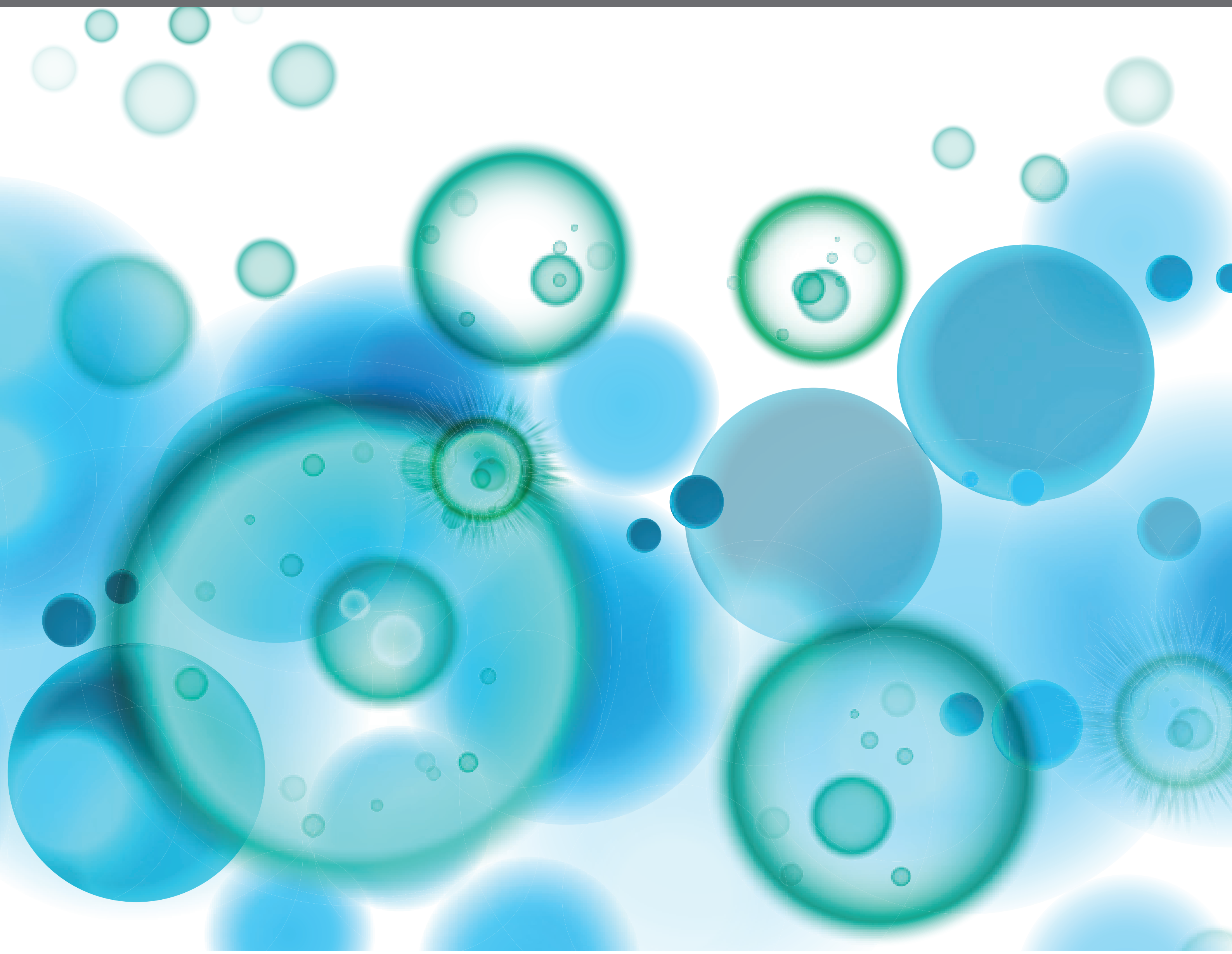
THE IL-17 CYTOKINE FAMILY IN TISSUE HOMEOSTASIS AND DISEASE

EDITED BY: Nicola Ivan Lorè, Kong Chen and Katarzyna Bulek
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-662-1
DOI 10.3389/978-2-88966-662-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

THE IL-17 CYTOKINE FAMILY IN TISSUE HOMEOSTASIS AND DISEASE

Topic Editors:
Nicola Ivan Lorè, IRCCS San Raffaele Scientific Institute, Italy
Kong Chen, University of Pittsburgh, United States
Katarzyna Bulek, Jagiellonian University, Poland

Citation: Lorè, N. I., Chen, K., Bulek, K., eds. (2021). The IL-17 Cytokine Family in Tissue Homeostasis and Disease. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-662-1

Table of Contents

frontiers in Immunology

EDITORIAL
published: 24 February 2021
doi: 10.3389/fimmu.2021.641986

Check for updates

Editorial: The IL-17 Cytokine Family in Tissue Homeostasis and Disease

Nicola I. Lorè[1,2], Kong Chen[3] and Katarzyna Bulek[4,5]*

[1] Division of Immunology, Transplantation, and Infectious Diseases, Emerging Bacterial Pathogens Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy, [2] Università Vita-Salute San Raffaele, Milan, Italy, [3] Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh, Pittsburgh, PA, United States, [4] Department of Immunology, Jagiellonian University, Kraków, Poland, [5] Department of Inflammation and Immunity, Cleveland Clinic, Cleveland, OH, United States

Keywords: IL-17 cytokine family, host-pathogen, cancer, autoimmunity, inflammatory disease

Editorial on the Research Topic

The IL-17 Cytokine Family in Tissue Homeostasis and Disease

The IL-17 cytokine family represents a wide class of pleiotropic inflammatory molecules that are structurally related. The IL-17 cytokines can modulate complex dynamic interactions between stromal and immune cells and determine the outcome of pathophysiological processes. Historically the most well-known cytokines across the IL-17 family are the IL-17A, IL-17F and IL-17E (also known as IL-25) while others such as IL-17B, IL-17C or IL-17D are emerging in modulating tissue homeostasis and disease (1). This cytokine family activates downstream signaling through the IL-17 receptor (IL-17R) family, which includes five members named IL-17receptor(R)A, IL-17RB, IL-17RC, IL-17RE, and IL-17RD (1, 2). In this context, IL-17RA can play a pleiotropic role by interacting with other IL17 receptors, including IL-17RC, IL-17RB, IL-17RE, and IL-17RD.

In this Research Topic you will find a number of original articles and reviews aiming at shedding light on the multifaceted role of IL-17 cytokines and their receptors in the field of immunity, host-pathogen interactions, autoimmunity and tumor immunology.

The mini review by Brevi et al. describes the role of the IL-17 family cytokines in the interplay between microbiota and epithelial cells that may contribute to the cancer pathogenesis. The authors purposely focused on IL-17B-to-F, which role is less understood, and discussed differences and similarities between these cytokines in the microbiota-immunity-cancer axis. Better understanding of these relationships may provide therapeutic strategies targeting IL-17-related diseases.

The emerging role of the IL-17B/IL-17RB axis in cancer has been discussed in review article of Bastid et al. with a particular attention on tumorigenesis and resistance to anticancer therapies. They described the expression and signaling pathways of the IL-17B/IL-17RB axis such as cellular sources and its role in inflammatory disease. They clearly highlighted how several reports proposed the potential role of IL-17B or its receptor in the outcome of different cancer types, such as breast carcinoma, gastric cancer, lung cancer, primary glioblastoma, lymphomas or acute myeloid leukemia. Moreover, they deeply described potential mechanisms of action by the IL-17B/IL-17RB axis not only in enhancing the proliferative, migratory and invasive properties of tumor cells, but also in impairing the anti-tumor immune response and favoring resistance to cancer treatments.

In the review article, Nies and Panzer discussed the latest discoveries about the identification, regulation, and function of the IL-17C/IL-17RE pathway. The authors described the mechanisms of IL-17C/RE driven inflammation in epithelial and Th17 cells and discussed its role in the context of infectious and autoimmune diseases. They summarized the role of the axis in bacterial, fungal, and viral infections. Moreover, they reviewed the first approaches to target IL-17C/IL-17RE axis, which they believed would be especially important for the treatment of autoimmune disorders.

OPEN ACCESS

Edited and reviewed by:
Silvano Sozzani,
Sapienza University of Rome, Italy

Correspondence:
Nicola I. Lorè
lore.nicolaivan@hsr.it

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *15 December 2020*
Accepted: *28 January 2021*
Published: *24 February 2021*

Citation:
Lorè NI, Chen K and Bulek K (2021) Editorial: The IL-17 Cytokine Family in Tissue Homeostasis and Disease. Front. Immunol. 12:641986. doi: 10.3389/fimmu.2021.641986

The study of Adams et al. shows the generation and the characterization of a novel humanized IgG1 antibody, named Bimekizumab, able to neutralize both IL-17A and IL-17F cytokines. In this context we can speculate that strategy to block both IL-17A and IL-17F cytokines may be useful as therapeutic option in diseases where the treatment anti IL-17RA failed. It's worth noting that the block of IL-17RA signaling may alter other inflammatory pathways modulated by other IL-17 receptors (e.g., IL-17RB or IL-17RC) and independent by IL-17A or IL-17F cytokines. This new monoclonal neutralizing antibody represents a new potential therapeutic strategy when both IL-17A and IL-17F contributes to disease progression.

Porphyromonas gingivalis can cause oral microbiome dysbiosis and contributes to the development of periodontitis. Using a mouse model, Bittner-Eddy et al. show that persistent oral *P. gingivalis* infection initiated an IL-17A-biased response dominated by Th17 cells and a distinct population of IL-17A-expressing Treg cells that changes into a late Th1 response with only sporadic phenotypic conversion from Th17 cells. Understanding the mechanism of Treg-Th17 transdifferentiation may provide novel targets to control the inflammatory disease processes.

Among the different potential biological function of IL-17A, Ramakrishnan et al. shows that IL-17A cytokine increases mitochondrial dysfunction in primary asthmatic bronchial fibroblasts. Moreover, IL-17 increased the expression of autophagy-related genes to a statistically significant extent in severe asthmatic fibroblasts than in healthy, suggesting a unique response to IL-17 stimulation in fibroblasts from patients with severe asthma. Overall, the data presented in this work suggest that IL-17 may be considered a potent inducer of pro-fibrotic phenotype through induction of autophagy in bronchial fibroblasts.

Chronic inflammation in obesity is believed to be associated with severity of asthma and Th17 cells have been shown to be associated with severe asthma due to their resistance to steroid treatment. Heialy et al. examined adipocytes responses to Th17 cytokines from lean and obese subjects and found out not only that stimulation leads to further inflammation in adipocytes obtained from obese subjects, but also that IL-17 may modulate adipocyte responses to steroids and obese adipocytes are not responsive to steroid treatment. Indeed, serum obtained from obese and morbidly obese asthmatic patients showed a significant decrease in GR-α/GR-β ratio, a marker for steroid resistance in adipocytes, and an increase in IL-17F and IL-13 compared to lean and overweight patients. To a certain extent, this study explains why steroid hyporesponsiveness is commonly described in obese asthmatics.

The mini review article of Capone and Volpe shed the light on the transcriptional regulators of T-helper 17 Cell differentiation in health and autoimmune diseases. The authors focused their attention on transcription factors modulating the levels of retinoic acid-related orphan nuclear receptors and IL-17A, with a particular attention on Th-17 population. The potential involvement of Th17-related transcriptional regulators has been deeply described in the context of autoimmune diseases, such as crohn's disease and multiple sclerosis. They concluded with an interesting overview on the potential therapeutic approaches of targeting transcriptional regulators of Th17 cells in experimental model of "autoimmune diseases" and clinical trials.

In the review article of Milovanovic et al., the critical role for IL-17 and T helper 17 cells have been discussed in the pathogenesis of chronic inflammatory and autoimmune diseases. In this context, the authors review and discussed the biological processes related to IL-17A signals and Th17 cells with a particular attention on environmental factors influencing the pathogenic potential of Th17 cells. Of interest they reviewed and discussed literature related to IL-17 cytokine and the cellular target as therapeutic approach in multiple sclerosis, Alzheimer's disease and ischemic brain injury. Overall this review article shows an interesting overview related to the role of IL-17 and its therapeutic potential targeting in the pathogenesis of neuroinflammatory and neurodegenerative diseases.

The research article of Chen et al. demonstrates that mTOR blockade can inhibit IL-17A and TNFα production and suggest that mTOR targeting may support an alternative therapeutic option in fighting of progression of spondyloarthritis disease. In particular, the targeting of mTOR is beneficial to inhibit IL-17A and TNFα protein production by human peripheral blood mononuclear cells from spondyloarthritis patients and to reduce IL-17A expression in inflamed joints using HLA-B27 tg rat model.

Cutaneous squamous cell carcinoma (cSCC) is the second most common type of non-melanoma skin cancer and genome-wide association study for cSCC suggests a role for aryl hydrocarbon receptor (AhR) and IRF4. Sato et al. investigated the role of AhR signal in keratinocytes for the development of cSCC using a two-stage chemically induced skin carcinogenesis mouse model and human cSCC samples. The authors showed that AhR ligands increase the expression of IL-17 downstream genes in normal human epidermal keratinocytes; the number of cutaneous SCC lesions is decreased in AhR deficient mice; and in patients' samples, atypical keratinocytes overexpress Th17 downstream genes in tumor lesions of cSCC as compared to normal keratinocytes at the marginal zone of the tumor. These data support the hypothesis that AhR ligands promote the development of cSCC through induction of Th17 cells.

Overall, this special topic highlights recent advances for a better understanding of IL-17 cytokine family in tissue homeostasis and disease. Here, we wanted to show the broad biological function mediated by IL-17 cytokine family ranging from immune defense against pathogens to the modulation of inflammation and tumor immunology, as observed in infection, inflammatory diseases or cancer. Moreover, several therapeutic approaches targeting the IL-17 cytokine family are available and they are paving the way for the development of specific strategies limiting the progression of different diseases including autoimmunity, chronic immune-mediated diseases, lung illnesses, and tumor immunology.

AUTHOR CONTRIBUTIONS

NL, KC, and KB edited the topic and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work has been supported by Fondazione Cariplo (grant n° 2016-0572) to NL; by National Science Centre in Poland (grant #2015/19/B/NZ6/01578) to KB.

ACKNOWLEDGMENTS

We wish to acknowledge all authors who have participated in this Research Topic and the reviewers for their insightful comments.

REFERENCES

1. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. *Immunity*. (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
2. Lorè NI, Bragonzi A, Cigana C. The IL-17A/IL-17RA axis in pulmonary defence and immunopathology. *Cytokine Growth Factor Rev*. (2016) 30:19–27. doi: 10.1016/j.cytogfr.2016.03.009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 26 February 2020
doi: 10.3389/fimmu.2020.00341

Check for updates

IL-17C/IL-17RE: Emergence of a Unique Axis in T_H17 Biology

Jasper F. Nies[1] *and Ulf Panzer*[1,2]*

[1] *Translational Immunology, III. Department of Medicine, University Medical Center Hamburg-Eppendorf Hamburg, Hamburg, Germany,* [2] *Hamburg Center of Translational Immunology (HCTI), University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

Therapeutic targeting of IL-17A and its receptor IL-17RA with antibodies has turned out to be a tremendous success in the treatment of several autoimmune conditions. As the IL-17 cytokine family consists of six members (IL-17A to F), it is intriguing to elucidate the biological function of these five other molecules to identify more potential targets. In the past decade, IL-17C has emerged as quite a unique member of this pro-inflammatory cytokine group. In contrast to the well-described IL-17A and IL-17F, IL-17C is upregulated at very early timepoints of several disease settings. Also, the cellular source of the homodimeric cytokine differs from the other members of the family: Epithelial rather than hematopoietic cells were identified as the producers of IL-17C, while its receptor IL-17RE is expressed on T_H17 cells as well as the epithelial cells themselves. Numerous investigations led to the current understanding that IL-17C (a) maintains an autocrine loop in the epithelium reinforcing innate immune barriers and (b) stimulates highly inflammatory T_H17 cells. Functionally, the IL-17C/RE axis has been described to be involved in the pathogenesis of several diseases ranging from infectious and autoimmune conditions to cancer development and progression. This body of evidence has paved the way for the first clinical trials attempting to neutralize IL-17C in patients. Here, we review the latest knowledge about identification, regulation, and function of the IL-17C/IL-17receptor E pathway in inflammation and immunity, with a focus on the mechanisms underlying tissue injury. We also discuss the rationale for the translation of these findings into new therapeutic approaches in patients with immune-mediated disease.

Keywords: IL-17C, IL-17RE, immunity, inflammation, Th17

OPEN ACCESS

Edited by:
Katarzyna Bulek,
Jagiellonian University, Poland

Reviewed by:
Peter A. Ward,
University of Michigan, United States
Piergiuseppe De Berardinis,
Istituto di Biochimica delle Proteine (IBP), Italy

***Correspondence:**
Ulf Panzer
panzer@uke.de

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity,
a section of the journal Frontiers in Immunology

Received: *05 December 2019*
Accepted: *12 February 2020*
Published: *26 February 2020*

Citation:
Nies JF and Panzer U (2020) IL-17C/IL-17RE: Emergence of a Unique Axis in T_H17 Biology. Front. Immunol. 11:341. doi: 10.3389/fimmu.2020.00341

INTRODUCTION

The discovery of T_H17 cells as a novel subset of $CD4^+$ T cells in 2005 (1) lead to a paradigm shift in the field of immunology. Our previously incomplete and inconsistent understanding of many diseases' pathogenesis was manifold enhanced thanks to rigorous examination of this new T cell lineage. These discoveries are not only important for basic immunological research, but drugs targeting T_H17-related molecules have had a significant impact on the treatment of immunological diseases (2–4).

As the name of the T_H17 cells was coined by their characteristic production of the highly inflammatory cytokine IL-17A upon activation, most scientific effort has been put into understanding the biological activity of this protein. However, five more cytokines with structural similarity to IL-17A have been identified (IL-17B-F). In this six-member cytokine family, IL-17A is best characterized, followed by the very closely related IL-17F. Structurally, all members of the IL-17 cytokine family are homodimers in their biologically active form, yet one heterodimer consisting of IL-17A and IL-17F (IL-17A/F) is described (5, 6). The proteins bind to heterodimeric receptor complexes to induce signaling in their target cells. Most of those complexes consist of the ubiquitously expressed subunit IL-17RA and a second, ligand-specific subunit (IL-17RB-RE) (7–11). IL-17D remains an orphan ligand in the cytokine family (**Figure 1**).

In line with the current understanding of T_H17 cells being a highly inflammatory lineage, IL-17A and F induce several inflammatory pathways. Most markedly, their binding to the receptor complex IL-17RA/RC, which is predominantly expressed on epithelial cells, leads to upregulation of cytokines, anti-bacterial peptides, and chemokines. The chemokines then recruit innate immune cells like neutrophils which potently enhance the inflammatory reaction. Thus, it is fair to say that by now we have got a good grasp of how IL-17A and F unfold their inflammatory effect.

The role of the remaining four IL-17 family members has long been considered rather elusive. However, the last years have shed a little more light on the IL-17C/RE axis, which unveiled some unique features.

In this review, we provide an overview of expression patterns and the functional importance of IL-17C and its receptor IL-17RE in immunological diseases, present a hypothesis of how the IL-17C/RE axis mediates its inflammatory effect, summarize intracellular signaling pathways, and give an outlook on translational approaches.

IDENTIFICATION OF THE IL-17C/RE AXIS

First Characterization of IL-17C

In 2000, the cytokine IL-17C has first been identified by a homology-search for proteins similar to IL-17A (12). *IL17C* is located on chromosome 16q24, is 1.1 kb long, and the protein IL-17C shares roughly 27% amino acid identity with IL-17A. Interestingly, after stimulation no induction of *IL17C* mRNA was observed in $CD4^+$ cells, which are the main source of IL-17A and F. This was the first evidence that IL-17C seems to assume a unique role in the IL-17 family. In an initial functional analysis of the protein, the authors showed that IL-17C stimulated the monocytic cell line THP-1 to release TNF-α and IL-1β.

IL-17C Is Expressed by Epithelial Cells and Not by Hematopoietic Cells

Unlike what is known about the other IL-17 family members, many studies suggest that *IL17C* is not expressed by leukocytes, but by non-hematopoietic cells.

The characteristic production of IL-17A by a subset of $CD4^+$ cells has led to the name of T_H17 cells, which emerged to be a distinct lineage apart from the classical dichotomy of T_H1 and T_H2 cells. However, not only $CD4^+$ T cells produce IL-17A, but also $CD8^+$ T cells (13), γδ T cells (14, 15), invariant natural killer T cells (iNKT) (16), group 3 innate lymphoid cells (ILCs) (17), and even B cells (18).

In contrast, *IL17C* is expressed by epithelial cells. In a model for psoriasis, keratinocytes are the main source of IL-17C (19). Several groups confirmed this *IL17C* expression in keratinocytes (20–23). Other epithelial cells producing the cytokine include colonic epithelial cells (9), resident kidney cells (24, 25), and respiratory epithelial cells (26–28).

Although this strong evidence points to epithelial cells as the main source of IL17C, its expression has also been found in leukocytes (29, 30) and smooth muscle cells (31).

IL-17C and the Microbiome

T_H17 biology is closely linked to the microbiome as it influences T_H17 cell development: Experiments with antibiotic treatment or germ-free mice drastically reduced intestinal T_H17 cells (32, 33).

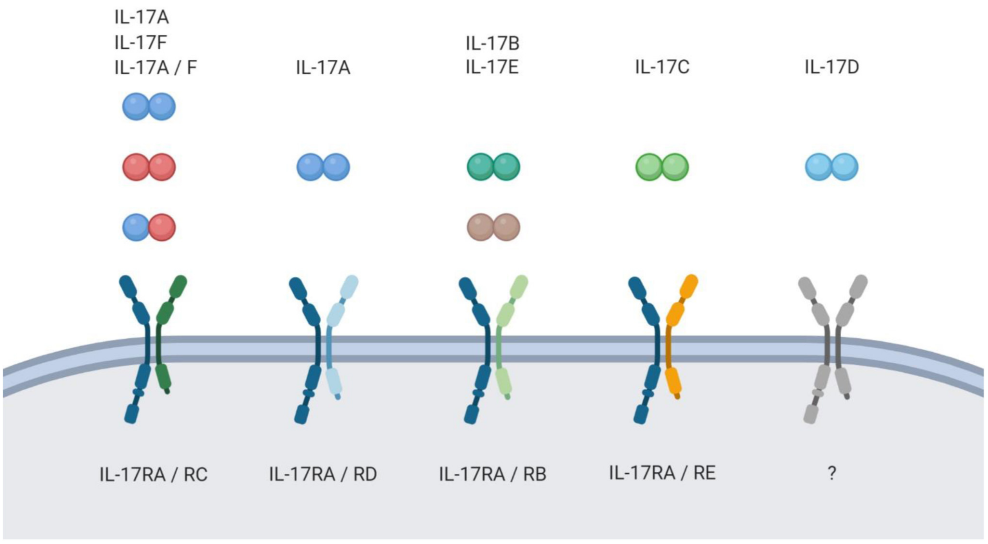


FIGURE 1 | The IL-17 family. Schematic overview of the IL-17 family members and their respective receptor complexes.

However, specific bacteria are required for proper induction of this cell type. Segmented filamentous bacteria (SFB) can potently induce the T_H17 cell development (34, 35), while *Bacteroides fragilis* suppresses this differentiation (36). Thus, changes in the gut flora influence the development of T_H17 cells, which can both aggravate or ameliorate extra-intestinal T_H17-driven autoimmunity (37).

Even though T_H17 cells themselves are not the source of IL-17C, intestinal bacteria still seem to play a role for *IL17C* expression in the gut. Antibiotic treatment of mice blocked the induction of *Il17c*. IL-23 and IL-22 were reported to be dispensable, but TLR-MyD88 signaling in gut resident cells was essential for the induction of *Il17c* expression. In the same experiment, the authors identified MyD88 as being essential for proper induction of *Il17a,* but not *Il17c*, in hematopoietic cells (38). Co-culture of murine colonic epithelial cells with *Citrobacter rodentium* induces IL-17C production in those cells. Specifically, Lipopolysaccharide (LPS) and flagellin are two pattern-associated molecular patterns (PAMPs) that can be recognized by toll-like receptors (TLRs) and culturing the cells with those components alone resulted in strong IL-17C production (9). A change in *Il17c* expression was not observed in any of the analyzed leukocyte populations (T lymphocytes, B lymphocytes, intraepithelial lymphocytes, lamina propria mononuclear cells). This finding was validated by the fact that no difference in *Il17c* induction was seen between wildtype and recombination-activating gene 1 (*Rag1*) deficient mice. Within the non-leukocytic cell populations in the colon, *Il17c* induction was indeed limited to only the colonic epithelial cells since no mRNA upregulation *Il17c* was seen in colonic stromal cells after infection (9). Those findings indicate that TLR activation by microbiota in the gut is important for both IL-17A and IL-17C, albeit the source of those cytokines is located to different cell types: Hematopoietic cells and gut resident epithelial cells, respectively.

Thus, epithelial cells are the main source of the cytokine in different tissues. That stands in stark contrast to the cellular source of other cytokines of the IL-17 family, which are mainly expressed by leukocytes.

IL17C Is Upregulated Early in Disease

Regarding the temporal expression of *IL17C*, current data point to an early upregulation during disease. *Il17c* mRNA was strongly upregulated after 4 days in the colons of bacterially infected mice, while *Il17a* expression peaked at day 12 (9). Ramirez-Carrozzi et al. analyzed the kinetics of *Il17c* expression in detail: *in vitro* stimulation of HCT-15 cells with heat-killed *E. coli* lead to a rapid expression of the cytokine after 1 h and murine skin challenged with imiquimod showed strong *Il17c* expression after 2 days. In the DSS-colitis model, the authors found induction of *Il17c* expression after 2 days in colons and mesenteric lymph nodes, but upregulation of *I17a* and *Il17f* mRNA transcripts was not detected before day 6 (19). In the nephrotoxic nephritis (NTN) mouse model for crescentic glomerulonephritis, we showed that *Il17c* is upregulated as early as 12 h after induction of the disease, while *Il17a* and *Il17f* expression starts after a couple of days (24).

IL-17C Binds to the Receptor Complex IL-17RA/RE

The group that first described IL-17C also suggested that IL-17C does not bind to IL-17RA, but to another receptor. Expressing a His-tagged and metabolically labeled form of the extracellular domain of IL-17RA in 293T cells, precipitation with IL-17A was observed as expected, but no precipitation could be detected during incubation with IL-17C (12). Six years later, another group discovered this receptor, which has been named IL-17RE. Murine IL-17RE shares 40% DNA and 18% amino acid sequence with IL-17RC (39). However, the authors did not yet identify a ligand binding to this receptor subunit. The receptor was found to be expressed in lung, kidney, stomach, intestine, and testis of mice and to have six different isoforms.

Several groups described that IL-17C is the specific ligand for IL-17RE in 2011: Transfection of 293T cells with the receptor subunits IL-17RA-RE revealed that IL-17C seems to bind exclusively to IL-17RE (40). Another group used a similar approach by analyzing the binding of Flag-tagged human IL-17C to HEK293 cells overexpressing each of the five IL-17 receptor subunits. In contrast to the findings of the first characterization of IL-17C (no binding to IL-17RA) (12), flow cytometer examination of the cells after incubation with IL-17C showed binding to both IL-17RA and IL-17RE, but none of the other receptor subunits. Also, no interactions were found between IL-17RE and any of the other IL-17 cytokine family members (19).

Song and colleagues used glutathione S-transferase precipitation to demonstrate that IL-17C associates not only with IL-17RE but with a heterodimeric receptor complex consisting of IL-17RA and IL-17RE (9). Using a blocking antibody against IL-17RA during stimulation of keratinocytes with IL-17C, a dose-dependent inhibition of IL-17C-induced G-CSF and β-defensin-2 expression was observed, underlining the functional dependence on IL-17RA (19).

The IL-17RA/RE Receptor Complex Is Expressed on Both Epithelial and T_H17 Cells

Interestingly, epithelial cells—the main source of IL-17C—express the specific receptor for the cytokine. Strong *Il17re* expression has been detected in keratinocytes and colon epithelial cells (19). Reynolds et al. report expression of the receptor subunit in the colonic epithelial cell line YAMC (41). *IL17RE* is also expressed in nerve fibers of human skin after HSV-2 reactivation (21).

Apart from epithelial cells, Chang et al. *first* described that *Il17re* is expressed on T_H17 cells. While numerous tissues express an isoform of IL-17RE that lacks the transmembrane domain, T_H17 cells expressed high amounts of full-length *IL17RE*. This expression is strongly enhanced when the cells are stimulated with a cytokine cocktail of IL-6, TGF-β, IL-1, and IL-23 and IL-17C also induced expression of the receptor on T_H17 cells (40). Validating this finding, our group found strong *Il17re* expression IL-17A$^+$ YFP$^+$ cells from IL-17A YFP$^+$ fate reporter

TABLE 1 | Sources of IL-17C and IL-17RE.

Protein	Cell type	References
IL-17C	Keratinocytes	(19–23)
	Resident kidney cells	(24, 25)
	Colonic epithelial cells	(9)
	Respiratory epithelial cells	(26–28)
	Smooth muscle cells	(31)
	Leukocytes	(29, 30)
IL-17RE	T_H17 cells	(24, 40)
	keratinocytes	(19)
	Colonic epithelial cells	(19, 41)
	Skin nerve fibers	(21)

Overview of cell types producing IL-17C and IL-17RE.

mice and in T_H17 polarized cells (compared to T_H0, T_H1, and Treg cells) (24).

Similar to *IL17C,* we reported that *Il17re* was upregulated 24 h after induction of NTN (24).

In summary, epithelial cells produce IL-17C at early timepoints in disease. The cytokine signals through the heterodimeric receptor complex IL-17RA/RE. This complex is expressed by several epithelial cells themselves. Secondly, T_H17 cells express *IL17RE* which indicates that this T cell lineage is also responsive to IL-17C (**Table 1**).

INFECTION AND AUTOIMMUNITY

Many studies report that *IL17C* expression is upregulated at an early stage in both infectious and autoimmune diseases. This suggests that it is involved in the innate first-line immunity in the pathogenesis of those conditions. Intriguingly, IL-17C also plays an important role in the initiation of the adaptive immune response later. First, we will take a closer look at the role of the IL-17C/RE immune axis in infectious diseases. Second, we will zoom in on autoimmune conditions.

IL-17C/RE Signaling Induces Innate Immune Functions in Bacterial, Fungal, and Viral Infections

Signaling through the IL-17C/RE is involved in host defense against foreign pathogenic microorganisms. In the following paragraphs, we will summarize the role of the axis in bacterial, fungal, and viral infections.

Bacterial Infections

The IL-17C/RE axis plays a significant role in several bacterial infection models. Mice infected with the intestinal pathogen *Citrobacter rodentium* showed upregulation of *Il17c* mRNA in the colon (9). *Ex vivo* cultured murine colon tissue and colonic epithelial cells showed marked mRNA expression of antibacterial peptides, inflammatory cytokines, and chemokines after stimulation with IL-17C. Clinically, lack of signaling through the IL-17C/RE axis modeled with *Il17re*$^{-/-}$ mice lead to decreased mRNA levels of said molecules and failure to clear the infection. This resulted in loss of body weight, higher intestinal and splenic weight, increased bacterial burden, and death. Interestingly, there was no difference when the cells were treated with IL-17A or F, which indicates that IL-17RE is dispensable for these two cytokines.

In a model of acute colitis, *I17c*$^{-/-}$ mice challenged with dextran sulfate sodium (DSS) had a significantly worse outcome than mice with physiological IL-17C production, which is reflected by earlier and more pronounced weight loss and colonic shortening. The authors explain this observation with the fact that IL-17C induced mRNA expression of tight-junction molecules, which are essential for the integrity of the colonic mucosal barrier (41).

Another group examined the role of IL-17RE in this model confirming those findings of the IL-17C/RE axis assuming a crucial role in protection against bacteria-driven DSS-induced colitis (19).

The immune axis also plays a role in the defense against airway infections with *Pseudomonas aeruginosa* and *Haemophilus influenza* (26, 27).

Fungal Infections

The impact of IL-17C has also been examined in fungal infections. Huang and colleagues reported that IL-17C is required for a lethal course of systemic infection with *Candida albicans* in mice since *II17c*$^{-/-}$ mice displayed increased survival and less severe functional and morphological kidney damage (25). This is in contrast to the function of IL-17A in this model: While *Il17a* overexpression protects the mice, lack of signaling through IL-17RA results in increased susceptibility to this fungal infection (42). Similarly, patients with Job's syndrome, a condition with T_H17 cell defects, are also at great risk to suffer from such fungal infections (43, 44). Another study reported that IL-17C is not involved in immunity to systemic, oral and dermal candidiasis (45). Even though *Il17c* mRNA expression was upregulated 2 days after exposure to the fungus, the group did not observe a difference in clearance of the infection or gene expression profiles between mice lacking IL-17C or IL-17RE compared to a wildtype control group.

Viral Infections

Two studies investigated the role of IL-17C/RE in viral infections. Peng et al. showed that IL-17C was the only IL-17 family cytokine that was induced in keratinocytes from human genital skin biopsies during recurrent HSV-2 reactivation. Also, cultured human keratinocytes produced IL-17C in response to infection with HSV-2. Since cutaneous nerve fibers expressed *IL17RE* and *ex vivo* application of IL-17C reduced apoptosis in the nerve cells, the authors hypothesize that keratinocyte-derived IL-17C serves as a protective agent for nerve fibers during HSV-2 reactivation in the skin (21). Another group recently analyzed the effects of IL-17C in *in vitro* virus-bacteria coinfection of human bronchial epithelial cells to assess the cytokine's role in COPD exacerbations. A challenge with both pathogens resulted in a synergistic induction of IL-17C. Interestingly, tissue from healthy smokers released little IL-17C upon exposure to the pathogens, but epithelial cells from COPD patients released significantly more. Thus, the IL-17C/RE axis might be involved in the pathogenesis of COPD exacerbations of mixed upper airway infections (46).

Several T_H17-Driven Autoimmune Diseases Are Exacerbated by IL-17C/RE

Inflammation orchestrated by T_H17 cells is a hallmark of various autoimmune conditions like rheumatoid arthritis, psoriasis, multiple sclerosis, autoimmune kidney diseases, and autoimmune hepatitis.

In 2007, Yamaguchi et al. attributed IL-17C a role in the pathogenesis of collagen-induced arthritis (30). Mice adoptively transferred with $CD4^+$ T cells, which were retrovirally transduced with either IL-17A, B, C, or F, had significantly higher arthritis scores than those that got cells transduced with an empty vector.

Several studies also evaluated the role of IL-17C in skin inflammation complementing the picture of IL-17C-induced auto-aggression. Johansen et al. first showed that *IL17C* mRNA and protein levels were increased in the skin of patients with psoriatic lesions compared to non-lesional skin (47). In fact, IL-17C is by far the most abundant IL-17 cytokine found in the skin of such skin lesions: Its protein levels were reported to be roughly 125-fold higher than those of IL-17A in the lesions (48). Transgenic mice lacking *Il17c*, *Il17ra,* or *Il17re* display a less severe course of imiquimod-induced psoriasis (19, 49) while an overexpression of *Il17c* in skin keratinocytes lead to spontaneous development of psoriasiform skin lesions (48). IL-17C also drives inflammation in atopic dermatitis as *IL17C* expression was increased in lesional skin of patients and blocking IL-17C with an antibody ameliorated skin inflammation in one mouse model for psoriasis and two models for atopic dermatitis (50).

Also, IL-17C/RE signaling aggravates the course of experimental autoimmune encephalitis (EAE) (40). *Il17c*$^{-/-}$ mice were less prone to develop the disease and those that did showed less pronounced clinical manifestations of the inflammation. Vice versa, increased signaling through the axis in transgenic mice overexpressing *Il17re* in $CD4^+$ cells lead to a worse clinical situation of the animals.

We have recently described that the serum levels of IL-17C are significantly higher in patients with ANCA-associated glomerulonephritis compared to a healthy control group, which was not true for IL-17A, F, and B. We showed the pro-inflammatory role of IL-17C in established mouse models for lupus nephritis and crescentic glomerulonephritis. In accordance with the mentioned previous studies, our experiments showed expression of *Il17re* by T_H17 cells and significantly less T_H17 cells in inflamed kidneys of both *Il17c*$^{-/-}$ and *Il17re*$^{-/-}$ mice (24).

Two studies investigated the involvement of the IL-17C/RE axis in autoimmune hepatitis. One group found evidence that IL-17C stimulates intrahepatic $CD4^+$ T cells to release IL-2 with subsequent NK-cell mediated liver damage. In this study, lesser levels of GOT and GPT in sera of *Il17c*$^{-/-}$ and *Il17re*$^{-/-}$ mice were found compared to wildtype mice (51). However, another group found no differences in GOT and GPT activities and granulocyte infiltration into the liver between *Il17c*$^{-/-}$ and wildtype mice in the same model (52).

Further diseases involving the IL-17C/RE axis include psoriasiform skin lesions in inflammatory bowel disease (IBD) patients under anti-TNF-α treatment (53), recurrent aphthous ulcers (20), LPS-induced endotoxin shock (52), and different forms of cancer (38, 54–56) (**Tables 2**, **3**).

MECHANISMS OF IL-17C/RE DRIVEN INFLAMMATION

Mechanistically, a body of evidence suggests that IL-17C exerts two important immunological effects: (a) In an autocrine feedback loop with epithelial cells, IL-17C strengthens innate barriers against infectious agents. (b) Boosting T_H17 cell function, IL-17C also stimulates the adaptive immune system to efficiently fight off infections. Yet, those pathways harbor the risk of T_H17-driven autoimmunity.

As IL-17A has been studied much more extensively as IL-17C and acts on epithelial cells, it is worthwhile to recapitulate the signaling of IL-17A through IL-17RA.

The similar expression of fibroblast growth factor and IL-17R (SEFIR) domain is highly conserved within the IL-17 receptor family and structurally similar to the Toll/IL-1R (TIR) domain found in TLRs and the IL-1β receptor (57). Yet, IL-17 signaling employs an adaptor protein unique to IL-17 signaling called ACT1, which also carries the SEFIR domain. The adaptor protein can then bind several intracellular signaling proteins to induce several conserved signaling pathways. Pathways activated by IL-17 receptor signaling include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (58), inhibitor of NF-κB ζ (IκBζ) (59), mitogen-activated protein kinase (MAPK) (60–62), and CCAAT/enhancer-binding protein (C/EBP) (63, 64). Together, these pathways mediate mitogenic signals and induce expression of pro-inflammatory cytokines and chemokines.

Another domain called TIR-like loop (TILL) domain is crucial for IL-17 signaling but is only present in the IL-17RA subunit (65). However, most of the other subunits of this family heterodimerize with IL-17RA to form a functional complex, which suggests that IL-17RA is the domain necessary for intracellular signaling. Also, the C/EBPβ activation domain (CBAD) on IL-17RA stimulates signaling through the transcription factor C/EBPβ (65), initiating one of the few known inhibitory mechanisms of IL-17 signaling (66).

A very important aspect of IL-17 signaling is synergism: *De novo* gene expression by IL-17A in target cells does not fully account for the observed strong inflammatory effect of the cytokine. Signaling through IL-17RA stabilizes mRNA transcripts of genes expressed by other strong inflammatory stimuli like TNF-α (59, 67). Ligand binding to IL-17RA recruits the kinase IKKi to phosphorylate ACT1. TRAF2 and 5 then bind to form a complex that can inhibit cleavage of mRNA (61, 68).

Thus, the full biological activity of IL-17A becomes apparent only in concert with other factors of the inflammatory milieu. Such synergetic effects have also been described between IL-17C

TABLE 2 | Main findings of experimental data on IL-17C and IL-17RE.

Disease model		Mice used	Main phenotype of investigated group	References
Experimental autoimmune encephalitis (EAE)		*Il17c*$^{-/-}$	Less clinical manifestation, lower mortality	(40)
		Il17re overexpressing CD4^{+} T cells adoptively transferred to wildtype C57Bl/6	Increase in EAE symptoms	
Nephrotoxic Nephritis (NTN)		*Il17c*$^{-/-}$ *Il17re*$^{-/-}$	Reduced functional and morphological kidney damage, less renal Th17 infiltration	(24)
Pristane-induced lupus nephritis		*Il17c*$^{-/-}$	Reduced functional and morphological kidney damage	(24)
Psoriasis	Imiquimod-induced	*Il17c*$^{-/-}$ *Il17re*$^{-/-}$	Less severe course of the disease	(19, 49)
	Il-17c-induced	*Il17c* overexpression in keratinocytes of wildtype C57Bl/6	Spontaneous development of psoriasiform skin lesions	(48)
	IL-23-induced	BALB/c	Reduced ear swelling and acanthosis under anti-IL-17C treatment	(50)
Con-A-induced autoimmune hepatitis		*Il17c*$^{-/-}$ *Il17re*$^{-/-}$	Lesser levels of GOT and GPT, attributed to inhibited NK-cell mediated liver damage	(51)
		Il17c$^{-/-}$	No difference in GOT and GPT levels or hepatic granulocyte infiltration	(52)
Collagen-induced Arthritis (CIA)		*Il17c* overexpressing CD4^{+} T cells adoptively transferred to DBA1 mice *Il17c* BM chimeric mice	Higher arthritis scores than control	(30)
Atopic dermatitis	MC903-induced	BALB/c	Less severe ear swelling under anti-IL-17C treatment	(50)
	Flaky tail	Flaky tail (Matt^{ma}/^{ma}Flg$^{ft/ft}$)	Less hair loss and excoriation and ameliorated blepharitis under anti-IL-17C treatment	
Dextrane sulfate sodium (DSS) induced colitis		*Il17c*$^{-/-}$ *Il17re*$^{-/-}$	More pronounced body weight loss and colonic shortening due to lesser expression of antibacterial, inflammatory, and tight-junction molecules	(19, 41, 52)
Citrobacter rodentium infection		*Il17re*$^{-/-}$	More body weight loss, higher intestinal and splenic weight, higher bacterial burden, higher mortality	(9)
Pseudomonas aeruginosa airway infection		*Il17c*$^{-/-}$	Increased survival	(26)
Systemic *Candida albicans* infection		*Il17c*$^{-/-}$	Increased survival and less severe kidney damage	(25)
Systemic, oral and dermal Candidiasis		*Il17c*$^{-/-}$ *Il17re*$^{-/-}$	No difference between knockout and wildtype groups	(45)
LPS-induced endotoxin shock		*Il17c*$^{-/-}$	Higher resistance to endotoxin-induced shock.	(52)

Summary of current data on the IL-17C/RE axis in mouse models.

and three other cytokines: TNF-α (19, 24, 48), IL-22 (9, 24), and IL-1β (19). However, the underlying molecular mechanisms have not specifically been studied for IL-17C/RE signaling.

IL-17C and the Epithelial Cell

The first site of IL-17C immunity is the epithelial cell. Group-specific innate signaling pathways like the activation of TLRs in response to PAMPs induce expression of *Il17c* (9, 19, 38). Activation of TLR is one of the first responses of the immune system after contact with pathogens, which explains the early upregulation of *IL17C* in the various infectious diseases. Intracellular MyD88 signaling induced by TLR2 and 5 agonists or IL-1β stimulated the expression of *IL17C* in mucosal epithelial cells (19). Another intracellular mechanism for *IL17C* expression in response to pathogens is activation of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) by *Staphylococcus aureus* (69).

There is strong evidence for a synergistic effect between TNF-α and IL-17A as IL-17A signaling stabilizes mRNA of target genes of TNF-α. Interestingly, one target gene that is synergistically induced by IL-17A and TNF-α is *IL17C* (70). However, stimulation of murine and human epithelial cells with TNF-α or IL-17A alone is also capable of upregulating *IL17C* expression (9, 19).

These findings are underlined by the fact that *IL17C* expression is decreased in skin biopsies of psoriasis patients under anti-TNF-α therapy (22). Likewise, IL-17RA blockade with Brodalumab lead to decreased levels of *IL17C* expression in psoriatic skin (71).

In terms of signaling cascades, TNF-α signaling seems to employ the p38 mitogen activated protein kinase (22) and the NF-κB pathway to enhance *IL17C* expression. Direct evidence of this are three bindings sites for NF-κB in the *IL17C* promotor (23).

TABLE 3 | IL-17C/RE data on human samples.

Disease	Main finding	References
Psoriasis	Elevated levels of *Il17c* mRNA and IL-17C protein in lesional patient skin. Impaired *Il17re* expression in those lesions.	(47)
	IL-17C most abundant IL-17 cytokine in lesional skin (125-fold of IL-17A)	(48)
ANCA-associated glomerulonephritis	IL-17C as the only IL-17 cytokine with elevated serum protein levels	(24)
Atopic dermatitis	Increased *Il17c* expression and positive immunohistochemistry staining for IL-17C in skin of atopic dermatitis patients	(50)
Recurrent aphthous ulcers (RAU)	Human oral keratinocytes stained positive for IL-17C in RAU lesions of patients and expressed TNF-α in response to IL-17C *in vitro*	(20)
Anti-TNF-α-induced psoriasiform skin lesions in Crohn's disease	High IL-17C protein concentrations in skin lesions	(53)
HSV-2 reactivation in genital skin	Protective effect of IL-17C on skin neurons	(21)
Pseudomonas aeruginosa airway infection	Enhanced inflammatory response to infection by human epithelial cell line	(27)
Virus-bacteria coinfection in COPD	Coinfection led to synergistic upregulation of *Il17c* in human bronchial epithelial cells; stimulation with IL-17C upregulated chemokines.	(46)

Summary of current human data on the IL-17C/RE axis.

Thus, both PAMPs and pro-inflammatory cytokines can induce *IL17C* expression. TNF-α and IL-17A are able to induce *IL17C* individually and a strong synergistic effect between the two cytokines drastically boosts the expression.

Binding of IL-17C to the IL-17RA/RE complex on the epithelial IL-17C-source cells forms an autocrine loop in the epithelium. Like IL-17A, IL-17C signaling through IL-17RA/RE employs the adaptor molecule ACT1 (40). The signaling cascade then activates the MAPK pathway by phosphorylation of p38, ERK, and JNK as well as the NF-κB pathway by phosphorylation of the p65 subunit and the NF-κB inhibitor IκBα (9). Also, signaling through L-17RA/RE on epithelial cells reinforces the mechanical epithelial barrier by expressing the tight-junction proteins occludin, claudin-1, and claudin-4 (41). Host defense mechanisms in epithelial cells induced by IL-17C include the expression of hBD2, S100A7/8/9, CXCL1/2/3, CCL20, TNFAIP6, and TNIP3 (19) as well as pro-inflammatory cytokines like IL-1β, IL-17A/F, IL-22, IL-6, IL-8, VEGF, and TNF-α (48). This expression profile is a potent response to actively fight off invading pathogens.

Thus, the autocrine loop of IL-17C in the epithelium is an early protective response against pathogenic alterations in the microbiome and other epithelial tissues.

IL-17C and the T_H17 Cell

The second site of action of IL-17C is the T_H17 cell. We have shown that the numbers of T_H17 cells significantly decrease in the absence of IL-17C or IL-17RE in a murine models of autoimmune kidney diseases (24). This effect of IL-17C on T_H17 cells might be due to increased proliferation or differentiation, inhibited apoptosis, or impeded exhaustion. Other groups have investigated these intracellular effects in more detail.

In the EAE model, T_H17 differentiation was induced by IL-17C/RE signaling via IκBζ (40). Signaling through IL-17RE lead to increased production of IL-17A, IL-17F, and IL-22. Song et al. showed that IL-17C induces the expression of anti-apoptotic factors *BCL2* and *BCL2L1* in intestinal epithelial cells (38). This anti-apoptotic effect of the IL-17C/RE axis was also seen in nerve fibers during HSV-2 reactivation (21). As mentioned before, signaling pathways of IL-17C in epithelial cells involve NF-κB and MAPK (9), which might also be true for T_H17 cells and would be indicative of an effect on proliferation of target cells.

Many groups have shown the pro-inflammatory role of IL-17C in disease settings that are known to be driven by a strong T_H17 cell activity. As IL-17C induces the expression of IL-17A in T_H17 cells (40), it may be that this effect of IL-17C is dependent on IL-17A. Indeed, blockade of IL-17A with an antibody abolished the difference in renal damage between wildtype and $Il17c^{-/-}$ mice in a model for crescentic glomerulonephritis (24). Thus, this stimulatory effect of IL-17C/RE on T_H17 cells leads to higher levels of T_H17 signature cytokines—above all IL-17A—which accounts for the strong inflammatory effect of IL-17C. As excessive T_H17 cell activity is linked to many autoimmune diseases, IL-17C-mediated stimulation of the T_H17 cell represents a cause for T_H17 autoimmunity upstream of main effector cytokines like IL-17A and F.

In terms of regulating IL-17C signaling, Monin et al. identified the endoribonuclease MCP-1 induced protein 1 (MCPIP1) as a negative regulator of both IL-17A and C signaling: In a model of imiquimod-induced skin inflammation, mice deficient in MCPIP1 showed increased inflammation and upregulation of IL-17A- and IL-17C-dependent genes, but unaltered levels of IL-17A and C. This indicates that MCPIP1 influences intracellular pathways downstream of IL-17 receptor signaling as opposed to modulation of the expression of IL-17 cytokines (72). The exact mechanism of this negative regulation on IL-17A and C signaling has not been described. However, previous studies have shown that MCPIP1 hampers TLR signaling in response to LPS by degrading mRNA of *Il6* (73) and interferes with

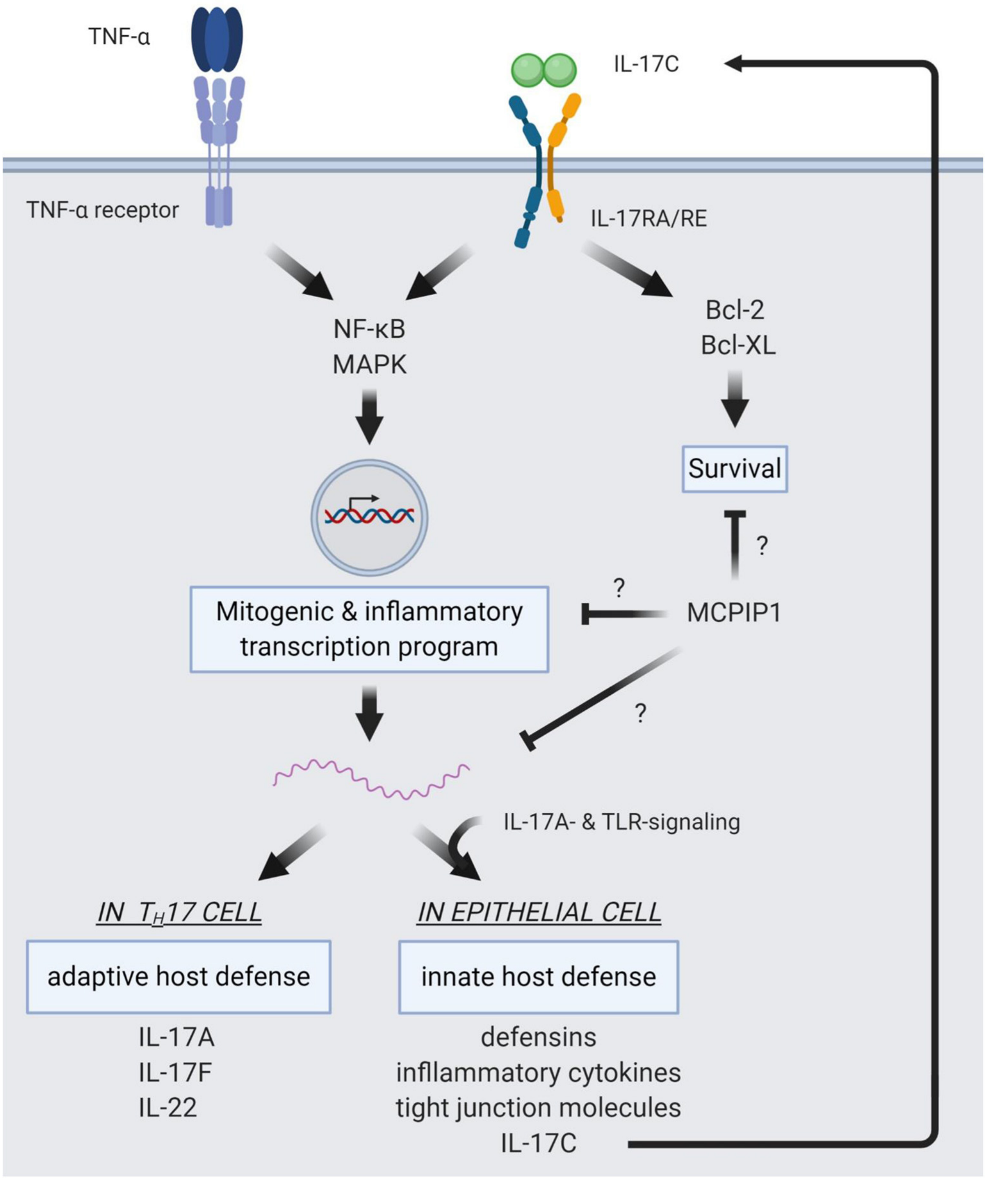


FIGURE 2 | Intracellular pathways of IL-17C signaling. IL-17C signaling induces NF-κB and MAPK signaling pathways. This induction shows a synergistic effect with TNF-α signaling, resulting in strong induction of a mitogenic and pro-inflammatory expression profile. In epithelial cells, target genes of IL-17C/RE signaling are defensins, inflammatory cytokines, and tight junction molecules to reinforce innate host barriers in response to pathogens. Also, IL-17C expression is upregulated in the epithelium and subject to a synergism of IL-17A- and TLR-signaling. IL-17C is then released from the epithelial cell and binds to the IL-17RA/RE receptor complex expressed on the same cell, forming an autocrine loop. In T_H17 cells, IL-17C induces expression of *IL17A*, *IL17F*, and *IL22*, boosting adaptive defense mechanisms. IL-17C also activates anti-apoptotic pathways via Bcl-2 and Bcl-X_L. MCPIP1 is a regulator of IL-17C/RE signaling, but the distinct mechanisms of this negative regulation are not yet elucidated.

MAPK and NF-κB signaling by deubiquitination of signaling molecules (74). Even more, MCPIP1 degrades *Il17ra* and *Il17rc* mRNA (75) and MCPIP1 deficiency boosts T_H17 effector functions (76), which underlines its regulatory effect in IL-17 signaling.

Figure 2 summarizes intracellular signaling pathways of IL-17C.

Taken together, IL-17C assumes a position at the interface of innate and adaptive immune system: It is upregulated during early stages of disease and reinforces innate defense lines in the epithelium via an autocrine loop. Its stimulatory action on the T_H17 cells induces the adaptive immune response and can trigger autoimmune disease (**Figure 3**).

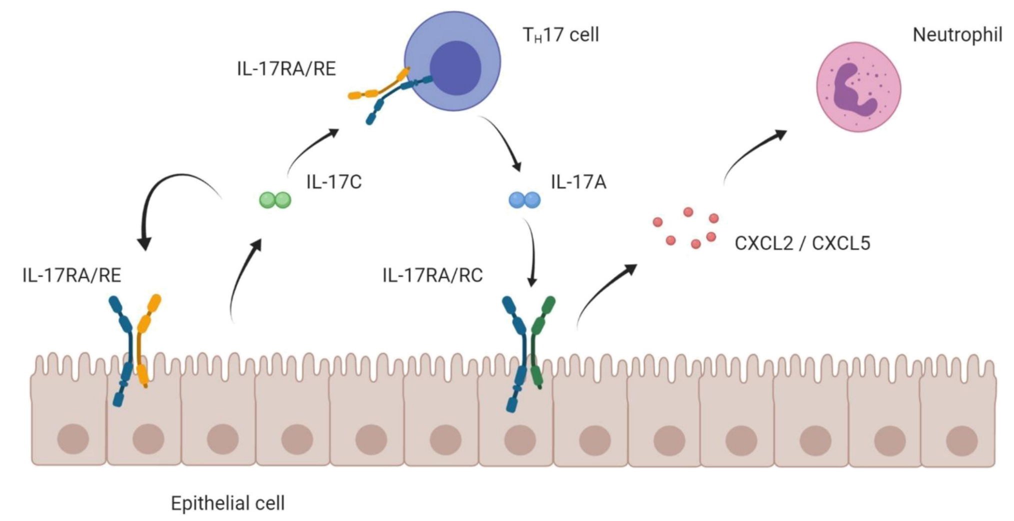


FIGURE 3 | Mode of action of IL-17C on epithelial and T_H17 cells. Schematic hypothesis of the pro-inflammatory mode of action of the IL-17C/RE axis. IL-17C is mainly expressed by epithelial cells. Expressing the IL-17RA/RE receptor complex, both the epithelial cell itself and T_H17 cells are targets for IL-17C. Boosting expression of *IL17A* in T_H17 cells, IL-17C indirectly enhances epithelial expression of chemokines that attract neutrophils, which ultimately cause a strong inflammatory reaction.

FIRST STEPS IN THERAPEUTIC TARGETING OF THE IL-17C/RE AXIS

Antibodies targeting T_H17 cell functions are already in clinical use for a host of autoimmune disorders like psoriasis, psoriatic arthritis, and IBD.

Ustekinumab is a monoclonal antibody directed against the p40 subunit which is shared by the cytokines IL-12 and IL-23. It has been shown to be very successful in the treatment of psoriasis and psoriatic arthritis (77, 78) and is approved for Crohn's disease (79).

Other antibodies directly targeting IL-17A (Secukinumab, Ixekizumab) or the receptor IL-17RA (Brodalumab) show astonishing effects in psoriasis patients (4, 80, 81). Other indications are psoriatic arthritis (2, 3, 82) and ankylosing spondylitis (83).

Neutralizing IL-17C is an intriguing approach in the treatment of autoimmune diseases as it might hamper T_H17 function in general and not only the impact signaling of the signature cytokine IL-17A. Indeed, the first clinical studies with an anti-IL-17C-neutralizing antibody have been started in patients with atopic dermatitis (84) after trials in murine models showed promising results (50).

Interestingly, targeting the cytokine IL-17A or its receptor IL-17RA aggravates symptoms in IBD patients (85, 86). This shows that intervening in those signaling pathways might not be as straightforward as initially thought. Thus, it might be possible that the protective role that IL-17C plays for the integrity of epithelial barrier function exceeds its pathological effect for T_H17 stimulation in autoimmunity. Disrupting the autocrine loop of the epithelial cells with an antibody might lead to unwanted adverse effects like gastrointestinal or respiratory infections. Inhibiting the T_H17 cell function obviously harbors the risk of a general susceptibility to infections with extracellular bacteria and fungi.

DISCUSSION

In summary, IL-17C is a homodimeric cytokine that is expressed by non-hematopoietic—mainly epithelial—cells. It binds to its heterodimeric receptor complex IL-17RA/RE that is expressed on both a variety of epithelial cells and T_H17 cells. Compared to other IL-17 cytokine family members, *IL17C* is upregulated at early stages of the diseases and plays two roles. (a) In an autocrine manner it sustains barrier integrity of epithelial cell layers and thus supports the innate immune system to keep infections in check. (b) By binding to IL-17RE on T_H17 cells, IL-17C also stimulates the adaptive immune response to potently fight off invading pathogens. The downside of this mode of action is the risk of immunological derailment, leading to autoimmune conditions.

Intracellular signaling of IL-17C/RE involves anti-apoptotic Bcl-2 and Bcl-X_L as well as the NF-κB and MAPK pathways to promote proliferation and host defense. The induction of *IL17C* has been shown to be dependent on TLR signaling and pro-inflammatory cytokines. *IL17C* expression is subject to a synergism between TNF-α and IL-17A, presumably due to mRNA stabilization by IL-17A. To date, the molecular mechanisms of described synergisms between IL-17C and other cytokines (TNF-α, IL-22, and IL-1β) have not specifically been investigated.

Being an inflammatory mediator upstream of T_H17 effector cytokines, IL-17C represents an interesting target for pharmacological intervention. The first clinical trials have been started for atopic dermatitis and data from human samples suggest transferability of experimental data to the clinical setting for some diseases (24, 48, 53).

The first translational approaches to pharmacologically exploit the IL-17C/RE axis are on the way. We believe that the main potential of such interventions lies in the treatment of autoimmune disorders. Yet, a lot of experimental data on more disease settings requires further analyses of human samples to investigate potential patient populations for this kind of treatment.

AUTHOR CONTRIBUTIONS

UP and JN wrote the manuscript and designed the figures.

FUNDING

UP and JN were supported by the Deutsche Forschungsgemeinschaft (SFB1192 project A1 and MGK).

ACKNOWLEDGMENTS

The authors apologize to all colleagues whose work could not be cited due to space restrictions.

REFERENCES

1. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*. (2005) 201:233–40. doi: 10.1084/jem.20041257
2. Mease PJ, McInnes IB, Kirkham B, Kavanaugh A, Rahman P, van der Heijde D, et al. Secukinumab inhibition of interleukin-17A in patients with psoriatic arthritis. *N Engl J Med*. (2015) 373:1329–39. doi: 10.1056/NEJMoa1412679
3. McInnes IB, Mease PJ, Kirkham B, Kavanaugh A, Ritchlin CT, Rahman P, et al. Secukinumab, a human anti-interleukin-17A monoclonal antibody, in patients with psoriatic arthritis (FUTURE 2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. (2015) 386:1137–46. doi: 10.1016/S0140-6736(15)61134-5
4. Langley RG, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, et al. Secukinumab in plaque psoriasis–results of two phase 3 trials. *N Engl J Med*. (2014) 371:326–38. doi: 10.1056/NEJMoa1314258
5. Wright JF, Guo Y, Quazi A, Luxenberg DP, Bennett F, Ross JF, et al. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J Biol Chem*. (2007) 282:13447–55. doi: 10.1074/jbc.M700499200
6. Chang SH, Dong C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res*. (2007) 17:435–40. doi: 10.1038/cr.2007.35
7. Toy D, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, et al. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol*. (2006) 177:36–9. doi: 10.4049/jimmunol.177.1.36
8. Rickel EA, Siegel LA, Yoon BR, Rottman JB, Kugler DG, Swart DA, et al. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J Immunol*. (2008) 181:4299–310. doi: 10.4049/jimmunol.181.6.4299
9. Song X, Zhu S, Shi P, Liu Y, Shi Y, Levin SD, et al. IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. *Nat Immunol*. (2011) 12:1151–8. doi: 10.1038/ni.2155
10. Ramirez-Carrozzi V, Ota N, Sambandam A, Wong K, Hackney J, Martinez-Martin N, et al. Cutting edge: IL-17B uses IL-17RA and IL-17RB to induce Type 2 inflammation from human lymphocytes. *J Immunol*. (2019) 202:1935–41. doi: 10.4049/jimmunol.1800696
11. Su Y, Huang J, Zhao X, Lu H, Wang W, Yang XO, et al. Interleukin-17 receptor D constitutes an alternative receptor for interleukin-17A important in psoriasis-like skin inflammation. *Sci Immunol*. (2019) 4:eaau9657. doi: 10.1126/sciimmunol.aau9657
12. Li H, Chen J, Huang A, Stinson J, Heldens S, Foster J, et al. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc Natl Acad Sci USA*. (2000) 97:773–8. doi: 10.1073/pnas.97.2.773
13. Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J Clin Invest*. (2013) 123:247–60. doi: 10.1172/JCI63681
14. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity*. (2009) 31:321–30. doi: 10.1016/j.immuni.2009.06.020
15. Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol*. (2007) 178:4466–72. doi: 10.4049/jimmunol.178.7.4466
16. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med*. (2007) 204:995–1001. doi: 10.1084/jem.20061551
17. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, Ivanov II, et al. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med*. (2009) 206:35–41. doi: 10.1084/jem.20072713
18. Bermejo DA, Jackson SW, Gorosito-Serran M, Acosta-Rodriguez EV, Amezcua-Vesely MC, Sather BD, et al. Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. *Nat Immunol*. (2013) 14:514–22. doi: 10.1038/ni.2569
19. Ramirez-Carrozzi V, Sambandam A, Luis E, Lin Z, Jeet S, Lesch J, et al. IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. *Nat Immunol*. (2011) 12:1159–66. doi: 10.1038/ni.2156
20. Al-Samadi A, Kouri VP, Salem A, Ainola M, Kaivosoja E, Barreto G, et al. IL-17C and its receptor IL-17RA/IL-17RE identify human oral epithelial cell as an inflammatory cell in recurrent aphthous ulcer. *J Oral Pathol Med*. (2014) 43:117–24. doi: 10.1111/jop.12095
21. Peng T, Chanthaphavong RS, Sun S, Trigilio JA, Phasouk K, Jin L, et al. Keratinocytes produce IL-17c to protect peripheral nervous systems during human HSV-2 reactivation. *J Exp Med*. (2017) 214:2315–29. doi: 10.1084/jem.20160581
22. Johansen C, Vinter H, Soegaard-Madsen L, Olsen LR, Steiniche T, Iversen L, et al. Preferential inhibition of the mRNA expression of p38 mitogen-activated protein kinase regulated cytokines in psoriatic skin by anti-TNFα therapy. *Br J Dermatol*. (2010) 163:1194–204. doi: 10.1111/j.1365-2133.2010.10036.x
23. Johansen C, Riis JL, Gedebjerg A, Kragballe K, Iversen L. Tumor necrosis factor α-mediated induction of interleukin 17C in human keratinocytes is controlled by nuclear factor κB. *J Biol Chem*. (2011) 286:25487–94. doi: 10.1074/jbc.M111.240671
24. Krohn S, Nies JF, Kapffer S, Schmidt T, Riedel JH, Kaffke A, et al. IL-17C/IL-17 receptor E Signaling in CD4(+) T cells promotes TH17 cell-driven glomerular inflammation. *J Am Soc Nephrol*. (2018) 29:1210–22. doi: 10.1681/ASN.2017090949
25. Huang J, Meng S, Hong S, Lin X, Jin W, Dong C. IL-17C is required for lethal inflammation during systemic fungal infection. *Cell Mol Immunol*. (2016) 13:474–83. doi: 10.1038/cmi.2015.56
26. Wolf L, Sapich S, Honecker A, Jungnickel C, Seiler F, Bischoff M, et al. IL-17A-mediated expression of epithelial IL-17C promotes inflammation during acute

Pseudomonas aeruginosa pneumonia. *Am J Physiol Lung Cell Mol Physiol*. (2016) 311:L1015–22. doi: 10.1152/ajplung.00158.2016

27. Pfeifer P, Voss M, Wonnenberg B, Hellberg J, Seiler F, Lepper PM, et al. IL-17C is a mediator of respiratory epithelial innate immune response. *Am J Respir Cell Mol Biol*. (2013) 48:415–21. doi: 10.1165/rcmb.2012-0232OC
28. Kusagaya H, Fujisawa T, Yamanaka K, Mori K, Hashimoto D, Enomoto N, et al. Toll-like receptor-mediated airway IL-17C enhances epithelial host defense in an autocrine/paracrine manner. *Am J Respir Cell Mol Biol*. (2014) 50:30–9. doi: 10.1165/rcmb.2013-0130OC
29. Hou C, Zhang Y, Yu S, Li Z, Zhai Q, Li Z, et al. Presence of interleukin-17C in the tissue around aseptic loosened implants. *Int Orthop*. (2013) 37:953–9. doi: 10.1007/s00264-013-1812-x
30. Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, et al. IL-17B and IL-17C are associated with TNF-α production and contribute to the exacerbation of inflammatory arthritis. *J Immunol*. (2007) 179:7128–36. doi: 10.4049/jimmunol.179.10.7128
31. Butcher MJ, Waseem TC, Galkina EV. Smooth muscle cell-derived interleukin-17C plays an atherogenic role via the recruitment of proinflammatory interleukin-17A+ T cells to the aorta. *Arterioscler Thromb Vasc Biol*. (2016) 36:1496–506. doi: 10.1161/ATVBAHA.116.307892
32. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. (2008) 4:337–49. doi: 10.1016/j.chom.2008.09.009
33. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, et al. ATP drives lamina propria T(H)17 cell differentiation. *Nature*. (2008) 455:808–12. doi: 10.1038/nature07240
34. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. (2009) 139:485–98. doi: 10.1016/j.cell.2009.09.033
35. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity*. (2009) 31:677–89. doi: 10.1016/j.immuni.2009.08.020
36. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*. (2011) 332:974–7. doi: 10.1126/science.1206095
37. Krebs CF, Paust HJ, Krohn S, Koyro T, Brix SR, Riedel JH, et al. Autoimmune renal disease is exacerbated by S1P-receptor-1-dependent intestinal Th17 cell migration to the kidney. *Immunity*. (2016) 45:1078–92. doi: 10.1016/j.immuni.2016.10.020
38. Song X, Gao H, Lin Y, Yao Y, Zhu S, Wang J, et al. Alterations in the microbiota drive interleukin-17C production from intestinal epithelial cells to promote tumorigenesis. *Immunity*. (2014) 40:140–52. doi: 10.1016/j.immuni.2013.11.018
39. Li TS, Li XN, Chang ZJ, Fu XY, Liu L. Identification and functional characterization of a novel interleukin 17 receptor: a possible mitogenic activation through ras/mitogen-activated protein kinase signaling pathway. *Cell Signal*. (2006) 18:1287–98. doi: 10.1016/j.cellsig.2005.10.010
40. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C. Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E. *Immunity*. (2011) 35:611–21. doi: 10.1016/j.immuni.2011.09.010
41. Reynolds JM, Martinez GJ, Nallaparaju KC, Chang SH, Wang YH, Dong C. Cutting edge: regulation of intestinal inflammation and barrier function by IL-17C. *J Immunol*. (2012) 189:4226–30. doi: 10.4049/jimmunol.1103014
42. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis*. (2004) 190:624–31. doi: 10.1086/422329
43. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med*. (2008) 205:1551–7. doi: 10.1084/jem.20080218
44. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature*. (2008) 452:773–6. doi: 10.1038/nature06764
45. Conti HR, Whibley N, Coleman BM, Garg AV, Jaycox JR, Gaffen SL. Signaling through IL-17C/IL-17RE is dispensable for immunity to systemic, oral and cutaneous candidiasis. *PLoS ONE*. (2015) 10:e0122807. doi: 10.1371/journal.pone.0122807
46. Jamieson KC, Traves SL, Kooi C, Wiehler S, Dumonceaux CJ, Maciejewski BA, et al. Rhinovirus and bacteria synergistically induce IL-17C release from human airway epithelial cells to promote neutrophil recruitment. *J Immunol*. (2019) 202:160–70. doi: 10.4049/jimmunol.1800547
47. Johansen C, Usher PA, Kjellerup RB, Lundsgaard D, Iversen L, Kragballe K. Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. *Br J Dermatol*. (2009) 160:319–24. doi: 10.1111/j.1365-2133.2008.08902.x
48. Johnston A, Fritz Y, Dawes SM, Diaconu D, Al-Attar PM, Guzman AM, et al. Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J Immunol*. (2013) 190:2252–62. doi: 10.4049/jimmunol.1201505
49. El Malki K, Karbach SH, Huppert J, Zayoud M, Reissig S, Schuler R, et al. An alternative pathway of imiquimod-induced psoriasis-like skin inflammation in the absence of interleukin-17 receptor a signaling. *J Invest Dermatol*. (2013) 133:441–51. doi: 10.1038/jid.2012.318
50. Vandeghinste N, Klattig J, Jagerschmidt C, Lavazais S, Marsais F, Haas JD, et al. Neutralization of IL-17C reduces skin inflammation in mouse models of psoriasis and atopic dermatitis. *J Invest Dermatol*. (2018) 138:1555–63. doi: 10.1016/j.jid.2018.01.036
51. Huang J, Yuan Q, Zhu H, Yin L, Hong S, Dong Z, et al. IL-17C/IL-17RE augments T cell function in autoimmune hepatitis. *J Immunol*. (2017) 198:669–80. doi: 10.4049/jimmunol.1600977
52. Yamaguchi S, Nambu A, Numata T, Yoshizaki T, Narushima S, Shimura E, et al. The roles of IL-17C in T cell-dependent and -independent inflammatory diseases. *Sci Rep*. (2018) 8:15750. doi: 10.1038/s41598-018-34054-x
53. Friedrich M, Tillack C, Wollenberg A, Schauber J, Brand S. IL-36gamma sustains a proinflammatory self-amplifying loop with IL-17C in anti-TNF-induced psoriasiform skin lesions of patients with Crohn's disease. *Inflamm Bowel Dis*. (2014) 20:1891–901. doi: 10.1097/MIB.0000000000000198
54. Jungnickel C, Schmidt LH, Bittigkoffer L, Wolf L, Wolf A, Ritzmann F, et al. IL-17C mediates the recruitment of tumor-associated neutrophils and lung tumor growth. *Oncogene*. (2017) 36:4182–90. doi: 10.1038/onc.2017.28
55. Liao R, Sun J, Wu H, Yi Y, Wang JX, He HW, et al. High expression of IL-17 and IL-17RE associate with poor prognosis of hepatocellular carcinoma. *J Exp Clin Cancer Res*. (2013) 32:3. doi: 10.1186/1756-9966-32-3
56. Ritzmann F, Jungnickel C, Vella G, Kamyschnikow A, Herr C, Li D, et al. IL-17C-mediated innate inflammation decreases the response to PD-1 blockade in a model of Kras-driven lung cancer. *Sci Rep*. (2019) 9:10353. doi: 10.1038/s41598-019-46759-8
57. Novatchkova M, Leibbrandt A, Werzowa J, Neubuser A, Eisenhaber F. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem Sci*. (2003) 28:226–9. doi: 10.1016/S0968-0004(03)00067-7
58. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, Comeau MR, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity*. (1995) 3:811–21. doi: 10.1016/1074-7613(95)90070-5
59. Karlsen JR, Borregaard N, Cowland JB. Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-α is controlled by IκB-zeta but neither by C/EBP-beta nor C/EBP-delta. *J Biol Chem*. (2010) 285:14088–100. doi: 10.1074/jbc.M109.017129
60. Qian Y, Liu C, Hartupee J, Altuntas CZ, Gulen MF, Jane-Wit D, et al. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat Immunol*. (2007) 8:247–56. doi: 10.1038/ni1439
61. Bulek K, Liu C, Swaidani S, Wang L, Page RC, Gulen MF, et al. The inducible kinase IKKi is required for IL-17-dependent signaling associated with neutrophilia and pulmonary inflammation. *Nat Immunol*. (2011) 12:844–52. doi: 10.1038/ni.2080
62. Huang G, Wang Y, Vogel P, Chi H. Control of IL-17 receptor signaling and tissue inflammation by the p38α-MKP-1 signaling axis in a mouse model of multiple sclerosis. *Sci Signal*. (2015) 8:ra24. doi: 10.1126/scisignal.aaa2147
63. Chang SH, Park H, Dong C. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J Biol Chem*. (2006) 281:35603–7. doi: 10.1074/jbc.C600256200

64. Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional cooperation between interleukin-17 and tumor necrosis factor-α is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem*. (2004) 279:2559–67. doi: 10.1074/jbc.M308809200
65. Maitra A, Shen F, Hanel W, Mossman K, Tocker J, Swart D, et al. Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. *Proc Natl Acad Sci USA*. (2007) 104:7506–11. doi: 10.1073/pnas.0611589104
66. Shen F, Li N, Gade P, Kalvakolanu DV, Weibley T, Doble B, et al. IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. *Sci Signal*. (2009) 2:ra8. doi: 10.1126/scisignal.2000066
67. Hartupee J, Liu C, Novotny M, Li X, Hamilton T. IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol*. (2007) 179:4135–41. doi: 10.4049/jimmunol.179.6.4135
68. Sun D, Novotny M, Bulek K, Liu C, Li X, Hamilton T. Treatment with IL-17 prolongs the half-life of chemokine CXCL1 mRNA via the adaptor TRAF5 and the splicing-regulatory factor SF2 (ASF). *Nat Immunol*. (2011) 12:853–60. doi: 10.1038/ni.2081
69. Roth SA, Simanski M, Rademacher F, Schroder L, Harder J. The pattern recognition receptor NOD2 mediates Staphylococcus aureus-induced IL-17C expression in keratinocytes. *J Invest Dermatol*. (2014) 134:374–80. doi: 10.1038/jid.2013.313
70. Chiricozzi A, Guttman-Yassky E, Suarez-Farinas M, Nograles KE, Tian S, Cardinale I, et al. Integrative responses to IL-17 and TNF-α in human keratinocytes account for key inflammatory pathogenic circuits in psoriasis. *J Invest Dermatol*. (2011) 131:677–87. doi: 10.1038/jid.2010.340
71. Russell CB, Rand H, Bigler J, Kerkof K, Timour M, Bautista E, et al. Gene expression profiles normalized in psoriatic skin by treatment with brodalumab, a human anti-IL-17 receptor monoclonal antibody. *J Immunol*. (2014) 192:3828–36. doi: 10.4049/jimmunol.1301737
72. Monin L, Gudjonsson JE, Childs EE, Amatya N, Xing X, Verma AH, et al. MCPIP1/regnase-1 restricts IL-17A- and IL-17C-dependent skin inflammation. *J Immunol*. (2017) 198:767–75. doi: 10.4049/jimmunol.1601551
73. Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature*. (2009) 458:1185–90. doi: 10.1038/nature07924
74. Liang J, Saad Y, Lei T, Wang J, Qi D, Yang Q, et al. MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF-κB signaling. *J Exp Med*. (2010) 207:2959–73. doi: 10.1084/jem.20092641
75. Garg AV, Amatya N, Chen K, Cruz JA, Grover P, Whibley N, et al. MCPIP1 endoribonuclease activity negatively regulates interleukin-17-mediated signaling and inflammation. *Immunity*. (2015) 43:475–87. doi: 10.1016/j.immuni.2015.07.021
76. Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K, et al. Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. *Cell*. (2013) 153:1036–49. doi: 10.1016/j.cell.2013.04.034
77. Croxtall JD. Ustekinumab: a review of its use in the management of moderate to severe plaque psoriasis. *Drugs*. (2011) 71:1733–53. doi: 10.2165/11207530-000000000-00000
78. McInnes IB, Kavanaugh A, Gottlieb AB, Puig L, Rahman P, Ritchlin C, et al. Efficacy and safety of ustekinumab in patients with active psoriatic arthritis: 1 year results of the phase 3, multicentre, double-blind, placebo-controlled PSUMMIT 1 trial. *Lancet*. (2013) 382:780–9. doi: 10.1016/S0140-6736(13)60594-2
79. Feagan BG, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, et al. Ustekinumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med*. (2016) 375:1946–60. doi: 10.1056/NEJMoa1602773
80. Griffiths CE, Reich K, Lebwohl M, van de Kerkhof P, Paul C, Menter A, et al. Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials. *Lancet*. (2015) 386:541–51. doi: 10.1016/S0140-6736(15)60125-8
81. Lebwohl M, Strober B, Menter A, Gordon K, Weglowska J, Puig L, et al. Phase 3 studies comparing brodalumab with ustekinumab in psoriasis. *N Engl J Med*. (2015) 373:1318–28. doi: 10.1056/NEJMoa1503824
82. van der Heijde D, Cheng-Chung Wei J, Dougados M, Mease P, Deodhar A, Maksymowych WP, et al. Ixekizumab, an interleukin-17A antagonist in the treatment of ankylosing spondylitis or radiographic axial spondyloarthritis in patients previously untreated with biological disease-modifying anti-rheumatic drugs (COAST-V): 16 week results of a phase 3 randomised, double-blind, active-controlled and placebo-controlled trial. *Lancet*. (2018) 392:2441–51. doi: 10.1016/S0140-6736(18)31946-9
83. Baeten D, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, et al. Secukinumab, an interleukin-17A inhibitor, in ankylosing spondylitis. *N Engl J Med*. (2015) 373:2534–48. doi: 10.1056/NEJMoa1505066
84. GlobeNewswire Inc. *Galapagos and Morphosys Report First Promising Signs of Clinical Activity in a Phase 1 Study with IL-17c Antibody Mor106 in Atopic Dermatitis Patients*. (2017). Available online at: https://www.globenewswire.com/news-release/2017/09/27/1133502/0/en/Galapagos-and-MorphoSys-report-first-promising-signs-of-clinical-activity-in-a-Phase-1-study-with-IL-17C-antibody-MOR106-in-atopic-dermatitis-patients.html
85. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*. (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
86. Targan SR, Feagan B, Vermeire S, Panaccione R, Melmed GY, Landers C, et al. A randomized, double-blind, placebo-controlled phase 2 study of brodalumab in patients with moderate-to-severe Crohn's disease. *Am J Gastroenterol*. (2016) 111:1599–607. doi: 10.1038/ajg.2016.298

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 27 February 2020
doi: 10.3389/fimmu.2019.02344

Check for updates

mTOR Blockade by Rapamycin in Spondyloarthritis: Impact on Inflammation and New Bone Formation *in vitro* and *in vivo*

Sijia Chen[1,2,3]*, Melissa N. van Tok*[1,2]*, Véronique L. Knaup*[1]*, Lianne Kraal*[1,2]*, Désiree Pots*[1,2]*, Lina Bartels*[1]*, Ellen M. Gravallese*[3]*, Joel D. Taurog*[4]*, Marleen van de Sande*[1,2]*, Leonie M. van Duivenvoorde*[1,2] *and Dominique L. Baeten*[1,2,5]*

[1] *Department of Experimental Immunology, Infection and Immunity Institute, Amsterdam University Medical Centers, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands,* [2] *Department of Clinical Immunology and Rheumatology, Amsterdam Rheumatology & Immunology Center (ARC), Amsterdam University Medical Centers, Academic Medical Center, 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,* [4] *Internal Medicine, Rheumatic Diseases Division, UT Southwestern Medical Center, Dallas, TX, United States,* [5] *UCB Pharma, Slough, United Kingdom*

OPEN ACCESS

Edited by:
Nicola Ivan Lorè,
IRCCS San Raffaele Scientific Institute, Italy

Reviewed by:
Gyorgy Nagy,
Semmelweis University, Hungary
Saijilafu,
First Affiliated Hospital of Soochow University, China

***Correspondence:**
Dominique L. Baeten
d.l.baeten@amsterdamumc.nl

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders,
a section of the journal Frontiers in Immunology

Received: *11 July 2019*
Accepted: *17 September 2019*
Published: *27 February 2020*

Citation:
Chen S, van Tok MN, Knaup VL, Kraal L, Pots D, Bartels L, Gravallese EM, Taurog JD, van de Sande M, van Duivenvoorde LM and Baeten DL (2020) mTOR Blockade by Rapamycin in Spondyloarthritis: Impact on Inflammation and New Bone Formation in vitro and in vivo. Front. Immunol. 10:2344. doi: 10.3389/fimmu.2019.02344

Introduction: Spondyloarthritis (SpA) is characterized by inflammation, articular bone erosions and pathologic new bone formation. Targeting TNFα or IL-17A with current available therapies reduces inflammation in SpA, however, treatment of the bone pathology in SpA remains an unmet clinical need. Activation of the mammalian target Of rapamycin (mTOR) promotes IL-17A expression and osteogenesis. Therefore, the inhibition of mTOR (with rapamycin) could be a promising therapeutic avenue in SpA.

Objectives: To investigate the effect of blocking mTOR on inflammation, bone erosions and new bone formation in SpA.

Methods: Peripheral blood mononuclear cells (PBMCs) from patients with SpA were stimulated with anti-CD3/CD28 in the presence or absence of rapamycin and the resulting cytokine expression was assessed. Fibroblast-like synoviocytes (FLS) from SpA patients were assessed for osteogenic differentiation potential in conditions with TNFα, IL-17A, or TNFα plus IL-17A, in the presence or absence of rapamycin. HLA-B27/Huβ2m transgenic rats were immunized with low dose heat-inactivated *Mycobacterium tuberculosis (M. tub)*, treated with 1.5 mg/kg rapamycin prophylactically or therapeutically and monitored for arthritis and spondylitis. Histology and mRNA analysis were performed after 5 weeks of treatment to assess inflammation and bone pathology.

Results: *In vitro* TNFα and IL-17A protein production by SpA PBMCs was inhibited in the presence of rapamycin. Rapamycin also inhibited osteogenic differentiation of human SpA FLS. *Ex vivo* analysis of SpA synovial biopsies indicated activation of the mTOR pathway in the synovial tissue of SpA patients. *In vivo*, prophylactic treatment of HLA-B27/Huβ2m transgenic rats with rapamycin significantly inhibited the development and severity of inflammation in peripheral joints and spine (arthritis and spondylitis), with histological evidence of reduced bone erosions and new bone formation around peripheral joints. In addition, therapeutic treatment with rapamycin significantly decreased severity of arthritis and spondylitis, with peripheral joint histology showing reduced inflammation, bone erosions and new bone formation. *IL-17A* mRNA expression was decreased in the metacarpophalangeal joints after rapamycin treatment.

Conclusion: mTOR blockade inhibits IL-17A and TNFα production by PBMCs, and osteogenic differentiation of FLS from patients with SpA *in vitro.* In the HLA-B27 transgenic rat model of SpA, rapamycin inhibits arthritis and spondylitis development and severity, reduces articular bone erosions, decreases pathologic new bone formation and suppresses IL-17A expression. These results may support efforts to evaluate the efficacy of targeting the mTOR pathway in SpA patients.

Keywords: spondyloarthtritis, mTOR, rapamycin, IL-17A, fibroblast-like synoviocytes, small molecule treatment, animal models, HLA-B27 tg rats

INTRODUCTION

Spondyloarthritis (SpA) is the second most prevalent form of chronic inflammatory arthritis. The hallmarks of SpA are joint inflammation, articular bone erosions and pathologic new bone formation (1). Tumor necrosis factor-α (TNFα) and Interleukin-17A (IL-17A) are key disease-modulating cytokines in SpA (1–4). Currently, one third of patients do not respond to available therapy and only 20% of patients achieve remission. Loss of therapeutic efficacy can occur over time and anti-TNFα therapy does not reduce bone formation in the advanced stages of SpA (5–8). Although anti-IL-17A therapy has been demonstrated to reduce bone formation the *M.tub*-induced HLA-B27 transgenic rat model (HLA-B27 tg rats) (9), a beneficial effect of anti-IL-17A therapy on human SpA bone pathology is not well-understood and remains to be formally established (3, 5). Thus, there is an unmet clinical need to find therapies targeting both inflammation and pathologic bone formation in SpA.

The etiology of the new bone formation in SpA remains unclear (1, 10). We and others (9, 11, 12) have previously demonstrated that fibroblast-like synoviocytes (FLS) isolated from the synovial tissue may act as bone precursor cells and differentiate *in vitro* to osteoblast-like-cells. Osteoblasts are the bone-forming cells responsible for bone matrix and bone mineralization (10). It has also been demonstrated that TNFα and IL-17A can accelerate osteogenic differentiation of FLS cells *in vitro* (9, 12).

The mammalian target of rapamycin (mTOR) has been demonstrated to play an important role in inflammation. For instance, mTOR, can be blocked using rapamycin, a small molecular drug that has been applied clinically to prevent graft rejection in kidney transplantation (13–15). mTOR has been demonstrated to activate T cells and regulate RORγ translocation in murine cells to induce IL-17A expression (16, 17). Blocking mTOR reduces the percentage of Th17 cells in an animal model of colitis (18). RNA sequencing of inflamed synovial tissue from patients with SpA demonstrated the expression of the PI3K-Akt-mTOR pathway (Chen and Ross et al. under review).

In addition to modifying inflammation, mTOR signaling is downstream of bone anabolic pathways and promotes osteoblastic maturation and mineralization (19).

We sought to examine the effect of mTOR blockade with rapamycin on inflammation as well as new bone formation in the pathobiological context of SpA. Since mTOR pathway regulates the expression of the disease modulating cytokine IL-17A and promotes osteogenic differentiation we hypothesized that treatment with rapamycin may modify both inflammation and pathologic bone formation in SpA pathogenesis. Initially, we determined the effect of rapamycin *in vitro* on primary human SpA cells. Specifically, we investigated if rapamycin could inhibit the production of cytokines by SpA peripheral blood mononuclear cells (PBMCs) and if rapamycin could reduce the rate of human SpA FLS to differentiate to osteoblast-like-cells. Next, we confirmed the activation of mTOR pathway in SpA synovitis. In addition, we determined the prophylactic and therapeutic treatment effect of rapamycin in the *M.tub*-induced HLA-B27 transgenic rat model (HLA-B27 tg rats), an experimental model of SpA (20–22). In the HLA-B27 tg rats, we assessed whether rapamycin would reduce the incidence and severity in inflammation of peripheral joints and spine (arthritis and spondylitis), bone erosions and pathologic new bone formation *in vivo.*

MATERIALS AND METHODS

Human Cells and Tissue

Patient material was obtained from spondyloarthritis (SpA) and rheumatoid arthritis (RA) patients. The SpA patients included in this study fulfilled the Assessment of Spondyloarthritis International Society (ASAS) criteria for peripheral SpA (23). The RA patients were included according to the American College of Rheumatology classification criteria (24). All patients provided written informed consent before enrollment in the study. This study was approved by the Ethics Committee of the Amsterdam University Medical Center, University of Amsterdam, the Netherlands.

In vitro Stimulation of Human PBMCs and SFMCs

Primary human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors ($n = 3$) and SpA patients ($n = 6$), and synovial fluid mononuclear cells (SFMCs) were obtained from inflamed knee joints from SpA patients ($n = 2$). At the time of inclusion, SpA patients had not taken biologic agents for at least 3 months. PBMCs and SFMCs were isolated by density gradient centrifugation on Lymphoprep (Nycomed).

PBMCs and SFMCs were pre-incubated with vehicle (0.001% DMSO) or rapamycin in IMDM (Lonza) for 30 min and stimulated with anti-CD3 (clone 1XE, 1: 1,000, Sanquin) and anti-CD28 (clone 15E8, 2 μg/ml, Sanquin) for 48 h. Cytokines were measured in supernatants by ELISA (IL-17A and TNFα, eBioscience) according to the manufacturer's recommendations. Counting of viable PBMCs was performed by flow cytometry on an LSR Fortessa X-20 instrument (BD). Exclusion of DAPI (10 nM, 46-Diamidino-2-Phenylindole, Dihydrochloride; Sigma) and PI (3 μM, Propidium iodide; Sigma) was used to indicate cell viability. Accudrop Beads (BD) were used as the counting standard. Per sample, 10,000 beads were added and PBMCs were counted during the acquisition of 6,000 beads.

In vitro Osteogenic Differentiation of SpA Fibroblast-Like Synoviocytes (FLS)

Primary human SpA FLS ($n = 11$) were obtained from synovial tissue biopsies according to standardized protocol (9, 25). FLS viability was assessed in the presence of vehicle (0.00005%DMSO) or rapamycin (5 nM) with WST-1 assay (Roche) and Trypan Blue 0.4% solution (Gibco) (26). For *in vitro* differentiation, SpA FLS (passage 3–8) were cultured in Xvivo Stempro medium (R&D systems) with 50 μM ascorbic acid and 10 mM β-glycophosphate, supplemented with TNFα (1 ng/ml), IL-17A (50 ng/ml), or both. Media was refreshed twice weekly. Cells were fixed with 4% formaldehyde at days 7, 14, and 21 of differentiation. Alkaline phosphatase (ALP) staining was performed at days 7 and 14. Alizarin red (2%) staining was performed at day 21 for the osteogenic conditions without cytokines and at day 14 for conditions supplemented with cytokines, consistent with prior published protocols (9). The percentage of ALP staining and alizarin red staining in the wells were scored semi-quantitively by 2 observers, as described previously (9).

Immunofluorescence

Frozen synovial tissue sections ($n = 20$ from SpA patients and $n = 20$ from RA patients) were fixed and blocked with 10% serum. Staining with isotypes or primary antibodies was performed overnight at 4°C, followed by incubation with Alexa Fluor 488/Alexa Fluor 594-conjugated secondary antibodies for 30 min at room temperature. Antibodies used: monoclonal rabbit IgG anti-human phospho-S6 (pS6, ser235/236, clone D57.2.2E, Cell signaling); monoclonal mouse IgG1 anti-human CD3 (clone UCH-T1; Thermo Scientific Pierce); and monoclonal mouse IgG1 anti-human CD45 (HI30; Biolegend) at a concentration of 5 μg/ml. Slides were mounted with Prolong Gold with DAPI (Thermo Fisher). Pictures were taken on an epifluorescence imaging microscope (Leica) and analyzed using ImageJ (1.50i) software. The quantity of pS6 staining was scored on a 3-point semiquantitative scale by two independent observers, who were blinded for the diagnosis of the patients, according to standardized methods (9, 27–29).

Animals

In order to generate *M.tub*-induced HLA-B27/Huβ2m transgenic rats (HLA-B27 tg rats), the Tg(HLA-B∗2705, B2M)21-3Reh and Tg(B2M)283-2Reh Lewis rat lines (30) were bred and housed at the animal research institute of AMC. F1 (21–3 × 283–2) male and female rats were used for experiments. Animal experiments were approved by the Amsterdam University Medical Center (AUMC) Animal Care and Use Committee.

Male rats were orchiectomized to prevent epididymo-orchitis (31), as described previously (22). To synchronize disease onset, 6-week-old, HLA-B27/Huβ2m transgenic rats were immunized with heat-inactivated *Mycobacterium tuberculosis (M. tub)* (Difco, Detroit, MI, USA) in 100 μl Incomplete Freund's Adjuvant (IFA) (Chondrex, Redmond, WA, USA) as described previously (21, 22).

In vivo Preventive and Therapeutic Treatment With Rapamycin

Rats were treated with 1.5 mg/kg rapamycin or vehicle intra-peritoneally, three times per week for 5 weeks. The prophylactic treatment ($n = 13$ vehicle vs. $n = 11$ rapamycin) started 1 week post-immunization with *M. tub,* which is ∼1–2 weeks before the onset of arthritis and spondylitis. The therapeutic treatment ($n = 5$ vehicle vs. $n = 4$ rapamycin) started 1 week after 50% of the animals developed arthritis. Vehicle-treated rats were caged separately from rapamycin treated rats.

Clinical Measurement of Arthritis and Spondylitis

The HLA-B27 tg rats were monitored for arthritis and spondylitis incidence and severity, as described previously (9, 21, 22). Clinical measurements were performed by an observer blinded for treatment, and included weight, macroscopic severity scores for arthritis (0–12) and spondylitis (0–3), and hind paw swelling measured by plethysmometry. For the severity analysis, cumulative clinical scores of all limbs were calculated. For plethysmometry, the change in swelling in cm^3 was normalized to the measurement on the day before the disease onset as observed clinically (prophylactic experiment) or to the day of treatment start (therapeutic treatment).

Histology

Rats were sacrificed after 5 weeks of treatment. Hind paws (peripheral joints) and the tail (axial joints) were isolated and fixed in 10% formalin, decalcified in Osteosoft (Merck) and embedded in paraffn. For hematoxylin and eosin (H&E) or Safranin O/Fast Green staining, 5 μm sections were stained and scored by two observers blinded to the identities of the treatment groups. Semi-quantitative scoring was performed for inflammation, bone erosions, periosteal new bone formation and hypertrophic chondrocytes (enchondral new bone formation) as described previously (21, 22). Images were obtained with a light microscope (Leica).

Gene Expression Analysis

Metacarpophalangeal (MCP) joints were homogenized in TRIzol and further processed for RNA isolation on columns (RNAeasy mini columns, Qiagen) according to manufacturer's protocol.

qPCRs were performed with SYBR green primers (Life technologies) for *IL-17A, IL-17F, IL-22, TNFα, IL-23, RORC, IFNγ, IL-4,* with *GAPDH* as the reference gene. Data were represented as relative fold-changes [according to the 2^{-ddCt} method (32)] to one reference control sample. GAPDH expression was detected in all samples with <22 Ct cycles. When a gene was not detectable, Ct 40 was used for the calculation of the relative fold.

Statistics

Graphpad Prism 7 was used to perform statistical analysis. For all normally-distributed continuous data, the one-way ANOVA was performed with multiple comparison adjustments according to Bonferroni. Survival curves were analyzed for arthritis and spondylitis incidence and compared with the Log-Rank (Mantel-Cox) test. The Area Under the Curve (AUC) was calculated for clinical scores and hind paw swelling and analyzed with a Mann–Whitney *U*-test. For non-normally distributed data and nominal data, Mann–Whitney *U*-test was used.

