT cell activation depends on the interaction between the invariant TCR antigen Vα24 with CD1d loaded with the prototypic antigen glycosphingolipid a-galactosylceramide (αGalcer). iNKT cells can also be activated independently by the cytokine environment. iNKT cells display different receptors: "type 1": IL-12 and IL-18 receptors"; type 3": IL-23 receptor; but also CD40L to interact with B cells. This combination allows for releasing various cytokines including TNFα, IFNα, IFNβ, IFNγ, IL-4, IL-17, and IL-22. iNKT cells also possess 2 markers specific to IL-17–producing cells, CD161 and CCR6. **(E)** Schematic overview of the main receptors and secreted cytokines of innate lymphoid cells (ILCs). ILCs are characterized by lack of markers specific for T cells, B cells, and other hematopoietic cells. ILC development depends mainly on IL-7. ILCs are tissue-resident cells and possess chemokine receptors for migration, CCR6 for ILC3. ILC3s are related to RORγT and produce IL-17, IL-22, TNFα, and granulocyte macrophage–colony-stimulating factor in response to IL-1β and IL-23. Human ILC3s are identified by the combination of NKp44 that may normally contribute to the increased efficiency of activated natural killer (NK) cells; CD117, a tyrosine kinase receptor, and CD127 (IL-7Rα).

Invariant Natural Killer T (iNKT) Cells

iNKTs were first described for their regulatory role in oncology and autoimmunity. On the one hand, they promote cell-mediated immunity against tumors and bacteria or viruses, and on the other, they limit cell-mediated immunity and allograft rejection. This tolerogenic role in autoimmunity has been described in different experimental models such as type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis (35); ozone-induced asthma (36); and collagen-induced arthritis (37), but there are discrepancies with opposite results depending on the treatment protocol or mouse strains.

In humans, these cells represent approximately 0.01% of peripheral blood mononuclear cells, with considerable inter-individual variability (approximately 100-fold). The iNKTs have a Vα24-Jα18-Vβ11 invariant TCR (loading lipids or glycolipids) and are CD1d-restricted (**Figure 2D**). Upon activation, they can release a large panel of pro- and anti-inflammatory cytokines. The combination of the type of molecules loaded on CD1d, the binding kinetics, and the signal strength determine cell polarization and the profile of pro- or anti-inflammatory released cytokines (38).

A small subpopulation of IL-17A–producing iNKTs has been described in mice. These IL-17$^+$ iNKT cells express the RORγT transcription factor IL-23R and the chemokine receptor CCR6 (39–42). iNKT cells are found in the thymus, spleen, liver, and lungs and are highly enriched in peripheral lymph nodes. In vivo, they can produce high concentrations of IL-17, within 2 to 3 hr after stimulation with lipopolysaccharide or lipopolysaccharide-activated dendritic cells [reviewed in (43)]. Recently, this specific population has been described in humans. The RORγT$^+$ iNKT subset of cells accounted for 2.1% of the parent population and produced IL-17A and IL-22. The IL-23R was not expressed on the surface of these cells, but the authors found IL-23R mRNA expression, which suggested that the expression of the receptor on the cell surface depended on the inflammatory context. Secretion of IL-17A needed the combined presence of IL-23 with invariant TCR Vα24 stimulation (44).

Gamma delta T Cells (γδ T Cells)

γδ T cells represent approximately 3% to 5% of all lymphoid cells found in blood (45) and 50% of the total intraepithelial lymphocyte population at mucosal and epithelial sites, especially in the gut. In contrast to αβ T cells, whose mode of action is based on the TCR (46), γδ T cells are sensitive to a variety of antigens (47), mainly phosphorylated metabolites, also called phosphorantigens, issuing from bacterial metabolic pathways (48). γδ T-cell differentiation is already advanced at their exit from the thymus, which limits their plasticity in the periphery. Thus γδ T cells are activated by the cytokine environment rather than their TCR. Differentiation and expansion of IL-17$^+$ γδ T cells would depend mainly on IL-7 and TGFβ. We can now distinguish at least 4 subpopulations of Tγδ lymphocytes based on their effector function: IL-17 producers, IFNγ producers, innate-like Tγδ (49), and γδ T regulatory cells described in 2009 by Otsuka et al., only in mouse (50). γδ T cells express chemokine receptors, cytokine receptors, and pattern recognition receptors, and the receptors have been found involved in activating γδ T cells, especially IL-17 (**Figure 2C**). γδ T cells possess receptors for IL-1, IL-6, IL-18, IL-23, and TGFβ1 promoting IL-17 production. Signaling pathways traditionally involved in IL-17 production are also described for γδ T cells: Toll-like receptor 2 and Dendritic Cell-associated C-type lectin 1 (dectin 1), as well as the internal AhR. Recently, other types of interaction have been found involved in the production of IL-17A: CD30/CD30L and CD27. B- and T-lymphocyte attenuator and Notch could regulate this production by inhibiting RORγT (51). In addition, models of IL-2– and IL-25–deficient mice have shown that IL-2 plays a key role in maintenance of IL-17A–producing cells. The IL-2 receptor α chain (CD25) but not β chain (CD122) is expressed on the surface of IL-17–producing γδ T cells (52). IL-17$^+$ γδ T cells share many phenotypic characteristics with Th17, particularly STAT3 and RORγT transcription factors as well as surface markers CCR6 and IL-23R. The cytokine environment mainly determines the differentiation and expansion of this IL-17–producing subpopulation of γδ T cells: IL-7, whose receptor IL-7Rα is more expressed in the CD27$^-$ cell fraction, and TGFβ, whose role seems to amplify the production of IL-17 (53). The role of the TCR on γδ T-cell effector functions is still debated.

Innate Lymphoid Cells (ILCs)

ILCs are divided into 4 groups according to the specific cytokines they produce and the specific transcription factors they express according to their profile of differentiation and function. ILCs are tissue-resident innate immune cells involved in the host defense against pathogens and in tissue remodeling. Group 1 ILCs (ILC1s) are defined by their expression of T-bet and the production of IFNγ and TNFα. ILC1s have cytotoxic properties and are mainly found in the intestine, lung, and skin. Group 2 ILCs (ILC2s) express GATA3 and type 2 cytokines (IL-5, IL-9, and IL-13) upon IL-33 and IL-25 stimulation. ILC2-secreted cytokines can promote M2 macrophage polarization. ILC2s reside in lung, intestine, gut, and skin. Group 3 ILCs (ILC3s) and lymphoid tissue inducer (LTi) cells both express RORγt and produce IL-17 and/or IL-22 (**Figure 2E**). LTi cells drive the formation of secondary lymphoid structure such as lymph nodes and Peyer's patches during fetal development. ILC3s are found in skin and are particularly in psoriasis lesions (54). ILC3s seem to be critical for gut homeostasis by modulating cell proliferation, cytokines and antimicrobial peptide production, permeability of the intestinal barrier, and interactions between microbiota and CD4$^+$ T cells. NKp44$^+$ ILC3s are found in the mouse intestine and have a protective effect against induced colitis with a "homeostasis keeper" function *via* IL-17 secretion (55, 56). In healthy individuals, 0.01% to 0.1% of circulating lymphocytes express a CD127$^+$ ILC phenotype. Recently, Lim et al. showed that most CD127$^+$ ILCs found in peripheral blood are ILC2s, with near absence of NKp44$^+$ ILC3s (57) and CD127$^-$ ILC1s (58). The

authors also showed that ILC subpopulations differentiated in tissues and persisted in blood as a precursor. In the human gut, ILCs are mainly represented by $CD127^+$ ILC1s that are involved in the defense against pathogens in response to danger signals (58).

In humans, different subpopulations with similar phenotypic characteristics can be found on the same site, which suggests a plasticity of these cells. The cytokine environment is at the origin of the trans-differentiation of ILC3s to ILC1s: the pro-inflammatory cytokines IL-12 and IL-18 induce a downregulation of RORγT associated with an upregulation of T-bet (59, 60). This type of trans-differentiation has been observed in Crohn's disease (61).

Neutrophils

Neutrophils are the first line of defense of the immune system, constituting a cellular barrier against fungi and bacteria but also against altered endogenous cells or molecules. Neutrophils are mature cells that are rapidly activated and functional but with a short lifespan (a few hours) (62). The essential immune functions and short lifespan of neutrophils demand their constant production in bone marrow, called granulopoiesis. This highly regulated mechanism produces 10^{11} neutrophils each day (63). As a first-line defense, neutrophils have various functional capacities that occur alone or in combination (64, 65) and allow for destruction of the pathogen: phagocytosis, oxidative stress, release of cytokines, and NETosis. Furthermore, neutrophils are able to interact with immune cells, favoring the maturation of dendritic cells and natural killer cells and Th1 and Th17 differentiation (66). Conversely, Th17 cell-derived cytokines (e.g., IL-17, CXC-chemokine ligand 8 [CXCL8; also known as IL-8], IFNγ, TNF, and granulocyte macrophage–colony-stimulating factor [GM-CSF]) favor recruitment, activation, and prolonged survival of neutrophils at inflammatory sites. Cellular interactions between lymphocytes and neutrophils are crucial. These exchanges participate in regulating the adaptive immune system as suggested by the migration of neutrophils to lymph nodes. Nevertheless, neutrophils have limited transcriptional capacity. The quantity of mRNA produced corresponds to only 5% of that of the other leucocytes (67). However, considering that neutrophils recruited to inflamed tissues greatly outnumber other leukocytes, the overall impact of neutrophil-derived cytokines in the inflammatory response could counterbalance this transcriptional limitation. Like other cell populations, sub-populations of neutrophils with very specific abilities seem to exist both under homeostatic and pathological conditions.

Granulopoiesis is finely regulated by GM-CSF. This phenomenon induces a release of other pro-inflammatory factors (such as cytokines, chemokines, and matrix metalloproteinases) by mesenchymal and myeloid cells, thus allowing neutrophil recruitment and activation: the "neutrostat" system (68). The direct production of IL-17A by neutrophils is a highly controversial subject. Taylor et al. provided evidence that neutrophils could produce IL-17A not only for auto-loop activation but also to amplify the phenomenon of oxidative burst and defense against fungi. After preincubation with a fungal stimulus and incubation in the presence of IL-6 and IL-23, IL-17A mRNA and protein expression by neutrophils was detected. The neutrophils expressed IL-23 and IL-6 receptors on their cell membrane. The authors then showed that IL-17A production depended on RORγt and Dectin2. However, the cytokine concentrations used to stimulate the neutrophils were very high, far from "physiologic" conditions (69). More recently, a work from Tamassia et al. failed to reproduce these results. The authors showed with multiple methodological approaches that even with the same stimulations, neutrophils could not express or induce secretion of IL-17A at any stages of maturation. They also demonstrated that the antibodies used for immunohistochemistry/immunofluorescence were not specific to IL-17A and could induce false positive results. Finally, they showed the lack of histone marks associated with active and poised regulatory elements at the IL-17A locus of neutrophils as compared with Th17 cells, which suggested the inability of neutrophils to express IL-17A mRNA (70). A recent work from our group also confirmed that stimulated neutrophils from SpA patients were unable to express IL-17 A or IL-17F, both at the mRNA and protein levels (personal data).

Mast Cells

Mast cells are innate tissue resident cells of the immune system. They are not fully differentiated and are able to survive months or years despite not circulating in their mature form. Mast cells possess lysosome-like secreting dense granules in their cytoplasm that are released upon activation (71, 72) and represent a unique array of immune-modulating molecules. The activation signals rely on various stimulation processes including IgE receptor crosslinking, complement activation, neuropeptides, and toxin stimuli. Activation of mast cells induces the exocytosis of pre-formed molecules stored in granules. Mast cells "communicate" with various cell types, including cells belonging to the innate and adaptive immune system such as lymphocytes, macrophages, dendritic cells, and neutrophils (73). In several mouse models, mast cells participate in neutrophil recruitment *via* IL-8 (74): synovium in collagen-induced arthritis (75), skin in bullous pemphigoid (76), and meninges in experimental autoimmune encephalomyelitis (77). The mast cell ability to produce IL-17 was first suggested in atherosclerosis. Indeed, carotid endarterectomy immunohistochemical analysis revealed IL-17A/F^+ mast cells in complicated plaques, with no observation of IL-17A/F^+ T cells (Th17 cells). The ability of mast cells to produce IL-17A has also never been confirmed in mice models. A recent work involving tonsil biopsies and synovial tissue suggested that mast cells were able to capture circulating IL-17A and release it *via* a dynamic mechanism of endo- and exocytosis (78). The authors demonstrated that mast cells did not possess the necessary transcriptional machinery allowing for IL-17A synthesis. Even after stimulation, they did not find mRNA for IL-17A, whereas in immunofluorescence assessment of tonsillar sections, IL-17A was present in the cytoplasm of mast cells within secretory vesicles. Dynamin 2 GTPase appeared to be the process *via* which IL-17A internalization occurred, a mechanism independent of IL-17A receptors. The externalization process

was found only indirectly, and unlike cytokines classically released by mast cells, only the release of IL-17A used this process (78).

Eosinophils

Eosinophils display bi-lobed nuclei and specific granules characteristic of this cell population compared to the other granulocytes (neutrophils and basophils). Eosinophils are specifically involved in type 2 immunity. They are mainly increased in response to helminth infection and in the context of allergic disease. Eosinophils are able to enhance the immune responses mediated by T helper cell type 2 (Th2) through the production of IL-4 and by acting as antigen-presenting cells. Eosinophils produce chemoattractants for DC and effector Th cells such as CCL17, CCL22, CXCL9, CXCL10, and Eosinophil-derived-neurotoxin (EDN) [reviewed in (79)]. Several teams reported a production of IL-17 by eosinophils in specific inflammatory conditions. Shimura et al. showed that IL-17A—but not IL-17F—was crucial in LPS-induced sepsis in mice (80). Eosinophils were also able to produce IL17A after monosodium urate crystals stimulation (81). Other groups showed that eosinophils were able to produce IL-17A in response to Aspergillus fugimatus together with IL-23, thus contributing directly to the modulation of the IL23/IL17 axis (82, 83). To date, the capacity of IL17 production by eosinophils has not been demonstrated in immune mediated inflammatory diseases.

THE ROLE OF IL-17 IN SPA: FROM ANIMAL MODELS TO HUMAN DISEASE

Sherlock et al. used the mini circle DNA technology with IL-23 overexpression to induce an "SpA-like" phenotype with enthesitis in B10 RIII mice. Bone remodeling was associated with increased expression of IL-17A and IL-22, the latter even more important for bone formation than IL-17A.

Another group used the SKG mouse model (housed under specific pathogen conditions) injected with β1,3-glucan to obtain a phenotype closer to axSpA. The SKG strain develops spontaneous IL-17-dependent autoimmune inflammatory arthritis under microbial conditions induced by pulmonary fungal infection. β-glucan is a component of fungal cell walls including Candida and Aspergillus. This combination leads to axial and peripheral arthritis in mice. From these results, spondylitis was IL-23–dependent, as was arthritis and ileitis (84, 85).

The HLA-B27 transgenic rat is a classical model of SpA with axial and peripheral arthritis, nail dystrophy, gut inflammation, and orchitis/epididymitis (86). Th17 cells are involved in this rat model of SpA (87). HLA-B27 transgenic rats immunized for *Mycobacterium tuberculosis* is another model suggesting that the onset of the SpA phenotype could depend on IL-23. Indeed, anti-IL-23R treatment used before the appearance of the symptoms prevented the development of the axial disease in these rats but not the same treatment injected after the appearance of the symptoms (88). These data, if translatable to human disease, would suggest that axSpA should be IL-23–dependent in the preclinical disease phase but IL-23–independent once the disease is established. The same group showed that fibroblast-like synoviocytes exposed to IL-17A differentiated into osteoblasts. Using the same rat model with anti-IL17A treatment, axial inflammation (spondylitis) decreased (albeit not significantly). Blocking IL-17A appeared to limit bone remodeling and especially the periosteal new bone formation and to reduce peripheral and axial inflammation (89).

In humans, genome-wide association studies have shed light on the IL-23/IL-17 axis: 6 of the 48 non-MHC loci are genetically associated with SpA-involved genes in this pathway (RUNX3, IL-23R, IL-6R, IL-1R2, IL-12B, tyrosine kinase 2) (90). These data suggest that the inflammatory response in SpA may result from a complex interaction between different immune cell types and the key role of the IL-23/IL-17 axis in chronic inflammation.

Appel et al (7). assessed the facet joints of axSpA patients undergoing a surgical procedure. Immunostaining of histological sections revealed that IL-17–producing cells were mainly neutrophils and, in smaller proportions, T lymphocytes. Of note, the study population included patients with advanced disease, requiring surgery.

Several groups have reported an increased proportion of Th17 in the peripheral blood of AS patients as compared with controls or patients with other inflammatory conditions. Other IL-17–secreting cells are increased in number in AS patients. In peripheral blood, a study reported a three-fold higher frequency of circulating γδ T cells and five-fold higher frequency of IL-23R–expressing γδ T cells in AS patients versus healthy controls and versus rheumatoid arthritis patients, respectively (8). In this study, γδ T cells were suggested to be the dominant IL-17 producers in AS.

Another study involving AS patients reported decreased number of MAIT cells in peripheral blood but increased subpopulation of IL-17$^+$ MAIT cells as compared with controls. In this study, MAIT cells appeared to concentrate in the synovial fluid, thus suggesting a migration of these cells to the inflammatory sites. MAIT cells produced large amounts of IL-17A under IL-7 stimulation but surprisingly not IL-23 stimulation (91). This increase in IL-17A$^+$ MAIT cells was recently confirmed in a cohort with exclusively axSpA (92).

THE ROLE OF IL-17 IN BONE FORMATION

Inflammatory and painful entheses (the sites of attachment of tendons, ligaments, fascia, or joint capsules to bone) are the distinctive pathological features of SpA. Bone formation in SpA is closely linked to the inflammatory processes at the spinal entheses. Periosteal appositions at the sites of past inflammatory entheses are observed, constituting the basis for bone formation. This ossification goes through several stages: apoptosis of chondrocytes, which are further replaced by osteoblasts into osteocytes partitioned in the matrix (93, 94). Nevertheless, the mechanisms leading to bone formation at the inflamed entheseal sites in SpA are not fully understood.

Among other hypotheses, mechanical stress could be a key trigger of entheseal inflammation and further new bone formation. This hypothesis was assessed in the $TNF^{\Delta ARE}$ mouse model in which chronic and deregulated TNF production leads to axial and peripheral arthritis associated with a Crohn's-like ileitis. In this TNF-driven mouse model of SpA, Erk1/2 signaling plays a crucial role in the mechanical stress-induced inflammation. New bone formation was strongly promoted at entheseal sites by biomechanical stress and was correlated with the degree of inflammation.

At the entheseal level, IL-17 amplifies the inflammation by promoting the secretion of pro-inflammatory cytokines by the resident mesenchymal cells. GM-CSF, IL-6, IL-8, and IL-17 are chemo-attractants for neutrophils contributing to activate the inflammatory loop (95–98). The cytokine micro-environment seems to be determinant in bone remodeling phenomenon. IL-17 combined with TNF increases calcified matrix formation from mesenchymal cells when they are exposed to conditions leading to bone formation (99). Ono et al. showed a direct role of IL-17A on bone healing after a fracture: there was an increase in IL-17A at the fracture site enhancing bone regeneration. IL-17A activated osteogenesis by differentiating the mesenchymal cells present at the fracture site. Osteoclastogenesis was not affected. The major source of IL-17A was γδ T cells and in particular Vγ6 (100). These data were confirmed *in vitro* by Osta et al (99). However, the authors pointed out that this osteogenic differentiation of mesenchymal cells was only possible by combining IL-17A and TNF, IL-17A alone having no effect (99). Moreover, the combination of these two cytokines induced a decreased expression of DKK1 and RANKL in mesenchymal cells, thus contributing to an increased osteogenesis. The authors suggested that the cellular environment (i.e., the presence or absence of osteoclasts) crucially determined the effect of these two cytokines: the presence of mesenchymal cells combined with the absence of osteoclasts at the entheseal level could explain why IL-17A and TNF could both contribute to bone formation in SpA (99).

THE ROLE OF INNATE IMMUNE CELLS SECRETING IL-17: A PATH TO UNDERSTANDING THE FAILURE OF IL-23 BLOCKING AGENTS IN AXSPA?

Sherlock et al. demonstrated that the overexpression of IL-23 in mice induced a SpA-like phenotype with enthesitis but not requiring a mechanical overload. They identified a specific subset of enthesis-resident T cells: CD3+, CD4, and CD8 double-negative and expressing IL-23R. These cells, upon IL-23 stimulation, secreted high amounts of IL-17 and IL-22, inducing typical features of SpA, such as enthesitis and new bone formation (95). Another study using the same mouse model identified these cells as γδ T cells (101). More recently, Cuthbert et al. investigated the presence of γδ T cells in normal axial entheses harvested during orthopedic procedures from patients with mechanical back pain (e.g., osteoarthritis or scoliosis). Two γδ T-cell subsets were identified (γδ1 and γδ2). The γδ1 subset lacked IL-23R transcripts but was able to express IL-17A upon PMA/ionomycin or CD3/CD28 stimulation. Neither Il-17A nor IL-17F transcripts were expressed upon IL-23 stimulation. These findings might suggest that γδ1 T cells could be involved in IL-17 production in spinal enthesis from SpA patients, independent of IL-23. This could be an attractive explanation for the failure of IL-23 blocking treatments in axial SpA (102). Using a similar approach on patients undergoing a surgical procedure, Cuthbert et al. reported the presence of ILC3s in spinal entheses, with the same characteristics as ILC3s collected from synovial fluid from SpA patients [i.e., IL-23R, STAT3, and RORγt transcript expression (103)]. IL-17A transcript expression by these cells was obtained on IL-23/IL-1β stimulation.

Gracey et al. showed an increased number of $IL\text{-}17^{+}$ MAIT cells in peripheral blood from AS patients as compared with controls (91). These cells were able to express IL-17A upon priming with IL-7 but not IL-23 stimulation. IL-17A expression was assessed by flow cytometry.

How do these results fit into the current context of clinical trials showing failure of drugs targeting IL-23 in axSpA and the successful approaches of IL-17A targeting drugs? Obviously, blocking the terminal cytokine IL-17A is a valuable approach in axSpA, whatever the source of production: Th17 cells, MAIT cells, γδ T cells, or ILC3s. In contrast, IL-23 blockade is not effective for axial disease, which suggests that cells involved in the axSpA pathogenesis might be able to express IL-17A independent of IL-23. Preliminary results from several groups suggest that MAIT cells and γδ1 T cells could be the culprits, with the proviso that the said studies were conducted in healthy individuals.

CONCLUSION

In this review, we have described the different IL-17–producing cells belonging to the innate immunity compartment. Lymphoid cells (iNKT cells, MAIT cells, γδ T cells, ILC3s) can produce IL-17 *via* engagement of their TCR. Nevertheless, these cells also possess a unique ability to produce IL-17 independent of the conventional TCR–antigen interaction, in response to their cellular and cytokine/chemokine environment. Thus, they have dual IL-17 production capacity that could be of prime importance in SpA pathogenesis. In addition, their ability to secrete IL-17 is immediate, not requiring differentiation or proliferation steps. They are at the ultimate stage of maturation and thus ready to "draw." Finally, as for other cells belonging to the innate immunity, their migratory capacity is also pronounced. These cells are found in greater quantity on all inflammatory sites, which suggests that they may be responsible for IL-17–driven inflammation in target tissues of SpA (i.e., spine, skin, gut, or joints). Their precise role in the pathophysiology of SpA remains to be better defined, but preliminary results from several teams converge to suggest their major importance in the pathophysiogenesis of SpA, especially MAIT cells and γδ1 T cells that are able to express IL-17A upon priming with various

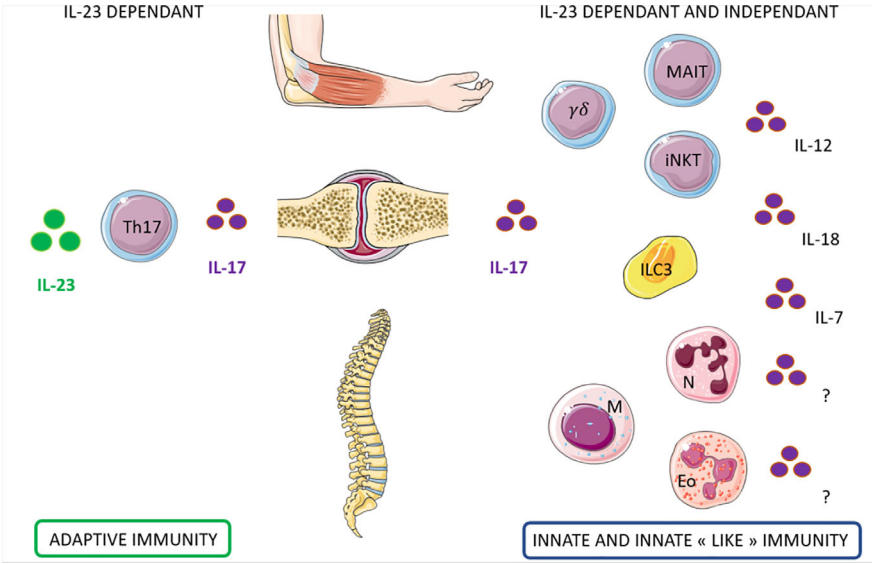

FIGURE 3 | Candidate cells that may contribute to IL-17 production dependent and independent of IL-23 in Spondyloarthritis. **Left:** Th17 cells contribute to the production of IL-17 through IL-23 dependant pathway for the entheseal and the peripheral involvement of Spondyloarthritis. **Right:** Innate immune cells may contribute to the production of IL17 through IL-23 but also independently. Several lymphoid cells (MAIT, γδT cells, iNKT, and ILC3) and myeloid cells (neutrophils, mast cells, eosinophils) has been identified as potential candidates. The cytokines that could induce this production independently from IL23 are still under investigation.

cytokines but independent of IL-23 (**Figure 3**). These results might be a path to understand why IL-17A blocking agents are effective in axSpA in contrast to IL-23–blocking drugs. These clinical results are a nice example of a reverse path from bedside to bench, where the results of therapeutic trials make us reflect more in depth on the pathophysiology of the disease.

AUTHOR'S NOTE

Servier medical art was used for the realization of the figures.

AUTHOR CONTRIBUTIONS

NR and CM-R contributed equally to this work and approved it for publication. All authors contributed to the article and approved the submitted version.

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OPINION
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Looking Beyond Th17 Cells: A Role for Tr1 Cells in Ankylosing Spondylitis?

Joanna McGinty, Nicola Brittain and Tony J. Kenna *

School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD, Australia

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INTRODUCTION

The chronic inflammatory arthritis ankylosing spondylitis (AS) is a highly heritable disease of complex genetics (1–3). The genetic association between AS and *HLAB27* has been studied for almost 50 years, and over the last decade or so large-scale genomics studies have defined variants outside the *HLA* that confer risk of developing AS (1, 2, 4). Key among these are genes involved in T cell activation (including *ERAP1, ERAP2, NPEPPS, UBE2E3, UBE2L3*), immune signaling (including *IL23R, IL6R, IL12B, TYK2*) and various transcription factors involved in functional differentiation of immune cells (including *TBX21, RUNX3 and EOMES*). Functional genomic approaches have implicated several immune cell types in disease processes, and studies support a role for microbial dysbiosis in disease pathogenesis. It is now obvious that AS is not only a genetically complex but also immunologically complex disease. To date, however, much effort has focused on 'low hanging fruit' from genomics studies, most notably *IL23R*. This important work has led to advances in treatment options for AS patients through inhibiting the pathogenic effects of IL-17. But trials of the IL-23 inhibitor Ustekinumab were not successful. Potential reasons for the failure of Ustekinumab Phase 3 trials in AS are many and include difficulties with outcome measures and trial design. But the failure has also sparked the community to re-evaluate subtleties in models of AS immunopathogenesis.

With many AS-associated genes implicated in various aspects of T cell biology, it is hard to pinpoint exact processes or pathways that are of critical importance in AS. Speculation needs to be supported by empirical observations from well-designed studies that push us beyond consideration of Th17 cell biology. Recently, Hanson and colleagues (5) provided evidence that AS patients exhibit significant reductions in the size of CD4 and CD8 T cell expansions globally in the peripheral blood, suggesting that perturbations in T cell survival, senescence, or regulation of clonal proliferation occur in AS patients during adaptive immune responses. This brings into focus what role regulatory T cells play in AS and indeed which populations of regulatory T cells may be of relevance to AS.

Regulatory T cells were originally described as a subset of immune cells critical for negative regulation of immune-mediated inflammation and prevention of autoimmune diseases. However, Tregs are also implicated in the suppression of both innate and adaptive immune cells towards allergens, organ transplants, commensals, food, and other innocuous environmental triggers (6).

$FoxP3^+$ Tregs may be thymically induced (tTregs), peripherally induced (pTregs) or induced in cell culture, in response to TGF-β. The tissue environment promotes Tregs to express tissue-specific transcription factors that cooperate with FoxP3, providing a specialized function and supporting Treg cell subset homeostasis (7). Tregs regulate their immune environment by contact-dependent

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Edited by:
Elisabetta Bianchi, Institut Pasteur, France

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***Correspondence:**
Tony J. Kenna
tony.kenna@qut.edu.au

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mechanisms, such as CD95 induction of conventional T cell apoptosis and CTLA4 downregulation of APC co-stimulatory function, as well as cytokine-mediated functions, including CD25 adsorption of IL-2 and IL-10 secretion which attenuates DC function and promotes Tr1 cell differentiation (8).

Tr1 cells, another subtype of regulatory T cells, do not constitutively express the transcription factor Foxp3. Upon TCR recognition of their cognate antigen at the site of tissue inflammation, Tr1 cells secrete large quantities of IL-10, which has many immunomodulatory effects on local immune cells (9). Both Tr1 and Treg cells serve a vital role in preventing deleterious immune responses with comparable mechanisms of suppression, yet Tregs are essential in the initial stage of immune suppression at the site of inflammation, while Tr1 cells are key for the maintenance of long-term tolerance and restoration of tissue homeostasis (10).

TR1 CELLS ARE IMPORTANT REGULATORS OF INFLAMMATION

Tr1 cells were first described in severe combined immunodeficiency disease (SCID) in the early 1990's (11). Since then, accumulating evidence implicates impaired function and reduced Tr1 cell numbers in immunopathogenesis of various immune-mediated diseases. Among these are inflammatory bowel disease (IBD), psoriasis, multiple sclerosis (MS), Grave's disease, Hashimoto's thyroiditis, and systemic lupus erythematosus (12–14). Broadly, IL-10 produced by Tr1 cells is a key regulator of TNF-mediated pathologies (15).

Naïve CD4+ T cells acquire a Tr1 phenotype upon cytokine signaling *via* IL-10, IL-21, or IL-27 which promotes STAT3 activation and subsequent priming of the *IL10* locus. Transcription factors that bind to *IL10* include EOMES, IRF4, c-Maf, Ahr, and Blimp-1 which act through multiple pathways to induce a stable production of IL-10 (10). In contrast, Th17 cells, although necessary for host defense against extracellular pathogens, when dysregulated become major pathogenic drivers of inflammation in many immune-mediated diseases. TGF-β and IL-6 are the key cytokines for initiating Th17 differentiation, which induces IL-23R expression as well as high secretion of the pro-inflammatory cytokine IL-17 (16, 17) (**Figure 1**). Microarray gene expression analysis comparing Tr1 cells and Th17 cells prior to IL-23 signaling identified the most predominantly overexpressed genes in Tr1 cells to be *IRF1, IRF8*, *PRDM1* (Blimp-1), and *TBX21* (18). IL-23 is secreted by various immune cells including dendritic cells (DCs) and macrophages in response to toll-like receptor signaling (19). Under homeostatic conditions, the presence of IL-23 in the distal small bowel promotes a localized cytokine environment that targets IL-23 sensitive intestinal cells which support mucosal barrier function and intestinal immunity. LAG-3, which is

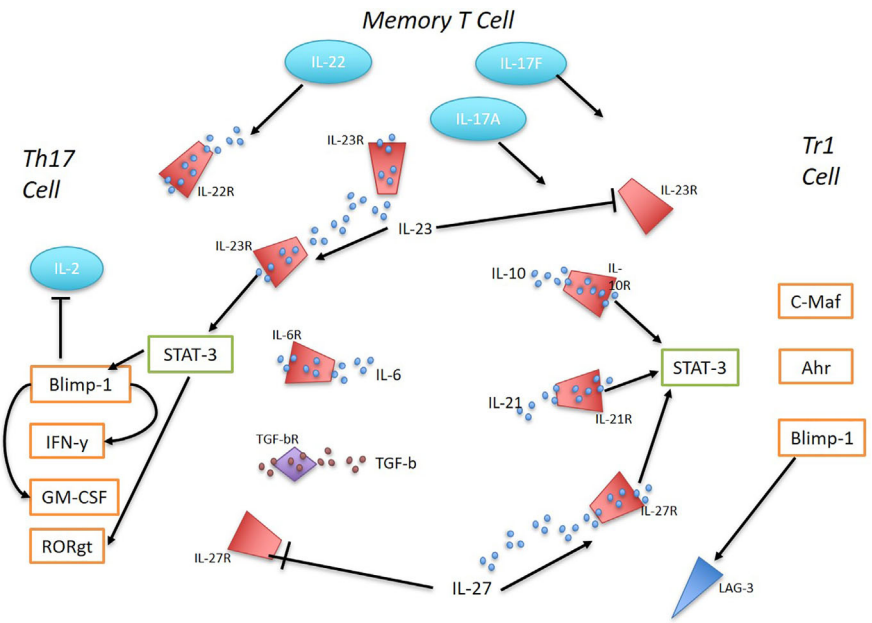

FIGURE 1 | The interplay between Th17 and Tr1 cells in inflammation. Ahr, c-Maf, and Blimp-1 promote the differentiation of Tr1 cells in response to IL-10, IL-21, and IL-27 *via* STAT3 activation. IL-27 was found to be sufficient to induce Blimp-1 in Tr1 cells and sustain high IL-10 production. IL-23 supresses Tr1 cells. Early Th17 cell development is initiated by TGF-B and IL-6, inducing IL-23R expression and IL-17 production. IL-23 signaling regulates Th17 cells and their pathogenicity. STAT-3 mediated IL-23-dependent Blimp-1 enhances Th17 pathogenic factors by increasing IL-23R expression, GM-CSF, IFN-y and repressing IL-2. RORγt expression is stabilized by IL-23R expression. IL-27 supresses Th17 cells. Memory T cells in response to TLR signaling produce IL-23. Memory T cells with Th17 pro-inflammatory phenotype secrete large amounts of 17A, IL-17F and IL-22.

expressed on natural regulatory T cells (Tregs), induced Tregs and Tr1 cells, has been shown to control intestinal IL-23 production by immunosuppression of CX3CR1+ tissue-resident macrophages and innate lymphoid cells (ILCs) type 3 (19, 20). *In vivo*, IL-23R signaling suppresses the differentiation of FoxP3+ Tregs and Tr1 cells and stabilizes the expression of RORγt, the Th17 signature transcription factor (16). IL-23 is a key factor for perpetuating and stabilizing Th17 cell activation and cytokine production as it induces strong proliferation of memory T cells that secrete large amounts of IL-17A, IL-17F, and IL-22 (16). IL-23-dependent signaling in Th17 cells induces Blimp-1 and in concert with T-bet, promotes pathogenicity by upregulating IL-23R expression GM-CSF and IFN-y while repressing IL-2 in a STAT3-mediated manner (21, 22).

Recent literature marks dysfunctional Tr1 cells and their reduced capacity to secrete large amounts of immune-mediating cytokine IL-10, as attributing to persistent inflammation in autoimmune disease contexts. Tr1 dysfunction is associated with inflammation in diseases genetically and clinically relevant to AS. For example, Tr1-derived IL-10 has a non-redundant role in preventing gut inflammation in IBD (22). In a mouse model of IBD, *IL23r* mRNA expression is detectable on both Tr1 cells and Th17 cells. Tr1 cells are responsive to IL-23 and downregulate IL-10 in response to IL-23R signaling (22). Clinical and genetic overlap between AS and IBD has been recognized for many years, and an increase in IL-23 has been well documented in both diseases (23, 24). Psoriasis, frequently concomitant with AS, is driven by chronic activation of autoreactive Th17 cells (25). Psoriasis patients exhibit an inverse relationship between disease severity and Tr1 and Treg cell numbers. Tr1 cells were not found in the skin of healthy controls; however, Tr1 cells were identified in the non-lesioned skin of psoriasis patients. Psoriatic lesions revealed an increase in activated $CD3^+CD4^+CD69^+$ T cells and a lack of Tr1 cells (26).

THE POTENTIAL ROLE OF THE IL-12/IL-23 AND IL-27 AXIS IN AS IMMUNOPATHOLOGY

IL-27 signaling induces Blimp-1-mediated IL-10 production in Tr1 cells, and in the absence of IL-23 signaling, Th17 cells respond to IL-27 and IL-12 signaling by secreting IL-10 in a Blimp-1 dependent manner. This demonstrates a potential for plasticity in Th17 cells as they lose their pathogenicity and adopt a Tr1-like phenotype which can contribute to homeostasis under certain conditions (18). In contrast, Th17 cells further stimulated by IL-23 demonstrated a commitment to the inflammatory phenotype (27), known to be implicated in many autoinflammatory conditions including AS (23). IL-27 levels are reported to be elevated in AS patients and to correlate with disease activity measures (28) which would seem to support development of Tr1 cells in AS patients. However, IL-23 counteracts IL-27 and IL-12-mediated effects on Tr1-development reinforcing the pro-inflammatory phenotype of Th17 cells (18). The balance between IL-23 *vs* IL-12/IL-27 signaling in CD4+ effector T cells determines whether tissue inflammation is perpetuated or resolved. It is our opinion that the immunomodulatory function of regulatory T cells is impaired in the context of AS, resulting in perpetual inflammation in the enthesis and ileum of patients with active disease. It is hypothesized that deficient IL-27 signaling, reduced IL-10 production by Tr1 cells and exacerbated IL-23 signaling promotes persistent IL-17 and other pro-inflammatory cytokine production and proliferation of pro-inflammatory T cell subsets. These three key factors that may diminish the capacity for immune regulation are linked to known AS genetic risk factors including *IL27, IL23R, TBX21*, and *EOMES* susceptibility mutations (1, 2). However, the link between AS susceptibility loci and development of Tr1 cells in AS has been further complicated recently. Pepelyayeva and colleagues reported reduced numbers of Tr1 cells in Erap−/− mice (29), a genotype that GWAS data associates with AS protection rather than risk (1). A better understanding of the role, if any, Tr1 cells play in AS patients may be a valuable step towards understanding how to control inflammation in this disease.

CAN TR1 CELLS OFFER ANY THERAPEUTIC POTENTIAL IN AS?

It is clear that Tr1 cells are an important regulator of general immune responses. Therapeutic manipulation of Tr1 cells, *ex vivo* or *in vivo* might be highly advantageous in several T cell-mediated diseases. Much progress has already been made in animal models, which proved that Tr1 cell-based therapies may be a feasible approach to treating inflammatory disorders in general.

Adoptive transfer of *in vitro* induced Ag-specific Tr1 cells efficiently prevents colitis induced in SCID mice by pathogenic T cells (30). In a pre-clinical model of type 1 diabetes Tr1 cells induced in the intestinal mucosa migrate to the periphery and control effector T cell responses and the development of diabetes (31). Studies in MS attributed deficiencies in IL-10-secreting Tr1 cells to decreased IL-27 and disruption of the CD46 pathway that promotes transformation of IFN-y-secreting Th1 cells into Tr1 cells. Exogenous IL-27 partially restored the number and function in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE) (32). IL-27 induced Tr1 cells have been described to have therapeutic potential in several autoimmune contexts by expanding their immunomodulatory function in active disease states.

Robust protocols have been established to generate clinical-grade human Tr1 cells (33, 34) and Tr1 cell-based therapies have been trialed in graft *versus* host disease (35) and refractory Crohn's disease (36) clinical trials.

DISCUSSION

Tr1 cells have an important role in autoimmune disease prevention, and investigating their potential to restore immune homeostasis in environments of persistent inflammation may be

beneficial in the context of AS. While the genetics of AS implicate Tr1 biology in disease processes discrepancies exist between *in vitro* data that suggest the cytokine environment in AS may be suitable for expansion of Tr1 cells (28) and *in vivo* data that show reduced Tr1 cells in mice that lack an important AS-susceptibility gene (29). A challenge from here is to define where and how Tr1 cells may be important in AS and to define pre-clinical models that will allow pre-clinical evaluation of their therapeutic potential in AS.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Interleukin-22 Influences the Th1/Th17 Axis

Hannes Lindahl * *and Tomas Olsson*

Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

Interleukin-22 (IL-22) is secreted by a wide range of immune cells and its downstream effects are mediated by the IL-22 receptor, which is present on non-immune cells in many organs throughout the body. IL-22 is an inflammatory mediator that conditions the tissue compartment by upregulating innate immune responses and is also a homeostatic factor that promotes tissue integrity and regeneration. Interestingly, the IL-22 system has also been linked to many T cell driven inflammatory diseases. Despite this, the downstream effects of IL-22 on the adaptive immune system has received little attention. We have reviewed the literature for experimental data that suggest IL-22 mediated effects on T cells, either transduced directly or *via* mediators expressed by innate immune cells or non-immune cells in response to IL-22. Collectively, the reviewed data indicate that IL-22 has a hitherto unappreciated influence on T helper cell polarization, or the secretion of signature cytokines, that is context dependent but in many cases results in a reduction of the Th1 type response and to some extent promotion of regulatory T cells. Further studies are needed that specifically address these aspects of IL-22 signaling, which can benefit the understanding and treatment of a wide range of diseases.

Keywords: interleukin 22 (IL-22), interferon gamma, interleukin 17 (IL-17), animal models, inflammatory disease, infectious disease

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***Correspondence:**
Hannes Lindahl
hannes.lindahl@ki.se

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INTRODUCTION

Interleukin-22 (IL-22) is often described as a cytokine that is expressed by immune cells but that exclusively acts on non-immune cells (1, 2). Its role is best understood at so called barrier surfaces such as the skin, lungs, and gut where the effects of IL-22 ligation typically involve proliferation, regeneration, or activation of innate immune mechanisms. Interestingly, the IL-22 system has also been linked to a range of T cell driven inflammatory diseases such as rheumatoid arthritis (RA), graft *versus* host disease (GvHD), and multiple sclerosis (MS) (2–4). However, relatively little is known about how IL-22 influences T cell polarization. The fact that the IL-22 receptor (IL-22R) has mostly been shown to be absent on immune cells has likely contributed to this (1). Although there

Abbreviations: IL, interleukin; RA, rheumatoid arthritis; MS, multiple sclerosis; IL-22R, IL-22 receptor; NKT cell, natural killer T cell; ILC, innate lymphoid cell; γδ T cell, gamma delta T cell; DC, dendritic cell; AhR, aryl hydrocarbon receptor; RORγt, RAR-related orphan receptor gamma t; TLR, Toll-like receptor; TGF-β, transforming growth factor beta; ICOS, inducible T cell co-stimulator; IL-22BP, IL-22 binding protein; TNF, tumor necrosis factor; IFNγ, interferon gamma; MAIT cell, mucosa associated invariant T cell; LTi cell, lymphoid tissue inducer cell; EAU, experimental autoimmune uveitis; APC, antigen presenting cell; CIA, collagen induced arthritis; LCMV, lymphocytic choriomeningitis virus; GvHD, graft *versus* host disease.

are an increasing number of reports of IL-22R expression on immune cells, in most cases any effect of IL-22 on T cells is likely indirect, transduced by mediators originating from IL-22 receptor expressing non-immune cells. IL-22 biology in general has been comprehensively described in excellent review articles (2, 4). Here, we have reviewed the literature for data that suggest effects of IL-22 on T helper cell polarization or secretion of the signature cytokines IFNγ and IL-17. Any experimental setup where IL-22 signaling has been specifically targeted has been screened for such data. By applying this perspective to the literature, we hope to promote further research on the role of IL-22 in shaping adaptive immunity in inflammatory and infectious diseases.

INTERLEUKIN 22

IL-22 was initially described as a cytokine produced by IL-9 activated T cells (5) and was later associated with the Th17 lineage (6). It has now become clear that IL-22 is not only frequently produced independently of IL-17 (7–9) but can also be produced by a wide range of other immune cells (**Table 1**). There are also reports of IL-22 being produced by non-hematopoietic cells (35, 36).

The most well-established inducer of IL-22 is IL-23 (6, 30). The IL-23 receptor has been detected on Th17 cells, natural killer T (NKT) cells, type 3 innate lymphoid cells (ILC3), gamma delta (γδ) T cells, macrophages, dendritic cells (DC), and neutrophils. Stimulation of these cells with IL-23 can induce production of IL-22 (11, 18, 29, 31, 32, 37, 38). Moreover, IL-1β can act both independently of and synergistically with IL-23 to induce IL-22 (18, 37, 39). IL-6 and TNF polarize naïve T cells to the Th22 lineage (8). The aryl hydrocarbon receptor (AhR) promotes the differentiation of several IL-22 producing cells such as Th17 cells, Th22 cells, and ILC3 (40, 41). Ahr is located in the cytoplasm where it senses endogenous and exogenous ligands, leading to nuclear translocation and transcription of *IL22* and other genes (41–43). Similarly, transcription factor RAR-related orphan receptor gamma t (RORγt) is also necessary for the differentiation of Th17 cells and ILC3 (40, 44). Furthermore, IL-22 induction by Toll-like receptor (TLR)2 ligation has also been described in innate lymphoid cells (45, 46).

Negative regulators of IL-22 production include transforming growth factor beta (TGF-β), IL-27, and Inducible T cell co-stimulator (ICOS), all of which transduce signals to the transcription factor c-Maf (47–49). Interestingly, IL-22 is one of few cytokines that has a dedicated soluble antagonist molecule to regulate its effects *in vivo*, IL-22 binding protein (IL-22BP), transcribed from the gene *IL22RA2* (**Figure 1**) (50, 51). Insights about the role of IL-22 can therefore also be gained from experiments in which IL-22BP levels have been manipulated.

Interleukin 22 Receptor

The IL-22R is a heterodimer of the subunit IL-10R2, which is expressed by most cells, and the more selectively distributed subunit IL-22R1 (**Figure 1**) (52–56). Ligation of IL-22R with IL-22 induces activation of the tyrosine kinases JAK1 and Tyk2, which in turn activates STAT3. STAT1 and STAT5 activation has also been reported (1, 57) as well as downstream signaling *via* the MAPK pathways (36, 58–60). IL-22R is expressed by epithelial and parenchymal cells in a wide range of tissues throughout the body. It is highly expressed at, but not limited to, barrier surfaces such as the skin, gut and lungs. Other tissues where IL-22 exerts effects include liver, thymus, pancreas, kidney, and synovium (25, 36, 61–63).

TABLE 1 | Cell types that express IL-22.

Cell type	Comments
Th1	Th1 polarization of human peripheral blood T cells *in vitro* induces IL-22 production (10).
Th17	In mice, expression of IL-22 is higher in Th17 cells compared to Th1 and Th2 (6, 11, 12). Colonization of germ-free mice with segmented filamentous bacteria promotes Th17 cells in the lamina propria of the small intestine (13).
Th22	Th22 is a distinct T helper subset that does not produce IFNγ, IL-4 or IL-17. It expresses CCR6 and skin homing CCR4 and CCR10. Differentiation is promoted by IL-6, TNF, and ligation of the AhR (7–9). *In vitro* and *in vivo*, Th22 cells show a marked plasticity toward IFNγ production under Th1 polarizing conditions (14).
CD8 T cells	IL-17 producing cytotoxic T cells often co-produce IL-22 (15–17).
γδ T cells	γδ T cells are an early source of IL-22 in several disease models (18, 19).
MAIT cells	MAIT cells from the female genital tract preferentially produce IL-17 and IL-22 after microbial stimulation in contrast to blood MAIT cells, which primarily produce IFNγ, TNF, and granzyme B (20).
NKT cells	Murine splenic NKT cells produce IL-22 after activation *in vitro* (21). IL-22 and IL-17 production by human NKT cells is largely segregated, implying distinct roles (22).
ILC3	NKp46$^+$ RORγt$^+$ ILCs are present in the intestines and are a source of IL-22, which has important local homeostatic and protective effects (23–26).
LTi cells	LTi cells are a rapid innate source of IL-22 involved in the development of secondary lymphoid tissue (27, 28).
Macrophages	IL-22 producing macrophages have been described in psoriatic skin (29).
Dendritic cells	*Ex vivo* and bone-marrow derived dendritic cells can express IL-22 in response to IL-23 (30, 31).
Neutrophils	Neutrophils can produce IL-17 and IL-22 in response to IL-23 (32, 33).
Mast cells	A subset of mast cells isolated from human skin affected with psoriasis or atopic dermatitis produce IL-22 (34).
Non-hematopoietic cells	Acinar cells of the lacrimal glands are the primary source of IL-22 in a mouse model of dry eye disease (35).

TNF, tumor necrosis factor; AhR, aryl hydrocarbon receptor; IFNγ, interferon gamma; γδ T, gamma delta; MAIT cells, mucosa associated invariant T cells; NKT cells, natural killer T cells; RORγt, RAR-related orphan receptor gamma t; ILC, innate lymphoid cells; LTi cells, lymphoid tissue inducer cells.

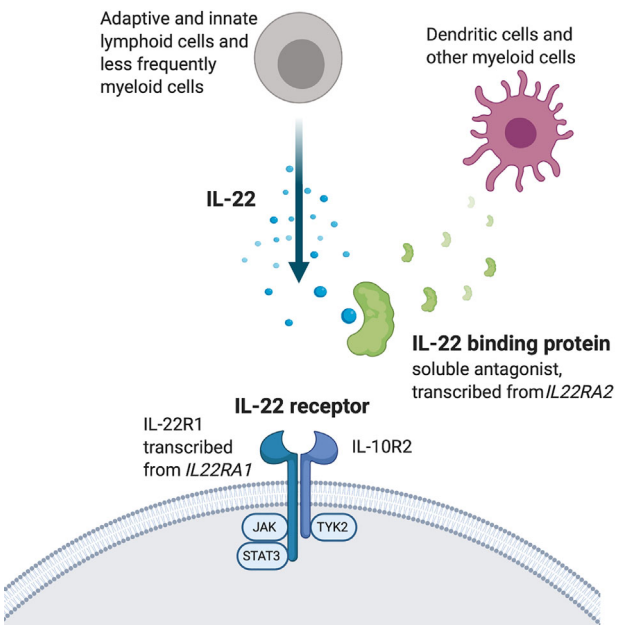

FIGURE 1 | The IL-22 system.

IL-22R has mostly not been detected on immune cells, which has established IL-22 as a cytokine that mediates one-way signaling from immune cells to tissue cells. However, there are reports of IL-22R expression on both myeloid and lymphoid cells and functional data on the effects of IL-22 on these cells. IL-22 has direct effects on splenic CD4 T cells, B cells and $CD11b^+$ cells in a mouse model of autoimmune arthritis (64–67) and on splenic $CD11b^+$ cells in a mouse model of autoimmune uveitis (68). Moreover, infiltrating mononuclear cells in the salivary glands of patients with primary Sjogren's syndrome express IL-22R1 shown by immunohistochemistry (69). Further characterization by flow cytometry showed IL-22R expression on macrophages and, to a lesser extent, on T and B cells both in salivary glands and in the circulation from primary Sjogren's syndrome patients but IL-22R was not detected at all in samples from non-specific chronic sialoadenitis. During the acute stage of *Mycobacterium tuberculosis* infection in mice, IL-22R is expressed primarily on epithelial cells. However, during the chronic stage both epithelial cells and recruited macrophages express IL-22R, which also was observed in human samples (46). IL-22 inhibits intracellular growth of *M tuberculosis* in human monocyte derived macrophages (70). Stimulating peripheral blood mononuclear cells from primary Sjogren's syndrome patients *in vitro* with IL-22 increased production of IL-17, which was not seen in non-specific chronic sialoadenitis (69). $CD14^+$ adipose tissue macrophages, but not circulating $CD14^+$ cells, express IL-22R1 shown by western blot and FACS (71). IL-22 strongly induces IL-1β from $CD14^+$ adipose tissue macrophages.

THE INFLUENCE OF INTERLEUKIN-22 ON THE TH1/TH17 AXIS

Inflammatory Diseases

Uveitis

The animal model experimental autoimmune uveitis (EAU) can be induced by immunization with retinal autoantigens and disease severity is evaluated by fundoscopy. Treatment with IL-22 before onset of EAU in mice results in reduced severity of disease and delayed onset (68). The protective effect of IL-22 in this model is associated with an overall reduction in eye-infiltrating cells with a proportional decrease in T cells and neutrophils. Interestingly, the IL-22R subunit *Il22ra1* is highly expressed by splenic $CD11b^+$ cells day 12 after immunization. When stimulated *in vitro* with IL-22, these cells produce less IL-6, IL-12, IL-23, and IL-1β but more IL-10 and TGF-β. Consistent with a tolerogenic phenotype, they also express less MHC class II, CD80, CD86, and CD40 but more PD-L1. IL-22 treated APCs yield less antigen specific T cell proliferation *in vitro* and induce less IFNγ and IL-17 production but more IL-10 production.

Dry Eye Disease

Dry eye disease is predominantly a Th17 cell driven autoimmune disorder resulting in ocular mucosal inflammation, which in severe cases can lead to damage to the cornea and vision loss. Both IL-17 and IL-22 are elevated in tear and lacrimal fluid in persons with dry eye disease (35). IL-17 levels are positively correlated and IL-22 levels negatively correlated with severity of disease. In a mouse model of dry eye disease IL-22 neutralizing antibody or *Il22* gene deletion both result in increased infiltration of Th17 cells and more severe disease, consistent with a protective effect of IL-22. IL-22R is expressed on the ocular surface and *in vitro* stimulation of corneal epithelial cells with IL-22 inhibits expression of inflammatory mediators, including the Th17-inducing cytokines IL-6 and IL-23.

Colitis

The homeostatic role of IL-22 in the gastrointestinal tract has received much attention (72). Activation of AhR alone promotes IL-22 secretion by intestinal leukocytes but it also acts synergistically with transcription factor RORγt (40). Steady state $Ahr^{-/-}$ mice have an increased number of intestinal Th17 cells as a result of commensal segmented filamentous bacteria expanding and locally inducing Th17 cells (73). Administration of IL-22 to $Ahr^{-/-}$ reduces Th17 cells to numbers that approach normal levels. The increased intestinal Th17 numbers in $Ahr^{-/-}$ mice do not lead to any overt gut pathology. However, when one allele of the RORγt gene is deleted in $Ahr^{-/-}$ mice IL-22 production is further decreased, which is associated with occasional observations of spontaneous colitis. The authors of this study suggest that in immunocompromised patients, that potentially have impaired IL-22 production, the normally innocuous segmented filamentous bacteria may expand and

cause intestinal autoimmunity through induction of pathogenic Th17 cells.

Arthritis

Collagen induced arthritis (CIA) is an animal model of RA. Daily administration of recombinant IL-22 starting before onset of arthritis reduces disease severity of CIA in DBA mice but does not alter incidence (74). The IL-22 treatment increases expression of IL-10 in the spleen and administration of neutralizing IL-10 antibody together with IL-22 cancels the protective effect of the latter. IL-22 induces secretion of IL-10 from $CD11b^+$ splenocytes harvested during early disease. Addition of IL-22 decreases IFNγ secretion induced *in vitro* by polyclonal T cell activation of splenocytes harvested from mice with early disease as well as from naïve mice. In contrast, stimulation with IL-22 increases IL-17 secretion from splenocytes restimulated *in vitro* with collagen.

In a follow-up study, Justa et al. showed that IL-22 has a dual role in CIA. Treatment with a neutralizing IL-22 antibody before onset of arthritis increases disease severity consistent with the previous study, whereas treatment after onset reduces disease severity (64). Surprisingly, they show that after disease onset IL-22 actually decreases *in vitro* IFNγ production from splenocytes after restimulation with collagen. No effect on IL-17 or IL-10 was observed in this context. However, IL-22 causes increased proportions of Th1 cells in the draining lymph nodes but still does not influence Th17 cells. In the affected joints the proportions of Th17 cells are increased as result of IL-22 signaling. *Il22ra1* mRNA and IL-22R1 protein expression is detected in $CD4^+$ splenocytes from mice with arthritis but not at baseline or during the initiation phase. $CD11c^+$ cells and CD4 T cells from the spleen of arthritic mice co-cultured in the presence of recombinant IL-22 results in less production of IFNγ but more IL-17. The protective effect of treatment with anti-IL-22 after disease onset is blunted when performing the experiment on $Ifng^{-/-}$ mice. In contrast, mice treated with anti-IL-22 before disease onset exacerbates disease, which is associated with decreased Th1 and unchanged Th17 proportions in draining lymph nodes but increased IL-17 production from cells from the affected joints. In summary, the pathogenic effect of IL-22 during late CIA is dependent on suppression of IFNγ, possibly mediated directly *via* IL-22R expression, which is upregulated on CD4 T cells during this phase of the disease.

A study by another group primarily focused on the influence of IL-22 on autoantibody formation in the context of CIA in C57BL/6 mice but also includes some data on T cells (75). They report that germinal center and autoantibody formation is reduced in $Il22^{-/-}$ mice and IL-22R expression is detected in follicular dendritic cell-like stromal cells. Furthermore, human lymphoid stromal cells produce B cell attracting chemokines CXCL12 and CXCL13 upon stimulation with IL-22. Regarding T cells, they show that, despite having less severe CIA, the $Il22^{-/-}$ mice have increased proportions of Th17 cells in the spleen ten days after immunization. The Th17 cells retain their pathogenic potential, which is shown by assessing secretion of IL-6 in co-cultures with synovial fibroblasts.

Psoriasis Arthritis

Psoriasis arthritis is characterized by skin lesions and articular inflammation and is often accompanied by osteoporosis. As with many of the inflammatory diseases Th17 cells have been shown to be critical in psoriasis arthritis (76). Central in the differentiation of Th17 cells is activation of transcription factor STAT3. In a novel model of psoriasis arthritis based on overactive STAT3 function specifically in CD4 T cells, the psoriatic skin phenotype and osteopenia were both ameliorated by either neutralizing IL-17 antibody or genetic deletion of *Il22* (77). The $Il22^{-/-}$ mice had no reduction in total T helper cell infiltration in the skin but had reduced proportions of Th17 cells consistent with a disease promoting role of IL-22. Increased proportions of Tregs but also Th1 cells were seen in the inflamed skin of the $Il22^{-/-}$ mice. Although an improvement in the osteoporosis phenotype was seen in the $Il22^{-/-}$ mice the proportion of $IL\text{-}17^+$ cells in the bone marrow was increased. Similarly, the proportions of Th1 cells and Tregs were also increased.

Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS and can be induced by immunization with myelin autoantigens, which leads to a T cell driven disease characterized by ascending paralysis. Although $Il22^{-/-}$ mice have no apparent phenotype in EAE experiments (78), mice lacking the endogenous antagonist molecule IL-22BP have less severe disease compared to wild type mice, suggesting a protective role for IL-22 (79). These seemingly contradictory findings can be reconciled if the presence of IL-22BP blocks IL-22 in wild type mice to such a degree that they are indistinguishable from $Il22^{-/-}$ mice. Consistent with the reduced EAE severity, IL-22BP knockout mice have decreased infiltration of $Ly6C^+$ inflammatory monocytes in the central nervous system (79). Although not statistically significant, a trend toward less $Il\text{-}17^+$ T cells infiltrating the CNS in IL-22BP knockout mice is seen. In a follow-up study the role of IL-22BP in EAE was further dissected using an inducible *in vivo* IL-22BP knockdown rat strain. Reducing IL-22BP expression before EAE immunization results in reduced incidence and severity of disease in conjunction with increased proportions of Tregs and decreased proportions of Th1 cells in the lymph nodes that drain the site of immunization (80). Taken together, IL-22 appears to have a protective effect in autoimmune neuroinflammation but, unlike many other animal models discussed in this article, the effect of endogenous IL-22 is normally blocked by IL-22BP. Homeostatic expression of this soluble antagonist molecule is detected in many tissues including secondary lymphoid organs where immune responses are initiated and in the CNS by microglia, making it an interesting pharmacological target in MS and other neuroinflammatory diseases.

Dermatitis

In contrast to EAE, IL-22BP knockout mice have more severe disease in an imiquimod induced model of psoriasis (81). This is

consistent with the well documented pathogenic role of IL-22 in rodent models of psoriasis (4). The draining lymph nodes of the IL-22BP knockout mice have larger proportions of $CD44^+$ $CD62L^-$ activated T cells. Also, proportions of both IFNγ producing and IL-17 producing CD8 T cells are elevated compared to wild type mice.

Allergic Airway Inflammation

The role of IL-22 has been investigated in a model of atopic dermatitis and allergic asthma in which mice are epicutaneously sensitized to the model antigen ovalbumin followed by intranasal challenge (82). Sensitization alone results in increased serum levels of IL-22 and after intranasal challenge increased *Il22* mRNA as well as increased eosinophil and neutrophil infiltration is detected in the airways. Applying the disease model to $Il22^{-/-}$ mice results in less eosinophils and neutrophils in the airways and reduces airway resistance after methacholine provocation. Cells acquired by bronchoalveolar lavage from $Il22^{-/-}$ mice contain more IFNγ producing type 1 ILCs compared to wild type mice. No change was observed regarding $IFN\gamma^+$ CD4 and CD8 T cells. The authors go on to show that IL-22 synergizes with TNF to drive the neutrophil dominated airway inflammation.

Hepatitis

IL-22 has well described protective and regenerative effects on the liver parenchyma after various insults (61, 83–85). In contrast, IL-22 has a disease promoting net effect on chronic hepatitis induced in a mouse strain, transgenically made to express hepatitis B virus antigens, that is treated with anti-CD137 to activate T cells. Using this model, $Il22^{+/+}$ mice have increased disease severity, infiltration of granulocytes and T cells compared to $Il22^{-/-}$ mice (86). Moreover, $Il22^{+/+}$ mice also have an altered balance between T cell responses compared to the $Il22^{-/-}$ mice, with higher hepatic and splenic Th17 numbers but unchanged Th1 numbers. Anti-IL-22 treatment reduces the hepatic stellate cell expression of CXCL10 and CCL20, ligands for CXCR3 and CCR6 respectively, both being chemokine receptors expressed on Th17 cells. IL-22 treated hepatic stellate cells have increased chemotactic potential in a transwell assay and specifically attracts Th17 cells.

Atherosclerosis

Th1 and Th17 cells both promote atherosclerosis, whereas Tregs are protective. The role of Th2 cells is unclear. Circulating IL-22 and Th22 cells are both elevated in patients with acute coronary syndrome (87). The role of IL-22 has been investigated using a mouse model in which atherosclerosis is induced by feeding $ApoE^{-/-}$ mice a so called western diet (88). By administering either recombinant IL-22 or neutralizing IL-22 antibody it was shown that IL-22 aggravates atherosclerosis, which was associated with increased macrophage and T cell infiltration in the blood vessels with a proportional increase in Th17 cells. IL-22 receptor is expressed in mouse aortic tissue and its expression is further elevated in the atherosclerosis prone mice.

Infectious Diseases

Chlamydial Lung Infection

Chlamydial organisms are obligate intracellular gram-negative bacteria that can cause pneumonia in humans, which is modeled in mice using *Chlamydia muridarum*. The role of IL-22 has been investigated in this disease model using either administration of recombinant IL-22 or neutralizing IL-22 antibody (89). In both cases IL-22 was shown to improve outcome associated with increased IL-17 production in infiltrating cells in the lungs and spleen *ex vivo* as well as in splenocytes after antigen specific activation *in vitro*. Conflicting results were obtained regarding IFNγ production. Anti-IL-22 treated mice also display lower IL-10 production *ex vivo* and after antigen specific stimulation *in vitro*.

Opportunistic Fungal Lung Infection

To mimic an opportunistic fungal lung infection in an immunocompromised patient, mice were treated with a neutrophil-depleting antibody prior to infection with *Aspergillus fumigatus* (90). Furthermore, to investigate the role of gut microbiota in this context, the mice were pre-treated with vancomycin prior to infection, which suppresses intestinal segmented filamentous bacteria and other gram-positive bacteria. Vancomycin pre-treatment did not affect disease severity in that study but did, however, reduce the presence of IL-17 and IL-22 in the lungs. They show that $Il22^{-/-}$ mice have increased colonization of intestinal segmented filamentous bacteria, consistent with Ivanov et al. (13). After infection with *A. fumigatus*, the amount of IL-17 in $Il22^{-/-}$ lung tissue appeared to be increased ($p = 0.06$), an effect that was obliterated by pre-treatment with vancomycin. Vancomycin pre-treatment of wild type mice followed by fecal transplant, neutrophil depletion, and *A. fumigatus* infection results in more Th17 cells infiltrating the lungs after fecal transplants from $Il22^{-/-}$ compared to wild type mice. Transferring serum from wild type mice colonized with segmented filamentous bacteria to uncolonized wild type mice infected with *A. fumigatus* results in a decrease in Th17 cells in the lungs compared to transferring serum from colonized $Il22^{-/-}$ mice. This effect is counteracted by premixing the serum with IL-1Ra (Anakinra). Wild type serum has decreased levels of IL-1α but similar levels of IL-1β compared to $Il22^{-/-}$ serum. Collectively making it plausible that IL-22 modulates serum IL-1Ra ligands leading to accumulation of Th17 cells in the lungs after fungal infection.

Tuberculosis

The immune response to *Mycobacterium tuberculosis* varies depending on the infecting strain. The hypervirulent W-Beijing lineage of *M tuberculosis* is a growing health threat that is often associated with human immunodeficiency virus and drug resistance. This infection is modeled in mice with the *M. tuberculosis* strain HN878 (46). In this context, $Il22^{-/-}$ mice have normal susceptibility to infection but have higher bacterial burden in the lungs during the chronic stage. IL-22 deficiency has no impact on alveolar macrophages, monocytes, or recruited macrophages in the lungs during the acute stage of disease but

reduces the numbers of IFNγ secreting CD4 and CD8 T cells as well as IL-17 secreting CD8 T cells. However, during the chronic stage the numbers of monocytes and recruited macrophages are lower in the *Il22*$^{-/-}$ mice consistent with the impaired bacterial clearance. Interestingly, IL-17, which has been shown to be required for protective immunity to this strain (91), is increased in CD8 T cells at this stage despite the poorer outcome. This implies that the lack of IL-22 and associated reduction in IFNγ production and increased bacterial burden are not compensated for by increased IL-17 production.

Malaria

Plasma levels of IL-22 are elevated in acute infection with *Plasmodium falciparum* in humans as well as in the murine malaria model *Plasmodium berghei* infection in C57BL/6 mice. *Il22*$^{-/-}$ mice have earlier onset of cerebral malaria compared to wild type mice despite similar parasite burden in the liver and decreased parasitemia (92). Similar results were seen in experiments using neutralizing IL-22 antibody *in vivo*. Day 3 after infection the *Il22*$^{-/-}$ mice have increased proportions of IFNγ^{+} cells in the spleen, evident in both CD4 and CD8 T cells as well as γδ T cells. Although no difference in IFNγ expression was observed day 6, IL-17 expression in CD4 T cells as well as γδ T cell is decreased at both time points in *Il22*$^{-/-}$ mice. APC and CD8 T cell co-cultures using bone marrow derived dendritic cells pulsed with antigen showed that both APCs and CD8 T cells from *Il22*$^{-/-}$ mice are primed to produce more IFNγ compared to cells from wild type mice. Both CD11c^{+} CD11b^{-} dendritic cells *ex vivo* and bone marrow derived dendritic cells from *Il22*$^{-/-}$ mice have increased lipopolysaccharide induced expression of CD80 and CD86. Adoptive transfer of splenocytes from OT1 mice, that have ovalbumin specific T cell receptors, into wild type or *Il22*$^{-/-}$ recipients followed by infection with a transgenic ovalbumin-expressing malaria strain results in more antigen specific T cell proliferation in the *Il22*$^{-/-}$ recipients.

Bacterial Colonization

The potential of IL-22 to influence the crosstalk between the microbiota and the immune system during non-inflammatory conditions in the mouse has been examined. *Staphylococcus epidermidis* was applied to the skin and using both gene-deleted mice and neutralizing antibody, the authors demonstrate IL-22 dependent upregulation of MHC class II expression on keratinocytes, which results in increased numbers of *S. epidermidis* specific Th1 in the skin but no difference was observed regarding Th17 cells (93). Genetic deletion of MHC class II specifically in keratinocytes reduces the Th1 cells but leaves Th17 cells unchanged. The authors speculate that this may be a result of differential requirements of Th1 and Th17 cells to local co-stimulation and chemokines produced by activated keratinocytes.

Viral Infection

The role of IL-22 in viral infections is less studied compared to bacterial infections. A study reports that IL-22 is secreted in liver and lymphoid organs within the first few days after intravenous administration of lymphocytic choriomeningitis virus (LCMV) (94). Here IL-22 is expressed mainly by γδ T cells and is dependent on the PI3K/mTOR pathway but not AhR signaling (94). IL-22R was detected on CD45^{-} cells in both the thymus and the spleen. Infecting *Il22*$^{-/-}$ mice with the LCMV results in increased expression in splenic CD4 T cells of activation marker CD44, chemokine receptor CXCR3, and proliferation marker ki67. Antigenic restimulation of splenic and liver T cells *in vitro* results in higher proportions of IFNγ^{+} CD4 and CD8 T cells. Overexpression of IL-22 leads to the opposite results, confirming that IL-22 dampens IFNγ^{+} T cell responses during acute (7 days) and persistent (60 days) LCMV infection in both lymphoid organs and the liver.

Immunization

In a study explicitly designed to study potential indirect effects of IL-22 on T helper cell responses, mucosal immunization with ovalbumin and the adjuvant cholera toxin was performed (95). In *Il22*$^{-/-}$ mice this results in greater antigen specific T cell responses to mucosal (intrarectal), but not systemic (intraperitoneal) immunization. Polyclonal and antigen specific restimulation *in vitro* of splenic T cells from the mucosally immunized *Il22*$^{-/-}$ mice results in elevated secretion of IFNγ and IL-17, but no difference was seen after systemic immunization. In this case the proposed mechanism is increased epithelial permeability in the mucosal membrane in the absence of the homeostatic trophic effects of IL-22 allowing more antigen to come in contact with the immune system.

Other Diseases

Multiple Myeloma

Circulating Th17 cells as well as serum levels of IL-22 and IL-17 are elevated in multiple myeloma patients compared to healthy controls, which has spurred investigations into the role of IL-22 in this disease. When peripheral blood mononuclear cells from multiple myeloma patients are cultured under Th1 polarizing conditions in the presence of IL-22 or IL-17, no effect on IFNγ was observed but when the two cytokines are combined IFNγ production is reduced (96). The authors suggest that the elevated circulating IL-22 and IL-17 in multiple myeloma patients may dampen Th1 responses potentially contributing to the observed immune dysfunction in this patient group.

Radiation Induced Thymic Injury

IL-22 produced by local lymphoid tissue inducer (LTi) cells after radiation induced thymic injury is essential for the regeneration of thymopoiesis (97). In this disease model, the upregulation of IL-22 in the thymus is dependent on IL-23 from dendritic cells, primarily of the CD103^{+} subset, and may be triggered by the loss of double positive thymocytes. IL-22R subunit *Il22ra1* is expressed on thymic epithelial cells and *in vitro* treatment of these cells with IL-22 results in improved survival and increased

proliferation. Administration of IL-22 to irradiated mice with or without subsequent hematopoietic stem cell transplantation results in increased thymic cellularity, including all developing thymocyte subsets and thymic epithelial subsets.

Lung Cancer

IL-22 and Th22 cells are elevated in sera and tumor samples from patients with lung cancer and high IL22R1 expression is an indicator of poor prognosis in non-small cell lung cancer. In a study aimed at elucidating the role of IL-22 in tumor-promoting inflammation a *Kras*-induced mouse lung cancer model was used in combination with genetic deletion of *Il22* (98). In the absence of IL-22 lung tumor burden is reduced. Characterization of the bronchoalveolar lavage fluid T cells showed reduced proportions of Tregs and increased proportions of IFNγ$^+$ CD4 and CD8 T cells. IL-22 is known to induce STAT3 activation, which has also been observed in non-small cell lung cancer and is furthermore associated with poor prognosis, thus being a plausible mechanism for the effects on tumor burden in the mouse model. However, this would not explain the observed effects of IL-22 on T cell phenotype. The authors propose that pharmacologic targeting of IL-22 may have potential as an add-on therapy to conventional treatments of *KRAS*-mutant lung cancer.

Liver Allograft Rejection

Both adaptive and innate immune cells influence acute liver transplant rejection. IFNγ and IL-17 secretion is involved but the role of IL-22 is largely unknown. In a rat model of acute liver allograft rejection treatment with IL-22 neutralizing antibody 12 h before sacrificing the animal day 1, representing ischemia-reperfusion-injury, results in worse liver function (99). In contrast, treatment with IL-22 antibody 24 h before sacrificing the animal on day 7, representing acute rejection, results in improved liver function. At both timepoints IL-22 promoted expression of anti-apoptosis and pro-regeneration associated genes, implying that another mechanism overrides these effects day 7 when clinical outcome is better compared to controls. IL-22 neutralization is associated with increased proportions of Tregs and decreased proportions of Th17 cells in the liver allografts day 7 but no difference was observed day 1. The authors propose that at both timepoints the effect of IL-22 is mediated *via* STAT3. During the ischemia-reperfusion-injury stage the protective effect on hepatocytes by induction of anti-apoptotic and reparative factors lead to better clinical outcome but at the acute rejection stage day 7 induction of hepatocyte chemokine secretion and Th17 type inflammation dominate leading to worse clinical outcome.

Graft *Versus* Host Disease

Several studies have examined the role of IL-22 in GvHD. In a model of acute GvHD performed by injecting C57BL/6 splenocytes into F1 progeny of C57BL/6 and D2 mice, neutralizing IL-22 antibody was administered simultaneously and results in increased survival as well as suppressed expansion of donor CD8 T cells and decreased disease-associated depletion of host cells, particularly B cells (100). This is associated with increased proportions of Tregs in the spleen and decreased proportions of IFNγ, IL-4, and TNF secreting CD4 T cells. Splenic CD11b$^+$ cells harvested from acute GvHD mice have upregulated IL-22R mRNA as well as protein and cells from IL-22 antibody treated mice have decreased expression of co-stimulatory molecules. Co-cultures with CD11b$^+$ cells from IL-22 antibody treated mice with normal C57BL/6 CD4$^+$ CD25$^-$ T cells promote Treg induction.

The role of IL-22 has also been investigated in a bone marrow transplantation model in which recipient mice were exposed to total body irradiation followed by injection of T cell depleted allogenic bone marrow (101). Treatment with recombinant IL-22 in this model accelerates thymic reconstitution. To induce GvHD the recipient mice were injected with allogenic T cells from the same donor mouse strain. Treatment with recombinant IL-22 has no effect on acute GvHD (day 7) but reduces severity of chronic GvHD (day 60), which is associated with increased numbers of natural Tregs in the thymus and the spleen and decreased numbers of Th1 cells in the spleen. No effect was seen on induced Tregs. The same group has shown using the same acute GvHD model with *Il22*$^{-/-}$ mice that recipient derived IL-22 reduces severity of disease and improves survival day 30 (102). The *Il22*$^{-/-}$ mice has higher proportions of Th1 cells in liver, spleen, and intestines. Tregs are reduced but no effect was seen on Th17 cells. IL-22R mRNA was detected in bone marrow derived dendritic cells and stimulation of these with recombinant IL-22 results in reduced expression of CD80 and IFNγ. Compared to wild type bone marrow derived dendritic cells *Il22*$^{-/-}$ counterparts co-cultured with wild type T cells results in higher proportions of Th1 cells and lower proportions of Tregs.

CONCLUDING REMARKS

Published data that demonstrate direct or indirect effects of IL-22 on T cell polarization or secretion of signature cytokines in the context of a wide range of diseases are compiled in this review (**Table 2**, **Figures 2** and **3**). The observed effects are not uniform, but the data suggest that IL-22 signaling often results in reduced Th1 type responses and it may also contribute to resolution of inflammation by promoting Tregs or IL-10 secretion.

IL-22 has an important role in the response to bacterial pathogens in several models of infectious diseases including Ci*trobacter rodentium* colitis (30, 40) and *Klebsiella pneumonia* (103) as well as *Mycobacterium tuberculosis* infections of the lungs (46). The proposed mechanisms have primarily been maintenance of physiological barriers and induction of antimicrobial peptides. Considering this, it is somewhat surprising that available data frequently describe an inhibitory effect of IL-22 on Th1 immune responses. In contrast, if one considers the inflammatory diseases, an inhibitory effect of IL-22 on inflammatory T cell responses is consistent with the observed net effect of IL-22 on most models. The notable exception is psoriasis and other dermatitides where

TABLE 2 | The influence of IL-22 on the Th1/Th17 axis.

Phenotype	Context	Effect	Influence of IL-22	References
Less IFNγ	Healthy human	–	Decreases IFNγ during Th1 differentiation of PBMCs when combined with IL-17	Prabhala et al. (96)
	EAU	pos	Decreases splenic T cell IFNγ *in vitro*	Ke et al. (68)
	Murine malaria	pos	Decreases splenic T cell IFNγ *ex vivo* and *in vitro*	Sellau et al. (92)
	CIA (treatment after onset)	neg	Decreases IFNγ or Th1 cells in spleen, draining lymph nodes, and APC/T cell co-culture	Justa et al. (64)
	CIA	pos	Decreases IFNγ after polyclonal activation of splenocytes *in vitro*	Sarkar et al. (74)
	EAE	pos	Decreases Th1 cells in draining lymph node cells 7 days after immunization	Lindahl et al. (80)
	GvHD (bone marrow)	pos	Decreases Th1 cells in the spleen day 60	Pan et al. (101)
	GvHD (splenocytes)	pos	Decreases donor Th1 cells in target tissues	Pan et al. (102)
	Allergic airway inflammation	pos	Decreases IFNγ in BAL cells	Leyva-Castillo et al. (82)
	Lung cancer	neg	Decreases Th1 and IFNγ$^+$ cytotoxic T cells in lung tumor tissue	Khosravi et al. (98)
	Vaccination	pos	Decreases IFNγ in restimulated splenocytes	Budda and Zenewicz (95)
	Psoriasis arthritis model	neg	Decreases Th1 cells in the skin and bone marrow	Yang et al. (77)
	LCMV infection	neg	Decreases Th1 and IFNγ$^+$ cytotoxic T cells in the liver and spleen	Yi et al. (94)
More IFNγ	Mycobacterial lung infection	pos	Increases IFNγ$^+$ T cells in the lungs during the acute stage of disease only	Treerat et al. (46)
	CIA (treatment before onset)	pos	Increases Th1 cells in draining lymph nodes	Justa et al. (64)
	Psoriasiform dermatitis	neg	Increases IFNγ$^+$ cytotoxic T cells in the draining lymph nodes after polyclonal stimulation *in vitro*	Lindahl et al. (81)
	Staphylococcal colonization on skin	–	Increases antigen specific Th1 cells	Tamoutounour et al. (93)
	GvHD	neg	Increases Th1 cells in the spleen	Wu et al. (100)
Less IL-17	Untreated mice	–	Decreases Th17 cells in the intestines	Qiu et al. (73)
	EAU	pos	Decreases splenocyte IL-17 expression *in vitro*	Ke et al. (68)
	Pulmonary fungal infection	–	Decreases Th17 cells in the lungs	McAleer et al. (90)
	Murine malaria	pos	Decreases Th17 cells in the spleen	Sellau et al. (92)
	CIA	pos	Decreases splenocyte IL-17 expression *ex vivo*	Corneth et al. (75)
	CIA (treatment before onset)	pos	Decreases Th17 cells in joints	Justa et al. (64)
	Vaccination	pos	Decreases IL-17 production in antigen stimulated splenocytes *in vitro*	Budda and Zenewicz (95)
	Psoriasis arthritis model	neg	Decreases Th17 cells in the bone marrow	Yang et al. (77)
	Dry eye disease model	pos	Decreases Th17 cells in the eyes	Ji et al. (35)
More IL-17	Mycobacterial lung infection	pos	Increases IL-17$^+$ cytotoxic T cells during the acute and chronic phase of the disease	Treerat et al. (46)
	Chlamydial lung infection	pos	Increases Th17 cells the lungs and spleen	Peng et al. (89)
	Chronic hepatitis	neg	Increases Th17 cells in the liver and spleen	Zhao et al. (86)
	CIA (treatment after onset)	neg	Increases Th17 cells in the joints and splenic APC/T cell co-cultures	Justa et al. (64)
	CIA	pos	Increases Th17 cells in the spleen after antigen specific restimulation	Sarkar et al. (74)
	Psoriasiform dermatitis	neg	Increases IL-17$^+$ cytotoxic T cells in the draining lymph nodes after polyclonal stimulation *in vitro*	Lindahl et al. (81)
	Psoriasis arthritis model	neg	Increases Th17 cells in the skin	Yang et al. (77)
	Atherosclerosis	neg	Increases Th17 cells in the blood vessel	Lin et al. (87)
	Liver allograft rejection – acute rejection	neg	Increases Th17 cells in the allografted liver	Zhang et al. (99)
More IL-10	EAU	pos	Increases splenic T cell IL-10 expression *in vitro*	Ke et al. (68)
	CIA	pos	Increases splenic T cell IL-10 expression *in vitro*	Sarkar et al. (74)
	Chlamydial lung infection	pos	Increases T cell IL-10 expression in the lungs, spleen, and lymph nodes	Peng et al. (89)
Less Tregs	GvHD	neg	Decreases Tregs in the spleen	Wu et al. (100)
	Psoriasis arthritis model	pos	Decreases Tregs in the skin and bone marrow	Yang et al. (77)
	Liver allograft rejection – acute rejection	neg	Decreases Tregs in the allografted liver tissue	Zhang et al. (99)
More Tregs	EAE	pos	Increases Tregs in the draining lymph nodes	Lindahl et al. (80)
	GvHD (bone marrow)	pos	Increases Tregs in the spleen and thymus.	Pan et al. (101)
	GvHD (splenocytes)	pos	Increases Tregs in the spleen, liver, and small intestines	Pan et al. (102)
	Lung cancer	neg	Increases Tregs in lung tumor tissue	Khosravi et al. (98)

T cell polarization or cytokine expression in experiments in which IL-22 signaling was specifically manipulated. The effect for each disease model is assigned as pos if IL-22 results in less severe disease and neg if IL-22 results in more severe disease. PBMC, peripheral blood mononuclear cells; EAU, experimental autoimmune uveitis; CIA, collagen induced arthritis; APC, antigen presenting cell; EAE, experimental autoimmune encephalomyelitis; GvHD, graft versus host disease; BAL, bronchioalveolar lavage; ILC, innate lymphoid cell; LCMV, lymphocytic choriomeningitis virus; Tregs, regulatory T cells.

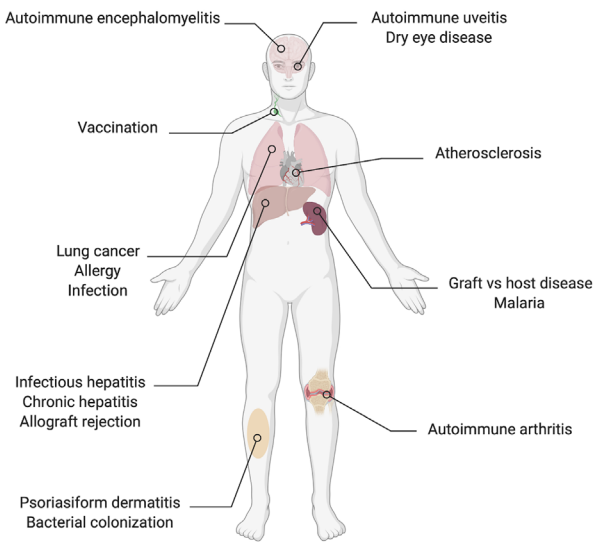

FIGURE 2 | Organs and conditions in which there are reported effects of IL-22 on the Th1/Th17 axis.

IL-22 has a pathogenic effect, which likely involves direct actions of IL-22 on keratinocytes leading to excessive proliferation, aberrant maturation as well as induction of inflammatory mediators (6, 11, 104–106).

Although not covered in this review, several studies have shown that IL-22 can promote B cell responses. CXCL13 is a central chemokine in B cell immune responses and is expressed in follicles of lymphoid tissues where it attracts B cells *via* the receptor CXCR5. *In vivo* neutralization of CXCL13 in mice reduces B cell recruitment to lymphoid follicles and inhibits formation of germinal centers (107). CXCL13 can be induced by IL-22 in tertiary lymphoid follicles (108) offering a plausible explanation to the elevated antibody levels in response to IL-22. In mice, IFNγ promotes isotype switching to IgG2a or IgG2c (109). The IL-22 mediated decrease in Th1 responses would thus be expected to be associated with a reduction in these isotypes which was the case in the study of Geboes et al. (66), but not in Justa et al. (64) or Corneth et al. (75). Although Th2 cells have not frequently been assessed in the included studies one can speculate that IL-22 induces a shift in the Th1/Th2 balance toward a Th2 and humoral immune response.

Both the proliferative and anti-apoptotic effects on epithelia and other tissues commonly attributed to IL-22 signaling as well as a potential inhibitory effect on T cell responses may lead to a permissive environment for tumor growth. IL-22 has been reported to both increase and decrease formation of tumors depending on tissue and model system but a tumor-promoting effect is more commonly observed (72). Interestingly, constitutive deletion of the IL-22 antagonist molecule IL-22BP in mice does not result in more tumor formation in steady state conditions but in the context of chronic colitis the unrestrained

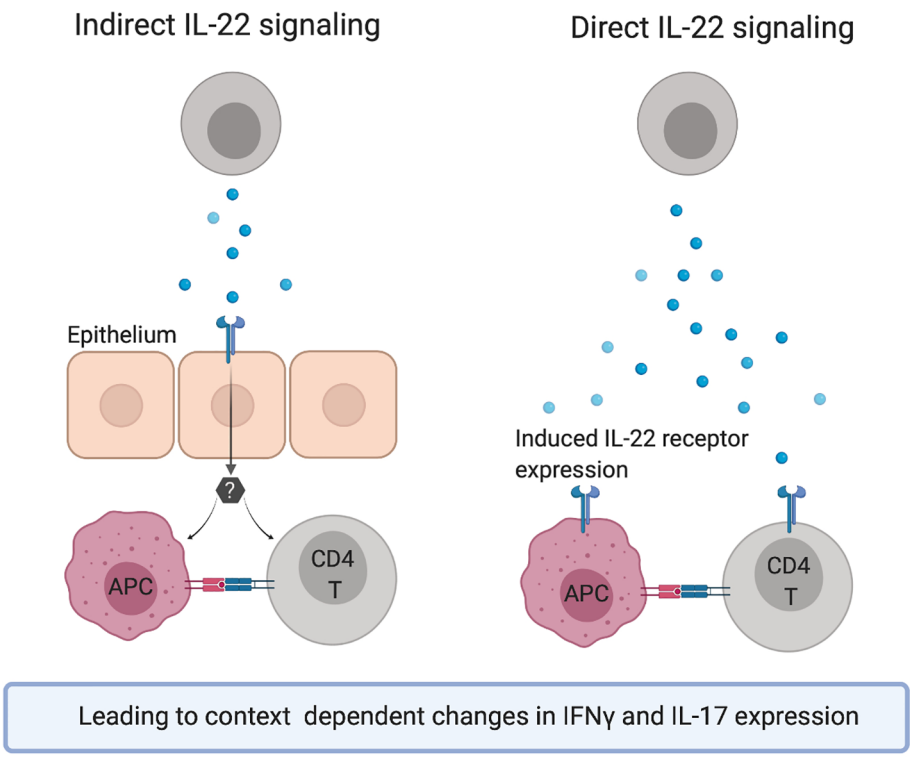

FIGURE 3 | Hypothetical mechanisms of action for the observed effects of IL-22 on the Th1/Th17 axis.

IL-22 signaling results in increased incidence of colon tumors. On the other hand, another study demonstrated a protective role of IL-22 in inflammation-induced colon tumors by improving the cellular response to DNA damage (110).

In summary, IL-22 signaling often reduces Th1 type immune responses. This characteristic of IL-22 may act in synergy with its protective effects on IL-22R expressing tissue cells to reduce collateral damage in the context of infection and inflammation. The long-term risk of tumor growth needs to be counterbalanced, which is the likely role of the endogenous IL-22 antagonist IL-22BP that is constitutively expressed in many tissues.

AUTHOR CONTRIBUTIONS

HL reviewed the literature and drafted the manuscript. TO revised the manuscript and approved the final version. All authors contributed to the article and approved the submitted version.

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Th17 Cell-Mediated Colitis Is Positively Regulated by Interferon Regulatory Factor 4 in a T Cell-*Extrinsic* Manner

Vera Buchele[1,2†], Patrick Konein[1,2], Tina Vogler[1,2], Timo Kunert[1,2], Karin Enderle[1,2], Hanif Khan[1,2], Maike Büttner-Herold[3], Christian H. K. Lehmann[2,4], Lukas Amon[2,4], Stefan Wirtz[1,2], Diana Dudziak[2,4], Markus F. Neurath[1,2], Clemens Neufert[1,2] and Kai Hildner[1,2]*

[1] Department of Medicine 1, University Hospital Erlangen, University of Erlangen-Nuremberg, Erlangen, Germany, [2] Deutsches Zentrum Immuntherapie (DZI), University Hospital Erlangen, University of Erlangen-Nuremberg, Erlangen, Germany, [3] Institute of Pathology, Department of Nephropathology, University Hospital Erlangen, Erlangen, Germany, [4] Laboratory of Dendritic Cell Biology, Department of Dermatology, University Hospital Erlangen, Friedrich-Alexander University of Erlangen-Nuremberg, Erlangen, Germany

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Lars Rogge,
Institut Pasteur, France

Reviewed by:
Jan Niess,
University Hospital of Basel, Switzerland
Sangwon Kim,
Thomas Jefferson University, United States

***Correspondence:**
Kai Hildner
Kai.Hildner@uk-erlangen.de

†Present address:
Vera Buchele,
Department of Transfusion Medicine and Hemostaseology, University Hospital Erlangen, University of Erlangen-Nuremberg, Erlangen, Germany

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Inflammatory bowel diseases (IBDs) are characterized by chronic, inflammatory gastrointestinal lesions and often require life-long treatment with immunosuppressants and repetitive surgical interventions. Despite progress in respect to the characterization of molecular mechanisms *e.g.* exerted by TNF-alpha, currently clinically approved therapeutics fail to provide long-term disease control for most patients. The transcription factor interferon regulatory factor 4 (IRF4) has been shown to play important developmental as well as functional roles within multiple immune cells. In the context of colitis, a T cell-intrinsic role of IRF4 in driving immune-mediated gut pathology is established. Here, we conversely addressed the impact of IRF4 inactivation in non-T cells on T cell driven colitis *in vivo*. Employing the $CD4^+CD25^-$ naïve T cell transfer model, we found that T cells fail to elicit colitis in IRF4-deficient compared to IRF4-proficient $Rag1^{-/-}$ mice. Reduced colitis activity in the absence of IRF4 was accompanied by hampered T cell expansion both within the mesenteric lymph node (MLN) and colonic lamina propria (cLP). Furthermore, the influx of various myeloids, presumably inflammation-promoting cells was abrogated overall leading to a less disrupted intestinal barrier. Mechanistically, gene profiling experiments revealed a Th17 response dominated molecular expression signature in colon tissues of IRF4-proficient, colitic $Rag1^{-/-}$ but not in colitis-protected $Rag1^{-/-}Irf4^{-/-}$ mice. Colitis mitigation in $Rag1^{-/-}Irf4^{-/-}$ T cell recipients resulted in reduced frequencies and absolute numbers of IL-17a-producing T cell subsets in MLN and cLP possibly due to a regulation of conventional dendritic cell subset 2 (cDC2) known to impact Th17 differentiation. Together, extending the T cell-intrinsic role for IRF4 in the

context of Th17 cell driven colitis, the provided data demonstrate a Th17-inducing and thereby colitis-promoting role of IRF4 through a T cell-extrinsic mechanism highlighting IRF4 as a putative molecular master switch among transcriptional regulators driving immune-mediated intestinal inflammation through both T cell-intrinsic and T cell-extrinsic mechanisms. Future studies need to further dissect IRF4 controlled pathways within distinct IRF4-expressing myeloid cell types, especially cDC2s, to elucidate the precise mechanisms accounting for hampered Th17 formation and, according to our data, the predominant mechanism of colitis protection in *Rag1*$^{-/-}$*Irf4*$^{-/-}$ T cell receiving mice.

Keywords: interferon regulatory factor 4 (IRF4), myeloid cells, Th17, intestinal inflammation, inflammatory bowel disease (IBD)

INTRODUCTION

Inflammatory bowel diseases (IBDs) clinically manifest with chronic affections of the gastrointestinal tract that are assumed to result from an inadequate immune response while antigens against which the immune attack may be specifically directed have not been identified so far (1). Besides some early-onset cases affecting infants that are due to a rather genetically circumscribed mutation as *e.g.* IL-10 receptor gene, onset of disease usually occurs in young to middle-aged adults and is triggered by so far rather undisclosed environmental factors that however epidemiologically seem to be associated with western life style habits as *e.g.* type of nutrition (1–3). Among the diverse and complex pool of immune cell subpopulations found within the inflamed gut of IBD patients, based on many preclinical experimental data sets, T cells are assumed to play a major pathogenetic role in mediating intestinal tissue inflammation (4–6). In fact, interleukin 17a (IL-17a) producing T helper (Th17) cells are one of the most prevalent T cell subsets in the inflamed gut tissue, suggesting a critical contribution to the pathogenesis of IBD (7). However, failure in clinical studies examining the efficacy of antibody mediated IL-17a and IL-17R blockade in IBD was therefore unexpected and may indicate that pro-inflammatory effects of Th17 cells are not or at least not exclusively mediated by the cytokine IL-17a alone with the latter putatively exerting rather barrier-protective effects in this context given the observation of disease aggravation following IL-17a neutralization in some patients (8, 9). Regardless, data on the biology and function of IL-23 in IBD argue for the overall colitogenic rather than inflammation-reducing nature of Th17 cells given the fact that IL-23 has been revealed to be one, if not the most important cytokine acting upstream of Th17 cells providing crucial signals for their survival and proliferation (10–13). Interestingly, recently IL-23 which expression is regularly upregulated in IBD tissues was suggested to be critically involved in driving alternative immune pathways specifically active in patients suffering from an anti-TNF-alpha blockade resistant disease (14). Overall, in addition to strategies that specifically block gut homing mediating molecules, IL-23 represents together with TNF-alpha one of the few already therapeutically established biological targets in clinical management of IBD further strengthening the case for the central pathogenicity of IL-23R^{+} Th17 cells in the context of IBD.