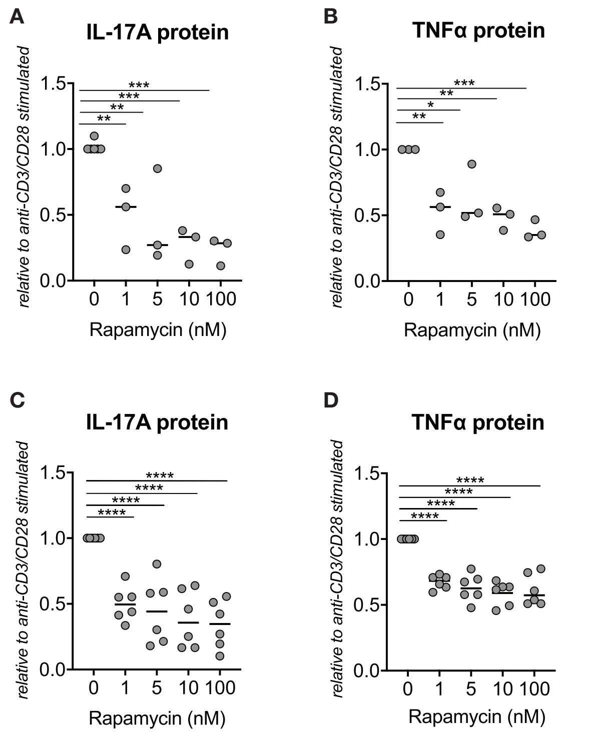


FIGURE 1 | Rapamycin reduces IL-17A and TNFα protein production by PBMCs from Spondyloarthritis (SpA) patients *in vitro*. **(A)** Rapamycin reduces IL-17A ($n = 3$) and **(B)** TNFα ($n = 3$) protein secretion by *in vitro* stimulated PBMCs from healthy donors. **(C)** Rapamycin inhibits production of IL-17A ($n = 6$) and **(D)** TNFα ($n = 6$) by PBMCs from patients with SpA. ****$p <$ 0.0001, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

RESULTS

Rapamycin Inhibits IL-17A and TNFα Protein Production by Human PBMCs From SpA Patients

As mTOR activation has been demonstrated to induce IL-17A expression in murine T cells (16), we hypothesized that rapamycin treatment would inhibit IL-17A production in human PBMCs. We therefore first examined the effect of rapamycin (0, 1, 5, 10, 100 nM) in healthy donor PBMCs stimulated with anti-CD3/CD28 antibodies. Rapamycin significantly reduced IL-17A and TNFα protein secretion by *in vitro* stimulated healthy donor PBMCs, with a reduction of 51.8% for IL-17A and 47.0% for TNFα at 1 nM, respectively (**Figures 1A,B**). Rapamycin did not induce cell death over the culture period of 48 h as measured by DAPI and PI staining by flow cytometry (**Supplemental Figure 1**).

Similarly, rapamycin significantly reduced IL-17A and TNFα production by human SpA PBMC: 1 nM of rapamycin induced a 50.0% reduction of IL-17A and 32.7% reduction of TNFα production (**Figures 1C,D**). A similar but non-significant trend was also observed when human SpA SFMCs were treated *in vitro* with rapamycin (**Supplemental Figures 2A,B**).

Rapamycin Reduces Osteogenic Differentiation of Human SpA Fibroblast-Like Synoviocytes (FLS)

In bone precursor cells, the mTOR pathway has also been reported to promote osteogenesis through regulation of bone anabolic pathways (19). To study a potential direct effect of mTOR inhibition by rapamycin on osteoblastic differentiation within the inflammatory context of SpA, we performed *in vitro* osteogenic differentiation assays with human FLS, in the presence and absence of key proinflammatory cytokines IL-17A and TNFα (9). During the differentiation process, we stained the cells for Alkaline phosphatase (ALP) and for mineralization with alizarin red. ALP is expressed early in the osteogenic differentiation process and mineralization is a characteristic of matured osteoblasts.

A dose-response experiment showed that ≥5 nM rapamycin consistently reduced osteogenic differentiation of human SpA FLS *in vitro*, as evidenced by alizarin red staining (data not shown). There was no increase in cell death after rapamycin treatment, as demonstrated by WST-1 assay and by Trypan Blue staining (**Supplemental Figure 3**). We confirmed this effect in FLS cells from additional SpA patients, finding that rapamycin (5 nM) treatment significantly reduced ALP staining, a marker

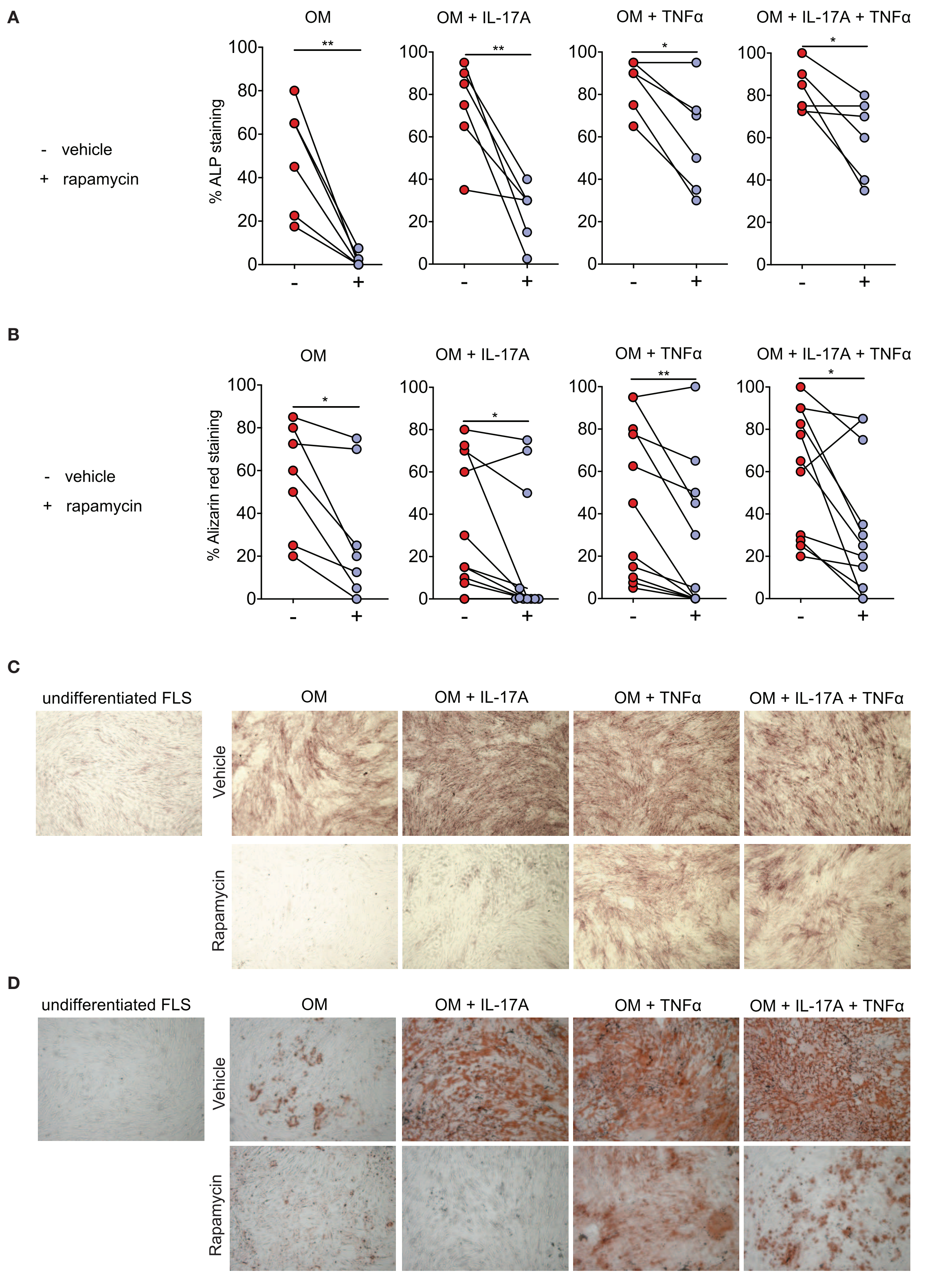


FIGURE 2 | Rapamycin reduces Spondyloarthritis (SpA) fibroblast-like synoviocytes (FLS) osteogenesis. **(A)** Rapamycin reduces osteogenic differentiation as evidenced by percentage of Alkaline phosphatase (ALP) staining at day 14 ($n = 6$), as well as by **(B)** percentage of alizarin red staining at at day 21 for OM ($n = 7$), and day 14 for the conditions with cytokines ($n = 11$). **(C)** Representative pictures of ALP staining and **(D)** alizarin red staining. OM: osteogenic media. $^{**}p < 0.01$ and $^{*}p < 0.05$.

for active (pre-) osteoblasts (**Figure 2A**), and alzarin red staining, a marker for mineralization (**Figure 2B**).

Rapamycin (5 nM) also significantly reduced osteogenic differentiation of human FLS in the presence of IL-17A and/or TNFα (**Figures 2A–D**). Consistent with prior report (9), IL-17A and TNFα accelerated human FLS osteoblastic differentiation as demonstrated by increased ALP staining (**Figure 2A**) and alizarin red staining (**Figure 2B**). Representative ALP (**Figure 2C**) and alizarin red (**Figure 2D**) pictures from one SpA FLS line are shown. These data indicate that rapamycin can reduce the osteoblastic differentiation rate of human SpA FLS *in vitro.*

mTOR Pathway Is Activated in Inflamed Synovial Tissues From Patients With SpA

We next assessed whether the mTOR pathway was activated in SpA synovitis. As phospho-S6 Ribosomal Protein (pS6) is a well-characterized downstream target of mTOR and indicates activation of the mTOR pathway, we stained for the presence of pS6 in inflamed SpA synovial tissue. Representative images are shown from 2 patients (**Figure 3A**). The majority of pS6 positive cells was observed in the sublining of the synovial tissue (**Figures 3A,B**). In addition to SpA synovial tissue, we also stained synovial tissue from rheumatoid arthritis (RA) patients, as the presence of pS6 and the expression of mTOR pathway have previously been described in RA synovitis (33).

pS6 levels are similar in SpA and RA synovial tissue groups as demonstrated by semiquantitative scoring (**Figure 3B**). In SpA synovial tissue, we observed colocalization of pS6 with CD3, a marker for T cells (**Figure 3C**). T cells from the synovial tissue have been demonstrated to express IL-17A (34). pS6 also stained CD45-negative cells (**Figure 3D**), indicating that the mTOR pathway is also activated in non-hematopoietic, stromal cells in SpA synovitis.

Prophylactic Treatment With Rapamycin Reduces Experimental Spondyloarthritis and New Bone Formation *in vivo*

Next, we tested the efficacy of rapamycin treatment in HLA-B27 tg rats, an experimental model of spondyloarthritis (9, 22, 35). In a prophylactic setting, 100% (**Figure 4A**) and 92% (**Figure 4D**) of animals developed arthritis and spondylitis, respectively, in the vehicle control group. Prophylactic rapamycin treatment significantly decreased the incidence of arthritis (36%, **Figure 4A**) and spondylitis (18%, **Figure 4D**). Moreover, arthritis severity (0.5 vs. 7.15 on a 0–12 scale, **Figure 4B**) and spondylitis severity (0.2 vs. 2.1 on a 0–3 scale, **Figure 4E**) were significantly reduced in the rapamycin vs. vehicle group. Plethysmometric analysis confirmed the significantly reduced swelling in the hind paws in the rapamycin treatment group (**Figure 4C**). Histological analysis of peripheral joints confirmed these clinical findings, demonstrating significantly reduced inflammation, bone and cartilage erosions, and new periosteal bone formation (**Figures 4F–H**), and a similar trend for the presence of hypertrophic chondrocytes (**Figure 4I**). The animals treated with rapamycin also had reduced inflammation in the spine (**Figure 4J**). Similar, but not significant, trends were observed for bone erosions, new bone formation and the presence of hypertrophic chondrocytes in the spine (**Figures 4K–M**). Prophylactic treatment data were pooled from two independent experiments.

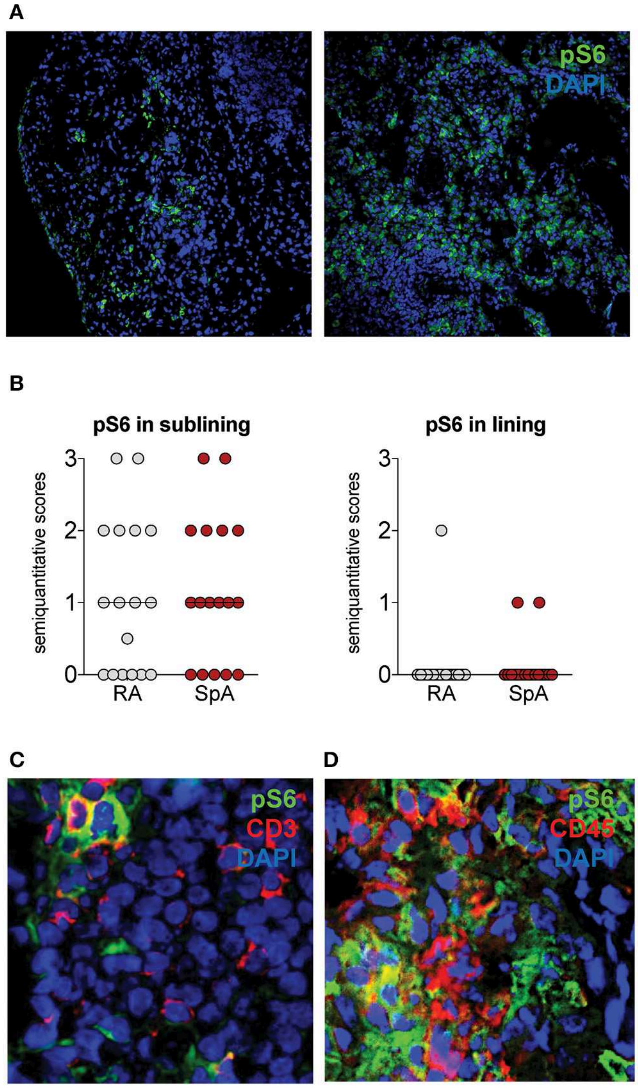


FIGURE 3 | pS6 is expressed in Spondyloarthritis (SpA) synovitis. **(A)** pS6 is mainly expressed in the sublining of the synovial tissue. Representative pictures from 2 SpA patients are shown. **(B)** Semiquantitative scoring of Ps6 in Rheumatoid Arthritis (RA, $n = 20$) and SpA ($n = 20$) synovial tissue. **(C)** pS6 partially colocalizes with CD3 by double immunofluorescence. **(D)** pS6 partially colocalizes with CD45.

Therapeutic Treatment With Rapamycin Attenuates Experimental Spondyloarthritis *in vivo*

We next assessed the effect of rapamycin on inflammation and bone pathology in a therapeutic setting. Treatment was started 1 week after 50% arthritis incidence (Day 30 after immunization with *M. tub*; **Figure 5**). In the vehicle group, the incidence

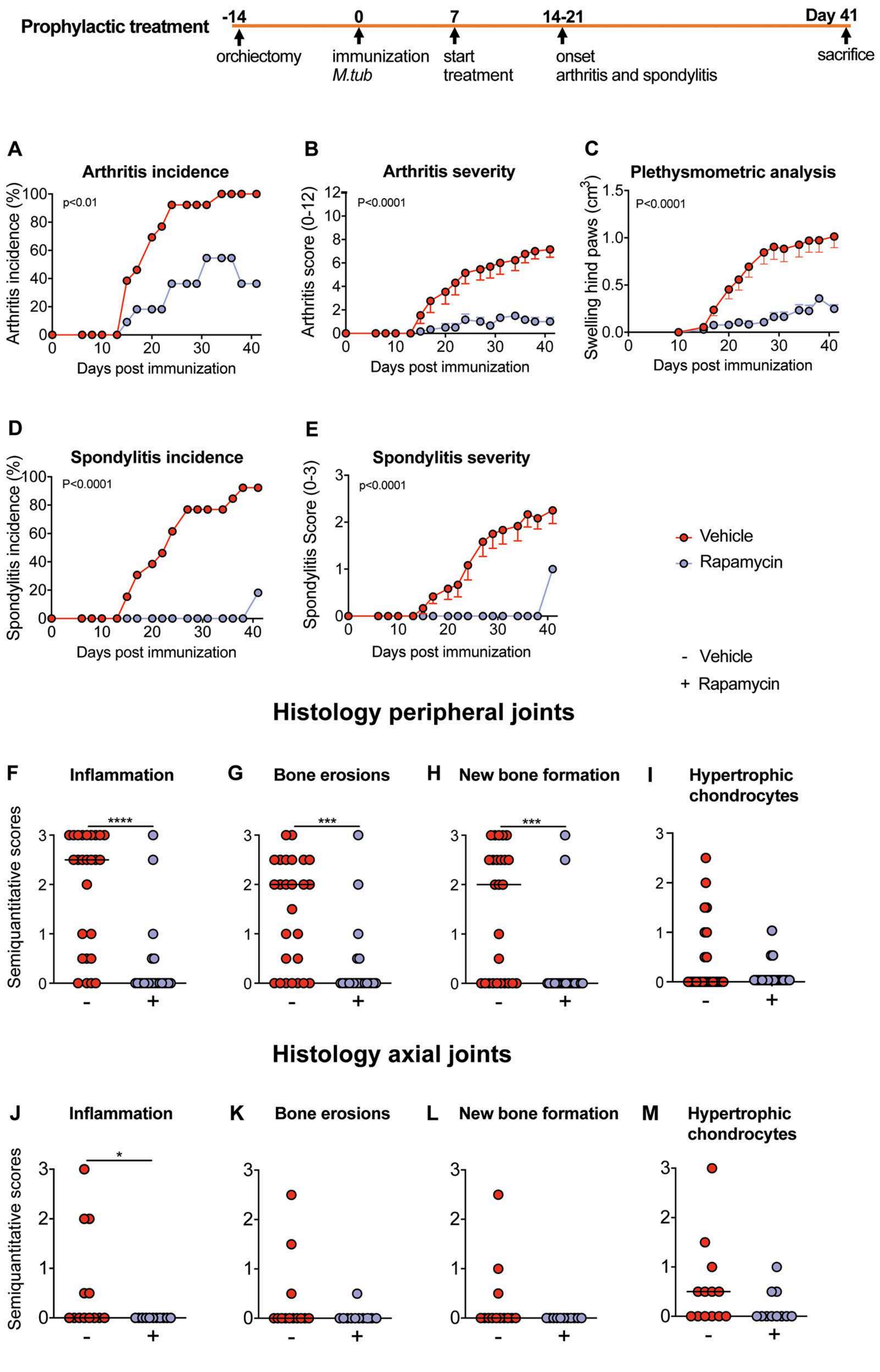


FIGURE 4 | Preventive treatment with rapamycin reduces experimental spondylarthritis *in vivo*. **(A)** Arthritis incidence. **(B)** Arthritis severity. **(C)** Plethysmometric analysis of the hind paws. **(D)** Spondylitis incidence and **(E)** spondylitis severity of vehicle ($n = 13$) and rapamycin treatment group ($n = 11$). **(F–I)** Semiquantitative scoring of inflammation, bone erosions and new bone formation in peripheral joint histology and in **(J–M)** axial histology. Mean ± SEM were presented in **(B,C,E)**. Median was depicted in **(F–M)**. *M.tub: Mycobacterium tuberculosis*. ****$p < 0.0001$, ***$p < 0.001$, and *$p < 0.05$.

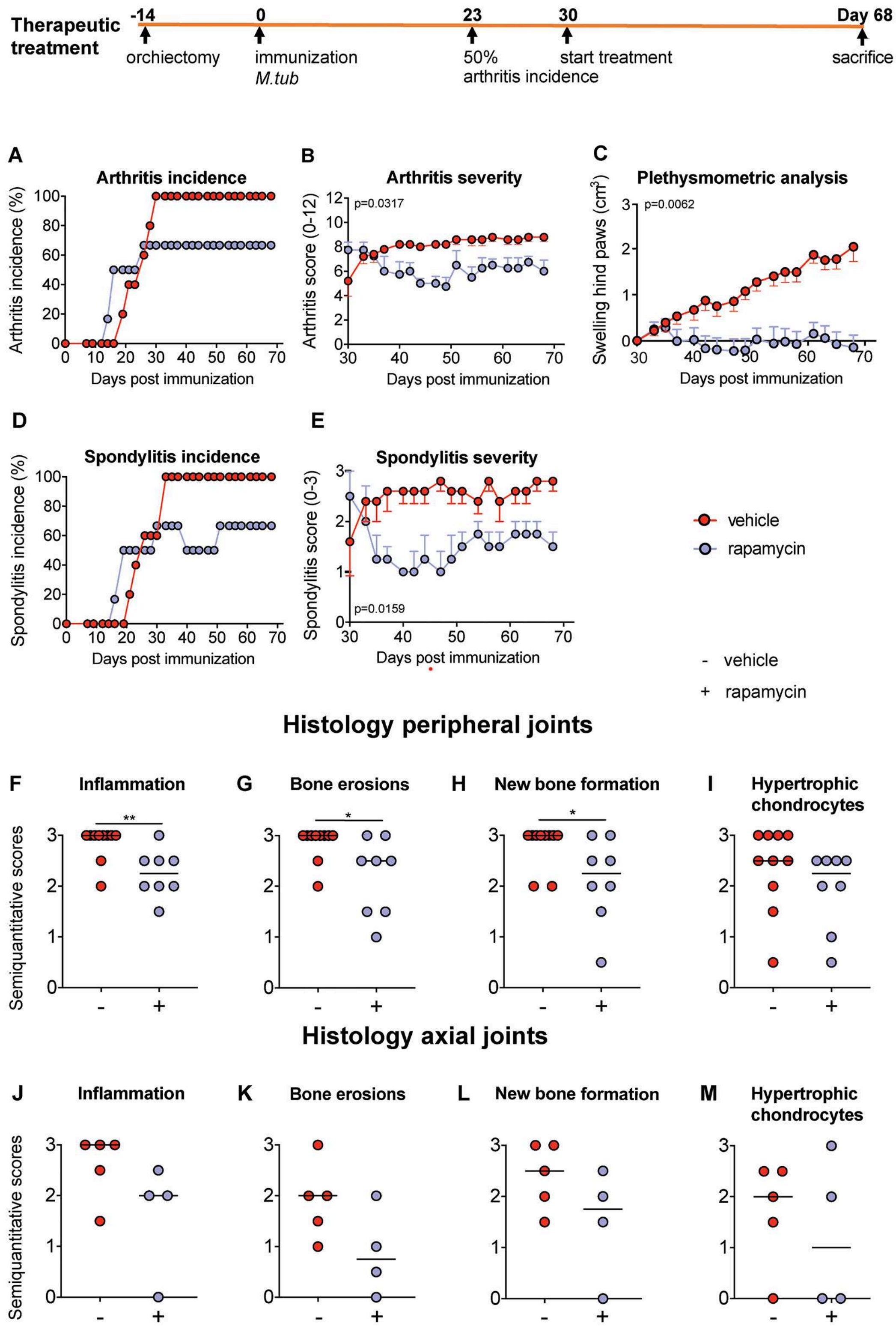


FIGURE 5 | Therapeutic treatment with rapamycin attenuates experimental spondyloarthritis *in vivo*. **(A)** The incidence of arthritis and **(D)** spondylitis are similar in the vehicle ($n = 5$) and rapamycin ($n = 4$) group at treatment initiation. In the diseased animals, treatment with rapamycin **(B)** diminished arthritis severity and **(E)** spondylitis severity. **(C)** Plethysmometric analysis of hindpaw swelling. **(F–I)** Semiquantitative scoring of inflammation, bone erosions and new bone formation in peripheral joint histology and in **(J–M)** axial histology. Mean ± SEM were presented in **(B,C,E)**. Median was depicted in **(F–M)**. *M.tub: Mycobacterium tuberculosis.* $^{**}p < 0.01$ and $^{*}p < 0.05$.

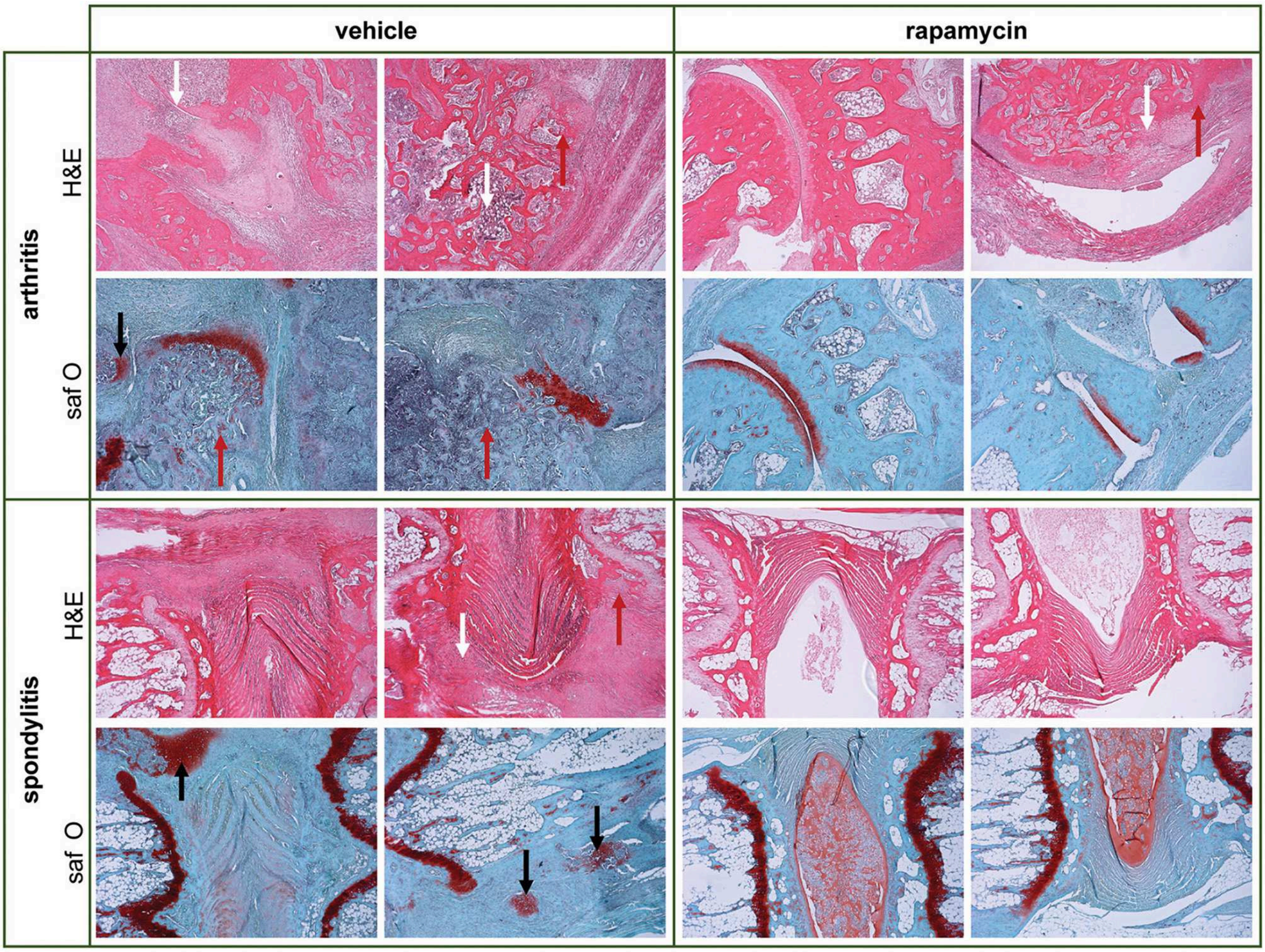


FIGURE 6 | Therapeutic treatment with rapamycin reduces inflammation, bone erosions and new bone formed in experimental spondyloarthritis. Representative hematoxylin and eosin (H&E) & Safranin O/Fast Green (saf O) pictures are shown for vehicle and rapamycin treated rats, to demonstrate aspects of pathology in the model. There is deformity of the joint anatomy with aspects of inflammation (white arrows), destruction/erosions (red arrows), and new bone formation (black arrows) in peripheral and axial joints. The quantification of the pathology is provided in **Figures 5F–M** for the therapeutic experiment (and in **Figures 4F–M** for the prophylactic experiment).

of arthritis and spondylitis continued to increase to 100% within 2 weeks after treatment initiation, whereas the incidence of both arthritis and spondylitis completely plateaued once rapamycin treatment was initiated (**Figures 5A,D**). Furthermore, therapeutic treatment with rapamycin significantly diminished arthritis severity (6 vs. 8.8; **Figure 5B**) and spondylitis severity (1.5 vs. 2.8; **Figure 5E**). In agreement with the clinical scores, plethysmometric analysis demonstrated a significant reduction in hind paw swelling in the rapamycin group compared to vehicle controls (**Figure 5C**). Histological analysis of peripheral joint tissues obtained 5 weeks after initiation of treatment confirmed the impact of rapamycin treatment on inflammation (**Figure 5F**), bone erosions (**Figure 5G**), and new periosteal bone formation (**Figure 5H**). A similar, but not significant trend was observed for hypertrophic chondrocytes in peripheral joints (**Figure 5I**). Histological analysis of the spine revealed similar trends (**Figures 5J–M**). Representative H&E and Safranin O staining of the peripheral joints and spine are shown in **Figure 6**.

Rapamycin Treatment Reduced IL-17A Expression in Inflamed Joints

We demonstrated that rapamycin inhibits IL-17A and TNFα production by human SpA PBMCs *in vitro*. To test the *in vivo* efficacy of rapamycin in the HLA-B27 tg rat model, we assessed the mRNA expression of key inflammatory cytokines such as IL-17A and TNFα in metacarpophalangeal (MCP) joints after therapeutic treatment with rapamycin. mRNA expression of *IL-17A, IL-17F*, and *IL-22* was significantly reduced in the MCP joints from the rapamycin vs. vehicle treatment group (**Figure 7**). This was paralleled by a trend toward reduction of the IL-17 master transcription factor *RORC*, but not the upstream Th17 differentiation cytokine *IL-23*. Also *IFNγ* mRNA expression was significantly reduced. There was a decreasing trend observed

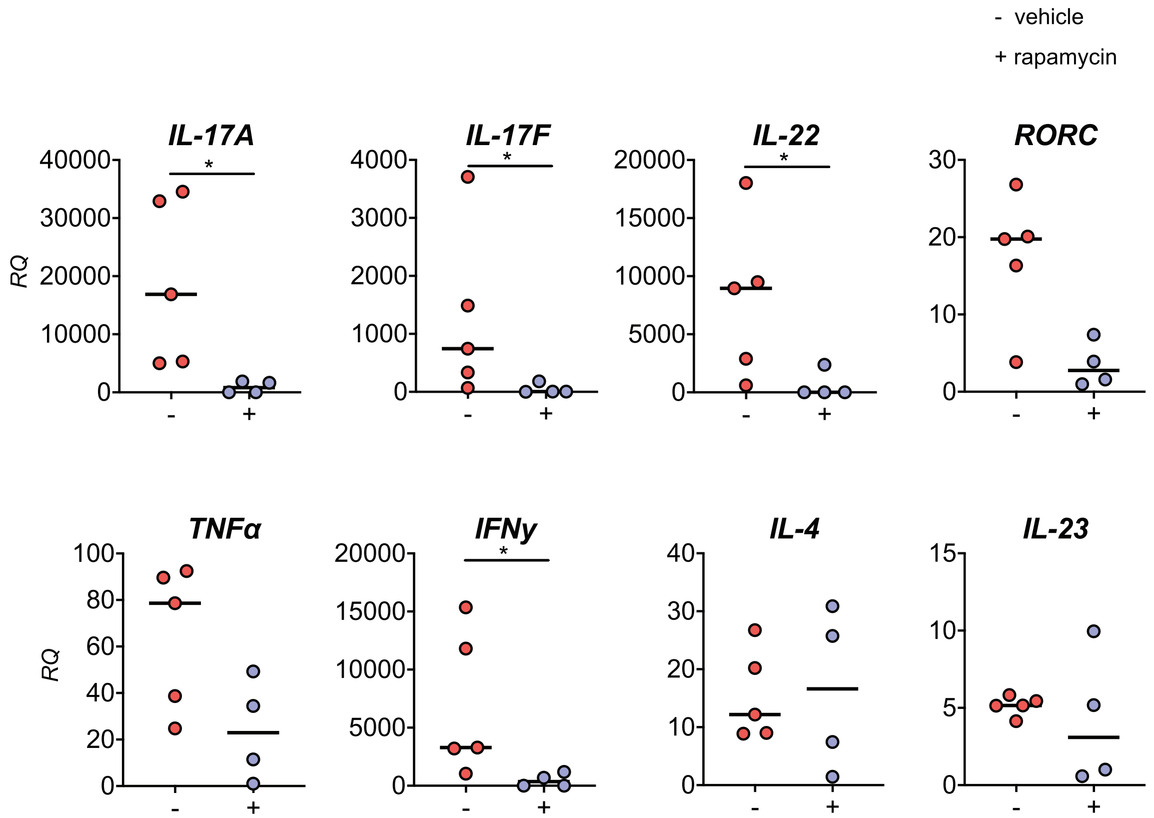


FIGURE 7 | Rapamycin treatment reduces IL-17A expression in inflamed joints. In the metacarpophalangeal (MCP) joints from HLA-B27 tg rats after the therapeutic treatment, mRNA expression of *IL-17A, IL-17F, IL-22,* and *IFNγ* are reduced in the rapamycin group. RQ, relative quantity. *$p < 0.05$.

for *TNFα,* whereas the prototypical Th2 cytokine *IL-4* was not modified.

DISCUSSION

We demonstrate here for the first time that targeting mTOR by rapamycin, prophylactically and therapeutically, reduces the incidence and severity of arthritis and spondylitis. Histology confirmed reduced inflammation, bone erosions and new periosteal bone formation in the peripheral joints with similar trends observed in the spine. These findings were supported by data we obtained *in vitro* in human SpA cells and from human synovial tissue *ex vivo*: rapamycin attenuated inflammatory cytokine production by human SpA PBMCs; rapamycin reduced human SpA fibroblast-like synoviocytes (FLS) osteogenesis rate, both in the presence and absence of TNFα and IL-17A; and the mTOR pathway is activated in human SpA synovitis.

Taken together, these data suggest that mTOR blockade by rapamycin attenuates inflammation, bone remodeling and new periosteal bone formation, which are all hallmarks of SpA pathogenesis. mTOR might be a promising therapeutic target in SpA patients, especially considering the effcacy of rapamycin in reducing new bone formation and bone erosions *in vivo.* This would address an important unmet clinical need in SpA patients to target bone pathology (1, 5).

Two potential explanations for the inhibitory effect of rapamycin on new bone formation in the HLA-B27 tg model are: it may either be the result of rapamycin reducing expression of the cytokine IL-17A, or a direct inhibitory effect of rapamycin on bone precursor cells. We have previously demonstrated that IL-17A promotes pathologic bone processes in the HLA-B27 tg rats and shown that IL-17A accelerates osteogenic differentiation of human SpA FLS *in vitro* (9). In line with these findings, others have reported that IL-17A accelerates osteogenic differentiation of FLS cells from reumatoid arthritis (RA) and osteoarthritis (OA) patients (12). We now demonstrate that FLS osteogenesis can be inhibited by rapamycin treatment, independently of IL-17A and TNFα cytokines. Rapamycin treatment in SpA PBMCs *in vitro* also inhibits production of IL-17A and TNFα. In addition to TNFα (1) and IL-17A (2, 3), IL-17F has recently been demonstrated to play an essential proinflammatory role in SpA pathogenesis, similarly to IL-17A (36). IL-17A and IL-17F m RNA expression were significantly reduced *in vivo* after rapamycin treatment.

Although we did not directly test this, rapamycin may suppress inflammation by promoting autophagy of misfolded HLA-B27. HLA-B27 heavy chain misfolding has been postulated to play a role in SpA pathogenesis (37, 38). In a HLA-B27 transgenic rat model (33-3 rats), rapamycin promoted autophagy-mediated degradation of misfolded B27 heavy chains *in vitro* and could thereby suppress the IL-23/IL-17 pathway (39–41). The presence of HLA-B27 misfolding and the effect of rapamycin treatment on autophagy remains to be tested in our model (21–3 × 283–2 rats).

Rapamycin may also have a direct effect on bone differentiation via mTOR's interaction with several anabolic bone pathways (19, 42, 43). mTOR is a kinase that forms mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (19), and these complexes integrate signals from a multitude of signaling pathways, including Wnt, PI3K-Akt, IGF, Notch, BMP, and mechanical stress (19, 44–52). Prolonged treatment with rapamycin has been demonstrated to inhibit both mTORC1 and mTORC2 (53), and likely alters bone pathway signaling. The exact mechanism of new bone formation in SpA remains to be elucidated (10, 54). In another model of ectopic bone formation, heterotopic ossification (HO), rapamycin treatment reduced ectopic bone by 50% (55). How the pathways involved in heterotopic ossification (HO) compare to those of SpA may be of interest.

In addition to bone precursor cells and osteoblasts, osteoclasts are also important cellular players in bone remodeling. We did not address osteoclasts in the present study, as the effect of rapamycin on osteoclasts has been studied previously. Inhibition of mTOR has been demonstrated to halt osteoclastogenesis (56, 57) and to improve joint erosions in a TNF-transgenic mice model of rheumatoid arthritis (33). These findings are in line with the attenuated bone erosions we observe in the HLA-B27 tg rats after rapamycin treatment, which could be explained by the inhibitory effect of rapamycin on IL-17A and TNFα production, as both these cytokines promote osteoclastogenesis (58–61).

Given that there is currently limited therapy in SpA, these results may support efforts to evaluate the efficacy of rapamycin treatment for SpA patients. Rapamycin has also been found to reduce inflammation in animal models of psoriasis (62) and colitis (18, 63). These disease manifestations can co-occur with SpA and have overlapping disease mechanisms with SpA (1). The side-effects of rapamycin have been characterized and include hyperlipidemia and osteonecrosis (64). Recently, low dose of rapamycin has been demonstrated to be effcacious in reducing musculoskeletal manifestations in mildly active SLE patients without serious side-effects (65). It is also promising that short-term side-effects such as dyslipidemia may subside after long-term mTOR blocking therapy (64). Moreover, there are new generation small molecules that target the mTOR pathway with potentially fewer side effects. The upstream PI3K/Akt/mTOR pathway may also present an additional set of potential targets for modulating mTOR activity in SpA pathology.

CONCLUSIONS

We provide a rationale for targeting the mTOR pathway in spondyloarthtritis by demonstrating that mTOR blockade with rapamycin inhibits IL-17A and TNFα production by SpA PBMCs and osteoblastic differentiation of human SpA FLS *in vitro*. In the HLA-B27 transgenic rat model of SpA, mTOR blockade reduces arthritis and spondylitis development and severity with decreased inflammation and bone defects with suppression of IL-17A. These results may support efforts to evaluate the efficacy of targeting the mTOR pathway in SpA patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Amsterdam University Medical Center, University of Amsterdam, the Netherlands. The patients/participants provided their written informed consent to participate in this study. Animal experiments were approved by the Amsterdam University Medical Center (AUMC) Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SC, LD, and DB contributed to study design, data collection, analysis, interpretation, and wrote the manuscript. MT, VK, LK, LB, and DP contributed to data collection, analysis, and interpretation and revised the manuscript. JT, EG, and MS contributed to analysis, interpretation of the data and critically revised the manuscript. All authors read and approved the submitted version of the manuscript.

FUNDING

This work was supported by a grant from the Dutch Arthritis Foundation, by a VICI grant from the Netherlands Organization for Scientific Research (NWO) and by the Consolidator Grant from the European Research Council (ERC).

ACKNOWLEDGMENTS

We thank Catherine Manning and Daniel Montoro for proofreading the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02344/full#supplementary-material

Supplemental Figure 1 | Viability of PBMCs ($n = 3$) in the presence of vehicle (DMSO) and rapamycin. **(A)** FACs gating strategy for DAPI and PI staining. **(B,C)** The percentage of viable cells are shown, normalized to (untreated) control conditions after 48 h of culture (mean ± SD, $n = 3$).

Supplemental Figure 2 | The effect of rapamycin on IL-17A and TNFα protein production by synovial mononuclear cells (SFMCs) from SpA patients ($n = 2$) *in vitro*. **(A)** IL-17A and **(B)** TNFα protein concentrations were measured in the supernatant.

Supplemental Figure 3 | Viability of fibroblast-like synoviocytes (FLS) in the presence of vehicle (DMSO) and rapamycin (5 nM). **(A)** Measurements by WST-1 assay after 30 min and **(B)** after 2.5 h (mean ± SD, $n = 4$). **(C)** The percentage of Trypan Blue-negative cells after 48 h of culture (mean ± SD, $n = 3$). ns, not significant.

REFERENCES

1. Dougados M, Baeten D. Spondyloarthritis. *Lancet.* (2011) 377:2127–37. doi: 10.1016/S0140-6736(11)60071-8
2. Baeten D, Sieper J, Braun J, Baraliakos X, Dougados M, Emery P, et al. Secukinumab, an interleukin-17A inhibitor, in ankylosing spondylitis. *N Engl J Med.* (2015) 373:2534–48. doi: 10.1056/NEJMoa1505066
3. Baraliakos X, Kivitz AJ, Deodhar AA, Braun J, Wei JC, Delicha EM, et al. Long-term effects of interleukin-17A inhibition with secukinumab in active ankylosing spondylitis: 3-year efficacy and safety results from an extension of the Phase 3 MEASURE 1 trial. *Clin Exp Rheumatol.* (2018) 36:50–5.
4. Gorman JD, Sack KE, Davis JC Jr. Long-term efficacy and safety of secukinumab 150 mg in ankylosing spondyloarthritis: 5-year results from the phase III MEASURE 1 extension study. *RMD Open.* (2019) 5. doi: 10.1136/rmdopen-2019-001005
5. Braun J, Baraliakos X, Deodhar A, Baeten D, Sieper J, Emery P, et al. Effect of secukinumab on clinical and radiographic outcomes in ankylosing spondylitis: 2-year results from the randomised phase III MEASURE 1 study. *Ann Rheum Dis.* (2017) 76:1070–7. doi: 10.1136/annrheumdis-2016-209730
6. van der Heijde D, Landewe R, Baraliakos X, Houben H, van Tubergen A, Williamson P, et al. Radiographic findings following two years of infliximab therapy in patients with ankylosing spondylitis. *Arthritis Rheum.* (2008) 58:3063–70. doi: 10.1002/art.23901
7. Zhang JR, Liu XJ, Xu WD, Dai SM. Effects of tumor necrosis factor-alpha inhibitors on new bone formation in ankylosing spondylitis. *Joint Bone Spine.* (2016) 83:257–64. doi: 10.1016/j.jbspin.2015.06.013
8. Finzel S, Kraus S, Schmidt S, Hueber A, Rech J, Engelke K, et al. Bone anabolic changes progress in psoriatic arthritis patients despite treatment with methotrexate or tumour necrosis factor inhibitors. *Ann Rheum Dis.* (2013) 72:1176–81. doi: 10.1136/annrheumdis-2012-201580
9. van Tok MN, van Duivenvoorde LM, Kramer I, Ingold P, Pfister S, Roth L, et al. Interleukin-17A inhibition diminishes inflammation and new bone formation in experimental spondyloarthritis. *Arthritis Rheumatol.* (2018) 71:612–25. doi: 10.1002/art.40770
10. Baum R, Gravallese EM. Bone as a target organ in rheumatic disease: impact on osteoclasts and osteoblasts. *Clin Rev Allergy Immunol.* (2016) 51:1–15. doi: 10.1007/s12016-015-8515-6
11. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum.* (2001) 44:1928–42. doi: 10.1002/1529-0131(200108)44:8<1928::AID-ART331>3.0.CO;2-P
12. Osta B, Roux JP, Lavocat F, Pierre M, Ndongo-Thiam N, Boivin G, et al. Differential effects of IL-17A and TNF-alpha on osteoblastic differentiation of isolated synoviocytes and on bone explants from arthritis patients. *Front Immunol.* (2015) 6:151. doi: 10.3389/fimmu.2015.00151
13. Weichhart T. Mammalian target of rapamycin: a signaling kinase for every aspect of cellular life. *Methods Mol Biol.* (2012) 821:1–14. doi: 10.1007/978-1-61779-430-8_1
14. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab.* (2014) 19:373–9. doi: 10.1016/j.cmet.2014.01.001
15. Webster AC, Lee VW, Chapman JR, Craig JC. Target of rapamycin inhibitors (TOR-I; sirolimus and everolimus) for primary immunosuppression in kidney transplant recipients. *Cochrane Database Syst Rev.* 2006:CD004290. doi: 10.1002/14651858.CD004290.pub2
16. Kurebayashi Y, Nagai S, Ikejiri A, Ohtani M, Ichiyama K, Baba Y, et al. PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORgamma. *Cell Rep.* (2012) 1:360–73. doi: 10.1016/j.celrep.2012.02.007
17. Ren W, Yin J, Duan J, Liu G, Tan B, Yang G, et al. mTORC1 signaling and IL-17 expression: defining pathways and possible therapeutic targets. *Eur J Immunol.* (2016) 46:291–9. doi: 10.1002/eji.201545886
18. Hu S, Chen M, Wang Y, Wang Z, Pei Y, Fan R, et al. mTOR inhibition attenuates dextran sulfate sodium-induced colitis by suppressing T cell proliferation and balancing TH1/TH17/Treg profile. *PLoS ONE.* (2016) 11:e0154564. doi: 10.1371/journal.pone.0154564
19. Chen J, Long F. mTOR signaling in skeletal development and disease. *Bone Res.* (2018) 6:1. doi: 10.1038/s41413-017-0004-5
20. Vieira-Sousa E, van Duivenvoorde LM, Fonseca JE, Lories RJ, Baeten DL. Review: animal models as a tool to dissect pivotal pathways driving spondyloarthritis. *Arthritis Rheumatol.* (2015) 67:2813–27. doi: 10.1002/art.39282
21. van Duivenvoorde LM, Dorris M, Satumtira N, van Tok MN, Redlich K, Tak PP, et al. Relationship between inflammation, bone destruction, and osteoproliferation in spondyloarthritis in HLA-B27/Huβ2m transgenic rats. *Arthritis Rheumatol.* (2012) 64:3210–9. doi: 10.1002/art.34600
22. van Tok MN, Satumtira N, Dorris M, Pots D, Slobodin G, van de Sande MG, et al. Innate immune activation can trigger experimental spondyloarthritis in HLA-B27/Hubeta2m transgenic rats. *Front Immunol.* (2017) 8:920. doi: 10.3389/fimmu.2017.00920
23. Rudwaleit M, van der Heijde D, Landewe R, Akkoc N, Brandt J, Chou CT, et al. The assessment of spondyloarthritis international society classification criteria for peripheral spondyloarthritis and for spondyloarthritis in general. *Ann Rheum Dis.* (2011) 70:25–31. doi: 10.1136/ard.2010.133645
24. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* (2010) 62:2569–81. doi: 10.1002/art.27584
25. Baeten D, Van den Bosch F, Elewaut D, Stuer A, Veys EM, De Keyser F. Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. *Clin Rheumatol.* (1999) 18:434–41. doi: 10.1007/s100670050134
26. Galluzzi L, Morselli E, Kepp O, Vitale I, Younes AB, Maiuri MC, et al. Evaluation of rapamycin-induced cell death. *Methods Mol Biol.* (2012) 821:125–69. doi: 10.1007/978-1-61779-430-8_9
27. Smith MD, Baeten D, Ulfgren AK, McInnes IB, Fitzgerald O, Bresnihan B, et al. Standardisation of synovial tissue infiltrate analysis: how far have we come? How much further do we need to go? *Ann Rheum Dis.* (2006) 65:93–100. doi: 10.1136/ard.2005.036905
28. Baeten D, Demetter P, Cuvelier C, Van Den Bosch F, Kruithof E, Van Damme N, et al. Comparative study of the synovial histology in rheumatoid arthritis, spondyloarthropathy, and osteoarthritis: influence of disease duration and activity. *Ann Rheum Dis.* (2000) 59:945–53. doi: 10.1136/ard.59.12.945
29. van Mens LJJ, van de Sande MGH, Menegatti S, Chen S, Blijdorp ICJ, de Jong HM, et al. IL-17 blockade with secukinumab in peripheral spondyloarthritis impacts synovial immunopathology without compromising systemic immune responses. *Arthritis Rheumatol.* (2018) 70:1994–2002. doi: 10.1136/annrheumdis-2018-EWRR2018.15
30. Tran TM, Dorris ML, Satumtira N, Richardson JA, Hammer RE, Shang J, et al. Additional human beta2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats. *Arthritis Rheum.* (2006) 54:1317–27. doi: 10.1002/art.21740
31. Taurog JD, Rival C, van Duivenvoorde LM, Satumtira N, Dorris ML, Sun M, et al. Autoimmune epididymoorchitis is essential to the pathogenesis of male-specific spondylarthritis in HLA-B27-transgenic rats. *Arthritis Rheum.* (2012) 64:2518–28. doi: 10.1002/art.34480
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
33. Cejka D, Hayer S, Niederreiter B, Sieghart W, Fuereder T, Zwerina J, et al. Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. *Arthritis Rheum.* (2010) 62:2294–302. doi: 10.1002/art.27504
34. Wade SM, Canavan M, McGarry T, Low C, Wade SC, Mullan RH, et al. Association of synovial tissue polyfunctional T-cells with DAPSA in psoriatic arthritis. *Ann Rheum Dis.* (2019) 78:350–4. doi: 10.1136/annrheumdis-2018-214138
35. van Tok MN, Na S, Lao CR, Alvi M, Pots D, van de Sande MGH, et al. The initiation, but not the persistence, of experimental spondyloarthritis is dependent on interleukin-23 signaling. *Front Immunol.* (2018) 9:1550. doi: 10.3389/fimmu.2018.01550
36. Glatt S, Helmer E, Haier B, Strimenopoulou F, Price G, Vajjah P, et al. First-in-human randomized study of bimekizumab, a humanized monoclonal antibody and selective dual inhibitor of IL-17A and IL-17F, in mild psoriasis. *Br J Clin Pharmacol.* (2017) 83:991–1001. doi: 10.1111/bcp.13185

37. Colbert RA, Tran TM, Layh-Schmitt G. HLA-B27 misfolding and ankylosing spondylitis. *Mol Immunol.* (2014) 57:44–51. doi: 10.1016/j.molimm.2013.07.013
38. Caffrey MF, James DC. Human lymphocyte antigen association in ankylosing spondylitis. *Nature.* (1973) 242:121. doi: 10.1038/242121a0
39. Navid F, Layh-Schmitt G, Sikora KA, Cougnoux A, Colbert RA. The role of autophagy in the degradation of misfolded HLA-B27 heavy chains. *Arthritis Rheumatol.* (2018) 70:746–55. doi: 10.1002/art.40414
40. DeLay ML, Turner MJ, Klenk EI, Smith JA, Sowders DP, Colbert RA. HLA-B27 misfolding and the unfolded protein response augment interleukin-23 production and are associated with Th17 activation in transgenic rats. *Arthritis Rheum.* (2009) 60:2633–43. doi: 10.1002/art.24763
41. Glatigny S, Fert I, Blaton MA, Lories RJ, Araujo LM, Chiocchia G, et al. Proinflammatory Th17 cells are expanded and induced by dendritic cells in spondylarthritis-prone HLA-B27-transgenic rats. *Arthritis Rheum.* (2012) 64:110–20. doi: 10.1002/art.33321
42. Pantovic A, Krstic A, Janjetovic K, Kocic J, Harhaji-Trajkovic L, Bugarski D, et al. Coordinated time-dependent modulation of AMPK/Akt/mTOR signaling and autophagy controls osteogenic differentiation of human mesenchymal stem cells. *Bone.* (2013) 52:524–31. doi: 10.1016/j.bone.2012.10.024
43. Singha UK, Jiang Y, Yu S, Luo M, Lu Y, Zhang J, et al. Rapamycin inhibits osteoblast proliferation and differentiation in MC3T3-E1 cells and primary mouse bone marrow stromal cells. *J Cell Biochem.* (2008) 103:434–46. doi: 10.1002/jcb.21411
44. Martin SK, Fitter S, Dutta AK, Matthews MP, Walkley CR, Hall MN, et al. Brief report: the differential roles of mTORC1 and mTORC2 in mesenchymal stem cell differentiation. *Stem Cells.* (2015) 33:1359–65. doi: 10.1002/stem.1931
45. Liu DM, Zhao L, Liu TT, Jiao PL, Zhao DD, Shih MS, et al. Rictor/mTORC2 loss in osteoblasts impairs bone mass and strength. *Bone.* (2016) 90:50–8. doi: 10.1016/j.bone.2016.05.010
46. Chen J, Tu X, Esen E, Joeng KS, Lin C, Arbeit JM, et al. WNT7B promotes bone formation in part through mTORC1. *PLoS Genet.* (2014) 10:e1004145. doi: 10.1371/journal.pgen.1004145
47. Xian L, Wu X, Pang L, Lou M, Rosen CJ, Qiu T, et al. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nat Med.* (2012) 18:1095–101. doi: 10.1038/nm.2793
48. Huang B, Wang Y, Wang W, Chen J, Lai P, Liu Z, et al. mTORC1 prevents preosteoblast differentiation through the notch signaling pathway. *PLoS Genet.* (2015) 11:e1005426. doi: 10.1371/journal.pgen.1005426
49. Karner CM, Lee SY, Long F. Bmp induces osteoblast differentiation through both Smad4 and mTORC1 signaling. *Mol Cell Biol.* (2017) 37:e00253-16. doi: 10.1128/MCB.00253-16
50. Esen E, Chen J, Karner CM, Okunade AL, Patterson BW, Long F. WNT-LRP5 signaling induces Warburg effect through mTORC2 activation during osteoblast differentiation. *Cell Metab.* (2013) 17:745–55. doi: 10.1016/j.cmet.2013.03.017
51. Shi Y, Chen J, Karner CM, Long F. Hedgehog signaling activates a positive feedback mechanism involving insulin-like growth factors to induce osteoblast differentiation. *Proc Natl Acad Sci USA.* (2015) 112:4678–83. doi: 10.1073/pnas.1502301112
52. Sen B, Xie Z, Case N, Thompson WR, Uzer G, Styner M, et al. mTORC2 regulates mechanically induced cytoskeletal reorganization and lineage selection in marrow-derived mesenchymal stem cells. *J Bone Miner Res.* (2014) 29:78–89. doi: 10.1002/jbmr.2031
53. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell.* (2006) 22:159–68. doi: 10.1016/j.molcel.2006.03.029
54. Lories RJ, Luyten FP, de Vlam K. Progress in spondylarthritis. Mechanisms of new bone formation in spondyloarthritis. *Arthritis Res Ther.* (2009) 11:221. doi: 10.1186/ar2642
55. Qureshi AT, Dey D, Sanders EM, Seavey JG, Tomasino AM, Moss K, et al. Inhibition of mammalian target of rapamycin signaling with rapamycin prevents trauma-induced heterotopic ossification. *Am J Pathol.* (2017) 187:2536–45. doi: 10.1016/j.ajpath.2017.07.010
56. Zhang Y, Xu S, Li K, Tan K, Liang K, Wang J, et al. mTORC1 inhibits NF-kappaB/NFATc1 signaling and prevents osteoclast precursor differentiation, *in vitro* and in mice. *J Bone Miner Res.* (2017) 32:1829–40. doi: 10.1002/jbmr.3172
57. Dai Q, Xie F, Han Y, Ma X, Zhou S, Jiang L, et al. Inactivation of regulatory-associated protein of mTOR (Raptor)/mammalian target of rapamycin complex 1 (mTORC1) signaling in osteoclasts increases bone mass by inhibiting osteoclast differentiation in mice. *J Biol Chem.* (2017) 292:196–204. doi: 10.1074/jbc.M116.764761
58. Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. *J Biol Chem.* (2000) 275:4858–64. doi: 10.1074/jbc.275.7.4858
59. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, et al. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med.* (2000) 191:275–86. doi: 10.1084/jem.191.2.275
60. Gravallese EM, Schett G. Effects of the IL-23-IL-17 pathway on bone in spondyloarthritis. *Nat Rev Rheumatol.* (2018) 14:631–40. doi: 10.1038/s41584-018-0091-8
61. Yago T, Nanke Y, Ichikawa N, Kobashigawa T, Mogi M, Kamatani N, et al. IL-17 induces osteoclastogenesis from human monocytes alone in the absence of osteoblasts, which is potently inhibited by anti-TNF-alpha antibody: a novel mechanism of osteoclastogenesis by IL-17. *J Cell Biochem.* (2009) 108:947–55. doi: 10.1002/jcb.22326
62. Burger C, Shirsath N, Lang V, Diehl S, Kaufmann R, Weigert A, et al. Blocking mTOR signalling with rapamycin ameliorates imiquimod-induced psoriasis in mice. *Acta Derm Venereol.* (2017) 97:1087–94. doi: 10.2340/00015555-2724
63. Yin H, Li X, Zhang B, Liu T, Yuan B, Ni Q, et al. Sirolimus ameliorates inflammatory responses by switching the regulatory T/T helper type 17 profile in murine colitis. *Immunology.* (2013) 139:494–502. doi: 10.1111/imm.12096
64. Nguyen LS, Vautier M, Allenbach Y, Zahr N, Benveniste O, Funck-Brentano C, et al. Sirolimus and mTOR inhibitors: a review of side effects and specific management in solid organ transplantation. *Drug Saf.* (2019) 42:813–25. doi: 10.1007/s40264-019-00810-9
65. Eriksson P, Wallin P, Sjowall C. Clinical experience of sirolimus regarding efficacy and safety in systemic lupus erythematosus. *Front Pharmacol.* (2019) 10:82. doi: 10.3389/fphar.2019.00082

Conflict of Interest: DB is an employee of UCB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 12 March 2020
doi: 10.3389/fimmu.2020.00348

Check for updates

Transcriptional Regulators of T Helper 17 Cell Differentiation in Health and Autoimmune Diseases

Alessia Capone[1,2] *and Elisabetta Volpe*[1]*

[1] Neuroimmunology Unit, IRCCS Fondazione Santa Lucia, Rome, Italy, [2] Department of Biology and Biotechnology Charles Darwin, Sapienza University, Rome, Italy

T helper (Th) 17 cells are a subtype of CD4 T lymphocytes characterized by the expression of retinoic acid-receptor (RAR)-related orphan receptor (ROR)γt transcription factor, encoded by gene *Rorc*. These cells are implicated in the pathology of autoimmune inflammatory disorders as well as in the clearance of extracellular infections. The main function of Th17 cells is the production of cytokine called interleukin (IL)-17A. This review highlights recent advances in mechanisms regulating transcription of IL-17A. In particular, we described the lineage defining transcription factor RORγt and other factors that regulate transcription of *Il17a* or *Rorc* by interacting with RORγt or by binding their specific DNA regions, which may positively or negatively influence their expression. Moreover, we reported the eventual involvement of those factors in Th17-related diseases, such as multiple sclerosis, rheumatoid arthritis, psoriasis, and Crohn's disease, characterized by an exaggerated Th17 response. Finally, we discussed the potential new therapeutic approaches for Th17-related diseases targeting these transcription factors. The wide knowledge of transcriptional regulators of Th17 cells is crucial for the better understanding of the pathogenic role of these cells and for development of therapeutic strategies aimed at fighting Th17-related diseases.

Keywords: T helper 17 cells, interleukin-17, retinoic acid receptor related orphan nuclear receptor γt, multiple sclerosis, Crohn's disease, rheumatoid arthritis, psoriasis

Edited by:
Nicola Ivan Lorè,
IRCCS San Raffaele Scientific Institute, Italy

Reviewed by:
Rami Bechara,
University of Pittsburgh, United States
Samuele Notarbartolo,
Istituto Nazionale Genetica Molecolare (INGM), Italy
Laura A. Solt,
The Scripps Research Institute, United States

****Correspondence:***
Elisabetta Volpe
e.volpe@hsantalucia.it

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *13 December 2019*
Accepted: *13 February 2020*
Published: *12 March 2020*

Citation:
Capone A and Volpe E (2020) Transcriptional Regulators of T Helper 17 Cell Differentiation in Health and Autoimmune Diseases. Front. Immunol. 11:348. doi: 10.3389/fimmu.2020.00348

INTRODUCTION

T helper (Th) 17 cells are a subtype of CD4 T lymphocytes, specialized in immune response against fungi and some extracellular bacteria (1–4). The interleukin (IL)-17A, originally named CTLA8, is the most representative cytokine produced by Th17 cells (3, 5, 6), also produced by cytotoxic T lymphocytes, and innate lymphocytes, including γδ T, natural killer T, and group 3 innate lymphoid cells (7).

The binding of IL-17A with its receptor activates the target cells, such as epithelial cells, endothelial cells, and fibroblasts (3, 4, 8) and induces CXCL1, CXCL2, and CXCL8, which attract myeloid cells such as neutrophils to the infected or injured tissue (9); IL-6 and G-CSF, which promote myeloid-driven innate inflammation (10); and β-defensins, S100A8, and lipocalin 2, which protect the host during acute microbial invasion (11).

In addition to IL-17A, Th17 cells produce IL-17F, IL-21, IL-22, and, in human, also IL-26 (3, 5, 6, 12), which collectively ensure an appropriate defense against pathogens. In fact, genetic defects in the Th17–cytokine pathways lead to severe mucocutaneous candidiasis (13–15).

However, a dysregulated activity of Th17 cells has been associated to autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis, psoriasis, and Crohn's disease (8, 16).

Given the relevance of Th17 cells in both physiological and pathological contexts, numerous studies investigated the molecular mechanisms regulating the transcriptional program of Th17 cells.

Majority of the Th17 transcription factors were discovered and validated through analysis of IL-17A expression in mice deficient for specific transcription factors, and mice containing a GFP reporter cDNA knocked-in at the site for initiation of the translation of specific transcription factors (17–21). Similarly, the *in vitro* expression of IL-17A was assessed in cells cotransfected with constructs overexpressing the specific transcription factors and reporter constructs containing regions upstream of the *Il17a* transcription start site (17, 19). More recently, modern technologies, such as chromatin immunoprecipation (ChIP) and single-cell RNA-sequencing, were allowed to better explore the functions of transcription factors in Th17 cells (22–24). However, although the expression of Th17 transcription factors was validated in human Th17 cells, most of the studies demonstrating their regulatory mechanism were performed in murine cells.

The first transcription factor discovered, designated as the "lineage defining transcription factor of Th17 cells," is RORγt, which is essential and sufficient to induce Th17 lineage fate in both human and mouse cells (5, 17, 25).

However, succeeding studies revealed that multiple transcriptional regulators contribute to full Th17 differentiation program through several mechanisms, including binding to specific regions of *Il17a* and *Rorc* genes, or interacting and synergizing with RORγt, or facilitating the recruitment of other proteins on *Il17a* or *Rorc* promoters.

Collectively, Th17 transcriptional regulators may contribute to Th17 functions in physiological and pathological contexts. Thus, in this review, we reported recent advances on the molecular mechanisms directly regulating transcription of *Il17a* and *Rorc*. Moreover, we discussed their involvement in autoimmune disorders associated to an exaggerated Th17 response. Finally, we discussed the recent therapeutic approaches targeting Th17 transcriptional regulators in Th17-related autoimmune diseases.

RETINOIC ACID-RECEPTOR-RELATED ORPHAN RECEPTOR (ROR) TRANSCRIPTION FACTORS IN TH17 CELLS

The retinoic acid-related orphan nuclear receptors (RORs) belong to a superfamily of ligand regulated transcription factors (26, 27). ROR transcription factors bind DNA response elements, called ROR response elements (ROREs) (26, 28), consisting of the consensus core motif AGGTCA preceded by a 5′ A/T-rich sequence located into regulatory regions of target genes (27).

The interaction of ROR factors with their specific ligands allows recruitment of cofactor proteins, which leads to the transcription of their target genes (29).

ROR family is composed of three members, RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3) (30–32), encoded by *Rora*, *Rorb*, and *Rorc* genes, respectively. *Ror* genes may encode different protein isoforms, among which RORα4 and RORγt are the unique isoforms expressed in cells of the immune system (29).

Interestingly, RORγt is expressed in thymocytes at the double-positive stage of T cell development, but is absent in mature thymocytes and in mature naive T cells in spleen and peripheral lymph nodes (33). In 2006, RORγt has been detected in IL-17-producing T cells (17), and it has been shown to play a central role in Th17 differentiation (17, 34).

Precursors or derivatives of cholesterol, such as desmosterol (35) and oxysterols (36), respectively, have been identified as activator ligands of RORγt, while bile acid synthesized from cholesterol called 3-oxoLC is an inhibitory ligand of RORγt (37).

RORγt regulates *Il17a* transcription by binding RORE sequences present in the 2-kb promoter fragment upstream of the transcription start site (38). In addition, the conserved non-coding sequences (CNS)2 (also called CNS5) located in the vicinity of the *Il17a* gene (approximately 5-kb upstream of promoter) (39) contains two ROREs, which are also conserved in human (39, 40). It has been demonstrated that RORγt binds CNS2 of the *Il17a* gene (**Figure 1**) and mediates *Il17a* transcription by controlling the chromatin remodeling. In fact, CNS2 is also bound by p300 and JmjC domain-containing protein (JMJD)3 that mediate permissive histone acetylation (41, 42) and remove repressive histone marker H3K27me3 (43–45), respectively, resulting in hyperacetylation of histone H3 (46, 47). Moreover, CNS2 interacts with *Il17a* promoter by forming a loop, and brings CNS2-associated histone remodeling enzymes to the promoter for the activation of *Il17a* transcription (39).

Similarly, it has been demonstrated that RORα4 overexpression promotes, while RORα4 deficiency impairs, *Il17a* expression (40). Interestingly, coexpression of RORα4 and RORγt causes the synergistic increase in IL-17A, indicating that RORα4 and RORγt work together to regulate Th17 cell differentiation (40, 48).

Given the high similarity of DNA-binding domains between RORα4 and RORγt, they activate *Il17a* transcription through the same molecular mechanism (40) (**Figure 1**).

However, RORα4 and RORγt are not sufficient to generate and specify the full Th17 program.

In fact, transcriptional regulators of RORγt, as well as other transcription factors that interact with RORγt, or bind the promoter or the intergenic regions of the *Il17a* locus, play a crucial role in the generation of Th17 cells.

OTHER TRANSCRIPTIONAL REGULATORS OF *RORC* AND *IL17A*

The transcription of RORγt is initiated by activation of the promoter RORC2 into the *Rorc* locus. RORC2 promoter contains nuclear factor of activated T cells (NFAT)-binding sequences, specific for NFAT and nuclear factor (NF)-kB proteins. Recently, it has been reported that the p65 NF-kB subunit and NFATc2 bind human *Rorc* promoter and promote a permissive chromatin

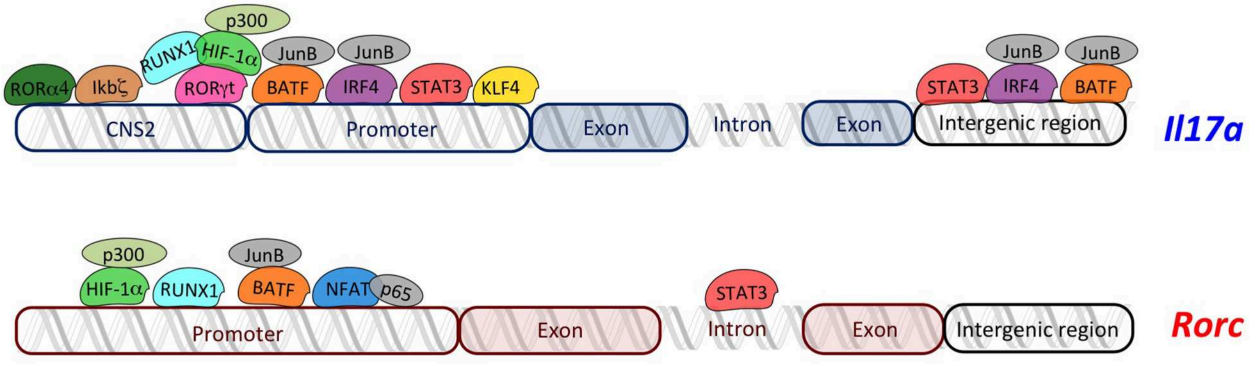


FIGURE 1 | Overview of transcriptional regulators of *Il17a* and *Rorc*. The transcriptional regulators of Th17 cells (RORγt, RORα4, ikbζ, RUNX1, HIF-1α, STAT3, IRF4, NFAT, KLF4, and BATF) regulate transcription of *Il17a* and *Rorc* by binding specific regions in their loci. Schema does not respect the real organization and structure of each gene locus.

conformation at RORC2 regulatory regions (49). Consistently, it has been reported that two NF-kB proteins, c-Rel and p65, activate the murine *Rorc* promoter (50).

Interestingly, the nuclear protein inhibitor of kB (IκB)ζ, which belongs to the IkB kinases and regulates activation of NF-kB pathway, binds CNS2 elements in *Il17a* locus (**Figure 1**), thus leading to an efficient recruitment of transcriptional coactivators with histone acetylase activity (18) and promoting *Il17a* expression without modulating expression of *Rorc* and *Rora* (51, 52).

CNS2 region of *Il17a* is bound by another transcriptional regulator called Runt-related transcription factor (RUNX)1, whose effect is dependent on RORγt. In fact, it has been demonstrated that RUNX1 interacts with RORγt to potentiate *Il17a* expression and is required for the full effect of RORγt on *Il17a* expression (38) (**Figure 1**). Additionally, RUNX1 plays a role in Th17 differentiation, independently of RORγt, by binding the promoter of the gene encoding RORγt through three conserved RUNX1-binding sites (53) (**Figure 1**).

Hypoxia-inducible factor (HIF)-1α is a key metabolic sensor (19, 54), which binds hypoxia response element (HRE, a conserved HIF)-1α-binding site) located in the proximal region of the *Rorc* promoter, in both human and mouse (19). Moreover, HIF-1α might physically associate with RORγt, serving as a coactivator for RORγt, thus contributing to *Il17a* expression without direct DNA binding on *Il17a* locus (19) (**Figure 1**). Further studies discovered that HIF-1α activates target genes by recruiting the factor p300, which possesses histone acetyltransferase activity and acetylates histones to "open" the chromatin structure (55). Indeed, the colocalized binding of RORγt, HIF-1α, and p300 occurs at the promoter of the *Il17a* gene (19).

Signal transducer and activator of transcription (STAT) 3 is another transcription factor regulating RORγt, and IL-17A (56) by interacting with the Stat-binding domains into the *Rorc* first intron, the *Il17a* promoter, and the intergenic region of the *Il17a* locus (**Figure 1**) (56–58). Moreover, STAT3 regulates positive epigenetic modifications, increasing permissive H3K4me3 marks on its target genes, including *Rorc*, *Rora*, and another gene encoding for transcriptional regulator of Th17 cells, called basic leucine zipper ATF-like transcription factor (BATF) (56).

BATF forms a heterodimer with JunB, and binds to the *Il17a* promoter as well as two conserved intergenic elements in the *Il17a* locus in Th17 cells (**Figure 1**). Interestingly, BATF synergizes with RORγt by binding to an overlapped conserved region recognized by RORγt into *Il17a* gene (20). Furthermore, the complex JunB and BATF also promotes the transcription of *Rorc* and *Rora* (58, 59) (**Figure 1**).

Genome-wide JunB-DNA binding analysis, using ChIP sequencing with anti-JunB antibody, revealed that JunB colocalizes in Th17 cells with another transcription factor, called interferon regulatory factor (IRF)4, involved in Th17 differentiation (21). In fact, IRF4 targets sequences enriched for activating protein 1 (AP-1)–IRF composite elements (AICEs) located into regulatory elements of the *Il17a* promoter (58, 60), which are cobound by BATF, an AP-1 factor (61). Thus, IRF4 and BATF bind cooperatively to structurally divergent AICEs to promote IL-17A activation in Th17 cells (61). Importantly, not only *Il17a* locus but also *Il21*, *Il22*, and *Il23r* loci contain one or more coincident binding peaks for IRF4 and BATF that were positioned in promoters and/or intronic regions, and ChIP assays verified the binding to these regions of both IRF4 and BATF complexed with JunB (61). The Kruppel-like factor (KLF)4 is another factor involved in the direct regulation of IL-17A, as demonstrated by ChIP analysis. In fact KLF4 binds the *Il17a* promoter and induces IL-17A expression, independently of RORγt (62).

Altogether, this information reveals a complex interconnected network of transcriptional regulators that finely regulates generation of Th17 cells.

The timing of transcriptional events leading to the full Th17 differentiation remains enigmatic. However, the transcriptional regulators activated upon T cell receptor engagement, such as NFAT, likely initiate the differentiation process by inducing RORγt transcription, and up-regulating receptor for polarizing cytokines, whose ligation leads to activation of other transcription factors. Among them, BATF, IRF4, and STAT3 are considered initiator transcription factors (24, 63). In fact, BATF and IRF4 are responsible of initial chromatin accessibility in *Il17a* locus and, with STAT3, of initiation of the transcriptional program that is then globally tuned by the lineage-specific transcription factor RORγt, which plays a pivotal deterministic role at key loci (24, 63). Then, RUNX1, HIF1a, and IκBζ can be considered cooperators of ROR nuclear receptors.

Importantly, there is high interconnectivity among transcription factors, including positive feedback loops reinforcing expression of initiator transcription factors BATF, IRF4, and STAT3 (24).

However, a negative feedback loop mediated by c-Maf, which is induced by initiator transcription factors, may limit Th17 response. In particular, c-Maf is a transcriptional regulator that, in Th17 cells, functions as a negative regulator, attenuating the expression of pro-inflammatory loci (e.g., *Batf*, *Rora*, *Runx1*, *Il1r1*, *Ccr6*, and *Tnf*) and positively regulating few loci linked to attenuating inflammation (e.g., *Il9*, *Il10*, *Lif*, and *Ctla4*). Another transcription factor known to limit Th17 response is Fosl2 exerting antagonistic effect to BATF, by competing for the same binding sites and by directly repressing BATF (24). STAT1 and STAT5 are known to inhibit Th17 polarization by directly binding *Rorc* or *Il17a* loci. In particular, STAT5 represses IL-17A induction by binding the *Il17a* locus, removing accessible histone marks, and displacing STAT3 occupancy (64, 65); STAT1 has been shown to bind upstream of the *Rorc* locus in human Hela cells (66).

TH17-RELATED TRANSCRIPTIONAL REGULATORS IN AUTOIMMUNE DISEASES

Given the crucial role of Th17 cells in autoimmune disorders, the altered expression of Th17 transcriptional regulators may be related to a persistent Th17 cell response typical of diseases, such as psoriasis, rheumatoid arthritis, Crohn's disease, and MS (16). The role of the transcription factors activating a Th17 response has been mainly investigated in the murine model of MS, the experimental autoimmune encephalomyelitis (EAE), where deletion of each specific Th17 transcription factor reduced the disease (17–21, 40, 62, 67, 68). However, the potential involvement of such transcription factors in human autoimmune diseases, as well as their expression in immune cells from patients, has not been largely investigated.

It has been reported that the levels of phosphorylated STAT3 (pSTAT-3) in lymphocytes are up-regulated in MS patients during relapse compared to healthy donors and MS patients in remission phase. Moreover, pSTAT-3 levels positively correlate with magnetic resonance imaging data, indicating that STAT3 activation is associated to disease activity (69). In contrast, the expression of RORγt analyzed at transcriptional (70) and protein level (71) does not differ between MS patients and healthy donors.

However, the activity of RORγt is ligand regulated and the putative natural ligands of RORγt are molecules of the cholesterol pathway. In this context, it has been reported that levels of oxysterols in relapsing-remitting MS patients were associated with conversion to secondary progressive-MS (72).

Moreover, an aberrant activation of STAT3 was found in intestinal T cells of Crohn's disease patients compared to healthy donors (73); the expression of IRF-4 was significantly increased in inflammatory cells of psoriasis patients than that in healthy controls (74); HIF-1α was found strongly expressed by immune cells in the intimal layer of the synovium in rheumatoid arthritis patients (75). However, the lack of correlations with clinical parameters in most part of these studies does not permit the definition of the role of the enhanced expression of those transcriptional regulators in human diseases.

Additionally, genetic abnormalities in Th17 transcriptional regulators may favor Th17 cell response and may influence susceptibility to autoimmune diseases. However, few studies demonstrate association between gene variants of Th17 transcription factors and Th17-related diseases. For instance, single-nucleotide polymorphisms (rs734232) affecting the consensus-binding site for RUNX1, or *Runx1* itself, are associated with susceptibility to rheumatoid arthritis and psoriasis (76–78), while *Stat3* gene was identified as risk locus for Crohn's disease and MS (79, 80).

THERAPEUTIC APPROACHES TARGETING TRANSCRIPTIONAL REGULATORS OF TH17 CELLS

Antibodies targeting IL-17A are approved for the treatment of psoriasis (81), while this approach is ineffective in MS, and deleterious in Crohn's disease (82). Recently, antagonists of Th17 transcriptional regulators have been proposed as potential new treatments of Th17-mediated diseases. Given the high cell specificity, RORγt is the transcription factor representing the ideal target for the manipulation of Th17 cell response. Several molecules targeting RORγt have been discovered and tested in murine models: digoxin, urosolic acid, and SR1001 reduce EAE severity (83–85); BI119 abrogates experimental colitis (86); SR2211 and JNJ-54271074 have therapeutic effect on experimental arthritis (87, 88); TMP778 and S18-000003 show efficacy in a psoriasis-like skin inflammation model (89, 90). In addition, other RORγt inverse agonists have been discovered (carbazole carboxamides, MG2778, TAK-828F, 6-substituted quinolines, A213) and tested as negative regulators of Th17 response (**Table 1**) (91–96).

Clinical studies testing the actual clinical efficacy and eventual side effects are active or completed. For instance, the oral compound VTP-43742 demonstrated efficacy through the reduction of clinical scores in psoriasis patients (NCT02555709). However, clinical data also showed liver toxicity, and VTP-43742 has been replaced with a new improved molecule VTP-45489 (**Table 1**). Similarly, other early clinical agents like GSK-2981278, JTE-151, JNJ-3534, ABBV-553, TAK-828, and AZD-0284 were either discontinued or suspended for further development (**Table 1**) (99). Currently, novel RORγt inhibitors are

TABLE 1 | List of the therapeutic approaches targeting transcriptional regulators of Th17 cells.

Compound	Target	Disease	Status	References
Digoxin	RORγt	Multiple sclerosis	Mouse model	(81)
Urosolic acid	RORγt	Multiple sclerosis	Mouse model	(82)
SR1001	RORγt	Multiple sclerosis	Mouse model	(83)
BI119	RORγt	Colitis	Mouse model	(84)
SR2211	RORγt	Arthritis	Mouse model	(85)
JNJ-54271074	RORγt	Arthritis	Mouse model	(86)
A213	RORγt	Psoriasis	Mouse model	(91)
TMP778	RORγt	Psoriasis	Mouse model	(87)
S18-000003	RORγt	Psoriasis	Mouse model	(88)
Carbazole carboxamides	RORγt	Autoimmune disorders	*in-vitro* cell models	(90)
MG2778	RORγt	Autoimmune disorders	*in-vitro* cell models	(92)
TAK-828F	RORγt	Autoimmune disorders	*in-vitro* cell models	(93)
6-substituted quinolines	RORγt	Autoimmune disorders	*in-vitro* cell models	(94)
VTP-45489	RORγt	Psoriasis	To be tested in clinical trial	(95)
VTP-43742	RORγt	Psoriasis	Phase II terminated for liver toxicity	(95)
GSK-2981278	RORγt	Psoriasis	Phase II terminated	(95)
JTE-151	RORγt	Autoimmune disorders	Discontinued for further development	(95)
JNJ-3534	RORγt	Autoimmune disorders	Discontinued for further development	(95)
ABBV-553	RORγt	Psoriasis	Phase I terminated for safety concern	(95)
TAK-828	RORγt	Autoimmune disorders	Discontinued for further development	(95)
AZD-0284	RORγt	Autoimmune disorders	Discontinued for further development	(95)
ABBV-157	RORγt	Psoriasis	Phase I recruiting	(96)
JTE-451	RORγt	Psoriasis	Phase I Active, not recruiting	(96)
ESR-114	RORγt	Psoriasis	Phase I completed	(96)
ARN-6039	RORγt	Multiple Sclerosis	Phase I completed	(96)
AUR-101	RORγt	Psoriasis	Phase II active, not recruiting	(96)
RTA-1701	RORγt	Autoimmune disorders	Phase I completed	(96)
GSK2981278	RORγt	Psoriasis	Phase II completed	(96)
SAR-441169	RORγt	Psoriasis	Phase I	(96)
ROR antagonists	RORγt	Inflammatory diseases	Phase I	(96)
2-benzoyl-phenoxy acetamide	HIF-1α	Arthritis	Mouse model	(97)
STA-21	STAT3	Psoriasis	Phase II completed	(98)

monitored in the clinical studies: ABBV-157 in psoriasis phase I (NCT03922607); JTE-451 and ESR-114 in psoriasis phase II (NCT03832738 and NCT03630939, respectively); ARN-6039 in MS phase I (NCT03237832); AUR-101 in psoriasis phase II (NCT04207801); RTA-1701 in healthy phase I (NCT03579030); GSK2981278 in psoriasis phase I (NCT03004846 and NCT02548052); SAR-441169 in psoriasis phase I; and ROR antagonists in inflammatory disease phase I (100) (**Table 1**).

Another promising target among Th17 transcription factors is HIF-1α. To date, the most advanced HIF pathway-targeted pharmaceuticals in terms of clinical development are cell-permeable prolyl hydroxylase inhibitors, evaluated for treatment of anemia. A number of HIF inhibitors have been developed also for cancer therapy (97) and are considered promising novel treatments for rheumatoid arthritis (101), such as the 2-benzoyl-phenoxy acetamide that acts as anti-arthritic agent in an experimental adjuvant induced arthritis rat model (98) (**Table 1**). However, none of the compounds targeting HIF-1α has been assessed in clinical trials for rheumatoid arthritis.

STAT3 is another potential drug target currently used for cancer therapy given its aberrant activation in many human tumors (102). Concerning Th17-related diseases, the small STAT3 inhibitor STA-21 has been tested on psoriasis patients in a nonrandomized study, and psoriatic lesions in six of the eight patients showed improvement after topical STA-21 treatment for 2 weeks (NCT01047943) (**Table 1**) (103). However, this effect is likely related to the inhibition of epidermal keratinocyte proliferation, rather than to immune cell activity (103).

Collectively, these data indicate that Th17 transcriptional regulators are promising targets for Th17-related diseases. However, given their broad expression in different cell types, it is crucial to develop inhibitors highly specific for immune cells to minimize off-target effects.

CONCLUSIONS

Since the discovery of Th17 cells, remarkable advances in the understanding of Th17 response have been reported. In particular, the study of the mechanisms regulating the transcription of *Rorc* and *Il17a* genes has advanced our understanding of the generation of Th17 cells. Moreover, small molecules interfering with these mechanisms provide promising results in pre-clinical research and clinical trials. Future studies further detailing the transcriptional program of Th17 cells could lead to the identification of pathways or regulators that are specifically activated during diseases. Advances in these points are critical for the development of new compounds that target more accurately the pathogenic effect of Th17 cells, and that could become new therapeutic strategies in Th17-related diseases.

AUTHOR CONTRIBUTIONS

AC drafted the manuscript. EV critically reviewed the manuscript and finalized the manuscript for submission. AC and EV approved the final version.

FUNDING

This work was supported by Progetto Giovani Ricercatori Italian Ministry of Health, Italy (cod. GR-2016-02361163) and FISM-Fondazione Italiana Sclerosi Multipla (cod. FISM2016/R/31) to EV.

REFERENCES

1. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. (2006) 24:677–88. doi: 10.1016/j.immuni.2006.06.002
2. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction effector functions of T(H)17 cells. *Nature*. (2008) 453:1051–7. doi: 10.1038/nature07036
3. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. (2005) 6:1133–41. doi: 10.1038/ni1261
4. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol*. (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
5. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, et al. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol*. (2008) 9:650–7. doi: 10.1038/ni.1613
6. Aujla SJ, Dubin PJ, Kolls JK. Th17 cells and mucosal host defense. *Semin Immunol*. (2007) 19:377–82. doi: 10.1016/j.smim.2007.10.009
7. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol*. (2010) 10:479–89. doi: 10.1038/nri2800
8. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *Am J Pathol*. (2012) 181:8–18. doi: 10.1016/j.ajpath.2012.03.044
9. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology*. (2010) 129:311–21. doi: 10.1111/j.1365-2567.2009.03240.x
10. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol*. (2014) 14:585–600. doi: 10.1038/nri3707
11. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. *Immunity*. (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
12. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol*. (2007) 8:950–7. doi: 10.1038/ni1497
13. Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med*. (2009) 206:299–311. doi: 10.1084/jem.20081463
14. Drummond RA, Lionakis MS. Organ-specific mechanisms linking innate and adaptive antifungal immunity. *Semin Cell Dev Biol*. (2019) 89:78–90. doi: 10.1016/j.semcdb.2018.01.008
15. Li J, Vinh DC, Casanova JL, Puel A. Inborn errors of immunity underlying fungal diseases in otherwise healthy individuals. *Curr Opin Microbiol*. (2017) 40:46–57. doi: 10.1016/j.mib.2017.10.016
16. Patel DD, Kuchroo VK. Th17 Cell Pathway in human immunity: lessons from genetics and therapeutic interventions. *Immunity*. (2015) 43:1040–51. doi: 10.1016/j.immuni.2015.12.003
17. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. (2006) 126:1121–33. doi: 10.1016/j.cell.2006.07.035
18. Okamoto K, Iwai Y, Oh-Hora M, Yamamoto M, Morio T, Aoki K, et al. IkappaBzeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature*. (2010) 464:1381–5. doi: 10.1038/nature08922
19. Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell*. (2011) 146:772–84. doi: 10.1016/j.cell.2011.07.033
20. Schraml BU, Hildner K, Ise W, Lee WL, Smith WA, Solomon B, et al. The AP-1 transcription factor Batf controls T(H)17 differentiation. *Nature*. (2009) 460:405–9. doi: 10.1038/nature08114
21. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol*. (2007) 8:958–66. doi: 10.1038/ni1500
22. Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature*. (2010) 467:967–71. doi: 10.1038/nature09447
23. Gaublomme JT, Yosef N, Lee Y, Gertner RS, Yang LV, Wu C, et al. Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. *Cell*. (2015) 163:1400–12. doi: 10.1016/j.cell.2015.11.009
24. Ciofani M, Madar A, Galan C, Sellars M, Mace K, Pauli F, et al. A validated regulatory network for Th17 cell specification. *Cell*. (2012) 151:289–303. doi: 10.1016/j.cell.2012.09.016
25. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol*. (2008) 9:641–9. doi: 10.1038/ni.1610
26. Giguere V, Tini M, Flock G, Ong E, Evans RM, Otulakowski G. Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. *Genes Dev*. (1994) 8:538–53. doi: 10.1101/gad.8.5.538
27. Jetten AM. Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nucl Recept Signal*. (2009) 7:e003. doi: 10.1621/nrs.07003
28. Medvedev A, Yan ZH, Hirose T, Giguere V, Jetten AM. Cloning of a cDNA encoding the murine orphan receptor RZR/ROR gamma and characterization of its response element. *Gene*. (1996) 181:199–206. doi: 10.1016/S0378-1119(96)00504-5
29. Zhang Y, Luo XY, Wu DH, Xu Y. ROR nuclear receptors: structures, related diseases, and drug discovery. *Acta Pharmacol Sin*. (2015) 36:71–87. doi: 10.1038/aps.2014.120
30. Hirose T, Smith RJ, Jetten AM. ROR gamma: the third member of ROR/RZR orphan receptor subfamily that is highly expressed

in skeletal muscle. *Biochem Biophys Res Commun*. (1994) 205:1976–83. doi: 10.1006/bbrc.1994.2902
31. Carlberg C, Hooft van Huijsduijnen R, Staple JK, DeLamarter JF, Becker-Andre RZRs M, a new family of retinoid-related orphan receptors that function as both monomers and homodimers. *Mol Endocrinol*. (1994). 8:757–70. doi: 10.1210/mend.8.6.7935491
32. Becker-Andre M, Andre E, DeLamarter JF. Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. *Biochem Biophys Res Commun*. (1993) 194:1371–9. doi: 10.1006/bbrc.1993.1976
33. Eberl G, Littman DR. Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. *Science*. (2004) 305:248–51. doi: 10.1126/science.1096472
34. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. (2007) 8:967–74. doi: 10.1038/ni1488
35. Hu X, Wang Y, Hao LY, Liu X, Lesch CA, Sanchez BM, et al. Sterol metabolism controls T(H)17 differentiation by generating endogenous RORgamma agonists. *Nat Chem Biol*. (2015) 11:141–7. doi: 10.1038/nchembio.1714
36. Soroosh P, Wu J, Xue X, Song J, Sutton SW, Sablad M, et al. Oxysterols are agonist ligands of RORgammat and drive Th17 cell differentiation. *Proc Natl Acad Sci USA*. (2014) 111:12163–8.
37. Hang S, Paik D, Yao L, Kim E, Jamma T, Lu J, et al. Bile acid metabolites control TH17 and Treg cell differentiation. *Nature*. (2019) 576:143–8. doi: 10.1038/s41586-019-1785-z
38. Zhang F, Meng G, Strober W. Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol*. (2008) 9:1297–306. doi: 10.1038/ni.1663
39. Wang X, Zhang Y, Yang XO, Nurieva RI, Chang SH, Ojeda SS, et al. Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity*. (2012) 36:23–31. doi: 10.1016/j.immuni.2011.10.019
40. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*. (2008) 28:29–39. doi: 10.1016/j.immuni.2007.11.016
41. Wang X, Pan L, Feng Y, Wang Y, Han Q, Han L, et al. P300 plays a role in p16(INK4a) expression and cell cycle arrest. *Oncogene*. (2008) 27:1894–904. doi: 10.1038/sj.onc.1210821
42. Liu X, Wang L, Zhao K, Thompson PR, Hwang Y, Marmorstein R, et al. The structural basis of protein acetylation by the p300/CBP transcriptional coactivator. *Nature*. (2008) 451:846–50. doi: 10.1038/nature06546
43. Agger K, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature*. (2007) 449:731–4. doi: 10.1038/nature06145
44. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell*. (2007) 130:1083–94. doi: 10.1016/j.cell.2007.08.019
45. Xiang Y, Zhu Z, Han G, Lin H, Xu L, Chen CD. JMJD3 is a histone H3K27 demethylase. *Cell Res*. (2007) 17:850–7. doi: 10.1038/cr.2007.83
46. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol*. (2009) 9:91–105. doi: 10.1038/nri2487
47. Akimzhanov AM, Yang XO, Dong C. Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. *J Biol Chem*. (2007) 282:5969–72. doi: 10.1074/jbc.C600322200
48. Sundrud MS, Rao A. Regulation of T helper 17 differentiation by orphan nuclear receptors: it's not just ROR gamma t anymore. *Immunity*. (2008) 28:5–7. doi: 10.1016/j.immuni.2007.12.006
49. Yahia-Cherbal H, Rybczynska M, Lovecchio D, Stephen T, Lescale C, Placek K, et al. NFAT primes the human RORC locus for RORgammat expression in CD4(+) T cells. *Nat Commun*. (2019) 10:4698. doi: 10.1038/s41467-019-12680-x
50. Ruan Q, Kameswaran V, Zhang Y, Zheng S, Sun J, Wang J, et al. The Th17 immune response is controlled by the Rel-RORgamma-RORgamma T transcriptional axis. *J Exp Med*. (2011) 208:2321–33. doi: 10.1084/jem.20110462
51. Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem*. (2001) 276:27657–62. doi: 10.1074/jbc.M103426200
52. Muta T. IkappaB-zeta: an inducible regulator of nuclear factor-kappaB. *Vitam Horm*. (2006). 74:301–16. doi: 10.1016/S0083-6729(06)74012-2
53. Liu HP, Cao AT, Feng T, Li Q, Zhang W, Yao S, et al. TGF-beta converts Th1 cells into Th17 cells through stimulation of Runx1 expression. *Eur J Immunol*. (2015) 45:1010–8. doi: 10.1002/eji.201444726
54. Semenza GL. Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE*. (2007) 2007:cm8. doi: 10.1126/stke.4072007cm8
55. Thompson PR, Wang D, Wang L, Fulco M, Pediconi N, Zhang D, et al. Regulation of the p300 HAT domain via a novel activation loop. *Nat Struct Mol Biol*. (2004) 11:308–15. doi: 10.1038/nsmb740
56. Durant L, Watford WT, Ramos HL, Laurence A, Vahedi G, Wei L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. (2010) 32:605–15. doi: 10.1016/j.immuni.2010.05.003
57. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci USA*. (2006) 103:8137–42. doi: 10.1073/pnas.0600666103
58. Hasan Z, Koizumi SI, Sasaki D, Yamada H, Arakaki N, Fujihara Y, et al. JunB is essential for IL-23-dependent pathogenicity of Th17 cells. *Nat Commun*. (2017) 8:15628. doi: 10.1038/ncomms15628
59. Yamazaki S, Tanaka Y, Araki H, Kohda A, Sanematsu F, Arasaki T, et al. The AP-1 transcription factor JunB is required for Th17 cell differentiation. *Sci Rep*. (2017) 7:17402. doi: 10.1038/s41598-017-17597-3
60. Biswas PS, Gupta S, Chang E, Song L, Stirzaker RA, Liao JK, et al. Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. *J Clin Invest*. (2010) 120:3280–95. doi: 10.1172/JCI42856
61. Glasmacher E, Agrawal S, Chang AB, Murphy TL, Zeng W, Vander Lugt B, et al. A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. *Science*. (2012) 338:975–80. doi: 10.1126/science.1228309
62. Lebson L, Gocke A, Rosenzweig J, Alder J, Civin C, Calabresi PA, et al. Cutting edge: The transcription factor Kruppel-like factor 4 regulates the differentiation of Th17 cells independently of RORgammat. *J Immunol*. (2010) 185:7161–4. doi: 10.4049/jimmunol.1002750
63. Yosef N, Shalek AK, Gaublomme JT, Jin H, Lee Y, Awasthi A, et al. Dynamic regulatory network controlling TH17 cell differentiation. *Nature*. (2013) 496:461–8. doi: 10.1038/nature11981
64. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity*. (2007) 26:371–81. doi: 10.1016/j.immuni.2007.02.009
65. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol*. (2011) 12:247–54. doi: 10.1038/ni.1995
66. Robertson G, Hirst M, Bainbridge M, Bilenky M, Zhao Y, Zeng T, et al. Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nat Methods*. (2007) 4:651–7. doi: 10.1038/nmeth1068
67. Liu X, Lee YS, Yu CR, Egwuagu CE. Loss of STAT3 in CD4+ T cells prevents development of experimental autoimmune diseases. *J Immunol*. (2008) 180:6070–6. doi: 10.4049/jimmunol.180.9.6070
68. Wang Y, Godec J, Ben-Aissa K, Cui K, Zhao K, Pucsek AB, et al. The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon-gamma-producing T helper 17 cells. *Immunity*. (2014) 40:355–66. doi: 10.1016/j.immuni.2014.01.002
69. Frisullo G, Angelucci F, Caggiula M, Nociti V, Iorio R, Patanella AK, et al. pSTAT1, pSTAT3, and T-bet expression in peripheral blood mononuclear cells from relapsing-remitting multiple sclerosis patients correlates with disease activity. *J Neurosci Res*. (2006) 84:1027–36. doi: 10.1002/jnr.20995
70. Edstrom M, Mellergard J, Mjosberg J, Jenmalm M, Vrethem M, Press R, et al. Transcriptional characteristics of CD4+ T cells in multiple sclerosis: relative lack of suppressive populations in blood. *Mult Scler*. (2011) 17:57–66. doi: 10.1177/1352458510381256

71. Capone A, Bianco M, Ruocco G, De Bardi M, Battistini L, Ruggieri S, et al. Distinct expression of inflammatory features in T helper 17 cells from multiple sclerosis patients. *Cells*. (2019) 8:533. doi: 10.3390/cells8060533
72. Fellows Maxwell K, Bhattacharya S, Bodziak ML, Jakimovski D, Hagemeier J, Browne RW, et al. Oxysterols and apolipoproteins in multiple sclerosis: a 5 year follow-up study. *J Lipid Res*. (2019) 60:1190–8. doi: 10.1194/jlr.M089664
73. Lovato P, Brender C, Agnholt J, Kelsen J, Kaltoft K, Svejgaard A, et al. Constitutive STAT3 activation in intestinal T cells from patients with Crohn's disease. *J Biol Chem*. (2003) 278:16777–81. doi: 10.1074/jbc.M207999200
74. Ni A, Chen H, Wu Y, Li W, Chen S, Li J. Expression of IRF-4 and IBP in skin lesions of patients with psoriasis vulgaris. *J Huazhong Univ Sci Technolog Med Sci*. (2012) 32:287–90. doi: 10.1007/s11596-012-0050-6
75. Hollander AP, Corke KP, Freemont AJ, Lewis CE. Expression of hypoxia-inducible factor 1alpha by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. *Arthritis Rheum*. (2001) 44:1540–4.
76. Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, et al. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature*. (2007) 446:685–9. doi: 10.1038/nature05673
77. Tokuhiro S, Yamada R, Chang X, Suzuki A, Kochi Y, Sawada T, et al. An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat Genet*. (2003) 35:341–8. doi: 10.1038/ng1267
78. Helms C, Cao L, Krueger JG, Wijsman EM, Chamian F, Gordon D, et al. A putative RUNX1 binding site variant between SLC9A3R1 and NAT9 is associated with susceptibility to psoriasis. *Nat Genet*. (2003) 35:349–56. doi: 10.1038/ng1268
79. International Multiple Sclerosis Genetics C, Wellcome Trust Case Control C, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*. (2011) 476:214–9. doi: 10.1038/nature10251
80. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet*. (2008) 40:955–62. doi: 10.1038/ng.175
81. McInnes IB, Mease PJ, Kirkham B, Kavanaugh A, Ritchlin CT, Rahman P, et al. Secukinumab, a human anti-interleukin-17A monoclonal antibody, in patients with psoriatic arthritis (FUTURE 2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. (2015) 386:1137–46. doi: 10.1016/S0140-6736(15)61134-5
82. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*. (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
83. Huh JR, Leung MW, Huang P, Ryan DA, Krout MR, Malapaka RR, et al. Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. *Nature*. (2011) 472:486–90. doi: 10.1038/nature09978
84. Xu T, Wang X, Zhong B, Nurieva RI, Ding S, Dong C. Ursolic acid suppresses interleukin-17 (IL-17) production by selectively antagonizing the function of RORgamma t protein. *J Biol Chem*. (2011) 286:22707–10. doi: 10.1074/jbc.C111.250407
85. Solt LA, Kumar N, Nuhant P, Wang Y, Lauer JL, Liu J, et al. Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature*. (2011) 472:491–4. doi: 10.1038/nature10075
86. Withers DR, Hepworth MR, Wang X, Mackley EC, Halford EE, Dutton EE, et al. Transient inhibition of ROR-gammat therapeutically limits intestinal inflammation by reducing TH17 cells and preserving group 3 innate lymphoid cells. *Nat Med*. (2016) 22:319–23. doi: 10.1038/nm.4046
87. Xue X, Soroosh P, De Leon-Tabaldo A, Luna-Roman R, Sablad M, Rozenkrants N, et al. Pharmacologic modulation of RORgammat translates to efficacy in preclinical and translational models of psoriasis and inflammatory arthritis. *Sci Rep*. (2016) 6:37977. doi: 10.1038/srep37977
88. Chang MR, Lyda B, Kamenecka TM, Griffin PR. Pharmacologic repression of retinoic acid receptor-related orphan nuclear receptor gamma is therapeutic in the collagen-induced arthritis experimental model. *Arthritis Rheumatol*. (2014) 66:579–88. doi: 10.1002/art.38272
89. Skepner J, Ramesh R, Trocha M, Schmidt D, Baloglu E, Lobera M, et al. Pharmacologic inhibition of RORgammat regulates Th17 signature gene expression and suppresses cutaneous inflammation *in vivo*. *J Immunol*. (2014) 192:2564–75. doi: 10.4049/jimmunol.1302190
90. Imura C, Ueyama A, Sasaki Y, Shimizu M, Furue Y, Tai N, et al. A novel RORgammat inhibitor is a potential therapeutic agent for the topical treatment of psoriasis with low risk of thymic aberrations. *J Dermatol Sci*. (2019) 93:176–85. doi: 10.1016/j.jdermsci.2019.03.002
91. Shibata A, Uga K, Sato T, Sagara M, Igaki K, Nakamura Y, et al. Pharmacological inhibitory profile of TAK-828F, a potent and selective orally available RORgammat inverse agonist. *Biochem Pharmacol*. (2018) 150:35–45. doi: 10.1016/j.bcp.2018.01.023
92. Tang L, Yang X, Liang Y, Xie H, Dai Z, Zheng G. Transcription factor retinoid-related orphan receptor gammat: a promising target for the treatment of psoriasis. *Front Immunol*. (2018) 9:1210. doi: 10.3389/fimmu.2018.01210
93. Huang Y, Yu M, Sun N, Tang T, Yu F, Song X, et al. Discovery of carbazole carboxamides as novel RORgammat inverse agonists. *Eur J Med Chem*. (2018) 148:465–76. doi: 10.1016/j.ejmech.2018.02.050
94. Dal Pra M, Carta D, Szabadkai G, Suman M, Frion-Herrera Y, Paccagnella N, et al. Targeting RORs nuclear receptors by novel synthetic steroidal inverse agonists for autoimmune disorders. *Bioorg Med Chem*. (2018) 26:1686–704. doi: 10.1016/j.bmc.2018.02.018
95. Barbay JK, Cummings MD, Abad M, Castro G, Kreutter KD, Kummer DA, et al. 6-Substituted quinolines as RORgammat inverse agonists. *Bioorg Med Chem Lett*. (2017) 27:5277–83. doi: 10.1016/j.bmcl.2017.10.027
96. Takaishi M, Ishizaki M, Suzuki K, Isobe T, Shimozato T, Sano S. Oral administration of a novel RORgammat antagonist attenuates psoriasis-like skin lesion of two independent mouse models through neutralization of IL-17. *J Dermatol Sci*. (2017) 85:12–9. doi: 10.1016/j.jdermsci.2016.10.001
97. Ban HS, Uto Y, Won M, Nakamura H. Hypoxia-inducible factor (HIF) inhibitors: a patent survey (2011-2015). *Expert Opin Ther Pat*. (2016) 26:309–22. doi: 10.1517/13543776.2016.1146252
98. Shankar J, Thippegowda PB, Kanum SA. Inhibition of HIF-1alpha activity by BP-1 ameliorates adjuvant induced arthritis in rats. *Biochem Biophys Res Commun*. (2009) 387:223–8. doi: 10.1016/j.bbrc.2009.01.086
99. Gege C. RORgammat inhibitors as potential back-ups for the phase II candidate VTP-43742 from vitae pharmaceuticals: patent evaluation of WO2016061160 and US20160122345. *Exp Opin Ther Pat*. (2017) 27:1–8. doi: 10.1080/13543776.2017.1262350
100. Sun N, Guo H, Wang Y. Retinoic acid receptor-related orphan receptor gamma-t (RORgammat) inhibitors in clinical development for the treatment of autoimmune diseases: a patent review (2016-present). *Expert Opin Ther Pat*. (2019) 29:663–74. doi: 10.1080/13543776.2019.1655541
101. Hua S, Dias TH. Hypoxia-inducible factor (HIF) as a target for novel therapies in rheumatoid arthritis. *Front Pharmacol*. (2016) 7:184. doi: 10.3389/fphar.2016.00184
102. Yang L, Lin S, Xu L, Lin J, Zhao C, Huang X. Novel activators and small-molecule inhibitors of STAT3 in cancer. *Cytokine Growth Factor Rev*. (2019) 49:10–22. doi: 10.1016/j.cytogfr.2019.10.005
103. Miyoshi K, Takaishi M, Nakajima K, Ikeda M, Kanda T, Tarutani M, et al. Stat3 as a therapeutic target for the treatment of psoriasis: a clinical feasibility study with STA-21, a Stat3 inhibitor. *J Invest Dermatol*. (2011) 131:108–17. doi: 10.1038/jid.2010.255

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 21 April 2020
doi: 10.3389/fimmu.2020.00718

The Emerging Role of the IL-17B/IL-17RB Pathway in Cancer

Jérémy Bastid[1], Cécile Dejou[1], Aurélie Docquier[1] and Nathalie Bonnefoy[2]*

[1] OREGA Biotech, Ecully, France, [2] IRCM, Institut de Recherche en Cancérologie de Montpellier, INSERM U1194, Université de Montpellier, Institut Régional du Cancer de Montpellier, Montpellier, France

Among inflammatory mediators, a growing body of evidence emphasizes the contribution of the interleukin 17 (IL-17) cytokine family in malignant diseases. Besides IL-17A, the prototypic member of the IL-17 family, several experimental findings strongly support the role of the IL-17B/IL-17 receptor B (IL-17RB) pathway in tumorigenesis and resistance to anticancer therapies. In mouse models, IL-17B signaling through IL-17RB directly promotes cancer cell survival, proliferation, and migration, and induces resistance to conventional chemotherapeutic agents. Importantly, recent work by our and other laboratories showed that IL-17B signaling dramatically alters the tumor microenvironment by promoting chemokine and cytokine secretion which foster tumor progression. Moreover, the finding that elevated IL-17B is associated with poor prognosis in patients with pancreatic, gastric, lung, and breast cancer strengthens the results obtained in pre-clinical studies and highlights its clinical relevance. Here, we review the current understanding on the IL-17B/IL-17RB expression patterns and biological activities in cancer and highlight issues that remain to be addressed to better characterize IL-17B and its receptor as potential targets for enhancing the effectiveness of the existing cancer therapies.

Keywords: IL-17, IL-17B, IL-17RB, inflammation, cancer, cancer therapy

OPEN ACCESS

Edited by:
Nicola Ivan Lorè,
San Raffaele Scientific Institute (IRCCS), Italy

Reviewed by:
Matteo Bellone,
San Raffaele Hospital (IRCCS), Italy
Zongbing You,
Tulane University, United States

****Correspondence:***
Nathalie Bonnefoy
nathalie.bonnefoy@inserm.fr

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity,
a section of the journal Frontiers in Immunology

Received: *10 February 2020*
Accepted: *30 March 2020*
Published: *21 April 2020*

Citation:
Bastid J, Dejou C, Docquier A and Bonnefoy N (2020) The Emerging Role of the IL-17B/IL-17RB Pathway in Cancer. Front. Immunol. 11:718. doi: 10.3389/fimmu.2020.00718

INTRODUCTION

The IL-17 cytokine family and its receptors play crucial roles in normal host immune responses. Their dysregulated expression has been associated with many human diseases, notably inflammation and cancer. The IL-17 family includes six members (IL-17A to IL-17F) with different sequence homology and functions (1). These cytokines exert their activities through binding to IL-17 receptors (IL-17R, IL-17RA to IL-17RE) that function as homo- or heterodimeric complexes. IL-17A is the prototypic member of the IL-17 family and is predominantly produced by T helper 17 (Th17) cells. IL17A binding to IL-17RA/IL17RC heterodimers leads to the production of cytokines and chemokines, such as tumor necrosis factor a (TNF-α), IL-6, CXCL8, and CXCL1, involved in mechanisms of the host defense against extracellular bacterial and fungal infections (2, 3). However, IL-17A overproduction has been associated with chronic inflammatory disorders, autoimmune diseases and cancer (2, 4–6). Among other members of the IL-17 family, IL-17B was originally described as increased during intestinal inflammation (7). Moreover, it stimulates TNF-α and IL-1β production by the human monocytic leukemia THP-1 cells (7) and promotes neutrophil migration upon intraperitoneal administration, suggesting a pro-inflammatory role (8). More recent findings strongly suggest a role for the IL-17B/IL-17RB pathway in tumorigenesis. For instance, in mouse models, IL-17B signaling through IL-17RB promotes cancer cell survival, proliferation, and migration (9–12), while in humans, elevated IL-17B expression has been associated with poor prognosis in patients with different cancer types (10–12).

In this review, we summarize the knowledge on the expression and biological activities of the IL-17B cytokine and its receptor, and then focus on their implication in tumorigenesis highlighting gaps that remain in our understanding of this topic.

IL-17B AND ITS RECEPTOR IL-17RB

IL-17B Expression

Following the discovery of IL-17A, which was originally named CTLA8, screens to identify homologous genes led to the discovery of the IL-17B, IL-17C, IL-17D, IL-17E (known as IL-25), and IL-17F cytokines. Human IL-17B was cloned in 2000 by homology-based screening of an expressed sequence tag database, followed by amplification from a fetal tissue cDNA library (7, 8). The IL-17B protein shares 88% of homology with its murine ortholog but only 29% homology with human IL-17A (8). IL-17B is secreted as a non-covalent dimer glycoprotein consisting of 180 amino acids and has a predicted molecular mass of 20.4 kDa as a monomer (2, 13). The human *IL-17B* gene was mapped to chromosome 5q32–34, and its mRNA is strongly expressed in adult pancreas, small intestine, stomach, testis and more weakly in spinal cord, prostate, colon and ovary (7, 8). IL-17B expression was also detected in rheumatoid synovial tissues from patients with rheumatic arthritis, where it is mainly produced by neutrophils (14), as well as in chondrocytes (15) neurons (16) and naive, memory and germinal center B cells (17). Importantly, the IL-17B and IL-17A expression profiles are very different. Indeed, IL-17B was never detected in activated CD4 T cells, particularly Th17 CD4 T cells that are the main IL-17A source (7).

IL-17B Receptor Expression

IL-17B binds to its receptor IL-17RB, a 47.9 kDa transmembrane protein (462 aa) that belongs to the IL-17 receptor family. IL-17RB has a SEFIR cytoplasmic domain implicated in homotypic dimerization and recruitment of signaling proteins (11, 18) (shared with IL-17RA) and a TRAF6-binding domain (not found in IL-17RA). IL-17B shares its receptor IL-17RB with IL-17E (also known as IL-25) that binds to the heterodimeric IL-17RA/IL-17RB complex (19). The binding affinity (KD) of IL-17B for IL-17RB is around 30-fold lower than that of IL-17E, with a similar association rate (Kon) but a substantially faster dissociation rate (Koff) (20).

IL-17RB is expressed in various endocrine tissues and in epithelial cells in different organs such as kidney and liver and mucosal tissues (8, 19, 21). Elevated IL-17RB expression is also found lung tissues from asthmatic patients and in skin lesions from patients with atopic dermatitis (22). IL-17RB expression in human innate type 2 lymphocytes, natural killer T (NKT) cells, and Th2 cells (20, 22) suggests a potential role in immune cells. In these human cells IL-17B promotes IL-33-driven type 2 immune responses, a function shared with IL-17E, but not with IL-17A (20).

IL-17RB Signaling Pathway

Data on the IL-17RB signaling pathway are limited and mainly described after binding of IL-17E. Upon ligand binding, IL-17RB activates the canonical NK-κB pathway as well as ERK, JNK, and p38 (19, 23, 24). Moreover, TRAF6 binds to IL-17RB independently of its ligand and participates in IL-17RB-dependent NF-κB activation (23).

IL17B/RB Pathway in Inflammatory Diseases

IL-17B was originally described as a proinflammatory cytokine (8, 9). Indeed, IL-17B is strongly expressed in the paws of arthritic mice and administration of a polyclonal anti-IL-17B antibody ameliorates collagen-induced arthritis in these mice (25). Moreover, IL-17B has been detected in rheumatoid synovial tissues from patients with rheumatic arthritis. In these tissues, IL-17B is produced by neutrophils and potentiates TNF-α effect on the production of cytokines and chemokines, such as IL-6, G-CSF, and CCL20, known to control immune cell trafficking to inflamed tissues (14). Interestingly, although IL-17B and IL-17E (IL-25) share a common receptor, IL-17RB, IL-17B, and IL-17E deficiency lead to opposite results in a model of acute colitis induced by dextran sulfate sodium. These results indicate that IL-17E has a pathogenic role in colon inflammation, whereas Il-17B has a protective role. Moreover, IL-17B inhibits IL-17E binding to IL-17RA– IL-17RB complexes on epithelial cells, and limits IL-17E-induced IL-6 production by colon epithelial cells (26). Altogether, these findings suggest that if both cytokines are concomitantly produce at the same site, IL-17B might restrict IL-17E/IL-17RB signaling. The two cytokines have opposite roles also in *Citrobacter rodentium* infection and allergic asthma (26). Similarly, in murine cancer models and patients, IL-17B exhibits protumor roles and IL-17E antitumor activities (see just below).

IL-17B/IL-17RB PATHWAY IN TUMORS

Expression and Prognosis

In the last decade, several reports highlighted the potential role of the IL-17B/IL-RB pathway in cancer (9–39). High expression of IL-17B or its receptor has been associated with poor patient prognosis in different cancer types (see **Table 1**). For instance, in a cohort of 69 patients with ductal invasive breast carcinoma, Furuta et al., were the first to show that an IL-17RB (referred to as IL-25R in this study) was upregulated in 19% of patients. Moreover, IL-17RB detection was significantly correlated with poor prognosis and high mortality rate in this cohort (27). These first results were then confirmed by Huang et al., in an independent cohort of 179 patients with breast cancer (28). In this study, the correlation between IL-17RB expression and poor prognosis was statistically significant even after adjustment for several clinical parameters (age, tumor size, lymph node status and estrogen receptor expression). The authors also observed that IL-17RB expression was associated with HER2 amplification and survival rate was lowest in patients with high expression of both IL-17RB and HER2 (28). Finally, in another cohort of 143 patients, we showed that not only IL-17RB but also IL-17B expression is associated with reduced patient survival. Then, we analyzed microarray data of 1809 patients with breast cancer, and found that high IL-17B expression was significantly correlated with poorer prognosis in the whole population and in the basal-like subtype, but not in other breast cancer subtypes. Conversely, IL-17A expression was associated with favorable outcomes in the whole population and in the different molecular subtypes from this cohort (10).

Besides breast cancer, Wu et al., showed that in a cohort of 111 patients with pancreatic cancer, high expression of IL-17RB expression strongly correlates with poor differentiation, metastasis, and tumor stage using the TNM staging system. They found that in patients with high IL-17RB expression, prognosis is worse and malignancy is enhanced (11). More recently, high IL-17RB expression was correlated with poor prognosis also in patients with gastric cancer, where the percentage of IL-17RB positive cancer cells is high in grade II to IV tumors and low in grade I tumors (31). In lung cancer, microarray dataset analysis also associated *IL-17B* and *IL-17RB* gene expression with poor patient survival. Moreover, immunohistochemistry analysis also showed that IL-17RB is up-regulated in patients with lung adenocarcinoma compared with normal lung tissues specimens and is associated with lymph node and distant metastasis as well as reduced progression-free-survival and overall survival (12). Finally, by ranking cytokine-encoding genes based on their survival predictive values in the Chinese Glioma Genome Atlas database ($n = 105$ patients), Cai et al., identified *IL-17B* as one of the six enriched genes (among 593) with the strongest predictive value for a poor overall survival in patients with primary glioblastoma (32).

Besides solid tumors, analysis of the profiling data of 730 immune response genes in 60 primary testicular lymphomas obtained with the Nanostring technology recently identified a 25-gene signature that characterizes patients with the shortest 5-year progression free survival. This signature is enriched in cytokines and cytokine receptors and includes IL-17B (33). Additionally, mRNA expression profiles analysis in the HemaExplorer database showed that IL-17B and IL-17RB are strongly expressed in acute myeloid leukemia (AML), compared with normal hematopoietic stem cells. In line with the *in-silico* analysis results, IL-17B, and IL-17RB mRNA and protein expression were significantly increased in AML blasts compared with cells from healthy controls. Particularly, their expression was dramatically increased in bone marrow supernatant from patients with AML compared with healthy donors (ELISA analysis) (38).

Thus, by combining bioinformatics analysis of gene expression datasets and protein analysis in several independent cohorts, these studies clearly show the association between a deregulated expression of the IL-17 and/or IL-17RB and poor prognosis in many different cancers. The IL-17B/IL-17RB signaling pathway role in tumorigenesis and treatment resistance is mediated through different mechanisms, not fully understood yet, as discussed in the next paragraph.

MECHANISMS OF ACTION

The IL-17B/IL-17RB pathway is considered as a signaling cascade that promotes cancer cell survival, proliferation and migration. The pro-tumor functions associated with the IL-17B/IL-17RB pathway are diverse and complex because they involve mechanisms that act directly on tumor cells, and also indirect mechanisms that lead to tumor microenvironment remodeling (see **Table 1** and **Figure 1**).

In vivo mouse models and *in vitro* cell assays indicate that in different tumor cell types, IL-17B signaling is critical for tumorigenesis promoting cancer cell survival and proliferation. Mechanistic studies in the MDA-MB 361, MDA-MB468, and MCF-7 breast cancer cell lines revealed that IL-17B promotes breast cancer cells survival *in vitro* by activating the ERK and NF-kB pathways and by enhancing the expression of anti-apoptotic Bcl-2 family members (10, 28). This leads to resistance to chemotherapeutic drugs, such as etoposide (a topoisomerase II inhibitor) (28), and paclitaxel (a spindle poison) (10). Similar results were recently obtained in leukemic cells by Guo et al., who demonstrated that the IL-17RB pathway promotes the survival of MOLM-13 AML cells by increasing ERK and NF-KB phosphorylation and Bcl-2 level and consequently, resistance to the purine analog Ara-C, the frontline chemotherapeutic agent for AML (38). Importantly, in each study, inhibition of the IL-17B/IL-17RB axis by downregulating receptor expression in tumor cells or by using neutralizing anti-IL-17RB antibodies restored chemosensitivity *in vitro* (28, 38) and *in vivo* (10). Similarly, IL-17B or IL-17RB silencing in cancer cells or treatment with antibodies targeting IL-17RB reduced proliferation of MDA-MB361 breast cancer cells and MOLM-13 AML cells *in vitro* and tumor growth *in vivo* in xenograft models based on these cell lines (28, 38). Interestingly, IL-17RB knockdown in MOLM-13 AML cells had a stronger effect than IL-17B knockdown *in vivo*, reflecting the potential contribution of the microenvironment-derived IL-17B to the signal delivered to IL-17RB-positive leukemia cells. As a corollary to this observation Bie et al., recently showed that non-tumor tissue-derived IL-17B promotes the proliferation and migration of MGC-803 gastric cancer cells (31). This also suggests that stimulation by any other IL-17RB-positive cells from the tumor microenvironment might indirectly contribute to the tumor progression. Indeed, Bie and colleagues showed that mesenchymal stem cells (MSCs) produce IL-6, IL-8, TGF-β, and CCL-5 following IL-17B stimulation and that supernatants collected from MSCs incubated with recombinant IL-17B promote the proliferation of MGC-803 gastric cancer cells *in vitro* (9). Thus, both direct IL-17RB signaling in the tumor cells and indirect IL-17RB signaling in cells present in the tumor microenvironment, such as MSCs, might contribute to promote tumor proliferation.

In addition to the effect on cancer cell proliferation and survival, the IL-17B/IL-17RB signaling pathway induces stemness and epithelial to mesenchymal transition (EMT) of MGC-803 gastric cancer cells through activation of the AKT/GSK-3β/β-catenin pathway and the up-regulation of Sox2, Oct4, and Nanog proteins. The relevance of these *in vitro* results to human gastric cancer is supported by the positive correlation between *IL-17RB* and *OCT4*, *NANOG*, *LGR5*, and *SALL4* mRNA expression in human gastric cancer tissues (31). Interestingly, IL-17RB signaling through the ERK/GSK-3β/β-catenin pathway has been associated also with EMT in lung cancer. Indeed,

TABLE 1 | IL-17B IL-17RB expression in cancers.

Tumor	Expression—Prognosis	Mechanism—Models	References
Breast	IL-17RB upregulation is correlated with poor prognosis.	ShRNA-dependent reduction of IL-17B decreases tumor growth and invasiveness of MDA-MB468 human breast cancer cells.	(27)
Breast	IL-17RB upregulation is correlated with poor prong/osis.	IL-17RB recruits TRAF6, activates NF-kB, upregulates Bcl-2, and induces resistance to etoposide. IL-17RB or IL-17B targeting with Abs attenuates human MDA-MB361 breast cancer cell colony formation *in vitro* and tumor growth *in vivo*.	(28)
Breast	High expression of IL-17B and IL-17RB is associated with poor prognosis. IL-17B upregulation is associated with poorer survival in patients with basal-like breast cancer.	MCF7 and MDA-MB468 human breast cancer cells that overexpress IL-17B are resistant to paclitaxel. Treatment with anti-IL-17RB antibodies restores breast tumor chemosensitivity *in vivo*.	(10)
Breast		TGF-β secreted by Treg cells up-regulates IL-17RB on 4T1 and EMT6 murine breast cancer cells via Smad2/3/4 signaling and increases their tumor growth and metastatic potential *in vivo.*	(29)
Pancreas	IL-17RB overexpression is associated with metastasis and poor clinical outcome.	Depletion of IL-17B or IL-17RB by shRNA or treatment with anti-IL-17B or anti-IL-17RB antibodies reduces CFPAC-1 and BxPC3 pancreatic cell line colony formation, invasion, tumor growth, and metastasis in xenograft models.	(11)
Gastric	IL-17RB expression in group 2 innate lymphoid cells (ILC2) is higher in peripheral blood mononuclear cells from patients with gastric cancer than in healthy donors.	IL-17RB expression by ILC2.	(30)
Gastric	Overexpression of IL-17RB correlates with poor prognosis. IL-17B level in serum is higher in patients with gastric cancer than in healthy donors.	IL-17B activates the AKT/β-catenin pathway and promotes stemness and EMT of MGC-803 human gastric cancer cells.	(31)
Glioblastoma	A signature with 6 enriched cytokines (incl. enriched expression of IL-17B) predicts poor overall survival		(32)
Primary testicular lymphoma	A signature with 25 enriched cytokines (including IL-17B) predicts poor survival.		(33)
Colon	IL-17B expression is increased in moderate and poorly differentiated tumors.	IL-17RB is expressed by colon epithelial cells. Neutrophils are the main source of IL-17B in the stroma.	(34)
Prostate	IL-17RB expression is higher in cancer-associated fibroblasts from prostate cancer patients than in fibroblasts from benign prostate hyperplasia.	IL-17RB expression by cancer-associated fibroblasts.	(35)
ATL	Overexpression of IL-17RB in leukemic cells.	Tax induces IL-17RB expression in a NF-kB dependent-manner in the HTLV-1 transformed T cell lines C8166 and MT-2.	(36)
Thyroid	IL-17RB is upregulated in thyroid cancer tissues compared with normal thyroid tissues.	IL-17B/IL-17RB signaling induces ERK activation, MMP-9 expression and promotes migration and invasion of SW1736 thyroid cancer cells. IL-17RB signaling contributes to tumor growth and metastasis formation of SW1736 tumor cell xenografts.	(37)
Lung	High IL-17B expression is associated with poor overall survival. High IL-17RB expression is associated with positive lymph nodes and distant metastases and positive distant metastases, and is predictive of disease-free survival and overall survival.	IL-17RB expression positively correlates with the invasion potential of lung cancer cell lines. IL-17RB promotes invasion/migration of H441 lung carcinoma cells through activation of the ERK signaling pathway, and its overexpression increases their metastatic potential *in vivo.*	(12)
AML	IL-17B and IL-17RB mRNA expression is significantly upregulated in patients with AML.	IL-17B/IL-17RB signaling drives MOLM-13 AML cell resistance to Ara-C (ERK/NF-kB/Bcl-2). Ara-C increases IL-17B expression.	(38)

Yang et al., recently demonstrated that in lung cancer cell lines, IL-17RB-mediated activation of the ERK pathway is critical to maintain the expression of Snail and Twist, two key transcription factors for EMT induction. Specifically, in A549 an CL1-5 lung cancer cell lines that spontaneously expressed high level of IL-17RB, Snail and Twist expression was decreased upon IL-17RB knockdown. These *in vitro* results were strengthened by immunohistochemistry analysis of a cohort of 139 primary lung tumors in which IL-17RB expression was positively correlated with Snail or Twist expression (12).

Activation of the ERK/GSK-3β/β-catenin pathway following IL-17RB stimulation also promotes the invasion and the migration of H441 and CL1-0 human lung cancer cells *in vitro.* This effect is lost by inhibiting ERK1/2 phosphorylation using the

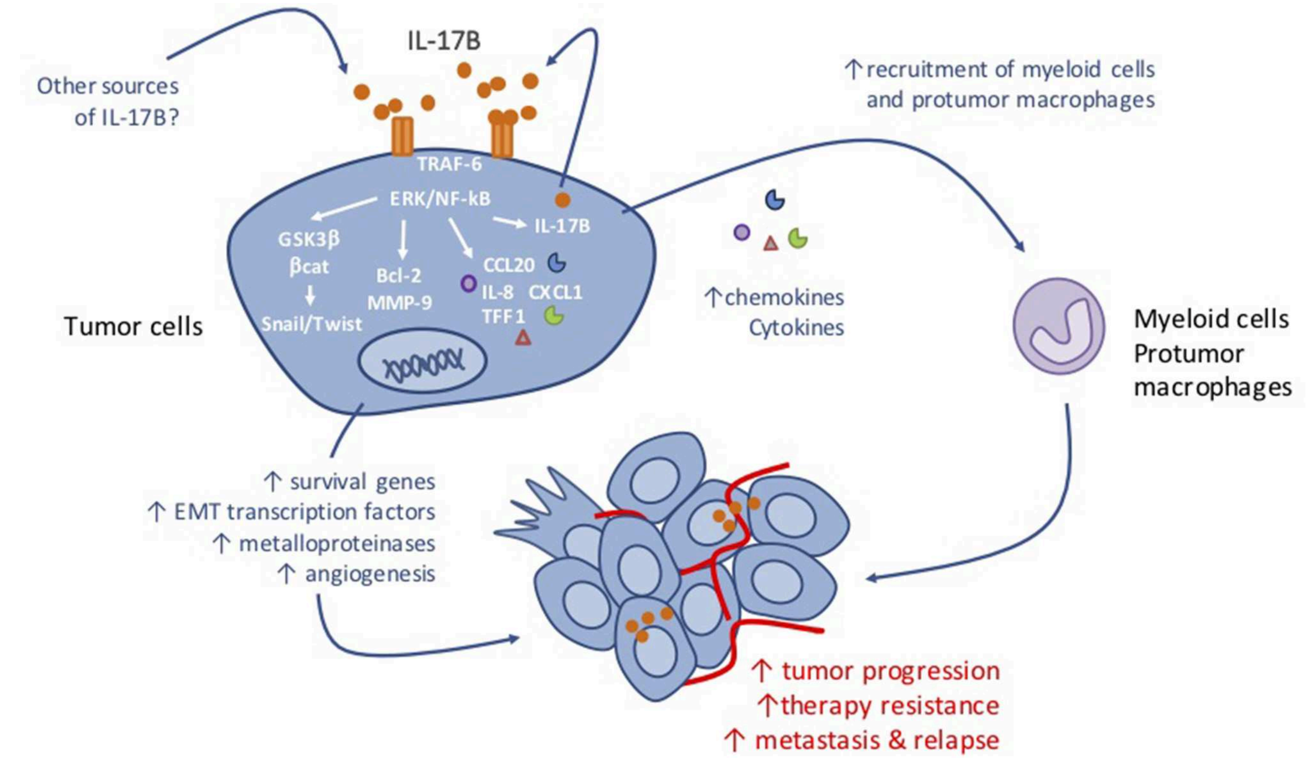


FIGURE 1 | Anticipated mechanism of action of IL-17B in tumorigenesis.

MEK/ERK inhibitor PD98059. Furthermore, the authors showed that IL-17RB overexpression in the H441 significantly increases in the number of metastatic nodules in the lung of xenografted mice. In patients with lung cancer, IL-17RB expression level correlates with lymph node and distant metastasis occurrence (12). These results connect the IL-17RB pathway to the control of metastasis formation, and support previous findings in thyroid (37) and pancreatic cancer (11). Indeed, in the SW1736 thyroid cancer cell line, IL-17B-dependent stimulation of IL-17RB induces ERK1/2 activation and increases expression of the matrix metalloproteinase MMP-9 expression, a key mediator of tumor invasion and metastasis formation. This results in an increased migration and invasion capacities of SW1736 cells both *in vitro* and *in vivo* (37). In pancreatic cancer, high IL-17RB expression has been associated with postoperative metastases in patients (11). Conversely, *IL-17RB or IL-17B* knockdown in CFPAC-1 and BxPC3 pancreatic cancer cell reduces their soft agar colony formation in soft agar and cell invasion *in vitro*. *In vivo* studies showed that tumor growth and metastasis formation are reduced in mice xenografted with *IL-17RB* or *IL-17B* knockdown cells compared with parental CFPAC-1 and BxPC3 cells. Similarly, treatment with an IL-17B neutralizing antibody showed reduced CFPAC-1 and BxPC3 tumor cell xenograft growth and metastasis formation *in vivo* (11). Interestingly in this study, Wu et al., found that the IL-17B/IL-17RB pathway supports tumorigenicity and metastasis formation of human pancreatic cancer cells through the activation of ERK1/2 signaling. This resulted in the expression of the pro-inflammatory cytokines and chemokines CCL20, CXCL1, IL-8, and TFF1 leading to the recruitment of macrophages in the tumor microenvironment and of vasculogenic endothelial cells to promote angiogenesis. In agreement, shRNA-mediated of CCL20, CXCL1, or TFF1 depletion in CFPAC-1 pancreatic cancer cells significantly decreased the percentage of macrophages that interact with tumor cells *in vivo*, while IL-8 depletion reduced CD31+ endothelial cell recruitment. Importantly, although TFF1 is predominantly expressed by cancer cells, the authors detected CCL20, CXCL1, and IL-8 in cancer cells and also in the surrounding stroma (11). Likewise, chemokines might also be secreted by tumor-infiltrating cells as a result of stimulation of IL-17RB-expressing stromal, and might contribute to macrophages and endothelial cell recruitment to promote cancer progression.

CONCLUSIONS AND FUTURE DIRECTIONS

Altogether, these studies clearly identified IL-17B and IL-17RB as key actors of cell tumorigenesis by enhancing the survival and the proliferative, migratory and invasive properties of tumor cells. Moreover, IL-17RB-mediated secretion of soluble factors will ultimately reshape the tumor microenvironment toward a macrophage-enriched infiltrate that might impair the anti-tumor immune response and favor resistance to treatments

(**Figure 1**). In fact, our own unpublished data in mouse models suggest that IL-17B-driven alterations in the TME are the major contributors of the anticancer effect after IL-17B neutralization. Therefore, targeting Il-17B or its receptor might represent an interesting therapeutic option for cancer therapy. However, as IL-17RB is a common receptor for both IL-17B and IL-17E that binds to the heterodimeric complex IL-17RA and IL-17RB (19), the anticancer effect of IL-17E (IL-25) must be taken into account. Indeed, unlike IL-17B, IL-17E (or IL-25) causes caspase-mediated apoptosis of breast cancer cells and reduces colony formation of IL17RB-expressing breast tumor cell lines *in vitro* (28). Furthermore, IL-17E (IL-25) markedly reduces growth of MDA-MB468 breast tumor xenografts *in vivo*, while IL-17B increases it (27). These results suggest that in cancer, like in mucosal inflammation (26) IL-17B and IL-17E might have opposite effects and that IL-17B is a negative regulator of IL-17E signaling, when they are concomitantly produced and co-expressed in a tissue. Therefore, targeting IL-17B rather than its receptor appears to be a better strategy for anti-cancer therapy. Although the effects of IL-17B neutralization remain to be better defined, the possibility of remodeling the tumor immune microenvironment, in particularly by decreasing the immunosuppression linked to the strong infiltration by macrophages and neutrophils, is an interesting mechanism in the context of resistance to new immunotherapies, such as checkpoint inhibitors and immunogenic chemotherapies, or radiotherapy.

AUTHOR CONTRIBUTIONS

JB and NB wrote the manuscript, CD and AD revised the manuscript.

FUNDING

This work was supported by institutional grants from INSERM, Université de Montpellier and by the French National Research Agency under the program Investissements d'avenir Grant Agreement LabEx MAbImprove.

REFERENCES

1. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. *Immunity.* (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
2. Kolls JK, Lindén A. Interleukin-17 family members and inflammation. *Immunity.* (2004) 21:467–76. doi: 10.1016/j.immuni.2004.08.018
3. Pappu R, Ramirez-Carrozzi V, Sambandam A. The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. *Immunology.* (2011) 134:8–16. doi: 10.1111/j.1365-2567.2011.03465.x
4. Beringer A, Noack M, Miossec P. IL-17 in chronic inflammation: from discovery to targeting. *Trends Mol Med.* (2016) 22:230–41. doi: 10.1016/j.molmed.2016.01.001
5. Cochaud S, Giustiniani J, Thomas C, Laprevotte E, Garbar C, Savoye AM, et al. IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2. *Sci Rep.* (2013) 3:3456. doi: 10.1038/srep03456
6. Chung AS, Wu X, Zhuang G, Ngu H, Kasman I, Zhang J, et al. An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nat Med.* (2013) 19:1114–23. doi: 10.1038/nm.3291
7. Li H, Chen J, Huang A, Stinson J, Heldens S, Foster J, et al. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc Natl Acad Sci.* (2000) 97:773–8. doi: 10.1073/pnas.97.2.773
8. Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, et al. A novel cytokine receptor-ligand pair: identification, molecular characterization, and *in vivo* immunomodulatory activity. *J Biol Chem.* (2000) 275:19167–76. doi: 10.1074/jbc.M910228199
9. Bie Q, Zhang B, Sun C, Ji X, Barnie PA, Qi C, et al. IL-17B activated mesenchymal stem cells enhance proliferation and migration of gastric cancer cells. *Oncotarget.* (2017) 8:1485. doi: 10.18632/oncotarget.14835
10. Laprevotte E, Cochaud S, du Manoir S, Lapierre M, Dejou C, Philippe M, et al. The IL-17B-IL-17 receptor B pathway promotes resistance to paclitaxel in breast tumors through activation of the ERK1/2 pathway. *Oncotarget.* (2017) 8:113360–72. doi: 10.18632/oncotarget.23008
11. Wu H-H, Hwang-Verslues WW, Lee W-H, Huang C-K, Wei P-C, Chen C-L, et al. Targeting IL-17B–IL-17RB signaling with an anti–IL-17RB antibody blocks pancreatic cancer metastasis by silencing multiple chemokines. *J Exp Med.* (2015) 212:333–49. doi: 10.1084/jem.20141702
12. Yang Y-F, Lee Y-C, Lo S, Chung Y-N, Hsieh Y-C, Chiu W-C, et al. A positive feedback loop of IL-17B-IL-17RB activates ERK/β-catenin to promote lung cancer metastasis. *Cancer Lett.* (2018) 422:44–55. doi: 10.1016/j.canlet.2018.02.037
13. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity.* (2011) 34:149–62. doi: 10.1016/j.immuni.2011.02.012
14. Kouri V-P, Olkkonen J, Ainola M, Li T-F, Björkman L, Konttinen YT, et al. Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatol Oxf Engl.* (2014) 53:39–47. doi: 10.1093/rheumatology/ket309
15. Kokubu T, Haudenschild DR, Moseley TA, Rose L, Reddi AH. Immunolocalization of IL-17A, IL-17B, and their receptors in chondrocytes during fracture healing. *J Histochem Cytochem Off J Histochem Soc.* (2008) 56:89–95. doi: 10.1369/jhc.7A7223.2007
16. Moore EE, Presnell S, Garrigues U, Guilbot A, LeGuern E, Smith D, et al. Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease. *Neuromuscul Disord NMD.* (2002) 12:141–50. doi: 10.1016/s0960-8966(01)00250-4
17. Ferretti E, Ponzoni M, Doglioni C, Pistoia V. IL-17 superfamily cytokines modulate normal germinal center B cell migration. *J Leukoc Biol.* (2016) 100:913–8. doi: 10.1189/jlb.1VMR0216-096RR
18. Novatchkova M, Leibbrandt A, Werzowa J, Neubüser A, Eisenhaber F. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem Sci.* (2003) 28:226–9. doi: 10.1016/S0968-0004(03)00067-7
19. Lee J, Ho W-H, Maruoka M, Corpuz RT, Baldwin DT, Foster JS, et al. IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *J Biol Chem.* (2001) 276:1660–4. doi: 10.1074/jbc.M008289200
20. Ramirez-Carrozzi V, Ota N, Sambandam A, Wong K, Hackney J, Martinez-Martin N, et al. Cutting edge: IL-17B uses IL-17RA and IL-17RB to induce type 2 inflammation from human lymphocytes. *J Immunol.* (2019) 202:1935–41. doi: 10.4049/jimmunol.1800696
21. Gaffen SL. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol.* (2009) 9:556–67. doi: 10.1038/nri2586
22. Wang Y-H, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med.* (2007) 204:1837–47. doi: 10.1084/jem.20070406
23. Maezawa Y, Nakajima H, Suzuki K, Tamachi T, Ikeda K, Inoue J, et al. Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling. *J Immunol Baltim Md 1950.* (2006) 176:1013–8. doi: 10.4049/jimmunol.176.2.1013
24. Wong CK, Cheung PFY, Ip WK, Lam CWK. Interleukin-25-induced chemokines and interleukin-6 release from eosinophils is mediated by p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and

nuclear factor-kappaB. *Am J Respir Cell Mol Biol.* (2005) 33:186–94. doi: 10.1165/rcmb.2005-0034OC

25. Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, et al. IL-17B and IL-17C are associated with TNF-α production and contribute to the exacerbation of inflammatory arthritis. *J Immunol.* (2007) 179:7128–36. doi: 10.4049/jimmunol.179.10.7128
26. Reynolds JM, Lee Y-H, Shi Y, Wang X, Angkasekwinai P, Nallaparaju KC, et al. Interleukin-17B antagonizes interleukin-25-mediated mucosal inflammation. *Immunity.* (2015) 42:692–703. doi: 10.1016/j.immuni.2015.03.008
27. Furuta S, Jeng Y-M, Zhou L, Huang L, Kuhn I, Bissell MJ, et al. IL-25 causes apoptosis of IL-25R-Expressing breast cancer cells without toxicity to nonmalignant cells. *Sci Transl Med.* (2011) 3:78ra31–ra31. doi: 10.1126/scitranslmed.3001374
28. Huang C-K, Yang C-Y, Jeng Y-M, Chen C-L, Wu H-H, Chang Y-C, et al. Autocrine/paracrine mechanism of interleukin-17B receptor promotes breast tumorigenesis through NF-κB-mediated antiapoptotic pathway. *Oncogene.* (2014) 33:2968–77. doi: 10.1038/onc.2013.268
29. Huang S, Wei P, Hwang-Verslues WW, Kuo W, Jeng Y, Hu C, et al. TGF-β1 secreted by Tregs in lymph nodes promotes breast cancer malignancy via up-regulation of IL-17RB. *EMBO Mol Med.* (2017) 9:1660–80. doi: 10.15252/emmm.201606914
30. Bie Q, Zhang P, Su Z, Zheng D, Ying X, Wu Y, et al. Polarization of ILC2s in peripheral blood might contribute to immunosuppressive microenvironment in patients with gastric cancer. *J Immunol Res.* (2014) 2014:1–10. doi: 10.1155/2014/923135
31. Bie Q, Sun C, Gong A, Li C, Su Z, Zheng D, et al. Non-tumor tissue derived interleukin-17B activates IL-17RB/AKT/β-catenin pathway to enhance the stemness of gastric cancer. *Sci Rep.* (2016) 6:25447. doi: 10.1038/srep25447
32. Cai J, Zhang W, Yang P, Wang Y, Li M, Zhang C, et al. Identification of a 6-Cytokine prognostic signature in patients with primary glioblastoma harboring M2 microglia/macrophage phenotype relevance. *PLoS ONE.* (2015) 10:e0126022. doi: 10.1371/journal.pone.0126022
33. Leivonen S-K, Pollari M, Brück O, Pellinen T, Autio M, Karjalainen-Lindsberg M-L, et al. T-cell inflamed tumor microenvironment predicts favorable prognosis in primary testicular lymphoma. *Haematologica.* (2019) 104:338–346. doi: 10.3324/haematol.2018.200105
34. Al-Samadi A, Moossavi S, Salem A, Sotoudeh M, Tuovinen SM, Konttinen YT, et al. Distinctive expression pattern of interleukin-17 cytokine family members in colorectal cancer. *Tumor Biol.* (2016) 37:1609–15. doi: 10.1007/s13277-015-3941-x
35. Eiro N, Fernandez-Gomez J, Sacristán R, Fernandez-Garcia B, Lobo B, Gonzalez-Suarez J, et al. Stromal factors involved in human prostate cancer development, progression and castration resistance. *J Cancer Res Clin Oncol.* (2017) 143:351–9. doi: 10.1007/s00432-016-2284-3
36. Lavorgna A, Matsuoka M, Harhaj EW. A critical role for il-17rb signaling in HTLV-1 tax-induced NF-κB Activation and T-cell transformation. *PLoS Pathog.* (2014) 10:e1004418. doi: 10.1371/journal.ppat.1004418
37. Ren L, Xu Y, Liu C, Wang S, Qin G. IL-17RB enhances thyroid cancer cell invasion and metastasis via ERK1/2 pathway-mediated MMP-9 expression. *Mol Immunol.* (2017) 90:126–35. doi: 10.1016/j.molimm.2017.06.034
38. Guo H-Z, Niu L-T, Qiang W-T, Chen J, Wang J, Yang H, et al. Leukemic IL-17RB signaling regulates leukemic survival and chemoresistance. *FASEB J.* (2019) 33:9565–9576. doi: 10.1096/fj.201900099R

Conflict of Interest: JB, CD, and AD are employees of OREGA Biotech, JB and NB are shareholders of OREGA Biotech.

frontiers in Immunology

ORIGINAL RESEARCH
published: 23 April 2020
doi: 10.3389/fimmu.2020.00677

Transient Expression of IL-17A in Foxp3 Fate-Tracked Cells in *Porphyromonas gingivalis*-Mediated Oral Dysbiosis

Peter D. Bittner-Eddy, Lori A. Fischer and Massimo Costalonga*

Division of Periodontology, Department of Developmental and Surgical Sciences, School of Dentistry, University of Minnesota, Minneapolis, MN, United States

In periodontitis *Porphyromonas gingivalis* contributes to the development of a dysbiotic oral microbiome. This altered ecosystem elicits a diverse innate and adaptive immune response that simultaneously involves Th1, Th17, and Treg cells. It has been shown that Th17 cells can alter their gene expression to produce interferon-gamma (IFN-γ). Forkhead box P3 (Foxp3) is considered the master regulator of Treg cells that produce inhibitory cytokines like IL-10. Differentiation pathways that lead to Th17 and Treg cells from naïve progenitors are considered antagonistic. However, it has been reported that Treg cells expressing IL-17A as well as IFN-γ producing Th17 cells have been observed in several inflammatory conditions. Each scenario appears plausible with T cell transdifferentiation resulting from persistent microbial challenge and consequent inflammation. We established that oral colonization with *P. gingivalis* drives an initial IL-17A dominated Th17 response in the oral mucosa that is dependent on intraepithelial Langerhans cells (LCs). We hypothesized that Treg cells contribute to this initial IL-17A response through transient expression of IL-17A and that persistent mucosal colonization with *P. gingivalis* drives Th17 cells toward an IFN-γ phenotype at later stages of infection. We utilized fate-tracking mice where IL-17A- or Foxp3-promoter activity drives the permanent expression of red fluorescent protein tdTomato to test our hypothesis. At day 28 of infection timeline, Th17 cells dominated in the oral mucosa, outnumbering Th1 cells by 3:1. By day 48 this dominance was inverted with Th1 cells outnumbering Th17 cells by nearly 2:1. Tracking tdTomato^{+} Th17 cells revealed only sporadic transdifferentiation to an IFN-γ-producing phenotype by day 48; the appearance of Th1 cells at day 48 was due to a late *de novo* Th1 response. tdTomato^{+} Foxp3^{+} T cells were 35% of the total live CD4^{+}T cells in the oral mucosa and 3.9% of them developed a transient IL-17A-producing phenotype by day 28. Interestingly, by day 48 these IL-17A-producing Foxp3^{+} T cells had disappeared. Therefore, persistent oral *P. gingivalis* infection stimulates an initial IL-17A-biased response led by Th17 cells and a small but significant number of IL-17A-expressing Treg cells that changes into a late *de novo* Th1 response with only sporadic transdifferentiation of Th17 cells.

Keywords: Foxp3, Treg cells, Th17 cells, fate-tracking, IL-17A, periodontitis, *Porphyromonas gingivalis*

OPEN ACCESS

Edited by:
Kong Chen,
University of Pittsburgh, United States

Reviewed by:
Jeremy McAleer,
Marshall University, United States
Heather Conti,
The University of Toledo, United States

****Correspondence:***
Massimo Costalonga
costa002@umn.edu

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *16 December 2019*
Accepted: *26 March 2020*
Published: *23 April 2020*

Citation:
Bittner-Eddy PD, Fischer LA and Costalonga M (2020) Transient Expression of IL-17A in Foxp3 Fate-Tracked Cells in Porphyromonas gingivalis-Mediated Oral Dysbiosis. Front. Immunol. 11:677. doi: 10.3389/fimmu.2020.00677

INTRODUCTION

Periodontitis is a destructive inflammatory disease that leads to progressive destruction of the soft tissues and alveolar bone supporting the tooth. This disease represents the sixth most prevalent disease worldwide (1). Severe periodontitis affects between 8 and 10% of the adult population in western and developing countries (2, 3). Periodontitis is associated with persistent colonization of the periodontal pocket by a consortia of microorganisms organized as a multispecies biofilm that contains symbionts, pathobionts and keystone bacterial pathogens like *Porphyromonas gingivalis* (*Pg*) (4). Virulence factors of microorganisms like *Pg* induce inflammation thereby altering the nutrient foundation of the microbial community resulting in population shifts within the consortia (5). Although poorly pathogenic in mono-colonized germ free mice, the dysbiosis induced by *Pg* in specific pathogen free mice (6) elicits an adaptive CD4^{+} T cell response against a wide spectrum of antigens originating from the expanded pathobiont population. The resulting immune response eventually leads to progressive destruction of the soft connective tissues and alveolar bone holding teeth in place (7). Understanding the immunopathogenesis of periodontitis is critical to strategies that seek to prevent, treat or predict future occurrence of disease.

We address the immunopathogenesis of periodontitis by determining how the innate and adaptive immune response behaves against new microbial threats entering the oral ecosystem. Here, activated CD4^{+} T and B cells are key players in modulating homeostasis of the bone supporting the tooth following the microbial insult (8–14) and reviewed in (5). CD4^{+} T helper (Th) 1, Th17 and T regulatory cells (Treg) often coexist in the same periodontal lesion. We currently do not know if these CD4^{+} T cells are generated and maintained as independent lineages or whether in the face of persistent dysbiosis and a chronic disease state they exhibit phenotypic plasticity and shift over time to different pathogenic potentials.

Situated proximal to the mucosal microbial biofilm in the periodontal pocket, epithelial and Langerhans cells (LCs) sample the microbial environment, recruit the subepithelial inflammatory infiltrate and modulate the adaptive response. We have established that Th17 differentiation of *Pg*-specific naïve CD4^{+} T cells *in vivo* is sustained by LCs (15). Current research suggests that in periodontitis Th17 cells and their signature cytokine, IL-17A, are central to bone destruction by promoting osteoclastogenesis (16–18). Although other evidence suggests that IL-17A can be protective (19), many suggest that IFN-γ-producing Th1 cells also drive alveolar bone destruction (8, 12, 20). Plasticity of Th17 cells is well documented (21–24), and a late developmental switch to IFN-γ expression in Th17 cells has been implicated in the pathologies of a number of inflammatory autoimmune diseases (25–28).

T regulatory cells (Treg) regulate the activity of T cells of several different phenotypes. The nuclear protein Forkhead box P3 (Foxp3) is considered the master regulator of Treg cells. However, the notion of Foxp3-expressing cells as a stable lineage of terminally differentiated Treg cells is controversial. Treg cells generally expressing IL-10 can also switch to IFN-γ-producing Th1-like cells (29) and even IL-17A-producing Th17-like cells (30) under certain inflammatory conditions [reviewed in (31–33)]. Currently, Treg cells are proposed as a heterogeneous pool, and while the majority of them are lineage stable, a minor uncommitted population does retain the capacity of reprogramming to a different phenotype [reviewed in (34)]. Although some evidence is present in humans (35), *in vivo* mouse models of inflammatory colitis provide the strongest evidence of Treg to Th17 reprogramming. In a murine model of inflammatory colitis CCR6^{+} Tregs producing retinoic acid orphan receptor (ROR) γt apparently drive the inflammation of the large intestine. In this model, CCL20 inhibits Foxp3 expression and directs former Tregs toward IL-17A expression. Analysis of peripheral blood from patients with ulcerative colitis suggests a similar process could occur in humans (35). Finally, IFNγ^{+} Th1/Tregs have been described in a murine model of atherosclerosis, suggesting Treg-Th1 plasticity could also occur (36).

What is unknown in periodontitis is whether differentiated CD4^{+} T cells modulate their response by re-programming cytokine expression when encountering persistent dysbiosis and heightened ability of pathobionts to cross the oral mucosal barrier. Here we tested the hypothesis that persistent *Pg* colonization creates the conditions to drive Treg and Th17 transdifferentiation. To test this hypothesis, we utilized Th17 and Treg lineage-tracing mice (23, 29) orally inoculated with *Pg* at 4-day intervals to mimic persistent dysbiosis. CD4^{+} T cells permanently labeled with a fluorescent reporter protein after activation of IL-17A or Foxp3 promoters were tested for expression of unorthodox cytokines. In this manuscript we present the relative proportion of Th17, Th17-derived Th1-like cells expressing IFN-γ, new Th1 cells, Treg and Treg-derived Th17-like that express IL-17A in murine oral mucosal and cervical lymph nodes over time after persistent oral colonization with *Pg*.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota, and performed on age and sex-matched (8 to 10 weeks) mice or littermates, where appropriate. All mice were housed in microisolator cages with food and water *ad libitum* in a specific pathogen free animal facility. C57BL/6J mice were originally obtained from Jackson Laboratories (Bar Harbor, ME, United States). Experimental IL-17A^{cre} fate-tracking mice were bred by us as previously described (37). Experimental tamoxifen-inducible Treg-fate-tracking animals (Foxp3^{cre} fate-tracking mice) were generated by crossing B6.Cg-Gt(ROSA)26Stm14(CAG-tdTomato)Hze/J (Jackson #007914) animals with Foxp3tm9(EGFP/cre/ERT2)Ayr/J animals [Jackson #016961, (38)] to generate F_1 hybrids. F_1 animals were then cross bred to generate F_2 breeders and experimental animals. F_2 experimental animals were Rosa26-tdTomato homozygous and either Foxp3-cre-ERT2 hemizygous (males) or Foxp3-cre-ERT2 homozygous (females). In these animals, removal of the floxed stop codon and expression of tdTomato red fluorescent protein is tightly regulated by administration of tamoxifen. Inducible activation of Foxp3-cre is critical here as Foxp3 is active developmentally, and stochastic expression of Foxp3-cre can lead to mice with non-specific expression of tdTomato in multiple cell lineages (37).

Administration of *P. gingivalis*

All mice were treated for 10 days with Sulfamethoxazole/Trimethoprim (SMZ-TMP), with a 4-day pause without antibiotics before administration of *Pg* or PBS as previously described (39, 40). The SMZ-TMP treatment is solely used to create an ecological niche in the oral microflora of mice enabling *Pg* colonization, and has no significant lasting or other effects. *Pg* strain ATCC 53977 (10^9 CFU/mouse) or PBS was administered in 2% carboxymethylcellulose via atraumatic oral gavage with a ball-tipped needle as previously described every 4 days until completion of the experiment (39, 40) to ensure persistent *Pg*-induced dysbiosis (6).

Administration of Tamoxifen

Tamoxifen (Millipore-Sigma, St Louis, MO, United States) was prepared at 40 mg/ml in olive oil with 10% (v/v) ethanol. 200 μl of tamoxifen stock solution was given per mouse via oral gavage on days 14, 15, and 17 of the experimental timeline (38). Tamoxifen was administered as a brief "pulse" after *Pg* inoculation at day 14 for two specific reasons. First, we needed to strike a balance between having a sufficient number of newly generated antigen-specific Foxp3 Tregs present in the mouse and allowing sufficient time for these now tdTomato-expressing cells to respond to dysbiotic changes induced by persistent *Pg*. Second, we want to limit the labeling of conventional CD4 T cells that may transiently express Foxp3 during early lineage commitment (41). Additionally, this system also avoids mislabeling of tissues due to stochastic activation of Foxp3 that we reported to occur during embryogenesis (37).

Identification of *P. gingivalis*-Specific Antigen-Experienced CD4+ T Cells in Cervical Lymph Nodes by Flow Cytometry

Single-cell suspensions were isolated by standard techniques from cervical lymph nodes of C57BL/6J mice following sustained *Pg* inoculation or PBS (sham) treatment. Cells were stained with viability dye Zombie Aqua (BioLegend, San Diego, CA, United States) and Fc receptors blocked using anti CD16/CD32 antibody (eBioscience; clone 93). Cells were stained using a gingipain-specific MHC class II tetramer (pR/Kgp:I-A^b) as previously described (42) and then with anti-mouse CD3 (BioLegend; clone 17A2), CD4 (eBioscience; clone RM4-5), CD8α (eBioscience; clone 53-6.7), CD44 (BioLegend; clone IM7) and B220 (eBioscience; clone RA3-6B2) fluorochrome-conjugated mAbs to distinguish antigen-experienced CD4+ T cells from B cells, CD8+ T cells and naïve lymphocytes. Cells were sorted on an LSR II flow cytometer (BD Biosciences, San Jose, CA, United States) and fluorescence emissions analyzed with FlowJo software (v10.4.1;Tree Star, Ashland, OR, United States).

Analysis of Immune Cells From Oral Mucosa by Flow Cytometry

Mice were given an intravenous injection of 1.25 μg FITC-conjugated rat anti-mouse CD45 monoclonal antibody (eBioscience San Diego, CA, United States; clone 30-F11) as described previously to exclude blood resident immune cells from subsequent analyses (43). Oral mucosa (maxillary and mandibular gingiva, buccal, and posterior hard-palate tissues) was harvested and single-cell suspensions generated as described (43). Cells were stained with Zombie Aqua, Fc receptors blocked with anti-mouse CD16/CD32 antibody followed by incubation with a panel of mAbs that included anti-mouse CD45 mAb conjugated to PE (eBioscience; clone 30-F11), anti-mouse CD3, CD4, CD8α, TCR γδ(BioLegend; clone GL3), and TCR β (BioLegend; clone H57-597) fluorochrome-conjugated mAbs. Cells were acquired on an LSR II flow cytometer and fluorescence emissions analyzed with FlowJo software (v10.4.1;Tree Star, Ashland, OR, United States).

Identification of Cytokines and Foxp3 in CD4+ T Cells Isolated From Oral Mucosa and Cervical Lymph Nodes

Single-cell suspensions from oral mucosal samples or cervical lymph nodes were harvested and cultured overnight in complete EHAA (Irvine Scientific, Santa Ana, CA, United States) supplemented with 2.5 μg/ml of PHA-L (Millipore-Sigma). Cells were polyclonally stimulated with PMA-Ionomycin in the presence of brefeldin A as previously described (42). After 6 h of stimulation, cells were incubated with Zombie Aqua, anti-mouse CD16/CD32 antibody and surface stained with the panel of mAbs described above. Cells obtained from oral mucosal tissues were not additionally stained using the gingipain-displaying MHC class II tetramer (pR/Kgp:I-A^b) (42) as insufficient gingipain-specific CD4+ T cells (*Pg*-specific) are present in this tissue type for robust analysis. For cells obtained from IL-$17A^{cre}$ fate-tracking and $Foxp3^{cre}$ fate-tracking mice, fixation was done in 1:1 ratio of 4% paraformaldehyde and BD Perm/Wash (Cat. #51-2091KZ; BD Biosciences, San Jose, CA, United States) to preserve tdTomato and GFP signals during permeabilization. Permeablized cells were then stained with IL-17A (eBioscience; clone eBio17B7), IFN-γ (Biolegend; clone XMG1.2), or IL-10 (Biolegend; clone JES5-16E3) fluorochrome-conjugated mAbs. Cells from $Foxp3^{cre}$ fate-tracking mice were incubated with anti-mouse Foxp3:AF488 mAb (Biolegend; clone MF-14) concurrently with cytokine mAbs. Cells were acquired on an LSR II flow cytometer and fluorescence emissions analyzed with FlowJo software (v10.4.1;Tree Star, Ashland, OR, United States).

Statistical Analysis

Data was analyzed and plotted using Prism 7 software (GraphPad Software, San Diego, CA, United States) and displayed as means ± SEM. Each data point is from at least three independent

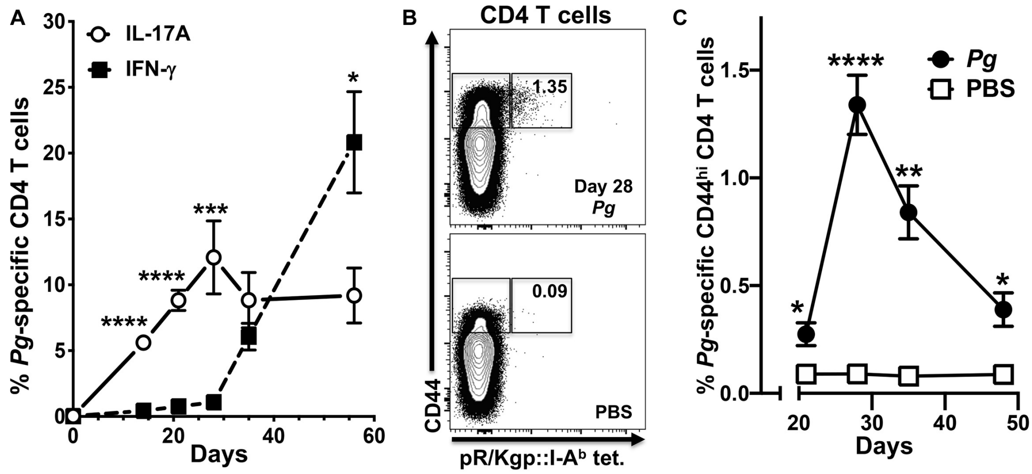


FIGURE 1 | The percentage of *P. gingivalis*-specific $CD4^+$ T cells expressing IFN-γ increases from day 28 in cervical lymph nodes. Single-cell suspensions from cervical lymph nodes of C57BL/6J mice orally inoculated with *Pg* or PBS were enriched for $CD4^+$ cells at defined time points and stimulated with PMA/ionomycin in the presence of brefeldin A. Cells were surface stained with anti-mouse CD3, B220, CD8α, CD4, CD44 fluorochrome-conjugated mAbs and pR/Kgp:I-A^b tetramer and then intracellularly with anti-mouse IL-17A and IFN-γ mAbs to identify antigen-experienced gingipain-specific $CD4^+$ T cells by flow cytometry (gated as $CD44^{bright}$ $CD3^+$ $CD4^+$ pR/Kgp-IA^{b+} $B220^-$ $CD8\alpha^-$). Data was pooled from three independent experiments totaling at least 8 mice per group and displayed as mean percentage ± SEM. **(A)** Summary data of total antigen-experienced pR/Kgp:I-A^b tetramer positive $CD4^+$ T cells that expressed either IL-17A or IFN-γ. **(B)** Representative FACS plots from a *Pg* and PBS mouse showing pR/Kgp:I-A^b tetramer positive $CD4^+$ T cells. Gates are drawn around antigen-experienced $CD4^+$ T cells identified as $CD44^{bright}$. The frequency of pR/Kgp:I-A^b tetramer positive cells identified as a percentage of the total antigen-experienced $CD4^+$ T cell population is given. **(C)** Summary data of percentage of antigen-experienced pR/Kgp:I-A^b tetramer positive $CD4^+$ T cells. Percentages were compared using two-tailed Student's *t*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

experiments. Means between two groups were compared using Student's *t*-test and between multiple groups with one-way ANOVA with *post hoc* analyses for multiple comparisons. *P*-values of 0.05 or less were considered significant. Unpaired or paired *t*-tests were performed as dictated by the data set being analyzed. Non-parametric comparisons were assessed with Mann-Whitney *U* test.

Data for the MFI of GFP was analyzed by fitting a mixed effects model with paired values (tdTomato +ve vs. tdTomato −ve in the same sample), rather than by repeated-measures ANOVA. Repeated measure ANOVA is unable to process missing values, which occurred in mice where $tdTomato^+$ $IL\text{-}17A^+$ or $tdTomato^+$ $IFN\text{-}\gamma^+$ populations were not detected. A *post hoc* Sidak's multiple comparisons test was utilized to determine *p*-values of comparisons found significant by the initial mixed-effects analysis.

RESULTS

IL-17A-Expressing *Pg*-Specific $CD4^+$ T Cells in Cervical Lymph Nodes Peak at Day 28 While IFN-γ Expressing Cells Increase From Day 28 to 56

We have shown previously that Th17 cells are predominant in the early adaptive $CD4^+$ T cell response to *Pg* in an oral model of periodontitis, and that this response is critically dependent on LCs (39). To study the dynamics of an evolving adaptive response to *Pg*, we tracked expression of IL-17A and IFN-γ in *Pg*-specific antigen-experienced $CD4^+$ T cells isolated from cervical lymph nodes (CLN) of *Pg* or PBS-treated C57BL/6J mice over the course of 56 days. The *Pg*-specific Th17 response increased and peaked at day 28, and was significantly greater than the minor Th1 response we observed at days 14–28 (**Figure 1A**). While the *Pg*-specific Th17 response plateaued from day 28 to 56, the frequency of *Pg*-specific Th1 cells increased substantially by day 35, and at the experimental endpoint (day 56) it was significantly higher than the frequency of *Pg*-specific Th17 cells (**Figure 1A**). The frequency of antigen-experienced *Pg*-specific $CD4^+$ T cells in CLN as a percentage of total $CD44^{hi}$ $CD4^+$ T cells (**Figure 1B**) peaked at day 28 and then declined but was still significantly higher than that observed in PBS treated mice at all time points examined (**Figure 1C**).

Total $CD4^+$ T Cell Response Against Oral *Pg* in the Oral Mucosa Evolves From an Initial Th17 Response to One Dominated by Th1

We next examined $CD4^+$ T cells recovered from the oral mucosa of C57BL/6J mice for expression of IFN-γ and IL-17A (**Figure 2A**). Here we chose to examine day 28 and day 48 based on changes we observed occurring in cells analyzed from cervical lymph nodes of *Pg* inoculated mice. In this analysis we found significantly more IL-17A expressing $CD4^+$ T cells at day 28 compared to day 48 and fewer cells expressing IFN-γ at day 28 compared to day 48 (**Figure 2B**). In contrast, the frequency of IL-17A expressing $CD4^+$ T cells decreased significantly by

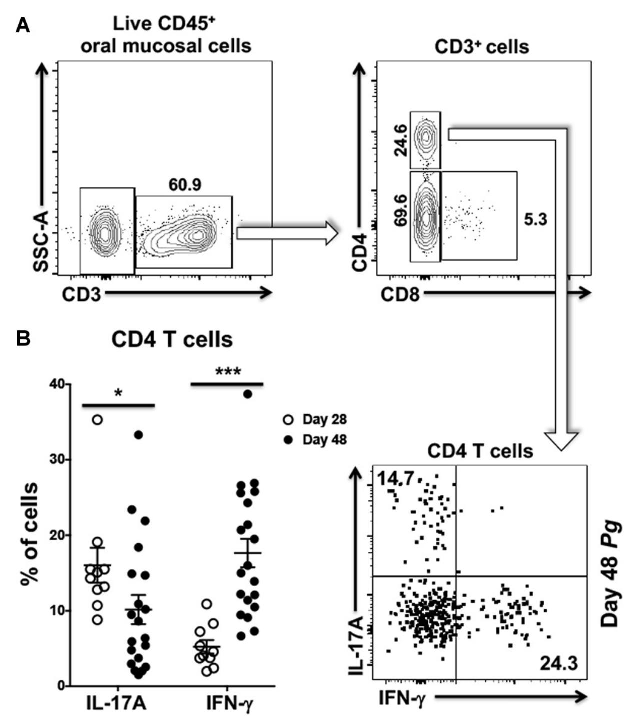


FIGURE 2 | Frequency of Th1 cells significantly increases within the oral mucosa of *P. gingivalis* inoculated mice by day 48. **(A)** Representative flow cytometry plots showing the gating strategy used to identify Th1 (IFN-γ+) and Th17 (IL-17A+) cells within the oral mucosa. Numbers alongside gates indicate% of cells within that gate. **(B)** Summary data showing individual data points from three experiments examining the frequency of Th1 and Th17 recovered from the oral mucosa of mice at day 28 and day 48. Means ± SEM are plotted. Groups were compared with two-tailed Student's *t*-test. $^{*}p < 0.05$, $^{***}p < 0.001$.

day 48 with a significant increase in the frequency of CD4+ T cells expressing IFN-γ. At day 28 Th17 cells were dominant in the murine oral mucosa out numbering Th1 cells by more than 3:1, but by day 48 this dominance was inverted with Th1 cells now outnumbering Th17 cells by close to 2:1. This phenotype shift in the overall mucosal CD4+ T cell response is consistent with the observations in cervical lymph nodes we reported earlier (**Figure 1**).

At Homeostasis tdTomato Expression Marks Three Distinct IL-17A+ T Cell Populations in the Oral Mucosa of IL-17A^{cre} Fate-Tracking Mice

The shift from a Th17 to a Th1-type response in cervical lymph nodes and in the oral mucosa suggested that a dynamic remodeling of the adaptive CD4+ T cell response to *Pg*-induced dysbiosis can occur over time. To examine whether this remodeling might involve transdifferentiation of Th17 cells to a Th1-like phenotype, we generated IL-17A^{cre} fate-tracking mice. IL-17A/Cre expression allows the de-repression of a tdTomato red fluorescent reporter protein thereby permanently labeling the entire progeny of all IL-17A-expressing cells (23, 37). In IL-17A^{cre} fate-tracking mice at homeostasis, we observed three distinct populations of CD3+ T cells in the oral mucosa that were marked by tdTomato expression (**Figure 3A**). These cells, therefore, must have had or currently have an active IL-17A promoter. Using this strategy, we identified significant numbers of conventional CD4+ T cells (Th17), γδ T cells and a population of CD3+ cells that have the β chain of the T cell receptor, but are marked as double-negative CD3+ T cells (CD3+ DN) due to the lack of CD4 and CD8 cell surface markers. This latter cell population may represent so-called mucosal-associated invariant T (MAIT) cells. Although we did not examine CD103 or CD69 expression in these CD3+ DN, NKT cells can be ruled out since they were negative for the diagnostic NK1.1 cell surface marker (data not shown). Notably, we did not observe tdTomato expressing CD8 T cells, also known as Tc17 cells. Permanent tdTomato labeling of cells that expressed IL-17A at any one time can reveal phenotypically plastic subpopulations that initiate the expression of unorthodox cytokines, like IFN-γ. At homeostasis, the three tdTomato+ CD3 T cells populations primarily expressed IL-17A with no evidence of plasticity toward a Th1-type phenotype (**Figure 3B**).

Sporadic IFN-γ Expression by IL-17A^{cre}-tdTomato+ T Helper Cells in the Oral Mucosa of *Pg* Inoculated Mice by Day 48

To examine potential *Pg*-induced transdifferentiation in CD3+ T cells subsets, we analyzed cells isolated from the oral mucosa of IL-17A^{cre} fate-tracking mice at day 28 and 48 and compared them to PBS control mice. In an initial characterization of *Pg* infection, we found significant infiltration of CD4+ T cells, γδ T cells and CD3+ DN cells into the oral mucosa at day 28 compared to PBS controls (**Figure 4A**). γδ T cells were more abundant than CD4+ T cells or CD3+ DN cells, although the latter cell types showed greater increases relative to PBS control mice. All three populations, however, declined by day 48 relative to day 28 despite sustained *Pg* inoculation.

We next examined the IL-17A^{cre}-tdTomato+ fraction amongst the CD4+ T cell, γδ T cell and CD3+ DN cell populations for expression of IL-17A and IFN-γ (**Figures 4B,C**). Expression of IFN-γ in these IL-17A^{cre}-tdTomato+ cells would be evidence of phenotype plasticity. High frequencies of γδ T cells expressing IL-17A, averaging just under 90%, and very few γδ T cells expressing IFN-γ were observed (**Figure 4C**). IL-17A expression was more variable in CD4+ T cells, and to a lesser extent in CD3+ DN cells, across all three groups (Day 28, 48, and PBS), but showed no significant differences (**Figures 4B,C**). Although we only found a trend of increased frequency of IL-17A^{cre}-tdTomato+ CD4+ T cells expressing IFN-γ at day 48 compared to day 28 ($p = 0.07$, Mann-Whitney *U* test), it is clear from three distinct outliers that Th17 plasticity can occur due to *Pg*-induced dysbiosis. In a sample size of 16 mice we found 3 mice that at day 48 had frequencies of IFN-γ expression in IL-17A-cre-tdTomato+ CD4+ T cells that exceeded 15% (**Figure 4B** – gray box). Moreover, in these 3 mice,

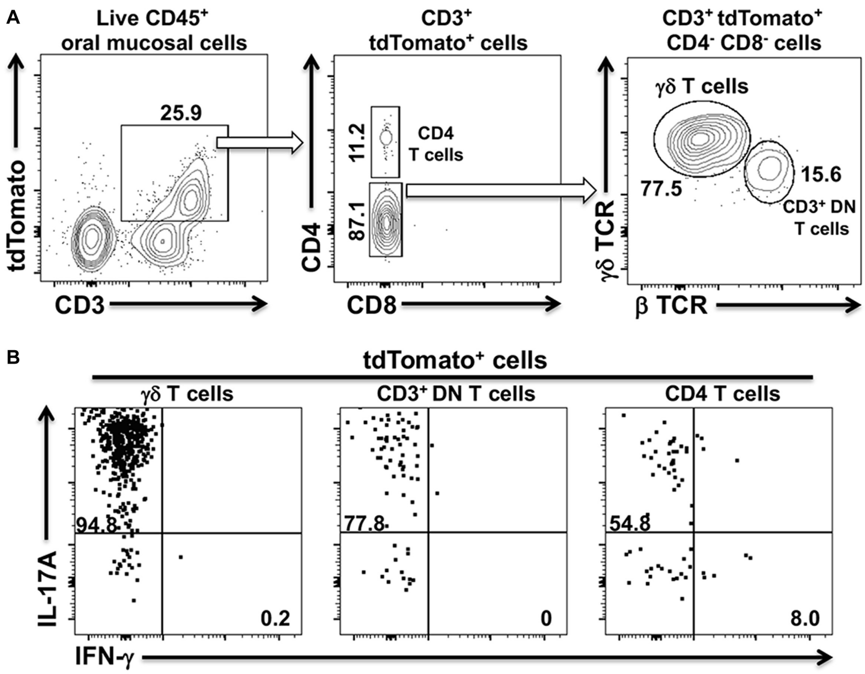


FIGURE 3 | tdTomato expression in three distinct CD3+ T cells populations within the oral mucosa of IL-17A^{cre} fate-tracking mice. Oral mucosa was harvested from IL-17A^{cre} fate-tracking mice and single cell suspensions prepared from the tissue for subsequent analysis by flow cytometry. Single cell suspensions were stained with vitality dye Zombie Aqua followed by a panel of anti-mouse mAbs to identify immune cell subsets expressing tdTomato. Cells were counted by flow cytometry and analyzed by FlowJo software. **(A)** Representative flow cytometry plots showing gating strategy to identify three tdTomato+ cell populations. Numbers indicate the% of cells within a particular gate. Live CD4+ T cells were identified as Zombie Aqua^{lo}, CD45+, CD3+, CD4+, CD8α−, NK1.1−; live γδT cells as Zombie Aqua^{lo}, CD45+, CD3+, γδ TCR+, β TCR−, CD4−, CD8α−, NK1.1−; live CD3+ DN T cells as Zombie Aqua^{lo}, CD45+, CD3+, β TCR+, γδ TCR−, CD4−, CD8α−, NK1.1−. **(B)** Representative flow cytometry plots showing expression of IL-17A and IFN-γ in the three CD3+ tdTomato+ cell populations. Single cell suspensions obtained from oral mucosa were cultured and stimulated with PMA/ionomycin in the presence of brefeldin A. Cells were surface stained with anti-mouse mAbs as in **(A)** and then intracellularly with anti-mouse IL-17A and IFN-γ mAbs.

IL-17A-cre-tdTomato+ CD4+ T cells that expressed both IFN-γ and IL-17A were also found (**Figure 4B**; upper right [Q2] flow cytometry dot-plot).

IL-17A^{cre} fate-tracking mice did not exhibit IL-17A^{cre}-tdTomato+ neutrophils in the oral mucosa following inoculation with *Pg* (data not shown) indicating that neutrophils do not, and have not, expressed IL-17A at any one time in their ontogeny. Furthermore, there was no influx of IL-17A expressing NKT cells or Tc17 cells to the oral mucosa (44, 45).

Fluorescent Foxp3+ Populations Generated in Foxp3^{cre} Fate-Tracking Mice

As we did not observe consistent transdifferentiation of Th17 cells to IFN-γ producing cells, we next sought to determine whether the CD4+ Treg response is reshaped after persistent exposure to oral *Pg*. Treg cells are a high frequency population in the murine oral mucosa producing anti-inflammatory cytokines like IL-10 at homeostasis. A shift toward production of pro-inflammatory cytokines such as IFN-γ and IL-17A could have significant implications for pathogenesis of periodontal disease.

Utilizing Foxp3^{cre} fate-tracking mice that track the fate of Foxp3-expressing Treg cells we have the potential to identify three populations of fluorescent cells in our experimental system due to tdTomato expression and GFP expression driven from the Foxp3 promoter (**Figure 5A**). The first population, single GFP+ cells (green fluorescent only) represents a population of Foxp3+ cells that differentiated only after tamoxifen has completely cleared from the host animal, so called late phase (tdTomato−) Treg cells (**Figure 5A**). Second, dual GFP+ (green fluorescent) and tdTomato+ (red fluorescent) cells, demonstrate currently active Foxp3 promoter driving expression of GFP and cre/ERT2-dependent tdTomato expression in the presence of tamoxifen (**Figure 5A**). Lastly, single tdTomato+ cells (red fluorescent) represent a population where the Foxp3 promoter was active at the time of tamoxifen administration but that is no longer active at day 28 or day 48 of timeline (**Figure 5B**).

We found that oral administration of tamoxifen on day 14, 15, and 17 generated a robust population of dual positive tdTomato+ GFP+ Treg cells in oral mucosal tissues at day 28 and day 48 (**Figures 5C**). Day 48 mice had higher frequencies of single GFP+ cells compared to day 28 mice (49 ± 5 versus 15 ± 6), consistent with a longer period of post-tamoxifen recruitment of Treg cells in these mice. Interestingly, the increased frequency of single GFP+ cells appeared to come at the expense of non-Treg CD4+ T cells (GFP− and tdTomato− population). In mice, active tamoxifen persists for 22 h (46), giving us the opportunity

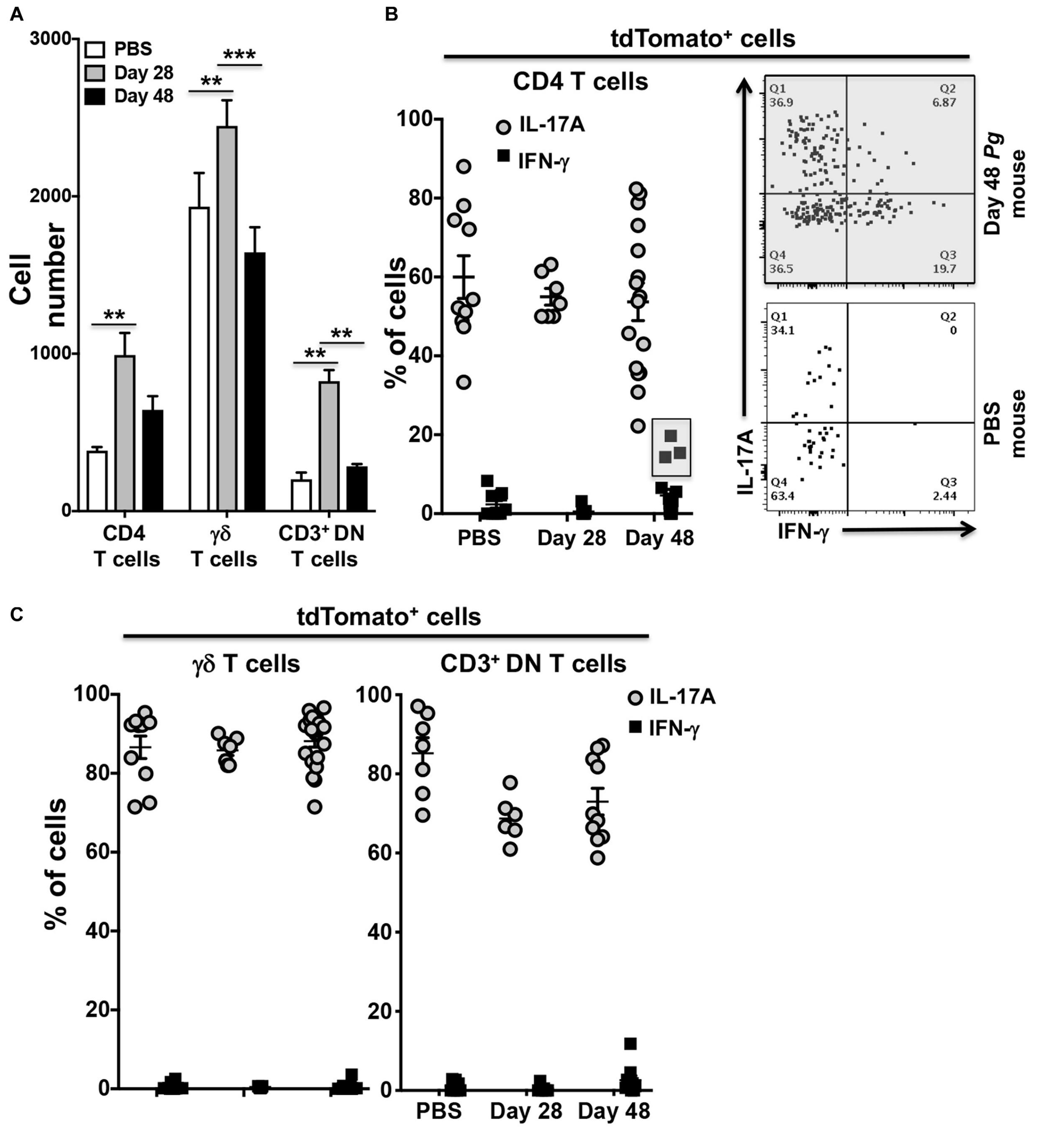


FIGURE 4 | Expression of IFN-γ by tdTomato+ CD4+ T cells present within the oral mucosa of *P. gingivalis* inoculated mice is sporadic. IL-17A^{cre} fate-tracking mice were pre-treated with SMZ and then orally inoculated with either *P. gingivalis* (4×10^9 cfu per ml) or vehicle (PBS). At day 28 or 48 oral mucosa was harvested and single-cell suspensions from single mice treated and stained with mAbs as described in **Figure 3**. **(A)** Summary data of total numbers of CD4+ T cells, CD3+ DN T cells and γδ T cells found in the oral mucosa of mice after 28 or 48 days. Cell types were identified from single cell suspensions as described in **Figure 3**. Cell numbers were normalized to 100,000 live non-immune cells to account for potential cell loss during processing and counting. Data are from 3 experiments with at least 2 mice per time point and are plotted with means ± SEM. Means analyzed by two-tailed Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$. **(B)** Summary data showing individual data points from three experiments examining the frequency of IL-17A and IFN-γ expression in IL-17A^{cre}-tdTomato+ CD4+ T cells recovered from the oral mucosa of IL17A fate-tracking mice. Representative flow cytometry plots from a single PBS and *P. gingivalis* inoculated mouse at day 48 showing evidence of IFN-γ expression in IL-17A^{cre}-tdTomato+ CD4+ T cells. **(C)** Summary data showing individual data points from three experiments examining the frequency of IL-17A and IFN- γ expression in IL-17A^{cre}-tdTomato+ CD3+ DN T cells and γδ T cells recovered from the oral mucosa of IL-17A^{cre} fate-tracking mice.

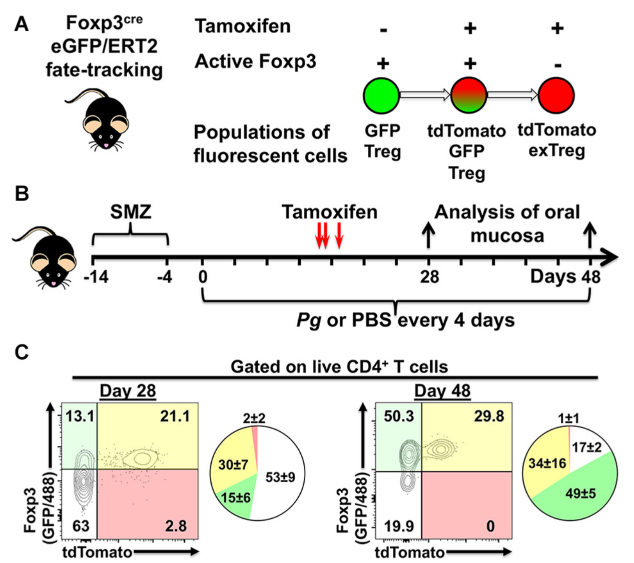


FIGURE 5 | Experimental design to identify populations of fluorescently labeled Treg cells. **(A)** Potential populations of fluorescent Treg cells found in tamoxifen-treated Foxp3^{cre}-GFP//ERT2 fate-tracking mice. **(B)** Experimental timeline for administration of *P. gingivalis* and, tamoxifen in experimental animals. **(C)** Representative flow cytometry plots gated on live CD4^{+} T cells from the oral mucosa of mice treated with tamoxifen and inoculated with *Pg* for 28 or 48 days. CD4^{+} T cells were identified as Zombie Aqua^{lo}, CD45^{+}, CD3^{+}, CD4^{+}, β TCR^{+}, CD8α^{-}. To augment GFP expressed from the Foxp3 promoter in these mice, cells were additionally stained with anti-mouse Foxp3 mAb conjugated to AF488. Numbers within the quadrants indicate% of the total CD4^{+} T cells gate. Summary pie charts show mean% ± SEM *N* = 4 mice.

with repeated administrations to simultaneously identify and track phenotype plasticity in early (tdTomato^{+}) and late phase (tdTomato^{-}) Treg cells in our experimental system. Additionally, delivering tamoxifen after the initiation of the adaptive immune response to *Pg*-induced dysbiosis also helps limit tdTomato-labeling of naïve cells committed to a Th17 phenotype that may transiently express Foxp3 (41).

Transient IL-17A Expression by tdTomato^{+} Foxp3-GFP^{+} T Cells in the Oral Mucosa of Foxp3^{cre} Fate-Tracking Mice

Examining cytokine expression of single red and dual red/green fluorescent cells after the administration of tamoxifen elucidated the extent of transdifferentiation of Foxp3^{+} Treg cells (**Figure 6A**). Foxp3^{cre} fate-tracking mice not treated with tamoxifen, used as FACS gating controls, had either negligible numbers or frequently no tdTomato^{+} cells related to endogenous estrogen levels (**Figure 6A**). Next, we compared expression of IL-17A, IFN-γ, and IL-10 in tdTomato^{+} cells isolated from *Pg* and sham treated Foxp3^{cre}-fate-tracking mice. Twenty eight days after initial *Pg* inoculation Foxp3^{cre}-tdTomato^{+} T cells were 35% (range 22.7 to 47.6) of the CD4^{+} T cells we detect in the oral mucosa. At day 28, the Foxp3^{cre}-tdTomato^{+} T cells that produced IL-17A were 3.9% (range 0.0 to 6.3). This was significantly higher than the 0.9% observed in sham-inoculated controls ($p < 0.001$). However, after 48 days of continuous *Pg* inoculation, the percentage of Foxp3^{cre}-tdTomato^{+} T cells producing IL-17A had dropped significantly to 1.3% when compared to day 28 ($p < 0.01$). This frequency was not significantly different from sham-inoculated controls, suggesting here that by day 48 *Pg* is no longer sustaining IL-17A expression in fate-tracked Treg cells (**Figure 6B**). Interestingly, although we did identify Foxp3^{cre}-tdTomato^{+} T cells expressing IFN-γ, the frequency was low and independent of *Pg* inoculation as there was no significant difference between sham- and *Pg*-inoculated mice at day 28 or 48 (**Figure 6C**). As expected we observed IL-10 expression in Foxp3^{cre}-tdTomato^{+} T cells at homeostasis but their frequency did not increase after *Pg*-induced dysbiosis (**Figure 6D**).

T Cells Producing IL-17A Maintain Expression of Foxp3 When Assessed by GFP Signal

Whether the observed kinetics of IL-17A expression in Foxp3^{cre}-tdTomato^{+} T cells was due to clonal contraction of transdifferentiated cells or reversion back to normal Treg phenotype was unclear. Therefore, we sought to determine whether these IL-17A producing Foxp3^{cre}-tdTomato^{+} cells were simultaneously expressing Foxp3 or whether expression of IL-17A was paralleled by cessation of Foxp3 expression. While all cells with active Foxp3 promoters at the time of tamoxifen administration would be tdTomato^{+}, only cells actively expressing Foxp3 at the time of analysis would be GFP^{+}. In order to normalize the analysis across multiple experiments, a paired analysis was utilized to compare the mean fluorescence intensity (MFI) of the GFP signal between IL-17A- and IFNγ-producing cells that are tdTomato positive or negative (**Figure 7**). As expected, in the IL-17A^{-} IFN-γ^{-} populations, Foxp3^{cre}-tdTomato^{+} T cells had a higher MFI of GFP than the tdTomato^{-} ones indicating an ongoing active Foxp3 promoter (**Figure 7**). Surprisingly though, in IL-17A-expressing populations the MFI of GFP was higher in Foxp3^{cre}-tdTomato^{+} that in Foxp3^{cre}-tdTomato^{-} T cells. Similarly, in the IFN-γ-expressing populations the GFP MFI was higher in Foxp3^{cre}-tdTomato^{+} that in Foxp3^{cre}-tdTomato^{-} T cells. This pattern indicates that the Foxp3 promoter continues to be active in IL-17A- and IFN-γ-expressing populations (**Figure 7**). In our experimental design, we used IL-17A mAbs conjugated to either APC or Brilliant Violet 421, so this result is not an artifact due to inadequate compensation of IL-17A signal spillover into the GFP or tdTomato channel. Lastly, Foxp3-GFP signal was still significantly higher in the Foxp3^{cre}-tdTomato^{+} population than in the corresponding tdTomato^{-} population at day 48 (data not shown). This indicates that Foxp3 promoter activity remains stable in Foxp3^{cre}-tdTomato^{+} cells for the duration of the experiment suggesting that Treg cells can shift to proinflammatory phenotypes while maintaining a transcription factor characteristic of regulatory T cells.

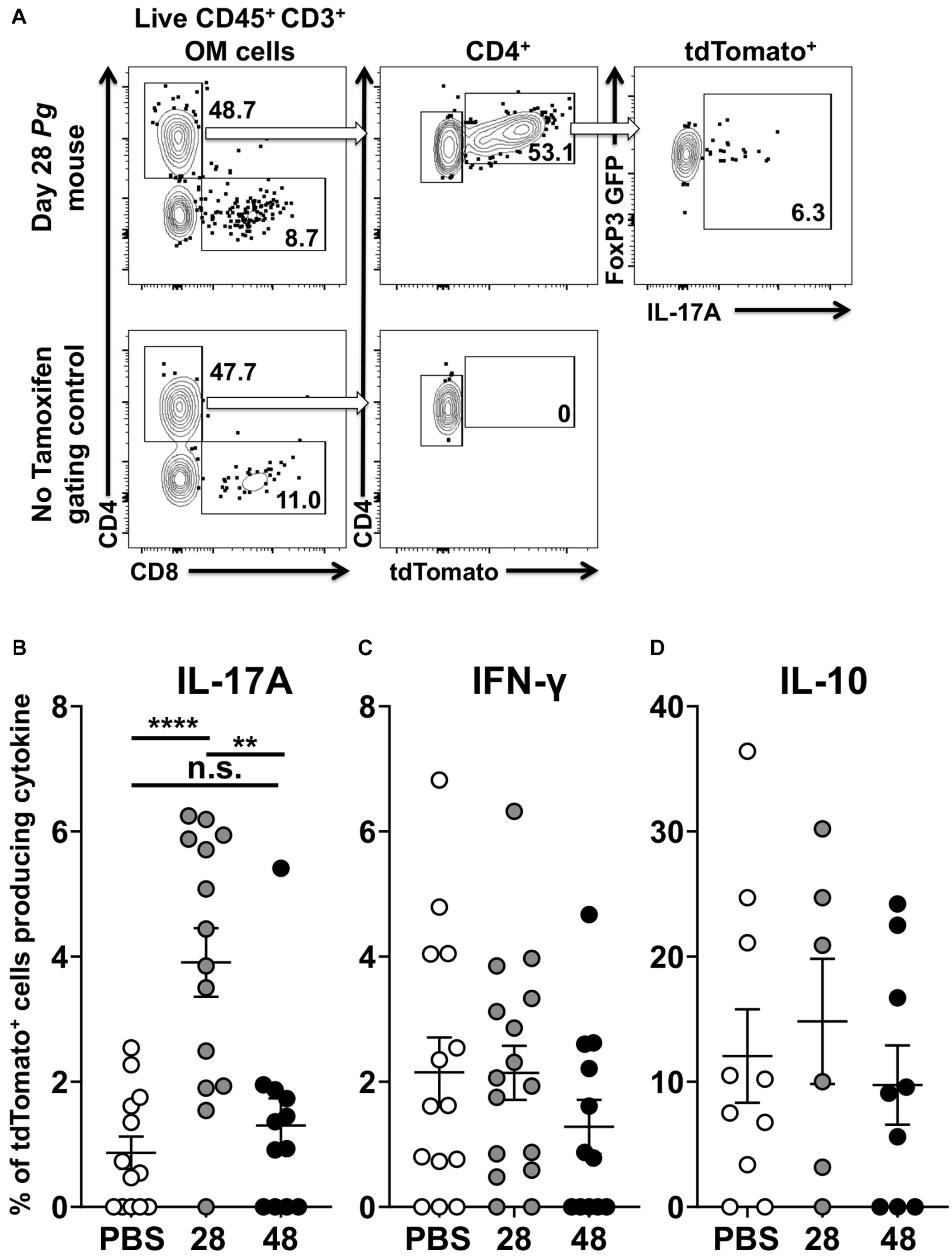


FIGURE 6 | *P. gingivalis* induces transient IL-17A expression in Treg cells in the oral mucosa. Single cell suspensions were isolated from oral mucosa of *P. gingivalis* inoculated mice at 28 or 48 days. Single cell suspensions were cultured and stimulated with PMA/ionomycin in the presence of brefeldin A. Cultured cells were surface stained with anti-mouse CD3, B220, CD8α, and CD4 fluorochrome-conjugated mAbs to identify tdTomato+ CD4+ Treg cells by flow cytometry (gated as CD3+ CD4+ tdTomato+ B220− CD8α−) and then intracellular stained with anti-mouse IL-17A, IFN-γ, and IL-10. **(A)** Representative flow cytometry plots showing gating strategy to identify the Foxp3^{cre}-tdTomato+ cell population within the oral mucosa also expressing Foxp3-GFP and IL-17A. Numbers indicate the % of cells within the associated gate. **(B–D)** Means of the frequency of tdTomato+ CD4+ T cells expressing IL-17A **(B)**, IFN- γ **(C)**, or IL-10 **(D)** at day 28 (gray circle) and 48 (black circle) were compared to sham controls (white circle) using two-tailed Student's *t*-test and presented as means ± SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$. Each circle represents two pooled oral mucosae from two mice.

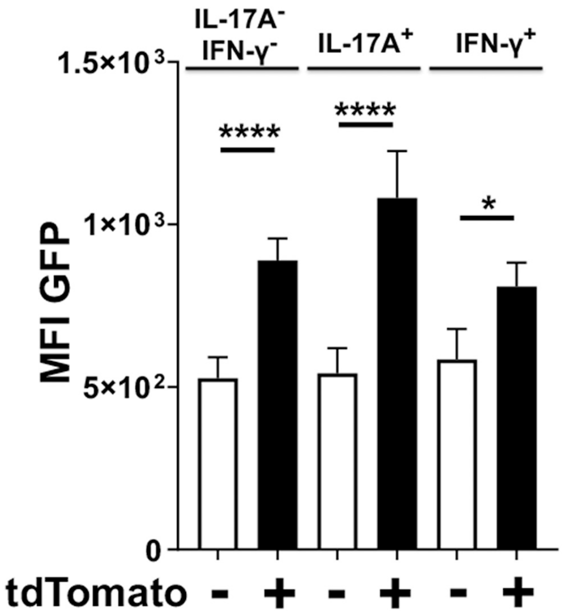


FIGURE 7 | Foxp3^{cre}-tdTomato$^+$ Treg cells producing IL-17A have an active Foxp3 promoter. Foxp3^{cre}-tdTomato$^+$ CD4$^+$ Treg cells from the oral mucosa were identified by flow cytometry (gated as CD3$^+$ CD4$^+$ tdTomato$^+$ B220$^-$ CD8α$^-$). As the GFP signal in these mice was weak, anti-mouse FoxP3-Alexa488 was added during intracellular staining to increase the Foxp3 signal. The mean fluorescence intensity (MFI) of Foxp3-dependent green fluorescent protein (GFP) in populations of tdTomato$^-$ (white bars) and tdTomato$^+$ cells (black bars) from 20 mice are compared and presented for cells expressing IL-17A, IFN-γ or neither of the two cytokines. The GFP MFI was compared by fitting a mixed effects model with paired values. A *post hoc* Sidak's multiple comparisons test was utilized to determine *p*-values of comparisons found significant by the initial mixed-effects analysis. *$p < 0.05$, ****$p < 0.0001$.

DISCUSSION

Our fate-tracking animal model with repeated inoculations of *Pg* accomplishes two objectives. First, it is representative of human periodontitis since the persistence of *Pg* is a characteristic of the disease. In humans, *Pg* is present at periodontitis sites in higher numbers than at healthy sites. *Pg* disappears below detectable levels after periodontal treatment and it reappears when disease returns and/or exacerbates (4, 47). Second, the adaptive response is best interpreted when the priming antigens are delivered synchronously. Since *Pg* is a keystone pathogen capable of inducing microbial dysbiosis in the oral cavity, the model allows the assessment of the dynamics of the immune response against *Pg* in the cervical LN as well as the local response against blooming pathobionts within the microflora over time. The phenotype of the adaptive response at these two locations is coherent.

Transdifferentiation of IL-17A^{cre} Fate-Tracked Cells

It has been widely reported that diseases can lead to a changing microenvironment resulting in localized changes to the inflammatory cytokine milieu that reshapes the adaptive immune response (22–24, 48). With such changes, adaptive effector or tissue memory CD4$^+$ T cell may be impacted through transdifferentiation leading to greater pathologic phenotypes (26, 49–52). We were interested in determining if the adaptive CD4$^+$ T cell response to sustained dysbiosis elicited by the keystone pathogen *Pg* resulted in transdifferentiation in Th17 and Treg cells in the oral mucosa. To address this question, we used two different fate-tracking reporter mouse strains to examine plasticity in Th17 or Treg cells, in a murine model of periodontitis (23, 37). Transdifferentiation of Th17 cells is well documented (21–23, 26, 53) and a late developmental switch to IFN-γ expression in Th17 cells has been implicated in the pathologies of a diverse group of inflammatory autoimmune diseases such as psoriatic arthritis, Crohn's disease, ulcerative colitis, type 1 diabetes, and multiple sclerosis (23, 26, 49–52, 54). Similarly, there have been reports that Treg cells also can transdifferentiate into IFN-γ-producing Th1-like cells (29, 30, 55–57).