Antigen-presenting cells (APCs) have been identified and characterized to be critical instructors and modulators of both pro- and anti-inflammatory T cell responses *in vivo* (15–18). In addition to providing co-stimulatory or -inhibitory signals, APCs do so largely by expressing and releasing cytokines as *e.g.* IL-12, IL-23, or TGF-*ß* all known to be crucial upstream regulators and promoters of pro-inflammatory or regulatory T cell differentiation programs (16, 17, 19, 20). T cells themselves are unable to express inflammation-promoting cytokines like IL-23 and IL-12. Hence, *e.g.* IL-23 is largely provided by APC as *e.g.* dendritic cells and monocytes with the latter shown to have the ability to differentiate into inflammatory dendritic cells in the context of mucosal inflammation (16, 19, 21–23). Dendritic cells are subdivided into conventional (cDCs) and plasmacytoid dendritic cells (pDCs). Based on the developmental dependence on specific transcriptional regulators and critical functional differences in respect to their differential abilities to induce and promote certain types of T cell responses, cDCs can be further differentiated into two major subsets, cDC1 and cDC2 (15, 24, 25). cDC1s have been shown to be particularly critical for the induction of anti-viral and anti-tumor CD8^{+} T cell responses in part by the preferential ability to release IL-12 and cDC1 development is dependent on the transcription factor axis IRF8/BATF3/ID2 (26–29). In contrast, development and functionality of cDC2 are largely dependent on the transcription factor IRF4 (16, 30, 31). Interestingly, cDC2s have been shown to represent a critical source for IL-23 expression *in vivo* suggesting that especially IRF4 dependent cDC2s might represent critical APC driving Th17 cell responses *in vivo* as *e.g.* in the context of colitis (15, 16, 19). While the T cell-intrinsic function of IRF4 in regard to its contribution to the manifestation of colitis has been thoroughly evaluated (32), the question whether IRF4 expressed by non-T cells is involved in the colitis manifestation and more specifically in the orchestration of the colitogenic T cell responses and if so in what way has not been studied in great detail so far. Hence, here we assessed the T cell-extrinsic role of IRF4 for the course of acute T cell driven intestinal inflammation employing the widely accepted CD4^{+}CD25^{-} naïve T cell transfer model system (33, 34). We found that IRF4 expressed in non-T cells is indispensable for the clinical, endoscopic, and histopathological colitis manifestation. Moreover, IRF4

deficiency within mice receiving IRF4-expressing T cells resulted in a decreased recovery rate of transferred T cells both in the draining mesenteric lymph node and the colonic lamina propria. Most importantly, formation of colitogenic T cells subsets and here foremost IL-17a expressing Th17 cell subsets were severely hampered in the absence of T cell-extrinsic IRF4 expression. Given its established, cell-intrinsic role during the regulation of Th17 fate decision and cDC2 development and function with the latter known to promote Th17 immune responses, IRF4 overall emerges as a key transcriptional regulator globally promoting Th17 cell driven gut inflammation.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were purchased from Janvier Labs, and congenic CD45.1/Ly5.1 B6.SJL-PtrprcaPepcb/BoyCrl mice and B6.PL-*Thy1*^{a}/CyJ mice were purchased from Charles River Laboratories and The Jackson Laboratory, respectively. B6.129S7-Rag1tm1Mom/J (termed *Rag1*$^{-/-}$ mice) and B6.129P2-*Irf4*^{tm1Mak}/J (termed *Irf4*$^{-/-}$ mice) were purchased from The Jackson Laboratory and intercrossed to generate *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice. Mice were maintained under specific pathogen-free conditions. Mice at 8 to 16 weeks of age were used. This study was carried out in accordance with the recommendations of the government of Lower Franconia in Bavaria, Germany. The protocol was approved by the government of Lower Franconia in Bavaria, Germany.

T Cell Transfer Colitis Model

Splenocytes were isolated from wildtype donor mice (C57Bl/6, CD45.1/Ly5.1 B6.SJL-PtrprcaPepcb/BoyCrl or B6.PL-*Thy1*^{a}/CyJ). For this purpose, the spleen was mashed through a 40 µm cell strainer and red blood cells were lysed with ACK buffer (0.15 M NH_4Cl, 1 M $KHCO_3$, 0.8 M Na_2EDTA; pH7.2). Naïve ($CD4^+CD25^-$) splenic T cells were isolated from spleen cell suspensions by two consecutive magnetic cell separation steps with the $CD4^+$ T cell isolation kit followed by the CD25 Microbead kit (Miltenyi Biotec) according to the manufacturer's instructions. Purity of cell isolates was confirmed by flow cytometry. To induce transfer colitis, 1×10^6 naïve T cells were injected i.p. into recipient mice. *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were used as IRF4-deficient recipients. Either *Rag1*$^{-/-}$*Irf4*$^{+/+}$ or *Rag1*$^{-/-}$*Irf4*$^{+/-}$ mice were used as IRF4-proficient T cell recipients due to a virtually indistinguishable colitis phenotype of wildtype (*Irf4*$^{+/+}$) and IRF4 heterozygous (*Irf*$^{+/-}$) mice, and this group is further referred in the manuscript as *Rag1*$^{-/-}$ mice.

Mini-Endoscopy of Mice

Mucosal inflammation of the colon after T cell transfer was assessed macroscopically by colonoscopy using an image 1^{TM} S3 mini-endoscope (Karl Storz) as previously described (35). For this purpose, mice were anesthetized and inflammation of the colon was assessed using a modified murine endoscopic index of colitis severity (MEICS) based on four parameters: thickening of the bowel wall, changes of the vascularity, granularity of the mucosal surface and stool consistency. Every parameter was scored from zero for no colitis to three for massive inflammation adding up to a maximal score of 12 as previously described (36).

Histopathological Analysis

Samples of the distal colon were rinsed with phosphate-buffered saline (PBS; Sigma-Aldrich) and fixed in 4.5% formaldehyde (Carl Roth) overnight. For histopathological analysis, sections of paraffin-embedded colon tissue were stained with hematoxylin and eosin (H&E), and inflammation was assessed by a pathologist in a blinded manner based on a slightly modified scoring system described by Erben et al. (37). Slides were analyzed using a Zeiss Axio Imager.A1 microscope and measurements were performed in micrographs taken with a Zeiss AxioCam MRc camera and Zeiss AxioVision (4.9.1.SP2) software. Briefly, histopathological changes were scored for four criteria: inflammatory density (0 = no/minimal, 1 <10%, 2 = 10–25%, 3 = 26–50%, 4 >50%), hyperplasia (0 <200 µm crypt length, 1 = 200–299 µm, 2 = 300–399 µm, 3 >400 µm), goblet cell loss (0 <20%, 1 = 21–35%, 2 = 36–50%, 3 >50%) and crypt abscesses (0 = none, 1 <one, 2 = one to two, 3 = three or more per quadrant in a circumferential colonic section). In addition, the location of inflammation was assessed (1 = only mucosa, 2 = extending into the submucosa, 3 = transmural), the crypt loss was scored from 0 to 2 (0 = none, 1 = one, 2 = two or more neighboring crypts lost), and the absence or presence of erosion, ulceration and irregular crypts was scored with 0 or 1, respectively. Scores of each criterion were summed up leading to a maximum total histology score of 21.

Immunohistochemistry

Samples of the distal part of the colon were frozen and embedded in Optimal Cutting Temperature compound (Sakura). Frozen tissue sections were fixed in 2% paraformaldehyde for 20 min. To prevent unspecific binding, sections were blocked for 1 h with blocking buffer consisting of 10% fetal calf serum (FCS; Pan-Biotech) and 1% bovine serum albumin (Sigma-Aldrich). Sections were double-stained with anti-F4/80-Alexa Fluor 488 (BM8; BioLegend) and anti-CD4-Alexa Fluor 647 (GK1.5; BioLegend) or rabbit anti-MPO (polyclonal; Abcam) in blocking buffer overnight at 4°C. For MPO staining, sections were additionally incubated with donkey anti-rabbit-IgG-Alexa Fluor 647 (Poly4064; BioLegend) for 2 h at room temperature in blocking buffer. Nuclei were counterstained with Hoechst 33342 (Life Technologies). Images were recorded using the confocal microscope Leica TCS SP5II with Leica LasX software.

Isolation of Colonic Lamina Propria Cells, Splenocytes, and Mesenteric Lymph Node Cells

Colonic lamina propria (cLP) cells were isolated by enzymatic digestion as described before (38). Briefly, the colon was rinsed

with PBS to remove intestinal content, opened longitudinally, and cut into small pieces. After washing colonic pieces twice with Hanks' Balanced Salt solution (HBSS; Sigma-Aldrich) supplemented with EDTA (0.5 mM), intestinal tissue was digested in HBSS containing DNase I (0.25 mg/ml), collagenase D (0.5 mg/ml), dispase II (3 Units/ml), and 5% FCS. Following filtering through a 40 μm cell strainer (Falcon) and washing of the digested tissue with PBS, cLP cells were enriched with density gradient centrifugation where 80% Percoll was overlaid with cells resuspended in 40% Percoll (GE Healthcare). cLP cells were washed with RPMI supplemented with 10% FCS prior to further analysis. Splenocytes and mesenteric lymph node (MLN) cells were isolated by enzymatic digestion of chopped tissues with DNase I (30 U/ml), collagenase B (0.25 mg/ml) in DMEM high glucose (Gibco) supplemented with 10% FCS for 1 h at 37°C. Cells were filtered through a 40 μm cell strainer and washed with PBS. Red blood cells were lysed with ACK buffer. All enzymes were purchased from Sigma-Aldrich.

Flow Cytometry Analysis

For the analysis of cell surface markers, isolated cells were stained with fluorochrome-conjugated antibodies dissolved in staining buffer (3% FCS in PBS) for 20 min at 4°C in the dark before analysis. For staining of intranuclear proteins, the FOXP3 Fix/Perm buffer set (BioLegend) was used according to the manufacturer's instructions. Briefly, following staining of cell surface markers, cells were fixed in fixation/permeabilization working solution for 40 min at 4°C in the dark before permeabilization and staining in permeabilization buffer for 40 min at 4°C in the dark. For intracellular cytokine staining, isolated cells (1×10^6/ml) were cultured in cell culture medium [DMEM high glucose containing 10% FCS, 100 U/ml penicillin/0.1 mg/ml streptomycin (Sigma-Aldrich), 1% non-essential amino acids (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich) and 0.5 mM β-mercaptoethanol (Gibco)] in the presence of 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μM ionomycin (Sigma-Aldrich) for 4 h at 37°C. 1 μg/ml brefeldin A (Sigma-Aldrich) was added for the last 3 h of culture. Thereafter, cells were stained for surface markers. Intracellular cytokine staining was performed as described previously (39). In brief, following fixation with 2% formaldehyde for 15 min, cells were permeabilized with 0.05% saponin (Sigma-Aldrich) in staining buffer. Intracellular cytokines were stained for 30 min at 4°C in the dark using fluorochrome-labeled antibodies dissolved in 0.5% saponin in staining buffer. To identify and exclude dead cells DAPI (Sigma-Aldrich) or the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used according to the manufacturer's instructions. Stained cells were measured in staining buffer on a FACSFortessa II (BD Biosciences) flow cytometer, and data were analyzed using FlowJo 10.7.1 software (Tree Star Inc). Cell aggregates were excluded from analysis using forward scatter-area *versus* forward scatter-height scatterplots. The following antibodies were used: anti-Ly6C (HK1.4), anti-Ly6G (IA8), anti-F4/80 (BM8), anti-CD3ϵ (17A2), anti-CD4 (GK1.5), anti-GM-CSF (MP1-22E9), anti-IL-17a (TC11-18H10.1), anti-IFN-γ (XMG1.2); anti-CD45.2 (104), anti-I-A/I-E (M5/114.15.2), anti-CD11c (N418), anti-CD103 (2E7), anti-CD11b (M1/70), anti-IRF-4 (IRF4.3E4), anti-CD172 (A7R34), anti-T-bet (4B10), anti-XCR1 (ZET), streptavidin-Brilliant-Violet 421 from BioLegend, anti-CD11b (M1/70), anti-RORγt (Q31-378), anti-I-A/I-E (M5/114.15.2), anti-CD45 (30F11), anti-CD19 (1D3), anti-CD3ϵ (17A2), anti-NK1.1 (PK136), anti-CD11c (HL3) from BD Biosciences, Anti-CD172a (P84) from Life Technologies, anti-Thy1.2 (30-H12), anti-GATA3 (REA174) and a custom made biotinylated lineage antibody cocktail (anti-B220, anti-CD3, anti-CD5, anti-CD11b, anti-GR1, anti-NK1.1, anti-SiglecF, anti-Ter119) from Miltenyi Biotec.

Quantitative Real-Time Polymerase Chain Reaction

Following RNA isolation from a sample of distal colonic tissue using the NucleoSpin RNA isolation kit (Macherey Nagel), cDNA of 1 μg RNA was reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories). RNA quality and concentration were measured with a Nanodrop ND-1000 (Thermo Fisher Scientific). Gene expression was measured by quantitative Real-Time polymerase chain reaction (qPCR) using iQ SYBR Green Supermix (Bio-Rad Laboratories) and the following primers (MWG Eurofins): *Hprt* forward 5′-TGG ATA CAG GCC AGA CTT TGT T-3′, reverse 5′-CAG ATT CAA CTT GCG CTC ATC-3′, *Ifng* forward 5′-ATC TGG AGG AAC TGG CAA AA-3′, reverse 5′-TGA GCT CAT TGA ATG CTT GG-3′, *Csf2* forward 5′-ATC AAA GAA GCC CTG AAC CT-3′, reverse 5′-GTG TTT CAC AGT CCG TTT CC-3′, *Tnf* forward 5′-CTT GTG GCA GGG GCC ACC AC-3′, reverse 5′-CCA TGC CGT TGG CCA GGA GG-3′, *Il-17a* forward 5′-GCT CCA GAA GGC CCT CAG A-3′, reverse 5′-AGC TTT CCC TCC GCA TTG A-3′, *Il1b* forward 5′-GTG ACG TTC CCA TTA GAC AA-3′, reverse 5′-TAT TTT GTC GTT GCT TGG TT-3′, *Rorc* forward 5′-CCG CTG AGA GGG CTT CAC-3′, reverse 5′-TGC AGG AGT AGG CCA CAT TAC A-3′, *Il23r* forward 5′-CAC AAC AAC TAC ACG TCC AT-3′, reverse 5′-TAC CAG TTT CTT GAC ATC GC-3′, *Batf* forward 5′-GGA AGA TTA GAA CCA TGC CTC-3′, reverse 5′-CCA GGT GAA GGG TGT CGG-3′, *Il6* forward 5′-CCG GAG AGG AGA CTT CAC AG-3′, reverse 5′-TTC TGC AAG TGC ATC ATC GT-3′, *Il12a* forward 5′-ACA GGG TGA TGG GCT ATC TG-3′, reverse 5′-GGG GAG ATG AGA TGT GAT GG-3′, *Il23r* forward 5′-CAC AAC AAC TAC ACG TCC AT-3′, reverse 5′-TAC CAG TTT CTT GAC ATC GC-3′. Primer sets for *Tbx21* and *Il23a* were purchased from QIAGEN. Samples were run on a CFX Connect and CFX96 Real-Time PCR detection system (Bio-Rad Laboratories). Data were analyzed with CFX Manager v3.1 (Bio-Rad Laboratories). Expression levels of target genes for each sample were normalized relative to the reference gene *Hprt*. Relative gene expression levels were calculated with the $2^{(-\Delta\Delta Ct)}$ method. Mean gene expression levels detected in $Rag1^{-/-}$ mice were arbitrarily set to one and gene expression levels of $Rag1^{-/-}Irf4^{-/-}$

mice were calculated and displayed in relation to the normalized *Rag1*$^{-/-}$ mice.

Statistical Analysis

Unpaired Student's *t* test was used for comparison of means of two datasets. $*p < 0.05$ was considered to be significant. Statistical analysis was performed with Graphpad Prism 8.3.0 software.

RESULTS

Rag1$^{-/-}$*Irf4*$^{-/-}$ Mice Fail to Develop Colitis Upon Transfer of Naïve CD4$^+$ T Cells

Previously, we and others have reported that in the T cell transfer colitis model inflammatory manifestations within the gut are strongly dependent on T cell-intrinsic expression of transcription factors proven to be indispensable for the differentiation of Th17 cells *in vitro* and *in vivo* (32, 40–42). In line with that, T cell restricted IRF4-deficiency severely compromised T cells' ability to induce colitis (32). Conversely, however, the selective role IRF4 expressed by non-T cells exerts in the immune pathogenesis of colitis remains largely unresolved. To decipher the unknown impact of IRF4 in this setting, we sought to create a genetic model in which IRF4 deficiency is restricted to the non-T cell compartment while T cells remain fully competent, *i.e.* T cells with preserved IRF4 expression abilities. In this experimental set-up, any kind of regulation of observed T cell colitogenicity would be secondary to the T cell-extrinsic IRF4 deficiency, *i.e.* clearly attributable to the altered functionality of non-T cells in the absence of IRF4. Thereby, this data set was suitable to help define the T cell-extrinsic role of IRF4 in the context of colitis. To achieve this goal, we crossed IRF4 germline deficient mice onto the Rag1-deficient background and elicited colitis in these mice upon naïve CD4$^+$ T cell transfer into *Rag1*$^{-/-}$*Irf4*$^{-/-}$, *Rag1*$^{-/-}$*Irf4*$^{+/-}$ or *Rag1*$^{-/-}$*Irf4*$^{+/+}$ mice with the last two groups serving as IRF4-sufficient control animals (termed *Rag1*$^{-/-}$ mice) (33, 34, 43). Strikingly, as documented by the dynamic changes of the body weight curves over time, after 7–10 days, *i.e.* upon completion of priming and start of substantial expansion of transferred CD4$^+$ T cells, IRF4-proficient recipient mice started to lose initially subtly but progressively increasing weight compared to IRF4-deficient *Rag1*$^{-/-}$ mice with a clear separation of both curves at the beginning of the fourth week after experimental start (**Figure 1A**).

To assess whether the observed weight loss is accompanied by or even due to an acute immune-mediated affection of the colonic barrier, we performed mini-endoscopic evaluation of the distal colon of both IRF4-sufficient and -deficient *Rag1*$^{-/-}$ mice *in vivo* prior sacrificing mice (**Figure 1B**). As displayed in **Figure 1B**, at this time point colonoscopy showed that IRF4-competent mice suffered from severe colitis in respect to all evaluated categories adding up to a mean sum score of around 8. In contrast, T cell receiving *Rag1*$^{-/-}$ mice deficient in IRF4 virtually lacked macroscopic signs of colitis evidenced by a sum score of <3 with a score of ≥3 representing the empiric cut-off value for mice suffering from clinically meaningful, endoscopic signs of colitis. To further validate the macroscopic results, we performed thorough histopathological studies by evaluating cross-sections derived from the distal colon, *i.e.* areas matching the mini-endoscopically assessed region (**Figure 1C**). Here, histopathological scoring and grading revealed that both goblet cell loss and crypt length increase commonly observed following severe forms of intestinal inflammation were decreased in *Rag1*$^{-/-}$*Irf4*$^{-/-}$ T cell receiving mice. Overall, histopathologically assessed parameters summarized in the histology score underscore and further extend the results obtained during endoscopic evaluation that *Rag1*$^{-/-}$ mice lacking IRF4 expression are largely protected from T cell mediated colon inflammation (**Figure 1C**). Overall data presented so far unequivocally demonstrate that IRF4 expression within non-T cells is indispensable for T cell mediated transfer colitis in *Rag1*$^{-/-}$ recipient mice based on both clinical, endoscopic, and histopathological scoring results.

Colitis Protection of IRF4 Deficient *Rag1*$^{-/-}$ Mice Is Associated With a Reduced Expansion of Transferred T Cells and Diminished Recruitment of Inflammatory Mononuclear Cells Into the Colon

To further deconstruct the mechanism underlying reduced manifestation of intestinal inflammation in *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice when compared to controls, we next focused on the quantitative and qualitative regulation of the cellular composition within the inflamed gut. First, we performed multi-color immunofluorescence (IF) staining of distal colon cross-sections matching previously histopathologically assessed areas (**Figure 2A**). We readily observed a striking reduction of the total immune cell infiltration in *Rag1*$^{-/-}$ mice lacking IRF4 expression. In line with our IF data, cell number calculations of isolated colonic lamina propria mononuclear cell preparations confirmed a significant reduction of total cLP cells in mice lacking IRF4 compared to IRF4-sufficient controls (**Figure 2B**). Furthermore IF imaging suggested that transferred CD4$^+$ T cells are less abundant in *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice compared to controls, and this finding was further substantiated by additional flow cytometry-based enumeration of CD4$^+$ T cells within the total live cLP-derived immune cell pool (**Figures 2A, B**). To further dissect whether this observation results from a general reduction of T cell activation/proliferation or is rather due to a gut homing phenotype due to hampered migratory properties of the T cells in the absence of IRF4 expression in non-T cells, we determined both the total cellularity and relative fraction of T cells within the mesenteric lymph node (MLN) cells by flow cytometry. Interestingly, similar to the cLP compartment, total numbers of MLN residing immune cells were reduced in *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice compared to controls (**Figure 2B**). Also, the absolute numbers of CD4$^+$ T cells were significantly diminished within the MLN suggesting that at least to a certain degree hampered systemic T cell activation and expansion may account for the lack of a colitis-mediating T cell pool in the absence of T cell-extrinsic IRF4 expression. However, despite that finding, the fraction of CD4$^+$ T cells within the MLN residing immune cell pool

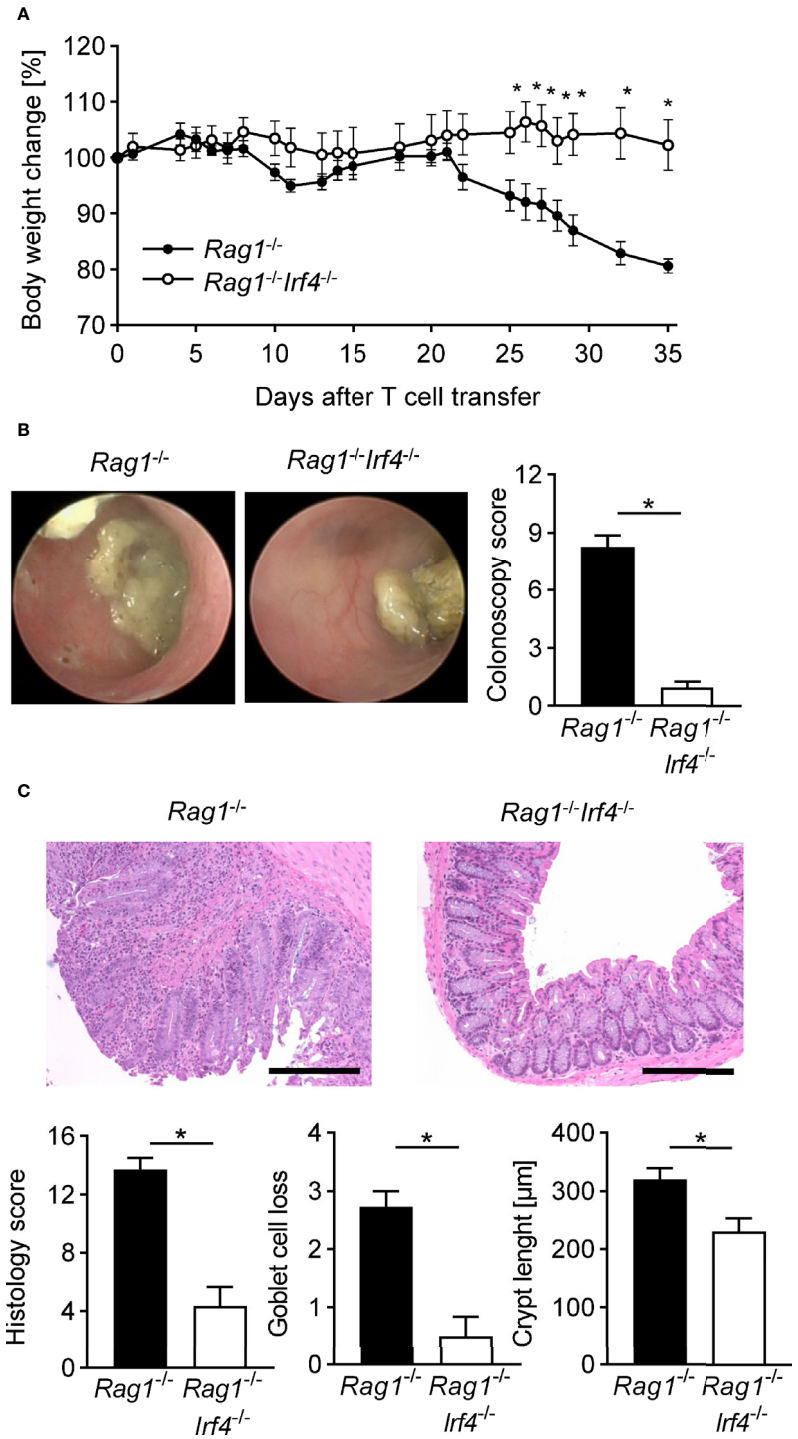

FIGURE 1 | Inactivation of IRF4 in non-T cells abrogates clinical, endoscopic, and histopathological signs of colitis. At day 0 *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were injected i.p. with 1 × 10^6 naïve ($CD4^+CD25^-$) T cells. **(A)** Percentage body weight changes compared to the original body weight at day 0 were assessed over time. A representative course of body weight changes per experimental group from one of four experiments is shown (*Rag1*$^{-/-}$ n = 6; *Rag1*$^{-/-}$*Irf4*$^{-/-}$ n = 5). **(B)** Colitis severity was analyzed by colonoscopy when T cell recipient *Rag1*$^{-/-}$ mice showed sustained weight loss of more than 10% of their initial body weight for at least one week (four to five weeks after T cell transfer). Colonoscopy score of three independent experiments and representative endoscopic images for every experimental group are shown. *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were sacrificed when colitis was established in T cell recipient *Rag1*$^{-/-}$ mice identified by weight loss and colonic inflammation *via* colonoscopy (five to six weeks after T cell transfer). **(C)** Histopathological scoring of the inflammation present in the distal colon. One representative H&E-stained histopathological cross-section of the distal colon per group is shown. Scale bars: 100 µm. **(B, C)** Data are combined from three individual experiments (*Rag1*$^{-/-}$ n = 11; *Rag1*$^{-/-}$*Irf4*$^{-/-}$ n = 12). Data were analyzed by Student's *t* test and are shown as mean ± SEM. *p <0.05.

was even relatively increased compared to control mice (**Figure 2B**). Hence, hampered efflux of $CD4^+$ T cells from the MLN due to incomplete priming and/or imprinting of gut homing properties may be also contributing to the reduced representation of colitogenic T cells in the cLP fraction.

Intestinal myeloid cells have been described to play a dichotomic, context-dependent role under homeostatic and inflammatory conditions by exerting both anti-inflammatory as well as pro-inflammatory functions (22, 23, 44). Especially blood-derived monocytes are recruited to the gut through signal cascades initially set in motion by the intestinal immigration of cytokine releasing effector T cells where they were shown to at least partially convert into cells displaying a $Ly6C^{hi}$ inflammatory phenotype (45–47). In fact, IF studies exploring the expression pattern of the surface glycoprotein and myeloid marker F4/80 revealed that colonic tissues of T cell receiving *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice contained visibly reduced F4/80 expressing cells compared to controls contrasting the virtually indistinguishable pattern of $F4/80^+$ and/or MPO^+ cells resp. in the absence of inflammation between IRF4 proficient *vs.* deficient mice (**Figure 2A**, **Supplementary Figure 1A**). Further resolution in regard to the cellular composition of the $F4/80^+$ cells was provided by additional in-depth flow cytometry analyses of cLP cell fractions. With the help of the shown gating strategy starting of $CD11b^+$ cells, we are able to distinguish $F4/80^+Ly6G^{neg}SSC^{low}Ly6C^{high}$ inflammatory monocytes from $F4/80^+Ly6G^{neg}SSC^{low}Ly6C^{neg}$ eosinophils and $F4/80^{neg}Ly6C^+Ly6G^+$ neutrophils (**Figure 2C**). Employing this gating approach, we readily found that the fraction of $CD11b^+$ cells is dramatically increased within the pool of live cLP cells derived from T cell treated *Rag1*$^{-/-}$ mice with intact IRF4 expression compared to IRF4-deficient mice (**Figure 2C**). Moreover, we found that mononuclear cell isolates from the cLP compartment contained both absolutely and relatively diminished fractions of both neutrophils, eosinophils and presumably inflammatory $Ly6C^{high}$ monocytes in *Rag1*$^{-/-}$ mice lacking IRF4 expression compared to IRF4-proficient controls (**Figure 2C**). In contrast, neither absolute nor relative IRF4-dependent regulation of either cell population prior T cell transfer, *i.e.* non-inflamed mice was detectable (**Supplementary Figure 1B**). Finally, similar to our studies on the T cell phenotype shown above in **Figures 2A, B**, we compared the composition of the MLN resident myeloid cell compartment in IRF4-sufficient *Rag1*$^{-/-}$ mice to *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice prior and after T cell transfer. We could not detect any relative or absolute IRF4-dependent difference within granulocyte subsets and $Ly6C^{high}$ monocytes in the absence of T cell induced colitis (**Supplementary Figure 1C**). Moreover, however contrasting our studies on the cLP compartment, the relative fractions of MLN residing neutrophils, eosinophils, and inflammatory monocytes were not increased in IRF4-competent compared to IRF4-deficient *Rag1*$^{-/-}$ mice after T cell transfer (**Figure 2D**). In addition, absolute numbers of neutrophils were virtually indistinguishable between T cell receiving *Rag1*$^{-/-}$ mice irrespective of their ability to express IRF4. However, in contrast, both absolute eosinophil as well as $Ly6C^{high}$ monocyte counts were significantly reduced in MLN of *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice compared to controls (**Figure 2D**). In summary, our data so far suggest that T cell-extrinsic inactivation of IRF4 leads to hampered T cell expansion both in MLN and cLP, while defective MLN exiting and/or gut homing abilities might add to the overall reduced recovery of putatively colitogenic cLP T cells. Furthermore, recruitment and/or local, *i.e.* intestinal expansion of neutrophils, eosinophils and $Ly6C^{high}$ monocytes are severely compromised upon T cell transfer in *Rag1*$^{-/-}$ mice lacking IRF4 expression compared to IRF4 competent controls.

IRF4 Induces a Colonic Th17 Gene Expression Signature in T Cell-Driven Colitis

Our results have so far established that IRF4 controls the expansion of transferred T cells and recruitment of a series of putatively pro-inflammatory acting myeloid cells in T cell mediated acute colitis through a T cell-extrinsic mechanism. To gain further insight into the molecular mechanism and tissue micromilieu acting in the colitic tissue on the immune cell network, we performed quantitative gene expression analyses employing a series of markers affiliated to distinct T helper cell subsets (**Figure 3**). As displayed in **Figure 3A**, expression of the prototypical Th1 cytokine IFN-gamma was not differentially expressed between IRF4-competent *vs.* -deficient *Rag1*$^{-/-}$ T cell recipient mice despite a trend towards reduced expression in the absence of IRF4. However, in contrast, colonic expression levels of IL-17, GM-CSF and TNF-alpha were significantly reduced in the absence of IRF4 compared to controls suggesting hampered representation of a Th17 cell enriched cytokine milieu *in situ*. To specifically assess whether Th17 cell differentiation inducing and/or Th17 cell promoting cytokines are differentially regulated, we compared colonic gene expression profiles of IL-12 (Th1) and IL-1*ß*/IL-6/IL-23 (Th17) between colitis-protected *Rag1*$^{-/-}$*Irf4*$^{-/-}$ and colitic IRF4-competent mice (**Figure 3B**). With the exception of *Il23a*, *i.e.* IL-23p19 the colonic tissue expression of all molecules was significantly reduced in the absence of IRF4 clearly indicating that IRF4 expressed by non-T cells might be critical for the generation of a Th17 prone microenvironment. In line with a predominately Th17 differentiation permitting tissue micromilieu, additional quantification of total IL-23R expression revealed significantly upregulated tissue levels in the presence of T cell extrinsically expressed IRF4 (**Figure 3B**). Overall enhanced IL-23R expression might indicate increased susceptibility of invading immune cells including T cells to be permissive for IL-23 mediated effects as Th17 cell differentiation and/or expansion. Since our data so far clearly suggested that molecules and pathways feeding into Th17 cell rather than Th1 cell differentiation are positively regulated by IRF4 through a T cell-extrinsic mechanism, we finally assessed tissue expression levels of transcription factors firmly connected to Th1 and Th17 differentiation, respectively (**Figure 3C**). Interestingly, tissue T-bet/*Tbx21* expression widely accepted to be a critical regulator of Th1 differentiation was virtually indistinguishable between both *Rag1*$^{-/-}$ cohorts irrespective of their ability to express IRF4 or not (48). In contrast, however, and in full agreement with the data presented above (**Figures 3A, B**), *Rag1*$^{-/-}$ mice deficient in IRF4

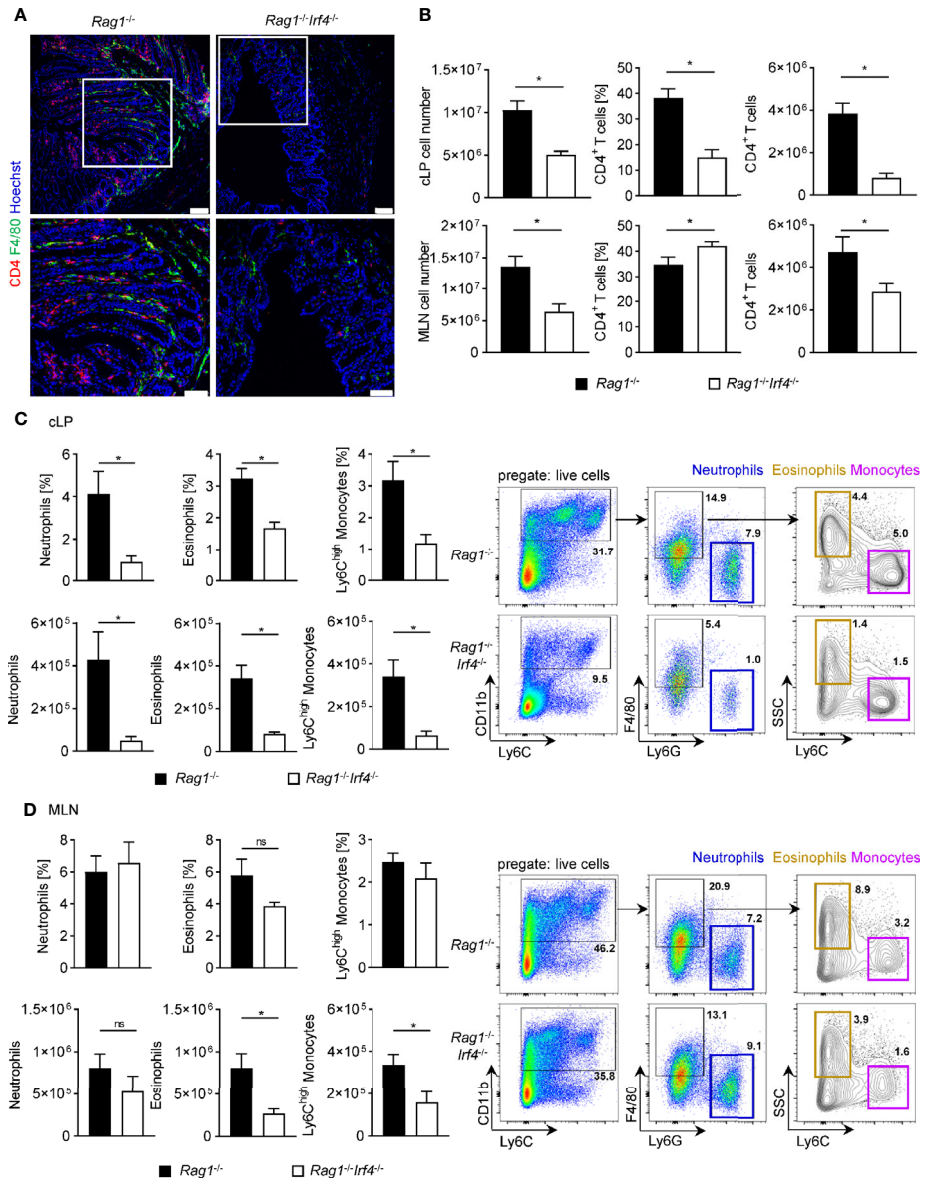

FIGURE 2 | Hampered expansion and influx of both recipient-derived myeloid and donor-derived T cells in T cell receiving *Rag1*$^{-/-}$ mice deficient in IRF4. *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were injected i.p. with 1 × 10^6 naïve (CD4$^+$CD25$^-$) T cells. When colitis was established in *Rag1*$^{-/-}$ mice five to six weeks after T cell transfer, both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were sacrificed, and the influx of T cells and inflammatory myeloid cells into the colon and mesenteric lymph node (MLN) was analyzed. **(A)** Representative immunofluorescence staining of F4/80$^+$ and CD4$^+$ cells in colonic cross sections at day 39 after T cell transfer. Upper panel: overview picture of the colonic tissue (scale bars = 75 µm); lower panel: higher magnification of the white boxed area within the colonic tissue of the upper panel (scale bars = 50 µm). **(B)** Absolute number of colonic lamina propria (cLP) and MLN was determined (left column). Frequencies (middle column) and absolute numbers (right column) of CD4$^+$ T cells (CD3$^+$CD4$^+$) within the total live immune cell pool were analysed by flow cytometry. In addition relative fraction (upper panel) and absolute cellularity (lower panel) of neutrophils (CD11b$^+$F4/80$^-$Ly6G$^+$Ly6C$^+$), eosinophils (CD11b$^+$F4/80$^+$Ly6G$^-$SSC^{high}Ly6C$^-$) and inflammatory Ly6C^{high} monocytes (CD11b$^+$F4/80$^+$Ly6G$^-$SSC^{low}Ly6C^{high}) within the total live immune cell pool of cLP **(C)** and MLN **(D)** cells were analyzed by flow cytometry. One representative flow cytometry plot is shown for every experimental group and illustrates the gating strategy for the indicated cell populations. Frequencies of cells in each sub-gate are calculated as a percentage of live cells. Data are combined from two individual experiments (*Rag1*$^{-/-}$ n = 7; *Rag1*$^{-/-}$*Irf4*$^{-/-}$ n = 8). Data were analyzed by Student's *t* test and are shown as mean ± SEM. ns, not significant. *$p < 0.05$.

showed a significantly reduced tissue expression of *bona fide* Th17 regulating transcription factors RORγt and BATF (39, 49). Collectively, our data demonstrate that IRF4 expression by non-T cells in T cell receiving *Rag1*$^{-/-}$ mice is crucial for the establishment of a Th17 prone microenvironment in the colon suggesting that IRF4 may control T cell mediated colitis through provision of Th17 cell differentiation enabling and promoting mechanisms outside of T cells.

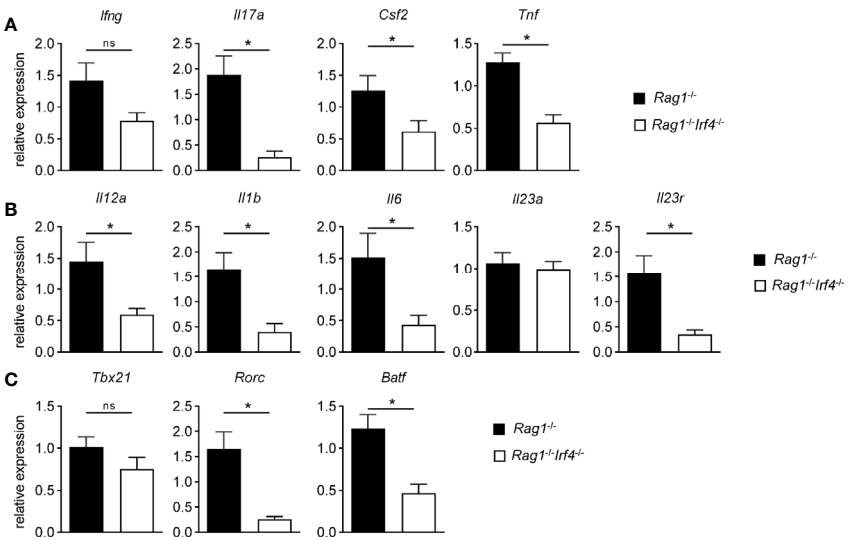

FIGURE 3 | IRF4 drives a pro-inflammatory, Th17 cell differentiation promoting and putatively presence of functional Th17 cells increasing molecular gene expression signature in colitis tissues in a T cell-extrinsic manner. *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were injected i.p. with 1 × 10^6 naïve (CD4$^+$CD25$^-$) T cells. When colitis was established in *Rag1*$^{-/-}$ mice five to six weeks after T cell transfer, both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were sacrificed. Gene expression levels of **(A)** *Ifng*, *Il17a*, *Csf2*, *Tnf*, **(B)** *Il12a*, *Il1b*, *Il6*, *Il23a*, *Il23r*, **(C)** *Tbx21*, *Rorc*, and *Batf* transcripts within colonic tissue were analyzed and quantitated by qPCR. Mean of gene expression levels detected in colonic tissues of *Rag1*$^{-/-}$ mice was arbitrarily set down to 1, and all other gene expression levels were normalized to the expression level detected within *Rag1*$^{-/-}$ mice. Data are combined from three individual experiments (*Rag1*$^{-/-}$ n = 13; *Rag1*$^{-/-}$*Irf4*$^{-/-}$ n = 13). Data were analyzed by Student's *t* test and are shown as mean ± SEM. ns, not significant. *$p < 0.05$.

IRF4 Is Indispensable for the Formation, Expansion, and Intestinal Homing of Th17 but Not Th1 Cells in a T Cell-Extrinsic Manner

To further test our hypothesis that missing IRF4 expression within non-T cells results in the abrogated formation of colitogenic Th17 cells and that this step is critical as it may mainly account for alleviated colitis manifestation in this group, we performed in-depth intracellular cytokine staining profiling experiments employing flow cytometry (**Figure 4**). To achieve this goal, following *ex vivo* restimulation we stained both MLN and cLP derived CD4$^+$ T cells for IFN-gamma, IL-17a, and GM-CSF expression and compared frequencies of Th1 and Th17 subsets resp. between *Rag1*$^{-/-}$*Irf4*$^{-/-}$ and IRF4-sufficient control mice. In accordance with our gene expression profiling experiments, T-bet dependent Th1 cells defined as IFN-gamma$^+$IL-17a$^-$ T cells were detected irrespective of the presence or absence of IRF4 expression and hence appear to develop independent of T cell-extrinsic IRF4 expression (**Figures 4A, B**). In sharp contrast, however, IL-17a producing T cell subsets including IL-17a single producing as well as IL-17a$^+$ T cells co-expressing either IFN-gamma or GM-CSF were broadly negatively affected in mice lacking T cell-extrinsic IRF4 expression (**Figures 4A, B**). Strikingly, the comparison of both MLN and cLP displayed an unequivocal pattern across organs in the absence of T cell-extrinsic IRF4 expression overall supporting the conclusion that reduced Th17 cell fractions in these mice reflect a rather general negative effect on Th17 cell differentiation and eventually Th17 cell pool than merely results from hampered colonic influx of in MLN otherwise appropriately skewed and functionally equipped Th17 cells.

Finally, to shed light on the mechanism putatively underlying altered T cell instruction and proliferation in the absence of IRF4, we screened for IRF4-dependent changes in the colonic innate immune cell pool in the steady state, *i.e.* prior T cell transfer. In regard to innate lymphoid cells (ILC) with presumably overall rather colitis-suppressive effects given the fact that ILC depletion leads to enhanced colitis manifestation (50, 51), we found that the colonic pool of ILC1, ILC2, and ILC3 forms in a virtually IRF4 expression independent manner (**Supplementary Figure 2**). Next, we assessed the antigen-presenting cell (APC) compartment in *Rag1*$^{-/-}$ *vs. Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice (**Supplementary Figure 3**). In support of the possibility that IRF4 deficient APCs may primarily account for the observed reduced ability to skew transferred naïve T cells into colitogenic Th17 cells, we confirmed data from previous reports (15, 16, 24) by demonstrating that the pool of Th17 responses promoting cDC2s but not cDC1s is reduced in the absence of IRF4 both in the spleen and MLN (**Supplementary Figures 3A, B**). Although, colonic DC populations were not regulated in the absence of IRF4 (**Supplementary Figure 3C**), flow cytometric expression profiling among colonic APCs interestingly revealed that predominately CD11b$^+$ cDCs, *i.e.* cDC2s regulated in both MLN and spleen, express higher levels IRF4 protein compared to CD103$^+$CD11b$^-$ cDC1 (**Supplementary Figure 3D**). This result implies that albeit

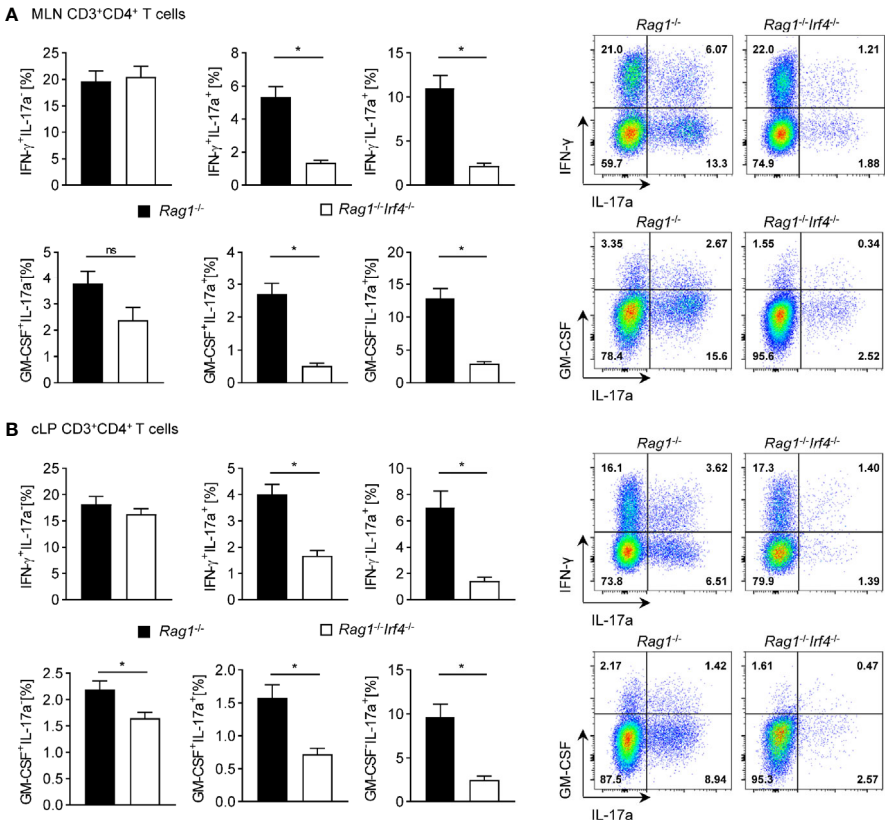

FIGURE 4 | Multiple Th17 cell subsets but not *bona fide* Th1 cells are critically dependent on the expression of functional IRF4 in *Rag1*$^{-/-}$ mice with established T cell driven colitis. *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were injected i.p. with 1 × 10^6 naïve ($CD4^+CD25^-$) T cells. When colitis was established in *Rag1*$^{-/-}$ mice five to six weeks after T cell transfer, both *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Irf4*$^{-/-}$ mice were sacrificed, and the cytokine profile of transferred $CD4^+$ T cells ($CD3^+CD4^+$) in the MLN **(A)** and in the cLP **(B)** was analyzed by intracellular flow cytometry after *ex vivo* restimulation. Frequencies of IFN-γ^+IL-17a$^-$, IFN-γ^+IL-17a$^+$, IFN-γ^-IL-17a$^+$ (upper panel) and GM-CSF^+IL-17a$^-$ GM-CSF^+IL-17a$^+$ GM-CSF^-IL-17a$^+$ (lower panel) expressing cell populations within the $CD4^+$ T cell pool were analyzed. One representative flow cytometry plot is shown for every experimental group. Data are combined from two individual experiments (*Rag1*$^{-/-}$ n = 7; *Rag1*$^{-/-}$*Irf4*$^{-/-}$ n = 8). Data were analyzed by Student's *t* test and are shown as mean ± SEM. ns, not significant. *$p < 0.05$.

not diminished in numbers, colonic cDC2 might be functionally affected (*e.g.* migratory abilities) upon IRF4 deletion and at least partially contribute to compromised induction of colitogenicity within transferred T cells.

Collectively, given the hampered colitis formation in T cell recipient *Rag1*$^{-/-}$ mice lacking IRF4, here we provide data giving crucial mechanistic insight into the molecular mechanisms by showing that IRF4 controls the formation of colitogenic BATF- and RORγt-dependent Th17 cell subsets in a T cell-extrinsic manner.

DISCUSSION

Clinical practice in IBD management has profoundly changed upon the availability of targeted therapies. While antibody-mediated blockade of the cytokine TNF-alpha represents a mainstay in the management of chronic inflammatory diseases including IBD, the expression of additional cytokines as *e.g.* IL-1ß, IL-6, IL-17, and IL-23 has been identified to be highly upregulated within inflamed tissues (7, 52–54). While clinical studies demonstrated that inhibition of some of those cytokines is effective at least in certain disease entities, in IBD only IL-23 targeting showed convincing inflammation-reducing effects and hence was approved for this indication (55). Molecularly, IL-23 employs multiple mechanism thereby affecting a series of immune cells to promote immune-mediated tissue inflammation (10–12, 56–58). However, IL-23 is predominately fostering pro-inflammatory T helper cells that share a common feature, *i.e.* the expression of IL-17a (10, 59). Hence, the identification of alternative targeting strategies going beyond IL-23 neutralization to contain Th17 cell driven tissue destruction remains a valuable goal for basic research enterprises. Recently, effective targeting of the transcriptional regulator GATA3, which is being widely accepted to promote type 2 tissue immune responses by regulating gene expression on a transcriptional level in multiple immune cell subsets implied in

the pathogenesis of allergic airway diseases, was reported to be achievable by local application of GATA-3-specific DNAzymes, thereby showing clinical efficacy in asthma patients (60, 61). Hence, targeting transcription factors to tackle mucosal inflammation may represent a challenging but putatively rewarding research area also in the context of Th17 mediated tissue inflammation. In the chain of events underlying the initiation of an inflammation cascade, transcription factors act rather upstream and thereby often control pathogenetically and functionally related networks acting across cell type borders. The identification and interference with a potential master switch within the Th17 network would hence represent a major advance for the field of targeted immunotherapy of immune-mediated chronic inflammatory disorders.

Until know, T cell-intrinsic expression of a series of transcriptional regulators like BATF, RORγt and IRF4 has been identified to be crucial for a T cell to differentiate into a pro-inflammatory Th17 cell (39, 49, 62). Interestingly, transcription factors usually regulate multiple milestones within a given cell type both by direct and indirect transcriptional effects. For example, besides regulating the expression of IL-17 cytokine family members directly, BATF is indispensable for continuous RORγt expression and hence controls through this regulation of the Th17 cell network also indirectly the expression of the IL-23 receptor in T cells (39, 63, 64). Among Th17 fate regulating transcription factors, especially IRF4 seems to exert crucial functions in a series of T cell-extrinsic immune cell populations including innate lymphoid cells (ILCs), dendritic cells, and monocytes (16, 19, 65–67). However, the functional role of IRF4 expressed in non-T cells in the context of intestinal inflammation has not gained much attention. We found here in this study that inactivation of IRF4 employing germ line deletion disables IRF4-proficient T cells to mediate disease in a widely accepted T cell dependent mouse model of acute colitis. Mechanistically, our analyses revealed that the establishment of a Th17 inducing cytokine milieu in the colon, as *e.g.* upregulated IL-1ß and IL-6 expression, required IRF4 while for IL-23 expression it was not. While the reduction of IL-1ß and IL-6 might be sufficient to explain hampered Th17 differentiation, the missing regulation of IL-23 expression in colitic tissue appears at first sight puzzling for two reasons: First, IRF4 dependent cDC2s have been described to be the major producer of IL-23 among DCs and thereby putatively impacting Th17 cell differentiation directly (16, 19). Second, to account for the hampered proliferation of differentiated Th17 cells as one possible explanation for the reduced colitis manifestation appears at first sight rather unlikely given the widely accepted dominant role of IL-23 in this context through the provision of critical survival and expansion signals especially for IL-23 receptor expressing Th17 cells (12, 13). However, although we have not tested that possibility directly, in the light of downregulated BATF/RORγt both known to regulate IL-23 receptor expression T cells primed in IRF4-deficient *Rag1*$^{-/-}$ mice in fact may lack IL-23R expression on their cell surface. Thereby T cells were unable to receive proliferation-promoting effects from IL-23, overall providing an explanation for their mitigated colitogenicity. In this scenario and supported by our data, colonic IL-23 levels are mounted by cells functioning at least in this respect independent of IRF4, resulting in similar total IL-23 tissue levels. In fact, T cells selectively lacking IL-23R through genetic inactivation similarly fail to mediate colitis in this model system (12). Hence, despite indistinguishable provision of IL-23, effects dependent on the IL-23/IL-23R interaction on T cells appear to be extinguished in the absence of IRF4 in non-T cells due to virtually absent IL-23R expression on developing effector T cells. In fact, IL-23R expression analysis within total colon tissues revealed strikingly reduced levels presumably due to decreased total T cell numbers in the colon but most likely also reflecting reduced IL-23 receptor copies expressed by a single cLP T cell. Future studies certainly need to further investigate the question which molecular signals derived from the remaining and putatively functionally compromised IRF4-deficient cDC2s may directly or indirectly account for proper induction of IL-23 receptor expressing, colitogenic Th17 cells. Interestingly, IL-6 was shown to induce both BATF and RORγt in a STAT3-dependent manner (42). Given reduced IL-6 tissue levels, IL-6 expression might be dependent on the presence of IRF4 within myeloid cells both in the MLN and/or cLP (16, 19). Hence, IRF4 deficiency-related reduction of IL-6 expression might additionally undermine Th17 differentiation. This scenario of IL-6 driven Th17 cell differentiation may be clinically relevant given the clinical observation that some patients do not respond to IL-23 blockade despite a Th17 dominated mucosal inflammation *in situ*. However, this hypothesis will require vigorous testing in the future especially in the light of rather moderate results that studies investigating the efficacy of an IL-6 blocking antibody (tocilizumab) in IBD reported (68, 69).

Hence, based on our current study, global interference with IRF4 expression and consecutively IRF4-dependent pathways might emerge as a promising therapeutic option gain control especially in those sub-cohorts of IBD patients suffering from continuous intestinal inflammation refractory to all currently available and clinically approved treatment regimens. Due to its documented, dual colitogenic role both in T cells and non-T cells, IRF4 targeting may be in this regard a very attractive molecular target to limit intestinal inflammation by inhibiting both development and functionality of Th17 cells.

Since the identification of the precise IRF4-dependent non-T cell immune cell(s) and/or signaling pathways putatively regulating T cell driven colitis was not the focus of this study and in fact will require already planned future experimentation, we can only speculate on that at this point. Although IRF4 has been clearly shown to impact ILC biology (65), antibody mediated ablation of ILC in fact leads to an aggravation of colitis (data not shown) and as previously published (50, 51) indicating that hampered ILC functionality is not likely to underlie the colitis protection observed in the absence of IRF4. In addition, in this study we provide experimental data that IRF4 deficiency does not alter the pool of colonic ILC1, ILC2 or ILC3 on Rag1$^{-/-}$ background. While this does not formally exclude an

ILC-mediated mechanism underlying colitis protection in the absence of IRF4, our and published data, however, are not in favor of the conclusion that regulation of ILC function is here involved. Based on the currently available literature, among myeloid cells known to express IRF4 the functionality of APC may appear to be most likely regulated in an IRF4-dependent manner and by this control colitogenic T cell differentiation (15, 16, 44). In $CD11b^+$ cDCs, *i.e.* cDC2, IRF4 regulates migration to (70) and survival within mucosal tissues (16, 19). As elaborated above in respect to the biology of IL-23, cDC2 cells are in part dependent on IRF4 as confirmed in this study and have been put forward to be critical in Th17 cell biology due to its preferential expression of IL-23 among cDCs (16, 19, 71, 72). Future studies including the usage of cDC-specific deletor mice, *i.e.* genetically engineered mice expressing cre recombinase under the control of the promotor of *Zbtb46* crossed to mice carrying a conditionally targeted IRF4 allele will be required to address that question (73, 74). Similarly, distinct functions within monocytes and monocyte-derived cells have been shown to be regulated by IRF4 as *e.g.* the ability to cross-present cell-associated antigens to $CD8^+$ T cells (66), fine-tuning TLR signaling (75), cytokine expression (76) or development and functionality of immunosuppressive myeloid cell populations (77). Here, with the help of novel genetic model systems, future studies need to decipher cell-type specific roles of IRF4 within ontogenetically distinct cDC and monocyte-derived cell populations in the context of colitis.

Collectively, our study unequivocally demonstrates that IRF4 expressed in non-T cells is a positive regulator of Th17 cell mediated colitis. Given the T cell-intrinsic role of IRF4 in driving Th17 cell differentiation *in vitro* (62) and also in the context of colitis *in vivo* (32), our data support the conclusion that inactivation of IRF4 universally protects against Th17 mediated colitis and suggest that strategies to interfere with IRF4 gene expression are of potential great interest to harness acute intestinal inflammation.

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 animal study was reviewed and approved by government of Lower Franconia.

AUTHOR CONTRIBUTIONS

VB, TV, PK, TK, and HK performed the experiments. VB analyzed and interpreted the data together with KH and TV. KE and CN established and performed the immunofluorescence stainings with the help of TV. MH performed the histopathological analyses. CL, LA, SW, and DD gave important advice and provided crucial reagents. MN gave important advice and helped with the interpretation and critical discussion of the results. KH directed the study and wrote the manuscript together with VB and valuable input from all authors. 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.2020.590893/full#supplementary-material

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Tristetraprolin Regulates T_H17 Cell Function and Ameliorates DSS-Induced Colitis in Mice

Hui Peng[1†], Huan Ning[1†], Qinghong Wang[1], Jinping Lai[2], Lin Wei[3], Deborah J. Stumpo[4], Perry J. Blackshear[4], Mingui Fu[5], Rong Hou[1], Daniel F. Hoft[1] and Jianguo Liu[1]*

[1] Division of Infectious Diseases, Allergy and Immunology, Department of Internal Medicine, Saint Louis University School of Medicine, Saint Louis University, St. Louis, MO, United States, [2] Department of Pathology, Saint Louis University School of Medicine, Saint Louis University, St. Louis, MO, United States, [3] Department of Immunology, School of Basic Medicine, Hebei Medical University, Shijiazhuang, China, [4] National Institute of Environmental Health Sciences, Research Triangle, NC, United States, [5] Shock/Trauma Research Center and Department of Basic Medical Science, School of Medicine, University of Missouri-Kansas City, Kansas City, MO, United States

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Lars Rogge, Institut Pasteur, France

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Dimitrios Tsitsipatis, National Institutes of Health (NIH), United States

***Correspondence:**
Jianguo Liu
jianguo.liu@health.slu.edu

[†]These authors have contributed equally to this work

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T_H17 cells have been extensively investigated in inflammation, autoimmune diseases, and cancer. The precise molecular mechanisms for T_H17 cell regulation, however, remain elusive, especially regulation at the post-transcriptional level. Tristetraprolin (TTP) is an RNA-binding protein important for degradation of the mRNAs encoding several proinflammatory cytokines. With newly generated T cell-specific TTP conditional knockout mice ($CD4^{Cre}TTP^{f/f}$), we found that aging $CD4^{Cre}TTP^{f/f}$ mice displayed an increase of IL-17A in serum and spontaneously developed chronic skin inflammation along with increased effector T_H17 cells in the affected skin. TTP inhibited T_H17 cell development and function by promoting IL-17A mRNA degradation. In a DSS-induced colitis model, $CD4^{Cre}TTP^{f/f}$ mice displayed severe colitis and had more T_H17 cells and serum IL-17A compared with wild-type mice. Furthermore, neutralization of IL-17A reduced the severity of colitis. Our results reveal a new mechanism for regulating T_H17 function and T_H17-mediated inflammation post-transcriptionally by TTP, suggests that TTP might be a novel therapeutic target for the treatment of T_H17-mediated diseases.

Keywords: tristetraprolin, RNA-binding protein, IL-17, mRNA decay, T_H17, DSS, colitis

INTRODUCTION

T_H17 cells play a pivotal role in the pathogeneses of several diseases, including autoimmune arthritis, multiple sclerosis, and inflammatory bowel disease (IBD) (1, 2). Differentiation of T_H17 cells requires T-cell receptor (TCR) signals plus transforming growth factor β (TGF-β) and interleukin 6 (IL-6) stimulation. Several transcription factors, including RORγt, Stat3 and interferon-induced factor 4 (IRF-4), have been shown to mediate T_H17 cell differentiation (3–7). In addition, IL-23 is essential for T_H17 cell survival/expansion and for generation of pathogenic T_H17 cells, although initial differentiation of T_H17 cells depends on IL-6 and TGF-β stimulation (8). As the signature cytokine produced by T_H17 cells, IL-17 contributes to the pathogenesis of T_H17-mediated inflammatory diseases, such as psoriasis (9), rheumatoid arthritis (10), and IBD (11, 12), as well as host defense against certain pathogens (13). Therefore, tightly controlling the development and function of T_H17 cells is essential for maintaining homeostasis. So far, most studies have focused on transcriptional regulation of T_H17 cell differentiation and function; much less is known about how T_H17 cells are regulated at the post-transcriptional level.

Tristetraprolin (TTP, also known as TIS11, ZFP36, and Nup475), a CCCH tandem zinc-finger protein (ZFP) coded by gene Zfp36, is involved in the regulation of inflammatory responses at the post-transcriptional level (14). Expression of TTP mRNA and protein is tightly regulated in macrophages under the control of TLR4 and other TLR signaling (15–17). Upregulation of TTP can reduce inflammatory responses in macrophages (18). TTP binds to AREs within the 3′ untranslated region (3′ UTR) of its target transcripts, causing destabilization of the mRNAs encoding tumor necrosis factor α (TNF-α) (19), granulocyte-macrophage colony-stimulating factor (GM-CSF) (20), cyclooxygenase 2 (21), IL-2 (22), IL-10 (23), and the chemokine CXCL1 (24), among others (25). The mRNAs encoding TNF-α and GM-CSF are stabilized in TTP-deficient mice and in cells derived from them (16, 20). Oversecretion of these cytokines in TTP knockout (KO) mice results in a severe systemic inflammatory response including arthritis, autoimmunity, and myeloid hyperplasia (26). We previously demonstrated that TTP inhibits IL-23 expression through promoting p19 mRNA degradation via AREs in the 3′ UTR (27). Molle et al. (28) found similar findings as ours and showed that IL-23 oversecretion in conventional $TTP^{-/-}$ mice causes an increase in T_H17 cells, and both IL-23 and IL-17A contribute to the chronic inflammation in conventional $TTP^{-/-}$ mice. Although TTP is one of the best characterized post-transcriptional regulators and ARE binding proteins, it remains largely unclear whether TTP affects T cells, specifically T_H17 cell development and function *in vivo*.

In this study, we generated T cell-specific TTP conditional KO ($CD4^{Cre}TTP^{f/f}$) mice to investigate the effects of TTP on T-cell development and function. Aging $CD4^{Cre}TTP^{f/f}$ mice developed spontaneous skin inflammation and displayed an increase in systemic IL-17A and skin T_H17 cells. $CD4^+$ T cells lacking TTP were more likely to develop into T_H17 cells compared with wild-type (WT) $CD4^+$ T cells. In fact, IL-17 productivity was enhanced in $TTP^{-/-}$ CD4 T cells compared with WT $CD4^+$ T cells at the single-cell level. This increased IL-17 production in $TTP^{-/-}$ $CD4^+$ T cells was mediated by increased IL-17A mRNA stability, demonstrating that TTP promotes the degradation of IL-17A mRNA. Furthermore, the $CD4^{Cre}TTP^{f/f}$ mice were prone to DSS-induced colitis with higher levels of serum IL-17A and T_H17 cells in mesenteric lymph node (LN) than WT mice. Neutralization of IL-17A reduced the severity of colitis in $CD4^{Cre}TTP^{f/f}$ mice. Therefore, our study reveals a novel post-transcriptional pathway through which TTP suppresses the function of T_H17 cells.

RESULTS

T Cell-Specific TTP Conditional KO Mice Develop Chronic Skin Inflammation During the Aging Processes

To determine the effects of TTP on T cells, we generated T cell-specific TTP conditional KO mice by crossing $TTP^{flox/flox}$ mice with mice expressing Cre recombinase transgene driven by the CD4 promoter ($CD4^{Cre}$) (29). Cre recombinase led to deletion of the exon 2 and the 3′ UTR of TTP in both CD4 and CD8 T cells due to CD4/CD8 coexpression transiently in the thymus (30). Exon 2 codes the tandem zinc-finger domain, which is responsible for the RNA-binding activity of TTP. Functional TTP KO specifically in CD4 T cells was evidenced in CD4 T cells lacking TTP expression and in macrophages with normal TTP expression (**Supplementary Figure 1A**). The percentages of thymic CD4 and CD8 T cells were similar between WT and $CD4^{Cre}TTP^{f/f}$ mice (**Supplementary Figure 1B**), and the numbers of macrophages and dendritic cells (DCs) in spleen were comparable between WT and $CD4^{Cre}TTP^{f/f}$ mice (**Supplementary Figure 1C**). Among most obvious phenotypes of the conventional $TTP^{-/-}$ mice are growth retardation and joint swelling (26). Somewhat surprisingly, the conditional $CD4^{Cre}TTP^{f/f}$ mice grew normally, with body weights that were identical to those of their WT littermates and with no signs of joint inflammation (**Figure 1A**). Enlargement of LNs and spleens was noted in the $CD4^{Cre}TTP^{f/f}$ mice (**Figure 1B**). Although the younger $CD4^{Cre}TTP^{f/f}$ mice did not show any signs of chronic inflammation, $CD4^{Cre}TTP^{f/f}$ mice started showing evident dermatitis within 10 months of birth, and 80% had dermatitis by 16 months of age (**Figure 1C**). Histological analysis of the affected skin indicated that the epidermal layer thickness was significantly increased, and more inflammatory cells were present in the affected skin (**Figure 1D**). Immunofluorescence staining showed an increase in $IL\text{-}17^+CD4^+$ cells in the skin lesions of $CD4^{Cre}TTP^{f/f}$ mice compared to WT mice (**Supplementary Figure 1D**). Next, we isolated cells from draining LNs of the affected skin and stimulated them with PMA and ionomycin for 4 h, followed by detection of IL-17– and interferon γ (IFN-γ)–producing CD4 T cells by flow cytometry. As shown in **Figure 1E**, there were higher percentages of IL-17–producing CD4 T cells in the draining LNs of $CD4^{Cre}TTP^{f/f}$ mice than in WT mice. When these LN cells were stimulated by anti-CD3 with or without anti-CD28 antibody (Ab), more IL-17A was secreted by LN cells of $CD4^{Cre}TTP^{f/f}$ mice than that of WT mice (**Figure 1F**), suggesting a correlation between IL-17 and the development of dermatitis in the aging $CD4^{Cre}TTP^{f/f}$ mice.

T Cell-Specific TTP Conditional KO Mice Have More IL-17–Producing Effector T Cells

T cells, especially T_H17 cells, are major producers of IL-17. To test whether TTP affected T_H17 cell function, we first checked CD4 T-cell proliferation. The proliferative capacity of CD4 T cells was similar between $CD4^{Cre}TTP^{f/f}$ mice and WT mice (**Figure 2A**). However, $CD4^+$ T cells from $CD4^{Cre}TTP^{f/f}$ mice (**Figure 2B**) and from conventional $TTP^{-/-}$ mice (**Supplementary Figure 2A**) were more likely to become $CD62L^-$ $CD44^+$ effector T cells compared with cells from WT mice, indicating that T cell-specific TTP deficiency leads to CD4 T-cell activation. Indeed, $CD4^+$ T cells from spleens of $CD4^{Cre}TTP^{f/f}$ mice secreted higher levels of IL-17A than WT cells (**Figures 2C,D**). Systemic IL-17A levels were also significantly elevated in $CD4^{Cre}TTP^{f/f}$ mice compared with their WT littermates (**Figure 2E**). Interestingly,

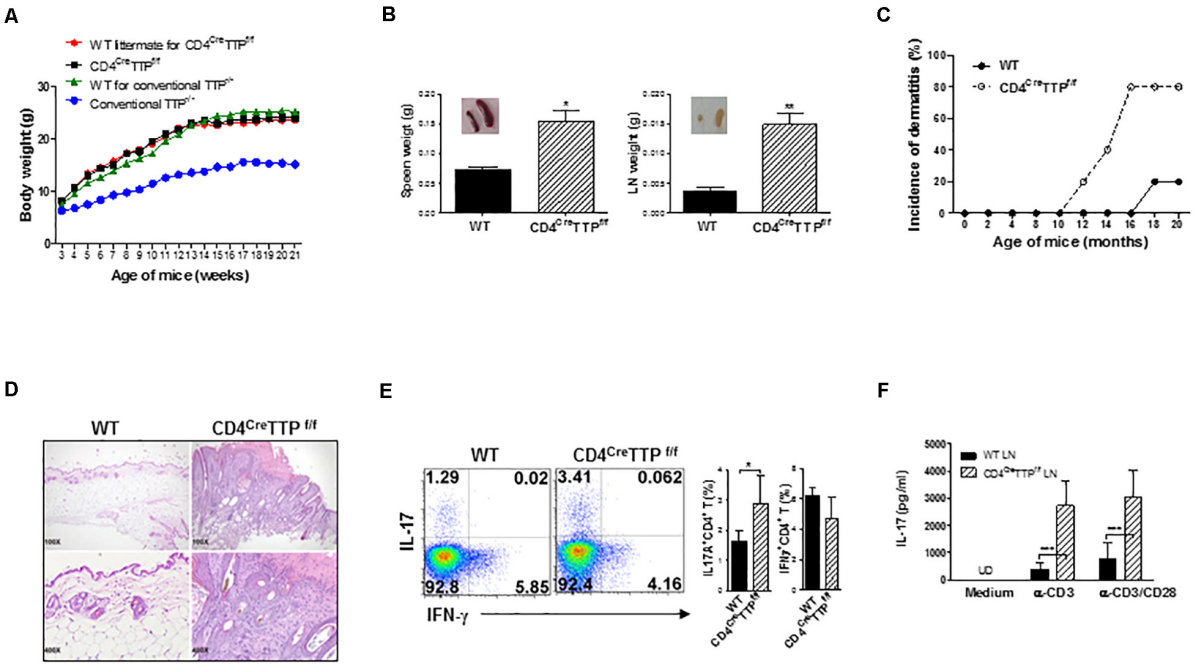

FIGURE 1 | Aging mice with specific deletion of TTP in T cells develop chronic skin inflammation. **(A)** Growth curves of $CD4^{Cre}TTP^{f/f}$ mice and wild-type (WT) littermates were monitored and recorded weekly. Data are presented as mean ± SD of 6 mice in each group. **(B)** Data represent weight (g) of spleens and LN (means ± SD) from three $CD4^{Cre}TTP^{f/f}$ mice and WT littermate at 11–13 months old. Inserted images were spleen and lymph node of $CD4^{Cre}TTP^{f/f}$ mice and WT littermates. **(C)** Percentages of dermatitis. Five mice in each group. **(D)** Skin sections from $CD4^{Cre}TTP^{f/f}$ mice and WT mice were analyzed with hematoxylin and eosin staining. Cells from draining lymph nodes (LN) of the affected skin in five WT and $CD4^{Cre}TTP^{f/f}$ mice were stimulated with PMA and ionomycin for 4 h and analyzed by flow cytometry for intracellular cytokine gated on $CD4^+$ T cells **(E)**, or stimulated with anti-CD3 (1 μg/mL) with or without anti-CD28 (1 μg/mL) Abs for 3 days for detection of IL-17A by ELISA **(F)**. Data shown are means ± SD from five mice. UD, undetectable. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

the increased serum IL-17A was not manifest until $CD4^{Cre}TTP^{f/f}$ mice were older than 16 weeks (**Figure 2E**). CD4 T cells purified from spleens of the conventional $TTP^{-/-}$ mice also showed a significant increase in IL-17–producing effector CD4 T cells when the mice were older than 8 months of age (**Supplementary Figure 2B**). In addition, the levels of IL-17 and IL-6 in culture supernatants of $CD4^+$ T cells (**Supplementary Figure 2C**) and IL-17A in serum (**Supplementary Figure 2D**) were increased significantly in conventional $TTP^{-/-}$ mice compared with WT mice. These data indicate that TTP plays a role in suppression of IL-17 secretion and in T_H17-mediated inflammation in aging mice.

T_H17 Cells Lacking TTP Have Increased per Cell Cytokine Productivity

To figure out whether TTP deficiency could enhance T_H17 cell differentiation, we differentiated naive CD4 T cells from WT and $CD4^{Cre}TTP^{f/f}$ mice into T_H1 and T_H17 subsets under T_H1 and T_H17 polarizing conditions and then measured intracellular IFN-γ and IL-17A with flow cytometry. IFN-γ–producing CD4 T cells were comparable between $TTP^{-/-}$ CD4 T cells and WT CD4 T cells under T_H0, T_H1, and T_H17 polarizing conditions (**Figure 3A** and **Supplementary Figure 3A**). Surprisingly, even the percentages of differentiated T_H17 cells were comparable between $TTP^{-/-}$ CD4 T cells and WT CD4 T cells (**Figure 3A** and **Supplementary Figure 3A**); the secretion of IL-17 by $TTP^{-/-}$ CD4 T cells was increased under all conditions (**Figure 3B**). In addition, when total CD4 T cells from WT and $TTP^{-/-}$ mice were cultured under T_H0 and T_H17 conditions, there was little increase of IL-17–producing CD4 T cells in cells lacking TTP (**Figure 3C** and **Supplementary Figure 3B**). This little increased $TTP^{-/-}$ T_H17 cells was in contrast to significantly increased levels of IL-17A produced by the $TTP^{-/-}$ CD4 T cells in culture supernatants (**Figure 3D**). These data suggest that the increased IL-17 secretion by $TTP^{-/-}$ $CD4^+$ T cells may not be due to an increase in T_H17 cell differentiation. Indeed, the mean fluorescence intensity of IL-17A was significantly increased in $TTP^{-/-}$ $CD4^+$ T cells compared with WT cells under T_H17 differentiation conditions (**Figure 3E**), indicating that each $TTP^{-/-}$ $CD4^+$ T cell produces much more IL-17A protein than WT cells. In addition, $TTP^{-/-}$ $CD4^+$ T cells polarized under T_H17 and T_H1 conditions expressed more IL-17 and IL-6 mRNA than WT cells, whereas IFN-γ mRNA expression was no difference (**Figure 3F** and **Supplementary Figures 4A,B**). Compared to WT cells, the expression of the T_H17 cell master transcription factor RORγt was decreased, Stat3 increased and Tbx21 mRNA kept no change in $TTP^{-/-}$ CD4 T cells under either T_H17 or T_H1 skewing conditions (**Figure 3F** and **Supplementary Figure 4B**), further supporting that T_H17

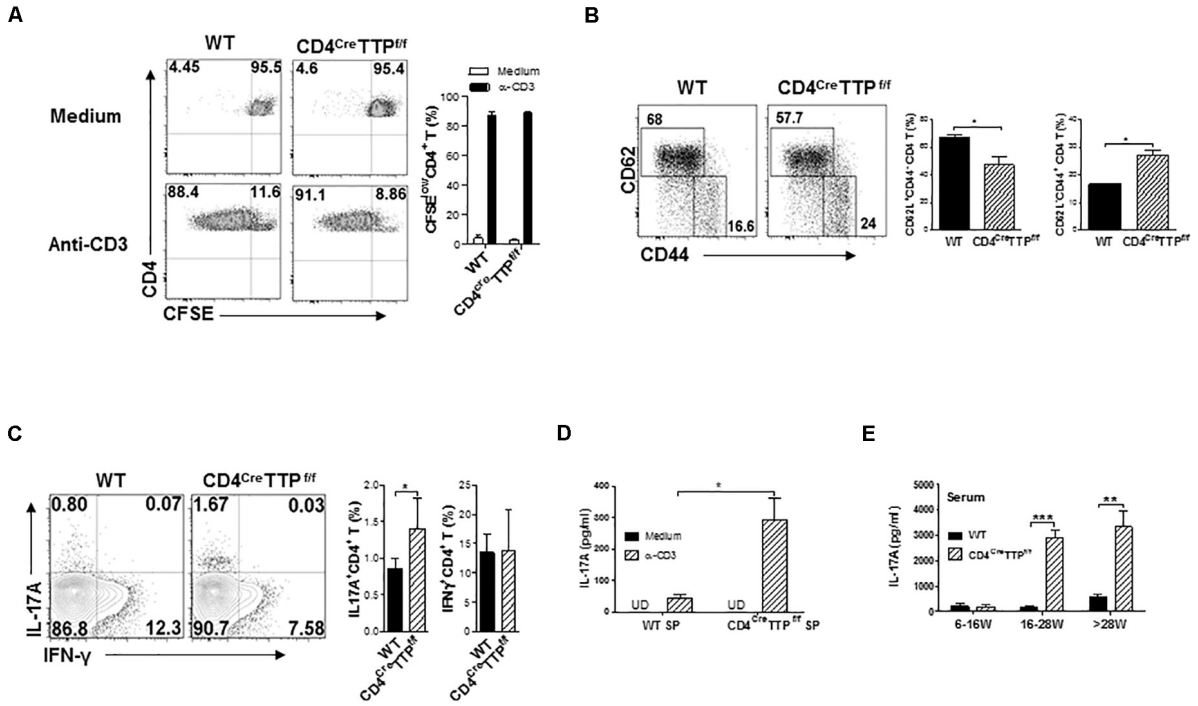

FIGURE 2 | T cell-specific TTP conditional knockout mice have increased IL-17–producing effector T cells. **(A)** Single spleen cells of wild-type (WT) and $CD4^{Cre}TTP^{f/f}$ mice aged 6–8 months were labeled with CFSE and cultured with anti-CD3 (1 μg/mL) Ab for 4 days before the proliferation was assessed by flow cytometry. Percentages of $CFSE^{low}$ $CD4^+$ T cells were summarized from three to four independent experiments. **(B)** Wild-type and $CD4^{Cre}TTP^{f/f}$ splenocytes were stained for CD44 and CD62L gated on $CD4^+$ cells. Percentages of $CD62L^-$ $CD44^+$ (effector) and $CD62L^+CD44^-$ (naive) $CD4^+$ T cells from four independent experiments were summarized and compared by *t*-test. **(C)** WT and $CD4^{Cre}TTP^{f/f}$ splenocytes were stimulated by PMA and ionomycin for 4 h and then analyzed by flow cytometry for intracellular cytokine gated on $CD4^+$ T cells. Fluorescence-activated cell sorting images represent one of five similar results. Percentages of $IL\text{-}17^+$ and IFN^+ CD4 T cells from four independent experiments were summarized and compared by *t*-test. **(D)** Cells from spleens (SP) of WT and $CD4^{Cre}TTP^{f/f}$ mice were stimulated with anti-CD3 Ab (1 μg/mL) for 3 days. Supernatants were collected and filtered for detection of IL-17A by ELISA. Data represent means ± SD from three to four mice in each group. **(E)** IL-17A in serum of WT and $CD4^{Cre}TTP^{f/f}$ mice aged between 6 and 30 weeks old was determined by ELISA. Data in E and F are expressed as means ± SD, with four to five mice per group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

cell differentiation is not enhanced in CD4 T cells lacking TTP. Overall, these data indicate that the increased IL-17 secretion in $TTP^{-/-}$ $CD4^+$ T cells is due mainly to an enhanced synthesis of IL-17 at the single-cell level.

TTP Controls the Effector Function of T_H17 Cells by Reducing IL-17A mRNA Stability

To explore the mechanisms of TTP-mediated inhibition of IL-17A, we measured and compared IL-17 mRNA stability between WT and $TTP^{-/-}$ T_H17 cells. As shown in **Figure 4A**, the half-life of IL-17A mRNA was increased from 36 min in WT T_H17 cells to 115 min in $TTP^{-/-}$ T_H17 cells. The half-lives of IL-10 and IFN-γ mRNAs were also increased in $TTP^{-/-}$ T_H17 cells compared to WT cells, whereas the TGF-β mRNA half-life remained similar (**Figure 4A**). In addition, the steady-state levels of IL-17A and TNF-α mRNA were also increased in $TTP^{-/-}$ CD4 T cells in response to TCR and costimulatory signals (**Supplementary Figure 5A**). The mRNA half-lives of transcription factors important for T_H17 cell development, such as IRF4 and IRF8, were actually decreased in $TTP^{-/-}$ T_H17 cells compared with WT cells (**Supplementary Figures 5B,C**), further indicating that TTP has minimal effects on T_H17 cell differentiation. To further confirm the inhibitory effects of TTP on IL-17A, we introduced TTP by adenoviral transduction into Jurkat T cells and found that overexpression of TTP inhibited the expression of IL-17A mRNA (**Figure 4B**). To determine whether IL-17A 3′ UTR mediated the IL-17 inhibition, we cloned the 3′ UTR of IL-17A mRNA downstream of luciferase gene and cotransfected a TTP expression vector with the IL-17A–3′ UTR luciferase-reporter construct into HEK293 cells, followed by measuring luciferase activity. TTP inhibited IL-17A–3′ UTR–mediated luciferase activity (**Figure 4C**), indicating that TTP promotes IL-17A mRNA degradation through its 3′ UTR. TTP is a ZFP containing a CCCH tandem zinc-finger domain. Zinc fingers of this type have been found in many RNA-binding proteins and are responsible for binding to the 3′ UTRs of target mRNAs. To determine whether these zinc fingers were responsible for TTP inhibition of IL-17A secretion, we cotransfected WT TTP, or two TTP zinc-finger mutants (TTPΔC124R and TTPΔC147R), with the IL-17A-3′ UTR–luciferase-reporter plasmid into HEK293 cells, followed by measuring luciferase activity. As shown in **Figure 4D**, the

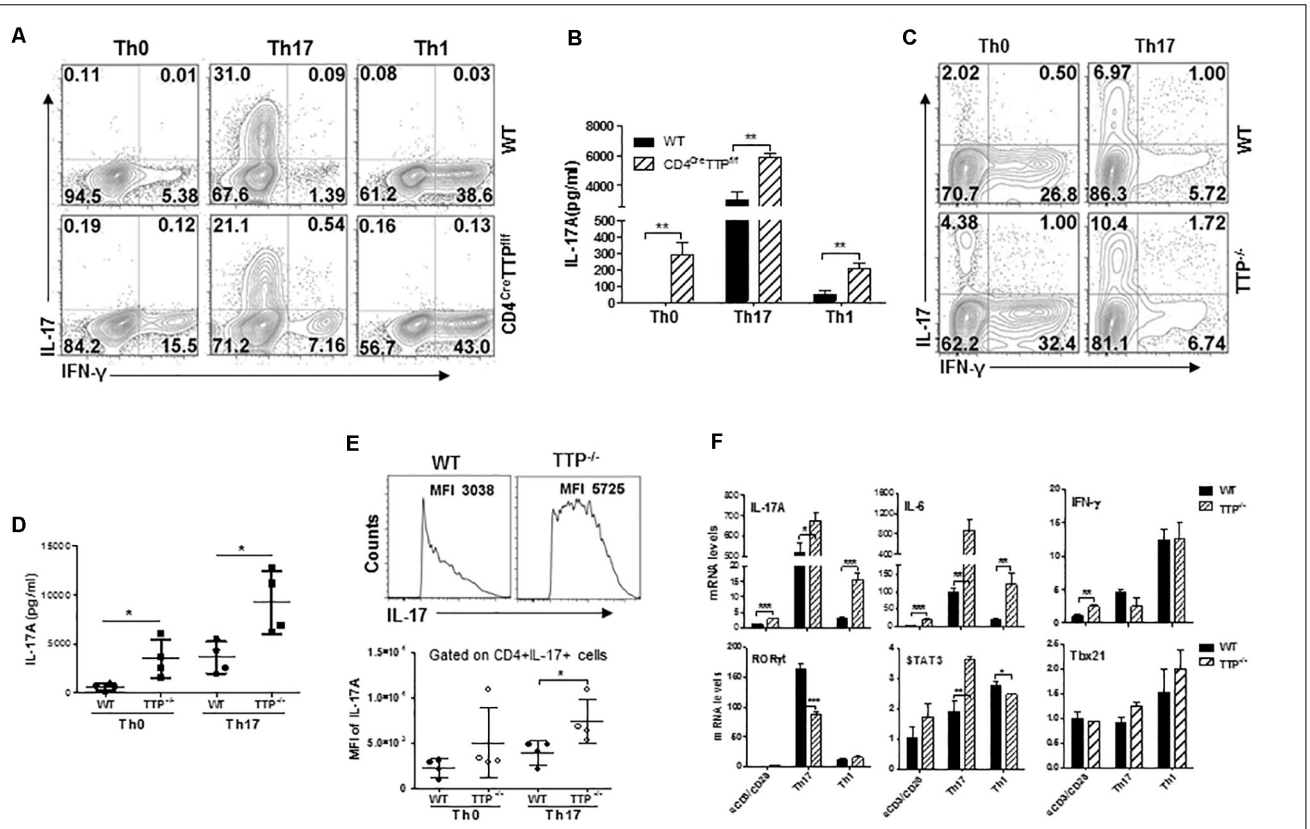

FIGURE 3 | CD4 T cells deficient in TTP produce more IL-17 than wild-type (WT) T cells at the single-cell level. **(A)** Naive $CD4^+$ T cells from $CD4^{Cre}TTP^{f/f}$ and WT mice aged 6–8 months were cultured under T_H1 or T_H17 polarizing conditions in 96-well plate coated with anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL) Abs for 3 days before stimulation with PMA and ionomycin for 4 h. Intracellular IL-17A and IFN-γ in $CD4^+$ T cells were analyzed by fluorescence-activated cell sorting (FACS). **(B)** Supernatants of the above cells were collected to measure IL-17A protein levels by ELISA (means ± SD from three independent experiments). **(C,D)** Total $CD4^+$T cells from WT and $TTP^{-/-}$ mice were stimulated with anti-CD3/CD28 Abs under T_H17 polarizing conditions for 3 days, rested for 3 days, and then treated with P/I for 4 h, followed by FACS analysis **(C)**. IL-17A levels in the supernatants of the above cells were detected by ELISA and summarized from four independent experiments **(D)**. **(E)** Mean fluorescence intensity (MFI) of intracellular IL-17A in $CD4^+$ T cells from WT and $TTP^{-/-}$ mice under T_H17 cell differentiation. Each dot represents one experiment. **(F)** IL-17A, IL-6, IFN-γ, *Rorc*, *Stat3*, and *Tbx21* mRNA expression in CD4 T cells under T_H1 and T_H17 cell differentiation for 4 days. Data shown are means ± SD from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

C147R TTP mutant lost its suppressive effect on IL-17A-3′ UTR–regulated luciferase activity, whereas the C124R mutant retained luciferase suppressive activity. These data suggest that TTP via zinc finger promotes IL-17A mRNA degradation, resulting in inhibition of IL-17 secretion by CD4 T cells.

T Cell-Specific TTP Conditional KO Mice Are Prone to DSS-Induced Intestinal Inflammation

T_H17 cells and IL-17 play important roles in intestinal inflammation (25, 26). DSS administration is commonly used to generate an acute mouse model of IBD. We used this model to determine the role of TTP in T_H17-mediated intestinal inflammation. We fed WT and $CD4^{Cre}TTP^{f/f}$ mice DSS-containing water and found that the $CD4^{Cre}TTP^{f/f}$ mice displayed severe colitis compared with WT mice. Specifically, the $CD4^{Cre}TTP^{f/f}$ mice developed significantly greater weight loss and severe bloody diarrhea than the WT mice (**Figures 5A,B**). All $CD4^{Cre}TTP^{f/f}$ mice died due to severe colitis by 8 days after drinking DSS water, whereas 80% of WT mice survived (**Figure 5C**). Colon lengths were also greatly shortened in $CD4^{Cre}TTP^{f/f}$ mice compared with WT mice (**Figures 5D,E**). Histological analysis showed more severe tissue damage and more infiltrating inflammatory cells in the colons from DSS-treated $CD4^{Cre}TTP^{f/f}$ mice compared with WT mice (**Figures 5F,G**). These results indicate that T cell-specific TTP KO mice are vulnerable to DSS-induced colitis.

IL-17 Mediates the DSS-Induced Colitis in T Cell-Specific TTP Conditional KO Mice

To determine whether IL-17 is responsible for the DSS-induced colitis in this model, we measured IL-17 levels in serum of the $CD4^{Cre}TTP^{f/f}$ mice and control WT mice fed DSS-containing water. Serum levels of IL-17A were higher in both WT and $CD4^{Cre}TTP^{f/f}$ mice fed DSS water than in those without DSS, with even greater IL-17A levels in the $CD4^{Cre}TTP^{f/f}$ mice (**Figure 6A**). Next, we analyzed CD4 T-cell

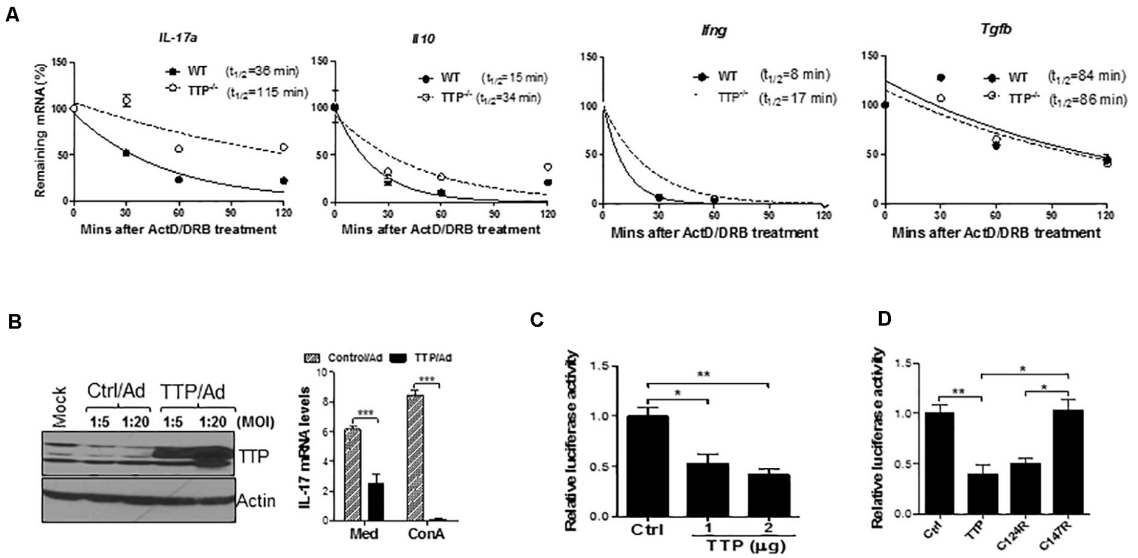

FIGURE 4 | TTP controls effector function of T_H17 cells by destabilizing IL-17 mRNA stability. **(A)** Splenic $CD4^+$ T cells of *TTP*$^{-/-}$ mice and wild-type (WT) littermates aged 6–8 months were stimulated under T_H17 polarizing conditions for 4 days and then actinomycin D (ActD) (5 μg/mL) and DRB (10 μg/mL) were added. Total RNAs were extracted at 30, 60, 90, and 120 min after adding ActD and DRB. cDNAs were reverse-transcribed and residual cytokine as well as GAPDH mRNA were measured by real-time quantitative PCR (qPCR). The levels of residual cytokine mRNAs were normalized to GAPDH mRNA at each time point and half-life of the mRNA determined by comparing to the levels of mRNA before adding ActD and DRB. **(B)** Jurkat cells were infected with control adenovirus (Ctrl/Ad) or TTP-expressing adenovirus (TTP/Ad) at MOI = 5 and 20 for 24 h, followed by adding ConA (5 ug/mL) and then measuring IL-17 mRNA by qPCR and TTP protein by immunoblotting. **(C)** HEK293 cells were transiently cotransfected with IL-17A 3′ UTR–driven luciferase construct along with CMV–TTP vector, as well as empty vector (Ctrl), followed by measurement of luciferase activity in cell lysates after 40 h. **(D)** HEK293 cells were transiently cotransfected with IL-17A 3′ UTR–driven luciferase construct along with WT TTP, as well as two TTP zinc-finger mutant constructs (C124R and C147R), followed by measurement of luciferase activity in cell lysates after 40 h. Data shown as relative levels compared to luciferase activity in cells transfected with the empty vector (Ctrl) from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

subsets and found a significant increase in IL-17A–producing $CD4^+$ cells from the mesenteric LNs (MLNs) of $CD4^{Cre}TTP^{f/f}$ mice compared to WT mice, whereas the frequency of IFN-γ–producing $CD4^+$ T cells was comparable (**Figures 6B,C**), further suggesting that IL-17 produced by the T_H17 cells may contribute to the colitis in $CD4^{Cre}TTP^{f/f}$ mice. To confirm a causal relationship between IL-17 and colitis, we administered a neutralizing Ab specifically directed against IL-17A to $CD4^{Cre}TTP^{f/f}$ mice fed DSS-water and then closely monitored their body weights. The $CD4^{Cre}TTP^{f/f}$ mice receiving IL-17A neutralizing Ab showed significantly less body weight loss (**Figure 6D**) and had longer colons (**Figure 6E**) than the mice receiving control immunoglobulin G (IgG). Histological analysis also showed reduced intestinal inflammation and more intact structure in the colons of $CD4^{Cre}TTP^{f/f}$ mice given IL-17A neutralizing Ab (**Figures 6F,G**). These results demonstrate that IL-17 produced by T_H17 cells contributes, at least partially, to the pathogenesis of DSS-induced colitis in $CD4^{Cre}TTP^{f/f}$ mice.