Initially we found that *Pg*-specific CD4$^+$ T cells identified in cervical lymph nodes that drain the oral mucosa switch phenotype from Th17 to a mix of Th17 and Th1 cells over a period of sustained oral colonization with *Pg*. The early Th17 response is consistent with what we have reported previously (39) and also in our analysis of CD4$^+$ T cells isolated from NALT following oral inoculation with *Pg* (43). The late-stage switch to a Th1-type response seems a direct consequence of a *de novo* adaption against a persistent threat. Consistent with localized changes in the cytokine milieu, differentiated Th17 cells may re-program their cytokine expression when encountering a persistent pathogen across the oral mucosal barrier. Data from IL-17A^{cre} fate-tracking mouse indicates that a *de novo* response to persistent *Pg* occurs with Th1 cells dominating Th17 cells in the oral mucosa at late time points. Lack of consistent transdifferentiation in Th17 cells to express IFN-γ rules out sustained local environmental changes in oral mucosa driving changes in memory Th cells. *Pg*-induced dysbiosis may be the driver of this new Th1 response. *Pg* is considered a keystone pathogen so that continued exposure in the oral cavity to this bacterium may drive other pathobionts to become more numerous or more prone to intracellular survival like *Fusobacterium nucleatum* or *Aggregatibacter actinomycetemcomitans* (58–61). Pathobionts may induce an IFN-γ mediated-response to deal with this new intracellular threat. Persistent *Pg* inoculation and heightened inflammation may also result in bacteria invading further into the oral mucosa. Bypassing resident LCs in the epithelium may allow invading pathobionts to be phagocytosed by DCs located in the lamina propria. Consistent with this idea, it is interesting to note that in the absence of LCs, mice mount a robust Th1 response to *Pg* (39).

Notwithstanding differences in homing receptors that dictate tissue residency, we found that *Pg*-specific Th17 cells in mesenteric lymph nodes dominated the adaptive CD4$^+$ T cell response even after persistent oral *Pg* presence (40). The contribution of recirculating memory Th1 cells potentially developed in the GI to the oral response is therefore unlikely. Moreover, the transition from an initial Th17 response to one dominated by infiltrating Th1 cells has also been reported for a number of other inflammatory diseases (23, 62–64). Interestingly, Harbour et al. report that, in addition to plasticity of Th17 cells driving inflammation in a mouse model of colitis, Th17 cells are also instrumental in driving pathogenic Th1 cells from naïve CD4$^+$ T cell precursors (51).

In addition to a switch to a Th1 dominated response, we observed evidence of sporadic transdifferentiation of Th17 cells. Clear evidence of plasticity in these Th17 cells is reinforced by the detection of cells that co-express IL-17A and IFN-γ, which is a hallmark of transdifferentiated Th17 cells (23, 26, 51, 65). The difference of these outliers could be explained by the local cytokine environment and the relative levels of cytokines such as IL-23, IL-1β, IL-12, and TGF-β that have been implicated in either maintaining or driving local transdifferentiation in Th17 cells (22, 26, 53, 65).

γδ T cells that express IL-17A also have the capacity to exhibit plasticity and in some disease states have hallmarks of active histone modifications in genes that drive IFN-γ expression (66–69). Proinflammatory cytokines IL-1β and IL-23 have been reported to act in concert to induce IFN-γ expression in γδ T cells (67) and microRNAs have been shown to regulate IFN-γ plasticity in γδ T cells (68). In our analysis of γδ T cells we found no evidence of IFN-γ expression in the tdTomato$^+$ γδ T fraction indicating that these cells do not exhibit plasticity in the oral mucosa following *Pg*-induced dysbiosis. Interestingly, of the 3 animals that did exhibit Th17 transdifferentiation to IFN-γ, we found no evidence of IFN-γ expression in the γδ T cells recovered from these same mice. This dichotomy suggests that there may be different signals driving transdifferentiation in mucosal Th17 and γδ T cell populations. Although IL-23 is important for maintaining Th17 cells, IL-23 in conjunction with IL-12 can drive transdifferentiation in Th17 cells by suppressing IL-17A expression and at the same time promoting IFN-γ expression through upregulation of T-bet (22, 23, 65, 70). Furthermore, pathogenic IFN-γ expression in transdifferentiated Th17 cells mediated by IL-23 is dependent on the basic-leucine zipper transcription factor, JunB (71, 72). Differential expression of JunB in Th17 cells and γδ T cells located in the oral mucosa of *Pg* inoculated mice may, therefore, account for our observation of lack of γδ T cell plasticity in the small number of mice where we observed it in Th17 cells.

In addition to Th17 and γδ T cells expressing IL-17A, we also identified a population of CD3$^+$ TCRαβ$^+$ T cells in the oral mucosa that were negative for CD4 and CD8 and expressed IL-17A. These so-called CD3$^+$ double negative (DN) T cells have never been reported as a source of IL-17A in the oral mucosa. The number of CD3$^+$ DN T cells increased in the oral mucosa in response to *Pg* but did not show evidence of IFN-γ expression. The origins of these CD3$^+$ DN T cells remains somewhat controversial, but it is known that they are a heterogenous T cell population with capabilities to express both pro and anti-inflammatory cytokines in steady state and during inflammation (73). CD3$^+$ DN T cells are typically found in low numbers in peripheral tissues but contributing IL-17A against viral and bacterial pathogens (74–76) and in autoimmune diseases such as psoriasis and Sjögren's syndrome (77, 78). In the context of periodontal disease, we do not know if CD3$^+$ DN T cells are contributing to host defense or exacerbating periodontitis by acting as an additional source of IL-17A. In a recent report, Sparber et al. described three lymphocyte sources of IL-17A in the murine tongue important for host defense against oropharyngeal candidiasis (79). One of these cell types, CD3$^+$ TCRαβ$^+$ T cells may include a population of CD3$^+$ DN T cells, but were not defined further with CD4 and CD8 markers. The authors also did not examine the oral mucosa. Interestingly though, a significant contribution to resistance to oropharyngeal candidiasis were IL-17A-expressing innate lymphoid cells (ILC), characterized as CD3$^-$, αβTCR$^-$ and γδTCR$^-$ (79). We did not observe ILC in our IL-17 fate-tracking mouse. This may reflect differences in residency of ILCs between the mouse tongue and oral mucosa (gingiva, buccal, and hard palate mucosa). Although this topic is subject to current debate (17, 80, 81), we also did not observe IL-17A^{cre}-tdTomato$^+$ neutrophils despite infection with *Pg* resulting in an influx of neutrophils. This suggests that, neutrophils are not a source IL-17A in our periodontitis model.

Transdifferentiation of Foxp3^{cre} Fate-Tracked Cells

Tamoxifen administration induced permanent tdTomato labeling in cells with concurrent expression of Foxp3 and GFP. Naïve T cells in a TGF-β environment that will eventually commit to a Th17 lineage can co-express Foxp3 and RORγt (41). Therefore, we chose to initiate tamoxifen administration only after the majority of precursor cells capable of becoming Th17 in response to oral *Pg*-induced dysbiosis were activated and no longer transiently expressing Foxp3. *Pg*-induced dysbiosis leads to expression of IL-17A in Foxp3^{cre}-tdTomato$^+$ Treg cells after 28 days of oral *Pg* persistence. Strikingly, IL-17A expression was transient since the frequency of IL-17A expressing Foxp3^{cre}-tdTomato$^+$ Treg cells was significantly reduced after 48 days of persistent oral *Pg* colonization. We did not observe significant IL-17A plasticity of Tregs in sham-treated mice (PBS mice), indicating that microbial dysbiosis is necessary to drive this transient transdifferentiation process. Potentially some newly differentiated Th17 cells transiently expressing Foxp3 could have been misidentified as transdifferentiated Treg cells in this system. However, this is unlikely since continued active Foxp3-driven GFP expression in IL-17A-producing Foxp3^{cre}-tdTomato$^+$ cells at day 28 argues against this possibility. Moreover, we identified a large population of Th17 that were tdTomato and GFP double negative indicating that transient or persistent Foxp3 expression in Th17 cells does not occur or occurs rarely in our system. If all developing Th17 cells had been actively producing Foxp3 at the time of tamoxifen administration these IL-17A-producing CD4 T cells would have been tdTomato$^+$ at days 28 and 48.

While both Foxp3 and RORγt transcription factor are upregulated in the presence of TGF-β, Foxp3 antagonizes RORγt and IL-17A production in the absence of concurrent IL-6 exposure (82–85). The relative ratio of Foxp3 to RORγt within a cell and environmental IL-6 may therefore determine the proinflammatory or regulatory activity of Foxp3$^+$ RORγt$^+$ cells. Populations of Foxp3$^+$ RORγt$^+$ cells with Th17 potential have been reported in human peripheral blood (86). These double positive cells were found to have significantly lower expression of Foxp3 than classical suppressive Tregs, although other groups have reported that human pro-inflammatory IL-17A^{+} Foxp3^{+} T cells have significantly higher expression of Foxp3 than classic Tregs (87). Suppressor Foxp3$^{int.}$ RORγt^{+} T cells have been reported in murine autoimmune diabetes but these double positive cells have been reported to produce IL-17 *in vitro* under polarizing conditions (88). Cyclical expression of IL-6 or transient expression of IL-6 by dendritic cells early during persistent exposure to *Pg* may explain the fleeting nature of the Treg plasticity we observed. *In vitro*, purified populations of CD4^{+} CD25^{+} Foxp3^{+} T cells are able to differentiate into Th17 cells in the presence of IL-6 with concurrent absence of exogenous TGF-β (89).

IL-17^{+} Foxp3^{+} T cells have been observed in human chronic inflammatory conditions. For example, IL-17^{+} Foxp3^{+} T cells have been observed in patients with inflammatory bowel disease, or more specifically, in patients with Crohn's disease but not ulcerative colitis (55, 56). Significantly, IL-17A^{+} Foxp3^{+} T cells are also found in human periodontal lesions but not in gingivitis (30). Lastly, Tregs from human rheumatoid arthritis patients were found to demonstrate increased plasticity toward a Th17-like phenotype (90). While among the studies that evaluated function, IL-17A^{+} Foxp3^{+} T cells were usually found to be suppressive (91, 92), not all studies are in agreement (30, 55, 56, 90). In psoriasis and systemic lupus erythematosus, IL-17^{+} Tregs are pro-inflammatory rather than suppressive (93, 94).

Interestingly, whilst the frequency of IL-17A-expressing Treg cells was up at day 28 and down at day 48, the expression of Foxp3 in the tdTomato^{+} population was stable across both time points. Foxp3^{cre} tdTomato^{+} T cells remained Foxp3-GFP^{+} and/or stained with anti-Foxp3 mAb, even when producing IL-17A or after 48 days of persistent exposure to *Pg*. This is in agreement with Rubtsov et al. (38) but in contrast to Miyao et al. (95) who found that Foxp3 expression is unstable and transient in conventional CD4^{+} T cells in adoptive transfer models. In this model, expression of IL-17A in cells co-expressing Foxp3 is not explained by transcriptional reprogramming and plasticity of Tregs but rather by transient Foxp3 expression in Th17 cells.

Some of the difference between studies may also be explained by differences in animal model or experimental design. For example, Miyao et al. exposed Foxp3^{+} cells to an inflammatory environment for a period of 4 days *in vitro*. Our model exposed cells to an inflammatory environment *in vivo* for a considerably longer period. It is therefore possible that a longer period of inflammation or more robust inflammatory stimulus is necessary to induce IL-17A production by Foxp3^{+} T cells.

The microbiome likely plays a role in Treg transdifferentiation, as various *Clostridium* species of human origin favor the induction of murine colonic Foxp3^{+} Treg cells co-expressing RORγt (55). At least one study has demonstrated that antibiotic treatment that reduces levels of periodontal pathogens reduces the number of IL-17^{+} Foxp3^{+} T cells from peripheral blood of periodontitis patients, again supporting a connection between inflammatory environment, microbiome, and Treg plasticity (87).

The late-stage response to *Pg*-induced dysbiosis is a switch to a *de novo* Th1-type response sustained by IFN-γ. Although Treg have been shown to produce IFN-γ in several models of disease (36, 96, 97) the frequency of Foxp3^{cre}-tdTomato^{+} cells expressing IFN-γ is small (∼2%) and importantly it does not differ between *Pg*-treated and sham-treated mice.

In summary, our data suggests that in a persistent dysbiotic environment driving inflammation the oral CD4^{+} T cell response evolves from one that is initially dominated by IL-17A to one that is predominantly IFN-γ. Such IFN-γ response is generated *de novo* by Th1 cells. Consistent with this shift in the response, we identified a small but significant population of Treg cells expressing IL-17A at day 28 that disappeared at day 48. The kinetics of the inflammatory response may control whether Treg cells will behave as pro- or anti-inflammatory actors. This evolving dysbiosis and inflammatory environment at day 48, post *Pg*, specifically induce Th17 cells into sporadic transdifferentiation and IFN-γ expression. Ultimately, understanding the nature of Treg-Th17 transdifferentiation may provide insights on how to control the inflammatory disease processes. Which components of the microbial biofilm or which host cell under the influence of such microbial environment are responsible for driving the transdifferentiation of Treg or Th17 in the oral environment remains to be elucidated.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Protocol ID #1810-36395A).

AUTHOR CONTRIBUTIONS

MC and PB-E provided the intellectual contribution, designed the experiments, and interpreted the data. PB-E, LF, and MC performed the experiments. All authors contributed to drafting the manuscript. PB-E and LF contributed equally to the interpretation of the results.

FUNDING

We acknowledge the University of Minnesota UFCR for flow cytometry resources. This research was supported by NIH grants R03 DE025882 (PB-E), R21 DE026209 (MC) and the Erwin M. Schaffer Chair for Periodontal Research and OVPR Grant-In-Aid #355802 at the University of Minnesota.

REFERENCES

1. Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. *J Dent Res*. (2014) 93:1045–53. doi: 10.1177/0022034514552491
2. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ. Periodontitis in US adults: national health and nutrition examination survey 2009-2014. *J Am Dent Assoc*. (2018) 149:576–88.e6. doi: 10.1016/j.adaj.2018.04.023
3. Neely AL, Holford TR, Loe H, Anerud A, Boysen H. The natural history of periodontal disease in humans: risk factors for tooth loss in caries-free subjects receiving no oral health care. *J Clin Periodontol*. (2005) 32:984–93. doi: 10.1111/j.1600-051X.2005.00797.x
4. Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett*. (2014) 162(2 Pt A):22–38. doi: 10.1016/j.imlet.2014.08.017
5. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol*. (2018) 16:745–59. doi: 10.1038/s41579-018-0089-x
6. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe*. (2011) 10:497–506. doi: 10.1016/j.chom.2011.10.006
7. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol*. (2014) 35:3–11. doi: 10.1016/j.it.2013.09.001
8. Baker PJ, Dixon M, Evans RT, Dufour L, Johnson E, Roopenian DC. CD4(+) T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. *Infect Immun*. (1999) 67:2804–9.
9. Baker PJ, Howe L, Garneau J, Roopenian DC. T cell knockout mice have diminished alveolar bone loss after oral infection with *Porphyromonas gingivalis*. *FEMS Immunol Med Microbiol*. (2002) 34:45–50.
10. Nurieva R, Yang XO, Chung Y, Dong C. Cutting edge: in vitro generated Th17 cells maintain their cytokine expression program in normal but not lymphopenic hosts. *J Immunol*. (2009) 182:2565–8. doi: 10.4049/jimmunol.0803931
11. Teng YT, Nguyen H, Hassanloo A, Ellen RP, Hozumi N, Gorczynski RM. Periodontal immune responses of human lymphocytes in *Actinobacillus actinomycetemcomitans*-inoculated NOD/SCID mice engrafted with peripheral blood leukocytes of periodontitis patients. *J Periodontal Res*. (1999) 34:54–61. doi: 10.1111/j.1600-0765.1999.tb02222.x
12. Teng YT, Mahamed D, Singh B. Gamma interferon positively modulates *Actinobacillus actinomycetemcomitans*-specific RANKL+ CD4+ Th-cell-mediated alveolar bone destruction in vivo. *Infection and Immunity*. (2005) 73:3453–61. doi: 10.1128/IAI.73.6.3453-3461.2005
13. Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, et al. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am J Pathol*. (2006) 169:987–98. doi: 10.2353/ajpath.2006.060180
14. Ernst CW, Lee JE, Nakanishi T, Karimbux NY, Rezende TM, Stashenko P, et al. Diminished forkhead box P3/CD25 double-positive T regulatory cells are associated with the increased nuclear factor-kappaB ligand (RANKL+) T cells in bone resorption lesion of periodontal disease. *Clin Exp Immunol*. (2007) 148:271–80. doi: 10.1111/j.1365-2249.2006.03318.x
15. Bittner-Eddy PD, Fischer L, Thieu K, Costalonga M. Langerhans cells drive gingipain-specific Th17 differentiation in murine periodontitis. *J Dent Res*. (2014) 93:43.
16. Won HY, Lee JA, Park ZS, Song JS, Kim HY, Jang SM, et al. Prominent bone loss mediated by RANKL and IL-17 produced by CD4+ T cells in TallyHo/JngJ mice. *PLoS One*. (2011) 6:e18168. doi: 10.1371/journal.pone.0018168
17. Eskan MA, Jotwani R, Abe T, Chmelar J, Lim JH, Liang S, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat Immunol*. (2012) 13:465–73. doi: 10.1038/ni.2260
18. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology in mice and humans. *Sci Transl Med*. (2018) 10:eaat0797. doi: 10.1126/scitranslmed.aat0797
19. Yu JJ, Ruddy MJ, Wong GC, Sfintescu C, Baker PJ, Smith JB, et al. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. *Blood*. (2007) 109:3794–802. doi: 10.1182/blood-2005-09-010116
20. Stashenko P, Goncalves RB, Lipkin B, Ficarelli A, Sasaki H, Campos-Neto A. Th1 immune response promotes severe bone resorption caused by *Porphyromonas gingivalis*. *Am J Pathol*. (2007) 170:203–13. doi: 10.2353/ajpath.2007.060597
21. Shi G, Cox CA, Vistica BP, Tan C, Wawrousek EF, Gery I. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J Immunol*. (2008) 181:7205–13.
22. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity*. (2009) 30:92–107. doi: 10.1016/j.immuni.2008.11.005
23. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol*. (2011) 12:255–63. doi: 10.1038/ni.1993
24. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature*. (2012) 484:514–8. doi: 10.1038/nature10957
25. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol*. (2009) 39:216–24. doi: 10.1002/eji.200838475
26. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci USA*. (2010) 107:14751–6. doi: 10.1073/pnas.1003852107
27. Feng T, Qin H, Wang L, Benveniste EN, Elson CO, Cong Y. Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. *J Immunol*. (2011) 186:6313–8. doi: 10.4049/jimmunol.1001454
28. Morrison PJ, Bending D, Fouser LA, Wright JF, Stockinger B, Cooke A, et al. Th17-cell plasticity in *Helicobacter hepaticus*-induced intestinal inflammation. *Mucosal Immunol*. (2013) 6:1143–56. doi: 10.1038/mi.2013.11
29. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature*. (2013) 499:485–90. doi: 10.1038/nature12297
30. Okui T, Aoki Y, Ito H, Honda T, Yamazaki K. The presence of IL-17+/FOXP3+ double-positive cells in periodontitis. *J Dent Res*. (2012) 91:574–9. doi: 10.1177/0022034512446341
31. Omenetti S, Pizarro TT. The Treg/Th17 Axis: a dynamic balance regulated by the gut microbiome. *Front Immunol*. (2015) 6:639. doi: 10.3389/fimmu.2015.00639
32. Kleinewietfeld M, Hafler DA. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Semin Immunol*. (2013) 25:305–12. doi: 10.1016/j.smim.2013.10.009
33. Sawant DV, Vignali DA. Once a treg, always a treg? *Immunol Rev*. (2014) 259:173–91. doi: 10.1111/imr.12173
34. da Silva Martins M, Piccirillo CA. Functional stability of Foxp3+ regulatory T cells. *Trends Mol Med*. (2012) 18:454–62. doi: 10.1016/j.molmed.2012.06.001
35. Kulkarni N, Meitei HT, Sonar SA, Sharma PK, Mujeeb VR, Srivastava S, et al. CCR6 signaling inhibits suppressor function of induced-Treg during gut inflammation. *J Autoimmun*. (2018) 88:121–30. doi: 10.1016/j.jaut.2017.10.013
36. Butcher MJ, Filipowicz AR, Waseem TC, McGary CM, Crow KJ, Magilnick N, et al. Atherosclerosis-driven treg plasticity results in formation of a dysfunctional subset of plastic IFNgamma+ Th1/Tregs. *Circ Res*. (2016) 119:1190–203. doi: 10.1161/CIRCRESAHA.116.309764
37. Bittner-Eddy PD, Fischer LA, Costalonga M. Cre-loxP reporter mouse reveals stochastic activity of the Foxp3 promoter. *Front Immunol*. (2019) 10:2228. doi: 10.3389/fimmu.2019.02228
38. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the regulatory T cell lineage in vivo. *Science*. (2010) 329:1667–71. doi: 10.1126/science.1191996
39. Bittner-Eddy PD, Fischer LA, Kaplan DH, Thieu K, Costalonga M. Mucosal langerhans cells promote differentiation of Th17 cells in a murine model of periodontitis but are not required for *Porphyromonas gingivalis*-driven

alveolar bone destruction. *J Immunol*. (2016) 197:1435–46. doi: 10.4049/jimmunol.1502693

40. Fischer LA, Bittner-Eddy PD, Costalonga M. Fetal weight outcomes in C57BL/6J and C57BL/6NCrl mice after oral colonization with *Porphyromonas gingivalis*. *Infect Immun*. (2019) 87:e00280-19. doi: 10.1128/IAI.00280-19
41. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature*. (2008) 453:236–40. doi: 10.1038/nature06878
42. Bittner-Eddy PD, Fischer LA, Costalonga M. Identification of gingipain-specific I-A(b) -restricted CD4+ T cells following mucosal colonization with *Porphyromonas gingivalis* in C57BL/6 mice. *Mol Oral Microbiol*. (2013) 28:452–66. doi: 10.1111/omi.12038
43. Bittner-Eddy PD, Fischer LA, Tu AA, Allman DA, Costalonga M. Discriminating between interstitial and circulating leukocytes in tissues of the murine oral mucosa avoiding nasal-associated lymphoid tissue contamination. *Front Immunol*. (2017) 8:1398. doi: 10.3389/fimmu.2017.01398
44. Park JY, Chung H, Choi Y, Park JH. Phenotype and tissue residency of lymphocytes in the murine oral mucosa. *Front Immunol*. (2017) 8:250. doi: 10.3389/fimmu.2017.00250
45. Bittner-Eddy P, Fischer L, Costalonga M. Langerhans cells indirectly suppress oral mucosal Tc17 Cells. *J Dent Res*. (2019) 98:2648.
46. Reid JM, Goetz MP, Buhrow SA, Walden C, Safgren SL, Kuffel MJ, et al. Pharmacokinetics of endoxifen and tamoxifen in female mice: implications for comparative in vivo activity studies. *Cancer Chemother Pharmacol*. (2014) 74:1271–8. doi: 10.1007/s00280-014-2605-7
47. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol*. (1998) 25:134–44.
48. Wang Y, Godec J, Ben-Aissa K, Cui K, Zhao K, Pucsek AB, et al. The transcription factors T-bet and Runx are required for the ontogeny of pathogenic interferon-gamma-producing T helper 17 cells. *Immunity*. (2014) 40:355–66. doi: 10.1016/j.immuni.2014.01.002
49. Bsat M, Chapuy L, Rubio M, Wassef R, Richard C, Schwenter F, et al. Differential pathogenic Th17 profile in mesenteric lymph nodes of Crohn's disease and ulcerative colitis patients. *Front Immunol*. (2019) 10:1177. doi: 10.3389/fimmu.2019.01177
50. Wade SM, Canavan M, McGarry T, Low C, Wade SC, Mullan RH, et al. Association of synovial tissue polyfunctional T-cells with DAPSA in psoriatic arthritis. *Ann Rheum Dis*. (2019) 78:350–4. doi: 10.1136/annrheumdis-2018-214138
51. Harbour SN, Maynard CL, Zindl CL, Schoeb TR, Weaver CT. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. *Proc Natl Acad Sci USA*. (2015) 112:7061–6. doi: 10.1073/pnas.1415675112
52. Reinert-Hartwall L, Honkanen J, Salo HM, Nieminen JK, Luopajarvi K, Harkonen T, et al. Th1/Th17 plasticity is a marker of advanced beta cell autoimmunity and impaired glucose tolerance in humans. *J Immunol*. (2015) 194:68–75. doi: 10.4049/jimmunol.1401653
53. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol*. (2013) 14:372–9. doi: 10.1038/ni.2552
54. Carbajal KS, Mironova Y, Ulrich-Lewis JT, Kulkarni D, Grifka-Walk HM, Huber AK, et al. Th Cell Diversity in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Immunol*. (2015) 195:2552–9. doi: 10.4049/jimmunol.1501097
55. Ueno A, Jijon H, Chan R, Ford K, Hirota C, Kaplan GG, et al. Increased prevalence of circulating novel IL-17 secreting Foxp3 expressing CD4+ T cells and defective suppressive function of circulating Foxp3+ regulatory cells support plasticity between Th17 and regulatory T cells in inflammatory bowel disease patients. *Inflamm Bowel Dis*. (2013) 19:2522–34. doi: 10.1097/MIB.0b013e3182a85709
56. Hovhannisyan Z, Treatman J, Littman DR, Mayer L. Characterization of interleukin-17-producing regulatory T cells in inflamed intestinal mucosa from patients with inflammatory bowel diseases. *Gastroenterology*. (2011) 140:957–65. doi: 10.1053/j.gastro.2010.12.002
57. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. (2009) 10:1000–7. doi: 10.1038/ni.1774
58. Dorn BR, Dunn WA Jr., Progulske-Fox A. *Porphyromonas gingivalis* traffics to autophagosomes in human coronary artery endothelial cells. *Infect. Immun*. (2001) 69:5698–708.
59. Rudney JD, Chen R, Sedgewick GJ. *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythensis* are components of a polymicrobial intracellular flora within human buccal cells. *J Dent Res*. (2005) 84:59–63.
60. Yamatake K, Maeda M, Kadowaki T, Takii R, Tsukuba T, Ueno T, et al. Role for gingipains in *Porphyromonas gingivalis* traffic to phagolysosomes and survival in human aortic endothelial cells. *Infect Immun*. (2007) 75:2090–100.
61. Rudney JD, Chen R, Zhang G. Streptococci dominate the diverse flora within buccal cells. *J Dent Res*. (2005) 84:1165–71.
62. Paust HJ, Turner JE, Riedel JH, Disteldorf E, Peters A, Schmidt T, et al. Chemokines play a critical role in the cross-regulation of Th1 and Th17 immune responses in murine crescentic glomerulonephritis. *Kidney Int*. (2012) 82:72–83. doi: 10.1038/ki.2012.101
63. Odobasic D, Gan PY, Summers SA, Semple TJ, Muljadi RC, Iwakura Y, et al. Interleukin-17A promotes early but attenuates established disease in crescentic glomerulonephritis in mice. *Am J Pathol*. (2011) 179:1188–98. doi: 10.1016/j.ajpath.2011.05.039
64. Krebs CF, Turner JE, Paust HJ, Kapffer S, Koyro T, Krohn S, et al. Plasticity of Th17 cells in autoimmune kidney diseases. *J Immunol*. (2016) 197:449–57. doi: 10.4049/jimmunol.1501831
65. Ahern PP, Schiering C, Buonocore S, McGeachy MJ, Cua DJ, Maloy KJ, et al. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity*. (2010) 33:279–88. doi: 10.1016/j.immuni.2010.08.010
66. Barros-Martins J, Schmolka N, Fontinha D, Pires de Miranda M, Simas JP, Brok I, et al. Effector gammadelta T cell differentiation relies on master but not auxiliary Th cell transcription factors. *J Immunol*. (2016) 196:3642–52. doi: 10.4049/jimmunol.1501921
67. Schmolka N, Serre K, Grosso AR, Rei M, Pennington DJ, Gomes AQ, et al. Epigenetic and transcriptional signatures of stable versus plastic differentiation of proinflammatory gammadelta T cell subsets. *Nat Immunol*. (2013) 14:1093–100. doi: 10.1038/ni.2702
68. Schmolka N, Papotto PH, Romero PV, Amado T, Enguita FJ, Amorim A, et al. MicroRNA-146a controls functional plasticity in gammadelta T cells by targeting NOD1. *Sci Immunol*. (2018) 3:eaao1392. doi: 10.1126/sciimmunol.aao1392
69. Sheridan BS, Romagnoli PA, Pham QM, Fu HH, Alonzo F III, Schubert WD, et al. gammadelta T cells exhibit multifunctional and protective memory in intestinal tissues. *Immunity*. (2013) 39:184–95. doi: 10.1016/j.immuni.2013.06.015
70. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med*. (2007) 204:1849–61. doi: 10.1084/jem.20070663
71. Carr TM, Wheaton JD, Houtz GM, Ciofani M. JunB promotes Th17 cell identity and restrains alternative CD4(+) T-cell programs during inflammation. *Nat Commun*. (2017) 8:301. doi: 10.1038/s41467-017-00380-3
72. Hasan Z, Koizumi SI, Sasaki D, Yamada H, Arakaki N, Fujihara Y, et al. JunB is essential for IL-23-dependent pathogenicity of Th17 cells. *Nat Commun*. (2017) 8:15628. doi: 10.1038/ncomms15628
73. Brandt D, Hedrich CM. TCRalphabeta(+)CD3(+)CD4(-)CD8(-) (double negative) T cells in autoimmunity. *Autoimmun Rev*. (2018) 17:422–30. doi: 10.1016/j.autrev.2018.02.001
74. Cowley SC, Meierovics AI, Frelinger JA, Iwakura Y, Elkins KL. Lung CD4-CD8- double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with *Francisella tularensis* live vaccine strain. *J Immunol*. (2010) 184:5791–801. doi: 10.4049/jimmunol.1000362
75. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A, et al. IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections. *J Immunol*. (2010) 184:1710–20. doi: 10.4049/jimmunol.0902796
76. Neyt K, GeurtsvanKessel CH, Lambrecht BN. Double-negative T resident memory cells of the lung react to influenza virus infection via CD11c(hi) dendritic cells. *Mucosal Immunol*. (2016) 9:999–1014. doi: 10.1038/mi.2015.91
77. Alunno A, Carubbi F, Bartoloni E, Bistoni O, Caterbi S, Cipriani P, et al. Unmasking the pathogenic role of IL-17 axis in primary Sjogren's syndrome:

a new era for therapeutic targeting? *Autoimmun Rev*. (2014) 13:1167–73. doi: 10.1016/j.autrev.2014.08.022
78. Ueyama A, Imura C, Fusamae Y, Tsujii K, Furue Y, Aoki M, et al. Potential role of IL-17-producing CD4/CD8 double negative alphabeta T cells in psoriatic skin inflammation in a TPA-induced STAT3C transgenic mouse model. *J Dermatol Sci*. (2017) 85:27–35. doi: 10.1016/j.jdermsci.2016.10.007
79. Sparber F, Dolowschiak T, Mertens S, Lauener L, Clausen BE, Joller N, et al. Langerin+ DCs regulate innate IL-17 production in the oral mucosa during Candida albicans-mediated infection. *PLoS Pathogens*. (2018) 14:e1007069. doi: 10.1371/journal.ppat.1007069
80. Li J, Casanova JL, Puel A. Mucocutaneous IL-17 immunity in mice and humans: host defense vs. excessive inflammation. *Mucosal Immunol*. (2018) 11:581–9. doi: 10.1038/mi.2017.97
81. Tamassia N, Arruda-Silva F, Calzetti F, Lonardi S, Gasperini S, Gardiman E, et al. A reappraisal on the potential ability of human neutrophils to express and produce IL-17 family members in vitro: failure to reproducibly detect it. *Front Immunol*. (2018) 9:795. doi: 10.3389/fimmu.2018.00795
82. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, et al. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J Biol Chem*. (2008) 283:17003–8. doi: 10.1074/jbc.M801286200
83. Ren J, Li B. The functional stability of FOXP3 and RORgammat in Treg and Th17 and their therapeutic applications. *Adv Protein Chem Struct Biol*. (2017) 107:155–89. doi: 10.1016/bs.apcsb.2016.10.002
84. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*. (2008) 29:44–56. doi: 10.1016/j.immuni.2008.05.007
85. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. (2006) 441:235–8. doi: 10.1038/nature04753
86. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
87. Rajendran M, Looney S, Singh N, Elashiry M, Meghil MM, El-Awady AR, et al. Systemic antibiotic therapy reduces circulating inflammatory dendritic cells and Treg-Th17 plasticity in periodontitis. *J Immunol*. (2019) 202:2690–9. doi: 10.4049/jimmunol.1900046
88. Tartar DM, VanMorlan AM, Wan X, Guloglu FB, Jain R, Haymaker CL, et al. FoxP3+RORgammat+ T helper intermediates display suppressive function against autoimmune diabetes. *J Immunol*. (2010) 184:3377–85. doi: 10.4049/jimmunol.0903324
89. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol*. (2007) 178:6725–9. doi: 10.4049/jimmunol.178.11.6725
90. Wang T, Sun X, Zhao J, Zhang J, Zhu H, Li C, et al. Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. *Ann Rheum Dis*. (2015) 74:1293–301. doi: 10.1136/annrheumdis-2013-204228
91. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood*. (2009) 113:4240–9. doi: 10.1182/blood-2008-10-183251
92. Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, et al. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci USA*. (2009) 106:4793–8. doi: 10.1073/pnas.0900408106
93. Jiang C, Wang H, Xue M, Lin L, Wang J, Cai G, et al. Reprograming of peripheral Foxp3(+) regulatory T cell towards Th17-like cell in patients with active systemic lupus erythematosus. *Clin Immunol*. (2019) 209:108267. doi: 10.1016/j.clim.2019.108267
94. Kannan AK, Su Z, Gauvin DM, Paulsboe SE, Duggan R, Lasko LM, et al. IL-23 induces regulatory T cell plasticity with implications for inflammatory skin diseases. *Sci Rep*. (2019) 9:17675. doi: 10.1038/s41598-019-53240-z
95. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity*. (2012) 36:262–75. doi: 10.1016/j.immuni.2011.12.012
96. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat Med*. (2011) 17:673–5. doi: 10.1038/nm.2389
97. McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. *J Immunol*. (2011) 186:3918–26. doi: 10.4049/jimmunol.1003099

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 May 2020
doi: 10.3389/fimmu.2020.01002

IL-17 Induced Autophagy Regulates Mitochondrial Dysfunction and Fibrosis in Severe Asthmatic Bronchial Fibroblasts

Rakhee K. Ramakrishnan[1], *Khuloud Bajbouj*[1], *Saba Al Heialy*[2,3], *Bassam Mahboub*[1,4], *Abdul Wahid Ansari*[1], *Ibrahim Y. Hachim*[1], *Surendra Rawat*[1], *Laila Salameh*[1,4], *Mahmood Y. Hachim*[1], *Ronald Olivenstein*[3], *Rabih Halwani*[1], *Rifat Hamoudi*[1] and *Qutayba Hamid*[1,3]*

[1] Sharjah Institute for Medical Research – College of Medicine, University of Sharjah, Sharjah, United Arab Emirates, [2] College of Medicine, Mohammed Bin Rashid University, Dubai, United Arab Emirates, [3] Meakins-Christie Laboratories, McGill University, Montreal, QC, Canada, [4] Rashid Hospital, Dubai Health Authority, Dubai, United Arab Emirates

OPEN ACCESS

Edited by:
Nicola Ivan Lorè,
IRCCS San Raffaele Scientific Institute, Italy

Reviewed by:
Saurabh Aggarwal,
University of Alabama at Birmingham, United States
Valentino Bezzerri,
Azienda Ospedaliero Universitaria Ospedali Riuniti, Italy

***Correspondence:**
Qutayba Hamid
qalheialy@sharjah.ac.ae

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity,
a section of the journal Frontiers in Immunology

Received: 31 January 2020
Accepted: 27 April 2020
Published: 21 May 2020

Citation:
Ramakrishnan RK, Bajbouj K, Al Heialy S, Mahboub B, Ansari AW, Hachim IY, Rawat S, Salameh L, Hachim MY, Olivenstein R, Halwani R, Hamoudi R and Hamid Q (2020) IL-17 Induced Autophagy Regulates Mitochondrial Dysfunction and Fibrosis in Severe Asthmatic Bronchial Fibroblasts. Front. Immunol. 11:1002. doi: 10.3389/fimmu.2020.01002

The accumulation of fibroblasts, their synthesis of extracellular matrix (ECM) proteins and their innate resistance to apoptosis are characteristics of subepithelial fibrosis observed in severe asthma. Interleukin-17 (IL-17) is an important regulator of airway remodeling in asthma. However, the contribution of IL-17 to the pro-fibrotic phenotype of bronchial fibroblasts is not well-characterized. In this study, we investigated whether IL-17 induced autophagy regulates mitochondrial and pro-fibrotic function in bronchial fibroblasts. The primary cultured bronchial fibroblasts isolated from non-asthmatic (NHBF) and severe asthmatic (DHBF) subjects were treated with IL-17 in order to ascertain its effect on mitochondrial function, mitochondrial quality control, and apoptosis using immunoblotting and flow cytometric analyses. At baseline, DHBF exhibited higher levels of mitophagy and mitochondrial biogenesis compared to NHBF. Immunohistochemical evaluation of bronchial biopsies showed intense PINK1 immunoreactivity in severe asthma than in control. IL-17 intensified the mitochondrial dysfunction and impaired the mitochondrial quality control machinery in NHBF and DHBF. Moreover, IL-17 augmented a pro-fibrotic and anti-apoptotic response in both group of fibroblasts. Inhibition of autophagy using bafilomycin-A1 reduced PINK1 expression in NHBF and restored the IL-17 mediated changes in PINK1 to their basal levels in DHBF. Bafilomycin-A1 also reversed the IL-17 associated fibrotic response in these fibroblasts, suggesting a role for IL-17 induced autophagy in the induction of fibrosis in bronchial fibroblasts. Taken together, our findings suggest that IL-17 induced autophagy promotes mitochondrial dysfunction and fibrosis in bronchial fibroblasts from both non-asthmatic and severe asthmatic subjects. Our study provides insights into the therapeutic potential of targeting autophagy in ameliorating fibrosis, particularly in severe asthmatic individuals.

Keywords: severe asthma, bronchial fibroblasts, mitochondria, autophagy, IL-17, fibrosis, mitochondrial dysfunction

INTRODUCTION

Fibroblasts, the effector cells of fibrosis, exhibit a bio-synthetic, contractile, adhesive, and pro-inflammatory phenotype for effective wound healing. As opposed to their self-limited and tightly regulated repair in response to tissue injury, under pathological conditions, persistent fibroblast activation paves way to extracellular matrix (ECM) accumulation, and remodeling along with their differentiation into apoptosis-resistant myofibroblasts. Chronic lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF), exhibit phenotypically different fibroblasts that are responsible for the loss of the typical airway architecture and impair airway function (1).

Increased airway fibroblast population, increased collagen deposition, airway smooth muscle hyperplasia, and hypertrophy are characteristic airway structural changes that selectively differentiate severe persistent asthma from milder forms of the disease (2). The fibroblast numbers and collagen deposition also negatively correlate with the extent of airflow limitation in patients with asthma (2). In addition to the central airways, higher myofibroblast numbers were reported in the alveolar and lung parenchyma of asthmatics (3, 4). Nonetheless, fibroblasts and their role in asthma pathogenesis have been relatively undervalued and understudied.

Th17 cells and their canonical cytokines, IL-17A and IL-17F, are key players in the pathogenesis of asthma and are closely associated with the more severe phenotypes (5). Airway tissues from patients with severe asthma demonstrated increased expression of Th17-associated cytokines, IL-17A, and IL-17F (6), together with increased expression of IL-8 and excess neutrophilia (7). IL-17 being a key mediator of neutrophilic inflammation, upregulated IL-17 expression can be considered a characteristic hallmark of severe asthma, known for exhibiting a Th2-low, and neutrophilic phenotype (8). IL-17 induced the secretion of pro-fibrotic cytokines and pro-inflammatory mediators, including IL-6, IL-11, IL-8, and GROα/CXCL1, by bronchial fibroblasts exerting their importance in regulating fibrotic and inflammatory responses in the airways (9). Furthermore, bronchial fibroblasts when co-cultured with CD4+ T cells promoted a Th17 profile in asthma (10). It was also reported that anti-IL-17 therapy in a murine asthma model exacerbated with lipopolysaccharide led to decreased oxidative stress and ECM remodeling (11) further implicating IL-17 in airway remodeling in asthma.

Abbreviations: ECM, Extracellular matrix; NHBF, Normal human bronchial fibroblasts; DHBF, Diseased human bronchial fibroblasts; COPD, Chronic obstructive pulmonary disease; IPF, Idiopathic pulmonary fibrosis; S-As, Severe asthmatic; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; FCCP, Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; qPCR, Quantitative Polymerase Chain Reaction; QC, Quality control; LC3B, Microtubule-associated protein 1 light chain 3B; LAMP2, Lysosomal-associated membrane protein 2; PINK1, PTEN-induced putative kinase 1; PRKN, Parkin; SIRT1, Sirtuin 1; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1 alpha; $\Delta\Psi$m, Mitochondrial membrane potential; BECN1, Beclin 1; ATG5, Autophagy related 5; SQSTM1/p62, Sequestosome-1; Baf-A1, Bafilomycin-A1; COL1A1, Collagen, type I, alpha 1; COL3A1, Collagen, type III, alpha 1; COL5A1, Collagen, type V, alpha 1; FN1, Fibronectin.

Increasing evidence suggests that mitochondrial dysfunction is key to the pathogenesis of asthma (12–14). Exposure to environmental oxidants such as tobacco smoke, diesel exhaust particles, and ozone caused an increase in cellular reactive oxygen species (ROS) levels which subsequently induced mitochondrial dysfunction in the lung (15). An experimental allergic model of asthma demonstrated mitochondrial dysfunction in lung mitochondria and associated mitochondrial structural changes in bronchial epithelium (13). Oxidative damage-induced mitochondrial dysfunction further exacerbated allergic airway inflammation (14). That said, this may be a strong indication that beyond its canonical function of ATP production, the non-canonical roles of mitochondria can also influence airway structure and function.

IL-17 mediated mitochondrial dysfunction has been studied in disease models, including rheumatoid arthritis (RA) (16) and vitiligo (17). However, there exists a gap in knowledge regarding the role of IL-17 in mitochondrial dysfunction in asthma, and more importantly in whether this affects airway remodeling in severe asthma. In the present study, we investigated the putative link between autophagy and IL-17A induced mitochondrial dysfunction and fibrosis in non-asthmatic and severe asthmatic (S-As) fibroblasts. IL-17A will henceforth be referred to as IL-17 in the rest of this study. Using bronchial fibroblasts isolated from S-As and non-asthmatic subjects, we show increased autophagy, mitochondrial dysfunction, and fibrotic gene expression in S-As fibroblasts which was exacerbated upon stimulation with IL-17, thereby contributing to the pathobiology of subepithelial fibrosis in severe asthma.

MATERIALS AND METHODS

Cell Culture

The primary bronchial fibroblasts were isolated from endobronchial tissue biopsies obtained from non-smoking patients with severe asthma or non-smoking healthy volunteers. The healthy and severe asthmatic subjects were age-matched to exclude the confounding effects due to age. The mean age of the healthy and severe asthmatic subjects was 43.7 ± 12.5 and 43.4 ± 8.3 years, respectively. These fibroblasts were obtained from the Quebec Respiratory Health Research Network (McGill University Health Centre/Meakins-Christie Laboratories Tissue Bank, Montreal, Canada), as described previously (18). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml of penicillin, and 100 ng/ml streptomycin in 75-cm^2 flasks. The cells were maintained at 37°C in 5% CO_2 with medium change performed every 2–3 days. The fibroblasts were passaged a maximum of eight times and experiments were conducted using fibroblasts at matched passages. All cell culture reagents were purchased from Sigma-Aldrich.

The cells were seeded in 6- or 12-well-plates for experiments and at 50% confluency, they were serum-starved in 1% FBS-supplemented DMEM for 24 hours (h). The cells were then stimulated with 25 ng/ml recombinant human IL-17A (Sigma) for 48 h (for mRNA) or 96 h (for protein). Autophagy inhibition was achieved by pre-treating cells with 10 nM bafilomycin-A1 (Santa Cruz) for 4 h prior to stimulation with IL-17. Co-treatment with 10 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (Tocris) for 2 h was used as a positive control to induce mitochondrial dysfunction.

Immuno/Western Blotting

The cells were lysed using 10X RIPA Buffer (abcam) after supplementation with 1x Protease Inhibitor Cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Total protein concentrations were determined using Protein Assay Kit II (Bio-Rad). Twenty micrograms total proteins were separated using either 12.5% gels or 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-rad). The proteins were transferred onto a nitrocellulose membrane (Bio-rad), blocked in skimmed milk for 1 h at room temperature, incubated overnight at 4°C with antibodies specific to LC3B (abcam), Mitophagy Antibody Sampler Kit (Cell Signaling Technology), LAMP2A (abcam), SIRT1 (Cell Signaling Technology), PGC1α (Novus Biologicals), and Survivin (abcam). β-actin (Sigma-Aldrich) or GAPDH (Cell Signaling Technology) were used as loading controls. The blots were developed using the Clarity Western ECL Substrate (Bio-Rad) in the ChemiDoc Touch Gel Imaging System (Bio-Rad). Image Lab software (Bio-Rad) was used to detect and quantify the protein bands.

Mitochondrial Assays

The mitochondrial mass and mitochondrial membrane potential-associated apoptosis were measured using MitoTracker Green (Invitrogen) and Mitochondrial Membrane Potential Apoptosis Kit, with Mitotracker Red & Annexin V Alexa Fluor 488 (Invitrogen), respectively. The cells were stained with 50 nM MitoTracker Green or 50 nM MitoTracker Red while protected from light for 30 min at 37°C in an atmosphere of 5% CO_2. The MitoTracker Red stained cells were thereafter washed and labeled with Annexin V-AF488 for 15 min at room temperature in the dark. The cells were then analyzed using the BD FACSAria III flow cytometer and the acquired data analyzed using FlowJo v10 software.

Immunohistochemistry

Paraffin slides of bronchial biopsy tissues obtained by fiberoptic bronchoscopy from non-asthmatic control subjects archived at the Biobank of the Quebec Respiratory Health Research Network Canada with MUHC REB number BMB-02-039-t (19), were obtained. Severe asthmatic subjects, who fulfilled the American Thoracic Society (ATS) criteria and were taking treatments based on the Global Initiative for Asthma (GINA), were recruited by the treating physician and nurse who obtained written informed consent, from the Severe Asthma Clinic in the Pulmonary Medicine department at Rashid Hospital, Dubai, UAE. The endobronchial biopsies were performed in accordance with a study protocol approved by the Dubai Scientific Research Ethics Committee with approval number DSREC-11/2017_04. The biopsies were collected and embedded as previously described (20).

Immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) biopsy samples was performed to determine the protein levels and distribution of PINK1 as previously described (20). Briefly, 3 μm thick sections were cut from the paraffin blocks and routine deparaffinization and rehydration steps performed. Heat-activated antigen retrieval was carried out using sodium citrate buffer at pH 6.0 and developed using HRP/DAB (ABC) Detection IHC kit (abcam), according to manufacturer recommendations. The slides were immunostained with rabbit anti-PINK1 (1:350 dilution; Novus Biologicals) antibody. The primary antibody was omitted to serve as technical negative control and appropriate positive control tissue was used. Nuclei were counterstained blue with hematoxylin (Thermo Scientific Shandon).

Quantitative Polymerase Chain Reaction (qPCR)

The total RNA was extracted using the Trizol (Invitrogen) method according to manufacturer instructions. RNA concentrations were measured using Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in the Veriti Thermal Cycler (Applied Biosystems). cDNA amplification was carried out using 5x Hot FirePol EvaGreen qRT-PCR SuperMix (Solis Biodyne) in QuantStudio 3 Real-Time PCR System (Applied Biosystems). The primers are listed in **Table 1**. Gene expression was analyzed using the Comparative Ct (ΔΔCt) method after normalization to the housekeeping gene 18 s rRNA. All results were expressed as fold change relative to NHBF for baseline measurements or the untreated controls for IL-17 treatment.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM) of 2–4 independent experiments. Data analyses

TABLE 1 | List of primer sequences used in qPCR.

Genes	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
IL-6	GAAAGCAGCAAAGAGGCAC	GCACAGCTCTGGCTTGTTCC
BECN1	ATGCAGGTGAGCTTCGTGTG	CTGGGCTGTGGTAAGTAATGGA
ATG5	GACCAGTTTTGGGCCATCAATC	GTGCAACTGTCCATCTGCAGC
LC3B	GAACGGACACAGCATGGTCAGC	ACGTCTCCTGGGAGGCATAG
SQSTM1	TTGTACCCACATCTCCCGCCA	TACTGGATGGTGTCCAGAGCCG
LAMP2	AACTTCAACAGTGGCACCCACC	AGTGATGTTCAGCTGCAGCCCC
PINK1	CCTGCGCCAGTACCTTTGTGT	TGGGTCCAGCTCCACAAGGATG
PRKN	CTCCAGCCATGGTTTCCCAGTG	CCAGGTCACAATTCTGCACAGTC
COL1A1	GATTGACCCCAACCAAGGCTG	GCCGAACCAGACATGCCTC
COL3A1	GATCAGGCCAGTGGAAATG	GTGTGTTTCGTGCAACCATC
COL5A1	GTCGATCCTAACCAAGGATGC	GAACCAGGAGCCCGGGTTTTC
FN1	CTGGGAACACTTACCGAGTGGG	CCACCAGTCTCATGTGGTCTCC
ACTA2	CTTCGTGTTGCCCCTGAAGAG	GCATAGAGAGACAGCACCGC
18s	TGACTCAACACGGGAAACC	TCGCTCCACCAACTAAGAAC

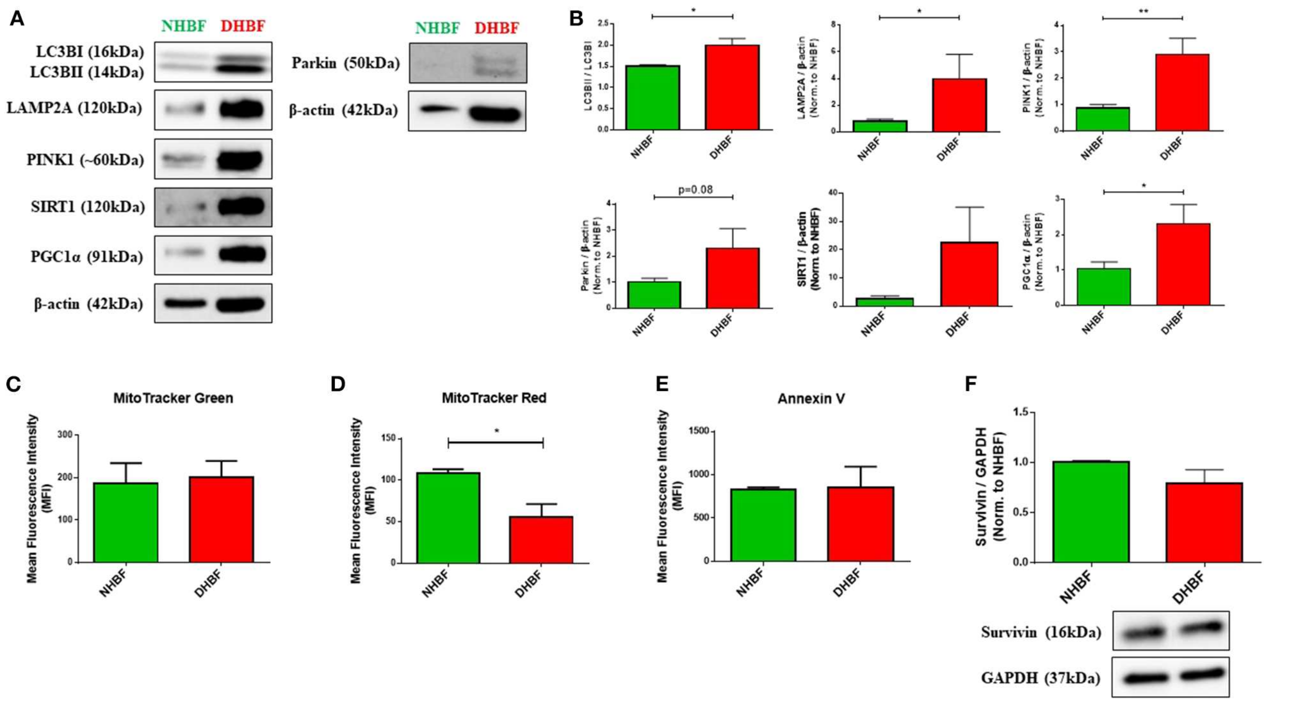


FIGURE 1 | Enhancement of mitochondrial quality control in severe asthmatic (S-As) fibroblasts. In order to measure the basal expression levels, non-asthmatic fibroblasts (NHBF), and S-As fibroblasts (DHBF) were serum-starved for 24 h and thereafter, cultured in DMEM complete medium for 24 h. The fibroblasts were then subjected to Western Blot or flow cytometric analysis. β-actin or GAPDH was used as loading control as indicated. **(A)** Representative immunoblots and **(B)** densitometric analysis of autophagy markers, with LC3B lipidation represented as the ratio of LC3BII to LC3BI, and LAMP2A expression, mitophagy markers, PINK1 and Parkin, and mitochondrial biogenesis markers, SIRT1 and PGC1α. Data representative of three independent experiments. **(C)** Bar charts indicating NHBF (green) and DHBF (red) fluorescence when stained with MitoTracker Green, **(D)** MitoTracker Red and **(E)** Annexin V followed by flow cytometric analysis. 10,000 events were analyzed in each flow cytometry experiment. Data representative of three independent experiments. **(F)** Densitometric analysis and representative immunoblots depicting expression of anti-apoptotic protein, Survivin, in NHBF and DHBF. Data presented as mean ± SEM relative to NHBF (where indicated). Statistical significance assessed by Mann Whitney test. *$p < 0.05$, **$p < 0.01$.

were performed using Mann Whitney test while comparing NHBF and DHBF, one-way ANOVA followed by Tukey's multiple comparison tests or unpaired t-test with multiple comparisons using the Holm-Sidak method for statistical analysis of the data using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). A $p < 0.05$ was considered statistically significant.

RESULTS

Enhancement of Mitochondrial Quality Control in S-As Fibroblasts

Being highly dynamic organelles, mitochondria are constantly under the surveillance of mitochondrial quality control (QC) mechanisms of mitophagy, and mitochondrial biogenesis to identify and resolve mitochondrial defects. To investigate the state of mitochondrial dysfunction in S-As fibroblasts, the mitochondrial QC mechanisms were examined in S-As fibroblasts (DHBF) and non-asthmatic fibroblasts (NHBF) isolated from endobronchial biopsy tissues. Mitophagy is triggered by the accumulation of PTEN-induced putative kinase protein 1 (PINK1) on the outer mitochondrial membrane as a result of mitochondrial depolarization. PINK1 then recruits E3 ubiquitin ligase Parkin which ubiquitinates mitochondrial surface proteins tagging them for autophagy-dependent lysosomal clearance. Since mitophagy is dependent on the autophagy machinery, we first examined the protein expression of autophagy marker, microtubule-associated protein 1 light chain 3 beta (LC3B), and lysosome-associated membrane protein 2A (LAMP2A), which is essential for lysosomal fusion with autophagic vacuoles (21). In comparison to NHBF, increased accumulation of LC3B, increased LC3B lipidation (conversion of LC3BI to LC3BII) ($p = 0.03$), and upregulated expression of LAMP2A ($p = 0.02$) were detected in DHBF (**Figures 1A,B**).

Western blot analysis also showed increased expression of mitophagy-specific proteins, PINK1 ($p = 0.004$) and Parkin ($p = 0.08$) in DHBF compared to NHBF (**Figures 1A,B**), which indicated increased levels of mitophagy in S-As fibroblasts. Higher expression of mitochondrial biogenesis markers, sirtuin 1 (SIRT1), and proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) ($p = 0.02$), was also detected in DHBF than in NHBF (**Figures 1A,B**). This activation of mitochondrial QC mechanisms in S-As fibroblasts is indicative of intrinsic mitochondrial damage.

MitoTracker dyes are a useful tool in assessing mitophagy (22). Therefore, we next evaluated the total mitochondrial

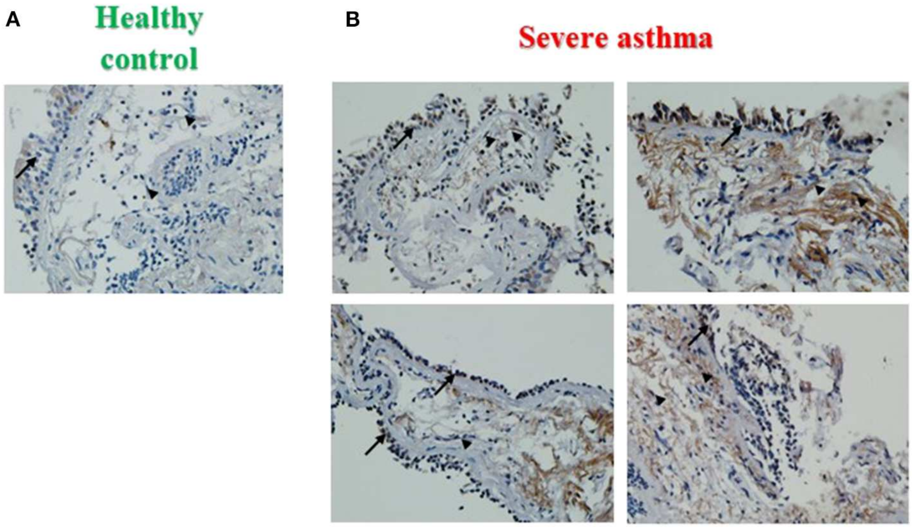


FIGURE 2 | Increased PINK1 expression in severe asthmatic bronchial biopsy tissues. Representative images taken at 40X magnification showing PINK1 immunostaining developed with 3,3′-diaminobenzidine diaminobenzidine (brown). Nuclei were counterstained with hematoxylin (blue). Representative bronchial biopsy sections from **(A)** healthy control showing weak, **(B)** severe asthmatic showing moderate to strong PINK1 protein expression. Arrows refer to bronchial epithelium. Arrowheads refer to fibroblasts.

mass using MitoTracker Green, a fluorescent dye that binds mitochondria independent of mitochondrial membrane potential ($\Delta\Psi$m), and the mitochondrial membrane potential was determined using the MitoTracker Red fluorescent probe. MitoTracker Green staining showed similar mitochondrial mass in NHBF and DHBF (**Figure 1C**), which supported the increased turnover of damaged mitochondria in S-As fibroblasts through enhanced mitophagy and biogenesis. Furthermore, MitoTracker Red staining also showed approximately 48% reduction in $\Delta\Psi$m in DHBF ($p = 0.03$) when compared to NHBF (**Figure 1D**), confirming the presence of mitochondrial abnormalities in S-As fibroblasts.

Studies have shown that mitochondrial damage and autophagy are closely related to cell death (23, 24). We, therefore, studied mitochondrial damage-mediated cell apoptosis by using Annexin V staining to detect apoptotic cells. Flow cytometric analysis showed comparable Annexin V staining in NHBF and DHBF (**Figure 1E**). This was also corroborated in the western blot analysis of anti-apoptotic protein survivin, which showed similar expression levels in NHBF and DHBF (**Figure 1F**). Taken together, these findings may imply that mitochondrial damage in S-As fibroblasts is associated with efficient recycling of mitochondria through mitophagy and biogenesis resulting in fibroblast resistance to apoptosis.

Increased PINK1 Expression in Severe Asthmatic Bronchial Biopsy Tissues

PINK1 is fundamental to mitochondrial homeostasis and serves as a sensor of mitochondrial damage (25). For further validation of the role of mitochondrial dysfunction in asthma progression, we next performed immunohistochemical staining of PINK1 on bronchial biopsies obtained from a cohort of six patients with severe asthma compared to 2 biopsies obtained from healthy control subjects. Our results showed that PINK1 protein levels were high (moderate to strong) in the bronchial epithelium as well as in fibroblasts of four out of the six (66.67%) severe asthmatic patients' samples (**Figure 2B**) compared to the healthy control group that showed low expression levels (negative or weak stain) (**Figure 2A**). PINK1 immunostaining across the 8 biopsies is summarized in **Table 2**. Thus, PINK1 serves a protective role in S-As fibroblasts by defending cells from damage-mediated mitochondrial dysfunction and cellular apoptosis.

TABLE 2 | PINK1 immunohistochemistry staining.

	Negative - weak	Moderate - strong
Healthy control	2 (100%)	0 (0%)
Severe asthma	2 (33.34%)	4 (66.67%)

IL-17 Increases Mitochondrial Dysfunction in Bronchial Fibroblasts

Since IL-17 is strongly implicated in the pathogenesis of severe asthma (5), we investigated the effects of IL-17 on mitochondrial mass and function in both non-asthmatic NHBF and severe asthmatic DHBF. Stimulation of bronchial fibroblasts with IL-17 at a concentration of 25 ng/ml was shown to activate inflammatory and remodeling processes (26). In view of the fact that bronchial airway tissue is subjected to chronic exposure to IL-17 in patients with severe asthma (6), NHBF and DHBF were incubated with 25 ng/ml of IL-17 for a duration of up to 96 h to study the long-term effect of prolonged exposure to IL-17. A 48-h

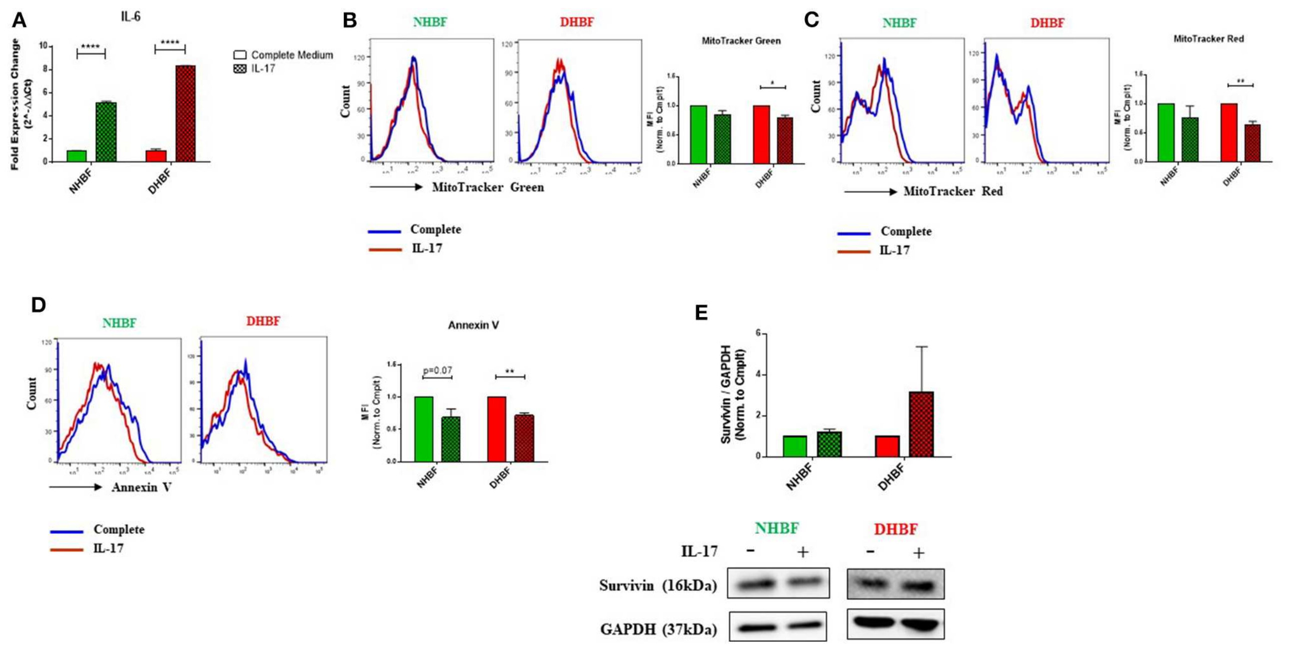


FIGURE 3 | IL-17 increases mitochondrial dysfunction in bronchial fibroblasts. NHBF and DHBF were serum-starved for 24 h and thereafter, cultured in DMEM complete medium without or with IL-17 (25 ng/ml) for up to 96 h. **(A)** mRNA expression of IL-6 in NHBF and DHBF upon IL-17 stimulation was analyzed by qPCR and expressed as fold expression change relative to the respective untreated control post normalization to housekeeping gene 18 s rRNA. Data representative of $n = 3$. **(B)** Representative histograms and bar charts indicating fluorescence in mean fluorescence intensity (MFI) in cells stained with MitoTracker Green, **(C)** MitoTracker Red, and **(D)** Annexin V followed by flow cytometric analysis. 10,000 events were analyzed in each flow cytometry experiment. Data representative of three independent experiments. **(E)** Densitometric analysis and representative immunoblots depicting expression of anti-apoptotic protein, Survivin, in NHBF (left panel) and DHBF (right panel) upon IL-17 stimulation. GAPDH was used as loading control. Data presented as mean ± SEM after normalization to the respective untreated control (Complete Medium). Statistical significance assessed by unpaired *t*-test with multiple comparisons using the Holm-Sidak method. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

exposure to IL-17 significantly increased the mRNA levels of IL-6 in both NHBF and DHBF (**Figure 3A**), which indicated 25 ng/ml of IL-17 to be an effective dose to study the pathology associated with IL-17 in NHBF and DHBF.

IL-17 mediated mitochondrial dysfunction is usually associated with mitochondrial depolarization (16). We, therefore, examined whether IL-17 caused changes in mitochondrial quantity and quality in NHBF and DHBF. MitoTracker Green and mitochondrial membrane potential-dependent MitoTracker Red staining showed an inclination towards a decline in NHBF upon stimulation with IL-17 (**Figures 3B,C**), where their normalized fluorescence decreased from 1 to 0.85 and 0.77, respectively. IL-17, however, significantly attenuated mitochondrial mass and $\Delta\Psi m$ in DHBF when compared to untreated cells (**Figures 3B,C**). In DHBF, IL-17 caused a drop in normalized MitoTracker Green and MitoTracker Red fluorescence from 1 to 0.79 ($p = 0.02$) and 0.63 ($p = 0.005$), respectively. These data suggest that IL-17 is a key pathological cytokine that potentially induces mitochondrial dysfunction in healthy bronchial fibroblasts and intensifies pre-existing mitochondrial damage in S-As fibroblasts.

Taking into consideration the effect of IL-17 on mitochondrial quantity and quality in bronchial fibroblasts, we next evaluated the significance of this change on cell fate. As seen in **Figure 3D**, flow cytometric analysis showed that IL-17 weakened Annexin V staining in both NHBF and DHBF, which suggested that IL-17 protected these fibroblasts from apoptosis. Western blot analysis further indicated that IL-17 treatment increased the expression of survivin, an anti-apoptotic protein, to a greater extent in DHBF than that in NHBF (**Figure 3E**). Taken together, these findings suggest that IL-17 mediated mitochondrial dysfunction may be associated with increased survival of bronchial fibroblasts.

IL-17 Impairs Mitochondrial Quality Control in Bronchial Fibroblasts

We next studied the impact of IL-17 on autophagy in NHBF and DHBF by culturing the fibroblasts with or without IL-17 for a duration of 48 and 96 h for mRNA and protein analyses, respectively. We then measured the mRNA levels of autophagy markers, BECN1, ATG5, LC3B, SQSTM1, and LAMP2. IL-17 significantly increased the mRNA expression of these markers in DHBF but no change was detected in NHBF, except for SQSTM1 which showed a statistically significant increase in NHBF as well (**Figure 4A**). Western blot analysis showed that IL-17 treatment increased LC3B lipidation (LC3BII/LC3BI ratio) from 1.23 to 1.56 in NHBF and from 1.68 to 2.5 ($p = 0.02$) in DHBF (**Figure 4C**). IL-17 also showed an increased trend in the protein expression of p62 in NHBF and DHBF (**Figures 4B,C**). These findings suggest that IL-17 further upregulates autophagy

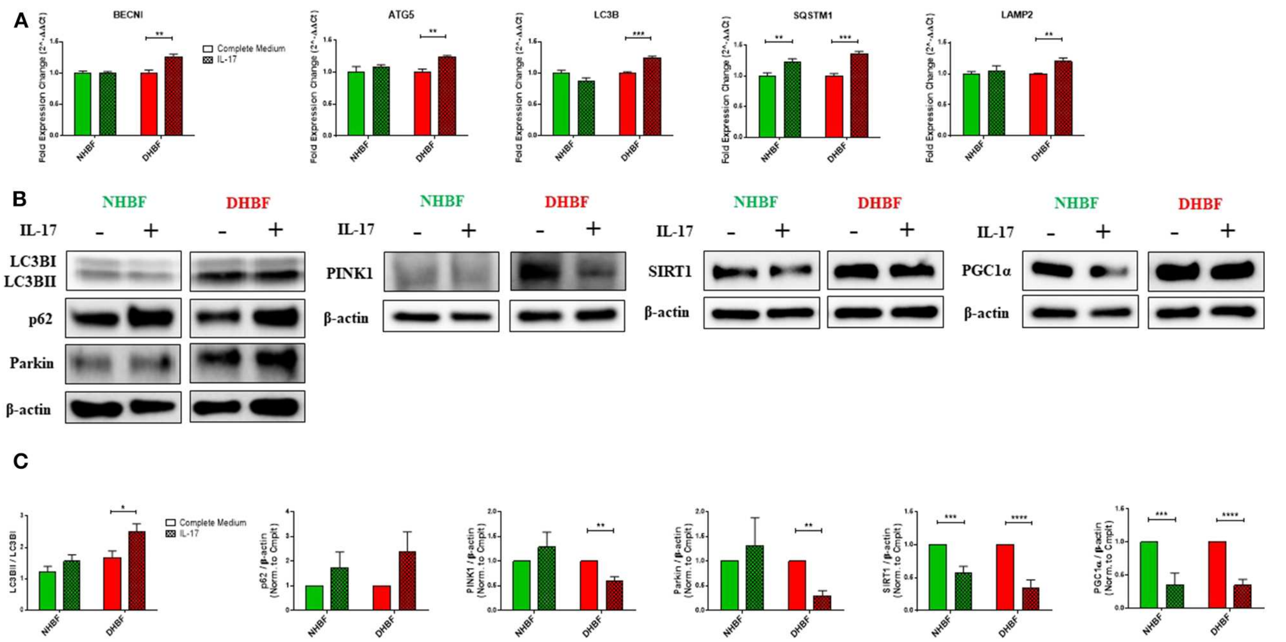


FIGURE 4 | IL-17 impairs mitochondrial quality control in bronchial fibroblasts. NHBF and DHBF were serum-starved for 24 h and thereafter, cultured in DMEM complete medium without or with IL-17 (25 ng/ml) for 48 h for mRNA analysis and 96 h for protein analysis. **(A)** mRNA expression of autophagy genes, BECN1, ATG5, LC3B, SQSTM1, and LAMP2 in NHBF and DHBF upon IL-17 stimulation was analyzed by qPCR and expressed as fold expression change relative to the respective untreated control post normalization to housekeeping gene 18 s rRNA. Data representative of $n = 3$. **(B)** Representative immunoblots and **(C)** densitometric analysis of autophagy markers, with LC3B lipidation represented as the ratio of LC3BII to LC3BI and p62 expression, mitophagy markers, PINK1 and Parkin, and mitochondrial biogenesis markers, SIRT1, and PGC1α. Data representative of three independent experiments. β-actin was used as loading control. Data presented as mean ± SEM after normalization to the respective untreated control (Complete Medium). Statistical significance assessed by unpaired *t*-test with multiple comparisons using the Holm-Sidak method. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

by elevating the expression of autophagy-related genes in S-As fibroblasts.

Next, we aimed to determine the direct effects of IL-17 on mitochondrial QC in bronchial fibroblasts. In NHBF, there was a trend toward an increase in the expression of PINK1 and Parkin with IL-17 treatment (**Figures 4B,C**). However, this was not accompanied by a corresponding increase in SIRT1 and PGC1α expression. In contrast, a marked decline in SIRT1 and PGC1α expression was noted in NHBF (**Figures 4B,C**). IL-17 thus, contributed to increased mitophagy and lowered biogenesis in non-asthmatic fibroblasts. Surprisingly, IL-17 stimulation led to a significant reduction in PINK1 and Parkin expression (**Figures 4B,C**) together with a significant drop in SIRT1 and PGC1α levels in DHBF (**Figures 4B,C**). These results suggest that chronic exposure to IL-17 disrupts the balance between mitophagy and mitochondrial biogenesis leading to impairment in the mitochondrial quality control machinery in bronchial fibroblasts.

Autophagy Triggers IL-17 Induced Mitochondrial Dysfunction

We observed that the enhanced autophagy levels in S-As fibroblasts was further elevated in response to IL-17. In order to study further the role of autophagy in IL-17 induced mitochondrial dysfunction, we pharmacologically inhibited autophagy in bronchial fibroblasts using bafilomycin-A1 (Baf-A1) that blocks autophagosomal fusion with lysosomes (27). Baf-A1, thus, causes the accumulation of autophagosomal vacuoles, which can be confirmed by the increased abundance of LC3BII and p62 in cells treated with Baf-A1. The bronchial fibroblasts were pre-treated with Baf-A1 at 10 nM for 4 h and then stimulated with IL-17 for up to 48 h for mRNA and up to 96 h for protein analyses. NHBF and DHBF were further co-incubated with 10 μM of FCCP in the final 2 h of treatment to induce mitochondrial uncoupling and to serve as a positive control for mitochondrial damage (28). FCCP treatment stimulated an increase in BECNI gene expression (**Figure 5A**) and increased accumulation of LC3BII and p62 proteins (**Figures 5B,C**) in NHBF, indicating the stimulation of autophagy machinery upon mitochondrial damage. Stimulation of PINK1 and PRKN gene expression was induced by FCCP in NHBF (**Figures 5D,E**), in agreement with the fact that FCCP induced mitochondrial uncoupling signals the removal of damaged mitochondria by increasing mitophagy. NHBF demonstrated a quick response to FCCP treatment within 2 h. Baf-A1 significantly decreased BECN1 gene expression (**Figure 5A**) and increased LC3B and p62 accumulation (**Figures 5B,C**) in NHBF, indicating successful inhibition of autophagy flux in NHBF. Baf-A1 treatment also suppressed mitophagy tagging in NHBF as a reduced trend in PINK1 and PRKN gene expression was observed (**Figures 5D,E**). Stimulation with IL-17 marginally increased the abundance of

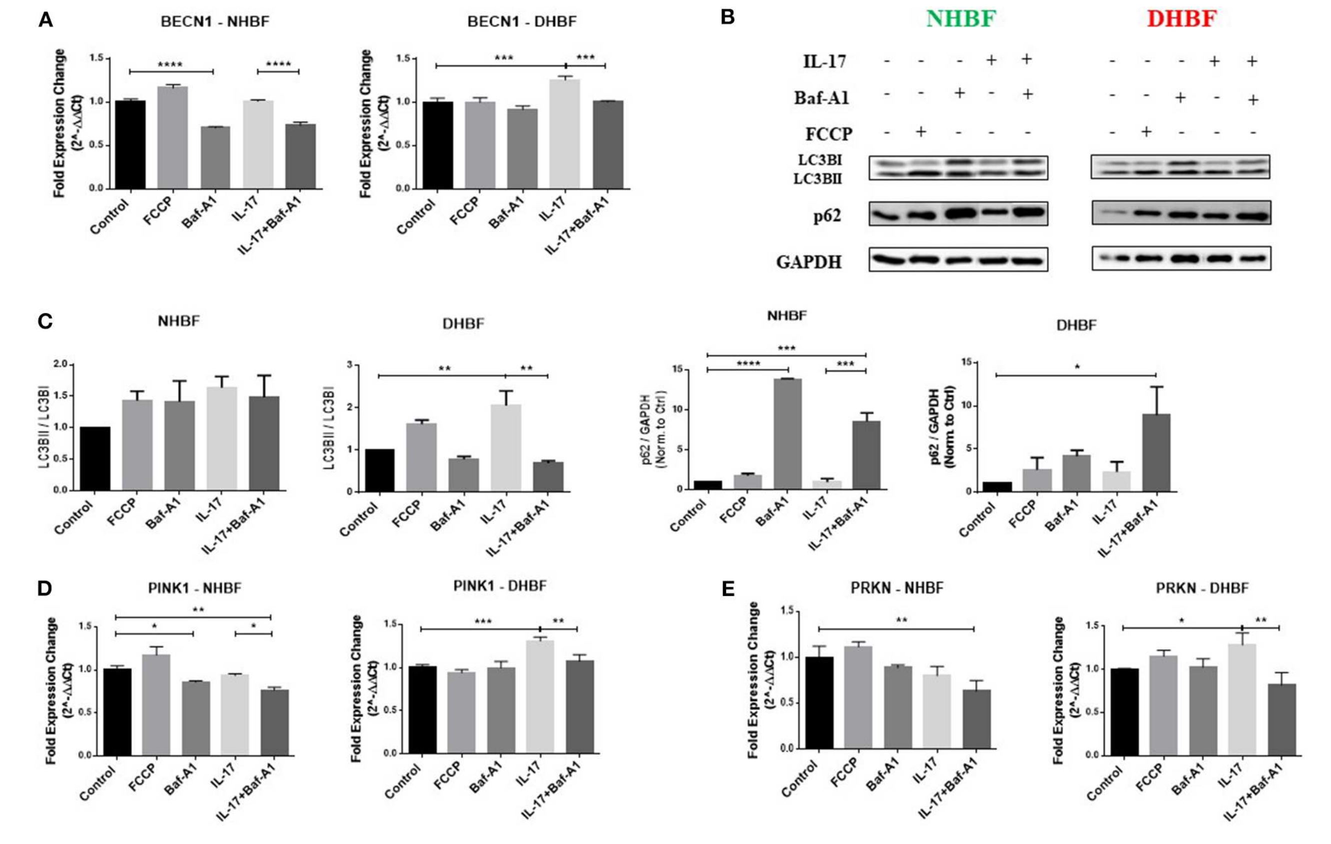


FIGURE 5 | Autophagy triggers IL-17 induced mitochondrial dysfunction. NHBF and DHBF were serum-starved for 24 h, pre-treated with Bafilomycin-A1 (10 nM) for 4 h and thereafter, cultured in DMEM complete medium without or with IL-17 (25 ng/ml) for 48 h for mRNA analysis and 96 h for protein analysis. The cells were co-incubated with FCCP (10 μM) in the final 2 h of treatment. **(A)** mRNA expression of autophagy gene, BECN1, in NHBF (left panel) and DHBF (right panel) was analyzed by qPCR and expressed as fold expression change relative to the respective untreated control post normalization to housekeeping gene 18 s rRNA. Data representative of $n = 3$. **(B)** Representative immunoblots and **(C)** densitometric analysis depicting expression of autophagy markers, LC3B and p62, in NHBF and DHBF. GAPDH was used as loading control. Data representative of two independent experiments. **(D)** mRNA expression of PINK1 and **(E)** Parkin in NHBF (left panel) and DHBF (right panel) was analyzed by qPCR and expressed as fold expression change relative to the respective untreated control post normalization to housekeeping gene 18 s rRNA. Data representative of $n = 3$. Data presented as mean ± SEM after normalization to the respective untreated control. Statistical significance assessed by 1-way ANOVA with Tukey's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

LC3BII and p62 in NHBF compared to time-matched untreated controls (**Figures 5B,C**). However, PINK1 and PRKN gene expression was not affected by IL-17 in NHBF (**Figures 5D,E**). Co-treatment with IL-17 and Baf-A1 reduced the PINK1 and PRKN gene expression to the lowest levels when compared to untreated controls (**Figures 5D,E**).

In DHBF, FCCP induced LC3BII and p62 accumulation (**Figures 5B,C**). However, PINK1 and PRKN gene expression was not affected by FCCP (**Figures 5D,E**), reinforcing the presence of depolarized mitochondria in DHBF. IL-17 induced activation of autophagy in DHBF as observed by elevated BECN1 gene expression (**Figure 5A**) and buildup of LC3BII and p62 proteins (**Figures 5B,C**) upon IL-17 treatment. A significant increase in PINK1 (1.3-fold) and PRKN (1.28-fold) gene expression (**Figures 5D,E**) by IL-17 was also noted in these fibroblasts, which suggested that IL-17 induced mitochondrial damage in DHBF signaling their detection and increased tagging by PINK1 and Parkin. Interestingly, co-treatment with IL-17 and Baf-A1 reversed the IL-17 mediated changes in PINK1 and PRKN mRNA levels (**Figures 5D,E**) in DHBF. Taken together, these findings suggest that IL-17 induced mitochondrial dysfunction in S-As fibroblasts is regulated by autophagy. Blocking autophagy reduced the expression of PINK1 and Parkin, indirectly suggesting an improvement in mitochondrial health resulting in a reduced demand for transcriptional regulation of PINK1 and Parkin-mediated mitophagy.

Autophagy Triggers IL-17 Induced Pro-fibrotic Phenotype

Finally, we aimed to investigate whether there was an association between autophagy and IL-17 induced fibrogenesis in bronchial fibroblasts. Since asthmatic airways are characterized by increased deposition of ECM proteins, including collagen types I, III, and V (COL1A1, COL3A1, COL5A1), and fibronectin (FN1) (29), we first characterized their baseline expression in NHBF and DHBF. As expected, the S-As fibroblasts exhibited significantly higher mRNA expression of collagen subtypes, COL1A1 ($p < 0.0001$), COL3A1 ($p < 0.0001$), and COL5A1 (p

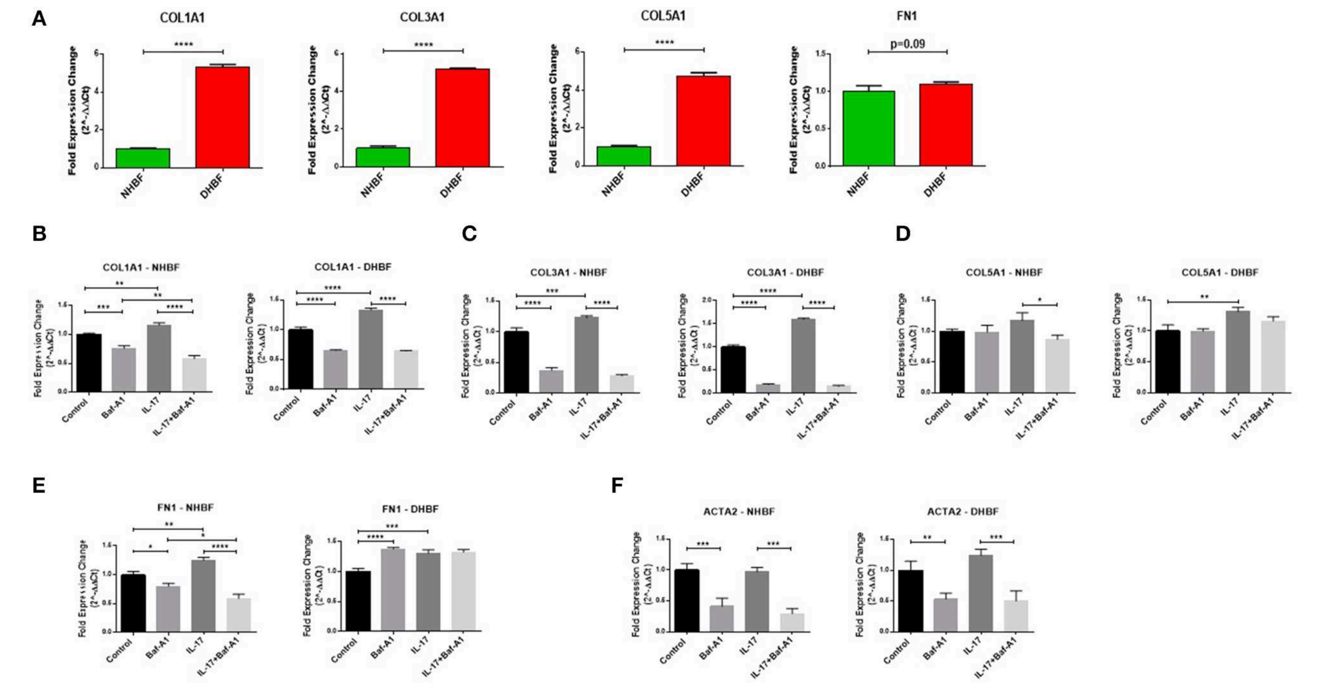


FIGURE 6 | Autophagy triggers IL-17 induced pro-fibrotic phenotype. NHBF and DHBF were serum-starved for 24 h, pre-treated with Bafilomycin-A1 (10 nM) for 4 h and thereafter, cultured in DMEM complete medium without or with IL-17 (25 ng/ml) for 48 h. **(A)** mRNA expression of fibrotic genes, COL1A1, COL3A1, COL5A1 and FN1, at baseline in NHBF and DHBF. Data representative of $n = 3$. mRNA expression of **(B)** COL1A1, **(C)** COL3A1, **(D)** COL5A1, **(E)** FN1, and **(F)** ACTA2, in NHBF (left panel) and DHBF (right panel) was analyzed by qPCR and expressed as fold expression change relative to the untreated control post normalization to housekeeping gene 18s rRNA. Data representative of n=3. Data presented as mean ± SEM after normalization to NHBF or the respective untreated control. Statistical significance assessed by **(A)** unpaired two-tailed *t*-test or **(B–F)** 1-way ANOVA with Tukey's multiple comparison tests. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

< 0.0001) when compared to NHBF (**Figure 6A**). Although a trend toward an increase in mRNA expression of FN1 was noted in DHBF relative to NHBF, it was not statistically significant. Thus, the severe asthmatic fibroblasts used in this study demonstrated an increased pro-fibrotic profile when compared to their healthy counterparts.

We then evaluated whether the mRNA expression of these fibrotic genes as well as α-smooth muscle actin (ACTA2), a marker of myofibroblast differentiation, in bronchial fibroblasts was dependent on autophagy or IL-17 by inhibiting autophagy flux using Baf-A1. As shown in **Figures 6B–F**, at basal levels, blocking autophagy using Baf-A1 significantly decreased the expression of COL1A1, COL3A1, FN1 and ACTA2 in NHBF, and COL1A1, COL3A1, and ACTA2 in DHBF. IL-17 increased the transcriptional levels of COL1A1 (1.3-fold), COL3A1 (1.6-fold), COL5A1 (1.3-fold), and FN1 (1.2-fold) in DHBF. IL-17 also increased the gene expression of some of these ECM components in NHBF, but to a lower extent than in DHBF. Thus, IL-17 induced a potent fibrotic response in both non-asthmatic and S-As bronchial fibroblasts. Interestingly, co-treatment with Baf-A1 reversed the IL-17 induced increase in COL1A1, COL3A1, COL5A1 and FN1 in NHBF, and COL1A1, COL3A1, and ACTA2 in DHBF compared to their respective untreated controls. These findings suggest that IL-17 is a potent inducer of pro-fibrotic phenotype through induction of autophagy in bronchial fibroblasts.

DISCUSSION

Fibrosis is a challenging pathophysiological condition to treat in asthmatics. In fact, the inability of current asthmatic drugs to reverse fibrosis adds to the burden in asthmatic patients (30). In the present study, we investigated the effects of IL-17 on mitochondrial dysfunction and fibrosis in non-asthmatic and S-As fibroblasts. To our knowledge, this is the first study to demonstrate that IL-17 accelerated mitochondrial dysfunction and fibrosis in bronchial fibroblasts, but to a significantly greater extent in S-As fibroblasts when compared to non-asthmatic controls. This induction was further shown to be regulated by the activation of autophagy in these fibroblasts. Our data suggested that the pre-existing mitochondrial damage and fibrotic phenotype in S-As fibroblasts were further amplified by IL-17.

Mitochondrial QC machinery is essential for mitochondrial homeostasis and induction of mitochondrial damage activates this machinery to re-establish homeostasis. We first studied the mitochondrial QC mechanisms of mitophagy and mitochondrial biogenesis as an indicator of mitochondrial damage. Enhanced autophagy in S-As fibroblasts was associated with increased levels of mitophagy and biogenesis compared to healthy controls (**Figures 1A,B**). This suggested enhanced mitochondrial damage in S-As fibroblasts resulting in their continuous turnover. This was confirmed by the reduced mitochondrial membrane potential in these fibroblasts when compared to their healthy counterparts (**Figure 1D**). Additionally, the comparable mitochondrial mass between the two groups of fibroblasts (**Figure 1C**) reinforced the continuous turnover of damaged mitochondria.

PINK1 accumulation is a characteristic of mitochondrial damage and intense PINK1 immunoreactivity was observed in S-As airway biopsies (**Figure 2B**), which further confirmed increased mitochondrial damage in S-As airways than in healthy airways. The observed increase in autophagy and PINK1 immunoreactivity in S-As fibroblasts may be attributed to their prolonged exposure to increased levels of IL-17, which is known to be upregulated in the airway tissue microenvironment in severe asthma (6).

Because of the increasing implications of the pro-inflammatory and pro-fibrotic roles of IL-17 in severe asthma, we investigated the involvement of IL-17 in the mitochondrial dysfunction observed in S-As fibroblasts. The effects of IL-17 were more evident in S-As fibroblasts than in their healthy counterparts. In our study, we showed a 5-fold increase in IL-6 expression in NHBF and an 8-fold increase in DHBF upon IL-17 stimulation (**Figure 3A**). IL-6 is an important regulator of pathogenesis in asthma and has also been implicated in subepithelial fibrosis and airway remodeling in asthma (31). IL-6 is also vital in Th17 biology as it is essential for the differentiation of naïve T cells into Th17 cells, which are key producers of IL-17 (32). Furthermore, IL-6 stimulation of neutrophils from asthmatics significantly increased their production of IL-17A and IL-17F cytokines (33). Therefore, the induction of IL-6 expression in turn in structural bronchial fibroblasts by IL-17 creates a feedforward loop that sustains tissue inflammation by recruiting neutrophils and other immune cells to inflamed lung tissues thereby contributing to persistent airway tissue remodeling (34).

Furthermore, IL-17 significantly decreased the mitochondrial membrane potential and mitochondrial mass in S-As fibroblasts (**Figures 3B,C**). At the same time, a tendency toward a drop in $\Delta\Psi m$ and mitochondrial mass was noted in the healthy fibroblasts (**Figures 3B,C**). These hallmark features indicated that an IL-17 rich microenvironment renders the healthy fibroblasts vulnerable to mitochondrial malfunction and intensifies the pre-existing mitochondrial dysfunction in S-As fibroblasts. Taken together, our findings suggest that S-As fibroblasts show greater susceptibility to IL-17.

IL-17 induced reduction in mitochondrial mass implies enhanced mitochondrial degradation through mitophagy. As expected, IL-17 increased the expression of autophagy-related genes (**Figure 4A**), LC3B lipidation and protein levels of p62 (**Figures 4B,C**), to a statistically significant extent in S-As fibroblasts than in healthy. Interestingly, IL-17 treatment induced an increase in mitophagy in the healthy but it did not reach statistical significance, while a significant decline in mitophagy was observed in the S-As fibroblasts upon IL-17 stimulation (**Figures 4B,C**). The associated decline in mitochondrial biogenesis in both group of fibroblasts (**Figures 4B,C**) reflect an impairment in the mitochondrial QC machinery as a result of prolonged exposure to IL-17. This supports the notion that due to the innate phenotypic heterogeneity between non-asthmatic and S-As fibroblasts, they display unique responses to IL-17 stimulation.

Autophagy has emerged as a key player of cell survival in asthma (35). Increased LC3B expression exerted a cytoprotective role and inhibited hypoxia-induced epithelial apoptosis in lung epithelial cells (36). In addition to disabling the mitochondrial QC machinery, IL-17 inhibited apoptosis by decreasing Annexin V staining (**Figure 3D**) and inducing an increase in the expression of anti-apoptotic protein, Survivin (**Figure 3E**) in both non-asthmatic and S-As fibroblasts. IL-17 was previously reported to impair apoptosis in RA synovial fibroblasts through the activation of autophagy (16) and our findings are in accordance with this study.

We speculate that as a result of IL-17 induced mitochondrial damage, the healthy and S-As fibroblasts endure this damage by increasing their basal autophagy to pathological levels. In the presence of IL-17, the declining $\Delta\Psi m$ in the otherwise healthy mitochondria in healthy fibroblasts trigger an increase in mitophagy to mitigate the damage. However, the increased mitophagy is not paralleled by an increase in biogenesis. On the contrary, the continuous dissipation of $\Delta\Psi m$ caused by IL-17 in mitochondria with pre-existing mitochondrial abnormalities overwhelms the mitophagy and biogenesis machinery resulting in their malfunction in S-As fibroblasts. Nevertheless, the decreased mitochondrial mass in these fibroblasts may suggest the recruitment of alternative mitochondrial degradation mechanisms, including proteasomes, intramitochondrial proteolytic systems or vacuole/lysosome-mediated pathway (37), which may help prevent the accumulation of dysfunctional mitochondria and hence, overcome mitochondria-induced apoptosis (38). This may explain the IL-17 induced increased persistence of the healthy and S-As fibroblasts despite their mitochondrial dysfunction.

The observation of increased autophagy and mitochondrial dysfunction brought about by IL-17 in S-As fibroblasts led us to hypothesize that IL-17 induced autophagy regulates mitochondrial dysfunction as well as promotes the fibrotic phenotype of these diseased fibroblasts. Accordingly, inhibition of autophagy using Baf-A1 reversed the IL-17 mediated increase in PINK1 and PRKN gene expression in S-As fibroblasts (**Figures 5D,E**). Co-incubation with IL-17 and Baf-A1 also brought about a significant reduction in PINK1 and PRKN gene expression in the healthy fibroblasts when compared to their untreated controls (**Figures 5D,E**). Since increased PINK1 and PRKN gene expression is induced upon mitochondrial damage, their decreased gene expression may imply a reduction in mitochondrial dysfunction.

It was however interesting to note that the IL-17 induction of PINK1 and PRKN gene expression in S-As fibroblasts (**Figures 5D,E**) did not correlate with the expression of their protein products (**Figures 4B,C**). Since IL-17 induced mitochondrial dysfunction in these fibroblasts, the transcriptional upregulation of PINK1 and PRKN represents a consequential response. However, it is plausible that IL-17 may also exert an influence on the post-transcriptional factors, including protein translation, post-translational events and protein degradation, thereby, impairing the mitochondrial QC machinery within these fibroblasts. To this regard, a study by Chowdhury et al. showed the ability of IL-17A to interact with microRNAs and thereby affect AUBps protein binding to mRNA facilitating either mRNA decay or stabilization (39). At the same time, the kinetics of IL-17 induced gene and protein expression is currently not well-understood and future studies using pulse-chase labeling may provide an improved understanding of this mechanism.

We, along with many others, have previously reported that subepithelial fibrosis in asthmatic airways is characterized by increased deposition of collagens, specifically collagen types I, III and V, and fibronectin (29, 40–42) as well as increased myofibroblast differentiation (43). Accordingly, the S-As fibroblasts demonstrated significantly enhanced gene expression of these collagen subtypes when compared to their healthy counterparts (**Figure 6A**). More importantly, we have also showed the association between increased levels of IL-17 and collagen types I and III in severe asthmatic bronchial tissues (42). In a study of orbital fibroblasts in thyroid-associated ophthalmopathy, IL-17 promoted the gene expression of collagen types I and III, and ACTA2 (44). In line with these previous studies, IL-17 augmented the gene expression of COL1A1, COL3A1, COL5A1, and FNI to a greater extent in S-As fibroblasts than their healthy counterparts (**Figures 6B–E**). Interestingly, IL-17 did not induce myofibroblast differentiation in bronchial fibroblasts (**Figure 6F**). This could perhaps be because parenchymal fibroblasts being major producers of α-smooth muscle actin (α-SMA) contributed largely to the myofibroblastic phenotype as opposed to bronchial fibroblasts (45). Although IL-17 has previously been reported to enhance the expression of pro-fibrotic genes, its ability to induce ACTA2 expression was found to vary from cell to cell. For instance, IL-17 alone could not induce ACTA2 expression in primary human hepatic stellate cells (46). On the other hand, IL-17A promoted ACTA2 expression in orbital fibroblasts in thyroid-associated ophthalmopathy (44). Alternatively, autophagy was found to regulate myofibroblast differentiation in bronchial fibroblasts.

A recent study demonstrated selective activation of autophagy in a cell context-dependent manner in asthma (47). In this study, autophagy was found to be critical in the development of airway remodeling with multiple autophagy markers showing positive staining in tissue sections of asthmatic airways. Moreover, inhibiting autophagy using chloroquine was found to attenuate airway inflammation, airway hyperresponsiveness and airway remodeling, including a reduction in α-SMA immunoreactivity in the airways, in allergic asthmatic mice. We have also previously reported that dysregulation of autophagy is associated with subepithelial fibrosis in the airways of refractory asthmatics (19). In this study, ATG5 gene expression positively correlated with COL5A1 expression in bronchial biopsies from refractory asthmatics. Interestingly, our results are in line with these earlier studies as we show that co-incubation of bronchial fibroblasts with Baf-A1 significantly reduced COL1A1, COL3A1, FN1, and ACTA2 gene expression (**Figures 6B–F**). Additionally, inhibition of autophagy also blocked the IL-17 mediated increase in pro-fibrotic gene signature in both groups of fibroblasts.

CONCLUSIONS

In summary, our data suggest that IL-17 induces mitochondrial dysfunction and pro-fibrotic signature through the activation of autophagy in bronchial fibroblasts. This provides insight into a potential pathway that contributes to fibrosis in severe asthmatic airways, and reveal the therapeutic potential of targeting autophagy to subdue fibrosis, particularly in severe asthmatic individuals.

DATA AVAILABILITY STATEMENT

The data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The primary bronchial fibroblasts, and paraffin slides from non-asthmatic controls used in our study were obtained from the Biobank at the Quebec Respiratory Health Research Network. The original study was approved by institutional review board (MUHC REB number BMB-02-039-t) and the subjects had provided written informed consent.

The severe asthmatic bronchial biopsies were obtained from patients recruited at the Severe Asthma Clinic in the Pulmonary Medicine department at Rashid Hospital. The study was approved by the Dubai Health Authority and Dubai Scientific Research Ethics Committee (DSREC-11/2017_04). The patients received a detailed description of the study from the nurse and researchers, and samples were collected after their written informed consent.

AUTHOR CONTRIBUTIONS

QH, RR, KB, and RHal: conceptualization. RR, AA, SR, LS, and MH: data curation. RR, AA, and IH: formal analysis. QH, BM, and RHam: funding acquisition and project administration. RR, KB, and RHal: investigation. RR: methodology and validation. QH, RHam, and RO: resources. QH, RHal, BM, and RHam: supervision. RR, AA, and IH: visualization. RR and SA: writing – original draft. RR, SA, KB, and RHal: writing – review and editing.

FUNDING

This study was supported by a collaborative grant (16010902009-P) from the University of Sharjah.

ACKNOWLEDGMENTS

We would like to thank Dr. Youssef Dairi and his team at Rashid hospital for their support in collecting bronchial biopsies from severe asthmatic patients. We would like to acknowledge Mr. Manju Jayakumar for his contribution in running the flow cytometry samples. We would also like to thank Dr. Swati Goel and Ms. Bushra Mdkhana for their assistance with immunoblotting experiments.

REFERENCES

1. Kis K, Liu X, Hagood JS. Myofibroblast differentiation and survival in fibrotic disease. *Expert Rev Mol Med*. (2011) 13:e27. doi: 10.1017/S1462399411001967
2. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med*. (2003)167:1360–8. doi: 10.1164/rccm.200209-1030OC
3. Weitoft M, Andersson C, Andersson-Sjoland A, Tufvesson E, Bjermer L, Erjefalt J, et al. Controlled and uncontrolled asthma display distinct alveolar tissue matrix compositions. *Respir Res*. (2014)15:67. doi: 10.1186/1465-9921-15-67
4. Boser SR, Mauad T, Araujo-Paulino BB, Mitchell I, Shrestha G, Chiu A, et al. Myofibroblasts are increased in the lung parenchyma in asthma. *PLoS ONE*. (2017) 12:e0182378. doi: 10.1371/journal.pone.0182378
5. Ramakrishnan RK, Al Heialy S, Hamid Q. Role of IL-17 in asthma pathogenesis and its implications for the clinic. *Expert Rev Respir Med*. (2019) 13:1057–68. doi: 10.1080/17476348.2019.1666002
6. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol*. (2009) 123:1185–7. doi: 10.1016/j.jaci.2009.02.024
7. Shannon J, Ernst P, Yamauchi Y, Olivenstein R, Lemiere C, Foley S, et al. Differences in airway cytokine profile in severe asthma compared to moderate asthma. *Chest*. (2008) 133:420–6. doi: 10.1378/chest.07-1881
8. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med*. (2009) 180:388–95. doi: 10.1164/rccm.200903-0392OC
9. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol*. (2001) 108:430–8. doi: 10.1067/mai.2001.117929
10. Loubaki L, Hadj-Salem I, Fakhfakh R, Jacques E, Plante S, Boisvert M, et al. Co-culture of human bronchial fibroblasts and CD4+ T cells increases Th17 cytokine signature. *PLoS ONE*. (2013) 8:e81983. doi: 10.1371/journal.pone.0081983
11. Camargo LDN, Righetti RF, Aristoteles L, Dos Santos TM, de Souza FCR, Fukuzaki S, et al. Effects of Anti-IL-17 on inflammation, remodeling, and oxidative stress in an experimental model of asthma exacerbated by LPS. *Front Immunol*. (2017) 8:1835. doi: 10.3389/fimmu.2017.01835
12. Heinzmann A, Thoma C, Dietrich H, Deichmann KA. Identification of common polymorphisms in the mitochondrial genome. *Allergy*. (2003) 58:830–1. doi: 10.1034/j.1398-9995.2003.00223.x
13. Mabalirajan U, Dinda AK, Kumar S, Roshan R, Gupta P, Sharma SK, et al. Mitochondrial structural changes and dysfunction are associated with experimental allergic asthma. *J Immunol*. (2008) 181:3540–8. doi: 10.4049/jimmunol.181.5.3540
14. Aguilera-Aguirre L, Bacsi A, Saavedra-Molina A, Kurosky A, Sur S, Boldogh I. Mitochondrial dysfunction increases allergic airway inflammation. *J Immunol*. (2009) 183:5379–87. doi: 10.4049/jimmunol.09 00228
15. Servais S, Boussouar A, Molnar A, Douki T, Pequignot JM, Favier R. Age-related sensitivity to lung oxidative stress during ozone exposure. *Free Radical Res*. (2005) 39:305–16. doi: 10.1080/10715760400011098
16. Kim EK, Kwon JE, Lee SY, Lee EJ, Kim DS, Moon SJ, et al. IL-17-mediated mitochondrial dysfunction impairs apoptosis in rheumatoid arthritis synovial fibroblasts through activation of autophagy. *Cell Death Dis*. (2017) 8:e2565. doi: 10.1038/cddis.2016.490
17. Zhou J, An X, Dong J, Wang Y, Zhong H, Duan L, et al. IL-17 induces cellular stress microenvironment of melanocytes to promote autophagic cell apoptosis in vitiligo. *FASEB J*. (2018) 32:4899–916. doi: 10.1096/fj.201701242RR
18. Panariti A, Baglole CJ, Sanchez V, Eidelman DH, Hussain S, Olivenstein R, et al. Interleukin-17A and vascular remodelling in severe asthma; lack of evidence for a direct role. *Clin Exp Allergy*. (2018) 48:365–78. doi: 10.1111/cea.13093
19. Poon AH, Choy DF, Chouiali F, Ramakrishnan RK, Mahboub B, Audusseau S, et al. Increased autophagy-related 5 gene expression is associated with collagen expression in the airways of refractory asthmatics. *Front Immunol*. (2017) 8:355. doi: 10.3389/fimmu.2017.00355
20. Ichikawa T, Panariti A, Audusseau S, Mogas AK, Olivenstein R, Chakir J, et al. Effect of bronchial thermoplasty on structural changes and inflammatory mediators in the airways of subjects with severe asthma. *Respir Med*. (2019) 150:165–72. doi: 10.1016/j.rmed.2019.03.005
21. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature*. (2000) 406:902–6. doi: 10.1038/35022595
22. Xiao B, Deng X, Zhou W, Tan EK. Flow cytometry-based assessment of mitophagy using MitoTracker. *Front Cell Neurosci*. (2016) 10:76. doi: 10.3389/fncel.2016.00076
23. Wang C, Youle RJ. The role of mitochondria in apoptosis*. *Ann Rev Gene*. (2009) 43:95–118. doi: 10.1146/annurev-genet-102108-134850
24. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol*. (2014) 15:81–94. doi: 10.1038/nrm3735
25. Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron*. (2015) 85:257–73. doi: 10.1016/j.neuron.2014.12.007
26. Dessalle K, Narayanan V, Kyoh S, Mogas A, Halayko AJ, Nair P, et al. Human bronchial and parenchymal fibroblasts display differences in basal inflammatory phenotype and response to IL-17A. *Clin Exp Allergy*. (2016) 46:945–56. doi: 10.1111/cea.12744
27. Ghavami S, Mutawe MM, Schaafsma D, Yeganeh B, Unruh H, Klonisch T, et al. Geranylgeranyl transferase 1 modulates autophagy and apoptosis in human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. (2012) 302:L420–8. doi: 10.1152/ajplung.00312.2011
28. Berezhnov AV, Soutar MP, Fedotova EI, Frolova MS, Plun-Favreau H, Zinchenko VP, et al. Intracellular pH modulates autophagy and mitophagy. *J Biol Chem*. (2016) 291:8701–8. doi: 10.1074/jbc.M115.691774
29. Wilson JW, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy*. (1997) 27:363–71. doi: 10.1046/j.1365-2222.1997.600864.x
30. Durrani SR, Viswanathan RK, Busse WW. What effect does asthma treatment have on airway remodeling? Current perspectives. *J Allergy Clin Immunol*. (2011) 128:439–48; quiz 49–50. doi: 10.1016/j.jaci.2011.06.002
31. Rincon M, Irvin CG. Role of IL-6 in asthma and other inflammatory pulmonary diseases. *Int J Biol Sci*. (2012) 8:1281–90. doi: 10.7150/ijbs.4874
32. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. (2007) 8:967–74. doi: 10.1038/ni1488
33. Halwani R, Sultana A, Vazquez-Tello A, Jamhawi A, Al-Masri AA, Al-Muhsen S. Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma. *J Asthma*. (2017) 54:893–904. doi: 10.1080/02770903.2017.1283696
34. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity*. (2011) 34:149–62. doi: 10.1016/j.immuni.2011.02.012
35. Jyothula SS, Eissa NT. Autophagy and role in asthma. *Curr Opin Pulm Med*. (2013) 19:30–5. doi: 10.1097/MCP.0b013e32835b1150
36. Tanaka A, Jin Y, Lee SJ, Zhang M, Kim HP, Stolz DB, et al. Hyperoxia-induced LC3B interacts with the Fas apoptotic pathway in epithelial cell death. *Am J Respir Cell Mol Biol*. (2012) 46:507–14. doi: 10.1165/rcmb.2009-0415OC
37. Mijaljica D, Prescott M, Devenish RJ. Different fates of mitochondria: alternative ways for degradation? *Autophagy*. (2007) 3:4–9. doi: 10.4161/auto.3011
38. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. *Circ Res*. (2012) 111:1208–21. doi: 10.1161/CIRCRESAHA.112.265819
39. Chowdhury S, Dijkhuis A, Steiert S, Lutter R. IL-17 attenuates degradation of ARE-mRNAs by changing the cooperation between AU-binding proteins and microRNA16. *PLoS Gene*. (2013) 9:e1003747. doi: 10.1371/journal.pgen.1003747
40. Ignotz RA, Massague J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. (1986) 261:4337–45.
41. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in

asthmatic airway inflammation. *J Allergy Clin Immunol*. (1998) 102:783–8. doi: 10.1016/S0091-6749(98)70018-1

42. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol*. (2003) 111:1293–8. doi: 10.1067/mai.2003.1557
43. Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol*. (1990) 3:507–11. doi: 10.1165/ajrcmb/3.5.507
44. Fang S, Huang Y, Wang S, Zhang Y, Luo X, Liu L, et al. IL-17A Exacerbates fibrosis by promoting the proinflammatory and profibrotic function of orbital fibroblasts in TAO. *J Clin Endocrinol Metabol*. (2016) 101:2955–65. doi: 10.1210/jc.2016-1882
45. Pechkovsky DV, Hackett TL, An SS, Shaheen F, Murray LA, Knight DA. Human lung parenchyma but not proximal bronchi produces fibroblasts with enhanced TGF-beta signaling and alpha-SMA expression. *Am J Respir Cell Mol Biol*. (2010) 43:641–51. doi: 10.1165/rcmb.2009-0318OC
46. Fabre T, Kared H, Friedman SL, Shoukry NH. IL-17A enhances the expression of profibrotic genes through upregulation of the TGF-beta receptor on hepatic stellate cells in a JNK-dependent manner. *J Immunol*. (2014) 193:3925–33. doi: 10.4049/jimmunol.1400861
47. McAlinden KD, Deshpande DA, Ghavami S, Xenaki D, Sohal SS, Oliver BG, et al. Autophagy activation in asthma airways remodeling. *Am J Respir Cell Mol Biol*. (2019) 60:541–53. doi: 10.1165/rcmb.2018-0169OC

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 03 June 2020
doi: 10.3389/fimmu.2020.00947

Interleukin-17 in Chronic Inflammatory Neurological Diseases

Jelena Milovanovic [1,2], Aleksandar Arsenijevic [1], Bojana Stojanovic [1,3], Tatjana Kanjevac [4], Dragana Arsenijevic [1,5], Gordana Radosavljevic [1], Marija Milovanovic [1] and Nebojsa Arsenijevic [1]*

[1] Faculty of Medical Sciences, Center for Molecular Medicine and Stem Cell Research, University of Kragujevac, Kragujevac, Serbia, [2] Department of Histology and Embriology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [3] Department of Pathophysiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [4] Department of Dentistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [5] Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

A critical role for IL-17, a cytokine produced by T helper 17 (Th17) cells, has been indicated in the pathogenesis of chronic inflammatory and autoimmune diseases. A positive effect of blockade of IL-17 secreted by autoreactive T cells has been shown in various inflammatory diseases. Several cytokines, whose production is affected by environmental factors, control Th17 differentiation and its maintenance in tissues during chronic inflammation. The roles of IL-17 in the pathogenesis of chronic neuroinflammatory conditions, multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), Alzheimer's disease, and ischemic brain injury are reviewed here. The role of environmental stimuli in Th17 differentiation is also summarized, highlighting the role of viral infection in the regulation of pathogenic T helper cells in EAE.

Keywords: IL-17, Th17, EAE, Alzheimer's disease, ischemic brain injury

OPEN ACCESS

Edited by:
*Nicola Ivan Lorè,
IRCCS San Raffaele Scientific Institute, Italy*

Reviewed by:
*Rongxin Zhang,
Tianjin Medical University, China
Avijit Ray,
AbbVie, United States*

****Correspondence:***
*Marija Milovanovic
marijaposta@gmail.com*

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *14 February 2020*
Accepted: *22 April 2020*
Published: *03 June 2020*

Citation:
Milovanovic J, Arsenijevic A, Stojanovic B, Kanjevac T, Arsenijevic D, Radosavljevic G, Milovanovic M and Arsenijevic N (2020) Interleukin-17 in Chronic Inflammatory Neurological Diseases. Front. Immunol. 11:947. doi: 10.3389/fimmu.2020.00947

INTRODUCTION

Interleukin-17 (IL-17) is the first-described and founder member of the IL-17 family of inflammatory cytokines, which contains six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. The gene that encodes IL-17A was discovered in 1993 as an RNA transcript homologous to a *Herpesvirus Saimiri* gene, and the protein, initially called CTLA-8, was cloned (1). However, IL-17 attracted widespread attention in 2005, when two independent groups simultaneously characterized a new population of T helper (Th) CD4+ cells that produced IL-17A, named Th17 (2, 3). T helper CD4+ cells were first marked as the principal source of IL-17, but it was later shown that CD8+ cells also produce this cytokine, and these cells are termed Tc17. Also, several types of innate immune cells such as γδ T, natural killer T (NKT), TCRβ+ natural Th17, and Type 3 innate lymphoid cells (ILC3) produce IL-17 (4). All of these IL-17-producing cells are termed "Type 17" cells.

The proinflammatory activities of IL-17 are key in anti-microbial protection of the host, but uncontrolled IL-17 activity is associated with different immunopathological conditions, autoimmune diseases, and cancer progression (5). A critical role for IL-17R signaling in protection against bacterial and fungal infections, particularly by Candida albicans and Klebsiella pneumoniae, has been described in various studies in mice (6). In humans, mutations in IL-17 signaling genes (ACT1, IL17RA, IL17RC) are associated with chronic mucocutaneous candidiasis (5, 7, 8). The same condition also develops in individuals with AIRE deficiency, a condition accompanied by the production of anti-IL-17 antibodies (9).

Anti-IL-17A antibodies have shown therapeutic effect in various inflammatory diseases. Several anti-IL-17 antibodies have been approved for the treatment of plaque psoriasis (10, 11). Positive effects of IL-17 blockade have been shown in clinical trials of ankylosing spondylitis and psoriatic arthritis (12). Anti-IL17R antibody treatment of Crohn's disease has been shown to worsen the disease (13, 14), whereas targeting cytokines that control the differentiation of Th17 cells and therefore IL-17 secretion with anti-p40 subunit antibodies (Ustekinumab, Briakinumab) and anti-IL-6 receptor antibody (Tocilizumab) showed efficacy (15–17). These findings indicate that IL-17, by maintaining the integrity of the intestinal barrier, plays a dominantly protective role that overcomes its potential for tissue destruction in inflammatory bowel disease (18). Clinical use of antibodies that target IL-17 signaling gave insights into functions of IL-17 in humans.

IL-17R SIGNALING

The family of IL-17 receptors contains five different receptors (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE) with common a cytoplasmic motif known as the SEFIR domain (19). IL-17 exists either as a homodimer or as a heterodimer, and both forms of the cytokine induce signals through dimeric IL-17RA and IL-17RC receptor complex (5). Binding of IL-17 to its receptor induces activation of several independent signaling pathways mediated by a cytosolic adaptor protein, Act1, and different TRAF proteins (5, 19, 20). IL-17 signaling mediated through TRAF6 and TRAF4 results in the transcription of inflammatory genes. Activation of TRAF6 by binding of IL-17 to its receptor leads to triggering of NF-κB, C/EBPβ, C/EBPδ, and MAPK pathways, while TRAF4 activation in complex with MEKK3 and MEK5 activates ERK5 (21). On the other hand, the mRNA stability of genes controlled by IL-17 is controlled IL-17-activated TRAF2 and TRAF5 (22).

Expression of IL-17R is ubiquitous, but the main targets of IL-17 are non-hematopoietic cells (23). IL-17 signaling induces the production of proinflammatory cytokines (IL-1, IL-6, G-CSF, GM-CSF, and TNF) and chemokines (CXCL1, CXCL2, CXCL5, CCL2, CCL7, CCL20, and IL-8), matrix metalloproteinases (MMP1, MMP3, MMP9, and MMP13), and anti-microbial peptides (β-defensins, S-100 proteins) (24, 25). The biological activities of IL-17 are often the result of synergistic or cooperative effects of IL-17 and other inflammatory cytokines (26). There are several mechanisms of negative regulation of IL-17 signal transduction. The negative regulators of IL-17 signaling are different ubiquitinases, deubiquitinases, kinases, endoribonuclease, and micro RNAs (21).

However, there is tissue-specific IL-17-dependent gene induction (27). In gut epithelium, IL-17 regulates the expression of several molecules that contribute to the preservation of continuous intestinal epithelium. In renal epithelial cells, IL-17 induces the expression of kallikrein 1 (28), while in salivary epithelium, it induces the expression of histatins (29), molecules that are involved in protection against *C. albicans*. IL-17-mediated osteolysis, which is detected in periodontitis and mouse models of arthritis or periodontal disease, is probably mediated by a receptor activator of NF-κB ligand (RANKL, TNFSF11, or osteoprotegerin ligand, OPGL) whose espression is induced by IL-17 (30, 31).

DIFFERENTIATION OF TH17 CELLS

Th17 cells are classified as an inflammatory subset of T helper cells that perform key roles at mucosal surfaces where they mediate protection from bacteria and fungi and also contribute to the regulation of the mutualistic microorganisms that constitute the microbiota (32, 33). However, Th17 cells are also one of the major factors in the pathogenesis of several autoimmune diseases, including autoimmune disease of the central nervous system, Multiple sclerosis (MS) (2, 3, 34–39). The process of differentiation of naive CD4+ cells into Th17 cells is very similar to that of Th1 differentiation, but transcriptional factors that mediate this are distinct and it require stimulation with the cytokines IL-1β, IL-6, IL-21, and TGFβ, which are produced by professional antigen-presenting cells (APCs) (32, 40–47). Cytokines produced by APCs stimulate the JAK-STAT3 axis and upregulate the expression of transcription factors RORγt and RORα, identified as markers of the Th17 lineage (48–52). The differentiation of Th17 cells is reduced in the states of IL-6, IL-21, TGFβ, or RORγt deficiency, which leads to reduced production of Th17 cytokines and impaired defense against extracellular bacteria and fungi but also attenuation of autoimmunity (41, 48, 53). However, an alternative mode for the differentiation of pathogenic Th17 cells in the absence of TGFβ signaling has been described *in vivo* in Experimental Autoimmune Encephalomyelitis (EAE) (54). Cytokines that induce Th1 and Th2 differentiation are described as the main inhibitors of Th17 differentiation. IL-2 is a key repressor of Th17 differentiation, as it activates transcription factor STAT5 and thus inhibits IL-17 production (55), while inhibition of IL-2 expression in T lymphocytes stimulates Th17 cell development (56, 57).

In animal models of autoimmune diseases, proinflammatory cytokines IL-1β and IL-23 have been shown to be enhancers and stabilizers of partially or completely differentiated effector Th17 cells, which dominantly express corresponding receptors for these cytokines, IL-1R1 and IL-23R (44, 58–61). In line with this observation, transfer of Th17 cells *in vitro* obtained by exposure to IL-6 and TGFβ does not induce EAE in mouse, while Th17 cells obtained by stimulation of naive cells with IL-1β, IL-6, and IL-23 achieve the pathogenic potential and are able to elicit EAE (55). In fact, it was later shown that IL-6 and TGFβ in Th17 cells induce production of anti-inflammatory cytokine IL-10, while IL-23 has a critical role in the induction of the endogenous cytokine TGFβ3. Suppression of IL-10 production in Th17 cells during their differentiation results in high expressions of T-bet, IL-23R, and GM-CSF, markers of Th17 cells with pathogenic potential (62, 63). Furthermore, IL-1 and IL-23 stimulation through JunB and SOCS family members (64, 65) affects the effector profile of Th17 cells and induces the development of highly pathogenic double-positive IL-17+ IFNγ+ and IL-17+

GM-CSF+ T cells (66–68). These pathogenic double-positive cells originate from Th17 cells. However, IL-23 is not required for the differentiation and maintenance of nonpathogenic Th17 cells in the gut and functional plasticity toward T follicular helper cells (66, 68). The novel genes Gpr65, Toso, and Plzp, identified by the single-cell RNA-sequencing analysis of *ex vivo* Th17 cells, are found to promote Th17 pathogenicity and to cause EAE and chronic inflammation in the CNS of mice, while CD5 antigen-like (CD5L) attenuates Th17 cell-mediated disease (69, 70).

Before activation, T cells do not express receptors for cytokines IL-1 and IL-23 (58, 71). During the initial phase of Th17 differentiation, IL-6 induces binding of RORγt to the Il1r1 locus and binding of STAT3 to the Il23r locus, leading to the expression of these genes (54). Phosphorylation of STAT3 increases the expression of the conserved miR-183/96/182 cluster, which in turn reduces the expression of Foxo1, a transcription factor that negatively regulates the expressions of IL-1R1 and IL-23R (72). The Major Transcriptional Effector of Notch Signaling, RBPJ, promotes IL-23R expression and induces pathogenicity of Th17 cells (73). The differentiation of Th17 is stabilized by positive feed-forward loop stimulation with IL-1β and IL-23, accompanied by upregulation of IL-1R and IL-23R (50, 74). The hallmark of effector Th17 cells is IL-23R expression, and its signaling promotes the expression of transcriptional factor Blimp-1, which induces the expression of several genes *in vivo*, leading to the enhanced pathogenicity of Th17 cells (75).

The differentiation of human Th17 cells *in vitro*, similar to mouse Th17 cells, requires IL-1, IL-6, IL-23, and TGFβ. Initially, a few studies demonstrated that TGFβ was not required, while stimulation with IL-1β, IL-6, and IL-23 was sufficient for induction of human Th17 cell differentiation (76, 77). Later studies showed that TGFβ, IL-23, and IL-1β (or IL-6) were the key factors needed for differentiation of human Th17 cells under serum-free conditions, since serum could be the source of TGFβ or aryl hydrocarbon receptor (AhR) ligands (78, 79). In line with findings regarding Th17 differentiation in mouse, IL-23 is also the key player in the differentiation of human Th17 cells; moreover, human CD4+ T cells express IL-23R before activation and immediately respond to IL-23, while IL-23 signals in accordance with stimulation with IL-1β further upregulate IL-23R expression (78, 80). Described dominant-negative mutations of STAT3 gene, which can be manifested by hyper-immunoglobulin E syndrome, inadequate Th17 cell differentiation, and reduced production of IL-17, supports the roles of STAT3 in the IL-6- and IL-23-mediated process of Th17 differentiation in humans (81, 82).

ENVIRONMENTAL FACTORS THAT AFFECT THE PATHOGENIC POTENTIAL OF TH17 CELLS

Different environmental factors modulate the reactions of the immune system and strongly accelerate the pathogenic potential of Th17 cells. T helper cells under Th17 culture condition increase expression of the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that senses environmental toxins and endogenous molecules such as metabolites of tryptophan, and the stimulation of this molecule induces the release of IL-17 and IL-22 by effector Th17 cells (83, 84). The transcription factor, hypoxia-inducible factor 1 (HIF-1), a key metabolic sensor, directly regulates expression of RORγt and IL-17 at the transcriptional level and promotes Th17 differentiation (85, 86). Signaling *via* kinase complex mTORC1 coordinates metabolic and transcriptional programs that regulate the development of pathogenic Th17 cells (87). Disrupted mTORC1 signaling in Th17 cells leads to upregulated expression of TCF-1 (transcription factor T-cell factor 1) and development of stemness-like features, while transdifferentiation in the Th1 is arrested. Mice with blocked mTORC1 activity are protected from EAE, while their Th17 cells do not express T-bet and IFN-γ (87).

A high-salt diet can enhance the differentiation of Th17 cells and thus contribute to the development of EAE (88, 89). Exposure to a high-salt diet induces the expression of serum glucocorticoid kinase 1 (SGK1), which promotes the expression of IL-23R and thus stabilizes pathogenic Th17 cells and enhances the production of GM-CSF (88, 89). Since mice with T-cell-specific deletion of *Sgk1* develop attenuated EAE, without exacerbation after exposure to a high-salt diet, it appears that a high-salt diet modulates EAE severity by its direct effect on T-cell differentiation (88).

TH17 AND IL-17 IN MULTIPLE SCLEROSIS AND EAE

Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS) that is characterized by damage to myelinated axons in the CNS, leading to the loss of myelin sheath. Inflammatory processes that cause myelin damage lead to the destruction of oligodendrocytes and axons, with subsequent axonal loss, and transient or permanent loss of neurologic functions, resulting in various types of disabilities of different severity (90). An overall reduction in CNS volume is very often seen in MS. Localized inflammatory foci can be found in the white matter in almost all areas of CNS, with a considerable number of plaques in the gray matter and anywhere in the CNS parenchyma, including the optic nerves, brainstem, periventricular white matter, and cervical spinal cord (91–93). The course of MS and clinical symptoms are highly variable and unpredictable, varying from a relatively benign illness with minimal impairment to a rapidly evolving and life-threatening disease that requires serious medical treatment (94).

The precise etiology of MS is unknown, but it is considered that both genetic and environmental factors play significant roles in its development (95). The pathogenesis of MS also remains elusive, but it is believed that MS is an autoimmune disease mediated by auto-reactive CD4+ T cells specific for myelin antigens. Autoreactive T cells initiate and perpetuate an inflammatory cascade, resulting in demyelination and axonal loss (96). The huge heterogeneity of disease course in patients with MS and in the histopathological features seen in the CNS indicates that multiple immunopathological pathways contribute to the disease development. Evidence from clinical studies suggests that inflammatory mediators, such as cytokines, play an essential role in the pathogenesis of MS (91, 97).

The pathogenesis of MS has been mostly described by analogy to EAE, an animal model of MS (98–100). In typical EAE induced by immunization with autoantigen, myelin-specific $CD4^+$ T cells are activated in the lymph organs in the periphery, develop encephalitogenic potential, and infiltrate the CNS, where they recognize specific autoantigens presented by local antigen-presenting cells (APCs) and reactivate. The inflammatory process in MS is initiated by binding of pathogen-associated molecular patterns (PAMPs) from pathogens or commensal bacteria and damage-associated molecular patterns (DAMPs) from dead or dying cells to pathogen recognition receptors (PRRs), leading to activation of innate immune cells and production of IL-1, IL-6, IL-12, IL-18, and IL-23, cytokines that promote the differentiation and expansion of encephalitogenic Th1 and Th17 cells (101, 102). Myelin-specific CD4+ T cells that enter the CNS are reactivated and expanded by the IL-1β and IL-23 produced by resident microglia and infiltrating inflammatory monocytes. Encephalityogenic Th1 and Th17 cells in the CNS produce inflammatory cytokines that activate glial cells to produce inflammatory mediators, matrix metalloproteinases, chemokines, and free radicals, which induce myelin damage, leading to manifestations of neurologic deficits (65, 66, 101, 103). One of the main differences between MS and animal models (EAE) is the localization of demyelination. In EAE demyelination, is mainly located in the spinal cord, whereas in MS, this process mainly affects the cerebral and cerebellar cortex (104). The dominant population of T cells in active MS lesions are CD8+ T cells, but in EAE, the primary encephalitogenic T cells and dominant population in CNS infiltrates are CD4+ T cells, with less evidence for the role of CD8+ T cells (105). Neurodegeneration is more typical for MS, while in EAE models, the dominant finding is neuroinflammation (106). In the later stages of MS, neurodegeneration appears to be independent of the inflammatory process, which cannot be found in the acute inflammatory EAE model (107). However, axonal and neuronal loss and demyelination with remyelination can be observed in EAE in Biozzi antibody high (ABH) mice (108). Despite the limitations of the EAE models, the main findings regarding MS pathogenesis have come from EAE studies, as has the design, development, and validation of many therapeutics used for the treatment of MS (109).

Cytokines play roles in the pathogenesis of MS and EAE and in the processes of inducing oligodendrocyte cell death, neuronal dysfunction, and axonal degeneration (110). Th17 cells are considered to be one of the key effectors of autoimmune inflammatory diseases, including MS and experimental disease EAE (2, 111–113). Increased expression of IL-17- and Th17-associated transcripts (Il6, Il17a) has been demonstrated in MS plaques collected at autopsy (114). Further, IL-17 was marked as the highest-ranking gene expressed in the CNS of MS patients at autopsy (114); this was before the discovery of Th17 cells. Also, another report indicated that MS is a primarily IL-17-mediated autoimmune disease (78). Later, the results of various studies showed that a single nucleotide polymorphism (SNP) in IL-23R gene is linked to several human autoimmune diseases, indicating that IL-23 signaling is an essential event in the development of pathogenic Th17 cells. It is known that IL-17 can stimulate the production of other proinflammatory cytokines and chemokines and thus evince a powerful proinflammatory effect (115). The concentration of IL-17 is significantly higher in the serum of MS patients with relapses and remissions than in normal, healthy subjects (116) and is in correlation with disease activity, as demonstrated by magnetic resonance imaging (117). Consistently with the increased concentration of IL-17 in liquor and peripheral blood of MS patients, the proportion of Th17 cells is also increased, especially during relapses, while there is no change in Th1 cells (118, 119). Th17 cells are able to cross the blood–brain barrier, and their presence in MS lesions is associated with enhanced neuroinflammation (120). It has been shown that IFN-γ-producing Th17 cells cross the blood–brain barrier and accumulate in the CNS during the active phase of MS (121). Besides CD4+ T cells, there is evidence that IL-17-producing CD8 T cells contribute to CNS tissue damage in EAE and are also present in the liquor of patients with MS (122, 123). Importantly, it has been documented that the cells that enter the CNS in the first wave of CNS infiltration are Th17 cells (124), followed by infiltration with other immune cells that further promote and sustain tissue inflammation. Also, the presence of IL-17- and IL-22-producing Th cells has been reported in the early stages of MS (125).

Beneficial effects of treatment with rituximab, blocking anti-CD20 antibody, in EAE are associated with decreased production of several cytokines, including IL-17 (126). Neutralization of IL-17 can significantly attenuate the progress of EAE by attenuating the induction of pathogenic cytokines (58). Also, EAE severity was ameliorated in IL-17-deficient animals (123, 127, 128), while the disease was mild, with delayed onset, in RORγt-deficient mice (48).

One study indicates that the beneficial effect of vitamin D supplementation in MS patients is mediated by alleviating the percentage of pathogenic T-cell subsets that produce IL-17 (129). It has also recently been shown that amelioration of MS by dimethyl fumarate is associated with suppression of IL-17+ CD8+ Tc17 cells (130). The beneficial effect of statins in some forms of MS could be due to their effect on Th17 cells (131). Phase IIa study has been conducted in order to investigate possible beneficial effects of Secukinumab, an IL-17A-neutralizing monoclonal antibody. No adverse effects of Secukinumab were detected, while the results of this study indicate that blocking IL-17A with an antibody may reduce MRI lesion activity in MS (132).

There are studies that demonstrate the importance of Th17 cells in EAE and MS, but there is also evidence that indicates that Th1 cells are the main mediators of neuropathology in the EAE model (113, 133). Several reports indicated that IFN-γ-deficient and IFN-γR-deficient mice, as well as anti-IFN-γ-treated mice, develop EAE (134, 135). Also, there are reports showing a protective role for IFN-γ in EAE, mediated by the supression of pathogenic Th17 cells (3). The presence of T cells that coexpress IL-17 and IFN-γ under inflammatory situations has been reported (136, 137). These Th1/Th17 cells were noticed in the CNS of mice with EAE (138). Data obtained from the mouse studies indicate that Th17 cells lacking Il17a generated *in vitro* are able to induce EAE upon adoptive transfer, similar to wild-type Th17 cells (127, 139). Finally, it seems that there is significant plasticity of Th17 cells, with evidence that lymphocytes obtained from the blood of MS patients have an increased potential to switch from IL-17-secreting Th17 cells to IFN-γ-secreting Th1, also called ex-Th17 cells (121).

Several studies have demonstrated that IL-23, a cytokine essential for the differentiation and expansion of Th17 cells, promotes EAE more robustly than IL-12, a cytokine that stimulates the development of INF-γ-producing Th1 cells (140). IL-23 is a covalent heterodimer of p40 (IL-12) and p19 (IL-23) subunits (70). IL-12 and IL-23 share the p40 subunit. The same cell types, mainly dendritic cells, produce both of these two cytokines, but their relative ratio depends on the nature of stimuli that activate dendritic cells (141). IL-12Rβ2-deficient mice with excluded IL-23 signaling are more susceptible to EAE, develop disease earlier, and have more severe disease, with greater demyelination and CNS inflammation, compared to WT mice (142). This result was contrary to findings in IL-12Rβ1-deficient mice (excluded IL-12 signaling) (143). Also, it has been shown that IL-23, not IL-12, plays the key role in the development of CNS autoimmune inflammation, affecting the subset of memory Th1 cells (144). It has also been shown that IL-23 induces differentiation of highly encephalitogenic Th cells that produce IL-17A (145).

In attempts to clearly define the roles of Th1 and Th17 subpopulations in MS pathogenesis, it was shown that the transfer of Th17 cells induces more severe EAE compared to Th1 cells (58). Another study showed that autoantigen-specific Th1 and Th17 cells were able to induce disease with similar severity but with different pathological findings (133). Th1-mediated neuroinflammation was characterized by macrophage infiltration, while, in Th17-mediated disease, neutrophil predominated in CNS infiltrates (133). Also, it was found that Th17 cells induce mainly brain damage, in contrast to Th1 cells, which dominantly induce spinal cord inflammation (146).

IL-17 mediates EAE development by the stimulation of IL-17R expressed on endothelial cells, astrocytes, microglia, and resident neuroectodermal cells (147). Mouse astrocytes express receptor for IL-17 (148) when stimulated with recombinant IL-17A *in vitro*, but also, *in vivo* in the EAE model, they produce various cytokines and chemokines, IL-6, TNFα, CCL2, CCL3, CCL20, CXCL1, CXCL2, CXCL9, CXCL10, and CXCL11 (IP-9) (149–151), that promote the influx of immune cells into the CNS and mediate neuroinflammation. Similarly, human astrocytes cultured with IL-17 *in vitro* produce IL-6, a cytokine that perpetuates the differentiation of CD4 naive cells into Th17 cells (152). The role of IL-17-mediated activation of astrocytes in EAE pathogenesis was confirmed by attenuation of EAE in animals with blocked IL-17 signaling in astrocytes (152). IL-17 also contributes to EAE development by affecting the activity of NG2+ oligodendrocyte precursor cells (OPCs) (153). Further, *in vitro* treatment of these cells with IL-17 strongly inhibits the maturation of oligodendrocytes and reduces their survival (154). Another study also indicates that IL-17 mediates apoptosis and inhibits differentiation of oligodendrocytes *in vitro* (155). IL-17 stimulates the maturation of primary OPCs and their participation in inflammatory processes (156). Microglial cells stimulated *in vitro* with IL-17 produce inflammatory mediators IL-6 and CXCL2, while only LPS pre-stimulated microglia exert enhanced cytotoxic effects (157). Further, microglial cells co-cultured with Th1/Th17 cells, but not Th1-only cells, produce high amounts of IL-1β, IL-6, and TNF-α, which promote further Th17 differentiation, neuroinflammation, and damage (158). IL-17 disrupts blood–brain barrier (BBB) tight junctions *in vitro* and *in vivo* in MS and promotes CNS inflammation (120). In an EAE model, it has been shown that IL-17 disrupts BBB by the induction of oxidative stress in endothelial cells accompanied by down-modulation of the tight junction molecule occludin (159). IL-17A levels are elevated in the CSF of relapsing-remitting MS patients, and this level correlates with the level of BBB dysfunction. Also, the treatment of BBB cell line hCMEC/D3 with a combination of IL-17A and IL-6 reduces the expression of tight junction-associated genes and disrupts monolayer integrity (160). Indirect evidence supports the role of IL-17 in direct neuronal damage. Different neuronal populations express IL-17 receptor (161). Direct contact, resembling immune synapses, of MOG-specific Th17 cells and neurons in demyelinating lesions associated with axonal damage has been shown by confocal, electron, and intravital microscopy, indicating the central role of Th17 cells in neuronal dysfunction (162).

IL-22, a cytokine whose production specifically induces IL-23, contributes to the pathogenicity of Th17 cells (163). It has been reported that IL-22 contributes to MS severity (120) as well as dysregulated expression of IL-22 and its antagonist, IL-22BP (164). Single nucleotide polymorphism in the IL-22R A2 gene is associated with MS risk (91, 165). IL-22 can contribute to MS pathogenesis by enhancing the expression of Fas in oligodendrocytes, resulting in oligodendrocytic apoptosis, and decreasing the expression of FOXP3 in T cells (166). Production of IL-22 is increased during the peak phase of EAE and is decreased during remission (167). However, beside involvement in many neurological inflammations, IL-22 may also be protective (168).

Although Th17 cells and their hallmark cytokines IL-17, IL-22, and IL-23 have been marked as the crucial players in the pathogenesis of MS and EAE, however, mice lacking IL-17 and IL-22 develop EAE (62, 169).

Findings indicating that GM-CSF has the key role in the encephalitogenic potential of Th17 cells in mice (74, 170, 171), specifically, increased levels of GM-CSF in the cerebrospinal fluid and serum of active MS patients with the relapsing-remitting type of the disease and increased secretion of GM-CSF from T cells isolated from the peripheral blood and brain lesion of MS, suggest that GM-CSF also plays an important role in MS development (172, 173). Unlike other cytokines, GM-CSF plays a non-redundant role in EAE development, and its secretion alone is able to provide development of autoaggressive and pathogenic MOG-specific T cells (170). GM-CSF-deficient Th cells are not able to induce EAE, indicating that the encephalitogenic potential of both Th1 and Th17 cells depends on their GM-CSF production (74).

In our previous studies, we have shown that overcoming resistance to induction of EAE with MOG_{35-55} peptide of BALB/c mice by infection with murine cytomegalovirus (MCMV) (174) or by deletion of ST2 gene (169) is associated with increased production of IL-17 in T cells.

Disease developed by MCMV-infected BALB/c mice is accompanied with an increase in IL-17-positive CD4+ and CD8+ cells in the central nervous system. Brain infiltrates in MCMV-infected BALB/c mice were more significant that in C57BL/6 mice, with a similar number of CD4+ and CD8+ cells, contrary to the dominantly CD4+ cells in C57BL/6 mice, which develop "typical" EAE (105). The encephalitogenic potential of CD4+ T cells in the CNS infiltrates of BALB/c mice is further documented by the detection of CCR6, the key molecule that mediates the initial infiltration of the CNS by Th17 cells (174, 175). Almost equal participation of IFN-γ- and T-bet (Th1)- and IL-17- and RORγt (Th17)-expressing cells was found in the CNS of MCMV-infected MOG_{35-55} immunized BALB/c mice, in contrast to almost exclusive CNS infiltration with Th1 cells in C57BL/6 mice infected with γHV-68 before EAE induction (174, 176). Further, CNS infiltrates of BALB/c mice infected with MCMV before MOG_{35-55} immunization contained CD8+ cells that express T1 and T17 transcriptional factors and corresponding cytokines, TNF-α and IFN-γ (Tc1) and IL-17 (Tc17 cells) (174).

Since cerebrospinal fluid of early-stage MS patients contains a greater number of Tc17 cells in comparison with peripheral blood, these cells are considered to be required for the accumulation of Th17 cells in the CNS in MS (177). No inflammatory T1 and T17 cells were found in the CNS of BALB/c mice immunized with MOG_{35-55} (174), while in the CNS of unimmunized BALB/c mice infected with MCMV neonatally, Tc1 cells (IFN-γ and T-bet+) dominated (178). CD8+ T cells isolated from CNS of MCMV-infected and MOG_{35-55}-immunized mice produced inflammatory cytokines in response to *in vitro* MOG_{35-55} peptide stimulation but were not specific for viral epitopes pp89 and m164 (174). These findings indicate that the newly developing autoimmune process in MOG_{35-55}-immunized BALB/c mice previously infected with MCMV attracts a new population of IL-17-producing CD8+ cells that participate in the development of autoimmunity (177). These findings are in line with previous reports that the expansion of myelin-specific CD8+ T cells follows CD4+ T cell-mediated initiation of the autoimmune process in CNS, thus contributing to tissue damage (179). The significant presence of IL-17-, CCR6-, and RORγt-positive CD4+ and CD8+ cells in the CNS of MOG_{35-55-}immunized BALB/c mice with non-productive MCMV infection in contrast to uninfected BALB/c mice immunized with MOG_{35-55}, with negligible number of these cells in the CNS, indicates that MCMV infection probably modulates the activation and differentiation of antigen-presenting cells in the periphery, changing their signature cytokines, and thus, after additional stimulus, enables the development of Th17/Tc17 cells with encephalitogenic potential (174).

Our results indicate that MCMV infection of BALB/c mice significantly affects dendritic cells in peripheral lymph nodes, thus enabling differentiation of encephalitogenic cells (174). In line with the well-known capacity of MCMV to encode an analog of chemokine CCL2 (180) that induces monocyte recruitment and viral dissemination (181), we found a higher percentage of CCR2+ dendritic cells in the peripheral lymph nodes of MCMV-infected mice (174). In contrast with a previous report that MCMV attracts monocytes that acquire immunosuppressive characteristics (182), we found higher percentages of dendritic cell-expressing markers of activation, CD86 and CD40, and Th1-promoting cytokine IL-12, indicating that MCMV infection of BALB/c mice increases the proportion of inflammatory dendritic cells in peripheral lymph nodes and thus enables the development of encephalitogenic T cells (174).

IL-17 IN ALZHEIMER'S DISEASE

Alzheimer's Disease (AD) is the most common neurodegenerative disorder causing cognitive impairment in the elderly (183, 184). The histopathological hallmarks of AD are amyloid plaques in the brain, mainly consisting of fibrillary forms of amyloid β peptide-40 (Aβ-40) and amyloid β peptide-42 (Aβ-42) (185). The fibrillary forms of amyloid β found in the amyloid plaques are obtained by a sequential cleavage from amyloid precursor proteins (186, 187). Highly insoluble Aβ peptides generated in the CNS play a crucial role in the pathogenesis of AD; they activate the complement pathway (168) and stimulate microglia to produce the proinflammatory cytokines and chemokines and thus induce accumulation of inflammatory cells into the CNS (188, 189). This proinflammatory process mediated by microglia leads to neurodegeneration (188, 190), although microglia play a protective role also, due to the clearing of Aβ aggregates by phagocytosis (191). Aβ peptides also increase the production of reactive nitrogen and oxygen species by microglial cells, leading to oxidative stress development, stimulation of Th17 cells, and IL-17 production (192, 193). It appears that the main roles of IL-17 in AD pathogenesis are the attraction of neutrophils and the stimulation of their function. It has been shown that Aβ aggregates mediate the chemotaxis and the recruitment of neutrophils in the CNS of mice overexpressing human mutant amyloid precursor protein (APP), which produce IL-17 and thus amplify neutrophil entry in the CNS (192), although mesenteric lymph nodes of these mice have lower production of IL-17 as a consequence of reduced differentiation of Th17 cells (194). Since neutrophils are the main targets of IL-17 in the CNS but are also very important sources of this cytokine, these cells, by promoting inflammation and CNS tissue damage, could have an important role in the development of AD pathology. Results from *in vitro* experiments indicate that IL-17 might promote autophagy in neurons and thus induce neurodegeneration (195).

There have been more reports about the role of innate immunity in AD than about adaptive immunity, but increased activation of T and B lymphocytes was recently demonstrated in a triple transgenic mouse model that replicated Aβ and tau neuropathology (196). Moreover, it has been shown that these cells produced high levels of IL-2, TNF-α, IL-17, and GM-CSF, indicating that neurodegeneration in these mice is associated with Th17 polarization (196). Increased expression of IL-17, IL-22, and RORγt has been found in the hippocampus, CSF, and serum of rats after intrathecal injection of Aβ-42 peptide (197). In the same study, Zhang et al. indicated that after disruption of the blood–brain barrier with Aβ-42 injection, Th17 cells enter into the brain (197). Tian et al. reported that postoperative cognitive dysfunction is associated with an enhanced level of IL17A in the hippocampus and suggested that IL-17-mediated damage of the hippocampus leads to Aβ1-42 accumulation and thus probably to cognitive decline (198). Increased expression of RORγt, IL-23, and IL-17 was found in the brains of Aβ-42-injected rats, while Treg-related cytokines TGF-β and IL-35 were decreased (199). Activated Th1 or Th17 cells in the brain produce inflammatory cytokines IFN-γ or IL-17 and thus heighten the inflammatory cascade, recruit and activate immune cells, and promote AD neuropathology (192, 200).

In MS, cytokines released by Th17 cells bind to their receptors on neurons and activate the apoptotic pathway, leading to neurodegeneration (201). Expression of Fas and FasL is also increased in the brain of AD rats (197, 202), and it could be assumed that Th17 cells activate the apoptotic pathway in neurons by Fas/FasL interaction and thus contribute to the development of neurodegeneration in AD (197, 203).

Elevated levels of IL-1β in the brains of AD mice homozygous for a destructive mutation of TLR4 cause up-regulation of IL-17 (204). In a very recent study, it has been shown that the administration of blocking anti-IL-17 antibody decreases the cognitive impairment and neuroinflammation induced by $A\beta_{1-42}$ injection into cerebral ventricles of adult CD1 mice, as suggested by reduced $A\beta_{1-42}$, glial fibrillary acidic protein (GFAP), S100 proteins, and inflammatory mediators and cytokines (205). This result supports the previously indicated role of IL-17 and related cytokines in promoting AD neuroinflammation and neurodegeneration (206). On the other hand, there is a study that indicates a protective role for IL-17 in an animal model of AD (207). Intracranially overexpressed IL-17 reduced cerebral amyloid angiopathy and improved anxiety and learning deficits (207). Further, it has recently been shown that ICR mice injected with IL-17 have an improvement in spatial learning as measured by the Morris water maze test, which is associated with the promotion of maturation of already-formed neuroblasts and the inhibition of neuroprogenitor proliferation (208).

The number of both CD4+ and CD8+ T cells in the brain parenchyma and vascular endothelium in humans with AD is higher than in healthy controls (209). Further, naive lymphocytes obtained from AD patients had increased production of Th17-related cytokine IL-21 and had higher expression of Th17 transcription factor RORγt, while monocytes obtained from the same patients produced higher amounts of IL-6 and IL-23 (210). A higher proportion of Th17 cells has been noticed in peripheral blood of patients with mild cognitive impairment due to AD pathology than in subjects with mild cognitive impairment due to pathologies other than AD and healthy controls (211). Also, higher concentrations of IL-17 and IL-23 were detected in the serum of AD patients than in healthy controls (212). IL-17 is reported to be a good plasma biomarker for distinguishing individuals with AD from cognitively healthy control subjects (213). Also, it was reported that the IL-17 concentration in cerebrospinal fluid could be used antemortem for identification of frontotemporal lobar degeneration with tau pathology (214).

It has been proposed that a desirable AD vaccine should induce Th2 and inhibit Th1/Th17 immune responses to Aβ in order to limit or prevent neuroinflammation and subsequent neurodegeneration (215).

A number of reports indicate the important role of IL-17 in AD pathogenesis; however, the precise mechanism of IL-17 upregulation in the CNS of AD patients is not known. It is possible that microbial infection, as was reported for respiratory infection (216) or inadequate immune surveillance in the gut (194), induces higher IL-17 production in the CNS, which later leads to deposition of amyloid-β. However, the opposite sequence of events is possible; that is, deposition of amyloid-β and inadequate clearance stimulate receptors of innate immune cells and induce production of IL-17, which perpetuates AD pathogenesis.

IL-17 IN ISCHEMIC BRAIN INJURY

Brain ischemia causes necrosis of the affected CNS tissue due to the loss of nutritional supply (217). Damaged CNS tissue releases damage-associated molecular patterns (DAMPs) that stimulate resident innate immune cells in the CNS, in the first line microglia (218). Activated microglia cells have a dual role: these cells play a beneficial role by phagocytosis of damaged tissue but also release inflammatory mediators TNF-α, IL-1β, IL-6, and IL-17, which enhance inflammation and tissue damage (219). DAMP molecules, released after ischemic brain damage, such as high mobility group 1 box 1 (HMGB1) (220, 221) and peroxiredoxin, induce IL-23 production in microglia/macrophages by activating TLR2 and TLR4, which subsequently induce the expression of IL-17 in other immune cells but also in microglia (222).

Activated immune cells after reperfusion additionally damage CNS tissue and significantly contribute to overall tissue damage after stroke. Adaptive immunity most probably contributes to inflammation development in CNS tissue after ischemia-reperfusion, especially T cells (223, 224). Similar was found in animal models: RAG1(–/–) mice, after stroke induced by transient middle cerebral artery occlusion, developed reduced damage of brain tissue, but detrimental effects of T cells in cerebral ischemia did not depend on antigen recognition or TCR costimulation (225). The presence of Th1 as well as Th17 cells was noticed in the brain lesions in ischemic stroke (226). These cells release proinflammatory cytokines and thus contribute to tissue damage (226).

Waisman et al. indicate IL-17 as one of the molecules that play a particular role in the delayed phase of the postinfarct inflammatory cascade (217). Increased expression of IL-17 mRNA in peripheral blood mononuclear cells was detected in patients after ischemic stroke, and its expression was in correlation with Scandinavian Stroke Scale scores (227). High

TABLE 1 | The main cellular source of IL-17 and its target cells in chronic inflammatory neurological diseases.

	Multiple sclerosis	Alzheimer's disease	Ischemic brain injury
Main source of IL-17	Th17	Neutrophils	γδ T cells
IL-17 target cells	• Astrocytes • BBB endothelial cells • Microglia/macrophages • Oligodendrocyte precursor cells	• Neutrophils • Neurons • Microglia/macrophages • BBB endothelial cells	• BBB endothelial cells • Astrocytes • Neurons • Microglia/macrophages
Main biological effects of IL-17	• BBB disruption • Induction of inflammation • Myelin damage	• Induction of inflammation • Deposition of amyloid-β	• BBB disruption • Induction of inflammation • CNS tissue damage

BBB, Blood–Brain Barrier.

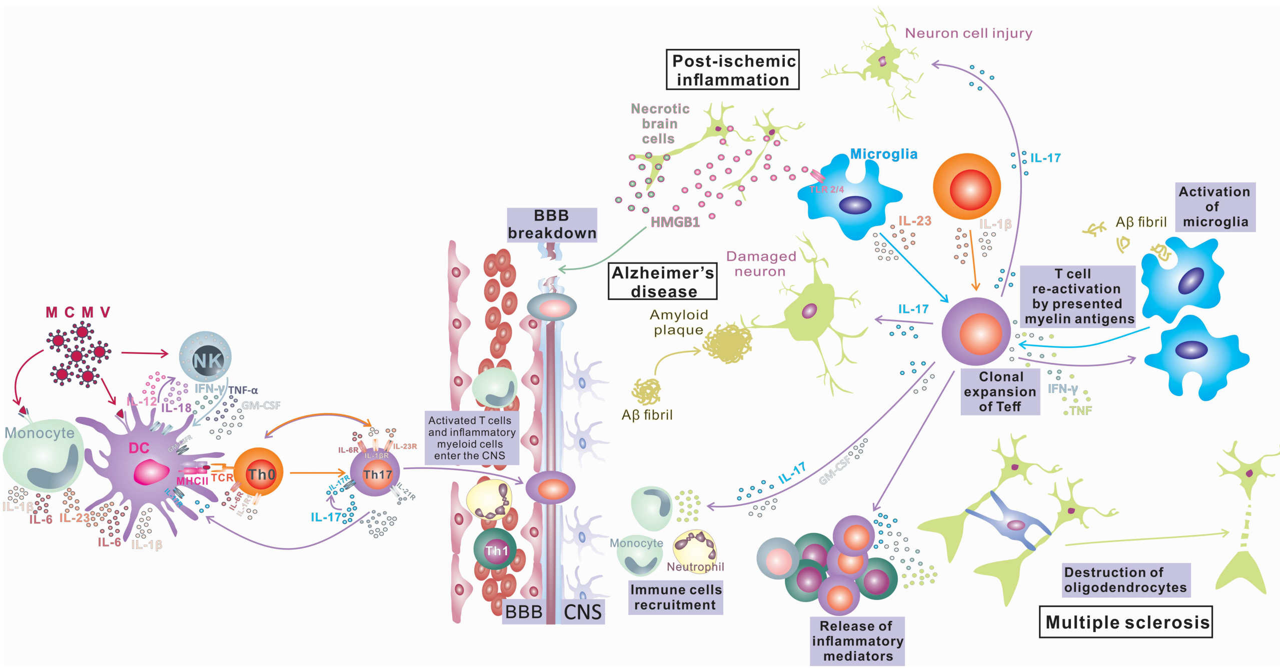


FIGURE 1 | IL-17 in Inflammatory Diseases of the Central Nervous System. Infection in the periphery (viral infections) activates innate immunity (monocytes/macrophages and NK cells) and induces a proinflammatory environment that changes the phenotype of antigen-presenting cells, which differentiate into inflammatory APCs that produce inflammatory cytokines IL-1, IL-6, IL-12, and IL-23. These APCs induce bystander activation of autoreactive T cells and their differentiation toward encephalitogenic T cells (IFN-γ, IL-17, TNF-α, Tbet, RORγt, CXCR3, and CCR6 positive) capable of entering the CNS, where after reactivation in contact with antigens presented by local tissue APCs, they proliferate and produce cytokines (IL-17, GM-CSF) that contribute to BBB disruption and recruitment of other immune cells into the CNS, finally inducing myelin damage (Multiple sclerosis). Peripheral infections can compromise the BBB and lead to an influx of IL-17-producing cells into the CNS. IL-17 can induce damage to neurons by direct cytotoxic effects or by recruitment of neutrophils and induction of inflammation, leading to deposition of amyloid fibrils and plaque formation (Alzheimer's disease). Also, the opposite order of events is possible, where microglia phagocytize amyloid fibrils and induce differentiation of T cells toward IL-17-producing cells, and the released IL-17 damages the BBB, recruits neutrophils, and induces inflammation and neuron damage, which exacerbates amyloid deposition (Alzheimer's disease). CNS tissue damaged by ischemia releases damage-associated molecular patterns (HMGB1) that stimulate microglia to release inflammatory mediators TNF-α, IL-1β, IL-6, and IL-17, which enhance inflammation and tissue damage. Activated microglia also can induce Th17 development. IL-17 released by innate immune cells or Th17 cells can enhance BBB damage, recruit immune cells, and enhance inflammation, inducing direct neuronal damage (postischemic inflammation).

expression of IL-17 was observed in ischemic injured brain tissue in experimental animals and also in postmortem analyzed human tissues (228–230). Also, higher expression of IL-17 at the mRNA and protein levels has been detected in the penumbral brain tissue 1, 3, and 6 days after reperfusion in mice (231). In an animal model of ischemic stroke, an increased number of IL-17-producing blood mononuclear cells were observed (232). In another study, IL-17 levels were elevated 3 days after reperfusion. This induction of IL-17 production was IL-23-dependent, and γδ T cells were indicated as the main source of IL-17 (233). In this study, it has been shown that IL-17 plays the main role in the stage of tissue damage after infarction, since IL-17-deficient mice had attenuated damage only on day 4 after ischemic insult (234). On the other hand, IL-23p19-deficient mice developed attenuated CNS tissue damage on day one after stroke induction (234), but γδ T-cell-deficient mice still develop brain injury after ischemia reperfusion induction (228). Although there is no clear evidence that Th17 cells play a role in tissue damage after stroke induction, activation of T cells and autoantigen-specific T cells, which exacerbates ischemic brain injury, was noticed in experimental animals (234). Also, an increase in the proportion of Th17 cells and a decrease in Treg cells in the periphery might contribute to CNS tissue damage after ischemia-reperfusion (235). Astrocytes are also marked as a source of IL-17 in inflammatory foci after brain ischemia-reperfusion (228, 236).

IL-17 may contribute to CNS tissue damage by several mechanisms, as described previously in other inflammatory diseases of CNS, affecting the cells that express IL-17 receptor, microglia, endothelial cells, astrocytes, and neurons, as summarized in **Table 1**. Although ischemia-reperfusion induces necrosis of blood–brain barrier, IL-17 could enhance BBB damage by the disruption of tight junctions (120) and by the promotion of monocyte migration across the BBB through an intracellular adhesion molecule (ICAM) 1-dependent mechanism (159). Levels of inflammatory cytokines IL-1β, TNFα, and matrix metalloproteinases, indicators of BBB damage, are decreased after stroke induction in IL-17-deficient mice (233). Increased expression of IL-17 receptor on neurons has been shown simultaneously with increased expression of IL-17 in CNS tissue after stroke induction, indicating the role of IL-17 in direct neuronal damage (230). This observation is supported by *in vitro* study (230). IL-17 also enhances autophagy in neurons and thus aggravates neuronal ischemic injuries (237). In synergy with TNF-α released by macrophages, IL-17 stimulates astrocytes to produce CXCL1, which recruits neutrophils into the CNS and thus enhances inflammation and damage (228). Astrocytes stimulated *in vitro* with TNFα and IL-17A show enhanced expression of several chemokines that have a role in the attraction of other immune cells, CCL20, CXCL2, CXCL9, CXCL10, and CXCL11, (153). IL-17, synergistically with IL-6, induces expression of CCL20 in astrocytes, which is a chemokine that attracts Th17 cells (149).

On the other hand, IL-17A induces the expression of molecules that have neuroprotective effects, brain-derived neurotrophic factors (BDNF), glia-derived neurotrophic factors (GDNF), and nerve growth factors (NGF), indicating that IL-17 might have a role in the reduction of damage (157). Recently, it has been shown that recombinant mouse IL-17A significantly attenuates damage of cortical astrocytes after stroke induction in a dose-dependent manner by inhibition of apoptosis (238).

CONCLUDING COMMENTS

Despite a large number of reports that indicate an important or, in some diseases, even indispensable role of IL-17 and Th17-related cytokines in inflammatory and degenerative neurological diseases, the precise mechanism of the pathogenic effect of IL-17 in the CNS is still elusive. Numerous *in vivo* and *in vitro* studies identify several types of CNS tissue cells as IL-17 targets and illustrate the effects of stimulation of these cells with IL-17 (summarized in **Figure 1**). However, the relative contributions of these processes to tissue damage and the development of inflammatory CNS diseases in humans are still undetermined. In order to gain new insights into the role of IL-17 in the pathogenesis and eventual new treatment of neuroinflammatory and neurodegenerative diseases, additional research in this field is required.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was funded by grants from the Serbian Ministry of Science and Technological Development, Grants No. ON175069, the Serbian bilateral project with PR China (06/2018), and the Faculty of Medical Sciences, University of Kragujevac (JP18/19 and JP19/19).

REFERENCES

1. Rouvier E, Luciani MF, Mattéi MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a Herpesvirus Saimiri gene. *J Immunol.* (1993) 150:5445–56.
2. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* (2005) 6:1123–32. doi: 10.1038/ni1254
3. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol.* (2005) 6:1133–41. doi: 10.1038/ni1261
4. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol.* (2010) 10:479–89. doi: 10.1038/nri2800
5. Amatya N, Garg AV, Gaffen SL. IL-17 signaling: the yin and the yang. *Trends Immunol.* (2017) 38:310–22. doi: 10.1016/j.it.2017.01.006
6. Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. *Cytokine.* (2013) 64:477–85. doi: 10.1016/j.cyto.2013.07.022
7. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Kim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* (2011) 332:65–68. doi: 10.1126/science.1200439
8. Boisson B, Wang C, Pedergnana V, Wu L, Cypowyj S, Rybojad M, et al. A biallelic ACT1 mutation selectively abolishes interleukin-17 responses in humans with chronic mucocutaneous candidiasis. *Immunity.* (2013) 39:676–86. doi: 10.1016/j.immuni.2013.09.002
9. Puel A, Döffinger R, Natividad A, Chrabieh M, Barcenas-Morales G, Picard C, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med.* (2010) 207:291–7. doi: 10.1084/jem.20091983
10. Sanford M, McKeage K. Secukinumab: first global approval. *Drugs.* (2015) 75:329–38. doi: 10.1007/s40265-015-0359-0

11. Blauvelt A, Papp KA, Griffiths CEM, Puig L, Weisman J, Dutronc Y, et al. Phase 3 Trials of Ixekizumab in Moderate-to-Severe Plaque Psoriasis. *N Engl J Med.* (2016) 375:345–56. doi: 10.1056/NEJMoa1512711
12. Miossec P, Kolls JK. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov.* (2012) 11:763–76. doi: 10.1038/nrd3794
13. Targan SR, Feagan B, Vermeire S, Panaccione R, Melmed GY, Landers C, et al. A Randomized, Double-Blind, Placebo-Controlled Phase 2 Study of Brodalumab in Patients With Moderate-to-Severe Crohn's Disease. *Am J Gastroenterol.* (2016) 111:1599–607. doi: 10.1038/ajg.2016.298
14. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut.* (2012) 61:1693–700. doi: 10.1136/gutjnl-2011-301668
15. Panaccione R, Sandborn WJ, Gordon GL, Lee SD, Safdi A, Sedghi S, et al. Briakinumab for treatment of Crohn's disease: results of a randomized trial. *Inflamm Bowel Dis.* (2015) 21:1329–40. doi: 10.1097/MIB.0000000000000366
16. Ito H, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, et al. A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology.* (2004) 126:989–96. doi: 10.1053/j.gastro.2004.01.012
17. Sandborn WJ, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, et al. Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med.* (2012) 367:1519–28. doi: 10.1056/NEJMoa1203572
18. Lee JS, Tato CM, Joyce-Shaikh B, Gulen MF, Cayatte C, Chen Y, et al. Interleukin-23-dependent IL-17 production regulates intestinal epithelial permeability. *Immunity.* (2015) 43:727–38. doi: 10.1016/j.immuni.2015.09.003
19. Novatchkova M, Leibbrandt A, Werzowa J, Neubüser A, Eisenhaber F. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem Sci.* (2003) 28:226–9. doi: 10.1016/S0968-0004(03)00067-7
20. Leonardi A, Chariot A, Claudio E, Cunningham K, Siebenlist U. CIKS, a connection to IκB kinase and stress-activated protein kinase. *Proc Natl Acad Sci USA.* (2000) 97:10494–9. doi: 10.1073/pnas.190245697
21. Song X, Qian Y. The activation and regulation of IL-17 receptor mediated signaling. *Cytokine.* (2013) 62:175–82. doi: 10.1016/j.cyto.2013.03.014
22. Hartupee J, Liu C, Novotny M, Li X, Hamilton T. IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol.* (2007) 179:4135–41. doi: 10.4049/jimmunol.179.6.4135
23. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol.* (2014) 14:585–600. doi: 10.1038/nri3707
24. Ruiz de Morales JMG, Puig L, Daudén E, Cañete JD, Pablos JL, Martin AO, et al. Critical role of interleukin (IL)-17 in inflammatory and immune disorders: an updated review of the evidence focusing in controversies. *Autoimmun Rev*. (2020) 19:102429. doi: 10.1016/j.autrev.2019.102429
25. Onishi RM, Gaffen SL. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology.* (2010) 129:311–21. doi: 10.1111/j.1365-2567.2009.03240.x
26. Shen F, Gaffen SL. Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy. *Cytokine.* (2008) 41:92–104. doi: 10.1016/j.cyto.2007.11.013
27. Chang SH, Dong C. Signaling of interleukin-17 family cytokines in immunity and inflammation. *Cell Signal.* (2011) 23:1069–75. doi: 10.1016/j.cellsig.2010.11.022
28. Ramani K, Garg AV, Jawale CV, Conti HR, Whibley N, Jackson EK, et al. The kallikrein-kinin system: a novel mediator of IL-17-driven anti-candida immunity in the kidney. *PLoS Pathog.* (2016) 12:e1005952. doi: 10.1371/journal.ppat.1005952
29. Conti HR, Baker O, Freeman AF, Jang WS, Holland SM, Li RA, et al. New mechanism of oral immunity to mucosal candidiasis in hyper-IgE syndrome. *Mucosal Immunol.* (2011) 4:448–455. doi: 10.1038/mi.2011.5
30. Kotake S, Udagawa N, Takahashi N, Matsuzaki K, Itoh K, Ishiyama S, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest.* (1999) 103:1345–52. doi: 10.1172/JCI5703
31. Gaffen SL, Hajishengallis G. A new inflammatory cytokine on the block: re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. *J Dent Res.* (2008) 87:817–28. doi: 10.1177/154405910808700908
32. Lee JY, Hall JA, Kroehling L, Wu L, Najar T. Serum amyloid a proteins induce pathogenic Th17 cells and promote inflammatory disease. *Cell.* (2020) 180:79–91.e16. doi: 10.1016/j.cell.2019.11.026
33. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. *Nature.* (2016) 535:75–84. doi: 10.1038/nature18848
34. Takatori H, Kanno Y, Chen Z, O'Shea JJ. New complexities in helper T cell fate determination and the implications for autoimmune diseases. *Mod Rheumatol.* (2008) 18:533–41. doi: 10.3109/s10165-008-0099-z
35. Vaknin-Dembinsky A, Balashov K, Weiner HL. IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J Immunol.* (2006) 176:7768–74. doi: 10.4049/jimmunol.176.12.7768
36. Wen SR, Liu GJ, Feng RN, Gong FC, Zhong H, Duan SR, et al. Increased levels of IL-23 and osteopontin in serum and cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol.* (2012) 244:94–6. doi: 10.1016/j.jneuroim.2011.12.004
37. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. *Immunity.* (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
38. Patel DD, Kuchroo VK. Th17 cell pathway in human immunity: lessons from genetics and therapeutic interventions. *Immunity.* (2015) 43:1040–51. doi: 10.1016/j.immuni.2015.12.003
39. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol.* (2017) 17:535–44. doi: 10.1038/nri.2017.50
40. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* (2006) 441:235–8. doi: 10.1038/nature04753
41. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-β induces development of the T(H)17 lineage. *Nature.* (2006) 441:231–4. doi: 10.1038/nature04754
42. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. *Immunity.* (2006) 24:179–89. doi: 10.1016/j.immuni.2006.01.001
43. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol.* (2007) 8:967–74. doi: 10.1038/ni1488
44. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity.* (2009) 17:576–87. doi: 10.1016/j.immuni.2009.02.007
45. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol.* (2011) 12:255–63. doi: 10.1038/ni.1993
46. Komuczki J, Tuzlak S, Friebel E, Hartwig T, Spath S, Rosenstiel P, et al. Fate-Mapping of GM-CSF expression identifies a discrete subset of inflammation-driving T helper cells regulated by cytokines IL-23 and IL-1β. *Immunity.* (2019) 21:1289–304.e6. doi: 10.1016/j.immuni.2019.04.006
47. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol.* (2009) 10:314–24. doi: 10.1038/ni.1698
48. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* (2006) 126:1121–33. doi: 10.1016/j.cell.2006.07.035
49. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors RORα and RORγ. *Immunity.* (2008) 28:29–39. doi: 10.1016/j.immuni.2007.11.016
50. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science.* (1994) 264:95–8. doi: 10.1126/science.8140422
51. Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat3 activation is responsible for IL-6-dependent T cell proliferation through

preventing apoptosis: generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol.* (1998) 161:4652–60.

52. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem.* (2007) 282:9358–63. doi: 10.1074/jbc.C600321200
53. Eugster HP, Frei K, Kopf M, Lassmann H, Fontana A. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Eur J Immunol.* (1998) 28:2178–87. doi: 10.1002/(SICI)1521-4141(199807)28:07<2178::AID-IMMU2178>3.0.CO;2-D
54. Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-β signalling. *Nature.* (2010) 467:967–71. doi: 10.1038/nature09447
55. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity.* (2007) 26:371–81. doi: 10.1016/j.immuni.2007.02.009
56. Kim HS, Jang SW, Lee W, Kim K, Sohn H, Hwang SS, et al. PTEN drives Th17 cell differentiation by preventing IL-2 production. *J Exp Med.* (2017) 214:3381–98. doi: 10.1084/jem.20170523
57. Quintana FJ, Jin H, Burns EJ, Nadeau M, Yeste A, Kumar D, et al. Aiolos promotes TH17 differentiation by directly silencing Il2 expression. *Nat Immunol.* (2012) 13:770–7. doi: 10.1038/ni.2363
58. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* (2005) 201:233–40. doi: 10.1084/jem.20041257
59. Schiffenbauer J, Streit WJ, Butfiloski E, LaBow M, Edwards C III, Moldawer LL. The induction of EAE is only partially dependent on TNF receptor signaling but requires the IL-1 type I receptor. *Clin Immunol.* (2000) 95:117–23. doi: 10.1006/clim.2000.4851
60. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med.* (2006) 203:1685–91. doi: 10.1084/jem.20060285
61. Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y. Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis. *Int Immunol.* (2006) 18:399–407. doi: 10.1093/intimm/dxh379
62. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, et al. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nat. Immunol.* (2007) 8:1390–7. doi: 10.1038/ni1539
63. Lee Y, Awasthi A, Yosef N, Quintana FJ, Xiao S, Peters A, et al. Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol.* (2012) 13:991–9. doi: 10.1038/ni.2416
64. Carr TM, Wheaton JD, Houtz GM, Ciofani M. JunB promotes Th17 cell identity and restrains alternative CD4+ T-cell programs during inflammation. *Nat Commun.* (2017) 8:301. doi: 10.1038/s41467-017-00380-3
65. Hasan Z, Koizumi SI, Sasaki D, Yamada H, Arakaki N, Fujihara Y, et al. JunB is essential for IL-23-dependent pathogenicity of Th17 cells. *Nat Commun.* (2017) 8:15628. doi: 10.1038/ncomms15628
66. Yasuda K, Takeuchi Y, Hirota K. Correction to: the pathogenicity of Th17 cells in autoimmune diseases. *Semin Immunopathol.* (2019) 41:299. doi: 10.1007/s00281-019-00746-3
67. Basu R, Whitley SK, Bhaumik S, Zindl CL, Schoeb TR, Benveniste EN, et al. IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance. *Nat Immunol.* (2015) 16:286–95. doi: 10.1038/ni.3099
68. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol.* (2013) 14:372–9. doi: 10.1038/ni.2552
69. Gaublomme JT, Yosef N, Lee Y, Gertner RS, Yang LV, Wu C, et al. Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. *Cell.* (2015) 163:1400–12. doi: 10.1016/j.cell.2015.11.009
70. Wang C, Yosef N, Gaublomme J, Wu C, Lee Y, Clish CB, et al. CD5L/AIM regulates lipid biosynthesis and restrains Th17 cell pathogenicity. *Cell.* (2015) 163:1413–27. doi: 10.1016/j.cell.2015.10.068
71. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* (2000) 13:715–25. doi: 10.1016/S1074-7613(00)00070-4
72. Ichiyama K, Gonzalez-Martin A, Kim BS, Jin HY, Jin W, Xu W, et al. The MicroRNA-183-96-182 cluster promotes t helper 17 cell pathogenicity by negatively regulating transcription factor foxo1 expression. *Immunity.* (2016) 44:1284–98. doi: 10.1016/j.immuni.2016.05.015
73. Meyer Zu Horste G, Wu C, Wang C, Cong L, Pawlak M, Lee Y, et al. RBPJ controls development of pathogenic Th17 cells by regulating IL-23 receptor expression. *Cell Rep.* (2016) 16:392–404. doi: 10.1016/j.celrep.2016.05.088
74. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Safavi F, et al. The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol.* (2011) 12:568–75. doi: 10.1038/ni.2031
75. Jain R, Chen Y, Kanno Y, Joyce-Shaikh B, Vahedi G, Hirahara K, et al. Interleukin-23-induced transcription factor blimp-1 promotes pathogenicity of T helper 17 cells. *Immunity.* (2016) 44:131–42. doi: 10.1016/j.immuni.2015.11.009
76. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol.* (2007) 8:942–9. doi: 10.1038/ni1496
77. Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol.* (2007) 8:950–7. doi: 10.1038/ni1497
78. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. *Nat Immunol.* (2008) 9:641–9. doi: 10.1038/ni.1610
79. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupé P, Barillot E, et al. A critical function for transforming growth factor-β, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol.* (2008) 9:650–7. doi: 10.1038/ni.1613
80. Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum.* (2007) 56:2936–46. doi: 10.1002/art.22866
81. Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature.* (2007) 448:1058–62. doi: 10.1038/nature06096
82. Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature.* (2008) 452:773–6. doi: 10.1038/nature06764
83. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature.* (2008) 453:65–71. doi: 10.1038/nature06880
84. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature.* (2008) 453:106–9. doi: 10.1038/nature06881
85. Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell.* (2011) 146:772–84. doi: 10.1016/j.cell.2011.07.033
86. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med.* (2011) 208:1367–76. doi: 10.1084/jem.20110278
87. Karmaus PWF, Chen X, Lim SA, Herrada AA, Nguyen TM, Xu B, et al. Metabolic heterogeneity underlies reciprocal fates of TH17 cell stemness and plasticity. *Nature.* (2019) 565:101–5. doi: 10.1038/s41586-018-0806-7
88. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature.* (2013) 496:513–7. doi: 10.1038/nature11984
89. Kleinewietfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, et al. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature.* (2013) 496:518–22. doi: 10.1038/nature11868
90. Weinshenker BG. Epidemiology of multiple sclerosis. *Neurol Clin.* (1996) 14:291–308. doi: 10.1016/S0733-8619(05)70257-7

91. Wang K, Song F, Fernandez-Escobar A, Luo G, Wang JH, Sun Y. The properties of cytokines in multiple sclerosis: pros and cons. *Am J Med Sci.* (2018) 356:552–60. doi: 10.1016/j.amjms.2018.08.018
92. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med.* (2007) 204:1849–61. doi: 10.1084/jem.20070663
93. Ge Y. Multiple sclerosis: the role of mr imaging. *AJNR Am J Neuroradiol.* (2006) 27:1165–76.
94. Hauser SL, Oksenberg JR. The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron.* (2006) 52:61–76. doi: 10.1016/j.neuron.2006.09.011
95. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest.* (2012) 122:1180–8. doi: 10.1172/JCI58649
96. Wu GF, Alvarez E. The immunopathophysiology of multiple sclerosis. *Neurol Clin.* (2011) 29:257–78. doi: 10.1016/j.ncl.2010.12.009
97. Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol.* (2000) 47:707–17. doi: 10.1002/1531-8249(200006)47:6<707::AID-ANA3>3.0.CO;2-Q
98. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann Neurol.* (2006) 60:12–21. doi: 10.1002/ana.20913
99. Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Ann Rev Immunol.* (2002) 20:101–23. doi: 10.1146/annurev.immunol.20.081701.141316
100. Furlan R, Cuomo C, Martino G. Animal models of multiple sclerosis. *Methods Mol Biol.* (2009) 549:157–73. doi: 10.1007/978-1-60327-931-4_11
101. McGinley AM, Edwards SC, Raverdeau M, Mills KHG. Th17 cells, γδ T cells and their interplay in EAE and multiple sclerosis. *J Autoimmun.* (2018) 87, 97-108.. doi: 10.1016/j.jaut.2018.01.001
102. Mills KH. TLR-dependent T cell activation in autoimmunity. *Nat Rev Immunol.* (2011) 11:807–22. doi: 10.1038/nri3095
103. Williams KC, Ulvestad E, Hickey WF. Immunology of multiple sclerosis. *Clin Neurosci.* (1994) 2:229–45.
104. Kutzelnigg A1, Faber-Rod JC, Bauer J, Lucchinetti CF, Sorensen PS, Laursen H, et al. Widespread demyelination in the cerebellar cortex in multiple sclerosis. *Brain Pathol.* (2007) 17:38–44. doi: 10.1111/j.1750-3639.2006.00041.x
105. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med.* (2000) 192:393–404. doi: 10.1084/jem.192.3.393
106. BD Trapp BD, Nave KA. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci.* (2008) 31:247–69. doi: 10.1146/annurev.neuro.30.051606.094313
107. Rudick RA, Fisher E, Lee JC, Simon J, Jacobs L. Use of the brain parenchymal fraction to measure whole brain atrophy in relapsing-remitting MS. Multiple Sclerosis Collaborative Research Group. *Neurology.* (1999) 53:1698–704. doi: 10.1212/WNL.53.8.1698
108. Hampton DW, Anderson J, Pryce G, Irvine KA, Giovannoni G, Fawcett JW, et al. An experimental model of secondary progressive multiple sclerosis that shows regional variation in gliosis, remyelination, axonal and neuronal loss. *J Neuroimmunol.* (2008) 201–202:200–11. doi: 10.1016/j.jneuroim.2008.05.034
109. Farooqi N, Gran B, Constantinescu CS. Are current disease-modifying therapeutics in multiple sclerosis justified on the basis of studies in experimental autoimmune encephalomyelitis? *J Neurochem.* (2010) 115:829–44. doi: 10.1111/j.1471-4159.2010.06982.x
110. Wujek JR, Bjartmar C, Richer E, Ransohoff RM, Yu M, Tuohy VK, et al. Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J Neuropathol Exp Neurol.* (2002) 61:23–32. doi: 10.1093/jnen/61.1.23
111. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, Toyka KV, et al. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol.* (2005) 237:123–30. doi: 10.1016/j.cellimm.2005.11.002
112. Traugott U, Raine CS, McFarlin DE. Acute experimental allergic encephalomyelitis in the mouse: immunopathology of the developing lesion. *Cell. Immunol.* (1985) 91:240–54. doi: 10.1016/0008-8749(85)90047-4
113. Dungan LS, McGuinness NC, Boon L, Lynch MA, Mills KH. Innate IFN-γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages. *Eur J Immunol.* (2014) 44:2903–17. doi: 10.1002/eji.201444612
114. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med.* (2002) 8:500–8. doi: 10.1038/nm0502-500
115. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity.* (2004) 21:467–76. doi: 10.1016/j.immuni.2004.08.018
116. Schofield C, Fischer SK, Townsend MJ, Mosesova S, Peng K, Setiadi AF, et al. Characterization of IL-17AA and IL-17FF in rheumatoid arthritis and multiple sclerosis. *Bioanalysis.* (2016) 8:2317–27. doi: 10.4155/bio-2016-0207
117. Hedegaard CJ, Krakauer M, Bendtzen K, Lund H, Sellebjerg F, Nielsen CH. T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. *Immunology.* (2008) 125:161–9. doi: 10.1111/j.1365-2567.2008.02837.x
118. Brucklacher-Waldert V, Stuerner K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain.* (2009) 132(Pt 12):3329–41. doi: 10.1093/brain/awp289
119. Durelli L, Conti L, Clerico M, Boselli D, Contessa G, Ripellino P, et al. T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-β. *Ann. Neurol.* (2009) 65:499–509. doi: 10.1002/ana.21652
120. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med.* (2007) 13:1173–5. doi: 10.1038/nm1651
121. Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, et al. Preferential recruitment of interferon-γ-expressing TH17 cells in multiple sclerosis. *Ann Neurol.* (2009) 66:390–402. doi: 10.1002/ana.21748
122. Schirmer L, Rothhammer V, Hemmer B, Korn T. Enriched CD161high CCR6+ γδ T cells in the cerebrospinal fluid of patients with multiple sclerosis. *JAMA Neurol.* (2013) 70:345–51. doi: 10.1001/2013.jamaneurol.409
123. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol.* (2008) 172:146–55. doi: 10.2353/ajpath.2008.070690
124. Korn T, Bettelli E, GaoW, Awasthi A, Jager A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature.* (2007) 448:484–7. doi: 10.1038/nature05970
125. Wing AC, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Sacramento PM, et al. Interleukin-17-and interleukin-22-secreting myelin-specific CD4+ T cells resistant to corticoids are related with active brain lesions in multiple sclerosis patients. *Immunology.* (2016) 147:212–20. doi: 10.1111/imm.12552
126. Brod SA. Ingested (Oral) Rituximab Inhibits Eae. *Cytokine.* (2016) 85:177–83. doi: 10.1016/j.cyto.2016.06.026
127. Segal BM. Th17 cells in autoimmune demyelinating disease. *Semin Immunopathol.* (2010) 32:71–7. doi: 10.1007/s00281-009-0186-z
128. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol.* (2006) 177:566–73. doi: 10.4049/jimmunol.177.1.566
129. da Costa DS, Hygino J, Ferreira TB, Kasahara TM, Barros PO, Monteiro C, et al. Vitamin D modulates different IL-17-secreting T cell subsets in multiple sclerosis patients. *J Neuroimmunol.* (2016) 299:8–18. doi: 10.1016/j.jneuroim.2016.08.005
130. Lückel C, Picard F, Raifer H, Campos Carrascosa L, Guralnik A, Zhang Y, et al. IL-17+ CD8+ T cell suppression by dimethyl fumarate associates with clinical response in multiple sclerosis. *Nat Commun.* (2019) 10:5722. doi: 10.1038/s41467-019-13731-z
131. Ntolkeras G, Barba C, Mavropoulos A, Vasileiadis GK, Dardiotis E, Sakkas LI, et al. On the immunoregulatory role of statins in multiple

sclerosis: the effects on Th17 cells. *Immunol Res.* (2019) 67:310–324. doi: 10.1007/s12026-019-09089-5
132. Havrdová E, Belova A, Goloborodko A, Tisserant A, Wright A, Wallstroem E, et al. Activity of secukinumab, an anti-IL-17A antibody, on brain lesions in RRMS: results from a randomized, proof-of-concept study. *J Neurol.* (2016) 263:1287–95. doi: 10.1007/s00415-016-8128-x
133. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM. IL-12–and IL-23–modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med.* (2008) 205:1535–41. doi: 10.1084/jem.20080159
134. Ferber IA, Brocke S, Taylor-Edwards C, Ridgway W, Dinisco C, Steinman L, et al. Mice with a disrupted IFN-γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol.* (1996) 156:5–7.
135. Heremans H, Dillen C, Groenen M, Martens E, Billiau A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-γ. *Europ J Immunol.* (1996) 26:2393–8. doi: 10.1002/eji.1830261019
136. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol.* (2007) 8:639–46. doi: 10.1038/ni1467
137. Lee YK, Mukasa R, Hatton RD, Weaver CT. Developmental plasticity of Th17 and Treg cells. *Curr Opin Immunol.* (2009) 21:274–80. doi: 10.1016/j.coi.2009.05.021
138. Abromson-Leeman S, Bronson RT, Dorf ME. Encephalitogenic T cells that stably express both T-bet and RORγt consistently produce IFNγ but have a spectrum of IL-17 profiles. *J Neuroimmunol.* (2009) 215:10–24. doi: 10.1016/j.jneuroim.2009.07.007
139. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol.* (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710
140. Touil T, Fitzgerald D, Zhang GX, Rostami AM, Gran B. Pathophysiology of interleukin-23 in experimental autoimmune encephalomyelitis. *Drug News Perspect.* (2006) 19:77–83. doi: 10.1358/dnp.2006.19.2.977443
141. Gerosa F, Baldani-Guerra B, Lyakh LA, Batoni G, Esin S, Winkler-Pickett RT, et al. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J Exp Med.* (2008) 205:1447–61. doi: 10.1084/jem.20071450
142. Zhang GX, Gran B, Yu S, Li J, Siglienti I, Chen X, et al. Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-β 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol.* (2003) 170:2153–60. doi: 10.4049/jimmunol.170.4.2153
143. Lyakh L, Trinchieri G, Provezza L, Carra G, Gerosa F. Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. *Immunol Rev.* (2008) 226:112–31. doi: 10.1111/j.1600-065X.2008.00700.x
144. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature.* (2003) 421:744–8. doi: 10.1038/nature01355
145. Fitzgerald DC, Ciric B, Touil T, Harle H, Grammatikopolou J, Das Sarma J, et al. Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. *J Immunol.* (2007) 179:3268–75. doi: 10.4049/jimmunol.179.5.3268
146. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med.* (2008) 14:337–42. doi: 10.1038/nm1715
147. Lin W, Wang N, Zhou K, Su F, Jiang Y, et al. RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling. *EMBO Rep.* (2018) 19:e44951. doi: 10.15252/embr.201744951
148. Das Sarma J, Ciric B, Marek R, Sadhukhan S, Caruso ML, Shafagh J, et al. Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. *J Neuroinflamm.* (2009) 6:14. doi: 10.1186/1742-2094-6-14
149. Meares GP, Ma X, Qin H, Benveniste EN. Regulation of CCL20 expression in astrocytes by IL-6 and IL-17. *Glia.* (2012) 60:771–81. doi: 10.1002/glia.22307
150. Yi H, Bai Y, Zhu X, Lin L, Zhao L, Wu X, et al. IL-17A induces MIP-1alpha expression in primary astrocytes via Src/MAPK/PI3K/NF-kB pathways:implications for multiple sclerosis. *J Neuroimmune Pharmacol.* (2014) 9:629–41. doi: 10.1007/s11481-014-9553-1
151. Xiao Y, Jin J, Chang M, Nakaya M, Hu H, Zou Q, et al. TPL2 mediates autoimmune inflammation through activation of the TAK1 axis of IL-17 signaling. *J Exp Med.* (2014) 211:1689–1702. doi: 10.1084/jem.20132640
152. Elain G, Jeanneau K, Rutkowska A, Mir AK, Dev KK. The selective anti-IL17A monoclonal antibody secukinumab (AIN457) attenuates IL17A-induced levels of IL6 in human astrocytes. *Glia.* (2014) 62:725–35. doi: 10.1002/glia.22637
153. Kang Z, Altuntas CZ, Gulen MF, Liu C, Giltiay N, Qin H, et al. Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity.* (2010) 32:414–25. doi: 10.1016/j.immuni.2010.03.004
154. Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, et al. Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nat Neurosci.* (2013) 16:1401–8. doi: 10.1038/nn.3505
155. Paintlia MK, Paintlia AS, Singh AK, Singh I. Synergistic activity of interleukin-17 and tumor necrosis factor-alpha enhances oxidative stress-mediated oligodendrocyte apoptosis. *J Neurochem.* (2011) 116:508–21. doi: 10.1111/j.1471-4159.2010.07136.x
156. Rodgers JM, Robinson AP, Rosler ES, Lariosa-Willingham K, Persons RE, Dugas JC, et al. IL-17A activates ERK1/2 and enhances differentiation of oligodendrocyte progenitor cells. *Glia.* (2015) 63:768–79. doi: 10.1002/glia.22783
157. Kawanokuchi J, Shimizu K, Nitta A, Yamada K, Mizuno, Takeuchia H, et al. Production and functions of IL-17 in microglia. *J Neuroimmunol.* (2008) 194:54–61. doi: 10.1016/j.jneuroim.2007.11.006
158. Murphy AC, Lalor SJ, Lynch MA, Mills KH. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav Immun.* (2010) 24:641–51. doi: 10.1016/j.bbi.2010.01.014
159. Huppert J, Closhen D, Croxford A, White R, Kulig P, Pietrowski E, et al. Cellular mechanisms of IL-17-induced blood-brain barrier disruption. *FASEB J.* (2010) 24:1023–34. doi: 10.1096/fj.09-141978
160. Setiadi AF, Abbas AR, Jeet S, Wong K, Bischof A, Peng I, et al. IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. *J Neuroimmunol.* (2019) 332:147–54. doi: 10.1016/j.jneuroim.2019.04.011
161. Reed MD, Yim YS, Wimmer RD, Kim H, Ryu C, Welch GM, et al. IL-17a promotes sociability in mouse models of neurodevelopmental disorders. *Nature.* (2020) 577:249–53. doi: 10.1038/s41586-019-1843-6
162. Siffrin V, Radbruch H, Glumm R, Niesner R, Paterka M, Herz J, et al. *In vivo* imaging of partially reversible Th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. *Immunity.* (2010) 33:424–36. doi: 10.1016/j.immuni.2010.08.018
163. Kreymborg K, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, et al. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol.* (2007) 179:8098–104. doi: 10.4049/jimmunol.179.12.8098
164. Perriard G, Mathias A, Enz L, Canales M, Schluep M, Gentner M, et al. Interleukin-22 is increased in multiple sclerosis patients and targets astrocytes. *J Neuroinflammation.* (2015) 12:119. doi: 10.1186/s12974-015-0335-3
165. Beyeen AD, Adzemovic MZ, Ockinger J, Stridh P, Becanovic K, Laaksonen H, et al. IL-22RA2 associates with multiple sclerosis and macrophage effector mechanisms in experimental neuroinflammation. *J Immunol.* (2010) 185:6883–90. doi: 10.4049/jimmunol.1001392
166. Zhen J, Yuan J, Fu Y, Zhu R, Wang M, Chang H, et al. IL-22 promotes Fas expression in oligodendrocytes and inhibits FOXP3 expression in T cells by activating the NF-κB pathway in multiple sclerosis. *Mol Immunol.* (2017) 82:84–93. doi: 10.1016/j.molimm.2016.12.020
167. Almolda B, Costa M, Montoya M, González B, Castellano B. Increase in Th17 and Treg lymphocytes and decrease of IL22 correlate

with the recovery phase of acute EAE in rat. *PLoS ONE*. (2011) 6:e27473. doi: 10.1371/journal.pone.0027473
168. Xin N, Namaka MP, Dou C, Zhang Y. Exploring the role of interleukin-22 in neurological and autoimmune disorders. *Int Immunopharmacol*. (2015) 28:1076–83. doi: 10.1016/j.intimp.2015.08.016
169. Milovanovic M, Volarevic V, Ljujic B, Radosavljevic G, Jovanovic I, Arsenijevic N, et al. Deletion of IL-33R. (ST2) abrogates resistance to EAE in BALB/C mice by enhancing polarization of APC to inflammatory phenotype. *PLoS ONE*. (2012) 7:e45225. doi: 10.1371/journal.pone.0045225
170. Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, et al. RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phasof autoimmune neuroinflammation. *Nat Immunol*. (2011) 12:560–7. doi: 10.1038/ni.2027
171. Haak S, Croxford AL, Kreymborg K, Heppner FL, Pouly S, Becher B, et al. IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J Clin Invest*. (2009) 119:61–9. doi: 10.1172/JCI35997
172. Carrieri PB, Provitera V, De Rosa T, Tartaglia G, Gorga F, Perrella O. Profile of cerebrospinal fluid and serum cytokines in patients with relapsing-remitting multiple sclerosis: a correlation with clinical activity. *Immunopharmacol Immunotoxicol*. (1998) 20:373–82. doi: 10.3109/08923979809034820
173. Rasouli J, Ciric B, Imitola J, Gonnella P, Hwang D, Mahajan K, et al. Expression of GM-CSF in T cells is increased in multiple sclerosis and suppressed by IFN-β therapy. *J Immunol*. (2015) 194:5085–93. doi: 10.4049/jimmunol.1403243
174. Milovanovic J, Popovic B, Milovanovic M, Kvestak D, Arsenijevic A, Stojanovic B, et al. Murine cytomegalovirus infection induces susceptibility to EAE in resistant BALB/c mice. *Front Immunol*. (2017) 8:192. doi: 10.3389/fimmu.2017.00192
175. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, et al. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol*. (2009) 10:514–23. doi: 10.1038/ni.1716
176. Casiraghi C, Shanina I, Cho S, Freeman ML, Blackman MA, Horwitz MS. γherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein–Barr virus and multiple sclerosis. *PLoS Pathog*. (2012) 8:e1002715. doi: 10.1371/journal.ppat.1002715
177. Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J Clin Invest*. (2013) 123:247–60. doi: 10.1172/JCI63681
178. Bantug GR, Cekinovic D, Bradford R, Koontz T, Jonjic S, Britt WJ. CD8+ T lymphocytes control murine cytomegalovirus replication in the central nervous system of newborn animals. *J Immunol*. (2008) 181:2111–23. doi: 10.4049/jimmunol.181.3.2111
179. Ji Q, Castelli L, Goverman JM. MHC class I-restricted myelin epitopes are cross-presented by Tip-DCs that promote determinant spreading to CD8? T cells. *Nat Immunol*. (2013) 14:254–61. doi: 10.1038/ni.2513
180. Saederup N, Aguirre SA, Sparer TE, Bouley DM, Mocarski ES. Murine cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination that increases inflammation at initial sites of infection. *J Virol*. (2001) 75:9966–76. doi: 10.1128/JVI.75.20.9966-9976.2001
181. Noda S, Aguirre SA, Bitmansour A, Brown JM, Sparer TE, Huang J, et al. Cytomegalovirus MCK-2 controls mobilization and recruitment of myeloid progenitor cells to facilitate dissemination. *Blood*. (2006) 107:30–8. doi: 10.1182/blood-2005-05-1833
182. Daley-Bauer LP, Wynn GM, Mocarski ES. Cytomegalovirus impairs antiviral CD8+ T cell immunity by recruiting inflammatory monocytes. *Immunity*. (2012) 37:122–33. doi: 10.1016/j.immuni.2012.04.014
183. Karantzoulis S, Galvin JE. Distinguishing Alzheimer's disease from other major forms of dementia. *Expert Rev Neurother*. (2011) 11:1579–91. doi: 10.1586/ern.11.155
184. Wray S, Fox NC. Stem cell therapy for Alzheimer's disease: hope or hype? *Lancet Neurol*. (2016) 15:133–5. doi: 10.1016/S1474-4422(15)00382-8
185. Gouras GK, Olsson TT, Hansson O. β-Amyloid peptides and amyloid plaques in Alzheimer's disease. *Neurotherapeutics*. (2015) 12:3–11. doi: 10.1007/s13311-014-0313-y
186. Gu L, Tran J, Jiang L, Guo Z. A new structural model of Alzheimer's Aβ42 fibrils based on electron paramagnetic resonance data and Rosetta modeling. *J Struct Biol*. (2016) 194:61–7. doi: 10.1016/j.jsb.2016.01.013
187. Lyons B, Friedrich M, Raftery M, Truscott R. Amyloid plaque in the human brain can decompose from Aβ(1-40/1-42) by spontaneous nonenzymatic processes. *Anal Chem*. (2016) 88:2675–84. doi: 10.1021/acs.analchem.5b03891
188. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann Transl Med*. (2015) 3:136. doi: 10.3978/j.issn.2305-5839.2015.03.49
189. Rudinskiy N, Fuerer C, Demurtas D, Zamorano S, De Piano C, Herrmann AG, et al. Amyloid-beta oligomerization is associated with the generation of a typical peptide fragment fingerprint. *Alzheimers Dement*. (2016) 12:996–1013. doi: 10.1016/j.jalz.2016.03.011
190. Perry VH, Nicoll JA, Holmes C. Microglia in neurodegenerative disease. *Nat Rev Neurol*. (2010) 6:193–201. doi: 10.1038/nrneurol.2010.17
191. Myhre O, Utkilen H, Duale N, Brunborg G, Hofer T. Metal dyshomeostasis and inflammation in Alzheimer's and Parkinson's diseases: possible impact of environmental exposures. *Oxid Med Cell Long*. (2013) 2013:726954. doi: 10.1155/2013/726954
192. Zenaro E, Pietronigro E, Della Bianca V, Piacentino G, Constantin G, Budui S, et al. Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. *Nat. Med*. (2015) 21:880–6. doi: 10.1038/nm.3913
193. Yin Y, Wen S, Li G, Wang D. Hypoxia enhances stimulating effect of amyloid beta peptide (25–35) for interleukin 17 and T helper lymphocyte subtype 17 upregulation in cultured peripheral blood mononuclear cells. *Microbiol Immunol*. (2009) 53:281–6. doi: 10.1111/j.1348-0421.2009.00120.x
194. Saksida T, Koprivica I, Vujičić M, Stošić-Grujičić S, Perović M. Impaired IL-17 production in gut-residing immune cells of 5xFAD mice with Alzheimer's disease pathology. *J Alzheimers Dis*. (2018) 61:619–30. doi: 10.3233/JAD-170538
195. Wang X, Zhang M, Liu H. LncRNA17A regulates autophagy and apoptosis of SH-SY5Y cell line as an in vitro model for Alzheimer's disease. *Biosci Biotechnol Biochem*. (2019) 83:609–21. doi: 10.1080/09168451.2018.1562874
196. St-Amo I, Bosoi CR, Paré I, Ignatius Arokia Doss PM, Rangachari M, Hébert SS, et al. Peripheral adaptive immunity of the triple transgenic mouse model of Alzheimer's disease. *J Neuroinflammation*. (2019) 16:3. doi: 10.1186/s12974-018-1380-5
197. Zhang J, Ke KF, Liu Z, Qiu YH, Peng YP. Th17 cell-mediated neuroinflammation is involved in neurodegeneration of aβ1-42-induced Alzheimer's disease model rats. *PLoS ONE*. (2013) 8:e75786. doi: 10.1371/journal.pone.0075786
198. Tian A, Ma H, Zhang R, Tan W, Wang X, Wang Yet al. Interleukin17A promotes postoperative cognitive dysfunction by triggering β-amyloid accumulation via the transforming growth factor-β (TGFβ)/smad signaling pathway. *PLoS ONE*. (2015) 10:e0141596. doi: 10.1371/journal.pone.0141596
199. Zhang Y, Liu M, Sun H, Yin K. Matrine improves cognitive impairment and modulates the balance of Th17/Treg cytokines in a rat model of Aβ1-42-induced Alzheimer's disease. *Central-Eur J Immunol*. (2016) 40:411. doi: 10.5114/ceji.2015.56961
200. Browne TC, McQuillan K, McManus RM, O'Reilly JA, Mills KH, Lynch MA. IFN-γ production by amyloid β-specific Th1 cells promotes microglial activation and increases plaque burden in a mouse model of Alzheimer's disease. *J Immunol*. (2013) 190:2241–51. doi: 10.4049/jimmunol.1200947
201. Tzartos JS, Craner MJ, Friese MA, Jakobsen KB, Newcombe J, Esiri MM, et al.IL-21 and IL-21 receptor expression in lymphocytes and neurons in multiple sclerosis brain. *Am J Pathol*. (2011) 178:794–802. doi: 10.1016/j.ajpath.2010.10.043
202. Tahmasebinia F, Pourgholaminejad A. The role of Th17 cells in auto-inflammatory neurological disorders. *Prog Neuropsychopharmacol Biol Psychiatry*. (2017) 79(Pt B):408–16. doi: 10.1016/j.pnpbp.2017.07.023
203. Giuliani F, Goodyer CG, Antel JP, Yong VW. Vulnerability of human neurons to T cell-mediated cytotoxicity. *J Immunol*. (2003) 171:368–79. doi: 10.4049/jimmunol.171.1.368
204. Jin JJ, Kim HD, Maxwell JA, Li L, Fukuchi K. Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease. *J Neuroinflammation*. (2008) 5:23. doi: 10.1186/1742-2094-5-23