DISCUSSION

T_H17 cells play important roles in chronic inflammation. Excessive T_H17 cell development and IL-17 production are associated with the pathogenesis of several diseases, including autoimmune arthritis, multiple sclerosis, and IBD (31, 32). So far, most studies have focused on the development of T_H17 cells and their transcriptional regulation by key transcription factors. Relatively little is known about how T_H17 cells are regulated at the post-transcriptional level. In this study, we have demonstrated that the RNA-binding protein TTP negatively regulates T_H17 cell function at the post-transcriptional level by enhancing degradation of the IL-17 mRNA. It has been reported that conventional $TTP^{-/-}$ mice develop a severe inflammatory syndrome with elevated levels of circulating inflammatory cytokines due to increased mRNA stability (22, 27, 28, 33–40), demonstrating that TTP is an important regulator for control of inflammatory responses. In order to evaluate the role of TTP in regulation of T-cell development and function, we generated CD4-specifc TTP conditional KO mice. The $CD4^{Cre}TTP^{f/f}$ mice exhibited different phenotypes from conventional $TTP^{-/-}$ mice. One obvious difference was that the $CD4^{Cre}TTP^{f/f}$ mice appeared to undergo normal growth, whereas the conventional $TTP^{-/-}$ mice had retarded growth (**Figure 1A**). Another difference in the $CD4^{Cre}TTP^{f/f}$ mice was the lack of the early onset and severe inflammatory arthritis universally seen in the conventional $TTP^{-/-}$ mice (26). Although younger $CD4^{Cre}TTP^{f/f}$ mice appeared normal, they did develop atopic dermatitis and had elevated skin T_H17 cells and serum levels of IL-17A when they aged past 5–8 months. Frasca et al. (41) showed that TTP levels were higher in activated B cells from old mice as compared

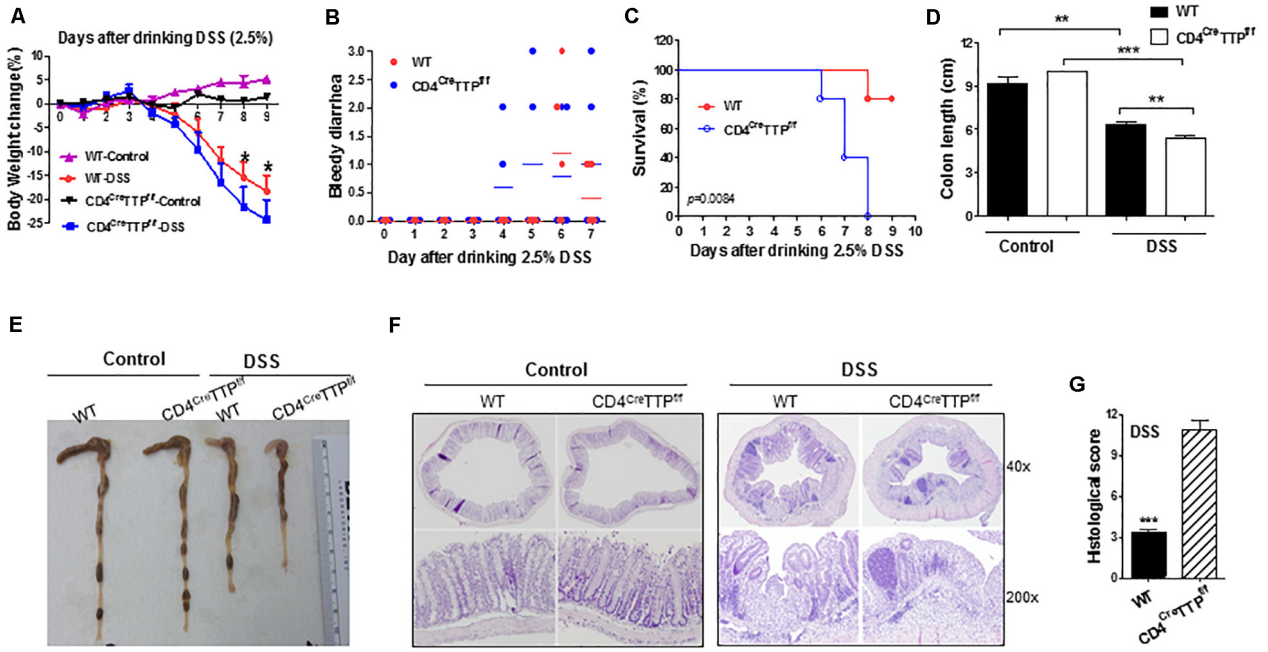

FIGURE 5 | T cell-specific TTP conditional knockout mice are prone to DSS-induced colitis. Age- and body weight-matched $CD4^{Cre}TTP^{f/f}$ and control mice (8–12 weeks old) were administered with DSS in drinking water (2.5% wt/vol) for 5 days. Each group had five mice. Average body weight changes **(A)**, bloody diarrhea **(B)**, and survival **(C)** shown at indicated time points. On day 9, the lengths of colons of mice from treated groups were measured **(D)** and photographed **(E)**. Sections of colons in each group were stained with hematoxylin and eosin **(F)**. **(G)** Histological scores of colons collected from the above mice. Data are from four mice per group and represented as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

with young mice. These data suggest that TTP plays a role in controlling lymphocyte activation and chronic inflammation during the aging processes.

The spontaneous chronic inflammation in conventional $TTP^{-/-}$ mice is considered to be at least partly TNF-α dependent, as TNF mRNA was stabilized in $TTP^{-/-}$ macrophages after stimulation with lipopolysaccharide (16, 30), and treatment of the conventional TTP KO mice with Abs to TNF-α (26), or breeding to TNF-α receptor–deficient mice (15), prevented development of the inflammatory syndrome. However, specific deletion of TTP in myeloid cells did not recapitulate the phenotypes of conventional $TTP^{-/-}$ mice (30), indicating the involvement of other cell types in the development of the inflammatory syndromes. Indeed, we found that both $CD4^+$ and $CD8^+$ T cells (**Supplementary Figure 6**) in the T cell-specific TTP-deficient mice produced much higher levels of IL-17A compared with the cells of WT mice. The increased IL-17A along with spontaneous dermatitis development in old $CD4^{Cre}TTP^{f/f}$ mice demonstrates a role for TTP in regulating T cell-mediated inflammation. Studies from our laboratory and others show that TTP targets IL-23 and IL-6 mRNA for degradation (27, 34, 36, 37). Because both cytokines are important for T_H17 cell differentiation, these collective results suggest that TTP may affect the development of T_H17 cells. Surprisingly, several key transcription factors important for T_H17 cell differentiation, such as RORγt, IRF4, IRF8, were not increased in $TTP^{-/-}$ T_H17 cells, indicating that TTP may affect T_H17 cell function rather than T_H17 cell development. Indeed, $TTP^{-/-}$ T_H17 cells produced more IL-17A than WT T_H17 cells at the single-cell level (**Figures 3A–E**), and the IL-17 mRNA stability was increased in $TTP^{-/-}$ T_H17 cells (**Figure 4A**). Our results indicate that TTP negatively regulates T_H17 cell function by promoting mRNA degradation of its signature cytokine IL-17. IFN-γ might also play a role in the development of skin inflammation in aged $CD4^{Cre}TTP^{f/f}$ mice, as the levels of IFN-γ were also increased in the T_H0 and T_H17 cells lacking TTP (**Figure 3A**), which is consistent with a previous report that TTP promotes IFN-γ mRNA degradation (35). Molle et al. (28) reported that IL-17 is involved in the generation of joint inflammation in conventional $TTP^{-/-}$ mice, as deletion of IL-17 in $TTP^{-/-}$ mice ameliorates the inflammation. They also reported that the differentiation of T_H17 cells from naive CD4 T cells lacking TTP was normal (28), which is in line with our observation. These data together suggest that TTP regulates T_H17 cell function by targeting IL-17 mRNA stability, without directly affecting T_H17 cell differentiation.

Signaling through many TLRs and other environmental stimuli can induce TTP expression in macrophages, which in turn prevents excessive cytokine production and inflammation. TTP suppresses the expression of proinflammatory cytokines by binding directly to AREs in the 3′ UTR of their mRNAs, leading to their deadenylation and decay. In primary CD4 T cells, TTP inhibits IL-17 expression by directly promoting mRNA degradation via the IL-17 3′ UTR (**Figures 4A,C**). Using human T-cell line HuT102 and Jurkat cells, Lee et al. (42) reported that TTP bound to several AREs in the 3′ UTR of IL-17 mRNA for degradation of human IL-17 transcript. These data suggest that

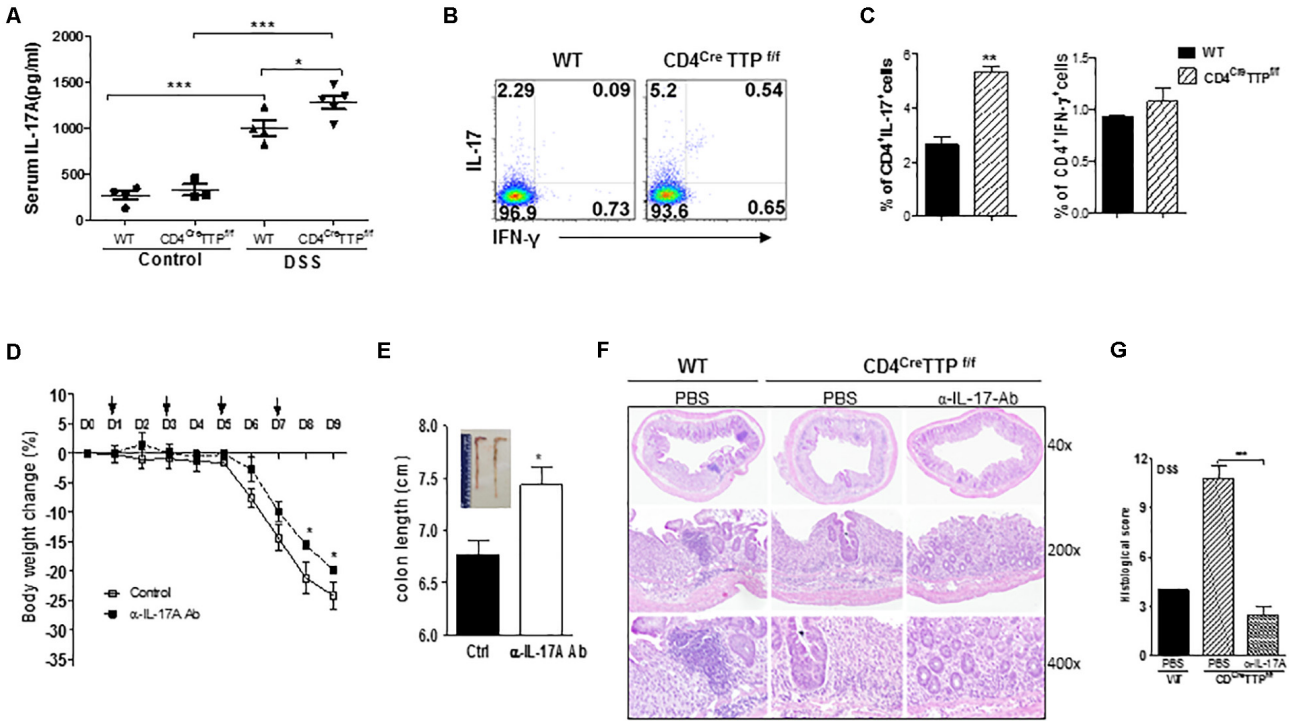

FIGURE 6 | IL-17 mediates DSS-induced colitis in the T cell-specific TTP conditional knockout mice. Wild-type and $CD4^{Cre}TTP^{f/f}$ mice were fed 2.5% DSS water for 5 days and then switched to normal water for additional 4 days. Serum was isolated at day 9 and used to measure IL-17A levels by ELISA **(A)**. Lymphocytes isolated from mesenteric LN at day 9 were analyzed for intracellular cytokine expression by flow cytometry gated as $CD4^+$ T cells **(B,C)**. $CD4^{Cre}TTP^{f/f}$ mice were received 150 mg of IL-17A–specific neutralizing mAb (*InVivo*MAb anti-mouse IL-17A, clone 17F3; BioXcell) intraperitoneally every other day starting on day 1 after DSS water administration. **(D)** Average body weight of the mice at indicated time points. **(E)** At day 9, colons were photographed (insert) and individual colon lengths were measured. **(F)** Sections of colons were analyzed with hematoxylin and eosin staining. **(G)** Histological scores of colons collected from the above mice. Data are from four or five mice per group and represented as mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between groups.

TTP regulates the expression of macrophage cytokines and T-cell cytokines through similar mechanisms. It is known that TTP regulates targeted gene expression through several mechanisms, such as mRNA localization or translation efficiency or other RNA modifications in addition to mRNA stabilization (25, 43). These mechanisms may also contribute to IL-17 regulation by TTP in T cells.

IBDs in human are characterized by chronic intestinal inflammation mediated by several factors. Both protective and pathogenic functions of IL-17 have been reported in different experimental models of colitis. Adoptive transfer of $IL\text{-}17^{-/-}$ $CD45RB^{hi}$ T cells into $RAG^{-/-}$ recipient mice induced a more severe wasting disease compared with WT counterparts (44). In contrast, IL-17 deficiency resulted in resistance to dextran sulfate sodium–induced colitis in mice, indicating a pathogenic role of IL-17 in intestinal inflammation (12). Our data show increased IL-17 in $CD4^{Cre}TTP^{f/f}$ mice, along with accelerated wasting disease compared with WT littermates. It has been reported that neutralizing IL-17 activity enhanced the development of DSS-colitis in WT mice (45). Therefore, we focused our study on whether IL-17 in the CD4 conditional TTP KO mice played a role in DSS-induced colitis by administering IL-17 neutralization Ab only to KO mice. As shown in **Figure 6D**, neutralizing IL-17 reduced the severity of wasting disease, supporting a pathogenic role for IL-17 in intestinal inflammation observed in the $CD4^{Cre}TTP^{f/f}$ mice. Notably, although neutralization of IL-17 reduced DSS-induced colitis, mice still developed significant weight loss. This suggests that other factors regulated by TTP may also contribute to DSS-colitis in the T cell-specific TTP conditional KO mice. Although CD4cre is broadly used to generating conditional gene targeting in CD4 T cells, several types of cells express CD4, including thymic CD8, CD4-expressing DCs, and monocytes. Therefore, the phenotypes observed in the $CD4^{Cre}TTP^{f/f}$ mice may be mediated by several types of cells. TNF-α is known to be important in the pathogenesis of intestinal inflammation. TNF-α expression was indeed higher in $TTP^{-/-}$ T_H17 cells than WT T_H17 cells (**Supplementary Figure 4B**). TNF expression in $TTP^{-/-}T_H17$ cells may also contribute to the DSS-induced colitis in the $CD4^{Cre}TTP^{f/f}$ mice. IL-6 could be involved, because TTP deficiency also led to increased IL-6 in the serum of CD4 T cell-specific TTP KO mice (**Figure 3F** and **Supplementary Figure 4A**). More study is needed to further investigate the roles of IL-6 and other players in the pathogenesis of intestinal inflammation mediated by TTP. T_H17 cell-produced GM-CSF plays an essential role in autoimmune inflammation (such as encephalitis) mediated mainly by a positive feedback loop between GM-CSF secreted

by T_H17 cells and the production of IL-23 by antigen-presenting cells (46, 47). We recently report that GM-CSF–producing CD4 T cells may be a unique subset of T helper cells, namely, T_H-GF-CSF, important for T cell-mediated inflammation (48). GM-CSF has been reported to mediate intestine inflammation (49). Considering TTP mediates GM-CSF expression (20), it is possible that GM-CSF may also contribute to the phenotypes in the $CD4^{Cre}TTP^{f/f}$ mice.

In summary, our results reveal a new mechanism for regulating T_H17 function and T_H17-mediated inflammation, through post-transcriptional regulation of IL-17 mRNA decay by TTP, suggesting that TTP might be a novel therapeutic target for treatment of the T_H17-mediated diseases.

MATERIALS AND METHODS

Mice

$CD4^{Cre}TTP^{f/f}$ mice were obtained by crossing mice expressing Cre recombinase under the control of the murine CD4 promoter ($CD4^{Cre}$) mice purchased from the Jackson Laboratory (Bar Harbor, ME, United States) (29) with the previously described $TTP^{flox/flox}$ mice (30). All mice were on the C57BL/6 background and were bred at the animal facility of Saint Louis University. Animal experiments were approved by the Institutional Animal Care and Use Committee at Saint Louis University and were performed according to federal and institutional guidelines.

Cell Lines

Jurkat cells were purchased from ATCC and cultured in RPMI-1640 with 10% (vol/vol) fetal calf serum (Sigma, St. Louis, MO, United States; endotoxin NMT 10.0 EU/mL) supplemented with 2 mM glutamine and 100 units/mL of penicillin and streptomycin (Sigma). HEK293T cells were purchased from ATCC and cultured in Dulbecco modified eagle medium (4.5 g/L) with 10% (vol/vol) fetal calf serum and 100 units/mL of penicillin and streptomycin purchased from Sigma as described above.

Antibodies and Flow Cytometry

The following Abs were purchased from BD Biosciences: anti-CD3-APC (clone 145-2C11), anti–CD4-PE (clone H129.19), anti–CD8-PE (clone 53-6.7), anti–CD4-PerCP (clone RM4-5), anti–CD44-FITC (clone IM7). For intracellular staining of cytokines, cells were stimulated for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/mL; Sigma) and ionomycin (0.5 μg/mL; Sigma) in the presence of Monensin (GolgiStop; 0.7 μL/mL; BD Biosciences) for 4 h and then fixed and permeabilized for intracellular cytokine testing according to the manufacturer's instructions (BD Biosciences). Antibodies used were as follows: anti-IFN-γ–APC (clone XMG1.2), anti–IL-17A–PE (clone TC11-18H10.1), anti–IL-17A–AF488 (clone TC11-18H10.1), anti-IFN-γ–AF700 (clone XMG1.2), from BD Biosciences. Cells were analyzed on a FACSCanto II or FACS LSR II (BD), and data were analyzed with FlowJo software (TreeStar).

T-Cell Stimulation and Differentiation

$CD4^+$ T cells, purified using the DynaBeads FlowComp mouse CD4 kit (Invitrogen) or EasySep Mouse Naive $CD4^+$ T Cell Iso Kit (Stemcell), were activated by plate-coated anti-CD3 (1 μg/mL; 145-2C11; Biolegend) and anti-CD28 (1 μg/mL; 37.51; Biolegend) Abs for 72 h in complete cell culture medium (RPMI 1640) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin–streptomycin, non-essential amino acids, sodium pyruvate, 10 mM HEPES, and 50 μM 2-mercaptoethanol. Supernatants were collected for measuring cytokines by enzyme-linked immunosorbent assay (ELISA) (BD Bioscience). For *in vitro* T-cell differentiation, $CD4^+$ T cells were activated with plate-coated anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL) Abs under the following conditions: for T_H1: anti-IL-4 20 μg/mL (11B11; BD), IL-12 10 U/mL (Peprotech); for T_H17: IL-6 50 ng/mL (Peprotech), TGF-β 1 ng/mL (Peprotech), IL-23 20 ng/mL (Peprotech), anti-IL-4 10 μg/mL (11B11; BD), and anti-IFN-γ 10 μg/mL (XMG1.2; BD). Cells were cultured for 4 days before activated with PMA (50 ng/mL; Sigma) and ionomycin (0.5 μg/mL; Sigma) for 4 h for analysis of cytokines and surface markers with flow cytometry.

Plasmid Constructs

Full-length 3′ UTR of murine IL-17A was retrieved from the website ARE site (Universitat Wien). A 656-bp fragment of the mouse IL-17A 3′ UTR amplified from cDNA of mouse T cells was cloned downstream of the luciferase gene in pGL3 control vector (Promega) between *Xba*I and *Fse*I sites. Luciferase-reporter gene expression is driven by SV40 promoter and has been used by us and others to study mRNA stability controlled by downstream inserted 3′ UTR or siRNA (27, 50–54). WT TTP and two mutant TTP plasmids were generated in Perry Blackshear's laboratory (55).

Luciferase Assay

Transient transfections were performed by electroporation. Briefly, for each condition 0.4 mL of HEK293T-cell suspension containing 1×10^7 cells was mixed with 3 μg of total DNA (including reporter, effector, internal control, and carrier DNA) and electroporated in 0.45-cm electroporation cuvettes (Gene Pulser II; Bio-Rad Laboratories, Hercules, CA, United States) at 975 microfarade and 300 V in RPMI 1640 medium without serum. The transfected cells from different cuvettes were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM glutamine, 10 μM chloroquine, and antibiotics and were added to 24-well plates and incubated for 40 h prior to harvesting. To measure luciferase activity, cells were pelleted by centrifugation and resuspended in 100 μL of lysis buffer containing 125 mM Tris-phosphate pH 7.8; 10 mM DTT; 10 mM 1-2-diaminocyclohexane-tetraacetic acid; 50% glycerol; and 5% Triton-X100. Luciferase activity was measured in cell lysates with Luciferase Reporter Assay system (Promega). Transfection efficiency was routinely monitored by β-galactosidase (β-gal) assay by cotransfection with 3 μg of pCMV-β-gal plasmid. Variability in β-gal activity between samples

was typically within 5%. Lysates were used for both luciferase and β -gal assays.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen, Life Technologies). Reverse transcription (RT) reactions were carried out as follows: 1-μg aliquots of total RNA were mixed with 1 μL oligo dT primers (0.5 mg/mL), 1 μL 10 mM dNTPs, and ddH_2O to equalize volumes of all samples at 12 μL. The mixture was heated at 65°C for 5 min, quenched on ice, spun down briefly, and 8 μL of a Master Mix was added. The RT Master Mix consisted of 4 μL 5 × first-strand buffer (Invitrogen), 2 μL 0.1 M DTT, 1 μL RNase inhibitor (40 U/μL; Invitrogen), and 1 μL Superscript II (200 μL/μL, Invitrogen). The reaction was incubated at 42°C for 60 min and then at 70°C for 15 min, followed by 4°C soak. To each sample (in 20 μL total volume), 80 μL ddH_2O was added; 5 μL diluted cDNA was used for each polymerase chain reaction (PCR) reaction of 25 μL volume. PCR was performed using two-step qRT-PCR with SYBR green (Invitrogen, Life Technologies) with ABI Prism 7700 Sequence detector/Real Time PCR machine. Results were analyzed using ΔΔCT method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous reference control. Primers for specific murine targets were as follows: *Ifn-γ*, forward, 5′-CCATCCTTTTGCCAGTTCCTC-3′, and reverse, 5′-ATGAACGCTACAC ACTGCATC-3′; *Il17a*, forward, 5′-CTCCAGAAGGCCCTCAGACTAC-3′, and reverse, 5′-GG GTCTTCATTGCGGTGG-3′; *Tgfb*, forward, 5′-TAAGAGGTC ACCCGCGTGCT-3′, and reverse, 5′-AAAGACAGCCACTC AGGCGTA-3′; Rorc, forward, 5′-AAGATCTGCAGCTTT TCCACA-3′, and reverse, 5′-TTTGGAACTGGCTTTCCATC-3′; *Stat3*, forward, 5′-CAGACTGGTTGTTTCCATTCAGAT-3′, and reverse, 5′-ACCCAACAGCCGCCGTAG-3′; *Tbx21*, forward, 5′-ATGCGTACATGGACTCAAAGTT-3″, and reverse, 5′-TTT CCAA GAGACCCAGTTCATTG-3′; *Il6*, forward, 5′-GGAAATT GGGGTAGGAAGGA-3′, and reverse, 5′-TGTGCAATGGCAA TTCTGAT-3′; GAPDH, forward, 5′-TGGCCTACATGGCCT CCA-3′, and reverse, 5′-TCCCTAGGCCCCTCCTGTTAT-3′.

mRNA Stability

$CD4^+$ T cells were cultured under T_H17 polarization conditions before adding actinomycin D (5 μg/mL; Sigma) and 5,6-dichlorobenzimidazole riboside (DRB) (10 μg/mL; Sigma), and then total RNAs collected at different times. cDNAs were used to measure the remaining mRNA for calculating the half-life of each mRNA as described previously (27, 56, 57). In brief, the half-lives of mRNAs were calculated separately in WT and TTP KO cells and then compared. The remaining levels of mRNAs at 30, 60, 90, and 120 min after blocking *de novo* RNA synthesis with ActD/DRB were first normalized to housekeeping GAPDH at each time point and then calculated against the levels of mRNAs at $t = 0$, which was set as 100%.

Enzyme-Linked Immunosorbent Assays

Serum from blood of WT and KO mice treated with or without DSS, as well as supernatants from CD4 T cells, was harvested at described in the text and stored at −70°C. Mouse IL-17 was detected using ELISA MAX™ Deluxe Set Mouse IL-17A kit (Biolegend) according to the manufacturer's instructions. Concentrations were calculated by regression analysis of a standard curve.

Western Blotting

Cells were lysed in RIPA buffer for 30 min on ice. Protein lysates (25 μg) were loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membrane and blocked in 5% non-fat milk in Tris buffer, pH 8.0, at room temperature for 2 h. The membrane was blotted with anti-TTP Primary Ab (clone 3A2; 1 mg/mL; Santa Cruz Biotechnology) in Tris buffer containing 5% milk powder and left overnight at 4°C. After extensive washing, blots were subjected to horseradish peroxidase–conjugated sheep anti-mouse Ig secondary Ab at a 1:5,000 dilution in 5% milk and then detected with enhanced chemiluminescence detection (PerkinElmer Life Sciences Inc., Boston, MA, United States).

DSS Colitis Model

Colitis was induced in mice with drinking water containing 2.5% DSS (MP Biomedicals) for 5 days and then replaced with normal water. Mice were monitored for weight changes, diarrhea, bloody stools, and overall health. Mice were removed from the study when their body weight loss exceeded 25% of their original body weight and counted as death.

Histologic Colitis Assessment and Colon Length

The entire colon was removed from cecum to anus, measured, and fixed in 10% neutral-buffered formalin. Five-micrometer-thick sections were stained with hematoxylin and eosin for microscopic examination. Histological scores were assigned by a pathologist blinded to the experimental groups based on the extent and severity of inflammation, ulceration, and hyperplasia of the mucosa. The final score was calculated as the sum of individual factors, multiplied by the extent of tissue involvement. Severity scores for inflammation were as follows: 0 = normal (within normal limits), 1 = mild (small, focal, or widely separated, limited to lamina propria), 2 = moderate (multifocal or locally extensive, extending to submucosa), and 3 = severe (transmural inflammation with ulcers covering >20 crypts). Multiplied factors as extent of lesions were as follows: 0 = normal (0% involvement), 1 = up to 25% involvement, 2 = 25–50% involvement, 3 = over 51–50% involvement, and 4 = over 76–100% involvement. Spleen and MLNs were mechanically dissociated, and red cells were lysed in ACK lysis buffer. Cell suspensions were washed, enumerated, and stored in RPMI 1640 containing 10% FBS (Cellgro, Herndon, VA, United States) on ice until used.

Statistical Analysis

Data were analyzed with Prism software 5.0 (GraphPad). For standard data sets, data were shown as mean ± SD, and an unpaired two-tailed Student's *t*-test was used. For multiple groups, one-way analysis of variance was used. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between indicated groups.

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 animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Saint Louis University.

AUTHOR CONTRIBUTIONS

JLi contributed to the concept and design and study supervision. JLi, HN, and HP contributed to the development of the methodology. DS, PB, HN, HP, and QW contributed to acquisition of the data (provided animals, plasmids and reagents, etc.). JL, HN, JLa, LW, and HP contributed to analysis and interpretation of the data (e.g., statistical analysis, biostatistics, and computational analysis). JL, HP, DH, MF, and RH contributed to writing, review, and revision of the manuscript. PB contributed to administrative, technical, and material support (reporting or organizing data and construction database). 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.2020.01952/full#supplementary-material

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REVIEW
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The Role of the IL-23/IL-17 Axis in Disease Initiation in Spondyloarthritis: Lessons Learned From Animal Models

Mohamed Mandour[1,2*], *Sijia Chen*[1,2,3] *and Marleen G. H. van de Sande*[1,2]

[1] *Department of Clinical Immunology and Rheumatology, Amsterdam Rheumatology & Immunology Center (ARC), Amsterdam University Medical Centers, Location Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands,* [2] *Department of Experimental Immunology, Infection and Immunity Institute, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, Amsterdam, Netherlands,* [3] *Division of Rheumatology, Inflammation, and Immunity, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, United States*

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Lars Rogge, Institut Pasteur, France

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***Correspondence:**
Mohamed Mandour m.a.f.m.m.mandour@amsterdamumc.nl

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Spondyloarthritis (SpA) is a spectrum of chronic inflammatory joint diseases that frequently presents with inflammation of the axial skeleton, peripheral joints, entheses, skin, and gut. Understanding SpA pathogenesis has been proven challenging due to the limited availability of human target tissues. In recent years, the interleukin (IL)-23/IL-17 pathway has been implicated in the pathogenesis of SpA, in addition to the Tumor Necrosis Factor Alpha (TNF-α) cytokine. The underlying molecular mechanisms by which the IL-23/IL-17 pathway triggers disease initiation, both in the joints as well as at extra-musculoskeletal sites, are not precisely known. Animal models that resemble pathological features of human SpA have provided possibilities for in-depth molecular analyses of target tissues during various phases of the disease, including the pre-clinical initiation phase of the disease before arthritis and spondylitis are clinically present. Herein, we summarize recent insights gained in SpA animal models on the role of the IL-23/IL-17 pathway in immune activation across affected sites in SpA, which include the joint, entheses, gut and skin. We discuss how local activation of the IL-23/IL-17 axis may contribute to the development of tissue inflammation and the onset of clinically manifest SpA. The overall aim is to provide the reader with an overview of how the IL-23/IL-17 axis could contribute to the onset of SpA pathogenesis. We discuss how insights from animal studies into the initiation phase of disease could instruct validation studies in at-risk individuals and thereby provide a perspective for potential future preventive treatment.

Keywords: spondyloarthritis, interleukin-23/IL-17 axis, HLA-B27, animal models, psoriatic arthritis

INTRODUCTION

Spondyloarthritis (SpA) is a chronic inflammatory joint disease characterized by inflammation and new bone formation which results in structural damage. Clinically, patients present with axial manifestations, such as sacroiliitis or spondylitis, and/or peripheral manifestations such as arthritis, dactylitis, or enthesitis. Besides, extra-musculoskeletal features, including psoriasis, inflammatory bowel disease, and uveitis, can be present (1). SpA comprises 2 subtypes: peripheral SpA, with psoriatic arthritis (PsA) as the prototypical disease, and axial SpA, which encompasses radiographic axSpA, in which patients have signs of sacroiliitis on X-ray and fulfill the modified New York criteria (2), and the non-radiographic type (3). The disease pathogenesis of SpA is incompletely understood. Genetic factors combined with exposure to microbes by the loss of barrier function in the skin or the gut, or local mechanical stress at entheseal sites, are suggested to induce an inflammatory cascade resulting in joint inflammation and new bone formation. HLA-B27 is the strongest genetic factor linked to SpA. Genetic association studies, animal studies, human ex-vivo and intervention studies have demonstrated that the tumor necrosis factor alpha (TNF-α) and interleukin (IL)-23/IL-17 pathways are key players in the inflammatory cascade, both in the initiation phase of SpA, and during chronic persistent disease (4–6) (**Figure 1**).

TNF-α is a pleiotropic, pro-inflammatory cytokine playing major roles in protection against infections and driving inflammation in immune mediated inflammatory diseases including SpA. It is produced by various immune (activated T cells, macrophages, monocytes, neutrophils) and non-immune cells (fibroblasts, endothelial cells) (7). TNF-α is produced as a transmembrane bound protein expressed on the cell surface of various cell types including lymphocytes and macrophages, which is cleaved into a soluble form by metalloprotease TNF-α converting enzyme (TACE), also called A Disintegrin and Metalloprotease 17 (ADAM17). Both the transmembrane and soluble forms of TNF-α are biologically active and can bind to the TNF-α receptors 1 and 2, but their downstream effects vary (8, 9).

IL-17A is a pro-inflammatory cytokine implicated in various inflammatory disorders. IL-17A by itself only has a modest proinflammatory effect but acts as a potent enhancer of inflammation through synergy with other proinflammatory cytokines such as TNF-α (10). IL-17A was the first to be characterized among the 6 conserved IL-17 proteins (IL-17A-F), followed by IL-17F, which is for 50% structurally similar to IL-17A (11, 12). The IL-17 receptor family comprises the subunits IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. After binding of IL-17A or F to their receptors, Act1 associates with the IL-17 receptor resulting in the activation of various downstream signaling pathways (13). IL-23 is the canonical cytokine that activates IL-17A production. It is a heterodimeric cytokine, which contains a P19 and a P40 subunit. The P19 unit is exclusive to IL-23 whereas the P40 subunit is shared with IL-12 (14). IL-23 signals through the IL-23R and IL-12β1 subunits resulting in activation of the JAK-STAT pathway mainly *via* STAT3 (15). More recently, it has been shown that T cells and innate (-like) lymphocytes can produce IL-17A in response to cytokines other than canonical IL-23. These alternative cytokines include IL-7 and IL-9, which classically are required to maintain peripheral innate(-like) T cell populations (16–18). Other diverse innate immune cells also produce IL-17, including γδ T cells, NKp46+ NK cells, intestinal Paneth cells, invariant natural killer T cells (iNKT), MAIT cells and neutrophils (19). The emergence of distinct pathways culminating in the secretion of IL-17A, in addition to the canonical IL-23/IL-17 pathway, underscores the importance of IL-17A in human health and disease (20). In SpA, IL-17A driven inflammation contributes to erosive joint damage and pathological new bone formation (21).

Although the IL-23/IL-17 axis has a central role in SpA pathology, antibodies targeting IL-17A and IL-23 have demonstrated different levels of efficacy in the various subtypes of SpA (22–24). Most strikingly, IL-23 inhibition in human radiographic axSpA patients was not effective (25), whereas IL-17A-blocking therapies decreased inflammation and disease severity (6, 26). These clinical findings are supported by findings in the *Mycobacterium* tuberculosis (*M.tb*) accelerated HLA-B27 transgenic rat (27) model. The disease phenotype in this model resembles human SpA as these rats develop signs of arthritis and spondylitis, with inflammation and new bone formation. When IL-23 inhibition was started after the rats had developed established disease with arthritis and spondylitis, there was a lack of efficacy just as observed in patients (25, 28). In contrast, blocking IL-23R just after immunizing the rats completely prevented inflammation and new bone formation (24). These findings suggest that it may be necessary to block IL-23 in the pre-clinical phase, before the disease phenotype becomes clinically apparent. Based on these findings in the rats, it could be hypothesized that there may be a pre-clinical disease stage also in human SpA where specific immune pathways are already activated before the disease becomes clinically manifest (**Figure 1**). This concept of a pre-clinical "at-risk phase" phase is already well-established in a different form of chronic arthritis, rheumatoid arthritis (RA) (29–31). Better understanding of the molecular alterations present in the at-risk phase of RA have resulted in initial treatment trials aiming to prevent onset of disease (32, 33).

For long, it has been speculated that extra-musculoskeletal tissue inflammation in SpA contributes to the initiation of arthritis and spondylitis and subsequent development of full-blown SpA. To address the potential tissue-specific role of IL-23/IL-17 in both joint and extra-musculoskeletal tissues, it would be ideal to

Abbreviations: ADAM17, A disintegrin and metalloprotease 17; AIA, adjuvant-induced arthritis; AxSpA, Axial spondyloarthritis; CIA, collagen-induced arthritis; DCs, Dendritic cells; HLA-B27/Huβ2m- tg, HLA-B27/human β2 microglobulin transgenic rat; IL-23R, Interleukin 23 receptor; IL-23, Interleukin 23; IL-17A, Interleukin 17A; ILC, Innate lymphoid cells; LN, Lymph node; MLN, Mesenteric lymph node; NF-κB, Nuclear factor kappa-Beta; PsA, Psoriatic arthritis; NSAIDs, Non-steroidal anti-inflammatory drugs; PLN, Popliteal lymph node; PRR, Pattern recognition receptors; PsO, Psoriasis; TACE, TNF-α converting enzyme; TLR, Toll like receptors; TNF-α, Tumour necrosis factor alpha; TNFR1, Tumor necrosis factor receptor 1; TTP, Tristetraprolin; RANKL, Receptor activator of nuclear factor κB ligand; STAT3, Signal transducer and activator of transcription 3; SpA, Spondyloarthritidis; SPF, Specific pathogen free; UPR, Unfolded protein response.

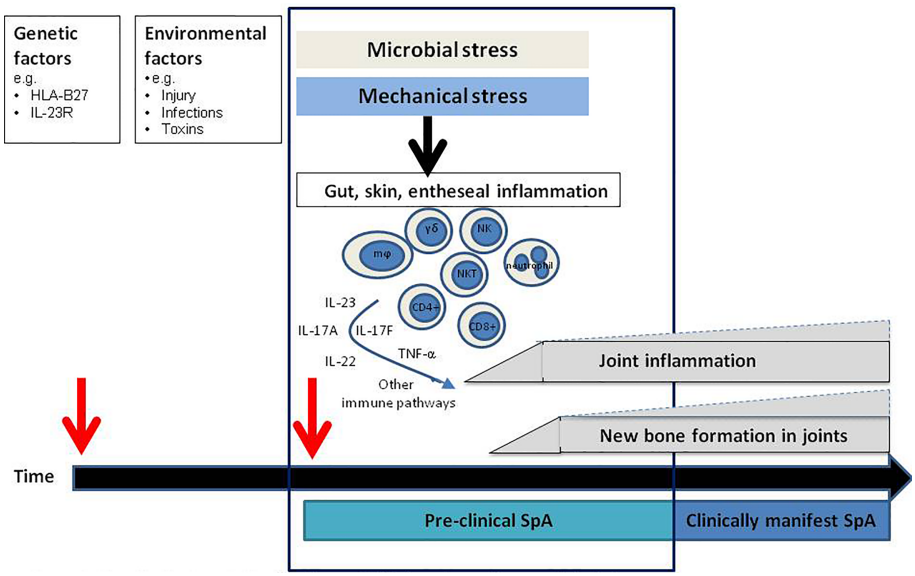

FIGURE 1 | Hypothetical model for the disease initiation phase: the pre-clinical phase. In the pre-clinical phase of disease innate immune triggering results in activation of specific pathogenic immune pathways site distant from the joints resulting in (subclinical) joint inflammation and new bone formation which subsequently develops into clinically manifest spondyloarthritis (SpA). mϕ, macrophages, NK, NK cells; NKT, NKT cells; γδ, gamma delta T cells.

examine these target tissues, however, this is challenging due to the relative lack of tissue samples that can easily be obtained from affected lesions. This is even more challenging in individuals who are at increased risk of SpA, before the onset of clinically manifest disease. Animal data could provide a good basis for future human studies allowing better understanding of the disease development, and on how extra-musculoskeletal tissues are involved in initiation of this disease. Increased understanding of the molecular pathways active in this very early stage of disease in SpA might identify novel potential treatment targets or even provide a basis for preventive treatment strategies aiming to further improve outcome for SpA patients.

Here, we provide an overview of findings from various experimental SpA animal models indicating how the IL-23/IL-17 axis is implicated in the initiation and progression of disease, with a focus on the major tissues involved in SpA pathogenesis.

EXPERIMENTAL MODELS OF SPA

Evidence from studies in animal models, human expression studies, and SpA genetic association studies, has indicated that the IL-23/IL-17-axis contributes to pathogenesis of chronic arthritis, spondylitis, and associated inflammatory manifestations (34).

There are several animal models that bear resemblance to human SpA (**Figure 2**) (35–37). These models differ in genetic modifications, disease mechanism and pathologic features. Collectively, they contribute to dissecting the pathogenic processes in SpA disease development and progression. Herein, we first briefly summarize the most commonly used experimental models of SpA. An thorough overview of these SpA animal models has been previously presented by Viera de Sousa et al. (35). There are more recent animal models developed since, which we are highlighting here as well (37).

HLA-B27/Huβ2m Overexpression

HLA-B27 is the major genetic risk factor for SpA (38, 39), and overexpression of HLA-B27 resulted in clear SpA-like features in rats (38), but not in mice (40). Over time, the HLA-B27 transgenic (tg) rat model has progressed from the HLA-B27/human β2 microglobulin (hβ2m) (tg) Lewis (21-4H) rats, characterized by orchitis, colitis and hind limbs arthritis, with psoriasis in up to 50% of the rats, to the HLA-B27/hβ2m-tg F344 (33-3) rats with similar clinical manifestations but with earlier disease onset. This was followed by the HLA-B27/hβ2m-tg Lewis rats (21-3 x 283-2)F1 line with lower HLA-B27 copy numbers, in which all male rats develop orchitis, followed by the development of arthritis (4–6 months age) in 70% of all male rats, and spondylitis (7–9 months age) in 50% of them (41). These rats also show signs of peripheral and axial new bone formation. Most recently, immunizing the (21-3 x 283-2)F1 rats with heat-inactivated *Mycobacterium tuberculosis* (*M.tb*) has been demonstrated to synchronize and accelerate the disease onset in both male and female rats. Arthritis and spondylitis development is visible in 80-100% of the rats 2-3 weeks post *M.tb* immunization (35).

TNF-α Overexpression

TNF-α is one of the key cytokines in SpA pathogenesis and there are several animal models that are based on TNF-α overexpression.

HLA-B27/Huβ2m overexpression

	arthritis	spondylitis	enthesitis	colitis	psoriasis	uveitis	new bone formation	bone loss	other
HLA-B27/Huβ2m- tg Lewis rats (21-4H)	x	x	x	x	x	x	x	x	dactylitis, epididymoorchitis
HLA-B27/Huβ2m- tg F344 rats (33-3)	x		x	x	x		x	x	dactylitis, epididymoorchitis
HLA-B27 Huβ2m- tg Lewis rats (21-3x283-2)F1	x	x			x		x	x	dactylitis, epididymoorchitis
Heat inactivated *M. tb* induced HLA-B27 Huβ2m tg Lewis rats (21-3x283-2) F1	x	x					x	x	wactylitis, epididymoorchitis

TNF-α overexpression

	arthritis	spondylitis	enthesitis	colitis	psoriasis	uveitis	new bone formation	bone loss	other
TNF $^{\Delta ARE}$ mice	x		x	x				x	
Transmembrane TNF tg mice (TgA86)	x	x	x				x	x	
Human TNF tg mice (Tg197)	x							x	weight loss

IL-23 /IL-17 related

	arthritis	spondylitis	enthesitis	colitis	psoriasis	uveitis	new bone formation	bone loss	other
IL-23mc induced B10.RIII mice	x x	x	x	x	x		x	x	
R26STAT3C$^{Stop(F)/(F)}$ CD4Cre mice	x		x		x			x	
K5.Stat3C:F759 mice	x				x				
Curdlan- induced SKG mice	x	x		x			x	x	weight loos, uveitis, dactylitis

Other models

	arthritis	spondylitis	enthesitis	colitis	psoriasis	uveitis	new bone formation	bone loss	other
Aging male DBA/1 mice	x		x		x		x		
IL27RA$^{-/-}$ p53$^{R172H/+}$ mice		x					x	x	
Tristetraprolin (TTP) KO mice	x		x		x				cachexia

FIGURE 2 | Animal models resembling human spondyloarthritis. HLA-B27/Huβ2m- tg, HLA-B27/human β2 microglobulin transgenic rat; TNF-α, Tumor necrosis factor alpha; IL-23, Interleukin-23; IL-17, Interleukin-17; M. tub, Mycobacterium tuberculosis; IL-23mc, Interleukin-23 minicircle; Human TNF tg mice, Human tumor necrosis factor transgenic mice.

In the TNF$^{\Delta ARE}$ mice, the overexpression of murine TNF-α leads to chronic peripheral polyarticular synovitis, enthesitis and colitis, without psoriasis, osteoproliferation or spinal involvement (42–44). The colitis severity varies from asymptomatic histological inflammation to severe ileitis depending on housing conditions. In contrast, mice that overexpress transmembrane TNF (tmTNF) (TgA86) (45) do develop new bone formation together with signs of peripheral arthritis and spondylitis, without gut or skin involvement (35). The human TNF-(Tg197) tg mice is a model characterized by destructive polyarticular synovitis (including sacroiliitis), with no spinal involvement, new bone formation, gut or skin inflammation (46). The differences in clinical presentation between the soluble and tmTNF overexpression models indicate that tmTNF drives the key clinical phenotype and pathologic bone formation of SpA.

IL-23/IL-17 RELATED ANIMAL MODELS OF SPA

Animal models played an important role in implicating the IL-23/IL-17 axis in SpA pathogenesis. In the IL-23 minicircle (mc) induced B10.RIII mice model, high systemic IL-23 expression results in axial and peripheral enthesitis with entheseal new bone formation, with no destructive synovitis at day 35 (47). Another model that is considered IL-23/IL-17 dependent is the SKG model with the ZAP-70^{W163C} mutation. This ZAP-70^{W163C} mutation is downstream of the T cell receptor signaling complex. This mutation affects positive and negative selection of T cells. Moreover, it alters the generation and function of CD25+CD4+ natural regulatory T (Treg) cells (48). SKG mice develop arthritis, enthesitis, spondylitis, peripheral new bone formation and Crohn's disease-like ileitis, but without clear signs of axial new bone formation. The disease onset can be synchronized by one IP injection of the fungal wall component curdlan. In this model, curdlan activates IL-23 release, inducing mucosal dysregulation and IL-17 and IL-22 cytokine expression, driving the SpA phenotype (49, 50).

Recently, IL-27RA$^{-/-}$ p53$^{R172H/+}$ mice have been demonstrated to show SpA-like disease (37). As a result of knocking out IL-27RA, these mice lack the inhibitory effect of IL-27 on Th17 lineage differentiation. IL-27 is a member of the IL-12 cytokine family and is known to down regulate IL-23, RAR-related orphan receptor (ROR)-γt, and Th17 differentiation (12, 47, 51), while p53 is known to negatively regulate osteoblast differentiation, bone formation and

remodeling pathway (52). The IL-27RA$^{-/-}$p53$^{R172H/+}$ mice demonstrate minimal axial inflammation and bone loss, with pathological bone formation at the intervertebral discs (37). Moreover, these mice also show skin inflammation (neutrophilic dermatitis) and additional organ pathology including chronic kidney nephropathy (37, 53).

Other models reflecting the importance of IL-17A production in SpA disease pathogenesis are the STAT3 overexpression models. STAT3 is a key regulatory factor inducing differentiation of naïve CD4$^+$ T cells into Th17 cells (54). The group of Yang Lu introduced the R26STAT3C$^{stopfl/fl}$ CD4Cre mouse model reflecting SpA with signs of psoriasis, driven by a specific hyperactive T cell-STAT3C allele (55, 56). The disease manifestations include spontaneous development of psoriatic skin lesions, enthesitis/tendinitis, and arthritis (57). Peri-articular bone erosion and osteopenia were also observed, without signs of new bone formation.

Similarly, overexpression of STAT3 in the K5.STAT3C:F759 murine model also results in spontaneous severe psoriatic cutaneous lesions and peripheral erosive arthritis (36).

OTHER MODELS

The aging male DBA/1 mice spontaneously develop brief arthritis, dactylitis, and enthesitis. In 10-12 weeks old mice progressive endochondral bone formation has been shown but still without axial or extra-articular pathologic changes (58, 59).

More recently, the Tristetraprolin (TTP) KO model has been shown to develop a severe, systemic inflammatory syndrome, with destructive arthritis, conjunctivitis, dermatitis, osteopenia, myeloid hyperplasia and cachexia (60). TTP is a RNA binding protein that has important endogenous anti-inflammatory effects, through destabilizing mRNAs encoding pro-inflammatory cytokines and suppressing their biosynthesis (e.g. TNF-α). The clinical and immunological phenotype associated with TTP deficiency was dependent on the IL-23/IL-17A axis (61). Most of the expressed phenotype was prevented using anti- (TNF-α) antibody treatment (60).

INVOLVEMENT OF IL-23/IL-17 AXIS IN THE INITIATION PHASE OF DISEASE

Although the major clinical presentation of SpA involves the joints (axial or peripheral), innate immune activation by either microbial stress caused by barrier dysfunction at the gut or the skin, or by mechanical stress caused by microtrauma at the entheses, is thought to trigger disease onset (27). The exact underlying mechanisms are not yet fully understood (**Figure 2**). To gain more insight into the disease initiation phase, of SpA, when first pathological molecular alterations occur, we assessed the result of IL-23 and IL-17 dysregulation in SpA experimental models. We highlight studies in which the disease initiation in SpA may be explained by IL-23/IL-17 activation systemically or locally in the entheseal areas close to the joints. Moreover, we will discuss other potential initiation sites of SpA by summarizing how gut and skin inflammation may relate to development of joint inflammation.

Sherlock et al. have shown that induction of high systemic IL-23 levels can induce enthesitis before development of new bone formation and synovial joint destruction. After induction of disease with IL-23mc, mice develop paw swelling at day 5-10 with entheseal inflammation (47). Although F4/80+ macrophages and myeloperoxidase (MPO)+ neutrophils were observed in the entheseal inflammatory infiltrate, IL-23 responsive RORγt+CD3+CD4-CD8- entheseal resident T cells, later identified as γδ T cells (62) were shown to be important for initiating inflammation, as this specific subset of T cells were shown to produce IL-17A and IL-17F, and IL-22 (47) and inhibition of IL-17 and/or IL-22 did decrease disease severity. Depletion of Th17 cells was shown not to change the disease course, indicating that indeed the resident entheseal T cells play an important role in driving the disease onset. In this model, the entheseal inflammation was followed by expansion of periosteal osteoblasts by day 18, accompanied by a molecular new bone formation gene expression profile. Entheseal new bone formation was observed at day 35 (47).

In the SKG model, curdlan (consisting of the fungal wall component beta-glucan) signaling *via* the dectin 1 pattern-recognition receptor, that specifically recognizes beta-glucans, was applied as a trigger of a SpA phenotype (49, 63). Curdlan injection induces ileal IL-23 secretion, which in turn provokes a state of mucosal dysregulation and cytokine imbalance. The mice develop (histological) inflammation at the axial and peripheral entheses 1 week after curdlan induction followed by development of clinical enthesitis, sacroiliitis, peripheral arthritis and colitis and uveitis with increased serum levels of IFN-γ, TNF-α, IL-6 and IL-17A. The surge of IFN-γ, IL-6 and IL-17A cytokines was shown to be IL-23 dependent (50, 64). The disease manifestations were shown to be IL-23 dependent and partially IL-17 dependent (64). Early IL-23 inhibition using anti-IL-23 mAb before disease induction in SKG mice resulted in a clear clinical improvement in these mice. There were no histological signs of arthritis and spondylitis (50, 64).

The importance of IL-23 in disease initiation was also found in the accelerated heat-inactivated *M.tb* induced HLA-B27 tg rat model. It was shown that there is a short increase in *IL-23R* expression and *RORC* gene expression shortly after immunization (24). Blocking IL-23R after immunization but before onset of clinical manifestations completely prevented arthritis and spondylitis development. This prophylactic treatment significantly suppressed the lymph nodes and splenic mRNA expression of various downstream cytokines, e.g. *Il-17A, Il-22, Mmp*3 and *Ccl20*. However, expression of other pro-inflammatory cytokines such as *Il-17F, Ifn-γ, Tnf-α*, and *Il-6* were not affected by IL-23R blocking. Although IL-23 was clearly implicated in disease initiation, the source and the cell type(s) responsive to IL-23 in this model need to be elucidated (24).

In the same accelerated HLA-B27tg rat model, prophylactic anti-IL-17A treatment significantly delayed the development and decreased the severity of spondylitis and arthritis. On histology there was less inflammation and less destruction in axial and peripheral joints in anti–IL-17A treated rats, but the disease

onset was not prevented (65). This is in contrast to the findings with anti-IL-23R blocking therapy, as anti-IL23R did prevent the disease onset.

Strikingly, in the B10.RII mice, the use of IL-17Amc DNA over-expression, as well as IL-17A blockade revealed no major role for IL-17A in driving arthritis in the B10.RIII model (47). Consistent with that, IL-17A deficiency in the IL-17A$^{-/-}$ SKG mice only resulted in a moderate reduction of the SKG phenotype (64). Altogether, the data from these SpA models indicate that IL-17A is likely not the only cytokine that contributes to the initiation of IL-23 dependent arthritis.

In addition to IL-17A, IL-22, as a downstream cytokine of IL-23, was shown to be relevant for the severity of enthesitis in both the IL-23mc and SKG mice model (47, 64, 66). *Ex vivo* gene expression profiling revealed the induction of murine *Il-6*, *Il-22* and *Cxcl1* by day 5 of IL-23 expression in the IL23mc model (47). Blocking IL-22 in SKG mice for 8 weeks from the first moment of clinical manifestations reduced Achilles tendon enthesitis, similar to the reduced severity of enthesitis observed in the IL-17A$^{-/-}$ SKG mice (64).

The aforementioned animal studies link IL-23 responsive cells to initiation of disease and development of clinical SpA manifestations. In humans innate immune cells have been reported to express IL-23R with inducible IL-17A/F expression (67). Recently also presence of conventional CD4+ and CD8+ T cells have been reported in human entheses, which upon stimulation could produce TNF-α and IL-17A. However, IL-17 production here is independent of IL-23 (67–69). If these cells could play a role in the onset of SpA in humans needs further investigation. This link between IL-23 responsive cells and initiation of disease in animal models direct further in depth characterization of both innate and adaptive immune cells from the moment of triggering of inflammation (possibly in the pre-clinical disease stage) to onset of clinically manifest spondyloarthritis to further elucidate which IL-23 responsive cells drive pro-inflammatory cytokine production, including IL-17A, and which cells drive IL-17 production independently of IL-23.

Altogether, IL-23 plays an important role in inducing SpA pathology as revealed in the different models. If immune dysregulation of IL-23 is also present in the pre-clincal phase of human SpA needs further investigation. If in the future we can identify individuals at very high risk of developing SpA, who would qualify for preventive treatment strategies, then anti-IL23 treatment could be suggested to be the first candidate to be tested as a preventive medicine. However, currently we lack good predictive tools that could identify those individuals at increased risk of developing SpA who would qualify for such a treatment strategy.

THE GUT-JOINT AXIS IN THE INITIATION OF SPONDYLOARTHRITIS

SpA patients often present with gut inflammation which led to the hypothesis that inflammation at the gut mucosa may initiate SpA pathogenesis (70, 71). Theoretically, gut inflammation could result in loss of intestinal barrier integrity allowing microbes and dietary antigens to enter into the bloodstream, and trigger immune dysregulation leading to joint inflammation (72–74). An in-depth review discussing the gut-joint axis in SpA was recently provided by Gracey et al. (71). Here we will discuss current literature on the potential crucial role of the IL-23/IL-17 axis in the transition of inflammation from the gut to the joint.

IL-17 has been considered to play a dual role in gut homeostasis. Whereas IL-23-independent IL-17A production by innate(-like) immune cells is considered to be protective in colitis by maintaining barrier function in the intestines (75, 76), IL-23-dependent IL-17A production by Th17 cells results in gut inflammation (76, 77). This might explain the opposing effects observed in clinical trial in active crohn's disease where IL-17A inhibition resulted in worsening of colitis, but treatment with anti-IL-12/IL-23p40 as well as p19 inhibition (78) improved inflammation (79). In addition to IL-17A, IL-17F has been demonstrated to promote inflammation in the intestines through its effect on the intestinal microbiome in mice (80). A study in experimental colitis provided evidence that inhibition of both IL-17A and IL-17F was necessary to attenuate colitis (81). IL-22 is another key player in intestinal host defense and maintaining mucosal homeostasis, which adds further complexity to role of the IL-17/IL-23 axis in gut inflammation. It induces direct intestinal expression of complement C3 and mucin genes, shares in the clearance of pathogens (82–84), *via* facilitating the production of cytokines and chemokines that mediate innate cell recruitment to infection sites (84). Furthermore, IL-22 has been shown to regulate hemopexin production, in order to impair bacterial growth (85).

Gut inflammation is a prominent feature in the high copy numbers HLA-B27 tg rat models (21-4H and 33-3 line) (38). Colitis is seen at 6 weeks age and increased *Il-23p19* expression occurred at the start of colitis associated with increased *Il-17A*, next to *Il-1, Il-6*, and *Tnf-α* expression. IL-23p19 and IL-17A transcripts were localized to CD11c+ antigen presenting cells and CD4+ T cells, respectively (86). This was accompanied by increased HLA-B27 expression and signs of HLA-B27 misfolding with an unfolded protein response (86). Mechanistically, it is thought that the increased HLA-B27 expression in activated macrophages results in an unfolded protein response with ER stress promoting IL-23 expression (86–88). This IL-23 could then induce pathogenic IL-17A production by Th17 cells. Ciccia et al. showed human evidence for HLA-B27's role in gut inflammation. They have observed that HLA-B27 misfolding occurs in the gut of AS patients and is associated with activation of autophagy. Autophagy appears to induce intestinal expression of IL-23 in the human gut (89).

Furthermore, Utriainen et al. (90) reported a significantly decreased subset of intestinal dendritic cells (DCs), which are involved in maintaining self-tolerance, in the mesenteric lymph nodes (MLNs) and gut draining lymph of HLA-B27-tg (33-3) rats. The deficiency of these tolerogenic DCs in combination with the improved ability of bone marrow-derived DCs to stimulate IL-17 production by CD4+ T cells could induce an immune mediated inflammatory response involving the IL-23/IL-17 axis. Possibly, the dysbalance in proinflammatory and

toloregenic DCs as well as the HLA-B27 misfolding as observed in these experimental animal models (86, 91, 92), might also be relevant for induction of disease in HLA-B27+ individuals.

In HLA-B27 tg (33-3) rats it was observed that HLA-B27 homodimer expression on various lymphocyte populations increases between week 6-23 of age, and is accompanied by colitis development and expansion of IL17+ CD4+ T cells and TNF+ CD4+ T cells. Of interest, presence of HLA-B27 homodimer expression on monocytes in gut draining MLN, but not on splenocytes, co-occurred with the expansion of Th17 cells, when colitis was first observed. Besides HLA-B27 misfolding, HLA-B27 homodimerization is proposed as another mechanism by which HLA-B27 can contribute to IL-17 production, which has been reviewed previously (93–95). HLA-B27 is normally present on the cellular surface with β_2m as a heterodimer, however when presented as a homodimer, this homodimer can interact with killer-cell immunoglobulin-like receptors (KIRs) on the surface of NK cells and T cells. This could result in direct activation of NK cells and T cells and aberrant cytokine production, contributing to an enhanced activation of the IL-23/IL-17 pathway (96).

The evidence for the role of the IL-23/IL-17 axis in colitis preceding joint inflammation was further provided by Glatigny (91). In the HLA-B27 tg (33-3 line) rats Glatigny et al. observed increased IL-17+TNF+ T cells in MLN which coincided with colitis, and later this was followed by the occurrence of the same cell types in PLN at the same time as the onset of arthritis. The increase in IL-17A+TNF+ T cells was paralleled by elevated mRNA expression levels of several genes indicating a Th17 phenotype (i.e. *Il-21, Il-22*, and *Rorc*) and increased serum levels of IL-17A (91).

These studies add to the concept that immune dysregulation in the gut precedes the onset of other clinical manifestations of SpA, possibly mediated by dysbiosis and/or colitis. They suggest that IL-23 produced in the gut could influence local activation of lymphoid cells in the entheses (47, 64). Various innate immune lymphoid cells able to produce these cytokines have been implicated in human SpA pathogenesis (reviewed in Gracey et al.) (71). If the IL-17+ T cells present in the gut mucosa are indeed pathogenic and if they play a role in the migration of immune cells from the gut to the joints needs further investigation. That lymphocytes can possibly egress from gut to joint prior to disease onset was suggested in a study in $TNF^{\Delta ARE}$ mice, reported as a conference abstract, where lymphocytes were shown to traffic from the colon to the joints (97). Ciccia et al. provided data, supporting active gut-joint trafficking in human axSpA. They showed an expanded gut-derived IL-17+ and IL-22+ ILC3 population expressing $\alpha4\beta7$ in the peripheral blood, synovial fluid and inflamed bone marrow of patients (98).

Dysbiosis is an alteration in the composition of complex commensal or microbiota that might be induced by a wide range of environmental factors (99). Related to colitis, dysbiosis is suggested as an important factor that drives pathologic immune pathways. In HLA-B27 tg rats (33-3) it was shown that gut metabolomic changes are already present before the onset of colitis suggesting that these alterations might be an initiating event, however if these changes affect the IL-23/IL-17 pathway was not investigated (100). Gill et al. investigated microbial dysbiosis in different HLA-B27 rat strains and found no common overlapping microbial pattern but showed that the genetic background determined the dysbiotic microbial pattern present in each model. Nevertheless, microbial dysbiosis (regardless of the pattern) provoked similar immune response with an obvious increase in IL-23 and IL-17, as well as IL-1, IFN-γ, and TNF-α cytokines coinciding with colitis as early as 2 months of age in the various strains (101).

Gut microbes triggering the immune system were also observed in the SKG mouse models of SpA. Observations in the $ZAP\text{-}70^{W163C}$ mutant SKG mice and TLR-4-deficient SKG mice have confirmed the role of gut microbiota and therelated colitis in SpA. In contrast to the germ free SKG mice, that are free of all microorganisms including those that are typically found in the gut, the curdlan treated specific pathogen fee (SPF) SKG mice show increased constitutive *Il-23* expression specifically in ileal tissue, associated with ER stress marker expression and MLNs cytokine production including *Il-6, Tnf-α, Ifn-γ* and *Il-17A (*64). Involvement of gut microbes was reported by Rehaume et al. who showed that (intraperitoneal) curdlan administration induced acute systemic inflammation with IL-6, TNF, MCP-1 production and neutrophil recruitment disregarding the presence of the SKG allele or microbiota, with the response diminishing earlier in the germ-free animals. Neutrophil IL-17A production in the peritoneal cavity was shown to be microbiota dependent, as was ER-stress induced increased ileal IL-23 expression, MLN IL-17A expression, goblet cell loss, and ileitis development. In contrast, the development of associated arthritis and spondylitis was not crucially dependent on the microbiota profile, but incidence of arthritis and its severity clearly differed with various microbial profiles (102). These studies provide a link of microbial innate immune triggering in gut to development of SpA features. These microbiome alterations may be further induced by the tissue inflammation in the gut, making it difficult to determine what the initiating factor was: the dysbiosis or the tissue inflammation? (103). In human SpA, dysbiosis has been seen both in patients (104) as well as in healthy HLA-B27+ individuals (105), and it remains speculative if dysbiosis lowers the threshold for gut inflammation, and the onset of SpA.

SKIN

Psoriasis is an extra-musculoskeletal manifestation often seen in SpA patients (4), in particular in a subtype of SpA, psoriatic arthritis (PsA). The proinflammatory role of IL-17A in the development of articular and cutaneous manifestations of PsA has been consistently established in animal models as well as in human PsA. Here, we highlight the animal models that describe the concomitant presence of psoriasis and musculoskeletal manifestations.

In wild type C57BL/6J mice, as early as 1 day after IL-17A mc gene transfer, clinical psoriatic changes were observed, accompanied by an inflammatory infiltrate of neutrophils. Moreover, gene expression of murine keratin 16 (*K16*), a marker of keratinocyte hyper-proliferation was highly expressed. It remains to be assessed which cells are responsible for both the skin inflammation as well as the joint and bone pathology (106).

Recently, additional novel animal models demonstrating the cutaneous, articular, and associated bone changes of psoriatic arthritis were developed. Yang et al. showed in the R26STAT3C$^{stopfl/fl}$ CD4Cre mice SpA like disease, which is driven by an amplified Th17 response downstream of T cell-specific expression of a hyperactive STAT3C allele (55, 56). Animals developed psoriasis-like skin lesions after 5 weeks with increased infiltration of single IL-17+, IL-22+ or combined IL-17/IL-22+ CD4+ T cells. The associated Achilles tendon enthesitis showed increased cell infiltration with both IL-23R and RORγt double positive CD4+ T cells. These findings imply that Th17 cells play an important role in the development of skin and entheseal inflammation (56).

The importance of STAT3 in psoriatic skin inflammation was also shown when specifically activated in keratinocytes in the K5.STAT3C:F759 mice model. Yamamoto et al. demonstrated that early occurrence of arthritis in K5.STAT3C:F759 mice was due to coincident skin inflammation induced by a hyper STAT3 activation. Mice showed increased serum IL-6 and IL-17A levels. IL-17A expression was elevated compared with F759 mice in inflamed joints, which contain increased numbers of CCR6+ CD4+ T cells compared to the LN compartment (36).

Although these 3 models clearly show involvement of the IL-23/IL-17 axis in development of psoriasis and arthritis, development of arthritis was not preceded by psoriasis but occurred simultaneously.

Skin inflammation preceding joint inflammation was addressed in F759 (not harboring the K5.STAT3C transgene) mice. These mice are known to spontaneously develop arthritis after 12-18 month. It was also shown that forced induction of psoriasis-like lesions by imiquimod application in these mice significantly accelerated arthritis development, suggesting that psoriatic inflammation facilitated arthritis development, possibly by activating the IL-17 pathway, as IL-17+ γδ T cells as well as Th17 cells were present in the inflamed skin. However, other options should not be ruled out as imiquimod can also have important systemic effects (36). Recently a study reported (currently only available in abstract) that psoriasis-like skin inflammation in C57/Bl6 male mice induced mild synovial joint inflammation. Similar results were obtained after dextran sodium sulphate (DSS) colitis induction in these mice (107).

These preliminary data indicate that the skin is a candidate location for triggering joint pathology. Besides follow-up studies in animal models resembling PsA, future longitudinal studies in patients with psoriasis at increased risk of development of PsA, are expected to provide further insight into the mechanisms triggering joint disease in PsA. Future studies could benefit from applying state of the art molecular imaging in combination with (serial) tissue biopsy analysis to repetitively assess tissue inflammation.

CONCLUSION AND FUTURE PERSPECTIVES

Literature on the animal models resembling human SpA indicates an overlapping and distinctive role of IL-17A and IL-23 in the initiation of disease. There is initial evidence for inflammation at gut and skin to precede joint inflammation in animal models of SpA with involvement of the IL-23/IL-17 axis. Importantly, in accordance with the insights from the *M.tb* HLA-B27 study (24, 27), IL-23 plays an important role in the onset of SpA-like pathology in many animal models, even before clinical joint manifestations are present, which is in clear contrast with the absence of any significant clinical effect as observed when blocking IL23R in diseased rats (24) or with blocking of IL-23p19 in AS patients (25). The data in animal models may help us to understand which pathways are dysregulated early in the disease process, in the pre-clinical phase, and if these dysregulations differ from observations in established disease.

While animal models provide an ideal basis for in-depth molecular analysis of target tissues in SpA to elucidate causal relationships between the upregulation of specific immune pathways and development of key SpA features, complimentary studies performing serial advanced molecular imaging or bio-sample collection in individuals with an increased risk of developing SpA or PsA, or in patients who have been recently diagnosed withSpA, are essentially required if we want to translate findings in animal models of SpA to human disease. The goal of studying the preclinical stage of the disease would be to find better diagnostic markers to allow an earlier diagnosis and open up opportunities for preventive treatment strategies (108–110). If IL-23 dysregulation in the pre-clinical phase of disease, as observed in various animal models, can be confirmed in human pre-clinical SpA, it could be speculated that IL-23 targeting may be effective in preventing disease, in contrast to the lack of effect observed with IL-23 blocking in established SpA. There is more recent evidence that Th17 cells in the synovial tissue are polyfunctional and produce multiple proinflammatory cytokines that synergize with IL-17A. Treatments targeting Th17 cells, or affecting multiple cytokines that synergize with IL-17A in pathogenic immune responses could also be considered for preventive trials, Whether these pathways are upregulated early in the disease pathogenesis remains to be elucidated. As an example, potential novel treatments such as TYK2 inhibition (108), mTOR inhibition by rapamycin (110), and PI3Kδ inhibition (109) have been shown *in vitro* and/or *in vivo* animal models to affect multiple cytokines relevant for SpA pathogenesis. Lastly, it remains to be studied if preclinical treatment in patients could prevent or affect pathologic bone formation, as this remains an unmet treatment need in SpA patients.

The IL-23/IL-17 axis is clearly implicated in disease initiation in SpA. However, which specific innate (-like) or adaptive immune cells are responsible for the pathogenic IL-17A production in the various target tissues, is still incompletely understood. Moreover, the effects of the plasticity of the Th17/Treg cells in initiating inflammation as well as the pathogenic role of pathways that enable IL-23 independent IL-17A production are still incompletely understood and prompt further research both in human SpA as in animal models resembling SpA.

To conclude, animal models have improved our understanding of the IL-23/IL-17 axis in SpA and continue to allow us to gain novel insights on SpA pathogenesis. Greater understanding of disease initiation in SpA animal models could

support human preclinical SpA studies, This may provide a basis for better future preventive approaches for our patients.

AUTHOR CONTRIBUTIONS

MM, SC, and MS: substantial contributions to conception and design, MM, SC and MS: drafted the article, over viewed it critically for important intellectual content and given final approval of the final version. All authors contributed to the article and approved the submitted version.

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REVIEW
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The IL-17/IL-23 Axis and Its Genetic Contribution to Psoriatic Arthritis

Matteo Vecellio[1,2*], *Vivien Xanath Hake*[2], *Connor Davidson*[1], *Maria Cristina Carena*[3], *B. Paul Wordsworth*[1†] *and Carlo Selmi*[2†]

[1] Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom, [2] Division of Rheumatology and Clinical Immunology, Humanitas Clinical and Research Center, IRCCS, Milan, Italy, [3] Leukocyte Biology Unit, Humanitas Clinical and Research Center, IRCCS, Milan, Italy

Psoriatic arthritis (PsA) is a chronic inflammatory disease belonging to the family of spondyloarthropathies (SpA). PsA commonly aggravates psoriasis of the skin and frequently manifests as an oligoarthritis with axial skeletal involvement and extraarticular manifestations including dactylitis, enthesitis, and uveitis. The weight of genetic predisposition to psoriasis and PsA is illustrated by the concordance rates in monozygotic twins which clearly demonstrate that genomics is insufficient to induce the clinical phenotype. The association of PsA with several single nucleotide polymorphisms (SNPs) at the IL23R locus and the involvement of Th17 cells in the immunopathogenesis of PsA clearly put the IL-23/IL-17 axis in the spotlight. The IL-23 and IL-17 cytokines have a pivotal role in the chronic inflammation of the synovium in PsA and are also prominent in the skin lesions of those with PsA. In this review, we focus on the genetic association of the IL-23/IL-17 axis with PsA and the contribution of these master cytokines in the pathophysiology of the disease, highlighting the main cell types incriminated in PsA and their specific role in the peripheral blood, lesional skin and joints of patients. We then provide an overview of the approved biologic drugs targeting the IL-23/IL-17 axis and discuss the advantages of genetic stratification to enhance personalized therapies in PsA.

Keywords: genetics, psoriatic arthritis, IL17, IL23, SNPs (single nucleotide polymorphisms), therapy

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Toshiyuki Yamamoto, Fukushima Medical University, Japan

****Correspondence:***
Matteo Vecellio
matteo.vecellio@ndorms.ox.ac.uk

[†]These authors have contributed equally to this work

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INTRODUCTION

Psoriatic arthritis (PsA) is a common inflammatory disease affecting the joints and it is usually accompanied by plaque psoriasis (Ps) (1). PsA occurs in up to 30% of patients with psoriasis (particularly those with nail involvement) and affects from 0,05 to 0,25% of the general population (2), making it the second most common form of chronic inflammatory arthritis after rheumatoid disease. To address the therapeutic choices and to envision the potential musculoskeletal and dermatological phenotypes, the GRAPPA (Group for Research and Assessment of Psoriasis and Psoriatic Arthritis) group identified six disease domains, i.e. peripheral arthritis, enthesitis, dactylitis, axial involvement, skin and nail psoriasis. Among these, peripheral arthritis and enthesitis are dominant and found in the vast majority of patients while the prevalence of axial PsA increases with disease duration, occurring in less than 5% of early referrals and up to 25–70% of patients with long-term disease course for PsA (2). PsA is in some cases characterized by axial

skeletal involvement, along with the more frequent oligoarthritis with mostly peripheral and asymmetric manifestations (3).

The pathogenetic link between psoriasis and PsA is highly representative of the mechanistic hypotheses of disease pathogenesis. Psoriatic skin is featured by hyperplasia of the epidermis and of the stratum corneum, infiltration of the epidermis by neutrophilic granulocytes (called Munro's micro abscesses) and infiltration of the dermis by T-cells, dendritic cells (DCs), and macrophages, which leads to the clinical features of raised erythematous silvery plaques (4). In a similar fashion, PsA is characterized by chronic inflammation which causes bone erosion and bone loss, but also new bone formation around the affected joints (5).

The increased number of osteoclasts found in the synovium in PsA is remarkably similar to rheumatoid arthritis (RA) and the persistent inflammatory synovitis causes progressive joint damage due to synovitis and erosion of articular cartilage, visible as radiological damage in almost half of the patients (6). The exaggerated inflammatory response lead to enthesitis, with the crucial contribution of IL-17 producing T-cells and enthesal resident cells, expressing IL-23R (7, 8) with biomechanical stress, HLA-B27, and a permissive microbiome as necessary factors (9).

The phenotypic features of PsA suggest that some of the genetics and molecular mechanisms of the disorder are shared across various different types of inflammatory diseases, including psoriasis, ankylosing spondylitis (AS), inflammatory bowel disease, and Behçet disease (10–12). Extra-articular manifestations of PsA include inflammation of the gastrointestinal tract (with a higher risk of inflammatory bowel disease) and the eye (uveitis), along numerous metabolic and neoplastic disturbances (13).

In this review we will address the contribution of genetics to susceptibility to PsA and its subsequent progression, with special emphasis on the IL-23/IL-17 axis and the genes and cells that this involves. We are well aware that genetics represents only a necessary but insufficient player in the disease etiology, as recapitulated by the low concordance rates in monozygotic twins for PsA. Of note, however, the same rates are for psoriasis among the highest in chronic inflammatory or autoimmune diseases.

PSA DIAGNOSIS

The diagnosis of PsA is largely based on features and the CASPAR criteria (14) are used in the research setting for more formal; classification purposes. In contrast to rheumatoid arthritis there are no specific markers or autoantibodies for the diagnosis of PsA: it is seronegative for both rheumatoid factor and antibodies to cyclic citrullinated peptides (CCP) unlike rheumatoid arthritis where these are positive in approximately 80% of cases. Overall PsA is equally distributed between males and females but axial manifestations (spondylitis) are about three times more common in males (15). PsA patients experience substantial functional impairment, decreased quality of life and reduced life expectancy, which highlights the importance of prompt implementation of appropriate treatment. Genetics and molecular studies have led to a better understanding of the etiology of the disease and in future may allow the development of more targeted individual approaches to therapy.

Laboratory tests typically but not invariably show increased raised inflammatory markers, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), whereas rheumatoid factor (RF) and CCP antibodies are negative, in contrast to rheumatoid arthritis (16). Recent studies have also shown a possible association with anti-LL37 (cathelicidin antimicrobial peptide) autoantibodies (17).

There is an association with the Human Leukocyte Antigen (HLA)-B27 that is most prevalent in those with sacroiliitis and axial skeletal involvement. Of particular interest, about 85% of PsA patients with bilateral sacroiliitis are positive for HLA-B27 in contrast to only 22% of cases with unilateral sacroiliitis (18, 19). Conversely, HLA-B17/Cw6 haplotype (strongly associated with psoriasis itself) is mostly associated with oligoarthritis (20).

HLA-B27 is the key genetic marker of ankylosing spondylitis (AS), commonly shared with axial-PsA (21). Although the prevalence of HLA-B27 is lower in patients with PsA (20%), it has been demonstrated that axial-PsA patients are significantly HLA-B27 positive compared to patients without axial involvement ($P < 0.001$) (22, 23).

Radiological imaging of PsA patients may show signs of both bone erosion and new bone formation with bone proliferation mostly found in the metacarpal and metatarsal bones and joints. Formation of new bones is mostly asymmetric, with deformities arising at digits and peripheral joints. Joint damage affects mostly the hands, wrists, feet, ankles, knees, and shoulders. Syndesmophytes may develop in the skeleton and calcification may appear at the entheses, specifically at the insertion sites (24). In contrast to rheumatoid arthritis, PsA may show different manifestations in the same anatomical site with osteolysis and bone deposition detected in the same hand. Dactylitis may be accompanied by bone erosion or new bone formation as well (25). In the spine the degree of new bone formation can be graded using scoring systems, such as the Bath Ankylosing Spondylitis Radiology Index (BASRI) and the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS) (26, 27).

THE GENETICS, EPIGENETICS, AND IMMUNOPATHOGENESIS OF PSA

There is a strong genetic component to psoriasis and PsA. Further, there is a significant degree of overlap in genetic predisposition between psoriasis, PsA, AS and the inflammatory bowel diseases (ulcerative colitis and Crohn Disease) (21, 28).

The genetic contribution to diseases like psoriasis and PsA has classically been investigated through twin studies. The concordance rate for psoriasis in MZ twins is between 20 to 64%, indicating that genetic factors account for roughly 70% of the population variance in the susceptibility to psoriasis (18). In polygenic diseases, there has also been an increasing focus on monozygotic (MZ) twins in order to assess the influence of epigenetics. This is true also for psoriasis and PsA (29). In addition to genetic predisposition, the onset and progression of

both psoriasis and PsA appear to be influenced by both the environment and epigenetics factors (30).

Genome-Wide Association Studies and Array-Based Technologies

PsA is a polygenic immune-mediated disease: genome-wide association studies (GWAS) have identified many genes/genomic loci increasing susceptibility for PsA, many of which are also common to psoriasis uncomplicated by arthritis; these include *HLA-A*, *HLA-B*, *HLA-C*, *IL23R*, *CSF2* (Colony Stimulating Factor 2 or granulocyte-macrophage colony stimulating factor), *TRAF3IP2* (TRAF3 Interacting Protein 2), *NOS2* (Nitric Oxide Synthase 2) (31, 32). The results of GWAS suggest that there are PsA-specific genetic variants which are independent of those previously identified in isolated psoriasis, specifically near *IL23R* and *TNFAIP3* (TNFα Induced Protein 3) (33).

GWAS are very informative for common and low frequency variants but they do not identify rare variants. Exome chips, such as the Illumina Exome BeadChip, allow the identification of coding variants and detection of rare SNPs (34). A very good alternative is the ImmunoChip, such as the Illumina Infinium genotyping chip, which contains 196,524 polymorphisms (718 small insertion deletions, 195,806 SNPs) and it is a "low-cost" option. It is designed to perform deep replication of major inflammatory and autoimmune diseases and fine mapping of established GWAS significant loci (35). In 2015, Bowes and colleagues used the ImmunoChip array to fine-map immune-related susceptibility loci including the known psoriasis risk loci, to define new PsA susceptibility loci. They identified a specific PsA variant at the *IL23R* locus, and a new PsA-specific association at chromosome 5q31 (36).

These associations may indicate roles for certain signaling pathways that are specific to the pathogenesis of PsA rather than psoriasis itself, but further investigation is needed to clearly understand their contribution.

PsA-Associated Genetic Variants and Their Immune-Pathological Role

Antigen presentation by MHC proteins is pivotal to acquired immunity, and MHC alleles are strongly associated genetically with both psoriasis and PsA. HLA class I alleles are associated with increased susceptibility for PsA, but the PsA-associated HLA alleles differ from those reported in psoriasis (37). For example, the association of *HLA-C*06*, which is consistently associated with psoriasis, is only very weakly associated with PsA. Conversely *HLA-B*08*, *HLA-B*27*, *HLA-B*38*, and *HLA-B*39* are the HLA alleles most consistently associated with PsA (38).

*HLA-B*27*, which is the pre-eminent genetic association with AS, is also consistently associated with PsA but not with psoriasis, thereby indicating different pathogenetic mechanisms in the two conditions despite the obvious disease-in-disease bias for case ascertainment. *HLA-B*27* is especially associated with axial skeletal disease and the strength of this association tends to show an inverse relationship with the number of peripheral joints involved (39).

Several other genes involved in the immune response are also associated with PsA. Thus, there are also several other non-HLA genetic associations involving components of the MHC class I antigen processing and presentation pathway: these include *ERAP1* and *ERAP2* (Endoplasmic Reticulum Amino-Peptidase-1 and -2). Others are involved in the innate immune response and the initiation of the immune reaction (e.g. *TLR4* - Toll-Like Receptor 4), or the differentiation and function of CD8+ T-cells (e.g. *RUNX3* - Runt-related transcription factor 3) (31, 40, 41). Many of these genes are also associated with increased susceptibility to AS, highlighting once again the considerable overlap between these two disorders in terms of their genetic susceptibility (42).

The GWAS era has also highlighted several PsA-associated single nucleotide polymorphisms (SNPs) located in *IL-23A*, *IL-23R*, *IL-12B*, *TYK2* (Tyrosine Kinase 2), and *TRAF3IP2* (which is a downstream target of the IL-17 receptor - IL-17R), implying a pivotal role for the IL-23/IL-17 axis in the pathogenesis of PsA disease pathogenesis (see **Table 1**) (5, 43). A crucial role for IL-17 family is undisputed as the levels of IL-17 and IL-17R were found increased in both psoriatic skin and synovial fluid of patients with PsA (44).

The contribution of the IL-23/IL-17 axis has greatly advanced our understanding of the pathogenesis of PsA. Th-17 cells produce the pro-inflammatory cytokine IL-17, and all the elements of the Th17 pathway, including MMP3 (Matrix Metalloproteinase 3), CCL1 and CCL20 (Chemokine Ligand 1 and 20) and IL6. The majority of these pro-inflammatory cytokines are upregulated in the blood, synovium, and skin of PsA patients (45, 46). A recent study has identified CXCR6 as a marker for IL-17+CD8+, specialized cells found in the synovial fluid of PsA patients. The presence of CXCR6+IL-17+CD8+ cells in PsA synovium may explain their contribution to the inflammatory environment (47).

TABLE 1 | Genetic variants related to the IL-23/IL-17 axis found associated with PsA through GWAS or consistently identified in targeted analysis studies.

Chromosome	Gene	SNP ID	Odds ratio	Function
1p31.3	*IL-23R*	rs11209026	0.6[#]	Th-17 signaling
		rs12044149	1.4[$]	
2q32.2	*STAT4*	rs7540214	N/A	Mediating response to IL-12 and Th-17/Th-1 differentiation
5q33.3	*IL12B*	rs2082412	1.4	Th-17 and Th-1 differentiation
		rs6887695	1.3[@]	
		rs4921482	1.4[$]	
		rs3212227	1.4[@]	
		rs918520	1.5*	
12q13.3	*IL23A*	rs2020584	N/A	Th-17 signaling
17q21.2	*STAT3*	rs744166		Mediating response to IL-12 and Th-17/Th-1 differentiation
5q33.1	*TNIP1*	rs8177833	1.8*	NFkB signaling
6q21	*TRAF3IP2*	rs33980500	1.7*	Th-17 signaling and NFkB signaling
		rs13190932	N/A	
19p13.2	*TYK2*	rs35251378	1.4*	NFkB, Interferon and Th-17 signaling

*Odds Ratio (OR) from Stuart PE et al. Am J Hum Genet. 2015 3; 97(6): 816–836.
[#]OR from Zhu K et al. Inflamm. Res. 2012; 61, 1149–1154.
[$]OR from Bowes et al. Nat Commun. 2015 5; 6: 6046.
[@]OR from Filer et al. Arthritis Rheum 2008; 58(12):3705-9.

IL-23 promotes the survival and expansion of Th-17 cells through its receptor IL23R and the related downstream signaling pathway, which is crucial to Th-17-mediated diseases like PsA. In 2009, a study conducted by Gladman group showed a protective effect for the *rs11209026* SNP in *IL-23R* (encoding the loss-of-function 381Gln allele in the cytoplasmic tail of IL23R) in a Canadian PsA cohort (48). This SNP is also associated with disease severity, while another variant, *rs12044149*, showed an independent peak of association with PsA, after conditioning for the top SNP associated with psoriasis overall (*rs9988642*) (36).

Th17-mediate inflammatory response also involves the signaling adaptor *TRAF3IP2 (*TRAF 3-interacting protein 2), which is downstream of IL-17R. The PsA-associated *TRAF3IP2* variant *rs33980500* alters the binding of the TNF receptor-associated factor 6 (*TRAF6*), which modulates immunoregulatory signals (49, 50).

IL-23 and IL-17 also have major effects on the activation of osteoclasts, which are the main responsible for bone erosion and where the cytokine RANKL (receptor activator of nuclear factor kappa-β ligand) is a critical factor in the promotion of osteoclasts differentiation. RANKL is also expressed by Th-17 lymphocytes and synovial fibroblasts. The dogma that Th17 cells were the primary responsible for bone resorption, was recently challenged in animal studies where cell specific deletion of RANKL indicated that RANKL expression was limited to synovial fibroblasts and B cells (51).

In PsA, bone deposition is crucial as bone resorption. This process arises from the aberrant proliferation, differentiation, and activity of osteoblasts, and several signaling pathways are involved, such as the bone morphogenetic protein (BMP) and the WNT signaling pathway, such as DKK1, and Sclerostin which are relevant for both PsA and AS (52).

The abnormal proliferation of keratinocytes promoting epidermal hyperplasia (53), also emphasizes the predominant functional role of the IL23/IL-17 axis in PsA pathogenesis and in PsA inflammatory cascade.

Lastly, Al Mossawi and colleagues recently demonstrated a marked increase of specific subsets of CD4+ and CD8 + T-cells producing GM-CSF in the blood and synovium of PsA patients. They also demonstrated an increased number of double-positive IL17/GM-CSF for CD4+, CD8+, γδ, and NK (natural killer) cells. The *CSF2* locus encoding GM-CSF, is also strongly associated genetically with PsA. Overall these results suggest a functional link between GM-CSF and the IL-17/IL-23 axis, opening important potential avenues for the treatment of PsA, including those targeting GM-CSF directly (54).

CELLULAR MECHANISMS IN PSA: THE MULTIFACETED ROLE OF IL-17 AND IL-23

The contribution of the IL-23/IL-17 axis to the development of PsA will be discussed in this section, highlighting Th-17 biology and the production of a pro-inflammatory *milieu* in the skin and in the synovium, and how this leads to the activation of osteoclasts, which are responsible for bone degradation, and of keratinocytes and neutrophils, which are implicated in the epidermal hyperplasia.

Cell Activation and Innate Immunity

Inflammation is one of the hallmarks of PsA and activation of the innate immune response is one of the physiological triggers leading to the inflammatory cascade. The transcription factor NF-κB has a major role in this cascade, as it promotes the transcription of several proinflammatory cytokine target genes. Downstream signaling, such as IFNs (Interferons) and TNFα strongly contribute to the immune response in PsA (55).

Several genes involved in these NF-κB pathways show genome-wide significant association with PsA, including *REL* (a subunit of NF-κB), *NOS2*, and *TNFAIP3* (31, 56). The IL-23/IL-17 axis might also influence the activation of the NF-kB pathway in other ways; these include the adaptor protein Act1, which when is phosphorylated binds to TRAF6 to activate the NF-κB activator protein 1 (AP-1), or the CCAAT-enhancer-binding protein (C/EBP) cascade (57).

Interferon signaling also plays a key functional role in the pathogenesis of PsA (58). It is therefore of interest that several interferon-related genes, including *TYK2*, *IFNL1R*, and *PTPN22*, exhibit genome-wide significant association with PsA (59). *TYK2*, for instance, encodes a tyrosine kinase that is involved in initiating IFNα signaling and, as mentioned above, it is also an activator of IL-23R.

TNFα clearly plays a major role in the inflammatory process of PsA as shown by the presence of raised levels of this cytokine at the sites of inflammation and the dramatic responses to treatment with anti-TNF biologics (60). It has a major role in innate immunity, inducing the production of inflammatory chemokines leading to the accumulation of activated T-cells, neutrophils, and monocytes. It is of some interest that significant association for psoriasis and PsA has been found for the SNPs *rs80267959*, *rs1800629*, and *rs361525* at the *TNFA* locus (61).

Cell Activation and Adaptive Immunity

The pathogenesis of PsA involves components of both the innate and adaptive immune system. Many different cell types are involved in these pathophysiological processes, including T-cells, neutrophils, keratinocytes, and synoviocytes (62).

The master cytokine in the pathogenesis of PsA is IL23. IL-23 is a heterodimer composed of two subunits p19 and p40 subunits, which bind to IL23R. While p19 is unique to IL-23, the p40 subunit is shared with IL-12 (63). The binding of IL-23 to IL23R leads to the recruitment of JAK2 and TYK2 kinases, which are able to activate IL23R (and its cognate IL12RB1), phosphorylate STAT3 (Signal Transducer and Activator of Transcription 3) and induce RORγT (RAR-related orphan receptor gamma) to promote the differentiation, survival, and expansion of Th-17 cells. IL-23 is essential not only in inducing the Th-17 phenotype but also in defining the level of pathogenicity (64).

Th-17 cells are among the possible drivers of the pathogenesis of PsA and the effector molecules they release are able to trigger different target cells such as osteoclasts, macrophages, and synovial fibroblasts. IL-17 is the major cytokine produced by

Th-17 cells, gd T cells and other various immune cells. IL-17 A is a homodimer disulfide-linked glycoprotein and it is the most widely studied member of the IL-17 cytokines family, which includes also IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (65). IL-17A and IL-17 F share 55% of homology and they can form heterodimers, which bind the receptor IL-17R. IL-17R is a dimeric complex consisting of two subunits, IL-17RA and IL-17RC subunits (66). The differential binding affinity of IL-17 for IL-17RA and IL-17RC is still not well define, especially under inflammatory conditions (67).

Th-17 cells differentiate from naïve T-cells depending on a combination of different cytokines along three distinct pathways (68): (i) IL-6 and Transforming Growth Factor-β (TGFβ) (in addition IL-1β and TNFα); (ii) IL-21 and TGFβ; (iii) IL-1β, IL-6, and IL-23 (69). Th-17 cells are characterized by the expression of the transcription factor RORγT with a specific gene signature which in addition to *IL17* also includes *IL6*, *TNF*, *CSF2*, *CCL20*, and *IL23R* (69).

T-cell subsets triple-positive for IL-17, GM-CSF, TNF, or IFN-γ were found increase in PsA synovial tissue. These enriched polyfunctional cells were also found associated with disease activity index (70).

Recently, an elegant study by Taams' group demonstrated that IL-17+CD8+T cells may contribute to inflammation and disease persistence in PsA. These cells were found increased in patients' synovium and have a Th-17 resembling transcriptomic profile characterized by high expression of CXCR6 (47).

JAK and STAT are the key signaling pathways transducing the cytokine signals in Th-17 cells (63). Following the inflammatory cascade mediated by IL-1β and IL-23, Th-17 cells release specific pro-inflammatory cytokines such as IL-17, GM-CSF, and IL-22. The function of Th-17 cells is tightly dependent on the balance of the effector molecules they produced, such as IL-23 and TGF-β, and on those cytokines promoting cell differentiation and maintenance (IL-23).

The effects of IL-23 on bone are conflicting. IL-23, in a Th-17 independent way, up-regulates the expression of the nuclear factor kappa-β ligand RANKL-receptor RANK in osteoclast precursors (from monocyte lineage cells), favoring osteoclast differentiation and proliferation (64, 71). IL-23 also induces the production of GM-CSF, which is an inhibitor of the differentiation of osteoclasts, thus limiting bone resorption (72). RANKL expression is also found in synovial fibroblasts where its deletion plays a key role in bone erosion (51).

Neoangiogenesis also appears to have an important part in the pathogenesis of PsA; new blood vessels are prominent in the synovial histology of the condition. It appears that the IL-17/IL-23 axis might play a role in the angiogenic process. As we have described, IL-17 produced by Th-17 cells up-regulates proinflammatory cytokines and prompts the recruitment of neutrophils and macrophages and endothelial cell migration. Further, keratinocytes, stimulated by IL-17 ligands, start to differentiate and proliferate aberrantly, producing proinflammatory adenosine monophosphate (AMP), chemokines, and angiogenic factors such as vascular endothelial growth factor (VEGF) (73).

IL-23 can also promote epidermal hyperplasia activating the proliferation of keratinocytes (increasing the expression of keratin 16) and by acting synergistically with IL-17 promotes the recruitment of neutrophils and the infiltration of IL-22 and IL-17 producing-cells into the lesioned skin (74). The response of keratinocytes and endothelial cells among others to IL-17 and IL-22 stimulation leads to the upregulation of chemokines such as CXCL1 and CCL20, pro-inflammatory cytokines, and anti-microbial peptides, such as LL-37 and β-defensins. IL-17 and IL-22 both promote keratinocyte proliferation and the recruitment of macrophages and neutrophils; they also decrease the expression of adhesion molecules (i.e. selectins and integrins) thus favoring the disruption of the skin barrier. IL-17 can also stimulate the expression of endothelial markers such as P- and E-selectins and adhesion molecules, including ICAM-1 (Intracellular Adhesion Molecule 1) and VCAM-1 (Vascular Cellular Adhesion Molecule 1), which enhances the mobilization of neutrophils (75).

Lastly, the expression of IL-17 and IL-23 is increased in the synovium (76, 77). Gene expression analysis of PsA synovium reveals a gene signature closer to PsA skin than to rheumatoid synovium (78). The recruitment of pathogenic IL-23/IL-17-producing CD4+ T-cells has been demonstrated to be higher in the joints, while the IL-17/IL-22 producing CD4+ T-cells are strongly detected in the skin and in the circulation (79, 80).

THERAPEUTIC APPROACHES IN PSA

Since the development of biologic therapies, the ultimate target for the treatment of any patient with psoriasis and/or PsA in modern times is complete remission (81). Initially these targeting TNFα were used with great effect but much effort has also been made to develop biological drugs targeting the IL-23/IL-17 axis. This axis, as specified previously explained, offers several plausible drug targets, such as the p40 subunit of the IL-23/IL-12 receptor, the p19 subunit of IL-23R, IL-17A and its specific receptor IL-17R (82).

In the treatment of PsA non-steroidal anti-inflammatory drugs (NSAID) and synthetic disease modifying antirheumatic drugs [sDMARDs, such as methotrexate (MTX), leflunomide, and sulfasalazine] remain the first-line therapies but biological molecules (bDMARDs) and targeted synthetic DMARDs (tsDMARDs) are used if therapy with NSAID and DMARDs fails to control the disease adequately.

Biologics (such as etanercept, infliximab, adalimumab, golimumab, certolizumab, ustekinumab, and secukinumab) or synthetic drugs (apremilast, tofacitinib and ixekizumab) are given in specific circumstances (62). The different drugs used in PsA (and in other conditions) with their mechanism of action are shown in **Table 2**.

A combination of two or more of these drugs is often administered in complex immunological diseases like PsA, and often results in increased efficacy compared with monotherapy (83).

In recent few years, a number of guidelines have been developed for the clinical assessment and management of PsA; these include recommendations from GRAPPA, EULAR (European League Against Rheumatology) and ACR/NPF (American College of Rheumatology/National Psoriasis Foundation) (84, 85).

TABLE 2 | Overview of the current treatments in PsA and related mechanism of action.

Category	Molecule	Mechanism of action	Approval and use	ACR20 in PsA (%)
sDMARDs	Methotrexate	Anti-metabolic	RA, Ps, and PsA	32–40
	Leflunomide	Inhibitor of pyrimidine synthesis	RA, Ps, and PsA	34
bDMARDs	Etanercept	TNFα blocker	AS, RA, and Ps	60–65
	Infliximab	TNFα blocker	AS, RA, Ps, and PsA	65
	Adalimumab	TNFα blocker	Ps and PsA	58
	Golimumab	TNFα blocker	PsA, AS, and RA	76
	Certolizumab	TNFα blocker	RA and Ps	52-63
	Brodalumab	IL-17RA inhibitor	Ps and PsA	39
	Ustekinumab	IL-12/IL-23 inhibitor	Ps and PsA	42–50
	Secukinumab	IL-17A inhibitor	AS, Ps, and PsA	54
	Guselkumab	IL-23 inhibitor	Ps, PsA	58
	Ixekizumab	IL-17A inhibitor	Ps, and PsA	60
tsDMARDs	Apremilast	PDE4 inhibitor	Ps and PsA	31
	Tofacitinib	JAK-1/3 inhibitor	PsA	50–53

sDMARDS, synthethic disease modifying anti-rheumatic drug; bDMARDs, biologic disease modifying anti-rheumatic drug; tsDMARDS, targeted synthetic disease modifying drug; ACR20, American College of Rheumatology 20 response; Ps, psoriasis; IL, interleukin; PDE4, phosphodiesterase type 4; TNFα, tumor necrosis factor α (15, 62, 82, 83).