205. Cristiano C, Volpicelli F, Lippiello P, Buono B, Raucci F, Piccolo M, et al. Neutralization of interleukin-17 rescues amyloid-β-induced neuroinflammation and memory impairment. *Br J Pharmacol.* (2019) 176:3544–57. doi: 10.1111/bph.14586
206. Solleiro-Villavicencio H, Rivas-Arancibia S. Effect of chronic oxidative stress on neuroinflammatory response mediated by CD4+T cells in neurodegenerative diseases. *Front Cell Neurosci.* (2018) 12:114. doi: 10.3389/fncel.2018.00114
207. Yang J, Kou J, Lalonde R, Fukuchi KI. Intracranial IL-17A overexpression decreases cerebral amyloid angiopathy by upregulation of ABCA1 in an animal model of Alzheimer's disease. *Brain Behav Immun.* (2017) 65:262–73. doi: 10.1016/j.bbi.2017.05.012
208. Tfilin M, Turgeman G. Interleukine-17 administration modulates adult hippocampal neurogenesis and improves spatial learning in mice. *J Mol Neurosci.* (2019) 69:254–63. doi: 10.1007/s12031-019-01354-4
209. Lemprière S. T cells on patrol in Alzheimer disease. *Nat Rev Neurol.* (2020) 16:128–9. doi: 10.1038/s41582-020-0317-7
210. Saresella M, Calabrese E, Marventano I, Piancone F, Gatti A, Alberoni M, et al. Increased activity of Th-17 and Th-9 lymphocytes and a skewing of the post-thymic differentiation pathway are seen in Alzheimer's disease. *Brain Behav Immunity.* (2011) 25:539–47. doi: 10.1016/j.bbi.2010.12.004
211. Oberstein TJ, Taha L, Spitzer P, Hellstern J, Herrmann M, Kornhuber J, et al. Imbalance of circulating Th17 and regulatory T cells in Alzheimer's disease: a case control study. *Front Immunol.* (2018) 9:1213. doi: 10.3389/fimmu.2018.01213
212. Chen J-M, Jiang G-X, Li Q-W, Zhou Z-M, Cheng Q. Increased serum levels of interleukin-18, -23 and -17 in chinese patients with Alzheimer's disease. *Dem Geriat Cogn Disord.* (2014) 38:321–9. doi: 10.1159/000360606
213. Doecke JD, Laws SM, Faux NG, Wilson W, Burnham SC. Blood-based protein biomarkers for diagnosis of Alzheimer disease. *Arch Neurol.* (2012) 69:1318–25. doi: 10.1001/archneurol.2012.1282
214. Hu WT, Chen-Plotkin A, Grossman M, Arnold SE, Clark CM. Novel CSF biomarkers for frontotemporal lobar degenerations. *Neurology.* (2010) 75:2079–86. doi: 10.1212/WNL.0b013e318200d78d
215. Marciani DJ. Alzheimer's disease vaccine development: a new strategy focusing on immune modulation. *J Neuroimmunol.* (2015) 287:54–63. doi: 10.1016/j.jneuroim.2015.08.008
216. McManus RM, Higgins SC, Mills KH, Lynch MA. Respiratory infection promotes T cell infiltration and amyloid-β deposition in APP/PS1 mice. *Neurobiol Aging.* (2014) 35:109–21. doi: 10.1016/j.neurobiolaging.2013.07.025
217. Wissman A, Hauptman J, Regen T. The role of IL-17 in CNS diseases. *Acta Neuropathol.* (2015) 129:625–37. doi: 10.1007/s00401-015-1402-7
218. Shekhar S, Cunningham MW, Pabbidi MR, Wang S, Booz GW, Fan F, et al. Targeting vascular inflammation in ischemic stroke: recent developments on novel immunomodulatory approaches. *Eur J Pharmacol.* (2018) 833:531–44. doi: 10.1016/j.ejphar.2018.06.028
219. Kigerl KA, de Rivero Vaccari JP, Dietrich WD, Popovich PG, Keane RW. Pattern recognition receptors and central nervous system repair. *Exp Neurol.* (2014) 258:5–16. doi: 10.1016/j.expneurol.2014.01.001
220. Hayakawa K, Qiu J, Lo EH. Biphasic actions of HMGB1 signaling in inflammation and recovery after stroke. *Ann N Y Acad Sci.* (2010) 1207:50–7. doi: 10.1111/j.1749-6632.2010.05728.x
221. Zhang J, Takahashi HK, Liu K, Wake H, Liu R, Maruo T, et al. Anti-high mobility group box-1 monoclonal antibody protects the blood-brain barrier from ischemia-induced disruption in rats. *Stroke.* (2011) 42:1420–8. doi: 10.1161/STROKEAHA.110.598334
222. Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, et al. Peroxiredoxin family proteins are key initiators of postischemic inflammation in the brain. *Nat Med.* (2012) 18:911–7. doi: 10.1038/nm.2749
223. Planas AM, Gómez-Choco M, Urra X, Gorina R, Caballero M. Brain-derived antigens in lymphoid tissue of patients with acute stroke. *J Immunol.* (2012) 188:2156–63. doi: 10.4049/jimmunol.1102289
224. Brait VH, Arumugam TV, Drummond GR, Sobey CG. Importance of T lymphocytes in brain injury, immunodeficiency, and recovery after cerebral ischemia. *J Cereb Blood Flow Metab.* (2012) 32:598–611. doi: 10.1038/jcbfm.2012.6
225. Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Scrhwarz T, et al. Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood.* (2010) 115:3835–42. doi: 10.1182/blood-2009-10-249078
226. Arumugam TV, Granger DN, Mattson MP. Stroke and T-cells. *Neuromolecular Med.* (2005) 7:229–42. doi: 10.1385/NMM:7:3:229
227. Kostulas N, Pelidou SH, Kivisäkk P, Kostulas V, Link H. Increased IL-1β, IL-8, and IL-17 mRNA expression in blood mononuclear cells observed in a prospective ischemic stroke study. *Stroke.* (1999) 30:2174–9. doi: 10.1161/01.STR.30.10.2174
228. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, et al. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood.* (2012) 120:3793–802. doi: 10.1182/blood-2012-02-412726
229. Li GZ, Zhong D, Yang LM, Sun B, Zhong ZH, Yin YH, et al. Expression of interleukin-17 in ischemic brain tissue. *Scand J Immunol.* (2005) 62:481–6. doi: 10.1111/j.1365-3083.2005.01683.x
230. Wang DD, Zhao YF, Wang GY, Sun B, Kong QF, Zhao K. et al. IL-17 potentiates neuronal injury induced by oxygen-glucose deprivation and affects neuronal IL-17 receptor expression. *J Neuroimmunol.* (2009) 212:17–25. doi: 10.1016/j.jneuroim.2009.04.007
231. Zhang J, Mao X, Zhou T, Cheng X, Lin Y. IL-17A contributes to brain ischemia reperfusion injury through calpain-TRPC6 pathway in mice. *Neuroscience.* (2014) 274:419–28. doi: 10.1016/j.neuroscience.2014.06.001
232. Li HL, Kostulas N, Huang YM, Xiao BG, van der Meide P, Kostuals V, et al. IL-17 and IFN-γ mRNA expression is increased in the brain and systemically after permanent middle cerebral artery occlusion in the rat. *J Neuroimmunol.* (2001) 116:5–14. doi: 10.1016/S0165-5728(01)00264-8
233. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, et al. Pivotal role of cerebral interleukin-17-producing $\gamma\delta$ T cells in the delayed phase of ischemic brain injury. *Nat Med.* (2009) 15:946–50. doi: 10.1038/nm.1999
234. Jin WN, Gonzales R, Feng Y, Wood K, Chai Z, Dong J-F, et al. Brain Ischemia Induces Diversified Neuroantigen-Specific T-Cell Responses That Exacerbate Brain Injury. *Stroke.* (2018) 49:1471–1478. doi: 10.1161/STROKEAHA.118.020203
235. Dolati S, Ahmadi M, Khalili M, Taheraghdam AA, Siahmansouri H, Babaloo Z, et al. Peripheral Th17/Treg imbalance in elderly patients with ischemic stroke. *Neurol Sci.* (2018) 39:647–54. doi: 10.1007/s10072-018-3250-4
236. Zhang Y, Xu D, Qi H, Yuan Y, Liu H, Yao S, et al. Enriched environment promotes post-stroke neurogenesis through NF-κB-mediated secretion of IL-17A from astrocytes. *Brain Res.* (2018) 1687:20–31. doi: 10.1016/j.brainres.2018.02.030
237. Liu T, Han S, Dai Q, Zheng J, Liu C, Li S, et al. IL-17A-mediated excessive autophagy aggravated neuronal ischemic injuries via Src-PP2B-mTOR pathway. *Front Immunol.* (2019) 10:2952. doi: 10.3389/fimmu.2019.02952
238. Dai Q, Li S, Liu T, Zheng J, Han S, Qu A, et al. Interleukin-17A-mediated alleviation of cortical astrocyte ischemic injuries affected the neurological outcome of mice with ischemic stroke. *J Cell Biochem.* (2019) 120:11498–509. doi: 10.1002/jcb.28429

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 04 August 2020
doi: 10.3389/fimmu.2020.01724

Contribution of IL-17 in Steroid Hyporesponsiveness in Obese Asthmatics Through Dysregulation of Glucocorticoid Receptors α and β

*Saba Al Heialy[1,2], Mellissa Gaudet[2], Rakhee K. Ramakrishnan[3], Andrea Mogas[2], Laila Salameh[3,4], Bassam Mahboub[3,4] and Qutayba Hamid[2,3]**

[1] College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates, [2] Meakins-Christie Laboratories, Research Institute of the McGill University Healthy Center, Montreal, QC, Canada, [3] Sharjah Institute for Medical Research, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates, [4] Pulmonary Medicine Department, Rashid Hospital, Dubai, United Arab Emirates

OPEN ACCESS

Edited by:
Kong Chen,
University of Pittsburgh, United States

Reviewed by:
Michelle L. Manni,
University of Pittsburgh, United States
Abdelilah Soussi Gounni,
University of Manitoba, Canada

***Correspondence:**
Qutayba Hamid
qalheialy@sharjah.ac.ae

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: 31 January 2020
Accepted: 29 June 2020
Published: 04 August 2020

Citation:
Al Heialy S, Gaudet M, Ramakrishnan RK, Mogas A, Salameh L, Mahboub B and Hamid Q (2020) Contribution of IL-17 in Steroid Hyporesponsiveness in Obese Asthmatics Through Dysregulation of Glucocorticoid Receptors α and β. Front. Immunol. 11:1724. doi: 10.3389/fimmu.2020.01724

Obesity is on the rise worldwide and is one of the most common comorbidities of asthma. The chronic inflammation seen in obesity is believed to contribute to this process. Asthma and obesity are associated with a poorer prognosis, more frequent exacerbations, and poor asthma control to standard controller medication. Difficult-to-treat asthma is associated with increased levels of Th17 cytokines which have been shown to play a central role in the upregulation of glucocorticoid receptor-beta (GR-β), a dominant-negative inhibitor of the classical GR-α. In this study, we studied the role of IL-17 cytokines in steroid hyporesponsiveness in obese asthmatics. We stimulated lean and obese adipocytes with IL-17A and IL-17F. Adipocytes obtained from obese patients cultured *in vitro* in the presence of IL-17A for 48 h showed a decrease in GRα/GRβ ratio as compared to adipocytes from lean subjects where GR-α/GR-β ratio was increased following IL-17A and IL-17F stimulation. At protein level, GR-β was increased in obese adipocytes with IL-17A and IL-17F stimulation. IL-8 and IL-6 expression was increased in IL-17-stimulated obese adipocytes. Pre-incubation with Dexamethasone (Dexa) led to a decrease in GR-α/GR-β ratio in obese adipocytes which was further affected by IL-17A whereas Dexa led to an increase in GR-α/GR-β ratio in lean adipocytes which was decreased in response to IL-17A. TGF-β mRNA expression was decreased in obese adipocytes in response to Th17 cytokines. We next sought to validate these findings in obese asthmatic patients. Serum obtained from obese asthmatic subjects showed a decrease in GRα/GRβ protein expression with an increase in IL-17F and IL-13 as compared to serum obtained from non-obese asthmatics. In conclusion, steroid hyporesponsiveness in obese asthmatic patients can be attributed to Th17 cytokines which are responsible for the dysregulation of the GRα/GRβ ratio and the inflammatory response.

Keywords: asthma, obesity, IL-17, steroid hyporesponsiveness, glucocorticoid receptors, inflammation

INTRODUCTION

Worldwide, incidence of obesity is on the rise at an alarming rate. It is estimated that by 2030, 38% of the world's population will be overweight and 20% will be obese (1). Obesity is simply defined as excess weight for height where the body mass index (BMI) is equal or greater to 30 kg/m^2 Obesity is associated with a multitude of metabolic abnormalities ranging from diabetes to cardiovascular disease and asthma (2, 3). It is believed that these associations are due mostly in part to the chronic inflammation associated with obesity. High levels of pro-inflammatory cytokines, such as interleukin (IL)-6 (4), IL-8 (5), and Tumor Necrosis Factor-alpha (TNF-α) (6) are seen in various models of obesity. Adipose tissue, which is composed mainly of adipocytes, is now recognized as an organ which contributes to systemic inflammation (7). Adipose explants from obese patients show an increase in mediators such as IL-6, TNF-α, angiotensinogen and complement C3 (8). Low-grade systemic inflammation has been shown to regulate adipogenesis and insulin resistance (9).

As adipose tissue has been shown to contribute to high levels of serum IL-6, this has prompted recent studies to focus on the role of Th17 cells in obesity. IL-6 is necessary for the polarization of CD4+ T cells into Th17 cells (10). The main role of Th17 cells is to clear bacteria and fungi. However, beyond their protective role, Th17 cells are implicated in many inflammatory conditions and are the major cellular source of IL-17 cytokines, most notably IL-17A and IL-17F (11). IL-17 has been shown to be upregulated in obese subjects (12). In a mouse model of diet-induced obesity, IL-17A production was enhanced by CD4+ T cells. Moreover, IL-17 is an important player in severe asthma (13). IL-17A and IL-17F production is increased with severity of the disease and Th17 cells are now recognized as the major T helper subset in severe asthma (14). The presence of IL-17 is crucial due to its role in steroid resistance through the dysregulation of glucocorticoid receptors.

In the United States, ∼60% of patients with severe asthma are obese (15). Asthma is a heterogeneous disease defined by many phenotypes. Understanding the mechanisms underlying the various asthma phenotypes is important in predicting therapy. Asthma associated with obesity is a complex phenotype which is characterized by worsening outcomes such as poor control and increased exacerbations akin to severe asthma (16). Overweight asthmatic children show a decreased response to inhaled budesonide compared to normal weight asthmatic children (17). An increasing body of literature show a reduction in steroid responsiveness in obese asthmatics compared to their lean counterparts (18, 19). Steroid hyporesponsiveness is one of the major characteristics of severe asthma and makes treatment of symptoms challenging. A study by Vazquez-Tello et al. showed that IL-17 cytokine stimulation of peripheral blood mononuclear cells (PBMCs) leads to an upregulation of the glucocorticoid receptor-beta (GR-β) (20). Alternative splicing of the GR transcript generates two isoforms of GR: GR-α and GR-β. GR-β is a dominant negative-regulator of the active GR-α and has been associated with steroid hyporesponsiveness.

Although studies have already shown a positive correlation between IL-17 and the inflammatory conditions of asthma and obesity individually, no studies to our knowledge have looked at the role of IL-17 in obese asthmatics. Moreover, the mechanism underlying the decreased responses to steroid in obese asthmatics has not been fully elucidated. We hypothesized that IL-17 cytokines are involved in steroid resistance described in obese asthmatics through the dysregulation of GR-α and GR-β.

MATERIALS AND METHODS

Pre-adipocyte & Adipocyte Cell Culture and Treatment

Subcutaneous human pre-adipocyte from lean and obese subjects were purchased from ATCC (VA, USA) & ZenBio (NC, USA). **Table 1** shows the data on pre-adipocytes obtained from lean and obese subjects. Pre-adipocytes were cultured in DMEM/Ham's F-12 (1:1, v/v) media (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) supplemented with: 0.01M HEPES pH 7.4 (Thermo Fisher Scientific Inc.), 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Pre-adipocytes were grown to 80% confluence in 10 cm dishes (Corning Inc., Corning, NY, USA) and detached using 0.05% Trypsin-EDTA (Thermo Fisher Scientific Inc.) and seeded in 6 well plates (Sigma-Aldrich, Ontario, Canada). Once confluency reached, the differentiation process was started using Adipocyte Differentiation Medium (ZenBio, NC,USA) for 7 days. The differentiation media was then changed to Adipocyte Maintenance Medium (ZenBio) as detailed in the Subcutaneous Human Adipocyte Manual ZBM0001.05 from ZenBio. Mature adipocytes were kept in culture for no longer than 2 weeks post differentiation.

Mature adipocytes were starved with DMEM/Ham's F-12 (1:1, v/v) media supplemented with: 0.01 M HEPES pH 7.4, 0.5% fetal bovine serum and 100 U/ml penicillin/streptomycin overnight. Cells were then stimulated with recombinant human IL-17A and F cytokines (100 ng/ml; R&D systems, Minneapolis, MN, USA) either alone or combined for 48 h. After stimulation culture media was collected and frozen for future experiments. Adipocytes were then processed for RNA extraction or protein lysis.

Mature adipocytes used in the experiments involving Dexamethasone (Dexa) were starved over night with DMEM/Ham's F-12 (1:1, v/v) media (Thermo Fisher Scientific Inc.) supplemented with: 0.01 M HEPES pH 7.4 (Thermo Fisher Scientific Inc.), 0.5% fetal bovine serum and 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific Inc.) with the

TABLE 1 | Data of adipocytes from lean and obese subjects.

	Lean adipocytes	Obese adipocytes
N	4	3
Age, year	37.8 ± 9.9	46 ± 4.4
BMI, kg/m^2	20.6 ± 2.2	33.4 ± 3.5

BMI, body mass index. Values shown are mean ± SE.

addition of 500 ng/ml of Dexa (Sigma-Aldrich). The following day, adipocytes were stimulated with 100 ng/ml of IL-17A (R&D systems) for 48 h. After stimulation the adipocytes were processed for RNA extraction.

Participant Selection

The study was approved by the ethical committee of Dubai Health Authority and Mohammed bin Rashid University of Medicine and Health Sciences Internal Review Board, Dubai, UAE. All participants provided written informed consent. Patients were recruited at Rashid Hospital, Dubai, UAE. Male and female moderate-to-severe asthmatic patients were >18 years of age, patients were diagnosed by spirometry and clinical history according to American Thoracic Society guidelines. Participants with a >20 pack-year smoking history or with a history of smoking within the last 6 months were excluded from the study.

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

Extraction of total RNA from adipocytes was performed using a phenol-chloroform extraction method (RiboZol RNA extraction reagent, VWR, Leicestershire, UK), as directed in the manufacturer's instructions. Contaminating DNA was removed from 4 μg of total RNA using the AccuRT Genomic DNA Removal Kit (Applied Biological Materials, Richmond, BC, Canada), following manufacturer's protocol. Reverse transcription was performed using the 5X All-In-One Reverse Transcriptase Mastermix (ABM). The TaqMan system was used to measure gene expression for GR-α, GR-β, and GAPDH as a house keeping (Applied Biosystems, Foster City, CA, USA). **Table 2** shows the list of forward and reverse primers used. The TaqMan reaction contained 2.5 μl of undiluted cDNA, 5 μl of TaqProbe 2× qPCR Mastermix-No Dye (ABM), 0.5 μl of ready-to-use probe, and 2 μl of nuclease free H_2O. mRNA expression of experiments using Dexamethasone was measured using a TaqMan reaction containing 1 μl of undiluted cDNA, 5 μl of TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μl of ready-to-use probe and 2.5 μl of nuclease free H_2O. Inflammatory marker gene targets (**Table 2**) were measured using EvaGreen qPCR Mastermix (ABM). The reaction was as follows: 5 μl of EvaGreen Mastermix, 2.5 μl of diluted cDNA (1/25), 0.6 μl of forward and reverse primers (10 μM) and 2.4 μl of nuclease free H_2O. Each sample was tested in duplicates and the qPCR amplification was performed using CFX96 thermal cycler (BioRad, Hercules, CA, USA) and cycler conditions for both TaqMan and EvaGreen qPCR were preformed according to manufacturer's protocol. The ΔΔCT method was used to measure gene expression for both detection methods: amount of target $= 2^{-\Delta\Delta CT}$.

GR-α and GR-β Protein Quantification

Mature adipocytes were cultured and treated as specified above. Cell culture media was collected and placed at −80°C for future experiments. Adipocytes were washed once with 500 μl PBS, PBS was removed gently using a pipette. 1 mL of PBS was then added to each well and placed at −80°C overnight, a second freeze-thaw cycle was conducted by simply thawing the frozen cells and placing the culture plate once more at −80°C overnight. The frozen adipocyte plate is thawed the next day and the cell lysate is removed from each well and centrifuged 5 min at 5,000 × g.

From the cell lysate, the protein concentration of GR-α and GR-β were quantified using a chemiluminescence immunoassay (CLIA) and ELISA kit, for each protein, respectively. The Human GR alpha (Glucocorticoid Receptor Alpha) CLIA Kit and the Human GR beta/Glucocorticoid Receptor Beta Elisa Kit (ELISAGenie, London, UK) were used to quantify the GR proteins. Assay procedure was followed according to manufacturer's protocol, except for the following steps: standards and cell lysates were incubated in assay plate over night at 4°C, Biotin-detection antibody was incubated at room temperature for 60 min, the HRP (CLIA)/SABC (ELISA) working solution was incubated at room temperature for 30 min, the Substrate Mixture (CLIA) was incubated 5 min at room temperature and the TMB (ELISA) substrate was incubated at room temperature.

GR-α and GR-β levels in serum were measured using the following ELISA kits: Human GR alpha/Glucocorticoid Receptor Alpha Elisa Kit (ELISAGenie) and the Human GR beta/Glucocorticoid Receptor Beta Elisa Kit (ELISAGenie). Assay procedure was followed according to manufacturer's protocol.

TABLE 2 | Forward and reverse primers inflammatory markers and their oligo sequences.

Primer name	Oligo sequence (5′ to 3′)
IL-8 forward	TCTGCAGCTCTGTGTGAAGGT G
IL-8 reverse	AATTTCTGTGTTGGCGCAGTG
IL-6 forward	ACCTTCCAAAGATGGCTGAAA
IL-6 reverse	GCTCTGGCTTGTTCCCTCACTAC
IL-17A forward	GAGGACAAGAACTTCCCCCG
IL-17A reverse	CATTGCCGTGGAGATTCCAAG
TNF-α forward	CCTCTTCTCCTTCCTGATCGT
TNF-α reverse	GGTTTGCTACAACATGGGCTA
TGF-β1 forward	TACCTGAACCCGTGTTGCTCTC
TGF-β1 reverse	GTTGCTGAGGTATCGCCAGGAA
IFN-γ forward	GTTTTGGGTTCTCTTGGCTGT
IFN-γ reverse	ATGTATTGCTTTGCGTTGGAC
IL-1β forward	TACATCAGCACCTCTCAAGCA
IL-1β reverse	CCACATTCAGCACAGGACTCT
GAPDH forward	GAAGGTGAAGGTCGGAGT
GAPDH Reverse	GAAGATGGTGATGGGATTTC

Cytokine Quantification

Cytokine concentrations in cell culture media secreted from treated adipocytes and serum samples was measured using a MILLIPLEX MAP Human High Sensitivity T Cell Panel—Immunology Multiplex Assay (EMDMillipore, Burlington, MA) with the following analytes: IL-4,-5,-6,-8, IFN-γ and a MILLIPLEX MAP Human TH17 Magnetic Bead Panel with the following analytes: IL2, IL-13, IL-17A, IL-17F. This assay was preformed according to manufacturer's protocol.

Statistical Analysis

Standard statistical two-tailed *t*-tests and one-way ANOVA using Tukey's multiple comparison test were performed to test for statistical significance between data groups using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). $p < 0.05$ was considered significant. Pearson correlation was used to study correlations.

RESULTS

GR-α/GR-β Ratio in Lean and Obese Adipocytes

Pre-adipocytes obtained from female lean ($n = 4$) and obese ($n = 3$) subjects ranging from 25 to 67 years of age (**Table 1**) were purchased and differentiated *in vitro*. The average BMI was 20.6 ± 2.2 kg/m^2 and 33.4 ± 3.5 kg/m^2 for the lean and obese adipocytes, respectively. Th17 cytokines, 100 ng/mL IL-17A and IL-7F in combination or IL-17A alone, were added for 48 h. Following stimulation, adipocytes were collected, and RNA was extracted. Adipocytes from lean subjects stimulated with IL-17A and IL-17F show a significant increase, with 2-fold change, in GR-α/GR-β ratio ($p = 0.0057$) (**Figure 1**). However, IL-17 stimulation of adipocytes from obese subjects shows a significant decrease in GR-α/GR-β ratio ($p = 0.03$) which has been described in asthmatic patients with steroid hyporesponsiveness (20). ELISA was used to confirm and assess protein levels of GR-α and GR-β. Cell lysates obtained from adipocytes from obese and lean subjects stimulated with IL-17A and IL-17F in combination or IL-17A alone show differential expression of GR-β (**Figure 2**). GR-β is highly increased ($p = 0.03$) in lean adipocytes when stimulated with IL-17A alone whereas GR-β is increased (not significant) in obese adipocytes only when stimulated with the combination of IL-17A and IL-17F compared to unstimulated cells (**Figure 2B**). GR-α is unchanged in response to IL-17 cytokine stimulation in both lean and obese adipocytes compared to unstimulated adipocytes (**Figure 2A**). Therefore, our data suggests that IL-17 cytokines may lead to an increase in GR-β mRNA and protein expression which contributes to the shift of the ratio of GR.

Steroid Unresponsiveness in Obese Adipocytes

We also sought to see the effect of IL-17 stimulation on steroid-treated adipocytes. Pre-incubation with Dexa (500 ng/mL) lead to an increase in mRNA expression of GR-α/GR-β ratio in lean adipocytes and a decreased ratio in obese adipocytes (**Figure 3**). This decrease in GR-α/GR-β suggests that obese adipocytes do not respond to Dexa. Interestingly, stimulation with IL-17A in pre-treated cells decreased the GR-α/GR-β ratio in both lean and obese adipocytes although not statistically significantly (**Figure 3**). This data suggests that IL-17 may modulate adipocyte responses to steroids and obese adipocytes are not responsive to steroid treatment.

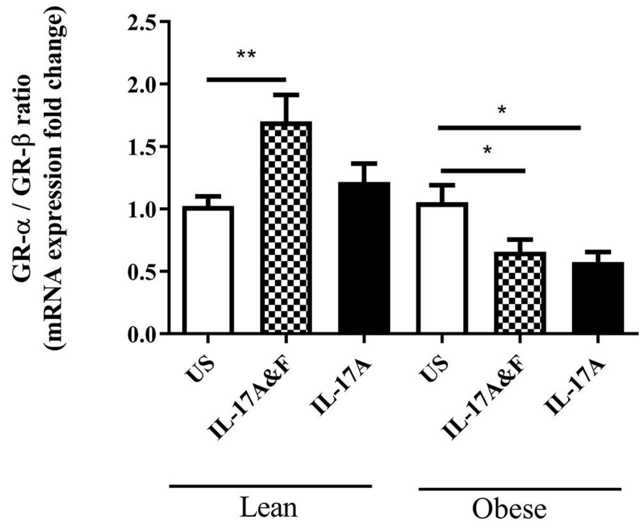


FIGURE 1 | Stimulation with IL-17A & F and IL-17A alone induces changes in GR-α/GR-β mRNA ratio. Adipocytes from lean and obese subjects were stimulated with 100 ng/mL of IL-17A and IL-17F in combination or IL-17A alone for 48 h. Cells were collected and qRT-PCR analysis was performed in duplicate using TaqMan probes to assess GR-α and GR-β mRNA expression. One independent experiment performed per subject. $n = 4$ lean subjects, $n = 3$ obese subjects, One-Way ANOVA, Mean ± SE; $^*P < 0.05$, $^{**}P < 0.01$.

Changes in Cytokine Profiles in Lean and Obese Adipocytes

Conditioned media was obtained from lean ($n = 4$) and obese ($n = 3$) subjects following IL-17 cytokine stimulation to assess cytokine production. mRNA expression was assessed at 48 h post-stimulation. The changes in IL-6 and IL-8 mRNA expression were observed post-stimulation in both lean and obese adipocytes (**Figure 4**). Interestingly, TGF-β mRNA expression was significantly decreased in obese adipocytes stimulated with IL-17 cytokines compared to unstimulated and IL-17-stimulated lean adipocytes (**Figure 4C**). TGF-β is an anti-inflammatory cytokine which is involved in obesity and asthma. To confirm these findings, multiplex assay was performed on conditioned media obtained 48 h post-stimulation to measure the levels of inflammatory cytokines (**Figures 5A–E**). At protein levels, the changes in cytokines expression were only significantly different in obese adipocytes stimulated with IL-17A and IL-17F in combination or IL-17A alone. IL-6, IL-8, and IFN-γ were significantly increased in obese adipocytes compared to unstimulated and IL-17-stimulated lean adipocytes. Our data suggests that IL-17 stimulation leads to further inflammation in adipocytes obtained from obese subjects. This is not observed in the adipocytes from lean subjects at protein level although changes were observed at mRNA level.

GR-α/GR-β Ratio in Serum of Obese and Non-obese Asthmatics

Following *in vitro* assays, we were interested to see if these findings were also observed in lean and obese asthmatics.

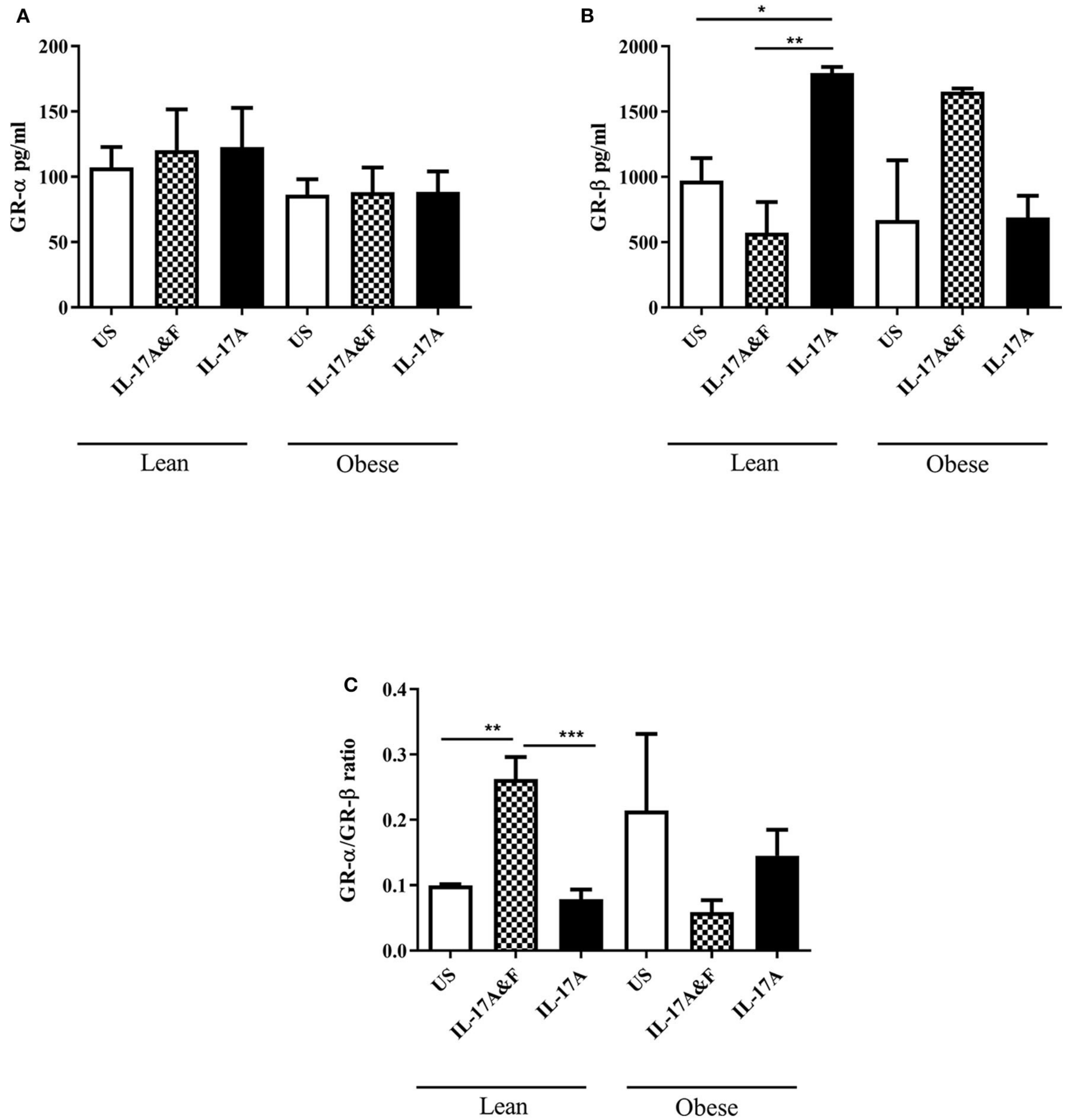


FIGURE 2 | IL-17A & F and IL-17A alone induces changes in protein levels of GR-β. GR-α **(A)** and GR- β **(B)** protein expression in adipocytes from lean and obese subjects following 48 h stimulation with IL-17A&F combination or IL-17A alone **(C)** Ratio of GR-α/GR-β. $n = 4$ lean subjects, $n = 3$ obese subjects, One-way ANOVA, Mean ± SE; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data is representative of three experiments.

Serum was obtained from 44 non-obese (lean and overweight) asthmatic patients and 57 obese (obese and morbidly obese) asthmatic patients. Demographic and clinical data of the patients is presented in **Table 3**. **Table 3** shows that all lung function parameters were comparable in lean and obese moderate-to-severe asthmatics. ACT scores were 16.2 ± 0.9 and 16.4 ± 0.6 for lean and obese asthmatics, respectively. Blood eosinophils were significantly ($p = 0.04$) decreased in obese asthmatics. Previous studies have shown discrepancies in eosinophil counts in blood, sputum and biopsies. In one study in mild-to-moderate asthmatics, there was no difference in blood eosinophil's in obese and lean subjects. However, sputum eosinophils were significantly decreased in sputum and increased in bronchial submucosa (21). ELISA was performed to assess GR-α/GR-β ratio. Data revealed that obese asthmatics had a significant decrease in GR-α/GR-β ratio compared to non-obese asthmatics (**Figure 6A**). The non-obese asthmatics show heterogeneity in their response. Despite this, the GR-α/GR-β ratio is significantly higher than the obese asthmatics. There was a negative correlation (r = −0.23) between GR-α/GR-β ratio and BMI ($p < 0.05$) as assessed by Pearson correlation (**Figure 6B**) When patients were categorized further into: lean ($n = 18$), overweight

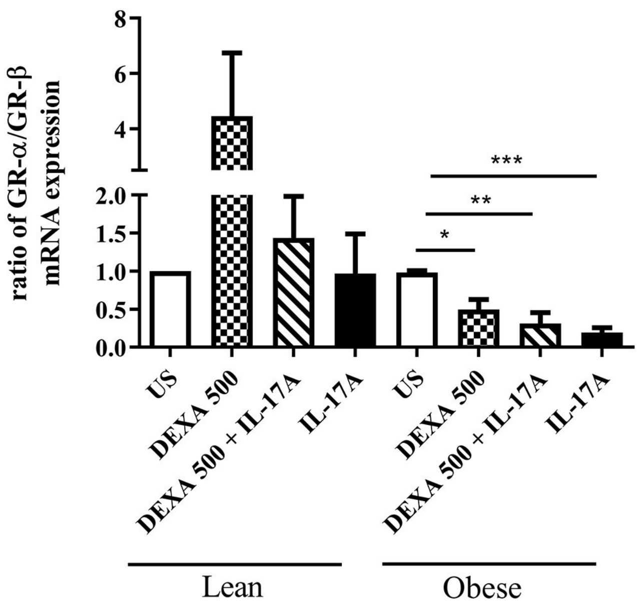


FIGURE 3 | Pre-treatment with Dexamethasone followed by IL-17A stimulation induces changes in GR-α/GR-β mRNA ratio. Adipocytes from lean and obese subjects were pre-treated with 500 ng/ml dexamethasone followed by a 48 h stimulation with 100 ng/ml of IL-17A. mRNA expression of GR-α and GR-β were measured by qRT-PCR in duplicates using TaqMan probes. One independent experiment preformed per subject: $n = 4$ lean subjects, $n = 3$ obese subjects, One-Way ANOVA, Mean ± SE; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

($n = 26$), obese ($n = 44$), and morbidly obese ($n = 13$) asthmatic patients, GR-α/GR-β ratio was significantly decreased in obese and morbidly obese asthmatic patients compared to overweight asthmatic patients ($p < 0.05$) (**Figure 6C**).

IL-17F and IL-13 Protein Expression Are Increased in Obese Asthmatics

We analyzed the protein expression of IL-17A, IL-17F, and IL-13 in serum of obese (obese and morbidly obese) and non-obese (lean and overweight) asthmatics (**Figures 7A–C**). There was no difference in serum IL-17A production in non-obese compared to obese asthmatics. However, there was a statistically significant increase in IL-17F ($p = 0.03$) and IL-13 ($p = 0.02$) production in obese compared to non-obese asthmatics. We then analyzed the IL-17F levels in the lowest quartile (<90 pg/mL) and highest quartile (>180 pg/mL). Interestingly, we found a significant difference between GR-α/GR-β ratio in the lowest and highest quartile. High levels of IL-17F were associated with decreased GR-α/GR-β ratio compared to low levels of IL-17F (**Figure 7D**).

DISCUSSION

Many studies have shown positive correlation between asthma and obesity. Moreover, clinical data suggests that obese asthmatics are refractory to conventional therapy. This study demonstrates, for the first time, that IL-17 plays a role in steroid resistance through the dysregulation of GR-α and GR-β expression in adipocytes. Our data suggests that IL-17 cytokines are also involved in the upregulation of pro-inflammatory mediators in the context of obesity. These findings were further strengthened by demonstrating a negative correlation between BMI and GR-α/GR-β ratio in serum from asthmatic patients. Serum obtained from obese and morbidly obese asthmatic patients showed a significant decrease in GR-α/GR-β ratio and an increase in IL-17F and IL-13 compared to lean and overweight patients.

Although most asthmatic patients respond well to conventional therapy, 25–35% of patients show no improvement in lung function in response to inhaled corticosteroids (22). Certain subsets of asthmatic patients such as active smokers (23) and obese patients have blunted steroid responses. The dual relationship between asthma and obesity is of interest as studies indicate that obesity does not necessarily cause asthma but may be a risk factor for the development and the severity of asthma. In both adults and children, the obese asthma phenotype tends to lead to more severe symptoms akin to the severe asthma phenotype. Of interest, 60% of severe asthmatic patients are obese (24). A body of literature has shown that one of the major players in steroid resistance in severe asthma is the defect in GR-α and thus dysregulation of the GR-α/GR-β ratio. Since this mechanism has been described in severe asthma, we were interested to see if this was also the case in obese asthmatics. We found that the ratio was decreased in obese asthmatics compared to non-obese asthmatics. This was statistically significant in obese and morbidly obese compared to overweight asthmatics. Interestingly, a correlation analysis revealed a statistically significant negative correlation between BMI and GR-α/GR-β ratio. This is of clinical relevance as BMI may predict the steroid responsiveness of asthmatic patients.

Having established that the ratio of GR-α/GR-β is altered in obese asthmatics, we were interested to see what mediators could be involved in this dysregulation. Obesity is associated with increased markers of inflammation in serum and adipose tissue in obese people with asthma. In the obese state, the adipose tissue is infiltrated with proinflammatory cytokines and adipokines. This led to the hypothesis that proinflammatory responses in the adipose tissue may lead to asthma. One of the major proinflammatory cytokines involved in obesity as well as asthma is IL-17A.The role of Th17 cells in obesity is relatively unexplored but evidence of accumulation of Th17 cells in a mouse model of diet-induced obesity has been described (25). Studies have suggested that obesity predisposes to the expansion of Th17 cells via IL-6 which may in turn exacerbate inflammatory conditions such as multiple sclerosis (26). However, the role of Th17 cells and its cytokines in obesity and, in particular, in obese asthma remains largely unknown. Therefore, we sought to study the role of IL-17A and IL-17F in steroid hyporesponsiveness. Adipocytes from lean and obese subjects were cultured in the presence of IL-17A and IL-17F in combination and IL-17A alone. IL-17A alone was used due to the overwhelming amount of literature that suggests a role for this proinflammatory cytokine in obesity. Adipocytes obtained from lean subjects stimulated

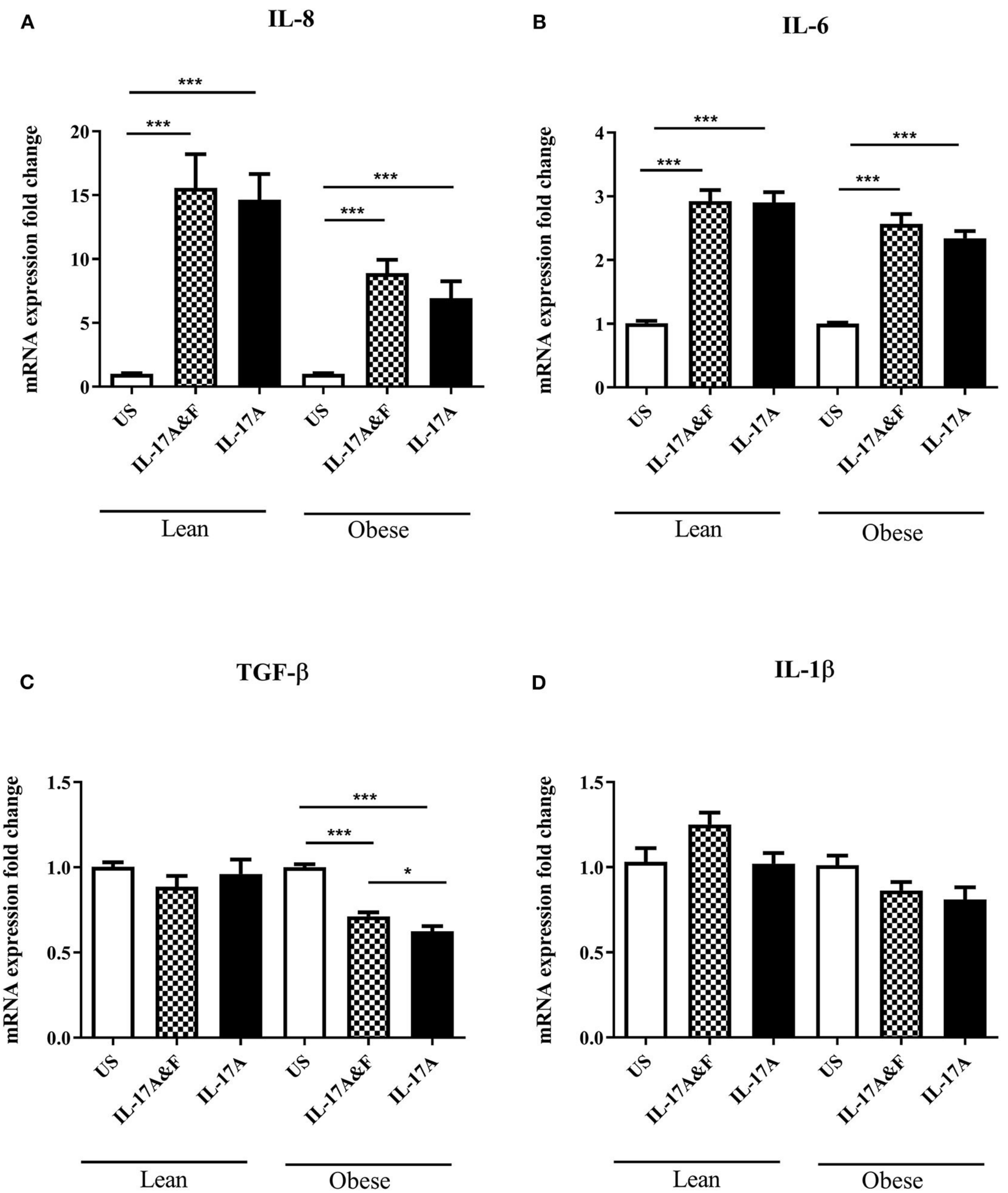


FIGURE 4 | Stimulation with IL-17A&F and IL-17A alone induces changes in mRNA expression of inflammatory mediators in adipocytes from lean and obese subjects. qRT-PCR analysis of detected mRNA expression of inflammatory markers: IL-8 **(A)**, IL-6 **(B)**, TGF-β **(C)**, IL-1β **(D)** in mature adipocytes after 48 h stimulation with combination of IL-17A&F or IL-17A alone. $n = 4$ lean subjects, $n = 3$ obese subjects, One-way ANOVA, Mean ± SE; *$P < 0.05$, ***$P < 0.001$.

in the presence of 100 ng/mL of IL-17A and IL-17F showed a large increase in GR-α/GR-β at mRNA level. This finding is in line with the potential dual role of IL-17 cytokines, where IL-17 may play a role in tissue homeostasis. However, adipocytes obtained from obese subjects which were stimulated with IL-17A and IL-17F showed a decrease in GR-α/GR-β ratio at mRNA level. Results obtained from protein differed in responses. Lean and obese adipocytes stimulated with IL-17 cytokines showed an increase in the negative regulator, GR-β. Although most studies on steroid resistance report a dysregulation with GR-α, our results showed no change in GR-α protein. Nonetheless, the overall effect of IL-17 cytokines is a decrease in the GR-α/GR-β ratio in both lean and obese adipocytes. Moreover, it would seem that obese adipocytes respond more to IL-17F. Very little amount of literature is available on the role of IL-17F in obesity as it is simply described as a closely related cytokine of IL-17A. Interestingly, in serum obtained from asthmatics patients, IL-17F protein expression is increased

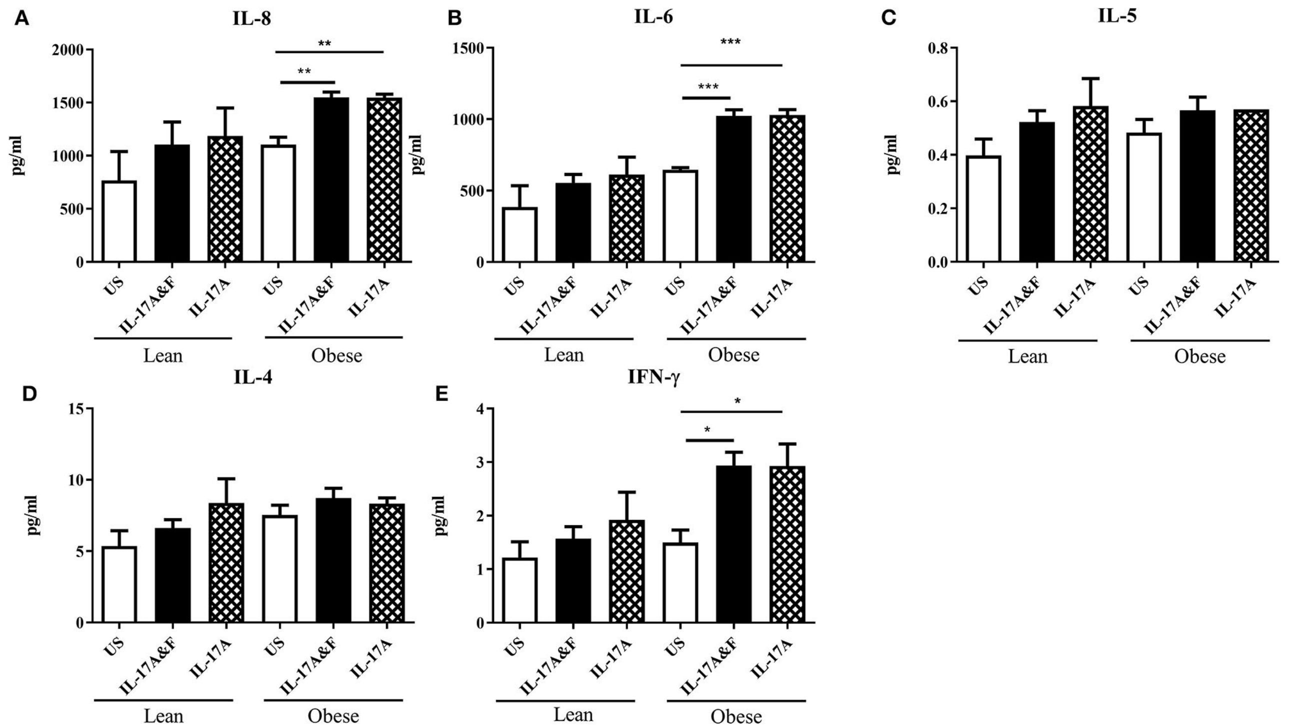


FIGURE 5 | Stimulation with IL-17A&F and IL-17A alone induces changes in inflammatory cytokine profiles in adipocytes from lean and obese subjects. A multiplex assay was used to measure the levels of inflammatory cytokines: IL-8 **(A)**, IL-6 **(B)**, IL-5 **(C)**, IL-4 **(D)**, and IFN-γ **(E)** secreted by mature adipocytes after 48 h stimulation with IL-17A & F combination or IL-17A alone. One independent experiment preformed per subject. $n = 4$ lean subjects, $n = 3$ obese subjects, One-way ANOVA, Mean ± SE; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 3 | Demographic and clinical data of patients.

	Non-obese	Obese
N	43	57
Age, year	36.3 ± 2.0	41.4 ± 2.1
BMI, kg/m^2	25.4 ± 0.5	36.5 ± 0.8
ACT score	16.2 ± 0.9	16.4 ± 0.6
FEV_1 (L)	2.6 ± 0.2	2.2 ± 0.1
FVC (L)	3.4 ± 0.2	2.7 ± 0.2
FEV_1/FVC (%)	76.1 ± 1.8	79.2 ± 1.6
Differentials (%)		
• Neutrophils	55.9 ± 2.4	56.0 ± 1.6
• Lymphocytes	30.9 ± 1.9	32.6 ± 1.5
• Monocytes	7.1 ± 0.4	7.0 ± 0.4
• Eosinophils	5.8 ± 0.7	3.9 ± 0.4
• Basophils	0.6 ± 0.1	0.5 ± 0.1

BMI, body mass index; ACT, Asthma Control Test; FEV_1, Forced expiratory volume in 1 s; FVC: forced vital capacity. Values shown are mean ± SE.

in obese and morbidly obese patients compared to lean and overweight patients whereas IL-17A is unchanged. The serum levels of Th17 cytokines are consistent with a previous study which showed that a healthier diet led to decreases in IL-17F but not IL-17A (27). Moreover, the levels of IL-17 were much higher than IL-17A. We also found an increase in IL-13 protein expression in serum of obese asthmatics compared to lean asthmatics. IL-13 is a pro-inflammatory cytokine involved in allergic asthma. IL-17A has been shown to enhance IL-13 activity (28). In mice, IL-13 treatment induced airway hyperresponsiveness and led to increased numbers of IL-17-producing CD4+ T cells (29). Increase in IL-13 has been reported in general obesity (30). Serum levels of IL-13 positively correlate with BMI (31). However, its role in the obese asthma phenotype is unknown and further investigations should be done to determine its exact function. In an animal model of obesity, it has been reported that BAL and serum IL-17A levels were not affected by the type of diet. However, pulmonary IL-17 mRNA levels were increased in high-fat diet animals compared to chow fed animals. Moreover, flow cytometry revealed an increase in IL-17A producing cells in the lungs (32). These results indicate that the changes in Th17 cytokines are observed locally within the lungs but obesity does not lead to increased systemic inflammation in asthma models. This warrants further investigations of IL-17-producing cells in the lung or adipose tissue of obese asthmatics compared to lean asthmatics. Demographic and clinical data for the lean and obese moderate-to-severe asthmatics revealed that lung function was similar in both populations whereas blood eosinophil's were decreased in obese asthmatics, This is in line with previous literature which has shown a negative correlation between BMI and blood eosinophil's in a population with high

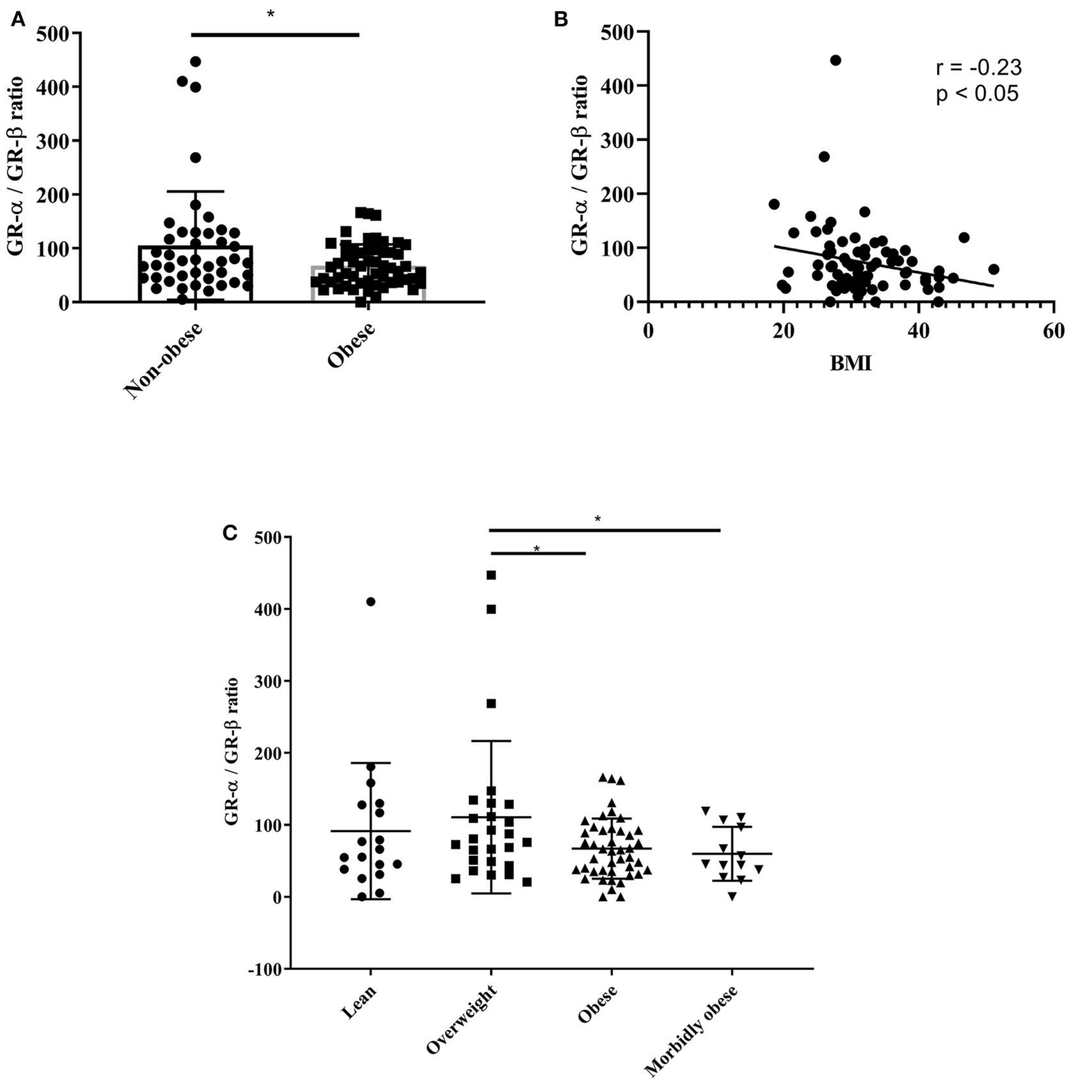


FIGURE 6 | GR-α/GR-β ratio is decreased in serum of obese asthmatic subjects. Serum was obtained from lean (BMI < 25 kg/m^2), overweight (BMI < 30 kg/m^2), obese (BMI < 35 kg/m^2), and morbidly obese (BMI > 35 kg/m^2) asthmatic subjects. **(A,C)** ELISA was used to assess protein levels of GR-α and GR-β. **(B)** Pearson correlation between BMI and GR-α/GR-β ratio. $n = 43$ non-obese subjects ($n = 18$ lean, $n = 26$ overweight), $n = 57$ obese subjects ($n = 44$ obese, $n = 13$ morbidly obese), Mean ± SE; *$P < 0.05$.

eosinophil's (33). The obese asthma phenotype is a complex phenotype where not all obese asthmatics share similar clinical features. However, studies have shown that clinical features that are common in patients with high BMI are late onset asthma, less airway eosinophil's and reduced atopy (34).

Adipose tissue, which is mainly composed of adipocytes, is a major source of proinflammatory cytokines such as IL-6, IL-8, IL-10, TNF-α, and IL-18 (35) thus linking obesity and inflammation. Due to their proinflammatory properties, IL-17 cytokines may be involved in the association between obesity and inflammation. Therefore, we were interested in examining the role of IL-17 in the production of inflammatory mediators by adipocytes obtained from obese and lean subjects. Adipocytes were stimulated with IL-17A and IL-17F for 48 h. Stimulation with IL-17 led to an increase in IL-6 and IL-8 mRNA. At protein level, this change was only observed in adipocytes from obese subjects. This is of interest as small adipocytes in lean individuals have been shown to promote homeostasis whereas large adipocytes from obese individuals promote inflammation and are involved in the recruitment of macrophages (36). This highlights a differential function for adipocytes in relation to body weight. In this study we found that obese adipocytes respond to IL-17 through the release of pro-inflammatory cytokines, which will lead to exaggerated inflammatory responses. Interestingly, steroid-treated obese adipocytes showed a decrease in GR-α/GR-β ratio which was further decreased in the presence of IL-17A. IL-17A was able to decrease the GR-α/GR-β ratio in steroid-treated lean adipocytes which did respond to Dexa. This finding suggests

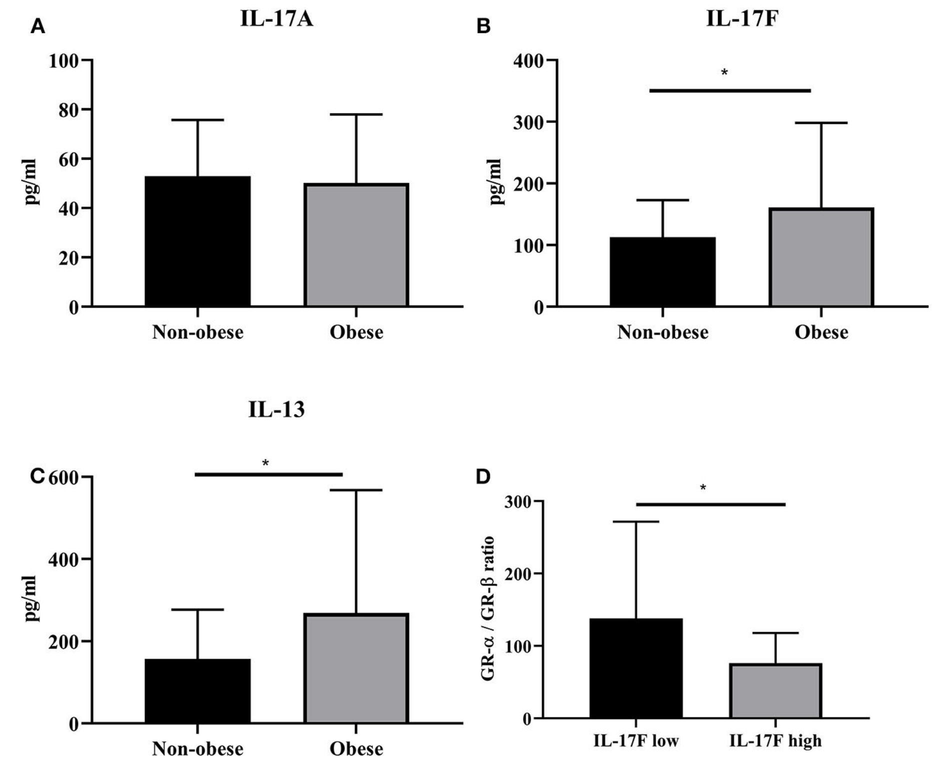


FIGURE 7 | IL-17F production is increased in serum of obese asthmatics. Serum was obtained from lean (BMI < 25 kg/m^2), overweight (BMI < 30 kg/m^2), obese (BMI < 35 kg/m^2), and morbidly obese (BMI > 35 kg/m^2) asthmatic subjects. **(A–C)** ELISA was used to assess protein levels of IL-17A, IL-17F, IL-13, respectively. **(D)** GR-α/GR-β ratio in subjects with high IL-17F and low IL-17F. $n = 43$ non-obese subjects, $n = 57$ obese subjects, Mean ± SE; $^*P < 0.05$.

that IL-17 is capable of altering responses to steroid. In a recent study on neutrophilic inflammation in asthma, it was shown that Dexa and IL-17A in combination synergistically induced the expression of the neutrophil promoting cytokine CSF3 and Dexa alone failed to alleviate neutrophilic inflammation (37).

In conclusion, our data suggest that IL-17 cytokines are involved in the inflammatory response seen in obese subjects. Moreover, IL-17 is involved in the dysregulation of glucocorticoid receptors which may explain steroid hyporesponsiveness commonly described in obese asthmatics. BMI can be used a predictor for steroid responsiveness. IL-17F and IL-13, which is increased in obese asthmatics, may be involved in the dysregulation of GR-α/GR-β ratio.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Dubai Health Authority Mohammed bin Rashid University of Medicine and Health Sciences Internal Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SA designed experiments, analyzed the samples, and contributed to the manuscript preparation. MG performed experiments, analyzed the samples, and contributed to the manuscript preparation. RR contributed to the manuscript preparation. AM performed ELISA experiments. LS collected samples from the patients. BM collected samples from the patients. QH contributed to the design of the experiments and manuscript preparation. All authors read and approved final version of the manuscript.

FUNDING

The work was funded by Mohammed Bin Rashid University of Medicine and Health Sciences Internal Grant (MBRU-CM-RG2017-10) and supported by L'Oreal-UNESCO for Women in Science grant.

REFERENCES

1. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes.* (2008) 32:1431–7. doi: 10.1038/ijo.2008.102
2. Khaodhiar L, McCowen KC, Blackburn GL. Obesity and its comorbid conditions. *Clin Cornerstone.* (1999) 2:17–31. doi: 10.1016/S1098-3597(99)90002-9
3. Mohanan S, Tapp H, McWilliams A, Dulin M. Obesity and asthma: pathophysiology and implications for diagnosis and management in primary care. *Exp Biol Med (Maywood).* (2014) 239:1531–40. doi: 10.1177/1535370214525302
4. Peters MC, McGrath KW, Hawkins GA, Hastie AT, Levy BD, Israel E, et al. Plasma interleukin-6 concentrations, metabolic dysfunction, and asthma severity: a cross-sectional analysis of two cohorts. *Lancet Respir Med.* (2016) 4:574–84. doi: 10.1016/S2213-2600(16)30048-0
5. Straczkowski M, Kowalska I, Nikolajuk A, Dzienis-Straczkowska S, Szelachowska M, Kinalska I. Plasma interleukin 8 concentrations in obese subjects with impaired glucose tolerance. *Cardiovasc Diabetol.* (2003) 2:5. doi: 10.1186/1475-2840-2-5
6. Hotamisligil GS, Spiegelman BM. Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes.* (1994) 43:1271–8. doi: 10.2337/diabetes.43.11.1271
7. Nishimura S, Manabe I, Nagai R. Adipose tissue inflammation in obesity and metabolic syndrome. *Discov Med.* (2009) 8:55–60.
8. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab.* (2001) 280:E745–51. doi: 10.1152/ajpendo.2001.280.5.E745
9. McArdle MA, Finucane OM, Connaughton RM, McMorrow AM, Roche HM. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. *Front Endocrinol.* (2013) 4:52. doi: 10.3389/fendo.2013.00052
10. Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem.* (2004) 279:2559–67. doi: 10.1074/jbc.M308809200
11. Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 cells: new players in asthma pathogenesis. *Allergy.* (2011) 66:989–98. doi: 10.1111/j.1398-9995.2011.02576.x
12. Sumarac-Dumanovic M, Stevanovic D, Ljubic A, Jorga J, Simic M, Stamenkovic-Pejkovic D, et al. Increased activity of interleukin-23/interleukin-17 proinflammatory axis in obese women. *Int J Obes.* (2009) 33:151–6. doi: 10.1038/ijo.2008.216
13. Ramakrishnan RK, Al Heialy S, Hamid Q. Role of IL-17 in asthma pathogenesis and its implications for the clinic. *Expert Rev Respir Med.* (2019) 13:1057–68. doi: 10.1080/17476348.2019.1666002
14. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol.* (2009) 123:1185–7. doi: 10.1016/j.jaci.2009.02.024
15. Tashiro H, Shore SA. Obesity and severe asthma. *Allergol Int.* (2019) 68:135–42. doi: 10.1016/j.alit.2018.10.004
16. Mosen DM, Schatz M, Magid DJ, Camargo CA Jr. The relationship between obesity and asthma severity and control in adults. *J Allergy Clin Immunol.* (2008) 122:507–11.e6. doi: 10.1016/j.jaci.2008.06.024
17. Forno E, Lescher R, Strunk R, Weiss S, Fuhlbrigge A, Celedon JC. Decreased response to inhaled steroids in overweight and obese asthmatic children. *J Allergy Clin Immunol.* (2011) 127:741–9. doi: 10.1016/j.jaci.2010.12.010
18. Goleva E, Covar R, Martin RJ, Leung DYM. Corticosteroid pharmacokinetic abnormalities in overweight and obese corticosteroid resistant asthmatics. *J Allergy Clin Immunol Pract.* (2016) 4:357–60.e2. doi: 10.1016/j.jaip.2015.11.013
19. Sobande PO, Kercsmar CM. Inhaled corticosteroids in asthma management. *Respir Care.* (2008) 53:625–33. discussion 33–4.
20. Vazquez-Tello A, Halwani R, Hamid Q, Al-Muhsen S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol.* (2013) 33:466–78. doi: 10.1007/s10875-012-9828-3
21. van der Wiel E, Ten Hacken NH, van den Berge M, Timens W, Reddel HK, Postma DS. Eosinophilic inflammation in subjects with mild-to-moderate asthma with and without obesity: disparity between sputum and biopsies. *Am J Respir Crit Care Med.* (2014) 189:1281–4. doi: 10.1164/rccm.201310-1841LE
22. Martin RJ, Szefler SJ, King TS, Kraft M, Boushey HA, Chinchilli VM, et al. The predicting response to inhaled corticosteroid efficacy (PRICE) trial. *J Allergy Clin Immunol.* (2007) 119:73–80. doi: 10.1016/j.jaci.2006.10.035
23. Tomlinson JE, McMahon AD, Chaudhuri R, Thompson JM, Wood SF, Thomson NC. Efficacy of low and high dose inhaled corticosteroid in smokers versus non-smokers with mild asthma. *Thorax.* (2005) 60:282–7. doi: 10.1136/thx.2004.033688
24. Schatz M, Hsu JW, Zeiger RS, Chen W, Dorenbaum A, Chipps BE, et al. Phenotypes determined by cluster analysis in severe or difficult-to-treat asthma. *J Allergy Clin Immunol.* (2014) 133:1549–56. doi: 10.1016/j.jaci.2013.10.006
25. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, et al. Obesity predisposes to Th17 bias. *Eur J Immunol.* (2009) 39:2629–35. doi: 10.1002/eji.200838893
26. Ji Z, Wu S, Xu Y, Qi J, Su X, Shen L. Obesity promotes EAE through IL-6 and CCL-2-mediated T cells infiltration. *Front Immunol.* (2019) 10:1881. doi: 10.3389/fimmu.2019.01881
27. Han YY, Forno E, Brehm JM, Acosta-Pérez E, Alvarez M, Colón-Semidey A, et al. Diet, interleukin-17, and childhood asthma in Puerto Ricans. *Ann Allergy Asthma Immunol.* (2015) 115:288–93.e1. doi: 10.1016/j.anai.2015.07.020
28. Hall SL, Baker T, Lajoie S, Richgels PK, Yang Y, McAlees JW, et al. IL-17A enhances IL-13 activity by enhancing IL-13-induced signal transducer and activator of transcription 6 activation. *J Allergy Clin Immunol.* (2017) 139:462–71.e14. doi: 10.1016/j.jaci.2016.04.037
29. Kinyanjui MW, Shan J, Nakada EM, Qureshi ST, Fixman ED. Dose-dependent effects of IL-17 on IL-13-induced airway inflammatory responses and airway hyperresponsiveness. *J Immunol.* (2013) 190:3859–68. doi: 10.4049/jimmunol.1200506
30. Schmidt FM, Weschenfelder J, Sander C, Minkwitz J, Thormann J, Chittka T, et al. Inflammatory cytokines in general and central obesity and modulating effects of physical activity. *PLoS ONE.* (2015) 10:e0121971. doi: 10.1371/journal.pone.0121971
31. Martínez-Reyes CP, Gómez-Arauz AY, Torres-Castro I, Manjarrez-Reyna AN, Palomera LF, Olivos-García A, et al. Serum levels of interleukin-13 increase in subjects with insulin resistance but do not correlate with markers of low-grade systemic inflammation. *J Diabetes Res.* (2018) 2018:7209872. doi: 10.1155/2018/7209872
32. Mathews JA, Wurmbrand AP, Ribeiro L, Neto FL, Shore SA. Induction of IL-17A precedes development of airway hyperresponsiveness during diet-induced obesity and correlates with complement factor D. *Front Immunol.* (2014) 5:440. doi: 10.3389/fimmu.2014.00440
33. Sunadome H, Matsumoto H, Izuhara Y, Nagasaki T, Kanemitsu Y, Ishiyama Y, et al. Correlation between eosinophil count, its genetic background and body mass index: the nagahama study. *Allergol Int.* (2020) 69:46–52. doi: 10.1016/j.alit.2019.05.012
34. Baffi CW, Winnica DE, Holguin F. Asthma and obesity: mechanisms and clinical implications. *Asthma Res Pract.* (2015) 1:1. doi: 10.1186/s40733-015-0001-7
35. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin immunol.* (2005) 115:911–9; quiz 20. doi: 10.1016/j.jaci.2005.02.023
36. Thomas D, Apovian C. Macrophage functions in lean and obese adipose tissue. *Metabolism.* (2017) 72:120–43. doi: 10.1016/j.metabol.2017.04.005

37. Ouyang S, Liu C, Xiao J, Chen X, Lui AC, Li X. Targeting IL-17A/glucocorticoid synergy to CSF3 expression in neutrophilic airway diseases. *JCI insight.* (2020) 5:e132836. doi: 10.1172/jci.insight.132836

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 August 2020
doi: 10.3389/fimmu.2020.01894

Bimekizumab, a Novel Humanized IgG1 Antibody That Neutralizes Both IL-17A and IL-17F

Ralph Adams[1†], Asher Maroof[2*†], Terry Baker[1], Alastair D. G. Lawson[3], Ruth Oliver[4], Ross Paveley[5], Steve Rapecki[1], Stevan Shaw[2], Pavan Vajjah[4], Shauna West[5] and Meryn Griffiths[6]

[1] Discovery Science, New Modality Therapeutics, UCB Pharma, Slough, United Kingdom, [2] Immuno-Bone Therapeutic Area, Immunology Research, UCB Pharma, Slough, United Kingdom, [3] Research Fellow, UCB Pharma, Slough, United Kingdom, [4] Development Science, QP/DMPK, UCB Pharma, Slough, United Kingdom, [5] Immuno-Bone Therapeutic Area, Immuno-Bone Discovery, UCB Pharma, Slough, United Kingdom, [6] Translational Medicine, TM Immuno-Bone, UCB Pharma, Slough, United Kingdom

OPEN ACCESS

Edited by:
Nicola Ivan Lorè, IRCCS San Raffaele Scientific Institute, Italy

Reviewed by:
Lars Rogge, Institut Pasteur, France
Katarzyna Bulek, Jagiellonian University, Poland

***Correspondence:**
Asher Maroof
Ash.Maroof@ucb.com

† These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *13 December 2019*
Accepted: *14 July 2020*
Published: *21 August 2020*

Citation:
Adams R, Maroof A, Baker T, Lawson ADG, Oliver R, Paveley R, Rapecki S, Shaw S, Vajjah P, West S and Griffiths M (2020) Bimekizumab, a Novel Humanized IgG1 Antibody That Neutralizes Both IL-17A and IL-17F. Front. Immunol. 11:1894. doi: 10.3389/fimmu.2020.01894

Interleukin (IL)-17A is a key driver of inflammation and the principal target of anti-IL-17 therapeutic monoclonal antibodies. IL-17A, and its structurally similar family member IL-17F, have been shown to be functionally dysregulated in certain human immune-mediated inflammatory diseases such as psoriasis, psoriatic arthritis, and axial spondyloarthritis. Given the overlapping biology of these two cytokines, we postulated that dual neutralization of IL-17A and IL-17F may provide a greater depth of clinical response in IL-17-mediated diseases than IL-17A inhibition alone. We identified 496.g1, a humanized antibody with strong affinity for IL-17A but poor affinity for IL-17F. Affinity maturation of 496.g1 to 496.g3 greatly enhanced the affinity of the Fab fragment for IL-17F while retaining strong binding to IL-17A. As an IgG1, the affinity for IL-17A and IL-17F was 3.2 pM and 23 pM, respectively. Comparison of 496.g3 IgG1 with the commercially available anti-IL-17A monoclonal antibodies ixekizumab and secukinumab, by surface plasmon resonance and in a human *in vitro* IL-17A functional assay, showed that 496.g3 and ixekizumab display equivalent affinity for IL-17A, and that both antibodies are markedly more potent than secukinumab. In contrast to ixekizumab and secukinumab, 496.g3 exhibited the unique feature of also being able to neutralize the biological activity of IL-17F. Therefore, antibody 496.g3 was selected for clinical development for its ability to neutralize the biologic function of both IL-17A and IL-17F and was renamed bimekizumab (formerly UCB4940). Early clinical data in patients with psoriasis, in those with psoriatic arthritis, and from the Phase 2 studies in psoriasis, psoriatic arthritis, and ankylosing spondylitis, are encouraging and support the targeted approach of dual neutralization of IL-17A and IL-17F. Taken together, these findings provide the rationale for the continued clinical evaluation of bimekizumab in patients with immune-mediated inflammatory diseases.

Keywords: anti-IL-17A, IL-17A, IL-17F, monoclonal antibody, bimekizumab, dual neutralization, dual targeting

INTRODUCTION

Interleukin (IL)-17A was the first identified member of a family of six structurally similar cytokines; IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F (1). Originally cloned in 1993 (2) as a cytokine derived from activated T cells, IL-17A is now recognized as a key pro-inflammatory cytokine in chronic immune-mediated inflammatory diseases, particularly psoriasis, and spondyloarthritis (3). The IL-17 axis has been shown to play an important role in defense against extracellular bacteria and fungi, through the induction of chemokines involved in the recruitment of neutrophils and monocytes. In a variety of murine disease models, IL-17A has also been demonstrated to have a crucial function in promoting chronic inflammation and autoimmunity (4–6). Among IL-17 family members, IL-17F is closest in sequence to IL-17A, sharing ∼50% structural homology (7). Expressed as homodimers, or as a heterodimer (IL-17A/F) (8, 9), both IL-17A and IL-17F signal through the same heterodimeric complex of IL-17 receptors A and C (IL-17RA/RC) (10). The majority of non-hematopoietic cells have the potential to respond to the localized production of IL-17A or IL-17F as a result of the ubiquitous expression of their specific receptors.

Both IL-17A and IL-17F cytokines are expressed by Th17 cells, as well as additional immune cell types, including CD8 T cells, natural killer T cells, lymphoid tissue inducer cells, innate lymphoid cells and γδ T cells (11). Although common pathways are involved in the differentiation of Th17 cells, emerging data suggest that the regulation of IL-17A expression is distinct from that of IL-17F (12). IL-17A has a stronger affinity for the IL-17RA/RC complex, and thus promotes a greater induction of pro-inflammatory genes than IL-17F. Early mouse model data showed that animals lacking either IL-17A or IL-17F exhibited distinct biology (13). In contrast to IL-17A, IL-17F was thought to drive models of lung inflammation, with little or no role in experimental autoimmune encephalitis (14). However, human translational data showed that, like IL-17A, IL-17F synergizes with tumor necrosis factor (TNF) to induce a pro-inflammatory gene signature that is qualitatively similar to that induced by the combination of IL-17A and TNF (15, 16). Dysregulated expression of IL-17A and IL-17F is associated with chronic inflammatory diseases such as psoriasis, psoriatic arthritis, rheumatoid arthritis, ankylosing spondylitis, and asthma (17–20). Although IL-17A is known to be the more potent of the two cytokines, IL-17F is the more abundantly expressed of the two in psoriasis and spondyloarthritis (21). Despite the paucity of data in murine models supporting a role for IL-17F in promoting inflammation, human genetic data in individuals with autosomal dominant mutations in IL-17F, suggest a previously underestimated role for this cytokine (22).

Inhibiting IL-17A has proven to be an effective therapeutic strategy in the clinic. Two anti-IL-17A antibodies, ixekizumab, and secukinumab, are approved for the treatment of patients with psoriasis, psoriatic arthritis, and ankylosing spondylitis (23, 24). Furthermore, head-to-head trials demonstrated the superior clinical efficacy of ixekizumab and secukinumab in psoriasis over established treatments, ustekinumab (anti-IL-12/IL-23 monoclonal antibody) and etanercept (soluble TNF receptor inhibitor), respectively (25, 26).

Given, IL-17A and IL-17F share overlapping biology, we postulate that IL-17F also contributes to chronic tissue inflammation, beyond the established role of IL-17A. This rationale supports our hypothesis that neutralization of both IL-17A and IL-17F may be more effective than inhibition of IL-17A alone to neutralize IL-17-driven pathology.

In this study we describe the generation and characterization of 496.g3 (known as bimekizumab, formerly UCB4940), a humanized monoclonal antibody with high affinity for both IL-17A and IL-17F.

METHODS

Preparation of Antibody Constructs and Expression

DNA encoding the light chain variable regions of 496.g1 and 496.g3 were cloned into UCB expression vectors containing DNA encoding human light chain Cκ. DNA encoding the shared heavy chain variable region of 496.g1 and 496.g3 was cloned into UCB expression vectors containing DNA encoding either human heavy chain γ1 C_H1 region to generate Fab or human heavy chain γ1 IgG regions to generate IgG1. Antibodies were transiently expressed in CHO-S XE cells, a CHO-K1 derived cell line (27).

Purification of Fab and IgG1

Fab and IgG proteins were purified from culture supernatants using affinity chromatography. Supernatants containing Fab were passed over a HiTrap Protein G column (GE Healthcare, Buckinghamshire, UK) and supernatants containing IgG were passed over a MabSelect™ SuRe™ column (GE Healthcare). Following a washing step with phosphate buffered saline (PBS) (pH 7.4), the bound material was eluted with 0.1 M glycine (pH 3.2) and neutralized with 2 m Tris-HCl (pH 8.5). Fractions containing Fab or IgG were pooled, quantified by absorbance at 280 nm, and concentrated using Amicon Ultra centrifugal filters (Merck Millipore, Massachusetts, USA). To isolate the monomeric fractions of Fab and IgG, we used size-exclusion chromatography over a HiLoad 16/60, Superdex 200 column (GE Healthcare) equilibrated with PBS (pH 7.4). Fractions containing monomeric Fab or IgG were pooled, quantified, concentrated, and stored at 4°C.

Enzyme-Linked Immunosorbent Assay (ELISA)

Standard ELISA plates (Nunc Maxisorp™, ThermoFischer Scientific, Massachusetts, USA) were coated with 1 μg/mL cytokine (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) in PBS pH 7.4 overnight. Plates were washed three times in wash buffer (PBS supplemented with 0.05% Tween20, SigmaAldrich, Missouri, USA) and tapped dry. Diluted antibody was added to the relevant wells and incubated for 1 h at room temperature. Plates were washed three times and a goat IgG-horseradish peroxidase conjugated antibody with specificity for human Fc (Jackson ImmunoResearch, Pennsylvania, USA) was added to each well. Plates were incubated for 1 h at room temperature. Plates were washed for a final time before the addition of TMB (3,3′,5,5′-tetramethylbenzidine) Stabilized Substrate (Promega, Southampton, UK) for 8 min, after which an equal volume of stop solution (1 M H_3PO_4) was added to each well. Absorbance was then measured at 450 nm using a plate spectrophotometer (Biotek Instruments).

Expression and Purification of IL-17A and IL-17F for Neutralization Bioassay

IL-17F was cloned into an in-house mammalian expression vector and expressed by transient transfection using the Expi293™ Expression System (Life Technologies). IL-17F protein was purified by cation exchange, followed by isolation of the dimer fraction by size exclusion chromatography.

IL-17A was cloned into an in-house mammalian expression vector upstream of the human IgG1 Fc coding region with a TEV (tobacco etch virus) cleavage site. IL-17A-Fc protein was expressed transiently in CHO-S XE cells. IL-17A protein was purified by Protein A affinity chromatography before the human Fc tag was removed using a TEV protease (produced in-house). A fraction containing untagged dimeric IL-17A protein was then isolated by size exclusion chromatography.

Neutralization Bioassay

The potency of antibody 496 variants, ixekizumab (Taltz®; Eli Lilly, Indiana, USA) or secukinumab (Cosentyx®; Novartis, Basel, Switzerland), for the neutralization of human IL-17A and IL-17F was determined using a human primary cell bioassay. Normal human dermal fibroblasts (NHDFs) derived from neonate foreskin (106-05n, Sigma-Aldrich, Missouri, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% of heat-inactivated low endotoxin fetal bovine serum and 2 mM L-glutamine (Invitrogen, California, USA). Cells were grown in T75 flasks until 80–90% confluent, before being removed using 0.25% Trypsin-EDTA and plated in 384 well-plates (Corning, New York, USA) at 1,250 cells per well. Cells were allowed to rest for 3 h before addition of cytokines and/or antibodies. IL-17A or IL-17F were incubated with TNF for 1 h prior to being added to the cells. IL-6 protein quantification was determined using homogeneous time resolved fluorescence (HTRF; Cisbio, Codolet, France), as per the manufacturer's instructions, using a recombinant IL-6 standard curve (R&D Systems, Minnesota, USA) detected at 18 h (+/− 2 h). IL-6 levels were plotted against inhibitor concentrations in Prism 6 (GraphPad, California, USA) to generate IC_{50} and IC_{90} values.

Surface Plasmon Resonance (SPR)

The binding affinities and kinetic parameters for the interactions of antibodies were determined by SPR on a Biacore T200 using Series S CM5 sensor chips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.05% v/v surfactant P20) was used as running buffer. All experiments were performed at 25°C. The antibody samples were captured using $F(ab')_2$ fragment-specific or Fcγ-specific Affinipure $F(ab')_2$ fragment goat anti-human IgG (Jackson ImmunoResearch, Pennsylvania, USA). Covalent immobilization of the capturing antibody was achieved by standard amine coupling chemistry to a level of 3,500–5,000 response units (RU).

Human IL-17A and IL-17A/F, cynomolgus macaque IL-17A and IL-17F (all generated at UCB) and human IL-17F (R&D Systems) were titrated over the captured purified antibody from 10 nM (IL-17F) or 5 nM (IL-17A and IL-17A/F) to 0.625 nM or 0.315 nM, respectively. Each assay cycle consisted of first capturing the antibody sample using a 1-min injection at a flow rate of 10 μL/min, followed by an association phase consisting of a 3-min injection of the IL-17 cytokine at a flow rate of 30 μL/min; dissociation was then monitored. After each cycle, the capture surface was regenerated at a flow rate of 10 μL/min with a 1-min injection of 40 mM HCl followed by a 30-sec injection of 10 mM or 5 mM NaOH. A blank flow-cell was used for reference subtraction and buffer-blank injections were included to subtract instrument noise and drift. Kinetic parameters were determined using Biacore T200 Evaluation Software V3.0.

RESULTS

Discovery and Characterization of 496.g1

Initially, we sought to generate an IL-17A therapeutic antibody. In brief, a panel of antibodies was raised in Sprague Dawley rats to human IL-17A. Using a single B cell selection method (28), the parental antibody 496 was identified for its strong binding to IL-17A and its ability to inhibit IL-17A-induced IL-6 production in the 3T3-NIH cell line (data not shown). Sequence alignment of IL-17 family members showed that IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F shared 20–50% homology to IL-17A at the amino acid level. To determine whether the humanized variant of 496, 496.g1, was cross-reactive with other IL-17 family members, an indirect ELISA using recombinant protein was performed. Relative to an isotype-matched control antibody, 496.g1 showed binding to IL-17A (EC_{90} 12.1 ng/mL) and IL-17F (EC_{90} 358.5 ng/mL). Little or no binding to human IL-17B, IL-17C, IL-17D, and IL-7E was observed (**Figure 1**).

Mathematical Modeling of IL-17A and IL-17F

To investigate the therapeutic potential of 496.g1, a target-mediated drug disposition model was used to predict the percentage of IL-17A or IL-17F bound to 496.g1 in skin (29). Allometric scaling, which considers the differences in body surface area and weight, was used to predict the pharmacokinetic parameters (clearance and volume of distribution) of 496.g1 in humans. In each case, the percentage of antibody that was ascribed to partition to the skin was 30% (30). The simulations predicted that, following a 160 mg IV dose of 496.g1 every 4 weeks, IL-17A was completely bound in plasma and >95% bound in skin compartments at trough or before the next dose was administered at steady state, but skin IL-17F showed <50% occupancy at the same timepoint (**Figure 2**). This was considered to be sub-optimal in humans as IL-17F signaling would not be completely inhibited. We had hypothesized that both IL-17A and IL-17F needed to be neutralized to attain optimal clinical

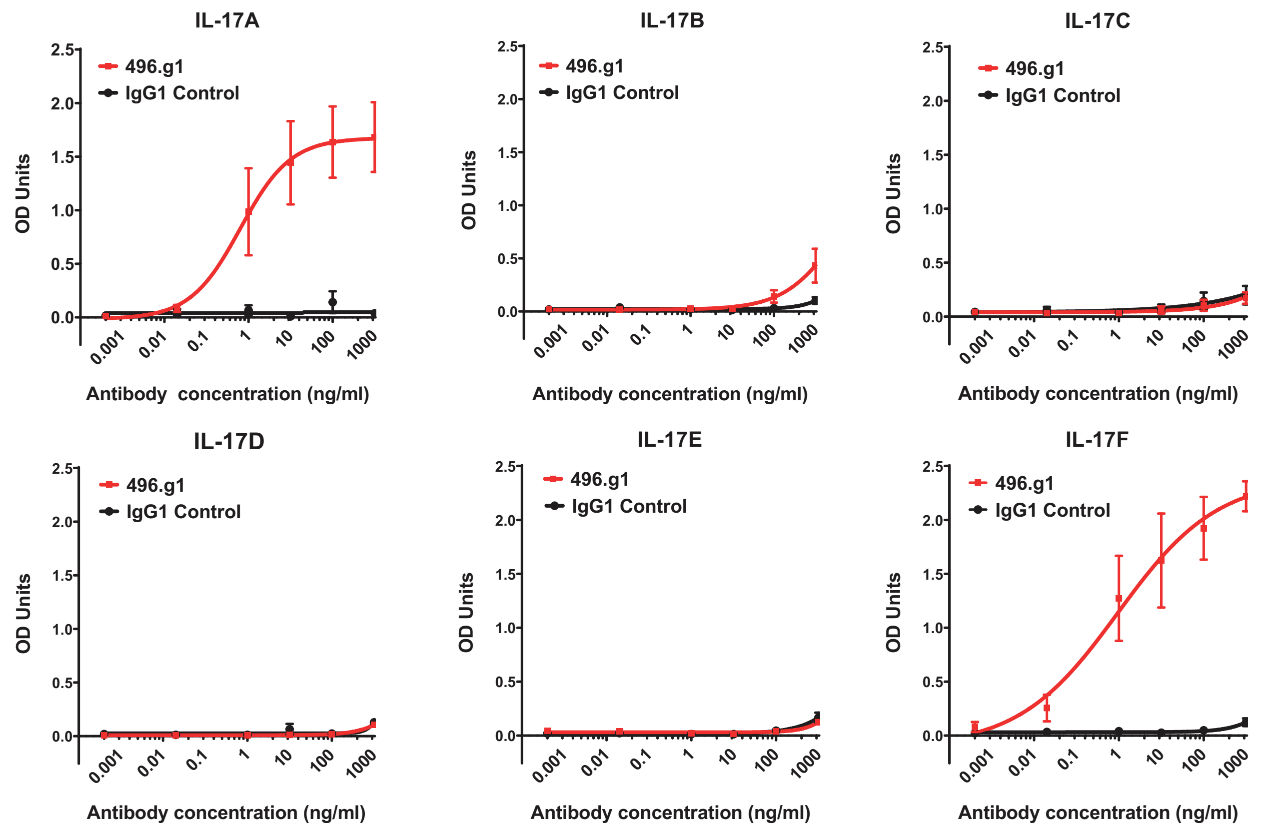


FIGURE 1 | Binding of 496.g1 to human recombinant IL-17A–F. Recombinant cytokines **(A–F)** were coated onto high-binding ELISA plates. Titrations of 496.g1 or isotype control antibody were added, starting at concentrations of 10 µg/mL. Optical density (OD) absorbance was measured at 450 nm. Values represent average absorbance and standard deviation of six technical replicates.

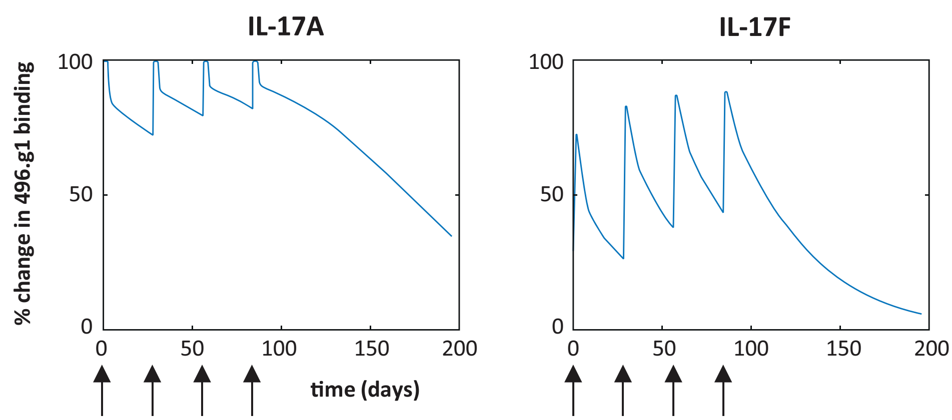


FIGURE 2 | Predicted percentage of IL-17A and IL-17F bound to 496.g1 in psoriatic skin based on a target-mediated drug disposition model. Simulations are based on a 160 mg dose IV every 4 weeks (arrow) and partitioning of 30% of the antibody into the skin and indicate insufficient binding of 496.g1 to completely inhibit IL-17F in psoriatic skin.

outcomes compared with inhibition of IL-17A alone. Therefore, it was decided to try to improve the affinity of 496.g1 for IL-17F, while maintaining affinity for IL-17A.

Characterization of 496.g3

To affinity mature 496.g1 for IL-17F, a crystal structure of 496.g1 Fab in complex with IL-17F was generated and a proprietary *in*

TABLE 1 | Binding affinities and kinetic parameters of 496.g1 and 496.g3 Fab fragments.

Cytokine	496	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	K_D (pM)
IL-17F	g1	4.00E+06	6.03E-03	1.51E-09	1,510
	g3	4.75E+06	1.64E-04	3.45E-11	35
IL-17A	g1	1.93E+06	5.63E-05	2.92E-11	29
	g3	1.44E+06	1.03E-05	7.20E-12	7

Association (k_a) and dissociation (k_d) rates and affinity constants (K_D) were determined by surface plasmon resonance.

silico design method applied to the interface (details of which can be found in patent application number WO2014198951A2). Using this method, we tested a series of mutation combinations, identifying five mutations in the light chain variable region of 496.g1 that increased binding affinity for IL-17F while also improving affinity for IL-17A, giving rise to antibody 496.g3 (**Supplementary Data**). As a purified Fab fragment, the affinity constants (K_D) of 496.g3 for IL-17F and IL-17A were shown to be 35 pM and 7 pM, respectively (**Table 1**). This compared favorably with the K_D of 496.g1 Fab for IL-17F and IL-17A at 1510 pM and 29 pM, respectively, showing a 43-fold increase in the affinity

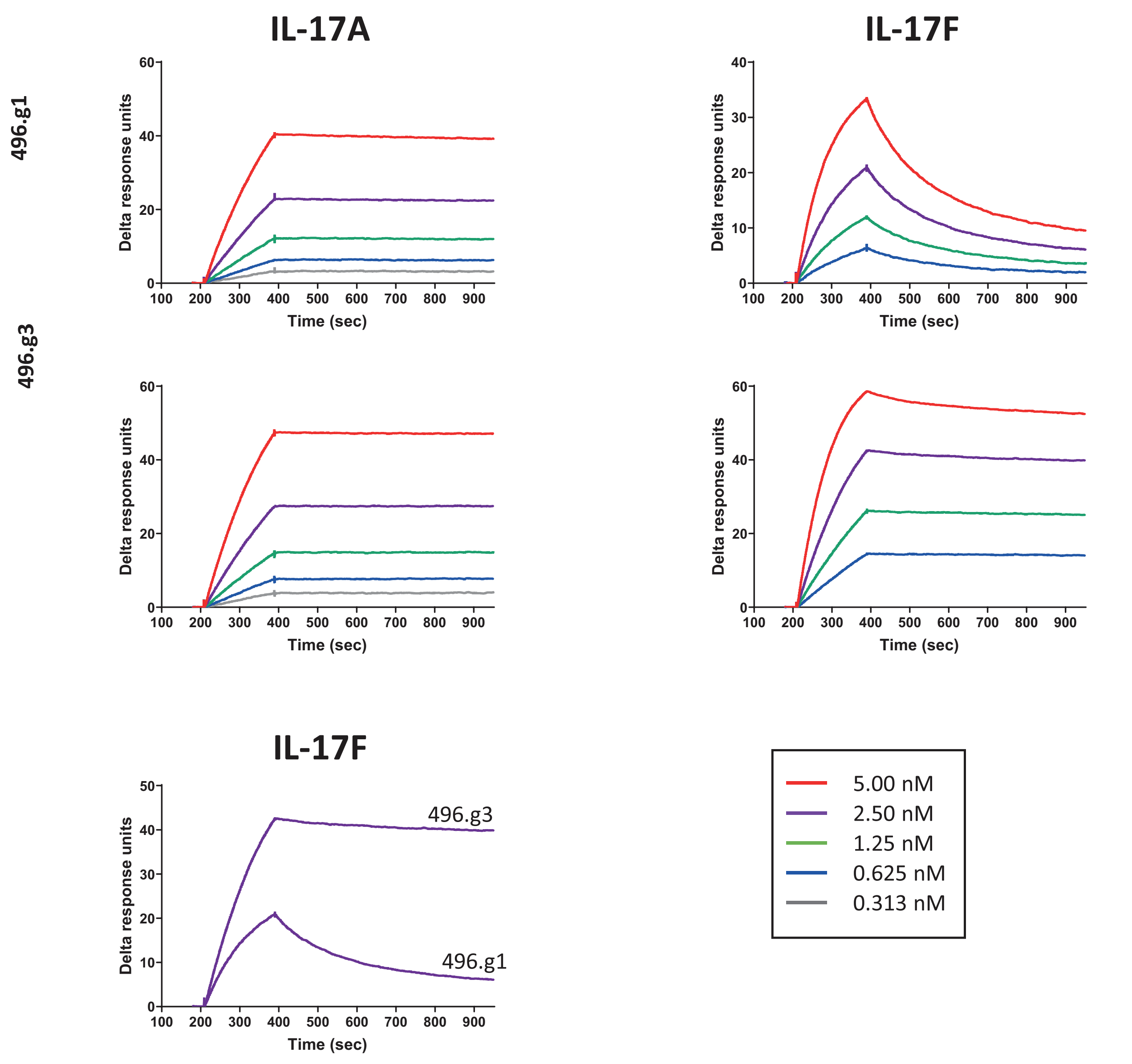


FIGURE 3 | Affinity of 496.g3 to IL-17A and IL-17F. Anti-human F(ab′)$_2$ was immobilized onto a CM5 sensor chip surface followed by the capture of either 496.g1 or 496.g3 Fabs. The association phase showed an increase in response over time following the injection of varying concentrations of IL-17A (5–0.313 nM) and IL-17F (5–0.625 nM) and this was followed by the dissociation phase when buffer replaced the IL-17A and IL-17F.

of 496.g3 Fab for IL-17F and a 4-fold increase in its affinity for IL-17A compared with 496.g1 (**Figure 3**).

In order to determine whether the improvement in affinity for IL-17F had resulted in a concomitant improvement in neutralization activity, the potency of 496.g3 in neutralizing IL-17A- or IL-17F-stimulated release of IL-6 from NHDFs was compared with that of 496.g1. Stimulation of cells by IL-17A or IL-17F was too weak for a qualitative assay. Similarly, TNF only weakly stimulated cells. However, when either IL-17A or IL-17F was combined with TNF, they acted synergistically to strongly elevate the assay signal. Both 496.g1 and 496.g3 inhibited IL-17A and IL-17F to the level of stimulation seen with TNF alone (**Figure 4**). Pre-incubation of either antibody with TNF had no effect on IL-6 stimulation (data not shown). Equivalent IC_{90}s were produced for 496.g1 and 496.g3 against IL-17A at 0.04 nM and 0.02 nM, respectively. Notably, 496.g3 was ∼10-fold more potent than 496.g1 against IL-17F, with IC_{90} values of 23.41 nM and 238.8 nM, respectively.

To enable toxicology and pharmacokinetic studies in primates, the affinity of 496.g3 for cynomolgus macaque IL-17A and IL-17F was determined. 496.g3 showed similar affinity for human and cynomolgus macaque IL-17A, with K_D values of 3.2 pM and 12 pM, respectively (**Table 2**). While the affinity for cynomolgus macaque IL-17F at 345 pM was weaker than for human IL-17F at 23 pM (**Table 2**), it was considered to be sufficient for 496.g3 characterization in primate studies. Further studies showed 496.g3 did not bind to mouse or rat IL-17A or IL-17F (data not shown).

Comparison With Approved Therapeutics

To compare the efficacy of 496.g3 in an *in vitro* neutralization assay against anti-IL-17A-specific antibodies ixekizumab and secukinumab, the relative inhibition of IL-17A, IL-17F, and IL-17A/F signaling was examined. This assay utilized IL-6 release as a surrogate marker of inflammatory activation. The potency curves were calculated relative to fibroblasts activated with TNF alone. 496.g3 and ixekizumab showed similar IC_{90} values for IL-17A at 2.5 ng/mL and 2.2 ng/mL, respectively, and for IL-17A/F at 179.2 ng/mL and 91 ng/mL, respectively. In contrast, secukinumab was significantly less potent against IL-17A at 956.2 ng/mL. Of note, only 496.g3 demonstrated inhibition of IL-17F (IC_{90} 137.8 ng/mL), as neither ixekizumab nor secukinumab bound IL-17F (**Figure 5A** and **Table 3**). These data are consistent with the reported affinity constants for ixekizumab and secukinumab, and the measured affinities for 496.g3 (**Table 4**).

Measurements of IL-17A and IL-17F in psoriatic lesional tissue and serum show that, on average, the level of IL-17F is 30-fold higher than that of IL-17A (21). These quantitative differences are also observed in the serum of patients with

TABLE 2 | Binding affinity and kinetic parameters of 496.g3 for human and cynomolgus macaque IL-17A and IL-17F.

Species	Cytokine	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	K_D (pM)
Human	IL-17F	2.99E+06	6.91E-05	2.31E-11	23
	IL-17A	4.59E+06	1.45E-05	3.17E-12	3.2
Cynomolgus macaque	IL-17F	4.27E+05	1.48E-04	3.45E-10	345
	IL-17A	1.64E+06	2.02E-05	1.23E-11	12

Association (k_a) and dissociation (k_d) rates and affinity constants (K_D) were determined by SPR.

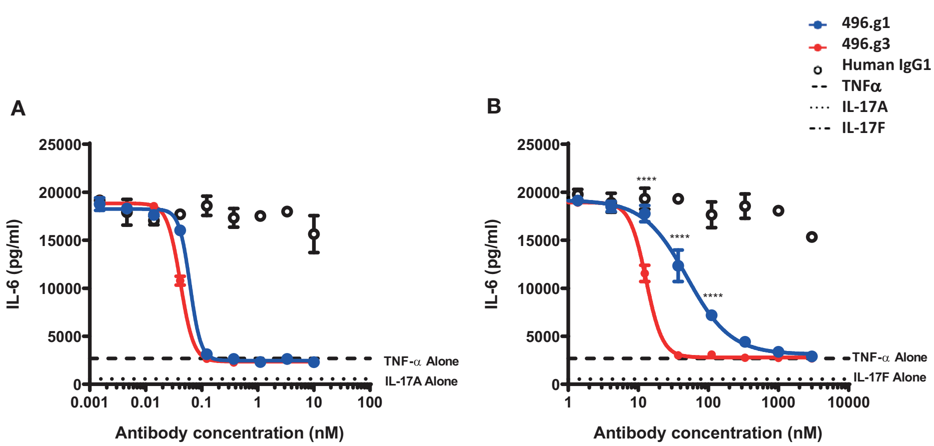


FIGURE 4 | Inhibition of IL-6 production from normal human dermal fibroblasts (NHDFs) stimulated with TNF in combination with IL-17A or IL-17F by IL-17-specific antibodies. **(A)** IL-17A (0.15 nM) or **(B)** IL-17F (25 nM) in combination with TNFα (0.025 nM) was pre-incubated with titrations of 496.g1, 496.g3 or an irrelevant antigen-specific human IgG1 isotype control for 1 h before addition to cells. NHDFs were subsequently stimulated for 18–20 h before IL-6 protein levels in the supernatant were determined by homogeneous time-resolved fluorescence. As shown in both figures, stimulation of NHDFs by TNF or IL-17A or IL-17F individually failed to elicit sufficient production of IL-6. Each condition was tested in duplicate and the data shown is representative of three separate experiments. Two-way ANOVA, Bonferroni post-test analysis was performed to compare inhibition of 496.g1 vs. 496.g3 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

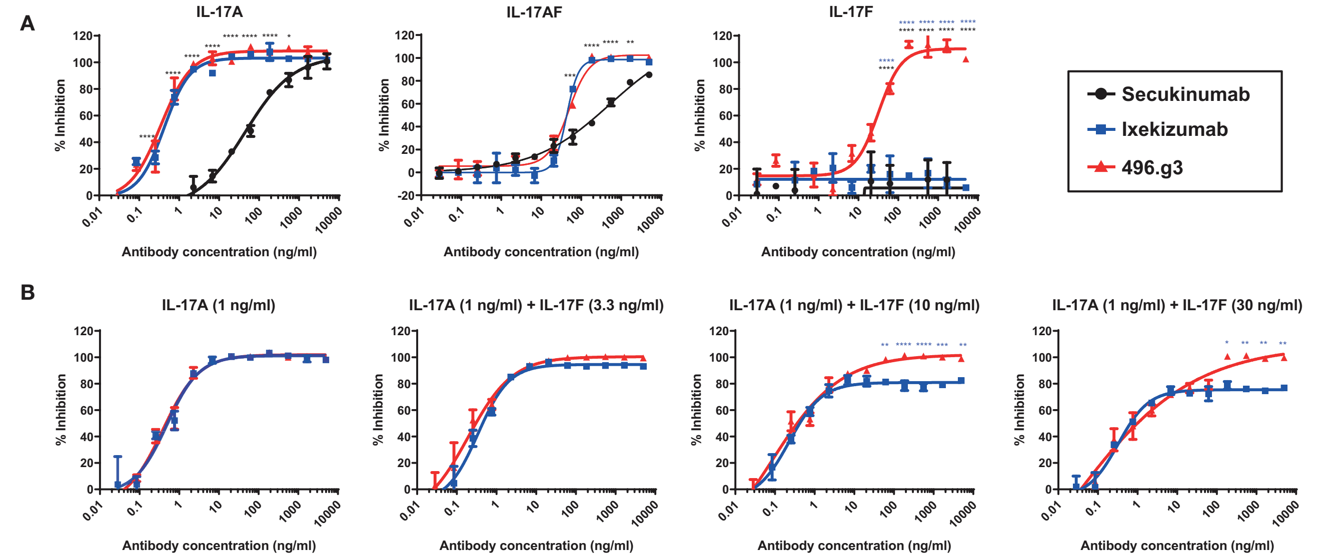


FIGURE 5 | Comparative efficacy of 496.g3 with the approved anti-IL-17A antibodies secukinumab and ixekizumab in inhibiting IL-6 production by normal human dermal fibroblasts (NHDFs). As seen in panel **(A)** 496.g3 was the only antibody that inhibited IL-6 production in response to stimulation by all three cytokines. In panel **(B)**, 496.g3 maintained complete inhibition of IL-6 production, while the efficacy of ixekizumab declined with each stepwise increase in the ratio of IL-17F to IL-17A. Potency curves were calculated relative to NHDFs activated with TNF alone. In both sets of experiments, IL-17A (1 ng/mL), IL-17A/F (30 ng/mL), or IL-17F (30 ng/mL) in combination with TNF (1 ng/mL) were pre-incubated with titrations of 496.g3, ixekizumab **(A,B)** and secukinumab **(A)** for 1 h before addition to NHDFs. The cells were subsequently stimulated for 18–20 h before IL-6 protein levels in the supernatant were determined by homogeneous time-resolved fluorescence. Each condition was tested in duplicate and the data shown is representative of three separate experiments. Two-way ANOVA, Bonferroni post-test analysis was performed to compare inhibition of 496.g3 vs. secukinumab (black stars) or ixekizumab (blue stars) (* $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$).

TABLE 3 | Comparative activity of 496.g3 with anti-IL-17A-specific antibodies secukinumab and ixekizumab in neutralizing IL-17A, IL-17F an IL-17A/F in an *in vitro* neutralization assay.

	IL-17A		IL-17A/F		IL-17F	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Secukinumab	45.0	956.2	525.9	65,535.0	ND	ND
Ixekizumab	0.4	2.2	43.2	91.0	ND	ND
496.g3	0.4	2.5	48.9	179.2	32.1	137.8

Calculation of IC_{50} and IC_{90} values for 496.g3, secukinumab and ixekizumab were determined by four parameter non-linear regression analysis for IL-17A, IL-17A/F, and IL-17F. IC_{50} and IC_{90} values are measured in ng/ml. ND, Not detectable.

TABLE 4 | Binding affinities of 496.g3, secukinumab and ixekizumab for human IL-17A, IL-17A/F, and IL-17F.

Cytokine	Secukinumab	Ixekizumab	496.g3
IL-17A	129†	1.8*	3.2†
IL-17A/F	2,400**	1.8*	26†^
IL-17F	NB†	NB*	23†

*Internal† and published *(31), **(32) binding affinities (pM) of 496.g3, secukinumab and ixekizumab for IL-17A, IL-17A/F, and IL-17F were generated by SPR. Affinity of 496.g3 for IL-17A and IL-17F was previously shown in* ***Table 2***. *^The kinetic parameters for binding to IL-17A/F are k_a = 3.19E+06, k_d = 8.17E-05 and K_D = 2.56E-11. NB, no binding.*

spondyloarthritis (21). To determine whether the ratio of IL-17F to IL-17A in psoriatic lesions is important to the differential therapeutic potential of 496.g3 vs. ixekizumab *in vitro*, a neutralization assay was performed using varying ratios of IL-17F to IL-17A. In line with the previous experiment (**Figure 5A**), in the presence of IL-17A 1 ng/mL, 496.g3 and ixekizumab showed similar levels of inhibition. As the ratios of IL-17F to IL-17A were increased to 3:1, 10:1, and 30:1, 496.g3 maintained complete inhibition of IL-17-driven signaling (**Figure 5B**). In contrast, the efficacy of ixekizumab reduced with each stepwise increase in the ratio of IL-17F to IL-17A (**Figure 5B**). To achieve full inhibition at the increased IL-17F:IL-17A ratio, a concomitant increase in the concentration of 496.g3 was required; this was expected given the requirement to neutralize two ligands. Collectively, these data emphasize that neutralization of both IL-17A and IL-17F is required to fully suppress inflammation driven by these two structurally similar IL-17 cytokines.

DISCUSSION

Antibody 496.g3 (bimekizumab) was generated to test our hypothesis that both IL-17A and IL-17F contribute to chronic tissue inflammation, and that dual neutralization of IL-17A and IL-17F may lead to superior clinical outcomes compared with inhibition of IL-17A alone.

IL-17A is the principal therapeutic target of the IL-17 family, which may be reflected in the roles of IL-17A and

IL-17F in mice. Studies using transgenic mice lacking either IL-17A or IL-17F showed that IL-17A plays a major role in the development of autoimmune diseases such as collagen-induced arthritis and Experimental Autoimmune Encephalitis (murine model of multiple sclerosis), and allergic diseases such as delayed-type hypersensitivity and contact hypersensitivity; in contrast, the role of IL-17F in these disease models was marginal (4, 5, 14, 33, 34). This is likely due to the 10,000-fold difference in potency between mouse recombinant IL-17A and IL-17F (data not shown). In contrast, the relative difference in potency between recombinant human IL-17A and IL-17F is ~100-fold (35).

In common with a number of other anti-IL-17A therapeutic antibodies that have entered the clinic, 496.g1, the antecedent of bimekizumab, was raised following immunization of rats with only IL-17A. 496.g1 possessed weak IL-17F neutralizing activity, which was subsequently exploited and enhanced through affinity maturation. As a standard IgG1, 496.g3 achieved dual targeting of both IL-17A and IL-17F (**Figure 6**). Biophysical characterization provided insights into how the molecule would behave in the manufacturing process. No unexpected or unwanted characteristics were identified during this process; indeed, 496.g3 showed good thermal stability and a favorable isoelectric point, which was comparable with other IgG monoclonal antibodies in the developmental and clinical landscape (36, 37).

Studies in human cells suggested a more prominent role for IL-17F in tissue inflammation (15). Treatment of synoviocytes from patients with rheumatoid arthritis with either recombinant IL-17A or IL-17F, in the presence of TNF, has been shown to induce a qualitatively similar gene signature (16). Transcriptional analysis of IL-17A- or IL-17F-stimulated dermal fibroblasts further reaffirmed the overlapping biology of these two cytokines, although IL-17A in either synoviocytes or dermal fibroblasts was the more potent cytokine (38). Indeed, using a more complex *in vitro* model of disease we have previously shown that dual neutralization of endogenous IL-17A and IL-17F produced by Th17 cells demonstrated greater suppression of inflammatory mediators compared to selective IL-17A blockade. Further, in addition to suppression of IL-6 we also observed significant inhibition of CXCL1, CXCL8, and CCL20, chemokines strongly linked to IL-17A and IL-17F biology (38). The limitations of our previous published research (38) with bimekizumab were two-fold. Firstly, 496.g3 was not compared against commercially approved anti-IL-17A antibodies, secukinumab, and ixekizumab, as performed in this study, but rather profiled against an in-house generated anti-IL-17A antibody. As our in-house anti-IL-17A antibody may behave differently to secukinumab and ixekizumab, it was more clinically relevant to compare against commercially approved antibodies. Further, in this study we also add more granularity on the specificity and potency of each antibody to specifically neutralize IL-17A, IL-17AF, or IL-17F when tested individually. Secondly, quantification of local and systemic levels of both IL-17 cytokines in patients with psoriasis, psoriatic arthritis, and ankylosing spondylitis revealed a greater abundance of IL-17F relative to IL-17A (>30-fold) (21). Significantly, we demonstrated, using an *in vitro* neutralization assay, that when the ratio of IL-17F to IL-17A was ≥10-fold the differential impact of 496.g3 over specific IL-17A inhibitors was observed. As expected, a higher concentration of 496.g3 was required to neutralize both IL-17A and IL-17F, when compared with IL-17A alone.

While the greater abundance of IL-17F and its shared overlapping biology with IL-17A suggest a role for this cytokine in promoting chronic tissue inflammation, our early clinical assessments of dual inhibition of IL-17A and IL-17F offers a direct approach to testing this hypothesis (39). Bimekizumab

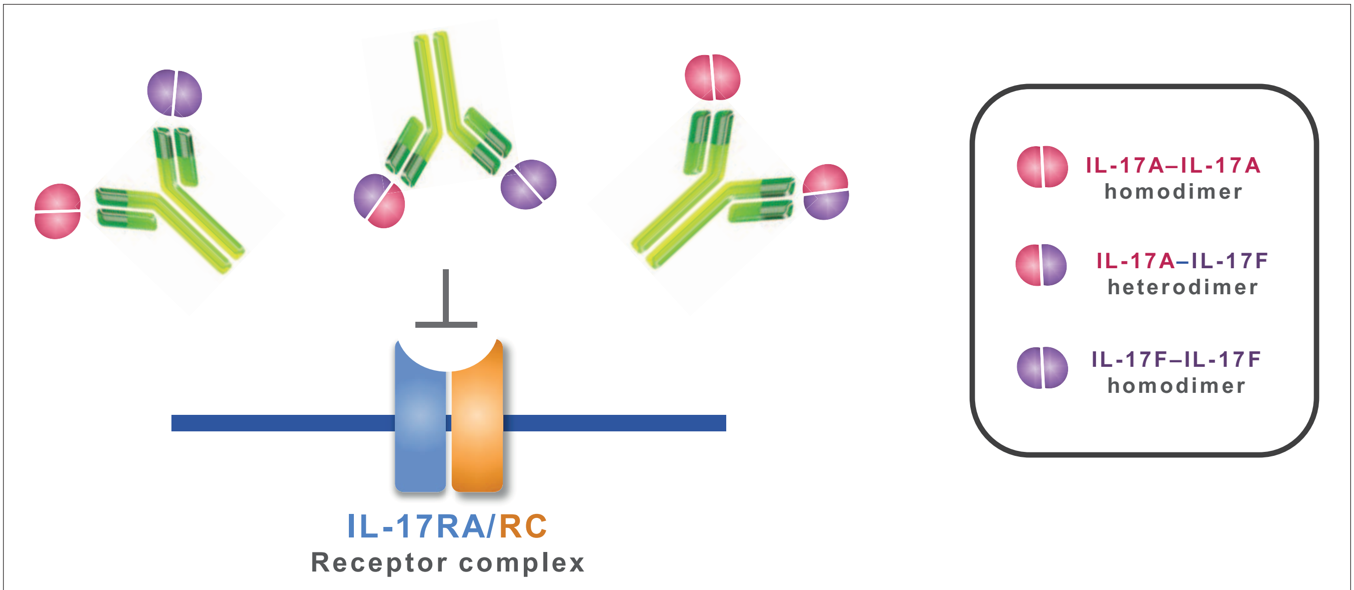


FIGURE 6 | Mechanism of action of bimekizumab. By selectively binding to IL-17A, IL-17F, and the IL-17A/F heterodimer, bimekizumab inhibits the activation of the IL-17RA/RC receptor complex by these cytokines, and the subsequent inflammatory cascade.

has completed Phase 1 and Phase 2 clinical trials in patients with psoriasis, psoriatic arthritis, and ankylosing spondylitis (38, 40–42). In the Phase 1 first-in-human, single-dose psoriasis study (NCT02529956), 26 patients received bimekizumab and 13 received placebo; bimekizumab treatment resulted in a rapid onset of clinically meaningful efficacy in measures of disease activity, which was maintained throughout the 20-week study in those receiving bimekizumab ≥160 mg (39). In the Phase 1b proof-of-concept study (NCT02141763) in patients with moderate to severe adult-onset psoriatic arthritis, 38 patients received bimekizumab and 12 patients received placebo (38). Bayesian analysis indicated a >99% probability that the American College of Rheumatology n (ACRn) index and ≥20% improvement in ACR index (ACR20) response at Week 8 were greater with bimekizumab vs. placebo and exceeded the pre-determined clinically relevant threshold. For the combined highest three doses, response rates at Week 8 were 80% (ACR20), 40% (ACR50), and 23% (ACR70), with maximal observed response rates for these endpoints of 80% (ACR20 at Week 8), 57% (ACR50 at Week 12), and 37% (ACR70 at Week 16). Moreover, in those patients with skin involvement, Week 8 Psoriasis Area Severity Index (PASI) response rates for PASI75 and PASI100 were 100% and 87%, respectively. Therefore, both pre-specified efficacy criteria in this study were met, demonstrating proof-of-concept. Further to this, results from BE ABLE 1, a 12-week, randomized, double-blind, placebo-controlled Phase 2b study in patients with moderate to severe psoriasis demonstrated superior efficacy of bimekizumab vs. placebo in all primary and secondary endpoints (40). Significant dose-dependent responses were observed and, in the highest dose groups, patients achieved high levels of skin clearance (PASI90) at Week 12. Of note, ∼50–60% of patients in the three highest dose groups achieved complete skin clearance (PASI100) following 12 weeks of bimekizumab treatment. Importantly, in all three clinical studies, the safety findings observed were consistent across bimekizumab dose groups and were as expected when considered in the context of anti-IL-17A antibodies. Preclinical and early clinical data are encouraging and further support the targeted approach of dual neutralization of IL-17A and IL-17F.

Results of the studies reported here demonstrate that bimekizumab, a monoclonal antibody, potently and selectively neutralizes IL-17A and IL-17F. With promising early clinical data in psoriasis, psoriatic arthritis, and ankylosing spondylitis, dual inhibition of IL-17A and IL-17F with bimekizumab offers a new therapeutic approach for the treatment of patients with immune-mediated inflammatory diseases.

DATA AVAILABILITY STATEMENT

Data from non-clinical studies is outside of UCB's data sharing policy and is unavailable for sharing.

AUTHOR CONTRIBUTIONS

RA, AM, TB, AL, RP, SR, SW, and MG were involved in antibody development and characterization, analyzed, and interpreted the data. RO and PV provided mathematical modeling. AM, AL, and SS provided conceptual and supervisory support. RA, AM, and MG drafted the manuscript. All authors contributed revisions, approved the final manuscript, contributed to the article, and approved the submitted version.

FUNDING

This study and technical editing support was funded by UCB Pharma.

ACKNOWLEDGMENTS

The authors would like to acknowledge Oliver Durrant, Andy Ventom and Alison Turner for work on the antibody characterization. The authors would like to acknowledge Simone E Auteri, MSc EMS PhD, and Susanne Wiegratz, MSc, of UCB Pharma, for publication coordination and to acknowledge Alexandra Webster, MSc, of iMed Comms, an Ashfield company, part of UDG Healthcare plc for technical editing support that was funded by UCB Pharma.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01894/full#supplementary-material

REFERENCES

1. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity.* (2011) 34:149–62. doi: 10.1016/j.immuni.2011.02.012
2. Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol.* (1993) 150:5445–56.
3. Schett G, Elewaut D, McInnes IB, Dayer JM, Neurath MF. How cytokine networks fuel inflammation: toward a cytokine-based disease taxonomy. *Nat Med.* (2013) 19:822–4. doi: 10.1038/nm.3260
4. Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol.* (2006) 177:566–73. doi: 10.4049/jimmunol.177.1.566
5. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol.* (2003) 171:6173–7. doi: 10.4049/jimmunol.171.11.6173
6. van Tok MN, van Duivenvoorde LM, Kramer I, Ingold P, Pfister S, Roth L, et al. Interleukin-17A inhibition diminishes inflammation and new bone formation in experimental spondyloarthritis. *Arthritis Rheumatol.* (2019) 71:612–25. doi: 10.1002/art.40770
7. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, et al. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* (2001) 20:5332–41. doi: 10.1093/emboj/20.19.5332
8. Chang SH, Dong C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res.* (2007) 17:435–40. doi: 10.1038/cr.2007.35

9. Wright JF, Guo Y, Quazi A, Luxenberg DP, Bennett F, Ross JF, et al. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J Biol Chem.* (2007) 282:13447–55. doi: 10.1074/jbc.M700499200
10. Gaffen SL. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol.* (2009) 9:556–67. doi: 10.1038/nri2586
11. Lim HJ, Jin HG, Woo ER, Lee SK, Kim HP. The root barks of morus alba and the flavonoid constituents inhibit airway inflammation. *J Ethnopharmacol.* (2013) 149:169–75. doi: 10.1016/j.jep.2013.06.017
12. Burns LA, Maroof A, Marshall D, Steel KJA, Lalnunhlimi S, Cole S, et al. Presence, function, and regulation of IL-17F-expressing CD4+ T cells. *Eur J Immunol.* (2019) 50:568–80. doi: 10.1002/eji.201948138
13. Chang SH, Dong C. IL-17F: regulation, signaling and function in inflammation. *Cytokine.* (2009) 46:7–11. doi: 10.1016/j.cyto.2008.12.024
14. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, et al. Regulation of inflammatory responses by IL-17F. *J Exp Med.* (2008) 205:1063–75. doi: 10.1084/jem.20071978
15. Hot A, Zrioual S, Toh ML, Lenief V, Miossec P. IL-17A- versus IL-17F-induced intracellular signal transduction pathways and modulation by IL-17RA and IL-17RC RNA interference in rheumatoid synoviocytes. *Ann Rheum Dis.* (2011) 70:341–8. doi: 10.1136/ard.2010.132233
16. Zrioual S, Ecochard R, Tournadre A, Lenief V, Cazalis MA, Miossec P. Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J Immunol.* (2009) 182:3112–20. doi: 10.4049/jimmunol.0801967
17. Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, et al. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest.* (2010) 138:1140–7. doi: 10.1378/chest.09-3058
18. Johansen C, Usher PA, Kjellerup RB, Lundsgaard D, Iversen L, Kragballe K. Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. *Br J Dermatol.* (2009) 160:319–24. doi: 10.1111/j.1365-2133.2008.08902.x
19. van Baarsen LG, Lebre MC, van der Coelen D, Aarrass S, Tang MW, Ramwadhdoebe TH, et al. Heterogeneous expression pattern of interleukin 17A (IL-17A), IL-17F and their receptors in synovium of rheumatoid arthritis, psoriatic arthritis and osteoarthritis: possible explanation for nonresponse to anti-IL-17 therapy? *Arthritis Res Ther.* (2014) 16:426. doi: 10.1186/s13075-014-0426-z
20. Yu HC, Lu MC, Huang KY, Huang HL, Liu SQ, Huang HB, et al. Sulfasalazine treatment suppresses the formation of HLA-B27 heavy chain homodimer in patients with ankylosing spondylitis. *Int J Mol Sci.* (2016) 17:46. doi: 10.3390/ijms17010046
21. Kolbinger F, Loesche C, Valentin MA, Jiang X, Cheng Y, Jarvis P, et al. Beta-defensin 2 is a responsive biomarker of IL-17A-driven skin pathology in patients with psoriasis. *J Allergy Clin Immunol.* (2017) 139:923–32.e928. doi: 10.1016/j.jaci.2016.06.038
22. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* (2011) 332:65–8. doi: 10.1126/science.1200439
23. PI I. *Taltz (ixekizumab) [package insert].* Utrecht: Eli Lilly (2019).
24. PI S. *Cosentyx (secukinumab) [package insert].* East Hanover, NJ: Novartis Pharmaceuticals Corporation (2018).
25. Griffiths CE, Reich K, Lebwohl M, van de Kerkhof P, Paul C, Menter A, et al. Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials. *Lancet.* (2015) 386:541–51. doi: 10.1016/S0140-6736(15)60125-8
26. Thaci D, Blauvelt A, Reich K, Tsai TF, Vanaclocha F, Kingo K, et al. Secukinumab is superior to ustekinumab in clearing skin of subjects with moderate to severe plaque psoriasis: CLEAR, a randomized controlled trial. *J Am Acad Dermatol.* (2015) 73:400–9. doi: 10.1016/j.jaad.2015.05.013
27. Cain K, Peters S, Hailu H, Sweeney B, Stephens P, Heads J, et al. A CHO cell line engineered to express XBP1 and ERO1-Lalpha has increased levels of transient protein expression. *Biotechnol Prog.* (2013) 29:697–706. doi: 10.1002/btpr.1693
28. Tickle S, Adams R, Brown D, Griffiths M, Lightwood D, Lawson A. High-throughput screening for high affinity antibodies. *J Assoc Lab Autom.* (2009) 14:303–7. doi: 10.1016/j.jala.2009.05.004
29. Davda JP, Hansen RJ. Properties of a general PK/PD model of antibody-ligand interactions for therapeutic antibodies that bind to soluble endogenous targets. *MAbs.* (2010) 2:576–88. doi: 10.4161/mabs.2.5.12833
30. Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. *J Pharm Sci.* (2004) 93:2645–68. doi: 10.1002/jps.20178
31. Liu L, Lu J, Allan BW, Tang Y, Tetreault J, Chow CK, et al. Generation and characterization of ixekizumab, a humanized monoclonal antibody that neutralizes interleukin-17A. *J Inflamm Res.* (2016) 9:39–50. doi: 10.2147/JIR.S100940
32. European Medicines Agency (EMA). Cosentyx Assessment Report (2014). Available online at: https://www.ema.europa.eu/en/medicines/human/EPAR/cosentyx (last accessed 10 August 2020).
33. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and−17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity.* (2009) 30:108–19. doi: 10.1016/j.immuni.2008.11.009
34. Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity.* (2002) 17:375–87. doi: 10.1016/S1074-7613(02)00391-6
35. Wright JF, Bennett F, Li B, Brooks J, Luxenberg DP, Whitters MJ, et al. The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol.* (2008) 181:2799–805. doi: 10.4049/jimmunol.181.4.2799
36. Garber E, Demarest SJ. A broad range of Fab stabilities within a host of therapeutic IgGs. *Biochem Biophys Res Commun.* (2007) 355:751–7. doi: 10.1016/j.bbrc.2007.02.042
37. Sagar D, Singh NP, Ginwala R, Huang X, Philip R, Nagarkatti M, et al. Antibody blockade of CLEC12A delays EAE onset and attenuates disease severity by impairing myeloid cell CNS infiltration and restoring positive immunity. *Sci Rep.* (2017) 7:2707. doi: 10.1038/s41598-017-03027-x
38. Glatt S, Baeten D, Baker T, Griffiths M, Ionescu L, Lawson ADG, et al. Dual IL-17A and IL-17F neutralisation by bimekizumab in psoriatic arthritis: evidence from preclinical experiments and a randomised placebo-controlled clinical trial that IL-17F contributes to human chronic tissue inflammation. *Ann Rheum Dis.* (2018) 77:523–32. doi: 10.1136/annrheumdis-2017-212127
39. Glatt S, Helmer E, Haier B, Strimenopoulou F, Price G, Vajjah P, et al. First-in-human randomised study of bimekizumab, a humanised monoclonal antibody and selective dual inhibitor of IL-17A and IL-17 F, in mild psoriasis. *Br J Clin Pharmacol.* (2017) 83:991–1001. doi: 10.1111/bcp.13185
40. Papp KA, Merola JF, Gottlieb AB, Griffiths CEM, Cross N, Peterson L, et al. Dual neutralization of both interleukin 17A and interleukin 17F with bimekizumab in patients with psoriasis: Results from BE ABLE 1, a 12-week randomized, double-blinded, placebo-controlled Phase 2b trial. *J Am Acad Dermatol.* (2018) 79:277–86.e210. doi: 10.1016/j.jaad.2018.03.037
41. Ritchlin CT, Kavanaugh A, Merola JF, Schett G, Scher JU, Warren RB, et al. Dual neutralization of IL-17A and IL-17F with bimekizumab in patients with active PsA: results from a 48-week phase 2b, randomized, double-blind, placebo-controlled, dose-ranging study. *Arthritis Rheumatol.* (2018) 70:4. doi: 10.1136/annrheumdis-2019-eular.4883
42. Van der Heijde D, Gensler LS, Deodhar A, Baraliakos X, Poddubnyy D, Farmer MK, et al. Dual neutralisation of IL-17A and IL-17F with bimekizumab in patients with active ankylosing spondylitis (AS): 12-week results from a phase 2b, randomised, double-blind, placebo-controlled, dose-ranging study. *Ann Rheum Dis.* (2018) 77:A70. doi: 10.1136/annrheumdis-2018-eular.7889

Conflict of Interest: RA, AM, TB, AL, RO, RP, SR, SS, PV, and MG are employees of UCB Pharma and hold stocks and/or stock options in UCB Pharma. SW is an employee of UCB Pharma.

frontiers in Immunology

ORIGINAL RESEARCH
published: 16 October 2020
doi: 10.3389/fimmu.2020.534323

Check for updates

Possible Roles of Proinflammatory Signaling in Keratinocytes Through Aryl Hydrocarbon Receptor Ligands for the Development of Squamous Cell Carcinoma

Yota Sato[1†], Taku Fujimura[1*†], Takanori Hidaka[1], Chunbing Lyu[1], Kayo Tanita[1], Shigeto Matsushita[2], Masayuki Yamamoto[3] and Setsuya Aiba[1]

[1] Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan, [2] Department of Dermato-Oncology/Dermatology, National Hospital Organization Kagoshima Medical Center, Kagoshima, Japan, [3] Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan

OPEN ACCESS

Edited by:
Kong Chen,
University of Pittsburgh, United States

Reviewed by:
Kamil R. Kranc,
University of Edinburgh,
United Kingdom
Kawaljit Kaur,
University of California, Los Angeles,
United States

***Correspondence:**
Taku Fujimura
tfujimura1@mac.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 12 February 2020
Accepted: 04 September 2020
Published: 16 October 2020

Citation:
Sato Y, Fujimura T, Hidaka T, Lyu C, Tanita K, Matsushita S, Yamamoto M and Aiba S (2020) Possible Roles of Proinflammatory Signaling in Keratinocytes Through Aryl Hydrocarbon Receptor Ligands for the Development of Squamous Cell Carcinoma. Front. Immunol. 11:534323. doi: 10.3389/fimmu.2020.534323

Aryl hydrocarbon receptor (AhR) provides a deeper insight into the pathogenesis of cutaneous squamous cell carcinoma (cSCC). AhR ligands, such as 6-formylindolo[3,2-b] carbazole (FICZ), and 7,12-Dimethylbenz[a]anthracene (DMBA), constitute major substrates for the cytochrome P450 (CYP) family, and influence the expression of various cytokine genes, including *IL-17* and *IL-23*-related genes via the AhR. On the other hand, proinflammatory cytokines could drive tumor progression through the TRAF-ERK5 signaling pathway in cSCC. From the above findings, we hypothesized that AhR ligands might enhance the mRNA expression of proinflammatory cytokines via the AhR, leading to the development of cSCC. The purpose of this study was to investigate (1) the immunomodulatory effects of FICZ and DMBA on normal human keratinocytes (NHKCs), focusing on IL-17, and related cytokines/chemokines (IL-23, IL-36γ, and CCL20), (2) the expression of these factors in AhR-dependent pathways using a two-stage chemically induced skin carcinogenesis mouse model, and (3) the expression of these factors in lesion-affected skin in cSCC. Both FICZ and DMBA augmented the expression of CYP1A1, p19, CCL20, and IL-36γ mRNA in NHKCs *in vitro*. Moreover, the mRNA expression of these proinflammatory factors, as well as IL-17, in mouse cSCC is significantly decreased in the AhR-(fl/fl) Krt5-(Cre) mice compared to wild type mice, leading to a decrease in the number of developed cSCC lesions. Furthermore, CCL20, IL-23, as well as IL-17, are detected in the lesion-affected skin of cSCC patients. Our study demonstrates a possible mechanism for the development of cSCC involving AhR-mediated signaling by epidermal keratinocytes and recruitment of Th17 cells.

Keywords: aryl hydrocarbon receptor, cutaneous SCC, IL-17, carcinogenesis, proinflammatory cytokines

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is the second most common type of non-melanoma skin cancer, and its risk factors have been widely reported (1, 2). For example, the precursor of cSCC is intraepithelial UV-induced damage, which can develop into actinic keratosis (AK) (3). Indeed, AK and SCC possess a similar genetic profile, such as alterations in the p53 gene (3), suggesting that AK could sequentially develop into cSCC. On the other hands, chemical exposure is another risk of cSCC. Recently, Chahal et al. reported a two-stage genome-wide association study for cSCC (4), suggesting the genome-wide significance of seven pigmentation-related loci and four susceptibility loci, including aryl hydrocarbon receptor (AhR) and IRF4. AhR is a dioxin receptor involved in anti-apoptotic pathways and progression of melanoma, whereas *IRF4* encodes a key transcription factor that controls M2 macrophage polarization (5). The authors concluded that these susceptibility loci provide a deeper insight into the pathogenesis of cSCC (4).

The exposure of keratinocytes to ultraviolet (UV) radiation leads to the intracellular production of 6-formylindolo[3,2-b]carbazole (FICZ), which exhibits high affinity for AhR (6, 7). FICZ is a tryptophan oxidation product formed by exposure to UV, and is metabolized by CYP1A1 (7). FICZ is a major substrate for the cytochrome P450 (CYP) family, and affects the expression of various genes via the AhR (8–10). Despite various reports about the physical and chemical properties of FICZ, its association with cSCC remains unclear.

7,12-Dimethylbenz[a]anthracene (DMBA), another type of AhR ligand that is typically found in cigarette smoke, is widely known to induce cutaneous SCC in mouse skin together with 12-O-Tetradecanoylphorbol 13-acetate (TPA) (11, 12). The majority of DMBA-induced SCCs possess mutations in oncogenes including Hras, Kras, and Rras (13, 14), which are detected in human SCC located in cervical, esophageal, and lung tissues, among others. Although these previous reports suggested the significance of oncogenic mutations caused by AhR ligands, the immunomodulatory effects of the AhR in cSCC are still unknown. Notably, AhRs are highly activated in Th17 cells (14), and AhR ligands enhance the differentiation of Th17 cells and IL-22 production via the AhR (15). Considering that IL-17 could drive the tumor progression through TRAF-ERK5 pathways in cSCC (16), we hypothesized that DMBA could induce IL-17 production from keratinocytes to drive the proliferation of keratinocytes, leading to the development of cSCC. In this report, we investigated the significance of AhR signaling in keratinocytes for the development of cSCC using a two-stage chemically induced skin carcinogenesis mouse model and human cSCC samples.

MATERIALS AND METHODS

Ethics Statement for Animal and Human Experiments

The protocol for the animal study was approved by the ethics committee at Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (permit number: 2017MdLMO-342-2). The research complied with the Tohoku University Graduate School of Medicine's Animal Experimentation Ethics guidelines and policies. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol for the human study was approved by the ethics committee at Tohoku University Graduate School of Medicine, Sendai, Japan (permit number: 2017-1-430), and Kagoshima Medical Center, Japan (permit number 29-2, 30-08).

Animals and Melanoma Cell Line

C57BL/6 mice and BALB/c mice (5 to 8 weeks old) were purchased from Japan Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in the animal facility at the Tohoku University Graduate School of Medicine. *Ahr*$^{fl/fl}$ mice and *Krt5*-Cre mice were kindly provided by Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan (6).

Reagents

The following antibodies (Abs) were used for immunohistochemical staining: mouse monoclonal Abs (LifeSpan BioScience, Seattle, WA, United States) against human CCL20 and human IL-36γ, rabbit polyclonal Abs (LifeSpan BioScience) against human CYP1A1 and human IL-23, and a goat polyclonal Ab (R&D Systems, Minneapolis, MN, United States) against human IL-17. The following antibodies were used for immunofluorescence (IF): mouse anti-human CD163 phycoerythrin-conjugated monoclonal antibody (R&D Systems), rabbit polyclonal anti-CCL22 antibody (R&D Systems), rabbit polyclonal anti-CXCL5 antibody (Lifespan Bioscience, Seattle, WA, United States), mouse anti-CXCL10 antibody (Lifespan Bioscience), Alexa Fluor 488-conjugated anti-mouse rat immunoglobulin (Ig)G (Abcam, Tokyo, Japan), and Alexa Fluor 488-conjugated anti-rabbit goat IgG (Abcam).

Tissue Samples and Immunohistochemical Staining

We collected archived formalin-fixed paraffin-embedded skin specimens and cryosections from cutaneous SCC patients treated in the Department of Dermatology at Tohoku University Graduate School of Medicine, Sendai, Japan, and Department of Dermato-Oncology/Dermatology at Kagoshima Medical Center, Kagoshima Japan. We employed immunohistochemical staining for 10 cases of squamous cell carcinoma and 10 cases of AK (**Table 1**). For cryosections, each sample was frozen in optimal cutting temperature embedding medium, and 6-μm sections were fixed with cold acetone for 30 min and then blocked with IF buffer (PBS, 5% bovine serum albumin). Thereafter, each section was incubated with the relevant antibodies. The slides were mounted in DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, United States) and examined using a Zeiss LSM 700 microscope equipped with a SPOT digital camera (Zeiss Japan, Tokyo, Japan).

Cell Culture and Stimulation

Normal human epidermal keratinocytes (NHKCs; Kurabo, Osaka, Japan) were cultured in HuMedia-KG supplemented with insulin (10 μg/mL), hEGF (0.1 ng/mL), hydrocortisone (0.5 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), and fetal bovine serum (0.4% v/v; Kurabo).

TABLE 1 | Characteristics of patients with cSCC and actinic keratosis.

	Age	Stage	Location	Histology
Invasive				
Case 1	31–40	T2N0M0 stage II	Scalp	Well differentiated
Case 2	51–60	T1N0M0 stage I	Cheek	Well differentiated
Case 3	71–80	T2N0M0 stage II	Scalp	Well differentiated
Case 4	61–70	T1N0M0 stage I	Forearm	Well differentiated
Case 5	71–80	T1N0M0 stage I	Nose	Well differentiated
Case 6	71–80	T1N0M0 stage I	Cheek	Well differentiated
Case 7	91–100	T2N0M0 stage II	Scalp	Well differentiated
Case 8	61–70	T2N0M0 stage II	Lower leg	Well differentiated
Case 9	61–70	T3N0M0 stage III	Scalp	Well differentiated
Case 10	71–80	T1N0M0 stage I	Scalp	Well differentiated
Case 11	61–70	T1N0M0 stage I	Penis	Well differentiated
Case 12	31–4-	T1N0M0 stage I	Scalp	Well differentiated
In situ				
Case 1	71–80	TisN0M0 stage 0	Forearm	Actinic keratosis
Case 2	81–90	TisN0M0 stage 0	Forearm	Actinic keratosis
Case 3	71–80	TisN0M0 stage 0	Ear	Actinic keratosis
Case 4	81–90	TisN0M0 stage 0	Cheek	Actinic keratosis
Case 5	61–70	TisN0M0 stage 0	Cheek	Actinic keratosis
Case 6	91–100	TisN0M0 stage 0	Medial canthu	Actinic keratosis
Case 7	81–90	TisN0M0 stage 0	Scalp	Actinic keratosis
Case 8	81–90	TisN0M0 stage 0	Preauricle	Actinic keratosis
Case 9	81–90	TisN0M0 stage 0	Cheek	Actinic keratosis
Case 10	91–100	TisN0M0 stage 0	Forearm	Actinic keratosis
Case 11	71–80	TisN0M0 stage 0	Scalp	Actinic keratosis
Case 12	91–100	TisN0M0 stage 0	Cheek	Actinic keratosis

Cells were cultured at 37°C in a 5% CO_2 atmosphere. Upon reaching 80% confluence, cells were treated with FICZ (10 nM), or DMBA (1 μ M) for 4 h.

RNA Extraction, Assessment of RNA Quality, and Reverse Transcription and Quantitative Real-Time PCR

Total RNA was extracted using a RNeasy Micro kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's instructions. The RNA was eluted using 4 μL of RNase-free water. Contaminating genomic DNA was removed by treating extracted RNA with DNase I (RNase-Free DNase Set; Qiagen). Reverse transcription was performed using a SuperScript VILO cDNA Synthesis kit (Invitrogen). Amplification reactions were performed using a Mx 3000P Real-Time Quantitative PCR System (Stratagene, Tokyo, Japan). The thermal cycling conditions were as follows: 3 min for polymerase activation at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. PCR products were maintained at 4°C. Relative mRNA expression levels were calculated for each gene and each time point after normalization against the expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA using the $\Delta\Delta$Ct method. Averaged data from at least three independent experiments are shown.

Cytokine Enzyme-Linked Immunosorbent Assays

Secretion of CCL20 (R&D Systems), IL-36γ (R&D Systems), and IL-23 (R&D Systems) in NHKCs was determined using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions.

Western Blotting

Normal human epidermal keratinocytes were seeded into 12-well plates and cultured as described above. Cells were collected and disrupted in lysis buffer (Cell Signaling Technology, Boston, MA, United States). After adding SDS sample buffer (Cell Signaling Technology), lysates were electrophoretically separated on a 12% polyacrylamide gel (ATTO Corp., Tokyo, Japan). Proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, United States). The membrane was blocked in 5% non-fat dry milk in Tris–buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 h at room temperature. After several washes with TBST, the membrane was incubated overnight at 4°C with primary mouse anti-human IL-36 beta antibody (R and D system; 1:1000), anti-human IL-36 gamma antibody (R and D system; 1:1000), anti-human p19 antibody (Proteintech, Tokyo; 1:1000), or anti-human tublin antibody (Proteintech; 1:1000). The membrane was washed several times in TBST followed by a 1-h incubation with horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, Dallas, TX, United States).

Two-Stage Chemically Induced Skin Carcinogenesis Mouse Model

7,12-Dimethylbenz[a]anthracene was purchased from Sigma-Aldrich, Merck Milipore, Billerica, MA, United States, and TPA was purchased from Calbiochem, Merck Milipore, Billerica, MA, United States. DMBA is used as a carcinogen and TPA as a promoter. At 6–8 weeks of age, the backs of the mice were shaved, and 2 days after shaving, DMBA (25 μg per mouse in 200 μL acetone) was applied to shaved dorsal back skin. 3 days after the first DMBA treatment, TPA (10 μg per mouse in 200 μL acetone) was applied. After four rounds of this single DMBA and TPA treatment, the mice were treated with TPA twice weekly for 20 weeks.

For qRT-PCR, the whole tumor was frozen with liquid nitrogen, then crushed with a Cryo-Press (MICROTEC, Chiba, Japan), as described previously (17). Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions.

Assessment of Immunohistochemical Staining

The intensity of immunohistochemical staining for each antibody was scored on a semiquantitative scale (**Table 1**).

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney U-test for comparison of values. The level of significance was set at $p < 0.05$.

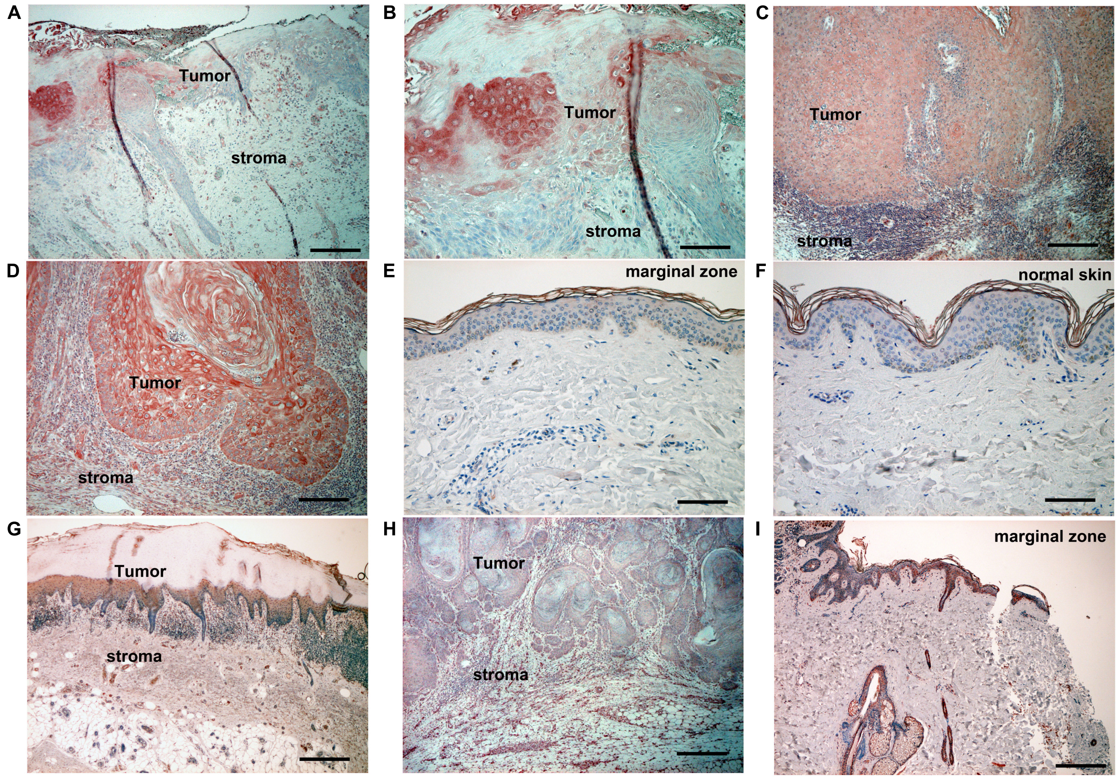


FIGURE 1 | Immunohistochemical analysis of CYP1A1 expression and AhR expression in lesion-affected skin of AK and cSCC. Sections of skin from lesion-affected areas of AK **(A,B,G)**, cSCC **(C,D,H)**, marginal areas around cSCC lesions **(E,I)**, or normal skin ([**F**]; nevus pigmentosus located at back) were deparaffinized and stained using anti-CYP1A1 antibodies **(A–F)**, or AhR antibodies **(G–I)**. The sections were developed with liquid permanent red. Scale bars, 100 μm **(B,D–F)**, 200 μm **(A,C,G–I)**. Representative specimens from analyses of 5 cases of actinic keratosis, 12 cases of cSCC, and 10 cases of nevus pigmentosus are shown.

RESULTS

Expression of CYP1A1 and Aryl Hydrocarbon Receptor and in Cutaneous Cell Carcinoma, Actinic Keratosis and Normal Skin

Since the AhR ligands, FICZ, and DMBA, are reported to promote carcinogenesis in SCC (11), we firstly employed immunohistochemical staining of CYP1A1 and AhR in 10 cases in each condition (cutaneous SCC, AK, and normal skin). Atypical keratinocytes in AK (**Figures 1A,B**) and cutaneous SCC (**Figures 1C,D**) expressed CYP1A1, whereas normal keratinocytes between the follicular bulbs in AK (**Figure 1A**), surgical margin of cutaneous SCC (**Figure 1E**), or normal skin (**Figure 1F**) did not express CYP1A1. Atypical keratinocytes in AK (**Figure 1G**), SCC (**Figure 1H**), and normal keratinocytes in basal layer (**Figure 1I**) of epidermis expressed AhR.

AhR Ligand Increases the Expression of CYP1A1, CCL20, p19, and IL-36γ mRNA in NHKCs

Considering that the atypical keratinocytes in SCC and AK express CYP1A1, AhR ligands stimulate NHKCs to increase the production of CYP1A1 as well as proinflammatory cytokines (18), and since IL-17 could drive the tumor progression through TRAF-ERK5 pathways in cSCC (16), we next examined the immunomodulatory effects of FICZ and DMBA on NHKCs, focusing on the expression of CYP1A1, CCL20, p19, p40, IL-36α, IL-36β, and IL-36γ mRNA at 4 h after stimulation *in vitro*. Both FICZ and DMBA augmented the expression of CYP1A1 as well as CCL20, p19, and IL-36γ mRNA (**Figure 2**). There was no significant increase in p40 and IL-36β mRNA expression (**Figure 2**).

FICZ and DMBA Increased the Production of CCL20, IL-36γ and p19 in NHKCs

As the results shown in **Figure 2A** suggest, FICZ and DMBA significantly increased the expression of CCL20, IL-36γ, and p19 in NHKCs. Therefore, we confirmed the production of CCL20, IL-36γ, and p19 protein in NHKCs treated with FICZ and DMBA *in vitro*. Production of CCL20 was significantly increased by treatment with FICZ and DMBA (**Figure 2B**). The production of IL-23 and IL-36β was detected by Western blot in each group (**Figure 2C**). The production of IL-36γ was not detected by ELISA and Western blot in each group.

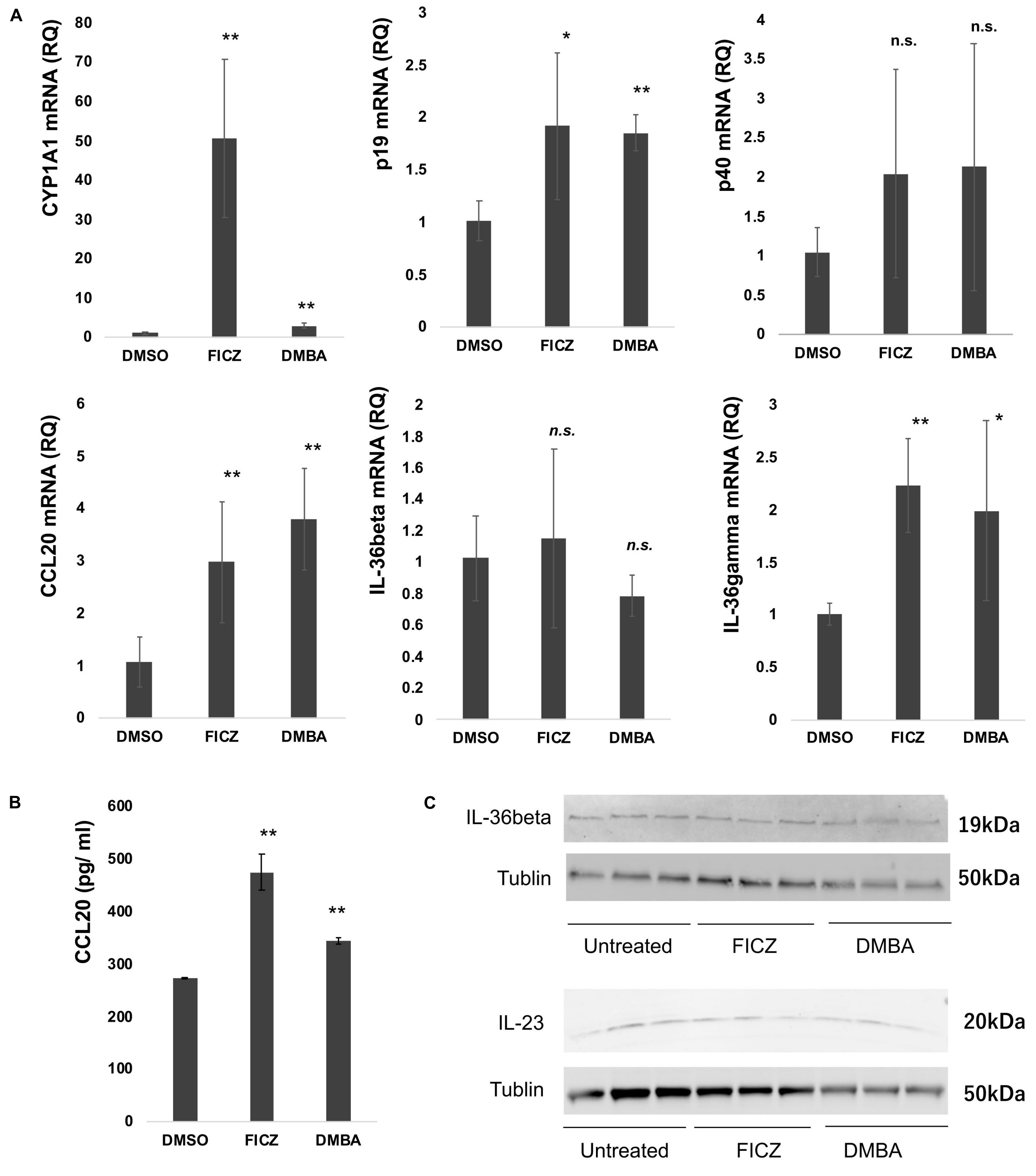


FIGURE 2 | Expression of CYP1A1, cytokine, and chemokine mRNA in normal human keratinocytes (NHKCs) stimulated with FICZ, or DMBA. NHKCs were cultured and treated with FICZ (10 nM), or DMBA (1 μM) as described in the Materials and Methods. At 4 h after stimulation, total RNA was recovered from NHKCs and amplified, labeled, and analyzed. Quantitative real-time PCR was conducted to determine the number of cDNA copies for each factor, and the mRNA expression level relative to that of untreated cells was calculated for each gene and time point after normalization against glyceraldehyde 3-phosphate dehydrogenase using the ΔΔCt method **(A)**. NHKC culture supernatant was harvested as described in the Materials and Methods and analyzed by ELISA **(B)**. Data from each donor were obtained from triplicate assays, and the mean ± SD was calculated. The means of at least three independent experiments are shown. IL-23 and IL-36β production was analyzed by western blotting as described in the Materials and Methods **(C)**. *$p < 0.05$, **$p < 0.01$.

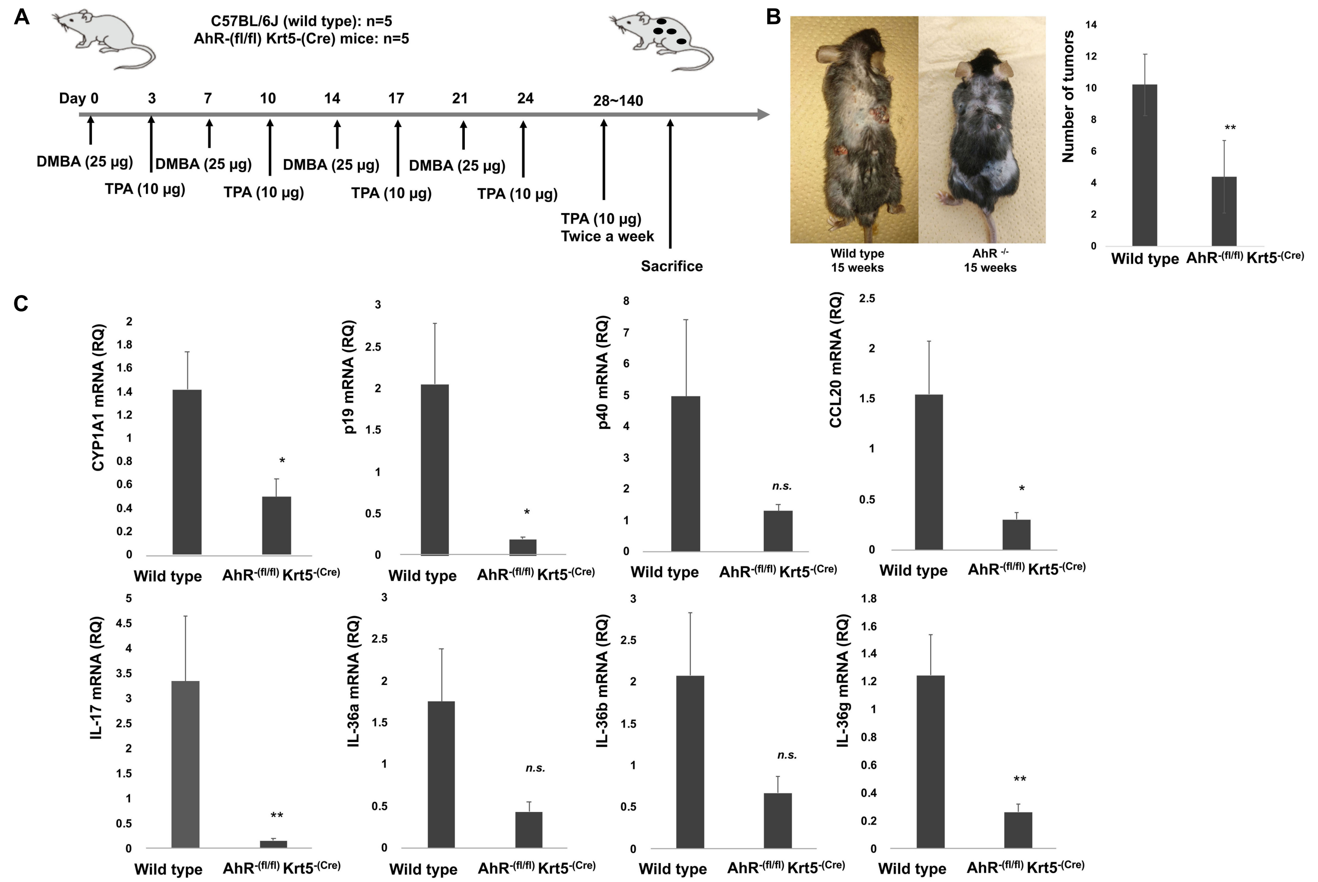


FIGURE 3 | AhR dependency in two-stage chemically induced skin carcinogenesis mouse model. Mouse cSCC in each mouse was induced as described in the Materials and Methods. The schematic representation of model of carcinogenesis **(A)**. The representative figure of DMBA-induced tumor in 5 wild type (AhR+/Krt5+) mouse and 5 AhR-(fl/fl) Krt5-(Cre) mouse **(B)**. The number of developed tumors were counted by two independent researchers, and the average number of induced tumors is calculated **(B)**. Expression of chemokines and cytokines in DMBA-induced tumor was analyzed by quantitative RT-PCR using the $\Delta\Delta$Ct method (n = 5). The data from each donor were obtained by triplicate assays, and then the mean ± SD was calculated **(C)**. *Marks a significant (p < 0.05) difference. **Marks a significant (p < 0.01) difference.

AhR Dependency of Two-Stage Chemically Induced Skin Carcinogenesis Mouse Model

To further examine the immunomodulatory mechanisms responsible for the AhR signal in the carcinogenesis of cutaneous SCC, we induced cutaneous SCC using a two-stage chemically induced skin carcinogenesis mouse model (**Figure 3A**). As shown in **Figure 3B**, the number of cutaneous SCC lesions is decreased in AhR-(fl/fl) Krt5-(Cre) mice, suggesting that the development of DMBA-induced cutaneous SCC depends on AhR signaling in keratinocytes. Since NHKCs increase the expression and production of Th17-related proinflammatory cytokines and chemokines *in vitro*, we evaluated these factors in established mouse cSCC. The mRNA expression of CYP1A1 (p = 0.002), CCL20 (p = 0.047), p19 (p = 0.029), CCL19 (p = 0.013), IL-36γ (p = 0.0076), and IL-17 (p = 0.0035) was significantly decreased in tumor from AhR-(fl/fl) Krt5-(Cre) mice compared with wild type mice (**Figure 3C**). On the other hand, there was no significant difference in mRNA expression of p40 (p = 0.1716), CCL22 (p = 0.1805), IL-36α (p = 0.063), and IL-36β (p = 0.099) in tumor from AhR-(fl/fl) Krt5-(Cre) mice compared with wild type mice.

Expression of CCL20, IL-23, IL-36γ, and IL-17 in Cutaneous cSCC

Considering that the cSCC mouse model described above suggested that the mRNA expression of Th17-related proinflammatory cytokines and chemokines depends on AhR signals in mouse cSCC *in vivo*, we further examined these cytokines and chemokines in cSCC patients. We employed IHC staining of CCL20, IL-23, IL-36γ, and IL-17 for samples from 10 cSCC patients. As demonstrated by the *in vitro* and *in vivo* model, atypical keratinocytes expressed CCL20 in tumor lesions of cSCC (**Figures 4a,b**), while normal keratinocytes at the marginal zone of the tumor did not express CCL20 (**Figure 4c**). Moreover, a substantial number of IL-23-producing cells were detected in the dermis of lesional skin of cSCC (**Figures 4d,e**), where a substantial number of IL-17-producing cells were also detected (**Figure 4g**). On the other hand, there

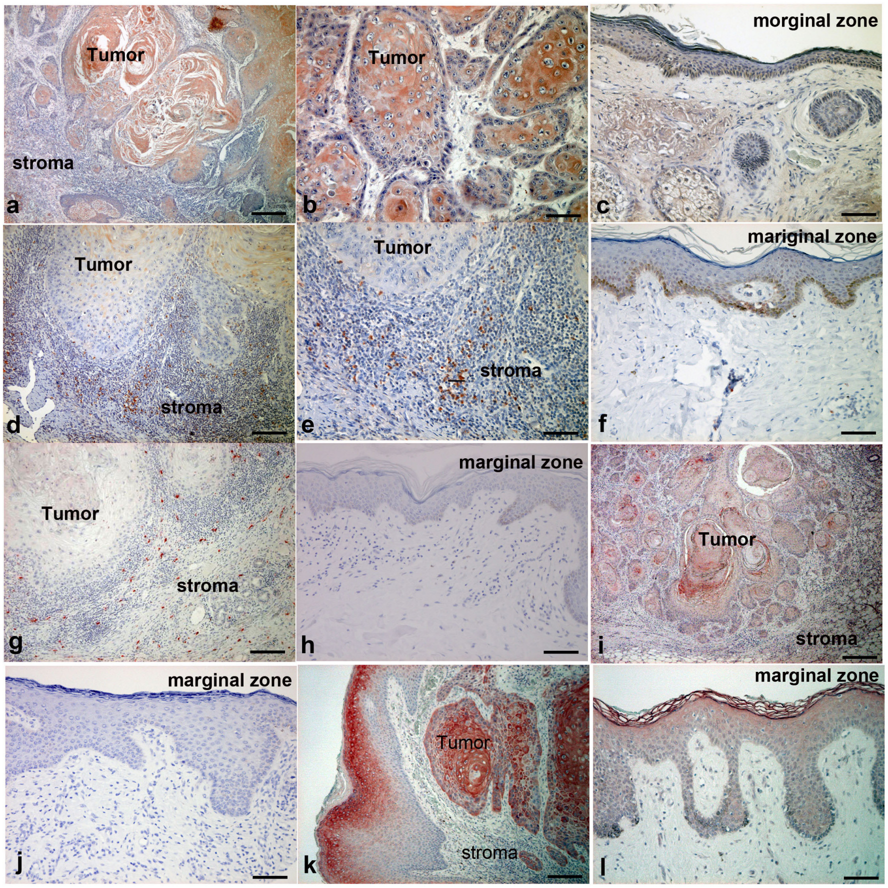


FIGURE 4 | Immunohistochemical analysis of CCL20, IL-23, IL-17, IL-36γ, and IL-17R expression in lesion-affected skin of cSCC. Sections of cSCC lesions were deparaffinized and stained using anti-CCL20 **(a–c)**, anti–IL-23 **(d–f)**, anti–IL-17 **(g,h)**, anti–IL-36γ **(i,j)**, or anti–IL-17R **(k,l)** antibodies. Sections were developed with liquid permanent red. Scale bars, 100 μm. Representative specimens from 12 cases of cSCC are shown. Scale bars, 50 μm **(b,i)**, 100 μm **(c,e–l)**, and 200 μm **(a,d)**.

were no IL-23-producing cells (**Figure 4f**) or IL-17-producing cells (**Figure 4h**) in the dermis of tumor marginal zone. These IL-23-producing cells and IL-17 producing cells were, at least in part, CD163 + tumor-associated macrophages (TAMs) and CD4 + IL-17 + Th17 cells, respectively, (**Supplementary Figure 1**). In addition, the expression of IL-36γ was detected in some of atypical keratinocytes (**Figure 4i**) but not in normal keratinocytes in the marginal zone of cSCC (**Figure 4j**). IL-17R is highly expressed in atypical keratinocytes in the lesional skin (**Figure 4k**), but only slightly expressed in the upper spiny layer of normal keratinocytes (**Figure 4l**).

DISCUSSION

Polycyclic aromatic hydrocarbons (PAHs) exert their biological effects via binding to the ligand-activated transcription factor AhR, which activates the expression of genes encoding detoxification enzymes (9). Chronic exposure of skin to PAHs, such as DMBA, induces chronic keratinocyte-specific activation of the AhR, which leads to the symptoms of atopic dermatitis with chronic inflammation (6). Another report also suggested that PAHs increase the expression of IL-5, IL-13, and IL-17 (19). These data suggest that chronic exposure of the skin to PAHs induces increased expression of both Th2 and Th17.

Recently, several reports suggested the significance of IL-17 in the development of cSCC. For example, Wu et al. reported that IL-17 signaling in keratinocytes drives IL-17-dependent sustained activation of the TRAF4-ERK5 axis, leading to keratinocyte proliferation and tumor formation in cSCC (16). In another report, IL-17 and IL-22 increased the proliferation and migration of CAL27 SCC cell lines, suggesting the contribution of IL-17 to the progression of SCC (20). Moreover, Gasparoto et al. reported the significant co-relation of IL-17 and the development of mouse cSCC (21). Furthermore, the significance of IL-17 is reported not only in cSCC, but also in other types of cancer. For example, IL-17 is positively associated with histologic grade and is described as a prognostic factor in breast cancer (22). More recently, we also reported the possible correlation between CCL20/IL-23/IL-17 axis in the development of extramammary Paget's disease (EMPD) (18). These reports suggested the significance of IL-17 in the carcinogenesis of cancers.

IL-23 plays important roles in inducing Th17 cell proliferation (23) even in the cancer microenvironment (24), and is also known to promote growth and proliferation of human SCC of the oral cavity (25). Indeed, the significance of IL-23 is reported in various cancer species (26). For example, in UVB-induced mouse skin cancer model, several proinflammatory cytokines, including IL-23, are increased by irradiation, suggesting the relationship between IL-23 and the development of mouse AK and cSCC (27). In addition to its role in SCC, IL-23 promotes tumor progression by the inhibition of apoptosis in breast cancer cell lines (26), and levels of IL-23 and IL-23R expression are positively correlated with tumor size, tumor-node-metastasis stage, and metastasis in breast cancer (26).

Considering that the IL-23/IL-17 pathogenic axis could be an anchor cytokine signal for the development of cSCC, and that the PAHs could induce an increased expression of Th17, we hypothesized that PAHs such as DMBA and FICZ could increase the expression of these proinflammatory cytokine-related factors. Indeed, both DMBA and FICZ increased the mRNA expression of CYP1A1, CCL20, p19, and IL-36γ in NHKCs *in vitro*. In parallel to data from the *in vitro* experiments, the mRNA expression of CYP1A1, CCL20, p19, and IL-36γ, as well as IL-17 in DMBA-induced cSCC from $AhR^{-(fl/fl)}$ $Krt5^{-(Cre)}$ mice, is significantly decreased compared with that of wild type mice. These results suggested the significance of the IL-23/IL-17 pathogenic axis in the development of cSCC. Indeed, immunohistochemical staining for the patients with cSCC revealed that atypical keratinocytes expressed CCL20 and IL-36γ, and a substantial number of IL-23-producing cells and IL-17-producing cells were detected in the lesional skin of cSCC. Moreover, the expression of IL-17R is higher in atypical keratinocytes than in the normal keratinocytes. Taken together, our data suggested that PAHs (FICZ and DMBA) that are classically known to induce cSCC could trigger the induction of IL-17-producing cells, leading to the development of cSCC.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tohoku University Graduate School of Medicine, Sendai, Japan (permit number: 2017-1-430) and Kagoshima Medical Center, Japan (permit number 29-2, 30-08). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Tohoku University Graduate School of Medicine for Animal Experimentation, Sendai, Japan (permit number: 2017MdLMO-342-2).

AUTHOR CONTRIBUTIONS

TF and TH conception and design. TF and TH development of methodology. YS, TF, CL, and KT acquisition of data. TF analysis and interpretation of data. TF writing, review, and/or revision of the manuscript. YS, TF, KT, and SM treating patients. TF, MY, and SA study supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported in part by the Japan Agency for Medical Research and Development (18cm01643h0001 and 19cm0106434h0002).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.534323/full#supplementary-material

Supplementary Figure 1 | Immunofluorescence staining of CD163 + TAMs and CD4 + T cells. Immunofluorescence staining of cSCC for IL-23 (green), CD163 (red), and DAPI (blue, nuclei; a), and IL-17 (green), CD4 (red), and DAPI (blue, nuclei). A merged image is also shown, with green and red combining into yellow. The isotype control IgG1 was stained as red or green. Representative specimens from 3 cases are shown.

REFERENCES

1. Thompson AK, Kelley BF, Prokop LJ, Murad MH, Baum CL. Risk factors for cutaneous squamous cell carcinoma recurrence, metastasis, and disease-specific death: a systematic review and meta-analysis. *JAMA Dermatol*. (2016) 152:419–28. doi: 10.1001/jamadermatol.2015.4994
2. Cheng J, Yan S. Prognostic variables in high-risk cutaneous squamous cell carcinoma: a review. *J Cutan Pathol*. (2016) 43:994–1004. doi: 10.1111/cup.12766
3. Fernandez Figueras MT. From actinic keratosis to squamous cell carcinoma: pathophysiology revisited. *J Eur Acad Dermatol Venereol*. (2017) 31(Suppl. 2):5–7. doi: 10.1111/jdv.14151
4. Chahal HS, Lin Y, Ransohoff KJ, Hinds DA, Wu W, Dai HJ, et al. Genome-wide association study identifies novel susceptibility loci for cutaneous squamous cell carcinoma. *Nat Commun*. (2016) 7:12048. doi: 10.1038/ncomms12048
5. Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*. (2010) 11:936–44. doi: 10.1038/ni.1920
6. Hidaka T, Ogawa E, Kobayashi EH, Suzuki T, Funayama R, Nagashima T, et al. The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. *Nat Immunol*. (2017) 18:64–73. doi: 10.1038/ni.3614
7. Ma Q. Influence of light on aryl hydrocarbon receptor signaling and consequences in drug metabolism, physiology and disease. *Expert Opin Drug Metab Toxicol*. (2011) 7:1267–93. doi: 10.1517/17425255.2011.614947
8. Di Meglio P, Duarte JH, Ahlfors H, Owens ND, Li Y, Villanova F, et al. Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions. *Immunity*. (2014) 40:989–1001. doi: 10.1016/j.immuni.2014.04.019
9. Morino-Koga S, Uchi H, Mitoma C, Wu Z, Kiyomatsu M, Fuyuno Y, et al. 6-formylindolo[3,2-b]carbazole (FICZ) accelerates skin wound healing via activation of ERK, but not aryl hydrocarbon receptor. *J Invest Dermatol*. (2017) 137:2217–26. doi: 10.1016/j.jid.2016.10.050

10. Morino-Koga S, Uchi H, Tsuji G, Takahara M, Kajiwara J, Hirata T, et al. Reduction of CC-chemokine ligand 5 by aryl hydrocarbon receptor ligands. *J Dermatol Sci*. (2013) 72:9–15. doi: 10.1016/j.jdermsci.2013.04.031
11. Okumura K, Kagawa N, Saito M, Yoshizawa Y, Munakata H, Isogai E, et al. CENP-R acts bilaterally as a tumor suppressor and as an oncogene in the two-stage skin carcinogenesis model. *Cancer Sci*. (2017) 108:2142–8. doi: 10.1111/cas.13348
12. Hidaka T, Fujimura T, Aiba S. Aryl hydrocarbon receptor modulates carcinogenesis and maintenance of skin cancers. *Front Med*. (2019) 6:194. doi: 10.3389/fmed.2019.00194
13. Nassar D, Latil M, Boeckx B, Lambrechts D, Blanpain C. Genomic landscape of carcinogen-induced and genetically induced mouse skin squamous cell carcinoma. *Nat Med*. (2015) 21:946–54. doi: 10.1038/nm.3878
14. McCreery MQ, Halliwill KD, Chin D, Delrosario R, Hirst G, Vuong P, et al. Evolution of metastasis revealed by mutational landscapes of chemically induced skin cancers. *Nat Med*. (2015) 21:1514–20. doi: 10.1038/nm.3979
15. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature*. (2008) 453:106–9. doi: 10.1038/nature06881
16. Wu L, Chen X, Zhao J, Martin B, Zepp JA, Ko JS, et al. A novel IL-17 signaling pathway controlling keratinocyte proliferation and tumorigenesis via the TRAF4-ERK5 axis. *J Exp Med*. (2015) 212:1571–87. doi: 10.1084/jem.20150204
17. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature*. (2008) 453:65–71. doi: 10.1038/nature06880
18. Sato Y, Fujimura T, Tanita K, Chunbing L, Matsushita S, Fujisawa Y, et al. Malassezia-derived aryl hydrocarbon receptor ligands enhance the CCL20/Th17/soluble CD163 pathogenic axis in extra-mammary Paget's disease. *Exp Dermatol*. (2019) 28:933–9. doi: 10.1111/exd.13944
19. Hong CH, Lee CH, Yu HS, Huang SK. Benzopyrene, a major polyaromatic hydrocarbon in smoke fume, mobilizes Langerhans cells and polarizes Th2/17 responses in epicutaneous protein sensitization through the aryl hydrocarbon receptor. *Int Immunopharmacol*. (2016) 36:111–7. doi: 10.1016/j.intimp.2016.04.017
20. Nardinocchi L, Sonego G, Passarelli F, Avitabile S, Scarponi C, Failla CM, et al. Interleukin-17 and interleukin-22 promote tumor progression in human non-melanoma skin cancer. *Eur J Immunol*. (2015) 45:922–31. doi: 10.1002/eji.201445052
21. Gasparoto TH, de Oliveira CE, de Freitas LT, Pinheiro CR, Ramos RN, da Silva AL, et al. Inflammatory events during murine squamous cell carcinoma development. *J Inflamm*. (2012) 9:46. doi: 10.1186/1476-9255-9-46
22. Fernandez-Garcia B, Eiro N, Miranda MA, Cid S, González LO, Domínguez F, et al. Prognostic significance of inflammatory factors expression by stroma from breast carcinomas. *Carcinogenesis*. (2016) 37:768–76. doi: 10.1093/carcin/bgw062
23. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity*. (2009) 31:331–41. doi: 10.1016/j.immuni.2009.08.001
24. Qian X, Gu L, Ning H, Zhang Y, Hsueh EC, Fu M, et al. Increased Th17 cells in the tumor microenvironment is mediated by IL-23 via tumor-secreted prostaglandin E2. *J Immunol*. (2013) 190:5894–902. doi: 10.4049/jimmunol.1203141
25. Fukuda M, Ehara M, Suzuki S, Ohmori Y, Sakashita H. IL-23 promotes growth and proliferation in human squamous cell carcinoma of the oral cavity. *Int J Oncol*. (2010) 36:1355–65. doi: 10.3892/ijo_00000620
26. Sheng S, Zhang J, Ai J, Hao X, Luan R. Aberrant expression of IL-23/IL-23R in patients with breast cancer and its clinical significance. *Mol Med Rep*. (2018) 17:4639–44. doi: 10.3892/mmr.2018.8427
27. Kremer JL, Melo GP, Marinello PC, Bordini HP, Rossaneis AC, Sábio LR, et al. Citral prevents UVB-induced skin carcinogenesis in hairless mice. *J Photochem Photobiol B*. (2019) 198:111565. doi: 10.1016/j.jphotobiol.2019.111565

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 10 November 2020
doi: 10.3389/fimmu.2020.565470

Check for updates

Much More Than IL-17A: Cytokines of the IL-17 Family Between Microbiota and Cancer

Arianna Brevi[1], Laura Lucia Cogrossi[1,2], Giulia Grazia[1], Desirée Masciovecchio[1], Daniela Impellizzieri[1], Lucrezia Lacanfora[1], Matteo Grioni[1] and Matteo Bellone[1]*

[1] Cellular Immunology Unit, Division of Immunology, Transplantation and Infectious Diseases, I.R.C.C.S. Ospedale San Raffaele, Milan, Italy, [2] Department of Medicine and Surgery, Vita-Salute San Raffaele University, Milan, Italy

The interleukin-(IL-)17 family of cytokines is composed of six members named IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A is the prototype of this family, and it was the first to be discovered and targeted in the clinic. IL-17A is essential for modulating the interplay between commensal microbes and epithelial cells at our borders (i.e., skin and mucosae), and yet, for protecting us from microbial invaders, thus preserving mucosal and skin integrity. Interactions between the microbiota and cells producing IL-17A have also been implicated in the pathogenesis of immune mediated inflammatory diseases and cancer. While interactions between microbiota and IL-17B-to-F have only partially been investigated, they are by no means less relevant. The cellular source of IL-17B-to-F, their main targets, and their function in homeostasis and disease distinguish IL-17B-to-F from IL-17A. Here, we intentionally overlook IL-17A, and we focus instead on the role of the other cytokines of the IL-17 family in the interplay between microbiota and epithelial cells that may contribute to cancer pathogenesis and immune surveillance. We also underscore differences and similarities between IL-17A and IL-17B-to-F in the microbiota-immunity-cancer axis, and we highlight therapeutic strategies that directly or indirectly target IL-17 cytokines in diseases.

Keywords: microbiota, Th17, autoimmunity, microbiome, gut, immunotherapy, arthritis, cancer

OPEN ACCESS

Edited by:
Katarzyna Bulek,
Jagiellonian University, Poland

Reviewed by:
Zoltan Vereb,
University of Szeged, Hungary
Massimo Costalonga,
University of Minnesota Twin Cities, United States

****Correspondence:***
Matteo Bellone
bellone.matteo@hsr.it

Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *25 May 2020*
Accepted: *15 October 2020*
Published: *10 November 2020*

Citation:
Brevi A, Cogrossi LL, Grazia G, Masciovecchio D, Impellizzieri D, Lacanfora L, Grioni M and Bellone M (2020) Much More Than IL-17A: Cytokines of the IL-17 Family Between Microbiota and Cancer. Front. Immunol. 11:565470. doi: 10.3389/fimmu.2020.565470

INTRODUCTION

Fine tuning of the interactions between eukaryotic and prokaryotic cells that literally share our body is essential for maintenance of health (1). In humans, the number of commensal, symbiont, and mutualistic microbes (i.e., microbiota) inhabiting the gut, skin, mucosae, and even some visceral organs, at least equals the number of eukaryotic cells (2). Nonetheless, the microbiome (i.e., the microbiota genomic repertoire) outnumbers the host's genome by 10 folds (3), and this may help explaining why the microbiota is so relevant for the correct functioning of our organs and tissues (4). The development of individual microbiota starts soon after birth (5), and it stabilizes within the first three years (6). Within the same time frame, the developing immune system has to deal with, and it is shaped by the microbiota (7). Indeed, the immune system adapts to antigens expressed by eukaryotic cells, through the mechanisms of central and peripheral immune tolerance, thus avoiding autoimmunity (8). The immune system also has to progressively cope with antigens expressed by the microbiota (9), a phenomenon we originally defined as adaptation to the "extended self" (10). Tolerance to the extended self is likely enforced by the perfect balancing between regulatory T cells (Tregs), which block excessive immune reactions (11), and T helper (Th) cells, rapidly intervening when a commensal species has overtly grown, or a new species appears within the microbiota. Indeed, antigens and metabolites generated in the presence of a defined microbiota modulate the expansion or contraction of Tregs and effector Th cells (12–14). For example, microbiota-immune system interactions skew mouse Th cells to produce interleukin-17 (IL-17) (15), and together with IL-22 (16), Th17 cells producing these cytokines protect the integrity of the gut mucosa, and stimulate the local maturation of immunoglobulin (Ig) A-producing plasma cells, thus restraining dwelling bacteria (17). Additionally, fibroblasts, endothelial cells, chondrocytes, and adipocytes respond to IL-17A by expressing antimicrobial proteins and peptides, and proinflammatory cytokines and chemokines involved in acute-phase responses and tissue remodeling (18, 19). As a consequence, skin and mucosae of organisms lacking IL-17A are more susceptible to fungal and bacterial infection (20).

An alteration or imbalance of the normal microbiota composition (i.e., dysbiosis) is a common characteristic of many human diseases, albeit it remains to be clarified if dysbiosis is cause or consequence of the disease. Obesity, type 2 diabetes, nonalcoholic fatty liver disease, periodontitis, rheumatoid arthritis, psoriatic arthritis, multiple sclerosis, and systemic lupus erythematosus are examples of diseases exacerbated or worsened by an altered gut flora (1, 4, 10, 14, 21). Interestingly, IL-17A has a relevant pathogenic role in all these diseases (10). For example, it is well known that the IL-12-IL-17 axis exerts an essential role both in the onset phase and at the time of bone destruction in autoimmune arthritis (22). Microbiome analysis in rheumatoid arthritis patients showed dysbiosis and a relative abundance of *Prevotella copri*, Gram negative bacteria that appear to favor the induction of Th17 cells (23) (**Table 1**). In mice, transfer of Th17 cells polarized by *P. copri*-stimulated dendritic cells induced arthritis (38). Both in humans (39) and in mice (40), cross-reactivity between bacteria and myelin antigens seems to activate Th17 cells that induce autoimmune demyelination. In experimental autoimmune encephalomyelitis (EAE), microbiota-induced Th17 lymphocytes migrated from the gut into the central nervous system, where they exacerbated the disease (41). Thus, control of pathogenic Th17 cells occurs in the gut. The mechanisms by which commensals modulate the immune response, and Th17 cells in particular, has only been partially defined. Very recently, Duscha et al. (42) showed that the availability of propionic acid in feces and blood of multiple sclerosis patients depends on intestinal microbiota composition, and 14-day supplementation of propionic acid in the diet correlated with Treg expansion in the intestine, and neurologic symptom amelioration. Interestingly, monoclonal antibodies blocking either IL-17 or IL-23 are already in the clinic or under investigation for the treatment of rheumatoid arthritis patients (43). Thus, IL-17A is a master regulator of host-microbiota interactions both in physiologic conditions and in immune-mediated inflammatory diseases (44, 45).

More recently, a microbiome has also been found in the blood and tumor of cancer patients (46), and microbiota-induced IL-17A has also been implicated in the pathogenesis of colon cancer, breast, pancreatic and ovarian carcinomas, and multiple myeloma (MM) (10, 47). The role of IL-17A in cancer has not been fully elucidated, and data are controversial. While in melanoma and ovarian cancer, Th17 cells activate anti-neoplastic cytotoxic T cell responses (48–50), they are

TABLE 1 | Microbes driving the production of IL-17 cytokines in inflammation and cancer.

	Microbes	Site	Cytokine produced	Producer cells	Outcome	Ref.
Physiological inflammatory response	*Tritrichomonas, Heligmosomoides polygyrus*	Intestine	IL-17E	Tuft cells	Activation of ILC2 and type-2 immunity in mice	(24)
	Citrobacter rodentium	Intestine	IL-17C IL-17B IL-17F	Epithelial cells	Induction of inflammation, promotion of epithelial barrier integrity in mice	(25–27),
	Listeria monocytogenes, *Influenza virus*	Intestine	IL-17D	Non-hematopoietic cells	Increased susceptibility to infection	(28)
Inflammatory diseases	*Pseudomonas aeruginosa*	Lungs	IL-17C	Epithelial cells	Induction of inflammation	(29)
	Bacteroides stercoris, *Bacteroides ovatus*, *Prevotella melaninogenica*	Lungs	IL-17B	Macrophages	Induction of pulmonary fibrosis in mice	(30)
	Fusobacterium nucleatum	Intestine	IL-17F	Epithelial cells	Correlates with progression of ulcerative colitis in humans and mice	(31)
	Prevotella copri, Prevotella nigrescens	Intestine	IL-17A	Th17	Correlates with enhanced rheumatoid arthritis in humans and mice	(32)
Cancer	*Proteobacteria, Verrucomicrobia*	Intestine	IL-17E	Macrophages	Correlates with progression of hepatocellular carcinoma in humans and mice	(33)
	Escherichia coli	Intestine	IL-17C	Epithelial cells	Colorectal cancer progression in mice	(34)
	Nontypeable *Haemophilus influenza*	Lungs	IL-17C	Epithelial cells	Progression of lung cancer in mice	(35)
	Bacteroides fragilis	Intestine	IL-17A	Th17 cells	Colorectal cancer progression in mice	(36)
	Prevotella heparinolytica	Intestine	IL-17A	Th17 cells	Multiple myeloma progression in mice	(37)

tumorigenic in a variety of mouse models of colon cancer (51), hepatocellular carcinoma (52), MM (37), and pancreatic cancer (53). The function of IL-17 may also vary depending on the disease phase, and in pancreatic cancer it has been proposed that IL-17-producing cells support tumor growth in the initial phases of the disease, while in advanced phases, IL-17A potentiates antitumor immunity (47). IL-17A can favor tumor growth either in a direct or in indirect manner. In mouse tumor cell lines expressing the IL-17R, IL-17A induced IL-6 production, which in turn activated signal transducer and activator of transcription (Stat) 3, eventually upregulating pro-survival and proangiogenic signals (54). On the other hand, IL-17A also recruits mouse innate immune cells like neutrophils and immature myeloid cells within the tumor, supporting the development of an immunosuppressive microenvironment, eventually favoring tumor growth (55–57).

Of relevance, modulation of the gut microbiota reduces expansion of Th17 cells and tumor progression both in solid and hematopoietic tumors (37, 58). For example, in mice affected by MM (59), a neoplasia of plasma cells accumulating primarily in the bone marrow together with an immune infiltrate (60), the gut microbiota enriched in *P. heparinolytica* induced Th17 cells locally, which migrated to the bone marrow and promoted aggressiveness of MM (**Table 1**). Indeed, both in humans and in mice neoplastic plasma cells express the IL-17 receptor (IL-17R) (37, 61), and IL-17 supports plasma cells survival and proliferation likely by inducing the autocrine release of IL-6 (54). Lack of IL-17A in MM mice, or treatment with antibiotics or monoclonal antibodies blocking IL-17/IL-17R interactions delayed disease progression (37). Thus, the microbiota-IL-17A axis is also relevant in cancer patients.

The gut microbiota may also influence response to therapy in cancer patients, and this is the focus of intense clinical investigation. For instance, the composition of the gut microbiota *per se* is sufficient to discriminate cancer patients who will or will not respond to antibodies blocking inhibitory immune checkpoints (62–64). Prospective clinical trials will better define the impact of microbiota modulation on cancer therapy.

IL-17A has been cloned in 1993 (65). At the beginning of this century, other molecules with sequence homology to IL-17A entered the IL-17 family, including IL-17B, IL-17C, IL-17D, IL-17E or IL-25, and IL-17F (66, 67). Each cytokine of the family acts as homodimer or heterodimer, and they interact with specific dimeric receptors (named IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE; **Figure 1**), with the exception of IL-17D, which remains orphan of its ligand (44). Binding of IL-17 cytokines to cognate IL-17Rs activates the shared SEFIR (SEF/IL-17R) cytoplasmic motif (68), which mediates the recruitment of Act1 (69). As detailed below, these steps are crucial for downstream recruitment and ubiquitination of TNF-receptor associated factor 6 (TRAF6), activation of nuclear factor κB (NF-κB), and expression of pro-inflammatory and anti-microbial molecules (70).

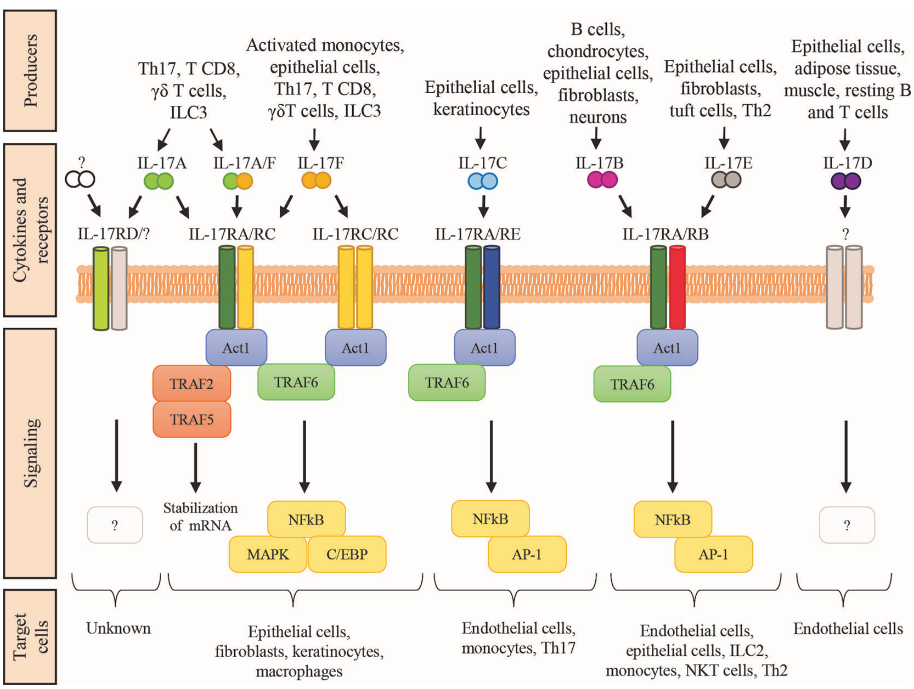


FIGURE 1 | The IL-17 family of cytokines. Schematic representation of the cytokines belonging to the IL-17 family, their respective receptor complexes coupled with intracellular signaling, and their target cells. Cytokines are reported in a mechanistic rather than alphabetic order. Producers each cytokine are also shown. AP-1, activator protein-1; C/EBP, CCAAT enhancer-binding protein; ILC, innate lymphoid cells; MAPK, mitogen-activated protein kinase; NKT natural killer T cells; Th2, T helper-2 cells; Th17, T helper-17 cells; TRAF, TNF-receptor associated factor; NF-kB, nuclear factor kB.

While the role of microbiota-driven IL-17A and Th17 cells in cancer have been extensively reviewed [e.g. (10, 20, 47)], a review dedicated to the role of the other cytokines of the IL-17 family in the microbiota-immunity-cancer axis is lacking. Thus, we intentionally overlooked the IL-17A/IL-17RA-RC pathway, and we have focused on IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F.

IL-17 SIGNALING

Cytokines of the IL-17 family are pleiotropic and exert potent and diverse *in vivo* functions through both canonical and noncanonical signaling pathways (68). Canonical signaling induces both transcriptional and post-transcriptional mechanisms involved in autoimmunity, hypersensitivity, and metabolic reprogramming of lymphoid tissues. Noncanonical signaling acts in synergy with other receptor systems, and it is mainly responsible for tissue repair and regeneration. Both mechanisms participate to host defenses, and tumor progression.

The IL-17Rs belong to a new subfamily of receptors consisting of 5 members: IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE which are single-pass transmembrane receptors with conserved domains (71) (**Figure 1**). Indeed, all members of the IL-17R family encode two extracellular fibronectin II-like domains and the conserved region SEFIR, which mediates the recruitment of the multifunctional adaptor Act1 (69). SEFIR is structurally related to the domain found in the toll-like receptor (TLR)/IL-1R (72). Functionally, the IL-17R is a heterodimeric complex composed of the IL-17RA in combination with other subunits that confer ligand or signaling specificity. IL-17A signals through IL-17RA in combination with IL-17RC. Whereas the IL-17RA subunit is ubiquitously expressed, IL17RC is mainly present on non-hematopoietic epithelial and mesenchymal cells. Interestingly, IL-17A interacts with its receptor as a homodimer or as a heterodimer with IL-17F (73). IL-17F could also bind this receptor complex as a homodimer. The difference between these three ligands is mainly in the potency of interaction: IL-17A>IL-17A-IL-17F> IL-17F (74). Also the IL-17RD has been proposed as an alternative receptor subunit for IL-17A, but not for IL-17F, and appears to favor the IL-17A-mediated recruitment of neutrophils (75). Finally, IL-17RA is also used by IL-17B, IL-17C, and IL-17E (also known as IL-25) (66). The detailed function of IL-17 receptors and their ligands remains partially elusive and requires further investigation.