For instance, EULAR recommends NSAID and local glucocorticoid injections, especially for enthesitis, in the early phase of the disease. If adverse prognostic factors are present or treatment fails, the administration of sDMARDs, such as MTX (or alternatively leflunomide or sulfasalazine) is recommended (86). If these fail to control the disease adequately or are poorly tolerated the administration of TNF inhibitors should be considered either in combination with DMARDs or not. Biologics should also be used in those with prominent axial disease or severe enthesitis. The continued use of TNF inhibitors should be evaluated carefully according to the patient's response and a switch to alternative biologics may be considered either where there is no initial benefit (primary failure) or where the response is lost after a period of time (secondary failure) perhaps due to the host generation of antibodies to the biologic (81).

Targeting the IL-17/IL-23 Pathway

The IL-17/IL-23 pathway and Th-17 cells have become a favorite target in PsA in recent years. For this purpose, several biological drugs have been developed (**Figure 1**). Ustekinumab, which is a monoclonal antibody targeting p40 subunit of IL-12/IL-23 has been used for skin and nail psoriasis but also for peripheral PsA in those patients who not respond not to DMARDs (87).

Two large clinical trials, PHOENIX 1 and 2, assessed the efficacy of ustekinumab in psoriasis patients (88) while PSUMMIT-1 and -2 examined its efficacy and safety in PsA (89). *Post-hoc* analyses confirmed the efficacy of ustekinumab not only on skin but also in improving PsA rheumatological manifestations and radiographic progression (90). The efficacy of ustekinumab in axial involvement for PsA or in axial SpA is believed to be marginal and the development programs have been thus discontinued. However, the final word on the efficacy of IL23 blockers in SpA remains uncertain as recent data on guselkumab showed efficacy on patient reported outcomes for PsA axial manifestations (91) and a recent phase IV study on ustekinumab reported that this drug is frequently used in patients with axial PsA (92). Inhibition of IL-17A has been achieved using secukinumab, a human monoclonal antibody targeting IL-17A, with efficacy in both PsA and ankylosing spondylitis (93, 94). In the treatment of PsA secukinumab is effective for dactylitis, enthesitis, skin and nail lesions, but its effects on joint disease is rather less, as shown in FUTURE 2 and 3 trials (95).

Targeting either IL-17 or its receptor in PsA patients include besides secukinumab, also ixekizumab and brodalumab. The efficacy of ixekizumab (also targeting IL-17A) was demonstrated in reducing active disease and radiologic progression in joints, as well as fulfilling the PsA criteria of skin response, as demonstrated in the head-to-head SPIRIT study (96). Furthermore, brodalumab, which is a human monoclonal antibody human anti-IL17RA, a pan inhibitor of IL-17A, IL-17F, and IL-25 is currently used in the treatment of psoriasis where shows a complete clearance of moderate-to-severe psoriasis (97). Brodalumab efficacy and safety was also assessed in PsA patients (98).

DISCOVER-1 and -2, a double-blind, randomized, placebo-controlled phase 3 trials proved the efficacy of Guselkumab a human monoclonal antibody specifically binding the p19 subunit of IL-23. The study has shown a substantial improvement in biological naïve patients with active disease, in particular in decreasing IL-17A, IL-17F, and CRP serum levels by week 16 achieving Psoriasis Score and Severity Index, PASI75 (99, 100).

Overall, following the blockade of the IL-23/IL-17 axis, clinical trials for Ps and PsA showed a good amelioration of the skin lesions while the joint response was much lower. A larger percentage of patients achieved the PASI75, PASI90 and PASI100 compared to proportion of patients fulfilling the ACR20, ACR50, or ACR70 criteria of response (101).

DISCUSSION AND CONCLUDING REMARKS: HOW GENETICS MIGHT BE CRUCIAL IN IDENTIFYING CREDIBLE THERAPEUTIC TARGETS

PsA is a complex polygenic disease with a genetic contribution that overlaps with other related conditions such as psoriasis, AS and IBD. Genetic variants associated with a specific disorder have the power to highlight genes or pathways that may contain credible

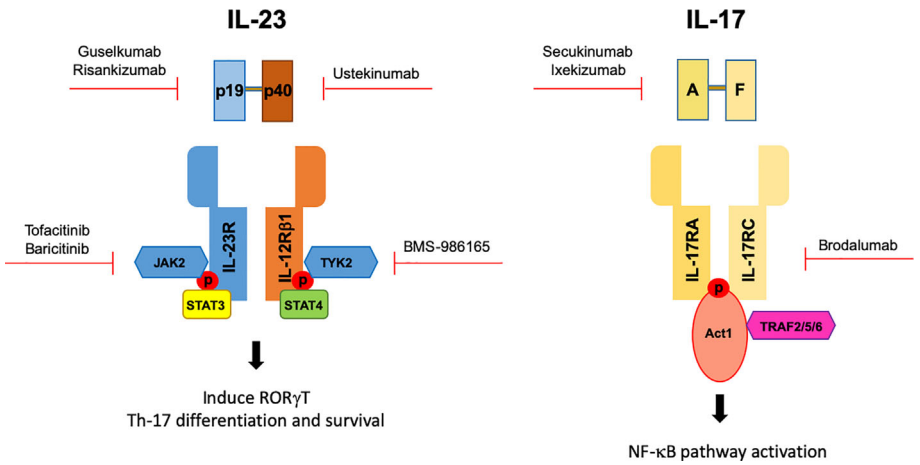

FIGURE 1 | Genetics studies allowed the identification of the IL-23/IL-17 axis having a crucial functional role in PsA pathogenesis. Genetics lead to the development of biological drugs targeting the IL-23/IL-17 axis in PsA. Red arrow indicates the target of different biologics.

targets for drug therapy. This is well exemplified for the IL-17/IL-23 axis and Th-17 cells with the development of biological drugs blocking IL-17 (i.e Secukinumab in AS), or IL-23 (i.e. Ustekinumab in psoriasis/PsA). Unfortunately, the process is challenging (102).

The first crucial point following a GWAS is to accurately assign associated SNPs to the genes they regulate in order to define credible pathways. For this purpose, several experimental functional assays have been developed. Functional disease-associated SNPs may affect the binding of transcription factors or the enrichment of regulatory markers: this is currently evaluated with *in vitro* Electrophoretic Mobility Shift Assays and *ex vivo* with Chromatin ImmunoPrecipitation). SNPs may have an effect on gene expression (evaluated with expression quantitative trait loci, eQTL studies) or on chromosome looping (assessed *via* chromosome conformation assays). Genome editing techniques are performed to define the consequences of harboring a risk variant on cellular function.

Second, these experiments must be performed considering cell-specificity (i.e. specific tissue or cell type) and condition-specificity (i.e. different stimulatory conditions) to provide significant insights into the pathogenesis of a specific disease and develop targeted therapies.

This approach will increase our understanding in defining credible pathways, specific genes, and cell populations for targeted therapy. The better molecular stratification we can achieve (for instance, patients with enrichment of associated SNPs in the IL-23/IL-17 pathway may respond more effectively to secukinumab or ustekinumab), the better the design of personalized therapeutic strategy will be. The final goal will be an advanced use of SNPs as pharmacogenetic markers, in order to define credible pathways to target and predict response to biological therapy (i.e. HLA-C*06 as a pharmacogenetic marker in response to Ustekinumab) (103).

AUTHOR CONTRIBUTIONS

MV, VH, BW, and CS conceived the manuscript. MV, VH, CD, MC, BW, and CS drafted the manuscript, and all the authors revised the final version prior to submission. All authors contributed to the article and approved the submitted version.

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REVIEW
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Perspectives on the Genetic Associations of Ankylosing Spondylitis

B. Paul Wordsworth[1,2]*, *Carla J. Cohen*[2], *Connor Davidson*[3] and *Matteo Vecellio*[1,2,3]

[1] Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford Institute of Musculoskeletal Sciences, Oxford, United Kingdom, [2] Botnar Research Centre, Nuffield Orthopaedic Centre, Oxford, United Kingdom, [3] Wellcome Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

Ankylosing spondylitis (AS) is a common form of inflammatory spinal arthritis with a complex polygenic aetiology. Genome-wide association studies have identified more than 100 loci, including some involved in antigen presentation (*HLA-B27*, *ERAP1*, and *ERAP2*), some in Th17 responses (*IL6R, IL23R, TYK2*, and *STAT3*), and others in macrophages and T-cells (*IL7R, CSF2*, *RUNX3*, and *GPR65*). Such observations have already helped identify potential new therapies targeting IL-17 and GM-CSF. Most AS genetic associations are not in protein-coding sequences but lie in intergenic regions where their direct relationship to particular genes is difficult to assess. They most likely reflect functional polymorphisms concerned with cell type-specific regulation of gene expression. Clarifying the nature of these associations should help to understand the pathogenic pathways involved in AS better and suggest potential cellular and molecular targets for drug therapy. However, even identifying the precise mechanisms behind the extremely strong HLA-B27 association with AS has so far proved elusive. Polygenic risk scores (using all the known genetic associations with AS) can be effective for the diagnosis of AS, particularly where there is a relatively high pre-test probability of AS. Genetic prediction of disease outcomes and response to biologics is not currently practicable.

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Maxime Breban,
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Nigil Haroon,
University of Toronto, Canada

***Correspondence:**
B. Paul Wordsworth
paul.wordsworth@ndorms.ox.ac.uk

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INTRODUCTION

Ankylosing spondylitis (AS) is the archetype of a group of inflammatory disorders known as spondyloarthropathies (SpA) because they often affect the spine (axial skeleton). Other forms of SpA (e.g., psoriatic arthritis, reactive arthritis and the enteropathic arthropathies associated with inflammatory bowel disease—IBD) also often involve the axial skeleton (axSpA) but sometimes just affect the peripheral joints (peripheral SpA). Any part of the spine may be involved in AS but the SI joints are the most commonly affected sites early in the disease. The demonstration of radiographic sacroiliitis is a formal prerequisite for the diagnosis of AS but may take many years to be apparent on plain films. Therefore, to diagnose early AS or axSpA (considered together here although there are semantic differences), magnetic resonance imaging (MRI) is preferred since it can detect the early inflammatory phase of the disease potentially many years before radiographic changes become apparent on X-rays (1, 2). AS is one of the commonest forms of arthritis in the developed and

developing world with a prevalence of up to one in 200 in Western Europe but it is much less common in some other parts of the globe, such as sub-Saharan Africa where its low prevalence generally reflects the low frequency of the immune response gene HLA-B27 with which it is so strongly associated—see below (3). In this review, we focus on AS and axSpA (defined by imaging criteria—either radiographs or MRI) as might be diagnosed using the algorithm presented by Taurog et al. (4). Unfortunately, despite increased awareness of the disease and the diagnostic utility of MRI, the diagnosis of AS is still missed all too often; only one-third of cases are diagnosed within a year of the onset of symptoms, and there is typically a delay of more than 6 years before the diagnosis is established (5, 6).

In contrast to the inflammation of the joint lining (synovitis) associated with many other arthropathies, such as rheumatoid arthritis, the characteristic pathology of AS is enthesitis. The entheses are anatomical sites that have evolved to tolerate heavy mechanical loads, such as fibrocartilaginous joints (including the SI joints), the osseous insertions of ligaments and tendons, and joint capsules. In AS, inflammation at these sites initially causes bone erosion but this is often followed by new bone formation, which creates "syndesmophytes" that bridge between adjacent vertebrae in the spine causing bony fusion (ankylosis). Over time this can lead to complete loss of spinal movement and the classic "bamboo spine" appearance on radiographs characteristic of the most severe cases (**Figure 1**). Some years ago, Sherlock and his colleagues shed some light on why the entheses might bear the brunt of the pathological attack when they demonstrated the presence of $CD3^+$ $CD4^-$ $CD8^-$ lymphocytes resident at the entheses expressing the interleukin (IL)-23 receptor (IL23R), and that a form of SpA resembling AS could be initiated in mice simply by liver-specific over-expression of IL23 alone without other cells being recruited to the affected tissues (7). Recently, γδ T-cells of both the Vδ1 and Vδ2 subsets have been demonstrated at the entheses that can be induced to produce IL-17, in the case of Vδ2 cells without the expression of IL23R (8). The relevance of IL23-driven pathways to the development of AS has also been amply demonstrated by numerous genetic associations with components of this pathway (see below).

As with many other common diseases, the nature versus nurture debate regarding the aetiology of AS has long been a source of interest and speculation. Of course, increased familial recurrence can reflect either environmental or intrinsic factors but the absence of obvious temporal clustering of cases within families and the fact that the disease tends to start at a broadly similar age (typically between 20 and 40 years of age) is more suggestive of genetic than environmental influences. It was the particularly strong familial nature of the disease that prompted Derek Brewerton (at the suggestion of his rheumatology colleague Dudley Hart at the Westminster Hospital) to look for genetic risk factors in AS rather than rheumatoid arthritis in the 1970's. By then it was already apparent that the pattern of AS recurrence risk among relatives of increasingly distant relatedness (very pronounced reduction in risk from first-degree to second-degree relatives, with more gradual reduction thereafter) was more consistent with a polygenic risk than either a monogenic or oligogenic contribution (9, 10). Despite this, such was the strength of the association between AS and the transplant antigen HLA-B27 (11, 12) that many erroneously assumed that AS was a monogenic disease. The classic way of investigating the genetic component of a disease by twin studies reveals a highly significant genetic contribution to AS, and one in which HLA-B27 is the major but by no means the only factor (**Figure 2**) (13). Armed with this limited but convincing information and the enthusiastic support of Sir John Bell and Mark Lathrop at the newly instituted Wellcome Trust Centre for Human Genetics a number of us from around the world therefore set out in the 1990's to try to identify at least some of

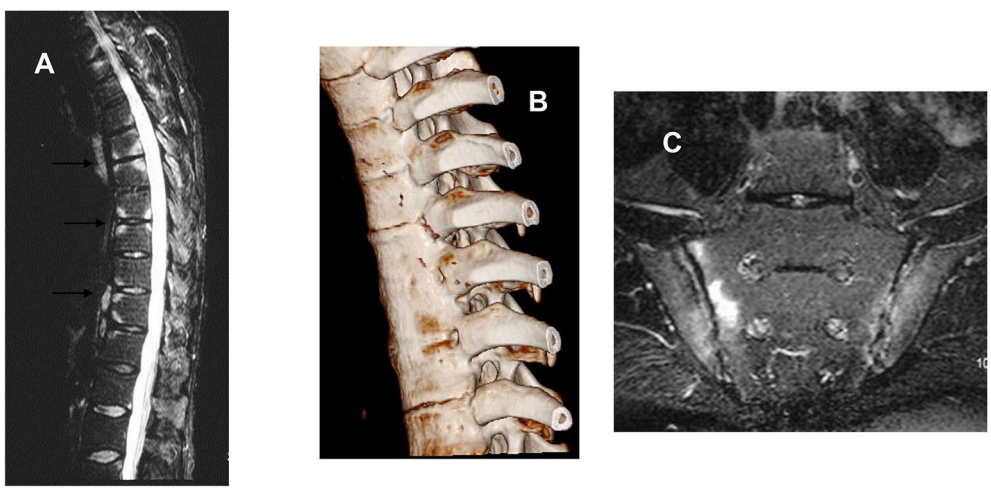

FIGURE 1 | **(A)** Sagittal magnetic resonance image of the thoracic spine of a 44-year-old man with active ankylosing spondylitis, showing high signal on these T2-weighted images consistent with inflammation at the vertebral corners consistent with the attachment of vertebral ligaments and discs. **(B)** Computed tomographic reconstruction of the thoracic spine of a 25-year-old man with AS since the age of 12. There is clear bony fusion between the adjacent vertebrae and also at the costovertebral joints. **(C)** Bilateral sacroiliitis shown by MRI (STIR sequence) worse on the sacral side of the right SI joint.

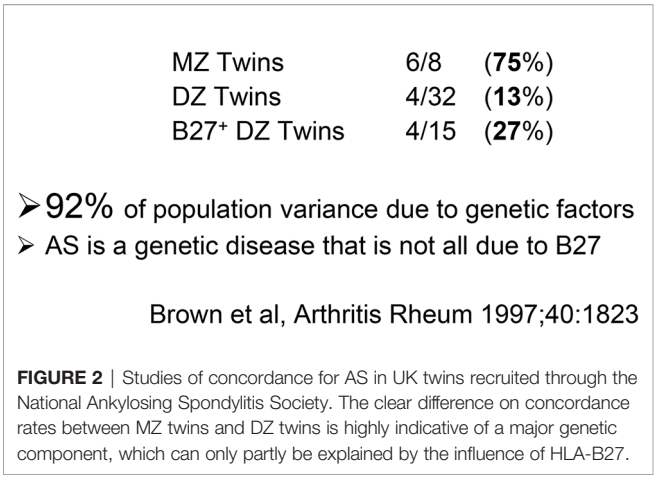

FIGURE 2 | Studies of concordance for AS in UK twins recruited through the National Ankylosing Spondylitis Society. The clear difference on concordance rates between MZ twins and DZ twins is highly indicative of a major genetic component, which can only partly be explained by the influence of HLA-B27.

the other genes that were involved. In this brief review we discuss selected examples of the progress that has already been made towards this goal and how this has helped to pin down some of the pathological processes involved in AS. We discuss some of the innovative methods that have been used to identify new genetic associations with AS and the problems in interpreting these associations at a functional level. We include brief discussions of how these findings could inform future drug target discovery and play a role in the diagnosis of AS, and personalizing therapeutics for individual patients.

GENOME-WIDE ASSOCIATION STUDIES IN AS

Prior to the late 1990s efforts to identify any non-HLA genes contributing to AS were limited to studies of so-called "candidate" genes for which there was (usually but not invariably) a compelling biological reason for why they might be involved. Naturally enough (given the association with HLA-B27), many of these candidates were broadly "immunological" in nature and, equally unsurprisingly, they were generally unrewarding. The transition to genome-wide approaches was perhaps somewhat offensive to some scientists, because it was essentially not "hypothesis-driven" in the classic Popperian philosophical sense—other than that we proposed that there were genes out there to be discovered. The initial studies in AS, based on a form of genetic linkage analysis of affected relative pairs proved too blunt an instrument for the job (despite the huge amount of work involved in recruiting several hundred affected sibling pairs and their nuclear families). Beyond demonstrating linkage to gene(s) in the major histocompatibility complex on chromosome 6 not very much else came up and certainly nothing that was categorically associated with AS even after applying meta-analysis (14–16). Worse still, it was obvious that this type of analysis had very little power to refine chromosomal intervals to the level of identifying individual genes and/or the polymorphisms in them that were disease-causing variants. It was not until technical advances allowed the application of much larger numbers (~500,000) of genetic markers known as single nucleotide polymorphisms (SNP) spanning the entire genome that the field really started to move on. Nonetheless, there were some exciting surprises even before this grand-scale technological revolution was fully in place. The following are just a few examples from the first decade of GWAS in AS.

Early Successes: Endoplasmic Reticulum Associated Aminopeptidase 1

The first GWAS in AS was published in 2007 as part of a broader attempt by the Wellcome Trust Case-Control Consortium to identify the genetic component of several common complex diseases, including cardiovascular disease, bipolar disease, inflammatory bowel disease, rheumatoid arthritis, tuberculosis, autoimmune thyroid disease, multiple sclerosis, and breast cancer (17). The number of AS cases was relatively small (~1,000) and the number of SNPs was modest (~14,500, of which 3,000 were in the major histocompatibility complex - MHC). Although the SNPs were gene-targeted non-synonymous variants (i.e., amino acid changing) this only gave a coverage of about one SNP per two gene loci, on average. By chance, one of the genes that registered association in this study had been allocated rather more than its fair share of SNPs—*ERAP1* (endoplasmic reticulum aminopeptidase 1 involved in processing peptide antigens for presentation by MHC class I molecules) had 5 non-synonymous (coding) SNPs. To this day *ERAP1* remains one of the most interesting and strongest associations ($p \sim 10^{-50}$) with AS outside the MHC (18–21). It is one of a family of aminopeptidases involved in the progressive cleavage of single amino acids from the amino-terminal end of peptides transported *via* the TAP (transporter associated with antigen processing) prior to associating with nascent MHC class I molecules. ERAP1 is crucial in shaping the available peptide repertoire, not only by providing peptides of the optimal length (8–9 amino acids) but also by influencing their amino-terminal residues that affect their binding to individual HLA allotypes, such as HLA-B27. A number of fascinating subsequent discoveries have been made about the nature of this genetic association with AS and the functions of ERAP1.

1. The association with *ERAP1* is synergistic with HLA-B27. Only around 84 per cent of AS cases in the UK are HLA-B27 positive and the association of AS with *ERAP1* is restricted to those who are HLA-B27 positive (20). Interestingly, HLA-B27 negative AS is associated with another aminopeptidase—*ERAP2*—which is adjacent to *ERAP1* on chromosome 5 but in a separate linkage disequilibrium block. Given the clear functional interdependence of MHC class I molecules and these aminopeptidases it is perhaps unsurprising that there should be such obvious genetic interaction but there are actually remarkably few similar examples to date in the literature. Indeed, it was this synergy between HLA-B27 and ERAP1 that prompted others to look successfully for similar MHC/ERAP interactions in psoriasis, a condition with well-described genetic overlap with AS and SpA (22). Subsequently, similar findings have also been described in Behcet's syndrome between *ERAP1* and *HLA-B*51* (23).

2. *ERAP2* actually turns out to be associated both with HLA-B27 positive and negative AS (although it needs rather highly powered studies to prove it). There is a high frequency *ERAP2* null allele that results in about a quarter of Europeans having no functional ERAP2 although precisely how this affects susceptibility to AS is not currently known (24).
3. Altering the expression of ERAP1 or ERAP2 has a profound impact on the repertoire of peptides bound to MHC class I molecules, including HLA-B27 (25, 26). But how this relates to the pathogenesis of AS is also unknown. Any potential "arthritogenic peptide" remains highly elusive.
4. *ERAP1* polymorphisms that afford protection against AS are common loss-of-function variants with reduced aminopeptidase activity that are also likely to influence this repertoire (18, 27, 28). Consequently, there would appear to be scope for developing small molecule inhibitors of ERAP1 (and possibly other aminopeptidases) in the quest for new therapies for the prevention or treatment of AS.

A Credible GWAS Hit: Interleukin-23 Receptor

The same early GWAS (17) that identified *ERAP1* also revealed the first evidence of association between AS and the *IL23R* locus on chromosome 1, encoding the IL23-specific component of the of the heterodimeric IL23 receptor (the other component, IL12RB1, can also combine with IL12RB2 to form the IL12 receptor) (29). In the main part of this study, the initial strength of the association was weak (as is often the case in such relatively poorly powered studies), but it was subsequently amply confirmed and strengthened (20, 21, 30). Further, this *IL23R* association is recapitulated in other diseases, such as psoriasis and inflammatory bowel disease (IBD), which commonly occur in individuals with AS and/or their relatives, highlighting a degree of shared genetic background between these conditions (22, 31). The main SNP primarily associated with AS, psoriasis and IBD (*rs11209026*) causes a loss-of function mutation in the cytoplasmic tail of IL23R that reduces IL-17 and IL22 production by Th17 effector cells (32, 33) and modulates responses to pattern recognition receptors (34). These findings suggest that IL-23 driven pathways are implicated in AS, a finding supported by the subsequent identification of several other genetic associations with components of the Th17 lymphocyte developmental pathway, including *IL6R*, *TYK2, STAT3, IL1R1/2*, and *IL12B* (encoding the p40 fragment of IL12 that dimerises either with p35 in IL12 or p19 to form IL23). Coffre et al. suggest that the effector functions of Th1 and Th17 cells are affected by multiple variants at genetic loci associated with the IL23-driven pathway, including *IL23R, IL12B, CCR6, IL17A/F, IFNG, IL12RB2, TBX21*, and *RORC* (35).

These findings support the case for targeting various components of the IL23 pathway as a means of treating AS. Further, since many of the same genetic associations are also found in psoriasis and IBD (36) similar therapeutic strategies might also be expected to be fruitful in these conditions. However, the results have proved somewhat unpredictable and indicate substantial complexity in the relevant biological pathways and their involvement not only in their effects in the various related forms of SpA but also on the associated skin and bowel disease. Thus, targeting of IL-17 (the main pro-inflammatory cytokine associated with terminally differentiated Th17 cells) with the therapeutic monoclonal antibodies secukinumab or ixekizumab has proved highly successful in AS (37, 38) and psoriasis (39) but not IBD (40). Targeting the p40 subunit common to both the IL23 and IL12 receptors (thereby blocking both IL12 and IL23) has proved disappointing in AS and axial SpA (41, 42) in contrast to its efficacy in psoriasis and IBD (43, 44). Finally, despite its success in treating psoriasis, psoriatic arthritis and IBD (45, 46) the therapeutic antibody risankizumab, which targets the p19 fragment of IL23, is ineffective in AS (47). It is therefore interesting that AS does not show the same genetic association with IL23 as psoriasis (36), perhaps suggesting that Il-23 itself is important in psoriasis while IL23R and downstream signalling pathways are rather more relevant to the pathogenesis of AS.

A Second Association at the IL23R Locus?

More detailed genomic studies have revealed other associations near *IL23R* independent of *rs11209026* in the intergenic region between *IL23R* and the neighbouring *IL12RB2* gene (tantalisingly encoding the IL12-specific component of the IL12 receptor - see above). The associated SNP - rs11209032 - lies in a regulatory region, including a transcription factor binding-site for TWIST1, and appears to increase Th1 cell differentiation but, so far, its role in the pathogenesis of AS is unclear (48, 49). The International Genetics of AS (IGAS) Immunochip study in 2013, which fine mapped ~200 loci of known importance in immune responses and inflammation, revealed that such complex associations with more than one SNP independently associated with AS at a given locus are not uncommon (21).

Other "Hits" With Immunological Relevance: IL7R (IL7 Receptor α Chain) CSF2 (Granulocyte-Macrophage Colony-Stimulating Factor), and GPR65 (G-Protein Coupled Receptor 65)

Unsurprisingly the IGAS Immunochip study identified or confirmed genome-wide associations with many other loci implicated in immune/inflammatory conditions (because, after all, that was what the "Immunochip" was designed to do). For example, the "suggestive" AS association with *rs6897932* in *IL7R* mirrored similar genome-wide significant associations of *IL7R* with multiple sclerosis and primary biliary sclerosis (21, 50, 51). The "C allele" affects differential splicing of the 6th exon in the transmembrane domain of IL7R and increases the amount of both membrane-bound and soluble IL7R. Soluble IL7R increases the half-life of IL7, which plays a key role in T-cell immunity. Synovial fluid monocytes from patients with SpA have increased levels of IL7R and a transcriptome profile that overlaps with IL-7-induced gene sets (52). Type 3 innate immune cells expressing

IL7R are also increased in the synovial tissues of patients with SpA, and these cells produce GM-CSF (granulocyte-macrophage colony-stimulating factor) after *in vitro* stimulation (53). Targeting GM-CSF with therapeutic antibodies has already been shown to be effective in rheumatoid arthritis (54) and would therefore appear to be an obvious target in SpA as well (one such antibody—namilumab—is currently under investigation in the Namaste Trial—ClinicalTrials.gov Identifier NCT036226658).

Further evidence supporting a role for GM-CSF in AS also comes from the genetic association with *GPR65* (encoding a G-protein-coupled receptor involved in proton sensing) in the IGAS Immunochip study (21). Although it was not appreciated at the time GPR65 plays an integral role in regulating GM-CSF expression. Single cell genomics reveals that it is also crucial to the pathogenicity of Th17-cells in murine experimental allergic encephalomyelitis (55). Th17-cells are pleiotropic; there are increased numbers of GM-CSF secreting $CD4^+$ and $CD8^+$ lymphocytes in the synovium and peripheral blood of patients with SpA, and also increased numbers of IL-$17A^+$/GM-CSF^+ double-positive $CD4^+$, $CD8^+$, γδ and NK cells. GM-CSF^+CD4^+ lymphocytes express GPR65 irrespective of whether they co-express IL-17A (53). Silencing *GPR65* in primary CD4-cells results in reduced GM-CSF expression and so it may also be an important potential therapeutic target for SpA.

A Plausible Association Without Functional Corroboration: NOS2 (Inducible Nitric Oxide Synthase)

The Immunochip study showed a convincing peak of association with SNPs upstream of the *NOS2* gene (21), which has previously been associated with susceptibility to infectious diseases, such as leishmaniasis, and inflammatory diseases in mice (56). *NOS2* is also associated with IBD where its expression in the gut mucosa is highly dysregulated (57). In contrast to mice, human macrophages appear not to have the same inducible up-regulation of *NOS2* (despite the application of many different conditions and stimuli, in the hands of one of us—CD). The *NOS2* genetic association appears solid and lies in a region upstream of the gene likely to have regulatory functions BUT (1) "Is this region actually regulating *NOS2* or another gene?", (2) "Are the conditions necessary to induce *NOS2* in human macrophages highly specific and different from those that we have tried so far?", or (3) "Is the effect on *NOS2* expression manifest in a different cell type from those we have explored to date?". With regard to the latter, it is interesting that around two-thirds of patients with AS have subclinical inflammation of the terminal ileum so perhaps the gut mucosa might be a more productive place to look (58).

A Strongly Associated Locus With Relationship to Immune Cell Development: RUNX3 (Runt-Related Transcription Factor 3)

The challenge of identifying a mechanistic explanation for genetic disease associations is hard enough when there is a clear functional effect arising from a protein-coding change, as in the case of *rs30187* in *ERAP1* or *rs11209026* in *IL23R*, or for that matter HLA-B27. Far more often the lead SNP in such associations lies outside the coding sequence, most likely in regions concerned with the regulation of gene expression—but "Which genes?" and "How are they regulated?" are huge issues. Such *cis*-regulatory elements are most likely to control the activity of neighbouring or nearby genes, but their influence could extend even megabases down the chromosome. These issues are well exemplified by the *RUNX3* association with AS.

RUNX3 is one of the family of multifunctional RUNX transcription factors that play key roles in the development and differentiation of many cell types, including many immune phenotypes. It has been strongly associated with AS by GWAS (20), and the lead SNP mapped more accurately in the Immunochip study to a region with characteristics of an enhancer upstream of the promoter (21, 59). Careful examination of this region reveals that there are at least two independent neighbouring AS-associated SNPs that affect the binding of different transcription factors. Further, despite the fact that they are only 500 base pairs apart, these two distinct SNPs appear to exert their influence in different cell types—*rs4648889* in CD8+ T-cells and *rs4265380* in monocytes (60). The challenge now is to translate this into a better understanding of the regulatory framework of genes involved and how this affects the pathogenesis of AS. Fortunately the science of "genomics" now provides a wealth of publicly available data relating to the regulation and expression of genes in specific cell types that facilitate these investigations. These include (1) eQTL (expression quantitative trait loci) mapping that relates gene expression to particular SNPs in particular cell types, such as monocytes (61), (2) areas of "open" chromatin (DNAse 1 hypersensitivity sites), (3) transcription factor binding-sites and (4) other chromatin modifications, such as histone methylation or acetylation, that indicate the activity status of genes and their enhancers (62, 63). All of these can potentially be used to cross-reference functional gene activity at the cellular level with disease-associated SNPs to pursue the ultimate aim of discovering relevant disease pathways and how they might be therapeutically manipulated.

In our lab, we have so far demonstrated that the *RUNX3* AS-associated SNP rs4648889 (above) mediates differential allelic binding of two regulatory factors/complexes to a putative enhancer in the region upstream of the promoter: (1) the transcription factor interferon regulatory factor (IRF) 5, which binds preferentially to the AS-protective "G" allele; and (2) components of the nucleosome remodeling and deacetylase (NuRD) complex (one of the four major ATP-dependent chromatin remodeling complexes that function as transcriptional repressors) bind preferentially instead to the AS-risk "A" allele at rs4648889 (64). Further work is necessary to confirm the functional consequences of this SNP on gene expression and the network of genes involved but preliminary experiments suggest that IRF5 knockdown in CD8+ T-cells reduces the expression of interferon gamma. Discovering new

drug targets by this type of reverse genetics represents a daunting challenge that will require many different approaches and techniques. Identification of the disease-associated SNPs by statistical techniques is hard enough but further progress towards a mechanistic explanation for these GWAS associations will undoubtedly require: (1) precise identification of the primary functional genetic variants involved (within an AS-associated LD block); (2) their effects on gene expression in specific cell types (transcriptomics); (3) their effects on protein translation (proteomics); and (4) how these vary in response to different stimuli (metabolomics). The majority of AS-associated loci exert only very small effects on predisposition to the disease, most likely through quite subtle regulatory effects on gene transcription. These will inevitably still need to be assessed in more complex cellular systems and relevant animal models. Nonetheless, even at this early stage of the investigation of *RUNX3* there are already hints that both CD8+ T-cells and monocytes might constitute plausible cellular targets for intervention in AS (60). Credible molecular targets have yet to emerge.

A Replicated GWAS Hit Without an Obvious Explanation: ANTXR2 (Anthrax Toxin Receptor 2)

Among the numerous genetic associations with AS are many that defy obvious explanation. The SNPs lying in an extended linkage disequilibrium block including the entire *ANTXR2* gene is an excellent example. The initial positive association found by the Triple A (Australo-Anglo-American) spondyloarthritis consortium (TASC) has been amply replicated in independent studies but it has been difficult to decide precisely which SNP is most closely associated with the disease (19, 65). Our limited knowledge of the biology of the protein does little to offer an explanation for its genetic association with AS. In addition to its role as a potential receptor for the anthrax toxin it appears to be involved in capillary morphogenesis. *ANTXR2* mutations also cause the rare monogenic hyaline fibromatosis syndrome (Online Mendelian Inheritance in Man catalogue number—228600), in which there are widespread subcutaneous nodules and other internal organ involvement, but none of this gives many clues as to whether or how it might be involved in AS. So far it is not even clear whether these SNPs are actually involved in the actions of *ANTXR2* or another gene in the vicinity. This is a common issue in providing mechanistic explanations for many GWAS "hits".

AS Genetics in Clinical Practice

Diagnostic Testing

A role for HLA-B27 in the diagnosis of AS is well established but its use should be implemented with care; the sensitivity and specificity of HLA-B27 testing is clearly related to the pre-test probability that an individual might have AS. Used as a screening test for AS on all individuals with low back pain in the community it is quite unhelpful, but if limited to individuals with clinical features suggestive of the condition it is very useful. People in whom the condition is suspected can be placed in a "suspicious" group according to their responses to a few simple questions. These include: (1) Chronicity (low back pain > 3 months), (2) Alternating buttock pain (indicative of SI joint inflammation), (3) Improvement with gentle exercise or anti-inflammatory analgesics, (4) Back pain interfering with sleep in the second half of the night, (5) Onset aged less than 40 years of age, (6) Affected first-degree relative, (7) Presence of co-morbidities known to be associated with AS, such as psoriasis, IBD or uveitis. Individuals with positive responses to these questions have a much higher pre-test probability of AS than others with low back pain in the community, and in those with 4 or more positive responses an additional positive HLA-B27 result may increase the likelihood of AS to over 90%. This can be further increased by the finding of SI joint inflammation on MRI. However, even with the combination of clinical questions, HLA-B27 testing and MRI the diagnosis is either missed or incorrect in about 5% of cases (6). The diagnosis is accurately made in only a third of patients in the first year of symptoms and is frequently delayed by 6 years or more (5). Brown et al. (66) have nicely reviewed the state of the art relating to biomarker development in AS, including genetic testing. They highlight the utility of HLA-B27 testing but suggest that polygenic risk scores (PRS), which additionally use all the other SNPs associated with AS, can give an even better positive predictive value (67). Using this approach, they and others have convincingly demonstrated that using 110 SNPs with reported genome-wide association to AS (including HLA-B27) is significantly more discriminatory than HLA-B27 alone in the diagnosis of AS. However, the difference is relatively small and of unproven clinical value (68). In contrast, a few well-chosen questions (see above) designed to identify those with high likelihood of AS/axSpA prior to implementing any sort of genetic testing are worth their weight in gold.

Prognosis

Prediction of the prognosis and outcomes of treatment in AS are long-term goals that could be facilitated by genetics since we already know that the severity of the disease is highly heritable and certainly not determined exclusively by HLA-B27 status (69). There is some evidence that outcomes from biologic therapies are better in HLA-B27 positive patients and that positive responses to secukinumab may be influenced by the *ERAP1* risk allele at *rs30187* (37, 66). However, these conclusions have been drawn from small studies and clearly require replication. We have also investigated a SNP in *TNFRSF1A* (encoding the p55 TNF Type 1 receptor) for its potential to influence not only susceptibility to AS but also its severity and responsiveness to anti-TNF biologics. The "G" allele of rs1800693 is associated with susceptibility to multiple sclerosis but protection against AS (20, 70, 71). It causes skipping of exon 6 resulting in a truncated soluble form of the protein with potential anti-inflammatory properties, mimicking the action of the anti-TNF fusion protein etanercept; this is particularly interesting given the possible association between anti-TNF biologic therapy and central nervous system demyelination (70, 72). However, the

rs1800693 polymorphism in *TNFRSF1A* neither appears to affect the severity of AS nor its response to anti-TNF biologics (73). In order to characterize such genetic influences on responses to therapy it may well be necessary to examine far larger case series than has been done to date.

Longstanding Conundrums

Why Does Not Everyone With HLA-B27 Get AS?

The aetiology of AS clearly involves other genetic and/or environmental factors than just HLA-B27. Twin studies indicate its polygenic nature, which is one explanation for why only around 5% of those with HLA-B27 develop AS. Estimates of broad-sense heritability suggest that over 90% of the population variance can be attributed to genetic factors (13) but this does not preclude the involvement of common environmental influences, such as infections, in its aetiology. It merely suggests that any such extrinsic factors are likely to be so common (like certain viral infections)? that they do not greatly influence the population variance (at least in developed Western societies). Whether this is always the case is a moot point. There are some exceptions to the general rule that the prevalence of AS mirrors that of HLA-B27 in the population. Thus, in The Gambia in tropical west Africa AS is exceptionally rare (as it is in much of sub-Saharan Africa) (3), but in contrast to many other African countries the frequency of HLA-B27 in The Gambia is at least 6% (not so very different from ~8% in the UK). The low Gambian prevalence of AS was initially attributed to the existence of an unusual HLA-B27 variant—*HLA-B*2703*—with potentially different functional characteristics from the *HLA-B*2705* allele, which is predominant in European populations (74). However, on closer inspection at least half of the B27-positive individuals in The Gambia actually carry the "European" *HLA-B*2705* allele, making it far from rare in that population (75). Another explanation for the rarity of the condition in this population is therefore necessary: perhaps there is some other genetic factor in this population or, more likely, something different about the Gambian environment that affords protection against the disease.

What About the Gut?

There has been much interest in the possibility of a link between the gut and AS for many years. One of us remembers the excitement at The Middlesex Hospital in London after early reports that faecal carriage of *Klebsiella* sp. was associated with active disease. However, these studies provoked strong views on either side, particularly relating to whether this could be explained on the basis of cross-reactive "autoimmune" responses (76, 77). Nevertheless, many lines of evidence point towards gut involvement in SpA and much current research. For example, IBD is often complicated by various forms of peripheral and axial arthritis, the onset of which may be before, concurrent or afterwards. Curiously, there are quite distinct clinical features to these various forms of arthritis. The type 1 peripheral arthropathy of IBD (similar to reactive arthritis in its asymmetric, pauciarticular, predominantly lower limb distribution) is strongly associated with HLA-B27 as is the axSpA associated with IBD, but the former runs a course mirroring activity of the IBD in contrast to the axSpA, which is independent of IBD activity (78, 79). In the type 2 peripheral arthropathy of IBD (polyarticular, upper and lower limb distribution), joint disease activity is also not linked to activity of the IBD and it has a distinct immunogenetic profile (not associated with HLA-B27 but rather with HLA-B44 (80). In our sample of ~8,500 cases of AS from the UK there is co-existent clinically overt IBD in ~10–15%, which is at least partly due to their shared genetic background (36). In other studies, two-thirds of those with AS without overt IBD exhibit subclinical histological gut inflammation (81). There is also some circumstantial evidence from long-term observational studies that a minority of individuals with reactive arthritis (usually a self-limiting condition triggered by infection in the gut or urogenital tract) may progress over time to axSpA/AS (82). Attempts to identify specific causative agents in the gut, such as *Klebsiella* sp., have largely proved unsuccessful but there is still much interest in the potential role that the gut microbiome might play in AS and its potential role in mediating local and systemic inflammation in SpA [reviewed in (66, 83)]. Wholesale sequencing of gut bacteria suggests that the gut microbiome in AS can be distinguished from the normal population and may have some correlation with disease activity (84–88). However, whether these results are truly disease specific must also take into account that the HLA alleles associated with AS (and also those associated with rheumatoid arthritis) have a significant impact on the host gut microbiome in healthy individuals too (89).

What Is the Evidence for a Specific Antigenic Stimulus in AS?

The strong HLA-B27 association with AS suggests that adaptive immune responses are important in its pathogenesis but any "arthritogenic peptide(s)" has so far proved elusive. Evidence for antigen-driven specific immune responses in the HLA-B27 associated arthropathies, is not new (90, 91) but the development of high throughput sequencing to assess the T-cell receptor repertoire has seen a recent resurgence of interest. Of particular interest, TCR binding motifs from some patients with AS show similarities with those identified previously in individuals with reactive arthritis (92–95). There is also evidence of a significant increase in CD8+ T-cell clonotypes specific for the Epstein-Barr and cytomegalovirus (92). In this regard it is therefore interesting that recent studies have identified conventional CD4+ and CD8+ T-cells resident at the entheses in humans that have regulatory phenotypes and reactivity against common viruses, including cytomegalovirus (particularly CD8+ T-cells) (96).

What Is the Role of HLA-B27 in AS?

Fifty years after it was first described the mechanism(s) underlying the strong association of AS with HLA-B27 still requires a truly convincing explanation. We have certainly learned a lot about this molecule in the intervening years—

crystal structure, the peptide repertoire it binds, its unusual folding characteristics, and interactions with receptors on innate immune cells—but where has this left us? Although there is some evidence of specific antigen presentation (see above) this is certainly inconclusive. Other theories have drawn on some of the atypical features of HLA-B27 among MHC class I molecules – in particular, its relatively slow folding and tendency to form homodimers. For a more detailed description of these theories the reader is referred elsewhere (97–99). Briefly, in addition to its role in antigen presentation HLA-B27 is unusual in its folding kinetics; unusual forms can accumulate in the endoplasmic reticulum causing an unfolded protein stress response, which can lead to IL23 production in dendritic cells. Similar responses have been observed in macrophages in the transgenic rat model of SpA (100). This theory provides a neat explanation for the apparent lack of antigen specificity in animal models of SpA (99) but is far from settled given the lack of evidence of UPR in gut epithelial cells from individuals with AS (100). HLA-B27 is also unusual in its ability to form homodimers or free heavy chains that can be recognized by killer-immunoglobulin-like receptors (KIR), which are mainly expressed on NK cells but also on CD4+ T-cells (101, 102). People with AS have a higher frequency of T-cells expressing this receptor and these are also polarized towards the Th17 phenotype that is associated with AS (97). Of interest, ERAP1 variants associated with protection against AS reduce HLA-B27 free heavy chain expression on monocytes and potentially reduce Th17 activity (103).

Bone Modeling and HLA-B27

Only a few tentative genetic associations that have been reported between AS and genes involved in bone modeling to date. Weak associations have been described with *RANK* (receptor activator of NF kappa B involved in osteoclast development) in Caucasians and *RANKL* (RANK ligand) in Chinese (104, 105). However, another recent paper suggests that HLA-B27 is involved in the activation of *TNAP* (encoding the enzyme alkaline phosphatase) in mesenchymal stem cells obtained from syndesmophytes of patients with AS. This led *in vitro* to accelerated mineralisation in a manner that was independent of the key osteoblast transcription factor RUNX2. Further, in an animal model, this process could be inhibited by bisphosphonates, a group of drugs commonly used in the treatment of osteoporosis, thereby holding considerable promise of a treatment that could retard the abnormal ossification and ankylosis associated with AS (106).

CONCLUDING REMARKS

It may be argued that so far, we have actually learned more about the treatment of common diseases from studying rare, phenotypically severe, monogenic conditions than from the genetics of common polygenic diseases like AS. There have certainly been some spectacular successes. First, the development of therapeutic RANKL (receptor activator of NFκB-ligand) antibodies (denosumab) for the treatment of osteoporosis, for which the insights came from very rare osteolytic bone diseases (familial expansile osteolysis—OMIM 174810) affecting the RANK/RANKL axis of osteoclast development (107). Second, anti-sclerostin antibodies (romosozumab) have also been successfully developed for the treatment of osteoporosis (108), based on the observation that loss-of-function mutations in sclerostin (a bone morphogenetic protein antagonist) were responsible for massive accumulation of bone in the rare recessive disorder, sclerosteosis (OMIM 269500). It is unsurprising that polygenic diseases have proved harder nuts to crack. Nevertheless, much progress has been made in AS already thanks to a hugely collaborative global effort (**Figure 3**).

If we have learnt anything about the study of complex diseases in the past 20 years, it is that size matters when it comes to genetic studies. With the assistance of various international consortia, we can generate sample sizes that now have the power reliably to detect loci increasing the risk of AS by 5% or less. Similar efforts will probably be essential to identify any genetic influences on therapeutic outcomes. Novel strategies for identifying susceptibility genes include increasing the power of such studies by combining cohorts of genetically related diseases, such as AS, psoriasis, IBD and sclerosing cholangitis. Individual loci identified in this way can then be individually tested in the specific disease subsets. The number of loci incriminated in AS has been increased to more than 100 in this way (36). Efforts to increase the number of cases for these studies have continued, and it is hoped that the latest GWAS from the IGAS consortium will present data from ~ 20,000 cases in the next 12 months. Translating these results into therapeutic targets will remain problematic but continuing advances in the field of functional genomics hold much promise for progress in this field (109). Detailed analysis and discussion of these issues is beyond the scope of this review, so the interested reader is referred to the 30th July issue of Nature that contains no fewer than 10 relevant articles on the subject [Nature 2020; vol 583: issue 7818]. As an example of what can be achieved, many of the

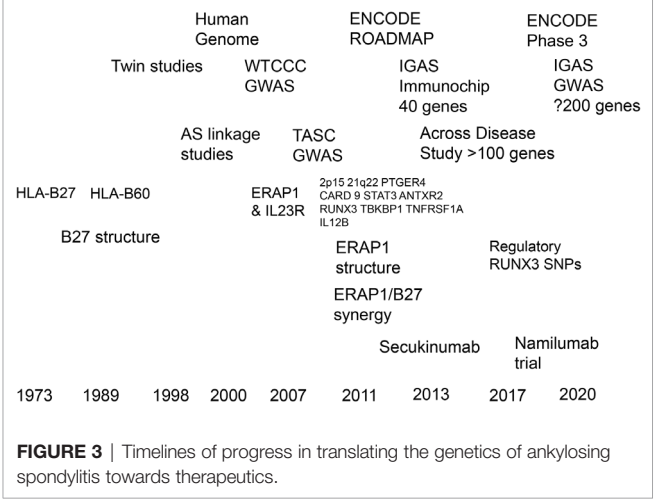

FIGURE 3 | Timelines of progress in translating the genetics of ankylosing spondylitis towards therapeutics.

associated genetic loci in another complex rheumatic disease—rheumatoid arthritis—have recently been shown to have complex chromatin interactions and effects on gene expression, specifically in T-cells. Further, using a multiomic approach, new genes not previously implicated by GWAS, such as *MYC* and *FOXO1* have been identified in the pathogenesis of the disease (110). In AS, even the original MHC association with HLA-B27 has been shown to be far more complex; there are numerous associations with both Class I and II alleles, and additional epistasis with ERAP1 (111). With a few exceptions (105–107, 112) most translational work in AS genetics has concentrated to date on its immunological and inflammatory contributions but, given that much of the pathology and the ensuing disability is caused by abnormal bone deposition, there is a strong case for investigating this aspect of the disease more intensively.

AUTHOR CONTRIBUTIONS

BPW and MV conceived the manuscript. BPW, MV, CD, and CJC drafted the manuscript. All authors contributed to the article and approved the submitted version.

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From the Genetics of Ankylosing Spondylitis to New Biology and Drug Target Discovery

Zaarour Nancy[1†], *Li Yan*[2†], *Shi Hui*[1], *Bowness Paul*[1] *and Chen Liye*[1*]

[1] *Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom,* [2] *Department of Rheumatology, The First Affiliated Hospital of Xiamen University, Medical College of Xiamen University, Xiamen, China*

Genome-wide association studies (GWAS) have identified 113 single nucleotide polymorphisms (SNPs) affecting the risk of developing ankylosing spondylitis (AS), and an on-going GWAS study will likely identify 100+ new risk loci. The translation of genetic findings to novel disease biology and treatments has been difficult due to the following challenges: (1) difficulties in determining the causal genes regulated by disease-associated SNPs, (2) difficulties in determining the relevant cell-type(s) that causal genes exhibit their function(s), (3) difficulties in determining appropriate cellular contexts to interrogate the functional role of causal genes in disease biology. This review will discuss recent progress and unanswered questions with a focus on these challenges. Additionally, we will review the investigation of biology and the development of drugs related to the IL-23/IL-17 pathway, which has been partially driven by the AS genetics, and discuss what can be learned from these studies for the future functional and translational study of AS-associated genes.

Keywords: ankylosing spondylitis, GWAS, functional genomics, IL-23/IL-17 axis, drug target, IL-1beta, genetics

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***Correspondence:**
Chen Liye
liye.chen@ndorms.ox.ac.uk

[†] *These authors have contributed equally to this work*

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INTRODUCTION

Ankylosing Spondylitis (AS) is a common form of immune-mediated arthritis that predominantly affects the sacroiliac and spinal joints and can result in excessive ossification of the affected tissues. Over the past decade the successful introduction of new treatments for AS (therapeutic monoclonal antibodies targeting tumor necrosis factor (TNF)-α and interleukin (IL)-17A) has highlighted some of the important pathological pathways involved. However, <50% of patients achieve good response (ASAS40) to either TNF-α or IL-17A blockade (1, 2). More importantly, there is no cure for AS and most patients require lifelong medication (with consequent potential adverse effects) to control their symptoms. Therefore, identifying novel therapeutic targets could have important benefits for patients with AS.

The value of genetics in drug discovery is increasingly appreciated (3, 4). The induction of IL-17A blockade in AS was partially driven by genetic studies showing multiple disease associations with genes involved in IL-23/IL-17A pathways (e.g., IL6R, IL23R, TYK2, IL1R1/2, IL27, STAT3) (5). Genome-wide association studies (GWAS) have already identified 113 single nucleotide polymorphisms (SNPs) affecting the risk of developing AS (6, 7). To date, there is a plausible explanation for only a minority of these genetic associations, substantially impeding their translation into therapeutic options.

The functional investigation of genetics association currently encountered a number of challenges: (1) difficulties in determining the causal genes regulated by disease-associated SNPs, (2) difficulties in determining the relevant cell-type(s) that causal genes exhibit their function(s), (3) difficulties in determining appropriate cellular contexts to interrogate the functional role of causal genes in disease biology. This review will discuss recent progress and remaining challenges. While appreciating the importance of identifying causal SNPs, limited by the length of this mini-review, we choose to refer readers to recent review rather than discuss this topic here (8). Following the identification of causal genes and related cellular contexts, immunological research is vital for drug discovery. We will use the IL-23/IL-17 pathway as an exemplar, in part driven by the AS genetics, and discuss what can be learned from these studies for the future functional and translational study of AS-associated genes.

AS GENETICS

Genetic contribution to the development of AS was first known following the discovery of HLA-B*27 as a strong genetic risk factor in 1973 (9–11). In fact, the association was so strong that HLA-B*27 was, for a long time, considered to be the sole genetic factor predisposing individuals to AS. Till 2007, powered by the technical development in SNP genotyping and statistical analysis for GWAS, the first AS GWAS was competed (12). Although with a relatively small sample size (1,000 patients and 1,500 controls), this study identified two key non-MHC genetic risks: IL23R and ERAP1. These findings were subsequently confirmed in a study with a larger cohort, which reported two additional associations with chromosome 2p15 and 21q22 (13). In the same year, a study focusing on 53 known genetic risks in Crohn's disease, a condition clinically related to AS, identified two additional AS-associated loci: 1q32 and STAT3 (14). In 2011, the striking epistasis between ERAP1 and HLA-B*27 was found, along with seven additional genetic loci with strong associations with AS (15). The most recent findings were reported from the Immunochip project with the strategy of high-density genotyping of immune-related loci, which, in part using "multi disease" methodology, has increased the number of SNPs independently affecting the risk of developing AS to 113 (6, 7).

Overall, a significant body of knowledge of AS genetics has been generated over the last decade. This rich and high-quality source of genetic risk associations in AS will, after appropriate decoding, provide critical sights in AS biology and new drug targets.

TRANSLATING GENETICS TO NEW BIOLOGY AND DRUG TARGET DISCOVERY

Recent Technical Advances and Opportunities

In attempting to reveal the functional basis of genetic risks associated with human diseases, various techniques have been developed over the past few years. We believe that expression quantitative trait loci (eQTL), promoter capture Hi-C (PCHi-C), and HiChIP constitute key advances for the prediction of causal genes through the annotation of genetic risks (**Figure 1**).

Expression quantitative trait loci (eQTL) identifies genomic variants that contribute to altered expression levels of mRNAs. eQTL have been carried out using various primary human immune cells (monocyte, macrophage, dendritic cell, CD4, CD8, Treg, Th1, Th2, Th17, Tfh, B-cell, NK and neutrophil) in different cellular contexts (resting and activation) (16–21). These data constitute a rich eQTL data resource which can be integrated with summary data from AS GWAS studies for the prediction of the causal genes (22, 23). Of note, eQTLs are only present in a proportion of GWAS SNPs (24, 25), highlighting the need for additional approaches to link SNP to gene.

The development of chromosome conformation capture (3C) and its related techniques, such as Hi-C, has allowed the detection of long-range regulatory DNA interactions (26, 27). To overcome the nature of complexity and high-cost of Hi-C, promoter capture Hi-C (PCHi-C) has been developed, combining Hi-C with hybridization-based capture of targeted genomic regions (28). PCHi-C has been carried out for various diseases using relevant tissues/organs and/or cells (29–31), but not yet in AS. One dataset, which we believe will be of particular value for AS research, provides high-resolution maps of promoter interactions at the genome-wide level in 17 human primary blood immune cell types (32). HiChIP is another technique derived from Hi-C which incorporates ChIP-seq—allowing the enrichment of chromatin looping events based on histone modifications (33). H3K27ac HiChIP has been applied to naïve CD4, Th17, and Treg cells to reveal T cell subtype-specific enhancer–promoter interactions (34). These enhancers often contact genes beyond their nearest neighbor gene–highlighting the importance of SNP annotation using functional genomic datasets. Thus, we believe that the integration of chromatin looping datasets and AS GWAS findings provides a potent approach to predict the causal genes.

Determination of *disease-relevant* cell-types for functional investigation is a key challenge impeding the translation of genetic findings to new biology and therapeutic options. Some causal genes identified by eQTL or chromatin looping datasets will be limited in their action to specific cell-type(s), guiding the selection of cells to be investigated (**Figure 1**). However, this information is not always available. In such a scenario interrogation of chromatin accessibility (DNase hypersensitivity assay or ATAC-seq), DNA methylation and histone modifications will be of great use following mapping with GWAS SNPs (**Figure 1**). The latter include enhancer (e.g., H3K4me1), promoter (H3K4me3), and active enhancer and promoter marks (H3K27ac).

Precise functional testing of predicted causal genes requires knowledge of cellular context(s). This is particularly important for genes where existing knowledge of function is limited, a common situation for GWAS hits. To this end, transcriptional data from patient-derived cells and/or particular disease-related cell-types, such as Th17 cells in AS, would be of great use. For example, if a causal gene is elevated in Th17 cells, one would predict it to be a possible Th17 regulator and test its function in a Th17 functional cellular assay. Single cell RNA

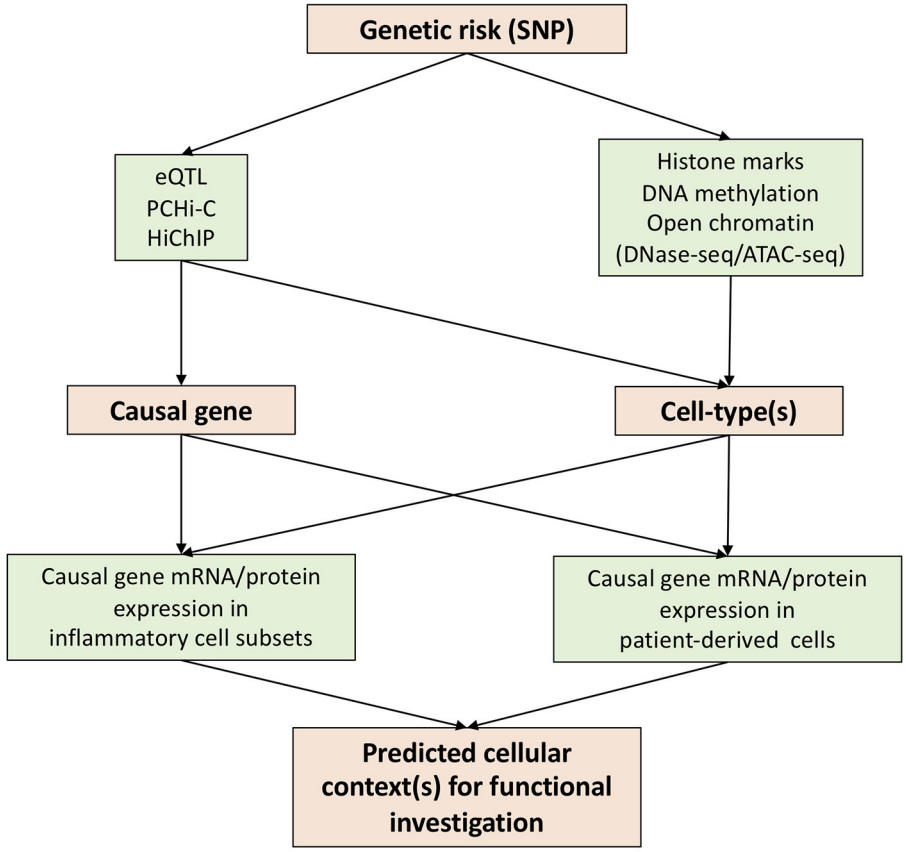

FIGURE 1 | Strategies to translate genetic risk to novel biology.

sequencing (scRNA-seq) would excel here in the provision of gene abundancy data for multiple cell subsets in patient blood or synovium. However, a well-known drawback of scRNA-seq is its inability to detect genes in low abundance. Antibody (CITE-seq) or oligo (BD Rhapsody)–based tagging of genes of interest might go some way to solve this problem. In addition, for genes with available antibodies, mass cytometry or CyTOF is an alternative approach to acquire the expression profile of a gene at the protein level.

Remaining Challenges and Possible Solutions

eQTLs are frequently different in different cell types. For example, the eQTL link of GAB2 gene with rs2511162 is found for naïve B cells and T cells, but not monocytes (19). Even within one cell-type, eQTLs can be highly context-specific. For example the AA genotype at rs1179625 is associated with higher basal mRNA HIP1 levels in naïve monocytes, but reduced HIP1 upregulation in lipopolysaccharides (LPS)-stimulated monocytes (16). Of note, the difference in context is not limited to resting vs. stimulation but may be highly time dependent for a cell-type treated with the same stimuli. For examples, the eQTL linking rs2275888 with IFNB1 gene transcription is present in monocytes after 2 h LPS-stimulation but not in resting monocytes or those cultured with LPS for 16 h (16). As mentioned in the previous section, cell-type and context specificity are also present in chromatin looping datasets (PCHi-C and HiChIP). Thus, although current eQTL and chromatin looping datasets have included various conditions for an individual cell-type, they cannot possibly cover all the complex and dynamic microenvironments present in human diseases including AS. Given the high probability of the presence of AS-specific genetic regulations, this knowledge will be crucial in advancing our understanding of the impact of genetic risks on AS biology and unraveling novel mechanisms and therapeutic options. To this end, we propose that functional genomics datasets should ideally be generated using cells from blood or even joint of patients with AS for the provision of disease-specific insights.

Evidence suggesting key roles for rare immune cell populations in AS has recently emerged,. For example invariant NK cells (iNKT) and γδ T cells have recently been reported to be a major source of IL-17 in the inflamed joint (35). These innate-like T cells are phenotypically and functionally different from conventional T cells, thus would likely have distinct gene expression mechanisms. Neither eQTL nor chromatin looping datasets have been generated for these un-conventional cell

types, and we propose that coordinated efforts to generate functional genomic datasets for these cells should be made by the scientific community.

Even within one cell-type, specific subsets might be highly relevant to the pathogenesis of human diseases. For example, using single cell RNA sequencing (scRNA-seq), MerTK+ synovial tissue macrophages have recently been shown to be key for the remission of rheumatoid arthritis after treatment cessation (36). Thus, scRNA-seq-based eQTL studies carried out using patient blood and/or tissue derived cells will be of great value. This approach was first reported in 2018 for a small cohort of 45 healthy donors (37). More recently, the single-cell eQTLGen consortium has been established and will provide standardized pipelines and guidelines for single-cell population genetics studies (38).

Most functional genomics data are at the DNA or RNA level. This does not invariably relate to cellular and cell surface protein expression. Advances in quantitative MS might allow QTL at the protein level. Indeed, quantitative proteomics has been utilized to advance knowledge in biology, such as the dynamic protein landscape of human Th17 differentiation (39), and the underlying mechanism of Myc controlling T cell proteomes and metabolic pathways (40).

It will also be important to contextualize the anatomical location of immune cells and their detailed functional interactions. The human tissue atlas will provide a framework and detailed spatial transcriptomic and protein expression studies of diseased tissue including entheses will undoubtedly enrich current knowledge. Without doubt the greatest knowledge gains will flow from the study of cells from inflamed tissues. We believe that obtaining these from human diseased tissues will be more informative given the limitations of current animal models and the rapid advances in single cell technology.

Moving from tissue level understanding to whole organism will be a further challenge. Animal models of AS have proved useful for studying specific pathogenic processes and offer opportunities for intervention. The HLA-B27 transgenic rat and the SKG mouse have both provided key insights, with the former model confirming the role of HLA B27, myeloid cells and gut flora in disease and the latter confirming the key role for the IL-23-17 pathway (see below). Considering both animal models and human studies it will also be important to distinguish the relative roles of tissue-resident and tissue-specific cells from those of circulating cells. We believe that using animal models to label leucocytes present in the gut mucosa (e.g., with photobleaching or fate mapping) and then follow their potential movement to joints and other inflammatory sites is likely to offer major insights into disease pathogenesis. Ultimately human experimental medicine studies will prove the key arbiters of target selection and will provide a rich source of data.

THE IL-23/IL-17 PATHWAY AND AS

IL-23/IL-17 Pathway

IL-23 is formed by P19 and P40 subunits with the later, along with P35, also forming IL-12 (41). IL-23 signals through the IL-23 receptor composed of IL-23R and IL-12Rβ1. IL-12 drives the differentiation of Th1 cells, whereas IL-23 is crucial for the survival and expansion of Th17 cells and can induce IL-17 production in memory T cells (42, 43). Additionally, IL-23 also induces IL-17 production by γδ T, NKT and innate lymphoid cells (44–46). In line with this, murine models support the T cell-mediated pathogenic role of IL-23 in inflammation in multiple organs, including joints, gut, brain (47–49). Of note, both IL-23 and IL-17A are required for the development of Spondyloarthritis-like pathology in SKG mice, a T-cell driven AS model with inflammation in arthritis, enthesitis, and ileitis (50).

Relevance to AS Genetics

More than 90% of genetic risk SNPs are present in non-coding regions. Thus, IL23R, where genetic risk loci reside both within coding (the cytoplasmic tail) and non-coding regulatory regions, represents the exception rather than the norm. The genetic association of IL23R loci with AS was first reported in 2007 (12), the first elucidated being a coding change SNP, rs11209026, associated with Arg or Gln at position 381 of IL-23R protein. Interestingly, the same SNP also affects the risk of developing inflammatory bowel disease (IBD) (51), a condition closely linked to AS. Indeed, a subgroup of patients with AS develops IBD and the sub-clinical gut inflammation has been reported in over 60% of patients with AS (52). The same SNP is also associated with psoriasis, another condition closely linked to AS. The protective variant R381Q is associated with reduced function of IL-23R and Th17 response in both CD4 and CD8 cells (53).

Pre-clinical/Clinical Development of Inhibitors Targeting IL-23/IL-17 Pathway

Antibodies blocking cytokines or receptors related to this pathway have been extensively tested in AS. IL-17A blockers have demonstrated efficacy and been approved for the treatment of AS (54, 55). In contrast, IL-23 inhibitors either targeting P40 or P19, have failed to show efficacy in clinical trials (56, 57). These results were unexpected considering the efficacy of IL-23 blockers for Crohn's disease, Psoriasis and psoriatic arthritis, conditions related to AS and with IL23R as a genetic risk (58–61). Of note, IL-17 inhibition was ineffective in Crohn's disease (62), suggesting the IL-23 biology beyond the simple induction of IL-17 cytokine secretion.

The success of IL-17A blocking and failure of IL-23 inhibition in AS suggested that IL-23 might not be the main driver of IL-17A production in AS. In human, IL-1β and IL-6 are required for the differentiation of Th17 cells (63). Of interest, IL-1β was essential in pathogen-induced Th17 differentiation to prime IL-17+IFN-γ+ "pathogenic" Th17 cells (64). Additionally, along with IL-23, IL-1β induces IL-17A production by γδ T and iNKT cells (45, 65), the major source of IL-17A in synovial fluid of patient with AS (35). The recruitment of IL-1β-producing myeloid cells has been shown to be a key factor driving the IL-17 secretion by γδ T and CD4 cells in the central nervous system (66). Two pieces of evidence link IL-1β to AS pathology: (1) both IL1R1 and IL1R2 are predicted genetic risks in AS (13), (2) monocytes in blood from patients with AS spontaneously produce IL-1β (67). Thus, we propose a model explaining the possible IL-1β-driven IL-17 biology in AS (**Figure 2**). Monocytes stimulated by bacteria

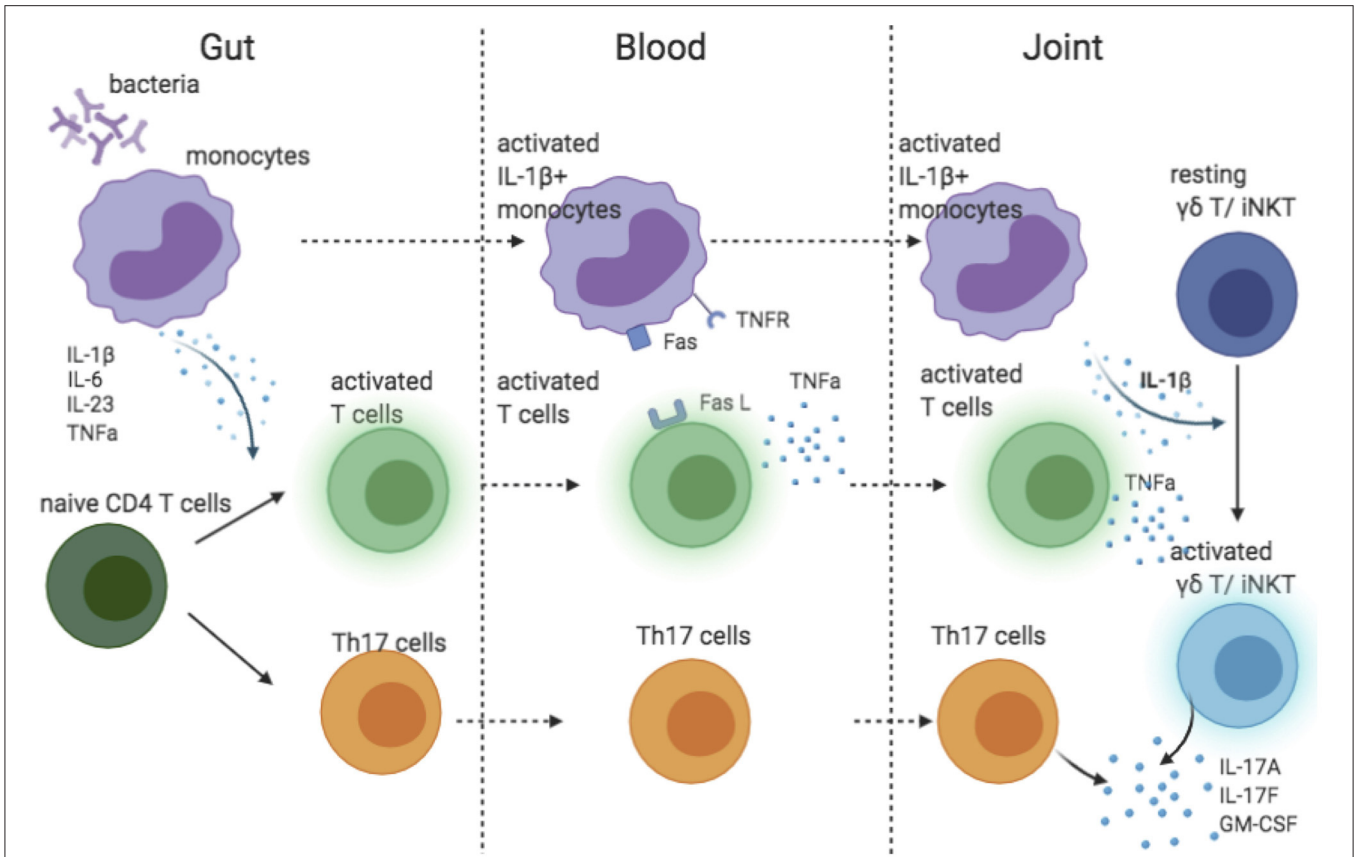

FIGURE 2 | Cartoon model suggesting possible role of IL-1 beta contributing to IL-17 response in AS.

in the gut produce pro-inflammatory cytokines that prime Th17 cells. Attracted by chemokines, IL-1β-secreting monocytes travel to the joint(s), where they activate γδ T and iNKT cells. Additionally, through a TNFR-Fas-caspase-8-dependent pathway, activated T cells also induce monocyte IL-1β secretion (68). However, IL-1β is unlikely to be the sole driver of IL-17 in AS because IL-1β inhibition was only effective for a subgroup of patients (69–71).

Lessons From IL-23/IL17

The therapeutic development of inhibitors targeting the IL-23/IL-17 pathway in AS highlights the notion that genetic risk alone is not necessarily the ideal guide to drug target identification and that downstream protein(s) might be better therapeutic options in some cases. Indeed the association of genetic risk with drug success in trails is substantially enhanced when proteins interacting with these risk-associated gene products are included (72). Considering the diseases that share IL23R risk associations, significant differences in therapeutic response to different agents have already emerged. The reasons why IL-23 neutralization proved highly beneficial in psoriasis but without efficacy (at least in initial trials) in Ankylosing Spondylitis, whereas IL-17 neutralization proved therapeutic in Psoriasis, psoriatic arthritis and ankylosing Spondylitis but not Crohn's disease have been discussed by Siebert and colleagues (73). Thus, it is increasingly clear that, following the identification of the causal genes, detailed understanding of the biological functions of the associated proteins in the context of both tissue site and stage of disease is crucial.

DISCUSSION

Exciting progress has been made in the genetics of AS, resulting in identification of over one hundred genetic variants that affect the risk of disease development. Entering the post-GWAS era, we have encountered multiple challenges and bottlenecks in the translation of GWAS findings to new biology and drug targets. With the rapid development of functional genomic techniques/methods and transcriptomic and phenotypic profiling of primary cells at single cell resolution, it is now possible to predict both causal genes and their relevant cell-type. This will allow us to more rigorously investigate the cellular contexts of disease pathogenesis and to functionally validate therapeutic targets. However, disease-specific functional genomic datasets and those for rarer immune cells are currently not available, representing opportunities for future research. The successful development of drugs targeting the IL-23/IL-17 axis for conditions genetically

associated with IL23R is a great example demonstrating the value of genetics in drug development. We also learned that causal genes are not always the best drug targets, highlighting the importance of establishing downstream pathways. Thus, an in-depth understanding of causal gene-related biology is absolutely crucial for the development of novel treatment options.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Role of the IL-23/IL-17 Pathway in Rheumatic Diseases: An Overview

*Claudia Schinocca[1], Chiara Rizzo[1], Serena Fasano[2], Giulia Grasso[1], Lidia La Barbera[1], Francesco Ciccia[2] and Giuliana Guggino[1]**

[1] Rheumatology Section, Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties, University Hospital "P. Giaccone", Palermo, Italy, [2] Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy

Interleukin-23 (IL-23) is a pro-inflammatory cytokine composed of two subunits, IL-23A (p19) and IL-12/23B (p40), the latter shared with Interleukin-12 (IL-12). IL-23 is mainly produced by macrophages and dendritic cells, in response to exogenous or endogenous signals, and drives the differentiation and activation of T helper 17 (Th17) cells with subsequent production of IL-17A, IL-17F, IL-6, IL-22, and tumor necrosis factor α (TNF-α). Although IL-23 plays a pivotal role in the protective immune response to bacterial and fungal infections, its dysregulation has been shown to exacerbate chronic immune-mediated inflammation. Well-established experimental data support the concept that IL-23/IL-17 axis activation contributes to the development of several inflammatory diseases, such as PsA, Psoriasis, Psoriatic Arthritis; AS, Ankylosing Spondylitis; IBD, Inflammatory Bowel Disease; RA, Rheumatoid Arthritis; SS, Sjogren Syndrome; MS, Multiple Sclerosis. As a result, emerging clinical studies have focused on the blockade of this pathogenic axis as a promising therapeutic target in several autoimmune disorders; nevertheless, a greater understanding of its contribution still requires further investigation. This review aims to elucidate the most recent studies and literature data on the pathogenetic role of IL-23 and Th17 cells in inflammatory rheumatic diseases.

Keywords: IL-23, IL-17, IL-23/IL-17 axis, inflammatory diseases, autoimmune diseases

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***Correspondence:**
Giuliana Guggino
giuliana.guggino@unipa.it

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REVIEW

Interleukin-23

Interleukin-23 (IL-23) is a member of the IL-12 cytokine family composed of the IL-23p19 subunit and the IL-12/23p40 subunit, the latter shared with IL-12, encoded by genes located on chromosomes 12q13.2 and 11q1.3, respectively (1–3).

IL- 23 is mainly secreted by activated macrophages and dendritic cells (DCs) located in peripheral tissues, such as skin, intestinal mucosa, joints and lungs (4–6).

Despite the protective role played by the IL-23/IL-17 axis against bacterial and fungal infections, extensive knowledge supports the contribution of its dysregulation in triggering chronic inflammation and autoimmunity, providing a solid substrate for the development of several autoimmune diseases like PsA, Psoriasis, Psoriatic Arthritis; AS, Ankylosing Spondylitis; IBD, Inflammatory Bowel Disease; RA, Rheumatoid Arthritis; SS, Sjogren Syndrome; MS, Multiple Sclerosis (3, 7–10) (**Table 1**).

The main role of IL-23 is to induce the differentiation of αβ T $CD4^+$ naïve cells (Th0 cells) in T helper type 17 (Th17 cells) (11, 12), which are considered pivotal players in autoimmunity (1, 9).

TABLE 1 | Therapeutic agents in rheumatic diseases.

	SpA	PsA	SS	SLE	RA	Target
Secukinumab	x	x	Ongoing trials	Ongoing trials	Ongoing trials	IL-17a
Ixekizumab	x	x			Ongoing trials	IL-17a
Brodalumab	Not approved				Not approved	IL-17R
Bimekizumab	Ongoing trials				Ongoing trials	IL-17a, IL-17f
Netakimab	Ongoing trials					IL-17a
Ustekinumab	Not approved	x		Ongoing trials		IL-12, IL-23
Guselkumab	Not approved	x			Not approved	IL-23
Apremilast	Not approved	x				PDE4
Tofacitinib	Ongoing trials	x			x	JAK1-JAK3 (JAK2)
Baricitinib					x	JAK1-JAK2
Upatacitinib	Ongoing trials				x	JAK1
Filgotinib	Ongoing trials					JAK1
Rituximab			Off label	Off label	x	CD-20
Tocilizumab			Not approved		x	IL-6R

SpA, Spondyloarthritis; PsA, Psoriatic Arthritis; SS, Sjogren Syndrome, SLE, Systemic Lupus Erhytematosus; RA, Rheumatoid Arthritis.

Although IL-12 and IL-23 are both members of the IL-12 family and have a similar structure, the role of these two cytokines in Th0 differentiation is totally different (13, 14); indeed, unlike IL-23, IL-12 induces differentiation of Th0 cells into T helper type 1 (Th1 cells) rather than into Th17 (**Figure 1**) (15–19). Both cytokines are produced by DCs and the balance of IL-12 and IL-23 production is controlled by prostaglandin E2 (PGE2), which promotes inflammatory responses (20, 21).

IL-12 and IL-23 act as a bridge between the innate and adaptive arms of the immune response (22). IL-12, produced by antigen presenting cells (APCs), is essential for the optimal proliferation and production of cytokines by Th1 cells in response to antigens. Overall, IL-12-induced IFN-γ is an effective activator of the antimicrobial functions of phagocytes and plays a critical role in resistance to many pathogenic bacteria, fungi and intracellular parasites (23).

Regarding the IL-23/IL-17 axis, γδ T cells and innate lymphoid cells (ILCs) constitutively express the IL-23 receptor (IL-23R), suggesting their prompt first-line response to IL-23, followed by cytokine secretion and subsequent activation of the adaptive immune response. Moreover, since Th0 cells do not express IL-23R, they require prior stimulation with transforming growth factor β (TGF-β), IL-6 and IL-21 to become responsive to IL-23 (24–29).

In response to IL-23-mediated activation, αβ T cells, γδ T cells and ILCs produce IL-17, IL-22, TNF-α, and interferon-gamma (IFN-γ); in addition, IL-23-activated γδ T cells make αβ T cells refractory to the suppressive activity of regulatory T cells (Treg) and they also prevent the conversion of conventional T cells into FOXP3+ Treg cells *in vivo* (30).

IL-23 Receptor

The IL-23 receptor (IL-23R) is a heterodimeric receptor composed of 2 subunits: IL-12Rβ1, in common with the IL-12 receptor (IL-12R) and IL-23Rα, specific to IL-23 signaling (31).

Therefore, T cells lacking IL-12Rβ1 cannot respond to IL-12 nor IL-23. Conversely, IL-23Rα-deficient T cells cannot respond to IL-23, while maintaining IL-12 signaling capability (32). IL-23Rα and IL-12Rβ1 chains are expressed on T cells, natural killer (NK) T cells, monocytes/macrophage and DCs (33).

The intracellular pathways require different signaling proteins: JAK2, Janus kinase 2; TYK2, tyrosine kinase 2; STAT3, STAT4, signal transducer and activator of transcription 3 and 4 (34, 35).

Specifically, IL-12Rβ1 binds to TYK2 inducing STAT4 phosphorylation which is essential for increasing IFN-γ production and subsequent Th1 cells differentiation. Instead, IL-23Rα interacts with JAK2, inducing STAT3 phosphorylation and leading to the upregulation of retinoid-related orphan receptor gamma tau (RORγt), crucial for the development of Th17 cells (19, 31, 36, 37).

Once activated, STATs homodimers translocate into the nucleus, where they bind to DNA in the promoter region of target genes, acting as downstream effectors in the IL-23/IL-12 signaling pathway (38, 39).

Finally, the evaluation of IL-23 functions *in vivo* in different mouse models supports the hypothesis that IL-23 may act in both lymph nodes and peripheral tissues to drive terminal differentiation of effector Th17 cells *in vivo*, promoting Th-17-mediated inflammation. Confirming these observations, in the absence of Il-23, Th17 cells experience "arrested development" leading to impaired function (40).

Interleukin-17A

Interleukin-17A (IL-17A) is the first described member of the IL-17 cytokine family, which includes six members, IL-17A to IL-17F (41, 42).

Many IL-17A-producing cells have been reported, including T $CD8^+$ cells (43), γδ T cells (44), and NK T cells (45); however, according to current knowledge, T $CD4^+$ cells (Th17) are the major source of IL-17.

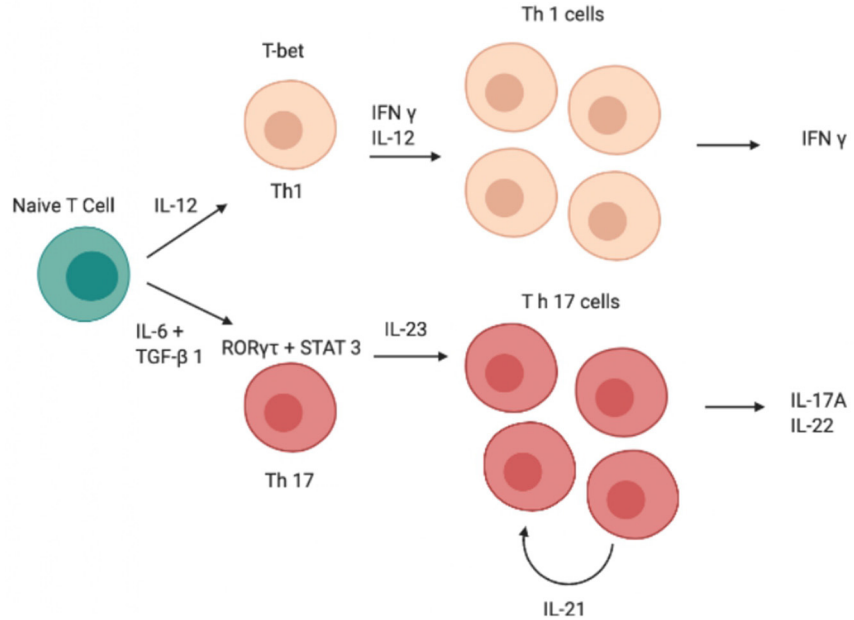

FIGURE 1 | Schematic image of the cascade of cytokines and transcription factors involved in the differentiation of Th1 and Th17 cells. IFN-γ, interferon-γ; IL, interleukin; RORγt, retinoid-related orphan receptor γt, STAT, signal transducer and activator of transcription; GATA, GATA transcription factor; TGF-β, transforming growth factor β, Th, T helper.

Although IL-23 has mainly been identified as the initiating factor for IL-17 expression from T cells (9, 46), Th0 cells do not constitutively express IL-23R, but they are still sensitive to IL-23 (47); in this context, it is reasonable to assume that IL-23R expression on these cells can be induced in the presence of other pro-inflammatory cytokines (48, 49).

Several findings clearly demonstrated that TGF-β and IL-6 are sufficient for Th17 differentiation *in vitro* and *in vivo,* in the absence of IL-23 (50–53).

Therefore, TGF-β, IL-6, and IL-21 seem to activate T lymphocytes and promote the initial differentiation of Th0 into Th17 cells, conferring responsiveness to IL-23 (50, 51, 54–60), which is a crucial step for Th17 cells stabilization and expansion (61).

Conversely, increased TGF-β levels coupled with the absence of inflammatory cytokines inhibit Th17 differentiation (62), as well as the common inhibitors of Th17 commitment (IFN-γ, IL-4, IL-25, IL-27) (54–56, 63–67).

Finally, IL-17-producing cells have been shown to express a wide range of heterogeneous cytokines such as IL-17A, IL-17F, IL-26 (62), and other proinflammatory mediators including IL-22, IL-21,IL-6, TNF-α, granulocyte colony-stimulating factors (GM-CSF), and chemokines (e.g., CCL20, CXCL8, CXCL1, CXCL10) (9).

IL-17 Receptor

IL-17 receptor (IL-17R) is expressed on many cell types, including epithelial cells, B and T cells, fibroblasts, monocytic cells, and bone marrow stroma (68).

IL-17 signaling activates nuclear factor κB (NFκB) activator adaptor protein (ACT1), which in turn acts on mitogen-activated protein kinases (MAPKs), including p38MAK (69), c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), Janus kinase (JAK), signal transducer and activator of transcription (STAT), phosphoinositol 3 kinase (PI3K) and induces several pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, CCL2), antimicrobial peptides (β-defensin), and matrix metalloproteinases (69–71).

In health conditions, IL-17 is one of the main contributors to the host defense against microbial infections (68, 72–74). Of note, the IL-17 pathway regulates antifungal immunity, in human and mice, inducing upregulation of proinflammatory cytokines, antimicrobial peptides and neutrophil-recruiting chemokines, which lead to limit fungal overgrowth (75).

Historically, $CD4^+$ T cells have been involved in protecting against *Candida albicans* infection were found in Human Immunodeficiency Virus (HIV) positive patients (76, 77).

Subsequently, Th17 subset was identified as $CD4^+$ T cells with reactivity to *Candida albicans* (37, 78).

Moreover, *ex vivo* studies on human T cells demonstrated that *Candida albicans* triggers Th17 cells which produce IL-17 and IFN-γ, but not IL-10. On the contrary, Staphylococcus aureus–activated Th17 cells produce IL-10, which can limit the immune system responses. These different responses could derive from the presence, in the priming phase, of different cytokine environments induced by each microbe (79).

The specific role of IL-17 in protection against the fungus *Candida albicans* was confirmed by evidence that IL-17RA deficient mice or mice and humans with defects along the IL-17

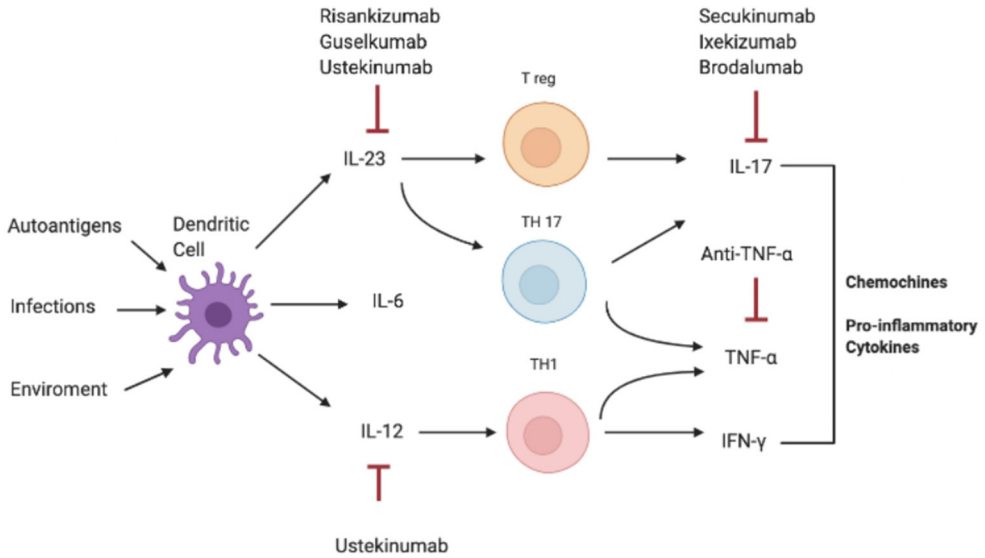

FIGURE 2 | Pathogenesis of IL-17-correlated disease and different targets of therapy. IFN-γ, interferon-γ; IL, interleukin; Th, T helper; TNF-α, Tumor necrosis factor.

signaling pathway, were susceptible to systemic *Candida albicans* infection (80–83).

Major roles of IL-17 include the promotion and initiation of chemotaxis and the recruitment and activation of neutrophils in inflamed tissues (71, 84, 85). Among its pleiotropic effects, the enhancement of angiogenesis (86) and the tissue remodeling through the production of angiogenic factors and matrix metalloproteases are worthy of note (87). IL-17 works in synergy with TNF-α causing release of the IL-6, TNF-α, and IL-1β, in order to amplify the multifaceted and complex inflammatory process (88).

Consistently, increased serum and tissue levels of IL-17 have been widely reported in inflammatory condition such as IBD, MS, and arthritis, compared to a non-pathological setting where IL-17A levels are extremely low or undetectable in human sera (**Figure 2**) (3, 10, 89–92).

Ankylosing Spondylitis (AS)

Ankylosing Spondylitis (AS) is the prototypical subset of SpA characterized by a predominant axial involvement (93). As a result, sacroiliitis is the clinical hallmark of disease and its identification through the most sophisticated imaging techniques, such as high-field magnetic resonance imaging (MRI), is extremely important in order to achieve an early diagnosis that can prompt a rapid treatment administration. The new classification criteria published by the Assessment in Spondylo-Arthritis international Society (ASAS) in 2009 have included MRI as the gold standard technique to identify active sacroiliitis, consisting in bone marrow oedema and osteitis, even in patients that have not developed radiographical signs of disease (94). According to these criteria, the presence of alterations (both in standard radiography or MRI) coupled with clinical, genetic and laboratory data, allow the classification of AS patients into radiological axial SpA (r-axSpA) or non-radiological axial SpA (nr-axSpA) (95).

New bone formation, determined by chronic inflammation involving the spine, leads to vertebral ankylosis, severe chronic pain and disability as major consequences of disease progression.

The beginning of the inflammatory process in AS relies on a complex, multifactorial interplay between genetic, epigenetic and environmental factors associated with a dysregulated immune response. The current understanding of AS pathogenesis suggests that the IL-23/IL-17 axis acts as the major driver in disease development, even if type 17 response could not entirely elucidate the mechanisms behind this rheumatic disease (96).

Genetics and epigenetics play a pivotal role in the pathogenesis of AS; siblings of AS patients have a higher risk of developing the disease and a high degree of concordance in twins is observed, 50–63% in monozygotic and 13–20% in dizygotic twins, respectively (97). The strongest genetic association is with the allele human leukocyte antigen B27 (HLA-B27) of the Major Histocompatibility Complex-I (MHC-I) gene, located on chromosome 6 (98).

Several theories have emerged to explain the possible pathomechanism related to HLA-B27, AS starting, and subsequent dysregulated activation of the IL-23/IL-17 axis via Th17 cells (99). Modifications in the shape of HLA-B27 affect the protein binding domain impairing both the antigen presentation process and the correct folding of the HLA-B27 molecule (100). The altered binding domain may lead to the presentation of self-peptides to cytotoxic $CD8^+$ T cells that in turn give rise to a pathological autoimmune response (101). On the other hand, the unfolded protein response (UPR) theory postulates that unconventional HLA-B27 variants homodimerize instead of heterodimerize; the misfolded proteins accumulate in the intracellular compartment triggering endoplasmic reticulum stress and increasing IL-23 production (102). In

fact, heterodimers expressed on APC surface directly interact with cell receptors on a wide range of immune cells such as NK, monocytes, B cells and promote a significant impact on Th17 stimulation (103). In particular, the aberrant expression of the allele of the HLA B 27 in spondylitis, could act by binding cells that contain a natural killer receptor for HLA B 27 homodimer, named killer cell immunoglobulin-like receptor 3DL2 (KIR3DL2), determining IL-17 production (104).

HLA-B27 alone accounts for almost the 25% of AS heritability and genetic-wide association studies (GWAS) have identified multiple genetic loci linked to disease pathogenesis (105, 106). In particular, single nucleotide polymorphisms (SNPs) in genes coding for aminopeptidases expressed in the endoplasmic reticulum (ER), such as ERAP1 and ERAP2, were identified in the past decade. These proteins trim peptides in the ER so that these molecules get to the right length, usually between 8 and 10 amino acids, to be presented by MHC-I molecules (107). Mutations in ERAP enzymes are supposed to lead to the formation of the so called "arthritogenic peptide" which, through mechanisms related to molecular mimicry, triggers immune cells to react against self-antigens located at joint and enthesal sites (108). ERAP1 and HLA-B27 effects are linked in an epistatic way, meaning that ERAP1 mutation effects are only observed in HLA-B27 positive patients (109).

SNPs directly affecting the IL-23/IL-17 axis were described in AS and further stress the importance of this pathway. The most relevant are located in the genes coding for IL-23R and STAT3 and TYK2, which are downstream targets of IL-23 signaling (110, 111).

However, it is not entirely clear how this polymorphism is responsible for pathogenesis, but this probably alters the amino acid sequence of the intracellular portion of IL-23R, and it could have a secondary regulatory function; indeed, protective alleles have also been identified in IL-23R, and some studies have detected polymorphisms in the IL-23R as a strong protective genetic factor (112).

The fundamental role played by the IL-23/IL-17 axis comes from several lines of evidence depicting a clear increase in IL-23 and IL-17 levels in the sera of AS patients (113). This observation is coupled with the increased number of Th17 cells in peripheral blood from AS patients (114). In this regard, the most intriguing and recent theories suggest that the interface between environment and immune system in AS can be represented by the gut epithelial barrier, where IL-17A exerts its functions in maintaining mucosal immunity and barrier functions (115). The intimate relationship between the articular disease and the gut is underlined by the detection of subclinical inflammation in up to 70% of AS patients on endoscopic examination, as well as on histological samples (116) and in about 10% of these occurs a clinical IBD suggesting a pre-clinical stage of IBD (115). Even bone marrow oedema in sacroiliac joints was correlated to gut inflammation, as emerged from the analysis of the Ghent cohort (117).

At gut level the huge number of adherent and invading bacteria, known as human microbiota, may be perturbed leading to both quantitative and qualitative alterations that affect the integrity of the gut-epithelial and vascular barriers in accordance with the "joint-gut axis theory" (118). The "leaky gut" allows the translocation of bacteria-derived peptides and primed immune cells to the interstitium and then to the bloodstream, eliciting a systemic abnormal inflammatory response. The derangement of the gut interface has been related to the alteration of the tight junction system and an increase in zonulin level was retrieved both in gut epithelium and peripheral blood (119). In AS patients dysbiosis was evidenced in comparison to healthy individuals and increased level of IL-23 are found in patients' gut and in particular in the terminal ileum (120). Several cell populations involved in epithelial immunity and joint/enthesal inflammation, such as Th17, ILC, γδ T cells, and mucosa-associate invariant T (MAIT) cells, as well as cells involved in mucosal homeostasis, such as Paneth cells, produce IL-23 at intestinal level. ILC3 are expanded in gut and differentiate upon IL-17 and TNF-α stimulation becoming an important source of IL-23 and IL-17 (121). Among the intraepithelial lymphocyte (IEL) compartment γδ T cells are the most represented population, accounting for approximately the 50% of IEL; on the other hand, they represent only the 3–5% of circulating T cells. Once activated with IL-23 these cells produce IL-17 (44). Their number was found increased in gut and γδ T cells obtained from AS patients show hyper-responsiveness to IL-23, due to IL-23R hyper-expression, with consequent discharge of higher amount of IL-17 when stimulated (122).

According to the gut-joint axis theory the intestinal activation of different immune cell subsets, among the ones above described, followed by their recirculation in blood may lead to their final localization in joint and enthesis where the inflammatory process is carried out. Intestine seems to be the major site of IL-23 production and is also the site where it acts most. Even gut- derived IL-17 producing ILC3 were found expanded at bone marrow, joint and peripheral blood level. ILC3 are also characterized by a significant expression of an integrin that regulates intestinal T cells homing, called α4β7. Moreover, the receptor of this integrin (named MADCAM1) was found upregulated in the gut and in bone marrow of AS patients, suggesting a chemoattraction process of ILC3 at inflammation site (121). Cuthbert et al. described resident γδ T cells at spinal enthesis where they contribute to IL-17 production in both IL-23 dependent and independent ways. In fact, a subpopulation of these cells lacking IL-23R was proven to produce IL-17 (123). In addition, IL-17 producing MAIT cells were found elevated in AS patients both in blood and synovial fluid (124, 125). To add more pieces to the already complex puzzle of IL-17 producing cells, a new population of $CD8^+$ T cells able to produce IL-17, named Tc17 cells, was described and was found increased in both peripheral blood and synovial fluid of AS patients (126). Other possible sources of IL-17 that deserve deeper investigation are tissue-resident memory T cells (TRM), mast cells and $CD3^-CD56^+$NK cells (127, 128). In axial tissues IL-23 producing cells are macrophages and DC while IL-17 sources are myeloid cells as neutrophils (129, 130).

Animal models have supported the actual knowledge on the role of the IL-23/IL-17 axis, even if human disease appears far more complex and no animal model can comprehensively elucidate it. Transgenic HLA-B27 rat model was used to

demonstrate the importance of the HLA-B27, gut microbiota and Th17 cells in the pathogenesis of SpA (131). These rats, grown in germ free condition do not develop SpA (132). Several mice models even exist and have contributed to the unraveling of the pivotal role played by IL-23 and IL-17 in activating T cells and driving disease development via type 17 immunity (133).

Taken together, the above resumed evidence underlines the importance of this axis in inducing and sustaining the multifaceted inflammatory process depicted in SpA, making it a central therapeutic target.

Advances in understanding the pathogenesis of SpA have prompted the development of biologic drugs designed to inhibit the IL-23/IL-17 axis. In fact, for more than 15 years, TNF-α inhibitors were the only biologic treatment available and, despite an initial great success in SpA management, it came out clearly that almost 40% of patients failed to reach a significative response. The new therapeutic agents interfering with the IL-23/IL-17 axis can be divided into monoclonal antibodies directly targeting IL-17, IL-23 or their receptors and small molecules inhibiting the intracellular pathways triggered by these cytokines (134).

Among monoclonal antibodies targeting IL-17, Secukinumab and Ixekizumab, respectively a fully human monoclonal IgG1/k antibody and a humanized IgG4 monoclonal antibody, are the only two molecules already marketed for SpA.

The MEASURE trials demonstrated the superiority of Secukinumab against placebo in providing sustained efficacy in relieving signs and symptoms of AS as well as in granting a good retention rate, as demonstrated in the 5-years extension study (135, 136). In the 2-years follow up in the MEASURE 1 trial no radiographic progression was evidenced in the 80% of patients included (137).

Ixekizumab was licensed for r-axSpA treatment in 2019 and for nr-axSpA in 2020, the COAST-V trial demonstrated a superior response rate in ASAS40 score at week 16 over placebo (138).

Several trials aimed to assess the therapeutic value of other IL-17 inhibitors, such as Netakimab and Bimekizumab, are currently ongoing (139, 140). The randomized controlled trial on Brodalumab, a humanized IgG2 monoclonal antibodies that binds IL-17R was discontinued because of the occurrence of high suicide ideation in the active group (141).

Up to date, no IL-23 targeting drug has been proven effective for AS (142, 143).

New treatments for AS are small molecules inhibitors that target intracellular proinflammatory pathways, as those triggered by cytokine stimulation. Among targeted synthetic DMARDs (tsDMARDs), JAK-inhibitors stand out as the most promising treatment (144). In particular, Tofacitinib, a pan-JAK inhibitor, Filgotinib and Upadacitinib, both JAK1 inhibitors, were shown to be superior to placebo in axSpA (145–147).

The inhibition of PDE4 through the small molecule Apremilast failed to achieve the primary end-point in the specifically designed phase 2 trial including 490 AS patients (148).

Future perspectives to implement the therapeutic options for AS patients include broader anti-inflammatory approaches with multi-cytokine blockade to overcome the inhibition of a single pathway, for example targeting simultaneously TNF-α and IL-17, as in an ongoing study in PsA (149).

Psoriatic Arthritis

Psoriatic arthritis (PsA) is a chronic, immune-mediated, inflammatory disease dominated by a heterogeneous phenotype that mainly affects peripheral and axial joints, entheses, skin, and nails, leading to juxta-articular new bone formation, bone erosions and abnormal keratinocyte proliferation (31, 150).

Interactions between genes and environmental triggers, including infections, trauma, stress, obesity and smoking are recognized as crucial for the onset of the autoimmune process in PsA. Furthermore, the disruption of the gut microbiota composition in PsA patients is supported by consolidate evidence (151, 152).

The main effector cells of the inflammatory cascade, both in joints and in plaques of patients with PsA, are DCs, macrophages, NK cells (153, 154), mast cells, neutrophils (155, 156), γδ T cells (157), T $CD4^+$ and T $CD8^+$ cells.

All the ones above described have a predominant IL-17 secretory phenotype (41, 158), defined by the production of cytokines such us IL-17, IL-22, and TNFa; however, T $CD4^+$ cells, as the major source of IL-17, are considered the cornerstone in the pathogenesis of psoriasis (157, 159).

Specifically, DC-derived cytokines, IL-23 and IL-12, drive the differentiation of distinct Th17 and Th1 cells, which are known to be implicated in the pathogenesis of PsA. Activated T cells move from the circulation to the target organs, and chronic inflammation occurs in the skin and joints.

Accordingly, increased numbers of Th17 cells were detected in the blood and affected skin of patients with psoriasis and in the blood and synovial fluid of patients with PsA. Furthermore, the assessment of the expression of IL-23, IL-17, and their related receptors in psoriatic skin lesions and inflamed synovium supports the concept of IL-23/IL-17 axis as a driving force of immune inflammation in psoriasis (160–164) PsA synovitis is characterized by significant infiltration of mononuclear cells, T and B cells, vascular proliferation and hyperplasia of synovial lining cells, similar to the pathological changes observed in RA (165). Additionally, ectopic lymphoid tissues were frequently found in PsA synovial membrane with microanatomical features for germinal center formation, capable of antibody production (166). The role of B cells in PsA is still elusive; a recent study reported that autoantibodies against a peptide sharing sequence homology with skin and entheseal autoantigens were detected in 85% of patients with PsA (167).

Several findings have clearly demonstrated the influence of IL-17 on bone metabolism. Despite the direct effects of IL-17 on osteoclasts, induction of osteoclastogenesis is mediated by the production of matrix metalloproteinases by macrophages and the activator of the NF-κB ligand receptor (RANKL) presented by osteoblasts (168–170).

Skin lesions from patients with psoriasis exhibit epidermal hyperplasia and infiltration with neutrophils, T $CD4^+$ and T $CD8^+$ cells, B cells, dendritic cells and mast cells, type 3 innate lymphoid cells (ILC3) and γδ T cells (171–174).

In contrast, IL-22, totally absent in synovial tissue, is highly expressed in entheses, where it promotes entheseal and periosteal bone formation through STAT3 activation, explaining the formation of enthesophyte and juxta-articular bone, hallmarks of PsA (175, 176). In skin lesions IL-22 drives keratinocyte hyperproliferation via STAT3 signaling (177) and prompts epithelial cells to release chemokines, such as IL-8 (178, 179), a key factor in neutrophil recruitment in psoriatic lesions and stimulates keratinocytes to secrete antimicrobial peptides (180, 181), preventing skin lesions from becoming infected (182).

In inflammation, Th17 cells also produce interleukin 9 (IL-9), which in turn induces the differentiation of Th17 cells and potentiates the suppressive effect of regulatory T lymphocytes via activation of STAT3 and STAT5. Our group demonstrated that IL-9 overexpression and T helper type 9 (Th9) polarization occur in the synovial tissue and peripheral blood of PsA patients. Furthermore, clinical improvement after treatment with TNFi and ustekinumab was associated with a significant reduction in circulating Th9 cells (183, 184).

Up to date, a wide range of therapeutic approaches have been proposed for PsA, depending on disease severity, including disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate; anti-tumor necrosis factor α (anti-TNF-α) agents, phosphodiesterase 4 (PDE4) inhibitors, IL-17 and IL-12/IL-23 inhibitors (185, 186).

The central contribution of IL-23/IL-17 axis in both PsA and psoriasis is confirmed by the efficacy of biologics neutralizing IL-17 or IL-23/IL-12 (187) as well as the effectiveness of TNF-α inhibition dependent on down-regulation of IL-17 pathway genes (188–190).

To date, several monoclonal antibodies have been approved for the treatment of PsA and can be introduce following the failure of non-steroidal anti-inflammatory drugs (NSAIDs) and/or conventional DMARDs, or an anti-TNF-α agent.

Ustekinumab is a human IgG1 monoclonal antibody that binds to the p40 subunit, shared with IL-12 and IL-23, and blocks downstream events of both the IL-12 and IL-23 signaling cascade through inhibition of IL-12Rβ1 binding (191). Ustekinumab has produced consistent and sustained clinical efficacy in two phase three clinical trials in PsA, PSUMMIT-1 and PSUMMIT-2, with data out to 52 weeks, and no new safety signals. PSUMMIT-1 included patients with active PsA despite conventional therapy who were all naïve to anti-TNF-α agents, whereas PSUMMIT-2 also included anti-TNF-α experienced patients (192).

Secukinumab, is a human IgG1κ monoclonal antibody that binds to IL-17A neutralizing its interaction with IL-17 receptors. It is currently approved in several countries for the treatment of PsA (193) and, in two phase 3 trials FUTURE1 and FUTURE 2, secukinumab has provided sustained improvements in disease signs and symptoms, assessing reduced radiographic progression in patients with active PsA through 2 years of therapy (194).

Ixekizumab is a humanized IgG4 monoclonal antibody targeting IL-17A, approved for the treatment of moderate-to-severe plaque psoriasis, active PsA, and active AS. Two phase three trials (SPIRIT-P1 and SPIRIT-P2) demonstrated that treatment with ixekizumab improved joint and skin disease compared to placebo (195, 196).

In addition, the SPIRIT-H2H study confirmed the superiority of ixekizumab over adalimumab in patients with Psa and inadequate response to csDMaRDs (197).

Over the past decade, the development of Janus kinase inhibitors (JAK inhibitors) has emerged as a new therapeutic option in autoimmune diseases, including PsA. The rationale of their use is suggested by the observation that the blockade of JAK receptor downregulates the production of the cytokines (TNF-α, IL-17, IL-6, IL-23) involved in the pathogenesis of PsA.

Tofacitinib is an orally administered inhibitor of predominantly JAK1 and JAK3, with functional selectivity to JAK2, used for the treatment of RA. Interestingly, its efficacy in managing treatment-resistant disease and ameliorating enthesitis, dactylitis, and radiographic progression has been reported. Consistently with previous observations, Tofacitinib could provide an alternative approach for PsA patients with inadequate response to DMARDs (198, 199).

Finally, Guselkumab, a monoclonal antibody targeting IL-23 via IL-23 p19 subunit, was recently approved for the treatment of PsA in adults with an inadequate response or intolerance to DMARDs therapy. Results for the use of Guselkumab derive from phase three clinical trials, DISCOVER-1 and DISCOVER-2, which demonstrated significantly better clinical and radiographic outcomes among PsA patients treated with the IL-23 inhibitor compared with placebo group.

Participants treated with Guselkumab achieved 20% improvement in American College of Rheumatology (ACR) response criteria at week 24 at rates of 52% in DISCOVER-1 and 64% in DISCOVER-2, whereas placebo-treated patients had rates of 22 and 33%, respectively (200, 201).

Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily affects synovial joints, accompanied by systemic inflammation and production of autoantibodies (202).

The synovitis in RA is characterized by an inflammatory infiltrate, consisting of leukocytes such as T and B cells, macrophages, granulocytes and dendritic cells, together with a synovial milieu dominated by proinflammatory cytokines and chemokines (203).

For a long time, RA was considered a Th1 dependent disease, until a significant amount of research in RA patients and experimental mouse models suggested that Th17 cells may play a central role in the pathogenesis of RA.

IL-17 is involved in both early and established RA disease, promoting activation of fibroblast-like synoviocytes (FLS), osteoclastogenesis, recruitment and activation of neutrophils, macrophages and B cells (204).

Synergism between IL-17 and TNF-α has been shown to activate the production of pro-inflammatory mediators, such as IL-1β, IL-6, IL-8, PGE2, and matrix metalloproteinases (MMPs), promoting progression of early inflammation toward a chronic arthritis (205, 206).

The differentiation of osteoclasts is induced significantly in the presence of IL-17 either directly (207), or indirectly, through upregulation of RANKL.

By the late 1990s studies had already shown that IL-17 expression had increased in the joint of RA patients compared to healthy individuals or osteoarthritis (OA) patients (89).

More recently, an increased proportion of chemokine receptor $CCR6^+$ Th17 cells has been described in the peripheral blood of treatment-naive patients with early RA (208), and higher frequencies of Th17 cells have been detected in the synovial compartment of RA patients, compared to OA patients (209).

Moreover, Th17 cells were associated with clinical parameters, such as disease activity score 28 (DAS28), C-reactive protein (CRP) levels and presence of anti-citrullinated protein antibodies (ACPAs), highly specific for RA (210, 211).

Direct evidence obtained from experimental mouse models confirms the critical role of the IL-23/IL-17 axis in the pathogenesis of arthritis. IL-23p19-deficient ($Il23a^{-/-}$) mice were protected against the development of collagen-induced arthritis (CIA), a mouse model of RA. IL-17-producing $CD4^+$ T cells were absent in the $Il23a^{-/-}$ mice despite normal induction of IFN-γ-producing $CD4^+$ Th1 cells (212).

Interplay between IL-23 and IL-17 production may be a critical immune pathway and a potential therapeutic target for a range of inflammatory arthritis.

Antibodies against IL-17 (Ixekizumab and Secukinumab) or IL-17R (Brodalumab) have been tested in RA patients (213–216). In a phase I RCT of RA patients treated with oral DMARDs, the addition of Ixekizumab improved RA signs and symptoms and disease activity scores such as DAS28, compared to placebo (213). This improvement was confirmed in a phase II study in which Ixekizumab was administered to patients who were naive to biological therapy or resistant to TNF-α inhibitors (217).

In a phase II study enrolling RA patients with inadequate response to methotrexate, greater decreases in DAS28 were observed with Secukinumab than with placebo (214).

Furthermore, patients with active RA who did not respond to DMARDs showed improvements after long-term treatment (52 weeks) with 150 mg of Secukinumab, with ACR50 rates increased from 45% to 16 to 55% at week 16 at week 52 (215). Conversely, Brodalumab showed no evidence of clinical benefit in RA patients in a phase Ib study (213).

In addition, the efficacy of Ustekinumab, a human anti-IL-12/23 p40 monoclonal antibody and human anti-IL-23 monoclonal antibody, Guselkumab, has been evaluated in patients with active RA not responsive to methotrexate therapy. However, no significant clinical improvement was recorded compared to the control group (218).

Recently, the double blockade of IL-17 and TNF-α has been studied using ABT-122, a variable double domain Ig that targets human TNF-α and IL-17 (219). A phase II study showed that there was no significant difference in the ACR20 response at week 12 by the double inhibition of IL-17 and TNF-α compared to treatment with only anti-TNF-α (220). Lastly, double blockade of IL-17A and IL-17F using Bimekizumab in RA patients with inadequate TNF-α response resulted in a greater reduction in DAS28-CRP at week 20 compared to anti-TNF-α inadequate response plus placebo group (221).

Sjögren's Syndrome (SS)

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by the lymphocytic infiltration into the exocrine glands, mainly salivary and lacrimal glands, and other tissues (222).

The role of the adaptive immune system in the pathogenesis of SS is supported by the presence of ectopic germinal centers in almost 25% of the patients, which promote local expansion of antigen-specific B cells, production of autoantibodies and hypergammaglobulinemia, as well as increased risk of developing non-Hodgkin lymphoma (NHL) (223, 224).

In this regard, the interaction between $CD4^+$ T cells and B cells appears to be a key step in the development of the disease (222).

Although SS has historically been considered a Th1-driven disease, subsequent studies have revealed that, in addition to Th1 cells, several subgroups of $CD4^+$ T cells are involved, follicular T helper cells (Tfh) and Th17. In healthy subjects, Th17 cells play an important role at mucosal barriers and are involved in immune responses. In SS, Th17 cells may be activated by dendritic cells in lymph nodes draining the salivary and lacrimal glands by the production of cytokines as TGF-β and IL-23. In the later phases of the disease, naïve T cells can also be polarized locally in Th17 cells by APC and cytokines as IL-6 and TGF-β (225). IL-23 production by macrophages is also important for maintenance and expansion of Th17 cells by STAT3 activation (226). The main effector cytokines of Th17 cells are IL-17 and IL-22. Recent studies reported that IL-17 protein and mRNA are present within lymphocytic infiltrates of minor salivary gland (MSG) tissue of SS patients.

Furthermore, IL-17 mRNA levels in MSG biopsies were related to the degree of inflammation (226–228).

Along with IL-17, the expression of IL-23 and IL-22 was also increased in the inflamed salivary glands of SS patients. IL-17 and IL-22 secretion in the exocrine glands promote inflammation and the induction of matrix metalloproteinases which may cause acinar damage (229).

Moreover, the presence of Th17 cells in SS infiltrate has been hypothesized to be crucial in B cell activation and formation of germinal centers within glands (230).

The IL-23/IL-17 axis also plays a role in the symptoms development, as confirmed by the increased levels of IL-17 and IL-6 protein and mRNA in tears and saliva from SS patients compared to non-SS controls. Additionally, the expression of Th17-associated cytokines correlated with ocular surface parameters, such as Schirmer I Test, break up time (BUT) and corneal fluorescent staining (CFS) (231).

A recent study showed that serum IL-17F levels were significantly increased in SS patients and were associated with high levels of autoantibodies and increased EULAR SS disease activity index (ESSDAI), compared to IL-17A, suggesting the possibility of several pathogenetic roles played by different IL-17 family members (232).

The role of the IL-23/IL-17 pathway in the pathogenesis of SS has been also supported by data from animal models. In C57BL/6.NOD-Aec1Aec2 mouse, a model of spontaneous SS,

genetic ablation of IL-17 reduced lymphocytic infiltration and restored glandular function, especially in female animals (233).

In addition, adoptive transfer of Th17 cells induced the development of experimental SS (ESS) in immunized IL-17 knockout mice (234).

Even if Th17 cells are the main source of IL-17 in SS, γδ T cells, NK cells, ILCs and $CD8^+$ T lymphocytes can also produce IL-17 (68). However, recent evidence has shown that $CD4^-$ $CD8^-$ (double negative, DN) T cells and mast cells can also participate in local IL-17 production in SS (235).

Rituximab treatment demonstrated a significant reduction in IL-17 expression in the salivary glands of SS patients, while factors that are important for maintenance of Th17 cells, as STAT3 and IL-23, are likely not affected (228).

Despite the increase in Th17 cells in SS is well-established, data on the amount of T reg cells are still controversial. Th17 and Treg cells are counter-regulated. IL-6 promotes Th17 differentiation by inhibiting T reg generation (51), while IL-2 acts in the opposite way (236). Since reduced IL-2 levels could underline Th17 upregulation (237), the effects of administration of recombinant IL-2 have been studied in SS. Reduction of glucocorticoid and Hydroxychloroquine (HCQ) in patients treated with IL-2 and restored Th17/Treg balance have been reported (238).

Regarding IL-6 inhibition, as the IL-6 signaling is important for the IL-17 cells differentiation, an effect of tocilizumab on Th17 cells is probable. However, the results of a randomized placebo-controlled study showed that Tocilizumab had no impact on the main symptoms of SS, although improvements in joint involvement were observed (239).

Long-term efficacy of Ustekinumab, a human monoclonal antibody directed against the p40 protein subunit shared by IL-12 and IL-23, in a patient suffering from psoriasis and SS has been recorded on both the cutaneous and joint component (240). However, the inhibition of IL-17 achieved by systemic administration of Secukinumab did not affect the severity of the dry eye (241). Given the pilot role of the IL-23/IL-17 axis in SS pathogenesis, further investigations are needed to confirm the selective blockade of Th17-associated cytokines as a potential therapeutic target.

Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease with multiple immunological abnormalities including dysregulation of both T and B lymphocytes and production of autoreactive antibodies directed toward nuclear self-antigens, with immune complex formation and tissue damage (242). A wide number of cytokines is involved in disease pathogenesis. Recently, the role of the IL-23/IL-17 axis has emerged in SLE and has been investigated either in humans or mice. Higher serum levels of IL-17 and IL-23 as well as increased number of Th17 cells has been demonstrated in patients with SLE compared with healthy controls (243, 244). Moreover, T cells that express IL-17 were found within infiltrates in kidney biopsies of patients with active lupus nephritis and significant proportion of these cells are double negative (DN) T cells, which are also expanded in the peripheral blood of patients with SLE, produce significant amounts of IL-17 and contribute to the disease pathogenesis (245). In a murine model of lupus, Zhang et al. detected DN T cells expressing high levels of IL-17A in the kidneys of mice with active nephritis. Interestingly, the lymphocytes isolated from these lupus-prone mice progressively express higher levels of IL-23 receptor as their disease worsens. Treating these lymphocytes *in vitro* with IL-23, they induce nephritis when transferred to non-autoimmune, control mice. Additionally, the kidneys of these recipient affected mice showed significant Ig and complement deposition in the glomeruli. This finding suggests that IL-23 also promoted an autoimmune B cell response. These data added to previous evidence indicate that DN T cells provide excessive help to B cells, resulting in abnormal production of pathogenic autoantibodies in SLE (246).

Consistently with previous findings, Chen et al. found that circulating Th17 frequency correlated with SLE activity, in particular with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and histological activity index in 24 lupus nephritis (LN) patients (247).

The link between IL-23/IL-17 axis and LN is also supported by the observation that high serum levels of IL-23 and IL-17 at baseline predict an unfavorable histopathological response and British Isles Lupus Assessment Group (BILAG)-non-responders had high IL-23, indicating that a number of LN-patients has a Th-17 phenotype that may influence response to treatment.

In addition to LN, high serum levels of IL-23 and Th17 cells are closely related to other SLE manifestations, including vasculitis, serositis, lymphopenia, central nervous system and cutaneous involvement and the production of autoantibodies (antinucleosome antibodies, antiphospholipid antibodies, and anti-SS-B/La antibodies) (244, 248–250).

Taken together, these findings suggest that Th17 cells are a promising therapeutic target for SLE.

Previous studies have shown that Hydroxychloroquine (HCQ), an essential drug for the treatment of SLE, can inhibit Th17 cell differentiation and production (251).

In a phase two trial of 102 adult patients with active SLE, the addition to standard-of-care treatment of Ustekinumab, a human IgG1k monoclonal antibody targeting both the IL-12 and IL-23 cytokines, resulted in better efficacy in clinical and laboratory parameters than placebo. In particular, 37 (62%) of 60 patients in the Ustekinumab group and 13 (33%) of 42 patients in the placebo group achieved a SLE disease activity index 2000 responder index-4 (SRI-4) response (difference 28%, $p = 0.006$) (252).

Few data are available in literature about the anti-IL-17A antibody (Secukinumab). A case of 62-year-old female who presented with psoriasis vulgaris and refractory lupus nephritis successfully treated with Secukinumab was reported. Further studies are needed to test the efficacy of drugs targeting.

CONCLUSION

This review provides an overview of the critical role played by the IL-23/IL-17 immune axis in a wide variety of inflammatory processes, and summarizes the current knowledge on cytokine milieu that regulates IL-17-producing cells. The growing body of evidence on the relevance of this intricate pathway in autoimmunity has allowed the implementation of treatment options in pathological conditions.

However, further studies are needed to clarify the complexity of IL-17 signaling, in order to allow the discovery of new potential therapeutic targets of inflammatory processes and the availability of cutting edge therapies designed on patients not responsive to standard treatments.

AUTHOR CONTRIBUTIONS

CS and SF: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review & editing, and visualization. GGr and FC: methodology, formal analysis, data curation, and writing—review & editing. CR: software, methodology, formal analysis, data curation, and writing—review & editing. LL: formal analysis, data curation, and writing—review & editing. GGu: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review & editing, visualization, supervision, and project administration. All authors had access to the study data and reviewed and approved the final manuscript.

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MINI REVIEW
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Clinical Trials Supporting the Role of the IL-17/IL-23 Axis in Axial Spondyloarthritis

Angela Ceribelli[1,2], *Francesca Motta*[1,2], *Matteo Vecellio*[1,3], *Natasa Isailovic*[1], *Francesco Ciccia*[4†] *and Carlo Selmi*[1,2*†]

[1] Division of Rheumatology and Clinical Immunology, Humanitas Clinical and Research Center - IRCCS, Rozzano (Mi), Italy, [2] Department of Biomedical Sciences, Humanitas University, Rozzano (Mi), Italy, [3] Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom, [4] Università degli Studi della Campania Luigi Vanvitelli, Naples, Italy

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***Correspondence:**
Carlo Selmi
carlo.selmi@humanitas.it

[†]These authors have contributed equally to this work

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The term spondyloarthritis (SpA) encompasses a heterogeneous group of inflammatory musculoskeletal diseases with several common genetic background and clinical features, including the possible involvement of the axial skeleton with peripheral mono- or oligo-arthritis and frequently coexisting skin, eye and intestinal manifestations. When the sacroiliac joints or other parts of the spine or thoracic wall are predominantly affected at magnetic resonance or X-ray imaging with inflammatory back pain, the disease is classified as axial SpA and the therapeutic choices are significantly different compared to cases of peripheral arthritis. Moving from the narrow effectiveness and safety profiles of non-steroidal anti-inflammatory drugs, there has been a significant research effort aimed at identifying new treatments based on our better understanding of the pathogenesis of SpA. Indeed, in parallel with the solid data demonstrating that IL-17 and IL-23 are key cytokines in the development of enthesitis and spondylitis, monoclonal antibodies interfering with this pathway have been developed for the treatment of axial SpA. Furthermore, the IL-17/IL-23 axis is key to extra-articular manifestations such as inflammatory bowel disease, uveitis, and psoriasis which are frequent comorbidities of SpA. Currently available drugs act through these mechanisms recognizing IL-23 and targeting IL-17, such as secukinumab and ixekizumab. These therapeutic approaches are now envisioned in the international treatment recommendations for psoriatic arthritis with an axial phenotype as well as for ankylosing spondylitis (AS). We will provide herein a concise comprehensive overview of the clinical evidence supporting the use of these and other drugs acting on IL-23 and IL-17 in axial SpA.

Keywords: Th17, enthesitis, spondylitis, biologics, HLA B27 allele

INTRODUCTION

The role of interleukin (IL)-17 and IL-23 in the pathogenesis of chronic inflammatory diseases such as spondyloarthritis (SpA) has been widely investigated over the past two decades, and this knowledge has led to the development of targeted therapies (1). IL-17 was first described in 1996 due to its effect on the production of IL-6 and IL-8 by rheumatoid arthritis (RA) synoviocytes (2) and the new cytokine was first named IL-17A but several other members of the family were subsequently identified, in particular IL-17F, which is approximately 50% homologous to IL-17A and both converge on TNFα, among other mediators (3, 4).

IL-23 induces IL-17A, IL-17F, IL-21 and IL-23 production (5, 6) thus playing an important role in the Th17 cell-mediated responses. IL-23 is part of the family of IL-12 cytokines and IL-12 and IL-23 share a common p40 subunit, coupled with a p35 chain for IL-12 and a p19 chain for IL-23 (7). IL-17 is a product of T cells, particularly Th17, even though several other cell types are able to produce IL-17, such as CD8+ T cells, γδ T cells, type 3 innate lymphoid cells (ILC3s) and natural killer T cells (8, 9). Other cell types such as neutrophils and mast cells do not express IL-17 mRNA but they can store exogenous IL-17 (10, 11). On the other hand, both IL-12 and IL-23 are produced by antigen-presenting cells, in particular dendritic cells, monocytes and macrophages, and based on the predominant presence of these cells in the inflamed tissues we can predict how the contribution of IL-23 is significant in a specific disease (5, 7) (**Figure 1**).

Both IL-23 and IL-17 have shown a significant therapeutic effect first in animal models and subsequently in patients affected by rheumatic conditions such as axial SpA (axSpA), i.e. a form of SpA that predominantly affects the axial skeleton, ranging from sacroiliitis to paradigmatic AS (1, 12). In particular, IL-17 inhibitors are currently approved as biological disease-modifying anti-rheumatic drugs (DMARDs) for axSpA, together with TNFα inhibitors, and currently include secukinumab [a human IgG1κ monoclonal antibody that binds to IL-17A (13)], ixekizumab [a humanized monoclonal antibody anti-17A (14)], while bimekizumab [a humanized monoclonal antibody anti-17A and IL-17F (15)] and netakimab [a humanized monoclonal antibody targeting interleukin-17A (16)] are being evaluated. On the other hand, IL-12/IL-23 inhibitors are effective against psoriasis (PsO) and psoriatic arthritis (PsA), predominantly on peripheral synovitis and enthesitis in axSpA (1), while the efficacy on axial manifestations remains inconclusive.

All biologic therapies currently approved for the treatment of axSpA are included in the recent recommendations approved in 2019 by the American College of Rheumatology, Spondylitis Association of America and Spondyloarthritis Research and Treatment Network (17), which strongly recommend to treat adult patients with active AS despite TNFα inhibitor (primary non-responder) with secukinumab or ixekizumab, while IL17 inhibitors are not recommended in the presence of inflammatory bowel disease (IBD) or recurrent uveitis.

In this concise review we will discuss the available clinical trials data and the recent advances in the discovery of new therapies for axSpA, with a specific focus on therapies targeting the IL-17/IL-23 axis.

CLINICAL TRIALS IN AXSPA FOR AVAILABLE DRUGS TARGETING THE IL-17A/IL-23 AXIS

In the last 5 years, new therapies for the treatment of axSpA have been approved thanks to the significant positive results in efficacy and safety obtained by these therapies targeting the IL-17/IL-23 axis.

Anti IL-17A Drugs

Two anti-IL-17A monoclonal antibodies, secukinumab and ixekizumab, are currently approved for the treatment of axSpA.

Secukinumab was initially approved for PsO treatment, later for PsA and axSpA. It was demonstrated that, compared with placebo, a significantly higher percentage of patients with active PsA achieved a 20% improvement in the American College of Rheumatology response criteria (ACR20) at week 24 when treated with secukinumab (18, 19). It provided a valid treatment for PsA entheseal disease (20), and was effective for signs and symptoms of axial disease in PsA and SpA patients. MAXIMISE evaluated the efficacy and safety of secukinumab (at the dosage of 300 and 150mg) in managing the axial manifestations of PsA, and results showed a rapid and significant improvement in ASAS20 responses at week 12 for axial manifestations and inadequate responses to NSAIDs (21) (ClinicalTrials.gov Identifier: NCT02721966). More recently, secukinumab proved efficacious for the treatment of non-radiographic axSpA (US FDA, June 2020) based on the results obtained from the PREVENT clinical trial (ClinicalTrials.gov Identifier: NCT02696031). By further searching the site clinicaltrials.gov for interventional studies in phase III aimed at blocking IL-17 in axSpA, we retrieved 7 studies focused on the use of secukinumab, for the analysis on the benefit of this drug on symptoms such as pain and on its safety in a 3-year follow-up period. One of these trials is focused on the speed of secukinumab-induced relief from pain in patients with axSpA (SKIPPAIN trial, ClinicalTrials.gov Identifier: NCT03136861), and its action on specific clinical manifestations such as axial involvement. Besides evaluating the efficacy of this drug, also its safety and tolerability are under evaluation for a follow-up period up to 3 years after marketing secukinumab.

Ixekizumab demonstrated efficacy for the treatment of moderate to severe plaque PsO since 2016. The indication was approved in 2017 for PsA, based on the reported ACR20 in a higher proportion of patients when compared with placebo (22) and showing efficacy also on entheseal disease (23). In 2019 it was approved for active radiographic axSpA thanks to its efficacy and safety outcome results (24, 25). More recently, ixekizumab was evaluated in the COAST-X trial in non-radiographic axSpA patients and it showed superiority to placebo at weeks 16 and 52, with similar rate of adverse events compared with previous ixekizumab studies. Results show that ixekizumab may be a

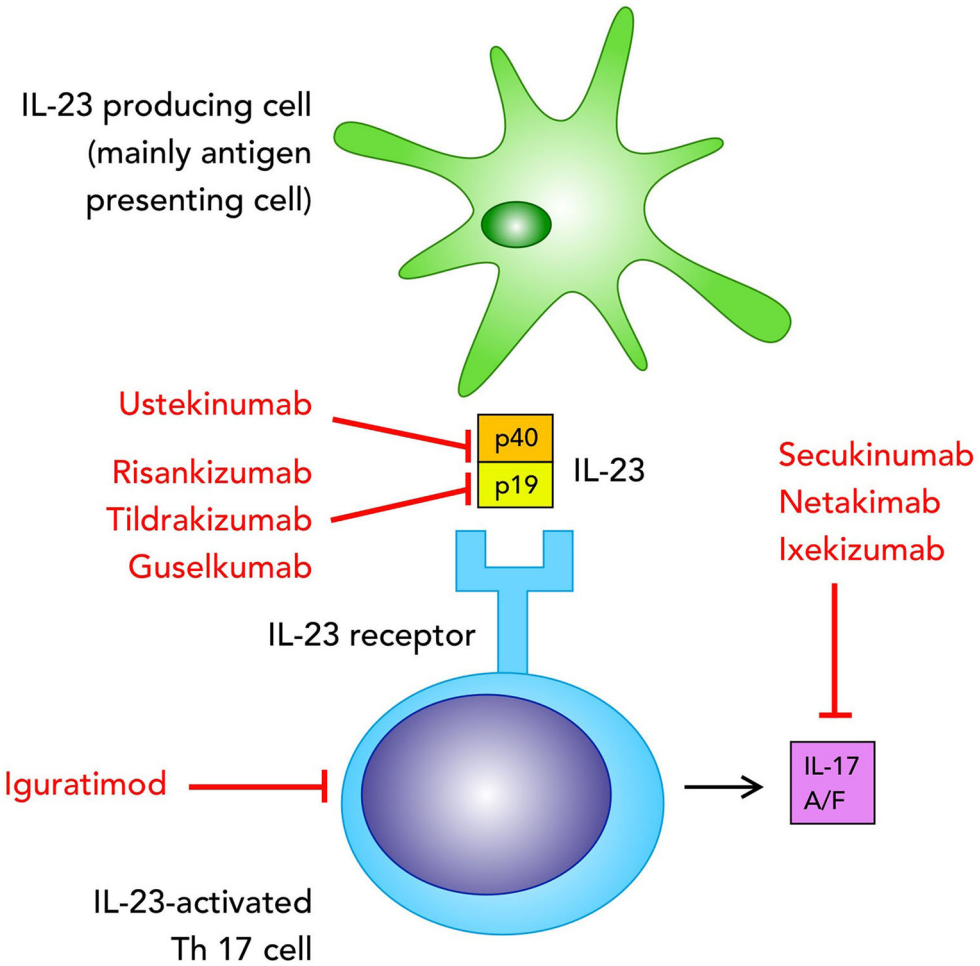

FIGURE 1 | Biologics targeting the IL-23/IL-17 pathway in axial spondyloarthritis, currently used in clinical practice or tested in clinical trials mentioned in the text.

therapeutic option for patients with non-radiographic axSpA who did not respond significantly or were intolerant to NSAIDs (24, 26), and this led to the U.S. FDA approval for the treatment of non-radiographic axSpA in June 2020.

Netakimab (BCD-085) is a novel molecule currently in phase III clinical trials in axSpA patients. In a Phase I clinical trial (ClinicalTrials.gov Identifier: NCT02380287), netakimab was evaluated for pharmacokinetics and safety in healthy volunteers, and results reported in October 2015. Thanks to the data from the Phase I trial, a randomized, placebo-controlled, double-blind Phase II trial (ClinicalTrials.gov Identifier: NCT02763111) was started to evaluate safety, effectiveness, and pharmacokinetic profile in 89 men and women with SpA. This drug was tested at doses of 40 mg, 80 mg, or 120 mg for 12 weeks and the trial results showed significant improvements in AS assessment score (ASAS), plus adverse events, withdrawal symptoms, and drug concentration. The results of this phase II trial (16) showed that netakimab was effective and generally safe in SpA patients, thus it was followed by the multicenter, randomized, placebo-controlled Phase III clinical trial (ClinicalTrials.gov Identifier: NCT03447704) which evaluated the safety and effectiveness of netakimab 120 mg against a placebo, for up to one year, in 228 patients with active SpA. Netakimab has been registered in spring 2020 for treatment of SpA and PsA in Russia. According to the Phase III clinical study BCD-085-5/ASTERA, 40% of patients with SpA achieve an ASAS40 response after 16 weeks of therapy (27).

A recent publication by Mease et al. showed the results of two phase III trials (AMVISION-1 and AMVISION-2) on the efficacy and safety of brodalumab, an IL-17 receptor subunit A inhibitor, compared with placebo, in patients with PsA (28). Results show that brodalumab confers a rapid and significant improvement in signs and symptoms of PsA versus placebo and it has a tolerance and safety profile similar to other IL-17 inhibitors (28).

Iguratimod (IGU), a novel small molecule with the effect similar to a non-steroidal anti-inflammatory drug and disease-modifying anti-rheumatic drug, acts through various

mechanisms such as inhibition of prostaglandin E2, TNFα, IL-17 production, macrophage migration inhibitory factor (MIF)-induced proinflammatory effects, osteoclastogenesis, and it promotes osteoblastic differentiation. IGU may be an effective treatment for axSpA as its mechanisms of action are related to those involved in the pathogenesis of axSpA, as shown in several small-scale clinical trials (29). IGU may be a suitable treatment for axSpA as clinical trials demonstrate a significant improvement for the ASAS20 response and reduction in inflammatory biomarkers in patients receiving IGU (29, 30).

Anti IL-23 Drugs

Ustekinumab, a human IL-12 and IL-23 antagonist that binds to their p40 subunit so that they subsequently cannot bind to their receptors to trigger pro-inflammatory cytokine release, is currently approved for use in moderate-severe PsO, PsA and Crohn's disease, while its efficacy in the treatment of axSpA was not demonstrated in 3 placebo-controlled trials, although its safety profile was consistent with studies in other indications (31). More recently, ustekinumab has been evaluated for the use in adult patients affected by moderate-severe ulcerative colitis, while its use failed in other autoimmune diseases such as multiple sclerosis (32), and encouraging data were provided in systemic lupus erythematosus.

Guselkumab, a monoclonal antibody targeting the IL-23 subunit alpha (p19 subunit) currently approved for the use in plaque PsO, is under evaluation for the use also in PsA with significant improvement in particular for manifestations such as enthesitis and dactylitis (33). The results of two phase III, randomized, double-blind, placebo-controlled studies (DISCOVER-1 and -2) on the efficacy of guselkumab in active PsA patients showed a significant improvement of the disease with ACR achievement over 24 weeks (34, 35).

Risankizumab is a monoclonal antibody that binds the p19 subunit of IL-23 and demonstrated efficacy in PsO and active peripheral PsA in preapproval trials (36), while a phase II study in axSpA did not show statistical difference from placebo and for this reason the further development of risankizumab in axSpA was not continued (37).

In February 2020, tildrakizumab, a monoclonal antibody targeting the IL-23 p19 subunit, was approved in Italy for treatment of severe plaque PsO thanks to the significantly positive results obtained in the reSURFACE 1 and 2 trials (22, 23). Efficacy and safety of tildrakizumab is under evaluation also for the treatment of active AS or non-radiographic axSpA (ClinicalTrials.gov Identifier: NCT 02980705). A phase II trial based on the use of tildrakizumab, is currently ongoing and results are still being analyzed, but the failure of two previous studies in axSpA with drugs with similar mechanism of action raises doubts these ongoing studies will be successful for treatment in axSpA (38).

Clinical trials focused on the inhibition of IL-23/IL-17 have been fundamental in PsO, while they showed mixed results in PsA and were even less conclusive in axSpA (39) (**Table 1** summarizes IL-17/IL-23 blocking molecules studied in clinical trials for the treatment of axSpA. **Figure 1** for their mechanisms of action). These results may mean that we still need to improve our knowledge of SpA, in particular for its pathogenesis, thus further head-to-head studies and more subtle evaluations of local tissue-specific mechanisms are required.

CLINICAL TRIALS IN AXSPA FOR AVAILABLE DRUGS TARGETING IL-17F

IL-17A shares a structural homology with IL-17F (55%) and has been reported to perform a similar biological function. Depending on the nature of the responder cell, the ligation of IL-17R triggers signaling pathways causing the activation of the transcription factors NFκB, IκBζ, AP1 and C-EBP, which induce transcription of several tissue-specific genes (40). However, recent studies have shown that IL-17F forms a homodimeric complex with receptor IL-17RC driving IL-17RA-independent and therefore (IL-17A-independent) signaling (41). The real distribution of the two IL-17RA and IL-17RC receptors on the surface of the cellular actors involved in the pathogenesis of AS is not clear, but it appears likely that some cell types may overexpress IL-17RC compared to IL-17RA, which could make IL-17F signaling through the symmetrical IL-17RC complex more relevant in some patients. Evidences that IL-17F is also increased in psoriatic skin and synovial cells of patients with peripheral SpA (42), support the rationale for targeting IL-17F as a therapeutic strategy as well.

In a recent phase IIb, randomized, double-blind, placebo-controlled, dose-ranging study, the efficacy of dual neutralisation of IL-17A and IL-17F with bimekizumab in patients with active AS has been studied. At week 12, significantly more bimekizumab-treated patients achieved ASAS40 vs placebo with a significant dose-response. At week 48, 58.6% and 62.3% of patients receiving bimekizumab 160 and 320 mg throughout the study achieved ASAS40, respectively with similar ASAS40 response rates being observed in re-randomized patients. Although these data are absolutely promising, they are not very different from the single inhibition of IL-17A as can be assumed from a theoretical point of view (34). Greater precision in patient selection, trivially assessing the peripheral distribution of IL-17RA and IL-17RC receptors, could increase the percentage of responders. However, further studies are required to determine the impact of double inhibition, IL-17A and IL-17F on the progression of radiographic progression in AS patients.

OTHER MECHANISMS OF ACTION IN AXSPA THERAPIES

A detailed discussion of the clinical efficacy of anti-TNFα goes beyond the aims of the present review article. The current recommendations for AxSpa and PsA include this class of drugs on the same line as other mechanisms of action and based on this TNF inhibitors will be mentioned as comparators, as shown in **Table 2** (43, 44).

TABLE 1 | Molecules blocking the IL-17/IL-23 axis, studied for the treatment of axSpA.

Drug	Target	Disease (clinical trial)	Cohort analyzed	Results
Secukinumab	Monoclonal antibody binding IL-17A	PsA (FUTURE, phase III)	Enrolled 996 active PsA Mean age: 48y F: 49% No prior anti-TNF therapy: 70% Enthesitis: 60% at baseline	Efficacy and safety on sign and symptoms of active PsA and entheseal disease
		axPsA (MAXIMISE, phase III)	Enrolled 498 active axPsA Mean age: 46y F ≈48% No prior bDMARD: 100% Enthesitis: SPARCC score ≈4.6 at baseline	Rapid and significant improvement in ASAS20 responses at week 12 for axial manifestations
		nr axSpA (PREVENT, phase III)	Enrolled 555 nr axSpA with sacroiliac inflammation at MRI Mean age 39y F ≈54% No prior anti-TNF therapy: 90% History of IBD ≈2%	Improvement compared to placebo-treated patients at week 16 for general health status, quality of life and safety
		axSpA (SKIPPAIN, phase III)	Enrolled 383 active axSpA Mean age 42y F 38%	Efficacy, safety, pain relief, improvement of axial disease
Ixekizumab	High-affinity IL-17A monoclonal antibody	PsA (SPIRIT-P1 and -P2, phase III)	Enrolled 363 active PsA Mean age ≈51y F ≈53% Previously treated with anti-TNFα with inadequate response or intolerant to anti-TNFα: 100% Enthesitis: ≈60% at baseline	Improvement in the signs and symptoms of patients with active PsA and entheseal disease
		nr axSpA (COAST-X, phase III)	Enrolled 303 active nr axSpA Mean age 40y F ≈52% No prior anti-TNFα therapy: 100% Enthesitis: ≈48% at baseline IBD: ≈1%	Efficacy
Netakimab (BCD-085)	Monoclonal antibody blocking IL-17	Registered for treatment of AS and PsA in Russia, from spring 2020	Phase II: enrolled 89 active AS Mean age ≈38y F ≈17% No prior anti-TNFα therapy: ≈85% Phase III: enrolled 228 active AS, no results posted	Effective and generally safe
Brodalumab	Monoclonal antibody against IL-17 receptor A	PsA (AMVISION-1 and -2, phase III)	Enrolled 962 active PsA Mean age ≈48y F ≈50% No prior bDMARDs: 70% Enthesitis: ≈67% at baseline	Improvement in signs and symptoms of PsA versus placebo, safety profile similar to other IL17 inhibitors
Iguratimod	Inhibitor of IL-17 production and additional mechanisms	AxSpA (small-scale clinical trials)	4 RCTs and 2 case series Active or refractory axSpA	Significant improvement for the ASAS20 response and reduction in inflammatory biomarkers
Bimekizumab	Monoclonal antibody against IL-17A and IL-17F	AS (Phase IIb clinical trial)	Enrolled 303 active AS Mean age ≈40y F ≈15% No prior bDMARDs: ≈90%	Significant improvement for the ASAS40 response at week 12
Guselkumab	Monoclonal antibody binding the p19 subunit of IL-23	PsA (DISCOVER-1 and -2, phase III)	DISCOVER-1: enrolled 381 active PsA Mean age ≈48y F 48% No prior use of bDMARDs: 69% Enthesitis: ≈58% at baseline DISCOVER-2: enrolled 741 active PsA Mean age ≈46y F ≈47% No prior use of bDMARDs: 100% Enthesitis: ≈68% at baseline	Rapid and significant improvement in PsA patients biologic-naive or previously treated with TNFα inhibitor treatment
Risankizumab	Monoclonal antibody that inhibits IL-23 by binding to its p19 subunit	AS (Phase II clinical trial)	Enrolled 159 active AS Mean age ≈38y F ≈28%	No statistical difference from placebo

(Continued)

TABLE 1 | Continued

Drug	Target	Disease (clinical trial)	Cohort analyzed	Results
Ustekinumab	Monoclonal antibody blocking the p-40 subunit of IL-12/IL-23	AxSpA (Phase III clinical trials)	No prior use of bDMARDs: 100% Enrolled 1018 active AS and nr axSpA Mean age ≈38y F ≈73% No prior use of bDMARDs: 100% in studies 1 and 2, 88% in study 3.	No demonstration of efficacy
Tildrakizumab	Monoclonal antibody that inhibits IL-23 by binding to its p19 subunit	AS, nr axSpA (Phase IIa clinical trial)	Enrolled 180 active AS or nr axSpA Mean age 39y F ≈23%	Failure of two previous studies with drugs with similar mechanism of action; efficacy and safety under evaluation in SpA

AxSpA, axial spondyloarthritis; axPsA, psoriatic arthritis with axial involvement; nr axSpA, non-radiographic axial SpA; AS, ankylosing spondylitis. ASAS, Assessment of Spondyloarthritis international Society. SPARCC, Spondyloarthritis Research Consortium of Canada enthesitis index. DMARD, disease-modifying anti-rheumatic drug. y, years. F, female.
In light gray, effective drugs; in black, non effective drugs; in dark gray, ongoing study.

Besides the anti-TNFα therapies approved in the last 20 years for the treatment of SpA patients (45–47), with proved efficacy also for the treatment of extraarticular manifestations such as uveitis (RAPID-axSpA ClinicalTrials.gov Identifier: NCT01087762) (48), biosimilars of TNFα inhibitors have become increasingly used, and observational studies of biologics-naïve patients with SpA have shown similar response and safety in patients treated with originators versus biosimilars, indicating comparable effects in clinical practice (49).

Oral small molecules targeting specific pro-inflammatory intracellular pathways are currently used in conditions such as PsA, as in the case of the phosphodiesterase 4 inhibitor apremilast, and the Janus kinase (JAK) inhibitors tofacitinib and baricitinib, plus filgotinib and upadacitinib still in development for PsA. The rationale for the use of JAKinibs in AS derives from studies in experimental models that have demonstrated a key role of the JAK/STAT pathway in the pathogenesis of the disease (50, 51). Moreover, *ex vivo* data also demonstrated the ability of several JAK inhibitors to inhibit Th17 responses in patients with AS. The potential efficacy of JAKinibs in the treatment of axial SpA is supported by recent phase II and III clinical trials. Tofacitinib, a JAK1 and 3 inhibitor, was tested in a phase II study in 208 AS patients (52). The ASAS20 response at week 12 occurred in 63%, 67%, and 40% in the tofacitinib 5 and 10 mg arms and placebo, respectively, and reduction of inflammation measured by MRI was demonstrated. The phase III study in AS is currently ongoing (ClinicalTrials.gov Identifier: NCT03502616). Upadacitinib, a selective JAK-1 inhibitor, has been also studied in AS patients with active disease and an inadequate response or contraindication to non-steroidal anti-inflammatory drugs in a double-blind, randomised controlled phase 3 trial, the SELECT-Axis study 1. In this study, significantly more patients in the upadacitinib group reached a higher ASAS40 response compared to placebo group at week 14 (52% *vs* 26%) (53). The TORTUGA study evaluated the efficacy of another selective JAK-1 inhibitor, filgotinib, in patients with AS (54). In this study, at week 12, 76% of patients receiving filgotinib achieved an ASAS20 response compared with 40% of patients assigned to placebo. ASAS40 was achieved by 38% patients assigned to filgotinib and by 19% patients assigned to placebo. Based on these studies, JAK blockade could represent a valid future therapeutic strategy in patients with AS.

DISCUSSION

From a pathological point of view, IL-23 is a crucial cytokine in the onset of disease manifestations such as enthesitis that may characterize peripheral manifestations in axSpA, as demonstrated by the fact that IL-23 is sufficient to induce the development of enthesitis and entheseal new bone formation in

TABLE 2 | Comparison of different biologic class efficacy in rheumatic diseases and disease subtypes.

	Anti-TNFα	Anti-IL-17	Anti-IL-23	JAK inhibitor	PDE4 inhibitors
RA	+	–	–	+	–
AxSpA	+	+	–	Ongoing studies	–
Disease activity					
Radiographic progression	+	+	–	Ongoing studies	–
PsA	+	+	+	+	+
Enthesitis	+	+	+	+	+
Peripheral arthritis	+	+	+	+	+
Dactylitis	+	+	+	+	+
Axial manifestations	+	+	–	Ongoing studies	–
Psoriasis	+	+	+	–	+
Nail	+	+	+	–	+
IBD	+	–	+	+	–
Acute anterior uveitis	+	–	–	–	–

AxSpA, axial spondyloarthritis; IBD, inflammatory bowel diseases; RA, rheumatoid arthritis; PsA, psoriatic arthritis; PsO, psoriasis.

the initial complete absence of synovitis (55). IL-23 stimulates the survival and expansion of Th-17 cells through the receptor IL-23R expressed by uncommitted CD4 and CD8 negative T cells, and this induces the related downstream signaling pathway crucial for the onset of Th-17-mediated diseases like PsA and axSpA (56).

Consistent with these mechanistic models, IL-17 inhibitors showed efficacy in axSpA treatment (13, 14) and case-control genome-wide-association studies demonstrated that an IL-23R polymorphism is associated with SpA (57). Moreover, the overexpression of IL-23 in mice can trigger a form of enthesitis which is similar to enthesitis observed in SpA patients (55). Therefore, it was unexpected to observe the results of two placebo-controlled trials in SpA showing that ustekinumab (31) and risankizumab (37) had no significant improvement on disease activity.

Inconclusive data from trials on IL-23 inhibitors may have several explanations, such as still unknown mechanisms of disease, different molecular effects compared to IL-17 blockers or confounding factors in the design of clinical trials, as heterogeneous enrollment with differences in the composition of the clinical cohorts where the monoclonal antibodies are being investigated (shown in **Table 1**). A possible additional mechanism of action of IL-17 and IL-23 involves bone metabolism, which is a pivotal pathogenic pathway in axSpA, related to inflammation. As for IL-17A, it has a potential effect on osteoblast differentiation that may depend on the cell type exposed, the differentiation stage of that cell and the timing and duration of cytokine exposure. On the contrary, IL-23 does not seem to have an effect on osteoblast activation (58).

Another difference in the molecular effects of the two cytokines is related to the timing, as IL-23 is thought to play a role in the early stages, making its blocking ineffective in established and symptomatic disease. In murine models the presence of IL-23 induced the conversion of non-pathogenic Th17 into pathogenic Th17 cells, an effect still to be well analyzed in humans (58).

The hypothesis of a non-linear relationship and an uncoupling of IL-23 and IL-17 can also be inferred from Crohn's diseases management, where IL-23 inhibition has some efficacy, while IL-17 blockade can worsen the outcomes.

A deeper knowledge of the IL-17/IL-23 axis should be pursued, as it appears to be one of the main pathways involved in the development of axSpA. In fact, several therapies currently used in conditions such as RA have not demonstrated efficacy in axSpA, as in the case of IL-1 or IL-6 inhibitors, T-cell modulators and B-cell ablators. Furthermore, apremilast, introduced in the recommendation for the management of peripheral PsA (43) was tested for active AS in a small proof-of-concept study (59), showing improvement of BASDAI in the treatment versus placebo arm, but the phase III trial failed to discriminate between treated and placebo patients (POSTURE study, ClinicalTrials.gov Identifier: NCT01583374). These results show that it is not enough to reduce inflammation in synovial tissues by using unspecific drugs, and that immunomodulatory agents do not all reach the target to reduce inflammation, in particular in a condition such as axSpA which involves many immunologic pathways in different anatomical areas and where inflammation is related to bone formation in specific sites. Additional chronic autoimmune diseases related to axSpA, such as PsA and IBD- related arthritis are currently evaluated to identify new therapeutic pathways and targets that may lead to disease remission.

Another significant aspect that we may consider for trials results analysis is that AS and axial PsA may be two different diseases with overlapping features rather than entities on the spectrum of the same disease (60, 61). In fact, AS patients with or without PsO seem to be consistently different from axSpA patients when it comes to their demographic features (15 years younger at their first manifestation of arthritis and first presented to the clinic 7 years earlier), genetic predisposition (much higher male predominance and four times more likely to be HLA-B27 positive), clinical characteristics (worse axial disease in AS versus axial PsA, whereas axial PsA has worse peripheral arthritis than AS patients) and radiographic alterations (worse grade of sacroiliitis on radiographs).

These aspects are crucial in the planning of clinical trials, when defining the population of patients to be included, as it may influence the results of specific treatments.

When choosing the biologic therapies for SpA patients, IL-17 inhibitors have always raised concern over the risk of IBD onset. In fact, the clinical development of secukinumab for Crohn's disease was stopped after a small sample size proof-of-concept study because of a higher rate of adverse events including relapse of pre-existing or new onset of IBD (62), and similarly in the phase-2 trial with brodalumab (63). The concern over elevated risk of IBD onset or worsening was minimized in a recent pooled safety analysis of 21 randomised-controlled trials plus post-marketing safety data (2014–2017) with secukinumab, across all rheumatological indications. This analysis reported an exposure adjusted incidence rate of IBD of <0.1- 0.4/100 person-years consistent with the background expected range of incidence rates for these patients (64–66). In conclusion, clinical trials have so far demonstrated the efficacy of anti-IL-17 therapies for axSpA treatment but data on IL-23 blocking remain to be elucidated after the earlier negative findings. This may be due to several reasons, such as the unique immunopathological microenvironment of axSpA, with IL-17 secretion in the absence of IL-23, at least in established disease. Another possible reason may be that many cell types different from conventional T cells can produce IL-17 in axSpA patients, partially independent of IL-23, and this should be extensively investigated. Currently, IL-17 and TNFα inhibitors are the only effective targeted therapies for axSpA and additional treatments need further testing in clinical trials to assess their efficacy and safety in axSpA.

AUTHOR CONTRIBUTIONS

AC and FM contributed to data collection and review organization. NI and MV contributed to finalizing the review writing. FC and CS supervised the review writing and organization. All authors contributed to the article and approved the submitted version.

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Impact of Janus Kinase Inhibition on the Treatment of Axial Spondyloarthropathies

Ariane Hammitzsch, Georg Lorenz and Philipp Moog*

Section of Rheumatology, Department of Nephrology, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany

Many immune cells and effector molecules (e.g. cytokines, Interferons, growth factors) utilize different combinations of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) molecules to transduce signals from the cell surface to the nucleus, where they regulate transcription. This pathway is basically involved in almost all inflammatory diseases and also in the interleukin (IL)-23/IL-17 cascade, which is an essential part of the pathogenesis of spondyloarthropathies (SpA). Upon evidence from *in vitro* and *in vivo* experiments indicating disease-modifying effects of JAK inhibition in inflammatory joint disease, numerous inhibitors of the JAK/STAT pathway (= JAKinibs) with different selectivity against the four members of the JAK family [JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2)] were developed. Trials in rheumatoid arthritis were successful with respect to efficacy and safety, and currently, three JAKinibs are approved for the treatment of rheumatoid arthritis in the European Union. Although new treatment options (anti-IL-23, anti-IL-17, and phosphodiesterase 4 inhibitors) have become available for spondyloarthritis and especially psoriatic arthritis (PsA) within the last years, most of them are biologics and do not address all disease manifestations equally. Therefore, multiple trials were initiated to evaluate JAKinibs in PsA and axial spondyloarthritis (axSpA). A trial of Tofacitinib (OPAL) was successful in PsA and has led to the inclusion of JAKinibs into the treatment algorithm. Currently many trials with JAKinibs are ongoing for PsA and axSpA, with one phase III trial of upadacitinib (selective JAK1 inhibitor) showing good therapeutic response in active radiographic axSpA.

Keywords: JAK – STAT signalling pathway, small molecule inhibitor, axial spondyloarthritis, preclinical efficacy and tolerability, safety profile

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****Correspondence:***
Ariane Hammitzsch
Ariane.Hammitzsch@gmail.com

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INTRODUCTION

Spondyloarthropathies (SpA) are a group of chronic inflammatory diseases, including axial SpA (axSpA) and psoriatic arthritis (PsA), as well as other less common forms like enteropathic or reactive arthritis. Besides skeletal manifestations (axial disease, peripheral arthritis, enthesitis, and dactylitis), the involvement of extra-articular organs (uveitis, psoriasis, and inflammatory bowel disease [IBD]) is a shared feature of these diseases (1). Current therapeutic options for SpA are limited compared with those for rheumatoid arthritis (RA), especially for axSpA, and mainly antibody-based, such as anti-tumor necrosis factor (TNF), anti-Interleukin (IL)-23 and anti-IL-17. Additionally, the therapeutic response greatly varies between the different diseases and affected

systems such as the spine, peripheral joints, skin, and eyes. Only 51.3% of axSpA patients respond to TNF inhibitors (TNFi), some loose response over time, and others are not eligible (2, 3). Janus kinase (JAK) and signal transducer and activator of transcription (STAT) molecules are central transmitters of pro- and anti-inflammatory signals in immune regulation (4). The IL-23/IL-17 pathway is highly important in the pathogenesis of SpA and is partly controlled by JAK (5, 6). Therefore, JAK inhibitors offer new treatment options for SpA. As these are currently more limited for axSpA compared with PsA, this review focuses on JAK inhibitors and their clinical application in axSpA.

JANUS KINASE AND SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION SIGNALING IN SPONDYLOARTHRITIS

JAK and STAT are central signal transducers for a great number of pro-inflammatory (e.g. IL-2, IL-7, IL-12, and IL-23) and anti-inflammatory cytokines (e.g. IL-10) influencing innate immune responses thought to be essential for the induction of SpA and adaptive immune functions maintaining and perpetuating the disease (7–9). This intracellular tyrosine kinase family consists of JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) and is coupled to STAT molecules (STAT1, STAT2, STAT3, STAT4, STAT5a and b, and STAT6) (7). Cytokine-receptor binding on the cell surface leads to autophosphorylation of JAK or phosphorylation of a partner JAK. Such activated JAK further phosphorylate sites of the intracellular domain of the receptor providing docking sites for STAT molecules. Dimers of STAT molecules phosphorylated by JAK migrate to the nucleus where they regulate gene expression. Different combinations of JAK and STAT are assigned to different cytokines and their receptors, providing a multitude of pathways and functions, as depicted in **Figure 1** (7). However, STAT can be activated by other kinases and exercise effects in an un-phosphorylated state and even extra-nuclear. JAK also act independently of STAT molecules, for example, by directly phosphorylating histones adding further to the complexity of JAK and STAT signaling in immune cell regulation (10).

With regard to the pathogenesis of SpA, JAKs are involved in the signaling of key cytokines within the IL-23/IL-17 pathway, and Genome-wide association studies have found single nucleotide polymorphisms (SNPs) for *IL23R*, *JAK2*, and *TYK2* in ankylosing spondylitis (AS) (11). IL-23, produced by activated myeloid cells, is important for the generation of IL-17 and IL-22 by target cells such as T helper cells 17 (Th17), gamma delta T cells (γδ T cells), or innate lymphoid cells (ILCs) type 3 (12). A combination of JAK2 and TYK2 transmits the IL-23 signal via STAT3 and, to a lesser extent, STAT4 (6, 13). IL-17A production is mainly JAK2-dependent, whereas IL-22 production requires TYK2 and JAK2 (14). By blocking IL-17 production, JAK inhibition subsequently affects the downstream effects of IL-17. Other cytokines favouring the development and maintenance of IL-17 producing cells include IL-6 (JAK1/JAK2/TYK2) and IL-21 (JAK1/JAK3) (7, 15). IL-22, another effector cytokine in the pathogenesis of SpA, uses the combination of JAK1 and TYK2 (7). Next to its protective functions at the epithelial barrier in the gut, IL-22 has pro-inflammatory and proliferative effects (synovial fibroblasts, keratinocytes) as well as osteoanabolic effects providing another interesting treatment target for SpA (16–18). Granulocyte-macrophage colony-stimulating factor, another pro-inflammatory cytokine produced by T cells and ILCs type 3 in SpA patients, signals via JAK2 (7, 19).

Respective of the various cytokines relying on JAK-STAT signaling, inhibition of this pathway offers multiple possibilities to modulate the immune and tissue response implicated in SpA. As cells of articular and extra-articular organs are utilizing this pathway, JAKinib will most likely affect the different sites of the disease. However, the protective pathways regulated by JAK-STAT might lead to adverse effects like viral infections by interfering with interferon signaling, as wells as with numbers and function of natural killer (NK) cells, cytotoxic T cells, and ILC (7).

ANIMAL AND PRECLINICAL DATA ON JANUS KINASE INHIBITORS IN SPONDYLOARTHROPATHIES

Although animal models for SpA do not fully replicate human disease, they have been useful in examining the molecular disease mechanisms and the role of JAK-STAT signaling. Enthesitis, an early and common feature in all forms of SpA, appears in mice with a myeloid cell-specific A20 (TNF-α-induced protein 3) deficiency (9, 20). The SpA-like arthropathy in this model is independent of TNF and relies on IL-1β and IL-6. Treatment with tofacitinib, an unselective JAK inhibitor, significantly reduced disease activity, confirmed by less inflammation of the synovial–entheseal complex on histology (20). In the SKG mouse model, which resembles human SpA if arthritis is initiated with curdlan and is dependent on IL-23 and Th17 cells, treatment with tofacitinib ameliorated established disease (21). Another experimental JAKinib also suppressed both inflammation and periosteal/entheseal bone formation in this model (22).

JAKinibs of different selectivity were shown to reduce Th17-type responses in CD4 T cells from patients with AS, PsA, and RA *ex vivo* with similar efficacy (23). Small interfering RNA-mediated knockdown of TYK2, signaling downstream of IL-23, was shown to be equally efficient in reducing type-17 cytokine secretion compared with JAK1 silencing or tofacitinib treatment (23). Several SNPs around the *TYK2* locus are associated with AS. Some of these exonic SNPs lead to loss-of-function variants of TYK2. One of these SNPs associated with multiple autoimmune diseases is protective, but does not impact on non-autoimmune domains such as susceptibility to infections (24). A highly specific TYK2 inhibitor, NDI-031407, blocked disease progression in the SKG mouse model (14). MRI imaging showed prevention of joint space narrowing and bone marrow edema. NDI-031407 also protected mice from bone marrow edema and enthesis-related synovitis in the IL-23 mini-circle model (mostly dependent

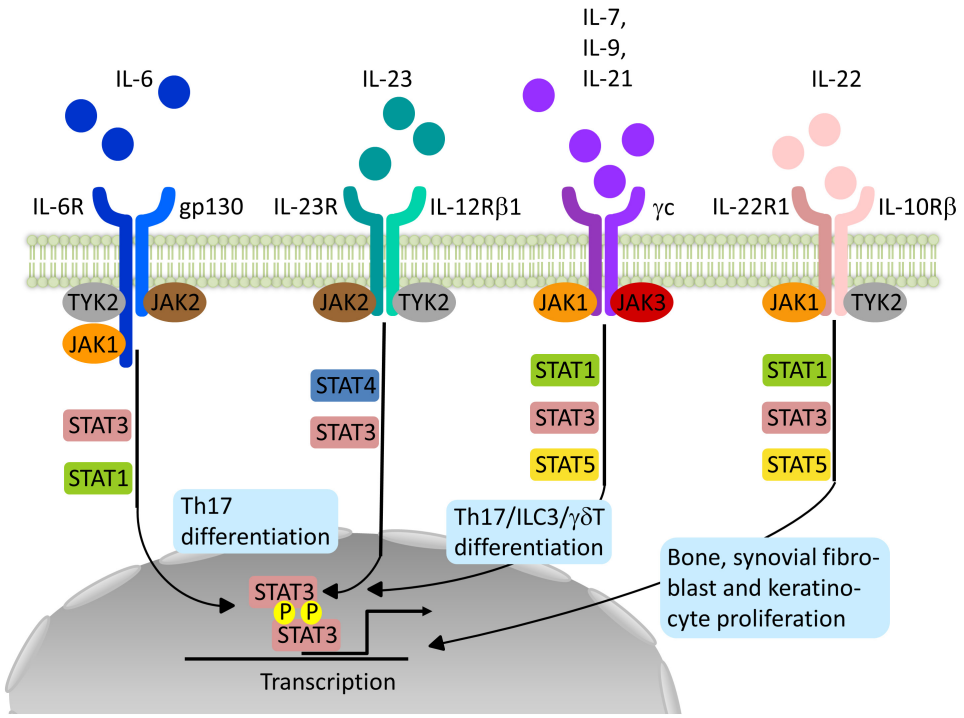

FIGURE 1 | Schematic representation of relevant JAK-STAT signaling pathways in the pathogenesis of Spondyloarthritis. Binding of the different Interleukins (IL) to their specific receptor subunits on different cell populations, e.g. T cells, innate lymphoid cells (ILC) or effector cells such as osteoblasts, fibroblasts or keratinocytes leads to activation of a specific JAK-STAT pathway. The different isoforms of JAK are coupled to specific receptor/cytokine pairs and allow for a targeted inhibition with a specific JAKinib. However, overlap exists allowing for unintended side effects or accumulative effects. JAK1-specific inhibitors for example affect signaling by IL-6, IL-21, IL-7, IL-9 and IL-22 targeting most of the relevant immune and effector cell populations in SpA pathogenesis.

on γδ T cells). It completely abrogated IL-22 production but only partially inhibited IL-17 production from γδ T cells upon stimulation with IL-1β and IL-23 (14). The frequency of a loss-of-function *TYK2* SNP (rs12720356) was significantly higher in AS patients with lower rates of spinal fusion, providing further evidence that targeting JAK could have effects on ankylosis.

With regard to the effects of JAKinib on bone metabolism, an important aspect in SpA, an experimental JAK2 inhibitor, AG490, reduced alkaline phosphatase activity in primary bone-derived cells from AS patients and healthy controls (25). On the other hand, tofacitinib and baricitinib increased bone mass in the K/BxN serum-transfer mouse model of RA-like arthritis and enhanced osteoblast function *in vitro* while sparing osteoclasts (26). These findings were confirmed in two RA patients treated with tofacitinib showing a substantial reduction in erosions of the metacarpophalangeal joints by micro-CT. Because activation of bone formation is deleterious in axSpA but useful in RA, further insight into the differential effects of JAKinib in these diseases is warranted.

Considering the combined data from animal models and clinical trials of anti-IL-23 antibodies in axSpA, TYK2 and JAK1 emerge as most promising targets of JAKinib for the treatment of axSpA, as they are involved in pathways relevant to the initiation (IL-23) and effector (IL-22) phase of the disease and especially in osteoproliferation (27). **Table 1** gives an overview of JAKinibs already tested in clinical trials and under preclinical evaluation.

CLINICAL DATA ON JANUS KINASE INHIBITORS IN SPONDYLOARTHROPATHIES

Tofacitinib (a pan-JAKinib, 196 biologic naïve patients) and filgotinib (a selective inhibitor of JAK1, no more than one TNF inhibitor, 107 patients, TORTUGA) have been trialed in phase II trials of active AS with an inadequate response to ≥2 or intolerance to non-steroidal anti-inflammatory drugs and high-sensitivity C-reactive protein (CRP) ≥3 mg/L (filgotinib trial) (50, 51). Upadacitinib (selective for JAK1) has been evaluated in a combined phase II/III trial (178 JAKinib and biologic naïve patients, SELECT-Axis 1) in active AS with the earlier mentioned inclusion criteria (52). The combined data on the efficacy on disease activity, functionality, and radiographic progression summarized below are extracted from these studies.

Efficacy on Disease Activity

After 12 weeks of treatment, Assessment in SpondyloArthritis International Society 20 (ASAS20) response rates were significantly higher for 5-mg tofacitinib twice daily (80.8%) and 200-mg filgotinib once daily (76%) compared with placebo (41.2 and 40%, respectively) but not for 2 mg (51.9%) or 10 mg (55.8%) of tofacitinib. ASAS40 response was significantly higher for all tofacitinib groups at week 12 and for 15-mg upadacitinib

TABLE 1 | Overview of JAK inhibitors tested in clinical trials and under preclinical evaluation for spondyloarthropathies and related diseases.

JAK inhibitor	Target	Trial/disease	Status	PMID/NCT trial number	Trial status
Tofacitinib	JAK1/JAK3	Rheumatoid Arthritis	Approved	(28–31)	
		Psoriatic Arthritis	Approved	(32, 33)	
		Axial Spondyloarthritis	Phase III	NCT03502616	Active
		Ulcerative Colitis	Phase III	(34)	
		Crohn's disease	Phase II	(35)	
		Psoriasis	Phase III	(36)	
		Uveitis	Phase II	NCT03580343	Active
Baricitinib	JAK1/JAK2	Rheumatoid Arthritis	Approved	(37, 38)	
Filgotinib	JAK1	Rheumatoid Arthritis	Phase III	(39)	
		Axial Spondyloarthritis	Phase III	NCT04483687, NCT04483700	Not started, Not started
		Psoriatic Arthritis	Phase III	NCT04115748, NCT04115839	Active, Recruiting
		Ulcerative Colitis	Phase III	NCT02914522	Completed
		Crohn's disease	Phase III	NCT02914561	Recruiting
		Uveitis	Phase II	NCT03207815	Recruiting
Upadacitinib	JAK1	Rheumatoid Arthritis	Approved	(40–42)	
		Axial Spondyloarthritis	Phase III	NCT04169373	Recruiting
		Psoriatic Arthritis	Phase III	NCT03104374, NCT03104400	Active, Active
		Ulcerative Colitis	Phase III	NCT03653026, NCT02819635	Recruiting, Recruiting
		Crohn's disease	Phase III	NCT03345836, NCT03345849	Recruiting, Recruiting
Pefacitinib	Pan-JAK	Rheumatoid Arthritis	Phase III	(43, 44)	
		Psoriasis	Phase II	(45)	
		Ulcerative Colitis	Phase II	(46)	
Deucravacitinib (BMS-986165)	TYK2	Psoriatic Arthritis	Phase II	NCT03881059	Active
		Psoriasis	Phase II	(47)	
Abrocitinib (PF-04965842)	JAK1	Psoriasis	Phase II	(48), NCT02201524	Terminated
Itacitinib (INCB039110)	JAK1	Rheumatoid Arthritis	Phase II	NCT01626573	Completed
		Psoriasis	Phase II	NCT01634087	Completed
PF-06651600	JAK3	Rheumatoid Arthritis	Phase II	NCT04413617	Not started
		Ulcerative Colitis	Phase II	NCT02958865	Recruiting
		Crohn's disease	Phase II	NCT03395184	Recruiting
SHR0302	JAK1	Rheumatoid Arthritis	Phase III	NCT04333771	Not started
		Axial Spondyloarthritis	Phase II/III	NCT04481139	Not started
		Ulcerative Colitis	Phase II	NCT03675477	Recruiting
		Crohn's disease	Phase II	NCT03677648	Recruiting
PF-06826647	TYK2	Psoriasis	Phase II	NCT03895372	Recruiting
		Ulcerative Colitis	Phase II	NCT04209556	Recruiting
Brepocitinib (PF-06700841)	JAK1/TYK2	Psoriatic Arthritis	Phase II	NCT03963401	Active
		Psoriasis	Phase II	NCT02969018	Completed
		Ulcerative Colitis	Phase II	NCT02958865	Recruiting
		Crohn's disease	Phase II	NCT03395184	Recruiting
NDI-031407	TYK2	SKG mouse model	Preclinical	(14)	
NDI-031232	TYK2		Preclinical		
SAR-20347	JAK1/TYK2	Psoriasis mouse model	Preclinical	(49)	

once daily compared with placebo at week 14 (52 vs. 26%). Tofacitinib (5-mg), filgotinib, and upadacitinib additionally lead to a significantly higher change of the mean Ankylosing Spondylitis Disease Activity Score (ASDAS) with rates of −1.4, −1.47 at week 12, and −1.45 at week 14, respectively, compared with placebo (−0.9, −0.57, and −0.54). Bath Ankylosing Spondylitis Disease Activity Index 50 (BASDAI50) response rates were significantly higher for all tofacitinib groups and upadacitinib with 42.3 to 46.2 and 45% vs. 23.5 and 23% in the placebo group. Enthesitis was significantly ameliorated by week 12 in 5- and 10-mg tofacitinib versus placebo.

The onset of response was slower with tofacitinib (approx. week 4) compared with TNF inhibitors but very rapid for filgotinib (week 1) and upadacitinib (week 2).

One limitation of the study of filgotinib is the relatively high proportion of patients with high high-sensitivity CRP at baseline, as elevated CRP is a known predictor of good response to therapy (53).

Efficacy With Regard to Functionality

Spinal mobility measured by Bath Ankylosing Spondylitis Metrology Index (BASMI) improved significantly with filgotinib compared with placebo by week 12 (−0.75 vs. −0.39). In the tofacitinib trial, significant improvement of BASMI was only achieved with 10 mg twice daily. In the upadacitinib trial, consistent improvements were seen with treatment for BASMI but did not meet significance based on multiplicity adjustment per the Hochberg procedure.

Efficacy With Regard to Radiographic Progression

Five- and 10-mg tofacitinib and filgotinib significantly improved Spondyloarthritis Research Consortium of Canada spine (−5.5 and −6.6, and −5.7) and sacroiliac joint (SIJ) scores (−3.2 and −3.6, and −3.52) compared with placebo (spine −0.1 and −0.52, SIJ −0.8 and 0.06). Upadacitinib had significant effects on the Spondyloarthritis Research Consortium of Canada spine score (−6.93 vs. −0.22).

A recent evaluation of the baseline and week 12 MRI scans from the TORTUGA trial found decreased SIJ erosion scores and increased backfill scores in the filgotinib group with increased erosion scores and no change in backfill scores in the placebo group, supporting the effects of filgotinib on structural lesions in axSpA (54).

Treatment-Emergent Adverse Events

Treatment-emergent adverse events (TEAEs) appeared slightly more often with 5- and 10-mg tofacitinib compared with 2 mg and placebo (53.8 and 51.9% vs. 44.2 and 43.1%) and upadacitinib (62 vs. 55% in the placebo group) but were similar in the filgotinib trial (31% both groups). The most common TEAEs in all trials were nasopharyngitis and upper respiratory tract infections. There were no malignancies, opportunistic infections, and cases of active tuberculosis or cases of extra-articular manifestations (IBD, psoriasis, and uveitis). Episodes of herpes zoster (HZ) were reported with tofacitinib and upadacitinib, and one non-serious venous thromboembolic event (VTE) with filgotinib.

Results from a phase III randomized controlled trial (RCT) of tofacitinib in active AS (NCT03502616) are expected this year.

Safety of Janus Kinase Inhibitors

In general, the long-term safety profile of JAKinibs is good and similar among the different inhibitors. Fears of high rates of opportunistic (including tuberculosis) and other infections have not been confirmed. Due to a lack of long-term data for JAKinib in SpA, data reported here are collected from clinical trials and post-marketing surveillance of RA. This seems feasible, as the three performed trials of JAKinibs in AS have so far shown similar safety profiles. However, it cannot be excluded that with longer observation periods and new trials leading to more exposed patients, new safety concerns may arise.

Overall incidence rates of serious infections are similar to those with biological disease-modifying antirheumatic drugs and range from 2.5 to 3.8 per 100 patient-years (55–58). However, a thorough screening of patients for tuberculosis before therapy is mandatory, with special alertness to extrapulmonary manifestations of tuberculosis (59). Reactivation of hepatitis B has been reported with JAKinib treatment, but treatment with tofacitinib in refractory cases under antiviral prophylaxis seems safe and effective (60, 61). The increased incidence of HZ is specific for JAKinib treatment and, for unknown reasons, seems to be more pronounced in Japan and Korea, ranging from 3.3 to 3.9 per 100 patient-years (26, 57, 58, 62, 63). The common risk factor for HZ over the different JAKinib was age (64, 65). Filgotinib so far has shown lower incidence rates of HZ and serious infections compared with other JAKinib, but long-term observations are lacking (66). This effect can possibly be attributed to less inhibition of JAK1-mediated signaling of interferon γ and IL-2, IL-4, and IL-15 (necessary for proliferation of NK cells) by filgotinib (67).

Regardless of a slightly increased risk for overall malignancies for RA patients compared with the general population, so far, no significant effects of JAKinib have been identified, excluding non-melanoma skin cancer (68). With respect to the two- to threefold increased risk for lymphoma in RA patients, the crude incidence rates with tofacitinib and baricitinib were low, with 0.10 (56). The effects of long-term use of JAKinib on the risk of cancer, for example, via interference with tumor surveillance through NK cells and interferon signaling are still unknown (57, 69).

As patients with RA, AS, and PsA generally have an increased risk for deep vein thrombosis (DVT), pulmonary embolism (PE), and venous thrombembolism (VTE) (risk ratios, 2.08, 2.17, and 1.96, respectively), special interest was given to such events in trials with JAKinibs (70–72). Incidence rates for DVT and PE with tofacitinib were 0.1 each (0.2 for PE with the 10-mg dose) and for VTE with upadacitinib 0.6 per 100 patient-years, and 0.1 and 0.2 per 100 patient-years with 100 and 200 mg of filgotinib (57, 58, 73). Therefore, a randomized safety endpoint study in moderate to severe RA comparing tofacitinib and TNF inhibitor has been implemented, including patients with at least one cardiovascular risk factor (NCT02092467). Nevertheless, the FDA and EMEA requested a warning of thrombosis for tofacitinib, baricitinib, and upadacitinib in 2019 (74, 75). A mechanistic explanation for the increased risk of thromboembolic events is still lacking. Despite a metanalysis of 30 RCTs on JAKinib in RA showing no significant differences in short-term major adverse cardiac events or VTE, a recent analysis of the World Health Organization global database (VigiBase) revealed a 2.3–3.4-fold increased risk for DVT and PE with tofacitinib and baricitinib in Europe (76, 77).

Use of a JAKinib has to be carefully evaluated in patients with risk factors for gastrointestinal perforation such as older age, history of diverticulitis or other gastrointestinal conditions, and use of prednisolone >7.5 mg/day or non-steroidal anti-inflammatory drugs (78, 79). The incidence rates per 1,000 patient-years for gastrointestinal perforation were 0.11 for tofacitinib and 0.04 for baricitinib (56). In analogy to tocilizumab, the risk of gastrointestinal perforation might be ascribed to the inhibition of IL-6 signaling by the different JAKinibs (57, 80).

Dose adjustments according to the metabolism of each drug should be considered for patients with moderate to severe hepatic or renal dysfunction. Laboratory changes in patients treated with JAKinibs are common and include changes

of hemoglobin, lymphocyte and platelet counts. However, it has been hard to separate the intrinsic effects of JAKinibs via concomitant JAK2 inhibition (main signaling JAK for erythropoietin and thrombopoietin receptors) and disease-driven inflammatory effects on erythro- and thrombopoiesis. Other common laboratory changes include elevation of serum transaminases, creatinine, high- and low-density lipoprotein cholesterols, but usually do not result in treatment cessation.

Teratogenic effects of JAKinib have been reported in preclinical animal studies, and so far pregnancy outcomes of 47 patients treated with tofacitinib during RCT are known (81–83). There were 25 healthy newborns, one congenital pulmonary valve stenosis, seven spontaneous abortions, eight medical terminations, and six pending or lost to follow-up (84). Therefore, JAKinibs are contraindicated during pregnancy and breastfeeding, requiring strict contraception in females of child-bearing age until at least 1 week after the last dose.

Next to the known TEAEs of conventional synthetic and biological disease-modifying antirheumatic drugs, e.g. infections, a special focus has to be placed on HZ, VTE and PE, and changes in blood cells and lipid metabolism with JAKinib treatment.

DISCUSSION AND PERSPECTIVE

Although the data from three RCTs of JAK inhibitors in active AS are very promising, studies evaluating patients who have failed TNFi or anti-IL-17 therapy will be of great interest to place JAKinibs in the treatment algorithm of axSpA. Other interesting issues are head-to-head comparisons with TNFi and efficacy in non-radiographic axSpA. For a better assessment of the long-term safety results of the SpA study extensions will have to be awaited. Also the differential effect of more selective JAKinibs on the various disease manifestations of SpA needs to be clarified. Interest focuses on the newly developed specific TYK2 inhibitor, BMS-986165, which has already completed a phase II trial for psoriasis and promises clinical efficacy in axSpA by preclinical data and translational research. It also needs to be elucidated if SpA patients might profit from different dosing strategies for induction and maintenance of remission, such as high loading doses and low maintenance doses. However, these new orally available agents will most likely soon be included in the treatment recommendations for axSpA and provide the clinician with options in patients who are not eligible or have contraindications to TNFi or anti-IL-17, such as allergic reactions, congestive heart failure, or concomitant demyelinating disease (TNFi) and concomitant active IBD (anti-IL-17) (85, 86). JAKinibs may also be advantageous in patients with repeated infections, as they have a shorter half-life compared with bDMARD or csDMARD. With regard to avoiding radiographic progression and chronic disability in axSpA patients, JAKinibs face the same challenges as other drugs. From long-term observations with TNFi, it became evident that a halt in radiographic progression probably can only be achieved with very early and prolonged treatment (for more than 4 years) (87, 88). Targeting new bone formation specifically might have too many adverse effects on general bone homeostasis.

Overall, JAKinibs seem safe when used in a well-screened patient population of SpA and under regular surveillance. They appear equally effective to biologic drugs by current evidence and have advantages besides their oral application and shorter half-life.

AUTHOR CONTRIBUTIONS

AH and PM drafted the manuscript. AH wrote the manuscript and created graphical illustrations with the input from GL and PM. All authors approved the final version of the manuscript.

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IL-12p40/IL-23p40 Blockade With Ustekinumab Decreases the Synovial Inflammatory Infiltrate Through Modulation of Multiple Signaling Pathways Including MAPK-ERK and Wnt

Renée H. Fiechter[1,2,3*†], *Henriëtte M. de Jong*[1,2,3†], *Leonieke J. J. van Mens*[1,2,3], *Inka A. Fluri*[1,2,3], *Sander W. Tas*[1,2,3], *Dominique L. P. Baeten*[1,2,3], *Nataliya G. Yeremenko*[1,2,3] *and Marleen G. H. van de Sande*[1,2,3*]

[1] *Amsterdam UMC, Department of Rheumatology and Clinical Immunology, Amsterdam Institute for Infection and Immunity, University of Amsterdam, Amsterdam, Netherlands,* [2] *Amsterdam UMC, Department of Experimental Immunology, Amsterdam Institute for Infection and Immunity, University of Amsterdam, Amsterdam, Netherlands,* [3] *Amsterdam Rheumatology and Immunology Center, Amsterdam, Netherlands*

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Lars Rogge,
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***Correspondence:**
Renée H. Fiechter
r.h.fiechter@amsterdamumc.nl
Marleen G. H. van de Sande
m.g.vandesande@amsterdamumc.nl

† *These authors have contributed equally to this work*

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Background: Psoriatic arthritis (PsA) is a chronic inflammatory joint disease within the spondyloarthritis spectrum. IL-12p40/IL-23p40 blockade reduces PsA disease activity, but its impact on synovial inflammation remains unclear.

Objectives: To investigate the cellular and molecular pathways affected by IL-12p40/IL-23p40 blockade with ustekinumab in the synovium of PsA patients.

Methods: Eleven PsA patients with at least one inflamed knee or ankle joint were included in a 24-week single-center open-label study and received ustekinumab 45 mg/sc according to standard care at week 0, 4, and 16. Besides clinical outcomes, synovial tissue (ST) samples were obtained by needle arthroscopy from an inflamed knee or ankle joint at baseline, week 12 and 24 and analyzed by immunohistochemistry, RNA-sequencing and real-time quantitative polymerase chain reaction (qPCR).

Results: We obtained paired baseline and week 12, and paired baseline, week 12 and 24 ST samples from nine and six patients, respectively. Eight patients completed 24 weeks of clinical follow-up. At 12 weeks 6/11 patients met ACR20, 2/11 met ACR50 and 1/11 met ACR70 improvement criteria, at 24 weeks this was 3/8, 2/8 and 1/8 patients, respectively. Clinical and serological markers improved significantly. No serious adverse events occurred. We observed numerical decreases of all infiltrating cell subtypes at week 12, reaching statistical significance for CD68+ sublining macrophages. For some cell types this was even more pronounced at week 24, but clearly synovial inflammation was incompletely resolved. IL-17A and F, TNF, IL-6, IL-8, and IL-12p40 were not significantly downregulated in qPCR analysis of W12 total biopsies, only MMP3 and IL-23p19 were significantly decreased. RNA-seq analysis revealed 178 significantly differentially expressed genes between baseline and 12 weeks (FDR 0.1). Gene Ontology and KEGG terms enrichment analyses identified overrepresentation of biological processes

as response to reactive oxygen species, chemotaxis, migration and angiogenesis as well as MAPK-ERK and PI3K-Akt signaling pathways among the downregulated genes and of Wnt signaling pathway among the upregulated genes. Furthermore, ACR20 responders and non-responders differed strikingly in gene expression profiles in a *post-hoc* exploratory analysis.

Conclusions: Ustekinumab suppresses PsA synovial inflammation through modulation of multiple signal transduction pathways, including MAPK-ERK, Wnt and potentially PI3K-Akt signaling rather than by directly impacting the IL-17 pathway.

Keywords: psoriatc arthritis, synovium, spondyarthropathies, ustekinumab, PI3K - AKT pathway, MAPK pathway, Wnt pathway, IL-23/IL-17 axis

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory joint disease in the spondyloarthritis (SpA) spectrum, with an unknown etiology, high morbidity, and a great impact on quality of life. The IL-23/IL-17 and TNF pathways play major roles in PsA pathogenesis. IL-23 is composed of two subunits: the p19 subunit, specific for IL-23, and the p40 subunit, shared between IL-23 and IL-12 (1). IL12B, the gene encoding for the p40 subunit, and IL23R, the gene encoding its receptor, are both associated with PsA (2). Many biological treatments targeting the IL-23/IL-17 or TNF pathways decrease PsA activity (3). However, these treatments fail to induce remission in all patients, they vary in their effect on the different clinical PsA/SpA features (4–9), and although they effectively decrease inflammation and erosion development, they cannot halt new bone formation; underlining that there is still a need for new treatment strategies targeting all domains of SpA and inducing remission in all patients. Ustekinumab, a monoclonal antibody blocking the p40 subunit shared by IL-12 and IL-23, reduces PsA activity and has been shown to inhibit radiographic progression (4, 5, 10, 11), but how it affects synovial inflammation remains largely unknown.

The synovium is the key target tissue in inflammatory arthritis. Years of synovial tissue biopsy studies have greatly improved our understanding of SpA and PsA disease pathophysiology (12–14). Moreover, small focused mechanism of action trials with in depth analysis of synovial tissue responses before and after treatment further elucidated which pathophysiological processes are affected by specific treatments (15–17). This increased our understanding of pathways driving the chronic inflammatory response and showed which pathways could potentially be targeted to improve treatment responses and outcome in the future. We, therefore, set up this mechanism of action study, aimed to assess how IL-12p40/IL-23p40 blockade by ustekinumab impacts the cellular infiltrate and molecular pathways in the PsA synovium.

STUDY DESIGN

The Medical Ethics Committee of the Academic Medical Center Amsterdam (METC 2014_359; NL50218.018.14) approved this 24-week single-center open-label investigator-initiated mechanism of action study of ustekinumab (45 mg/sc at week 0, 4, and 16 according to standard care) which included eleven patients; all were clinically diagnosed with psoriatic arthritis, fulfilled the CASPAR classification criteria and gave written informed consent before enrollment.

Patients

All patients had active disease with arthritis of at least a knee or an ankle joint. Patients were aged ≥18 years. Treatment with ustekinumab was indicated by the treating physician, but not initiated yet. A maximum of 50% of patients were allowed to have received TNFα inhibitors previously. Exclusion criteria were: contraindications for needle-arthroscopy such as joint replacement or the use of anticoagulation drugs, any therapy by intra-articular injections within 4 weeks before baseline, any intramuscular injection with corticosteroids within 2 weeks before baseline, and the use of an investigational drug or device within 4 weeks before baseline. Thirteen patients were screened for eligibility; eleven patients were included in this trial and participated up to and including the week 12 study visit. The needle-arthroscopy with synovial tissue biopsy sampling at 12 weeks was performed in nine patients. Due to anxiety, one patient declined another arthroscopy, but did complete all study visits. Another patient withdrew from the study after the week 12 study visit. Leading up to the 24 weeks study visit, two patients were excluded since intra-articular corticosteroids were given for persistent arthritis in a knee or finger joint. Technical difficulties prevented biopsy sampling in one patient at 24 weeks. In total, synovial tissue biopsy sampling was performed in six patients at 24 weeks, while eight patients were included in the efficacy analyses. All patients were males. The mean age and SD of our patients was 51.3 ± 11.1 years, their median symptom duration was 7 years [interquartile range (IQR) 2–14 years]. Several patients were on stable doses of concomitant medication at inclusion and remained so during the study: five (45.5%) patients used NSAIDs, one (9.1%) used oral corticosteroids (5 mg daily) and three (27.3%) used cDMARDs. Only two patients (18.2%) had previously been treated with a TNFα inhibitor.

Clinical Assessments

Study visits were conducted at baseline and 4, 8, 12, and 24 weeks after baseline. Demographics, relevant medical history, and medication were recorded at baseline together with specific

disease characteristics, such as duration of disease complaints, date of diagnosis, pattern of psoriatic arthritis complaints, and a family history of spondyloarthritis related diseases. At every visit, patients global disease activity and total pain on a Visual Analog Scale (VAS) of 0–100 mm, and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) were recorded. We also recorded Physician global disease activity (VAS 0–100), 78/76 tender/swollen joint counts, enthesitis according to the Leeds Enthesitis Index (LEI), presence or absence of dactylitis per digit, Psoriasis Area and Severity Index (PASI) and the presence or absence of psoriatic nail dystrophy per digit. At every study visit, inflammatory markers C-reactive protein (CRP) and Erythrocyte Sedimentation Rate (ESR) were measured. Safety was assessed through routine safety lab, physical examination and registration of side effects. The primary efficacy endpoint was the number of patients meeting the American College of Rheumatology 20% improvement (ACR20) response at 12 weeks. Other clinical response outcome measures, such as ACR50, ACR70, European League Against Rheumatism (EULAR) Disease Activity Score (DAS) response and Psoriatic Arthritis Response Criteria (PsARC) response were assessed at both 12 and 24 weeks.

Synovial Sampling by Arthroscopy

Synovial tissue biopsy samples were obtained by needle-arthroscopy from an inflamed knee or ankle at baseline and repeated in the same joint after 12 and 24 weeks, as described previously (18). Biopsy samples were immediately snap-frozen either en bloc in TissueTec (Sakura Finetek) for histologic evaluation or directly for RNA extraction (19).

Immunohistochemical Staining

Cryostat sections (5 μm) were subsequently fixed with acetone, blocked with casein (Merck KGaA) and incubated with primary antibodies for 1 h at room temperature. Primary antibodies included CD3, LN10 (Vector laboratories); CD15, HI98 (Biolegend); CD55, 67 (Bio-connect); CD20, L26; CD68, EBM-11; CD138, MI15; CD163, Ber-MAC3 and vWF, polyclonal, A0082 (all Dako). Isotype- and concentration-matched antibodies were taken along as negative controls. After blocking for endogenous peroxidases, sections were incubated with horseradish-peroxidase-conjugated secondary antibodies [goat anti-mouse HRP, P0447 or swine anti-rabbit HRP, P0399 (both Dako)] for 30 min followed by staining with DAB chromogen (Biolegend). Two independent observers (NY and RF) scored the sections semiquantitatively on a 5-point scale, unaware of the corresponding patient and time point. CD68 was scored for lining and sublining separately.

cDNA Library Generation and Next-Generation Sequencing

Total RNA was extracted from snap-frozen synovial biopsies using RNA Stat-60 (Tel-Test Inc), treated with DNase I (Invitrogen) and cleaned using RNeasy columns (79254, Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were measured using Qubit RNA BR Assay kit (Life Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies), correspondingly. cDNA libraries were constructed with Illumina TruSeq™ RNA Sample Preparation Kit (Illumina) using 1 μg of total RNA with RNA integrity number (RIN) ≥7. Briefly, the protocol consisted of polyA-RNA enrichment, RNA fragmentation, reverse transcription of fragmented RNA into cDNA, adapters ligation onto both ends of the cDNA fragments, and amplification of cDNA fragments by PCR. Resulting cDNA libraries were paired-end sequenced on Illumina NovaSeq 6000 by Macrogen (Seoul, Korea) to obtain around 60 million reads per sample. Macrogen then performed quality control of raw sequence reads (FastQC v0.11.7), removed low quality reads (Trimmomatic 0.38), mapped the reads to the reference genome (USCS hg19 assembly) with HISAT2 v2.1.0, assembled the transcript with Stringtie v1.3.4d, and calculated raw transcription profiles as a read count for each gene and each sample.

Identification of Differentially Expressed Genes (DEGs)

We identified DEGs through count-based pipe-line edgeR (20); we analyzed and visualized DEGs data with edgeR, ggplot2, pheatmap, and enhanced Volcano packages using R v3.6.3 and RStudio v1.2.50 by BioBelka Genomics (Amsterdam). For pathway enrichment analysis, we used The Database for Annotation, Visualization and Integrated Discovery (DAVID) with default settings (21). We chose two to three genes from pathways and terms relating to pathophysiological processes and confirmed those by qPCR analysis.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was reverse transcribed using a RevertAid H Minus First-Strand cDNA synthesis kit (Thermo Scientific) and analyzed by real-time qPCR with TaqMan gene expression assays for IL6 (Hs00174131_m1), IL8 (Hs00174103_m1), IL17A (Hs00174383_m1), IL17F (Hs00369400_m1), CCL20 (Hs00355476_m1), matrix metalloproteinase 3 (MMP3) (Hs00968305_m1), CXCL6 (Hs00605742_g1), TNFα (Hs00174128_m1), IL23p19 (Hs00372324_m1), IL12p40 (Hs01011518_m1), CD20 (Hs00544819_m1) IL12p35(Hs01073447), IL27p28(Hs00377366_m1), EBI3(Hs01057148_m1), FGF5 (Hs03676587_s1), DKK3 (Hs00247429_m1), WNT9a (Hs01573829_m1), FGF14 (Hs00738588_m1), PGF (Hs00182176_m1), NTF3 (Hs00267375_s1), CD70 (Hs00174297_m1), FOLR1 (Hs06631528_s1), VEGFC (Hs01099203_m1), CREB3L1 (Hs05025625_m1), SEMA5A (Hs01549381_m1), MUC1 (Hs00159357_m1), GLUL (Hs00365928_g1), ANGPTL1 (Hs00559786_m1), NOV (Hs00159631_m1), NOG (Hs00271352_s1), IER2 (Hs01109355_m1), AZIN1 (Hs00210634_m1) (Thermofisher) using Real-Time PCR QuantStudio3 PCR System (Applied Biosystems). Expressions of all genes were normalized to the expression of GAPDH (4310884E) as housekeeping gene and presented as fold change relative to the reference sample.

Statistical Analysis

Clinical data are represented as median and interquartile range (IQR) unless mentioned otherwise. We analyzed clinical outcomes, real-time qPCR and immunohistochemistry using non-parametric statistics with the Wilcoxon matched pairs signed-rank test and considered values under 0.05 significant.

RESULTS

Safety

During this study, 12 adverse events occurred, but no serious adverse events: eight patients had a common cold; one patient shortly experienced back, arm and leg pain after a bicycle ride; another had reoccurring arthritis of his unbiopsied knee for which he received intra-articular corticosteroids after his third arthroscopy; lastly, one patient reported headache and another stomach ache. Severe infections did not occur.

Efficacy

The primary efficacy endpoint, ACR20 response at week 12, was reached in six out of 11 (54.5%) patients. Two (18.2%) patients achieved an ACR50 response and one (9.1%) patient an ACR70 response at 12 weeks. At week 24, three out of eight (37.5%) patients achieved an ACR20 response, two (25.0%) an ACR50 response and one (12.5%) an ACR70 response. PsARC response was achieved by two (18.2%) patients at week 12 and by three (37.5%) patients at week 24. Of the eleven patients, two (18.2%) patients had a good response according to the EULAR DAS response, three (27.3%) a moderate response and six (54.5%) were non-responders after 12 weeks of treatment. Of the eight patients at week 24, one (12.5%) patient had a good EULAR DAS response, two (25.0%) a moderate response and five (62.5%) were non-responders to treatment with ustekinumab. **Table 1** shows changes in disease activity parameters in the separate domains, revealing that the improvement of psoriatic skin disease was most prominent. Two out of eleven (18.1%) patients had dactylitis at baseline, which changed to 1/11 (9.1%) and 0/8 (0.0%) patients after 12 and 24 weeks of ustekinumab treatment, respectively. Four out of eleven (36.4%) patients had enthesitis at baseline, which changed to 3/11 (27.3%) and 1/8 (12.5%) patients after 12 and 24 weeks of ustekinumab treatment, respectively.

Immunomodulation of Synovial Inflammation by IL-12p40/IL-23p40 Blockade

In order to assess whether IL-12p40/IL-23p40 blockade impacts synovial inflammation in PsA we examined immune cell infiltration before and after ustekinumab treatment by immunohistochemistry (**Figure 1**). We observed a numerical decrease of all infiltrating cell subtypes at week 12, except for CD138+ plasma cells, reaching statistical significance for CD68+ macrophages ($p = 0.020$) in the synovial sublining. This numerical decrease tended to be even more pronounced at week 24 for some cell types, but clearly ustekinumab treatment did not completely resolve the synovial inflammation. A small numerical decrease was found for von Willebrand factor (vWF)-positive endothelial cells and no differences were found for CD55+ cells present in the lining layer or CD138+ plasma cells (**Supplementary Figure 1**).

Next, we investigated the expression of IL-17A and F, key cytokines downstream of IL-23, by real-time qPCR analysis of PsA synovium at the three time points and observed no significant alterations (**Figure 2A**). The expression of TNF, the other key pathogenic cytokine in PsA, also did not change. Looking further downstream, we found no significant differences for the pro-inflammatory cytokines IL-6 or IL-8, only mRNA levels of MMP3, showed a borderline significant decrease at week 12 ($p = 0.047$) (**Figure 2A**).

These findings show that despite the clear impact on inflammatory infiltration by histology, gene expression analysis failed to demonstrate any consistent and robust impact of p40 blockade by ustekinumab on the IL-23/IL-17 and TNF pathways.

Effect of IL-12p40/IL-23p40 Blockade on IL-12 Family Members in the Inflamed PsA Synovial Tissue

IL-12 and IL-23 belong to the IL-12 cytokine family, which is postulated to have feedback mechanisms (1). We, therefore, assessed whether IL-12p40/IL-23p40 blockade impacts the expression of IL-12 family members. qPCR analysis revealed that the expression of most IL-12 family members, including IL-12p40 itself, did not significantly change at both time points (**Figure 2B**). However, mRNA expression of the IL23A gene—encoding IL-23p19, the subunit that heterodimerizes with IL-23p40 to form the IL-23 cytokine—did decrease significantly at week 12 as compared to baseline ($p = 0.020$), but not at week 24 of the treatment.

Effect of IL-12p40/IL-23p40 Blockade on Whole PsA Synovial Tissue Transcriptome

To better understand the discrepancy between the histological improvement and the absence of impact on the IL-23/IL-17 and TNF pathways, and to investigate whether p40 blockade by ustekinumab affects other pathways in PsA synovitis, we performed an unbiased RNAseq analysis, on seven paired synovial biopsies obtained at baseline and after 12 weeks of treatment. A multidimensional scaling (MDS) plot based on 500 most variable genes failed to show a distinct separation between pre- and post-treatment groups, suggesting that biological variability between patients is larger than ustekinumab's treatment effect (**Figure 3A**). In total, 178 genes, including 27 upregulated and 151 downregulated genes, were identified as significantly differentially expressed in PsA synovium in response to the IL-12p40/IL-23p40 blockade using FDR cutoff 0.1 (**Figure 3B** and **Table 2**). Hierarchical clustering analysis of total differentially expressed genes (DEGs) revealed a better separation of pre- and post-treatment groups (**Figure 3C**). To gain more insight on biological processes modulated in PsA synovium in response to ustekinumab, we performed Gene Ontology (GO) terms and KEGG pathway enrichment analyses using the online software DAVID (https://david.ncifcrf.gov/). The downregulated DEGs were significantly enriched in Biological Processes (BP), such

TABLE 1 | Disease activity parameters as observed for the total study population.

	Baseline ($n = 11$)	Week 4 ($n = 11$)	Week 8 ($n = 11$)	Week 12 ($n = 11$)	Week 24 ($n = 8$)	p^*	p^{**}
SJC76 (*n*)	2 (1–3)	1 (0–2)	1 (0–2)	2 (1–3)	2 (1–2)	0.394	0.715
TJC78 (*n*)	1 (0–5)	1 (0–4)	1 (1–2)	1 (1–2)	0 (0–1)	0.168	0.034
DAS28 (0–10)	2.8 (2.6–3.4)	2.2 (1.6–3.6)	1.7 (1.5–2.8)	2.0 (1.3–3.1)	2.0 (1.0–3.0)	0.026	0.036
PASI BSL>0 (0–72)	3.4 (1.8–15.7)[#]	2.4 (1.9–4.3)[#]	1.5 (0.5–2.0)[#]	0.8 (0–1.8)[#]	0 (0–4.1)^	0.020	0.046
VAS patient global (0–100)	28 (16–55)	38 (23–68)	27 (10–63)	22 (12–46)	45 (3–51)	0.423	0.779
VAS patient pain (0–100)	16 (0–47)	22 (5–70)	19 (5–46)	17 (4–38)	12 (4–56)	0.241	0.888
BASDAI (0–10)	3.2 (2.4–4.7)	3.2 (1.5–5.7)	3 (1.5–4.6)	1.9 (1–3.8)	2.1 (0.4–4.2)	0.008	0.161
VAS physician (0–100)	48 (30–55)	34 (22–44)	20 (6–28)	15 (8–33)	8 (4–27)	0.007	0.021
CRP (mg/L)	8.5 (1.7–16.4)	2.3 (0.7–9.7)	2.2 (1.5–8.1)	1.6 (0.6–3.0)	1.0 (0.6–7.0)	0.008	0.012
ESR (mm/hr)	20 (8–35)	8 (5–28)	5 (2–20)	6 (2–17)	7 (2–16)	0.008	0.027

All values are presented as median (IQR). The p-value for the comparison of baseline and week 12 (*) or week 24 (**). [#]n = 9, ^n = 6. SJC, swollen joint count; TJC, tender joint count; PASI, psoriasis area severity index; BSL, baseline; VAS, visual analog scale; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

as voltage-dependent calcium channel activity (GO:1901843), myoinositol transport (GO:0015798), response to reactive oxygen species (GO:1901031), chemotaxis (GO:0050930; GO:0050918; GO:0060326; GO:0060754), migration (GO:0030335), positive regulation of endothelial cell proliferation (GO:0001938) and angiogenesis (GO:0001525); Cellular Components (CC), such as plasma membrane (GO:0009897; GO:0005887; GO:0005886), cell junctions (GO:0030054), and extracellular space (GO:0005615; GO:0005576); and in Molecular Functions (MF), such as growth factor activity (GO:0008083) and receptor binding (GO:0005102). In the KEGG pathway analysis, the downregulated DEGs were enriched in hypertrophic and dilated cardiomyopathy (hsa05410 and hsa05414), PI3K-Akt (hsa04151) and MAPK (hsa04010) signaling pathways (**Figure 3D** and **Table 2**). Wnt signaling (GO:0016055), cell-cell signaling (GO:0007267), and mechanical stimulus involved in sensory perception of pain (GO:0050966) GO terms were overrepresented within the upregulated DEGs. Ustekinumab thus seems to modulate both general inflammatory processes such as chemotaxis and angiogenesis, and specific molecular pathways such as MAPK and Wnt signaling, and potentially also PI3K-Akt signaling.

To validate the results of the RNA sequencing and pathway analysis, we determined expression levels of selected genes by qPCR at baseline and after 12 weeks of the treatment. qPCR analysis confirmed changes in the expression levels for most of the selected DEGs resulted from the RNAseq analysis: CXCL6 ($p = 0.016$), FGF5 ($p = 0.016$), FGF14 ($p = 0.016$), NTF3 ($p = 0.031$), CD20 ($p = 0.046$), SEMA5A ($p = 0.078$), VEGFC ($p = 0.016$), FOLR1 ($p = 0.031$) for downregulated DEGs; DKK3 ($p = 0.030$), WNT9A ($p = 0.078$), MUC1 ($p = 0.078$), CD70 ($p = 0.031$), and CREB3L1 ($p = 0.047$) for upregulated DEGs (**Figure 4**).

ACR20 Responders and Non-responders Differ in Gene Expression Profiles

Strikingly, a *post-hoc* exploratory analysis of RNAseq data revealed a marked difference in gene expression profiles between ACR20 responders and non-responders. MDS plot based on 500 most variable genes shows a noticeable separation of ACR20 responders and non-responders groups (**Figure 5A**). The number of DEGs in the ACR20 non-responders group was very low, comprising only 19 DEGs, while the number of DEGs in the ACR20 responders group was increased compared to the total group, comprising 632 vs. 178 DEGs respectively (**Figure 5B**). Accordingly, only a few GO terms and KEGG pathways were enriched in downregulated DEGs of ACR20 non-responders, such as ephrin receptor signaling pathway (GO:0048013) and axon guidance (hsa04360); while numerous terms and pathways were enriched in the DEGs of ACR20 responders, including negative regulation of myofibroblast differentiation (GO:1904761), regulation of ERK1 and ERK2 cascade (GO:0070372) and PI3K-Akt signaling (hsa04151) for downregulated DEGs and collagen fibril organization (GO:0030199), osteoblast differentiation (GO:0001649), positive regulation of protein kinase B signaling (GO:0051897), positive regulation of fibroblast proliferation (GO:0048146) and complement and coagulation cascades (hsa04610) for upregulated DEGs (**Figure 5C**). To confirm the in RNAseq analysis observed differences between ACR20 responders and non-responders, we assessed the expression of genes that responded significantly different to ustekinumab treatment between both groups (**Figure 6A**). qPCR analysis confirmed that expressions of angiopoietin-like 1 (ANGPTL1), antizyme inhibitor 1 (AZIN1) and nephroblastoma overexpressed (NOV) changed in opposite directions for ACR20 responders and non-responders in response to the treatment (**Figure 6B**). Changes in expression of glutamine synthetase (GLUL), immediate early response 2 (IER2) and noggin (NOG) were less clear in the qPCR analysis as compared to the RNAseq data (**Figure 6B**). Similarly to the total group the PI3K-Akt-mTOR and MAPK-ERK signaling pathways were modulated in the ACR20 responders. However, in contrast to the total group, in the ACR20 responders p40 targeting with ustekinumab resulted in modulation of specific remodeling pathways such as myofibroblast and osteoblast differentiation and fibroblast proliferation.

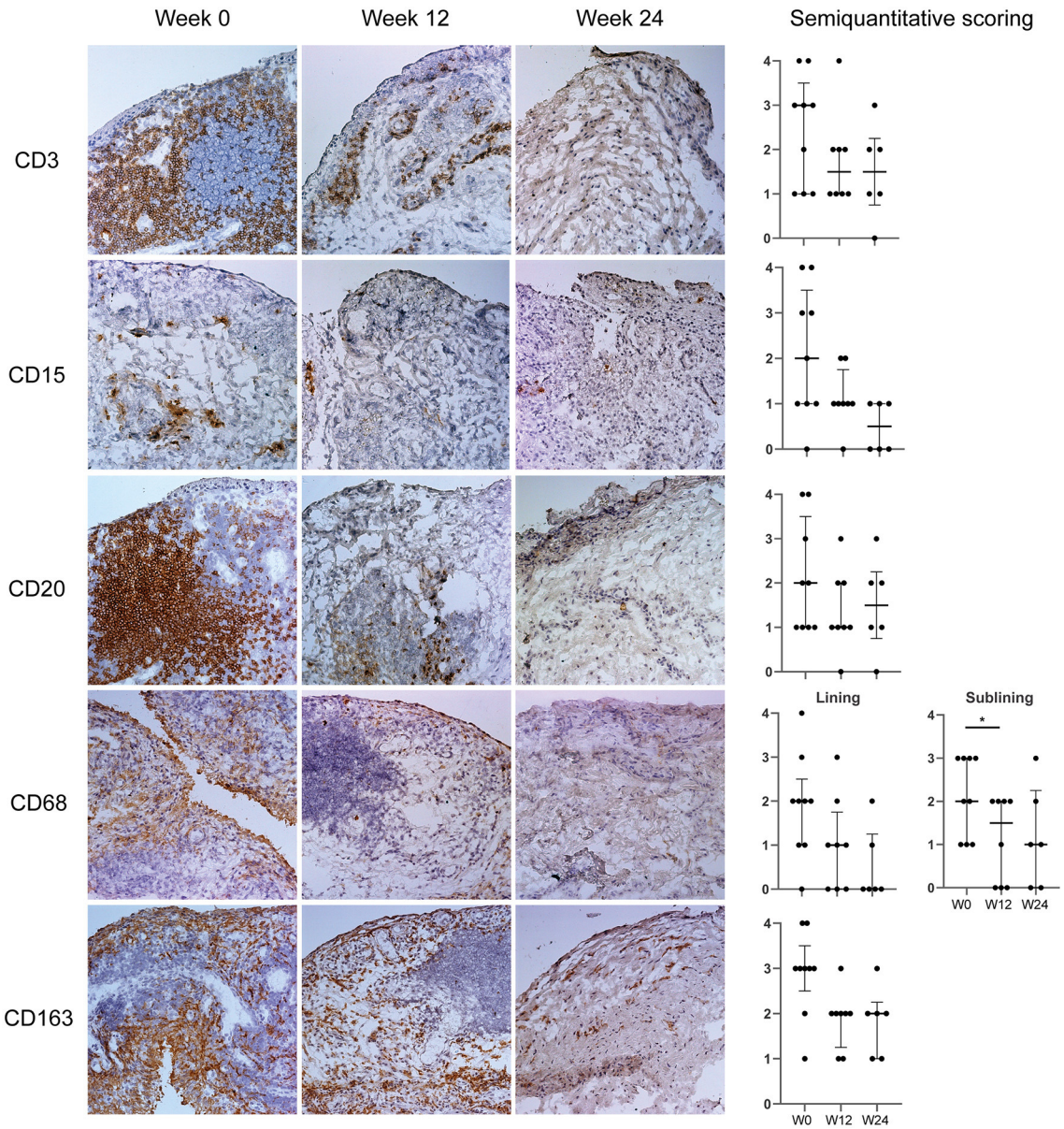

FIGURE 1 | IL-12p40/IL-23p40 blockade by ustekinumab decreases the synovial infiltrate. **(Left)** Representative paired cryosections of synovial tissue obtained before (Week 0, W0, $n = 9$) and after 12 (W12, $n = 8$), and 24 (W24, $n = 6$) weeks of ustekinumab treatment, immunohistochemically stained for CD3 (T-cells), CD15 (neutrophils), CD20 (B-cells), CD68 (macrophages), and CD163 (alternatively activated macrophages). Original magnification x 20. **(Right)** Blind semiquantitative scoring. CD68 was scored for lining and sublining separately. Bars show median and interquartile ranges. Numerical decreases are seen for all cells, reaching significance for CD68 sublining macrophages after 12 weeks of ustekinumab treatment. $^*P < 0.05$.

DISCUSSION

To advance our knowledge of PsA pathophysiology and disease management, we set up this mechanism of action study, aimed to assess how IL-12p40/IL-23p40 blockade by ustekinumab impacts the cellular infiltrate and molecular pathways in the PsA synovium.

Within the limitations of this mechanistic trial design, we observed that of our PsA patients 55 percent reached the primary efficacy outcome, an ACR20 response after 12 weeks of ustekinumab treatment. This aligns with previous PsA studies with ustekinumab (4, 5) and thus enables us to study the impact of ustekinumab on infiltrating cells and molecular pathways in the PsA synovium.

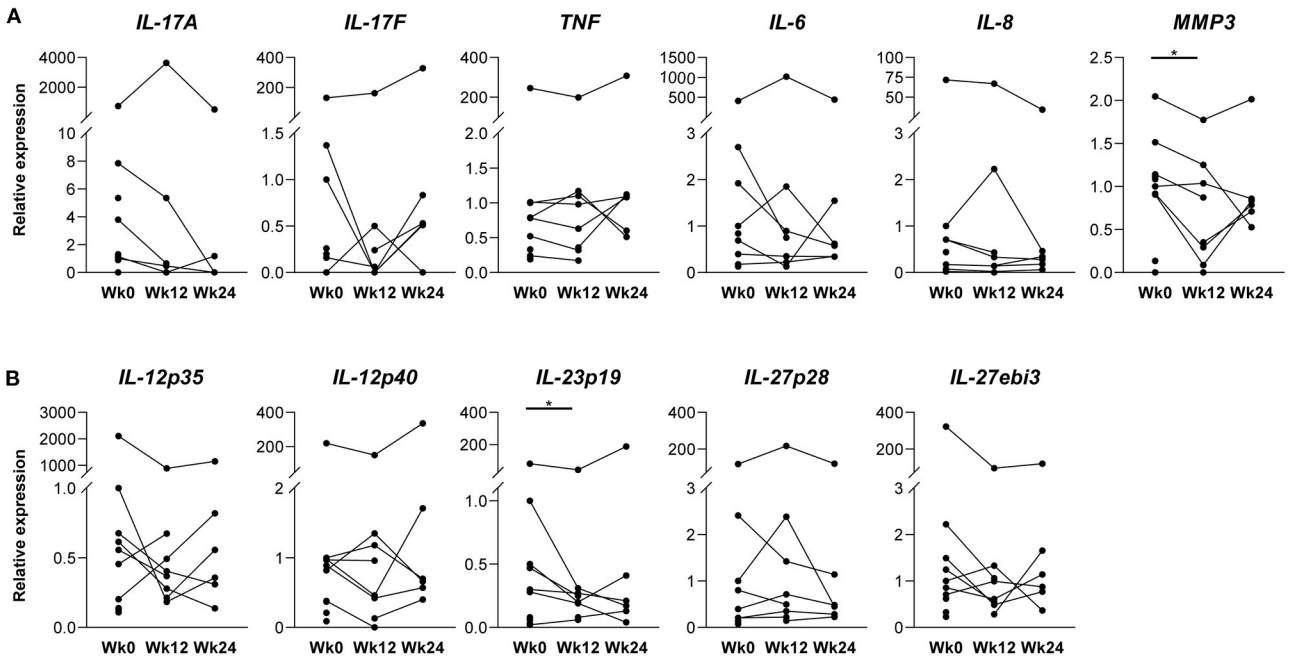

FIGURE 2 | Key inflammatory cytokines and IL-12 family member subunits in synovial tissue before and after ustekinumab treatment. mRNA levels were assessed in total synovial biopsies before (W0, $n = 10$) and after 12 (W12, $n = 8$), and 24 weeks (W24, $n = 6$) of ustekinumab treatment by a real-time quantitative polymerase chain reaction, normalized to the expression of GAPDH housekeeping gene and presented as fold change relative to the reference sample. **(A)** Relative mRNA expression of interleukin (IL)-17A, IL-17F, tumor necrosis factor (TNF), IL-6 and IL-8 show no significant alterations after ustekinumab treatment, only matrix metalloproteinase 3 (MMP3) reaches borderline significance ($p = 0.047$) after 12 weeks of ustekinumab treatment. **(B)** Relative mRNA expression of IL-12p35, IL-12p40, IL-23p19, IL-27p28, and IL-27ebi3 show no effect of IL12-p40 blockade on mRNA expression of IL-12 family subunits, except for IL-23p19, which is significantly decreased after 12 weeks of ustekinumab treatment ($p = 0.020$). *$P < 0.05$.

Baseline synovial samples resembled previous descriptions of inflamed SpA synovial tissue with increased cellular infiltration and hypervascularity (22). Importantly, 12 weeks of ustekinumab treatment resulted in a numerical decrease of all infiltrating immune cells, except for plasma cells, and a significant decrease in CD68-positive sublining macrophages and MMP3 levels, both previously identified biomarkers for treatment response in SpA (12, 17, 23). In line with earlier studies investigating the effects of TNF and IL-17A inhibition (15, 16, 22), IL-12p40/IL-23p40 inhibition decreased PsA synovial inflammation, but did not normalize synovial histology. A persistent synovial cellular infiltrate can be observed in PsA patients in both clinical and ultrasound remission after effective treatment (22). Consequently, it could be hypothesized that either longer treatment is needed, or that targeting a single inflammatory molecule suffices to reduce clinical inflammation in PsA, but fails to completely reverse the complex disease pathophysiology on the cellular and molecular level. Frequent relapse of SpA after treatment cessation pleads for the latter (24–26).

Comparing synovial biopsies before and after different targeted treatments on the molecular level may enlighten us on (novel) pathways that are mainly IL-23/IL-17 or TNF driven, and on processes that remain active in clinically quiescent joints, as well as in patients with persistent disease activity after treatment. mRNA expression of IL-17A, IL-17F, and TNF, key cytokines of the two major pathways driving PsA/SpA disease, seemed unaffected by IL-12p40/IL-23p40 blockade with ustekinumab in our cohort. Previously, we have shown that synovial TNF expression is also unaffected by anti-IL-17A treatment (15), indicating that the IL-23/IL-17 pathway does not regulate the TNF pathway in the synovium. This may explain the differential effects of these treatments in individual patients. Since IL-23 is thought to be upstream of IL-17, reduced IL-17 expression after ustekinumab treatment would have been likely and was seen in psoriatic skin (27). Perhaps our study population was too small to observe a decline on the group level, as IL-17A expression did decrease in some patients. But besides IL-17A and IL-17F levels, levels of downstream cytokines IL-6 and IL-8 also did not decrease on the group level after ustekinumab treatment. A possible explanation for the lack of effect on IL-17A and F expression levels observed in our study could be that IL-17 can not only be produced in a IL-23-dependent fashion by Th17 cells, but also independent of IL-23 by innate like lymphocytes (28, 29), and that these IL-23 independent sources are more important in psoriatic synovium than skin. Unfortunately, we were unable to perform functional studies for verification. Similarly, anti-IL-23R treatment did not affect IL-17A/F expression in a therapeutic setting in an experimental SpA rat model (30). So our results might underline that the IL-23/IL-17 pathway does not function purely linear (31, 32), and that IL-12p40/IL-23p40 blockade

TABLE 2 | Gene ontology and KEGG pathway enrichment analysis of differentially expressed genes upon IL12p40/IL23p40 blockade with ustekinumab.

DEG class	Term	GO terms	DEG (total)	*p* Value	FE	Genes
Up						
	GO BP					
		GO:0050966~detection of mechanical stimulus involved in sensory perception of pain	2 (8)	0.010	182.5	KCNA1. NTRK1
		GO:0016055~Wnt signaling pathway	3 (187)	0.025	11.7	DKK3, WNT9A, AMOTL2
		GO:0007275~multicellular organism development	4 (521)	0.030	5.6	CHRD, DKK3, CREB3L1, WNT9A
		GO:0007267~cell-cell signaling	3 (254)	0.043	8.6	WNT9A, CCL13, CD70
		GO:0097191~extrinsic apoptotic signaling pathway	2 (42)	0.054	34.8	TNFSF12-TNFSF13, CD70
		GO:0030855~epithelial cell differentiation	2 (70)	0.088	20.9	MUC1, AKR1C2
		GO:0071300~cellular response to retinoic acid	2 (70)	0.088	20.9	WNT9A, MUC1
	GO CC					
		GO:0005615~extracellular space	8 (1347)	0.001	4.5	CHRD, DKK3, CSN1S1, WNT9A, CCL13, MUC1, TNFSF12-TNFSF13, CD70
		GO:0005887~integral component of plasma membrane	7 (1415)	0.007	3.8	KCNA1, MUC1, TNFSF12-TNFSF13, SLC6A9, SLC6A7, CD70, NTRK1
		GO:0031410~cytoplasmic vesicle	3 (235)	0.035	9.7	AMOTL2, KCNA1, NTRK1
		GO:0005576~extracellular region	6 (1610)	0.047	2.8	DKK3, CSN1S1, WNT9A, COL8A2, CCL13, TNFSF12-TNFSF13
		GO:0016324~apical plasma membrane	3 (291)	0.052	7.8	AMOTL2, KCNA1, MUC1
	GO MF					
		GO:0005328~neurotransmitter:sodium symporter activity	2 (18)	0.023	81.6	SLC6A9, SLC6A7
		GO:0005102~receptor binding	3 (353)	0.077	6.2	CCL13, TNFSF12-TNFSF13, CD70
Down						
	GO BP					
		GO:0050930~induction of positive chemotaxis	3 (15)	0.005	27.3	VEGFC, NTF3, PGF
		GO:0045766~positive regulation of angiogenesis	5 (115)	0.010	5.9	C5, SEMA5A, VEGFC, PGF, DDAH1
		GO:0034220~ion transmembrane transport	6 (210)	0.019	3.9	GABRB2, GRIA3, NEDD4L, NALCN, ANO5, ATP8A1
		GO:0015798~myo-inositol transport	2 (3)	0.022	91.0	PGAP1, SLC5A3
		GO:1901843~positive regulation of high voltage-gated calcium channel activity	2 (3)	0.022	91.0	FGF14, CACNA2D1
		GO:0050918~positive chemotaxis	3 (35)	0.027	11.7	SEMA5A, VEGFC, NTF3
		GO:1901031~regulation of response to reactive oxygen species	2 (4)	0.029	68.3	SESN1, SESN3
		GO:0007229~integrin-mediated signaling pathway	4 (99)	0.035	5.5	ITGAV, ITGBL1, ITGA1, ERBIN
		GO:0038027~apolipoprotein A-I-mediated signaling pathway	2 (5)	0.036	54.6	ITGAV, ABCA1
		GO:0007267~cell-cell signaling	6 (254)	0.038	3.2	FGF14, SEMA5A, CXCL6, NTF3, FGF5, PGF
		GO:0060754~positive regulation of mast cell chemotaxis	2 (6)	0.043	45.5	VEGFC, PGF
		GO:0030335~positive regulation of cell migration	5 (184)	0.045	3.7	ITGAV, SEMA5A, NTF3, CEMIP, ATP8A1
		GO:0051781~positive regulation of cell division	3 (47)	0.046	8.7	VEGFC, FGF5, PGF
		GO:0007155~cell adhesion	8 (459)	0.050	2.4	ANOS1, ITGAV, ITGBL1, ERBIN, SEMA5A, CNTNAP3, EPHA3, COL19A1
		GO:0008284~positive regulation of cell proliferation	8 (466)	0.054	2.3	HTR2A, RUNX2, ITGAV, VEGFC, PHIP, NTF3, FGF5, PGF

(Continued)

TABLE 2 | Continued

DEG class	Term	GO terms	DEG (total)	*p* Value	FE	Genes
		GO:0070588~calcium ion transmembrane transport	4 (119)	0.056	4.6	ITGAV, CACNB4, PKD2L1, NALCN
		GO:0003073~regulation of systemic arterial blood pressure	2 (8)	0.057	34.1	ADAMTS16, DDAH1
		GO:1904262~negative regulation of TORC1 signaling	2 (9)	0.064	30.3	SESN1, SESN3
		GO:0050764~regulation of phagocytosis	2 (9)	0.064	30.3	ITGAV, CACNB4
		GO:0001525~angiogenesis	5 (223)	0.080	3.1	NRXN1, ITGAV, VEGFC, PGF, C1GALT1
		GO:0060326~cell chemotaxis	3 (65)	0.081	6.3	C5, SEMA5A, CXCL6
		GO:0001938~positive regulation of endothelial cell proliferation	3 (69)	0.090	5.9	SEMA5A, VEGFC, PGF
		GO:0010745~negative regulation of macrophage derived foam cell differentiation	2 (13)	0.090	21.0	ITGAV, ABCA1
		GO:0048010~vascular endothelial growth factor receptor signaling pathway	3 (72)	0.096	5.7	ITGAV, VEGFC, PGF
		GO:0033700~phospholipid efflux	2 (14)	0.097	19.5	ABCA1, APOC1
		GO:0007268~chemical synaptic transmission	5 (240)	0.098	2.8	HTR2A, NRXN1, GABRB2, CACNB4, GRIA3
		GO:0035725~sodium ion transmembrane transport	3 (73)	0.099	5.6	PKD2L1, SLC4A7, NALCN
		GO:0030054~cell junction	10 (459)	0.004	3.2	NRXN1, GABRB2, OLFM3, CXADR, ERBIN, PSD3, GRIA3, HMCN1, DCP2, CACNB4
		GO:0009897~external side of plasma membrane	6 (213)	0.016	4.1	ITGAV, ITGA1, ABCA1, MS4A1, CD28, SCUBE1
		GO:0005887~integral component of plasma membrane	18 (1415)	0.017	1.8	CLEC2D, NRXN1, FOLR1, GABRB2, TLR10, EPHA3, ABCA1, MS4A1, CD28, SLC5A3, HTR2A, ITGAV, CXADR, MYZAP, PRSS35, SLC4A7, GPR88, SLC2A12
		GO:0000139~Golgi membrane	10 (591)	0.021	2.4	FOLR1, FAM198B, ZDHHC21, UGCG, XYLT1, MGAT4A, MGAT4C, FKTN, ATP8A1, C1GALT1
		GO:0005886~plasma membrane	39 (4121)	0.027	1.4	CACNA2D1, CLEC2D, GABRB2, TLR10, CACNB4, GPR174, ABCA1, CD28, ATP8A1, HTR2A, FCRL1, CXADR, CNTNAP3, MYZAP, PREP0, PRSS35, NEDD4L, SLC4A7, IKZF3, ENOX1, NALCN, FOLR1, NRXN1, ANOS1, ITGA1, ERBIN, EPHA3, GRIA3, SLC5A3, CACNB4, CEMIP, DYNC2H1, ZDHHC21, ITGAV, SEMA5A, PKD2L1, ANO5, GPR88, SLC2A12
		GO:0036449~microtubule minus-end	2 (4)	0.027	72.3	NIN, ASPM
		GO:0009986~cell surface	9 (542)	0.033	2.4	FOLR1, CLEC2D, NRXN1, ITGAV, CXADR, ITGA1, CD28, PKD2L1, SCUBE1
		GO:0016323~basolateral plasma membrane	5 (180)	0.036	4.0	FOLR1, CXADR, ERBIN, PRSS35, SLC4A7
		GO:0005615~extracellular space	16 (1347)	0.043	1.7	ANOS1, MS4A1, SCUBE1, FGF5, SIAE, OLFM3, CXADR, C5, MYZAP, VEGFC, CXCL6, TCTN1, PRSS35, FKTN, ENOX1, PGF

(Continued)

TABLE 2 | Continued

DEG class	Term	GO terms	DEG(total)	*p* Value	FE	Genes
		GO:0005576~extracellular region	18 (1610)	0.049	1.6	ANOS1, ITGBL1, XYLT1, EPHA3, APOC1, GZMK, FGF5, CEMIP, COL19A1, FGF14, CXADR, C5, CNTNAP3, VEGFC, CXCL6, NTF3, PGF, RSPO3
		GO:0043235~receptor complex	4 (127)	0.057	4.6	PEX5L, LRP1B, ITLN1, PKD2L1
		GO:0070062~extracellular exosome	27 (2811)	0.065	1.4	CACNA2D1, GABRB2, NEBL, HMCN1, ATAD2, ANAPC2, SIAE, DDAH1, ATP8A1, C5, ITLN1, PRSS35, NEDD4L, PHIP, MGAT4A, FOLR1, CAMK4, ITGA1, MS4A1, APOC1, CACNB4, DYNC2H1, AGMAT, KNL12, ITGAV, SEMA5A, PI15
		GO:0097431~mitotic spindle pole	2 (10)	0.067	28.9	NIN, ASPM
		GO:0061700~GATOR2 complex	2 (10)	0.067	28.9	SESN1, SESN3
	GO MF					
		GO:0005102~receptor binding	8 (353)	0.012	3.2	NRXN1, CXADR, C5, ITGA1, ERBIN, AOC1, NTF3, RSPO3
		GO:0008201~heparin binding	5 (160)	0.026	4.4	FGF14, ANOS1, CXCL6, PGF, RSPO3
		GO:0008083~growth factor activity	5 (162)	0.027	4.4	FGF14, VEGFC, NTF3, FGF5, PGF
		GO:0008454~alpha-1,3-mannosylglycoprotein 4-beta-N-acetylglucosaminyltransferase activity	2 (4)	0.028	70.9	MGAT4A, MGAT4C
		GO:0005245~voltage-gated calcium channel activity	3 (40)	0.032	10.6	CACNA2D1, ITGAV, CACNB4
		GO:0046872~metal ion binding	22 (2069)	0.050	1.5	CACNA2D1, NRXN1, ITGA1, EBF1, ZMAT1, RASA2, BCL11A, ZBTB41, DDAH1, AGMAT, ZNF876P, ITGAV, ZNF506, ZBTB44, PDE3A, MGAT4A, MGAT4C, IKZF3, IKZF2, ZNF107, TRIM2, C1GALT1
		GO:0008509~anion transmembrane transporter activity	2 (9)	0.061	31.5	AOC1, SLC4A7
		GO:0003676~nucleic acid binding	12 (985)	0.084	1.7	CLEC2D, TTC14, ZMYM1, ZNF506, ZBTB44, BCL11A, TDRD9, ZBTB41, ENOX1, IKZF3, IKZF2, ZNF107
		GO:0001618~virus receptor activity	3 (70)	0.086	6.1	HTR2A, ITGAV, CXADR
		GO:0008144~drug binding	3 (76)	0.099	5.6	FOLR1, HTR2A, TOP2A
		GO:0005272~sodium channel activity	2 (15)	0.100	18.9	PKD2L1, NALCN
	KEGG					
		hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4 (67)	0.011	8.4	CACNA2D1, ITGAV, ITGA1, CACNB4
		hsa05410:Hypertrophic cardiomyopathy (HCM)	4 (78)	0.017	7.2	CACNA2D1, ITGAV, ITGA1, CACNB4
		hsa05414:Dilated cardiomyopathy	4 (84)	0.020	6.7	CACNA2D1, ITGAV, ITGA1, CACNB4
		hsa04010:MAPK signaling pathway	6 (253)	0.031	3.3	FGF14, CACNA2D1, CACNB4, RASA2, NTF3, FGF5
		hsa04014:Ras signaling pathway	5 (226)	0.072	3.1	FGF14, VEGFC, RASA2, FGF5, PGF
		hsa04151:PI3K-Akt signaling pathway	6 (345)	0.091	2.4	FGF14, ITGAV, ITGA1, VEGFC, FGF5, PGF

Twenty seven significantly upregulated and 151 significantly downregulated genes in response to the treatment with ustekinumab (FDR 0.1) were imported into DAVID database. GO terms and KEGG pathway analysis were performed to identify biological processes significantly enriched ($p < 0.05$) among anti- IL12p40/IL23p40 regulated genes in the synovium of patients with PsA. DEG, differentially expressed genes; FE, fold enrichment; GO BP, gene ontology term biological process; GO CC, gene ontology term cellular component; GO MF, gene ontology term molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

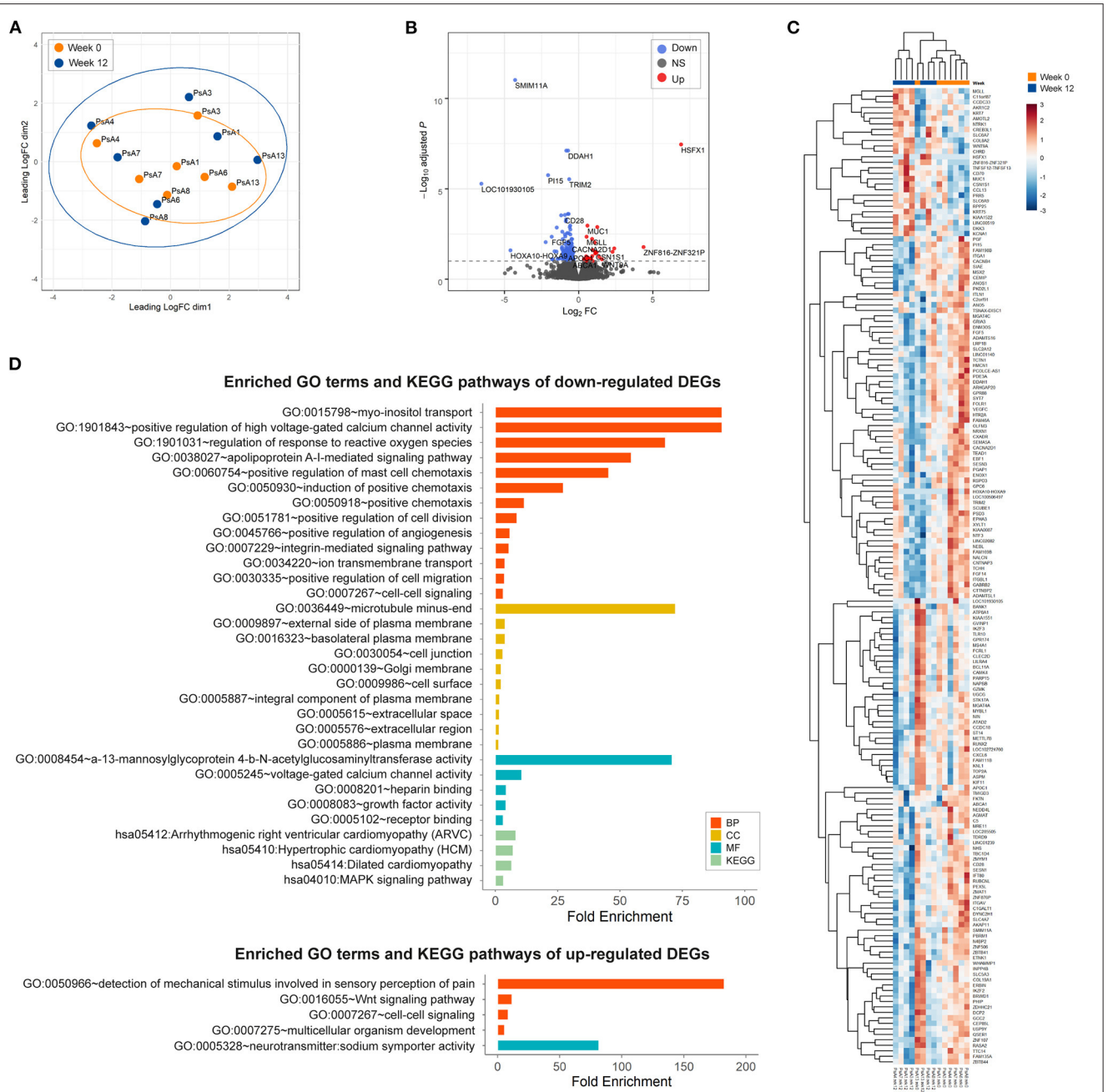

FIGURE 3 | Differential gene expression and pathway analysis of changes in psoriatic arthritis synovium 12 weeks after treatment with ustekinumab. **(A)** Multidimensional scaling (MDS) plot of gene expression (logCPM) in pre-treatment (red, week 0, $n = 7$) and post-treatment (blue, week 12, $n = 7$) synovium reveals no distinct separation by the treatment effect. **(B)** A volcano plot identifying significantly differentially expressed genes (DEGs) in response to IL-23p40/IL-12p40 blockade with ustekinumab (FDR < 0.1, dashed line): red, upregulated genes; blue, downregulated genes; black, non-differentially expressed genes. **(C)** Hierarchical cluster analysis of all significant genes modulated by the treatment separates pre- and post-treated groups. Normalized scaled log2 gene expression levels are shown. **(D)** The enriched ($p < 0.05$) gene ontology (GO) terms and KEGG pathways of upregulated and downregulated DEGs in psoriatic arthritis synovium in response to the treatment with ustekinumab identified by DAVID. DEGs between pre- and post-treated groups were categorized into the three main categories of gene ontology classification: BP, biological process; CC, cellular component; MF, molecular function KEGG Kyoto Encyclopedia of Genes and Genomes.

exerts clinical effect through other ways than through modulating IL-17 levels.

The IL-12 cytokine family is postulated to have feedback mechanisms (1). However, we did not observe consistent changes as blocking of p40 only decreased IL-23p19 mRNA in the synovium, which suggests that cells producing IL-23, but not IL-12, may be affected by ustekinumab treatment. IL-23 is mainly produced by activated antigen-presenting cells, like dendritic

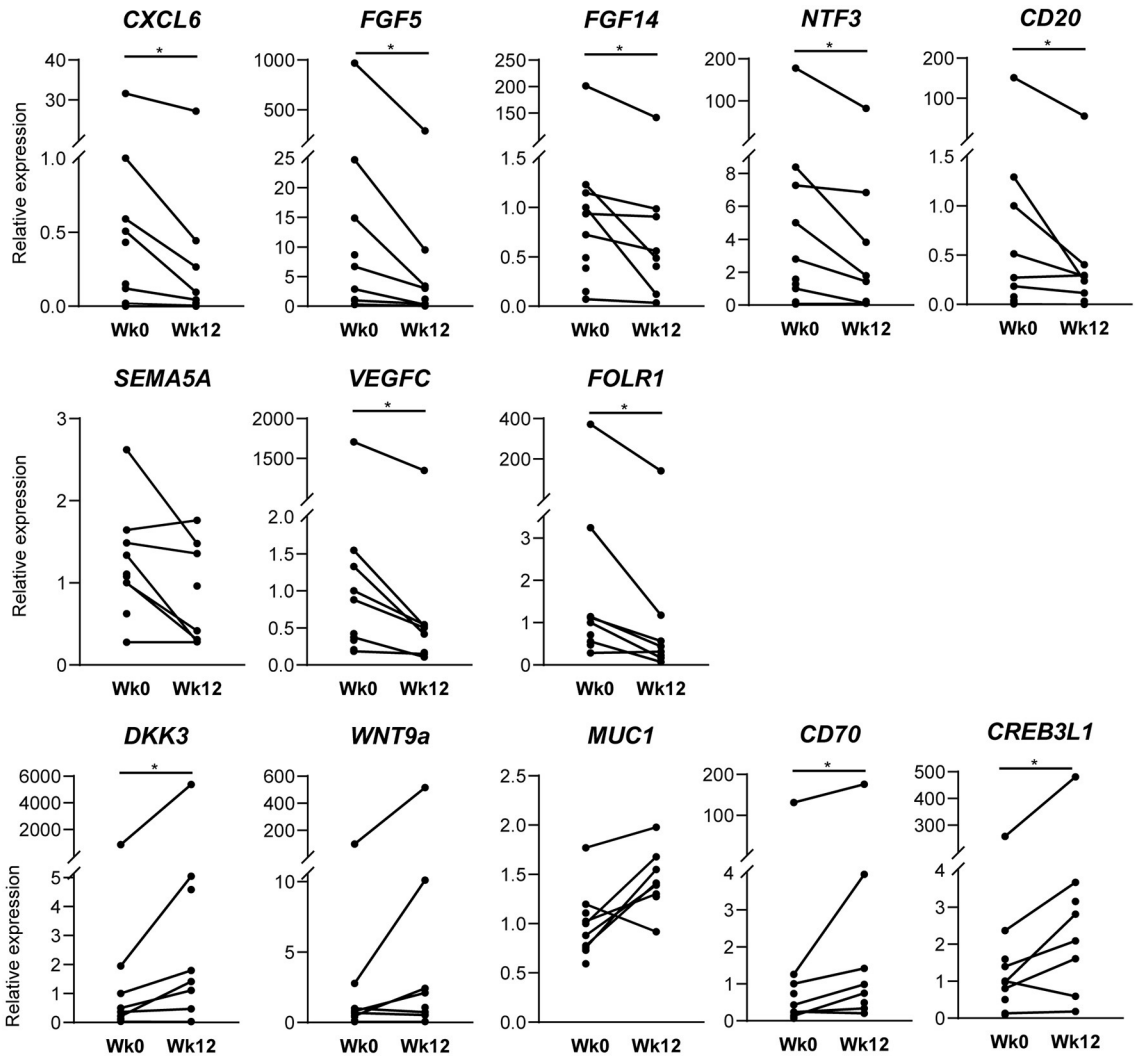

FIGURE 4 | Confirmation of RNA sequencing analysis by qPCR. Real-time quantitative polymerase chain reaction analysis of synovial tissue of baseline ($n = 10$) and after 12 weeks ($n = 8$) of ustekinumab treatment confirms the differential expression of genes found in RNA sequencing: CD20, CXCL6, FGF5, fibroblast growth factor 5; FGF14; FOLR1, folate receptor 1; NTF3, neurotrophin 3; SEMA5A, semaphorin 5A; VEGFC, vascular endothelial growth factor C for downregulated DEGs; CD70, CREB3L1, CAMP Responsive Element Binding Protein 3 Like 1; DKK3, Dickkopf WNT Signaling Pathway Inhibitor 3; MUC1, mucin 1; WNT9A, WNT family member 9A for upregulated genes $^*p < 0.05$.

cells and macrophages (33, 34), and macrophages were indeed decreased in our cohort. Yet, as we assessed mRNA expression levels in whole tissue, further *in vitro* studies are required to analyze and confirm which individual cells producing IL-23 are specifically affected.

To better understand the discrepancy between the histologic improvement and the apparent absence of impact on the IL-23/IL-17 and TNF pathways, we performed an unbiased RNAseq analysis of whole tissue followed by GO terms and KEGG pathway enrichment analyses to investigate whether p40 blockade affects other pathways in PsA synovitis. GO terms showed involvement of various pathways including chemotaxis and angiogenesis, two known prominent features in inflamed synovial tissue in SpA (22, 35, 36) in the downregulated DEGs. Specifically, gene expression of MS4A1, encoding CD20 expressed by B cells, and of CXCL6, involved in the attraction of neutrophils, were downregulated after ustekinumab treatment based on RNAseq and qPCR analysis, and numerical (albeit no significant) reductions of CD20+ B cells and CD15+ neutrophils were seen by IHC. Pro-angiogenic VEGFC and placental growth factor (PGF) were downregulated after ustekinumab treatment in RNAseq and confirmed for VEGFC by qPCR; the anti-angiogenic factor angiopoietin-like 1 (ANGPTL1) was upregulated in ACR20 responders in RNAseq and qPCR. However, expression of the MUC1 gene, which is upregulated in hypoxia and involved in hypoxia-induced angiogenesis (37), was increased after ustekinumab treatment. IHC staining for vWF showed a small but non-significant reduction. TNF blockade also affects synovial

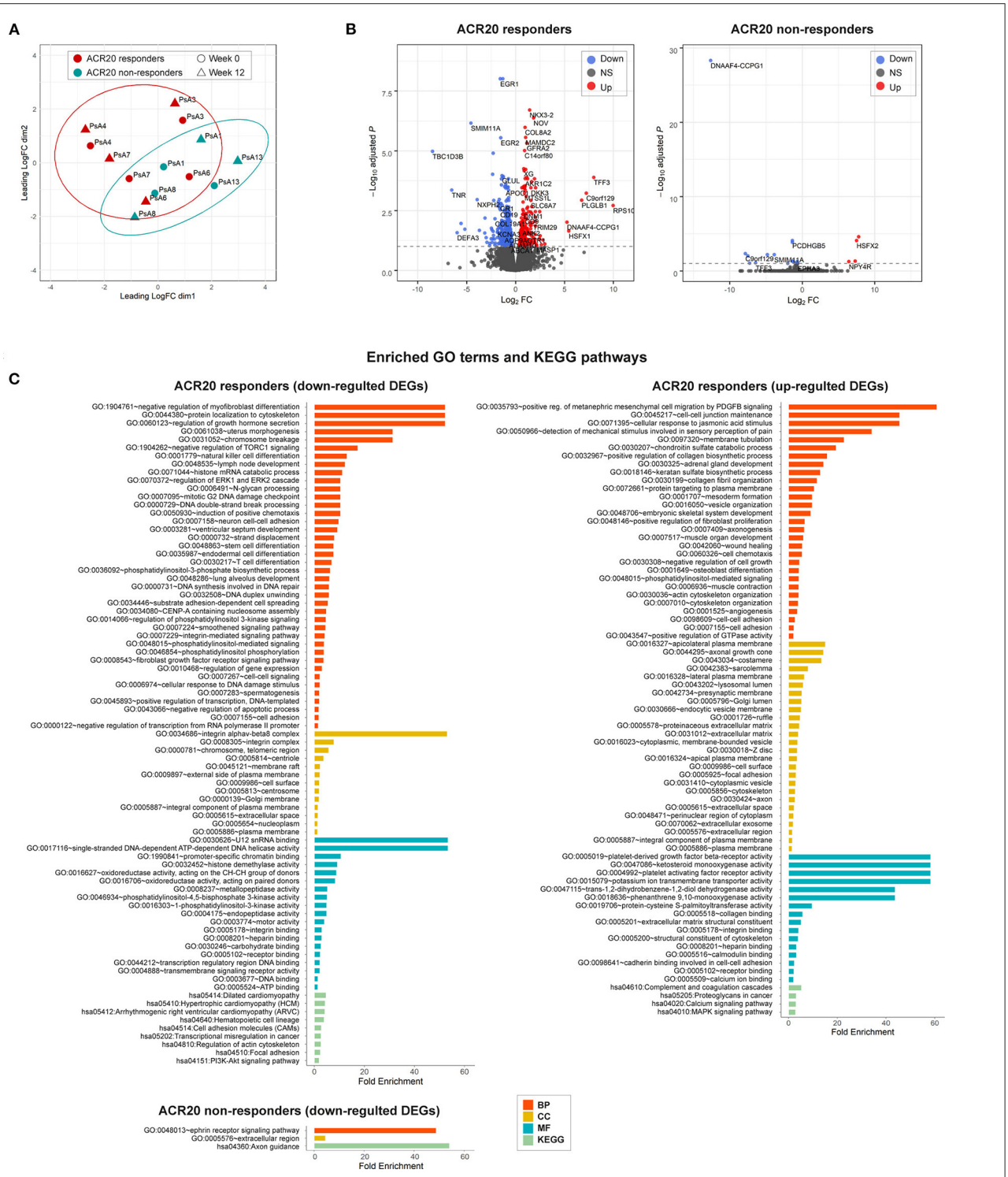

FIGURE 5 | Differential gene expression and pathway analysis of ACR20 responders and non-responders to ustekinumab treatment in a *post-hoc* explorational analysis. **(A)** Multidimensional scaling (MDS) plot of gene expression (logCPM) in American College of Rheumatology 20% improvement criteria (ACR20) responders (red, $n = 4$) and ACR20 non-responders (blue, $n = 3$) reveals separation of the two groups. **(B)** A volcano plot identifying significantly differentially expressed genes (DEGs) of ACR20 responders and non-responders to IL-23p40/IL-12p40 blockade with ustekinumab (FDR < 0.1, dashed line): red, upregulated genes; blue, downregulated genes; black, non-differentially expressed genes. **(C)** The enriched ($p < 0.05$) gene ontology (GO) terms and KEGG pathways of upregulated and downregulated DEGs in psoriatic arthritis synovium in response to the treatment with ustekinumab identified by DAVID for ACR20 responders and non-responders. DEGs between pre- and post-treated groups were categorized into the three main categories of gene ontology classification: BP, biological process; CC, cellular component; MF, molecular function.

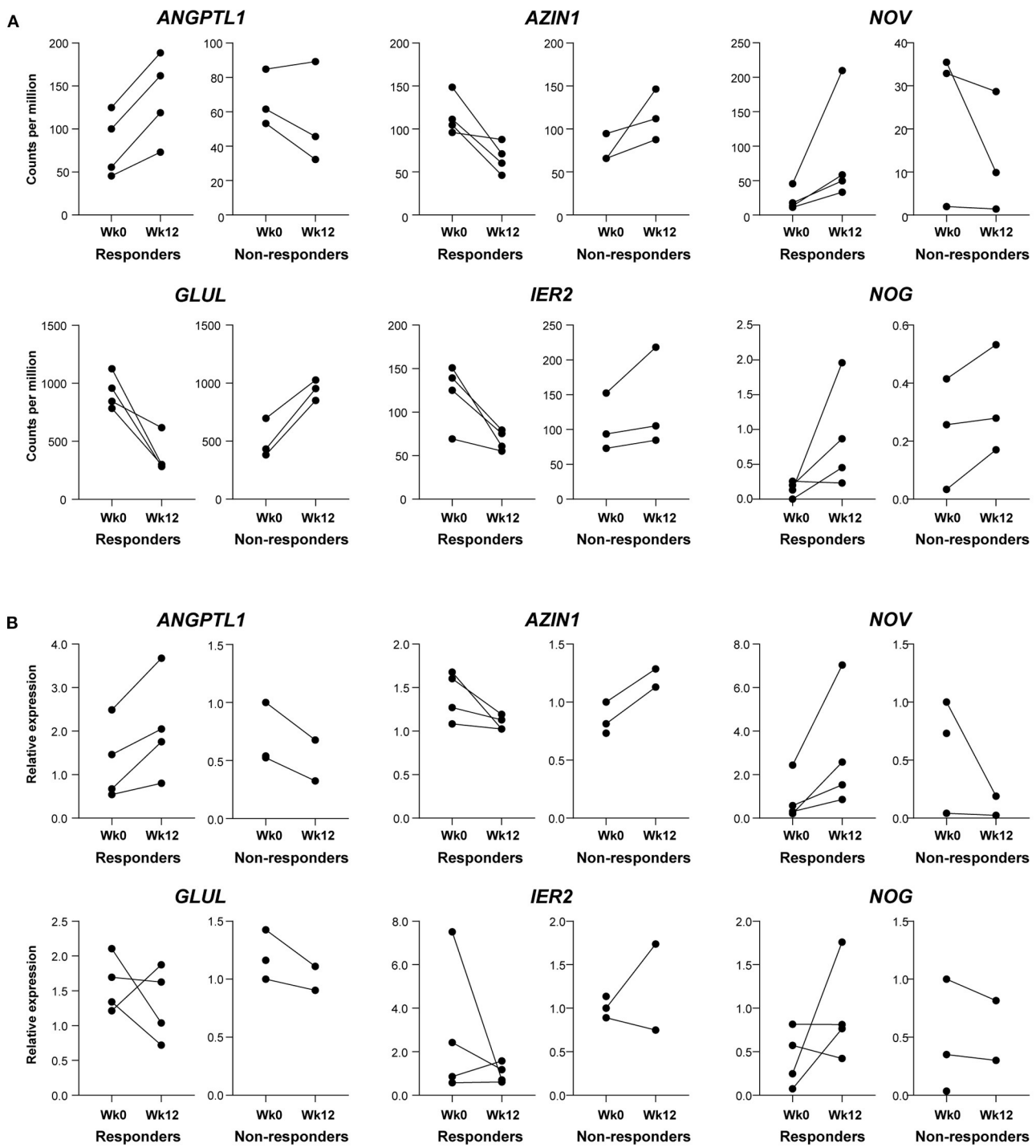

FIGURE 6 | Confirmation of difference in gene expression profiles of ACR20 responders and non-responders by qPCR. **(A)** Genes responding significantly different to ustekinumab treatment between ACR20 responders ($n = 4$) and non-responders ($n = 3$) in RNAseq analysis that were selected for validation. **(B)** Real-time quantitative polymerase chain reaction analysis of synovial tissue of baseline and after 12 weeks of ustekinumab treatment for angiopoietin-like 1 (ANGPTL1), antizyme inhibitor 1 (AZIN1), glutamine synthetase (GLUL), immediate early response 2 (IER2), noggin (NOG), and nephroblastoma overexpressed (NOV) confirm that ANGPTL1, AZIN1, and NOV levels go in opposite directions for ACR20 responders and non-responders.

angiogenesis (17, 22, 38). Whereas, previously, we observed that synovial vWF was unaffected by IL-17 blockade (15).

Besides cellular infiltration and angiogenesis, pathway analysis also revealed modulation of (myo)fibroblasts and osteoblasts, and involvement of the Wnt, PI3K-Akt-mTOR and MAPK-ERK signaling pathways.

A clear impact of ustekinumab on fibroblast and myofibroblast pathways can be seen through enrichment of GO terms fibroblast growth factor receptor signaling pathway, positive regulation of fibroblast proliferation, and negative regulators of myofibroblast differentiation, together with decreased gene and mRNA expression of several FGFs. As we previously reported that SpA synovium has a myofibroblast gene signature compared to RA synovium (39), and here we show that myofibroblasts are affected after treatment, it will be interesting to further investigate the role of myofibroblasts in PsA pathology and to see how p40 blockade specifically affects PsA synovial fibroblasts in future studies.

The effects of p40 blockade on the Wnt signaling pathway and osteoblast differentiation is a very interesting finding, since the IL-23/IL-17 pathway and Wnt pathway are intimately involved in SpA bone pathology [reviewed in (40)]. Previously, we found multiple genes involved in Wnt signaling to be highly expressed in SpA compared to RA synovium (39). Genes of the Wnt signaling pathway were also differentially expressed when comparing PsA to healthy synovium (41). The ligand WNT9A, here upregulated after ustekinumab treatment, suppresses chondrogenesis and is an important factor for joint maintenance (42). Moreover, ustekinumab treatment significantly increased mRNA expression of CREB3L1 gene encoding a transcription factor involved in bone formation (43); CREB3L1 deficiency can cause severe osteogenesis imperfecta (44). Animal studies already suggested a role for IL-12p40 in bone remodeling, since IL-12p40 depletion in mice stimulated bone regeneration and increased bone mass, while IL-12p35 depletion (solely targeting IL-12) impaired bone regeneration and increased bone loss (45). Clinically, ustekinumab-treated PsA patients had significantly lower radiographic progression (i.e., lower Sharp/van der Heijde score on joint space narrowing and erosions) than the placebo-treated group (10). Though we assessed the synovial response, not the bone response to ustekinumab, our results collide with literature that ustekinumab might affect bone remodeling pathways in PsA and should be further investigated as systemic bone loss and local new bone formation remain major unmet needs in PsA and SpA management.

Several GO terms and the KEGG pathway for the PI3K-Akt-mTOR signaling pathway were overrepresented in the total group and ACR20 responders. Since the PI3K-Akt-mTOR signaling pathway can regulate Th17 cell differentiation (46), it is considered an interesting treatment target for SpA. We previously showed that the PI3K-AKT-mTOR signaling pathway is activated in SpA synovium and selective inhibition of PI3Kδ reduced the inflammatory response of immune cells, and skin and synovial fibroblasts in SpA (47). Inhibition of PI3Ks was also effective in murine psoriasis (48) and collagen-induced arthritis models (49). mTOR blockade with rapamycin inhibited arthritis and affected bone remodeling in a SpA rat model and also inhibited osteogenic differentiation of SpA patients' synovial fibroblasts *in vitro* (50). Based on the recent data from our team and from others (46–50), we expect that the observed modulation of the PI3K-Akt-mTOR pathway might take place by innate-like (MAIT and γδ-T cells) and adaptive (Th17) cells or synovial fibroblasts, although this remains to be formally tested in future studies. The potential effects of ustekinumab on the PI3K-Akt-mTOR pathway in our study supports the concept that specific targeting of the PI3K-Akt-mTOR pathway could be beneficial in human SpA.

The intricate mitogen-activated protein kinase (MAPK) signaling network transduces intercellular signals from the cell membrane to the three MAPK families of extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, which regulate gene expression important for cell proliferation, differentiation, survival, and apoptosis (51). The MAPK signaling pathway and ERK1 and ERK2 cascade were modulated by ustekinumab in either the total group or ACR20 responders. Genes of the MAPK signaling pathway are differentially expressed in PsA vs. healthy synovium (41). Activated MAPKs are present in PsA synovium and TNF blockade reduces activation of ERK and JNK, but not of p38 (52). Previously, we found active MAPKs expression in the synovium of early SpA patients, but lower than in early RA patients for ERK and JNK (53). Even though post-transcriptional modification regulates MAPK activation, our results reveal that ustekinumab modulates the MAPK-ERK signaling pathway at the transcriptional level, which might aid in controlling PsA disease. Ustekinumab thus seems to affect various inflammatory molecular pathways in the PsA synovium. Future studies may show how p40 blockade directly modulates these pathways.

In a *post-hoc* exploratory analysis, ACR20 responders and non-responders differed strikingly in their molecular response to ustekinumab in an unbiased analysis, namely multidimensional scaling of the total transcriptome. We could validate differential expression between the groups for three out of six genes by qPCR, namely ANGPTL1, NOV and AZIN1. ANGPTL1, upregulated in responders, suppresses the PI3K-Akt and MAPK-ERK signaling pathways and inhibits angiogenesis (54, 55). Expression of NOV, encoding a small secreted regulatory protein, was upregulated in responders; NOV overexpression attenuates the PI3K-Akt-mTOR pathway and decreases MMP production (56), while mice without NOV protein display an osteoarthritis-like disease (57). AZIN1 was downregulated in responders; AZIN1 promotes polyamine production, essential for cell growth, and differentiation, but AZIN1 overactivation can increase the invasive potential of fibroblasts (58) and can lead to cancer, inflammation or diabetes (59). Since qPCR only analyzes one mRNA variant and not all RNAs like RNAseq, it is difficult to say for the unconfirmed genes, whether the RNAseq was false positive or the qPCR analysis was too narrow. Although we could observe a difference despite our small sample sizes, 4 vs. 3 patients in RNAseq, respectively, because of our small sample sizes, we cannot draw firm conclusions. Confirmation in larger cohorts may allow us to find biomarkers, i.e., differential expression of specific genes at baseline, which may predict individual treatment response to ustekinumab.

To our knowledge, this is the first study to assess the effects of ustekinumab on the cellular infiltrate and molecular pathways in synovial tissue biopsies taken from PsA patients before and after treatment. Whole tissue RNA sequencing enabled us to see the overall synovial tissue response to IL-12p40/IL-23p40 blockade, but kept us from the effector cells of the observed response. RNA sequencing at the single-cell level, multiplexed spatial imaging of the synovial tissue and flow cytometric assays of freshly isolated synovial cells before/after the treatment combined with *in vitro* analyses establishing the direct effect of ustekinumab on IL-17-producing cells would further elucidate how the IL-23/IL-17 pathway contributes to PsA pathophysiology. Nevertheless, these analyses were out of the scope of the current study. Though joint counts improved for the majority of patients in our study, we observed only limited joint responses on the group level due to the nature of our study population: a rather small group of mostly oligoarthritic patients. However, our study population resembles the general PsA population since patients visiting the outpatient clinic more often have oligoarticular than polyarticular disease. We observed only numerical differences for most cellular markers, presumably due to the small sample size. Given our small sample size of only male participants, our results should be confirmed in larger cohorts containing both sexes.

Comparing our RNAseq data to the molecular effects of TNF and/or IL-17A inhibition might discriminate treatment-specific effects from general effects of reduced inflammation in the synovial tissue. If these treatment-specific effects complement each other—for instance targeting both inflammation and bone remodeling -, combined therapy approaches may be explored in PsA and SpA in the future.

Taken together, this mechanism of action study indicates that blocking of the p40 subunit shared by IL-12 and IL-23 by ustekinumab suppresses synovial inflammation in PsA through modulation of MAPK-ERK, Wnt and potentially also PI3K-Akt-mTOR signaling pathways rather than by directly impacting the IL-17 pathway. It also implies that certain PsA patients might benefit more from this treatment than others, although this has to be verified and confirmed on larger cohorts.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA693312.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of the Academic Medical Center Amsterdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.611656/full#supplementary-material

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The IL-23/IL-17 Pathway in Inflammatory Skin Diseases: From Bench to Bedside

Taoming Liu, Sheng Li, Shuni Ying, Shunli Tang, Yuwei Ding, Yali Li, Jianjun Qiao and Hong Fang**

Department of Dermatology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

Interleukin-17 (IL-17) is an essential proinflammatory cytokine, which is mainly secreted by the $CD4^+$ helper T cells (Th17 cells) and subsets of innate lymphoid cells. IL-17A is associated with the pathogenesis of inflammatory diseases, including psoriasis, atopic dermatitis, hidradenitis suppurativa, alopecia areata, pityriasis rubra pilaris, pemphigus, and systemic sclerosis. Interleukin-23 (IL-23) plays a pivotal role in stimulating the production of IL-17 by activating the Th17 cells. The IL-23/IL-17 axis is an important pathway for targeted therapy for inflammatory diseases. Emerging evidence from clinical trials has shown that monoclonal antibodies against IL-23, IL-17, and tumor necrosis factor are effective in the treatment of patients with psoriasis, atopic dermatitis, hidradenitis suppurativa, pityriasis rubra pilaris, pemphigus, and systemic sclerosis. Here, we summarize the latest knowledge about the biology, signaling, and pathophysiological functions of the IL-23/IL-17 axis in inflammatory skin diseases. The currently available biologics targeting the axis is also discussed.

Keywords: IL-17 family, IL-23, IL-23/IL-17 axis, psoriasis, targeted therapy

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****Correspondence:***
Jianjun Qiao
qiaojianjun@zju.edu.cn
Hong Fang
fanghongzy@zju.edu.cn

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INTRODUCTION

Interleukin-17A (IL-17A) is cloned from a T cell hybridoma activated in rodents (1) and is related to several immune-mediated disorders, such as autoimmune (2), oncogenic (3), and infectious (4) diseases. The T helper 17 (Th17) cells constitute a unique subset of $CD4^+$ T cells and are the major source of IL-17 (5). IL-17A triggers cellular reactions not only in the keratinocytes, but also in some other cells, including neutrophils, endothelial cells, fibroblasts, and osteoclasts (6–10). In keratinocytes, the binding of IL-17A to IL-17 receptor (IL-17R) A, IL-17C, or IL-17RD stimulates keratinocyte proliferation. Subsequently, the release of inflammatory mediators and chemokines leads to inflammatory reaction (11, 12).

The cytokines, interleukin-23 (IL-23) and IL-17, have been confirmed to markedly affect chronic inflammation (10, 13–16). In addition, the discovery of the IL-23/IL-17 pathway has contributed to a clearer understanding of the underlying mechanism of inflammatory diseases. At present, therapies for inflammatory diseases have advanced from general immunosuppression to biologics against the IL-23/IL-17 signaling pathway, such as IL-17, IL-12/23 and IL-23 inhibitors. In this review, we highlight the potential implications of dysregulation of the IL-23/IL-17 axis in chronic

inflammatory skin diseases, including psoriasis, hidradenitis suppurativa (HS), atopic dermatitis (AD), alopecia areata (AA), pityriasis rubra pilaris (PRP), pemphigus, and systemic sclerosis (SSc).

SEARCH STRATEGY AND SELECTION CRITERIA

In this review, we are not intended to comprehensively review all pathways identified through human and murine laboratory studies or all clinical trials and case series in various inflammatory skin diseases. Nevertheless, we intent to focus on those targets of IL-23/IL-17 pathway demonstrated to be effective or potentially effective for treating human inflammatory skin diseases. We searched the published literature from PubMed and ClinicalTrial.gov with the search terms including 'IL-17,' 'psoriasis,' 'atopic dermatitis,' 'hidradenitis suppurativa,' 'alopecia areata,' 'pityriasis rubra pilaris,' 'pemphigus,' 'systemic sclerosis,' 'secukinumab,' 'ixekizumab,' 'brodalumab,' 'bimekizumab,' 'ustekinumab,' 'tildrakizumab,' 'guselkumab,' and 'risankizumab'. We mainly focused on publications written in English between September 1, 2010, and September 15, 2020. We chose the references depending on the basis of their originality and relevance to the topic.

IL-17 SIGNALING IN INFLAMMATORY AND AUTOIMMUNE DISEASES

Th17 cells are known to play an important role in inflammatory and autoimmune diseases (17–19). In general, IL-23 is involved in the activation of Th17 cells to induce the production of IL-17A, IL-17F, tumor necrosis factor (TNF), and IL-6 (20). Binding to its receptors, IL-23 contributes to the phosphorylation of receptor-associated JAKs and specific Tyr residues, and this is followed by activating the transcription of IL-17 and other genes. The participation of IL-23 is crucial in the differentiation of IL-17-expressing phenotypes, *via* activating the transcription factor retinoid-related orphan receptor-γt (ROR-γt) and signal transducer and activator of transcription 3 (STAT3) (21–23).

IL-17 induces expression of downstream genes by stimulating activation of pathways, including canonical nuclear factor-κB (NF-κB), CCAAT/enhancer-binding protein (C/EBP) family, and mitogen-activated protein kinase (MAPK) (**Figure 1**). The key complex, which is consisted of IL-17A/A, IL-17A/F, or IL-17F/F cytokine and IL-17RA or IL-17RC, is the start hallmark of IL-17 signaling transduction (24, 25). Moreover, IL-17RD is also found to be a functional receptor for IL-17A groups. Together with IL-17RC, IL-17RD acts on the downstream of proinflammatory gene expression of IL-17 signaling (12). IL-17R is characterized by a unique structure in its cytoplasmic tail, termed SEF/IL-17R (SEFIR) domain (26). IL-17 signaling recruits Act1 to IL-17R through interaction platform of SEFIR domain (27). Then Act1 (also known as an E3 ligase) promotes activation of distinct downstream signaling cascades by tumor-necrosis factor receptor–associated factor (TRAF) 6 (28). TRAF6 then recruits and stimulates the transforming growth factor β-activated kinase 1 and the inhibitor of kappa B kinase complex, resulting in activation of NF-κB, C/EBPβ, C/EBPδ, and MAPK pathway (29–31). IL-17R-Act1 complex binds with MEKK3 and MEK5, leading to keratinocyte proliferation (32). Act1 binds with TRAF2-TRAF5 to maintain the mRNA stability targeting IL-17 gene (33). In contrast, TRAF3 triggers a negative reaction in activation of NF-κB and MAPK pathway, resulting in suppressing the formation of IL-17R-Act1-TRAF6 (34). TRAF6, in combination with A20 (an anti-inflammatory protein) when presented, blocks the activation of NF-κB and MAPK to negatively regulate IL-17 signaling (35).

PSORIASIS

Role of IL-17 Family Members in Psoriasis

In patients with psoriasis, the IL-17 concentrations increase not only in the skin lesions and peripheral blood, but also in the nonlesional and uninvolved skin (36–40). There is evidence indicating that the main sources of IL-17A in patients with psoriasis are the neutrophils (41), Th17 cells (42), mast cells (43, 44), $CD8^+$ T cells (45), αβ T (46), γδ T cells (47), and innate lymphoid cells (48, 49) in the skin lesions.

Psoriasis autoantigens, such as LL37 (50), NFKBIZ (51), ADAMTSL5 (52), and CARMA2 (53), play a crucial role in the production of IL-17A and are involved in the pathogenesis of psoriasis. In psoriasis, the combination of LL37 with the patient's own DNA leads to the activation of the Toll-like receptor 9 (54). The self-DNA-LL37 complex acts on Toll-like receptor 7 in the plasmacytoid dendritic cells (DCs) and triggers the activation of the classical myeloid DCs (55) (**Figure 2**). Subsequently, the myeloid DCs produce IL-12 and IL-23. IL-23 induces the differentiation of the $CD4^+$ T cells into the Th1 cells and Th17 cells by stimulating the transcription factor ROR-γt and STAT3 (56, 57). Thereafter, the activated Th17 cells secrete Th17 cytokines (IL-17A, IL-22, and TNF-α), leading to the development of a positive feedback loop. IL-17 plays a key role in stimulating the NF-κB and MAPK signaling pathways (53, 58), contributing to a high expression of proinflammatory factors (CC-chemokine ligand 20 and CC-chemokine receptor 6) (14, 38). These proinflammatory cytokines and chemokines recruit the inflammatory cells and stimulate keratinocyte proliferation (59).

IL-17A plays an essential role in inflammation, metabolism, and bone/joint damage (8, 10, 15, 60–62). The IL-17 levels and Th17 cell frequencies are high in the skin, synovial fluid, and synovium tissue of the patients with psoriatic arthritis (PsA) (18, 63–65). In the synovium of patients with PsA, the mast cells and $CD8^+$ T cells are the main sources of IL-17A (66). The aberrant expression of IL-17A directly affects the osteoclast precursors, leading to bone destruction in PsA (67). In addition, IL-17A interacts with the mediators of the Wnt signaling pathway in the osteoblasts and osteocytes, thus, preventing bone formation (10).

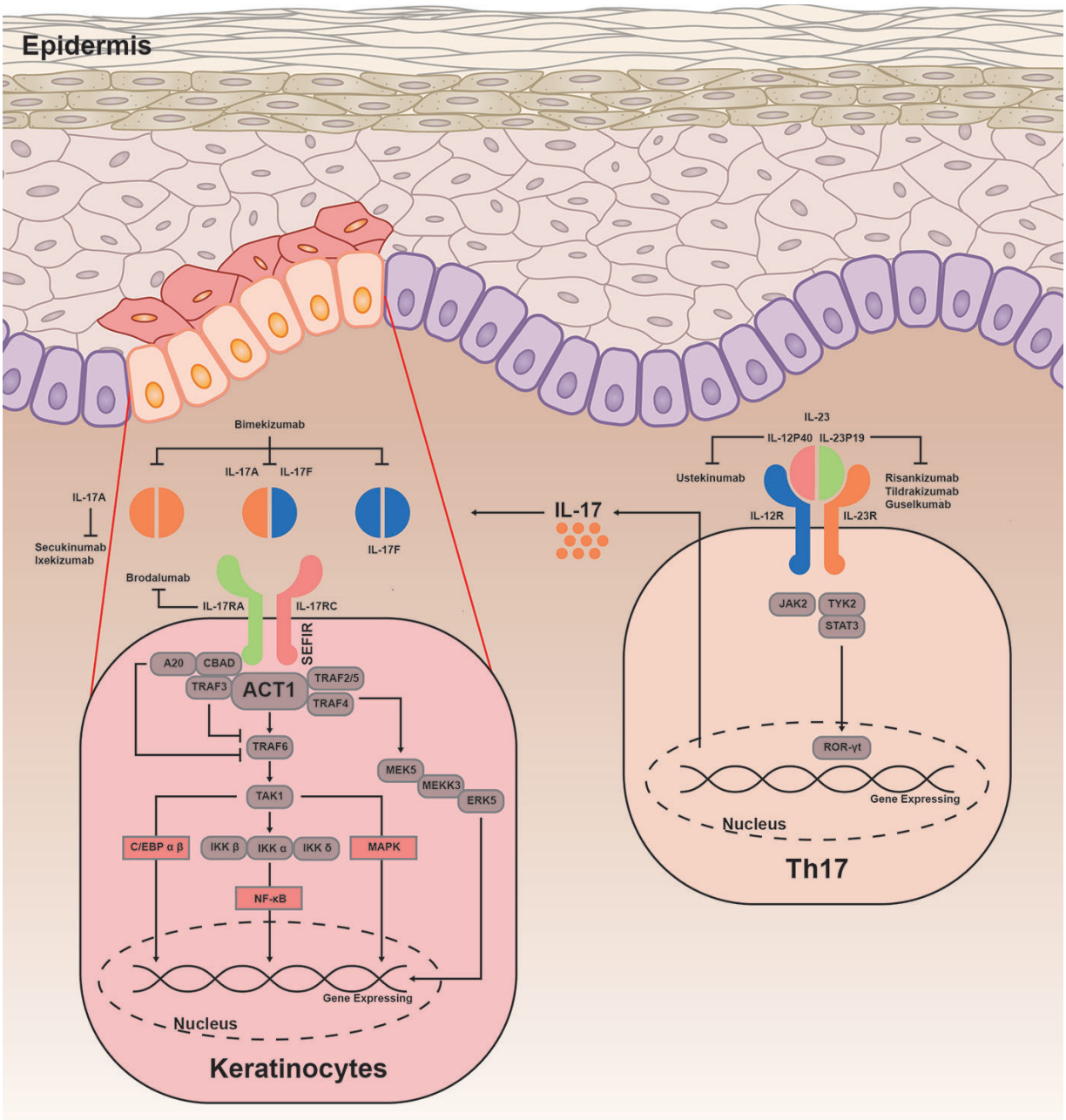

FIGURE 1 | IL-23/IL-17 signaling transduction. IL-23 is important in differentiation of Th17 cells, by promoting the production of IL-17A, IL-17F, TNF, and IL-6. IL-23 is heterodimeric and composed of IL-12p40 and IL-23p19. Binding to its receptors, IL-23 involves in phosphorylation of JAKs and TYK, as well as phosphorylation and dimerization of STAT3. Subsequently, STAT3 homodimers regulates the expression of ROR-γt to promote the gene expression. The combination of IL-17A/A, IL-17A/F, or IL-17F/F cytokine with IL-17RA and IL-17RC is found to be a crucial complex of immune response. IL-17R acts on Act1 through interaction platform of the SEFIR domain. Upon ligand binding, Act1 activates NF-κB, C/EBP family, and MAPK pathway by inducing various TRAF proteins. Act1 is essential for mediating ubiquitination of TRAF6, then TRAF6 triggers a positive reaction in multiple different pathways. TRAF6 recruits and stimulates the TAK1 and IKK complex, leading to activation of NF-κB pathway. IL-17R-Act1 complex together with TRAF4, MEKK3, and MEK5 to promote activation of ERK5. In addition, ACT1-TRAF2-TRAF5 complex is capable to maintain the mRNA stability targeting the IL-17 gene. The inhibitors A20 and TRAF3 are linked with IL-17RA, dependent on the CBAD. C/EBP, CCAAT/enhancer-binding proteins; NF-κB, canonical nuclear factor-κB; MAPK, mitogen-activated protein kinase; TRAF, tumor necrosis factor receptor associated factor; TAK1, transforming growth factor-β activated kinase 1; IKK, inhibitor of kappa B kinase; ERK5, extracellular signal-regulated kinase 5; RORγt, retinoid-related orphan receptor-γt; STAT3, signal transducer and activator of transcription 3; JAK2, Janus activated kinase 2; TYK2, tyrosine kinase 2.

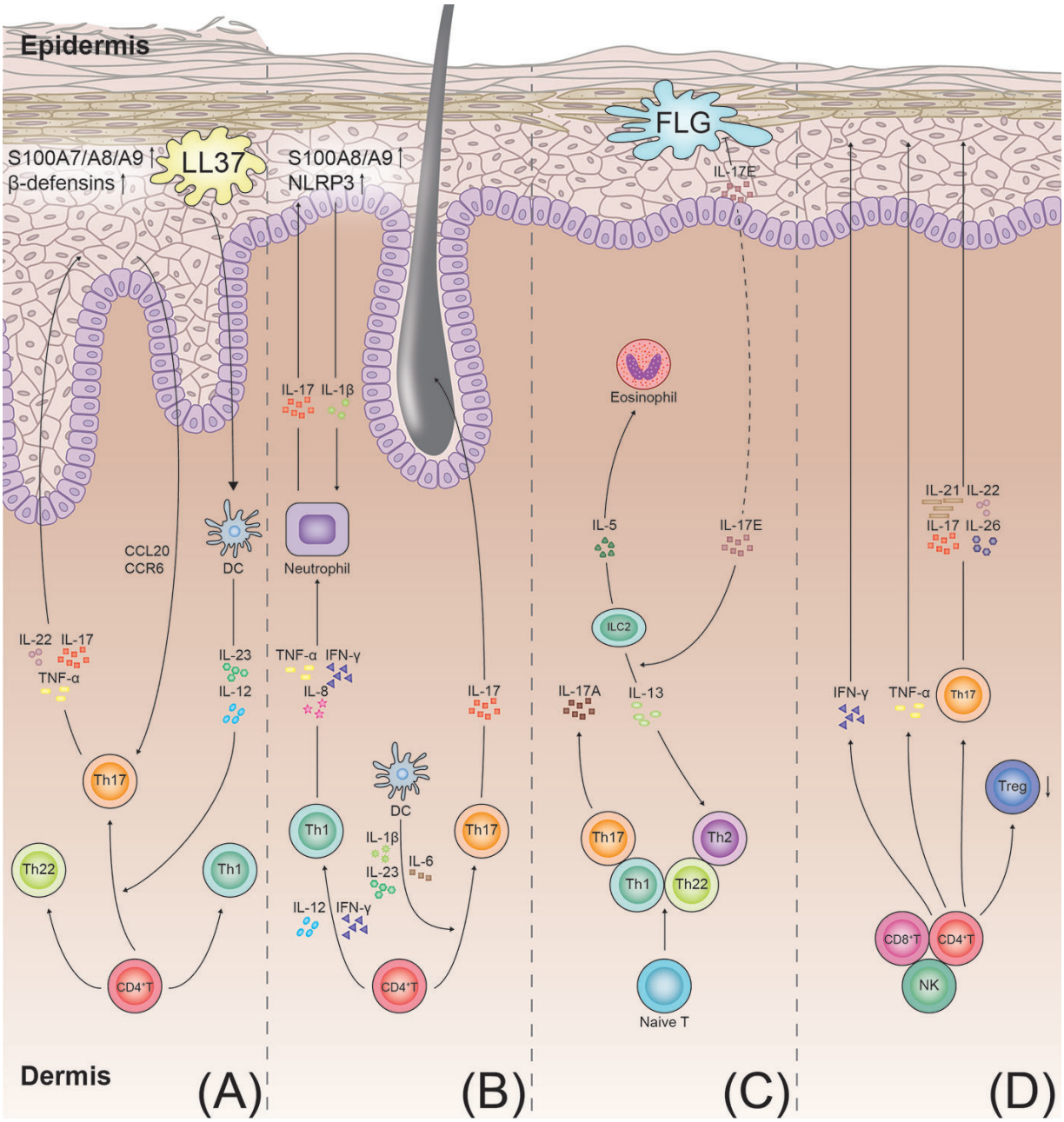

FIGURE 2 | T-cell immune axis and associated cytokines in the pathogenesis of psoriasis, hidradenitis suppurativa, atopic dermatitis, and alopecia areata. **(A)** Psoriasis develops through the aberrant activation of the dendritic cells producing IL-12 and IL-23. The dendritic cells induce the differentiation of the Th 17 cells and Th1 cells. IL-23 promotes the Th17 cells to secrete IL-17, IL-22, and TNF-α. In keratinocytes, IL-17 also stimulates production of antimicrobial peptides (S100A7/A8/A9 proteins and beta defensins). These cytokines promote keratinocyte proliferation and neutrophil recruitment, resulting in the formation of psoriatic plaques. **(B)** In hidradenitis suppurativa, the T cells involved in the pathogenesis of hidradenitis suppurativa include the Th1 and Th17 cells. IL-23 induces the differentiation of the Th17 cells and overexpression of IL-17. IL-17 induces the expression of the proinflammatory proteins (S100A8/A9) and NLRP3 in the keratinocytes. More inflammatory cytokines are recruited to the follicular unit and perilesional skin. **(C)** In atopic dermatitis, with impairment of the skin barrier in patients with atopic dermatitis, the damaged keratinocytes produce inflammatory cytokines (IL-17E). The cytokines stimulate the ILC2s to secrete type 2 cytokines (IL-5 and IL-13). IL-17E also inhibits the synthesis of FLG. IL-4, IL-13, and IL-31 directly stimulate the sensory nerves to promote pruritus. **(D)** In alopecia areata, the elevated IFN-γ levels in the perifollicular area activate the differentiation of the $CD4^+$ T cells into various types of T cells, as shown. Th17 cells act on the hair follicle by producing proinflammatory mediators (IL-17, IL-21, IL-22, and IL-26), ultimately leading to the disruption of hair growth. TNF, tumor necrosis factor; NLRP3, NACHT, LRR, and NACHT, LRR, and PYD domains-containing protein 3; FLG, filaggrin; ILC2s, type 2 innate lymphoid cells; DC, dendritic cell.

Psoriatic inflammation is not restricted to the skin or joints. Metabolic disorders, such as hyperglycemia (68) and cardiovascular risks (69), are also associated with psoriasis.

Apart from IL-17A, the other IL-17 family members (IL-17C, E, and F) may also be involved in the pathogenesis of psoriasis (70–72). In contrast to that in the nonlesional psoriatic skin, mRNA expression of IL-17A, IL-17C, IL-17E, and IL-17F is increased in the lesional psoriatic skin (39, 70, 73). The IL-17C and IL-17E levels are higher than the IL-17A levels in the skin lesions in the psoriatic animal models (7, 73). Through the STAT3 pathway, the binding of IL-17E to IL-17RB induces keratinocyte proliferation to amplify skin inflammation in the psoriatic animal models (70). However, the effects of IL17-C on the mechanisms of psoriasis are not clear. Both IL-17F and IL-17A share homomeric and heterodimeric proteins with 50% sequence identity. The molecular structure and function of IL-17F are highly similar to those of IL-17A (74). Bimekizumab (an inhibitor of both IL-17A and IL-17F) is more effective than a blockade of IL-17A or IL-17F alone, especially for suppressing neutrophil chemotaxis and activating the synoviocytes or human dermal fibroblasts *in vitro* (71).

Targeted Therapy in Psoriasis

The targeted biologics in the treatment of psoriasis are listed in **Supplementary Table**. Biologics targeting IL-23 or IL-17A have shown remarkable effects in the treatment of psoriasis. The anti-IL-17 agents approved by the FDA include secukinumab (anti-IL-17A), ixekizumab (anti-IL-17A), brodalumab (anti-IL-17RA), and bimekizumab (anti-IL-17A and -17F). Biologics against IL-23 include ustekinumab (anti-IL-12/23p40), tildrakizumab (anti-IL-23p19), guselkumab (anti-IL-23p19), and risankizumab (anti-IL-23p19).

Targeting IL-17

Secukinumab

Secukinumab, a human immunoglobulin G1 monoclonal antibody against IL-17A, is an effective and safe biologic for psoriasis, involving skin, nails (75), and PsA (76). The data from the phase III randomized trials (ERASURE and FIXTURE) showed that secukinumab at doses of 300 or 150 mg is effective and safe for the treatment of moderate-to-severe psoriasis up to week 52 (**Supplementary Table**) (77). Secukinumab maintains significant powerful and long-lasting effects on the patients receiving the 300 mg secukinumab treatment every 4 weeks. The Psoriasis Area and Severity Index (PASI) 90/100 was 66.4%/41% at 156 weeks (78).

In the FUTURE 2 study, patients with PsA receiving secukinumab therapy achieved excellent and sustained improvement in the PASI90 and American College of Rheumatology 50 (ACR50) response at week 24 (**Supplementary Table**) (79). The results for patients who continued the study showed that, at week 104, the ACR50 response rates were 50.6 and 36% with the 300 and 150 mg doses of secukinumab, respectively (80). Secukinumab is important in suppressing synovitis and structural bone changes in patients with PsA at week 24, and low rates of radiographic progression are maintained at week 52 with secukinumab (81, 82).

Secukinumab has significant and long-term efficacy for the treatment of nail psoriasis. Therefore, the improvement in the Nail Psoriasis Severity Index (NAPSI) in the secukinumab 300 and secukinumab 150 groups was 73 and 63.6% at week 128, respectively (75). In summary, secukinumab shows excellent and sustained efficacy for the treatment of patients with moderate-to-severe plaque psoriasis and patients with psoriasis with or without arthritis and nail involvement.

Ixekizumab

Ixekizumab, a humanized immunoglobulin G4 monoclonal antibody, selectively blocks IL-17A. A multicenter trial (UNCOVER-3) reported that ixekizumab shows long-term efficacy for treating moderate-to-severe plaque psoriasis and that the treatment effects are strongly sustained for up to 156 weeks (**Supplementary Table**) (83, 84). Similarly, ixekizumab maintains promising clinical improvements in the scalp, nails, and palm (83).

According to the data from phase III studies (SPIRIT-P1 and SPIRIT-P2), ixekizumab is associated with improvements in disease prognosis and physical function in patients with active PsA, particularly, in those who are refractory to therapies or have an inadequate response to the anti-TNF therapies (**Supplementary Table**) (85, 86). To date, blockade of IL-17A is being advocated as first-line for treatment of PsA by the European League Against Rheumatism in 2019 (87). In general, the aforementioned studies suggest that ixekizumab is effective in controlling psoriasis and PsA, particularly in patients with lesions in hard-to-treat areas or in those who are refractory to treatments.

Brodalumab

Brodalumab, a fully human immunoglobulin G2 IL-17RA antagonist, leads to a rapid improvement in the molecular, histological, and clinical features of psoriasis at week 12 (88). The AMAAGINE-1 study showed that brodalumab shows sustained efficacy (120 weeks) in the treatment of moderate-to-severe plaque psoriasis (**Supplementary Table**) (89, 90). Brodalumab inhibits a broader range of targets, namely, IL-17AA, IL-17AF, IL-17FF, IL-17C, and IL-17E *via* IL-17RA, compared with secukinumab and ixekizumab. An open-label study involving 39 patients with moderate-to-severe psoriasis revealed that brodalumab treatment may be effective for the patients who did not respond to secukinumab, ixekizumab, or ustekinumab (91, 92).

Moreover, the data from an open-label study have indicated significant beneficial effect of brodalumab on psoriatic erythroderma (n = 18) and generalized pustular psoriasis (n = 12; **Supplementary Table**) (93). However, the sample size of this study was small; hence, multicenter trials with large sample sizes of patients with psoriatic erythroderma or generalized pustular psoriasis should be conducted.

Bimekizumab

Bimekizumab, a humanized monoclonal IgG1 antagonist neutralizing both IL-17A and IL-17F, is effective for PsA and moderate-to-severe plaque psoriasis (71, 94). Two phase II trials (BE ABLE 1 and BE ABLE 2) reported the safety and efficacy of bimekizumab for the treatment of moderate-to-severe plaque

psoriasis (**Supplementary Table**) (94, 95). Patients with active PsA, who were administered bimekizumab, showed marked improvements in their condition at week 48 (**Supplementary Table**) (96).

Adverse Events of Targeting IL-17 Therapy

The most commonly noted treatment-emergent adverse events (TEAEs) are infections, nasopharyngitis, headache, and diarrhea in patients treated with IL-17 inhibitors compared with those treated with a placebo (76, 97–99). A systematic review speculated that it was safe to use IL-17 antagonists (secukinumab, ixekizumab, and brodalumab) for patients with psoriasis with latent tuberculosis infection (100). However, eczematous eruptions were reported in some patients after treatment with biologics against IL-17A (secukinumab or ixekizumab) for 3–4 months (101). To date, the mechanism underlying the onset of eczematous adverse events after anti-IL-17A treatment is not clear. Both the Th1 and Th2 responses are involved in the pathogenesis of eczema. This may be due to the anti-IL-17 biologics mainly inhibit the Th17 cytokines and mediate an imbalance in the Th2/Th17 immune response, thus leading to eczematous eruptions (102–104). The deficiency of Th17 cells, IL-17RA, and IL-17F are essential for host defense against fungal pathogens in mucocutaneous and oral epithelial cells (105–109). The risk of chronic mucocutaneous candidiasis increases in patients received IL-17 blockades (secukinumab, ixekizumab, brodalumab, or bimekizumab) (94, 96, 110).

Targeting IL-23

Ustekinumab, a humanized IgG1 monoclonal antibody against the p40 subunit of IL-12 and IL-23, is approved for treating adult and pediatric patients with moderate-to-severe plaque psoriasis (111). Two phase III trials (PHOENIX 1 and PHOENIX 2) reported rapid and sustained efficacy of ustekinumab when administered at doses of 45 mg or 90 mg every 12 weeks for patients with moderate-to-severe plaque psoriasis (**Supplementary Table**) (112, 113).

According to the phase IV trial (VIP-U), ustekinumab may reduce aortic vascular inflammation transiently (at week 12) and downregulate the expression of the inflammatory cytokines (TNF-α, IL-1β, IL-17A, IL-18, and IL-6) sustainably (at week 52) (114). In 25 patients with psoriasis, inflammation in the liver, spleen, and artery decreased after treatment with ustekinumab, as indicated by the radiography findings (115). A summary analysis indicated that ustekinumab has long-term (5 years) safety with respect to patients with moderate-to-severe psoriasis (116).

Risankizumab, a humanized IgG1 monoclonal antibody inhibits the p19 subunit of IL-23 (117). In the landmark UltIMMa-1 and UltIMMa-2 studies, 150 mg risankizumab proved beneficial in the treatment of moderate-to-severe psoriasis compared with a placebo and ustekinumab (**Supplementary Table**) (118).

Tildrakizumab is a humanized IgG1 monoclonal antagonist, targeting IL-23p19. The data from reSURFACE 1 and reSURFACE 2 have indicated significant safety and efficacy of tildrakizumab for the treatment of chronic plaque psoriasis; moreover, tildrakizumab was well-tolerated by the patients (**Supplementary Table**) (119). A pooled analysis of three trials showed that tildrakizumab maintains beneficial impact and low rates of serious TEAEs. The PASI75 scores of patients continuously treated with 100 mg and 200 mg tildrakizumab at 64 weeks were 86 and 83%, respectively (120).

Guselkumab, a human monoclonal anti-IL-23p19 antagonist, is used for the treatment of PsA (121). Two phase III trials (DISCOVER-1 and DISCOVER-2) showed that patients with PsA treated with guselkumab showed excellent and rapid, improvements in their condition; moreover, guselkumab treatment was safe for these patients. The results of the trials revealed that, at week 24, the ACR20 response rates were 52 and 64% for guselkumab administered every 4 weeks and 8 weeks, respectively (**Supplementary Table**) (122, 123).

In summary, the aforementioned clinical trials reported that anti-IL-23 antibodies can successfully control psoriasis and PsA. However, the long-term follow-up data on anti-IL-23p19 biologics are limited. Multicenter studies with large sample sizes should be conducted in the future to evaluate the long-term efficacy and safety of these antagonists.

HIDRADENITIS SUPPURATIVA

Role of IL-17 in Hidradenitis Suppurativa

HS is a Th1/Th17-driven inflammatory skin disease (**Figure 2**) (124). The histopathological analysis of skin biopsy samples has revealed that the frequencies of the Th17 cells and regulatory T (Treg) cells are elevated in the lesional HS skin (19). The levels of inflammatory cytokines (IL-17, IL-23, IL-1β, TNF-α, and IL-12) are high in the lesional, perilesional, and uninvolved skin of patients with HS (125, 126). The serum levels of IL-17 and S100A8/A9 are higher in the patients with HS than in healthy individuals (127, 128).

The neutrophils and Th17 cells are the major sources of IL-17 in HS; in contrast, the keratinocytes are a key source of proinflammatory molecules, including S100A8/A9, NACHT, LRR, and PYD domains-containing protein 3, and caspase-1 (19, 129). Notably, HS showed histopathological changes characteristic of epidermal psoriasiform hyperplasia, follicular plugging, and infiltration of low-grade leucocytes in the uninvolved skin of perilesional HS (130). In such microenvironments, the IL-17-stimulated keratocytes show upregulation of the expression of the proinflammatory proteins (S100A8/A9) and promotion of the release of IL-1β by activation of the NACHT, LRR, and PYD domains-containing protein 3. Consequently, large amounts of proinflammatory molecules are recruited to promote the influx of the neutrophils, which, in turn, upregulate the release of IL-17 and S100A8/A9; thus, a positive-feedback loop of the inflammatory response is maintained (**Figure 2**) (129, 131).

Targeted Therapy in Hidradenitis Suppurativa

An open-label and single-site exploratory trial has reported the efficacy of targeting IL-17A with secukinumab in the treatment of

HS. The nine patients administrated of 300 mg secukinumab once a week from baseline for 5 weeks and then every 4 weeks. At 24 weeks, 67% patients with HS achieved Hidradenitis Suppurativa Clinical Response (HiSCR) score (132). Very recently, an open-label pilot cohort study on 10 patients assessed the well tolerability and clinical response of brodalumab in the treatment of moderate to severe HS. It demonstrated that patients received 210 mg brodalumab achieving HiSCR at week 12, and HiSCR improvement occurred as early as week 2 (133). An open-label study indicates that, at week 40, a moderate-to-marked improvement of the modified Sartorius score was achieved in 82% (14/17) of patients with HS receiving IL-12/23 biologic ustekinumab therapy (134). The data on the efficacy and safety of biologics for treating HS are limited, and further studies with adequate sample sizes are required to establish the effective and long-term impact of treatment.

ATOPIC DERMATITIS

Traditionally, AD was considered a Th2 immune response with elevated levels of IgE. Studies have revealed that the Th1, Th2, Th22, and Th17 cells are involved in the pathogenesis of AD (**Figure 2**) (135, 136). It has been demonstrated that Th22 and Th17 immune responses contribute to chronic skin lesions of AD, especially in pediatric, intrinsic, and Asian patients (137–140).

IL-17E (also called IL-25) level increases in the epidermis in patients with AD (141). In keratinocytes, the null mutation of filaggrin gene (FLG) is associated with the skin barrier dysfunction, increasing the risk of AD (142, 143). FLG synthesis is suppressed by IL-17E in the keratinocytes (144). Moreover, the data from mouse models indicated that IL-17E induces the type 2 innate lymphoid cells to produce type 2 cytokines (IL-5 and IL-13) (145).

To investigate the efficacy of anti-IL-17A biologics in AD, a randomized phase II trial was conducted involving 41 patients who were administered secukinumab. However, the trial results showed that at week 16, both clinical assessments (the Scoring Atopic Dermatitis index and Eczema Area and Severity Index) and lesional skin immunohistochemical analysis of patients receiving secukinumab revealed no significant improvement compared with those receiving a placebo (146). This trial demonstrated that IL17 is not a pivotal contributor to the pathogenesis of AD, even in the subsets of patients with higher Th17 activation.

Although ustekinumab showed promising efficacy in a review which included published case reports and case series (147), no efficacy was observed in randomized controlled trials of targeting IL-12/23 for treating patients with AD (148, 149). Additional studies with large sample sizes and may show the efficacy of ustekinumab in treating AD.

ALOPECIA AREATA

AA is a common inflammatory skin disorder, which is characterized by nonscarring hair loss *via* infiltration of the $CD8^+$ T cells and increase in the levels of cytokines (IFN-γ, TNF-α, IL-17 and IL-4; **Figure 2**) (150). IL-2, IFN-γ, IL-10, IL-13, and IL-17A are expressed at high levels in the serum of patients with AA, while the level of transforming growth factor -β1 is decreased (151, 152). The Th17 cell frequencies and IL-17 levels significantly increased both in the peripheral blood and scalp lesions in patients with AA; however, the frequency of Treg cells decreased (153, 154). Studies have reported that patients with AA do not show any response to the administration of anti-IL-23/IL-12 ustekinumab (n = 4) or anti-IL-17A secukinumab (n = 7) (155, 156). Therefore, it cannot be concluded the contribution of Th17/IL-17 in the pathogenesis of AA. Further clinical trials with large sample size may reveal the value of IL-17 as a target of AA.

PITYRIASIS RUBRA PILARIS

PRP is a rare acquired inflammatory skin disease. The levels of Th17 and Th1 cytokines increase in the lesional skin of the patients with PRP, including IL-17A, IL-17F, IL-22, TNF, IL-6, IL-12, IL-23, and IL-1β (157). The IL-23/Th17 axis seems to be important in the pathogenesis of PRP due to the clinical and histopathologic improvement in the targeting IL-12/23 and IL-17A (ustekinumab, secukinumab, and ixekizumab) treatment of patients with PRP (157–160). In a single-armed trial, analyzing changes in the clinical signs and symptoms (using PASI scores) showed that PASI50, PASI75 and PASI90 response rates were 58, 42, and 17% respectively during ixekizumab treatment of PRP at week 24 (160). For those 5 patients who failed to conventional therapies, all of them have achieved clinical improvement from ustekinumab, particularly, changes in decreased erythema, follicular hyperkeratosis, and scaling during a 15-month follow-up period (161).

PEMPHIGUS

In the serum and lesional skin of patients with pemphigus vulgaris, the levels of IL-23 and IL-17 increase, both are significantly correlated with diseases severity (162, 163). The frequency of $CD4^+IL\text{-}17^+$cells and the level of IL-23R mRNA show increases in the serum of patients with pemphigus foliaceus (164), in contrast to showing decreases in newly diagnosed patients with pemphigus vulgaris (165). This may be a result of the Th17 cells have plasticity and converting to Th1-like Th17 cells (165–167). There are some reports that the frequency of Th17 cells and level of IL-17 show decreases in other autoimmune and inflammatory diseases, such as lipopolysaccharides responsive beige-like anchor protein deficiency (165, 167).

SYSTEMIC SCLEROSIS

The imbalance and dysfunction of Th17/Treg cells are crucial to the generation of SSc (168). Quantitative analysis of Th17

cytokines in lesional skin of SSc showed that the expression of IL-17A, IL-13, IL-22, and IL-26 mRNA are higher compared with healthy control (169, 170). The levels of circulating Th17 cells and IL-17 elevated in serum of patients with SSc. They are in correlation with disease severity and collagen overproduction (171, 172). The elevated levels of IL-17A act on dermal vascular smooth muscle cells to promote vascular fibrosis in the patients with SSc, *via* activating extracellular signal-regulated kinase 1/2 signaling pathway (173).

CONCLUSION

In summary, the Th17/IL-17 axis has been identified as a key factor in skin inflammatory diseases, such as psoriasis, HS, AD, PRP, pemphigus, and SSc. Neutralizing IL-17 or IL-23 in psoriasis, HS and PRP has shown promising clinical improvements. Additional studies are required to identify whether IL-17 is involved in the pathogenesis of AA, PRP, pemphigus, and SSc, which may lead to the development of targeted strategies for efficiently ameliorating or specifically eliminating these debilitating diseases.

AUTHOR CONTRIBUTIONS

TL, YL, JQ, and HF contributed to conception, literature search, and manuscript writing. SL, SY, ST, and YD created graphical illustrations. TL, JQ, and HF contributed to manuscript revision and read the submitted version. 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.2020.594735/full#supplementary-material

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Major Role of the IL17/23 Axis in Psoriasis Supports the Development of New Targeted Therapies

Hélène Bugaut[1,2,3*] *and Sélim Aractingi*[1,2,4]

[1] Faculté de médecine, Université de Paris, Paris, France, [2] Service de dermatologie, Hôpital Cochin, Paris, France, [3] U932 Immunité et cancer, Institut Curie, Paris, France, [4] Equipe Biologie cutanée, Institut Cochin, Inserm, UMRS1016, Paris, France

Psoriasis is a frequent, chronic disease characterized by cutaneous inflammatory plaques and/or arthritis. It may be associated with few other diseases, mainly Crohn's disease and metabolic syndrome. The medical and psychosocial burden of psoriasis remains high even since biological treatments arose, stressing that efforts to decipher its physiopathology are constantly needed. Tumor-necrosis factor α, interleukin (IL) 12 and IL17 have been previously associated with psoriasis and successfully targeted by monoclonal antibodies. IL17 in particular has been initially described as a T helper (Th) 17—produced cytokine, but it is now established that other cell types, such as γδ T lymphocytes, Mucosal-Associated Invariant T (MAIT) cells and Innate Lymphoïd Cells (ILC) 3 are also important sources of IL17 in the skin in response to inflammatory stimuli. Th17 phenotype has been shown to be stabilized by IL23, which is synthetized by macrophages and dendritic cells in response to Toll Like Receptors and C-type Lectin Receptors stimulation. Recent data also reported a crucial role for IL23 in MAIT17 and ILC3 homeostasis. Genome-wide association studies have found a significant link between IL23 receptor polymorphism and psoriasis susceptibility. IL23 signals through Janus kinase 2 and Tyrosine kinase 2, against which specific inhibitors are currently being tested. Monoclonal antibodies against IL17 and IL23 are only the beginning of a new avenue in psoriasis treatment. This review focuses on the molecular basis underlying IL23/IL17 axis blockade in psoriasis, and on future targets in this pathway.

Keywords: IL23, IL17, psoriasis, skin, Th17

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Case Western Reserve University,
United States

***Correspondence:**
Hélène Bugaut
helene.bugaut@curie.fr

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INTRODUCTION

Psoriasis is a chronic inflammatory disease involving the skin and/or the joints. Psoriasis prevalence in adults ranges from 0.51 to 11.43% worldwide (1) but is mainly considered to affect 2–3% of the population, with similar frequency in males and females (2). Skin lesions are featured by relapsing cutaneous erythro-squamous patches in its most frequent form, namely psoriasis vulgaris (PV). These will target electively peculiar locations such as scalp, palms and soles but also sacrum or large folds. Arthritis (PA, psoriatic arthritis) is associated to skin lesions in 1 to 15% of psoriasis patients (3). Of note, PA may either concern the peripheral articulations being therefore close to the

characteristics of rheumatoid arthritis or in the contrary involve the spine, close to ankylosing spondylitis.

Psoriasis belongs to the spectrum of autoinflammatory diseases. Even in psoriasis without PA, its medical and psychosocial burden is high. Indeed, several studies using robust tools indicate that in all countries psoriasis may severely affect the quality of life (QOL) of these patients (4). In addition, psoriasis is significantly associated with metabolic syndrome, cardiovascular comorbidities and more rarely Crohn's disease (5).

Since the 1990s, genetic and immunological studies have impressively dissected the mechanisms of psoriasis. Briefly, the disease appears to result from the interaction of genetic background and environmental triggers. Susceptibility loci mainly belong to HLA class I and II genes but also to various genes implicated in interleukin (IL) 17, IL23 and nuclear factor-kappa B (NFκB) pathways. In susceptible individuals, especially in response to physical traumatisms, autoantigens from keratinocytes, such as DNA, conjugate with anti-microbial peptides like cathelicidin/LL-37 (6), and activate plasmacytoid dendritic cells (pDCs) in the dermis. pDCs then secrete type I interferon and tumor-necrosis alpha (TNFα), which will activate classical dendritic cells (cDCs). These cDCs will produce IL12 and IL23, and skew the education of naïve T cells into T helper (Th) 1, Th17 and Th22 cells. Tumor-necrosis alpha (TNFα), IL17, and IL22 produced by these CD4+ T cells will then promote secretion of pro-inflammatory chemokines by keratinocytes, proliferation of epithelial cells and hyperkeratosis, and the recruitement of more inflammatory immune cells, accounting for the erythemato-squamous clinical lesions (7).

Resulting from this knowledge, targeted therapies have been a turnover in the management of psoriasis. TNFα blocking agents have initially paved the way, followed by monoclonal antibodies directed against IL12/23, IL17, and IL23. This highlights the crucial role of the IL17/23 cytokine pathway in psoriasis pathogenesis. Constant efforts are required to decipher the molecular mechanisms behind this disease, since new treatments are still needed for refractory and severe cases.

GENETIC VARIANTS HIGHLIGHT CRITICAL IMMUNOLOGICAL PATHWAYS

Heritability might account for as much as 68% of psoriasis susceptibility in Europeans (8). The first genetic linkage analyses in familial psoriasis (9) demonstrated the role of major histocompatibility complex alleles, mainly *HLA-C*06:02*, and were further confirmed by genome-wide association studies (GWAS) (10). Further insights into psoriasis genetics confirmed the importance of several immunological pathways among variants (11). Various mutations activating the pro-inflammatory NFκB pathway downstream of IL17 receptor (IL17R), such as in *TRAF3 Interacting Protein 2* (*TRAF3IP2*), which encodes ACT1, a protein that allows signal transduction from the IL17R and downstream activation of NFκB, in *CARD14* (an activator of NFκB), and in TNFAIP3 (tumor necrosis factor alpha induced protein 3, also called A20) and TNFAIP3 Interacting Protein 1 (TNIP1), have also been associated with an increased risk of developing psoriasis by GWAS studies, in Asian and Caucasian populations (10, 12–20).

Th17 lymphocytes are a major source of IL17, and they require IL23 to maintain their phenotype and to produce large amounts of IL17 (21–24). Polymorphisms in both subunits of IL23, IL23A(p19) and IL12B(p40), and in IL23R, have been associated with an increased risk of psoriasis in North Americans, Europeans and Asians (10, 12, 25). Signaling downstream of IL23 requires the signal transducer and activator of transcription protein 3 (STAT3). Polymorphisms have also been found by a GWAS meta-analysis in this gene (13). *Interferon regulatory factor 4* (*IRF4*), another gene whose variants are associated with psoriasis (13), encodes a transcription factor that binds to the IL17 promoter and regulates Th17 pathogenic properties (26, 27). IRF4 also drives the differentiation of conventional dendritic cells (cDCs) into cDC2, which produce IL23 and promote Th17 (28, 29).

Eventually, clues for the implication of other sources of IL17 than Th17 in psoriasis pathogenesis may also be suggested by the association of *Runt-related transcription factor 3* (*RUNX3*) polymorphisms to psoriasis susceptibility (13). RUNX3 is indeed a critical transcription factor for innate lymphoid cells (ILC), in particular for ILC3, the IL17 producing subset (30).

The vast majority of available genetic association studies highlight the role of the immune system in the pathophysiology of psoriasis. Even if some other genes such as IL-36 receptor antagonist (*IL-36RN*) are implicated (31, 32), the IL-17/23 axis seems to play a cardinal role.

IL17 AS A CENTRAL EFFECTOR IN PSORIASIS

An extensive amount of evidence now places IL17 as a key player in psoriasis pathogenesis (33, 34). In addition to genetic association studies, the efficacy of monoclonal antibodies targeting IL17 is a strong argument for the implication of this cytokine (35, 36).

Six isoforms of IL17 exist, and IL17A and IL17F are deemed to be the most pathogenic in psoriasis (37). IL17 receptors are heterodimers of IL17RA and a ligand specific subunit (IL17RB–E). IL17 receptors are widely expressed on epithelial cells (38). Upon the recognition of its ligand, IL17R recruits ACT1, which binds to tumor necrosis factor receptor 6 (TRAF6) (20). Downstream signaling involves mitogen activated protein kinase (MAPK), NFκB and C/EBPβ/δ pathways (19, 39–41). IL17 drives secretion of inflammatory chemokines, cytokines and antimicrobial peptides by keratinocytes, such as chemokine (C-C motif) ligand 20 (CCL20), IL-8 and β-defensin2 (42–45). IL17 indeed seems to play a key role in skin local immunity, as inborn deficiencies of IL17 or IL17R are responsible for chronic mucocutaneous candidiasis in humans (46). Pro-inflammatory mediators then recruit more Th17 lymphocytes, for example through CCL20/CCR6 signaling (47), and neutrophils, and

increase local inflammation, resulting in the erythematous lesions characteristic of psoriasis.

Th17 cells were the first described source of IL17 (21, 48) and as such have been implicated in the pathogenesis of psoriasis (49). Th17, along with Th1, are found in the dermis of psoriatic lesions, and produce IL17 and IL22 (50), which in turn drives inflammatory and antimicrobial molecules secretion by keratinocytes (51). In mice, Th17 differentiate from naïve T CD4+ lymphocytes upon IL6 and transforming growth factor β stimulation; this process is amplified by IL1β and TNFα. Th17 cell survival and expansion depends on IL23 (21). IL23R is induced in Th17 by IL6 signaling through Janus kinases (JAK) JAK1, JAK2 and tyrosine kinase 2 (TYK2), STAT3 and RAR-related orphan receptor gamma t (RORγt) (52–54). IL23 signals through JAK/STAT3, resulting in enhancement of the Th17 phenotype (22). In humans however, Th17 require IL23 and IL1β for their differentiation (51). Keratinocytes also produce cytokines such as IL1β which amplifies Th17 generation (55).

Of note, Th17 are not the only source of IL17 in psoriasis. Other innate subsets, such as unconventional T cells, produce this key cytokine and might represent new therapeutic targets (56).

Innate Cells Are Key Sources of IL17

Gamma delta T lymphocytes (Tγδ) are abundant innate-like T lymphocytes in the dermis. They can be divided into T-box expressed in T cells+ (T-bet)+, IFNγ-producing Tγδ and RORγt +, IL17-producing Tγδ (57–59). The IL17-producing subset predominates in the dermis, expresses IL23R, depends on STAT3 signaling and is a major source of pathogenic IL17 in psoriasis (60–62).

Mucosal-associated invariant T cells (MAIT) are recently characterized innate-like T lymphocytes which recognize metabolites produced by bacteria and fungi. They are abundant in barrier tissues and especially in the skin. Although rare in mice, they represent 1–10% of T lymphocytes in human blood, skin and intestine (63–65). In mice, they are also divided into MAIT1 and MAIT17 subsets, expressing T-bet and RORγt and producing IFNγ and IL17, respectively (66). MAIT17 rely on IL23 for their homeostasis and activation (67, 68) and are enriched in psoriatic skin lesions (64).

Another important type of unconventional cells are innate lymphoid cells (ILC). They have a lymphoid morphology, do not rely on recombination-activating genes (RAG) for their development, and lack myeloid, dendritic and T/B markers. Type 1 ILC encompass NK cells and ILC1, express T-bet and secrete IFNγ; ILC2 are characterized by GATA3 expression and IL5 and IL13 secretion; while ILC3 express RORγt and require IL23 to produce IL17 (69). ILC3 - like Th17 cells, IL17-producing Tγδ cells and MAIT17 cells - are increased in blood and cutaneous lesions of psoriasis patients (70, 71).

Several reports of IL17 secretion by neutrophils through extracellular traps production in psoriasis have been published (72–74). Neutrophils seem to express IL23R and RORγt (75), but their contribution to IL17 production in psoriasis is still largely unknown.

Eventually, keratinocytes themselves are able to produce IL17C, enhancing inflammation in psoriasis in an autocrine way (37, 44, 76, 77).

IL23 Is a Key Regulator of IL17 Production

In many IL17-producing cell types, IL23 plays a pivotal role in IL17 secretion (78). IL23 induces Th17 phenotype in humans (51) or maintains this phenotype in mice (21–24). IL23 is required for IL17 production by skin Tγδ (62), MAIT17 (67, 68), ILC3 (69) and maybe by neutrophils (75). The receptor for IL23 is a heterodimer of IL23R, which signals through JAK2, and of IL12Rβ1, which signals through TYK2. Both activate STAT3, resulting in RORγt expression and IL17 secretion (22, 79).

It seems that in the gut, contrary to the skin, IL17 might play a protective role on the epithelial barrier, and its secretion seems to be at least partially IL23-independent (80). This difference may account for the worsening of Crohn's disease symptoms in psoriasis patients treated with antiIL17, which is not found so far during IL23 blockade (81).

IL23 is mostly produced by cDC2 in mice, which correspond to CD1c+ DC in humans. cDC2 are driven by the transcription factor IRF4 and promote Th17 differentiation in mice and humans (29). This IL23 production depends on Toll Like Receptors and C-type Lectin Receptors stimulation, and neurogenic locus notch homolog protein 2 (NOTCH2) signaling, in different models of inflammation including psoriasis (51, 82, 83).

The whole IL23/JAK/STAT3/RORγt/IL17 pathway plays a central role in psoriasis pathogenesis and is a key target of many recent and developing treatments for psoriasis.

Targeting the IL23/IL17 Axis in Psoriasis

The development of new psoriasis treatments has nicely demonstrated *in vivo* the essential role of the IL23/IL17 axis in psoriasis (**Figure 1**). Ustekinumab, an antip40 (common to IL12 and IL23) antibody, represented the second generation of monoclonal antibodies developed in psoriasis after antiTNFα antibodies. It induces a nonspecific inhibition of Th1 and Th17 with a high efficiency (Psoriasis Area Severity Index improvement ≥ 75% (PASI75) at week 12: 67%), but that is reached slowly, usually in 3–6 months (84–86).

More recently, a third generation of monoclonal antibodies became available: secukinumab and ixekizumab targeted IL17A, whereas bimekizumab blocked both IL17A and IL17F and brodalumab inhibited IL17R. Their efficacy was also high (PASI75 at week 12: 77–86%) but reached much faster, in 1–3 months (35, 36, 87–89). However, unexpected flare-ups of Crohn's disease happened in a minority of patients, whereas it was not the case during TNFα and IL12/IL23 inhibition (81, 90). Even if this over-risk is not fully confirmed (91), several studies now suggest that IL17 might play a protective role in the gut, where secretion by Tγδ and ILC3 might predominate, whereas IL17 is endowed with pro-inflammatory functions in the skin (44, 80, 92). Other expected side effects include diffuse candidiasis, as suggested by studies from inborn errors in IL17 signaling (46). A warning about suicide risk restricted to

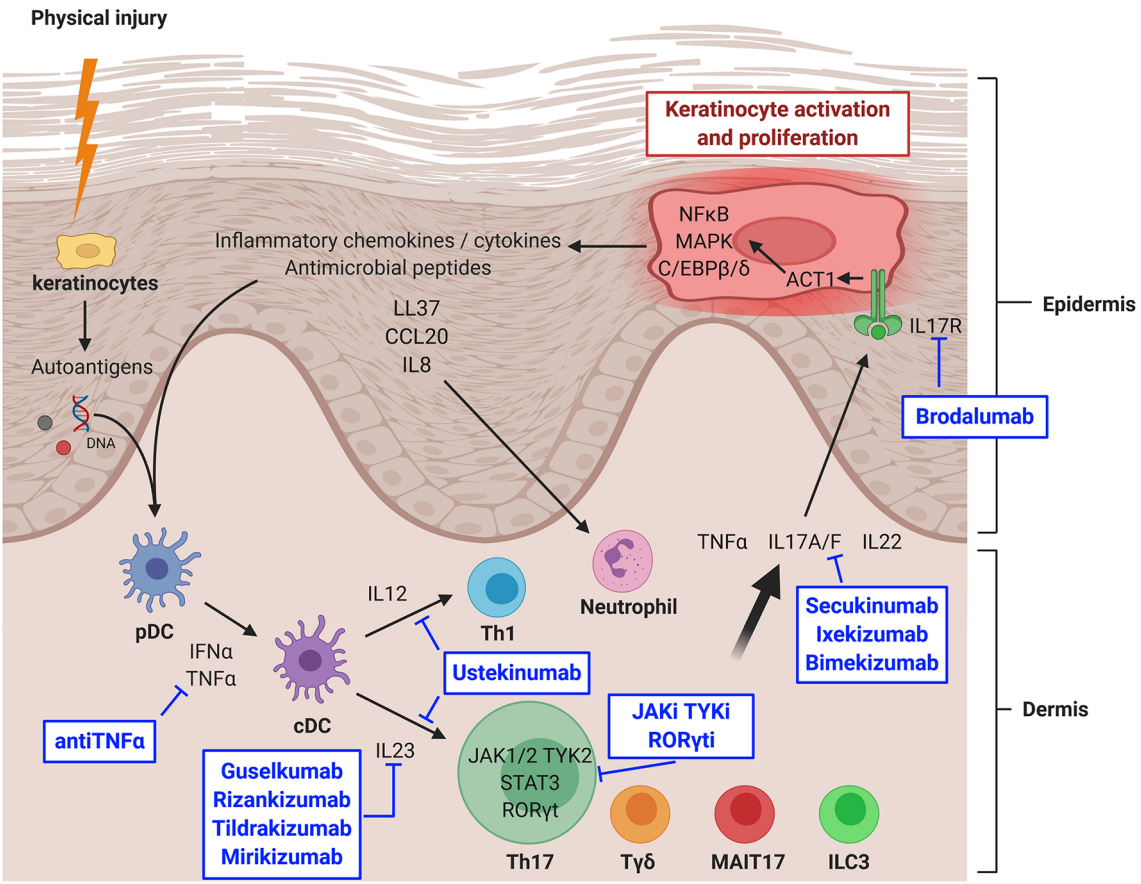

FIGURE 1 | IL23/IL17 axis in psoriasis and targeted therapies. CCL20, chemokine (C-C motif) ligand 20; cDC, classical dendritic cell; IFNα, interferon alpha; IL, interleukin; IL17R, IL17 receptor; ILC, innate lymphoid cell; JAK, Janus kinase; JAKi, JAK inhibitor; MAIT, mucosal associated invariant T cell; MAPK, mitogen activated protein kinase; NFκB, nuclear factor-kappa B; pDC, plasmacytoid dendritic cell; RORγt, RAR-related orphan receptor gamma t; RORγti, RORγt inhibitor; STAT, signal transducer and activator of transcription protein; Tγδ, gamma delta T lymphocyte; Th, T helper lymphocyte; TNFα, tumor necrosis factor alpha; TYK, tyrosine kinase; TYKi, TYK inhibitor. Created with BioRender.com.

brodalumab (93) has been described but does not seem to be confirmed by more recent follow-ups.

The latest generation of monoclonal antibodies in psoriasis is represented by specific antiIL23 treatments such as guselkumab, rizankizumab, tildrakizumab and mirikizumab. Their efficacy is very high (PASI90 at week 16: 67–75%) and reached as quickly as when using antiIL17 antibodies, but without the previous side effect of IBD flare (94–99).

New therapeutic strategies in psoriasis now tend toward small molecules targeting JAKs, in order to prevent signaling downstream of IL23 and IL6. Tofacitinib, which blocks JAK1, JAK2 and JAK3, is tested in several clinical trials (100–105). Results are interesting but side effects, especially cytopenias, are pushing toward more selective JAK inhibitors (106). Specific TYK2 inhibitors are also under development, with an encouraging phase II trial (107), and several phase III trials ongoing (ClinicalTrials.gov identifiers NCT04036435, NCT03924427, NCT04167462, NCT03624127, and NCT03611751). Masitinib, the TYK c-kit inhibitor, is also undergoing a phase II trial (ClinicalTrials.gov identifier NCT01045577).

Inhibitors of RORγt are also under development, with an ongoing phase II trial (ClinicalTrials.gov identifier NCT04207801) while another one was terminated for adverse events (ClinicalTrials.gov identifier NCT03329885). RORγt inhibition could be relevant as it does not seem to affect Tγδ nor ILC3, which could spare the protective role of IL17 on the intestinal barrier (78, 108). Concerns about a risk of deep immunosuppression have been raised since RORγt is required at the early stage (double positive stage) of thymic development for all T lymphocytes (109, 110). Opportunist candidiasis and mycobacterial infections might also be a concern, since they are encountered in patients with inborn deficiencies in RORγt (111). Finally, conditional knock-out mice for RORγt develop lymphomas (112), which are thus closely monitored in clinical trials.

A promising strategy might be to use the topical route to avoid these potential serious side effects. Topical tofacitinib has shown promising results in a phase II trial (113). A topical formulation of a JAK1 and TYK2 inhibitor is currently undergoing a phase II trial (ClinicalTrials.gov identifier

NCT03850483). Topical RORγt inhibitors are still in phase I or preclinical development (114, 115).

CONCLUSION

IL23/IL17 axis plays a crucial role in psoriasis. Innate-like sources of IL17, such as Tγδ, MAIT and ILC3 are broadening the scope of pathogenic cells beyond classical Th17. Therapeutic targets now encompass IL23, JAK, RORγt and IL17 steps in this pathway, opening new avenues for resistant psoriasis treatment.

AUTHOR CONTRIBUTIONS

HB had written the first draft of the manuscript. SA had reviewed the manuscript extensively. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of the IL23/IL17 Pathway in Crohn's Disease

Heike Schmitt[1,2], *Markus F. Neurath*[1,2] *and Raja Atreya*[1,2*]

[1] First Department of Medicine, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany, [2] Deutsches Zentrum Immuntherapie, Erlangen, Germany

Crohn's disease (CD) is a chronic relapsing disorder of the gastrointestinal tract and represents one of the main entities of inflammatory bowel disease (IBD). CD affects genetically susceptible patients that are influenced by environmental factors and the intestinal microbiome, which results in excessive activation of the mucosal immune system and aberrant cytokine responses. Various studies have implicated the pro-inflammatory cytokines IL17 and IL23 in the pathogenesis of CD. IL23 is a member of the IL12 family of cytokines and is able to enhance and affect the expansion of pathogenic T helper type 17 (Th17) cells through various mechanisms, including maintenance of Th17 signature genes, upregulation of effector genes or suppression of repressive factors. Moreover, IL17 and IL23 signaling is able to induce a cascade of pro-inflammatory molecules like TNF, IFNγ, IL22, lymphotoxin, IL1β and lipopolysaccharide (LPS). Here, IL17A and TNF are known to mediate signaling synergistically to drive expression of inflammatory genes. Recent advances in understanding the immunopathogenetic mechanisms underlying CD have led to the development of new biological therapies that selectively intervene and inhibit inflammatory processes caused by pro-inflammatory mediators like IL17 and IL23. Recently published data demonstrate that treatment with selective IL23 inhibitors lead to markedly high response rates in the cohort of CD patients that failed previous anti-TNF therapy. Macrophages are considered as a main source of IL23 in the intestine and are supposed to play a key role in the molecular crosstalk with T cell subsets and innate lymphoid cells in the gut. The following review focuses on mechanisms, pathways and specific therapies in Crohn's disease underlying the IL23/IL17 pathway.

Keywords: Crohn's disease, anti-TNF therapy, IL17/IL23 axis, intestinal immunity, inflammation, resistance to apoptosis, non-responder

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***Correspondence:**
Raja Atreya
raja.atreya@uk-erlangen.de

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CROHN'S DISEASE

CD representing one of the major forms of inflammatory bowel diseases (IBD), is a chronic inflammatory condition affecting the gastrointestinal tract (1). The global annual incidence of IBD is rising and it is estimated that the incidence of IBD in European countries is 3-8.5/100,000, and as many as 2.2 million people in Europe suffer from IBD (2). All parts of the gastrointestinal tract can be affected whereas the terminal ileum and the colon are the most frequent localizations (3). CD is thought to be the result of the interaction between genetic susceptibility, environmental factors and the intestinal

microflora causing abnormalities in mucosal immune response and altered epithelial barrier function (1, 4). CD is associated with significant morbidity and has a marked impact on the patient's quality of life as the most common symptoms include abdominal pain, diarrhea, rectal bleeding, weight loss, fever, and fatigue. Extra-intestinal inflammation manifests frequently in the eyes, liver, skin and joints, reflecting the systemic nature of this debilitating disease. Moreover, the majority of patients eventually develop penetrating or stricturing complications leading to repeated surgeries and disability (5, 6). The pathogenesis of CD is complex. Recent studies have greatly improved our understanding of the pathophysiology of CD, leading to major advances in the treatment and diagnosis of CD (7, 8). Earlier treatment goals focused on reducing clinical symptoms, but in the course of time and the development of new-targeted therapies, the initial goal of achieving clinical remission, shifted to steroid-free remission, endoscopic remission and mucosal healing, which have all become an integral part of successful CD treatment (9, 10). The first class of substances approved for the treatment of CD were anti-TNF antibodies (infliximab, adalimumab and certolizumab pegol). In the next few years, antibodies against the integrin alpha4beta7 (vedolizumab) and interleukin 12 (IL12) and interleukin 23 (IL23) through their common p40 subunit (ustekinumab) have been approved for CD therapy (11, 12). Moreover, recently published data demonstrate that the treatment with the selective IL23p19 inhibitors risankizumab or brazikumab leads to high response rates in CD patients that did not respond to previous anti-TNF therapy (13, 14). Although the aforementioned-targeted therapies have achieved great clinical success, it was found that only a subgroup of CD patients benefit from these treatments. In addition, there are currently no clinically compatible predictive biomarkers for individual guidance of drug therapy. Therefore, it is of utmost clinical importance to gain a deeper understanding of the respective modes of action of each therapeutic substance class to ensure that each patient is provided with the most effective and appropriate therapy (15, 16).

IL23 SIGNALING

IL23 is a heterodimer cytokine consisting of the p40 subunit (shared with IL12) and the unique p19 subunit (IL23A) encoded by the IL23 gene (17). IL23 belongs to the IL12 cytokine family whereas the human p19 is a four alpha-helix protein with 70% similarity to its mouse orthologue (18). The heterodimer cytokine IL12 is built by the two subunits p40 (shared with IL23) and p35. IL23 signals through its heterodimeric receptor complex consisting of the two subunits IL12Rβ1 and IL23R, while IL12 signals through its heterodimeric receptor complex consisting of the two subunits IL12Rβ1 and IL12Rβ2. The shared p40 subunit of IL12 and IL23 signals through IL12Rβ1 whereas the unique subunit IL23p19 signals through IL23R and the unique IL12p35 interacts with IL12Rβ2 (19) (**Figure 1**).

IL23 binding to its receptor activates Janus kinase 2 (jak2) and tyrosine kinase 2 (tyk2), which then phosphorylates the receptor to form a docking site leading to the subsequent phosphorylation of signal transducer and activator of transcription 3 (STAT3) for the p19 subunit and STAT4 for the p40 subunit. The initiation of IL23R signaling leads to the activation of several pathways, which are centrally involved in the pathogenesis of CD, for example P38 MAPK, PI3K-Akt or the NFκB pathway. This activation leads to the release of CD associated cytokines like IL17A, IL17F or IL22 (20–22) (**Figure 1**).

IL23 IN CROHN'S DISEASE

Different studies have shown that a multitude of cytokines play an important role in the development and perpetuation of CD. It has been proven that IL23 in particular is mainly involved in the pathogenesis of CD (23, 24). Genome-wide association study (GWAS) have analyzed the polymorphism in the gene encoding IL23R and linked it to the pathogenesis of IBD, indicating the important role of IL23 in mucosal inflammation. In addition, the elevated levels of IL23 in the mucosa of CD patients further emphasizes its key role in the pathogenesis of IBD (25). IL23 is mainly expressed by CD14+ intestinal macrophages that are key players in mediating the perpetuation of inflammation by infiltrating into the inflamed intestine in CD patients (26–28). Dendritic cells and epithelial cells were also shown to produce IL23 (29). This is supported by a recently published study showing that mucosal TNFR2-expressing CD4+ T cells circumvent anti-TNF-induced apoptosis by coexpressing IL23R, which is activated by the upregulated IL23 production of mucosal CD14+ macrophages. Here, IL23 caused the activation of pSTAT3 in CD4+ mucosal T cells, which results in resistance to apoptotic signals. The activated T cells are characterized by the release of high amounts of Th1 and Th17 cytokines. These TNFR2+IL23R+T cells expand and accumulate in the mucosa of anti-TNF-refractory CD patients, where they perpetuate chronic intestinal inflammation (28) (**Figure 2**). These data imply that anti-TNF resistant patients could benefit from therapies specifically targeting IL23.

THE ROLE OF IL23 IN THE DEVELOPMENT OF TH17 CELLS

CD4+ helper T cells are pivotal players in the pathogenesis of CD and, depending on the cytokine milieu, differentiate into regulatory and effector T cells i.e. Th1, Th2, Th17, follicular helper T cells (Tfh) and regulatory T-cells (Tregs). Until the discovery of other T cell lineages, Th1 and Th2 were longtime considered to be the only cells arising from progenitor CD4+ helper T cells (30). The Th1/Th2 paradigm offered a framework for understanding the pathogenesis of IBD and several other chronic inflammatory diseases. However, the distinguishing proof of Th17 cells has greatly extended the understanding of autoimmunity and inflammation and provided missing scientific links that could not be solely explained by Th1 and Th2 cells. Specific signal transduction mechanisms, several transcription factors and milieu specific cytokine patterns are responsible for the polarization of progenitor CD4+ helper cells (31). Distinct

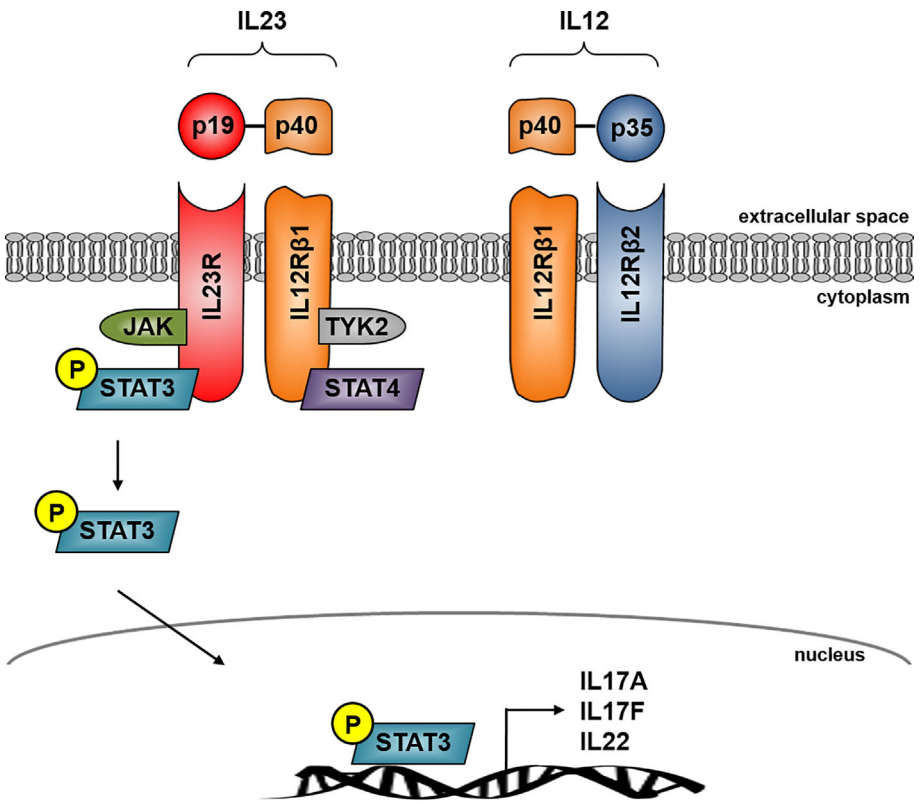

FIGURE 1 | IL23 signaling in Crohn's disease. IL23 is a heterodimer consisting of the unique subunits p19 and p40, the latter is shared with IL12. IL23 signals through its heterodimeric receptor complex consisting of the two subunits IL12Rβ1 and IL23R whereas IL23R is the unique subunit and IL-12Rβ1 shares the IL12 receptor complex. The IL23R complex signals through JAK kinase and STAT transcription factors. IL23 binding to its receptor activates Jak2 and Tyk2 kinases which then phosphorylates the receptor to form a docking site leading to the subsequent phosphorylation of STAT3 for the p19 subunit and STAT4 for the p40 subunit. IL23R signaling activates several pathways leading to transcription of several effector cytokine genes in CD including IL17A, IL17F and IL22.

from the development of Th1 and Th2 cell lineages, Th17 cell differentiation is prompted by the synergistic work of STAT3 and the transcription factor retinoid acid related-orphan nuclear receptor gamma (RORγt). The activation of RORγt causes the expression of IL17 and IL23 receptor (IL23R), leading to the production of IL23 by various immune cells, like dendritic cells or monocytes/macrophages, which in return increases the expression of RORγt and IL17 *via* STAT3 (32). The IL23R is absent on naïve CD4+ helper T cells leading to the idea that IL23 alone is not able to induce Th17 cell development. Indeed, it was shown that IL23 is especially important for maintenance and expansion of the Th17 lineage *via* a positive feedback loop that upregulates IL17, RORγt, TNF, IL1 and IL6. This positive feedback is centrally involved in the expansion of pathogenic pro inflammatory Th17 cells in CD (33–35) (**Figure 3**).

TH17 CELLS AND IL17 IN THE PATHOGENESIS OF CROHN'S DISEASE

The IL17 cytokine family consists of six ligands, IL17A to IL17F and is the key cytokine produced by Th17 cells. Besides IL17, Th17 cells also produce IL21, IL22, IFNγ and TNF (36). The discovery of the IL23/Th17 pathway paved the way for a better and deeper understanding of the pathogenesis of CD and the involved immune cells leading to the successful development of novel therapeutic substance classes targeting this specific pathway (37). Several studies revealed that IL17 producing cells mainly accumulate in the submucosa and muscularis propria of CD patients (38). Flow cytometric analysis of mucosal cells further demonstrated the increase of IL17 producing T cells in CD patients compared to controls. Interestingly, some of these cells also coexpressed IFNγ, a more Th1 related cytokine. Subsequent stimulation of these cells with IL12 elevated the expression of the Th1 related markers Tbet and IFNγ and decreased the Th17 related markers RORγT and IL17. These results clearly indicate that IL17 producing T cells from CD patients can be polarized from Th1 cells (39, 40). Animal models have also been used to evaluate the role of Th17 cells in the pathogenesis of IBD. Zhang and colleagues could demonstrate by using IL17RA knockout mice in a trinitrobenzenesulfonic (TNBS) induced colitis model that IL17 is essential for the development of colonic inflammation. Accordingly the application of the IL17RA IgG1 fusion protein in mice with TNBS-colitis significantly decreased colonic inflammation and protected the mice from weight loss (41). Studies in the dextran-sulfate sodium (DSS)-induced colitis

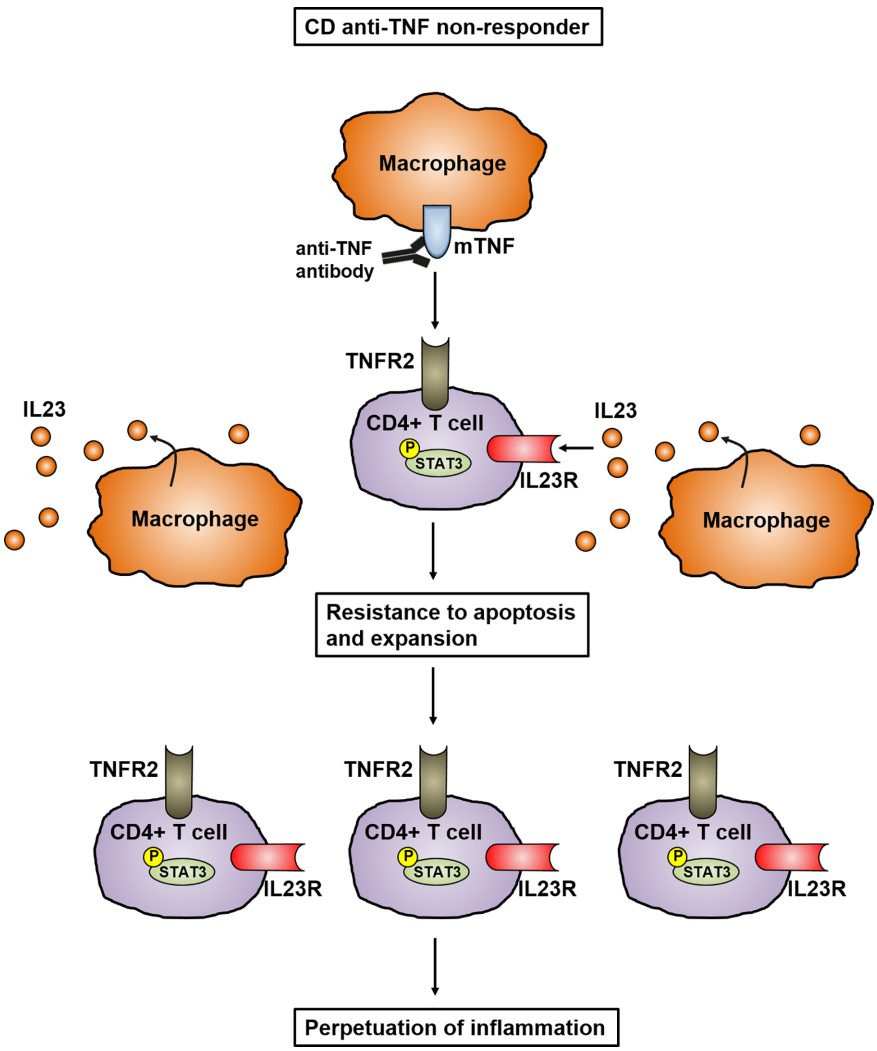

FIGURE 2 | Model of IL23 mediated resistance to apoptosis of mucosal CD4+ T cells in anti-TNF refractory Crohn's disease patients. In anti-TNF refractory patients, TNFR2 bearing gut CD4+ T cells express the IL23R. Heightened production of IL23 from CD14+ macrophages leads to binding to the IL23R on CD4+TNFR2+ T cells and induction of STAT3 activation. This activation leads to the expansion of CD4+IL23R+TNFR2+ T cells that are resistant to apoptosis induction by anti-TNF antibodies, resulting in the perpetuation of mucosal inflammation.

model revealed that IL17F deficiency leads to colitis reduction, whereas IL17A deficiency resulted in a more severe course of the disease (36, 42, 43). In line with this, a monoclonal antibody against IL17A (secukinumab) failed to show therapeutic efficacy in the treatment of CD, moreover a high rate of adverse events and increased severity of the disease compared to the placebo group was reported (43).

TH17 PLASTICITY AND ITS RELEVANCE IN CHRONIC INFLAMMATION

Polarized T cells have the ability to change their phenotype and repolarize towards various fates. This innate flexibility is termed plasticity (44). The plasticity of cells can be influenced by several factors like the cytokine environment, metabolites or different microbial components. The cytokine milieu drives T cell subset development and also induces plasticity through the activation of distinct and specific STAT molecules and multiple transcription factors like FOS-like antigen (Fosl2) or interferon regulatory factor (IRF4) (45, 46). The plasticity of Th1-Th17 has been reported to play an essential role in the regulation of intestinal immune responses (47). Several studies indicate that the development of IBD is associated with both Th1 and Th17 cells. The accumulation of Th1 and Th17 cells in the mucosa of IBD patients results in elevated IFNγ and IL17 levels compared to healthy controls. IFNγ+ IL-17+ co-expressing cells are considered to be Th17 cells that transform into Th1 lymphocyte progenitor cells, demonstrating the important role of Th17/Th1 plasticity in the pathogenesis of chronic intestinal

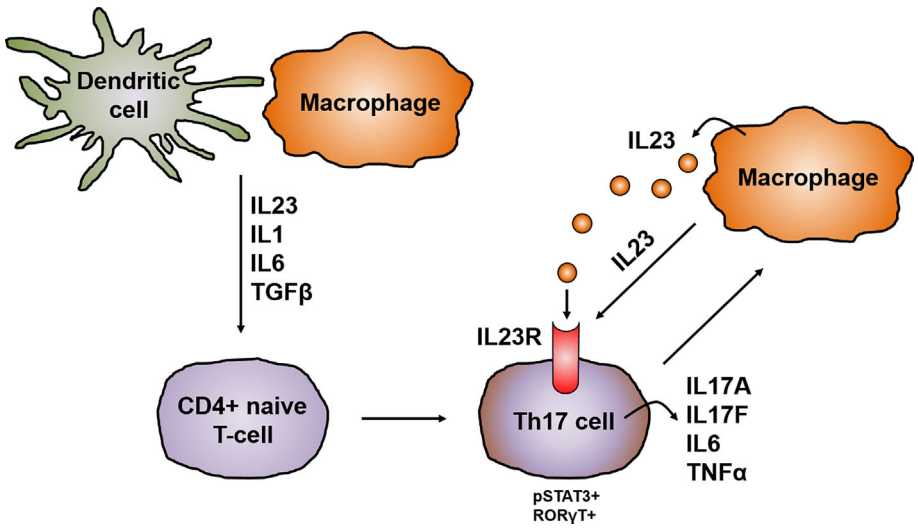

FIGURE 3 | IL23 in the development and activation of Th17 cells. In chronic inflammation, antigen-presenting cells like dendritic cells and macrophages are the main producers of IL23, which promotes together with other cytokines like IL1, IL6 and TGFβ the development of IL17 producing pathogenic Th17 cells. The differentiation of Th17 cells is prompted by the synergistically working of STAT3 and RORγt leading to the upregulation of the IL23R on Th17 cells and the release of other pro-inflammatory cytokines like IL17A, IL17F, IL6 or TNFα. This in turn leads to the production of IL23 mainly by macrophages. IL23 is on the one hand important for the maintenance and expansion of the Th17 lineage and in addition acts mainly on macrophages in an autocrine manner.

inflammation (48). IL23 signaling can drive the conversion of Th17 to Th1 cells by shifting the secretion of IL17A to IFNγ *in vivo* (49). Here, IL23 may suppress IL17 expression and enhance IFNγ release through a STAT4/T-bet-dependent pathway, particularly under conditions of decreased TGFβ expression, a sustained inducer of IL17A and IL17F (50). Moreover, a murine model with CD4+ T cells lacking the IL23R has revealed that IL23R signaling induces colitis, associated with the induction of IFNγ and IL17A co-expressing cells (51). Interestingly it was also shown that Th17 derived Th1 cells express CD161, which is a surface marker on Th17 cell progenitors (52). Studies have also demonstrated that IFNγ+ IL17+ coexpressing T cells from CD patients express the IL23R and therefore are centrally involved in the pathogenesis of CD (28). Therapies targeting IL17, IFNγ or IL23 might therefore also have an influence on Th17-dervied Th1 cells. The above described research findings demonstrate that Th17/Th1 cells play a pivotal role in the development and pathogenesis of IBD.

IL23 AND IL17 RESPONSIVE CELLS

Different studies have demonstrated that the interaction with IL23 and its receptor mainly leads to phosphorylation of STAT3, building up a positive feedback loop that triggers gene expression important for Th17 cell activation and effector functions (53). IL23 is essential for the maturation and expansion of Th17 cells in humans and mice and is indispensable for their initial differentiation from naive CD4+ T cells to fully pathogenic Th17 cells (54). These Th17 cells massively infiltrate the inflamed intestine of CD patients, where they produce pro-inflammatory cytokines like IL17 and thereby perpetuating the inflammatory process (55). Besides Th17 cells, a variety of innate immune cells respond to IL23, including subsets of γδ T cells, natural killer T (NKT) cells, intrathymically primed "natural" Th17 cells and innate lymphoid cells (ILC) (54). These innate immune cell subsets are collectively referred to as "type 17 cells" and are located in non-lymphoid organs where they are able to respond immediately to tissue damage or pathogen invasion. Stimulation of Th17 cells and type 17 cells with IL1β and IL23 induces local tissue inflammation, characterized by type 17 signature cytokines such as IL17, IL22 and GM-CSF (56). Furthermore, it was shown that IL23 is able to induce IL17 expression in RAG – deficient mice (lacking B and T cells), demonstrating that innate IL17 producing cells are an integral part in IL17 based immune responses (57). Several publications indicate that these IL23 dependent innate IL17 producing cells are mainly found in the skin and mucous membranes where they play a central role in homeostasis (58–60).

ILC3 cells express the transcription factor RORγt and are important players in protecting against extracellular pathogens in the gastrointestinal mucosa. IL23 responsive ILCs are located in human mucosa-associated lymphoid tissue, for example the intestinal Peyer's patches (59). ILC3 cells are considered to be responsible for gastrointestinal mucosal homeostasis in the physiological state through moderate production of IL22, IL17, and GMCSF. A dysregulation of ILC3 cells cause the overexpression of the inflammatory cytokines IL22 and IL17. Subsequently, neutrophils are recruited and cleave epithelial cadherin and junctional adhesion molecule-like molecules (JAMLs), resulting in elevated epithelial permeability (61). Moreover, these cells have also been linked to the pathogenesis of IBD, as they express the IL23R, leading to overproduction of several effector cytokines like IL12, IFNγ and IL17 by these cells

in an IL23 depending manner (62, 63). In line with these data, Geremia and colleagues could demonstrate that IL23 responsive ILCs accumulate in the mucosa of CD patients where they produce inflammatory cytokines leading to intestinal inflammation (23).

γδ T cells are mainly found in mucosal and skin surfaces, more precisely in the intestinal intraepithelial compartment, and also show a broad expression of IL23R. They play a central role in the mucosal barrier due to their expression of pattern recognition receptors (PRRs) such as CLEC7A or TLR2 (64). Since peripheral γδ T cells are capable of recognizing both self- and non-self-ligands, it is assumed that these cells can be separated into two main categories of "antigen-experienced" and "antigen-naïve" γδ T cells (65). Recently, studies have demonstrated that γδ T cells are key innate IL17-producing cells in autoimmune inflammation and infectious diseases (66, 67). After stimulation with IL23, γδ T cells start to secrete IL22, IL21 and IL17. Their role in the pathogenesis of CD is not fully understood but studies in several mouse colitis models suggest an important role of γδ T cells in this context and also in other chronic inflammatory diseases (68).

CD1d- expressing NKT cells are mainly found in the human intestine where they recognize lipids from commensal microbes. Based on their T cell receptor (TCR) characteristics, NKT cells are stratified into two main subsets, type I and type II NKT cells (69). They are centrally involved in the regulation of intestinal homeostasis and inflammation (70). After stimulation with IL23, NKT cells produce large amounts of IL22 and IL17. Several murine colitis models have indicated that the contribution of NKT cells can be protective or pathogenic. Here, the kind of inflammatory stimuli and lipid antigens play a crucial role in determining the immune response (69). Various clinical studies indicated reduced levels of type I NKT cells in the intestine and peripheral blood of CD and UC patients (71, 72). In contrast, another study revealed an accumulation of type II NKT cells in the lamina propria of UC patients (73).

Thus, the discovery of the IL23/IL17 pathway has led to fundamental changes in our understanding of cellular immunity and essentially contributed to the development of clinical trials and therapeutic strategies targeting the IL23/IL17 pathway in CD.

THE IMPACT OF IL23R POLYMORPHISM ON TH17 CELL FUNCTION

GWAS studies have revealed more than 200 risk variants associated with IBD, most of them affect CD and UC. The majority of disease-related single nucleotide polymorphisms (SNPs) occur in non-coding regions of the genome (74, 75). Interestingly, the variants in the IL23R are protein-coding and are therefore an exception in contrast to the large portion of non-coding risk variants. In 2006, a study by Duerr and colleagues revealed a link between variants of the IL23R gene on chromosome 1p31 and ileal Crohn's disease (24). Especially the coding variant R381Q has been linked with functional consequences to T cell immunity. CD patients carrying the protective variant of the IL23R produce reduced levels of IL17 and IL22 after IL23 stimulation, resulting in lower frequencies of circulating Th17 cells (76). It could further be shown that T cells from these patients display a diminished IL23 mediated phosphorylation of STAT3 and release less IL17 after exposure to *Borrelia burgdorferi*, a strong inducer of Th17 responses (77). A case-control study with 201 CD patients demonstrated that the development of CD is associated with the IL23R variant G149R (78). In contrast, further studies noted by using a candidate gene approach that SNPs in IL23R leads to high activation of the IL23/IL17 pathway, which was also linked with increased risk for CD and UC (77). These insights in the gene polymorphism of IL23R also affects the strategy of treatment. It was shown that IL23R genotype status determine early response to infliximab (79). Taken together, the recent years of research suggest that disease protective variants of the IL23R are more associated with reduced IL23R activity, whereas disease associated variants are more linked to elevated IL23R signaling.

THERAPEUTIC APPROACHES TARGETING IL23 AND IL17 SIGNALING

The recent finding of the critical role of IL23 and IL17 in the pathogenesis of IBD and other immune-mediated diseases has led to the development of new therapeutic approaches targeting these cytokines and corresponding receptors (56, 80, 81). First studies were conducted with anti-p40 antibodies (the shared subunit of IL23 and IL12) such as briakinumab (82) or ustekinumab (83). In another study, ustekinumab treated CD patients with a moderate to severe disease course displayed an increased rate of response and remission to ustekinumab induction and maintenance treatment compared to the placebo treated group (12, 84). Anti-TNF treated CD patients with severe psoriasisform lesions and dermal Th17 cell infiltrates were additionally treated with ustekinumab, leading to a remarkable suppression of skin lesions (85). The promising results of ustekinumab treatment emphasizes the important role of the interaction of IL23/IL23R and IL17/IL17R in the pathogenesis of CD. The blockade of the selective IL23p19 subunit (which is not shared with IL12) allows normal Th1 responses that are mediated by IL12. In contrast to directly antagonizing IL17 function, an IL23 blocking antibody should inhibit the IL23 dependent development and proliferation of pathogenic Th17 cells, which subsequently leads to the reduction of pro-inflammatory cytokines associated with this cell type, such as IL17, IL21 and IL22. Based on the clinical efficacy of IL23 specific inhibitors in psoriasis, more recent studies evaluated the effects of IL23p19 blockade in CD. Risankizumab is a humanized monoclonal antibody targeting the p19 subunit. In a phase 2 trial, 121 patients with active CD were randomized to receive different doses of risankizumab or placebo. After 12 weeks, a significantly higher proportion of patients, which were treated with 600mg risankizumab, achieved clinical remission in comparison to the placebo group. Analysis of mucosal samples

revealed that risankizumab treatment leads to the suppression of various genes linked to the IL23/IL17 axis (13, 86, 87). The treatment of risankizumab also leads to the maintenance of remission at week 26 in treated CD patients (86). Brazikumab, another p19 blocker, is a fully human IgG2 IL23 antibody and was tested in a phase 2 study with active CD patients that failed previous anti-TNF therapy (14). In this study, clinical improvement of CD patients 8 and 24 weeks after initiation of brakizumab therapy could be achieved in comparison to the placebo treated group. In addition, patients receiving brazikumab had greater reductions in serum IL22 levels than placebo treated patients, again emphasizing the importance of the IL23/IL17 axis in the pathogenesis of CD (14). Here, patients with elevated baseline IL22 serum levels had a higher probability of achieving clinical remission upon brazikumab treatment.

Further late-stage clinical studies targeting p19 are currently being conducted (e.g. with the p19 neutralizing antibodies risankizumab (13), brazikumab (14), mirikizumab (88) or guselkumab (89)). The p19 antibody tildrakizumab has not yet been tested in CD patients, but has proven therapeutic efficacy in phase 3 trial in psoriasis patients (90). The oral peptide PTG-200 that selectively antagonizes the IL23R was well tolerated in a phase 1 trial in healthy volunteers (91) and will be tested in CD phase 2 trials. A summary of the pharmaceutical compounds can be found in **Table 1**.

As mentioned above, blocking IL17 signaling directly in CD patients might also influence the Th1 immune response, including microbial defense. Two different strategies blocking IL17 in CD patients with moderate to severe CD have been evaluated. Secukinumab directly targets IL17A whereas brodalumab blocks the IL17R subunit IL17RA. Secukinumab therapy did not meet the primary endpoint but rather led to worsening of disease and furthermore a heightened incidence of severe adverse such as fungal infections were reported compared to the placebo treated group (43). Similarly, brodalumab treatment in CD was prematurely stopped as numerical worsening of CD in the antibody treated group was found (92) Interestingly, both antibodies show high efficacy in the treatment of psoriasis (93–95) (**Figure 4**).

In contrast to IL23, different murine models of colitis suggest a protective role for IL17A. It was shown that the neutralization of IL17A in a dextran sodium sulfate (DSS) murine colitis model resulted in elevated tissue damage (96) and T cells, lacking IL17A or IL17R, transferred into RAG-1 deficient mice, led to increased severity of the colitis course (97). Interestingly, it was further demonstrated that IL17A is able to promote epithelial barrier function by regulating proteins like occluding, which is an important tight junction protein. This protection leads to less excessive gut permeability after epithelial injury in a colitis mouse model (98). In this study, colonic IL23R+ γδ T cells were the main producers of gut-protective IL17A. Moreover, the protective effect of IL17 was also present in the absence of IL23, indicating an IL23 independent release of protective IL17A from IL23R+ γδ T in this context (98). While several studies clearly could not demonstrate any efficacy for neutralizing IL17A or IL17RA in CD, the current understanding of the mechanism of IL17 mediated protective effects in both mouse and man is still elusive.

JANUS KINASE (JAK) INHIBITORS IN IBD

Most pathways that are involved in IBD are characterized by the massive production of pro-inflammatory cytokines by different immune cells, leading to the inflammation of the mucosa or the disruption of the intestinal barrier (99). JAKs are cytoplasmic tyrosine kinases that transform extracellular processes into various intracellular immune and inflammatory processes (100). One central role of cytokines is the contribution to transcellular signaling by activating the JAK signal transducer and activator of transcription JAK/STAT pathway (101). The IL23 signaling pathway includes the activation of members of the JAK family of tyrosine kinases and the several downstream transcription factors of the STAT family. IL23R signaling is linked to Jak2 and Tyk2 leading to the phosphorylation of STAT3 (**Figure 1**).

JAK inhibitors influence several inflammatory pathways and are therefore a promising target for inflammatory diseases like IBD. However, blocking JAKs in CD or UC patients showed contradictory results (102).

Tofacinitib is a pan-JAK inhibitor that demonstrated efficacy in patients with moderate to severe UC (103). In contrast, Tofacitinib has not reached the primary endpoint in CD patients leading to the discontinuation of clinical trials for the treatment of CD patients with Tofacitinib (104, 105).

TABLE 1 | Targeted therapies directed against IL12, IL17, IL23 or their respective receptors.

Drug	Route	Target	Current stage of development
Ustekinumab	IV/SC	p40	Approved for induction and maintenance therapy (12)
Risankizumab	IV/SC	p19	Phase 2 study (13)
Brazikumab	IV/SC	p19	Phase 2a study (14)
Mirikizumab	IV/SC	p19	Phase 2 study (88)
Guselkumab	SC	p19	Phase 2 study (89)
Briakinumab	IV/SC	p19	Phase 2b study (82); did not meet primary endpoint, halted development
Tildrakizumab	SC	p19	No Crohn's disease data
PTG-200	Oral	IL23R	Phase 1 study (91)
Secukinumab	IV	IL17	Phase 2a study (43); worsening of disease, halted development
Brodalumab	IV	IL17R	Phase 2a study (92); worsening of disease, halted development

IV = intravenous; SC = subcutaneous.

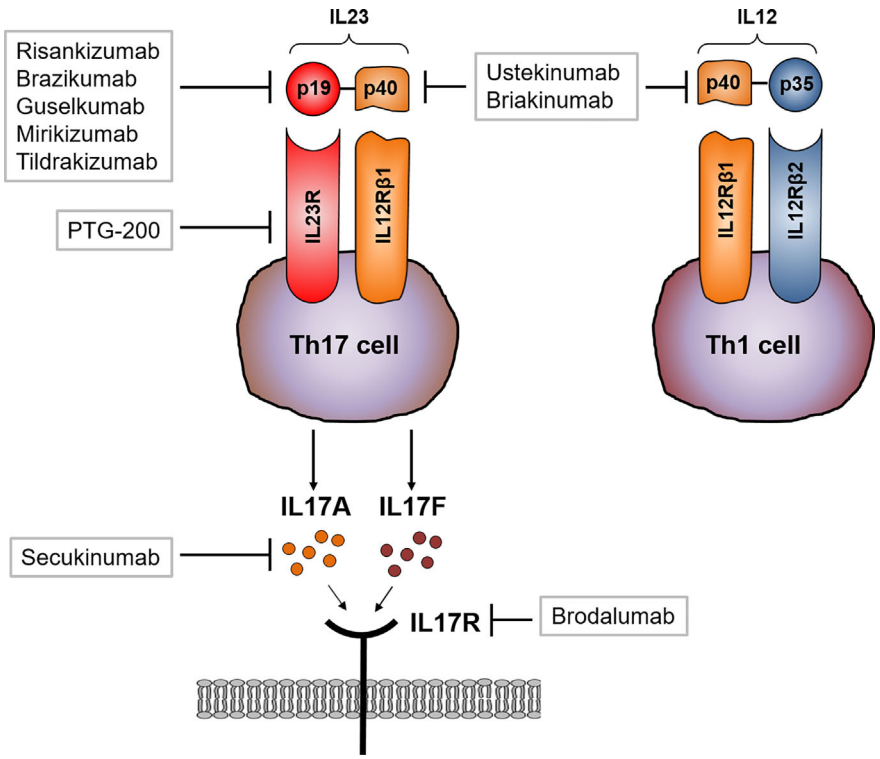

FIGURE 4 | Therapeutic approaches targeting IL23 and IL17 signaling. Ustekinumab and briakinumab specifically blocks the IL12/IL23 subunit p40 in CD patients whereas risankizumab, brazikumab, guselkumab and mirikizumab selectively block the unique subunit p19. Activated Th17 cells produce large amounts of IL17. Secukinumab directly binds to IL17A and thereby inhibits the interaction with the IL17 receptor (IL17R). Brodalumab directly binds to the IL17R causing an inhibition of IL17 ligand binding (A and F) to their receptor.

Filgotinib has a 28-fold more selectivity for JAK1 compared with JAK2 and is therefore regarded as a JAK1 inhibitor (106). The efficacy of Filgotinib for the induction of remission in moderate to severe CD patients was evaluated in the randomized, placebo-controlled, multicenter phase II study (107) and showed promising efficacy data.

Upadacitinib is an oral JAK1 selective inhibitor with a 74-fold more selectivity for JAK1 over JAK2. The efficacy of Upadacitinib for the induction and maintenance of remission in moderate to severe CD patients was studied in a randomized, placebo-controlled multicenter phase II trial (108) and similarly demonstrated convincing signs of effectiveness. Subsequent studies will have to clarify whether more specific JAK inhibition is able to achieve high efficacy, while providing a convincing safety profile.

Altogether, JAK inhibitors represent an attractive therapeutic category of molecules for targeting IL23 downstream. Therefore, JAK inhibitors may represent an effective treatment for IBD, although potential benefits in efficacy and safety for CD need further evaluation.

CONCLUSION

The discovery of the IL23/IL17 axis has changed our fundamental understanding of the pathology of chronic inflammatory diseases like CD and described a new way of how immune responses can trigger intestinal tissue damage. Until the discovery of other T cell lineages, Th1 and Th2 were longtime considered to be the only cells arising from progenitor CD4+ helper T cells. It was shown that IL23 is especially important for maintenance and expansion of the Th17 lineage *via* a positive feedback loop that upregulates IL17, RORγt, TNF, IL1 and IL6. This positive feedback is centrally involved in the expansion of pathogenic pro inflammatory Th17 cells in CD. GWAS have analyzed the polymorphisms in the gene encoding IL23R and linked it to the pathogenesis of IBD, indicating the important role of the IL23/IL17 axis in mucosal inflammation.

The current availability of the specific anti-p40 antibody ustekinumab and the expected arrival of specific anti-p19 antibodies broaden our therapeutic armamentarium in the treatment of Crohn's disease, but inevitably leads us to the questions which patients would likely benefit the most from these compounds. Clinical trial results have indicated that prior exposure to anti-TNF therapy seems to be associated with lesser probability of responding to subsequent ustekinumab therapy in comparison to anti-TNF naïve patients (12). We still await data regarding respective effectiveness of p19 inhibitors in primarily anti-TNF naïve patients, but upregulation of the IL-23R on mucosal T cells of anti-TNF non-responders, rendering these cells more responsive to increased IL23p19 production from

CD14+ mucosal macrophages, indicate the potential for anti-IL23p19-specific therapies in anti-TNF non-responders (28, 109). Recent studies have shown that the mucosal cytokine profiles shift during the course of disease (110). It could be shown that early mucosal inflammation before endoscopic recurrence showed an abundance of Th1-related cytokines and TNF and slightly increased IL17A expression in the terminal ileum. Transition from this stage to endoscopic recurrence was marked by high levels of Th1 cytokines, marked increase in IL17A, and induction of IL6 and IL23, while established lesions were characterized by a mixed Th1–Th17 profile with low levels of TNF (111). Furthermore, IL12p40 and Th1 cytokines demonstrated higher mucosal expression in recently diagnosed pediatric in comparison to patients with long-standing Crohn's disease (112). These data might indicate that anti-p40 blockade might be particularly effective in early disease, while p19 inhibition might rather be positioned in the treatment of more established lesions. Currently conducted head-to-head trials of ustekinumab and p19 inhibitors might help us to determine the optimal place of these substances in our treatment algorithm (113). Clinical practice will also answer the important question whether patients will still benefit from anti-IL23p19 antagonism if they have previously failed to benefit from anti-IL12p40 antibody therapy, and vice versa (114). Even with the upcoming availability of p19 inhibitors in addition to the already available anti-p40 antibody, there is still the currently unmet clinical need to establish predictive markers of response to identify the subgroup of IBD patients that have a heightened probability of response to respective treatments (115). In IL23p19 inhibitors, there has so far been only a report that indicated that higher serum IL22 concentrations were associated with a greater likelihood of response to brazikumab (14). These findings must however be validated in subsequent studies and other p19 inhibitors as well before they are able to enter daily clinical practice. Only improved understanding of the mucosal immune milieu and the development of biomarkers will enable us to develop personalized approaches to treatment and future algorithms for biological therapy in these patients.

AUTHOR CONTRIBUTIONS

HS wrote the manuscript. MN and RA assessed the articles and their relevance to the above topics. RA supervised and drafted the manuscript and is the corresponding author. All authors contributed to the article and approved the submitted version.

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The IL23-IL17 Immune Axis in the Treatment of Ulcerative Colitis: Successes, Defeats, and Ongoing Challenges

Daniele Noviello[1†], *Riccardo Mager*[2†], *Giulia Roda*[2,3], *Riccardo G. Borroni*[3,5], *Gionata Fiorino*[2,3] *and Stefania Vetrano*[2,4*]

[1] Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy, [2] Inflammatory Bowel Disease (IBD) Center, Laboratory of Gastrointestinal Immunopathology, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy, [3] Inflammatory Bowel Disease (IBD) Center, Department of Gastroenterology, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy, [4] Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Italy, [5] Dermatology, Humanitas Clinical and Research Center-IRCCS, Rozzano, Italy

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***Correspondence:**
Stefania Vetrano
stefania.vetrano@hunimed.eu;
Stefania.vetrano@humanitasresearch.it

[†]These authors have contributed equally to this work

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Ulcerative colitis (UC) is a chronic relapsing disorder of the colonic tract, characterized by a dysregulated innate and adaptive immune response to gut microbiota that contributes to the perpetuation of intestinal inflammatory processes. The Interleukin (IL) 23/IL17 axis has been reported to play a key role in UC pathogenesis promoting Th17 cells and cytokines-related immune response. Recently, the blockade of IL23/IL17 pathways has been raised enormous interest in the treatment o several chronic inflammatory disorders. In this review, we summarize the emerging results from clinical trials that evoked both promise and discouragement in IL23/IL17 axis in the treatment of UC. Targeting IL23 p40 through Ustekinumab results safe and effective to induce and maintain clinical remission, low inflammatory indexes, mucosal healing, and a better quality of life. Studies targeting IL23 p19 through Mirikizumab, Risankizumab, Brazikumab and Guselkumab are still ongoing. To date, no clinical studies targeting IL17 pathway are ongoing in UC. IL-17 targeting is thought to have a context-dependent biological effect, based on whether cytokine is selectively targeted or if its function is dampened by the upstream block of IL23.

Keywords: ulcerative colitis, IBD, IL23, IL17, ustekinumab, mirikizumab, rinsankizumab

INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing inflammatory bowel disease (IBD), involving the rectum and a variable extent of the colon (1). It affects mainly young subjects (2^{nd}-4^{th} decade of life, mainly), presenting with bloody diarrhoea (1, 2). Aminosalicylates and low-bioavailability corticosteroids are the main choice of treatment for mild to moderate disease, while systemic corticosteroids, immunosuppressants, monoclonal antibodies and small molecules are used in moderate to severe UC (3). Despite the fact that these agents have been proven to induce and maintain clinical and endoscopic remission (4, 5), the majority of patients lose response over time (5–8) and colectomy is needed in up to 15% of patients (9). Moreover, UC is a progressive and

disabling disease with long term complications and often these agents fail to modify the course of the disease (10, 11).

Although still not completely clear, scientific evidences support a multifactorial pathogenesis characterized by a dysregulated immune response to gut microbiota which leads to progressive destructive damage and defective repair of the gastrointestinal tract (3). Immune cells, in fact, have primarily been explored as therapeutic targets to resolving their aberrant function in these patients. An excessive Th2 immune response with increased amounts of Interleukin (IL)13 and IL5 (12) is considered as a hallmark of UC. However, more recently, IL17-producing T cells, an independent lineage from Th1 or Th2 cells capable of promoting immune-mediated inflammatory responses in various immunological disorders (13–16), have been identified as new players in UC pathogenesis (16). The evidence that IL23 amplifies Th17 cell responses has opened new avenues to explore IL23/IL17 axis as promising therapeutic targets in IBD.

Several mouse models of colitis have shown an enhanced production of IL23 (17–20) and IL17 (21–23). Accordingly, all observational studies, despite the heterogeneity of the sample size (12 to 102 patients) and of the disease severity (active or remission), confirmed high levels of IL23 and IL17A in the serum of UC patients (24–30). Increased levels of IL23 correlated with disease severity in 40 UC patients (24). Nevertheless, data regarding both IL23 and IL17 expression in the inflamed mucosa remain controversial, probably due to the inhomogeneity in terms of sample size, clinical and endosopical activity and location of the biopsy (25, 29, 31). Moreover, the blockade of IL23 and to a certain extent IL17 effectively suppressed gut inflammation in various mouse models of colitis (18, 21, 32–37). These evidences supported the involvement of IL23 and IL17 in UC pathogenesis, therefore strategies aiming at their suppression have been posed as promising strategy for the treatment of UC.

In this review, we summarize briefly the IL23/IL17 pathway focusing on the emerging results from clinical trials that have evoked both promise and discouragement in IL23/IL17 axis in the treatment of UC.

IL23/IL17 PATHWAYS

IL23 is a heterodimeric cytokine of the IL12 family composed of a specific p19 subunit and a shared p40 subunit (38, 39). Mainly produced by monocytes, macrophages, activated dendritic upon Toll-like receptor signaling (40), IL23 is capable to induce a strong proinflammatory effect through the activation of various target cells, beyond the aforementioned Th17 cells (41). IL23 binds to a heterodimeric receptor complex, IL23R, composed of the β1 subunit of IL12 (IL12Rβ1) and an IL23 specific subunit, (IL23Rα). Selected single nucleotide polymorphisms (SNPs) on IL23R gene have been associated to increased risk for the development of UC (42, 43), and to influence the phenotype of the disease (44). Interestingly, stratification by ethnicity revealed that some SNPs were high associated with UC in the Caucasian population, but not in Asians (45, 46), as if the mutation in IL23R increased predispositions for developing UC in certain geographic area.

Upon IL23 stimulation, the receptor activates the Jak-Stat signaling cascade promoting production of proinflammatory cytokines. Jak kinase 2 and tyrosine kinase 2 become activated and trigger the translocation of STAT3-STAT4 dimer to the nucleus, where in turn activates gene expression (39). IL23R is expressed on T cells, innate lymphoid cells, intraepithelial lymphocytes, natural killer cells, intestinal epithelial cells and granulocytes (41, 47). IL23-activated Th17 cells produce a variety of cytokines, including tumor necrosis factor-α (TNFα), INFγ, IL6, IL17A, IL17F, IL21 and IL22 (48, 49).

IL17 is a cytokine family which comprises six proteins, IL17A to IL17F. **IL17A,** broadly distributed and produced by several cell types, is a potent pro-inflammatory cytokine that amplifies inflammatory response by sustaining the release of others inflammatory mediators such as TNF-α, IL-6 (16) and by inducing neutrophil-related genes like CXC-chemokine ligand 1 (CXCL1), CXCL2 and CXCL5 involved in the inflammatory processes (50). Activated neutrophils may prompt a positive feedback producing IL17A and IL22, as a result (51). Conversely, IL17A promotes antimicrobial or epithelial barrier genes like regenerating (REG) proteins, S100 proteins, lipocalin 2, lactoferrin, β-defensins, and claudin, zona occludens 1 (52, 53). **IL17F** shares almost half the structure of IL17A (54) and its effect on pro-inflammatory genes (CXCL1, IL6, CCL2, CCL7, and Matrix Metalloproteinases 13) with a less extent (23). **IL17B**, **IL17C** and **IL17D** are expressed mainly on epithelial cells and exert pro-inflammatory functions *in vitro*, but their exact biological roles have not yet been fully elucidated (55–57). Finally, **IL17E**, also known as IL25, is involved in Th2 cell responses against parasites (58).

INTERPLAY BETWEEN IL23/IL17 AXIS AND GUT MICROBIOTA

The intestinal microbiota, in addition to having an enormous influence on nutrition, metabolism and physiology of the host, is also widely accepted as an immunomodulator of the development and maintenance of a healthy host immune system. The gut microbiota, in fact, has a pivotal role in the generation and functional training of innate and adaptive of immune cells, including Th17 cells, the most abundant CD4 T cells in mucosal tissues (59, 60). Accordigly, adult germ-free mice have fewer Th17 cells and smaller Peyer's patches in their small intestine (61) confirming the crucial role of gut microbiota in the development of immune system. The transcription factor RORγt has been described to be important for Th17 differentiation by regulating the expression of Th17 genes and for the IL23/IL17 axis, key regulatory cells of the intestinal mucosal firewall, which provides a functional barrier of defense against microbial and dietary antigens by the presence of a mucus layer; the integrity of epithelial cells; and the release of antimirobial peptides and immunoglobulin A (62). Changes in the composition of

microbial communities referred as dysbiosis can dictate intestinal immune response triggering immune diseases (63). Both commensal bacteria and pathogens can induce IL23 production by activated dendritic cells (64, 65). Recently, Martínez-López M et al. showed that the Mincle-Syk signaling axis is involved in the sensing of mucosal-associated bacteria through dendritic cells, which induce IL-6 and IL-23 first and then IL-17 and IL-22 production (66). Consequently, the absence of a functional Mincle-Syk axis is associated with impaired intestinal immune barrier function (66). Similarly, *segmented filamentous bacteria* (*SFBs*), *Cytophaga-Flavobacter-Bacteroidetes* are responsible for Th17 induction in the gut of adult mice (59, 67). Although the underlying mechanism is not well known, *SFB* overgrowth in mice with RORγt, IL-17 or IL-17R depletion has been found (68, 69). However, further studies are needed to address the active interplay between human IL23/IL17 and gut microbiota, for which limited studies are still available.

TARGETING IL23

Anti-IL12/IL23 p40

Recently, **ustekinumab** (UST, Janssen-Cilag), a fully human IgG1κ monoclonal antibody against the shared p40 subunit of IL-12 and IL23 has been recently approved by EMA and FDA for treating of moderate to severe active UC who have had an inadequate response with, lost response to, or were intolerant to either conventional therapy or a biologic or have medical contraindications to such therapies. UST efficacy and safety has been investigated in a phase 3 trial (UNIFI) among 523 patients with moderate to severe active UC. Intravenous (IV) UST was more effective than placebo (15.6% vs 5.3%) for inducing clinical remission in patients at week 8. Subcutaneous (SC) UST q12w or q8w was more effective than placebo (38.4% or 43.8% vs 24%) for maintaining clinical remission in responders at induction at week 44. No significant differences were observed in patients with or without previous treatment failure with biologics (70). Among 116 delayed responders (pts achieving clinical response at week 16 continuing UST 90mg SC q8w) 74.1% were in clinical remission at week 44 and increased to 79.3% at week 92, among them 94.6% were corticosteroid free (71).

Very recently, further results from additional analysis on UNIFI data have showed its efficacy beyond clinical remission. Dose adjustment, based on the clinical judgement of disease activity, from UST q12w to q8w increased clinical remission rates (72). Reductions in stool frequency and rectal bleeding achieved after induction have been reported through 2 years of UST SC maintenance (73). Patients with mucosal healing, defined as Mayo endoscopy subscore ≤1 and histological improvement based on the Geboes score, after induction had significantly lower disease activity than those without at week 44, retained through week 92. A trend for lower inflammation measured by CRP and faecal calprotectin was also reported (74). Patients health-related quality of life (HRQoL), assessed using The Inflammatory Bowel Disease Questionnaire (IBDQ), and the The Short Form (36) Health Survey (SF-36), improved in most patients after UST induction therapy and was retained through week 92. 55.6% of patients were in IBDQ remission at week 92, 67.5% of them already in remission at maintenance baseline, and improvement in SF-36 (≥ 5 points) was achieved in half the patients (75). A pharmaeconomics analysis revealed that UST treatment in moderate to severe UC is cost effective *vs.* placebo over 1 year (76).

Real-world studies on UST efficacy are in progress. In the ENEIDA registry, among 47 patients previously exposed to biologics (>70% to >2), clinical response was achieved in 36% at week 8 (77). In the GETAID cohort, among 103 patients, most of them already exposed to anti-TNF and vedolizumab drugs, UST was effective in inducing steroid-free clinical remission and clinical remission in 35.0% and 39.8% respectively, at weeks 12–16. The endoscopic activity, assessed using the Ulcerative Colitis Endoscopic Index of Severity (UCEIS), showed a significant improvement from baseline, 3.8 ± 1.9 *vs.* 5.0 ± 1.2 (78). In two tertiary IBD centers in the US, among 66 patients almost all exposed to biologics or tofacitinib UST was effective in inducing clinical remission in 45% and 33% endoscopic and histologic remission at 1 year (79). Prior immunogenicity to anti-TNF did not confer a significantly risk of immunogenicity to UST in a cohort of 152 IBD patients, as the majority of real-worlds patients have likely failed anti-TNF biologics (80). Among 400 patients who received continuous UST in the induction, maintenance and LTE UNIFI trial, 22 (5.5%) patients developed antibodies to UST that were often transient and did not appear to affect efficacy or advers effects (81). Conversely, a smaller study found a strong association between antibodies to UST and clinical remission (82). Few case reports described the efficacious use of IV UST alone or in combination with cyclosporine as rescue therapy for acute severe UC (83, 84).

Phase 4 trials and observational studies are ongoing. BioIBD (NCT03885713) and i-BANK (NCT03809728) aim to identify predictive and prognostic biomarkers of natural history and response to biotherapies, including UST. VERDICT (NCT04259138) aims to define the optimal treatment target among corticosteroid-free symptomatic remission, or plus endoscopic remission, or plus histological remission. HARIR (NCT03006198) aims to explore the disease characteristics, treatment and outcomes in the emerging regions of North Africa, the Middle East, and Western Asia.

Because IL23 involvement and not IL12 seems pivotal in UC pathogenesis, a more selective generation of antibodies towards IL23 p19 is under investigation: mirikizumab, risankizumab, brazikumab and guselkumab.

Anti-IL23 p19

Mirikizumab (LY3074828, Eli Lilly) is a humanized immunoglobulin G4–variant monoclonal antibody against the p19 subunit of IL23. In the phase 2 trial, mirikizumab did not achieve the primary endpoint, namely clinical remission at week 12, but it was more effective than placebo (59.7% *vs.* 20.6%) in inducing a clinical response with the 200-mg dose group showing the largest benefit. However, significant improvement in stool frequency and rectal bleeding were observed within week 2 and continued through week 52 (85). In the maintenance

study, SC mirikizumab q4w in responders at induction increased clinical response and remission rates up to 80.9% and 46.8% at week 52, respectively (86, 87). Further analysis of the phase 2 trial data showed that endoscopic improvement and histologic remission were achieved respectively in up to 30.6% and 45.2% at the end of the induction phase and increased to 42.6% and 66.0% at week 52 (86, 87). Those results are consistent with significant improvements in patients HRQoL, assessed using the SF-36 v2, after 12 weeks of induction and sustained during the maintenance treatment (88). Absence of urgency is associated with improved clinical, endoscopic, histologic outcomes and better QoL assessed through the IBDQ (89, 90). In addition to the standard outcomes, additional exploratory biomarkers have been studied. IL17A and IL-22 plasma concentrations were reduced in clinical responders by 113.5%/57.4% at week 12 and further reductions were observed at week 52, leading to normal or near normal circulating levels (91). The genetic expression of biological pathways UC-specific and involved in resistance to anti-TNF showed a significant modulation in the inflamed tissue of UC treated with mirikuzumab for 12 weeks (92). Specifically, the different expression of genes involved in cell adhesion and leukocyte trafficking from UC inflamed tissue correlate better with histopatology than endoscopy and Mayo score (93).

Several phase 3 trials are ongoing. LUCENT 1 (NCT03518086) and LUCENT 2 (NCT03524092) are randomized, double-blind, placebo-controlled studies for induction and maintenance treatment, respectively, in patients with moderate to severe UC. LUCENT 3 (NCT03519945) is the long-term open-label extension program (NCT03519945). LUCENT-ACT (NCT04469062) is a randomized, double-blind, parallel-arm, placebo- and active-controlled treat-through study that aims to evaluate mirikizumab efficacy and safety compared to vedolizumab and placebo. SHINE 1 (NCT04004611) is a phase 2 Multicenter, Open-Label trial in Children and Teenagers (2 to 17 years).

Risankizumab (BI655066/ABBV066, AbbVie) is a humanized monoclonal antibody against the p19 subunit of IL23. To date, no results are available about its efficacy and safety in UC.

A Phase 2/3 randomized, double-blind, placebo-controlled trial for induction treatment (NCT03398148) and a Phase 3 randomized, double-blind, placebo-controlled trial for maintenance treatment (NCT03398135) are ongoing in moderate to severe UC. A phase 1 (NCT04254783) aims to evaluate the effect of IV infusions on pharmacokinetics of cytocrome p450 substrate.

Brazikumab (MEDI2070, AMG 139, AstraZeneca) is a human monoclonal antibody against the p19 subunit of IL23. To date, no results are available about its efficacy and safety in UC. An induction phase 2 multicenter, randomized, double-blind, double-dummy, placebo and active-controlled, parallel-group (NCT03616821, EXPEDITION) is ongoing in moderate to severe UC; vedolizumab is the active comparator. A phase 2 open-label extension study (NCT04277546) in patients of NCT03616821 trial who previously completed or discontinued brazikumab due to lack of efficacy after Week 10 is ongoing.

Guselkumab (CNTO 1959, Janssen-Cilag) is another human monoclonal antibody against the p19 subunit of IL23. To date, no results are available about its efficacy and safety in UC. A phase 2b/3, randomized, double-blind, placebo-controlled, parallel-group NCT04033445, QUASAR) is ongoing in patients with moderately to severely active UC. Combination therapy with guselkumab and golimumab is under investigation for the first time in a Phase 2a randomized, double-blind, active-controlled study (NCT03662542, VEGA in patients with moderate to severe UC).

To date, data on the safety profile of monoclonal antibodies targeting IL23 in UC comes only from UST e mirikizumab studies. UST safety profile has been relatively favorable in UNIFI. The incidence of serious adverse events and infections was similar to that with placebo, both in the induction and in the maintenance study. No malignancies, opportunistic infections or tuberculosis occurred (70). In the phase 2 trial, mirikizumab safety profile appeared consistent with other IL23-targeting biologics (86). The most frequent AEs were nasopharyngitis, worsening of UC, anemia, headache, nausea, cough, and worsening of gastroenteritis during induction; worsening of UC, nasopharyngitis, headache, upper respiratory tract infection, arthralgia, hypertension, and influenza during maintenance (86). UST exposure throughout pregnancy recorded no apparent safety signals (94). In particular, among 478 maternal pregnancies exposed to UST, 11 of them with UC, the prevalence of live births, spontaneous abortions and congenital anomalies were consistent with the general population and anti-TNF therapies. Real world data from the ENEIDA registry, the GETAID cohort and a US population were consistent with the known safety profile of UST (77–79). Three cases of leukocytoclastic vasculitis related to UST have been reported (79, 95, 96). To date, there are no data on safety for Risankizumab, Brazikumab and Guselkumab in UC.

A summary of all current clinical trials of anti-IL23 monoclonal antibodies in UC is shown in **Table 1**.

TARGETING IL17

To date, no clinical studies targeting IL17 pathway are ongoing in UC. However, preclinical models, human genetic evidences and clinical studies with anti IL17A/F in other immune-mediated inflammatory diseases (IMID) support a role of this pathway in the intestinal inflammation. In various mouse models of colitis, the blockade of IL17 pathways through monoclocal antibodies or genetic deletion led to contrasting results: from a protective to an irrelevant and even to an harmful effect have been described (21–23, 97, 98). Genetic studies on UC patients have showed that selected haplotype and polymorphisms in IL17 genes are associated with an increased susceptibility to UC (43, 99–102) and its severity (103, 104), although other studies did not detect these associations (105).

Targeting IL17 in Crohn's Disease

After the successful results of anti IL23 p40 and p19 monoclonal antibodies in reducing intestinal inflammation in Crohn's Disease (CD) (70, 106) another form of IBD, targeting of the

TABLE 1 | Main characteristics of all ongoing clinical trials about monoclonal antibodies targenting IL23 p40 and p19 in UC.

Drug	Study Phase	Sample size (estimated primary completion date)	Primary endpoints	Active comparator	Reference
Ustekinumab	IV BioIBD	Recruiting (August 2021)	Identification of predictive Biomarkers for response to biologic therapies at induction	infliximab/ adalimumab/ golimumab/ vedolizumab	NCT03885713
	IV i-BAnk	Recruiting (April 2021)	Identification of prognostic and predictive biomarkers of patients who have lost response to biotherapies	anti-TNF/ ustekinumab/ vedolizumab	NCT03809728
	IV VERDICT	Recruiting (November 2024)	Determination of the optimal treatment target among corticosteroid-free symptomatic remission, or plus endoscopic remission, or plus histological remission	/	NCT04259138
	Observational HARIR	140	Tracking biologics along the silk road: number of participants with clinical response, remission and quality of life.	remicade/simponi/ stelara	NCT03006198
Mirikizumab	III LUCENT 1	Recruiting (September 2020)	Efficacy and safety of Mirikizumab to induce clinical remission at week 12	/	NCT03518086
	III LUCENT 2	Recruiting (March 2021)	Efficacy and safety of Mirikizumab to mantain clinical remission at week 40	/	NCT03524092
	III LUCENT 3	Recruiting (August 2023)	Evaluate the Long-Term Efficacy and Safety of Mirikizumab to mantain clinical remission at week 52	/	NCT03519945
	III LUCENT-ACT	Recruiting (March 2024)	Efficacy and safety of Mirikizumab to induce clinical remission compared to vedolizumab and placebo at week 12	vedolizumab	NCT04469062
	II	Recruiting (July 2022)	Evaluate Mirikizumab pharmacokinetics	/	NCT04004611
Risankizumab	II/III	Recruiting (March 2022)	Efficacy and safety of Risankizumab to induce clinical remission at week 12	/	NCT03398148
	III	Recruiting (December 2022)	Efficacy and safety of risankizumab to mantain clinical remission at week 52	/	NCT03398135
	I	Recruiting (July 2021)	Effect of Intravenous (IV) Infusions of Risankizumab on Pharmacokinetics of Cytochome P450 Substrates	/	NCT04254783
Bradizikumab	II	Recruiting (August 2020)	Evaluate the Efficacy and Safety of Bradizikumab to induce clinical remission compared to vedolizumab and placebo at week 10	vedolizumab	NCT03616821
	II open label	Enrolling by invitation	Safety of Bradizikumab	/	NCT04277546
Guselkumab	II/III QUASAR	Recruiting (June 2022)	Efficacy and safety of Guselkumab to induce clinical remission at week 12	/	NCT04033445
	II VEGA	Recruiting (November 2020)	Efficacy and safety of Guselkumab compared to vedolizumab and placebo to induce clinical remission at week 12	combination	NCT03662542

IL23/IL17 axis was thought to be a good strategy for both CD and UC. Further immunomodulation of the axis was attempted with the use of selective anti IL17A antibody and anti IL17R, secukinumab and brodalumab, respectively. Hueber et al. recruited 59 patients with moderate to severe CD and allocated them in a 2:1 ratio to receive secukinumab or placebo (107). The trial was prematurely stopped because an interim analysis found significantly higher rates of serious adverse events in the treatment group compared to placebo group (107). There is no trial evaluating the effect of Secukinumab in patients with UC.

Thus, future studies are evaluating therapeutic strategies combining IL17A and IL17F blockade.

Targeting IL17 in the IMID

Secukinumab (Novartis), is an important pharmacological agent in the therapeutic armamentarium against psoriasis (PsO), psoriatic arthritis (PsA) and axial spondyloartropathies (axSpA). Schreiber et al. conducted a retrospective analysis of 21 trials evaluating the exposure adjusted incidence rates (EAIRs) of CD, UC and IBD unclassified (IBD-U) in 7355 patients with PsO, PsA or axSpA treated with secukinumab with a cumulative exposure of 16,226.9 person-years (108). In the PsO cohort (n=5,181) they reported 10 new-onset cases of UC and 4 exacerbations of UC (among 10 patients with a known history of UC) during the study treatment (EAIRs of 0.13 per 100 PY), in the PsA cohort (n=1,380) there were 2 new-onset UC cases and 1 exacerbation (among 2 patients with a known history of UC) (EAIRs of 0,08 per 100PY), in the Ankylosing Spondilytis (AS) cohort (n=794) they reported 3 new cases of UC and 1 exacerbations (among 3 patients with a known history of UC) (EAIRs of 0,2) [10]. In their analysis, the incidence rates of IBD were in the range of the background rates of the respective conditions, considering that patients with PsO, PsA and axial spondyloarthropathies have a 1 to 3 fold increased risk of developing IBD compared to the general population (109–111). Of notice, among 48 patients with an history of IBD at baseline, 11 had an exacerbation during the study period (22,9%) (108).

Ixekizumab (Eli Lilly and Co) is an anti-IL17A with proven efficacy in PsA, PsO and axSpA. Genovese et al. evaluated the result of 21 trials in PsA, PsO and axSpA patients exposed to

Ixekizumab (112). Among the PsO patients (n=5898) there were 17 cases of UC (14 *de novo* and 3 exacerbations, IR 0.1 per 100 PY), in the PsA cohort (n=1401), they reported 1 case of new onset UC (IR <0.05 per 100 PY), in the axSpA population, 3 patients experienced *de novo* UC and 3 patients with a known history of UC reported an exacerbation (112). Unfortunately, the authors didn't report the number of patients with a known IBD history at baseline.

Bimekizumab (UCB) is an anti IL17A and IL17F antibody currently in phase 3 clinical trials for AS, PsA and PsO [NCT03928743, NCT04109976, NCT03766685]. Dual neutralization of inteleukin-17A and inteleukin-17F with bimekizumab was tested in phase 2 studies in patients AS (113), PsA (114) and PsO (115). In the AS study, the authors report 4 cases of IBD (2 cases of UC) among 243 patients who received Bimekizumab (EAIRs for UC was 0.77 per 100 PY) (113). Notably, in the PsA and PsO study no cases of IBD were reported (in the PsA study 2 patients had an history of IBD (114, 115).

A summary of all current drugs available towards IL23/IL17 axis is shown in **Figure 1**.

DISCUSSION

Since the discovery of the pivotal proinflammatory effect of TNF in the immune pathogenesis of UC, immunologists and clinicians have worked jointly to identity new targets as potential therapeutic strategies. The finding that the heterodimers alpha 4 and beta 7 integrins could mediate lymphocytes binding to the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) selectively in the gut has been another step forward. More recently, the increasing evidences of the IL23-IL17 axis involvement in UC pathogenesis have opened to several potential therapeutic options. Together, the pathways between the Janus kinase (JAK) family of tyrosine kinases and the signal transducer and activator of transcription (STAT) family of DNA-binding proteins have unlocked the potential to affect multiple pro-inflammatory cytokine-dependent pathways at once.

Several drugs with different mechanisms of action are currently available for patients with moderate-to-severe UC refractory to conventional therapies (116). However, a limited number of patients achieves and mantains remission on the long-term and a significative portion of patients develop complications such as proximal extension, strictures, pseudopolyposis, gut dysmotility, anorectal dysfunction, colectomy, hospitalization and colorectal cancer (3). Anti-TNFs (*e.g.* infliximab, adalimumab, golimumab) are all effective and safe in moderate-to-severe UC, both in patients naïve to biologics or previously exposed (4), and, in the case of infliximab, there is also evidence supporting their role as a rescue therapy in severe UC refractory to steroids (117, 118). Vedolizumab, a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin, is also effective in moderate-to-severe UC for those who are naïve or refractory to anti-TNFs (119). More recently, Janus Kinase inhibitors (anti-JAK) have been also approved for the same setting of patients (8).

Targeting IL23 have shown promising results from the clinical point of view. UST has been recently approved in moderate-to-severe disease, showing good efficacy profile both for induction and maintenance of clinical remission, mucosal healing, and histological response. On the other side, the safety profile of IL-23 shows no increased risks of side effects compared to placebo. UST is effective and safe both in naïve patients and in patients previously exposed to other monoclonal antibodies, positioning UST as first or second choice in the therapeutic algorithm. Preliminary data on other anti IL-23 agents also show promising results in terms of efficacy and safety. More data are needed on the long-term outcomes, such as prolonged remission, corticosteroid-free remission, hospitalization and colectomy rates, and safety, as well as direct comparison with other drugs approved for the same indication to understand the best positioning of those agent in the therapeutic algorithm.

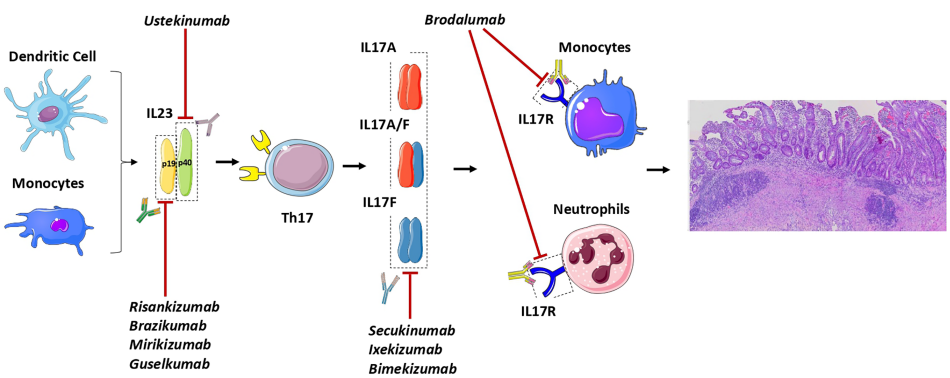

FIGURE 1 | General outlook of the IL23/IL17 axis in Ulcerative Colitis and of the drugs active on this pathway. The monoclonal antibodies risankizumab, brazikumab, mirikizumab and guselkumab recognize specifically IL23 by binding to the p19 its subunit. On the other hand, ustekinumab bind to the p40 subunit which is shared by IL23 and also IL12. Downstream blockade of the pathway can be achieved with monoclonal antibodies targeting IL17 (secukinumab, ixekizumab and bimekizumab) and the IL17R (brodalumab) which is mainly present on neutrophils and monocytes.

However, based on the paradigm shift towards precision medicine promoted by ECCO Scientific Workshop Steering Committee 2021 (120), patient specific characteristics should be considered more than drug characteristics. Given the beneficial effects in psoriasis and arthritis, UST treatment may be prioritized in IBD patients with extraintestinal manifestations. In addition, IL17A or IL-22 plasma concentrations at baseline could be eventually used to select patients as higher level of these citokynes were predictive for anti IL-23p19 success in CD patients (121).

To date, no clinical studies targeting IL17 pathway are ongoing in UC. However, the ineffective results of clinical trials on inhibition of IL17 in CD and the trigger effect on IBD onset or flare in patients treated for other IMID could be the clinical unmask of its context-dependent dual nature (16). IL17, independent from IL23, is involved in the local control of barrier integrity and defense against extracellular pathogens such as fungi and bacteria (122). In fact, genetic deficiency of IL17RA or IL17F is associated with chronic mucocutaneous candidiasis (122) and secukinumab -induced IL17F inhibition results in increased incidence of Candida spp. infection (107). IL17, dependent from IL23, exerts the known proinflammatory effect, successfully targeted in the other IMID. In addition, the remaining cytokines of the IL17 family may take part promoting inflammation in barrier organs or favoring repair of the gut mucosa after resolution of inflammation (50). An altered gut microbiota could be another possible explanation. In fact, Yeh et al. showed that treatment with secukinumab, but not UST, in psoriatic patients was associated at phylum level with increased *Proteobacteria* and decreased *Bacteroidetes* and *Firmicutes*, at family level with increased *Pseudomonadaceae* and *Enterobacteriaceae*, at order level with increased *Pseudomonadales* (123).

CONCLUSIONS

Targeting IL23/IL17 is a promising new therapeutic approach for the treatment of UC. Currently, more than 150 clinical studies have been registered with the intention of discovering effective treatments, we will have to wait for the outcomes of these studies for better clarify the efficacy of this approach, and whether a profile of a patient's gene variations can guide the selection of this treatment. Moreover, further studies are nedeed for the optimization of therapy, which demands a deeper understanding of disease mechanisms and drug modes of action that could support patient selection and treatment stratification.

AUTHOR CONTRIBUTIONS

DN and RM wrote the first draft and created table and figure. SV conceived the study and supervised the project. GR, RB, and GF critically reviewed the content of the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Adipokines, Cardiovascular Risk, and Therapeutic Management in Obesity and Psoriatic Arthritis

Sabrina Porta[1], *Matilde Otero-Losada*[2], *Rodolfo A. Kölliker Frers*[1,2], *Vanesa Cosentino*[1], *Eduardo Kerzberg*[1] *and Francisco Capani*[2,3,4]*

[1] Rheumatology Department, J. M. Ramos Mejía Hospital, Buenos Aires, Argentina, [2] Biomedical Research Center, Interamerican Open University, National Research Council (CAECIHS-UAI. CONICET), Buenos Aires, Argentina, [3] Department of Biology, University John F. Kennedy, Buenos Aires, Argentina, [4] Universidad Autónoma de Chile, Santiago, Chile

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Miguel Angel González-Gay,
University of Cantabria, Spain

***Correspondence:**
Francisco Capani
franciscocapani@hotmail.com

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Psoriatic arthritis is a chronic inflammatory disease with skin and joint pathology as the dominant characteristics. Scientific evidence supports its systemic nature and relevant relationship with obesity, metabolic syndrome, and associated conditions. Metabolic syndrome and obesity share common signaling pathways with joint inflammation, reinforcing the idea that adipose tissue is a major contributor to disease development and severity. The adipose tissue is not a mere energy store but also an endocrine organ participating in the immune response. In the search for the best therapeutic strategy for a patient, we should appraise the adipose tissue as an endocrine and immune organ responsible for mild chronic inflammation. Today, our challenge is not only to achieve disease remission but to control the associated comorbidities as well. In light of the high prevalence of obesity in psoriatic arthritis patients and the importance of the adipose tissue in the development of chronic inflammation, we aimed to identify the most relevant articles in this regard published in English until June 2020 using the PubMed database. Search terms included psoriatic arthritis, in combination with metabolic syndrome, obesity, adipokines, cardiovascular disease, and treatment. This review summarizes the current evidence regarding the role of adipose tissue as an adipokine-secreting endocrine organ, discussing its influence on disease development and severity, and ultimately in meeting successful disease management.

Keywords: psoriatic arthritis, metabolic syndrome, cardiovascular risk, obesity, adipokines, pathophysiology, treatment

INTRODUCTION

Psoriasis is a chronic inflammatory disease mainly compromising skin with systemic involvement associated with various comorbidities (1). With 6% to 40% percent of psoriatic patients experiencing psoriatic arthritis (PsA) (2), it equally affects men and women, starting usually at the age of 40 (3). The first signs appear on the skin usually 10 years before arthritis is diagnosed (4, 5).

Rheumatoid factor and cyclic citrullinated anti-peptide are absent, so PsA is considered a seronegative arthritis. Five PsA phenotypes may be distinguished based on the articular compromise pattern: predominantly axial, symmetric polyarthritis, asymmetric oligoarthritis, predominantly distal interphalangeal damage, and mutilating arthritis (6, 7).

Two distinguishing features of the disease are inflammation at the tendon and ligament insertions in the bone or enthesitis, and dactylitis when inflammation affects one finger entirely, risking the tendon sheath (8, 9).

The diagnosis of PsA is mainly clinical, reckoning on the classification criteria for psoriatic arthritis (CASPAR) (10, 11) (CASPAR) (10, 11).

Besides synovitis, enthesitis, dactylitis, and spondylitis, PsA typically presents non-musculoskeletal manifestations as anterior uveitis and inflammatory bowel disease. Comorbidities like obesity, cardiovascular disease, metabolic syndrome (MetS), and its components (obesity, diabetes mellitus, hypertension, and dyslipidemia) are also more frequent than in the general population (12).

Although the etiopathogenesis of PsA is multifactorial and not entirely known, there is an interaction between genetic susceptibility factors, mainly human leukocyte antigens (HLA) B27, together with other HLA-B loci and the HLA-Cw * 0602 allele) and environmental triggers (such as mechanical stress, dysbiosis, trauma, smoking, or infection), leading to dysregulation of immunoinflammatory pathways and the development of the disease (3).

In the last decades, a substantial body of evidence indicates that cutaneous psoriasis and psoriatic arthritis patients are at higher risk of developing cardiovascular disease. It is known that adipose tissue is metabolically active and an important source of inflammatory adipokines. Obesity and atherogenesis are low-grade inflammatory diseases and a link with associated PsA. Inflammatory mediators of obesity may represent one added key cardio-metabolic risk factor in PsA subjects (13).

The prevalence of immune-mediated diseases like psoriasis has increased in industrialized countries in the last decades, likely accountable to environmental factors, considering that the genetic predisposition has not changed (14). Western lifestyle, sedentary, with high fat and carbohydrate diets, and excessive sodium consumption favor the development of overweight and obesity (15).

In light of the high prevalence of obesity in psoriatic arthritis patients (16) and the importance of the adipose tissue in the development of chronic inflammation, we aimed to identify the most relevant articles in this regard published in English until June 2020 using the PubMed database. Search terms included psoriatic arthritis, in combination with metabolic syndrome, obesity, adipokines, cardiovascular disease, and treatment.

This review summarizes the current understanding of the role of the adipose tissue as an adipokine-secreting endocrine organ, discussing its influence on disease development and severity. Ultimately, robust evidence acknowledges considering the relevance of the adipose tissue in meeting successful disease management.

PATHOGENESIS OF PSORIATIC ARTHRITIS

Multiple factors participate in PsA development in an as yet not fully understood fashion. Some environmental factors like infections or mechanical stress give way to a genetically predisposed terrain, activating both the innate and adaptive immune systems. Genetic variants the major histocompatibility complex molecules and inflammatory mediators like the IL-12B gene, which encodes the p40 subunit of IL-23 and IL-12, the IL-23A gene, encoding for the p19 subunit of IL-23, and the IL-23 receptor gene (IL-23R), which encodes a common domain of IL-23R and IL-12R could favor the IL-23/IL17 axis activation, predisposing to the disease (17).

Chronic infections have raised great interest. In dysbiosis, microbiome proliferation, distorting its balance with the immune system, aberrant activation of the latter could favor cells recruitment, T helper (Th) 17 lymphocytes, in particular (17, 18). Activated Th17 lymphocytes might migrate from the intestinal mucosa and lymph nodes to the skin and joint tissue, causing local inflammation.

Repeated trauma causes tissue damage at the enthesis, i.e., tendon, ligament, and joint capsule insertion to the bone, activating the innate immune system by Damage-Associated-Molecular-Patterns (DAMPs) recognition (17, 19).

The growing importance of entheses in PsA pathophysiology has encouraged research studies. The enthesis is no longer considered a simple focal insertion but referred to as the 'organ of enthesis' (20). A healthy enthesis is usually anti-inflammatory, unlike the synovial membrane, which is proinflammatory regarding cellular composition and structure. When a mechanically stressed enthesis is injured, the associated inflammatory reaction manifests prominently within the juxtaposed synovium. The "synovio-entheseal complex" concept supports that specific factors at the joint level are relevant as danger signals, activating innate immune responses (20).

Regarding the adaptive immune system, IL17 A and IL23 appear key in PsA development.

Interleukin-17 not only activates innate-immunity cells but epithelial and endothelial cells, keratinocytes, chondrocytes, osteoblasts, and osteoclasts as well. Its ability to increase the level of IL1-IL6, IL8, TNF-α, matrix metalloproteinase-9 (MMP-9), granulocyte-macrophage colony-stimulating factor (GM-CSF), inducible nitric oxide synthase (iNOS), and receptor activator of nuclear factor-kappa B (RANK) applies to its participation in the inflammatory cascade (17).

In psoriatic patients, IL-17 handles CCL2 aberrant expression on keratinocytes, which recruits inflammatory cells, leading to hyperkeratosis and cell dysfunction (17).

Interleukin-17 and IL-23 act synergically in joint injury development, playing complementary roles in PsA pathogenesis. The respective intracellular signaling cascades are different, although IL-23 activates Th17 lymphocytes.

Antibodies against the keratinocyte cytoskeleton and enthesis sites have been identified, posing new theories as to disease development (21), while TH2-type response participation in seronegative arthritis has not been classically considered.

Psoriatic Arthritis and Cardiovascular Risk

Patients with PsA have increased cardiovascular risk, while the debate about the compromised metabolic pathways remains open (22). The so-called traditional cardiovascular risk factors, as hypertension, obesity, smoking, dyslipidemia, and metabolic syndrome, and hence the cardiovascular risk are increased in PsA patients (23, 24). Psoriasis is a systemic chronic inflammatory condition, where a high level of pro-inflammatory cytokines spreads beyond skin limits. Then, endothelial damage might be responsible for the increase of atherosclerotic disease in PsA patients (22). The 'two plaques for one syndrome' theory has been put forward. It states that a similar cytokine profile and inflammatory infiltrate is found in plaques both in skin psoriasis and atheroma (25).

We reported evidence supporting the contribution of classic cardiovascular risk factors and systemic inflammatory milieu in disease development. We found an increased level of C-reactive protein (CRP) and soluble intercellular adhesion molecule-1 (sICAM-1)in PsA patients without cardiovascular history or traditional risk factors compared with healthy subjects, the same as in recent-onset PsA patients, in the absence of cardiovascular risk factors (13).

Psoriatic Arthritis and Metabolic Syndrome

Psoriatic arthritis is undeniably a systemic disease, well expressed by the concept of psoriatic disease, having joint compromise as the central and most relevant characteristic (26). Within the spectrum of extra-articular manifestations, MetS stands out. Over the years, the diagnostic criterion for MetS, a cluster of coexistent hypertension, obesity, insulin resistance, and dyslipidemia, has undergone changes, each of which posing per se cardiovascular risk (27) Joint inflammation and MetS share common signaling pathways, which result in cardiovascular disease (3, 28). Atherosclerosis, MetS, and PsA show a common pattern of T-cell activation. They increase cytokine production with a Th-1 profile as tumor necrosis alpha (TNF-α), interleukin 1 beta (IL1-β), interleukin 10 (IL-10), and interferon (IFN) (3, 29). These cytokines induce insulin resistance in skeletal muscle, increase hepatic synthesis of pro-coagulant factors, inhibit lipoprotein lipase, and increase fatty acid oxidation, and atherogenic lipoprotein serum level. Cytokine production, insulin resistance, and endothelial dysfunction favor the formation and deposition of atheroma plaques in a deleterious positive feedback cycle (30). Proinflammatory cytokines not only promote vascular wall recruitment of macrophages and T lymphocytes precipitating atheroma plaque formation and deposition but induce foam cell lysis. This rupture or plaque accident releases cellular debris and thrombi to circulation with the consequent at distance damage often presented as acute myocardial infarction and cerebrovascular accident. The relationship between joint inflammation and atherosclerosis has led to considering PsA a cardiovascular risk factor on its own (31). Furthermore, PsA patients having no cardiovascular risk factors showed increased carotid intima-media thickness (IMTc) compared with control subjects (32, 33). In 2015, Di Minno et al.'s meta-analysis reported a higher carotid IMT and prevalence of carotid plaques, and reduced flow-mediated dilatation in 898 PsA patients compared with 1140 control (34). Although lifestyle changes are the fundamental pillar in MetS management, pharmacological intervention is often necessary. However, confirmed PsA diagnosis should refocus the attention to the inflammatory activity since psoriasis intrinsically favors endothelial dysfunction, insulin resistance, and a prothrombotic environmental background. Accordingly, the adequate MetS control should also consider joint disease remission.

Psoriatic Arthritis and Obesity

The complex, multifactorial relationship between obesity and psoriasis is not clear. Common pathophysiological mechanisms might account for pinning a vicious cycle that results in both. Whether one causes the other or they express different aspects of a same disorder is still undetermined.

While well-known factors may be accountable for arthritis development in PsA patients like severe skin compromise, nail compromise, genetic predisposition, and obesity, the latter has the advantage that can be changed.

Functional disability in PsA reduces physical activity, favoring weight gain, and, conversely, obesity prior to arthritis development is also true. This supports that chronic inflammation is likely involved in PsA development.

Two hypotheses explain how obesity favors PsA development in genetically predisposed subjects (35). One hypothesis proposes that adipose tissue acts as a source of inflammatory mediators like adipokines and proinflammatory cytokines, including TNF-alpha and IL-6 (30). The other one puts forward that overweight might would stress the enthesis due to the increase in the mechanical load with the following microtrauma, enticing an aberrant inflammatory response and PsA development (20). Obese patients had a higher prevalence of tuft resorption, Achilles and calcaneal spurs, and pelvic enthesitis compared with normal-weight subjects, reinforcing this hypothesis. Obesity was related to a late PsA onset, while normal-weight was associated with the HLA-B27 allele and an earlier onset of the disease (36).

Proinflammatory cytokines increased in obese patients are particularly involved in the pathophysiology of PsA and other inflammatory diseases.

Obesity is related to systemic inflammation and shares several pathways with PsA. Obesity, inflammatory status, and PsA are definitely related. A high level of inflammatory adipokines as found in obese patients may favor PsA expression in predisposed people.

Table 1 shows most of the few publications addressing the role of adipokines in PsA.

Despite certain aspects to be clarified, we can affirm that not only psoriasis increases the risk of developing obesity but also obesity is associated with higher prevalence and severity of psoriasis, even in the pediatric population (48, 49).

Studying 943 patients diagnosed with cutaneous psoriasis (CPs) showed BMI at 18 years was predictive of PsA. In the

TABLE 1 | Representative studies linking PsA with adipokines published between 2009 and 2019.

	Objective and characteristics	Main results
Eder et al. (37)	To compare MetS prevalence and levels of related biomarkers between 203 PsA patients and 155 controls.	MetS was higher in PsA (36.5%) compared with PsC (27.1%) p=0.056). Adiponectin was significantly associated with PsA (p=0.005), the use of anti-tumour necrosis factor α therapy (p=0.03) and active joint count (p=0.001).
Feld et al. (38)	To compare the prevalence of MetS and levels of related biomarkers between 74 PsA patients and 82 control subjects in a Mediterranean population.	MetS was higher in PsA patients compared with the control group: 54.8% versus 36.6%, respectively (P = 0.02). Leptin levels and leptin/adiponectin ratio were higher in PsA patients compared with controls: 83.4 versus 51.7 ng/mL (P = 0.001) and 6.3×10^{-3} versus 4.1×10^{-3} (P = 0.015), respectively.
Xue et al. (39)	To examine TNF-α, OPG, RANKL, leptin, adiponectin, resistin, chemerin, and omentin in 41 PsA patients, 20 PsO patients, and 24 healthy controls.	Compared with healthy controls, PsA patients had higher TNF-α, RANKL, OCs, leptin, and omentin, but lower adiponectin and chemerin.Increased serum levels of TNF-α, RANKL, leptin, and omentin were positively correlated with OCs numbers. Adiponectin level negatively correlated with OCs numbers. TNF-α, RANKL and leptin were positively correlated with disease activity.Only TNF-α was positively correlated with radiographic damage scores.
Caso et al. (40)	To investigate possible differences and correlations between adipokines and clinical expression in 42 PsA patients with clinically evident psoriasis (group 1) and 38 PsA patients without psoriasis (group 2)	A positive association was shown between leptin levels and female sex (β = 0.3, p = 0.001), BMI (β = 0.8, p < 0.0001), tender joint count (β = 0.23, p = 0.05), and patient pain-VAS score (β = 0.4, p = 0.049). In group 1, serum concentration of leptin was associated with female sex (β = 0.41, p < 0.0001) and BMI (β = 0.6, p = 0.012), whereas in group 2, a positive association was shown between leptin levels and BMI (β = 0.7, p = 0.003) and CRP (β = 0.35, p = 0.012). With regard to resistin, in the multivariate model, only the association between resistin and IL-6 was found (β = 0.33, p = 0.002). The association between resistin and IL-6 was confirmed in group 1 (β = 0.46, p = 0.004) but not in group 2.
Dikbas et al. (41)	To examine serum levels of adiponectin, resistin and visfatin, and their associations with disease activity and insulin resistance in 28 PsA patients and 39 healthy controls.	Levels of adiponectin, resistin and visfatin were higher in PsA patients compared with healthy controls (P < 0.05). Adiponectin (P = 0.001, OR = 3.1, 95% CI = 1.6–6.0), resistin (P = 0.06, OR = 1.8, 95% CI = 1.2–2.9) and visfatin (P = 0.03, OR = 3.9, 95% CI = 1.1–13.9) may contribute to pathogenesis of PsA.
Peters et al. (42)	To examine the effect of TNFα blockade therapy on adiponectin in 171 patients with rheumatoid arthritis (RA).	The mean ± SD absolute change in adiponectin levels was –0.23 ± 4.6 μg/ml in PsA patients treated with combined onercept 50 mg and onercept 100 mg (vs placebo, p=0.60) and 0.28 ± 3.23 μg/ml in RA patients treated with adalimumab (vs baseline, p=0.66).
Fassio et al. (43)	To evaluate secukinumab effect on different adipokines in 28 PsA patients.	In the male group, both resistin (p= 0.016) and chemerin (p= 0.028) showed a significant decrease compared with baseline after 6 months of therapy. A positive correlation was found for the overall values of CRP with resistin (p<0.001, R^2 = 0.326) and chemerin (p<0.001, R^2 = 0.251), and a weak negative correlation for adiponectin (p<0.046, R^2 = 0.036).
Chandran et al. (44)	To compare markers of cartilage metabolism, MetS and inflammation in 201 OA patients, 77 PsA patients, and 76 controls.	Levels of resistin, HGF, insulin, leptin, CRP, IL-6, IL-8, TNF-α, MCP-1, NGF were varied across the three groups (p<0.001). In multivariate analysis, resistin (OR = 1.26, 95% CI = 1.07 to 1.48), MCP-1 (OR = 1.10, 95% CI = 0.07 to 1.48), and NGF (OR < 0.001, 95% CI = <0.001 to 0.25) were independently associated with PsA vs OA.
Colak et al. (45)	To compare the relationship between disease activity and vaspin, neutrophil gelatinase-associated lipocalin (NGAL), and apolipoprotein levels in 50 PsA patients and 36 healthy controls.	The levels of vaspin, (391.63 ± 436.4 vs 176.67 ± 122.75, p = 0.001), NGAL (5.2 ± 2.67 vs 1.94 ± 2.09, p = 0.014), and apolipoprotein B/A1 ratio (0.78 ± 0.21 vs 0.66 ± 0.27, p = 0.023) were higher in PsA patients. Apolipoprotein A1 was lower in PsA patients compared with healthy controls, p= 0.017). Patients with MetS had s higher NGAL, Apo B, and Apo B/A1 ratio (p < 0.05). NGAL levels were negatively correlated with disease duration and psoriatic arthritis (p < 0.05). There was a positive correlation for Apo B, Apo B/A1 and BMI with WC (p < 0.05). Patients with MetS had higher scores of DAPSA and PASI (p < 0.05).
Wagner et al. (46)	To determine serum biomarker associations with clinical response to golimumab treatment in the first 100 PsA patients at baseline, week 4, and week 14.	A smaller subset of proteins (adiponectin, apolipoprotein CIII, serum glutamic oxaloacetic transaminase, and TNF α) was associated with a 75% improvement in the psoriasis area and severity index score (PASI75) at week 14,
Johnson et al. (47)	To compare serum biomarkers in 143 PsA patients with 180 PsO.	The level of TNF-α was higher in PsA patients compared with PsO (P < 0.001). A high TNF-α level was associated with increased odds of PsA (multivariate adjusted OR = 2.25, 95% CI = 1 41–3, p = 61). Patients with PSA showed a mild decrease in median total adiponectin and high molecular-weight (HMW) adiponectin. An inverse association was found between high total adiponectin and PsA (multivariate adjusted OR = 0.61, 95% CI = 0.39–0.96). An inverse association of PsA with total or HMW adiponectin was found only in participants reporting alcohol intake.

HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; OPG, osteoprotegerin; RANKL, receptor activator of the nuclear factor-kB ligand; OCs, osteoclast precursors; PsAJAI, Psoriatic Arthritis Joint Activity Index; PASI, psoriasis area and severity index score; HMW, high molecular-weight; RA, rheumatoid arthritis; HGF, hepatocyte growth factor; NGF, nerve growth factor; MCP-1, monocyte chemoattractant protein 1; OA, osteoarthritis; DAPSA, Disease Activity for Psoriatic Arthritis; NGAL, neutrophil gelatinase-associated lipocalin; Apo, apolipoprotein; WC, waist circumference; PsO, poriasis; PsA, psoriatic arthritis.

United Kingdom, the risk of developing PsA was studied based on the relationship between the BMI measured after the diagnosis of PsC and PsA development.

Compared with PsC patients with a BMI <25, those with a BMI of 25–29.9, 30.0–34.9 and ≥35.0 had age and sex-adjusted RRs of 1.1, 1.24, and 1.52, (p for trend <0.001) (50). One 14-year follow-up prospective study showed that BMI, weight change from early adulthood, waist circumference, hip circumference, and waist-hip ratio were associated with an increased risk of developing PsA in all the participants and in PsA women in particular (51).

Figure 1 shows the interaction between obesity, adipokines, in the development of psoriasis and psoriatic arthritis and cardiovascular disease.

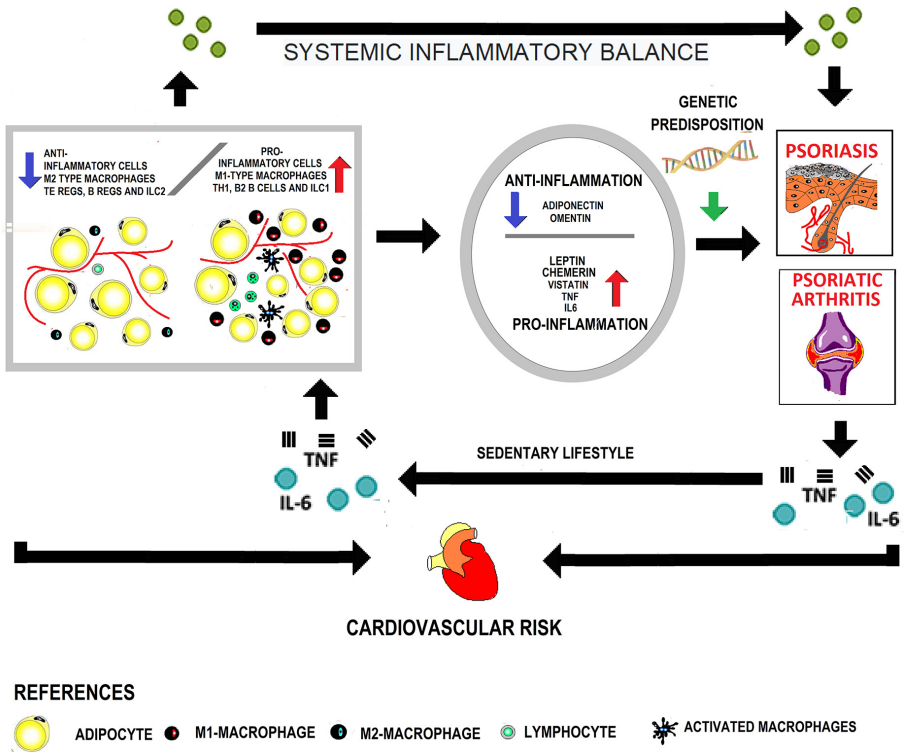

FIGURE 1 | Interaction between obesity and adipokines in the development of psoriasis, psoriatic arthritis, and cardiovascular disease. In obese patients, the adipose tissue presents cellular changes due to greater infiltration of pro-inflammatory cells with a decrease in the anti-inflammatory cell population. In this way, it becomes dysfunctional and acts as an endocrine organ, increasing the secretion of pro-inflammatory adipokines. A systemic inflammatory state is generated that, in genetically predisposed subjects, favors the appearance of skin psoriasis. On the other hand, the increased mechanical load at the enthesis sites contributes to the onset of psoriatic arthritis. The inflammation characteristic of the disease added to the sedentary lifestyle secondary to joint involvement perpetuates the growth of adipose tissue. Finally, both due to the presence of classical and non-classical factors associated with obesity, cardiovascular risk increases.

CHARACTERISTICS, DISTRIBUTION, AND FUNCTION OF THE ADIPOSE TISSUE

Obesity is considered as having a body mass index (BMI) above 30 kg/m2 (52). Its deleterious impact on health has been widely studied, often embedded in the well-known MetS. Metabolic syndrome is a cluster of risk factors leading to microvascular dysfunction and chronic cerebral hypoperfusion, brought by the coexistence of diabetes type 2 or insulin resistance, abdominal obesity, hypertension, and dyslipidemia, mainly a low level of high-density lipoprotein-cholesterol, (HDL) (53–55).

Although weight gain and a high BMI imply an increase in adiposity, studying fat distribution is crucial given the many functions of adipose tissue.

Two types of adipose tissue may be recognized based on cellular structure, location, color, vascularity, and function. Newborns have brown adipose tissue, which has multilocular adipocytes with a large number of mitochondria. Brown adipose tissue is vital at this life stage when heat production is essential for survival. Adults show only white adipose tissue, which is basically an energy store made up of triglycerides within adipocytes (54).

The white adipose tissue presents different distribution patterns, traditionally named as android and gynoid. Android phenotype typically has fat centrally distributed mainly in the abdomen, chest, shoulder, and nape of the neck. Gynoid fat distribution refers to fat peripherally accumulated mainly at the buttocks and hips (54).

The android or gynoid distribution pattern depends on the subcutaneous adipose tissue which contributes to regulate body temperature acting as a thermal insulator.

However, there is another fraction of the white adipose tissue, the visceral adipose tissue, which occupies the space between the internal organs, allowing their proper juxtaposition.

There are important differences between subcutaneous and visceral fatty tissue, because of the expression of different genes related to insulin resistance, inflammation, and a different pattern of adipokines secretion (56).

These different secretion patterns have local repercussions involving autocrine or paracrine mechanisms and systemic ones. In this way visceral fat directly affects the liver, its increase being responsible for the development of insulin resistance, dyslipidemia, glucose intolerance and cardiovascular hypertension (54).

Adipose Tissue as an Endocrine and Immune-System Organ

Adipose tissue is made up not only of adipocytes which represent one-third of total fat. Adipose tissue also comprises fibroblasts, macrophages, stroma cells, and pre-adipocytes (54) Then, adipose tissue is not a mere energy store but also an endocrine organ participating in the immune response (57).

Adipose tissue is responsible for synthesizing adipokines which form a heterogeneous group including hormones like leptin and adiponectin, cytokines like TNF-α, IL -6, omentin, visfatin, and other proteins like the plasminogen activator (PAI) -1, angiotensinogen and resistin.

The adipokines family includes anti-inflammatory and proinflammatory adipokines. Under normal metabolic conditions, they are balanced. However, their plasma profile changes in response to different challenges, like metabolic variations. A deficit in calorie supply during starvation causes that proinflammatory adipokines decrease, and anti-inflammatory adipokines increase.

Proinflammatory Adipokines

The hormone leptin is produced mainly by adipocytes and regulates whole-body energy homeostasis. Fat mass and inflammatory status determine leptin levels (30).It reduces food intake and increases energy expenditure, acting directly on the hypothalamus and other brain regions (58). Obese patients typically show a high circulating level of leptin and leptin resistance (57) Leptin pro-angiogenic properties have been described directly related to endothelial dysfunction and atherosclerosis development (59). Leptin also modulates the immune response as an inflammatory molecule capable of activating adaptive immunity cells (57).

High level of leptin found in PsA patients, considering leptin properties, has suggested that leptin might be involved in bone erosion, activating osteoclasts in these patients (22) perhaps due to its cytokine-like structure (30). Leptin correlates with metabolic syndrome features and inflammation in patients with moderate-to-severe psoriasis (60).

Chemerin is involved in inflammation, adipogenesis, angiogenesis, and dyslipidemia. It participates in preadipocyte differentiation into adipocytes and in the hyperplasia and hypertrophy of mature adipocytes (61).

Visfatin is another adipokine related to abdominal obesity that increases pro-inflammatory factors in monocytes, promoting T lymphocyte activation. Correlation between visfatin concentration and insulin resistance, measured by the HOMA (Homeostatic Model Assessment for Insulin Resistance)-index was found in patients with ankylosing spondylitis, chronic inflammatory arthritis closely related to psoriatic arthritis (62). At the vascular level, visfatin upregulates vascular endothelial growth factor (VEGF) secretion and downregulates metalloproteins expression (63).

The increased cardiovascular risk in obese patients may be related to altered fibrinolysis. Adipocytes produce PAI-1, and obese patients have a high PAI level correlated with insulin resistance (64).

Both IL-6 and TNF-α promote insulin resistance inhibiting lipoprotein lipase (LPL), decreasing triglyceride hydrolysis into free fatty acids, and increasing triglyceride storage in adipocytes, resulting in larger fat cells (57). Besides, TNF-α inhibits preadipocytes conversion to mature adipocytes (65) and promotes atherosclerotic plaque formation, favoring leukocyte recruitment and activation, endothelial cell expression of adhesion molecules, and triggering arterial wall inflammatory cascade (30).

Unlike TNF-α, whose change in local production has not been confirmed, the expression of IL-6 in visceral fat was higher than in subcutaneous fat (30).

Anti-inflammatory Adipokines

Adiponectin is exclusively synthesized and secreted by adipose tissue. Its protective role in the development of MetS and atherosclerosis has been suggested as it improves insulin sensitivity and fatty acid oxidation (63). Patients with chronic diseases show low adiponectin levels resulting from a decreased synthesis in adipose tissue after the increase in IL-6 and TNFα cytokines. A low adiponectin level is associated with insulin resistance, vasodilation, endothelial damage, and diastolic failure and with a high CRP level in obese patients (30). In addition, adiponectin receptor mRNA (AdipoR1/R2) expresses to a lesser extent as well (66). Adiponectin concentration was inversely correlated with triglycerides/HDL and total cholesterol/HDL cholesterol ratios, and with high fasting plasma glucose level in rheumatoid arthritis, the prototypical inflammatory arthritis. Therefore, in rheumatoid arthritis, low adiponectin level clustered with metabolic syndrome features implicated in accelerated atherosclerosis development (67).

Omentin, produced by the vascular stroma cells of the visceral adiposity, increases insulin sensitivity, stimulating insulin-mediated glucose uptake in human adipocytes (68).

Adipose Tissue Changes in Obesity

In obese patients, adipokines synthesis and secretion are altered. Adipocytes are dysfunctional and increase the secretion of pro-inflammatory adipokines (69), triggering systemic mild chronic inflammation in the adipose tissue at first, then spreading away all through (57). Leptin secretion increases secondary to the increase in adipocyte lipid content. Though aimed to reduce caloric intake by acting at the central nervous system, leptin secretion leads to an imbalance at the adipose tissue. Accordingly, infiltration and accumulation of proinflammatory M1-type macrophages increase, replacing the normally found type 2 macrophages. Not only IL6 and TNF-α increase but insulin resistance develops as well. Accompanying M1 macrophages, proinflammatory cells (Th1, B2 B cells and ILC1) increase and anti-inflammatory immune cells (Tregs, Bregs, and ILC2) decrease in adipose tissue, perpetuating chronic inflammation (57). In sum, the adipose tissue drives an imbalance between anti-inflammatory and pro-inflammatory adipokines favoring the latter, increasing insulin resistance, propitiating diabetes, MetS, and cardiovascular disease.

Faced with this scene, two ways to improve the inflammatory state in adipose tissue and insulin sensitivity appear: weight loss and white adipose tissue gaining characteristics of brown adipose

tissue in the so-called beiging process. The resulting beige or brite (brown in white) adipocytes (59) can burn lipids and restore normal insulin sensitivity, reducing the proinflammatory environment. Exposure to cold, β-adrenergic stimulation and some cytokines like IL33, IL13 and IL4 activate this mechanism.

In obesity, this process is slowed down, hampering insulin sensitivity restoration. Interleukin-17 is postulated to inhibit adipocyte differentiation and glucose absorption, and stem cell differentiation into adipocytes (57).

Although IL-17–producing T cells represent only 10% of the total immune cells in adipose tissue, the increase in fatty acids regulates the differentiation of CD4 + T cells into IL-17 T cells through acetyl Co-A carboxylase 1, increasing IL-17 T cells in obese patients, limiting the ability of fatty tissue to turn into brown tissue, decrease the inflammatory state and improve insulin sensitivity (57).

Obese patients have higher levels of IL-17 and IL-23 compared with normal weight counterparts unrelated to abdominal fat, insulin-resistance, or leptin level (70).

Presumably, IL-17 synthesized by visceral fat might favor vascular smooth muscle cells expression of eotaxin, related to carotid intima-media thickening as a sign of subclinical atherosclerosis (71).

Bone Marrow-White Adipose Tissue, Adiponectin, and the Bone

Bone marrow is a substantial reservoir of white adipose tissue alongside visceral and subcutaneous white fat. Adipocytes stand for up to 70% of total human bone marrow (72). Marrow adipose tissue (MAT) contributes to local and systemic metabolic processes with adipokine secretion (73). Its relevance as a paracrine organ, and its increase when bone mass decreases, have encouraged hypotheses regarding its role in bone health and metabolism. Bone MAT releases adiponectin in response to caloric restriction, highlighting its relevance as an endocrine organ (74).

Contrary to expectations, PsA with high BMI values show high adiponectin levels. Far from improving metabolism as in other tissues, adiponectin seems to have pro-inflammatory properties at the joints, increasing damage. Adiponectin proved a biomarker of radiographic progression independent of metabolic status in rheumatoid arthritis patients (75–77).

Regarding the involved mechanisms, adiponectin might act on osteoblasts, reducing the new bone formation and on osteoclasts, increasing bone resorption (73). Adiponectin and leptin receptors are found in osteoblasts, osteoclasts, and osteoclast precursors. Ultimately, the real impact of these adipokines in bone remodeling is still controversial (73).

In addition, adiponectin induces the release of proinflammatory cytokines like IL-6, monocytic chemotactic protein 1 (MCP-1), prodegradative enzymes like metalloproteinases, and nitric oxide in chondrocytes. Synovial fibroblasts have adiponectin receptors whose stimulation also contributes to promoting a prodegradative microenvironment (78–80).

Different adiponectin isoforms might account for the anti-inflammatory and pro-inflammatory properties of adiponectin (81).

RELATIONSHIPS AND CHANGE IN THERAPEUTICS

Nonbiological Systemic Agents

Currently, peripheral joint compromise is initially treated with synthetic disease-modifying drugs like methotrexate, leflunomide, sulfasalazine, and cyclosporine. Acute episodes are often controlled with non-steroidal anti-inflammatory drugs (NSAIDs), which should be used with caution in patients with cardiovascular disease or cardiovascular risk factors. Similarly, small corticosteroids dose for as short as possible should be used (82).

Disease-modifying antirheumatic drugs (DMARDs) should have a positive impact on cardiovascular disease in PsA patients since their pharmacological action reduces systemic inflammation.

Methotrexate (MTX) is a folate antagonist that inhibits the activity of dihydrofolate reductase activity and is one of the most widely used synthetic drugs in PsA treatment. Unfortunately, its effects on the cardiovascular are diametrically opposed, and, eventually, its prolonged administration may increase serum homocysteine level (83) and cardiovascular risk in patients with previous hyperhomocysteinemia (84). Fortunately, increasing homocysteine levels by MTZ can be prevented with folic acid administration (83) while keeping the anti-inflammatory properties that reduce cardiovascular risk. Large observational studies have shown that MTX reduces cardiovascular disease incidence in PsA and rheumatoid arthritis patients. Low or moderate cumulative doses and concomitant use of folic acid have shown more beneficial than high cumulative doses (85). However, the risk of hospitalization for ischemic heart disease among people who received MTX was comparable to that of people receiving other non-biologic antipsoriatic drugs like oral retinoids, cyclosporine, azathioprine, and mycophenolate (86).

Regarding liver disease, it may beiatrogenic or due to the disease itself. Leflunomide and MTX can induce nonalcoholic steatohepatitis (NASH). NASH was more frequent in MTX-treated patients and in patients with PsA compared with rheumatoid arthritis (87). However, PsA patients treated with an anti–TNF-α/MTX association had a lower risk of liver fibrosis compared with those treated with MTX alone (87).

The presence of liver disease should be considered when selecting the PsA treatment (16). Hepatic steatosis, found in 28.1% of the studied sample, was an independent predictor for not achieving the MDA (hazard ratio [HR], 1.91; 95% confidence interval [CI], 1.04–3.38) as reported by a prospective study (88). Patients with liver disease should not be treated with MTX, leflunomide, sulfasalazine, and NSAIDs. Whenever these drugs are administered, careful monitoring of transaminases and liver function should be performed.

Biological Agents

Proteins and/or their derivatives targeting specific molecular cascade steps involved in the pathophysiology of different diseases are defined as biological agents (89). According to their molecular structure, they are classified as recombinant human cytokines and growth factors, and fusion antibody proteins and monoclonal antibodies (89).

Fusion proteins are molecules formed by a naturally occurring receptor bound to an immunoglobulin structure. Monoclonal antibodies may be (a) chimeric antibodies, having 30% murine genes fused with human antibodies, (b) humanized antibodies, with 10% murine sequences, and (c) human antibodies derived solely from human immunoglobulin genes.

The development of biological drugs and small molecules pharmacologically active has expanded the therapeutic arsenal in PsA management. This has improved life quality and prognosis in patients refractory to methotrexate, sulfasalazine, leflunomide, or cyclosporin A, conventional immunosuppressive agents.

The complexity and systemic characteristics of PsA demand considering both the ongoing disease activity and extra-articular manifestations, and comorbidities before coming to a drug choice.

Evidence-based, obesity reflects mild chronic systemic inflammation. Worse, visceral fat might add to PsA intrinsic inflammation, secreting pro-inflammatory cytokines. As a result, it affects the therapeutic response and increases cardiovascular risk and the likelihood of expressing the disease in genetically predisposed subjects.

Abdominal obesity not only increases systemic inflammation in a positive feed-back cycle but is relevant to the therapeutic response.

Anti TNF α Agents

The TNF-α cytokine is key in PsA pathophysiology. Currently, five anti-TNFα molecules are available. Infliximab, adalimumab, and golimumab are monoclonal antibodies. Etanercept is a genetically manufactured dimeric protein resulting from the fusion of the soluble extracellular domain of human TNF-2α (TNFR2/p75) bound to the human IgG1 Fc domain. Certolizumab pegol is a humanized murine monoclonal antibody Fab fragment bound to two polyethylene glycol molecules.

Psoriatic arthritis patients treated with TNF-α underwent a larger decrease in IMTc, a marker of subclinical atherosclerotic disease, compared with those treated with synthetic disease-modifying drugs (6). However, obesity is a negative predictive factor for achieving minimum disease activity (MDA) in patients treated with biological therapies. Treatment with TNF-α inhibitors may induce weight gain in PsA patients (90).

In 2012, Di Minno et al. found that obesity was prevalent in patients not reaching the MDA end-point compared with those who did (64.0% versus 25.5%, p <0.001). Obesity was associated with an increased risk of not reaching MDA (HR = 4.90, 95% CI = 3.04–7.87, p<0.001). The HR for not reaching MDA was 3.98 (95% CI = 1.96–8.06, p< 0.001) and 5.40 (95% CI = 3.09–9.43, p<0.001) in subjects with BMI <30 kg/m^2 and <30 to 35 kg/m^2. In 98 subjects achieving MDA at 12 months of study, obesity was associated with a low probability of maintaining MDA at 24 months of follow-up (HR = 2.04, 95% CI = 1,015–3.61, p = 0.014) (91). In psoriatic arthritis, the disease activity index for PsA (DAPSA) score was associated with increased risk of subclinical atherosclerotic disease (92).

Assuming the negative effect of obesity in the disease's control, the same group of researchers studied the effect of a low-calorie diet and weight loss in 126 obese patients starting anti–TNF-α administration. After adjusting for any other clinical and laboratory factors, the dietary intervention proved a strong and independent predictor of MDA achievement (93).

This has probably been related to drug elimination. Patients weighing over100 kg eliminate the drug 55% faster and have a 35% larger distribution volume, reaching lower minimum levels at the time of dosing (94).

In obese patients with metabolic syndrome, the TNF-α blockade may be less effective. The circulating T helper (Th) 17 level and IL-1 cells, elevated in obese patients, can predict the response inadequate to Anti TNF-α (95).

The Danish and Icelandic biological treatment registry included 1943 patients with PsA. Of these, 1271 (65%) had a BMI, with 408 (32%) obese. Obese patients had a higher disease activity and poor adherence to treatment, men in particular, with the median duration of Anti-TNF-α treatment of 2.5 years (95% CI = 1.7–3.2) in the obese vs 5.9 (4.1–7.7) in the non-obese. (P <0.01). Obesity came out as a risk factor for abandoning treatment [HR = 1.6 (95% CI = 1.3–2.0)] (96).

Six-month treatment with adalimumab increased insulin sensitivity and reduced CRP and retinol-binding protein-4 (RBP-4) in a prospective study of 29 patients with moderate-to-severe psoriasis (97, 98). These results were supported by a meta-analysis that included 38 randomized controlled trials (RCTs) with 18024 patients reporting adverse events in adults with plaque psoriasis who received at least one dose of biological therapy, conventional systematic therapy, or placebo. No risk differences for major cardiovascular events were associated with the use of AntiTNF-α (odds ratio [OR] = 0.67, 95% CI = 0. 10–4.63) (99).

Anti IL-17 Agents

Among the biological agents acting through IL-17 inhibition, we find secukinumab and ixekizumab, which are anti–IL-17A monoclonal antibodies. Secukinumab efficacy in obese patients is controversial. In a retrospective observational study including 136 patients with cutaneous psoriasis from 10 dermatology centers in Spain with a 52-week follow-up, secukinumab efficacy was lower in patients with a BMI above 30 (100).

There is scarce information on secukinumab efficacy in obese patients with psoriatic arthritis.

Recently, Pantano et al., carried out a prospective analysis of 100 patients with PsA, divided into those with a BMI above or below 25. After 6 months of treatment, overweight and obese patients had an even better response to secukinumab compared with normal weight patients. Analyzing serum IL-17 levels in 20 obese and 20 non-obese patients, higher serum levels of IL-17 were found in the former (101). This finding was in line with the literature since obesity promotes the expansion of IL17-producing T cells in adipose tissue, and higher levels of IL17 and IL23 were found in this study.

A recent retrospective study of 290 PsA patients, 310 PsC patients and 600 healthy controls found that obesity was more frequent in either PsC (36.5% vs. 22%, OR = 2.1, 95% CI = 1.5–2.8, p <0.01) or PsA (27.6% vs. 22%, OR = 1.4, 95% CI = 1.0–1.9, p <0.05) compared with controls with no inflammatory disease. Curiously, obesity was more frequent in PsC (36.5%) than in PsA (27.6%) (OR = 1.5, 95% CI = 1.1 to 2.1, p <0.05). A family history of PsA (OR = 3.6, 95% CI = 1.1–12.4), axial compromise

(OR = 4.4, 95% CI = 1.0–15.4), and dyslipidemia (OR = 3.5, 95% CI = 1.5–8.6) were independently associated with obesity after correcting for probable confounding factors (102).

The effect of IL-17 on cardiovascular risk has been the subject of debate, likely due to its dual anti-atherogenic and pro-atherogenic action, depending on the inflammatory context (22).

An observational study including 195 secukinumab-treated patients with a 2-year follow-up reported that 2% of patients experienced a CV event (103). However, the safety data of the published meta-analysis did not risk differences in the risk of major cardiovascular events associated with the use of anti-IL-17A agents (secukinumab and ixekizumab) (OR = 1·00, 95% CI = 0·09–11·09) (99).

Anti-IL-12/IL-23

Ustekinumab is the first biological drug specifically targeted at IL-12/IL-23 approved to treat PsA. It binds to the p40 subunit, which is shared by IL-12 and IL-23, interfering with Th1 and Th17-dependent proinflammatory cytokines´ production (95).

Recent studies have shown that PsA patients´ response to ustekinumab was effective and sustained, regardless of body weight (104).

In a study of 79 patients diagnosed with cutaneous psoriasis, patients treated for 7 months with infliximab had an increase (P <0.001) in the mean BMI (2.1 ± 4.5%) and body weight. (2.5 ± 3.3 kg) compared with those treated with ustekinumab (0.1 ± 3.3%; 0.6 ± 1.1 kg) (105).

The pivotal, randomized, placebo-controlled, phase III PSUMMIT I trial not only confirmed the efficacy of ustexinumab in PsA but, unlike other biological agents, those with a bodyweight below or equal to 100 kg had higher response rates than those weighing over 100 kg. In a *post hoc* analysis by weight group, patients over 100 kg treated with the 90 mg dose showed a trend to higher American College of Rheumatology (ACR) and Psoriasis Area Severity Index (PASI) response rates than those treated with the 45-mg dose (106). Whether these differences are due to suboptimal dosing is to be determined (95).

An observational study of 50 patients with PsA, of whom 28% were obese, studied the efficacy of using ≥3 doses of ustekinumab. Fifty-four percent of patients treated with ustekinumab achieved an MDA response (107).

Regarding ustekinumab on cardiovascular risk, the debate is still open. Contrary to expectations, as IL-12 and IL-23 are found in atheroma plaques, the first data obtained from clinical studies using inhibitors reported a higher rate of major cardiovascular events in the ustekinumab group (108). However, later on, there was no increase in the number of events in the study, so the risk was even lower than in the general population (108).

Two meta-analyses have been carried out to assess the risk of major cardiovascular events (109, 110). Although one possible explanation has been that the methods used for data analysis were different, the results of both meta-analyses turned out contradictory (22). One of them reported no statistically significant differences in the risk of major cardiovascular events compared with placebo (OR = 4.48, 95% CI = 0.24–84.77, p = 0.32) (99)

Randomized long-term follow-up controlled trials are necessary to make recommendations (22).

CONCLUSION

Psoriasis, a systemic disease entangling MetS, cardiovascular disease, and joint involvement, puts forth obesity and PsA as indirect indicators of disease severity (111). The high prevalence of MetS and its components in PsA patients stresses the importance of performing anamnesis, thorough physical examination, and carotid arteries' Doppler evaluation, screening subclinical atherosclerotic disease. Counseling on lifestyle changes pointing to a well-balanced, healthy diet, smoking cessation, increasing physical activity, sleep care, and so on, is essential and should take part in their comprehensive assessment. Ultimately, non-classical factors' influence on cardiovascular disease development should not be underestimated, demanding rigorous control of disease activity.

Provided the large available therapeutic arsenal, the choice of treatment should consider the individual characteristics, obesity, and metabolic syndrome, in particular.

Optimal care of PsA patients is a huge challenge for the treating physicians. Therapeutic management should not be limited to the care of the skin and joints. The systemic and inflammatory nature of the disease makes the search for comorbidities imperative. Adequate cardiovascular risk assessment and strict bodyweight control should be considered therapeutic objectives. These will not only modify the patient's prognosis but influence therapeutic efficacy and have to be considered. In this way, the comprehensive management of the patient will be decisive in the choice of the therapeutic scheme.

AUTHOR CONTRIBUTIONS

SP, RK: writing—original draft. VC, EK: supervision. MO-L: review and editing—grammar, style, and language. FC: funding acquisition, writing—review. All authors contributed to the article and approved the submitted version.

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IL-23 Inhibition in Ankylosing Spondylitis: Where Did It Go Wrong?

Dominique Baeten[1,2*] *and Iannis E. Adamopoulos*[3]

[1] Clinical Immunology and Rheumatology, Amsterdam University Medical Center, Amsterdam, Netherlands, [2] Immunology Therapeutic Area, UCB, Slough, United Kingdom, [3] Department of Medicine, Division of Rheumatology and Clinical Immunology, Beth Israel Medical Deaconess Center, Boston, MA, United States

Axial spondyloarthritis is a prevalent form of chronic arthritis which is related to psoriatic arthritis and skin psoriasis. TNF and IL-17A as well as IL-17F are key cytokines contributing to the pathobiology of this disease, as evidence by the therapeutic efficacy of inhibition of these factors. Despite the evidence that IL-23 acts as an upstream driver of Th17 cells, the T lymphocytes producing IL-17, and that IL-23 inhibition shows profound efficacy in psoriasis, blocking IL-23 failed to show any evidence of clinical efficacy in axial spondyloarthritis. In this viewpoint article, we revisit the reasons-to-believe in a role of IL-23 in the pathobiology of axial spondyloarthritis, discuss what we have learned on the pathobiology of this disease in general and on the function of the IL-23/IL-17 axis in particular, and share a handful of lessons learned that are of relevance for the translation of emerging biological insights into clinical therapeutics.

Keywords: interleukin-23, interleukin-17, ankylosing spondylitis, axial spondyloarthritis, Th17

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Matteo Vecellio,
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****Correspondence:***
Dominique Baeten
d.l.baeten@amsterdamumc.nl

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INTRODUCTION

Axial spondyloarthritis (AxSpA) is a prevalent form of chronic arthritis affecting mainly the axial skeleton (1). As other forms of spondyloarthritis, including psoriatic arthritis, it also often affects peripheral joints. Both axial and peripheral disease is characterized by a combination of chronic inflammation (including synovitis, enthesitis, and osteitis), focal bone destruction, and exaggerated pathological new bone formation leading to joint ankylosis. Finally, a significant proportion of patients also display extra-articular manifestations such as psoriasis, Crohn's disease or colitis ulcerosa, and acute anterior uveitis.

The pathobiology of AxSpA remains incompletely understood but a few concepts have been firmly established. First, AxSpA does not display the prototypical features of classical autoimmune diseases such as female predominance, genetic association with MHC class II variants and molecules involved in T- or B-lymphocyte activation, presence of autoantibodies, and good clinal response to T- or B-cell targeted therapies. It is therefore considered as a hyperinflammatory disorder driven by an abnormal inflammatory (potentially innate immune) response to different forms of stress leading to uncontrolled tissue inflammation and damage (2). Second, AxSpA is strongly associated with HLA-B27 and overexpression of human HLA-B27 in rats leads to AxSpA-like disease (3). HLA-B27 could contribute to the pathobiology of the disease by antigen-presentation to cytotoxic T cells, intracellular misfolding leading to endoplasmic reticulum stress and abnormal cytokine production (including IL-23), and/or formation of heavy chain homodimers which can directly trigger NK and T cells and possibly other cell types to produce cytokines such as IL-17 (4). Finally, pro-

inflammatory cytokines such as TNF and IL-17 are critical drivers of the chronic inflammation as demonstrated by clinical efficacy of drugs blocking these cytokines. Within the IL-17 family, several IL-17A blockers have proven impact on chronic inflammation in AxSpA; preliminary evidence also suggests that IL-17A blockade may be more effective than TNF inhibition in halting pathological new bone formation (5, 6). More recently, IL-17F has been proposed to contribute beyond IL-17A in the pathobiology of both inflammation and new bone formation in spondyloarthritis (7–9). Also, other cytokines produced by so-called Th17 cells, including GM-SCF, have been implicated in the pathobiology of SpA (10), raising the question of the therapeutic value of targeting upstream activators of Th17 cells rather than IL-17 itself.

This concept has been amply explored in contrived *in vitro* and animal models, where IL-23 has been identified as a key factor in the differentiation, activation, and pathogenicity of Th17 cells (11). More importantly, drugs targeting either the IL-23–specific p40 subunit or the p19 subunit which is shared between IL-23 and IL-12 have shown impressive efficacy in skin psoriasis (12). Head-to-head studies even demonstrated the superiority of IL-23 inhibition over IL-17 inhibition in this disease, in line with a "cascade model" where "upstream" IL-23 drives downstream effector cytokines including but not restricted to IL-17A.

IL-23 INHIBITION IN AXIAL SPONDYLOARTHRITIS: WHAT THE CLINICAL TRIALS TAUGHT US

Considering the clinical and pathobiological link between psoriasis and spondyloarthritis and the efficacy of IL-17 blockade in both conditions, a randomized, placebo-controlled phase II clinical trial assessed the safety and efficacy of the anti-p19 antibody risankizumab in ankylosing spondylitis, the prototypical subform of AxSpA (13). Whereas no safety or intolerance signals were identified, the study failed to show any evidence for clinically significant improvement of the primary and secondary endpoints. Several lines of evidence concord to indicate that the unexpected outcome of this PoC trial is indeed true. First, the patient population is comparable to the patient populations included in other AS trials such as the anti-IL-17A trials. Second, the design of the trial including the endpoints are also well aligned with other AS trials. Third, the active drug, risankizumab, has proven efficacy in psoriasis and its PK profile was as expected. Fourth, primary, secondary, and exploratory endpoints consistently indicated lack of efficacy, and there was not even a trend toward a dose-response. Finally, a subsequent study testing the anti-p40 drug ustekinumab, which has also proven efficacy in psoriasis, also failed in ankylosing spondylitis (14). Taken together, these data provide a wealth of human pharmacological evidence that IL-23 may not be a relevant driver of the pathobiology and clinical symptoms of active, established AxSpA.

IL-23 AND AXIAL SPONDYLOARTHRITIS: REVISITING THE EVIDENCE

This unexpected clinical finding made us revisit the scientific reason-to-believe in IL-23 blockade in AxSpA. Beyond the previously mentioned understanding of the basic biology of the IL-23/IL-17 axis and the similarities and overlap between psoriasis and AxSpA, there were three main lines of evidence that had been considered. First, genome-wide association studies (GWAS) studies have clearly established that SNPs in the IL-23R are a susceptibility factor for ankylosing spondylitis (15) and several other genetic risk factors associated with ankylosing spondylitis also point toward the IL-23/IL-17 axis. However, the relative risk of these associations is moderate to low, several variants of IL-23R are associated with different diseases (including ankylosing spondylitis, psoriasis, Crohn's disease and ulcerative colitis), and the functional consequences of these variants remain incompletely understood. Second, over-expression of IL-23 in mice was reported to induce a spondyloarthritis-like phenotype by acting on RORgamma+ CD3+ CD4− CD8− entheseal T cells (16). However, this finding turned out to be hard to reproduce by other labs. On the contrary, we had demonstrated previously that systemic IL-23 exposure induced chronic arthritis, severe bone loss, and myelopoiesis in the bone marrow and spleen, which resulted in increased osteoclast differentiation and systemic bone loss (17), a phenotype which is not compatible with AxSpA. Third, there was indirect clinical evidence which, unfortunately, is methodologically flawed. A pilot study reported profound efficacy of the anti-p40 antibody ustekinumab in AS (18), but the uncontrolled, open-label design is completely flawed in a disease such as AS where the clinical outcomes are largely patient and physician dependent. Similarly, studies with ustekinumab in psoriatic arthritis reported a significant improvement in the BASDAI, a well-validated patient-reported outcome used in ankylosing spondylitis trials, and concluded that this drug was also effective for the axial symptoms in this disease. However, these studies ignored completely the fact that BASDAI, albeit having been developed for AS, does not in any way capture specifically axial disease and in fact is even a very good patient-reported outcome for peripheral arthritis (19).

In brief, the supporting evidence to believe in a central role for IL-23 in the pathobiology of AxSpA was, at best, circumstantial. More specifically, there is a striking lack of functional data to underpin if and how IL-23 contributes to the pathobiology of AxSpA. Studies with targeted therapies across an array of inflammatory conditions have indeed taught us that a single pathway or even cytokine with well understood basic biology can function in completely different way depending on the exact contact and thereby can drive clearly distinct pathobiology (20). In line with this, inhibition of IL-23 in collagen-induced arthritis and SKG mice ameliorated experimental arthritis but did not abolish pathology suggesting that other pathways remain active (21, 22). Besides the IL-23/IL-17 axis, we have now also demonstrated this for TNF: whereas the soluble form of TNF, signaling exclusively through the TNF-R1, drives profound

synovitis and bone destruction reminiscent of what we observe in human RA, the transmembrane form of the same cytokine drives osteoproliferative axial and peripheral joint inflammation as seen in SpA (23). Similarly, mutations in specific molecules of the IL-1 pathway lead to quite distinct autoinflammatory syndromes such as DIRA and CAPS, which affect other tissues and organs (24). All these examples highlight the importance to understand not only the basic biology of the cytokine pathway, but also to decipher its exact function and relative contribution in a particular disease context.

REVISITING THE PATHOBIOLOGY OF AXSPA

What have we now learned on the functional role of IL-23 in AxSpA? Two recent findings deserve further exploration in this context. First, could it be that IL-23 contribute to the disease initiation but becomes redundant in established disease? In other words: could IL-23 contribute to derail an IL-17 response which, once evolved to a state of chronic inflammation, persists even in the absence of IL-23? This concept is supported by our findings in the HLA-B27 transgenic rat model of SpA. The phenotype, histology, and pathobiology of this model recapitulates faithfully human SpA, is driven by the major genetic risk factor of human AS (HLA-B27) and responds well to both TNF and IL-17 blockade (25). Targeting IL-23R in this model lacked any efficacy in a therapeutic setting but did partially prevent disease onset in a prophylactic setting (26). Albeit intriguing from a scientific angle, this hypothesis may not be helpful from a clinical angle as it is at present impossible to capture and diagnose AxSpA patients in the early or even preclinical phase.

A second major insight is that IL-17 is not only produced by canonical Th17 cells but also by a variety of innate lymphocytes including MAT cells, gamma delta T cells, iNKT and ILC3 cells (27, 28). Those cell populations have been suggested to be less dependent on IL-23 for their IL-17 production: albeit IL-23 can certainly drive IL-17 production in these cells, other cytokines such as IL-1 and IL-18 are even more potent and IL-23 appears to be redundant in the presence of these other cytokines (29). This observation fits also with the fact that those innate immune cells were recently shown to amplify myelopoiesis *via* GM-CSF (30) and M-CSF signaling (31), which diversify the pathological signals of IL-23, extend them to other pathways including IL-18, and thereby render inhibition of IL-23 less effective in established disease. There is little information on the potential role of IL-18 in AxSpA. In contrast, multiple studies have demonstrated the association of IL1 gene cluster polymorphisms with AS, in particular polymorphisms in IL-1R2 (15) and IL-1A (32). Small scale proof-of-concept clinical trials with anakinra, a soluble IL-1 decoy receptor construct, yielded mixed results (33, 34) but it remains to be determined to what extend this relates to the biology, the therapeutic molecule, the trial design, and/or the target population. Collectively, it is therefore plausible that IL-23–independent pathways modulate the disease outcomes observed in AxSpA patients.

LESSONS LEARNED

In conclusion, the genetic, experimental, functional, and clinical studies on the role of IL-23 in ankylosing spondylitis have yielded a number of important lessons with broader relevance. First, the IL-23/IL-17 axis is not a linear "cascade." Rather IL-23 and IL-17 display partially overlapping but also partially distinct biology and pathobiology. Second, with the exception of monogenic diseases with high penetrance and rare extreme phenotypes, human genetic and expression studies are great tools to create hypotheses but are not fit for purpose to proof or disprove these. Third, animal models are still essential to help us to understand the biology of a pathway but one should remember that they are pathway models and, unfortunately, only sporadically disease models. Fourth, the function of a pathway and even a single inflammatory mediator is highly context dependent. Therefore, the development and validation of disease-relevant functional models is today one of the most critical factors needed to secure a rapid and adequate translational of emerging insights in basic immunology into novel therapeutics.

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.

AUTHOR CONTRIBUTIONS

DB and IEA drafted this manuscript, reviewed the content, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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REVIEW
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Why Inhibition of IL-23 Lacked Efficacy in Ankylosing Spondylitis

Dennis McGonagle[1,2*], *Abdulla Watad*[1,3,4], *Kassem Sharif*[1,3,4] *and Charlie Bridgewood*[1]

[1] Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, United Kingdom, [2] National Institute for Health Research (NIHR), Leeds Biomedical Research Centre (BRC), Leeds Teaching Hospitals, Leeds, United Kingdom, [3] Department of Medicine 'B', Zabludowicz Center for Autoimmune Diseases, Sheba Medical Center, Tel-Hashomer, Israel, [4] Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

The term spondyloarthritis pertains to both axial and peripheral arthritis including ankylosing spondylitis (AS) and psoriatic arthritis (PsA), which is strongly linked to psoriasis and also the arthritis associated with inflammatory bowel disease. The argument supporting the role for IL-23 across the spectrum of SpA comes from 4 sources. First, genome wide associated studies (GWAS) have shown that all the aforementioned disorders exhibit IL-23R pathway SNPs, whereas HLA-B27 is not linked to all of these diseases-hence the IL-23 pathway represents the common genetic denominator. Secondly, experimental animal models have demonstrated a pivotal role for the IL-23/IL-17 axis in SpA related arthropathy that initially manifests as enthesitis, but also synovitis and axial inflammation and also associated aortic root and cutaneous inflammation. Thirdly, the emergent immunology of the human enthesis also supports the presence of IL-23 producing myeloid cells, not just at the enthesis but in other SpA associated sites including skin and gut. Finally, drugs that target the IL-23 pathway show excellent efficacy for skin disease, efficacy for IBD and also in peripheral arthropathy associated with SpA. The apparent failure of IL-23 blockade in the AS which is effectively a spinal polyenthesitis but evidence for efficacy of IL-23 inhibition for peripheral enthesitis in PsA and preliminary suggestions for benefit in axial PsA, raises many questions. Key amongst these is whether spinal inflammation may exhibit entheseal IL-17A production independent of IL-23 but peripheral enthesitis is largely dependent on IL-23 driven IL-17 production. Furthermore, IL-23 blocking strategies in animal models may prevent experimental SpA evolution but not prevent established disease, perhaps pointing towards a role for IL-23 in innate immune disease initiation whereas persistent disease is dependent on memory T-cell responses that drive IL-17A production independently of IL-23, but this needs further study. Furthermore, IL-12/23 posology in inflammatory bowel disease is substantially higher than that used in AS trials which merits consideration. Therefore, the IL-23 pathway is centrally involved in the SpA concept but the nuances and intricacies in axial inflammation that suggest non-response to IL-23 antagonism await formal definition. The absence of comparative immunology between the different skeletal sites renders explanations purely hypothetical at this juncture.

Keywords: IL-23, psoriatic arthritis, ankylosing spondylitis, enthesis, IL-17

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Lars Rogge,
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Saikat Majumder,
University of Pittsburgh, United States

****Correspondence:***
Dennis McGonagle
D.G.McGonagle@leeds.ac.uk

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INTRODUCTION

The seminal clinical observations by Moll and Wright in the 1970s classified several diseases under the umbrella term of Spondyloarthorpathy (SpA) based on shared clinical and immunological features (1). These conditions included ankylosing spondylitis (AS), psoriatic arthritis (PsA) (and by extension of the psoriasis spectrum), inflammatory bowel disease (IBD) associated arthropathy including Crohn's disease and ulcerative colitis, enterogenic and urethrogenic reactive arthritis and anterior uveitis which is also associated with these conditions (2, 3). The common theme across these disorders was axial inflammation, peripheral lower limb oligoarthritis, enthesitis in some cases, a link to infection or intestinal dysfunction and negativity for rheumatoid factor (4, 5). A unified pathological understanding for the SpA associated arthropathy was not proposed in the original iteration of the concept.

Following on shortly after the Moll and Wright's SpA concept was the discovery of HLA-B27 that was associated with AS, PsA axial disease, IBD related axial arthritis, anterior uveitis and reactive arthritis (6–8). However, IBD itself or IBD related peripheral arthropathy was not associated with HLA-B27. The clinical features of Bechet's disease (BD) resulted in the investigators also proposing this to represent a member of the SpA concept in a paper that has been cited highly over four decades (9). The absence of sacroiliitis and the lack of a strong association with HLA-B27 meant that BD was never widely adopted in this proposed classification scheme. However, alluded to in the following discussion, GWAS studies have shown that the IL-23 pathway related genetic polymorphisms occur along the entire SpA arthropathy spectrum including ankylosing spondylitis and psoriatic arthritis, in psoriasis and inflammatory bowel disease and indeed in BD, thus completely vindicating the entire concept alluded to by Moll and Wright (9, 10).

CURRENT THERAPY IN AS

The current therapy options in AS include an anti-TNF agents for subjects that fail to respond to NSAIDS (Non-steroidal anti-inflammatory drugs). If anti-TNF is contraindicated or if there is loss of efficacy to anti-TNFs, one of two anti-IL-17A blockers can be considered with the provision that these agents should not be used for active associated IBD (11). The JAK inhibitors are likely to enter the clinical arena in AS in the coming years (12). Although guselkumab and ustekinumab may have some efficacy in PsA related axial disease (13, 14) there is no evidence for efficacy of this class of agent in AS from trials with ustekinumab and other p19 blocker risankizumab (15, 16).

IL-23/IL-17 AXIS

When naïve T-cells encounter a cognate antigen in lymphoid tissues, they have the ability to differentiate into effector T-cells, depending on the local microenvironment. This involves MHC peptide presentation to the T-cell receptor (TCR) (signal 1) and then co-activation with CD80/86 binding to CD28 (signal 2) on T-cells (17). In humans, cytokine stimuli such as IL-1β, IL-6, IL-21, and/or IL-23 can drive IL-17 production from T-cells, with the best described of these being CD4+ Th17 cells and CD8+ Tc17 cells (18). These IL-23 activated T-cells also secrete a range of other cytokines such as IL-17F, IL-22 and TNF (18).

THE IL-23 PATHWAY GENETIC ARGUMENT IN SPA SPECTRUM DISORDERS

Remarkably, IL-23R polymorphisms have been reported across all of the aforementioned categories of disease but not in classical autoimmunity (**Figure 1**). Furthermore, several SNPs related to the IL-23 pathway including those in the IL-23 cytokine itself, downstream JAK2 and Tyk2, STAT3 and IL-17RA signalling have also been reported across all of these diseases (5, 9, 19). A wealth of other genetic polymorphism data has strongly vindicated these findings insofar that classical autoimmune diseases have a completely different non-IL-23 pathway related genetic architecture (20). The IL-23R pathway SNPs are also associated with IBD (21) and BD (22), thus reinvigorating the historical ties with SpA as suggested by Moll and Wright and colleagues. The SNP in the IL-23R (R381Q) confers protection from IBD, AS and psoriasis (23–25). At a functional level, it results in a loss of function and less STAT3 activation and thus less induced IL-17 from T-cells (26, 27). Thus, it appears that "completely normal" IL-23 pathway signalling and functioning, which is comparatively higher than in subjects with the R381Q polymorphism is linked to AS. It might be theorised that anti-IL-23 therapy would reduce this further and align it with production levels associated with the protective allele. However, this has not been corroborated from trials in AS. While IL-23 pathway is genetically implicated in all the aforementioned tissues, the difference in relative contribution of IL-23 and other cytokines to the different SpA associated diseases shows differential efficacy as demonstrated by clinical trials (**Figure 2**).

TISSUE MICROANATOMY OF IL-23 PATHWAY AND ANIMAL MODELS

It is well established that the synovium is the primary target of inflammation in RA with autoimmunity directed against citrullinated synovial proteins driving an inflammatory reaction culminating in periarticular joint destruction and erosion, with the well-recognised polyarticular joint destruction phenotype. In the mid-1990s, MRI studies showed that enthesitis was evident in both swollen small and large joints in PsA and SpA in general (28). This resulted in the enthesitis based model for SpA whereby it was proposed cytokine mediated primary inflammatory reactions at the enthesis triggered an adjacent

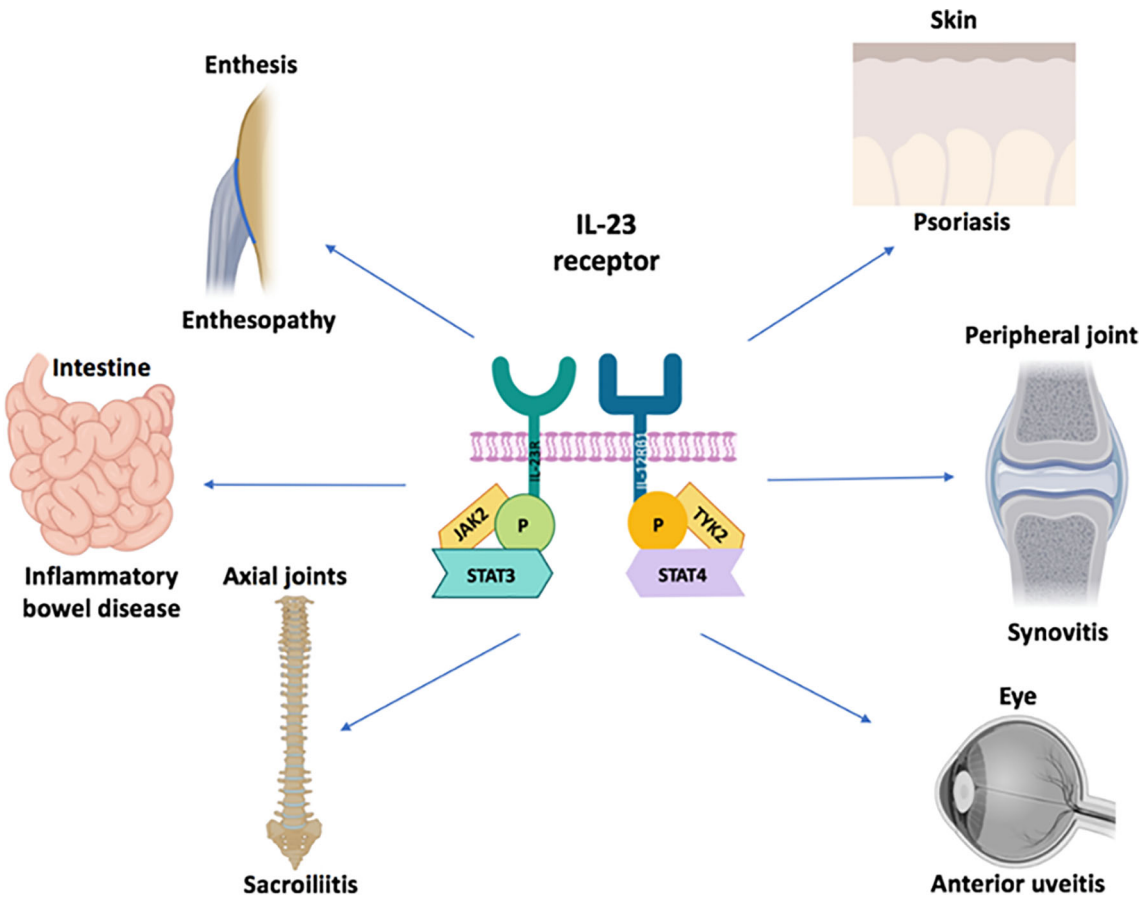

FIGURE 1 | IL-23 receptor polymorphism associated disorders. The IL-23 cytokine pathway has been firmly linked to ocular, cutaneous, intestinal disease and arthropathy at the genetic and cellular immunology level. There is considerable immunological heterogeneity in different organ responses. For example, the TNF-Fc fusion protein, etanercept does not work for IBD and is generally not effective for anterior uveitis. The role of IL-23 blockers for anterior uveitis awaits further definition but it is generally considered that anti-IL-17A blockers are not effective in this SpA domain All the agents of that antagonise the IL-23/17 axis show remarkable efficacy for psoriasis compared to joint disease. With respect to gut disease, the IL-17 blockers have an important role in barrier function at this site which may explain exacerbations of IBD under anti-IL-17A therapy. All three cytokines including TNF, IL-17A and IL-23 may be equally important for peripheral arthritis in PsA and also for enthesitis in the peripheral skeleton because good responses are seen following therapeutic antagonism. The anti-p40 IL-12/23 may be less effective than the other three categories of drugs for Psoriatic arthritis although further studies are needed. Finally, only TNF and IL-17 blockers have shown efficacy in the axial skeleton where IL-23 blockade with either p40 or p19 blockers has not worked. The site-specific compartmentalisation of immunity has come into sharp focus in the past few years and likely reflects tissue specific factors and microbiota interactive factors shaping diverse immune responses.

synovitis, tenosynovitis and osteitis (29–31). It was subsequently shown in animal models that dysregulated TNF production at the enthesis triggered polyarticular joint destruction, which further validated enthesitis as a mechanism of disease (32, 33).

A seminal paper by Sherlock et al. demonstrated that the normal murine enthesis harboured an IL-23R expressing cell population (34). This model was later confirmed to be Tyk2 dependent (Tyk2 mediates IL-23 signalling) (35) while the same paper found Tyk2 SNPs correlate with human AS disease progression. In the IL-23 minicricle model, the distal over-expression of the IL-23 cytokine in the murine liver using DNA minicircle technology resulted in a rapid onset of peripheral enthesitis that subsequently spread to the synovium and bone leading to polyarticular joint destruction. In the Sherlock model of IL-23 dependent enthesitis (34), it was subsequently shown by Reinhardt et al. that the majority of IL-17A producing cells in the normal murine enthesis were IL-23R expressing γδ T-cells (36). This population of cells are heterogeneous and carry out diverse functions including early innate immune responses, priming of adaptive immunity as well as prominent roles in tissue repair (37). The role of IL-23 in the SpA concept was strongly cemented in this model by the induction of psoriasiform skin inflammation, aortic root inflammation and also the development of axial inflammation (34). Other investigators using the same minicircle technology emphasised the role of severe synovitis and bone erosion and rheumatoid arthritis like features (38).

Of course, there are several independent animal models showing the pivotal role of IL-23 in experimental gut inflammation and reactive type arthritis (39, 40). A body of emergent research has also linked intestinal microbiota to the

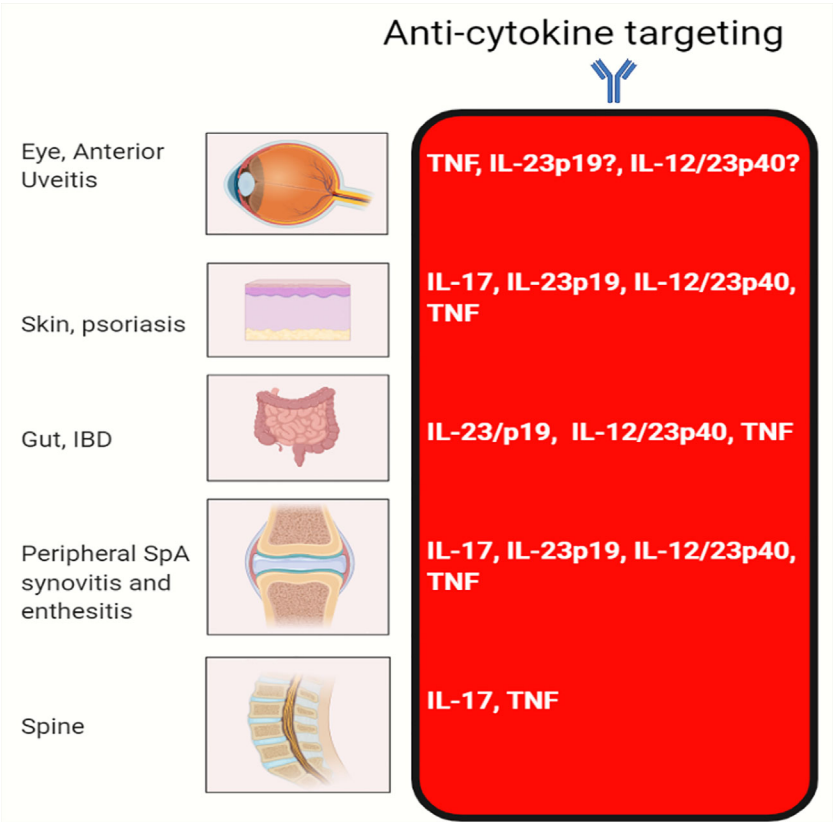

FIGURE 2 | Efficacy of cytokine blocking in different organs. The classical MHC class II associated autoimmune diseases that are characterised by autoantibody production segregate in families and individuals and show a female preponderance. The SpA group of diseases do not show substantial sex differences, have MHC class 1 genetic associations, a lack of specific confirmed disease associated autoantibodies and disease localisation to sites of injury or physical stressing. It is into this mix that genetics and experimental immunology have firmly confirmed the key role for the IL-23 pathway. Given that IL-23 regulates both IL-22 and IL-17, we believe that in addition to immunity including anti-fungal immunity *via* IL-17 regulation that the IL-23 pathway fine tunes tissue repair at sites of injury and physical or chemical stressing as for example in the intestine. TNF-Fc fusion protein, etanercept does not work for IBD and generally not effective for anterior uveitis.

IL-23/17 axis interdependence and cross-regulation with this being an area of active research (41–44). Another example of an IL-23 dependent SpA comes from the SKG mouse model that exhibits many of the SpA features in an IL-23/17 axis dependent pathway (45). The SKG contains a point mutation in the ZAP-70 gene, yielding reduced T-cell receptor signalling and following administration with fungal or bacterial adjuvants develops multi-organ inflammation and a SpA like disease (45). Collectively, these models support the idea that inflammation that is topographically enthesis centred drives disease (31).

EMERGING IMMUNOLOGY OF THE IL-23 PATHWAY AND IL-23/17 AXIS IN HUMAN SpA

Clinical trials in man dissect human disease immunopathogenesis and it is important to turn to these, in order to better understand human enthesitis. First, IL-17A blockers have proven efficacy for both peripheral and axial SpA including evidence for efficacy for isolated enthesitis as a secondary outcome measure (46–50). Likewise, the published literature shows efficacy for IL-12/IL-23 p40 blockers for peripheral PsA and for isolated enthesitis (30, 51–53). Recent studies have also shown that IL-23p19 blockade is effective for peripheral synovitis and related enthesitis (54, 55). These findings alone point towards a biological role for IL-23 at the non-axial peripheral enthesis (56), but what is the biological basis for this?

Following on from the Sherlock et al. study (34), our group investigated the presence of IL-23/17 axis cytokines at the normal human spinal enthesis. We defined normal spinal enthesis bone and soft tissue resident IL-23R expressing group 3 innate lymphoid cells (57). IL-23/IL-1β stimulation of normal human enthesis tissue resulted in upregulation of IL-17A and IL-17F transcript (57). Moreover, in humans we previously reported the presence of macrophages in acute enthesitis (58). This raised the possibility that local IL-23 production may be possible at the human enthesis and it was subsequently shown that the normal enthesis contains IL-23 inducible protein production from CD14+ myeloid cell following bacterial or fungal stimulation (59). We also found that this IL-23 secretion could be attenuated by the addition

of PDE4 blockers which may be relevant translationally since antagonism of this pathway shows efficacy for peripheral enthesitis in PsA in man (56). Both TNF and IL-17A are able to also induce osteogenesis *in vitro* in MSC from the spinal enthesis (60, 61).

COMPLEXITY OF THE IL-23 PATHWAY IN THE SPINE AND OTHER SpA FEATURES

Since the failure of IL-23 blocking in the AS, there has been great scientific speculation into the reason why (62). Remarkably, although the SpA group of conditions are closely interlinked, they also exhibit a differential immunopathology between different sites that is best encapsulated in the non-efficacy of therapies in some domains (**Table 1**). For example, the TNF fusion protein etanercept shows efficacy for the skeleton but not in the gut (68). Likewise, IL-17A blockers show impressive efficacy in the skin and good efficacy in the skeleton but are ineffective in the gut and in some circumstances are associated with IBD exacerbation (69). Laboratory research following the failed human trials of anti-IL-17A in Crohn's disease led to observations that γδ T-cell IL-17A production in the gut is produced independent of IL-23R signalling where IL -17 signalling was required for maintaining intestinal occludin junctions (70).

Given the aforementioned efficacy of IL-17 blockade in axial disease and the non-efficacy of IL-17A inhibition in the gut, the question arises as to whether there may be pathway for IL-17 production in the spine that is independent of IL-23 that may account for the curious reported lack of efficacy for IL-23 pathway inhibition in axial disease. Two trials of IL-23 pathway blockade including p40 and p19 blockade failed to show efficacy in AS, although marginal non-statistically significant improvements in CRP and subtle MRI improvements were evident under p40 antagonism (15, 16). There are two phase II trials of p19 blockers showing efficacy in psoriatic arthritis peripheral arthropathy including peripheral enthesitis (54, 55, 71). This has thrown up a new conundrum- how can a drug work for peripheral skeletal enthesitis but not axial enthesitis that underpins most of the AS pathology outside the sacroiliac joint. One important difference may be the presence of synovio-entheseal complexes in the peripheral skeleton but not in the spine (72).

EMERGENT CELLULAR PLAYERS IN THE NON-LINEARITY BETWEEN IL-23 AND IL-17 PATHWAYS IN SpA

Human γδ T-cells are classified into two major groups- δ1 and δ2 (73). We have explored the concept that there may be heterogeneity in these populations in man. Both the normal spinal entheseal soft tissue and peri-entheseal bone have resident γδ T-cell populations with these being more numerous in the peri-entheseal bone (74). In the enthesis resident γδ T-cell populations, we found that the δ1 population lacked IL-23R expression but that the δ2 population expressed this receptor. Only the δ2 population upregulated IL-17A in response to IL-23 signalling. However, both populations could be induced to express IL-17A upon PMA or anti-CD3/CD28 stimulation (74). Hence, the complexity of the IL-23 pathway extends to the spine and our results indicate that IL-17A, a key cytokine in AS and spinal inflammation, may not depend exclusively on IL-23. Accordingly, the IL-23/IL-17A axis is a two-sided coin with IL-17A production independent of IL-23 having very different biological consequences for gut and skeletal inflammation with IL-17A blockade in the former being detrimental but potentially beneficial in the latter (75, 76). In recent times, other theories have emerged of IL-17 secretion independent of IL-23. Mucosal-associated invariant T (MAIT) cells, are specialised innate-like T-cells that serve to bridge innate and adaptive immunity. MAITs are activated by conserved bacterial ligands which are derived from vitamin B biosynthesis, which are presented by the

TABLE 1 | Spondyloarthritis spectrum disease heterogeneity in immunotherapy responses.

Pathway	Agent	Adverse Event	Immunopathology	Recommendations	References
TNF	Infliximab, Etanercept, Adalimumab, Certolizumab pegol, Golimumab	Peripheral arthralgia in IBD therapy, Paradoxical psoriasis	Paradoxical upregulation of interferon pathways	Switch to IL-23 or IL-17 (except in IBD) inhibitors	(63, 64)
TNF	(Etanercept)	Uveitis, lack of efficacy in IBD	Mechanism unclear but in gut might be linked to fact antibodies may be linked to antibody dependent cytotoxicity for myeloid cells	Switch to a different TNF blocker	(63, 64)
IL-17	Secukinumab, Brodalumab, Ixekizumab	Inflammatory bowel disease	Dysregulation of the intestinal epithelial permeability which is regulated by IL-17A (tight junction).	Switch to TNF or IL-23 inhibitors	(65)
IL-23 (p40 and p19 blockers)	Ustekinumab, Rasinkizumab	Lack of evidence for efficacy in ankylosing spondylitis	Not understood but likely IL-17A production independently of IL-23		(15, 16)
α4β7 integrin	Vedolizumab	Sacroiliitis and synovitis	Abnormal intestinal barrier function and access of bacterial antigens, cytokines, adjuvants and pathogen-associated molecular pattern molecules to the systemic circulation and deposition in the peripheral skeleton at regions of entheseal tissue.	Switch to TNF or IL-12/23 blockers	(66, 67)

MHC-class I like MR1 to the TCR (77). Following TCR activation and also stimulation with IL-12 and IL-18, MAIT cells have been shown to secrete IL-17 that is independent of IL-23 (78). The human enthesis also contains conventional T-cells, both CD4+ and CD8+. Both entheseal CD4+ and CD8+ are able to secrete IL-17A following TCR stimulation (anti-CD3/CD28) without the need for additional IL-23 stimulation (79).

IL-23 BLOCKADE FOR THE PREVENTION OF SpA

The failed phase II trial of risankizumab in AS and the failed phase III ustekinumab trial in AS are responsible for these emergent immune insights (80, 81). This has been explored in the experimental SpA model induced in HLA-B27/Huβ2m transgenic rats that spontaneously develop SpA (82). These animals were either treated prophylactically with anti-IL-23R prior to disease onset or with control injections. Conversely the disease was allowed to fully manifest and then the animals were treated with anti-IL23R antibody or control. These experiments showed that IL-23 blockade was capable of preventing disease evolution but incapable of suppressing arthritis when fully established (82). How, exactly this relates to humans is unclear as the nuances of this rat model and its applicability to human SpA are not completely defined (4). For example, the findings might suggest a key role for memory T-cells that could produce IL-17A independent of IL-23 signalling. However, a role for CD8+ T-cells in HLA-B27 experimental SpA has never been substantiated (83), whereas the genetics of human SpA including HLA-B27, ERAP-1 and several other SNPs tends to incriminate this pathway (4).

There is some preliminary evidence supporting these animal models in humans. It has been recently shown that blocking of the IL-23 pathway with ustekinumab in psoriasis results in the regression of subclinical peripheral enthesopathy (84). Whether IL-23 blocker utilisation in psoriasis subjects will prevent axial inflammation evolution is an interesting and open question. It is worth pointing out that a secondary analysis of the pivotal phase III ustekinumab studies in PsA, showed efficacy in axial PsA including improvement in spinal domain pain (13).

Recent studies in abstract form have shown that patients with PsA enlisted in trials for polysynovitis, but also where 20% of patients had radiographic sacroilitis and back pain, that p19 blockade with guselkumab was associated with improvements in axial symptoms (14). These trials point to the possibility of inflammatory spinal disease immunological heterogeneity with some cases of PsA axial inflammation exhibiting a direct role for IL-23, which is stronger and different from that seen in AS.

SOME LOOSE ENDS WITH RESPECT TO IL-23 IN THE SPINE

It is unlikely that p19 blockade is interfering with the function of the poorly characterised cytokine IL-39, that also shares the p19 subunit (p19+EBI3) (85). Indeed, this cytokine remains a theoretical cytokine in humans with no evidence for either its formation or its function *in vivo* (85, 86). Hence, at this time it seems that the sole role of p19 blockade in main is on IL-23 and not another as yet ill-characterised cytokine, but further work is needed.

Most of the spinal inflammation in AS occurs in the peri-entheseal bone where disease localisation to this site is related to the HLA-B27 genetic status (58). Our work in human spinal entheses shows a much higher production or induction of IL-23 from the bone side of the enthesis (59). Whether this translates into therapeutics remains an open question and maybe higher doses of p19 blockers are needed to alleviate axial inflammation?

The failed trial of ustekinumab in AS used the 45mg and 90mg dosing regimen but the higher dose was associated with a non-significant CRP reduction and minor improvements in MRI lesions (81). The dose of ustekinumab used in Crohn's disease includes and 6 mg/kg intravenous loading dose (87) which is potentially the equivalent of 18 months of ustekinumab at the 90mg sc regimen for AS in the failed study. Clearly there is room for dose escalation to formally evaluate these questions. Also, it has been suggested that p40 blockers may restrain the immunoregulatory effects of IL-12 in the skin (88) and likewise there is uncertainty about any negative impact that p40 blockers could be exerting outside of the IL-23 pathway. However, the negative p19 study in AS argues against this.

It must be clearly articulated that translational therapy in man, and not laboratory experimental science is leading the understanding of these pathways. It is noteworthy that p40 blockers were associated with efficacy for axial symptoms in PsA, but it must be acknowledged that HLA-B27 negative axial PsA might represent a different disease from AS (89). A clinical short cut to understanding the dosing issues around IL-23 blockers may come from an evaluation of Crohn's disease therapy dosing on subjects with concomitant axial disease. Unfortunately, there is no comparative immunology between the spinal and peripheral entheses at this time so this is still largely conjectural.

CONCLUSIONS

For the purposes of this article the term SpA was taken to include the protean manifestations associated with axial inflammation including skin and gut involvement where it has clearly been shown that IL-23 SNPs are a common denominator across the different conditions. It is also clearly evident in experimental and human systems that the IL-23/IL-17 axis is involved in skin, gut and entheseal biology (90). A differential immunopathology exists within these disease domains reflecting the context dependent biology of different tissues that is currently best understood in terms of the barrier function role of IL-17A in the gut. The biological basis for IL-17 production in the spine that is seemingly independent of IL-23 needs verification, and if confirmed raises a vital question as to why IL-17A is so crucial to spinal immunobiology.

This non-linearity between IL-23 and IL-17 also appears to exist in the human spine but this knowledge is presently very rudimentary. Nevertheless, there is preliminary evidence suggesting that the downstream IL-17A pathway in axial biology is regulated in both IL-23 and IL-23 independent pathways. Further work is needed in man including IL-23 posology and careful assessment of disease subtypes and objective measures of inflammation including CRP and MRI appearances. The emergent biology of the IL-23/17 axis in the human skeleton strongly suggests that hidden within the current complexity is an IL-23 pathway, there may be a SpA subgroup with axial inflammation that might still be exploitable therapeutically with antagonism of this pathway.

AUTHOR CONTRIBUTIONS

CB, AW, KS and DM all contributed to scientific discussion, writing and figure making for the paper. All authors contributed to the article and approved the submitted version.

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