Canonical Signaling

The canonical IL-17 signaling pathway is initiated by SEFIR (**Figure 1**), which mediates Act1 recruitment (69). Act1 is crucial for IL-17 signaling, and it acts as adaptor and as RNA-binding protein (RBP) by forming several ribonucleoprotein particles (RNPs) (69, 76, 77). As adaptor, Act1 triggers multiple signaling cascades *via* the tumor TRAF-binding motif, which recruits different TRAF protein to initiate separate downstream pathways. The TRAF-binding motif is a distinct C-terminal region present only in IL-17RA. An analogous domain in other IL-17R family members is not found (68). Downstream recruitment and ubiquitination of TRAF6 leads to the activation of nuclear factor-kB (NF-kB), the CCAAT enhancer-binding proteins (C/EBPs) family, and the mitogen-activated protein kinase (MAPK) pathways (p38, ERK, and JNK) responsible for transcriptional regulation (44, 70). The TRAF6-mediated signaling is controlled by several regulatory mechanisms to hamper IL-17-induced inflammation. For instance, upregulation of IL-17 signaling *via* NFkB is associates with susceptibility to autoimmune syndromes, including psoriasis and experimental autoimmune encephalomyelitis (78, 79). Additionally, TRAF3 or TRAF4 compete with TRAF6 for the TRAF-binding motif on Act1, leading to reduced IL-17-induced expression of pro-inflammatory mediators, and Act1 is degraded by the proteasome in the presence of prolonged IL-17 stimulation (80). Thus, NF-κB and MAPK pathways downregulate IL-17 signaling. Conversely, C/EBP family activation potentiates the IL-17-inflammatory response through a feed-forward mechanism with other transcription factors like IκBζ. IκBζ modulation is crucial to control the IL-17-dependent responses, and it is one of the few targeted genes so far investigated. Indeed, most of the C/EBP-dependent genes involved in the IL-17 pathway remain elusive.

Act1 also acts as RBP upon TRAF2-TRAF5 complex engagement to control the stability and translation of mRNA from IL-17-target genes in response to IL-17 stimulation. IL-17 signaling results in the formation of multiple RNPs, associated with mRNA-stabilizing or mRNA-destabilizing factors, for post-transcriptional regulation of gene expression (81). Interestingly, IL-17 increases the half-life of mRNA to induce the efficient production of effector proteins.

Noncanonical Signaling

Noncanonical IL-17 signaling is characterized by synergistic interactions of IL-17 signals with other ligands, like cytokines or microbial products, that lead to activation of diverse signaling pathways (82–84). As few examples, NF-kB is activated upon interaction of IL-17 with tumor necrosis factor-α (TNF-α) or lymphotoxin, and the signal transducer and activator of transcription 1 (STAT1) when IL-17 interacts with interferon-γ (IFN-γ). Interaction between IL-17 and IL-13 activates STAT6, whereas the SMADs family is triggered by the interaction with tumor growth factor-β (TGF-β). Finally, IL-17 interactions with bacterial lipopolysaccharide or fungal products, like candidalysin, activates c-Fos (82–84).

IL-17 also controls tissue homeostasis by integrating signals from the IL-17R and growth factor receptors in a high cell type- and context-specific manner. In particular, an integration of IL-17 receptor signaling has been described with the epidermal growth factor receptor (EGFR), the fibroblast growth factor receptor (FGFR), NOTCH1, and with components of the C-type lectin receptors. The EGFR cascade is mainly identified in skin stem cells, and it is involved in wound healing and tumorigenesis (85, 86). Interactions between IL-17 and FGFR have been described in mouse colonic epithelial cells during tissue repair caused by colon inflammation (87). In mice, IL-17 signaling also engages the NOTCH1 receptor to promote neuroinflammation through expansion and differentiation of oligodendrocyte progenitor cells (88, 89). Finally, signaling integrations between IL-17 and components of C-type lectin receptors have been reported in keratinocytes during psoriasis (90).

Altogether, these findings support the existence of a complex and yet partially explored net of signaling pathways downstream IL-17 cytokine secretion and interaction with their receptors.

CYTOKINES OF THE IL-17 FAMILY OTHER THAN IL-17A IN HEALTH AND DISEASE

Cytokines of the IL-17 family are crucial components of the inflammatory response, and they are essential for normal host immune responses. Both in humans and in mice, IL-17 cytokines are produced by a vast array of cell types, and act on a multitude of cellular targets (44, 66, 67, 91–93), eventually inducing production of pro-inflammatory cytokines, chemokines, and prostaglandins (94) (**Figure 1**). Cytokines of the IL17 family exert non-redundant, and even opposing functions to promote elimination of intruders, and tissue reconstitution. They are also involved in many human pathologies including inflammatory immune mediated diseases and cancer.

Cytokines within the IL-17 family share 16-50% amino acid identity with IL-17A, with IL-17F being the most similar (50%) and IL-17E the most divergent (16%). The similarity between IL-17 cytokines is higher in the C terminus and in five spatially conserved cysteine residues. N terminus sequences of IL-17B, IL-17C, and IL-17E are substantially different from those found in IL-17A and IL-17F because of a longer extension of the former three proteins (95), suggesting that the N terminus is involved in receptor specificity (96).

Because IL17F has the highest homology with IL-17A, binds to the same complex IL17RA-RC, and activated Th17 cells to produce both IL-17A and IL-17F (97), we followed a mechanistic rather than an alphabetic order to describe cytokines of the IL-17 family, and we started from IL-17F. As we will see, not all these cytokines are produced by immune cells, but all of them either directly or indirectly impact the immune system. We refer the interested reader to excellent reviews for a comprehensive description of these cytokines (44, 66, 67, 91–93).

IL-17F

The *Il17f* gene is closely located to the *Il17a* gene both in humans (chromosome 6) and mice (chromosome 1), whereas genes encoding the other members of the IL-17 family are located in different chromosomes (18). The protein has a molecular mass of 18045 Da and is composed of 163 amino acids. IL-17F can form homodimer or heterodimer with IL17A (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL17F#summaries). Many genetic variants have been identified for the *Il17f* gene: most of them are missense mutations and some of them are pathogenetic (https://gnomad.broadinstitute.org/gene/ENSG00000112116?dataset=gnomad_r2_1). For example, the heterozygous missense mutation S95L (c.284C>T) in the *Il17f* gene has been found in patients with chronic mucocutaneous candidiasis, an infection caused by *Candida albicans* that affects nails, skin, and oral and genital mucosae. The S95L IL-17F mutant (IL-17FS95L) is normally expressed and forms homo- and heterodimers with IL-17F, IL-17FS95L, and IL-17A. However, IL-17FS95L is severely hypomorphic and exerts a dominant-negative effect by impairing the binding of its complexes to the receptor (98).

IL-17A and IL-17F are mainly produced by activated $CD4^+$ T cells leading to the definition of a distinct T cell subset named Th17 (66). The differentiation of Th17 cells in humans is induced by several cytokines including IL-1β, IL-21, IL-23 and TGF-β that activate the Stat3- and the IRF4-dependent expression of retinoic acid receptor-related orphan receptor-γt (ROR-γt). Th17 cells comprise IL-17A and IL-17F double positive cells, but also populations that are only positive for IL-17A or IL-17F have been identified, suggesting that the mechanisms regulating IL-17A and IL-17F production are different. Interestingly, in mice the expression of Il17a but not Il17f is strictly coupled to the T cell receptor (TCR) signaling through the inducible T cell kinase (Itk)-mediated nuclear factor of activated T cells (NFAT) recruitment (99). These data demonstrate that in mice Itk specifically links TCR signaling to Il17a expression, thus regulating Th17 cell cytokines through NFATc1.

As for IL-17A, IL-17F is also expressed in innate lymphoid cells (ILCs), γδ T cells, natural killer T (NKT) cells and $CD8^+$ T cells, but IL-17F is exclusively produced by activated monocytes and epithelial cells (25, 100, 101). Both IL-17A and IL-17F bind to the IL-17RA-RC heterodimer, and they induce a qualitatively but not quantitatively similar signal, being IL-17A far more potent than IL-17F. Both IL-17A and IL-17F can be secreted as disulfide-linked homodimers or heterodimers (18). Heterodimers exhibit intermediate levels of potency in inducing IL-6 and CXCL1 when compared to homodimeric cytokines (102).

Both cytokines act in synergy with TNF-α (103), and in mice contribute to inflammation and protection at barrier surfaces, with overlapping yet distinct roles (**Figure 2**) (25). *In vitro*, IL-17F preferentially associates with IL-17RC homodimers, leading to IL-17RA-independent signaling (104). The expression profiles of IL-17RA and IL-17RC are different among tissues and cell types, with IL-17RC preferentially expressed in non-immune cells (25). In mouse models, the constitutive expression of IL-17RC in intestinal epithelial cells (25) explains the more pathogenic effects of IL-17F than IL-17A on microbiota during colitis (**Figure 2**) (105, 106).

IL-17C

IL-17C is mainly known for its pro-inflammatory and antibacterial functions at epithelial sites in synergy with IL-17F (107). The *Il17c* gene is located in the long arm of human chromosome 16 (16q24.2). The IL-17C protein has a molecular mass of 21765 Da, and it is composed of 197 amino acids (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL17C). IL-17C share 23% amino acid homology with IL-17A (108), and while it binds a heterodimeric receptor formed by IL17RA and IL17RE, the IL-17RE subunit is the specific functional receptor for IL-17C (26). Most of the genetic variants of the *Il17c* gene are

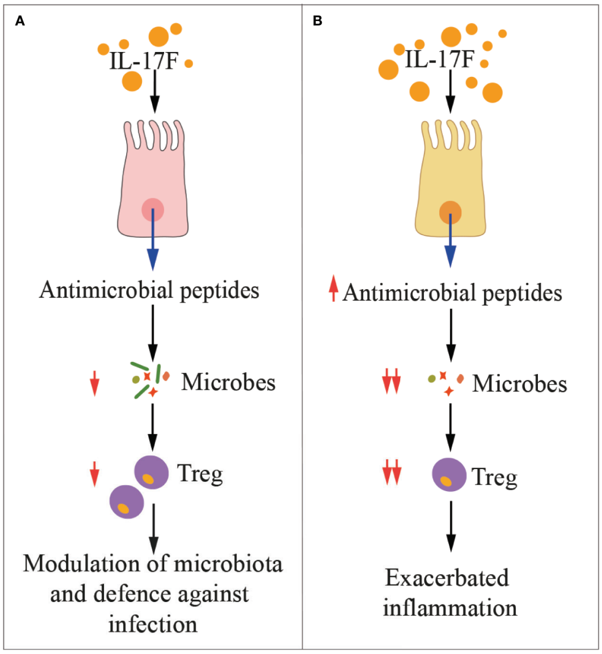


FIGURE 2 | IL-17F in health and disease. The cartoon summarizes the main functions of IL-17F in physiologic conditions **(A)** and during inflammation **(B)**. Treg, regulatory T cells.

missense mutations (https://gnomad.broadinstitute.org/gene/ENSG00000124391?dataset=gnomad_r2_1), and just few of them have clinical significance (https://www.ncbi.nlm.nih.gov/clinvar/?term=il17c%5Bgene%5D).

Both in humans and in mice, the IL-17C is produced by several cells, including intestinal, tracheal and lung epithelial cells and keratinocytes, which also express the IL-17RA-RE heterodimer (107). Thus, IL-17C acts locally in an autocrine manner to protect the mucosa or to induce epithelial inflammatory responses (**Figure 3**) similarly to IL-17A and IL-17F (107). For example, stimulation of mouse epithelial cells by *Escherichia coli* or pathogen-associated molecular patterns (PAMPs) activates a MyD88-dependent intracellular signaling, eventually inducing IL-17C production, which activates expression of chemokines, granulocyte-colony stimulating factor (GM-CSF), AMPs, and IL-1β in an autocrine fashion (78). Additional target genes of IL-17C in epithelial cells encode antimicrobial peptides like S100A7/8/9, β-defensin2, immune-activating molecules CXCL1/2/3 and CCL20, and proinflammatory cytokines as well as occludin, claudin-1, and claudin-4, which are involved in the formation of epithelial tight junctions (107, 109). Interestingly, Wolf et al. showed that in mice, *Pseudomonas aeruginosa*-induced IL-17C expression in lung epithelial cells by a IL-17A-dependent mechanism, thus demonstrating a network within the family of IL-17 cytokines that regulates each other expression (29).

IL-17C induces the expression of IL-1β and TNF-α in monocytes (67). IL-17RA-RE is also expressed on activated Th17 cells, and when triggered by IL-17C, it favors IL-17A, IL-17F, and IL-22 production by mouse Th17 cells, potentiating the adaptive immune response against pathogens and in autoimmunity (80, 110). Song et al. also identified the IL-17C/IL-17RE pathway as a pivotal regulator of innate immunity to intestinal bacterial pathogens in mice (26). Thus, IL-17C induces inflammation, but also promotes tissue healing.

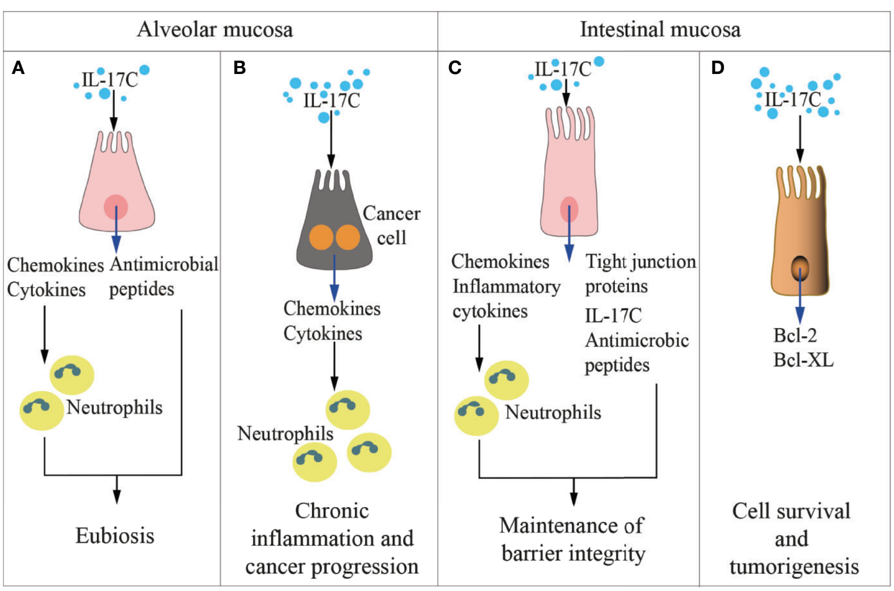


FIGURE 3 | IL-17C in health and disease. The cartoon summarizes the functions of IL-17 C in the alveolar (left panels) and the intestinal (right panels) mucosae in physiologic conditions **(A, C)** and during inflammation and cancer development **(B, D)**.

IL-17C is involved in several human diseases. IL-17C levels are elevated in psoriatic lesions, and it significantly affects the abundance of F4/80$^+$ macrophages within inflamed psoriatic plaques (107, 111, 112). Interestingly, IL-17C appears to also have a role in pathogenesis of atherosclerosis. Smith et al. reported that the mouse vasculature is an important source of IL-17C in atherosclerosis (113). Here, IL-17C exerts a pro-inflammatory role (114), by favoring the accumulation of pro-atherogenic Th17 cells within the aorta, which in turn affect the recruitment of monocytes and neutrophils to the plaque (115). Inflammatory glomerulonephritis also appears dependent on IL-17C, and Krohn et al. reported that patients affected by acute anti-neutrophil cytoplasmatic antibody-associated crescentic glomerulonephritis had significantly elevated serum levels of IL-17C (but not IL-17A, F, or E) (116). Additionally, they showed that glomerulonephritis ameliorated in mice lacking IL-17C and/or its receptor IL-17RE, and associated with a reduced Th7 response (116). We expect that in the next years IL-17C will be found involved in many more human diseases.

IL-17B

The human *Il17b* gene was cloned together with *Il17c* and is located on the long arm of human chromosome 5 (5q32). The translated protein has a molecular mass of 20437 Da and is composed of 180 amino acids (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL17B). At the N terminus, there is an 18–20-amino acid sequence containing a hydrophobic motif, which functions as secretory signal sequence (117). IL-17B is secreted as a noncovalent dimer (18). Among the IL-17 family members, IL-17B has 29% homology with IL-17A (108).

Also for *Il17b*, most of the genetic variants are missense mutations (https://gnomad.broadinstitute.org/gene/ENSG00000127743?dataset=gnomad_r2_1). Almost nothing is known about their consequences, except for three specific conditions: a neurodevelopmental disorder of clinical uncertain significance, an hereditary cancer-predisposing syndrome and keratoconus, an eye condition that affects the shape of the cornea (https://www.disgenet.org/browser/1/1/2/27190/), and it is due to C176Y, C124Y protein changes. (https://www.ncbi.nlm.nih.gov/clinvar/?term=IL17b%5Bgene%5D).

IL-17B was found to be originally expressed in adult pancreas, small intestine, and stomach, but not in T cells (118, 119). IL-17B is also highly expressed in chondrocytes and neurons, although low IL-17B mRNA has been detected in several organs (120). According to recent investigations, IL-17B is weakly expressed by the epithelium, whereas IL-17B is strongly expressed in a healthy colon by connective tissue cells (121). IL-17B expression, especially in the epithelial and stromal compartments, is increased in colorectal cancer (121).

IL-17B and IL-17E (also named IL-25) share the same heterodimeric receptor IL-17RA/RB, and may exert redundant or contraposed effects, depending on the tissue context, as detailed below. The signaling pathway downstream IL-17RA-RB receptor is poorly detailed, and mainly described upon IL-17E binding. Li et al. showed that *in vitro* IL-17B does not induce IL-6 expression, but induces monocytes to produce TNF-α and IL-1β (118), and in mice it favors neutrophils recruitment

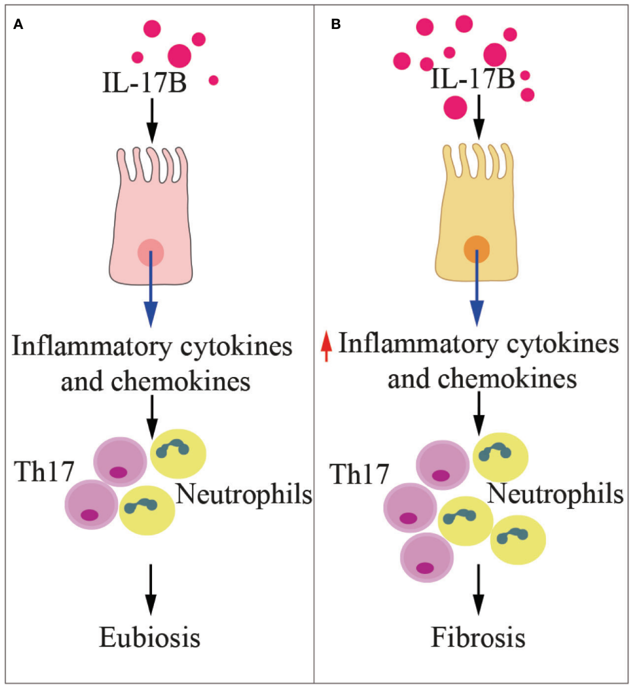


FIGURE 4 | IL-17B in health and disease. The cartoon summarizes the functions of IL-17B in physiologic conditions **(A)** and during inflammation **(B)**. Th17, T helper 17 cells.

(**Figure 4**) (119). *In vitro*, IL-17B promotes chemotaxis of IL-17RB-positive B cells by downregulating RGS16, the negative controller of CXCR4 and CXCR5 chemokine receptors (122). *In vivo*, IL-17B promotes embryonic development, tissue regeneration, and chemotaxis of B cells through IL-17RB in an autocrine fashion (93).

IL-17B has been investigated in several inflammatory diseases. While Ryan et al. (123) found genetic variants at the IL-17B locus in a 409 cases of coeliac disease and 355 controls, they did not find evidence that this locus was associated with the disease. Patients affected by systemic lupus erythematosus in the active phase showed higher levels of serum IL-17B than patients in the inactive phase (124). In community-acquired pneumonia, patients also showed higher serum levels of IL-17B when compared to healthy controls (125). IL-17B induced the expression of IL-8 in human bronchial epithelial cells through the activation of Akt, p38 mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK), and NF-κB signaling pathways. Finally, in mice affected by pneumonia, high IL-17B levels significantly correlated with IL-8 concentrations (125). IL-17B also is the predominant cytokine of the IL-17 family in the rheumatoid synovia, it is locally produced by neutrophils, and it contributes to tissue destruction by enhancing TNF-α -induced production of G-CSF and IL-6 in fibroblasts (126). Interestingly, treatment with IL-17B neutralizing antibodies ameliorated collagen-induced arthritis in mice (127). Altogether, these findings demonstrate that IL-17B is a proinflammatory cytokine involved in inflammation and autoimmunity.

High levels of IL-17B have also been associated with poor prognosis in patients with pancreatic, lung or breast cancer, suggesting that the same signaling is exploited by cancer cells for survival, proliferation, and migration (93). Moreover, a relationship between IL-17B and stemness has been found in gastric cancer (128). Conversely, high levels of IL-17B appear to exert antiangiogenic activities *in vitro* (129).

IL-17E

The *Il17e* gene is located on the long arm of human chromosome 14 (14q11.2). The molecular mass of the IL-17E protein, also named IL-25, is 20,330 Da, and it is composed of 177 amino acids. The IL-25 gene has two types of alternative spliced mRNA transcripts encoding two distinct subtypes (subtypes 1 and 2). Subtype 2 is different from subtype 1 for a shorter N end (130). To date, no studies have reported differences in the physiological role of the two subtypes. IL-17E shares only 17% homology with IL-17A, being the most distant among the cytokines of the IL-17 family (108). The human and mouse IL-17E genes share 80% homology (131). Genetic variants of *Il17e* gene are mostly missense mutations (https://gnomad.broadinstitute.org/gene/ENSG00000166090?dataset=gnomad_r2_1), and no specific clinical conditions have been associated to them (https://www.ncbi.nlm.nih.gov/clinvar/?term=IL25[gene]).

Intestinal tuft cells are the main producers of IL-25 (132). Additional sources of IL-17E exist, such as activated Th2 cells within the gastrointestinal tract and in other mucosal tissues (133), alveolar epithelial cells (134), alveolar macrophages (135), mesenchymal stem cells derived from the placenta and bone marrow (136), and mouse bone marrow-derived mast cells (137). IL-25 has been also found expressed in the murine central nervous system (138) as well as in the bronchial submucosa from asthmatic patients (139). In mice, IL-17E is also produced by brain capillary endothelial cells (140).

The receptor for IL-25 is composed of the IL-17RA and IL-17RB subunits (120). Thus IL-25 and IL-17B share the same receptor, and depending on tissue context, the two cytokines may exert redundant or contrasting effects. IL-17E stands among IL-17 family members for promoting the production of IL-4, IL-5 and IL-13 by innate type-2 immune cells (132, 141), nuocytes (142), T helper-2 cells, and NKT cells, thus contributing to the host defense against nematodes, but also to allergic reactions (133, 143). For example, after helminthic infection in mice, tuft cells-derived IL-17E induce ILC2 to produce IL-13, which activates epithelial cell progenitors resulting in the remodeling of the intestinal tissue and the induction of type-2 response (**Figure 5**) (132). Indeed, IL-17RA-RB triggering by IL-17E leads to TRAF6-mediated activation of NF-kB (144) and to the nuclear recruitment of the Th2 master regulator, GATA-3, in T cells (145). Additionally, IL-25 production is triggered in bronchial epithelial cells by rhinovirus infection, which causes local recruitment of eosinophils, neutrophils, basophils, and T and non-T type 2 cells, thus exacerbating asthma (146). IL-17E also amplifies a Th2 cell-dependent pathway in mice, thus promoting allergy (147).

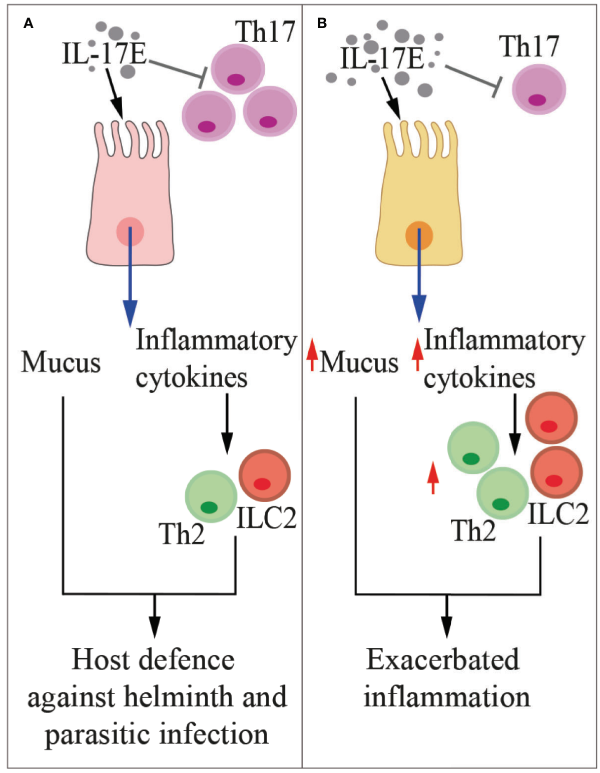


FIGURE 5 | IL-17E in health and disease. The cartoon summarizes the functions of IL-17E in physiologic conditions **(A)** and during inflammation **(B)**. Th2, type-2 T helper cells, ILC2, type-2 innate lymphoid cells.

While *in vitro* IL-17B elicits type 2 cytokine secretion (148), in several inflammatory conditions it antagonizes the pro-inflammatory activity of IL-17E by competing for the same receptor (27). IL-13 induced by IL-17E also inhibits IL-23, IL-1β, and IL-6 expression in activated DCs, thus blocking the induction of pathogenetic Th17 cells in autoimmune diseases (138). Interestingly, the IL-17E levels in the intestinal mucosa and serum of patients with active inflammatory bowel disease negatively correlated with endoscopic disease activity and C-reactive protein level (149), thus suggesting a protective role for IL-25 in this pathology. Indeed, IL-25 significantly inhibited the *in vitro* production of TNF-α, IFN-γ, and IL-17A by $CD4^+$ T cells, but it promoted IL-10 secretion (149).

IL-17E appears to exert a dual role also in cancer. In a variety of human tumor xenograft models, including melanoma, breast, lung, colon, and pancreatic cancers IL-17E has an antitumor effect (150). However, IL-17E, likely released by epithelial tuft cells in the presence of intestinal dysbiosis, can promote the progression of hepatocellular carcinoma by favoring alternative activation of macrophages and their CXCL10 secretion in the tumor microenvironment (33). The role of IL-17E in tumors needs to be further investigated.

IL-17D

IL-17D is the least investigated member of the IL-17 family (66). The *Il17d* gene is located on the long arm of the human chromosome 13 (13q12.11). The translated protein has a molecular mass of 21893 Da and is composed of 202 amino acids, making it the largest IL-17 (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IL17D&keywords=il17d). Like all IL-17 family members, IL-17D also has four cysteine residues that may allow homodimer formation through interchain disulfide linkages (96). Whether it forms heterodimer is not known. IL-17D, unlike other members of the IL-17 family, shows an extended C-terminal domain, which may mediate a unique receptor interaction. Most *Il17d* genetic variants are missense mutations, but little is known about their phenotypes (https://gnomad.broadinstitute.org/gene/ENSG00000172458?dataset=gnomad_r2_1).

IL-17D was originally found to be highly expressed in skeletal muscle, adipose tissue, brain, heart, lung and pancreas (96). Curiously, resting $CD4^+$ T cells and resting $CD19^+$ B cell, but not activated T cells, express low levels of the cytokine, which is orphan of its receptor (96), although hints are coming from the sea lamprey. Investigators have found that IL-17D, which is the most expressed IL-17 in this ancient fish, interacts with IL-17RA in B-like cells (151).

IL-17D does not stimulate the proliferation of immune cells of its own, but, in response to stress, it induces endothelial cells to produce IL-6, IL-8 and GM-CSF (96). Recent studies have shown that IL-17D expression is regulated by the transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2), sensor for oxidative and xenobiotic stress (152). The Nrf2-mediated expression of IL-17D in response to carcinogenic stimuli initiates antitumor immune responses in mice by activating natural killer (NK)-mediated immune surveillance (152). IL-17D is required for optimal antiviral immunity as well: also in this case, viral infection induces Nrf2 and IL-17D, causing local oxidative stress and antiviral responses (152). Thus, IL-17D should contribute to protecting us from viruses and cancer. Whether IL-17D participates in immunity against other pathogens, such as intracellular bacteria, remains to be defined (153).

As for the other members of the IL-17 family, also IL-17D is implicated in autoimmunity. IL-17D RNA has been detected in rheumatoid nodules, where IL-17A is absent, but not in peripheral blood mononuclear cells or in synovial fluid from patients with rheumatoid arthritis (154). Conversely, IL-17D lacks in psoriatic skin (155), thus suggesting that the pathogenic mechanisms downstream IL-17D are heterogenous.

All together, these findings demonstrate that cytokines of the IL17 family exert non-redundant, and even opposing functions spanning from elimination of intruders or neoplastic cells and tissue reconstitution with limited collateral damage at the inflammation site, to pro-inflammatory and pro-tumoral activities.

CYTOKINES OF THE IL-17 FAMILY OTHER THAN IL-17A AND THE MICROBIOTA

As for IL-17A, also other cytokines of the IL-17 family are involved in maintaining homeostasis at the interface between microbiota and barrier epithelia (**Table 1**). In addition, overproduction of some of these cytokines may lead to immune mediated inflammatory diseases, and even propel cancer.

IL-17F is one of the major regulators of commensal microbiota in the intestine (**Figure 2**), where it is constitutively expressed and induces the production of antimicrobial peptides (i.e., defensins) (25). Whereas IL-17F appears to have a marginal pathogenic role in immune mediated inflammatory diseases, it exerts a crucial function in host defense against infections, as for example, against *Citrobacter rodentium* (25), a Gram negative enteropathogenic bacterium, which is equivalent of *E. coli* in humans. Defensins also extensively modulate the gut microbiota, and Tang et al. clearly showed that IL-17F-induced production of defensins constrained growth of commensal bacteria directly involved in the expansion of Tregs (105). As consequence, chemically-induced colitis was milder in mice deficient of IL-17F than that of IL-17A-deficient or wild type mice (105). Interestingly, in this experimental setting, IL-17A and IL-17F exerted opposing roles. Indeed, IL-17A was protective against colitis mainly by ensuring integrity of the gut mucosa, while IL-17F was proinflammatory. These experimental evidences have been validated in humans by showing that IL-17F RNA was elevated in colon biopsies from patients affected by ulcerative colitis, and together with IL-6 and TNF-α, they support the generation of a local inflammatory environment (31). Additionally, blockade of the IL-17A pathway in patients with bowel syndromes worsened the pathology (156). Thus, IL-17F may protect against pathogens, but also limit the local immunosuppressive activity of Tregs, eventually unleashing undesired inflammation.

Also the IL-17C is involved in maintenance of epithelial barrier integrity (**Figure 3**), where it is selectively induced by inflammatory or bacterial *noxae* (91). While IL-17C and IL-17A appear to exert overlapping functions (107), IL-17C is mostly produced by epithelial cells at very early time points, and acts both in autocrine and exocrine fashions by inducing the expression of tight junction proteins (109), proinflammatory cytokines and antimicrobial peptides (26, 107). On the contrary, IL-17A is also produced by immune cells like Th17 cells, $\gamma\delta$ T cells, iNK T cells, macrophages, and ILCs. As an example, during infection with *C. rodentium*, IL-17C is upregulated in colon epithelial cells, and protects the mucosa in synergy with IL-22 (26), IL-17B (27), and IL-17F (25). IL-17C also attenuated inflammatory diseases like colitis, but it increased inflammation in psoriasis (107) and EAE (110) underlying the dual role exerted by this family of cytokines. At odds with IL-17A that controls fungal proliferation and infection, and whose blockage has been associated with fungal overgrowth and candidiasis (157), IL-17C is dispensable for immunity against candidiasis (158).

IL-17B is produced by epithelial cells in response to the abnormal expansion of pathobionts (i.e., commensals that in particular circumstances become pathogenic) within the microbiota (**Figure 3**). Its function is to protect the tissue and favor healing (93). Also in the course of allergic asthma, chemically-induced colitis or infection with *C. rodentium*, IL-17B exerts protective anti-inflammatory functions by interfering with IL-17E-induced IL-4 and IL-13 from type 2 Th cells and IL-6 from colon epithelial cells (27).

On the other hand, IL-17E, which also targets IL-17RA-RB, is essential for protecting the intestinal mucosa from parasitic infections (**Figure 4**) (159, 160). IL-17E production by tuft cells is constitutive and increases upon infection with natural mouse parasites like *Tritrichomonas* and *Heligmosomoid polygyrus*, resulting in stimulation of lamina propria ILC2 and mucosal tissue remodeling (**Table 1**). Schneider et al. (24) showed that *Tritrichomonas* favors fiber fermentation and intestinal accumulation of the short-chain fatty acid succinate, eventually inducing mouse intestinal tuft cells to release IL-17E, which in turn boosts type-2 immunity. Additionally, IL-17E produced by mouse intestinal epithelial cells upon microbiota stimulation limits the expansion of local Th17 cells (161) and IL-22 production by ILCs (162), thus identifying a delicate equilibrium among microbiota, adaptive immunity, and ILCs.

The expression of both IL-17E and IL-17B is upregulated during acute colonic inflammation (**Figures 4** and **5**), suggesting a dwelling activity between the two cytokines (27). Whereas IL-17B inhibits signaling of IL-17E but not of IL-17A or IL-17F, IL-17E does not interact with the IL-17RB homodimer, which remains available for IL-17B binding (27). Thus, the balance between IL-17B and IL-17E has to be fine-tuned to limit local inflammation and preserve mucosal integrity from the aggression of pathogens and pathobionts.

Also a dysregulated lung microbiota can drive IL-17B production, as it has been shown in a mouse model of bleomycin-induced lung fibrosis (30). More in details, the authors elegantly showed that depletion of the lung microbiota by antibiotics blocked bleomycin-induced lung fibrosis and death in mice. They also demonstrated that outer membrane vesicles locally released by *Bacteroides stercoris*, *B. ovatus*, and *Prevotella melaninogenica*, which were found enriched in the lung microbiota of mice treated with bleomycin, induced IL-17B production in the lung, thus favoring local immune cell infiltration and activation of profibrotic genes. These effects eventually increased bleomycin-induced mouse mortality. IL-17A and IL-17B have also been found in the bronchoalveolar lavage fluid of patients affected by lung fibrosis, but antibiotic treatments did not appear to be beneficial in limiting acute exacerbation in these patients (163, 164).

The role of IL-17D in the crosstalk between microbes and the immune system is less defined. Whereas IL-17D appears redundant in the context of inflammation induced by lipopolysaccharide, allergic agents or in EAE, it suppresses the function of DCs in inducing CD8$^+$ T cell responses, thus favoring infection by *Listeria monocytogenes* or influenza virus (28). However, IL-17D also activates NK-mediated immune surveillance (152), thus potentiating innate immunity. Further investigation is required to clarify how these IL-17D-mediated mechanisms impact microbiota-host interactions.

CYTOKINES OF THE IL-17 FAMILY OTHER THAN IL-17A, MICROBIOTA, AND CANCER

As for IL-17A, the role of IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F in cancer remain controversial. While IL-17D and IL-17E appear to exert preponderant tumor-protective activities, IL-17B, IL-17C, and IL-17F are tumor promoting, either through a direct effect on tumor cells, or by modulating the tumor microenvironment.

Anti-Tumor Activities of IL-17D and IL-17E

While IL-17D is released by several epithelial cells in response to pathogenic noxae, IL-17D expression in tumors does not appear compatible with their growth. In an elegant study, O'Sullivan et al. (165) showed that IL-17D derived from non-immunoedited cancer cells induces endothelial cells to produce monocyte chemoattractant protein 1 (MCP1), which is responsible for NK cell recruitment, eventually leading to tumor rejection. The same group also showed that mice deficient for IL-17D are more susceptible to viral infections and tumors (152). Nrf2 was shown to be responsible for IL-17D dependent recruitment of NK cells, and induction of Nrf2 by agonists led to regression of already established tumors *in vivo* (152). Thus, IL-17D might be an essential mechanism of immunoediting, and loss of IL-17D production might select for more aggressive tumors. Taking into account the propensity of IL-17A to propel tumor growth either in an autocrine (61, 166) or paracrine fashion (37), findings on IL-17D suggest that this cytokine counterbalances the pro-tumor activity of Th17 cells and IL-17A, and strategies to increase IL-17D might find clinical application. Gene expression analyses on human samples will define potential correlates between tumor immune infiltrate and expression of both IL-17A and IL-17D.

The potent pro-inflammatory activity of IL-17E also appears to be exploited against cancer. Purified IL-17E has been shown to delay growth of a variety of tumor xenografts when given alone or in combination with several drugs (150). The authors also documented accrual of eosinophils and activation of B cells in IL-17E-treated mice (150), but these mechanisms require further investigation.

IL-17E, which is also produced by mammary epithelial cells, has been shown to engage the IL-17RB on human mammary cancer cells, and to induce their caspase-dependent apoptosis. Interestingly this effect was restricted to neoplastic cells, because they express much more IL-17RB than normal mammary cells, and IL-17RB *in vivo* is expressed in high amounts in tumors from patients with poor prognosis (167). The authors also showed that purified IL-17E inhibited the growth of human mammary cancer cells xenografted in the mammary fat pad of mice (167). Additionally, administration of a synthetic compound able to induce IL-25 production by tumor associated fibroblasts suppressed growth of mammary tumor metastases in mice (168).

The effects of IL-17E might be context-dependent. It has been reported that the addition of cisplatin to cervical cancer cell cultures induced IL-17E and IL-17RB down-regulation, eventually inhibiting *in vitro* growth, migration, and invasion (169), Thus, IL-17E might exert a tumor-promoting activity, unless the latter depends on IL-17B, which also interacts with IL-17RB. *In vivo* data in genetically modified mice will clarify the effect of the two cytokines in cervical cancer.

Pro-Tumor Activities of IL-17B, IL-17C, and IL-17F

IL-17B acts as tumor promoter in several solid and hematopoietic malignancies (**Figure 4**). Furuta et al. showed that the IL-17B/IL-17RB signaling is critical for breast tumorigenesis, and that IL-17RB expression correlates with poor prognosis in breast cancer patients (167). Engagement of IL-17B with its receptor induces Nf-kB-mediated upregulation of Bcl-2 expression, and resistance of mammary cancer cells to etoposide (170). Because IL-17B and IL-17E share the same receptor heterodimer, and IL-17E induces apoptosis in mammary cancer cells (167), an opposing role for IL-17B and IL-17E can be hypothesized in breast cancer. It will be necessary to understand how two similar cytokines engaging the same receptor deliver anti- or pro-apoptotic signals.

Up-regulation of IL-17RB expression was also found in pancreatic cancer, where expression of IL-17RB associated with metastasis incidence and reduced progression free survival (171). IL-17RB triggering induced CCL20/CXCL1/IL-8/TFF1 chemokine expressions *via* the ERK1/2 pathway, thus promoting macrophage and endothelial cell recruitment at primary sites, cancer cell invasion and survival at distant sites. *In vivo*, anti-IL-17RB monoclonal antibodies inhibited tumor metastasis and prolonged survival in a mouse xenograft model (171). Others confirmed a direct tumor-promoting activity of IL-17B in gastric cancer (172), thyroid cancer (173), and in acute myeloid leukemia (174).

A direct connection between local microbiota, cytokine production and tumorigenesis has been reported for IL-17C (**Figure 3**). Song et al. found that IL-17C is upregulated in human colorectal cancers (34), and alterations in the microbiota (**Table 1**) drove IL-17C upregulation specifically in murine intestinal epithelial cells, eventually supporting their survival and neoplastic transformation (34). In line with these findings, it has been reported that both intra- and peri-tumoral expression of IL-17RE predict early and late recurrence in hepatocellular carcinoma (175).

IL-17C, which promotes neutrophilic inflammation (**Figure 3**), was also found abundant in human lung cancer samples, and IL-17C is a negative prognostic factor in patients with lymph node metastasis (35). Patients with chronic obstructive pulmonary disease are highly susceptible to non-small cell lung cancer, and often harbor IL-17C-inducing nontypeable *Haemophilus influenza* in their lungs (**Table 1**). In IL-17C-deficient mice, nontypeable *Haemophilus influenza* induced less neutrophil lung infiltrates and promoted less tumorigenesis (35), thus linking IL-17C to bacteria and lung cancer.

IL-17A and IL-17F share the same heterodimeric receptor (IL-17RA-RC). Tang et al. showed that mice deficient for IL-17F, and not mice deficient for IL-17A, resist chemically induced colitis, and this correlates with a different gut microbiota (105). *Fusobacterium nucleatum*, which has been linked to chronic inflammation and cancer (176), aggravates intestinal inflammation in mice by targeting caspase activation and recruitment domain 3 through NOD2, eventually activating the IL-17F/NF-κB pathway (31). Because colitis often anticipates colon cancer, a microbiota-modulated, tumor promoting role for IL-17F can be hypothesized, and it needs to be proven in *in vivo* experimental settings.

Strong correlations have been found between IL-17RA, microbiota, and cancer, and most of them have been attributed to IL-17A. As IL-17B, IL-17C, IL-17E, and IL-17F also exploit the subunit IL-17RA to deliver their intracellular signals, mice selectively deficient for either these cytokines or the IL-RC, IL-RB, and IL-RE will help in better understating the role of the different cytokines of the IL-17 family in the microbiota-immunity-cancer axis.

STRATEGIES TO TARGET CYTOKINES OF THE IL-17 FAMILY

Several strategies are being adopted in the clinic to impact the microbiota-IL-17 axis (**Table 2**). They include diets, prebiotics, probiotics or even fecal microbiota transplantation in effort to transiently or permanently modify the microbiota and eventually the immune response. Additionally, monoclonal antibodies directed against IL-17A or other cytokines and receptors of the

TABLE 2 | Therapeutic strategies under investigation to target cytokines of the IL-17 family.

Therapeutic Agent	Target Molecule	Impact on disease	Clinical Trial Number/Ref.
Brodalumab	IL-17RA	Reduced symptoms in rheumatoid arthritis and psoriatic arthritis patients	NCT00771030 NCT01059448 NCT00950989 NCT02024646 NCT02029495 NCT04183881 NCT01516957
Bimekizumab	IL-17A-IL-17F	Reduced symptoms in psoriatic arthritis patients Reduced chemical-induced colitis in mice	NCT02969525 (105)
Anti-IL-17RB	IL-17RB	Delayed pancreatic tumor growth and metastasis formation in mice	(171)
MOR106	IL-17C	Reduced atopic dermatitis in mice Ineffective against human atopic dermatitis	(177) NCT03864627 NCT03568071 NCT03689829 NCT02739009
Antibiotics	↓ IL-17B ↓ IL-17C ↓ IL-17F	Reduced bleomycin-induced lung fibrosis in mice Reduced colon cancer formation in mice Reduced chemical-induced colitis in mice	(30, 34, 105)
Q2-3	↑ IL-17E	Reduced breast cancer metastasis in mice	(168)
tBHQ	↑ IL-17D	Delayed growth of B16 melanoma, Burkitt's lymphoma and MCA-induced sarcoma in mice	(152)

Brodalumab, fully human IgG2 monoclonal antibody against IL-17RA; Bimekizumab, humanized IgG1 monoclonal antibody against both IL-17A and IL-17; MOR106, fully human IgG1 monoclonal antibody against IL-17C; Q2–3, synthetic dihydrobenzofuran lignan; tBHQ, Tert-butylhydroquinone; MCA, methylcholanthrene.

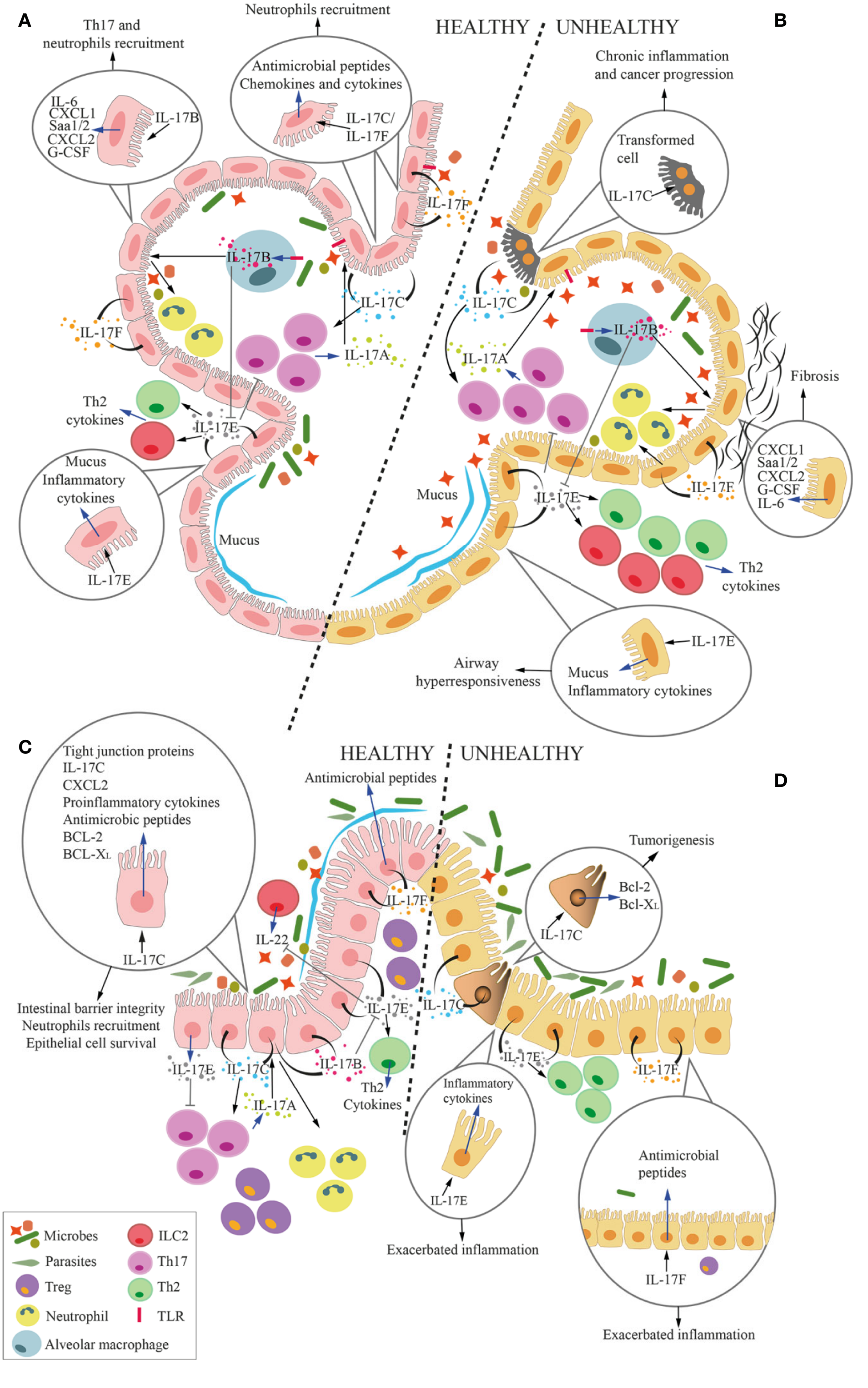


FIGURE 6 | Continued

FIGURE 6 | Overall function of IL-17 cytokines at the microbiota-host interface in the lung and the gut. Cartoon summarizing the overall role of IL-17 cytokines at the interface between microbiota and alveolar **(A, B)** and intestinal mucosa **(C, D)** in health **(A, C)** and disease **(B, D)**. Circles within the panels enlarge and focus on several effects of IL-17 cytokines on epithelial cells. Blue arrows represent the secretion of cytokines or the expression of genes by cells, whereas black arrows represent the stimulation of the cell by the cytokines. **(A, C)** In physiologic conditions, IL-17A, which is mostly released by Th17 cells, keep growth of commensal microbes residing in the lumen of the respiratory tract or the intestine under control. Mucosal epithelial cells secrete IL-17B, IL-17C, IL-17E and IL-17F in response to stimuli coming from the local microbiota. More in details, IL-17B, produced by alveolar macrophages (MØs) under TLR4-mediated stimulation, act on epithelial cells inducing release of several factors, among which IL-6, serum amyloid A1 and A2 (Saa1/2), CXCL1, CXCL2, and G-CSF. Some of these factors favor local recruitment of Th17 cells and neutrophils, which also contribute to maintain an adequate balance in the microbiota composition. Additionally, IL-17B induces monocytes to release TNF-α and IL-1β, which also favor neutrophil recruitment (not shown). IL-17C and IL-17F are released by epithelial cells, and act in autocrine and paracrine fashion inducing the production of antimicrobial peptides, but also chemokines and cytokines that favor neutrophil recruitment. IL-17C also promotes Th17 cell responses, and it supports barrier integrity through tight junction formation in epithelial cells. Also IL-17C induces expression of TNF-α and IL-1β in monocytes (not shown). IL-17E favors the induction of type 2 responses by Th2 cells and ILC2, whereas IL-17B blocks this action, thus avoiding excessive type 2 immune responses. While IL-17D activates NK-mediated immune surveillance (not shown), its relationship with lung and gut microbiota remains unknown. Therefore, IL-17D is not depicted in the figure. IL17E inhibits IL-23, IL-1β and IL-6 expression in activated dendritic cells (not shown), thus blocking the induction of pathogenic Th17 cells. Healthy alveolar epithelial cells also secrete mucus in response to IL-17E to protect the epithelium from bacterial adhesion. **(B, D)** In pathologic conditions, excessive IL-17A causes local inflammation. In response to the expansion of pathobionts, MØs release more IL-17B, which acts on epithelial cells to induce pro-inflammatory signals (IL-6, Saa1/2, CXCL1, CXCL2, and G-CSF), which may induce lung fibrosis. Stimuli from pathogenic bacteria unleash IL-17C hyperproduction, leading to chronic inflammation and tumorigenesis, also through the upregulation of Bcl-2 and Bcl-XL. Excessive IL-17E signaling is associated with stronger Type 2 immune reaction (Th2 and ILC2) that exacerbate airways hyperresponsiveness and gut inflammation. Unbalanced IL-17F in the gut induces the release of excessive antimicrobial peptides, which constrains Treg-inducing bacteria, therefore promoting gut inflammation.

IL-17 family are used or are under investigation. These strategies are tested both in inflammatory diseases (21, 178–182) and cancer (183–185).

In the field of cancer, almost 200 clinical trials are ongoing that aim either at identifying the microbiota accompanying malignancies, or at testing microbiota-modulating strategies. The composition of the gut microbiome is being analyzed in breast cancer (NCT03885648), colorectal cancer (NCT03385213), lung cancer (NCT04333004), thyroid cancer (NCT03543891), hepatocellular carcinoma (NCT02599909), and glioblastoma (NCT03631823) among others. There is also interest for the microbiota of the lung in lung cancer (NCT03068663), of the oronasal cavities in hematopoietic malignancies (NCT02949427), or even the intratumor microbiota as for breast cancer (NCT03586297) and prostate cancer (NCT03947515). Several clinical trials are designed to modify the microbiota and increase susceptibility to chemotherapy (NCT04138979), radiotherapy (NCT02559349), or immunotherapy (NCT04116775). A more extensive list of clinical trials on this issue is out of the scope of this review, and can be found at (www.clinicaltrials.gov). Many of them interfere with the interplay between microbiota and the immune system, thus impacting all the cytokines of the IL-17 family.

Monoclonal antibodies against IL-17A or IL-17RA are already available to patients affected by psoriasis and arthritis (45), and might even find application in malignancies (10, 47). Results from one clinical trial with anti-IL17A monoclonal antibodies in MM patients are longed for (NCT03111992). Brodalumab, a monoclonal antibody against IL-17RA is also under investigation in patients affected by rheumatoid and psoriatic arthritis (**Table 2**), and it might also find application in cancer patients.

Few approaches have instead been proposed to target cytokines of the IL-17 family other than IL-17A. Because IL-17F can be tumor promoting (31), it will be interesting to investigate the anti-tumor activity of Bimekizumab especially in colorectal cancer (105) (**Table 2**). Bimekizumab is a monoclonal antibody against both IL17A and IL-17F, which is currently investigated in psoriatic arthritis patients (186). Anti-IL17RB monoclonal antibodies might impact metastatic pancreatic cancer, has it has been shown in a mouse model (171). IL-17C is an interesting target in colorectal cancer because IL-17C has been found upregulated in these tumors, and in mice IL-17C was modulated by the gut microbiota (34). MOR106 is a humanized monoclonal IgG1 antibody against IL-17C, which has been developed to treat atopic dermatitis (177). Unfortunately, the clinical development program of MOR106 in atopic dermatitis was ended because of disappointing results. Because blocking IL-17C signaling significantly reduces the number and extension of colonic tumors in mice, MOR106 might be investigated in human colorectal and lung cancer (35). MOR106 would be of advantage in respect to anti-IL17A because IL-17C/IL-17RE signaling is dispensable for immunity to systemic, oral, and cutaneous candidiasis (158). Thus, either blocking the IL-17C/IL-17RE axis or acting on the gut microbiome might be beneficial to cancer patients (**Table 2**).

Q2-3, a synthetic dihydrobenzofuran lignan that stimulates production of IL-25, which competes with IL-17B for the IL-17RB receptor, reduces myeloid derived suppressor cell infiltration and metastasis appearance in a mouse model of breast cancer (168), suggesting its potential application to prevent breast cancer metastasis in humans (**Table 2**). Nonetheless, targeting the IL-17RB in cancer should be carefully investigated in breast cancer, because it could interfere with the anti-tumor activity of IL-17E (167).

Finally, Nrf2, a cellular checkpoint of xenobiotic and oxidative stress (187) is an interesting molecule, as it delays tumor growth by stimulating IL-17D production in tumor cells, which recruits NK cells within the tumor (**Table 2**) (152). An advantage of such compound is that it activates the tumor autocrine Nrf2/IL-17D signaling, by inducing cellular stress without producing reactive oxygen species. As an example, Tert-butylhydroquinone (tBHQ) has been tested in preclinical models of B16 melanoma, human Burkitt's lymphoma, and in the MCA-induced sarcoma, where activated Nrf2 and IL-17D production, resulting in delayed tumor progression (152). Nrf2 agonist are currently in clinical trials (e.g., NCT03182959, and NCT03934905).

CONCLUSIONS AND PERSPECTIVES

While characterized by a common genetic origin, cytokines from the IL-17 family demonstrate a wide heterogeneity in functions as well as in cellular source, and kinetic of production and secretion (**Figure 6**).

An intriguing evidence is that even if some of these cytokines share the same receptor, they may exert opposite downstream activities. For instance, blocking IL-17A is detrimental rather than curative in the murine model of chemically-induced colitis, and blockage of IL-17F either alone or with IL-17A resulted in disease amelioration (105). Thus, blocking IL-17RA impacts all IL-17 cytokines but IL-17D, and might exert unpredictable/undesired effects.

The same unpredictable/undesired effects might occur when attempting to modulate the microbiome. Examples are available of unexpected side effects of patients treated with probiotics (181).

All together these findings suggest that even if very fascinating and promising, the actual knowledge on the role of IL-17 cytokines in cancer is preliminary. A plethora of information about these cytokines in health and disease is waiting to be unveiled in next years.

AUTHOR CONTRIBUTIONS

AB and MB developed the concept of the review. All authors participated to retrieve the relevant literature, wrote and prepared the manuscript. DM and LC prepared the figures. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC; grant #IG21808 to MB). AB was supported by a fellowship from the Fondazione Italiana per la Ricerca sul Cancro/AIRC (grant #22316).

ACKNOWLEDGMENTS

This work has been conceived and generated during the lockdown due to the COVID-19 pandemic, and it has involved the Cellular Immunology Unit as a whole. We thank Nadia Messina for her ideas and support.

REFERENCES

1. Lynch SV, Pedersen O. The Human Intestinal Microbiome in Health and Disease. *N Engl J Med* (2016) 375:2369–79. doi: 10.1056/NEJMra1600266
2. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell* (2016) 164(3):337–40. doi: 10.1016/j.cell.2016.01.013
3. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* (2006) 124(4):837–48. doi: 10.1016/j.cell.2006.02.017
4. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. *Nat Med* (2018) 24 (4):392–400. doi: 10.1038/nm.4517
5. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* (2010) 107(26):11971–5. doi: 10.1073/pnas.1002601107
6. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. *Nature* (2012) 486(7402):222–7. doi: 10.1038/nature11053
7. Kundu P, Blacher E, Elinav E, Pettersson S. Our Gut Microbiome: The Evolving Inner Self. *Cell* (2017) 171(7):1481–93. doi: 10.1016/j.cell.2017.11.024
8. Bellone M. *Autoimmune Disease: Pathogenesis*. Chichester, United Kingdom: eLS, John Wiley & Sons, Ltd (2015). doi: 10.1002/9780470015902.a0001276.pub4
9. Lambrecht BN, Hammad H. The immunology of the allergy epidemic and the hygiene hypothesis. *Nat Immunol* (2017) 18(10):1076–83. doi: 10.1038/ni.3829
10. Bellone M, Brevi A, Huber S. Microbiota-Propelled T Helper 17 Cells in Inflammatory Diseases and Cancer. *Microbiol Mol Biol Rev* (2020) 84(2). doi: 10.1128/MMBR.00064-19
11. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. *Nat Rev Immunol* (2016) 16(5):295–309. doi: 10.1038/nri.2016.36
12. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. *Cell Host Microbe* (2018) 23(6):716–24. doi: 10.1016/j.chom.2018.05.003
13. Durack J, Lynch SV. The gut microbiome: Relationships with disease and opportunities for therapy. *J Exp Med* (2019) 216(1):20–40. doi: 10.1084/jem.20180448
14. Honda K, Littman DR. The microbiome in infectious disease and inflammation. *Annu Rev Immunol* (2012) 30:759–95. doi: 10.1146/annurev-immunol-020711-074937
15. Ivanov II, Honda K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe* (2012) 12(4):496–508. doi: 10.1016/j.chom.2012.09.009
16. Wolk K, Sabat R. Interleukin-22: a novel T- and NK-cell derived cytokine that regulates the biology of tissue cells. *Cytokine Growth Factor Rev* (2006) 17(5):367–80. doi: 10.1016/j.cytogfr.2006.09.001
17. Hirota K, Turner J, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of TH17 cells in Peyer's patches is responsible for the induction of T cell–dependent IgA responses. *Nat Immunol* (2013) 14:372–9. doi: 10.1038/ni.2552
18. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity* (2011) 34(2):149–62. doi: 10.1016/j.immuni.2011.02.012
19. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol* (2014) 14(9):585–600. doi: 10.1038/nri3707
20. Veldhoen M. Interleukin 17 is a chief orchestrator of immunity. *Nat Immunol* (2017) 18(6):612–21. doi: 10.1038/ni.3742
21. Tilg H, Zmora N, Adolph TE, Elinav E. The intestinal microbiota fuelling metabolic inflammation. *Nat Rev Immunol* (2020) 20(1):40–54. doi: 10.1038/s41577-019-0198-4
22. Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* (2006) 203(12):2673–82. doi: 10.1084/jem.20061775
23. de Aquino SG, Abdollahi-Roodsaz S, Koenders MI, van de Loo FA, Pruijn GJ, Marijnissen RJ, et al. Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response. *J Immunol* (2014) 192(9):4103–11. doi: 10.4049/jimmunol.1301970
24. Schneider C, O'Leary CE, von Moltke J, Liang HE, Ang QY, Turnbaugh PJ, et al. A Metabolite-Triggered Tuft Cell-ILC2 Circuit Drives Small Intestinal Remodeling. *Cell* (2018) 174(2):271–84 e14. doi: 10.1016/j.cell.2018.05.014
25. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* (2009) 30(1):108–19. doi: 10.1016/j.immuni.2008.11.009

26. Song X, Zhu S, Shi P, Liu Y, Shi Y, Levin SD, et al. IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. *Nat Immunol* (2011) 12(12):1151–8. doi: 10.1038/ni.2155
27. Reynolds JM, Lee YH, Shi Y, Wang X, Angkasekwinai P, Nallaparaju KC, et al. Interleukin-17B Antagonizes Interleukin-25-Mediated Mucosal Inflammation. *Immunity* (2015) 42(4):692–703. doi: 10.4049/jimmunol.1103014
28. Lee Y, Clinton J, Yao C, Chang SH. Interleukin-17D Promotes Pathogenicity During Infection by Suppressing CD8 T Cell Activity. *Front Immunol* (2019) 10:1172. doi: 10.3389/fimmu.2019.01172
29. Wolf L, Sapich S, Honecker A, Jungnickel C, Seiler F, Bischoff M, et al. IL-17A-mediated expression of epithelial IL-17C promotes inflammation during acute Pseudomonas aeruginosa pneumonia. *Am J Physiol Lung Cell Mol Physiol* (2016) 311(5):L1015–L22. doi: 10.1152/ajplung.00158.2016
30. Yang D, Chen X, Wang J, Lou Q, Lou Y, Li L, et al. Dysregulated Lung Commensal Bacteria Drive Interleukin-17B Production to Promote Pulmonary Fibrosis through Their Outer Membrane Vesicles. *Immunity* (2019) 50(3):692–706 e7. doi: 10.1016/j.immuni.2019.02.001
31. Chen Y, Chen Y, Cao P, Su W, Zhan N, Dong W. Fusobacterium nucleatum facilitates ulcerative colitis through activating IL-17F signaling to NF-kappaB via the upregulation of CARD3 expression. *J Pathol* (2020) 250 (2):170–82. doi: 10.1002/path.5358
32. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *eLife* (2013) 2:e01202. doi: 10.7554/eLife.01202
33. Li Q, Ma L, Shen S, Guo Y, Cao Q, Cai X, et al. Intestinal dysbacteriosis-induced IL-25 promotes development of HCC via alternative activation of macrophages in tumor microenvironment. *J Exp Clin Cancer Res* (2019) 38 (1):303. doi: 10.1186/s13046-019-1456-9
34. Song X, Gao H, Lin Y, Yao Y, Zhu S, Wang J, et al. Alterations in the microbiota drive interleukin-17C production from intestinal epithelial cells to promote tumorigenesis. *Immunity* (2014) 40(1):140–52. doi: 10.1016/j.immuni.2013.11.018
35. Jungnickel C, Schmidt LH, Bittigkoffer L, Wolf L, Wolf A, Ritzmann F, et al. IL-17C mediates the recruitment of tumor-associated neutrophils and lung tumor growth. *Oncogene* (2017) 36(29):4182–90. doi: 10.1038/onc.2017.28
36. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* (2009) 15(9):1016–22. doi: 10.1038/nm.2015
37. Calcinotto A, Brevi A, Chesi M, Ferrarese R, Garcia Perez L, Grioni M, et al. Microbiota-driven interleukin-17-producing cells and eosinophils synergize to accelerate multiple myeloma progression. *Nat Commun* (2018) 9(1):4832. doi: 10.1038/s41467-018-07305-8
38. Maeda Y, Kurakawa T, Umemoto E, Motooka D, Ito Y, Gotoh K, et al. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. *Arthritis Rheumatol* (2016) 68 (11):2646–61. doi: 10.1002/art.39783
39. Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* (2005) 23:683–747. doi: 10.1146/annurev.immunol.23.021704.115707
40. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* (2011) 479(7374):538–41. doi: 10.1038/nature10554
41. Esplugues E, Huber S, Gagliani N, Hauser AE, Town T, Wan YY, et al. Control of TH17 cells occurs in the small intestine. *Nature* (2011) 475 (7357):514–8. doi: 10.1038/nature10228
42. Duscha A, Gisevius B, Hirschberg S, Yissachar N, Stangl GI, Eilers E, et al. Propionic Acid Shapes the Multiple Sclerosis Disease Course by an Immunomodulatory Mechanism. *Cell* (2020) 180(6):1067–80. doi: 10.1016/j.cell.2020.02.035
43. Zwicky P, Unger S, Becher B. Targeting interleukin-17 in chronic inflammatory disease: A clinical perspective. *J Exp Med* (2020) 217(1). doi: 10.1084/jem.20191123
44. Amatya N, Garg AV, Gaffen SL. IL-17 Signaling: The Yin and the Yang. *Trends Immunol* (2017) 38(5):310–22. doi: 10.1016/j.it.2017.01.006
45. Beringer A, Miossec P. Systemic effects of IL-17 in inflammatory arthritis. *Nat Rev Rheumatol* (2019) 15(8):491–501. doi: 10.1038/s41584-019-0243-5
46. Poore GD, Kopylova E, Zhu Q, Carpenter C, Fraraccio S, Wandro S, et al. Microbiome analyses of blood and tissues suggest cancer diagnostic approach. *Nature* (2020) 579(7800):567–74. doi: 10.1038/s41586-020-2095-1
47. Vitiello GA, Miller G. Targeting the interleukin-17 immune axis for cancer immunotherapy. *J Exp Med* (2020) 217(1). doi: 10.1084/jem.20190456
48. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* (2009) 31(5):787–98. doi: 10.1016/j.immuni.2009.09.014
49. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, et al. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* (2009) 114(6):1141–9. doi: 10.1182/blood-2009-03-208249
50. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: immune correlates associated with clinical benefit in patients with resected high-risk stage IIIc/IV melanoma. *Clin Cancer Res* (2011) 17(4):896–906. doi: 10.1158/1078-0432.CCR-10-2463
51. Xu M, Pokrovskii M, Ding Y, Yi R, Au C, Harrison OJ, et al. c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* (2018) 554(7692):373–7. doi: 10.1038/nature25500
52. Gomes AL, Teijeiro A, Buren S, Tummala KS, Yilmaz M, Waisman A, et al. Metabolic Inflammation-Associated IL-17A Causes Non-alcoholic Steatohepatitis and Hepatocellular Carcinoma. *Cancer Cell* (2016) 30 (1):161–75. doi: 10.1016/j.ccell.2016.05.020
53. McAllister F, Bailey JM, Alsina J, Nirschl CJ, Sharma R, Fan H, et al. Oncogenic Kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia. *Cancer Cell* (2014) 25(5):621–37. doi: 10.1016/j.ccr.2014.03.014
54. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J Exp Med* (2009) 206(7):1457–64. doi: 10.1084/jem.20090207
55. Benevides L, da Fonseca DM, Donate PB, Tiezzi DG, De Carvalho DD, de Andrade JM, et al. IL17 Promotes Mammary Tumor Progression by Changing the Behavior of Tumor Cells and Eliciting Tumorigenic Neutrophils Recruitment. *Cancer Res* (2015) 75(18):3788–99. doi: 10.1158/0008-5472.CAN-15-0054
56. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau CS, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* (2015) 522(7556):345–8. doi: 10.1038/nature14282
57. Chung AS, Wu X, Zhuang G, Ngu H, Kasman I, Zhang J, et al. An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nat Med* (2013) 19(9):1114–23. doi: 10.1038/nm.3291
58. Li J, Sung CY, Lee N, Ni Y, Pihlajamaki J, Panagiotou G, et al. Probiotics modulated gut microbiota suppresses hepatocellular carcinoma growth in mice. *Proc Natl Acad Sci U S A* (2016) 113(9):E1306–15. doi: 10.1073/pnas.1518189113
59. Chesi M, Robbiani DF, Sebag M, Chng WJ, Affer M, Tiedemann R, et al. AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer Cell* (2008) 13(2):167–80. doi: 10.1016/j.ccr.2008.01.007
60. Calcinotto A, Grioni M, Jachetti E, Curnis F, Mondino A, Parmiani G, et al. Targeting TNF-alpha to neoangiogenic vessels enhances lymphocyte infiltration in tumors and increases the therapeutic potential of immunotherapy. *J Immunol* (2012) 188(6):2687–94. doi: 10.4049/jimmunol.1101877
61. Prabhala RH, Pelluru D, Fulciniti M, Prabhala HK, Nanjappa P, Song W, et al. Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. *Blood* (2010) 115 (26):5385–92. doi: 10.1182/blood-2009-10-246660
62. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* (2018) 359(6371):104–8. doi: 10.1126/science.aao3290

63. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359(6371):91–7. doi: 10.1126/science.aan3706
64. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* (2018) 359(6371):97–103. doi: 10.1126/science.aan4236
65. Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol* (1993) 150(12):5445–56.
66. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 Family of Cytokines in Health and Disease. *Immunity* (2019) 50(4):892–906. doi: 10.1016/j.immuni.2019.03.021
67. Brembilla NC, Senra L, Boehncke WH. The IL-17 Family of Cytokines in Psoriasis: IL-17A and Beyond. *Front Immunol* (2018) 9:1682. doi: 10.3389/fimmu.2018.01682
68. Li X, Bechara R, Zhao J, McGeachy MJ, Gaffen SL. IL-17 receptor-based signaling and implications for disease. *Nat Immunol* (2019) 20(12):1594–602. doi: 10.1038/s41590-019-0514-y
69. Qian Y, Liu C, Hartupee J, Altuntas CZ, Gulen MF, Jane-Wit D, et al. The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat Immunol* (2007) 8(3):247–56. doi: 10.1038/ni1439
70. Ogura H, Murakami M, Okuyama Y, Tsuruoka M, Kitabayashi C, Kanamoto M, et al. Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity* (2008) 29(4):628–36. doi: 10.1016/j.immuni.2008.07.018
71. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol* (2002) 71(1):1–8. doi: 10.1189/jlb.71.1.1
72. Novatchkova M, Leibbrandt A, Werzowa J, Neubuser A, Eisenhaber F. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem Sci* (2003) 28(5):226–9. doi: 10.1016/S0968-0004(03)00067-7
73. Wright JF, Bennett F, Li B, Brooks J, Luxenberg DP, Whitters MJ, et al. The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol* (2008) 181(4):2799–805. doi: 10.4049/jimmunol.181.4.2799
74. Wright JF, Guo Y, Quazi A, Luxenberg DP, Bennett F, Ross JF, et al. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J Biol Chem* (2007) 282(18):13447–55. doi: 10.1074/jbc.M700499200
75. Mellett M, Atzei P, Horgan A, Hams E, Floss T, Wurst W, et al. Orphan receptor IL-17RD tunes IL-17A signalling and is required for neutrophilia. *Nat Commun* (2012) 3:1119. doi: 10.1038/ncomms2127
76. Chang SH, Park H, Dong C. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J Biol Chem* (2006) 281(47):35603–7. doi: 10.1074/jbc.C600256200
77. Bulek K, Liu C, Swaidani S, Wang L, Page RC, Gulen MF, et al. The inducible kinase IKKi is required for IL-17-dependent signaling associated with neutrophilia and pulmonary inflammation. *Nat Immunol* (2011) 12 (9):844–52. doi: 10.1038/ni.2080
78. Garg AV, Ahmed M, Vallejo AN, Ma A, Gaffen SL. The deubiquitinase A20 mediates feedback inhibition of interleukin-17 receptor signaling. *Sci Signal* (2013) 6(278):ra44. doi: 10.1126/scisignal.2003699
79. Zhong B, Liu X, Wang X, Chang SH, Liu X, Wang A, et al. Negative regulation of IL-17-mediated signaling and inflammation by the ubiquitin-specific protease USP25. *Nat Immunol* (2012) 13(11):1110–7. doi: 10.1038/ni.2427
80. Shi P, Zhu S, Lin Y, Liu Y, Liu Y, Chen Z, et al. Persistent stimulation with interleukin-17 desensitizes cells through SCFbeta-TrCP-mediated degradation of Act1. *Sci Signal* (2011) 4(197):ra73. doi: 10.1126/scisignal.2001653
81. Liu C, Qian W, Qian Y, Giltiay NV, Lu Y, Swaidani S, et al. Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. *Sci Signal* (2009) 2(92):ra63. doi: 10.1126/scisignal.2000382
82. Ruddy MJ, Wong GC, Liu XK, Yamamoto H, Kasayama S, Kirkwood KL, et al. Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem* (2004) 279(4):2559–67. doi: 10.1074/jbc.M308809200
83. Song X, Dai D, He X, Zhu S, Yao Y, Gao H, et al. Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. *Immunity* (2015) 43(3):488–501. doi: 10.1016/j.immuni.2015.06.024
84. Verma AH, Richardson JP, Zhou C, Coleman BM, Moyes DL, Ho J, et al. Oral epithelial cells orchestrate innate type 17 responses to Candida albicans through the virulence factor candidalysin. *Sci Immunol* (2017) 2(17). doi: 10.1126/sciimmunol.aam8834
85. Chen X, Cai G, Liu C, Zhao J, Gu C, Wu L, et al. IL-17R-EGFR axis links wound healing to tumorigenesis in Lrig1(+) stem cells. *J Exp Med* (2019) 216 (1):195–214. doi: 10.1084/jem.20171849
86. Wu L, Chen X, Zhao J, Martin B, Zepp JA, Ko JS, et al. A novel IL-17 signaling pathway controlling keratinocyte proliferation and tumorigenesis via the TRAF4-ERK5 axis. *J Exp Med* (2015) 212(10):1571–87. doi: 10.1084/jem.20150204
87. Shao X, Chen S, Yang D, Cao M, Yao Y, Wu Z, et al. FGF2 cooperates with IL-17 to promote autoimmune inflammation. *Sci Rep* (2017) 7(1):7024. doi: 10.1038/s41598-017-07597-8
88. Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, et al. Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nat Neurosci* (2013) 16(10):1401–8. doi: 10.1038/nn.3505
89. Wang C, Zhang CJ, Martin BN, Bulek K, Kang Z, Zhao J, et al. IL-17 induced NOTCH1 activation in oligodendrocyte progenitor cells enhances proliferation and inflammatory gene expression. *Nat Commun* (2017) 8:15508. doi: 10.1038/ncomms15508
90. Langley RG, Elewski BE, Lebwohl M, Reich K, Griffiths CE, Papp K, et al. Secukinumab in plaque psoriasis–results of two phase 3 trials. *N Engl J Med* (2014) 371(4):326–38. doi: 10.1056/NEJMoa1314258
91. Nies JF, Panzer U. IL-17C/IL-17RE: Emergence of a Unique Axis in TH17 Biology. *Front Immunol* (2020) 11:341. doi: 10.3389/fimmu.2020.00341
92. Chang SH, Dong C. IL-17F: regulation, signaling and function in inflammation. *Cytokine* (2009) 46(1):7–11. doi: 10.1016/j.cyto.2008.12.024
93. Bie Q, Jin C, Zhang B, Dong H. IL-17B: A new area of study in the IL-17 family. *Mol Immunol* (2017) 90:50–6. doi: 10.1016/j.molimm.2017.07.004
94. Patel DD, Kuchroo VK. Th17 Cell Pathway in Human Immunity: Lessons from Genetics and Therapeutic Interventions. *Immunity* (2015) 43(6):1040–51. doi: 10.1016/j.immuni.2015.12.003
95. Gerhardt S, Abbott WM, Hargreaves D, Pauptit RA, Davies RA, Needham MR, et al. Structure of IL-17A in complex with a potent, fully human neutralizing antibody. *J Mol Biol* (2009) 394(5):905–21. doi: 10.1016/j.jmb.2009.10.008
96. Starnes T, Broxmeyer HE, Robertson MJ, Hromas R. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. *J Immunol* (2002) 169(2):642–6. doi: 10.4049/jimmunol.169.2.642
97. Akimzhanov AM, Yang XO, Dong C. Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. *J Biol Chem* (2007) 282(9):5969–72. doi: 10.1074/jbc.C600322200
98. Okada S, Puel A, Casanova JL, Kobayashi M. Chronic mucocutaneous candidiasis disease associated with inborn errors of IL-17 immunity. *Clin Transl Immunol* (2016) 5(12):e114. doi: 10.1038/cti.2016.71
99. Gomez-Rodriguez J, Sahu N, Handon R, Davidson TS, Anderson SM, Kirby MR, et al. Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase. *Immunity* (2009) 31 (4):587–97. doi: 10.1016/j.immuni.2009.07.009
100. Starnes T, Robertson MJ, Sledge G, Kelich S, Nakshatri H, Broxmeyer HE, et al. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol* (2001) 167(8):4137–40. doi: 10.4049/jimmunol.167.8.4137
101. Kawaguchi M, Onuchic LF, Li XD, Essayan DM, Schroeder J, Xiao HQ, et al. Identification of a novel cytokine, ML-1, and its expression in subjects with asthma. *J Immunol* (2001) 167(8):4430–5. doi: 10.4049/jimmunol.1201505
102. Chang SH, Dong C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res* (2007) 17(5):435–40. doi: 10.1038/cr.2007.35

103. Hot A, Miossec P. Effects of interleukin (IL)-17A and IL-17F in human rheumatoid arthritis synoviocytes. *Ann Rheum Dis* (2011) 70(5):727–32. doi: 10.1136/ard.2010.143768
104. Goepfert A, Lehmann S, Blank J, Kolbinger F, Rondeau JM. Structural Analysis Reveals that the Cytokine IL-17F Forms a Homodimeric Complex with Receptor IL-17RC to Drive IL-17RA-Independent Signaling. *Immunity* (2020) 52(3):499–512 e5. doi: 10.1016/j.immuni.2020.02.004
105. Tang C, Kakuta S, Shimizu K, Kadoki M, Kamiya T, Shimazu T, et al. Suppression of IL-17F, but not of IL-17A, provides protection against colitis by inducing Treg cells through modification of the intestinal microbiota. *Nat Immunol* (2018) 19(7):755–65. doi: 10.1038/s41590-018-0134-y
106. Kamiya T, Tang C, Kadoki M, Oshima K, Hattori M, Saijo S, et al. beta-Glucans in food modify colonic microflora by inducing antimicrobial protein, calprotectin, in a Dectin-1-induced-IL-17F-dependent manner. *Mucosal Immunol* (2018) 11(3):763–73. doi: 10.1038/mi.2017.86
107. Ramirez-Carrozzi V, Sambandam A, Luis E, Lin Z, Jeet S, Lesch J, et al. IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. *Nat Immunol* (2011) 12(12):1159–66. doi: 10.1038/ni.2156
108. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* (2004) 21(4):467–76. doi: 10.1016/j.immuni.2004.08.018
109. Reynolds JM, Martinez GJ, Nallaparaju KC, Chang SH, Wang YH, Dong C. Cutting edge: regulation of intestinal inflammation and barrier function by IL-17C. *J Immunol* (2012) 189(9):4226–30. doi: 10.1074/jbc.M910228199
110. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, Dong C. Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E. *Immunity* (2011) 35(4):611–21. doi: 10.1016/j.immuni.2011.09.010
111. Johnston A, Fritz Y, Dawes SM, Diaconu D, Al-Attar PM, Guzman AM, et al. Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J Immunol* (2013) 190(5):2252–62. doi: 10.4049/jimmunol.1201505
112. Martin DA, Towne JE, Kricorian G, Klekotka P, Gudjonsson JE, Krueger JG, et al. The emerging role of IL-17 in the pathogenesis of psoriasis: preclinical and clinical findings. *J Invest Dermatol* (2013) 133(1):17–26. doi: 10.1038/jid.2012.194
113. Smith E, Prasad KM, Butcher M, Dobrian A, Kolls JK, Ley K, et al. Blockade of interleukin-17A results in reduced atherosclerosis in apolipoprotein E-deficient mice. *Circulation* (2010) 121(15):1746–55. doi: 10.1161/CIRCULATIONAHA.109.924886
114. Butcher MJ, Waseem TC, Galkina EV. Smooth Muscle Cell-Derived Interleukin-17C Plays an Atherogenic Role via the Recruitment of Proinflammatory Interleukin-17A+ T Cells to the Aorta. *Arterioscler Thromb Vasc Biol* (2016) 36(8):1496–506. doi: 10.1161/ATVBAHA.116.307892
115. Butcher MJ, Gjurich BN, Phillips T, Galkina EV. The IL-17A/IL-17RA axis plays a proatherogenic role via the regulation of aortic myeloid cell recruitment. *Circ Res* (2012) 110(5):675–87. doi: 10.1161/CIRCRESAHA.111.261784
116. Krohn S, Nies JF, Kapffer S, Schmidt T, Riedel JH, Kaffke A, et al. IL-17C/IL-17 Receptor E Signaling in CD4(+) T Cells Promotes TH17 Cell-Driven Glomerular Inflammation. *J Am Soc Nephrol* (2018) 29(4):1210–22. doi: 10.1681/ASN.2017090949
117. Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, et al. Human IL-17: a novel cytokine derived from T cells. *J Immunol* (1995) 155 (12):5483–6.
118. Li H, Chen J, Huang A, Stinson J, Heldens S, Foster J, et al. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc Natl Acad Sci U S A* (2000) 97(2):773–8. doi: 10.1073/pnas.97.2.773
119. Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, et al. A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity. *J Biol Chem* (2000) 275(25):19167–76. doi: 10.1074/jbc.M910228199
120. Lee J, Ho WH, Maruoka M, Corpuz RT, Baldwin DT, Foster JS, et al. IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *J Biol Chem* (2001) 276(2):1660–4. doi: 10.1074/jbc.M008289200
121. Al-Samadi A, Moossavi S, Salem A, Sotoudeh M, Tuovinen SM, Konttinen YT, et al. Distinctive expression pattern of interleukin-17 cytokine family members in colorectal cancer. *Tumour Biol* (2016) 37(2):1609–15. doi: 10.1007/s13277-015-3941-x
122. Ferretti E, Ponzoni M, Doglioni C, Pistoia V. IL-17 superfamily cytokines modulate normal germinal center B cell migration. *J Leukoc Biol* (2016) 100 (5):913–8. doi: 10.1189/jlb.1VMR0216-096RR
123. Ryan AW, Thornton JM, Brophy K, Daly JS, McLoughlin RM, O'Morain C, et al. Chromosome 5q candidate genes in coeliac disease: genetic variation at IL4, IL5, IL9, IL13, IL17B and NR3C1. *Tissue Antigens* (2005) 65(2):150–5. doi: 10.1111/j.1399-0039.2005.00354.x
124. Robak E, Kulczycka-Siennicka L, Gerlicz Z, Kierstan M, Korycka-Wolowiec A, Sysa-Jedrzejowska A. Correlations between concentrations of interleukin (IL)-17A, IL-17B and IL-17F, and endothelial cells and proangiogenic cytokines in systemic lupus erythematosus patients. *Eur Cytokine Netw* (2013) 24(1):60–8. doi: 10.1684/ecn.2013.0330
125. Zhou J, Ren L, Chen D, Lin X, Huang S, Yin Y, et al. IL-17B is elevated in patients with pneumonia and mediates IL-8 production in bronchial epithelial cells. *Clin Immunol* (2017) 175:91–8. doi: 10.1016/j.clim.2016.12.008
126. Kouri VP, Olkkonen J, Ainola M, Li TF, Bjorkman L, Konttinen YT, et al. Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatol (Oxford)* (2014) 53(1):39–47. doi: 10.1093/rheumatology/ket309
127. Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, et al. IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. *J Immunol* (2007) 179(10):7128–36. doi: 10.4049/jimmunol.179.10.7128
128. Bie Q, Sun C, Gong A, Li C, Su Z, Zheng D, et al. Non-tumor tissue derived interleukin-17B activates IL-17RB/AKT/beta-catenin pathway to enhance the stemness of gastric cancer. *Sci Rep* (2016) 6:25447. doi: 10.1038/srep25447
129. Sanders AJ, Guo X, Mason MD, Jiang WG. IL-17B Can Impact on Endothelial Cellular Traits Linked to Tumour Angiogenesis. *J Oncol* (2010) 2010:817375. doi: 10.1155/2010/817375
130. Buning C, Genschel J, Weltrich R, Lochs H, Schmidt H. The interleukin-25 gene located in the inflammatory bowel disease (IBD) 4 region: no association with inflammatory bowel disease. *Eur J Immunogenet* (2003) 30(5):329–33. doi: 10.1046/j.1365-2370.2003.00411.x
131. Liu Y, Shao Z, Shangguan G, Bie Q, Zhang B. Biological Properties and the Role of IL-25 in Disease Pathogenesis. *J Immunol Res* (2018) 2018:6519465. doi: 10.1155/2018/6519465
132. von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature* (2016) 529(7585):221–5. doi: 10.1038/nature16161
133. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* (2001) 15 (6):985–95. doi: 10.1016/S1074-7613(01)00243-6
134. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* (2007) 204(7):1509–17. doi: 10.1084/jem.20061675
135. Kang CM, Jang AS, Ahn MH, Shin JA, Kim JH, Choi YS, et al. Interleukin-25 and interleukin-13 production by alveolar macrophages in response to particles. *Am J Respir Cell Mol Biol* (2005) 33(3):290–6. doi: 10.1165/rcmb.2005-0003OC
136. Wang WB, Yen ML, Liu KJ, Hsu PJ, Lin MH, Chen PM, et al. Interleukin-25 Mediates Transcriptional Control of PD-L1 via STAT3 in Multipotent Human Mesenchymal Stromal Cells (hMSCs) to Suppress Th17 Responses. *Stem Cell Rep* (2015) 5(3):392–404. doi: 10.1016/j.stemcr.2015.07.013
137. Ikeda K, Nakajima H, Suzuki K, Kagami S, Hirose K, Suto A, et al. Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* (2003) 101(9):3594–6. doi: 10.1182/blood-2002-09-2817
138. Kleinschek MA, Owyang AM, Joyce-Shaikh B, Langrish CL, Chen Y, Gorman DM, et al. IL-25 regulates Th17 function in autoimmune inflammation. *J Exp Med* (2007) 204(1):161–70. doi: 10.1084/jem.20061738
139. Letuve S, Lajoie-Kadoch S, Audusseau S, Rothenberg ME, Fiset PO, Ludwig MS, et al. IL-17E upregulates the expression of proinflammatory cytokines in

lung fibroblasts. *J Allergy Clin Immunol* (2006) 117(3):590–6. doi: 10.1016/j.jaci.2005.10.025
140. Sonobe Y, Takeuchi H, Kataoka K, Li H, Jin S, Mimuro M, et al. Interleukin-25 expressed by brain capillary endothelial cells maintains blood-brain barrier function in a protein kinase Cepsilon-dependent manner. *J Biol Chem* (2009) 284(46):31834–42. doi: 10.1074/jbc.M109.025940
141. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* (2010) 107(25):11489–94. doi: 10.1073/pnas.1003988107
142. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* (2010) 464(7293):1367–70. doi: 10.1038/nature08900
143. Terashima A, Watarai H, Inoue S, Sekine E, Nakagawa R, Hase K, et al. A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J Exp Med* (2008) 205(12):2727–33. doi: 10.1084/jem.20080698
144. Maezawa Y, Nakajima H, Suzuki K, Tamachi T, Ikeda K, Inoue J, et al. Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling. *J Immunol* (2006) 176(2):1013–8. doi: 10.4049/jimmunol.176.2.1013
145. Swaidani S, Bulek K, Kang Z, Gulen MF, Liu C, Yin W, et al. T cell-derived Act1 is necessary for IL-25-mediated Th2 responses and allergic airway inflammation. *J Immunol* (2011) 187(6):3155–64. doi: 10.4049/jimmunol.1002790
146. Beale J, Jayaraman A, Jackson DJ, Macintyre JDR, Edwards MR, Walton RP, et al. Rhinovirus-induced IL-25 in asthma exacerbation drives type 2 immunity and allergic pulmonary inflammation. *Sci Transl Med* (2014) 6(256):256ra134. doi: 10.1126/scitranslmed.3009124
147. Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, et al. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J Allergy Clin Immunol* (2006) 118(3):606–14. doi: 10.1016/j.jaci.2006.04.051
148. Ramirez-Carrozzi V, Ota N, Sambandam A, Wong K, Hackney J, Martinez-Martin N, et al. Cutting Edge: IL-17B Uses IL-17RA and IL-17RB to Induce Type 2 Inflammation from Human Lymphocytes. *J Immunol* (2019) 202(7):1935–41. doi: 10.4049/jimmunol.1800696
149. Su J, Chen T, Ji XY, Liu C, Yadav PK, Wu R, et al. IL-25 downregulates Th1/Th17 immune response in an IL-10-dependent manner in inflammatory bowel disease. *Inflammation Bowel Dis* (2013) 19(4):720–8. doi: 10.1097/MIB.0b013e3182802a76
150. Benatar T, Cao MY, Lee Y, Lightfoot J, Feng N, Gu X, et al. IL-17E, a proinflammatory cytokine, has antitumor efficacy against several tumor types in vivo. *Cancer Immunol Immunother* (2010) 59(6):805–17. doi: 10.1007/s00262-009-0802-8
151. Han Q, Das S, Hirano M, Holland SJ, McCurley N, Guo P, et al. Characterization of Lamprey IL-17 Family Members and Their Receptors. *J Immunol* (2015) 195(11):5440–51. doi: 10.4049/jimmunol.1500892
152. Saddawi-Konefka R, Seelige R, Gross ET, Levy E, Searles SC, Washington AJr., et al. Nrf2 Induces IL-17D to Mediate Tumor and Virus Surveillance. *Cell Rep* (2016) 16(9):2348–58. doi: 10.1016/j.celrep.2016.07.075
153. Seelige R, Washington AJr., Bui JD. The ancient cytokine IL-17D is regulated by Nrf2 and mediates tumor and virus surveillance. *Cytokine* (2017) 91:10–2. doi: 10.1016/j.cyto.2016.11.017
154. Stamp LK, Easson A, Lehnigk U, Highton J, Hessian PA. Different T cell subsets in the nodule and synovial membrane: absence of interleukin-17A in rheumatoid nodules. *Arthritis Rheumatol* (2008) 58(6):1601–8. doi: 10.1002/art.23455
155. Johansen C, Usher PA, Kjellerup RB, Lundsgaard D, Iversen L, Kragballe K. Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. *Br J Dermatol* (2009) 160(2):319–24. doi: 10.1111/j.1365-2133.2008.08902.x
156. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* (2012) 61(12):1693–700. doi: 10.1136/gutjnl-2011-301668
157. Lopez-Ferrer A, Vilarrasa E, Puig L. Secukinumab (AIN457) for the treatment of psoriasis. *Expert Rev Clin Immunol* (2015) 11(11):1177–88. doi: 10.1586/1744666X.2015.1095092
158. Conti HR, Whibley N, Coleman BM, Garg AV, Jaycox JR, Gaffen SL. Signaling through IL-17C/IL-17RE is dispensable for immunity to systemic, oral and cutaneous candidiasis. *PloS One* (2015) 10(4):e0122807. doi: 10.1371/journal.pone.0122807
159. Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, et al. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* (2006) 203(4):1105–16. doi: 10.1084/jem.20051615
160. Owyang AM, Zaph C, Wilson EH, Guild KJ, McClanahan T, Miller HR, et al. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med* (2006) 203(4):843–9. doi: 10.1084/jem.20051496
161. Zaph C, Du Y, Saenz SA, Nair MG, Perrigoue JG, Taylor BC, et al. Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med* (2008) 205(10):2191–8. doi: 10.1084/jem.20080720
162. Sawa S, Lochner M, Satoh-Takayama N, Dulauroy S, Berard M, Kleinschek M, et al. RORgammat+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat Immunol* (2011) 12(4):320–6. doi: 10.1038/ni.2002
163. Hammond M, Clark AB, Cahn AP, Chilvers ER, Fraser WD, Livermore DM, et al. The Efficacy and Mechanism Evaluation of Treating Idiopathic Pulmonary fibrosis with the Addition of Co-trimoxazole (EME-TIPAC): study protocol for a randomised controlled trial. *Trials* (2018) 19(1):89. doi: 10.1186/s13063-018-2453-6
164. Macaluso C, Maritano Furcada J, Alzaher O, Chaube R, Chua F, Wells AU, et al. The potential impact of azithromycin in idiopathic pulmonary fibrosis. *Eur Respir J* (2019) 53(2). doi: 10.1183/13993003.00628-2018
165. O'Sullivan T, Saddawi-Konefka R, Gross E, Tran M, Mayfield SP, Ikeda H, et al. Interleukin-17D mediates tumor rejection through recruitment of natural killer cells. *Cell Rep* (2014) 7(4):989–98. doi: 10.1016/j.celrep.2014.03.073
166. Prabhala RH, Fulciniti M, Pelluru D, Rashid N, Nigroiu A, Nanjappa P, et al. Targeting IL-17A in multiple myeloma: a potential novel therapeutic approach in myeloma. *Leukemia* (2016) 30(2):379–89. doi: 10.1038/leu.2015.228
167. Furuta S, Jeng YM, Zhou L, Huang L, Kuhn I, Bissell MJ, et al. IL-25 causes apoptosis of IL-25R-expressing breast cancer cells without toxicity to nonmalignant cells. *Sci Transl Med* (2011) 3(78):78ra31. doi: 10.1126/scitranslmed.3001374
168. Yin SY, Jian FY, Chen YH, Chien SC, Hsieh MC, Hsiao PW, et al. Induction of IL-25 secretion from tumour-associated fibroblasts suppresses mammary tumour metastasis. *Nat Commun* (2016) 7:11311. doi: 10.1038/ncomms11909
169. Cheng J, Gu CJ, Zhang B, Xie F, Yuan MM, Li MQ, et al. Cisplatin inhibits the growth, migration and invasion of cervical cancer cells by down-regulating IL-17E/IL-17RB. *Int J Clin Exp Pathol* (2017) 10(9):9341–51.
170. Huang CK, Yang CY, Jeng YM, Chen CL, Wu HH, Chang YC, et al. Autocrine/paracrine mechanism of interleukin-17B receptor promotes breast tumorigenesis through NF-kappaB-mediated antiapoptotic pathway. *Oncogene* (2014) 33(23):2968–77. doi: 10.1038/onc.2013.268
171. Wu HH, Hwang-Verslues WW, Lee WH, Huang CK, Wei PC, Chen CL, et al. Targeting IL-17B-IL-17RB signaling with an anti-IL-17RB antibody blocks pancreatic cancer metastasis by silencing multiple chemokines. *J Exp Med* (2015) 212(3):333–49. doi: 10.1084/jem.20141702
172. Bie Q, Zhang B, Sun C, Ji X, Barnie PA, Qi C, et al. IL-17B activated mesenchymal stem cells enhance proliferation and migration of gastric cancer cells. *Oncotarget* (2017) 8(12):18914–23. doi: 10.18632/oncotarget.14835
173. Ren L, Xu Y, Liu C, Wang S, Qin G. IL-17RB enhances thyroid cancer cell invasion and metastasis via ERK1/2 pathway-mediated MMP-9 expression. *Mol Immunol* (2017) 90:126–35. doi: 10.1016/j.molimm.2017.06.034
174. Guo HZ, Niu LT, Qiang WT, Chen J, Wang J, Yang H, et al. Leukemic IL-17RB signaling regulates leukemic survival and chemoresistance. *FASEB J* (2019) 33(8):9565–76. doi: 10.1096/fj.201900099R
175. Liao R, Sun J, Wu H, Yi Y, Wang JX, He HW, et al. High expression of IL-17 and IL-17RE associate with poor prognosis of hepatocellular carcinoma. *J Exp Clin Cancer Res* (2013) 32:3. doi: 10.1186/1756-9966-32-3

176. Brennan CA, Garrett WS. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. *Nat Rev Microbiol* (2019) 17(3):156–66. doi: 10.1038/s41579-018-0129-6
177. Vandeghinste N, Klattig J, Jagerschmidt C, Lavazais S, Marsais F, Haas JD, et al. Neutralization of IL-17C Reduces Skin Inflammation in Mouse Models of Psoriasis and Atopic Dermatitis. *J Invest Dermatol* (2018) 138(7):1555–63. doi: 10.1016/j.jid.2018.01.036
178. Cammarota G, Ianiro G, Kelly CR, Mullish BH, Allegretti JR, Kassam Z, et al. International consensus conference on stool banking for faecal microbiota transplantation in clinical practice. *Gut* (2019) 68(12):2111–21. doi: 10.1136/gutjnl-2019-319548
179. Lynch SV, Ng SC, Shanahan F, Tilg H. Translating the gut microbiome: ready for the clinic? *Nat Rev Gastroenterol Hepatol* (2019) 16(11):656–61. doi: 10.1038/s41575-019-0204-0
180. Kolodziejczyk AA, Zheng D, Elinav E. Diet-microbiota interactions and personalized nutrition. *Nat Rev Microbiol* (2019) 17(12):742–53. doi: 10.1038/s41579-019-0256-8
181. Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, et al. Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. *Cell* (2018) 174(6):1406–23. doi: 10.1016/j.cell.2018.08.047
182. Skelly AN, Sato Y, Kearney S, Honda K. Mining the microbiota for microbial and metabolite-based immunotherapies. *Nat Rev Immunol* (2019) 19 (5):305–23. doi: 10.1038/s41577-019-0144-5
183. Elinav E, Garrett WS, Trinchieri G, Wargo J. The cancer microbiome. *Nat Rev Cancer* (2019) 19(7):371–6. doi: 10.1038/s41568-019-0155-3
184. Helmink BA, Khan MAW, Hermann A, Gopalakrishnan V, Wargo JA. The microbiome, cancer, and cancer therapy. *Nat Med* (2019) 25(3):377–88. doi: 10.1038/s41591-019-0377-7
185. Zitvogel L, Daillere R, Roberti MP, Routy B, Kroemer G. Anticancer effects of the microbiome and its products. *Nat Rev Microbiol* (2017) 15:465–77. doi: 10.1038/nrmicro.2017.44
186. Ritchlin CT, Kavanaugh A, Merola JF, Schett G, Scher JU, Warren RB, et al. Bimekizumab in patients with active psoriatic arthritis: results from a 48-week, randomised, double-blind, placebo-controlled, dose-ranging phase 2b trial. *Lancet* (2020) 395(10222):427–40. doi: 10.1016/S0140-6736(19)33161-7
187. Ma Q. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol* (2013) 53:401–26. doi: 10.1146/annurev-pharmtox-011112-140320

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership