# REGULATION OF IMMUNITY BY NON-IMMUNE CELLS

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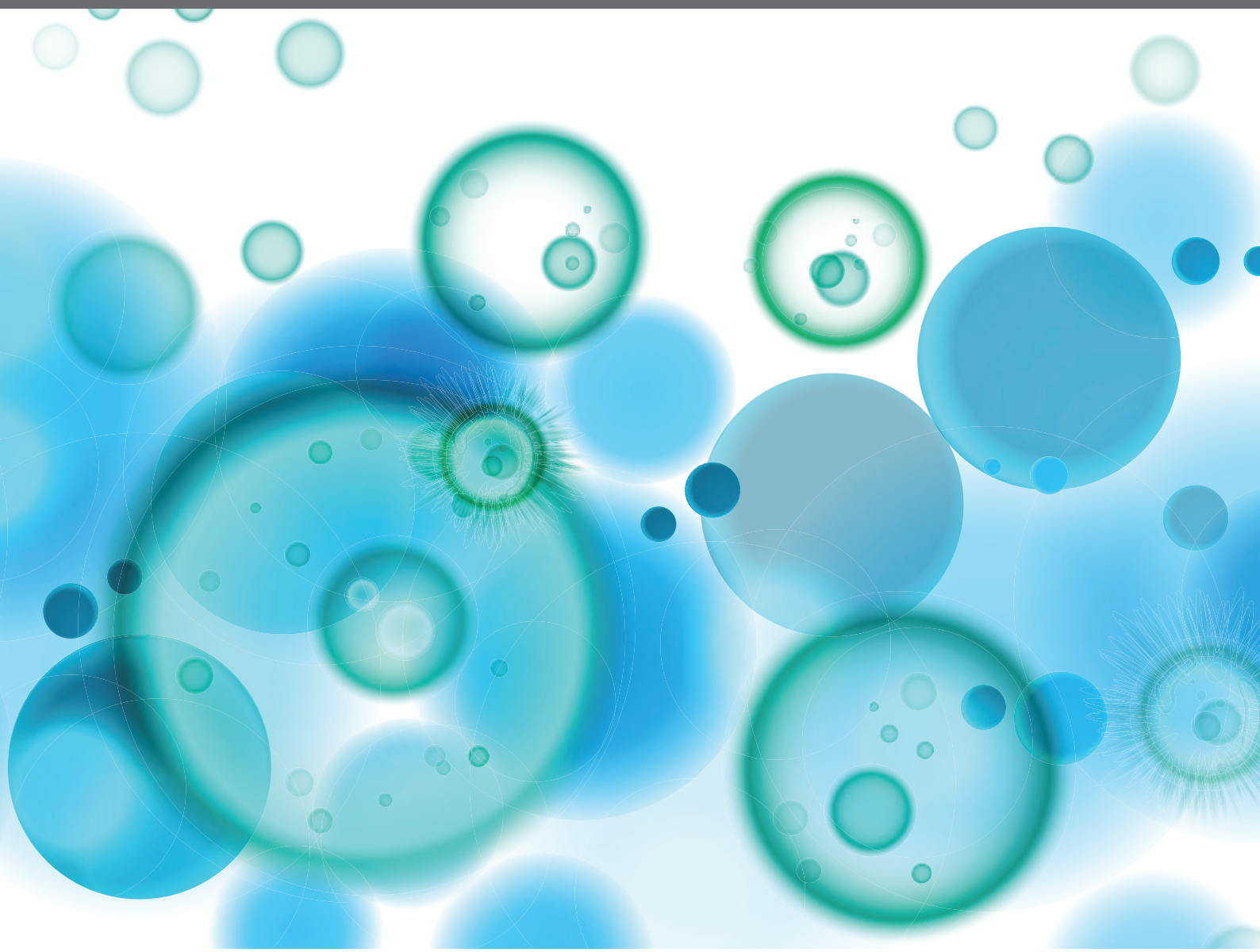

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# REGULATION OF IMMUNITY BY NON-IMMUNE CELLS

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EDITORIAL
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# Editorial: Regulation of Immunity by Non-Immune Cells

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**Keywords: nonimmune cell, mucosal tissue, skin, fat, stroma, joints, blood vessels**

**Editorial on the Research Topic**

**Regulation of Immunity by Non-Immune Cells**

## INTRODUCTION

The immune system is a highly sophisticated system that governs a major part of the protective and regenerative responses of the host against external and internal microbes, poisons, and other dangers. Classical innate and acquired immune cells include dendritic cells, macrophages, neutrophils, eosinophils, innate lymphoid cells, T cells, and B cells. B and T cells are activated by specific antigens; they organize the most effective type of immune response by eliminating harmful events. Phagocytic cells, such as dendritic cells and macrophages, play essential roles in both (i) the antigen-specific activation of lymphocytes and (ii) the situation-specific functional deviations of lymphocytes. In contrast, non-immune cells, which include epithelial cells, epidermal keratinocytes, mesenchymal cells, stromal cells, synoviocytes, and neurons, are expected to participate in the host's defense system not just as structural architectures, but also as regulators and effectors of its protective immune response. For example, the molecules released from damaged non-immune cells can affect several types of immune responses. Furthermore, the *de novo* production of bioactive mediators by non-immune cells, in response to several stimuli, can be involved in these processes. Therefore, defects in the abilities of non-immune cells to mediate immune regulation may be involved in the pathogenicities of a series of inflammatory diseases. During the process of immune regulation, non-immune cells must sense each type of danger signal appropriately, interpret it precisely, and activate the most appropriate type of immune response to overcome said danger. However, the precise cellular mechanisms involved in this process have not yet been fully elucidated. Thus, there is a need for a review series that explores the consideration of a common mechanism of immunity regulation by non-immune cells.

Here, we provide a cutting-edge collection of Review and Mini Review articles that discuss the roles and mechanisms of immune modulation by non-immune cells, beyond organs, tissues, and cell types. Our goal is to stimulate discussion regarding the common and essential roles of non-immune cells in orchestrating their host's protective responses and inflammatory processes, and to further our knowledge in this field.

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## MUCOSAL TISSUES

The gastrointestinal tracts are constitutively exposed by countless numbers of antigens, including commensal and pathogenic microorganisms. The epithelial monolayer that covers the gastrointestinal tract contains luminal antigens in the lumen. Therefore, the disruption of the epithelial layer predisposes patients to the development of inflammatory bowel disease (IBD). Internal nuclear factor-κB (NF-κB) signaling is a molecular signaling mechanism that regulates the homeostasis of intestinal epithelial cells (IECs). Garcia-Carbonell et al. summarized the molecular network of epithelial NF-κB signals downstream of the tumor necrosis factor (TNF) receptor. They also described the role that NF-κB signaling plays in IECs regarding the maintenance of the epithelial barrier system. They highlighted that the dysregulation of NF-κB signaling predisposes the development of IBD.

Goto discussed that IECs have the potential to modulate the mucosal immune system and construct a physical barrier. They also described the immunomodulatory effects of IECs in response to luminal antigens, especially to commensal and pathogenic microorganisms. At the same time, IECs produce antimicrobial molecules, including bactericidal peptides, immunoglobulin (IgA), and carbohydrate moieties. These molecules are induced by cytokines and molecules that are produced by immune cells in the lamina propria. Therefore, IECs work as bidirectional transducers of signals from luminal antigens and gut immune cells to maintain gut homeostasis.

Xue et al. highlighted that in the intestine, non-immune cells, such as IECs and enteric neurons, produce neurotransmitters such as dopamine. Neurotransmitters have been reported to have various immunomodulatory functions that are thought to be associated with the development and regulation of immunological diseases. They also provided evidence that intestinal dopamine, induced by commensal microbes, suppresses liver injury through the D1-like receptor-protein kinase A (PKA) signals in invariant natural killer T (iNKT) cells (Xue et al.). This suggests that the interplay between gut microbes and the host's nervous and immune systems plays an important role in the prevention of autoimmune hepatitis.

Kurashima et al. discussed that, in the intestine, mesenchymal stromal cells, IECs, and immune cells create a network system. Although the interplay between IECs and immune cells has been extensively investigated, our understanding of the characteristics and functions of intestinal stromal cells is limited. They took a broad view in summarizing evidence linking the roles of stromal cells, which modulate the function and differentiation of IECs, and mucosal immune cells (Kurashima et al.). One mechanism that highlights the role of stromal cells in the modulation of the immune system is that stromal cells activated by lymphotoxins produce cytokines and chemokines, which in turn recruit lymphocytes. This subsequently induces the organogenesis of secondary lymphoid tissues, such as Peyer's patches and mesenteric lymph nodes.

Hirahara et al. highlighted that the lungs also contain representative mucosal tissue composed of epithelial cells, stromal cells, and immune cells. They provided an elegant illustration of the molecular mechanism underlying the induction of inducible bronchus-associated lymphoid tissue (iBALT). In the context of allergic inflammation, interleukin (IL)-33 activates ST2+ memory CD4+ T cells to induce IL-5 and amphiregulin. These cytokines accelerate fibrosis and the pathology of asthma. Therefore, the interactions between immune and epithelial/mesenchymal cells are critical for the development of chronic lung inflammation.

## SKIN

Zhang revealed that epidermal keratinocytes are located at the outermost position in the skin and are the first responders to external agents and skin injuries. They focused on the potential roles of type 1 interferons (IFNs) in psoriasis, which is one of the most common chronic inflammatory diseases. Skin injuries can rapidly induce IFNβ from keratinocytes and IFNα from dermal plasmacytoid dendritic cells, through distinct mechanisms. IFNβ derived from keratinocytes promotes dendritic cell maturation and subsequent T-cell proliferation, triggering the development of psoriasis.

Dainichi et al. discussed that keratinocytes can be involved in the organization of immune responses of the skin through two phases: the initiation of primary immune responses and the propagation of secondary responses; this gives rise to the loop of chronic inflammation. TNF receptor-associated factor 6 (TRAF6) is a ubiquitin E3 ligase that is essential for various receptor signaling pathways that activate NF-κB and mitogen-activated protein kinase (MAPK). The above-mentioned mini review describes the roles of TRAF6 in epithelial tissues, including keratinocytes, in both epithelial primary and secondary responses.

## FAT AND STROMA

West highlighted that stromal cells complement the functions of classical immune cells by sensing pathogens and tissue damage, coordinating leukocyte recruitment and function, and promoting immune response resolution and tissue repair. Several members of the IL-6 cytokine family mediate crosstalk between stromal and immune cells; they play diverse roles in numerous inflammatory and homeostatic processes. The above-mentioned review summarizes our current understanding of how IL-6 family cytokines control stromal-immune crosstalk in healthy and diseased hosts, and how these interactions can be leveraged for clinical benefit.

Wong et al. prepared a mini review discussing the subcutaneous tissue, which forms an uninterrupted layer throughout the body in humans. Obesity leads to the upregulation of pro-inflammatory adipokines, and to the downregulation of anti-inflammatory adipokines from adipocytes. This results in the activation of the stromal vascular fraction, such as macrophages, in adipose tissue. Said mini review sheds light on the crosstalk between adipose and immune cells in psoriasis.

## JOINTS

Yoshitomi discussed the synovial tissue: a membranous, non-immune, organ-lining joint cavity. Fibroblast-like synoviocytes (FLSs) are the dominant non-immune cells in synovial tissues (Yoshitomi). FLSs mainly contribute to joint destruction in chronic inflammatory diseases, such as rheumatoid arthritis (RA), *via* multiple mechanisms. Their mini review describes the new findings and mechanisms underlying the regulation of immune reactions by non-immune FLS, and highlights their roles in the development of chronic inflammation.

## BLOOD VESSELS

Hu et al. highlighted that the mechanisms of atherogenesis are currently largely undefined. However, it has been demonstrated that disease progression involves crosstalk between immune cells and both endothelial cells and vascular smooth muscle cells (VSMCs). VSMCs maintain the integrity of the arterial wall in the media layer of arteries. They also participate in the remodeling of the arterial wall in atherosclerosis, throughout all stages of the disease. The said mini review focuses on the roles that VSMCs play in atherosclerosis immunity by organizing artery tertiary lymphoid organs (ATLOs).

## CONCLUDING REMARKS

We thank the contributors and dedicated reviewers for their efforts and generous enthusiasm. Although this Research Topic does not fully capture the full breadth of activity in the regulation of immunity by non-immune cells, we hope that the series of articles identified will stimulate the communication and integration of this concept, among both immunologists and non-immunologists. We believe that our article makes a significant contribution to the literature because of its timeliness, succinctness, and the need to highlight this important direction of study.

## AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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# Peripheral Dopamine Controlled by Gut Microbes Inhibits Invariant Natural Killer T Cell-Mediated Hepatitis

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Neurotransmitters have been shown to regulate immune responses, and thereby are critically related to autoimmune diseases. Here we showed that depletion of dopaminergic neurons significantly promoted activation of hepatic iNKT cells and augmented concanavalin A (Con A)-induced liver injury. The suppressive effect of dopamine on iNKT cells was mediated by D1-like receptor-PKA pathway. Clearance of gut microbiota by antibiotic cocktail reduced synthesis of dopamine in intestines and exacerbated liver damage, and that could be restored by recovery of gut microbiota or replenishment of D1-like receptor agonist. Our results demonstrate that peripheral dopamine controlled by gut microbes inhibits IL4 and IFNγ production in iNKT cells and suppresses iNKT cell-mediated hepatitis. Together, we propose a gut microbe-nervous system-immune system regulatory axis in modulating autoimmune hepatitis.

**Keywords: dopamine, D1-like receptors, hepatitis, iNKT, gut microbes**

## INTRODUCTION

Recent studies indicate a crosstalk between nervous system and immune system. Neurotransmitters, neuropeptides, cytokines and their receptors are main mediators in the neuro-immune network. Dopamine, a critical transmitter, has been shown to regulate immune responses in periphery, and has been related to tumor immunity and several autoimmune diseases, including inflammatory bowel diseases, multiple sclerosis and rheumatoid arthritis (1–3). Dopamine receptors have been detected in both innate immune cells and adaptive immune cells. According to the downstream signaling, these receptors can be classified into two subclasses (4). D1-like dopamine receptors (DRD) including DRD1 and DRD5 activate adenylyl cyclase, whereas D2-like receptors including DRD2, DRD3, and DRD4 inhibit adenylyl cyclase. These receptors show distinct affinity for dopamine: DRD3 > DRD5 > DRD4 > DRD2 > DRD1 (5). Thus, dopamine displays complex regulatory effect on immune responses, depending on dopamine concentration, subtype of receptors and type of immune cells. As an important immune regulator, peripheral dopamine is mainly produced by autonomic nervous system, gut epithelial cells, and immune cells including dendritic cells, regulatory T cells, B cells, and macrophages (5). About 50% dopamine is produced in gastrointestinal tract by enteric neurons and intestinal epithelial

cells, and thus results in higher level of dopamine in hepatic portal vein (6). Crosstalk between gut and liver has been demonstrated by plenty of studies. Progression of liver diseases is associated with molecules and cells derived from gut (7). The contributions of gut derived dopamine to liver immunity and autoimmune liver diseases remain to be elucidated.

As the most abundant innate lymphocytes in liver, invariant NKT (iNKT) cells play crucial roles in liver immunity and are critically linked to liver diseases (8). Different from conventional T cells, iNKT cells are tissue-resident cells and contribute to the first line of body defense. Upon stimulation, they release large amount of Th1 and Th2 cytokines, and bridge innate and adaptive immunity. Several factors modulating iNKT cell functions have been characterized, including antigens, cytokines, antigen presenting cells (APCs), and metabolites (9–12). Interestingly, nervous system has also been suggested to regulate the behavior of iNKT cells *in vivo* (13). During hepatocyte regeneration, sympathetic nervous system induces expansion of NKT cells (14). Additionally, norepinephrine inhibits apoptosis of NKT cells and restores hepatic NKT cell numbers in ob/ob mice (15). These findings demonstrate a link between nervous system and iNKT cells. However, the influences of dopamine on hepatic iNKT cell functions and iNKT cell related liver diseases are still unclear.

Here, we demonstrate that dopamine plays an important role in suppressing autoimmune hepatitis. Depletion of dopaminergic neurons using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) significantly augmented the concanavalin A (Con A)-induced hepatitis. Dopamine inhibited IL4 and IFNγ production in iNKT cells through D1-like receptor-PKA pathway, and thus suppressed the iNKT cell-mediated liver damage. Moreover, synthesis of peripheral dopamine was controlled by gut microbes. Clearance of gut microbes using antibiotics reduced dopamine synthesis in guts, and consequently promoted Con A-induced liver injury. Restoring dopamine synthesis via transferring gut microbes or replenishing D1-like receptor agonist ameliorated the liver damage in antibiotics-treated mice. Our study proposes a regulatory axis from gut microbes to neurotransmitter and then to autoimmune hepatitis.

# MATERIALS AND METHODS

## Mice and Treatment

WT mice were purchased from the Beijing Vital River Laboratory Animal Technology. *Jα18*$^{-/-}$ mice and *Vα14 Tg.cxcr6*$^{gfp/+}$ mice have previously been described and were provided by Dr. Bendelac. All male mice used were in C57BL/6 background and between 6 and 10 weeks of age, and were maintained under pathogen-free conditions. All animal procedures were approved by the USTC Institutional Animal Care and use Committee.

To investigate the influence of A68930 on iNKT cell functions *in vivo*, mice were injected intraperitoneally with α-GalCer (2 μg/mouse, Avanti Polar Lipids, Alabama) and A68930 (8 mg/kg, Sigma-Aldrich, Munich, Germany), 4 h before tissue collection. For the control group, mice were injected with α-GalCer only. To induce hepatitis, Con A (Sigma-Aldrich, Munich, Germany) was injected into the mice at a concentration of 15 mg/kg with or without A68930 or A77636 (8 mg/kg) (Sigma-Aldrich, Munich, Germany). Tissue samples and serum were collected after injecting Con-A for 12 h. To delete dopaminergic neurons *in vivo*, MPTP (20 mg/kg, Sigma-Aldrich, Munich, Germany) or PBS buffer as control was injected intraperitoneally for 4 times with 2 h intervals. Twenty-four hours after the last injection, these mice were injected with Con A with or without A77636 to induce liver damage. Livers were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological examination. Alanine aminotransferase and aspartate aminotransferase in serum were measured by a Chemray-240 Automated Chemistry Analyzer (Rayto, Shenzhen, China).

## Clearance and Restoration of Gut Microbiota

Four antibiotics in drinking water were used to treat mice for 2 weeks: vancomycin (1 g/L), streptomycin (2 g/L), metronidazole (2 g/L), and ampicillin (2 g/L). To restore the gut microbiota, antibiotics-treated mice were co-housed with age-matched normal mice for 4 weeks without antibiotics treatment.

## Stimulation of iNKT Cells

iNKT cells, gating as GFP$^{hi}$ cells, were sorted from livers of *Vα14 Tg.cxcr6*$^{gfp/+}$ mice (**Figure S1**), and were stimulated with α-GalCer-pulsed (1 μg/ml) RBL.CD1d cells or plate-coated anti-CD3 (10 μg/ml, Biolegend, San Diego, California) plus anti-CD28 (1 μg/ml, Biolegend, San Diego, California) antibodies in the presence of indicated reagents for 16 h. Cytokines in supernatants were measured by cytometric bead array. To measure intracellular cyclic adenosine monophosphate (cAMP), cells were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) plus ionomycin (1 μM) for 4–6 h.

## Intracellular Cytokine and Camp Staining

After surface staining, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany) and permeabilized with PBS buffer containing 0.1% saponin (Sigma-Aldrich, Munich, Germany) and 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Munich, Germany). Then, cells were stained with antibodies against intracellular cytokines or cAMP. Anti-TCRβ (H57-597), anti-IFNγ (XMG1.2), and anti-IL4 (11B11) were purchased from Biolegend (San Diego, California). Anti-cAMP was purchased from Abcam (Cambridge, England). PBS57-CD1d tetramer was provided by the NIH Tetramer Core Facility. Cells were analyzed with a FACSVerse flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data was analyzed with FlowJo 7.6 software (Tree star, Ashland, Oregon).

## High-Performance Liquid Chromatography (HPLC)

To measure the dopamine in portal vein, portal venous blood was collected from control or MPTP treated mice. Two hundred fifty microliter serum was mixed with 125 μl 2 M perchloric acid ($HCLO_4$), and then was centrifuged at 5,000 g for 10 min. The supernatant was collected and injected into the HPLC system (Antec Scientific, Zoeterwoude, Netherlands) for

dopamine analysis. Dopamine standard was used to determine the concentration.

## Real-Time PCR

Total RNA was extracted from stimulated cells with ReliaPrep™ RNA Cell Miniprep System (Promega, Fitchburg, Wisconsin). cDNA was synthesized from total RNA using Reverse Transcription System (Promega, Fitchburg, Wisconsin). Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega, Fitchburg, Wisconsin). *Actin* was used as an internal control gene. The primer sequences used were as follows:

*drd1* F 5′ GGATGTGCATCGAGGTGAATG; *drd1* R 5′CGA TGAGGCACAGCTCATT 3′; *drd2* F 5′ CAGATGCTTGCCATT GTTCT 3′; *drd2* R 5′ CAGCAGTGCAGGATCTTCAT 3′; *drd3* F 5′ GTGGCTCGGGGCCTTCATTG 3′; *drd3* R 5′ GGGCACTGT TCACGTAGCCA 3′; *drd4* F 5′ GTGTTGGACGCCTTTCTTCG 3′; *drd4* R 5′ GGGTTGAGGGCACTGTTGA 3′; *drd5* F 5′ CTGC GAGCATCCATCAAG 3′; *drd5* R 5′ CACAAGGGAAGCCAGT CC 3′; *IL4* F 5′ATGGAGCTGCAGAGACTCTT 3′; *IL4* R 5′ AAA GCATGGTGGCTCAGTAC 3′; *Ifng* F 5′ ATGAACGCTACACA CTGCATC 3′; *Ifng* R 5′ CCATCCTTTTGCCAGTTCCTC 3′; *Th* F 5′ GACAGTCCTCACACCATCCG 3′; *Th* R 5′ GACAGTCCT CACACCATCCG 3′.

## Western Blot

Cells or tissues were harvested and lysed with sample buffer and boiled for 10 min. Proteins were separated by electrophoresis and detected by western blot. Antibodies against CREB, pSer133-CREB, IκBα, pSer32-IκBα, TH, and Actin were purchased from Cell Signaling Technology (Danvers, Massachusetts), Sigma-Aldrich (Munich, Germany), Abcam (Cambridge, England), or Proteintech (Chicago, Illinois).

## Bacterial Genomic DNA Extraction and Amplification of 16S rRNA

Fresh feces were collected from the experimental mice, bacterial genomic DNA was extracted using the YuanPingHao Bio stool kit (Beijing, China). The amounts of different gut bacteria were measured by qPCR using primers specific for their 16S rRNA as previously described (16). Group-specific primers were used as follows: *Eubacterium rectale-Clostridium coccoides* (Erec), UniF338, 5′ACTCCTACGGGAGGCAGC 3′, C.cocR491, 5′GCTTCTTTAGTCAGGTACCGTCAT 3′; *Bacteroides* (Bact), BactF285, 5′GGTTCTGAGAGGAGGTCCC 3′, UniR338, 5′ GCTGCCTCCCGTAGGAGT 3′; *mouse intestinal Bacteroides* (MIB), Uni516F, 5′ CCAGCAGCCGCGGTAATA 3′, MIBR677, 5′ CGCATTCCGCATACTTCTC 3′; *Enterobacteriaceae* (Ent), 515F, 5′GTGCCAGCMGCCGCGGTAA 3′, 826R, 5′GCCTC AAGGGCACAACCTCCAAG 3′; Eubacteria (All bacteria), UniF340, 5′ACTCCTACGGGAGGCAGCAGT 3′, UniR514, 5′A TTACCGCGGCTGCTGGC3′.

## Statistical Analyses

Error bars represent SEM. Statistical analyses were performed using student's *t*-test (GraphPad Software). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered statistically significant.

# RESULTS

## Depletion of Dopaminergic Neurons Augments Con A-Induced Liver Injury

Previous studies indicate that large amount of peripheral dopamine is detected in hepatic portal vein (6). To demonstrate the role of dopamine in autoimmune hepatitis, we depleted peripheral dopamine by injecting mice with dopaminergic neuron-specific neurotoxin MPTP (17). MPTP efficiently depleted dopaminergic neurons as indicated by reduced expression of tyrosine hydroxylase, a key enzyme for dopamine biosynthesis, in brains (**Figure 1A**). Moreover, the concentration of dopamine in portal vein (**Figure 1B**) and mRNA of tyrosine hydroxylase in gut (**Figure S2**) were also significantly reduced by MPTP. It is well-known that iNKT cells are the main mediators in Con A-induced acute autoimmune hepatitis (18). Although depletion of dopaminergic neurons by MPTP did not influence the Con A-induced expression of CD69 in hepatic iNKT cells (**Figure 1C**), it significantly elevated their IFNγ production (**Figure 1D**). In agreement with previous findings that iNKT cells and IFNγ play important roles in the development of Con-A induced hepatitis (19, 20), exacerbated hepatocyte necrosis (**Figure 1E**) and increased alanine aminotransferase (ALT) as well as aspartate aminotransferase (AST; **Figure 1F**) were detected in MPTP treated mice after Con-A injection. These results demonstrated severer Con A-induced liver injury in MPTP treated mice than in control mice, suggesting a role of dopamine in suppressing autoimmune hepatitis.

## Dopamine Inhibits IL4 and IFNγ Production in iNKT Cells

To investigate whether dopamine could directly regulate iNKT cell functions, different doses of dopamine were added to iNKT cells that were activated by α-GalCer-pulsed RBL.CD1d cells. Dopamine inhibited both IL4 and IFNγ production from iNKT cells in a dose-dependent manner (**Figure 2A**). Similar results were obtained when NKT cells were activated by plate-coated anti-CD3 plus anti-CD28 antibodies (**Figure 2B**) or by PMA plus ionomycin bypassing T cell receptor (TCR) signaling (**Figure 2C**). These results indicate that dopamine could inhibit production of cytokines in iNKT cells in a TCR-independent manner. Next, we investigated whether dopamine inhibited IL4 and IFNγ production at transcriptional level. Reduced *Il4* and *Ifng* mRNA were detected in dopamine treated cells, suggesting a transcriptional inhibition by dopamine (**Figure 2D**). To exclude the possibility that diminished cytokine production was caused by cell death, we measured lactate dehydrogenase release in culture medium. Lactate dehydrogenase is a cytosolic enzyme and is released into culture medium when the plasma membrane is damaged. In our studies, dopamine did not increase the lactate dehydrogenase in medium, indicating normal cell viability after dopamine treatment (**Figure S3A**).

## Dopamine Inhibits IL4 and IFNγ Production in iNKT Cells Through D1-Like Receptors

Dopamine exerts its effects through five distinct receptors (DRD1 to DRD5). Except for DRD2, mRNA of other four

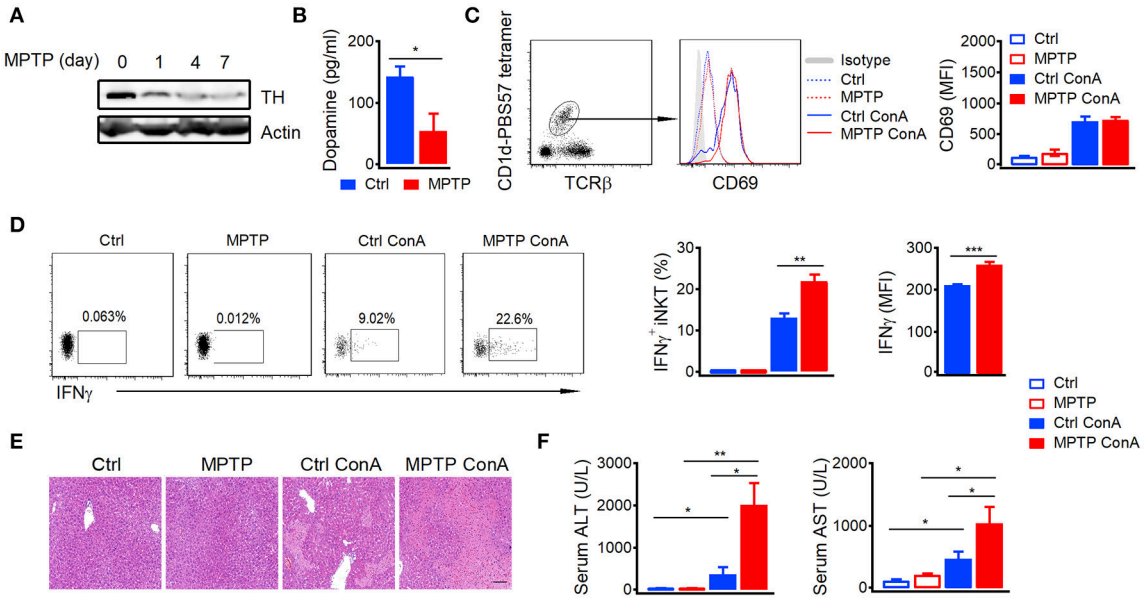


**FIGURE 1 |** Depletion of dopaminergic neurons promotes Con A-induced liver injury.**(A)** Expression of TH in brains of mice injected with MPTP (20 mg/kg) for indicated times. **(B)** Dopamine in portal vein of control mice and MPTP treated mice (24 h later). **(C,D)** CD69 expression **(C)**, percentages of IFNγ$^+$ hepatic iNKT cells and mean fluorescence intensity of IFNγ **(D)** in mice injected with Con A (15 mg/kg) with or without MPTP (20 mg/kg) pretreatment. **(E)** Hematoxylin and eosin staining of liver tissues from mice described in **(C,D)**. Bar, 100 μm. **(F)** ALT and AST in serum of mice described in **(C,D)**. $n = 5$ mice per group. Error bars represent SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MFI, mean fluorescent intensity; ConA, Concanavalin A.

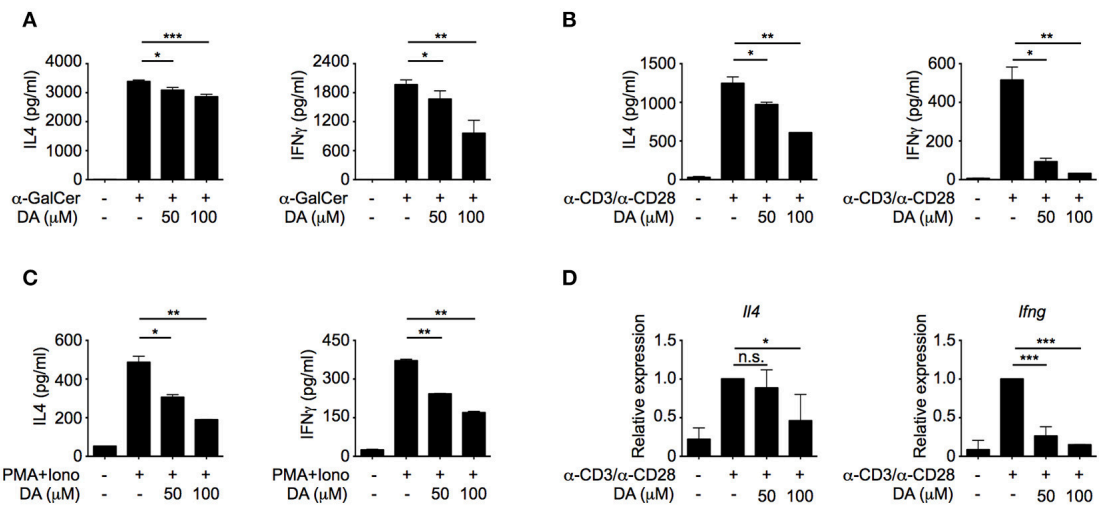


**FIGURE 2 |** Dopamine inhibits production of IL4 and IFNγ in iNKT cells. **(A–C)** IL4 and IFNγ production in iNKT cells activated by α-GalCer-pulsed RBL.CD1d cells **(A)**, anti-CD3 plus anti-CD28 **(B),** and PMA plus ionomycin **(C)** in the absence or presence of different doses of dopamine. Data are representative of three independent experiments. **(D)** mRNA levels of *Il4* and *Ifng* in iNKT cells activated by anti-CD3 plus anti-CD28, in the absence or presence of different doses of dopamine. Data are representative of three independent experiments. Error bars represent SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. DA, dopamine; PMA, phorbol myristate acetate; Iono, ionomycin.

receptors were detected in iNKT cells, including DRD1, DRD5, DRD3, and DRD4, although DRD3 level was relatively low (**Figure 3A**). To determine which receptor was involved in the suppressive effects of dopamine, selective receptor agonists had been used to activate distinct receptor, respectively. Interestingly, dopamine D1-like receptor agonist A77636, activating DRD1, and DRD5, significantly inhibited IL4 and IFNγ production, whereas agonists of DRD2, DRD3, and DRD4 showed no effect on cytokine production (**Figure 3B**). To further prove that dopamine inhibited cytokine production through

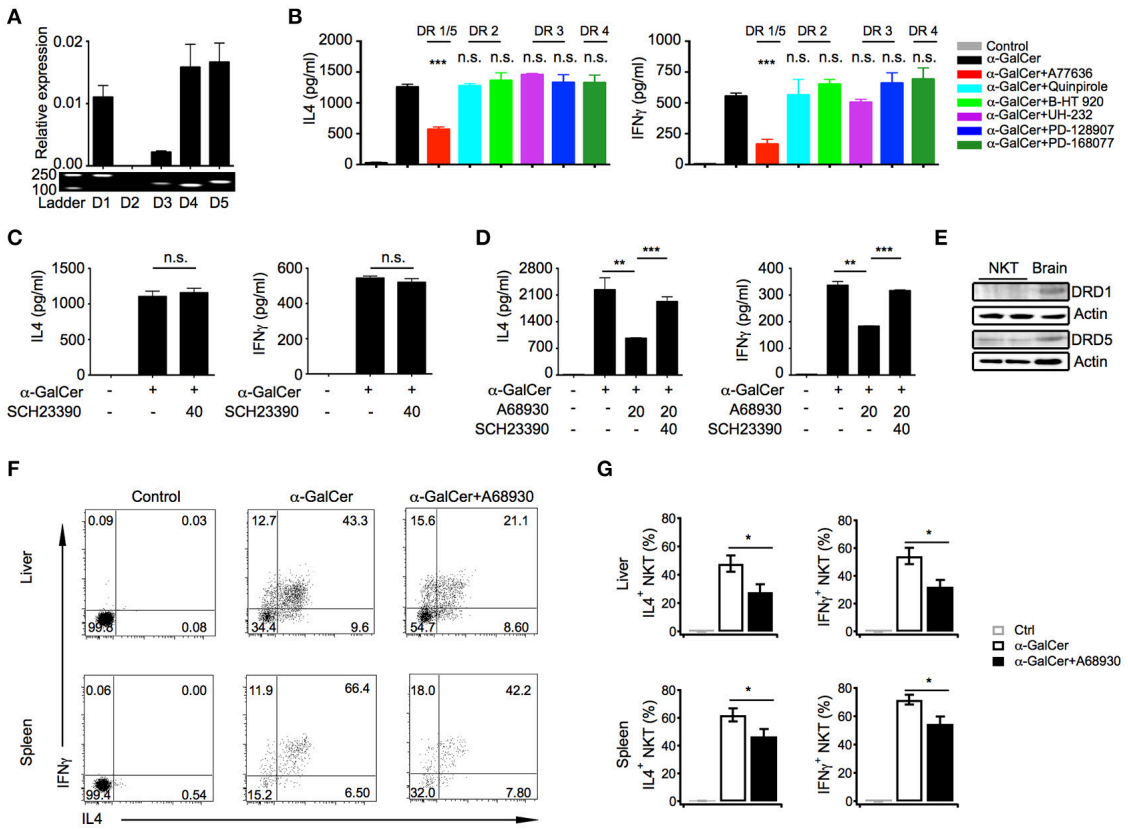


**FIGURE 3 |** Dopamine inhibits cytokine production in iNKT cells through D1-like receptors. **(A)** mRNA level and fragments of various dopamine receptors in iNKT cells. **(B)** Influences of indicated selective receptor agonists (20 μM) on production of IL4 and IFNγ in iNKT cells. iNKT cells were activated by α-GalCer-pulsed RBL.CD1d cells. **(C)** Influences of SCH23390, antagonist of D1-like receptors, on IL4 and IFNγ production in iNKT cells. **(D)** Influences of A68930, agonist of D1-like receptors, on IL4 and IFNγ production in iNKT cells pretreated with or without SCH23390 for 3 h. **(E)** Protein levels of DRD1, DRD5, and β-actin in iNKT cells. Brain was used as positive control. Data are representative of more than three independent experiments. **(F)** Intracellular staining of IL4 and IFNγ in hepatic and splenic iNKT cells after injecting α-GalCer (2 μg/mouse) with or without A68930 (8 mg/kg) for 4 h. In negative controls, mice were injected with PBS buffer ($n = 8$ mice per group). **(G)** Percentages of $IL4^+$ and $IFN\gamma^+$ iNKT cells as in **(F)**. Error bars represent SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. DR, dopamine receptor.

D1-like receptors, we blocked D1-like receptors with antagonist SCH23390. Although SCH23390 did not influence IL4 and IFNγ production in iNKT cells (**Figure 3C**), it abrogated the inhibitory effect of D1-like receptor agonist A68930 (**Figure 3D**). These results confirmed the specificity of agonist for D1-like receptors. All D1-like receptor agonists showed no influence on iNKT cell viability (**Figure S3B**). However, there is no selective agonist that could distinguish DRD1 from DRD5. Although mRNA of DRD1 and DRD5 were detected at similar level in iNKT cells, DRD5 rather than DRD1 was clearly detected at protein level (**Figure 3E**). Taken together, dopamine might inhibit cytokine responses in iNKT cells through D1-like receptors.

In order to investigate the role of dopamine in regulating iNKT cell functions *in vivo*, α-GalCer was injected into mice with or without D1-like receptor agonist A68930. A68930 significantly reduced percentages of $IL4^+$ and $IFN\gamma^+$ iNKT cells in livers and in spleens (**Figures 3F,G**). These results confirmed suppressive effect of dopamine on iNKT cell functions *in vivo*.

## Dopamine Inhibits iNKT Cell Functions via cAMP-PKA Pathway

D1-like receptors are coupled to $G_s$, which activates adenylyl cyclase and promotes production of cAMP (21). Consistent with previous studies, dopamine significantly increased cAMP level in activated iNKT cells (**Figure 4A**). cAMP has been shown to negatively regulate cytokine production in a variety of cells (22). It is possible that suppressive effect of dopamine was due to elevation of cAMP in iNKT cells. We increased intracellular cAMP concentration in iNKT cells with forskolin, a specific activator of adenylyl cyclase, and observed significant reduction of IL4 and IFNγ in a dose dependent manner (**Figure 4B**). Furthermore, cAMP analog 8-Br-cAMP inhibited IL4 and IFNγ production as well (**Figure 4C**). These results demonstrated that increased intracellular cAMP was responsible for the inhibitory effect of dopamine on iNKT cell functions. Protein kinase A (PKA) is a cAMP-dependent protein kinase, which has been previously shown to inhibit T cell functions (23). In our studies, we detected phosphorylation of CREB-S133 (**Figure 4D**), a substrate of PKA, in dopamine treated

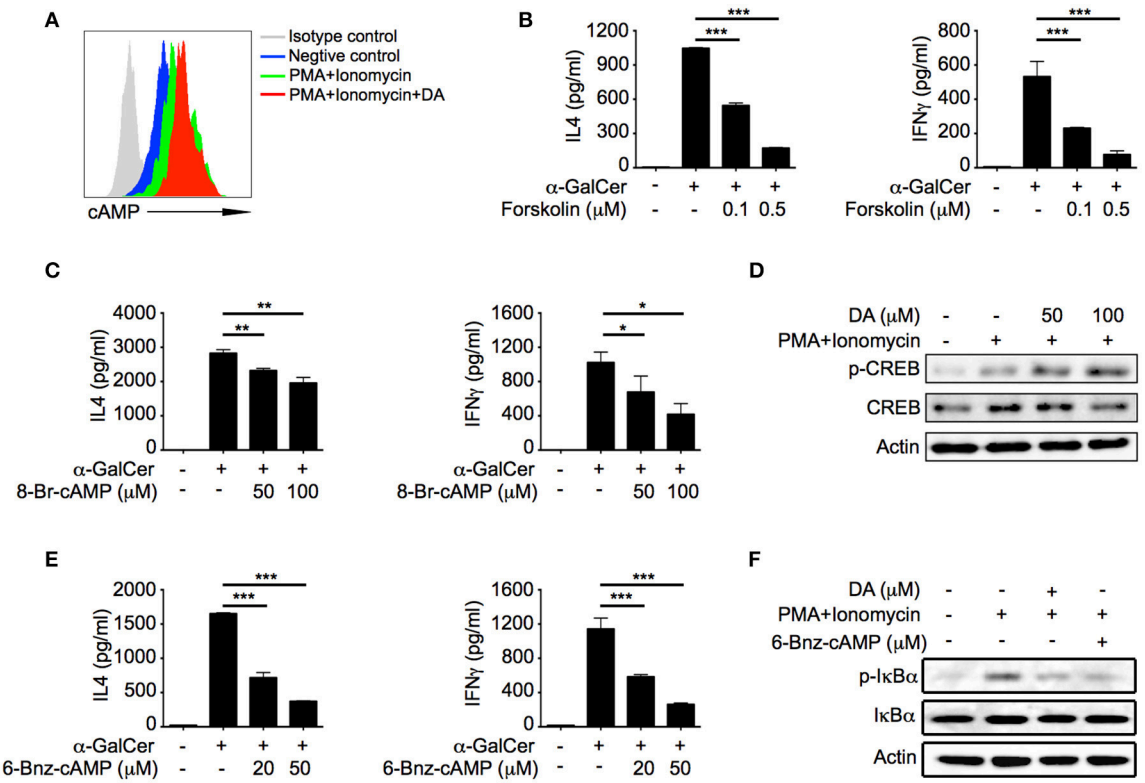


**FIGURE 4 |** Dopamine inhibits iNKT cell functions via cAMP-PKA pathway. **(A)** cAMP level in iNKT cells in the presence of indicated reagents. Gray line, isotype control; blue line, medium; green line, PMA plus Ionomycin; red line, PMA plus ionomycin plus dopamine (50 μM). **(B,C)** IL4 and IFNγ production in α-GalCer activated iNKT cells in the presence of different doses of forskolin **(B)** and 8-Br-cAMP **(C)**. **(D)** Immunoblot analysis of p-CREB, CREB, and β-actin in iNKT cells activated by PMA plus ionomycin in the absence or presence of dopamine. **(E)** IL4 and IFNγ production in α-GalCer activated iNKT cells in the presence of different doses of 6-Bnz-cAMP. **(F)** Immunoblot analysis of p-IκBα, IκBα, and β-actin in iNKT cells activated by PMA plus ionomycin in the absence or presence of dopamine (50 μM) or 6-Bnz-cAMP (50 μM). Data are representative of three independent experiments. Error bars represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PMA, phorbol myristate acetate; DA, dopamine.

cells, which indicated activation of PKA. To investigate whether dopamine inhibited production of cytokines in iNKT cells via activating PKA, we measured cytokine responses in the presence of 6-Bnz-cAMP, a specific activator of PKA. 6-Bnz-cAMP significantly inhibited IL4 and IFNγ production (**Figure 4E**). These results confirm that dopamine inhibits iNKT cell functions by activating cAMP-PKA pathway. Again, forskolin, 8-Br-cAMP, and 6-Bnz-cAMP did not increase the lactate dehydrogenase in medium (**Figure S3C**). It has been reported that PKA could inhibit TCR signaling via activating CSK (24, 25). However, dopamine suppressed cytokines in iNKT cells when TCR proximal signaling was bypassed by PMA plus ionomycin (**Figure 2C**). Next, we investigated the influences of dopamine and PKA activator on signal pathways downstream TCR. Activation of NFκB was significantly inhibited by dopamine and 6-Bnz-cAMP, as indicated by reduced phosphorylation of IκBα (**Figure 4F**). It has been shown previously that activation of NFκB is important for IL4 and IFNγ production in iNKT cells (26). Together, our results demonstrate an inhibitory effect of dopamine on iNKT cell functions via repressing NFκB signal pathway.

## D1-Like Receptor Agonists Inhibit iNKT Cell-Mediated Liver Injury

In agreement with the suppressive effects of A68930 on cytokine production in iNKT cells (**Figure 3D**), it dramatically inhibited Con A-induced IL4 and IFNγ production in hepatic iNKT cells (**Figure 5A**). Consistently, Con A-induced liver injury was significantly prevented by A68930, as indicated by reduced pro-inflammatory cytokine production in liver (**Figure 5B**), diminished ALT and AST (**Figures 5C**), and ameliorated histological damage (**Figure 5D**). In iNKT cell deficient *Jα18*$^{-/-}$ mice, Con A caused much less liver damage than in WT mice, as indicated by the lower levels of ALT and AST (**Figure 5E**). These results are consistent with previous findings that iNKT cells are main mediators of Con A-induced liver injury. Importantly, A68930 only reduced ALT and AST in WT mice but not in *Jα18*$^{-/-}$ mice (**Figure 5E**). Therefore, the suppressive effect of A68930 on Con A-induced liver injury is attributed to its inhibitory effect on iNKT cell functions.

Additionally, deficiency of dopamine caused severer liver damage after Con-A injection (**Figure 1**), which was abrogated by another D1-like receptor agonist A77636. In MPTP treated

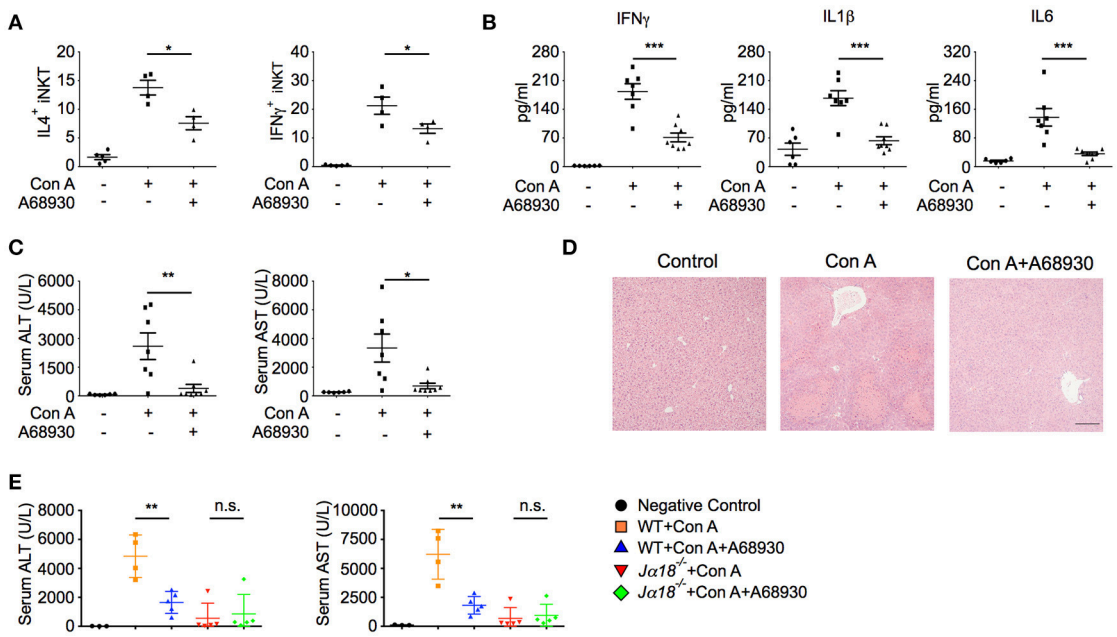


**FIGURE 5 |** A68930 inhibits Con A-induced liver injury. **(A–C)** Percentages of IL4$^+$ and IFNγ$^+$ iNKT cells in livers ($n$ = 4–5 mice per group), TNFα, IL1β, and IL6 in liver homogenates **(B)**, ALT and AST in serum **(C)** of mice injected with Con A (15 mg/kg) alone or with both A68930 (8 mg/kg) and Con A ($n$ = 6–8 mice per group). **(D)** Hematoxylin and eosin staining of liver tissues from mice described in **(B)**. Bar, 100 μm. **(E)** ALT and AST in serum of WT mice and *Jα18*$^{-/-}$ mice treated with indicated reagents ($n$ = 3–5 mice per group). Error bars represent SEM. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001. ConA, Concanavalin A; ALT, alanine transaminase; AST, aspartate aminotransferase.

mice, A77636 showed no influence on Con-A induced CD69 expression (**Figure 6A**) but reduced Con-A induced IFNγ production in hepatic iNKT cells (**Figure 6B**), diminished serum ALT and AST (**Figure 6C**), and ameliorated histological damage (**Figure 6D**). These results further confirmed that the exacerbated liver injury in MPTP mice was due to the insufficient dopamine.

## Gut Microbes Promote Dopamine Synthesis and Suppress Con A-Induced Liver Injury

In agreement with previous findings that peripheral dopamine mainly derives from gut (5, 6, 27), much higher level of tyrosine hydroxylase protein was detected in small intestines than in livers (**Figure 7A**). It has been shown that bacteria are able to either influence the production of neurotransmitters or generate many neurotransmitters directly including gamma-aminobutyric acid (GABA), norepinephrine (NE), and 5-hydroxytryptamine (5HT) (28). To investigate the influence of gut microbes on peripheral dopamine synthesis and hepatic iNKT cell functions, we cleared gut microbiota with antibiotic cocktail containing four antibiotics. Antibiotic cocktail successfully cleared major bacterial groups in feces, and co-housing antibiotics-treated mice with normal mice recovered gut microbiota (**Figure 7B**). Importantly, antibiotics significantly reduced tyrosine hydroxylase at mRNA level and protein level in small intestines, but showed no effect in livers (**Figures 7C,D**). Recovery of gut microbiota in antibiotics-treated mice restored expression of intestinal tyrosine hydroxylase (**Figures 7C,D**). These results indicate that gut microbes promote synthesis of dopamine in guts. Moreover, in agreement with the role of dopamine in suppressing iNKT cell functions *in vivo* (**Figure 1C**), clearance of gut microbes significantly elevated CD69 expression and IFNγ production in hepatic iNKT cells after Con A injection (**Figures 8A,B**). Furthermore, increased ALT and AST (**Figure 8C**), and exacerbated hepatocyte necrosis (**Figure 8D**) were observed in these mice without gut microbiota. Consistent with the recovery of intestinal tyrosine hydroxylase after co-housing (**Figures 7C,D**), co-housed mice reduced IFNγ production in hepatic iNKT cells and showed ameliorated liver damage in response to Con A (**Figures 8B–D**). Overall, in agreement with their role in promoting synthesis of peripheral dopamine, gut microbes suppress iNKT cell-mediated liver damage. Notably, in antibiotics-treated mice, replenishment of dopamine D1-like receptor agonist A77636 significantly reduced Con A-induced liver injury, as indicated by decreased IFNγ production in hepatic iNKT cells, diminished ALT and AST level, and improved histological damage (**Figures 7B–D**). These results further confirm that reduced dopamine synthesis in microbiota-cleared mice contributed to the severer iNKT cell-mediated liver injury.

## DISCUSSION

Dopamine has been previously shown as an immune modulator. Distinct effects of dopamine on T cells have been reported,

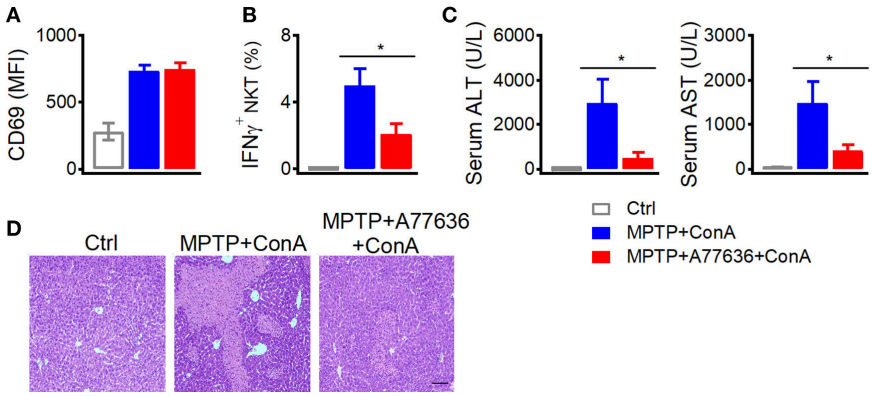


**FIGURE 6 |** A77636 inhibits Con A-induced liver injury in MPTP treated mice. **(A–D)** Mean fluorescence intensity of CD69 in hepatic iNKT cells **(A)**, percentages of IFNγ$^+$ hepatic iNKT cells **(B)**, ALT and AST in serum **(C)**, hematoxylin and eosin staining of liver tissues **(D)** from MPTP treated mice receiving Con A (15 mg/kg) or A77636 (8 mg/kg) plus Con A ($n$ = 4–5 mice per group). Bar, 100 μm. Error bars represent SEM. *P < 0.05. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MFI, mean fluorescent intensity; ConA, Concanavalin A; ALT, alanine transaminase; AST, aspartate aminotransferase.

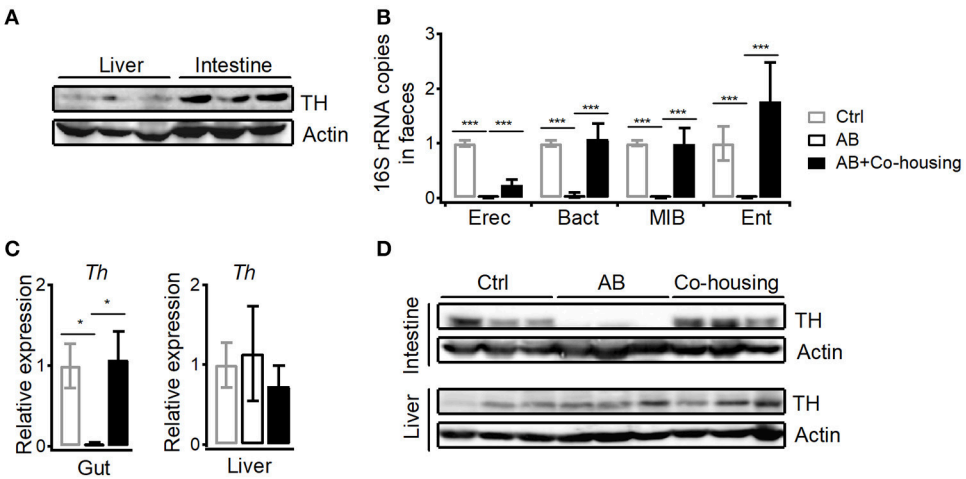


**FIGURE 7 |** Gut microbes promote dopamine synthesis in small intestines. **(A)** Expression of TH in livers and in small intestines. Data are representative of three independent experiments. **(B)** 16S rRNA of four bacterial groups in feces of control mice, antibiotics-treated mice, and antibiotics-treated mice co-housed with normal mice (*Eubacterium rectale-Clostridium coccoides*, Erec; *Bacteroides*, Bact; *mouse intestinal Bacteroides,* MIB; *Enterobacteriaceae,* Ent). **(C,D)** mRNA **(C)** and protein **(D)** of TH in small intestines and in livers from mice described in **(B)**. $n$ = 10–15 mice per group. Error bars represent SEM. *$P$ < 0.05, ***$P$ < 0.001. TH, tyrosine hydroxylase; AB, antibiotic; MFI, mean fluorescent intensity.

and those are mediated through different subtypes of receptor (29, 30). It has been reported that activation of D1-like receptors inhibits suppressive functions of regulatory T cells, and promotes differentiation of T helper 2 (Th2) and especially T helper 17 (Th17) in naïve CD4$^+$ T cells (31). Dopamine released by dendritic cells increases their IL12 and IL23 production through DRD5 in an autocrine manner, and consequently promotes Th17 differentiation (32), which contributes to inflammatory bowel diseases and multiple sclerosis. On the other hand, some studies report an inhibitory effect of dopamine on proliferation and cytotoxicity of CD4$^+$ and CD8$^+$ T cells through D1-like receptors (33). Interestingly, stimulation of DRD2 and DRD3, respectively elevates IL10 and TNFα production in resting human T cells (29), whereas activation of DRD3 reduces IL4 and IL10 mRNA but increases IFNγ mRNA in human activated CD4$^+$ and CD8$^+$ T cells (30). In addition to shaping T cell differentiation and function, dopamine could trigger T cell quiescence through DRD4, and that suggests a new way to treat lupus based on the higher expression of DRD4 in systemic lupus erythematosus patients (34, 35). Here, we show that dopamine represses IL4 and IFNγ production through D1-like receptors in iNKT cells (**Figure 3B**). D1-like receptors are coupled to $G_s$, which increases cAMP concentration via activating adenylyl cyclase, whereas D2-like receptors are coupled to $G_i$, which inhibits adenylyl cyclase and reduces intracellular cAMP (21). Although agonists of D1-like receptors could not distinguish

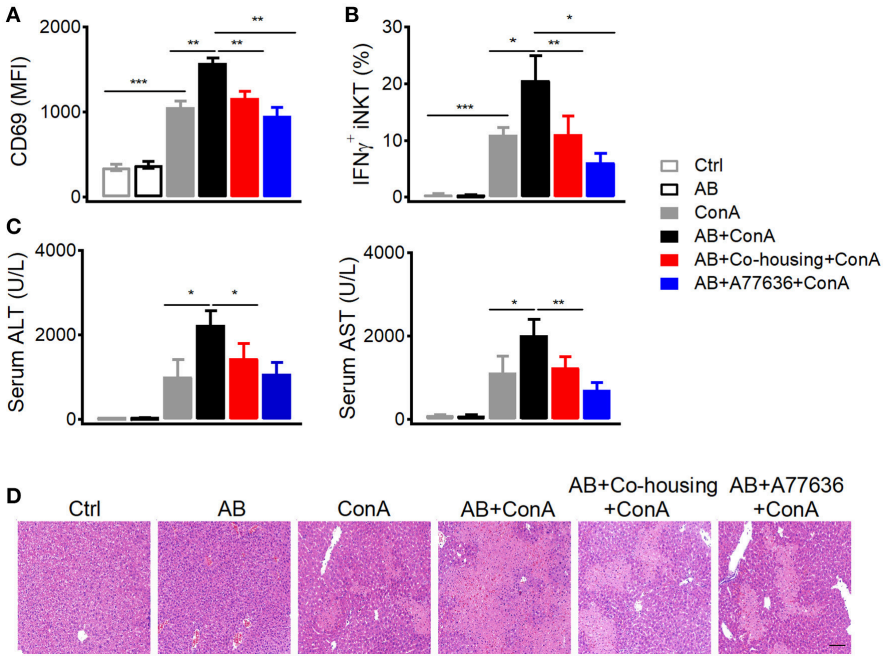


**FIGURE 8 |** Gut microbes inhibit Con A-induced liver injury. **(A–D)** Mean fluorescence intensity of CD69 in hepatic iNKT cells **(A)**, percentages of IFNγ$^+$ hepatic iNKT cells **(B)**, ALT and AST in serum **(C)**, hematoxylin and eosin staining of liver tissues **(D)** from indicated mice after Con A (15 mg/kg) or A77636 (8 mg/kg) plus Con A injection ($n$ = 10–15 mice per group). Bar, 100 μm. Error bars represent SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. AB, antibiotic; MFI, mean fluorescent intensity; ConA, Concanavalin A; ALT, alanine transaminase; AST, aspartate aminotransferase.

DRD1 from DRD5, we detected expression of DRD5 protein but not DRD1 protein in iNKT cells (**Figure 3E**), indicating a predominant role of DRD5 in mediating suppressive effect of dopamine. Although mRNA of DRD3 and DRD4 were detected in iNKT cells, their receptor agonists showed no effect on cytokine production. It is possible that protein levels of these receptors are not correlated with mRNA levels. Moreover, we show that dopamine inhibits cytokine production in iNKT cells by activating cAMP-PKA pathway downstream DRD5. It has been previously shown that PKA activates CSK and therefor inhibits Lck activation (24, 25). We do not exclude this possibility. Additionally, our results indicate that activation of PKA by dopamine could inhibit cytokine responses in iNKT cells in a TCR proximal signaling independent manner. When iNKT cells were activated by PMA plus ionomycin bypassing TCR signaling, activation of PKA significantly inhibited NFκB activation (**Figure 4F**), which is a key transcriptional factor regulating iNKT cell functions (36, 37).

As an important neurotransmitter, dopamine has been critically linked to major depressive disorder. Lower amount of dopamine has been detected in patients suffering from depression (38). On the other side, the potential association between depression and chronic liver diseases, such as alcoholic liver disease and non-alcoholic fatty liver disease, has been suggested by several studies (39). Chronic liver disease patients show more severe depressive tendencies (40). Additionally, influences of depression on non-alcoholic fatty liver disease have also been reported, and non-alcoholic fatty liver disease patients with depression show worse therapeutic outcomes (41). It is possible that dopamine could be a mediator linking depressive disorder to liver diseases. As important liver resident cells, pathogenic roles of iNKT cells have been demonstrated during the development of non-alcoholic fatty liver disease, alcoholic liver disease, and autoimmune liver disease (8, 42, 43). Here, we show suppressive effects of dopamine on IL4 and IFNγ production from hepatic iNKT cells, and demonstrate a protective role of dopamine in inhibiting iNKT cell-mediated autoimmune hepatitis. Our results are consistent with previous findings that IFNγ plays critical roles in Con-A induced liver hepatitis (20), and IL4 promotes iNKT cell-mediated liver injury via an autocrine fashion (18). According to previous studies, dopamine regulates other immune cell functions as well (44). However, those effects would be minor in our models, as indicated by the results that A68930 failed to improve Con A-induced liver injury in *Jα18*$^{-/-}$ mice (**Figure 5E**). Overall, our studies suggest that suppressive effect of dopamine on cytokine production in iNKT cells would help to keep the immune tolerance in liver, and reduction of dopamine might contribute to the liver injury.

Although dopamine is a neurotransmitter, it could be synthesized peripherally. We detected tyrosine hydroxylase in guts, which is in agreement with previous findings that high amount of dopamine is detected in hepatic portal vein (6). According to previous studies, multiple cells in liver are able to produce dopamine, including dendritic cells, regulatory T cells, B cells, macrophages, and autonomic nervous system (5). However, small intestines expressed much higher amount of tyrosine hydroxylase than livers (**Figure 7A**). These results indicate that guts are main source of dopamine for regulating

liver immunity, and explain the findings that although antibiotics reduced expression of tyrosine hydroxylase in small intestines but not in livers (**Figure 7D**), they significantly inhibited Con A-induced cytokine production in hepatic iNKT cells (**Figure 8B**). Gut microbiota has been previously shown to promote liver diseases via activating TLRs, modulating choline metabolism, and altering gut bile acids (45, 46). Conversely, our results (**Figures 8C,D**) and previous studies with bacteria free mice indicate a protective role of gut microbes in inhibiting liver injury (47). In addition to long chain fatty acids that have been shown to be synthesized by gut bacteria and reduce liver damage (48), we demonstrate a role of peripheral dopamine, synthesis of which is promoted by gut microbes, in suppressing liver injury. Due to the clearance of gut microbiota in our experiments, we could not exclude the possibility that some strains of bacteria might positively regulate the synthesis of dopamine, whereas other strains might regulate negatively. Moreover, it is still unclear whether the dopamine could be directly produced by gut microbes. It is rational to expect that dysbiosis of gut microbiota would influence biosynthesis of peripheral dopamine and consequently contributes to liver diseases.

In summary, we demonstrate that peripheral dopamine controlled by gut microbes inhibits IL4 and IFNγ production in iNKT cells and suppresses iNKT cell-mediated hepatitis. This microbes-dopamine-autoimmune hepatitis regulatory axis has to be further confirmed in human patients, and might provide new insight for clinical treatments.

## AUTHOR CONTRIBUTIONS

RX, HZ, JP, ZD, and WZ performed experiments. LB, RX, HZ, ZT, RZ, and ZZ designed the experiments. LB, RX, and HZ analyzed the data, prepared the figures, and wrote the manuscript. All authors read, commented, and approved final version of manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02398/full#supplementary-material

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# Immune Cell-Epithelial/Mesenchymal Interaction Contributing to Allergic Airway Inflammation Associated Pathology

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The primary function of the lung is efficient gas exchange between alveolar air and alveolar capillary blood. At the same time, the lung protects the host from continuous invasion of harmful viruses and bacteria by developing unique epithelial barrier systems. Thus, the lung has a complex architecture comprising a mixture of various types of cells including epithelial cells, mesenchymal cells, and immune cells. Recent studies have revealed that Interleukin (IL-)33, a member of the IL-1 family of cytokines, is a key environmental cytokine that is derived from epithelial cells and induces type 2 inflammation in the barrier organs, including the lung. IL-33 induces allergic diseases, such as asthma, through the activation of various immune cells that express an IL-33 receptor, ST2, including $ST2^+$ memory ($CD62L^{low}CD44^{hi}$) $CD4^+$ T cells. $ST2^+$ memory $CD4^+$ T cells have the capacity to produce high levels of IL-5 and Amphiregulin and are involved in the pathology of asthma. $ST2^+$ memory $CD4^+$ T cells are maintained by IL-7- and IL-33-produced lymphatic endothelial cells within inducible bronchus-associated lymphoid tissue (iBALT) around the bronchioles during chronic lung inflammation. In this review, we will discuss the impact of these immune cells-epithelial/mesenchymal interaction on shaping the pathology of chronic allergic inflammation. A better understanding of pathogenic roles of the cellular and molecular interaction between immune cells and non-immune cells is crucial for the development of new therapeutic strategies for intractable allergic diseases.

**Keywords: iBALT, pathogenic Th2 (Tpath2) cells, fibrosis, Amphiregulin (AREG), osteopontin (OPN, Spp1), inflammatory eosinophils**

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## MUCOSAL INFLAMMATION AND IBALT

### Induction of Inducible Bronchus-Associated Lymphoid Tissue (iBALT)

Pulmonary immunity is the first line of defense against pathogenic microorganisms and a unique local immune system that is distinct from systemic immunity. Following pulmonary inflammation, highly organized lymphoid structures known as iBALT are induced adjacent to the bronchus and next to a vein and artery. Similar to the secondary lymphoid organs (SLOs), iBALT consists of separated B cell areas with follicular dendritic cells (FDCs), where germinal centers can develop,

and T cell areas harboring dendritic cells (DCs) as well as high endothelial venules (HEVs). It has been proposed that iBALT serves as an inducible lymphoid tissue for pulmonary immune responses. Since iBALT is not formed in a pre-programed way and induced by various types of pulmonary inflammation, it has been recognized as the tertiary lymphoid organs. The induction of iBALT depends on the type of antigens and the magnitude of immune responses, and its cell number and size depend on the duration of antigenic exposure (1, 2). iBALT persists in the lung for months after inflammation resolved and plays an important role in the protection against re-invasion of pathogens.

A previous study examined the adaptive immune response of mice that forms iBALT but with the complete absence of SLOs in the body following inoculation with influenza virus (3). Surprisingly, mice without SLOs are still able to respond and clear a challenge infection, and influenza-specific virus-neutralizing antibodies are generated and maintained by the presence of iBALT. Thus, iBALT may be sufficient to protect against pathogens. It is known that modified vaccinia virus Ankara (MVA)-induced iBALT is able to become a general priming site against various pulmonary antigens [i.e., not only against the iBALT-initiating antigen but also against unrelated antigens (4)].

Given that the structure of iBALT is similar to that of conventional SLOs, there are many similarities in the molecular mechanisms underlying the development of these lymphoid organs. Lymphotoxin (LT)-alpha-deficient mice have defects in the development of LNs or Peyer's patches because of the reduction of homeostatic chemokines (CCL19, CCL21, and CXCL13) production by specialized stromal cells that express LTβR and tumor necrosis factor receptor 1 (TNFR1) (5–8). These homeostatic chemokines attract lymphocytes and promote lymphoid organ structure development. The development of iBALT also requires LT signaling, which leads to homeostatic chemokine production during chronic inflammation. CXCL13 and CCL19 have been shown to be controlled by LT-dependent mechanisms in a smoke-exposed chronic obstructive pulmonary disease (COPD) model (8). In addition, *plt/plt* mice, which lack CCL19 and CCL21, and *Cxcl13*$^{-/-}$ mice show attenuated iBALT formation after influenza infection. Furthermore, *Cxcl13*$^{-/-}$*plt/plt* mice fail to form detectable iBALT formations, suggesting that the combination of homeostatic chemokines is crucial for iBALT formation (9).

Several differential mechanisms exist in the development of iBALT and conventional lymphoid organs. For example, the transcriptional regulators inhibitor of DNA binding 2 (Id2) and RAR-related orphan receptor gamma t (RORγt), which are essential for the differentiation of lymphoid tissue inducer cells and organogenesis of peripheral lymph nodes and Peyer's patches, are dispensable for iBALT organogenesis (10–13). Formation of both iBALT and omentum milky spots were associated with CXCL13, but this was dispensable for tear-duct associated lymphoid tissue (TALT) (11, 14, 15). Interestingly, it has been reported that the expression of CXCL13 and CCL19 is controlled by IL-17-producing Th17 cells and is required for iBALT development, whereas LT signaling is required in the maintenance of iBALT formation (11). Furthermore, iBALT organogenesis has been shown to be induced by CD11b$^{+}$CD11c$^{+}$ DCs, which produce homeostatic chemokines and LT in response to influenza or vaccinia virus infection (4, 16). In addition, CCR7$^{+}$ Treg cells have been reported to suppress the development of iBALT (17).

Specialized stromal cells and lymphatic vessels are found in iBALT and play a critical role in the regulation of iBALT structure and various immune function. For example, in the B cell area of iBALT, there is a network of CXCL13-expressing FDCs as well as CXCL12-producing follicular stromal cells (18). In contrast to CXCL12 and CXCL13, CCL21 is found on HEVs in T cell areas but not in B cell follicles. Similar to the secondary lymphoid organs, lymphatics are developed in the T cell area and surrounding B cell follicle in the iBALT and likely facilitate the uptake of antigens (19–21). However, it is difficult to determine histologically whether these lymphatics are afferent and carry antigens and lymphocytes from distal portions of the lung toward iBALT or whether efferent and carry lymphocytes from iBALT and return them to the general circulation.

A previous report showed by the live imaging of *ex vivo* lung tissue that labeled antigen-pulsed DCs can migrate from airways into iBALT and prime T cell responses (4), suggesting that DC migration to iBALT is an important mechanism by which antigens are acquired for local immune responses. Epithelial antigen-transporting M cells are detected in iBALT of some species (22–24) and are also involved in the uptake of antigens in iBALT.

Several mechanisms underlying the recruitment of immune cells into already developed iBALT structure have been reported. The migration of naive T cells to lymph nodes via HEVs was shown to be mediated by adhesion molecules, such as integrins or selectins, as well as by chemokine receptors (25). Similarly, the migration of lymphocytes into iBALT is mediated by adhesion molecules and chemokines. Lymphocyte homing is controlled by L-selectin/Peripheral node addressin (PNAd), α4β1 integrin/Vascular cell adhesion molecule 1 (VCAM1), and the lymphocyte function-associated antigen 1 (LFA-1) adhesion pathway, whereas α4β7 integrin and Mucosal vascular addressin cell adhesion molecule (MAdCAM) are not involved in the spontaneous iBALT formation of autoimmune-prone NOD mice (26). Similar mechanisms have been found in iBALT associated with lung carcinoma in which B cells and some T cells expressed L-selectin and α4 integrin, suggesting that these adhesion molecules are involved in the migration of lymphocytes into human iBALT (27). Relevance of chemokine receptors on iBALT-infiltrating lymphocytes has been shown to be involved in trafficking of lymphocytes toward and within the lung (28, 29). In addition, multiple chemokines (CCL17, CCL19, CCL21, CCL22, CXCL13, and IL-16) are expressed in tumor-induced iBALT in lung cancer patients and their respective receptors (CCR7, CCR4, and CXCR5) are expressed on T cells within iBALT. Interestingly, we previously reported that specialized Thy1$^{+}$ lymphatic endothelial cells in iBALT have the ability to produce IL-7 as well as the T cell attractant chemokines CCL19 and CCL21 (30) (**Figure 1**). Furthermore, these Thy1$^{+}$IL-7$^{+}$ lymphatic endothelial cells also produce IL-33, an inflammatory

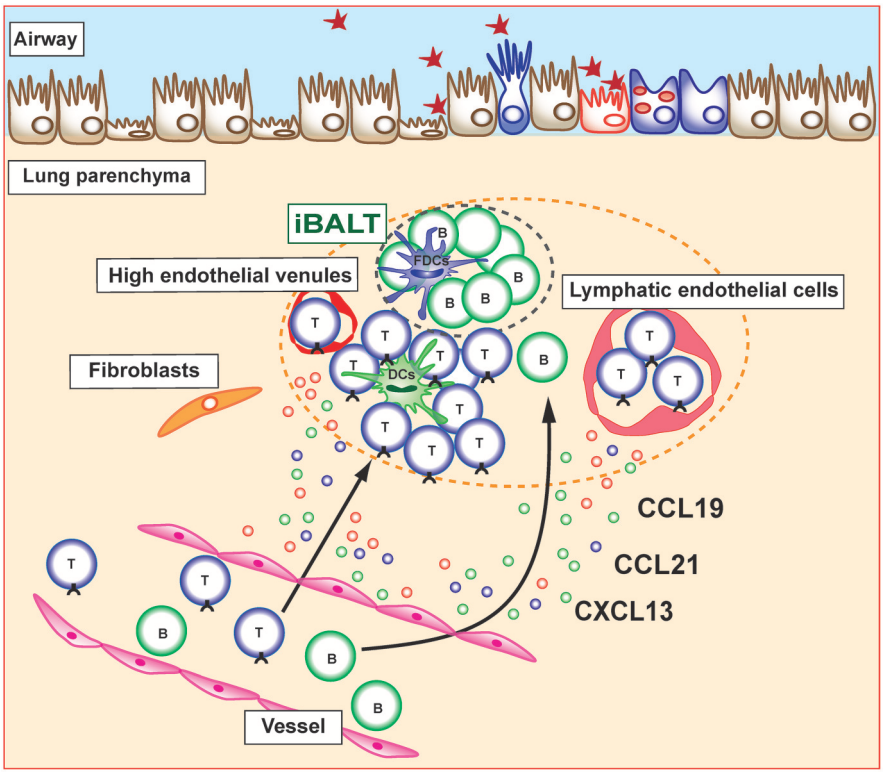


**FIGURE 1 |** Induction and maintenance of inducible bronchus-associated lymphoid tissue (iBALT). Inducible bronchus-associated lymphoid tissue (iBALT) is formed in the lung during chronic inflammation and consist of various type of immune cells, including T cells, B cells, dendritic cells (DCs), and follicular dendritic cells (FDCs). CCL19, CCL21, and CXCL13 are key chemokines for the induction and maintenance of iBALT structure. Stromal cells including fibroblasts, vascular endothelial cells, and lymphatic endothelial cells are the major producers of these chemokines in the lung parenchyma.

cytokine involved in the pathogenesis of allergic immune responses. It is expected that there are other unknown stromal cells that produce lymphocyte attractant chemokines within iBALT and regulate the trafficking of lymphocytes into iBALT. Further detailed analyses focusing on their characterization are required.

For the analysis of iBALT, various methods associated with iBALT formation have been investigated. BALT is not detectable in the lungs of naïve mice, but it has been reported that spontaneous iBALT formation is found in chemokine receptor CCR7-deficient mice (17). iBALT can also be induced by the intranasal administration of viruses, including influenza virus, poxvirus MVA, and murine herpes virus-68 (4, 20, 31). Furthermore, lipopolysaccharide (LPS) intranasal administration in newborn mice (11) or repetitive inhalations of heat-killed bacteria (18, 32) induce iBALT. iBALT formation has also been shown to be associated with chronic airway inflammation. Mouse models of chronic cigarette smoke-induced COPD model or severe antigen-induced experimental asthma model are also associated with iBALT formation in the lungs (33), and the iBALT areas increase with increased antigen exposure. The types of inflammation-inducing antigen appear to determine the cellular component that forms and is required for the maturation of iBALT (18). For instance, mice intranasally treated with poxvirus MVA develop highly organized iBALT with densely packed B cell follicles containing a network of FDCs with CXCL12-producing follicular stromal cells. In contrast, *Pseudomonas aeruginosa* treatment leads to iBALT without FDCs but still containing CXCL12-positive follicular stromal cells within B cell follicles. Thus, the type of antigen or magnitude of inflammation may affect the nature of iBALT formed in the lung.

Further detailed analyses of classification of iBALT are required to explore their distinct function in pulmonary immunity.

## Pathologic Roles of iBALT in Allergic Airway Inflammation: The Interaction of Pathogenic Th2 (Tpath2) Cells and Lymphatic Endothelial Cells in iBALT

In contrast to the protective role of iBALT in infectious diseases, iBALT structures are frequently detected in the lung tissue obtained from patients with asthma (2, 34) and are evident in mouse models of experimental asthma (30, 35, 36). It has been shown in clinical analyses that the cases with asthma tend to have larger and greater numbers of lymphoid cell aggregates in the lung than non-asthma cases, regardless of asthma severity (34). More recently, iBALT-like lymphoid structures were detected in

a biopsy of the inner bronchial wall in mild asthma patients (37). Several mouse models for allergic airway inflammation have been shown to be accompanied by the formation of iBALT. We and others have found that the induction of severe allergic lung inflammation by transferring highly Th2-polarized respiratory allergen-specific TCR transgenic CD4 T cells followed by repeated intranasal challenges with allergen induced iBALT in the lung (30, 35). The overexpression of IL-5 in mice was reported to develop iBALT formation, although whether or not this response is controlled via a direct effect on B cells remains to be determined (38). A histological examination of the lung treated with anti-IL-4 and anti-IL-13 antibodies also revealed that IL-4 and IL-13 have the potential to induce iBALT formation (35). Thus, it appears that allergic lung inflammation facilitates the formation of iBALT, which is involved in the pathogenesis of asthma.

Accumulating evidence has shown that iBALT is the structure supporting the maintenance of immunological memory. In a mouse model of allergic lung inflammation, antigen-specific IgE-secreting plasma cells have been shown to reside adjacent to the germinal centers in iBALT (39). Similarly, patients with allergic bronchopulmonary aspergillosis also develop iBALT areas, some of which have allergen-specific IgE-expressing B cells in the germinal centers (40). Furthermore, we recently demonstrated that the lung-resident antigen-specific memory Th2 cells preferentially reside in iBALT and are sufficient to induce asthmatic symptoms (30). Similarly, it has also been shown that lung-resident memory Th2 cells are sufficient to induce airway hyperresponsiveness by experimental blockade of lymphocyte migration (41). Unique clinical evidences from the patients with lung transplantation contributed to prove the concept proposed from mice experiments (42). The recipients without chronic airway inflammation developed asthma after the transplantation of the asthmatic lung. In contrast, the recipient with chronic airway inflammation did not suffer from asthma after the non-asthmatic lung transplantation. These findings suggest that asthma is a local disease and iBALT formation is involved in it.

Memory-type pathogenic Th2 cells are the principal cell population responsible for the pathogenesis of chronic allergic inflammation and the rapid relapse of acute allergic inflammation upon allergen re-exposure in mice (36). The areas of iBALT may efficiently collect antigen-bearing DCs or macrophages via lymphatics or by other mechanisms, promoting their concentration in areas devoted to T and B cell priming and simultaneously depleting them from the rest of the lung. In fact, plasmacytoid DCs in patients with asthma or moderate COPD are found to be concentrated in iBALT areas of the lung (43) where they may promote the local differentiation of Tregs. The mechanisms that regulate the maintenance of lung-resident memory Th2 cells have been incompletely defined. Tissue-resident memory CD4 T cells have been considered to be actively transported to the SLOs in order to interact with IL-7-producing stromal cells (44). However, parabiosis experiments in which mouse pairs are surgically conjoined to create shared circulation systems provide direct evidence for the retention of memory CD4 T cells in lung tissues (45). We recently clarified that the maintenance of lung-resident antigen-specific memory Th2 cells is dependent on IL-7-producing lymphatic endothelial cells (LECs), which localize within iBALT (30). In fact, antigen-specific memory Th2 cells are maintained in an IL-7-dependent manner but with an antigen-independent mechanism. Furthermore, Th2 cells, which are not specific to iBALT-initiating antigen, are still maintained within the iBALT and strongly contribute to the pathology of local pulmonary inflammation. Notably, IL-7-producing LECs increase their number after lung inflammation with the increased formation of iBALT, consistent with the fact that robust lymphangiogenesis occurs in mouse lungs after pulmonary infection and the lymphatic network is restricted to regions of iBALT (46). Thus, it appears that allergic asthmatic responses can cause iBALT formation and modify the lung microenvironment to promote the survival of antigen-specific memory Th2 cells within iBALT. Interestingly, the analysis of IL-7-producing LECs reveals the unique characteristic of this cell population as a producer of IL-33. It has been shown that IL-33 directly instructs memory type pathogenic Th2 (Tpath2) cells to enhance IL-5 production and induces eosinophilic inflammation (47). Interestingly, the adoptive transfer of *Il-1rl1*$^{-/-}$ Th2 cells, which have a defective expression of IL-33 receptor (ST2), can induce the formation of iBALT and thus can be maintained within the structure, but the ability to produce IL-4, IL-5, and IL-13 in *Il-1rl1*$^{-/-}$ Th2 cells dramatically decreases (48). This result shows that the IL-33-ST2 signaling pathway is critical for ensuring that Tpath2 cells maintain the ability to secrete Th2 cytokines. Thus, IL-7- and IL-33-producing LECs may provide a niche for Tpath2 cells to maintain their survival and pathogenicity in the lung (**Figure 2**). These findings suggest that IL-7- and IL-33-producing LECs within iBALT contribute to the trafficking, activation, and survival of Tpath2 cells and have a potential regulatory function in chronic allergic airway inflammation.

In fact, ectopic lymphoid structures are often developed within upper-respiratory airways with inflammation. Eosinophilic chronic rhinosinusitis (ECRS) is a chronic upper-respiratory airway allergic disease characterized by massive eosinophilic infiltration and high IL-5 levels associated with the formation of recurrent nasal polyps (49). We detected memory Th ($CD45RO^{+}CD4^{+}$) cells along with antigen-presenting cells and IL-7 and IL-33-producing LECs in ectopic lymphoid tissues of patients with ECRS, similar to that found in iBALT in mice (30). These findings suggest that similar cellular and molecular mechanisms underlying the maintenance of immunological memory at ectopic lymphoid structures may operate in the upper- and lower-respiratory airway, which is involved in the pathogenesis of chronic airway inflammation.

## PATHOGENESIS OF TISSUE FIBROSIS IN ALLERGIC AIRWAY INFLAMMATION

Memory Th2 cells are considered to be a key cell population in the pathogenesis of chronic inflammatory disorders including asthma. However, the specific subpopulations of memory Th cells and the factors that induce chronic airway inflammation, particularly fibrotic responses, remain unclear due to limited numbers of antigen-specific memory Th cells recoverable from

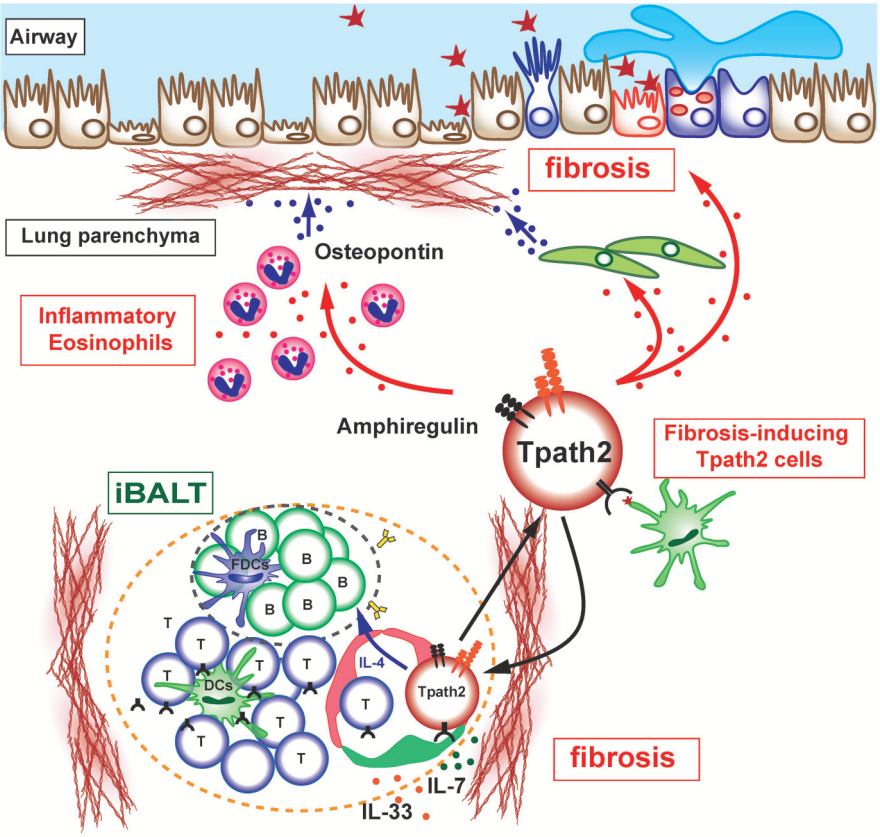


**FIGURE 2 |** Pathogenic interaction between immune cells and epithelial/mesenchymal cells in allergic airway inflammation. The selective localization and survival of memory-type Tpath2 cells within iBALT are supported by IL-7$^{+}$ lymphatic endothelial cells (LECs). These IL-7$^{+}$ LECs are also IL-33$^{+}$ and may therefore induce memory Th2 cells to be more pathogenic. A subpopulation of memory-type pathogenic Th2 (Tpath2) cells produces Amphiregulin, reprogramming eosinophils to the inflammatory state via the production of osteopontin and facilitating the fibrotic responses in the airway.

inflammatory respiratory tissues. Several investigators, including us, have recently identified phenotypically and functionally distinct memory Th2 cell subsets that produce large amounts of IL-5 in addition to IL-4 and IL-13 (36, 50, 51). Amphiregulin, a ligand for epithelial growth factor receptor (EGFR), has crucial roles in inflammation, tissue repair and fibrotic responses (52). Amphiregulin has been reported to be produced by effector Th2 cells (53). We recently identified an Amphiregulin-producing subpopulation of memory Tpath2 cells that is critical for shaping the pathology of fibrosis during chronic allergic inflammation (54). We found that Amphiregulin is a crucial mediator in the induction of fibrotic responses in chronic allergic inflammation. Amphiregulin-EGF receptor (EGFR)-mediated signaling promotes the reprogramming of eosinophils to an inflammatory state with enhanced production of osteopontin, a major component of the non-collagenous ECM in fibrotic tissues and a contributor to the pathogenesis of fibrosis (**Figure 2**). Amphiregulin also stimulates lung epithelial cells to undergo proliferation and enhanced mucin production (53, 55) and promotes the proliferation of lung fibroblasts (56). Moreover, Amphiregulin by macrophage induces TGF-β activation and the trans-differentiation of pericytes into myofibroblasts (57). Thus, our findings indicate that the cellular and molecular interaction between immune cells and non-immune cells is crucial for the development of fibrotic responses in the lung during chronic allergic inflammation.

Various types of immune cells including effector Th2 cells, regulatory T cells, macrophages, and ILC2s contribute to the tissue homeostasis in different organs. In the liver, Amphiregulin is associated with liver regeneration and fibrotic responses (58). In the heart, Amphiregulin from Ly6C$^{lo}$ macrophages is involved in the adaptive response to pressure overload (59). Moreover, recent research showed that Amphiregulin from Treg cells contributes to the suppression of neurotoxic astrogliosis in the brain (60). We await careful studies to elucidate the physiological and pathogenic roles of Amphiregulin in various tissues.

## CONCLUSION

We briefly reviewed the impact of the immune cell-epithelial/mesenchymal interaction on the pathology of chronic allergic inflammation. Tertiary lymphoid tissue, such as iBALT, is a key structure involved in the maintenance of memory T cells at mucosal sites. These ectopic lymphoid structures and the

heterogeneity of memory-type pathogenic T (Tpath) cells may contribute to the induction of resistance to certain therapies that is observed in various immune-mediated inflammatory diseases. The dysregulation of type 2 immune responses via various stimuli among immune cells and epithelial/mesenchymal cells at mucosal sites induces many allergic inflammatory diseases, such as asthma. It will therefore become increasingly important to understand the precise roles played by immune cells and non-immune cells in overcoming intractable immune-mediated inflammatory diseases.

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

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REVIEW
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# Coordination of Immune-Stroma Crosstalk by IL-6 Family Cytokines

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Stromal cells are a subject of rapidly growing immunological interest based on their ability to influence virtually all aspects of innate and adaptive immunity. Present in every bodily tissue, stromal cells complement the functions of classical immune cells by sensing pathogens and tissue damage, coordinating leukocyte recruitment and function, and promoting immune response resolution and tissue repair. These diverse roles come with a price: like classical immune cells, inappropriate stromal cell behavior can lead to various forms of pathology, including inflammatory disease, tissue fibrosis, and cancer. An important immunological function of stromal cells is to act as information relays, responding to leukocyte-derived signals and instructing leukocyte behavior in kind. In this regard, several members of the interleukin-6 (IL-6) cytokine family, including IL-6, IL-11, oncostatin M (OSM), and leukemia inhibitory factor (LIF), have gained recognition as factors that mediate crosstalk between stromal and immune cells, with diverse roles in numerous inflammatory and homeostatic processes. This review summarizes our current understanding of how IL-6 family cytokines control stromal-immune crosstalk in health and disease, and how these interactions can be leveraged for clinical benefit.

**Keywords: stromal cells, cytokines, inflammation, fibrosis, immune**

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## THE DIVERSE ROLES OF STROMAL CELLS IN IMMUNITY AND INFLAMMATION

The term "stroma" refers to the non-parenchymal components of tissues that form a supportive matrix in which parenchymal cells reside (1). While a confusingly broad array of cell types have been described as "stromal cells," in this review they are defined as non-hematopoietic, non-epithelial mesenchymal cells, including fibroblasts, myofibroblasts, bone marrow stromal cells, and the specialized fibroblast-like stromal cells of secondary lymphoid organs. Other mesenchymal populations such as endothelial cells, adipocytes, and muscle cells, while of great interest, are largely omitted from this discussion for the sake of brevity and clarity. Long considered to be mere structural entities without specialized functions, an explosion of data in the last two decades has established stromal cells as key regulators of both protective and pathological immune responses (2).

Regulation of immune function by stromal cells has been most extensively studied in the context of secondary lymphoid organs. First identified in 1992, podoplanin (PDPN)$^+$ fibroblastic reticular cells (FRC) form a dense reticular network in lymph nodes that facilitates leukocyte migration and antigen presentation (2–5). By producing soluble chemokines, cytokines, and other factors—such as CCL19 (C-C motif chemokine ligand 19), CCL21, and IL-7 (interleukin 7)—FRC are crucial for controlling leukocyte recruitment, survival, and proliferation. FRC-like stromal cells play similar roles in other lymphatic tissues, such as in tertiary lymphoid organs of the intestinal mucosa (6, 7).

In non-lymphoid tissues, stromal cells can exert similar effects to those of the secondary lymphoid organs by acting as scaffolds for leukocyte migration and by producing a diverse array of cytokines and chemokines (2).

Importantly, the immunological functions of stromal cells can vary substantially depending on their host organ and physiological context. For example, lymph node FRC recruit CCR7 (C-C chemokine receptor type 7)$^+$ T cells (naïve and central memory) and CCR7$^+$ dendritic cells (DC) to lymph nodes by producing the chemokines CCL19 and CCL21, as well as the pro-survival cytokines IL-7 and IL-15, thereby coordinating T cell activation and maintenance (4). In contrast, stromal cells in peripheral tissues generally lack expression of CCL19 and CCL21; accordingly, naïve and central memory T cells are infrequent in the periphery. However, expression of various pattern recognition and cytokine receptors by non-lymphoid tissue stromal cells allows them to sense microbial molecules and endogenous danger signals (1, 8, 9). In response, they produce chemokines [including CCL20 and CXCL10 (C-X-C motif chemokine ligand 10)] that attract effector T cells to sites of inflammation. Furthermore, inducible expression of leukocyte adhesion molecules including ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) allows tissue-resident stromal cells to further influence the balance between leukocyte recruitment, retention, and recirculation (1, 2, 9). Finally, stromal cells contribute directly to immune response resolution and tissue repair, the latter being one of their best studied functions. Examples of "pro-resolution" factors produced by stromal cells include NOS2 (nitric oxide synthase 2) and NO (nitric oxide), which are released by lymph node FRC to constrain T cell proliferation (10–12), and IDO1 (indoleamine 2,3-dioxygenase 1) produced by peripheral stromal cells, which similarly limits T cell proliferation by depleting the critical T cell metabolite tryptophan (13, 14). Thus, stromal cells in different tissues collectively regulate the strength, quality, and duration of immune responses via diverse and complementary mechanisms.

As with most immunological processes, communication between stromal and immune cells is highly dependent on cytokines. Stromal cells bear receptors to a variety of biologically diverse cytokines that represent virtually all branches of innate and adaptive immunity, including innate inflammatory cytokines [e.g., TNF (tumor necrosis factor) and IL-1β], Th1 cytokines [e.g., IFN-γ (interferon gamma)], Th2 cytokines (e.g., IL-13), Th17 cytokines (e.g., IL-17A), and tolerogenic cytokines [e.g., TGF-β (transforming growth factor beta)] (7, 9, 15, 16). In turn, stromal cells can be prodigious producers of other cytokines and chemokines, such as IL-6 (1, 2, 7, 9). In recent years, cytokines of the IL-6 family have gained increasing attention for their roles in various homeostatic and pathological processes, which in many cases can be attributed to their ability to co-ordinate immune-stroma crosstalk. This review aims to provide a focused update on the contributions of IL-6 family members to immune-stromal interactions.

## AN OVERVIEW OF THE IL-6 CYTOKINE FAMILY

The IL-6 family includes IL-6, IL-11, IL-27, IL-31, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine factor 1 (CLCF1) (17, 18). With the exception of IL-27, which is a heterodimeric protein comprised of IL-27p28 and EBI3 (Epstein-Barr virus-induced gene 3) (19), IL-6 family members are compact 4-helix bundle cytokines made from a single polypeptide. Glycoprotein 130 (gp130, encoded by the *IL6ST* gene) is a crucial receptor subunit utilized by all members of the IL-6 family except IL-31. While gp130 expression is relatively ubiquitous in a wide variety of tissues and organs, cell-type specificity for different IL-6 family members is bestowed by the more restricted expression patterns of ligand-specific co-receptors, including IL-6R (IL-6 receptor), IL-11R (IL-11 receptor), IL-27Rα (IL-27 receptor alpha), OSMR (OSM receptor), LIFR (LIF receptor), and CNTFRα (CNTF receptor alpha). Three distinct forms of receptor-ligand complexes have been described (**Figure 1**). First characterized was that of IL-6, which engages IL-6R along with two subunits of gp130. Intriguingly, although this implies the formation of a trimeric complex, a series of cooperative interactions can ultimately produce an interlocked hexamer comprised of two subunits each of IL-6, IL-6R, and gp130 (20). A similar structure is likely formed in response to IL-11/IL-11R interaction (21, 22). In this arrangement, only gp130 drives signal transduction, due to an absence of intracellular signaling motifs in IL-6R and IL-11R. In contrast, OSMR, LIFR, and IL-27Rα form heterodimers with gp130 in the presence of their cognate ligands (23–28). Unlike IL-6R and IL-11R, OSMR, LIFR, and IL-27Rα are capable of driving signal transduction via their own suite of signaling motifs. Finally, CNTF and CLCF1 drive formation of a trimeric complex that includes gp130, LIFR, and CNTFRα (29–31). The gp130-independent outlier of the family, IL-31, engages a heterodimeric complex of IL-31Rα (previously known as gp130-like receptor) and OSMR (18). Notably, while mouse OSM binds with high affinity only to the gp130/OSMR heterodimer, human and rat OSM can bind with high affinity to either gp130/OSMR or gp130/LIFR heterodimers (32–34). Thus, in rats and humans, manipulation of LIFR would be expected to affect both OSM and LIF signaling (as well as CLCF1, CT-1, and CNTF), while manipulation of OSMR would influence OSM and IL-31 signaling. As a corollary, changes in human or rat OSM bioavailability would influence cells that express OSMR and/or LIFR, while changes in LIF or IL-31 would affect only LIFR- or IL-31Rα-expressing cells, respectively.

All members of the IL-6 family drive signal transduction via receptor-associated Janus kinases (primarily JAK1 and JAK2), which phosphorylate a variety of conserved tyrosine residues in the cytoplasmic domains of signaling receptor subunits (gp130, OSMR, LIFR, IL-27Rα, and IL-31Rα) (17, 18, 35). Several downstream signaling pathways are activated in response, including signal transducer and activator of transcription

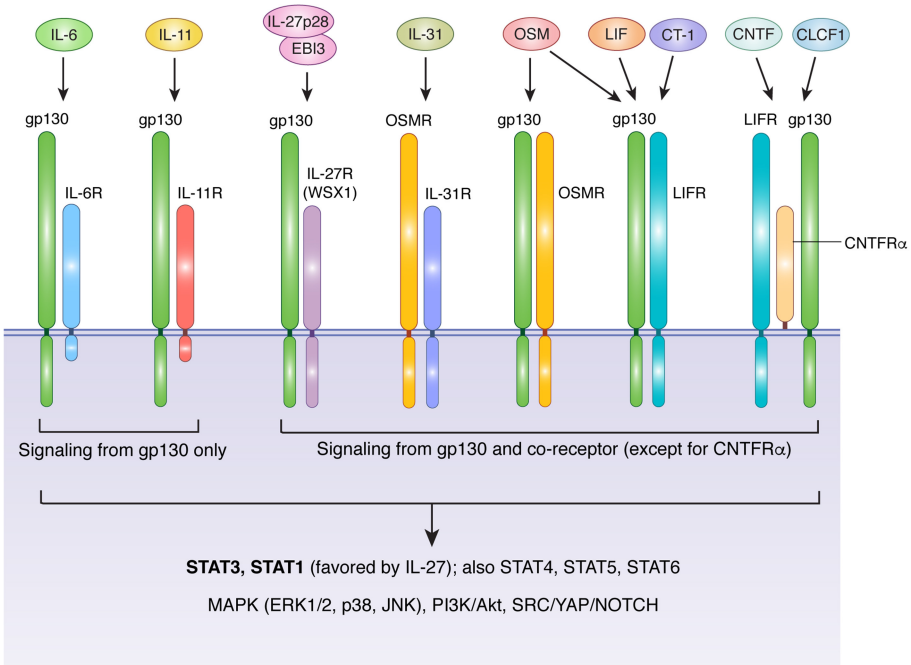


**FIGURE 1 |** Receptor usage of IL-6 family cytokines. With the exception of IL-31, IL-6 family cytokines transduce signals via receptor complexes that include gp130 and one or more additional ligand-specific subunits. IL-6 and IL-11 signaling requires IL-6R and IL-11R, respectively. The cytoplasmic domains of these receptor are short and lack signaling motifs, making gp130 the sole source of signal transduction downstream of IL-6 and IL-11. The heterodimeric cytokine IL-27 (comprised of IL-27p28 and EBI3) requires a complex of gp130 and IL-27RA. LIF and CT-1 use a heterodimeric complex of gp130 and LIFR, while CNTF and CLCF1 signal via a trimeric complex of gp130, LIFR, and CNTFRα, a GPI-anchored protein that does not directly contribute to signaling beyond facilitation of ligand binding. OSM displays species-specific receptor usage. In humans and rats, OSM signals via either gp130/OSMR or gp130/LIFR complexes, while in mice OSM is primarily recognized by OSMR. IL-31 does not require gp130, and instead uses a complex of OSMR and IL-31R. Aside from IL-6R, IL-11R, and CNTFRα, all receptors in the IL-6 family are capable of directly activating signal transduction in response to ligand binding. IL-6 family cytokines employ classical JAK-mediated signaling. Major downstream mediators include STAT3 (the main STAT for all except IL-27), STAT1 (activated preferentially by IL-27 and to a lesser extent by other IL-6 family members), additional STATs that depend on cell type and physiological context (including STATs 4, 5, and 6), the MAPK cascade, PI3K/Akt/mTOR signaling, and SRC/YAP/NOTCH signaling. Akt, protein kinase B; CLCF1, cardiotrophin-like cytokine factor 1; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin 1; EBI3, Epstein-Barr virus induced 3; ERK, extracellular signal-regulated kinase; gp130, glycoprotein 130, also known as IL-6 signal transducer; IL, interleukin; IL-6R, IL-6 receptor; IL-11R, IL-11 receptor; IL-27RA, IL-27 receptor; CNTFRα, CNTF receptor; LIF, leukemia inhibitory factor; LIFR, LIF receptor; MAPK, mitogen activated protein kinase; JAK, janus kinase; JNK, c-jun n-terminal kinase; mTOR, mammalian target of rapamycin; OSM, oncostatin M; OSMR, OSM receptor; PI3K, phosphatidylinositol-3-kinase; STAT, signal transducer and activator of transcription; SRC, Proto-oncogene tyrosine-protein kinase Src; YAP, yes-associated protein.

(STAT) proteins (including STAT1, STAT3, STAT4, STAT5, and STAT6), the mitogen-activated protein kinase (MAPK) cascade, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, and the SRC/YAP/NOTCH pathway (**Figure 1**). While signal transduction by individual IL-6 family members is broadly similar, the relative strength of activation of specific pathways can differ depending on the cytokine, cell type, and physiological context. For example, unlike gp130, OSMR efficiently recruits the adapter protein SHC, allowing OSM to drive more potent activation of the MAPK pathway than IL-6, which signals via SHP-2 bound to gp130 (35, 36). Similarly, although STAT3 is generally considered to be the dominant STAT protein activated by the IL-6 family, IL-27 preferentially activates STAT1 (37). Further complexity is provided by the capacity of IL-6, IL-11, and CNTF to signal via soluble receptor forms in a process known as *trans* signaling. In this process, soluble versions of IL-6R, IL-11R, or CNTFRα are produced either through proteolytic cleavage of membrane-bound receptors, or via expression of alternatively spliced mRNA; in either case, the soluble receptor form can dimerize with its cognate ligand in solution, and subsequently produce a functional signaling complex in association with membrane-bound gp130 (18, 38–40). Cells thus require only gp130 to be sensitive to *trans* signaling, which allows many cell types that lack IL-6R, IL-11R, or CNTFRα to respond to these cytokines. In the case of IL-6, *trans* signaling is thought to be a critical mechanism by which IL-6 promotes disease pathogenesis, particularly arthritis and colorectal cancer (18, 41, 42). Thus, while many similarities exist between IL-6 family cytokines, differences in their receptor usage, signal transduction profiles, and patterns of receptor expression collectively foster a substantial degree of functional pleiotropy. Indeed, IL-6 family members influence cell survival, proliferation, differentiation, metabolism, and migration, thus contributing to a plethora of physiological processes that are critical for both homeostasis and pathology.

## EXPRESSION OF IL-6 FAMILY CYTOKINES BY STROMAL CELLS

Although some members of the IL-6 family are produced primarily by hematopoietic cells (notably OSM and IL-27), stromal cells can be important sources of several others, including IL-6, IL-11, and LIF. Diverse factors appear to regulate the expression of these cytokines by stromal cells, including microbial sensing, detection of endogenous alarmins, stimulation by other cytokines (including those within the IL-6 family itself), and cell stress (**Figure 2**). Although these inputs are known drivers of cytokine production, the critical drivers *in vivo*, particularly under physiological conditions, are rarely well defined.

In response to infection or an inflammatory challenge, IL-6 production is rapidly increased by stromal cells. Depending on their location and the nature of the challenge, this could be due to direct sensing of danger signals, responses to other inflammatory cytokines, or both. As an NF-κB (nuclear factor kappa B) response gene (43), IL-6 is induced by stromal cells downstream of several pattern recognition receptors including, but probably not limited to, toll-like receptor (TLR)2, TLR4, and NOD2 (nucleotide binding oligomerization domain 2) (44–46). The NF-κB activating cytokines IL-1β, IL-17A, and TNF (tumor necrosis factor alpha) are also potent inducers of stromal IL-6 production, and can do so in synergy with one another (43, 47–54). Although NF-κB is thought to be the dominant driver of IL-6 production downstream of these cytokines, contributions by MAPK signaling have also been observed. Indeed, signaling by alternative pathways such as the MAPK and PI3K cascades may underlie the ability of cytokines like OSM (55, 56), IL-4 (49), and TGF-β (54, 57) to promote stromal IL-6 expression, since these are not classical activators of NF-κB. Beyond cytokines and danger signals, cadherin-11 (CDH11), a mesenchymal cadherin that engages in homophilic interactions between adjacent cells, has also been shown to drive IL-6 production via NF-κB and MAPK signaling (53). Indeed, blockade of CDH11 attenuates inflammation in mouse models of arthritis, an effect that may be due in part to reduced IL-6 production by CDH11$^+$ synovial fibroblasts (53). Finally, IL-6 is a well-known product of the senescence-associated secretory phenotype (SASP) in fibroblasts, a feature associated with aging and cancer (58). Indeed, IL-6 produced by prostate tumor fibroblasts in response to metabolic stress has been proposed to mediate malignant progression (59).

Less is known about the regulation of LIF and IL-11 expression by stromal cells, but the mechanisms involved may be similar

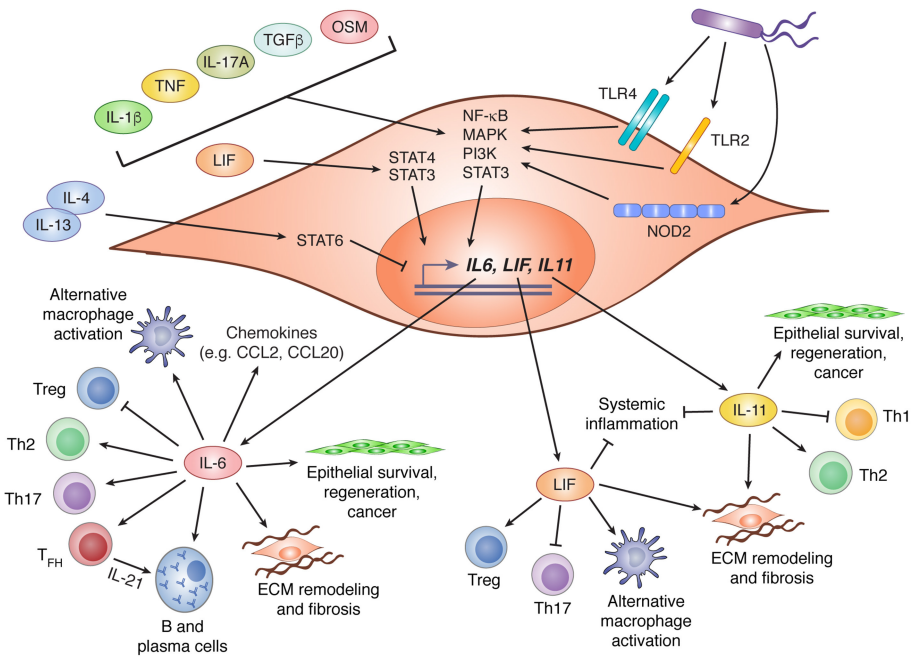


**FIGURE 2 |** IL-6 family cytokine production by stromal cells and their biological effects. Stromal cells are important contributors to production of three members of the IL-6 family: IL-6, LIF, and IL-11. Expression of these cytokines is regulated by various stimuli including recognition of bacterial products via TLR2, TLR4, or NOD2, and diverse cytokines that drive activation of NF-κB, MAPK, PI3K, and STAT3. LIF has been shown to promote IL-6 expression via STAT4 signaling, while IL-4 and IL-13 can suppress LIF and IL-11 expression through activation of STAT6. Following production by stromal cells, IL-6, LIF, and IL-11 can influence diverse biological processes including CD4$^+$ T cell polarization, regulation of chemokine production, promotion of alternative macrophage differentiation, and tissue remodeling through effects on stromal and epithelial cells. In this figure, arrows indicate stimulatory effects, and capped lines indicate inhibitory effects. All processes illustrated are described further in the main text. CCL, C-C motif chemokine ligand; ECM, extracellular matrix; IL, interleukin; LIF, leukemia inhibitor factor; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor kappa B; NOD2, nucleotide-binding oligomerization domain-containing protein 2; OSM, oncostatin M; PI3K, phosphatidylinositol-3-kinase; STAT, signal transducer and activator of transcription; $T_{FH}$, T follicular helper cell; TGFβ, transforming growth factor beta; Th, T helper; TLR, toll-like receptor; Treg, regulatory T cell.

to those of IL-6. Like IL-6, LIF and IL-11 expression by stromal cells can be induced by IL-1β, TNF, and TGF-β (60–64). Notably, induction of both IL-11 and LIF in response to TGF-β stimulation of cancer-associated fibroblasts is thought to promote tumor progression (61, 62). Intriguingly, IL-4 and IL-13 were shown to counteract TNF and IL-1β-induced expression of LIF and IL-11, but not IL-6, by gingival fibroblasts (64). This effect was dependent on STAT6, and provides a potential mechanism for selective modulation of individual IL-6 family members.

## RESPONSIVENESS OF STROMAL CELLS TO IL-6 FAMILY CYTOKINES

Stromal cells express the necessary receptor subunits to respond to the majority of gp130-dependent IL-6 family cytokines. In general, gp130 and OSMR are ubiquitously expressed by stromal cells from essentially all anatomical locations studied thus far. OSM is therefore a major activating factor of stromal cells, as well as various other mesenchymal populations including endothelial cells, muscle cells, adipocytes, and osteoblasts (34, 56, 65). Expression of other ligand-specific receptor subunits is more variable and depends on the cell type, anatomical location, and physiological context. IL-6R, for example, tends to be expressed at relatively low levels, and stromal cells are correspondingly less sensitive to classical IL-6 signaling than OSM. Indeed, expression of OSMR mRNA in human colon fibroblasts is roughly 10x higher than that of IL-6R (55). However, inflammatory conditions that yield soluble IL-6R can increase stromal cell sensitivity to IL-6 due to *trans* signaling. Responsiveness of stromal cells to LIF appears to vary widely depending on anatomical location. For example, LIF induces contractile and inflammatory phenotypes in dermal and synovial fibroblasts, respectively, but has little effect on colon fibroblasts (55, 62, 63, 66). Sensitivity of stromal cells to IL-11 and IL-27 has also been documented (67–73). In contrast, IL-31Rα does not seem to be expressed by most stromal cells at physiologically relevant levels (74, 75).

## CONTROL OF INFLAMMATION AND ADAPTIVE IMMUNITY BY THE IL-6-STROMA AXIS

Exposure of stromal cells to factors such as microbial ligands or inflammatory cytokines can drive IL-6 production during both acute and chronic inflammation. Following infection of mice by *Toxoplasma gondii*, for example, IL-6 expression was shown to be elevated in a population of bone marrow stromal cells characterized by high VCAM-1 and low CD146 expression, and stroma-derived IL-6 was required for the increased myelopoiesis that occurs as part of the host response to infection (76). Bone marrow stromal cells also induce IL-6 in response to viral infections such as CMV (cytomegalovirus) (77). During *Helicobacter hepaticus*-driven colitis in mice, non-hematopoietic stromal cells are the dominant intestinal producers of IL-6, with expression levels that substantially exceed those of MHC-II$^+$ myeloid cells (55). Interestingly, IL-6 expression may be a feature of specific intestinal stromal cell subsets with distinct ontogeny or activation states. For example, human OSMR$^{high}$ intestinal stromal cells were found to be enriched in IL-6 expression relative to their OSMR$^{low}$ counterparts (55), consistent with the well-established ability of OSM to induce IL-6 expression in mesenchymal cells (78–86). Single-cell RNA-sequencing has similarly revealed substantial heterogeneity in the intestinal stromal cell compartment. High IL-6 expression is enriched in a stromal cell subset that is rare in healthy individuals, but dramatically expanded in patients with inflammatory bowel disease (IBD) (87). Notably, these cells were further characterized by expression of a variety of additional immunostimulatory molecules, including IL-33 and the FRC-associated chemokines CCL19 and CCL21, implying a specialized role in immune regulation (87). Notably, a disease-associated single nucleotide polymorphism (SNP) in the human *IL6* promoter was shown to control production of IL-6 by fibroblasts, but had no effect on IL-6 expression by CD14$^+$ monocytes, suggesting that host genetics can also play an important role in determining stromal IL-6 output (88).

Following initiation of acute inflammation, IL-6 can act on several cell types to shape the quality of the ensuing immune response. For example, IL-6 controls the balance between inducible regulatory T cell (Treg) and Th17 differentiation following activation of naïve CD4$^+$ T cells (41). Although stromal cells have not conclusively been demonstrated to contribute to this process, FRC-derived IL-6 has been suggested to support the development and maintenance of B cell responses. Medullary FRC were shown to be important regulators of plasma cell homeostasis, in part by producing the plasma cell survival factor IL-6 (89, 90). IL-6 is also necessary for the differentiation of follicular helper T cells ($T_{FH}$), which drive the maturation of B cells and the generation of protective antibody responses (91, 92). Importantly, IL-6 induces production of IL-21 by $T_{FH}$ cells, which is critical for both $T_{FH}$ maintenance and plasma cell differentiation in germinal centers (93, 94). Publicly available data provided by the ImmGen project suggest that FRC constitutively express IL-6, and do so at levels that far exceed those of other lymph node-resident cell types (95). Thus, FRC-derived IL-6 is likely to be a central linchpin in the regulation of both T cell and B cell responses in secondary lymphoid organs.

In inflamed peripheral tissues, IL-6 controls the temporal switch from recruitment of granulocytes to preferential recruitment of mononuclear cells by modulating chemokine and cytokine production in local mesenchymal cells, including the suppression of TNF and IL-1β production, possibly via STAT3-mediated repression of NF-κB signaling (96, 97). IL-6 promotes the differentiation of monocytes into macrophages rather than dendritic cells *in vitro*, but genetic IL-6 deficiency does not affect dendritic cell frequencies *in vivo* (98–101). However, IL-6 appears to mediate alternative macrophage differentiation *in vivo* and inhibits inflammatory cytokine production and microbicidal activity by macrophages (102–105). IL-6 can also promote survival and regeneration of damaged epithelia during inflammatory challenges, a feature that can be subverted to promote cancer progression (106). Thus, while IL-6 is important for initiation of immune responses, it also promotes resolution

of inflammation and tissue repair (54, 62). Notably, IL-6 protects mice from the lethal inflammatory effects of Staphylococcal enterotoxin B (SEB; a model of toxic shock), in direct contrast with TNF (107).

Although IL-6 is an important regulator of physiological immune responses, excess or chronic IL-6 production can promote inflammatory or fibrotic pathology. IL-6 has been implicated in a variety of inflammatory diseases, but is perhaps best studied in the context of arthritis, a condition that can be effectively treated via blockade of IL-6 signaling (42). Synovial fibroblasts in inflamed joints are thought to be the major source of IL-6, which is likely produced in response to a variety of inflammatory factors including TNF, IL-1β, LIF, and CDH11 (47, 53, 63). IL-6 is necessary for pathology in pre-clinical models of antigen-induced and spontaneous arthritis, in which it orchestrates a variety of inflammatory processes including activation of CCL2 production by synovial fibroblasts, differentiation of autoinflammatory Th17 cells, and bone erosion via increased osteoclastogenesis (108–111). IL-6 has also been shown to promote CCL20 production by fibroblasts, which may further promote recruitment of inflammatory Th17 cells (112). IL-6 appears to promote arthritis primarily via *trans* signaling, likely because synovial fibroblasts and activated $CD4^+$ T cells do not express sufficient IL-6R to respond to IL-6 alone (108, 109). Indeed, CCL2 production following IL-6 stimulation of synovial fibroblasts requires the presence of soluble IL-6R, or a chimeric IL-6/IL-6R protein known as "hyper-IL-6" (108). IL-6 has also been shown to mediate fibrosis in the skin, lung, and heart (113–116). Notably, in a phase 2 clinical trial of patients with systemic sclerosis, a disease characterized by skin fibrosis, treatment with actemra (tocilizumab; anti-IL6R) dramatically attenuated fibrotic behavior and transcriptional signatures in dermal fibroblasts, along with significant attenuation of disease severity (113).

## REGULATION OF INFLAMMATION AND HEMATOPOIESIS BY THE OSM-STROMAL CELL AXIS

OSM is a pleiotropic cytokine with roles reported in a plethora of homeostatic and disease settings (34, 56, 65). Unlike IL-6, OSM is not generally produced at significant levels by stromal cells, but is instead a product of various hematopoietic cell types, including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, mast cells, and T cells (34, 56, 65). OSM is further distinguished from IL-6 by the cellular distribution of its specific receptors (OSMR and LIFR in humans; OSMR in mice), which are largely restricted to non-hematopoietic cell types, notably epithelial cells, fibroblasts, endothelial cells, adipocytes, and neurons (34, 56, 65). OSM thus provides a means for leukocytes to deliver information to non-hematopoietic cells in inflamed or damaged tissues.

While little is known about the role of OSM in infectious disease or other host-defense settings, OSM can clearly influence hematopoietic homeostasis. OSM is necessary for the maintenance of granulocyte-macrophage, erythroid, megakaryocyte, and multipotential hematopoietic progenitor populations in the bone marrow, an effect that likely involves stimulation of bone marrow stromal cells by OSM (117–120). The ability of OSM to drive expression of CXCL12 (SDF1) in stromal cells may partly explain its effects on hematopoiesis (119, 121–123). However, aging studies have shown that OSM-deficient mice develop progressive hematological defects that include reduced numbers of circulating leukocytes, erythrocytes, and platelets, along with pronounced bone marrow adiposity. OSM was shown to suppress adipose differentiation of murine $PDGFR\alpha^+$ $Sca1^+$ mesenchymal stem cells, thereby preventing the development of "fatty" marrow and safeguarding the hematopoietic niche (120). Several additional studies have confirmed that OSM acts on stromal progenitors to suppress adipocyte differentiation in favor of osteoblast development (124–130), suggesting that OSM plays a fundamental role in regulating the bone marrow microenvironment. Notably, overexpression of OSM in bone marrow stromal cells promotes the development of lethal myeloproliferative neoplasms and bone marrow fibrosis in mice (131, 132).

Numerous studies have implicated OSM in the pathogenesis of inflammatory conditions, including arthritis, inflammatory bowel disease, psoriasis, and allergic airway inflammation. Intra-articular adenoviral delivery of OSM causes arthritis-like pathology characterized by robust leukocyte infiltration, synovial hyperplasia, and erosion of bone and cartilage (133–135). Consistent with these findings, antibody blockade of OSM can reduce pathology in the collagen-induced and pristane-challenge pre-clinical models of inflammatory arthritis (136). Synovial fibroblasts respond to OSM by producing a wide variety of inflammatory factors including cytokines (e.g., IL-6), chemokines, (e.g., CCL2, CCL13, CXCL1), and leukocyte adhesion factors such as ICAM-1 (78, 82, 83, 134, 137–139). Furthermore, cytokine receptors such as IL1R1 (IL-1 receptor, type 1), gp130, and OSMR are induced by OSM, suggesting that OSM can sensitize synovial fibroblasts to additional cytokine stimulation. OSM can synergize with TNF and IL-1β to promote increased cytokine and chemokine expression, as well as high MMP (matrix metalloprotease) to TIMP1 (tissue inhibitor of metalloproteases) ratios to promote tissue damage. Remarkably, OSM alone drives high TIMP1 expression (134, 135, 138, 140, 141), and OSM only promotes net tissue degradation when acting in synergy with TNF, IL-1β, or IL-17A, suggesting that its pathogenicity may depend on the presence of other inflammatory factors (135, 137, 138, 142).

Emerging data suggest an important role for OSM-stromal cell interactions in barrier tissues, such as skin and intestinal mucosa. Dermal fibroblasts express extracellular matrix components such as collagens and glycosaminoglycans in response to OSM, and display an interferon-like response featuring upregulation of the viral RNA sensors RIG-I and MDA5 (143–146). While sufficient to induce skin inflammation, OSM may not be required for psoriasis-like pathology, as it is dispensable in the aldara (imiquimod) challenge model of psoriasis (55, 147, 148). OSM and OSMR are also overexpressed in the lesional skin of patients with atopic dermatitis, but whether OSM signaling is required for pathogenesis of this condition is unclear (147). A potentially non-redundant inflammatory role

for OSM has been described for IBD, however. Unlike other barrier tissues such as the skin, normal epithelial cells do not express appreciable amounts of OSMR in either the mouse or human intestine (55). Intestinal fibroblasts, however, are highly sensitive to OSM and express a range of inflammatory factors in response to OSM stimulation, including IL-6, CCL2, CXCL10, and ICAM1. Like synovial fibroblasts, intestinal stromal cells show a synergistic inflammatory response to combined OSM and TNF treatment (55). Notably, genetic OSM deficiency or OSM blockade using an OSMR-gp130 fusion protein attenuates colitis in a dysbiosis-driven model of IBD that is refractory to anti-TNF therapy. Furthermore, OSM, OSMR, and stromal OSM response genes are highly predictive of resistance to anti-TNF therapy (e.g., infliximab and golimumab) in IBD patient cohorts, suggesting that high OSM expression drives an inflammatory axis that is mechanistically distinct from that of TNF (55). Intriguingly, OSM was not found to affect the early acute kinetics of colitis induction, but instead interfered with the resolution of inflammation, thereby contributing to disease chronicity and cumulative tissue damage. Experiments involving adenoviral overexpression of OSM in models of acute chemically induced colitis have yielded conflicting results, suggesting that the impact of OSM in these systems may be context dependent (149).

Consistent with data from studies of skin and joint inflammation, exogenous OSM promotes a robust inflammatory and tissue remodeling response in the lung (150–153), and blockade of OSM reduces disease severity in a mouse model of asthmatic airway inflammation (154). OSM stimulation of lung tissue induces a pronounced eosinophilia driven by OSM-induced expression of eotaxin (CCL11) in lung fibroblasts (152, 155). Unusually for an IL-6 family cytokine, OSM was shown to activate STAT6 in lung fibroblasts and activate CCL11 expression in synergy with IL-4 (155). OSM can also synergize with IL-4 to promote VCAM-1 expression by lung fibroblasts, which may promote increased eosinophil adhesion (156). Interestingly, the OSM-induced response in mouse lung tissue appears to differ between inbred strains. While intratracheal OSM promoted similar fibrotic changes in the lungs of BALB/c and C57BL/6 mice, only C57BL/6 animals induced an inflammatory Th2 response (151). Strain-dependent effects are known to occur in other inflammatory disease settings such models of colitis, in which C57BL/6 mice often display differing disease susceptibility compared to animals from the BALB/c, 129.SvEv, or C3H/HeJBir backgrounds (157–165). This demonstrates the importance of considering strain-dependent effects when interpreting *in vivo* studies of OSM or other IL-6 family cytokines. Furthermore, such findings emphasize the challenges inherent in predicting the biology of humans (an outbred population) based on mouse model data. It is noteworthy that single nucleotide polymorphisms in the *OSM* and *OSMR* genetic loci are associated with risk of developing IBD (*OSM* and *OSMR*) and the IgA nephropathy form of glomerulonephritis (*OSM*), but the functional significance of these risk variants is not yet understood (166–168).

## LIF AND IL-11: STROMAL FACTORS WITH IMMUNOREGULATORY AND FIBROTIC PROPERTIES

Unlike OSM, stromal cells appear to be important physiological sources of LIF and IL-11, both of which are produced in response to TGFβ stimulation (62, 66, 68, 169). While some studies have suggested pro-inflammatory roles for these cytokines (63, 170, 171), substantial evidence suggests that they also exert important anti-inflammatory effects. Treatment with recombinant LIF was demonstrated over 20 years ago to protect mice from mortality and to reduce systemic TNF production in models of endotoxin-induced septic shock (172). Similar findings were later reported in LIF-deficient ($Lif^{-/-}$) mice. Compared to wild type mice, $Lif^{-/-}$ animals had significantly greater mortality after endotoxin challenge, with dramatically increased levels of circulating TNF and IL-6 and reduced levels of IL-10, suggesting a potent anti-inflammatory effect (173). Treatment of mice and rats with recombinant IL-11 similarly reduced mortality, organ damage, and systemic cytokine production (TNF, IL-1β, and IFN-γ) in models of acute endotoxemia (174, 175). Recombinant IL-11 treatment likewise prevented T cell-driven liver injury in response to concanavalin-A, and protected mice from necrotizing pancreatitis following challenge with cerulein (176, 177).

Both LIF and IL-11 are reported to influence $CD4^+$ T cell differentiation, favoring Treg development and Th2 development, respectively. Whereas IL-6 suppresses Treg development and promotes Th17 differentiation in synergy with TGFβ, LIF appears to have the opposite effect and can directly oppose the pro-Th17 activity of IL-6, possibly through induction of the negative regulatory factor SOCS3 (178, 179). Notably, LIF and IL-6 were identified as factors in human ovarian cancer ascites fluid that promoted differentiation of monocytes into anti-inflammatory macrophages with low IL-12 and high IL-10 production (100). *In vitro* culture models of human and mouse $CD4^+$ T cells have demonstrated that IL-11 represses Th1 polarization and promotes expression of IL-4, IL-5, and IL-10, which are associated with Th2 immunity (180, 181). IL-11 was also shown to inhibit IL-12 production from monocytes, which likely further attenuates Th1 development (180). IL-11 treatment was highly effective for preventing mortality in a mouse model of graft-vs.-host disease, in which it blocked expression of Th1-related cytokines (IL-12, IL-2, and IFN-γ), and induced production of IL-4 (182). Consistent with this Th2-promoting effect, IL-11 signaling is necessary for airway inflammation in mice challenged with inhaled OVA antigen or transgenic animals that overexpress IL-13 in the lung (170, 183). Following OVA challenge, $Il11ra^{-/-}$ mice showed dramatic reductions in lung-infiltrating Th2 cells, IL-13 expression, IgE production, and eosinophilia (170). Thus, while IL-11 can attenuate acute inflammation and Th1 responses, it may be pathogenic in conditions driven by Th2-polarized immunity.

Although LIF and IL-11 appear to have host-protective anti-inflammatory properties in the context of acute inflammatory challenges, they may be deleterious in setting of chronic

inflammation and tissue remodeling. Like IL-6, IL-11 can drive a STAT3-mediated regenerative program in epithelial cells, a function that promotes malignancy in cancers of the intestine, stomach, and mammary gland (61, 184–187). A recent study made the surprising observation that synovial fibroblasts express LIF in response to inflammatory cytokines such as TNF and IL-17A, and autocrine LIF signaling drives STAT4, which synergizes with TNF/IL-17A-derived signals (NF-κB and C/EBPβ) to promote potent expression of IL-6 (63). LIF may therefore be an important driver of pathogenic IL-6 production in patients with rheumatoid arthritis. In cancer, LIF has been shown to activate cancer-associated fibroblasts to promote contractility, extracellular matrix remodeling, and cancer cell invasiveness (62, 66). Several studies have examined developmental abnormalities in transgenic mice that express either LIF or bovine OSM (a LIFR ligand in mice) downstream of the *Lck* promoter. These mice display a dramatic re-organization of lymph node structure, extrathymic T cell development, and myelosclerosis, suggesting that chronic overproduction of LIF in hematopoietic organs can have profound consequences on hematopoiesis (188–194). Transgenic overexpression of IL-11 in mouse lung tissue provokes lymphocyte and myeloid infiltration and considerable subepithelial fibrosis (171). IL-11 was also shown to be mitogenic for lung fibroblasts isolated from either healthy donors or those with idiopathic pulmonary fibrosis (IPF). More recently, two studies that incorporated both human cell culture systems and pre-clinical mouse models highlighted the importance of IL-11 for fibrosis in the lung, heart, and kidney (68, 169). Intriguingly, IL-11 production in primary fibroblasts was shown to be induced by various pro-fibrotic stimuli (including PDGF, TGFβ, IL-13, and OSM), and was critical for the adoption of a fibrogenic phenotype, suggesting that IL-11 mediates fibrosis in response to a wide variety of upstream stimuli (68, 169).

## STROMAL CONNECTIONS WITH IL-27

As noted above, IL-27 differs structurally from other IL-6 family members, being a heterodimer comprised of IL-27p28 and EBI3 (19). IL-27 can be thought of as an IL-6 family member primarily because it depends on gp130 as a receptor component (28, 37, 195). However, the structure of IL-27 is more comparable to that of IL-12 family members, all of which are similarly composed of heterodimers; IL-12 is comprised of IL-12p35 and IL-12p40, IL-23 of IL-12p40 and IL-23p19, and IL-35 of IL-12p35 and EBI3 (37, 195). Compared to its siblings in the IL-6 family, relatively little is known about the physiological interactions between IL-27 and stromal cells. In general, IL-27 has been studied in the context of communication between leukocyte populations, in which IL-27 can act in either an immunostimulatory or immunoregulatory capacity (37). For example, IL-27 synergizes with IL-12 to promote T-bet and IFN-γ expression by $CD4^+$ T cells, thus promoting Th1 polarization (19, 196–199). However, IL-27 can also drive differentiation of T-bet expressing regulatory T cells (200), an effect that is in direct contrast to IL-6, which suppresses Treg differentiation. In both mice and humans, IL-27 is also thought to be a key driver of the immunoregulatory Tr1 subset of $CD4^+$ T cells, which specializes in IL-10 production to suppress inflammation (201–204). Because the well-studied hematopoietic effects of IL-27 are beyond the scope of this discussion, the reader is referred to recent reviews of the subject (37, 195).

IL-27 can directly influence stromal cells, although studies of this process are scarce. Like OSM, IL-27 can induce cytokine and chemokine production by fibroblasts from the synovium and lung, and does so in synergy with TNF or IL-1β (70, 71). Factors induced in this manner include IL-6, CCL2, CXCL10, and ICAM-1 (70, 71). IL-27 has also been shown to enhance the sensitivity of pulmonary fibroblasts to lipopolysaccharide by promoting TLR4 expression (73). Finally, high circulating IL-27 concentrations are observed in patients with systemic sclerosis, and IL-27 stimulation of skin fibroblasts induced proliferation and collagen synthesis, implying a possible role for IL-27 in the fibrotic pathology of this disease (72). Thus, although IL-27 is best studied in the context of leukocyte-leukocyte interactions, it has the potential to regulate processes beyond those of the hematopoietic system through direct effects on stromal populations. A key challenge of future studies will be to determine whether IL-27 signaling in the stroma is functionally important *in vivo*.

## EFFECTS OF IL-6 FAMILY CYTOKINES ON OTHER NON-HEMATOPOIETIC CELL TYPES

This review has dealt specifically with the impact of IL-6 family cytokines on a narrow group of mesenchymal cells that collectively include various phenotypes of fibroblasts. However, it should be noted that several other non-hematopoietic cell types can respond strongly to IL-6 family members and likely mediate at least some of their effects *in vivo*. Such populations include endothelial cells, adipocytes, muscle cells, chondrocytes, epithelial cells, and glial cells. Importantly, the functional effects of IL-6 family cytokines on fibroblasts overlap broadly with those reported for other cell types, suggesting a degree of functional conservation between non-hematopoietic populations. For example, both OSM and IL-6 are implicated as potent drivers of endothelial activation; in response to these cytokines, endothelial cells upregulate expression of chemokines and cytokines that include CCL2 and IL-6, as well as adhesion factors such as ICAM-1, VCAM-1, P-selectin, and E-selectin (79, 205–208). In this fashion, OSM and IL-6 are thought to promote leukocyte recruitment to inflamed tissues via stimulation of the endothelium. Because OSM and IL-6 exert similar effects on stromal cells, it is possible that these cytokines control the duration and intensity of inflammatory responses through coordinated action on multiple non-hematopoietic cell types. OSM similarly induces chemokine and cytokine production in chondrocytes, osteoblasts, and smooth muscle cells (80, 85, 86, 209, 210).

The relative importance of these different cell populations for the execution of IL-6 family effects is poorly understood and probably context-dependent. For example, OSMR is highly

expressed only by stromal cells in the intestinal mucosa, and it appears likely that these are the major mediators of OSM biology in this tissue (55). In contrast, OSMR is expressed broadly in many other tissues, making the identification of cell-type specific roles for OSM in these sites more challenging (34, 56, 65, 211). The ability of IL-6 and IL-11 to signal via both classical and *trans* mechanisms adds further complexity, since potential responder cells require only the ubiquitously expressed gp130 to recognize the *trans* forms of these cytokines. As such, we know little about the cell type requirements for IL-6/IL-11 functions *in vivo*, particularly under conditions of inflammatory pathology (18, 41). Finally, the ability of IL-6 family cytokines to modulate responses to other inflammatory mediators (e.g., TNF, IL-1β, IL-17A, IL-4) means that their effects *in vivo* likely depend on the composition of the broader cytokine milieu, as well as the relative abundance of cell types capable of receiving signals from both IL-6 family members and their synergy partners. Beyond characterizing the "receivers" of IL-6 family cytokines, we are similarly limited in our understanding of their *in vivo* sources. For example, IL-6 has traditionally been thought of as a primarily leukocyte-derived factor, but this concept is challenged by our growing awareness of non-hematopoietic cell types as sources of this cytokine (as well as factors such as LIF and IL-11). There is thus a clear need for carefully constructed *in vivo* studies that abrogate expression of individual IL-6 family receptors or ligands in specific cell types. This is increasingly feasible due to the growing availability of transgenic mice with floxed alleles of IL-6 family members and cell type-restricted Cre recombinase. Single-cell RNA-sequencing technology will also be a powerful tool to deconvolute the IL-6 family network *in vivo*.

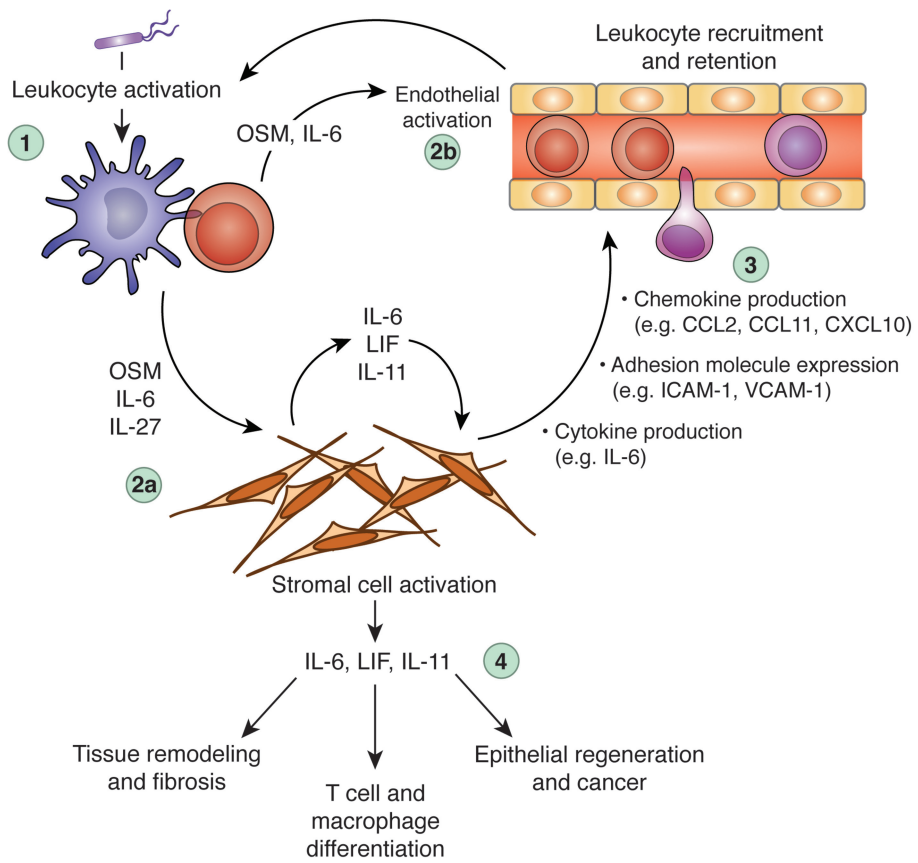


**FIGURE 3 |** Regulation of inflammation and tissue repair processes via cross-talk between leukocytes, the IL-6 family, and stromal cells. Dysregulation of IL-6 family cytokine expression can promote chronic inflammation, tissue remodeling, and fibrosis. In a hypothetical scenario of acute inflammation, an initial inflammatory insult (e.g., microbial sensing) triggers leukocyte activation and production of inflammatory mediators, including OSM, IL-6, and IL-27 (step 1). These cytokines can act alone or in synergy with other inflammatory mediators (e.g., TNF, IL-1β, IL-17A, etc.) to activate tissue-resident stromal cells (step 2a) and the local vasculature (step 2b). Stromal cells stimulated by IL-6 family members produce a wide array of additional inflammatory mediators and adhesion molecules, including IL-6, CCL2, CXCL10, and ICAM-1. These stroma-derived factors collectively promote leukocyte recruitment and retention (step 3), to potentiate the inflammatory response. Stromal cells stimulated by leukocyte-derived IL-6, OSM, or IL-27 (particularly in combination with other inflammatory cytokines) can produce IL-6 family cytokines in turn, including IL-6, LIF, and IL-11, which can further stimulate stromal cells through autocrine feedback or act on additional cell types to modulate leukocyte behavior (e.g., T cell polarization), tissue remodeling (e.g., matrix deposition), and tissue regeneration (step 4). When appropriately regulated, these processes constitute beneficial responses to tissue injury or infectious challenge. When dysregulated, however, these processes can become self-sustaining, leading to chronic inflammation and associated pathology. CCL, C-C motif chemokine ligand; CXCL10, C-X-C motif chemokine ligand 10; IL, interleukin; ICAM-1, intercellular adhesion molecule 1; LIF, leukemia inhibitor factor; OSM, oncostatin M; VCAM-1, vascular cell adhesion molecule 1.

## TARGETING IL-6 FAMILY CYTOKINES IN THE CLINIC

IL-6 is well established as a valuable clinical target, and antibodies that block either IL-6 (e.g., siltuximab) or IL-6R (e.g., tocilizumab or sarilumab) are routinely used for treatment of inflammatory arthritis, juvenile idiopathic arthritis, multi-centric Castleman disease, cytokine release syndrome (commonly encountered in the setting of tumor immunotherapy), and giant cell arteritis (212–217). Despite these successes, clinical development of agents that target other members of the IL-6 family has been modest [reviewed here (18)]. A humanized anti-OSM monoclonal antibody (GSK315234) was tested in a phase II trial of rheumatoid arthritis and found to be well tolerated, but without significant clinical activity (218). However, the limited efficacy of this antibody was ascribed to its relatively weak target affinity when compared to the native OSM receptor complex, and clinical studies with a next-generation high-affinity anti-OSM antibody are currently underway, focused this time on systemic sclerosis (ClinicalTrials.gov Identifier: NCT03041 025). Based on its immunoregulatory properties, a trial of recombinant IL-11 in rheumatoid arthritis was also conducted, but without success (219).

A concept that emerges from the previous sections of this review is the substantial degree of functional similarity and cross-regulation between IL-6 family members. As such, targeting a single member of the IL-6 family for treatment of aetiologically complex diseases may be insufficient to fully engage the biology of interest. For example, although many patients with arthritis benefit from anti-IL6R therapy, this benefit is usually restricted to an incomplete decrease in disease activity, suggesting that other inflammatory pathways are in play (213, 216). Indeed, virtually all members of the IL-6 family (IL-6, OSM, LIF, IL-11, and IL-27) have been implicated as drivers of fibrosis. This raises the possibility that combinatorial blockade of two or more factors may be necessary to effectively neutralize IL-6 family biology in a given disease setting. Although clinical experience with JAK inhibitors (which block signaling from broad spectra of cytokines) demonstrates that simultaneous targeting of multiple cytokine pathways can be achieved (220), the efficacy of targeting multiple IL-6 family members would nevertheless require careful balancing against potential unexpected toxicities, particularly in the case of antibodies with slow rates of systemic clearance. The probability of success with such approaches will likely be increased by a combination of biomarker-guided patient stratification [e.g., OSM expression in IBD (55)] and the careful selection of combination candidates based on gene expression patterns in human tissues and mechanistic insights derived from carefully conducted pre-clinical studies.

## CONCLUDING REMARKS

Increasing evidence indicates that stromal cells are integral to the biology of IL-6 family cytokines, both in healthy and pathological scenarios. Stromal cells both produce and sense IL-6 family cytokines, and can therefore not only respond to leukocyte-derived signals (e.g., OSM or IL-27), but also instruct leukocyte behavior (e.g., by producing IL-6, IL-11, or LIF) to influence the course of immunological processes (**Figure 3**). Although it is clear that IL-6 family cytokines play diverse roles *in vivo*, it is rare to identify specific cell types that are responsible for either cytokine production or cytokine responses. As such, although it is apparent that interactions between IL-6 family cytokines and stromal cells occur, whether these interactions are necessary *in vivo* is generally unclear. For example, while stromal cells appear to be important sources of IL-6 in models of inflammatory disease, whether they are indispensable sources of IL-6 has not been formally demonstrated. Similarly, while it is likely that stromal cells are key mediators of OSM biology in the intestine, the possibility that other mesenchymal cell types (or a hitherto unknown hematopoietic cell population) are involved cannot be ruled out. Greater use of genetic tools in animal studies, such as cell-type specific deletion of specific cytokine or cytokine receptor genes, is required to draw a more accurate roadmap of the functional connections between IL-6 family cytokines, individual cell types, and distinct biological processes. Rendering this map in greater detail will be critical for translating our knowledge of IL-6 family biology into clinical benefit.

## AUTHOR CONTRIBUTIONS

NW conceived and wrote the manuscript, and prepared figures for this review.

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REVIEW
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# Immune Control by TRAF6-Mediated Pathways of Epithelial Cells in the EIME (Epithelial Immune Microenvironment)

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In the protective responses of epithelial tissues, not only immune cells but also non-immune cells directly respond to external agents. Epithelial cells can be involved in the organization of immune responses through two phases. First, the exogenous harmful agents trigger the primary responses of the epithelial cells leading to various types of immune cell activation. Second, cytokines produced by the immune cells that are activated directly by the external agents and indirectly by the epithelial cell products elicit the secondary responses giving rise to further propagation of immune responses. TRAF6 is a ubiquitin E3 ligase, which intermediates between various types of receptors for exogenous agents or endogenous mediators and activation of subsequent transcriptional responses via NF-kappaB and MAPK pathways. TRAF6 ubiquitously participates in many protective responses in immune and non-immune cells. Particularly, epithelial TRAF6 has an essential role in the primary and secondary responses via driving type 17 response in psoriatic inflammation of the skin. Consistently, many psoriasis susceptibility genes encode the TRAF6 signaling players, such as ACT1 (*TRAF3IP2*), A20 (*TNFAIP3*), ABIN1 (*TNIP1*), IL-36Ra (*IL36RN*), IkappaBzeta (*NFKBIZ*), and CARD14. Herein, we describe the principal functions of TRAF6, especially in terms of positive and regulatory immune controls by interaction between immune cells and epithelial cells. In addition, we discuss how TRAF6 in the epithelial cells can organize the differentiation of immune responses and drive inflammatory loops in the epithelial immune microenvironment, which is termed EIME.

**Keywords: TRAF6, keratinocyte, EIME, IL-17, NF-kappaB, MAPK**

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## INTRODUCTION

The epithelial tissues compose the outermost surface of an organism. Epithelial cells are the first line confronting the exogenous harmful factors, such as toxins and infectious agents. Upon the attack of the offending agents, the epithelial cells not just release their cellular contents, but also respond to each danger by triggering different sets of transcriptional cascades that stimulate a specific type of immune responses. The immune cells that respond directly to the external agents and indirectly to the epithelial cell products are activated and produce a specific set of immune mediators; these in turn activate the epithelial cells again and propagate protective response, which is most effective

to solve life-threatening dangers in each situation. Consequently, as well as immune cells, epithelial cells are thought to be involved in the decision and organization of each type of immune responses (1). Therefore, the defect in this shield gives rise to chronic inflammatory skin diseases (1, 2). The mechanistic roles of immune cells and their signaling pathways in the decision of the type of immune responses have been extensively explored. However, the roles of signaling pathways of epithelial cells in the decision of the type of immune responses and their propagation have not been fully understood.

We have demonstrated that tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) in the epithelial keratinocytes is essential for driving interleukin (IL)-17-mediated psoriatic inflammation (3). The induction and propagation of type 17 immune responses are fully dependent on the epithelial TRAF6 in the skin of an animal model induced by topical imiquimod. Meanwhile, mice lacking TRAF6 specifically in the gut epithelium show an exacerbation of dextran sulfate sodium (DSS)-induced colitis suggesting a protective role of epithelial TRAF6 in barrier homeostasis and innate protective responses in the gut, which are also mediated by the T helper ($T_H$)17 cytokines (4). Taken together, the TRAF6 signaling pathways in the epithelial tissues are expected to play a pivotal role in IL-17-mediated inflammatory and protective responses. Thus, one can speculate that other signaling pathways in epithelial cells are essential in other type of immune responses, and the balance of the dominant cell signaling pathway in epithelial cells may play considerable roles in the decision of immune types. However, the counterpart signaling molecule of TRAF6 in the type 17 immune responses in the epithelial cells in type 1 or 2 immune responses remains obscure.

Here, we describe principal functions of TRAF6 and its roles in immune cells and non-immune epithelial cells. In addition, we provide recent insights into the regulatory mechanisms of the epithelial TRAF6 pathways with the contribution of other ubiquitin E3 ligases, deubiquitinases, and other molecules in type 17 immune responses. Moreover, we propose the function of epithelial TRAF6 in the inflammatory loop of IL-17 through organizing the type 17 epithelial immune microenvironment (EIME).

# TRAF6

## Molecular Function of TRAF6

TRAF6 was identified for the first time in 1996 as the new TRAF family member that mediates IL-1 signaling (5) as well as CD40 signaling (6). TRAF6 is a signaling adaptor functioning as an E3 ubiquitin ligase. Ubiquitin signaling is mainly mediated by the ubiquitin conjugation system that conjugates polyubiquitin chains (ubiquitin polymers) to proteins (7). This conjugation is mediated through a cascade of reactions catalyzed by 3 enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin–protein ligase (E3) (**Figure 1A**). The E3 ligase functions as a scaffold for the binding of both the E2 and target molecule and facilitates the transfer of ubiquitin from the E2 to the target protein. A Ubc13–Uev1a E2 complex generates Lys63 (K63)-linked polyubiquitin chains

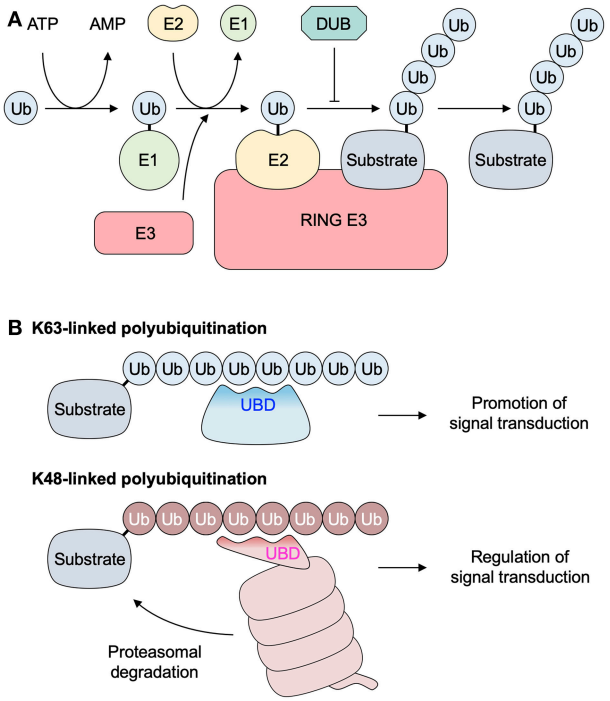


**FIGURE 1 |** Polyubiquitination system. **(A)** Three enzymes catalyze the polyubiquitination of the substrate protein through a cascade of reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). The E1 enzyme first forms a thiol ester bond with a ubiquitin. The activated ubiquitin is transferred to the E2. A RING E3 enzyme (such as TRAF6) functions as a scaffold for the binding of both the E2 and the target molecule, and facilitates the transfer of ubiquitin from the E2 to the target protein. **(B)** Lys63 (K63)-linked polyubiquitination promotes intracellular signal transduction via the association between a substrate protein with K63-linked ubiquitin chains and a protein with a UBD. K48-linked ubiquitin chains are recognized by proteasome, and subsequent proteasomal degradation of the substrate protein is involved in regulation of intracellular signal transduction in several ways. DUB, deubiquitinase; RING, really interesting new gene; Ub, ubiquitin; UBD, ubiquitin binding domain.

together with the RING E3 ligase, such as TRAF6. Binding K63-linked polyubiquitin chains to the target molecules plays a crucial role in a variety of immunological functions via regulating intracellular signal transduction (8–10). While various types of polyubiquitin chains are involved in the ubiquitin signaling, the role of K63-linked polyubiquitin chains are well-characterized in nuclear factor κB (NF-κB) pathways (7). K63-linked chains recruit proteins through their selective binding of a ubiquitin-binding domain (UBD) whereas K48-linked chains induce the proteasomal degradation of the substrate proteins regulating signal transduction (**Figure 1B**).

## Phenotypes of TRAF6 Deficient Mice

TRAF6-deficient (*Traf6*$^{-/-}$) mice appear normal at birth but become progressively runted, and typically die by 3 weeks of age (11–13). Therefore, TRAF6, as well as TRAF2 and TRAF3 (14, 15), is essential for perinatal and postnatal survival. *Traf6*$^{-/-}$ mice exhibit severe osteopetrosis, thymic atrophy, lymph node deficiency, and splenomegaly (11, 12). Spleens from

*Traf6*$^{-/-}$ mice are markedly disorganized, with a complete lack of normal T and B cell areas. Small clumps of lymphocytes are scattered throughout splenic sections, but distinct peri-arteriolar or lymphoid collections are absent. Assays *in vitro* demonstrated that TRAF6 is crucial not only in IL-1 and CD40 signaling but also in lipopolysaccharide (LPS) signaling (13). These findings established unexpectedly diverse and critical roles for TRAF6 in perinatal and postnatal survival, bone metabolism, innate immune responses, and cytokine signaling. Further investigation using conditional gene knockout techniques has clarified the immunological phenotypes of TRAF6 deficiency in each immune and epithelial cell subset (described and discussed in chapters 4 and 5, respectively).

## UPSTREAM MOLECULES

TRAF6 is a transducer of a number of receptor signaling pathways. In these pathways, there are TRAF6-binding motifs in the signaling adaptors and receptor molecules, such as IL-1 receptor-associated kinases (IRAKs), mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1), mitochondrial antiviral signaling protein (MAVS), NF-κB activator 1 (ACT1), CD40, and receptor activator of NF-κB (RANK) (9, 16) (**Figure 2**).

### IL-1 and TLR Pathways

The roles of TRAF6 in the MyD88-dependent pathways, such as IL-1 and Toll-like receptor (TLR) pathways, have been extensively investigated (17) (**Figure 2A**). Upon ligand binding, the IL-1 receptor (IL-1R) and MyD88-dependent TLRs (TLR1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13) recruit IRAKs via the adaptor MyD88 to trigger the recruitment of TRAF6 and subsequent formation of receptor-associated signaling complexes and ubiquitination of the components. In Toll–IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF)-dependent TLR pathways (such as those of TLR3 and TLR4), TRAF6 is recruited to TRIF and receptor-interacting protein kinase (RIPK)1 kinase, which activates TGF-β-activated kinase 1 (TAK1) in response to TRAF6 activation (18) while TRAF3 has a more important role than TRAF6 in TRIF-dependent signaling (19).

The production of IL-1β requires 2 signals: the priming signal 1 that induces the transcription of IL-1β and nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3), and the activating signal 2 that primes NLRP3 inflammasome and subsequent IL-1β maturation through their processing cascades. In addition to the transduction of the signal 1, it has been reported that TRAF6 is involved in signal 2 (20). TRAF6 promotes NLRP3 oligomerization as well as the interaction between NLRP3 and apoptosis-associated speck-like protein containing a caspase recruitment domain-containing protein (CARD) (ASC) in its ubiquitin E3 ligase activity-dependent manner. Deficiency of TRAF6 specifically inhibits IL-1/TLR priming-initiated caspase-1 cleavage, pyroptosis, and secretion of presynthesized IL-18 (20).

As well as phagocytes and immune cells, epithelial cells express IL-1R family receptors (21) and most TLRs (22, 23). Epithelial cell-specific deletion of *Myd88* has demonstrated intrinsic roles of epithelial IL-1 and TLR pathways in host defense (24–27) and carcinogenesis (28, 29).

### NLR and RLR Pathways

NOD-like receptors (NLRs) recognize bacterial muramyl dipeptide (MDP) and viral RNAs (30) and activate NF-κB via promoting TRAF6 to enhance NF-κB essential modulator (NEMO)/ IκB kinase (IKK) γ polyubiquitination (31) whereas TRAF2/5, but not TRAF6, are essential in NOD1/2-mediated NF-κB activation (32) (**Figure 2B**). In cytosolic retinoic-acid-inducible gene-I (RIG-I)-like receptor (RLR) pathways, the binding of RIG-I to viral RNAs induces its oligomerization with MAVS that recruits TRAF6 and triggers the activation of the downstream signaling pathways (33) (**Figure 2C**). It has been demonstrated that double-stranded (ds) RNA induces an antiviral defense status in epidermal keratinocytes through MDA5/RIG-I-mediated signaling (34). In addition, keratinocyte MAVS pathway is activated by a cathelicidin-derived antimicrobial peptide LL37 and dsDNA and involved in interferon (IFN)-β expression in psoriasis and during wound repair (35).

### Mitochondrial ROS Production

TRAF6-mediated mitochondrial reactive oxygen species (mtROS) production is well-demonstrated in macrophages: mtROS production triggered by TLR signaling involves the translocation of TRAF6 to mitochondria, where it engages and ubiquitinates ECSIT (36) (**Figure 2C**). This process is necessary for the increase in mtROS production. By LPS stimulation, ECSIT forms a complex with TRAF6 and TAK1 leading to the activation of NF-κB (37). Consistently, ECSIT- or TRAF6-deficient macrophages exhibit decreased levels of TLR-induced ROS and defective intracellular bacteria killing (36). It has also been demonstrated that TRAF6 is involved in mtROS production and subsequent apoptosis in human intestinal epithelial cell line Henle-407, and a Salmonella protein SopB binds to TRAF6 and prevent ROS-induced apoptosis of epithelial cells by retarding TRAF6 recruitment to mitochondria (38). In addition, oxidative stress-induced activation of apoptosis signal-regulating kinase 1 (ASK1) and subsequent activation of the MAPK pathway depends on TRAF6 (39). However, the mechanism of TRAF6 mitochondrial translocation or its interaction with a ROS–ASK1–TRAF6 pathway remain enigmatic (9, 40). The RLR signaling is in part potentiated by mtROS induction (40) (**Figure 2C**).

### CBM Complex

The signalosome-dependent pathways that include T cell receptor (TCR), B cell receptor (BCR), and C-type lectin receptor (CLR) pathways are mediated by the formation of signalosomes — CARD–BCL10–MALT1 (CBM) complexes (41–43). TCR and BCR pathways signal via CARD11/CARD-containing MAGUK protein 1 (CARMA1) while CLR pathways signal via CARD9 (**Figure 2D**). However, it remains unclear whether the formation of CBM complex is involved in TLR pathways as deficiency in B-cell lymphoma/leukemia 10 (BCL10) or caspase 8, which takes

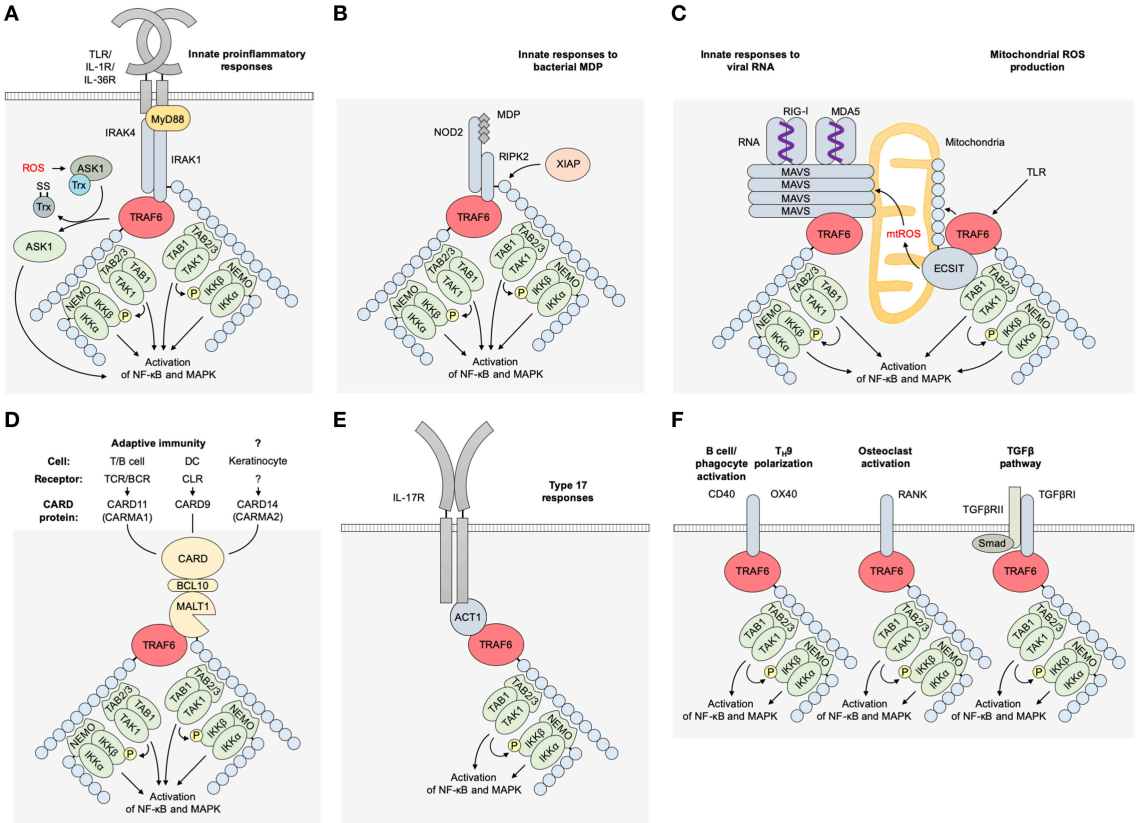


**FIGURE 2 |** Receptor signaling pathways upstream of TRAF6. **(A)** TLR/IL-1 family pathways. Receptor–ligand bindings cause the association between IRAK4/1 and TRAF6 and subsequent activation of TRAF6 in a MyD88-dependent manner. TRAF6 E3 activity mediates K63-linked ubiquitination of IRAK1, NEMO/IKKγ, and TRAF6 itself, resulting in the activation of NF-κB and MAPKs. **(B)** An NLR pathway. The binding of bacterial MDP or viral RNAs to NOD2 results in the association between RIPK2 and TRAF6, and subsequent activation of TRAF6. K63-linked ubiquitination of RIPK2 is expected to be mediated by another E3 ligase XIAP. **(C)** An RLR pathway. The binding of viral RNAs to RIG-I or MDA5 mediates MAVS polymerization at mitochondria and subsequent binding and activation of TRAF6. mtROS is also involved in the MAVS polymerization. TLR signaling mediates mtROS production via TRAF6 mitochondrial translocation and subsequent binding and polyubiquitination of ECSIT. **(D)** CBM signalosome complex-dependent pathways. The formation of a CBM complex is triggered by activation of CARD proteins: CARD11 (CARMA1) in the TCR/BCR pathway in T/B cells, respectively; CARD9 in the CLR pathway in DCs; and CARD14/CARMA2 in keratinocytes although its upstream receptor remains unidentified. TRAF6 is associated with the CBM complex, and TRAF6 E3 ligase activity mediates K63-linked ubiquitination of MALT1, NEMO/IKKγ, and TRAF6 itself. **(E)** An IL-17 pathway. The ligation of IL-17 cytokines to IL-17R recruits ACT1, which bridges the IL-17R and TRAF6 and promotes the E3 ligase activity of TRAF6. ACT1 also associates with BAFFR in B and T cells and CD40 in B cells and phagocytes, and is expected to regulate these receptor signaling pathways. **(F)** Other TRAF6-dependent pathways. A CD40 pathway in B cells, phagocytes and other cells; an OX40 pathway in T cells; a RANKL pathway in osteoclasts; and a TGFβRI pathway in various cells. ACT1, NF-κB activator 1; ASK1, apoptosis signal-regulating kinase 1; BCL10, B-cell lymphoma/leukemia 10; CARD, caspase recruitment domain-containing protein; DC, dendritic cell; ECSIT, evolutionarily conserved signaling intermediate in Toll pathways; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase, MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; MDP, muramyl dipeptide; mtROS, mitochondrial reactive oxygen species; MyD88, myeloid differentiation primary response protein 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domain; RANK, receptor activator of NF-κB; nucleotide-binding oligomerization domain; RANKL, RANK ligand; RIG-I, retinoic-acid-inducible gene-I; RLR, RIG-I-like receptor; TAB, TAK1 binding protein; TAK1, transforming growth factor-β-activated kinase 1; TGFβRI, transforming growth factor-β receptor I; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor associated factor 6; XIAP, X-linked inhibitor of apoptosis.

part in the formation of CBM complex, but not MALT1, abolishes the LPS-induced NF-κB activation (44). The CBM complex of CARD14/CARMA2 is expected to bind with TRAF6 and get involved in IL-17 pathways in keratinocytes whereas the upstream receptors of the CARD14 remain unknown (45). The formation of the CBM complexes results in the TRAF6 recruitment, which facilitates the polyubiquitination of the components of CBM complexes and their downstream molecules.

## IL-17 Pathways

The binding of IL-17A and/or IL-17F to the heterodimeric IL-17R leads to the recruitment of ACT1, which allows the incorporation of TRAF6 into the ACT1–TRAF6 signaling complex and then "downstream" activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways (46–48) (**Figure 2E**). The IL-17R family and ACT1 share sequence homology in their intracellular region with Toll-IL-1 receptor (TIR) domains, and it is involved in their homotypic interaction

(46, 49). ACT1 binds to TRAF6 effectively among TRAF family proteins (50). The formation of the IL-17-mediated ACT1–TRAF6 complex is required for IL-17-mediated NF-κB and c-Jun N-terminal kinase (JNK) activation but not for extracellular signal-regulated kinase (ERK) (p44/ERK1/MAPK3 and p42/ERK2/MAPK1) activation (51), or p38-mediated *Cxcl1* mRNA stabilization (52), which indicates the existence of an IL-17-induced and ACT1-mediated but TRAF6-independent pathway. The epithelial IL-17 pathway is expected to organize a unique "loop" in the EIME (discussed in chapter 9).

### Others

Other upstream molecules of TRAF6 have essential roles mainly in non-epithelial cells. CD40 and RANK directly recruit TRAF6 upon the activation of their receptor signaling pathways (9). TRAF6 directly interacts with transforming growth factor (TGF)-β receptor I (TGFβRI) and mediates Smad-independent activation of downstream pathways (53, 54). TRAF6 also functions as an inhibitor of TGF-β-induced Smad2/3 activation in the TGFβR pathway (55). In $T_H9$ differentiation, an OX40–TRAF6 binding promotes the TRAF6 E3 ligase activity resulting in non-canonical NF-κB activation (9) (**Figure 2F**).

## TRAF6 IN IMMUNE CELLS, PHAGOCYTES AND BLOOD CELLS

### Dendritic Cells

TRAF6 regulates the critical processes required for maturation, activation, and development of dendritic cells (DCs) (13). In response to LPS or CD40 stimulation, TRAF6-deficient DCs fail to upregulate surface expression of major histocompatibility complex (MHC) class II and B7.2, or to produce inflammatory cytokines. In addition, LPS-treated TRAF6-deficient DCs do not exhibit an enhanced capacity to stimulate naive T cells while splenic DC development is severely impaired as the CD4+CD8α- splenic DC subset is nearly absent in TRAF6-deficient mice (13).

TRAF6 in DCs has been shown to be critical for gut microbiota-dependent immune tolerance (56). DC-specific deletion of TRAF6 in *CD11c-Cre Traf6* $^{flox/flox}$ mice leads to diminishing gut commensal microbiota-dependent DC expression of IL-2 and results in reduced numbers of regulatory T (Treg) cells associated with spontaneous development of $T_H2$ cells, eosinophilic enteritis, and fibrosis in the small intestine (56).

### T Cells

Generation of *CD4-Cre Traf6* $^{flox/flox}$ mice made specific deletion of TRAF6 in T cells possible (both in CD4+ T cells and CD8+ T cells at the CD4+ CD8+ double positive stage during T cell development) (57). TRAF6-deficient T cells exhibit hyperactivation of a phosphatidylinositol 3 kinase (PI3K)–Akt pathway compared with wild-type T cells and become resistant to suppression by CD4+CD25+ Treg cells (57). In addition, TRAF6-deficient CD8+ T cells exhibit altered metabolism of fatty acids, such as metformin. As a result, T cell-specific deletion of TRAF6 generates defective long-lived memory CD8+ T cells, which are rescued by metformin treatment (58).

### B Cells

TRAF6 is originally identified as the TRAF family protein that directly associates with the cytoplasmic region of CD40 and its intracellular signaling and thus that plays crucial roles in B-cell function (6). The CD40–TRAF6 binding is important for IL-6 production, upregulation of CD80/B7.1, IL-6-dependent production of immunoglobulin by B cells (59), and subsequent affinity maturation and generation of long-lived plasma cells (60). Also, TRAF6 mediates T cell-independent (CD40-independent) immunoglobulin responses. The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through a TLR-like MyD88–TRAF6 pathway (61). Consistent with these results, B cell-specific deletion of TRAF6 in *CD19-Cre Traf6* $^{flox/flox}$ mice results in a reduced number of mature B cells in the bone marrow and the spleen, impaired T cell-dependent and independent immunoglobulin responses, and defective generation of B-1 B cells (62).

### Macrophages

CD40–TRAF6 signaling in macrophages mediates downstream activation of IKK–NF-κB and ERK MAPK pathways, which are involved in many phagocytic functions, such as IL-12 induction, autophagic vacuole–lysosome fusion in synergy with TNF signaling, and atherogenesis (9). Besides, TLR/RLR–TRAF6 signaling in macrophages induces the production of mtROS (described in section 3.3).

### Osteoclasts

RANK ligand (RANKL)–TRAF6 signaling is critical for osteoclast development and maintenance via the activation of NF-κB, MAPK, and Akt pathways, in addition to the expression of nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which is an osteoclast master regulatory transcription factor (9).

### Hematopoietic Stem Cells

TRAF6-dependent basal NF-κB activation is required for hematopoietic stem cell homeostasis in the absence of inflammation (63).

## TRAF6 SIGNALING PATHWAYS IN BARRIER TISSUES

### TRAF6 in the Skin

The skin is the outer protective wrapping of the body and continuously defends against external dangers and pathogens (1). The epidermis is the outermost layer of the skin that acts as a physical barrier and regulator of the protective responses (1). Epidermal keratinocytes express TRAF6, which participates in many intracellular signaling pathways, such as TLR pathways, IL-1 pathways, and IL-17 pathways; all of which are involved in the host defense system and inflammatory processes.

Results of animal experiments suggest that epidermal TRAF6 is required for the initiation and propagation of IL-17-mediated psoriatic inflammation (3). The development of psoriatic dermatitis induced by topical imiquimod is abolished in *K5-Cre Traf6* $^{flox/flox}$ mice lacking TRAF6 in keratinocytes (3). TRAF6 depletion in keratinocytes impairs subsequent activation of skin

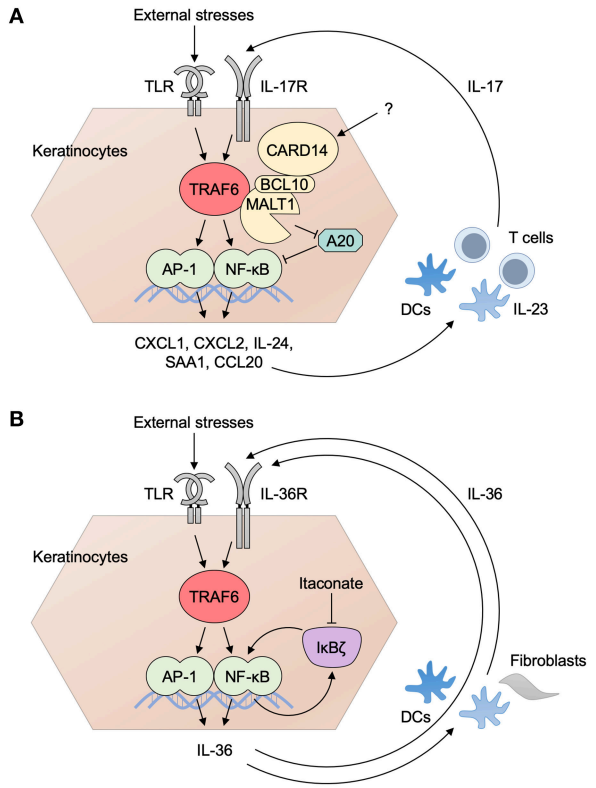


**FIGURE 3 |** Inflammatory loops in the EIME of psoriasis. **(A)** The inflammatory loop of IL-17 in psoriasis. TRAF6-dependent activation of danger signals (signal 1: such as TLR pathways) induces the production of psoriasis mediator from keratinocytes. Subsequent activation of the IL-23/IL-17 axis in DC–T cell interaction gives rise to the production of IL-17 (signal 2) that drives further activation of keratinocytes. CARD14 associates with ACT1 and TRAF6 and is involved in IL-17 signaling. Activated MALT1 degrades A20 and suppresses its regulatory roles for ubiquitin signaling. **(B)** The inflammatory loop of IL-36 and IκBζ in psoriasis. IL-36R is an IL-1R family receptor and its signaling pathway is expected to be TRAF6-dependent. The activation of an IL-36 pathway by the ligation of IL-36 to its receptor triggers the expression of IκBζ. It promotes the transcriptional expression of itself, as well as that of a series of psoriasis mediators inducing the production of IL-17 from immune cells. IL-36 from keratinocytes binds to IL-36R in keratinocytes and other cells, such as DCs and fibroblasts, and drives the loop of IL-36 pathway. Transcriptional regulation by IκBζ is in part mediated by histone methylation. Itaconate inhibits the protein induction of IκBζ and ameliorates psoriatic inflammation. AP-1, activator protein 1; BCL10, B-cell lymphoma/leukemia 10; CARD, caspase recruitment domain-containing protein; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; DC, dendritic cell; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; NF-κB, nuclear factor κB; SAA, serum amyloid A; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor associated factor 6.

resident DCs and their production of IL-23, and hinders IL-17A production of Vγ4+ γδ T cells in the skin. Moreover, TRAF6-null keratinocytes were resistant to the stimulation with either imiquimod or IL-17 *in vitro*, with subsequent absence of their psoriasis mediators for DC recruitment and activation. These results suggest that keratinocyte TRAF6 machinery is required for both the primary response to imiquimod and the secondary responses to IL-17 cytokines produced by T cells in this animal model. This is consistent with the idea that keratinocytes have critical roles in both the primary response to external dangers and the secondary propagation of inflammatory loop mediated by IL-17 cytokines in psoriasis (1).

## TRAF6 in the Gut

Mice lacking TRAF6 in intestinal epithelial cells (IECs) (*Villin-Cre Traf6* $^{flox/flox}$) show an exacerbated phenotype in DSS colitis: a model for intestinal bowel diseases (4). On the other hand, depletion of TLR signaling in IECs by ablation of MyD88 and TRIF in *Villin-Cre Myd88* $^{flox/flox}$ *Ticam1* $^{flox/flox}$ mice does not affect the severity of DSS colitis (4). In addition, germfree mice are known to be more susceptible to DSS colitis (64). These findings suggest that microbiota–host interactions may control the intestinal homeostasis, and TLR-independent intestinal epithelial TRAF6 signaling could have a beneficial role in this animal model.

## TRAF6 in Epithelial Primary and Secondary Responses

During host protection and inflammation, epithelial cell responses are divided into 2 phases: (i) primary responses to external triggers and (ii) secondary responses to internal immune mediators (1). Studies using *K5-Cre Traf6* $^{flox/flox}$ mice suggest that TRAF6 governs both the primary and secondary responses of keratinocytes, and the both are required for the initiation and propagation of psoriatic inflammation (3). Both response to imiquimod and IL-17A are defective in TRAF6-null keratinocytes, and compensation of primary responses by subcutaneous injection with IL-23 is not sufficient for the full development of psoriatic inflammation. TLR/IL-1–TRAR6 pathways are expected to trigger the primary responses while IL-17–TRAF6 pathways mediate the secondary responses. In epithelial cells, TRAF6 thus plays a unique role as "a hub" among TLR/IL-1 pathways and IL-17 pathways in the type 17 response by effective protein–protein interaction and synergistic activation of these pathways whereas the precise molecular mechanism remains to be elucidated. Furthermore, one may be tempted to speculate common and fundamental roles for TRAF6 signaling in epithelial cells as discussed in the next section.

## Epithelial TRAF6 in Protective Responses in the EIME

The results of epithelial TRAF6 depletion in the skin and in the gut are seemingly opposing because epidermal TRAF6 depletion results in the abolishment of imiquimod-induced inflammation (3) whereas IEC-specific TRAF6 depletion results in exacerbation of DSS-induced inflammation (4). However, IL-17 cytokines have major protective roles in mucocutaneous fungal infections (65–67) although they are related to the pathogenesis of psoriasis and its animal models. On the other hand, intestinal barrier integrity is maintained by IL-17 cytokines (68) and the IL-17 cytokines are related to the reduction of DSS colitis (69). Thus, it is a plausible idea that epithelial TRAF6 is uniquely involved in local, IL-17-mediated, protective responses in the skin and the gut despite its pathogenetic role in IL-17-mediated psoriatic inflammation (**Figure 3A**).

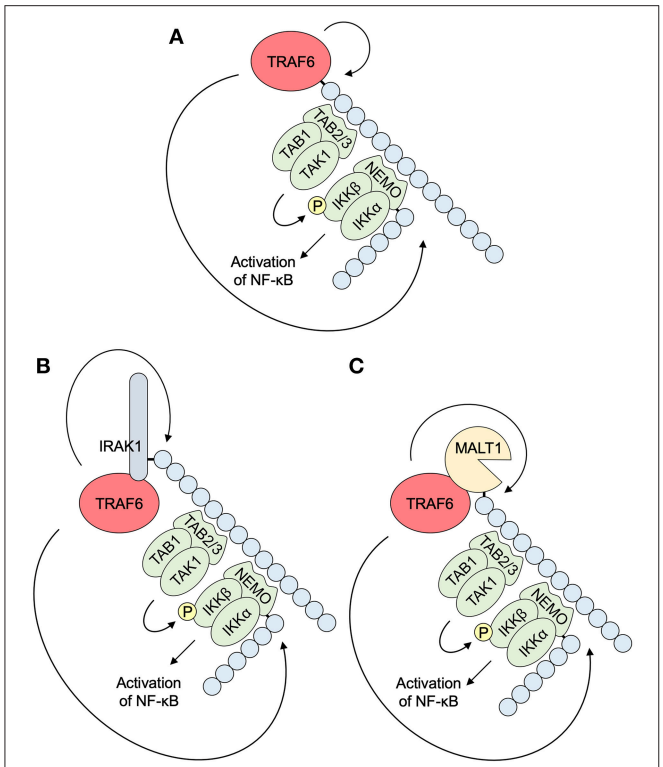


**FIGURE 4 |** NF-κB pathways downstream of TRAF6. **(A)** K63-linked self-ubiquitination by E3 ligase activity of TRAF6 accumulates TAB2/3 with a UBD and triggers subsequent association and activation of TAB1 and TAK1. NEMO/IKKγ also binds to K63-linked ubiquitin chains via a UBD, and associates with IKKα and IKKβ. NEMO ubiquitination by TRAF6 promotes the formation of an IKK complex. TAK1 activation is followed by the activation of MAPK pathways. IKKβ phosphorylation by TAK1 results in the activation of NF-κB pathways. **(B)** TRAF6 E3 ligase activity polyubiquitinates IRAK1, an upstream molecule of TRAF6 in IL-1/TLR pathways (see **A**). **(C)** TRAF6 E3 ligase activity polyubiquitinates MALT1, a component of a signalosome in TCR/BCR pathways, CLR pathways, and others (see **Figure 2D**). IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; TAB, TAK1 binding protein; TAK1, transforming growth factor-β-activated kinase 1; TRAF6, tumor necrosis factor receptor associated factor 6.

Collectively, epithelial TRAF6 is expected to have a pivotal role in the initiation and propagation of type 17 immune and protective responses, which are required at the outermost part of the body and distinctive in epithelial tissues. Especially, its involvement in the secondary responses to internal immune mediators characterizes the definitive role of epithelial TRAF6 in the EIME. In turn, TRAF6 is not just involved in homeostatic barrier protection and host defense, but also can be involved in the chronic inflammation via driving a "loop" of inflammation, as discussed at the latter part of this review.

## DOWNSTREAM EFFECTORS OF TRAF6

NF-κB pathways (**Figure 4**) and MAPK pathways (**Figure 5**) are the major downstream effectors of TRAF6 in epithelial cells (8–10).

### NF-κB

NF-κB is the ubiquitous and inducible transcription factor that induces host and cell-protective responses (70). Upon activation, TRAF6 catalyzes the generation of K63-linked polyubiquitin chains on itself (**Figure 4A**), or other target proteins, such as IRAK1 (**Figure 4B**), MALT1 (**Figure 4C**), and NEMO/IKKγ (**Figures 4A–C**). These chains recruit TAK1 binding protein (TAB) 2/3 that contains a UBD. TAB2/3 in turn recruits ubiquitin-dependent kinase TAK1. Its downstream kinase IKKγ also has an UBD and is recruited to the K63-linked chains and forms an IKK complex with IKKα and IKKβ. These events assemble a signaling complex that facilitates TAK1 and IKK activation. TAK1 phosphorylates and activates IKKβ, which activate a transcription factor, NF-κB (7, 10, 71). TAK1 is also involved in the activation of MAPKs and a transcription factor, activating protein-1 (AP-1), as described in the next section. TRAF6 is also involved in the activation of the NF-κB pathway via the attachment of K63-linked chains to BCL10 and MALT1, which recruit the IKK complex, in CBM signalosome complex-dependent pathways (71, 72) (**Figure 4C**).

For non-canonical NF-κB activation, TRAF6 is required for the activation of an NF-κB inducing kinase (NIK)-dependent IKKα-RelB-p52 pathway (73). NF-κB pathways can affect the activation of the MAPK pathways, as described below.

### MAPK

MAPK is a kinase family, which includes p38, JNK, and ERK (74, 75). These kinases have distinct roles in cell stress responses and cell proliferation. The MAPK activation is controlled by a three-layered kinase cascade: a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MKK), and a MAPK (**Figure 5**).

TRAF6 is involved in the activation of p38 and JNK through multiple MAP3Ks. ASK1 is a MAP3K of the p38 and JNK MAPK pathways (76, 77). An ASK1–MAPK pathway is preferentially activated in response to various types of cellular stresses. ASK1 forms a complex, which is constitutively inactive by the association with thioredoxin, yet TRAF2 and TRAF6 interact with and activate ASK1. $H_2O_2$-induced ASK1 activation and cell death are strongly reduced in the cells derived from *Traf2*$^{-/-}$ and *Traf6*$^{-/-}$ mice (39). Moreover, TRAF6 is involved in the activation of other MAP3Ks, such as MAPK–ERK kinase kinase (MEKK)1/3 and TAK1. In response to IL-1 and LPS, evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) interacts with TRAF6 and mediates the processing of MEKK1 and subsequent activation of NF-κB and JNK (78). TRAF6 also forms a complex with MEKK3, which activates NF-κB, JNK, and p38 but not ERK (79). TAK1 does not only contribute to NF-κB activation via IKKβ phosphorylation but also to AP-1 activation via an MKK7-JNK pathway (80) and an MKK6-p38 pathway (81). The K63-linked polyubiquitination of TAK1, likely catalyzed by TRAF6, leads to the formation of TRAF6–TAK1–MEKK3 complex resulting in effective activation of TAK1 and MEKK3 whereas MEKK3 can also be activated in a TAK1-independent manner (82).

p38 and JNK control gene transcription via activation of a transcription factor AP-1 while p38 MAPKs control post-transcriptional and epigenetic regulation of gene expression via

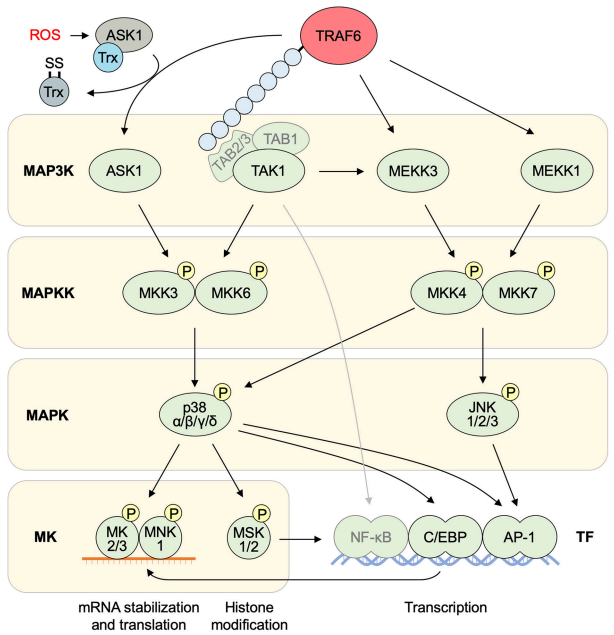


**FIGURE 5 |** MAPK pathways downstream of TRAF6. MAPK pathways are regulated by the phosphorylation cascade of MAP3Ks, MAPKKs, and MAPKs, resulting in the activation of MKs and a transcription factor AP-1. TRAF6 mediates the activation of MAP3Ks that are mainly involved in p38 and JNK MAPK activation. TRAF6 activates ASK1 MAP3K under oxidative stresses by removing thioredoxin. TRAF6-mediated K63-linked ubiquitin chains recruit TAB2/3 and subsequent activation of TAK1, which is also involved in the activation of NF-κB pathways. ASK1 and TAK1 are involved in the activation of p38 MAPK. p38 regulates gene expression at both transcriptional and post-transcriptional levels. C/EBP is a major transcription factor downstream of p38 MAPK. The E3 ligase activity of TRAF6 is required for the activation of other MAP3Ks MEKK3 and MEKK1, which are mainly involved in the activation of JNK MAPK, resulting in AP-1-mediated gene transcription. AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPK kinase kinase; MEKK; MAPK/ERK kinase kinase; MK, MAPK-activated protein kinase; MKK, MAPK kinase; MNK, MAPK-interacting kinase; MSK, mitogen- and stress-activated kinase; NF-κB; ROS, reactive oxygen species; NF-κB, nuclear factor κB; TAB, TAK1 binding protein; TAK1, TGF-β-activated kinase 1; TRAF6, tumor necrosis factor receptor associated factor 6; Trx, thioredoxin.

activation of a set of MAPK-activated protein kinases (MKs): MK2, MK3, and MAPK-interacting kinase (MNK) 1 regulate mRNA stability and translation; mitogen- and stress-activated kinase (MSK) 1 and MSK2 modulate histone modification (74, 75).

## PI3K–Akt Pathway

Several lines of evidence has suggested the links between TRAF6 and phosphoinositide 3-kinase (PI3K)–Akt pathway in various ways. RANKL and CD40 signaling pathway does not only activate NF-κB and MAPK, but also PI3K–Akt pathway via TRAF6 (83, 84): RANK and CD40 recruit TRAF6, Src family kinases, Cbl family-scaffolding proteins, and PI3K in a ligand-dependent manner, resulting Akt activation. TGFβ also activates PI3K-Akt signaling via TRAF6 in prostate cancer cells (85). In addition, LPS-induced activation of Akt depends on TRAF6 in platelets (86). In human airway epithelial cells, it was also demonstrated that two independent signaling pathways are involved in IL-17 signaling: one involves Akt1–TRAF6–TAK1–NF-κB activation, and the other is related to the Janus kinase (JAK)-associated PI3K signaling pathway (87). Studies using kidney epithelial collecting duct cells suggested that TRAF6 mediates K63-linked polyubiquitination and subsequent activation of Akt, which is required for cell adhesion via α3β1 and α6 integrins (88). Of note, however, it remains obscure whether TRAF6-dependent Akt activation has an essential role for epithelial cells in the EIME.

## C/EBP

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors, and have pivotal roles for cellular proliferation and differentiation, metabolism, and inflammation (89). In the cooperative IL-6 gene transcription by IL-17 and TNF in a bone or fibroblast cell line, both the NF-κB and C/EBP sites in the IL-6 promoter are found to be important, and C/EBPδ, and C/EBPβ appeared to be important for this cooperative transcription (90). In human hepatoma cells, IL-17 induces C/EBPβ activation via TRAF6 and TRAF6-dependent p38 MAPK (**Figure 5**) and ERK1/2 activation (91). C/EBPβ is expressed by terminally differentiated keratinocytes in psoriasis lesional skin and in 3D-cultured human keratinocytes treated with IL-17 (92). On the other hand, studies using stroma cell line ST2 have shown that C/EBPβ phosphorylation by ERK and glycogen synthase kinase 3β (GSK3β) exerts an inhibitory effect on IL-17-induced gene expression (93). In addition, *Cebpb*$^{-/-}$ mice are resistant to IL-17-mediated experimental autoimmune encephalomyelitis (EAE) (94) and susceptible to systemic candidiasis (95) but resistant to oropharyngeal candidiasis (OPC) (95). Specifically, C/EBPβ contributes to immunity to mucosal candidiasis during cortisone immunosuppression in a manner linked to β-defensin 3 expression (95). These findings suggest that the TRAF6–C/EBP pathway is not essential for the expression of some IL-17-response genes, such as *Defb3*, but for others, in the EIME.

In addition to C/EBP, many molecules are involved in the regulation of the TRAF6 pathway in epithelial cells. Particularly, the regulation of the IL-17–TRAF6 pathway has been extensively investigated. In the next chapter, we summarize the regulatory mechanism of epithelial signaling pathway downstream of TRAF6. We focus on the IL-17–TRAF6 pathway, which has a characteristic role in epithelial tissues.

# REGULATORY MECHANISMS OF THE IL-17–TRAF6 PATHWAY

## ACT1

Deficiency of ACT1 in fibroblast results in a selective defect in IL-17-induced activation of an NF-κB pathway and abrogates IL-17-induced cytokine and chemokine expression (49). The N-terminal domain of ACT1 is essential for the interaction with TRAF6 and for IL-17-mediated NF-κB activation in mouse embryonic fibroblasts (MEFs) (96). ACT1-deficient mice develop much less inflammatory disease in both EAE and DSS-induced colitis due to the impaired IL-17-induced expression of inflammation-related genes in ACT1-deficient astroglial cells or

gut epithelial cells (46). In humans, a biallelic missense mutation (T536I) in ACT1 abolishes the homotypic interaction between IL-17R and ACT1, resulting in impaired IL-17 responses with chronic mucocutaneous candidiasis (97).

The precise structure of the ACT1–TRAF6 complex remains obscure. The crystal structure of TRAF6 complexed with TRAF6-binding peptides from CD40 and RANK has proposed the TRAF6-binding motif (98). It is shared in CD40, RANK, and IRAK1, yet there are marked structural differences between receptor recognition by TRAF6 and that by other TRAFs (98). In ACT1, three TRAF6-binding motifs have been suggested: at amino acid residues (in human ACT1) 15–20 (96) and 37–42 (50) in the N-terminal region and 327–334 in the Ser–Gly–Asn–His (SGNH) hydrolase region (99).

The contribution of epithelial ACT1 in the development of psoriasis is still a matter of debate. On the one hand, D19 in the N-terminal region is critical for IL-17 signaling and interaction with TRAF proteins, IKKε [also known as IKKi; inducible IKK, discussed in section IKKε (IKKi)], and a chaperone heat shock protein (Hsp)90 (section Hsp90) (100). On the other hand, a D19A mutation is concluded to be a loss-of-function variant associated with psoriasis susceptibility (101–103). Also, ACT1-deficiencient mice spontaneously develop IL-22-dependent dermatitis (100). However, the hyperactive type 17 response related to the D19A mutation seems to be epithelial cell-independent, because mice with a T cell-specific deficiency in ACT1 (*Lck-Cre Traf3ip2* $^{flox/-}$) also developed a hyperactive type 17 response, suggesting a T cell-intrinsic phenotype.

## Other TRAF Proteins

TRAF3 is supposed to have a negative role in IL-17 signaling (104) (**Figure 6**). Treatment with TRAF3 siRNAs enhances IL-17 signaling in HeLa cells, and exacerbates EAE driven by IL-17 in mice. The enhanced IL-17 signaling in *Traf3*$^{-/-}$ MEFs are reversed by transfection with TRAF3. TRAF3 is assumed to inhibit IL-17 signaling by competing with ACT1 to interact with IL-17R. Nuclear Dbf2-related kinase 1 (NDR1) interacts with TRAF3 and prevents its binding to IL-17R, and consequently, NDR1 functions as a positive regulator of IL-17 signaling (105). The expression of NDR1 in the colon mucosal epithelial cells of ulcerative colitis patients is increased, suggesting the positive regulation of IL-17 signaling and production of inflammatory mediators (105).

TRAF4 is also suggested to be a negative regulator of IL-17 signaling (**Figure 6**) supported by the evidence that TRAF4 deficiency increases IL-17 signaling in mouse primary kidney cells and exacerbates EAE in mice (106). Therefore, we could conclude a restricting role for TRAF4 in IL-17 signaling, probably due to the competition of TRAF4 with TRAF6 for the interaction with ACT1. Besides, an IL-17-dependent TRAF4-ERK5 axis is suggested to drive a positive feedback loop of p63-mediated TRAF4 expression in keratinocyte proliferation (107).

TRAF2 and TRAF5 interact with ACT1 and activate downstream MAPK signaling (99). The TRAF2/5–ACT1 interaction are dependent on the ACT1 phosphorylation at S311, adjacent to a putative TRAF-binding motif.

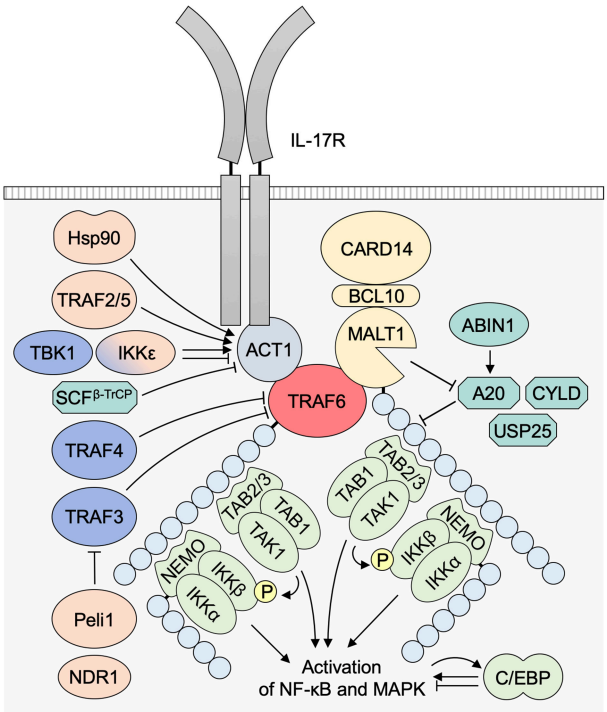


**FIGURE 6 |** The regulatory mechanisms of the IL-17–TRAF6 pathway. TRAF2 and TRAF5 interact with ACT1 and activate downstream MAPK signaling. The TRAF2/5 binding with ACT1 is dependent on ACT1 S311 phosphorylation by IKKε. IKKε and TBK1 can also be involved in the suppression of the association of ACT1–TRAF6 binding. Hsp90 is a chaperone protein of ACT1. The binding between Hsp90 and ACT1 is required for the IL-17 signaling and is dependent on ACT1 D19. SCF$^{\beta-TrCP}$ E3 ubiquitin ligase complexes are involved in the K48-linked polyubiquitination and degradation of ACT1. TRAF3 is expected to inhibit IL-17 signaling by competing with ACT1 to interact with IL-17R. TRAF4 is also suggested to be a negative regulator for IL-17 signaling, probably due to the competition of TRAF4 with TRAF6 for the interaction with ACT1. Peli1 opposes to TRAF3, by promoting TRAF6-induced K63-linked ubiquitination of c-IAP, which then ubiquitinates TRAF3 with K48 linkage, resulting in TRAF3 proteasomal degradation. NDR1 interferes TRAF3–TRAF6 interaction. A20 is a negative regulator for ubiquitin signaling via dual activity: (1) deubiquitinase activity to K63-linked chains and (2) K48-linked ubiquitinase activity mediating proteasomal degradation of substrate signaling molecules. ABIN1 promotes A20 activity. CYLD and USP25 are other deubiquitinases. C/EBP has both stimulatory and regulatory roles in the transcription of IL-17-response genes. ABIN1, A20 binding and inhibitor of NF-κB-1; ACT1, NF-κB activator 1; BCL10, B-cell lymphoma/leukemia 10; CARD, caspase recruitment domain-containing protein; C/EBP, CCAAT/enhancer binding protein; Hsp, heat shock protein; IKK, IκB kinase; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; NDR1, Nuclear Dbf2-related kinase 1; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; SCF$^{\beta-TrCP}$, Skp1-cullin-1-F-box protein β-transducin repeat-containing protein; TAB, TAK1 binding protein; TAK1, transforming growth factor-β-activated kinase 1; TBK1, TANK-binding kinase 1; TRAF, tumor necrosis factor receptor associated factor; USP25, ubiquitin-specific protease 25.

## IKKε (IKKi)

IKKε (IKKi) forms a complex with ACT1 and mediates IL-17-induced phosphorylation of ACT1 at S311, which is required for the IL-17-mediated ACT1–TRAF2/5 interaction but not for ACT1–TRAF6 interaction (99) (**Figure 6**). The IL-17-mediated ACT1 S311 phosphorylation by IKKε and subsequent formation

of ACT1–TRAF2/5 interaction is involved in IL-17 signaling. IKKε also participates in a TLR3/4–TRIF–TANK-binding kinase 1 (TBK1) pathway (108). Despite the requirement of IKKε for the ACT1 S311 phosphorylation in IL-17 signaling, IKKε and TBK1 phosphorylate ACT1 in three other Serine sites to suppress the association of ACT1 with TRAF6 and downstream NF-κB activation (109). IKKε-deficiency in airway epithelial cells reduces IL-17-induced JNK and p38 activation, and expression of IL-17-response genes (including *Cxcl1, Cxcl2, and Il6*), suggesting that IKKε is a modulator of IL-17 signaling through its effect on ACT1 phosphorylation and ACT1–TRAF interaction in epithelial cells. However, a precise mechanism for balancing between the ACT1–TRAF6 and the ACT1–TRAF2/5 interactions and its physiological role remain elusive.

## Hsp90

Hsp90 is a molecular chaperone protein essential for activating many signaling proteins in the eukaryotic cell (110). It has been observed that Hsp90 interacts with ACT1, but does not with the D19A loss-of-function mutation variant of ACT1 (104). In addition, Hsp90 inhibitors abolish the interaction of Hsp90 or TRAF proteins, and IL-17 signaling. Consequently, the activity of Hsp90 is required for the IL-17 signaling, and the interaction between Hsp90 and ACT1 N-terminus is critical for TRAF6-dependent IL-17-mediated response in epithelial cells (**Figure 6**).

## Other E3 Ligases: Peli and $SCF^{\beta-TrCP}$

Peli (Pellino) is a family of signal-responsive E3 ubiquitin ligases regulating innate immune responses by K48 and K63-linked polyubiquitination (111). The family encompasses 3 members (Peli1, 2, and 3) that are ubiquitous and interact with TRAF6, IRAK1/4, and TAK1.

Peli1 controls both the downstream and upstream TRAF6 signaling pathway. In downstream of TRAF6, Peli1 is involved in polyubiquitination of RIPK1 and subsequent activation of TAK1–IKK–NF-κB signaling in macrophages (112). The activation of Peli1 is mediated by TBK1 and IKKε in a TRIF-dependent TLR pathway (108). In upstream of TRAF6, Peli1 functions in the polyubiquitination of IRAK1 and is required for IL-1 signaling although the precise mode of action remains unclear (113). Peli1 promotes microglial TRAF6-mediated MAPK activation in EAE (114). Specifically, Peli1 mediates TRAF6-induced K63-linked polyubiquitination of c-IAP [c-inhibitor of apoptosis protein: a member of other E3 ubiquitin ligase family IAP (115)], which then ubiquitinates TRAF3 with K48 linkage, resulting in TRAF3 degradation and thereby removing its suppression of the signaling for MAPK activation (**Figure 6**). Blockade of IRAK1–Peli1–TRAF6 signaling by TGF-β-mediated Smad6–Peli1 interaction is involved in the anti-inflammatory effects of TGF-β signaling (116).

Moreover, Peli1 is possibly involved in the development of psoriasis. Peli1 expression is enhanced in the epidermis of psoriasis lesions, and doxy-inducible $Peli1^{tg}$ mice spontaneously develop psoriatic inflammation, which depends on Peli1 overexpression in radioresistant cells, with increased expression levels of IL-17 and IL-22 in the skin (115). In addition, imiquimod-induced psoriatic dermatitis is impaired in Peli1 deficient mice. These results suggest possible regulatory roles of Peli1 in IL-17 signaling in epithelial cells. In these mice, however, the involvement of Peli1 in keratinocyte-specific TRAF6 signaling remains unexplored (115).

Peli2 and Peli1 have redundant E3 ligase activities with TRAF6 in IL-1, TLR, and RANKL signaling (117). The IL-1β-induced formation of K63-polyubiquitin chains and ubiquitylation of IRAK1, IRAK4, and MyD88 are abolished in TRAF6/Peli1/Peli2 triple-knockout (KO) cells, but not in TRAF6 KO or Peli1/2 double-KO cells. In E3 ligase-inactive TRAF6 (L74H) mutant MEFs, TLR responses are reduced in the early phase but abolished in the late phase whereas RANKL signaling is unaffected. Thus, we may suggest that TRAF6 poses E3 ligase activity-dependent and independent roles.

Peli3 negatively regulates TLR3 signaling via polyubiquitination of TRAF6 as poly(I:C)-induced polyubiquitination of TRAF6 is defective in MEFs lacking Peli3, resulting in enhanced TLR3-mediated production of type I IFNs (118) and suggesting possible regulatory roles of Peli in TRAF6 signaling in epithelial cells.

Skp1-cullin-1-F-box (SCF) that contains the F-box protein β-transducin repeat-containing protein ($SCF^{\beta-TrCP}$) is an E3 ubiquitin ligase complex. It was demonstrated that $SCF^{\beta-TrCP}$ is involved in the desensitization of IL-17 signaling though ACT1 polyubiquitination and degradation (119). Persistent stimulation with IL-17 in HeLa cells stimulates ACT1 phosphorylation and subsequent K48-linked polyubiquitination and degradation through $SCF^{\beta-TrCP}$, resulting in the IL-17 desensitization. However, similar regulatory mechanisms remain unknown in epithelial cells.

## Deubiquitinases: A20, CYLD, and USP25

A20 is a ubiquitin editing enzyme and is a negative regulator of innate immune responses. Single nucleotide polymorphisms (SNPs) in *TNFAIP3* encoding A20 confer risk to several inflammatory or autoimmune diseases, such as psoriasis, Crohn's disease, rheumatoid arthritis, and systemic lupus erythematosus (120, 121). A20 regulates polyubiquitination via its dual roles (**Figure 6**): deubiquitinating enzyme activity removing K63-linked polyubiquitin chains resulting in reduction of ubiquitination signaling; and ubiquitin E3 ligase activity that promotes K48-linked polyubiquitination and subsequent proteasome-mediated degradation of the substrate signaling molecules (121). In MEFs, A20 is associated with TRAF6 in an IL-17-dependent manner and restricts the IL-17-dependent activation of NF-κB and MAPKs (122). It has also been suggested that TNF–A20 signaling axis is responsible for TNF-mediated IL-17 inhibition in CD4+ T cells, which is related to disease exacerbation in inflammatory bowel diseases and multiple sclerosis in addition to paradoxical reactions in psoriasis as a response to anti-TNF therapies (123). Mechanistically, A20 ovarian tumor (OTU) domain at the N-terminus, which has a deubiquitinase activity, binds to TRAF6 and dismantles K63-linked polyubiquitin chains from TRAF6 (124). However, it is not followed by A20-mediated K48-linked polyubiquitination and subsequent degradation of TRAF6. In addition, the

deubiquitinase activity of A20 is dispensable for NF-κB signaling in macrophages; as loss of deubiquitinase function mutation of A20 (C103A) does not affect ubiquitination and K63-linked ubiquitination levels in TRAF6 (125). Therefore, epithelial TRAF6 may not be a major target of A20 in regulating type 17 immune responses and the precise mechanisms of the cell-specific roles of A20 and their controls remain to be addressed.

*TNIP1*, encoding A20 binding and inhibitor of NF-κB-1 (ABIN1), is also associated with susceptibility to psoriasis (120). ABIN1 directly binds to A20 and NEMO/IKKγ and negatively restricts TNF and TLR-induced signals (126) (**Figure 6**). Loss of ABIN1 in keratinocytes (*K14-Cre Tnip1* $^{flox/flox}$) leads to deregulation of IL-17-induced gene expression and exaggerated chemokine production *in vitro* and overt psoriasis-like inflammation *in vivo* (127). In contrast, ABIN1 lentiviral overexpression inhibits the expression of genes for IL-17 and TNF signaling pathways in human keratinocytes *in vitro* (128). Thus, epithelial homeostasis and dysregulation of the polyubiquitination system is critical for the IL-17-mediated chronic inflammation such as psoriasis.

CYLD is another deubiquitinase that removes K63 and Met1 (M1)-linked polyubiquitin chains from several signaling mediators and thus dampens NF-κB-dependent gene expression (129) (**Figure 6**). CYLD has been demonstrated to negatively regulate TRAF6-mediated ubiquitination (130, 131). CYLD is required for down-regulation of RANKL signaling in osteoclasts by inhibiting TRAF6 ubiquitination (132). However, despite its significant role in modulating tumor development (including cylindroma) (133), contribution of epithelial CYLD in regulating innate and type 17 immune responses needs to be further investigated.

Of note, both A20 and CYLD deubiquitinases are cleaved by MALT1 that is activated by an upstream component of TRAF6 signaling: a CBM signalosome (134, 135) (**Figure 6**). Inactivation of MALT1 protease activity causes reduced stimulation-induced T cell proliferation, impaired IL-2 and TNF production, as well as defective $T_H17$ differentiation *in vitro* (136). Consequently, the development of $T_H17$-dependent EAE is attenuated in MALT1 protease activity-deficient mice despite their development of a multiorgan inflammatory pathology characterized by $T_H1$ and $T_H2/0$ responses (136). The administration of a MALT1 protease inhibitor mepazine also attenuates EAE (137). Possible contribution of CARD14 in cleaving these deubiquitinases is discussed in the first section of the next chapter.

Ubiquitin-specific protease 25 (USP25) is a newly identified deubiquitinase that negatively regulates IL-17-triggered signaling (138). IL-17 induces the association of USP25 with TRAF5 and TRAF6, and USP25 removes K63-linked ubiquitination in TRAF5 and TRAF6. USP25 deficiency enhances the expression of inflammatory mediators in lung epithelial cells and MEFs. Consistently, *Usp25*$^{-/-}$mice show greater sensitivity to IL-17-induced pulmonary inflammation and EAE (138).

# PLAYERS IN EPITHELIAL TRAF6 PATHWAYS IN PSORIASIS

## CARD14

Being a member of CARD family protein, CARD14 can bind with BCL10, MALT1, and TRAF proteins including TRAF6 (139). Also, it is involved in activation of innate immune responses by the formation of a CBM signalosome with subsequent activation of NF-κB and MAPK pathways (140, 141). CARD14 is known to be selectively expressed in the epidermis, and its gain-of-function mutations are found in the familial type of psoriasis (142). However, the receptor signaling pathways upstream of CARD14 in keratinocytes remain unspecified, yet the keratinocyte treatment with CARD14 siRNA reduces the MALT1 protease activity (143). In addition, CARD14 is involved in IL-17 pathways in keratinocytes (45). Pathogenic CARD14 mutants (such as CARD14 E138A or ΔE138) result in spontaneous formation of signalosome assembly in keratinocytes *in vitro* and development of psoriatic dermatitis *in vivo* (45, 144). IL-17 stimulates CARD14 interaction with TRAF6 and ACT1 in keratinocyte cell line HaCaT while IL-17 induces lower *Ccl20*, *S100a8*, and *S100a9* expression in CARD14-deficient mouse keratinocytes compared to wild-type cells *in vitro* (45).

Moreover, it has been demonstrated that the CARD14 E138A mutant activates MALT1 protease activity (145). Some pathogenic CARD14 mutants are all more potent than wild-type CARD14 in inducing A20 and CYLD cleavage but others are not (140). These results may tempt us to consider that defective regulation by A20 and CYLD for the IL-17–TRAF6-mediated responses is central for the development of psoriasis in patients with CARD14 pathogenic mutations. Consequently, CARD14 has definitive and multiple roles in the cascade/loop of IL-17–TRAF6-mediated chronic inflammation in the skin of psoriasis patients (**Figure 3A**).

It is of interest to note that loss-of-function mutations in *CARD14* have been reported in 3 families with a severe variant of atopic dermatitis (146) whereas *Card14*$^{-/-}$ mice do not have spontaneous AD (45).

## NF-κB Pathways vs. MAPK Pathways

IKK–NF-κB and p38/JNK–AP-1 pathways pose major impacts on TRAF6 downstream. Both NF-κB and p38 MAPK are activated in the epidermis of the lesional skin from psoriasis patients (147–149). Therefore, it seems confusing that both NF-κB and AP-1 deficiency in the epidermis result in spontaneous development of psoriatic inflammation in mice (150–152). However, the former is a very quick outcome after birth whereas the latter shows more chronic changes suggesting a secondary outcome. In addition to the AP-1-mediated gene transcription, p38 regulates the expression of inflammatory cytokines and chemokines via their mRNA stabilization and translation (74, 75), suggesting that the phenotype of mouse epidermal AP-1 deficiency does not fully represent that of p38 deficiency. Therefore, the unbalanced homeostasis of the TRAF6 signaling pathways with attenuated NF-κB activation and dominant MAPK activation in the epidermis might contribute to triggering type 17 innate response and giving rise to the increased susceptibility to psoriasis.

As for the balance between the NF-κB and MAPK activation, it is noteworthy that microRNA (miR)-146a is expressed in a NF-κB dependent manner and inhibits the transcription of TRAF6 and IRAK1, leading to negative feedback regulation of the TRAF6–NF-κB pathway (153, 154). Accordingly, miR-146a deficiency leads to hyperexpression of TRAF6 and IRAK1. Therefore, it is plausible to assume that the defective NF-κB activation in response to TLR/IL-1 or IL-17 signaling may initiate dominant activation of p38/JNK MAPKs (**Figure 7**). At present, however, it has not yet been determined whether cutaneous activation of p38/JNK MAPKs is sufficient for the induction of IL-17-dependent psoriatic inflammation.

## IL-36 Cytokines

Many IL-1 family cytokines, including IL-1α/β, IL-18, IL-33, and IL-36α/β/γ, share their molecular structure and protein maturation mechanism. The ligation to their functional receptors signals through MyD88-dependent TRAF6 signaling pathways (155) (**Figure 2A**). Imiquimod-induced skin inflammation is partially reduced in mice deficient for both IL-1α/IL-1β or for IL-1R1, but not in IL-1α- or IL-1β-deficient mice, demonstrating the redundant activity of IL-1α and IL-1β for skin inflammation (156). Limited clinical efficacy of anti-IL-1 or IL-1R antibodies in psoriasis also suggest possible redundancy of the IL-1 family (157).

Ligation of IL-36α/β/γ, but not the endogenous IL-36R antagonist (IL-36Ra), to IL-36R, activates NF-κB, and p38 MAPK pathways (158) (**Figure 3B**). Loss-of-function mutations in *IL36RN* encoding IL-36Ra are found in familial-type generalized pustular psoriasis (159). IL-36R-deficient ($Il36r^{-/-}$) mice are protected from the imiquimod-induced expansion of dermal IL-17-producing γδ T cells and psoriatic dermatitis, and IL-36R on radioresistant resident cells is crucial for these responses (160). In addition, RNA-seq analysis of normal human epidermal keratinocytes reveals that IL-1β and IL-36 responses in keratinocytes share MyD88-dependent gene signature (161). Therefore, IL-36–TRAF6 signaling in keratinocytes might be a critical event in this animal model and human psoriasis while the production of IL-36α/β/γ is not so affected in imiquimod-induced dermatitis in mice lacking TRAF6 in keratinocytes (3). Intriguingly, IL-36 also induces IκBζ expression in keratinocytes in a MyD88-dependent manner and is required for the expression of various psoriasis-related genes (162) as described in the next section.

## IκBζ

IκBζ is the inducible nuclear protein that functions as a regulator of IL-1/TLR-mediated gene expression, such as *Il6, Il12b*, and *Csf2*, but not *Tnf* (163). *NFKBIZ* encoding IκBζ resides in a psoriasis susceptible locus (164). IL-17 induces IκBζ expression in keratinocytes in a p38 MAPK-dependent manner (165, 166) while TNF and IL-17-mediated synergistic induction of *DEFB4*, but not *CCL20* or *IL8* expression, depends on IκBζ in human keratinocytes (166) although *CCL20* and *IL8* are also IκBζ target genes (162, 167). In addition, IκBζ is directly recruited to the promoter regions of psoriasis-associated target genes (167) whereas the loss of IκBζ expression alters H3K4 tri-methylation and switch/sucrose non-fermenting (SWI/SNF) complex recruitment, thereby influencing promoter accessibility at IκBζ target genes (168, 169). Moreover, imiquimod-induced psoriatic dermatitis is fully abolished in IκBζ-deficient mice (167) whereas intradermal injection of IL-36α induces psoriatic dermatitis that is dependent on IκBζ (162). Furthermore, dysregulation of IκBζ function might be involved in the chronicity of IL-17-mediated inflammation because it has been shown that IκBζ has opposite regulatory roles at initial and resolution phases of inflammation via the DNA methylation by Tet2 (170). Consistently, dimethyl itaconate can selectively regulate secondary, but not primary, transcriptional responses to TLR stimulation via inhibition of IκBζ protein induction by ATF3 while dimethyl itaconate ameliorates IL-17–IκBζ-driven skin pathology in a mouse model of psoriasis (171). Therefore, IL-36R–TRAF6–IκBζ-IL-36 and IL-17R–TRAF6–IL-17 loops might be key features of the chronic inflammation in the EIME of psoriatic dermatitis (**Figures 3A,B**). Of note, IκBζ-deficient mice spontaneously develop atopic dermatitis-like inflammation with increased levels of serum IgE (172).

## RIPKs

A RIPK family is composed of 7 kinases characterized by their roles in balancing inflammation and cell death in terms of canonical and non-canonical NF-κB, MAPK, and apoptotic and non-apoptotic cell death pathways (44, 173). Despite a characteristic capacity of RIPK family members to bind to TRAF proteins, RIPK2 and RIPK4, but not RIPK1, interact with TRAF6 and get involved in TRAF6-mediated NF-κB and MAPK activation (174–177). Only a few reports have suggested a link between psoriasis and keratinocyte RIPK4 (178, 179) whereas RIPK2 might be involved in gut mucosal innate responses (31).

Among RIPKs, only RIPK2 (also known as CARD3) has a CARD domain at the C terminus (173). TLR4-induced activation of NF-κB and p38 MAPK impaired in mouse macrophages lacking RIPK2 and the kinase activity of RIPK2 is dispensable in these signaling pathways (180). RIPK2 is known to function in NLR signaling via CARD–CARD homotypic interactions between NOD1/2 and RIPK2 (173). NOD2 overexpression-induced activation of TRAF6–NF-κB signaling is inhibited by RIPK2 siRNA. NOD2 and RIPK2 share a common E2 complex to ubiquitinate NEMO/IKKγ and activate NF-κB in MEFs and human intestinal microvascular endothelial cells (31, 181). The activation of TRAF6 is lost with major Crohn's disease-associated NOD2 allele L1007insC, suggesting involvement of RIPK2 in the linkage between the mucosal innate responses and the gut microbiota (31).

RIPK4 tips toward NF-κB signaling for inflammation especially in skin cells. RIPK4 has been shown to regulate epidermal differentiation and cutaneous inflammation. Mice with epidermis-specific expression of RIPK4 ($K14\text{-}Ripk4^{tg}$) are specifically sensitive to phorbol-12-myristate-13-acetate (PMA)-driven TNF-independent inflammation (182). Consistently, the PMA-induced expression of proinflammatory mediators is inhibited by RIPK4 siRNA treatment in human keratinocytes (183). RIPK4 expression levels are higher in keratinocytes in psoriasis lesions than in healthy control skin, and stimulation

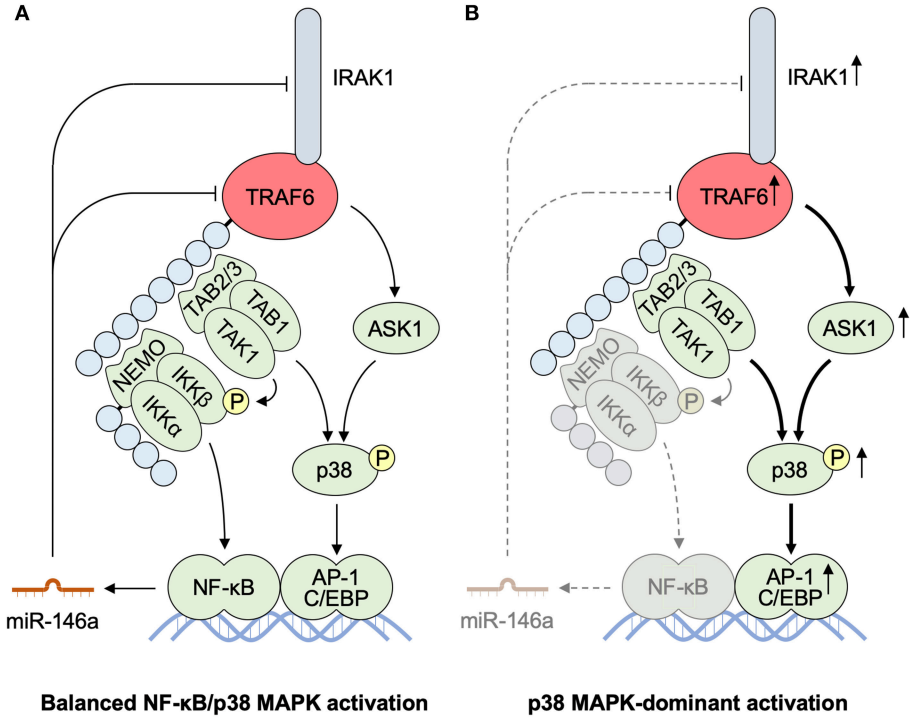


**FIGURE 7 |** MicroRNA-mediated TRAF6 regulation balancing NF-κB and MAPK activities. **(A)** miR-146a is produced in an NF-κB-dependent manner. miR-146a interferes the transcription and translation of IRAK1 and TRAF6 as a mediator of the negative feedback pathway for an NF-κB pathway. **(B)** Defective activation of an IKK–NF-κB pathway results in the reduced production of miR-146a. The impaired miR-mediated regulation of IRAK1 and TRAF6 accelerates further activation of a p38 MAPK pathway, resulting in p38 MAPK-dominant activation. AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; C/EBP, CCAAT/enhancer binding protein; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; miR, microRNA; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; TAB, TAK1 binding protein; TAK1, transforming growth factor-β-activated kinase 1; TRAF6, tumor necrosis factor receptor associated factor 6.

with IL-17 induces RIPK4 expression in keratinocytes (178, 179). In addition, RIPK4 interacts with STAT3 and enhances IL-17-mediated STAT3 phosphorylation and CCL20 expression in HaCaT cells. RIPK4 mutations are associated with popliteal pterygium syndrome (Bartsocas-Papas type) showing limb and skin abnormalities (184), which is considered to be a close resembling of IKKα deficiency (185, 186). Consistently, RIPK4 associates with IKKα and IKKβ and activates them in a kinase-dependent manner (187). Keratinocyte-specific ablation of RIPK4 (*K14-Cre Ripk4*$^{tm1c/tm1c}$) also results in delayed keratinization and stratum corneum maturation (188). Either RIPK4 or IKKα down-regulation in primary keratinocytes interferes with expression of *Ovol1* (189, 190). However, the involvement of TRAF6 in RIPK4 functions in keratinocytes remains largely unknown.

## EPITHELIAL TRAF6 SIGNALING IN THE EIME OF TYPE 17 RESPONSES

The epithelial tissues organize the microenvironment for the induction and propagation of situation-specific inflammation in the adjacent tissues beneath the epithelium (1, 2). This microenvironment is composed of 5 factors. Four out of them are unique in the epithelial microenvironment: (1) microbiota, (2) barriers, (3) epithelial cells, and (4) sensory nerve endings; while the 5th (immune cell society) completes the EIME. Interaction of these factors in the EIME governs the protective and regenerative responses of the epithelium. Transcriptional regulation of the epithelial cells has a central role in the organization of the EIME because both the primary responses to external agents and the secondary responses to the immune activation of epithelial cells produce inflammatory mediators essential for the amplification and propagation of effective immune responses. Accordingly, the dysregulated activation of the EIME can lead to the development of chronic inflammatory diseases in the skin, the gut, and the lung.

TRAF6 is considered to be a central factor that drives the transcriptional responses of epithelial cells in triggering and propagating type 17 immune responses, in host defense and inflammatory diseases. TRAF6 plays critical roles in the primary responses of epithelial cells to external agents that induce type 17 innate and immune responses. In addition, the epithelial cells have IL-17R while TRAF6 has an inevitable role in the IL-17 signaling. Moreover, the activation of TRAF6 signaling and downstream of NF-κB and MAPK pathways effectively promotes the transcription of proinflammatory mediators that mediate the activation of the IL-23/IL-17 axis and drive the inflammatory

loop of IL-17 in the EIME (**Figure 3A**). Furthermore, TRAF6 is expected to be essential for driving the inflammatory loop of IL-36–IκBζ that plays indispensable roles in organizing the type 17 EIME in the skin for the development psoriatic inflammation (**Figure 3B**). Consequently, several players contribute to the harmonious regulation of TRAF6 signaling in the epithelial cells in order to orchestrate the architecture of type 17 innate and immune responses as described in this review.

TRAF6-dependent signaling in keratinocytes does not seem to play critical roles in $T_H1$ or $T_H2$-type inflammation (3). K5-*Cre Traf6* $^{flox/flox}$ mice develop hapten-induced $T_H1$-type contact hypersensitivity as well as wild-type mice despite a significant but partial attenuation of *Ifng* expression in the skin. In addition, expression of *Il4* mRNA and serum IgE levels in papain-induced skin inflammation are comparable between K5-*Cre Traf6* $^{flox/flox}$ mice and wild-type mice. To our knowledge, however, there have been no additional information about the roles of epithelial TRAF6 in $T_H1$, $T_H2$, or Treg response, or counterpart molecules in epithelial signaling pathways for the induction of each immune responses.

## CONCLUDING REMARKS

The multilateral studies into the molecular functions and their regulatory mechanisms of TRAF6 have depicted various aspects of TRAF6 definitive roles in the immune system and inflammatory diseases. The new insights on TRAF6 signaling in epithelial cells during different immune responses provide us with the evidence for other potential roles rather than serving as barriers. One may expand the idea to the correspondence of specific cell signaling in the epithelial cells to certain type of immune response or chronic inflammation. However, these insights may raise more questions than answers: (1) Is epithelial TRAF6 signaling also essential for type-17 protective immune responses against microbes, such as Candida or segmented filamentous bacteria? (2) Is epithelial TRAF6 critical in the protective responses and inflammatory diseases in other epithelial organs such as the respiratory system or the urinary tract? (3) Does epithelial TRAF6 have definitive roles in $T_H1$, $T_H2$, or Treg response or differentiation in some situations? (4) Otherwise, what is the epithelial counterpart signaling molecule in type 1, type 2, or regulatory immune responses? (5) What "balance" of the downstream effectors can represent the decision on the consequent immune types? (6) What are the mechanisms responsible for the community of the EIME loop of inflammation in chronic inflammatory diseases?

The progress in our understandings of the regulation of immune responses by TRAF6 in the epithelial cells will arrow us to develop new therapeutic strategies. CD40–TRAF6-specific nanobiologics have been demonstrated to be effective *in vivo* (191, 192). These preceding investigations should enhance developing the new drugs that can modulate TRAF6 E3 ubiquitinase activities or protein–protein interactions with TRAF6 for specific purposes such as effective cutaneous vaccinations and treating chronic inflammatory diseases.

## AUTHOR CONTRIBUTIONS

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

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# Vascular Smooth Muscle Cells Contribute to Atherosclerosis Immunity

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Vascular smooth muscle cells (VSMCs) constitute the major cells in the media layer of arteries, and are critical to maintain the integrity of the arterial wall. They participate in arterial wall remodeling, and play important roles in atherosclerosis throughout all stages of the disease. Studies demonstrate that VSMCs can adopt numerous phenotypes depending on inputs from endothelial cells (ECs) of the intima, resident cells of the adventitia, circulating immune cells, hormones, and plasma lipoproteins. This plasticity allows them to perform multiple tasks in physiology and disease. In this minireview, we focus on a previously underappreciated activity of VSMCs, i.e., their impact on atherosclerosis immunity via formation of artery tertiary lymphoid organs (ATLOs).

**Keywords: atherosclerosis, vascular smooth muscle cells, endothelial cells, intima, adventitia, artery tertiary lymphoid organs**

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## PLASTICITY OF VSMCs IN PHYSIOLOGY AND DISEASE

Vascular smooth muscle cells (VSMCs) are the major constituents of medium- and large-sized arteries. Although mechanisms of atherogenesis largely remain to be defined, studies have demonstrated that disease progression involves crosstalk between immune cells with both ECs and VSMCs. Some of these interactions promote plaque growth while others attenuate the size, cellular composition, and stability of atherosclerotic plaques (1, 2) (**Figure 1**, **Table 1**). VSMCs show remarkable plasticity in response to vascular injury, inflammation, and lipoprotein accumulation during disease progression via reprogramming gene expression and a shift to a proliferative, pro-migratory, and activated phenotype, i.e., *phenotype switching* (5). In **Figure 1**, we depict critical molecular switches that have been proposed to be important regulators of disease progression (3, 6, 8, 11). During atherosclerosis initiation, blood-derived monocytes, which have been recruited into the intima, accumulate lipid giving them a foamy appearance. These foam cells contribute to *fatty streak* formation which constitutes the earliest and possibly reversible stage of atherosclerotic plaques. Fatty streaks gradually develop into atheromas/plaques ultimately leading to expanded plaques that contain VSMCs, T cells and myeloid cells (**Figure 1**) (33). Intriguingly, the composition of an atherosclerotic plaque rather than its size determines its stability as fibrous cap thickness and necrotic core size are potential hallmarks of a stable vs. an unstable plaque, respectively (4, 9). VSMCs in the intima layer are traditionally viewed as beneficial during atherogenesis because they produce extracellular matrix components, thereby promoting formation of stronger fibrous caps resulting in protection against plaque rupture (4, 9). It is increasingly apparent that

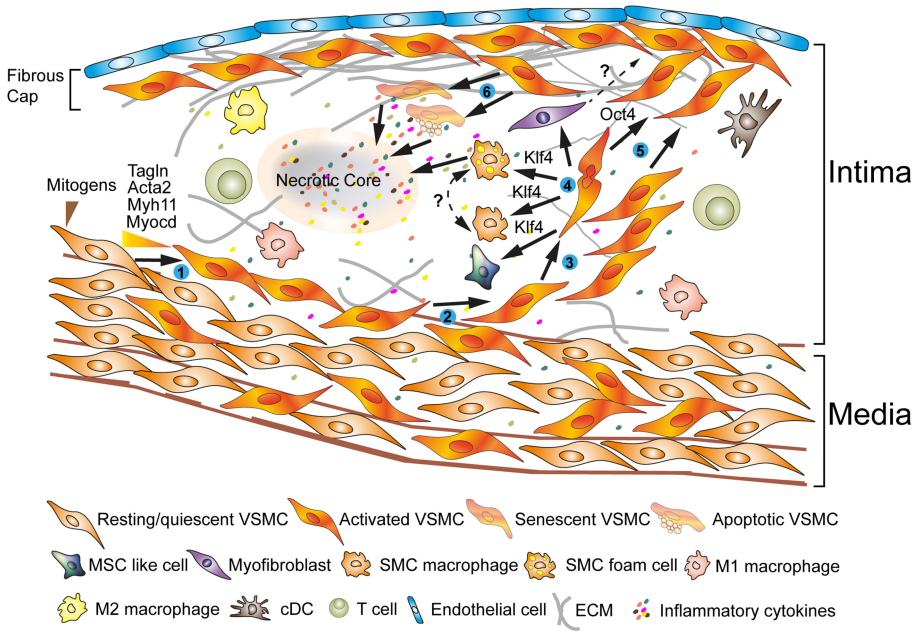


**FIGURE 1 |** Phenotypes of VSMCs during atherosclerosis progression reveal extraordinary plasticity. ECs and VSMCs in atherosclerotic plaques show activated phenotypes (❶) (3, 4). Some of the VSMCs originate in the media following recruitment into the intima; other VSMCs may be bone marrow-derived or they may originate from myeloid cells in the circulation (❷) (3). A fraction of VSMCs proliferate (❸) (3, 5). VSMCs secrete extracellular matrix components; the pluripotent transcription factor, i.e., Klf4, may play major roles in a process referred to as *phenotype switching* (❹) (6, 7). In attempts to shield the atherosclerotic plaque from lethal rupture, Oct4 may control a process that has been termed remigration to form a fibrous cap (❺) (4, 8–10). Senescence and apoptosis trigger the generation of additional inflammatory cytokines to form a necrotic core initiating a vicious cycle with lethal clinical consequences during the late stages of the disease (❻) (3, 4, 11).

VSMCs undergo a plethora of structural and functional phenotypical transformations and may even completely lose their native features to acquire characteristics of other cell types including macrophages. Data indicate that VSMCs can acquire dichotomic phenotypes with a Janus head-type nature, i.e., pro- vs. anti-atherogenic properties, depending on the tissue environment and action of risk factors (3). VSMCs release cytokines to stimulate adjacent ECs to express adhesion molecules and release cytokines, and/or enhance chemotaxis of monocyte/macrophages into the plaque (3, 34, 35) (**Table 1**). These data indicate that accumulation of VSMCs in the fibrous cap or intima are beneficial, whereas their loss or transition into an inflammatory phenotype are detrimental, and that the balance between VSMCs proliferation/migration vs. death/senescence determines atheroprogression vs. plaque stability (3). Various additional aspects of VSMC biology in health and disease were recently covered by a series of excellent reviews (https://academic.oup.com/cardiovascres/issue/114/4) and will not be covered here. Below, we therefore focus attention on the role of the adventitia and the potential impact of VSMCs in ATLO formation.

## VSMCs AFFECT ADVENTITIA IMMUNE RESPONSES IN HYPERLIPIDEMIC MICE

During the last two decades, it became increasingly apparent that the adventitia is a highly complex and immunologically active tissue harboring cells as diverse as stromal cells, nerves, lymph vessels, vasa vasora, and resident leukocytes/progenitor cells all of which have the ability to affect disease progress (36, 37). We observed that the adventitia of $ApoE^{-/-}$ mice undergoes major restructuring events during all stages of atherosclerosis: Both innate and adaptive immune cells accumulate adjacent to the neighboring atherosclerotic plaques (**Figure 2**). Moreover, our studies in experimental mice were corroborated in human diseased arteries: We and others observed ATLO-like structures in the adventitia of patients presenting with atherosclerotic aortic aneurysms (38, 39) and more recently, Akhavanpoor et al. observed well-developed ATLOs in the adventitia of a considerable percentage of patients afflicted with ischemic heart disease (40). In murine atherosclerosis, lymph vessel-, high endothelial venule (HEV)-, and blood vessel neogenesis are prominent features of this restructuring process (16, 41).

## AGING OR CHRONICITY OR BOTH?

Importantly, aging was found to be a major determinant of adventitia restructuring and sculpting. Fully developed ATLOs form during advanced stages of atherosclerosis but their early stages, i.e., T/B cell aggregates, emerge in parallel to the formation of atherosclerotic plaques (16, 27, 41, 42). Indeed, these early ATLOs form at the age of ∼30 weeks and reach more advanced stages at around 52 weeks to peak at 78 weeks (16, 41). Similar age-dependent developmental stages of TLOs have been observed

**TABLE 1 |** Key inflammatory mediators involved in VSMC-mediated atherosclerosis immunity.

| | Cellular sources | Cellular targets | Roles in SMC dynamics | Impact in local immunity | Roles in atherosclerosis | References |
|---|---|---|---|---|---|---|
| IL-1α | Apoptotic SMC, EC, T cell, macrophage | SMC, EC, monocyte | Activation, proliferation, remodeling | EC activation, monocyte influx | Damaging | |
| IL-1β | SMC, macrophage, DC, EC, platelet | SMC, EC, macrophage | Differentiation, proliferation, migration, ECM production, calcification, KLF4 expression | Monocyte/macrophage recruitment and activation, fibrous cap formation, adhesion molecule expression in EC, macrophage polarization | Damaging | (12) |
| IL-4 | Th2 cell, mast cell | SMC, macrophage, Th2 cell | Proliferation | Macrophage proliferation, M2 macrophage polarization, Th2 cell proliferation, and differentiation | Protective | (3) |
| IL-6 | Senescent SMC, EC, T cell, macrophage | SMC, EC, T cell, B cell | Inflammation, apoptosis, calcification | Monocyte influx, myeloid cell differentiation, activation | Controversial | (3, 13) |
| IL-8 | EC, monocyte, T cell | SMC, monocyte, neutrophil, T cell | Inflammation, apoptosis | Leukocyte retention | Damaging | |
| IL-17 | Th17 cell, gd T cell, NK cell, neutrophil | SMC, EC, macrophage, T cell | Inflammation, ECM destabilization | Leukocyte and neutrophil accumulation, EC adhesion molecule expression, MMP release | Controversial | (14) |
| IL-18 | SMC, EC, macrophage | SMC, EC, T cell, macrophage, NK cell | Adhesion molecule expression, ECM remodeling | Adhesion molecule expression, pro-inflammatory cytokine production | Damaging | |
| IFN-α | SMC, T cell, macrophage | SMC, EC, macrophage | Proliferation, migration, ECM remodeling | Adhesion molecule expression, cytokine production | Damaging | |
| TNF-α | SMC, T cell macrophage | SMC, monocyte, neutrophil, T cell | ECM production and remodeling, apoptosis, calcification | Neutrophil activation, proinflammatory cytokine production | Damaging | (15) |
| **Chemokines** | | | | | | |
| CCL-2/MCP-1 | SMC, EC, macrophage, T cell | SMC, monocyte, neutrophil | ECM remodeling, inflammation, migration | Monocyte recruitment and activation, neutrophil recruitment, pro-inflammatory cytokine release | Damaging | |
| CCL-19/21 | SMC | T cell, macrophage | ECM remodeling | T-cell and B-cell recruitment, proinflammatory cytokine release, macrophage egress | ATLO neogenesis, damaging | (13, 16, 17) |
| CXCL-12/SDF-1α | SMC, EC, macrophage, platelet | SMC, EC, monocyte, neutrophil | SMC progenitor recruitment, ECM remodeling | EC proliferation, adhesion molecule expression in EC, circulating progenitor recruitment, monocyte influx, plasma cell survival, neutrophil homeostasis, plaque stability | Protective | (18, 19) |
| CXCL-13 | SMC, macrophage | SMC, B cell, macrophage | Anti-apoptosis | B cell recruitment, maturation, proliferate, survival and affinity maturation, macrophage apoptosis, and polarization | ATLO neogenesis, Protective | (16, 20, 21) |
| CX3CL-1 | SMC, EC | SMC, T cell, monocyte, platelet | migration, survival | Monocyte/macrophage and T cell recruitment and adhesion, monocyte survival, platelet–monocyte complex formation | Damaging | |
| MIF | SMC, EC, macrophage, T cell | SMC, monocyte | Migration, EMC production, and remodeling | Monocyte influx | Damaging | |

*(Continued)*

**TABLE 1** | Continued

| | Cellular sources | Cellular targets | Roles in SMC dynamics | Impact in local immunity | Roles in atherosclerosis | References |
|---|---|---|---|---|---|---|
| **Growth factors** | | | | | | |
| IGF-1 | SMC, EC | SMC, EC, macrophage | Proliferation, migration, apoptosis, ECM production | EC migration, survival, adhesion molecule expression, macrophage chemotaxis | Protective | (22) |
| PDGF-BB/DD | SMC, EC, platelet macrophage | SMC, EC, fibroblast | Differentiation, proliferation, migration, maturation, ECM production, KLF4 expression, autophagy, survival | EC dysfunction, leukocyte accumulation | Damaging | (23, 24) |
| TGF–β | SMC, EC, T cell, B cell, macrophage | SMC, EC, Treg, B cell | Differentiation, proliferation, migration, ECM production | Fibrous cap remodeling, EC activation, angiogenesis, anti-inflammatory cytokine production, wound healing | Protective | (25, 26) |
| **Others** | | | | | | |
| Lymphotoxin (TLO inducing cytokine) | Ly6C$^{hi}$ monocyte, T cell, LTi cell | SMC, EC, macrophage, T cell, B cell | SMC activation, trans-differentiation | Angiogenesis, stromal cell differentiation, leukocyte accumulation | TLO neogenesis, protective | (16, 27, 28) |
| CD-40L (costimulatory molecule) | SMC, EC, T cell, platelet | SMC, EC, macrophage, T cell | Activation, inflammation | Proinflammatory cytokine production, macrophage polarization, MMP release | Damaging | (13) |
| NLRP-3 (inflammasome) | SMC, macrophage | SMC, macrophage, neutrophil | ECM remodeling, inflammation | Macrophage priming, pro-inflammatory cytokine release | Damaging | (29) |
| Leukotrien-B4 (lipid mediator) | Macrophage, foam cell, dendritic cell | SMC, EC, macrophage | Proliferation, migration, ECM remodeling | EC activation, leucocyte chemotaxis, and activation | Damaging | |
| Annexin-A1 (lipid mediator) | EC, macrophage, neutrophil | SMC, neutrophil, T cell, macrophage | Migration, ECM production | Neutrophil and monocyte recruitment, apoptosis, phagocytosis, T cell activation, M2 macrophage, polarization | Protective | (13, 30) |
| Ephrin-A2 (guidance molecule) | SMC, EC, macrophage | SMC, EC, macrophage | proliferation, ECM production | Angiogenesis, fibrous cap thickness, monocyte adhesion | Damaging | |
| Netrin-1 (guidance molecule) | SMC, EC, macrophage | SMC, EC, macrophage | Migration, ECM remodeling | EC NO release, macrophage retention, leukocyte trafficking | Protective | (31, 32) |

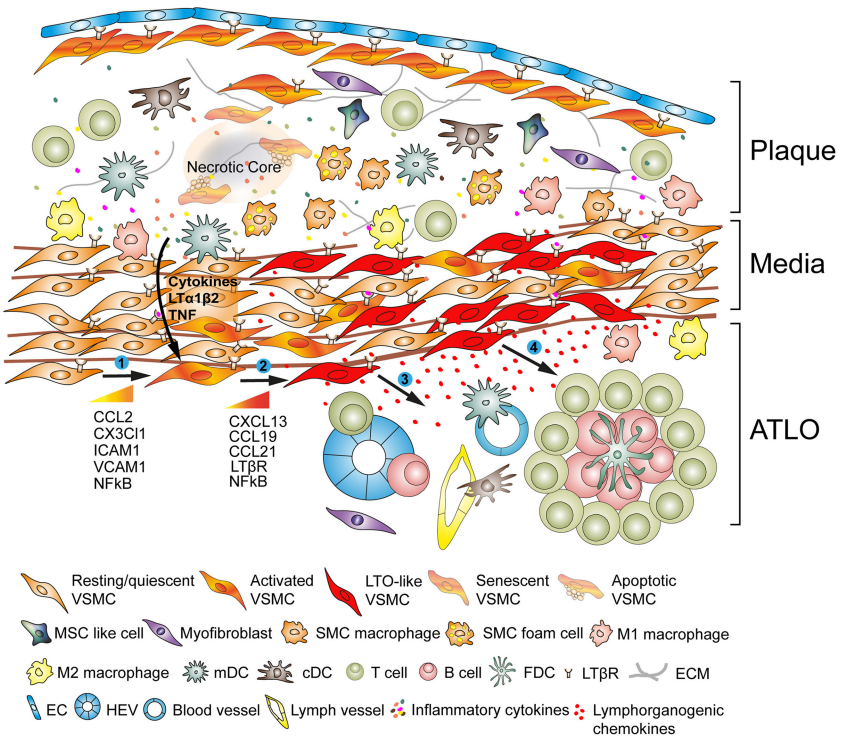


**FIGURE 2 |** Vascular smooth muscle cells (VSMC) participate in adventitia immunity during plaque formation. VSMCs sandwiched between atherosclerotic plaques and the adventitia adopt a lymphoid tissue organizer-like phenotype following activation via plaque-derived cues (❶) and subsequently transdifferentiate into LTo-like cells (❷). By means of their proliferative and cytokine-expressing phenotype they affect the restructuring and sculpting of the adventitia including angiogenesis, HEV formation, and lymph vessel neogenesis (❸). *Phenotype switching* also results in the expression and secretion of lymphorganogenic chemokines, i.e., CXCL13 and CCL21, thereby promoting ATLO formation depicted schematically in the lower part of the graph (❹).

in other chronic unresolvable diseases (1, 42). These data indicate that ATLO development is age-dependent and aging is positively associated with adventitial sculpting (**Figure 2**). However, the relation between aging and chronicity is difficult to study in murine atherosclerosis as atherosclerotic plaque development begins in the aortic arch, then travels sown the aorta to reach the abdominal aorta at later disease stages thus taking extended periods of time. More work is needed to clarify the important issue whether immune cell and/or VSMC senescence contributes to ATLO formation and which cells types, i.e., VSMCs, ECs, and/or immune cells are involved. Studies in other forms of TLO formation such as those occurring in the lung and the gastrointestinal tract would indicate that it is the chronicity of a persistent inflammatory tissue reaction rather than *bona fide* aging events of the immune system that is crucially contributing to the formation of ATLOs (43, 44).

## NEWLY FORMED ADVENTITIA STRUCTURES PARTICIPATE IN ATLO FUNCTION BY PROMOTING LYMPHOCYTE RECIRCULATION

Advanced ATLO stages include T/B cell aggregates, germinal centers within activated B cell follicles, lymph vessels, HEVs in T cell areas, and extracellular conduit meshworks (16). These major changes resemble structures reminiscent of those found in secondary lymphoid organs where they promote fundamental aspects of immune responses toward antigen including recirculation of naïve lymphocytes to find their cognitive antigens/autoantigens, and organizing affinity maturation of B cells toward potential autoantigens. Using adoptive lymphocyte transfer studies, we observed that ATLOs greatly enhance lymphocyte recruitment into the arterial wall by both promoting immigration and concomitant attenuation of emigration of antigen-inexperienced lymphocytes (27). Subsequent to their recruitment, T cells become activated, begin to proliferate, and some of the CD4 T helper cells are converted into induced T regulatory cells. These data reveal that the immune system in the adventitia is highly responsive toward the underlying inflammatory tissue reaction of the arterial wall. Moreover, B cells which form germinal centers in activated B cell follicles appear to undergo a germinal center reaction in the presence of follicular dendritic cells where some are converted into memory B cells while others leave the germinal centers to become plasma cells (45). When taken together, these data indicate that ATLOs rather than secondary lymphoid organs (as previously thought) organize atherosclerosis immunity.

## ATLO FORMATION IS HIGHLY TERRITORIALIZED INDICATING THAT SIGNALS ARE TRANSMITTED FROM PLAQUES TO THE ADVENTITIA VIA LYMPHOID TISSUE ORGANIZER-LIKE VSMCs

Another aspect of adventitia restructuring during atherogenesis is that ATLO formation is highly territorialized being largely restricted to adventitia segments adjacent to atherosclerotic plaques in the abdominal aorta in mice (16, 45). ATLOs are only occasionally found in the adventitia of the innominate artery or of the aortic arch where atherosclerosis is most prominent. Immune cells in the thoracic segments of the aorta are mainly composed of T cell aggregates compared to ATLOs in the abdominal aorta (16, 41). The exact mechanism for territoriality of ATLOs formation in the abdominal aorta is still unknown. TLOs appear to be a feature of many chronic unresolvable inflammatory diseases and are prominent hallmarks of autoimmune diseases (42). The cellularity and structures of TLOs in atherosclerosis are similar to TLOs in many other chronic diseases including cancer-associated TLO formation (46, 47). The field of TLO biology has dramatically expanded in recent years raising the important possibility that new therapeutic target may be identified via understanding of TLO's function in each of TLO-associated diseases. However, the various types of TLOs reveal some disease-specific features, which may ultimately determine whether the associated immune responses are harmful or protective. Such disease-specific characteristics possibly arise through one of several mechanisms including organ specificity and the nature of tissue damage (1, 42). The development of lymphoid organs is a complex process which involves hematopoietic *lymphoid tissue inducer* (LTi) cells, non-hematopoietic stromal *lymphoid tissue organizer* (LTo) cells and LTβR signaling. Our *in-vitro* studies of mouse aorta VSMCs indicated that upon appropriate stimulation they can serve as LTo-like cells (20), originally identified during embryonic development during the formation of secondary lymphoid tissues including lymph nodes and spleen (48, 49) (see below). It is important to note that ATLOs appear to involve VSMCs as important participants whereas other TLOs involve other LTo-like mesenchymal cells (50). The common denominator of all forms of TLOs, however, appears to be–unlike secondary lymphoid organs–a chronic inflammatory tissue reaction which drives the immune system to form these lymphoid structures close to or in some instances within the diseased tissue (42, 43).

## VSMCs DIRECTLY CONTRIBUTE TO ADVENTITIA IMMUNITY IN ATHEROSCLEROSIS

In view of the highly territorialized nature of ATLOs adjacent to atherosclerotic plaques, we reasoned that VSMCs may be involved in ATLO formation and by the same token may adopt a functional role in atherosclerosis progression. VSMCs highly express the LTβR constitutively (16), whereas its ligand $LT\alpha_1\beta_2$ is expressed on various immune cells termed LTi cells (51). VSMCs are hypothesized to be activated through the LTβR-LT signaling pathway by LTi cells involved in secondary lymphoid tissue neogenesis. The origin of LTi cell has not yet been clearly determined though activated macrophages and other immune cells in the intima plaque are candidates for this activity in LTO neogenesis. In response to cellular and soluble mediators VSMCs appear to undergo a distinct type of phenotype switching to a LTo-like phenotype by paracrine secretion of the lymphorganogenic chemokines, i.e., CXCL13 and CCL21, thereby attracting immune cells, e.g., macrophages/dendritic cells, T cells, and B cells to the local adventitia milieu leading to formation of ATLO in the adventitia (27, 41, 45) (**Table 1**). Global or VSMC-specific deficiency of LTβR in aged hyperlipidemic $ApoE^{-/-}$ mice demonstrated increased atherosclerotic plaque formation indicating that the VSMC LTβR has the ability to attenuate development of atherosclerosis under some experimental conditions (27). Other studies, however, showed that young global $ApoE^{-/-}$ $LTbR^{-/-}$mice maintained under a high fat diet (HFD) revealed a lower aortic plaque burden than their normal diet-fed $ApoE^{-/-}$ counterparts (52). These apparent contradictory data indicate that the roles of LTβR in young mice and under conditions of excessive hyperlipidemia may be different in the two models. Alternatively, the high fat diet which leads to dramatic and possibly intoxicating levels of plasma lipids, may be responsible for these differences. Since we do not regularly use a high fat diet in $ApoE^{-/-}$ mice, these discrepancies remain unresolved issues though it has been shown that the immune system may be overwhelmed by extreme levels of plasma lipids [reviewed in Mohanta et al.(42)]. These data call for further studies to examine the molecular basis for the apparent dichotomy of the LTβR in atherosclerosis progression in young vs. aged mice and/or early vs. advanced atherosclerosis. In addition, bone marrow-derived macrophages may function as LTβR-independent LTi cells and trigger the expression of CCL19, CCL20, and CXCL16 by VSMCs (53) (**Table 1**). Thus, VSMCs may participate in the formation of TLOs in atherosclerosis by upregulation of lymphorganogenic chemokines to promote immune cell aggregates in the adventitia. As mentioned above, ATLOs are common in humans burdened by ischemic coronary heart disease (40) and in human atherosclerotic aortic aneurysms (38, 39). When taken together, the occurrence of ATLOs in experimental mice and in human atherosclerosis raise important questions regarding the nature of atherosclerosis as an autoimmune disease: it is conceivable that during atherogenesis autoimmune T cells and autoimmune B cells directed against yet to be identified autoantigens are generated. Isolation of such autoimmune lymphocytes including sequencing of their T cell receptors and/or B cell receptors would allow to test major hypotheses in atherosclerosis. Indeed, atherosclerosis research would undergo a major shift and testing of individual autoimmune lymphocyte clones in functional *in vitro* and *in vivo* systems would become possible.

## CONCLUSION

Atherosclerosis is an inflammatory disease of arteries. VSMCs communicate with both the ECs and immune cells in the atherosclerotic plaques and resident cells in the adventitia. This ability allows VSMCs to affect atherosclerosis immunity in major ways including the formation of ATLOs. Further work is required to delineate the role of VSMCs during different stages of atherosclerosis, determine their role in young vs. aging arteries, and elucidate further their dichotomic roles in disease progression. A better understanding of these processes may open the way to develop therapeutic strategies for future intervention in clinically important late-stages of the disease.

## AUTHOR CONTRIBUTIONS

All authors contributed to the design, writing, and editing of the submitted manuscript, and approved it for publication.

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REVIEW
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# Dysregulation of Intestinal Epithelial Cell RIPK Pathways Promotes Chronic Inflammation in the IBD Gut

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Crohn's disease (CD) and ulcerative colitis (UC) are common intestinal bowel diseases (IBD) characterized by intestinal epithelial injury including extensive epithelial cell death, mucosal erosion, ulceration, and crypt abscess formation. Several factors including activated signaling pathways, microbial dysbiosis, and immune deregulation contribute to disease progression. Although most research efforts to date have focused on immune cells, it is becoming increasingly clear that intestinal epithelial cells (IEC) are important players in IBD pathogenesis. Aberrant or exacerbated responses to how IEC sense IBD-associated microbes, respond to TNF stimulation, and regenerate and heal the injured mucosa are critical to the integrity of the intestinal barrier. The role of several genes and pathways in which single nucleotide polymorphisms (SNP) showed strong association with IBD has recently been studied in the context of IEC. In patients with IBD, it has been shown that the expression of specific dysregulated genes in IECs plays an important role in TNF-induced cell death and microbial sensing. Among them, the NF-κB pathway and its target gene TNFAIP3 promote TNF-induced and receptor interacting protein kinase (RIPK1)-dependent intestinal epithelial cell death. On the other hand, RIPK2 functions as a key signaling protein in host defense responses induced by activation of the cytosolic microbial sensors nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2). The RIPK2-mediated signaling pathway leads to the activation of NF-κB and MAP kinases that induce autophagy following infection. This article will review these dysregulated RIPK pathways in IEC and their role in promoting chronic inflammation. It will also highlight future research directions and therapeutic approaches involving RIPKs in IBD.

**Keywords: IBD, apoptosis, RIPK, A20, RIPK1, NOD2, autophagy**

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Inflammatory bowel disease (IBD) is an inflammatory process with a chronic relapsing course that is characterized pathologically by intestinal inflammation and epithelial injury that affects the different gastrointestinal (GI) linings (1). IBD includes different inflammatory pathologies of the gastrointestinal track. The more prevalent IBD pathologies are Crohn's Disease (CD) and Ulcerative Colitis (UC) (1). Pathogenesis of IBD is multifactorial, involving genetic predisposition, disturbance of the commensal microbiota, epithelial barrier defects, dysregulated immune responses, and environmental factors (2). The gastrointestinal tract (in particular, the terminal ileum and colon) also contains a massive bacterial load that has the potential to initiate an acute inflammatory intestinal response if the mucosal barrier is breached and bacteria gain access to the lamina propria, as occurs in IBD (2).

The receptor interacting protein kinase (RIPK) proteins are key molecules for the maintainance of a healthy intestinal barrier (3). The RIPK family contains seven members that share a homologous serine-threonine kinase domain but has different functional domains (4). RIPK1 contains a death-domain in the C-terminal portion that allows its recruitment to different signaling complexes. RIPK2 is characterized by its caspase activation and recruitment domain (CARD). RIPK3, like RIPK1, has a RIP homotypic interaction motif (RHIM), which is necessary for RIPK1 and RIPK3 dimerization. RIPK4 (or DIK or PKK) and RIPK5 (or SgK288) contain ankyrin repeats in the C-terminal tail. Finally, RIPK6 (or LRRK1) and RIPK7 (LRRK2) have leucine-rich repeats (LRR) that could play a role in the recognition of inflammatory-associated molecular patterns. In this review, we will focus on the epithelial barrier and how an aberrant response to TNF stimulation, exarcebated, IBD-associated microbial sensing, and abnormal regeneration and healing of the injured mucosa by dysregulated RIPK pathways in IEC can critically affect the health of the intestinal barrier.

## INTESTINAL EPITHELIAL BARRIER

The intestinal epithelium forms the physical, protective, and host defense barrier against the harmful luminal microenvironment, while providing selective permeability for absorption of nutrients (5). The epithelium is covered by a single-cell layer composed of different subtypes of specialized intestinal epithelial cells (IECs) including enterocytes, goblet cells, enteroendocrine cells, Paneth cells, M cells, cup cells, and Tuft cells, all of which differentiate from common epithelial stem cells (5). These IECs types are functionally different and essential for maintaining intestinal homeostasis by separating the intestinal lumen from the underlying lamina propria and by controlling the crosstalk between luminal microbiota and subjacent immune cells (**Figure 1**). IECs not only function as a physical barrier through *enterocytes* (the largest cell population in IECs), but also through other specific functions. *Paneth cells*, for instance, are specialized secretory epithelial cells located in the crypt of the small intestine and contribute to the host defense secreting anti-microbial peptides that are diluted in the mucus enhancing the antimicrobial barrier and shape the commensal bacterial population (6–9). Paneth cells are characterized by an extensive endoplasmatic reticulum and Golgi apparatus with big secretory granules containing a wide variety of peptides, especially those with antimicrobial activity including defensins.

*Goblet cells* are the second most abundant cells in IECs and are specialized in mucus secretion (10). Mucins are highly O-glycosylated molecules that have gel-like properties and cover the inner walls of the gut lumen. Mucins form a bistratified mucus barrier, which becomes denser as it nears IECs, thus preventing bacteria from penetrating the barrier (11). At the same time, the mucus provides digestible glycans as a stable source of energy for the commensal microbiome (12–14). Intestinal goblet cells also sense luminal material that can be taken up delivered to lamina propria CD103+CD11c+ dendritic cells (DC) (15, 16) through goblet cell-associated antigen passages (GAPs). The DCs that interact with regulatory T cells have been suggested to induce tolerance to food antigens. Other cells, such as *enteroendocrine cells*, release a variety of humoral and paracrine mediators that can induce different immunoregulatory effects including cellular recruitment, activation, phagocytosis, antigen presentation and cytokine secretion (17, 18). Additionally, *tuft cells*, critical in the initiation of type-2 immune responses, are typically activated during intestinal protozoa or helminth parasite infections. *Microfold (M) cells* are epithelial cells specialized in phagocytosis and transcytosis of gut lumen antigens and pathogenic or commensal microorganisms across the intestinal epithelium toward the underlying gut-associated lymphoid tissues (GALT). M cells are also critical in maintaining a healthy intestinal barrier and control the crosstalk between luminal microbiota and subjacent immune cells.

IECs ability to act as a protective physical barrier is mediated by the formation of protein complex connections between adjacent cells, including tight junctions (TJ) and adherent junctions (AJ), which form the apical junction complex (AJC), as well as desmosomes, which are located in the basolateral membrane (19). These dynamic complexes are susceptible to endogenous and exogenous factors, such as cytokines, nutrients, and bacteria (19). TJs are the apical complexes of the AJC, connecting and sealing adjacent cells. TJ complexes are composed of junctional adhesion molecules (JAM), claudins, occludins, and zonula occludens (ZO), which seal neighboring cells together (20). AJs, composed of cadherins, form the second AJC loop, maintaining cell-to-cell connections; however, AJ are not critical for creating paracellular tightness (20). Finally, desmosomes connect intermediate filaments of neighboring cells, conferring mechanical strength to cell-to-cell junctions. They are formed by desmoplakin, plakoglobin, plakophilin, desmocollin, and desmoglein (21, 22). Tight junctions are critical for maintaining barrier function during IEC shedding, which occurs continuously from villus tips or colonic surfaces as a result of migration of the epithelial cell up the crypt–villus axis from stem cells at the base of the crypt (23). Normal cell shedding never causes a breach in the epithelial barrier because of the redistribution of tight junction proteins that facilitates the closure of the gap (24). However, in pathological conditions, when multiple neighboring cells are shed at the same time or cell death is activated, or turnover is increased a proper rearrangement of cell-to-cell contact cannot take place. Consequently, breaches appear in the intestinal epithelial barrier, which causes intestinal inflammation (23).

## RIPK PROTEINS ARE CRITICAL TO MAINTAINANCE OF BARRIER FUNCTION

### The Role of Autophagy Mediated by Nod2/RIPK2 in Maintaining Intestinal Homeostasis

Autophagy is a cell stress response that causes the encapsulation of cellular contents for subsequent degradation and recycling (25). Although the first barrier against bacterial and parasitic invasion of the intestine is the mucus layer, some pathogens

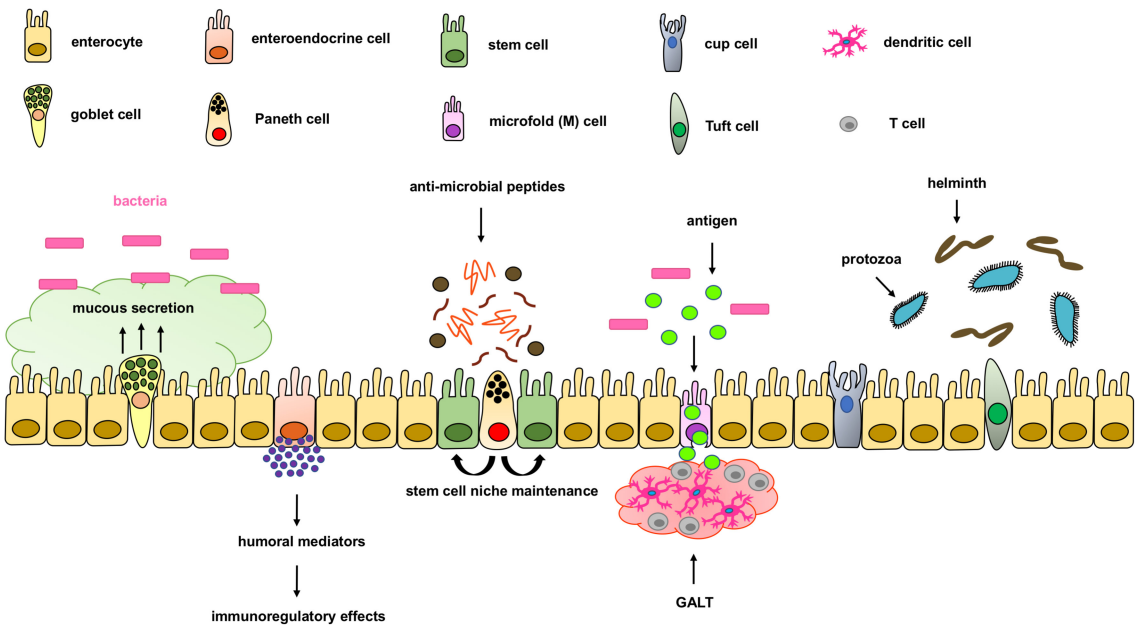


**FIGURE 1 |** Components of the intestinal epithelial cell barrier. In the intestinal epithelial cell barrier we can find different specialized cell types including; enterocytes, goblet cells, enteroendocrine cells, paneth cells, stem cells, microfold (M) cells, and cup and tuft cells. Briefly, enterocytes are the most common cells with nutrient absorption functions; goblet cells secrete mucus to form an extra intestinal protective layer; enteroendocrine cells secrete humoral mediators with immunoregulatory effects; paneth cells secrete antimicrobial peptides and maintain the stem cell niche; microfold cells play an important role in transporting lumen antigens into the associated intestinal lymphoid structures; and Tuft cells play an important innate role against helminth and protozoan infections.

can penetrate this layer to reach the IECs. In this situation, autophagy plays an important role by recognizing and degrading intracellular pathogens, thus functioning as an innate barrier to infection. It has already been shown that knockdown of autophagy genes in *Caenorhabditis elegans* and *Dictyostelium discoideum* increases *Salmonella typhimurium* intracellular replication, decreases animal lifespan, and results in apoptotic-independent death (26).

NOD2 (nucleotide-binding oligomerization domain-containing protein 2) is a critical element in regulating autophagy in IECs (27). NOD2, a cytosolic pattern recognition receptor, is activated by the peptidoglycan fragment muramyl dipeptide (MDP) to generate a proinflammatory immune response (28, 29). Over 30 cellular proteins interact with NOD2 directly and influence or regulate its functional activity (30). Among them, NOD2 recruits ATG16L1 (autophagy-related protein 16 like 1) to the plasma membrane at the bacterial entry site to induce phagophore formation. ATG16L1 then forms a complex with ATG5 and ATG12 to induce the lipidation of LC3 (microtubule-associated protein 1A/1B-light chain 3), forming an autophagosome and inducing autophagy (27). Additionally, upon activation, NOD1 and NOD2 recruit RIPK2 through CARD domains (31, 32), inducing RIPK2 k63-polyubiquitination in lysine 209 by cIAPs and the LUBAC complex (33–35). This leads to RIPK2 activation, which depends on autophosphorylation in residues Ser176 and Tyr474, an essential and enhancing site respectively (36, 37), and downstream activation of transforming growth factor beta-activated kinase 1 (TAK1) (38–40). TAK1 consecutively phosphorylates the IKK complex triggering NF-κB and MAPK pathway activation.

Travassos et al. showed that NOD1 and NOD2 can recruit ATG16L1 to the plasma membrane at the bacterial entry site in different cell types including the mouse intestinal epithelial cell line Mode-K through a RIPK2-independent mechanism (41). The role of RIPK2 as a kinase in autophagy induction downstream of NOD2 has also been investigated. In different cells, including the cell-like HCT116, RIPK2 kinase function is required for the phosphorylation of the protein kinase ULK1 at Ser555, and for the deactivation of the protein phosphatase 2A (PP2A) complex that negatively regulates autophagy induction downstream of p38 activation (42, 43). In dendritic cells, NOD2 is also able to trigger autophagy through RIPK2-mediated recruitment of ATG5, ATG7, and ATG16L1 (44). Anand et al. showed how activated RIPK2 promotes increased autophagosome formation by activating MAPK/ERK kinase 4 (MEKK4)–p38 signaling and/or extracellular signal-regulated kinase 1 (ERK1) and ERK2 signaling, which upregulates basal levels of autophagy (43).

Autophagy also plays an important role in protecting IECs from cell death. NOD2 is highly expressed in intestinal stem cells, and its activation by MDP triggers stem cell survival and strong cytoprotection against oxidative stress-mediated cell death (45). This could be due to NOD2s ability to activate the NF-κB pathway, which has protective effects in the intestinal epithelium (46). Animals lacking ATG16L1 in the epithelium

were more susceptible to DSS-induced colitis, and the pathology was exacerbated when these animals were infected with murine norovirus (MNV). Further histological analysis and organoid experiments show that ATG16L1 protects cells from necroptosis by removing aberrant mitochondria and impairing downstream reactive oxygen species (ROS) accumulation (47). In another model of colitis induced by *Helicobacter hepaticus*, mice with a deletion of ATG16L1 in IECs had worse histopathology than their littermates. IECs in affected mice were more susceptible to TNF-induced apoptotsis, increasing inflammation and pathology of the models (48). Overall, it has been shown that autophagy adds another layer of protection from foreign organisms by preventing pathogen proliferation and dissemination to extraintestinal sites (49). It also has a protective role in IECs, and defects in the autophagy pathway increase the susceptibility of the intestine to inflammation, inducing cell death and intestinal epithelial barrier breakdown.

## RIPK1 AND RIPK3 ARE CRITICAL IN MAINTAINING AN EQUILIBRIUM BETWEEN CELL SURVIVAL AND CELL DEATH DOWNSTREAM OF TNF

### RIPK1 and RIPK3

Two RIPK proteins have key kinase-dependent functions in deciding beneficial or deleterious effects downstream of TNF: RIPK1 and RIPK3 are two key molecules in the assembly of TNFR complexes that may trigger cell death (**Figure 2**).

- RIPK1 was the first protein of the RIPK family identified, interacting with apoptosis antigen 1 (APO-1 or FAS) through its death domain (DD), giving it its "receptor-interacting protein" name (50). Through its DD, RIPK1 can also bind other receptors such as the TNF-receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (DR4 and DR5) and death receptor 3 (DR3 or TRAMP). RIPK1 interacts with other adaptor proteins such as TRADD, Fas-associated protein with death domain (FADD), Toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF), RIP-associated ICH-1 (ICE (interleukin-1β-converting enzyme)/CED-3 homolog 1) protein with a death domain (RAIDD), TNF receptor associated factor (TRAF)1, TRAF2, TRAF3, and A20 (50–60). Furthermore, it can also interact with RIPK3, through its RIP homotypic interaction motif (RHIM) domain, as well as with focal adhesion kinase, MEKK1 and MEKK3 (61–64).

- RIPK3: similar to RIPK1; it contains an N-terminal kinase domain and a RHIM domain in the C-terminal part, that allows RIPK1/RIPK3 interactions (4). However, its C-terminal domain is completely different from other RIPK proteins. This could explain its ability to interact with the liver glycogen phosphorylase (PYGL), glutamate ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), enhancing its enzymatic activity (4). These are metabolic enzymes required for ATP production, with PYGL releasing glucose-1-phosphate from liver glycogen, and GLUL and GLUD1 playing crucial roles in the use of glutamate and glutamine as substrates for ATP through oxidative phosphorylation; this suggests a link between RIPK3 and metabolism (65). In fact, RIPK3 orchestrates necroptosis, "an active cell death pathway" that requires both adenosine triphosphate (ATP) and ROS (66). Through RIPK3-enhanced aerobic respiration, mitochondria could both produce energy to execute necroptosis while increasing the amounts of ROS required for the RIPK1/RIPK3 and later MLKL (mixed lineage kinase domain-like) complex formation and activation downstream of TNF (66).

Genetic studies has helped to understand the role of these kinases in cell death. Mice lacking RIPK1 show defects in multiple tissues, triggering systemic inflammation leading to perinatal death 1-3 days after birth (67). Simultaneous deletion of TNFR1 prolonged up to 12 days post-delivery the survival in *Ripk1*$^{-/-}$ *Tnfr1*$^{-/-}$ (68). Deletion of RIPK3, mixed lineage kinase domain like pseudokinase (MLKL) or caspase-8 in *Ripk1*$^{-/-}$ did not improve the phenotype, with those mice dying soon after delivery (69, 70), suggesting that when just apoptosis or necroptosis are blocked downstream of TNF, the other pathway gets activated. In a similar manner, triple deletion of RIPK1, RIPK3, and TNFR1 allows mice survive until adulthood. Yet, shortly after birth they present intestinal apoptosis, which could contribute to the mortality associated with blood bacteremia (69). Accordingly, simultaneous deletion of RIPK1, RIPK3, and caspase-8 or FADD protect the mice and prevented any macroscopic and microscopic signs of intestinal pathology, but mice developed autoimmune lymphoproliferative syndrome (69, 70). Since double deletion of FADD and RIPK1 induces perinatal death (71) but additional deletion of RIPK3 protect the animals, these results suggest that some other mechanism, independent of RIPK1, activates RIPK3. In fact, DNA-dependent activator of interferon regulatory factors (DAI) can interact and activate RIPK3 independently of RIPK1 (72). *Ripk1*$^{-/-}$ *Ripk*$^{-/-}$ and *Trif*$^{-/-}$ or *Ifnar*$^{-/-}$ animals were generated, and although it conferred certain protection compared with *Ripk1*$^{-/-}$ *Ripk*$^{-/-}$, those mice did not survive past weaning (70). Finally, mice lacking RIPK1 in IECs specifically (RIPK1$^{\Delta IEC}$), develop severe intestinal inflammation associated with IEC apoptosis leading to early post-birth death. Similarly, tamoxifen-induced deletion of *Ripk1* leads to rapid weight loss and mice death. Crypt cells from RIPK1$^{\Delta IEC}$ failed to grow into organoids (69), so *in vitro* deletion is required to grow RIPK1 deficient intestinal organoids (73). Unexpectedly, in those intestinal organoids the NF-κB pathway remained intact downstream of TNF, although they undergo massive cell death (73).

Two mice models with point mutations in the kinase domain were generated to study the kinase role of RIPK1 without compromising its scaffold function. RIPK1$^{K45A}$ and RIPK1$^{D138N}$ (74, 75) mice were born at expected Mendelian ratios and showed no abnormalities, pointing out the importance of RIPK1 function as a scaffold protein. Fibroblasts and macrophages derived from these mice were stimulated with TNF and were shown to be protected from cell death demonstrating the role of the kinase domain from RIPK1 in triggering cell death (74).

Unlike RIPK1$^{-/-}$, RIPK3$^{-/-}$ mice are indistinguible from their littermates and exhibit normal downstream pathway activation from TNFR1 and TLRs (76). However, knock-in mice harboring a kinase death form of RIPK3 (RIPK3$^{D161N}$) die at

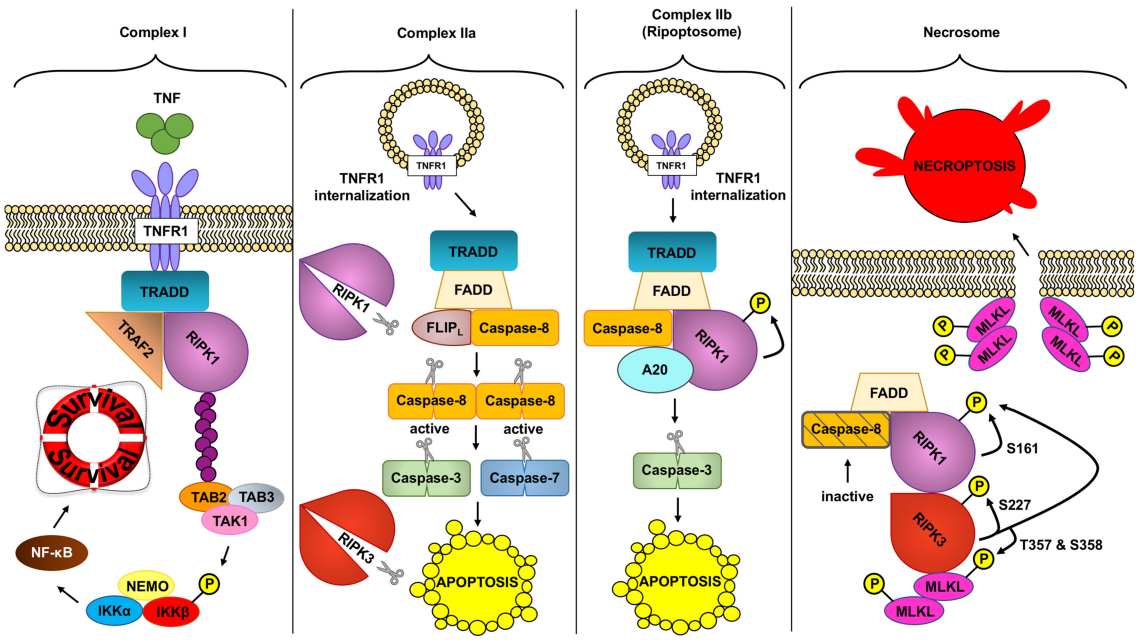


**FIGURE 2 |** Different TNFR complex formed downstream of TNF. TNF (tumor necrosis factor) can induce the formation of different complexes with diverse outcomes depending on the conditions. In a homeostatic situation, TNF triggers the formation of complex I, where RIPK1 (receptor interacting protein kinase 1) acts as a scaffold protein allowing the activation of the pro-inflammatory and protective pathway NF- κB (nuclear factor kappa-light-chain-enhancer of activated B cells). In pathologic conditions, complex IIa can be formed leading to the activation of caspase-8. Active caspase-8 cleaves RIPK1 and RIPK3 (receptor interacting protein kinase 3) and downstream caspases to induce apoptosis. Unlike complex IIa, complex IIb depends on the kinase role of RIPK1 to activate caspase-8 and execute apoptosis. Inhibition of RIPK1 kinase and ROS (reactive oxygen species) prevents this type of cell death. Finally, if caspases are inhibited and the NF- κB pathway is not activated, TNF can trigger the formation of the necrosome. This complex depends on the kinase activity of RIPK1 and RIPK3 to activate MLKL (mixed lineage kinase domain like). MLKL in turn will create the necroptotic pore in the plasma membrane inducing necroptosis, a regulated type of necrosis.

embryonic day 11.5 due to high amounts of cell death in the yolk sac vasculature. The authors of this study show how this cell death was dependent on caspase-8. Kinase death RIPK3, but not wild-type RIPK3, interacted with FADD, RIPK1, and caspase-8. Similarly, expression of RIPK3$^{D161N}$ in the adult intestine also led to diarrhea and massive weight loss due to caspase activation, and downstream apoptosis of IECs (77). Similar results were obtained when a RIPK3 inhibitor was given to mice (78). On the contrary, another RIPK3 kinase death animal line (RIPK3$^{K51A}$) did not present any embryonic abnormalities, and the mice were shown to be viable, fertile, and immunocompetent, as well as able to rescue the embryonic lethality seen in caspase-8 knock-out mice (78). However, RIPK3 inhibitors still induced apoptosis on cells expressing the RIPK3$^{K15A}$. Altogether, this data suggests that RIPK3 inhibition through small molecules or the presence of the D161N mutation induces conformational changes in RIPK3 that promote apoptosis. Although those results could be secondary to a change in the RIPK3 structure due to the D161N mutation, they suggest that RIPK3 kinase inhibition leads to apoptosis.

## RIPK1 Functions in TNFRI Complex I Downstream of TNF

*TNF* is one of numerous genes implicated in IBD pathogenesis stimulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). It codes for the prototypical inflammatory cytokine tumor necrosis factor (TNF), which has various functions in the intestine (79). TNF is synthesized as a transmembrane protein that forms homotrimeric structures, and is cleaved by a disintegrin and metalloprotease domain 17 (ADAM17) or by TNF-converting enzyme (or TACE), which releases its soluble form (80). TNF is able to bind two receptors: TNFR1 and TNFR2, which differ in their structure and expression pattern, as well as in the signaling pathways that they induce once they are engaged (80). TNFR1 is expressed in all cell types, whereas TNFR2 is mostly restricted to immune and endothelial cells. Both receptors are able to activate the NF-κB pathway through different signaling cascades as a result of strikingly different intracellular domains. TNFR1 contains a cytoplasmic death domain (DD), which is a conserved sequence of 80 amino acids that forms a distinctive fold (81, 82) and allows the recruitment of TNFR1-associated death domain protein (TRADD). TNFR2 lacks the death domain and recruits TNFR-associated factor 1 (TRAF1) and TRAF2, rather than TRADD (82–84). Both TNFR1 and TNFR2 can lead to NF-κB activation.

TNF has important protective functions in intestinal epithelial cells (**Figure 3**): (a) TNF modifies the first physical barrier of the intestine: the mucus layer. Through TNFR2, TNF sensitizes goblet cells to prostaglandin E2, a known mucus secretagoge, and protects the epithelium by increasing mucus secretion (85, 86), (b) TNF is able to induce the expression

of the polymeric immunoglobulin receptor (pIgR), which is necessary for the transcytosis of secretory IgA into the mucus, and prevents bacterial translocation into the lamina propria (87), (c) TNF is critical in wound healing, which is an important step in resolving injury and preventing chronification of underlying inflammation. Two different steps occur during this process: spreading and migration of cells through the basement membrane, and redifferentiation and proliferation of cells. Through TNFR2 dependent activation of focal adhesion kinase, TNF is able to induce epithelial migration (88) and cell proliferation (89). TNF can also support wound healing and cell survival through TNF-induced TACE activation, which subsequently liberates ErbB ligands that promote cell survival (90), (d) through TNFR1, TNF is able to activate the NF-κB pathway and assemble the TNFR1 complex I, which promotes IEC survival.

The assembly of the TNFR1 complex I is key in intestinal barrier maintenance. In most cell types, including IEC, transient TNF signaling inhibits apoptosis due to the assembly of the TNFR complex I, and activation of IκB kinase (IKK) β-dependent NF-κB (91). Upon the binding of TNF to homotrimers of TNFR1, the adaptor molecule TRADD is recruited to the cytoplasmatic TNFR1 domain. In a step-wise process, RIPK1, TRAF2, cellular inhibitor of apoptosis protein 1 (cIAP1) or cIAP2, and linear ubiquitin chain assembly complex (LUBAC) are recruited to form signaling complex I. TRAF2 and cIAP1/2 mediate K63-linked ubiquitination of the complex. In this situation, the kinase RIPK1 acts as a scaffold protein that allows for docking of the adaptor proteins TAK1-binding protein 2 (TAB2) and (TAB3) and the kinase TAK1 through RIPK1 K63-ubiquitins (92). Meanwhile, the LUBAC complex mediates M1-ubiquitination of some components in the complex I, such as RIPK1 and NF-kappa-B essential modulator (NEMO) (93, 94). The IKK complex is also recruited to the complex, and after phosphorylation of IKKβ by TAK1, mediates the activation of the canonical NF-κB pathway and the resulting upregulation of anti-apoptotic genes such as BCL2 (B-cell lymphoma 2) and FLIP (FLICE-like inhibitory protein), to promote cell survival and cell proliferation (80).

Several previous works have highlighted the critical role of this pathway on IEC survival. Early work by Egan et al. shows that deletion of IKKβ in IECs promotes the gut damage from ionizing radiation (IR) (95). Furthermore, when LPS, a known activator of the NF-κB pathway, is administered prior to IR, IECs are also protected from massive apoptosis, suggesting the IKK complex, the main protein complex downstream of TNF, provides protective effects to IECs (95). Similarly, IKKß was shown to be protective in a model of colitis induced by *C. difficile* and dextran sodium sulfate (DSS) (96, 97). Although deletion of IKKß or IKKß alone does not induce spontaneous colitis, IECs lacking NEMO or TAK1 develop colon pathology, in a complete or partial TNF dependent manner, respectively, including IEC apoptosis, demonstrating that the NF-κB pathway plays key homeostatic roles (98, 99). Interestingly, unlike $NEMO^{IEC-KO}$, $TAK1^{IEC-KO}$ develops intestinal inflammation, perhaps due to the ability of TAK1 to activate other protective pathways such as the Mitogen-activated protein kinases pathway (MAPK). Nevertheless, activated/nuclear NF-κB is present in both IECs and the lamina propria macrophages of active IBD areas (100). To determine the pathogenic function of persistent NF-κB activation, which occurs in IBD (100), we generated $Ikk\beta(EE)^{IEC}$ mice in which a constitutively active IKKβ(EE) variant is expressed in IEC from the villin promoter (101). Surprisingly, instead of being resistant to TNF-induced mucosal erosion, $Ikk\beta(EE)^{IEC}$ mice displayed severe TNF-dependent epithelial layer destruction when challenged with various stimuli that induce TNF production, or when given exogenous TNF (101). The mechanism by which constitutive IKKβ/NF-κB activation renders mouse IECs susceptible to TNF-induced killing rather than preventing it is unknown, but is likely to be relevant to understand the effect of NF-κB chronic activation in IECs of active IBD lesions.

## RIPK1/RIPK3 and the Assembly of TNFR Dependent Ripoptosome/Necroptosome

As reviewed above, transient TNF signaling inhibits apoptosis due to the assembly of TNFR1 complex I and IKKβ-dependent NF-κB activation (91). However, TNFR1–TRADD signaling can result in cell death in special circumstances, when complex I shifts toward complex IIa, IIb, or the necrosome to induce different types of TNF-induced cell death (**Figure 2**).

### Complex IIa

Ubiquitin removal from RIPK1, through deubiquitination by cylindromatosis (CYLD), or ubiquitination-impairment by cIAP1/2 depletion (102–104), alters the formation of complex I, allowing its disassembly and TNFR1 internalization (105). TRADD, FADD, pro-caspase-8 (caspase-8), and FLICE-like inhibitory protein (FLIPs) are then recruited to the TNFR1. In this complex, the long isoform of FLIP ($FLIP_L$) and the pro-caspase-8 form a heterodimeric caspase that cleaves and inactivates RIPK1 and RIPK3, as well as CYLD, to prevent necroptosis (106–108). This TRADD-dependent complex IIa also allows caspase-8 homodimerization and activation, resulting in activation of the executioners caspase-3 and caspase-7, which trigger apoptosis. This pathway, in normal conditions, would be inhibited due to previous NF-κB activation and expression of anti-apoptotic genes (108–110) but as mentioned above, ablation of IKKβ (95, 111), or its regulatory subunit NEMO (98), renders IEC susceptible to TNF-induced death.

### Complex IIb or Ripoptosome

TNFR complex IIb or the Ripoptosome has been described to occur downstream of TNF when cIAP1/2 is depleted through SMAC mimetics (SM) (112–114). SMAC (second mitochondria-derived activator of caspase) is a pro-apoptotic mitochondrial protein that inhibits IAPs. The exact mechanism that triggers the formation of complex IIb instead of IIa is unknown, although in this case the activation of NF-κB does not prevent apoptosis (115). TNF treatment together with TAK1 pharmacological inhibition also triggers RIPK1-dependent apoptosis, in a similar manner as TNF plus SM treatment, suggesting that TAK1 recruitment to cIAP1/2-ubiquitinated RIPK1 inhibits RIPK1-dependent apoptosis (115). In fact, IKKα and IKKβ, the downstream kinases of TAK1, inhibit RIPK1 in association with the Ripoptosome through direct phosphorylation of RIPK1 (116). Complex IIb, the Ripoptosome, is composed of RIPK1,

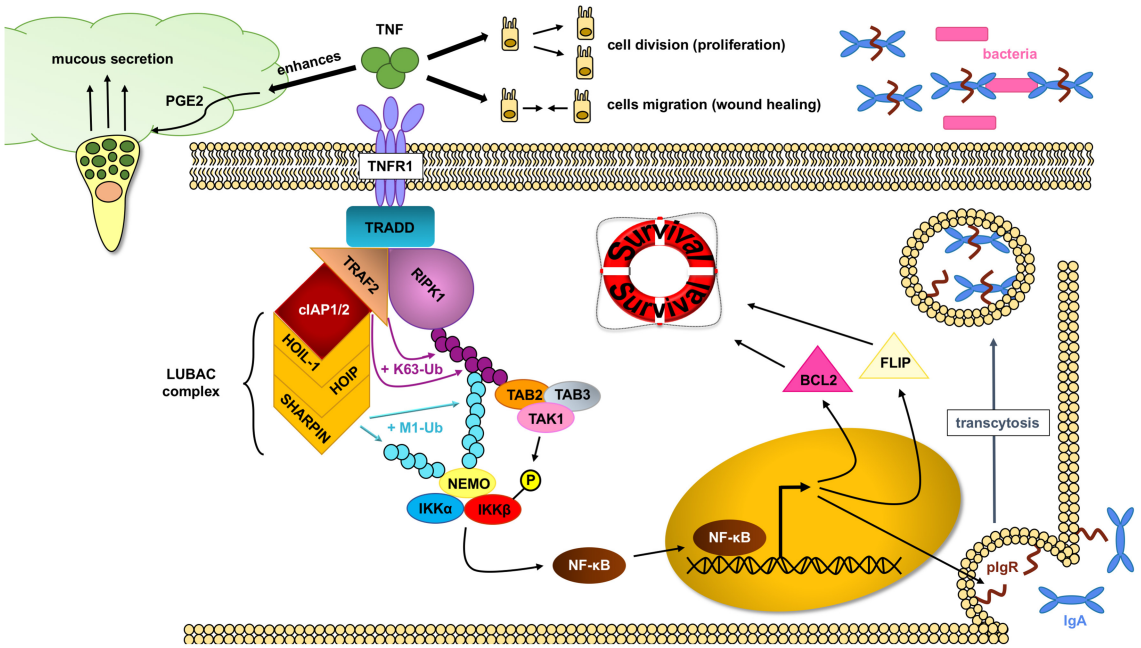


**FIGURE 3 |** NF- κB pathway activation through RIPK1 protects IECs. In homeostatic conditions, TNF (tumor necrosis factor) plays an important role in maintaining an intact intestinal epithelial barrier. Upon binding to the TNFR1 (tumor necrosis factor receptor 1), the TNFR complex I is formed, where RIPK1 (receptor interacting protein kinase 1) serves as an scaffold protein upon which the key IKK (IκB kinase) complex will bind and activate. The IKK complex will in turn induce the translocation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factors into the nucleus and allow gene expression, including cell survival factors. TNF can also help in the wound healing process by inducing cell proliferation and migration as well as enhance the intestinal barrier by favoring mucus production and impairing bacterial translocation through pIgR (polymeric immunoglobulin receptor) induction.

FADD and caspase-8, and A20 (117). It is independent of TRAILR1/DR4, TRAILR2/DR5, and Fas/CD95 activation (112). TLR3 can also potentially induce complex IIb. TLR3 activation induces apoptotic cell death downstream of TRIF that depends on a complex formed also by RIPK1, caspase-8, and FADD, although it is unknown whether this requires RIPK1 kinase activity (118, 119). Of note, TNFR1 activation is dispensable if cell death is triggered by etoposide, a genotoxic stress inducer that also depletes cIAPs, although in this case complex IIb formation occurs 6 h after the treatment, 4 h later than when triggered downstream of TNF. Complex IIb requires the kinase activity of RIPK1 to induce cell death although the exact mechanism of its activation or its role as a kinase is unknown; in fact no targets for the kinase activity of RIPK1 have been described apart from itself (113).

### TNF-Induced Necroptosis

Necroptosis can be triggered through different stimuli. Most studies on necroptosis have been performed after TNF, FAS, or TLRs stimulation, but it can also be triggered by intracellular events, such as viral infection through Z-DNA or Z-RNA sensing via Z-DNA binding protein 1 (ZBP1/DAI) (72). For instance, downstream of TNF, when caspases are not fully activated or their activity is blocked (ex: by viral inhibitors), the protein kinase RIPK3 is recruited and forms the necrosome, which will lead to necroptotic cell death (120, 121). Once engaged, RIPK1 and RIPK3 undergo auto and transphosphorylation leading to their activation. Interestingly, although RIPK3 can also phosphorylate RIPK1, RIPK1 does not phosphorylate RIPK3 (120). The requirement for RIPK1 and RIPK3 trans and autophosphorylation can explain the formation of RIPK1/3 amyloid structures through RIPK1 and RIPK3 RHIM domains, a required step for RIPK3 autophosphorylation (122, 123). All these signals will converge into MLKL phosphorylation and activation, and subsequent cell death (124). Phosphorylated MLKL binds to the inner leaflet of the plasma membrane and forms the necroptotic pore, executing necroptosis (125–127). Although RIPK1 can be autophosphorylated at S14/15, S20, S161, and S166, (128), only S161 has been shown to be required to induce necroptosis (129). RIPK1 phosphorylation on S89 or MK2 mediated phosphorylation of S321 impair RIPK1 mediated cell death (130, 131). Phosphorylation of S227 in RIPK3 allows the binding of RIPK3 to MLKL (124). In addition, MLKL is phosphorylated by RIPK3 at T357 and S358 residues in human, and S345, S347, and T349 residues in mouse. These phosphorylation sites are necessary for necroptosis since mutation of both sites inhibits necroptotic cell death (124, 132).

## DYSREGULATION OF INTESTINAL EPITHELIAL CELL RIPK PATHWAYS PROMOTES CELL DEATH IN IBD

The increased areas of epithelial cell death associated with IBD are especially prevalent in UC compared to CD and

controls (133). This epithelial cell death increases the chances of antigen translocation and subsequent triggering of inflammatory responses (134–137). These epithelial cell deaths present features of apoptosis and necrosis. Necrosis has long been recognized as a major trigger of inflammation; as cells die, their cellular contents activate the host immune response. Apoptotic cells will also increase the intestinal permeability by overwhelming the capacity of phagocytes to clear apoptotic cells, and by preventing proper tight junction function and intestinal barrier remodeling of patches of shedding cells. Interestingly, patients with active UC, who ultimately require surgery, had higher apoptotic indices than UC patients that were receiving medication. Also, electron microscopy on rectal biopsies of patients with CD and UC compared with normal controls showed patches of necrotic cells in four out of seven CD patients (135). Notably RIPK3 is expressed at high levels in the terminal ileums of patients with CD (138).

Several IBD pathogenic factors can promote IEC cell death in these patients. Among them, genetic predisposition, disturbance of the commensal microbiota, and dysregulated immune responses can contribute to epithelial barrier defects and promote chronic inflammation in the IBD gut.

## Genetic Predispositions to IBD

Although family history is a risk factor for developing IBD, the concordance rate in monozygotic twins is only 10–15% in UC, and 30–35% in CD, suggesting that non-genetic factors might play a bigger role (139). Nonetheless, the first genome-wide association study (GWAS) for Crohn's disease, undertaken in 2005 in Japan, identified the susceptibility locus of the tumor necrosis factor super family 15 gene (*TNFSF15*) (140). Subsequently, several other studies have identified, in different ethnic cohorts, 235 genetic markers in 200 susceptibility loci (141–143). Of the 163 identified loci in the Caucasian population, 110 appear to be relevant to both CD and ulcerative colitis (*TNFAIP3, IRGM, TNFSF15*), 23 appear to be specifically related to CD (*ATG16L1, NOD2*), and 30 appear to be specifically related to UC (*IRF5, NFKB1*). IL-23R has also been shown to be related to CD in several studies (144–146), with rs1343151 and rs7517847 variants decreasing the risk of developing the disease. Although, most identified SNPs lack functional data, the identification of these genes elucidates the critical pathways in IBD pathogenesis.

a. The first genetic risk variant identified for CD was the *NOD2* gene (147, 148). Hugo et al. found three different polymorphisms in *NOD2*; one is a frameshift mutation (L1007C) which causes a truncated protein transcript, and two are non-synonymous polymorphisms (R702W and G908R). Carriage of one copy of any risk allele confers a modestly increased risk of developing CD (2 to 4-fold). However, having two copies or a combination thereof is associated with a 20- to 40-fold increased risk. Another SNP in the autophagy gene ATG16L1, which is associated with CD, is responsible for a threonine to alanine substitution at amino acid 300 (T300A) that increases the odds ratio (OR) for CD to 1.62 in the Spanish population (149). Finally, another important gene related to autophagy is *IRGM* (immunity-related guanosine triphosphatase family M protein). It encodes a GTP-binding protein that induces autophagy and plays an important role in innate immunity against intracellular pathogens. Two flanking SNPs (rs13361189 and rs4958847) have been better associated with increased susceptibility to CD with an OR of 1.34 and 1.33; the first SNP alone also confers a small association with UC (OR: 1.16) (149).

   Impaired autophagy disturbs the function of IECs and influences the inflammatory and immune responses, ROS production, and endoplasmatic reticulum (ER) stress, promoting the occurrence and development of IBD (150–153). Furthermore, it is noted that autophagy can play a role in the release and degradation of the damage-associated molecular pattern molecules (DAMPs), contributing to the alleviation of IBD (154–156). ATG16L1 deletion also increases the chance of IECs necroptosis (47), and deletion of another autophagy protein, ATG5, results in impaired intestinal permeability and protection against *Toxoplasma gondii* infection (157). Finally, mice deficient in *Nod2* and *Atg16l1* showed Paneth cell defects and susceptibility to intestinal inflammation (158, 159). These results highlight the importance of the Paneth cell, that releases antimicrobial peptides, supports stem cells, and regulates AMP production (*Nod2*) and granule exocytosis (*Atg16l1*), in the pathogenesis of the disease. Importantly, similar phenotypes have been observed in human disease, and patients with Crohn's disease carrying the ATG16L1$^{T300A}$ mutation showed granular abnormalities in Paneth cells (159).

   Other authors looked into the role of the NOD2 L1007insC polymorphism, which results in a frameshift mutation that generates a truncated Nod2 protein. This mutation prevents peptidoglycan and MDP-dependent activation of the NF-κB pathway, and localization of NOD2 into the plasma membrane (28, 46, 160). NOD2 L1007insC did not prevent NOD2-ATG16L1 interaction, but did prevent its localization in the plasma membrane, impairing wapping of invading bacteria by autophagosomes. Furthermore, in different human epithelial cell lines, deletion of ATG16L1 or reconstitution with the common coding variant ATG16L1$^{T300A}$ abrogated capture and degradation of intracellular Salmonella (161, 162). Recently, Murthy et al. showed a relation between autophagy, cell death and inflammation. The authors demonstrated that caspase-3 enhances the cleavage of ATG16L1T300A, an SNP strongly associated with incidence of CD. They propose that the presence of T300A apoptotic stimuli enhances ATG16L1 cleavage, triggering cytokine production and inflammation (163). Another work has also shown how ATG16L1 prevented necroptosis in IECs (47).

b. RIP1 and RIP3: Although no SNPs in these proteins have been associated with IBD, the effect of RIPK1 deficiency in humans was studied by Cuchet-Lourenço et al. (3). In this study, they found four patients from three unrelated consanguineous families carrying homozygous

loss-of-function mutations in RIPK1. The four patients had lymphopenia, suffered from recurrent viral, bacterial and fungal infections, early-onset inflammatory bowel disease, involving the upper and lower gastrointestinal tract, and developed arthritis (3). Stimulation of skin fibroblasts with TNF and poly(I:C) *in vitro* showed similar results to those seen in mice, with impaired activation of downstream signaling pathways from TNFR1 and TLR3 and increased cell death through necroptosis.

c. NF-κB pathway: SNPs in ubiquitously expressed genes encoding NF-κB-regulated molecules show strong association with IBD (164, 165). NF-κB stimulates transcription of numerous genes implicated in IBD pathogenesis, including *TNF*. TNF inhibition is one of the main therapeutic options in IBD (100), leading to reduced IEC apoptosis and enhanced mucosal repair (91). In IECs, transient TNF signaling inhibits apoptosis due to IKKβ-dependent NF-κB activation (91). On the other hand, corresponding ablation of IKKβ (95, 111), or its regulatory subunit NEMO (98), renders IEC susceptible to TNF-induced death. However, IKK or NF-κB deficiencies have never been reported in IBD.

d. A20 is a NF-κB-responsive gene that is thought to be involved in negative feedback regulation of NF-κB activation in response to many proinflammatory stimuli (166, 167). A20 contains an ovarian tumor (out) domain with deubiquitinating activity (DUB) in the amino-terminal region and seven carboxy-terminal zinc finger (ZnF) domains. A20-deficient mice have a severe inflammatory phenotype, with hypersensitivity to TNF, and die prematurely due to severe multiorgan inflammation and cachexia (168). Although several reports describe that A20 terminates the NF-κB pathway through its DUB activity by breaking down the docking sites in the TNFR1 complex I, A20 knock-in mice bearing an inactivating mutation in DUB (C103A) or ZnF4 domains do not exhibit the severe inflammatory phenotype of full A20-knockout mice (169, 170), suggesting that the function of A20 to modulate the NF-κB is not dependent on its deubiquitinase activity.

Several studies have linked SNPs of *TNFAIP3*, which codes for the immunoregulatory protein A20, with susceptibility to multiple autoimmune human diseases. These diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, type 1 diabetes, coeliac disease, Crohn's disease, coronary artery disease in type 2 diabetes, and systemic sclerosis (171). Most of the SNPs related with IBD are located in non-exon areas, implying that they most likely play a role in RNA synthesis or maturation. The minor rs5029941 (alanine to valine substitution) allele is associated with increased risk for IBD with an OR of 3.75, while the rs7753394, located upstream to the coding region, has an OR of 1.21 in heterozygotes and 1.48 in homozygotes for CD. Finally the rs2327832 allele increases the OR for UC to 1.26 (172). Interestingly, the rs6927172 variant was associated with increased A20 expression, decreased TNF levels, and non-response to anti-TNF therapy in both CD and UC (173). On the other hand, the rs6927210, rs7753394, and rs7773904 variants were linked to improved response to anti-TNF drugs (174).

Given that A20 SNPs in other diseases, such as SLE, have been related to lower expression or function (175, 176), and that A20 deletion in the whole mouse or in different compartments, including the intestine, induces spontaneous inflammation (168, 174, 177, 178), it is thought (but not proven) that SNPs in the *TNFAIP3* gene are associated with IBD decrease A20 expression. In IEC, deletion of A20 on those cells renders the mice more susceptible to the DSS colitis model with higher amounts of apoptotic cells in the epithelial colon (179). While the previous study did not show spontaneous intestinal inflammation, combined deletion of A20 in IEC and the myeloid compartment induces spontaneous colitis and ileitis with the presence of apoptotic cells in the crypt compartment (174). Additionally, overexpression of A20 in the IEC protects the intestinal epithelial barrier after LPS challenge and prevents colitis induced by DSS but not TNBS (180, 181).

However, A20s role in cell death seems to be more dependent on cell type than its NF-κB regulatory function. An A20 specific deletion in B and T cells actually protects them from FAS and TCR (T-cell receptor) induced cell death (178, 182). Also. two independent works have looked into the RNA expression of A20 in IBD. Although Arsenescu et al. found a decrease in the RNA levels of A20, as well as other typical proinflammatory markers of IBD in non-inflamed IBD tissue compared with control samples (183), Vereecke et al. found that A20 levels of non-responder patients to anti-TNF therapy was higher both before and after treatments compared to controls and responders. Accordingly, levels of A20 in patients that responded to anti-TNF drugs diminished to basal levels after therapy. These results could suggest that the upregulation of A20 is triggering intestinal inflammation. We have recently showed that A20 protein levels in UC and CD are increased in IECs. Using transgenic mice that overexpress A20 in the IEC, we showed that increased and prolonged recruitment of A20 to the TNFR complex I favors a shift from complex I toward complex IIb, probably through maintenance of RIPK1 linear-poly-ubiquitinated status and inducing RIPK1-dependent apoptosis in IEC (117). Of interest, we also detected A20 in the ripoptsome complex (117). Concomitant with that, pharmacological and genetic RIPK1 kinase blockade prevented apoptosis, suggesting a new therapeutical treatment for IBD.

## Microbiome

The gut microbiome, including bacteria, fungi, virus, and other organisms, shapes host functions in both normal and disease conditions. The clinical observation that antibiotics have a modest effect in IBD (184–187) suggests that the microbiome could play a role in shaping the disease. In fact, bacterial dysbiosis has been shown to occur in IBD (188–191) with consistent reports of decreased biodiversity, both α diversity and species richness, a measure of the total number of species in a community. However, a specific role of bacterial dysbiosis in IBD is yet to be discovered. In fact, a recent paper by Halfvarson showed that inflammation was not directly correlated with increased dysbiosis (188). A similar concept was suggested by another study, which shows that there is

reduced diversity in inflamed vs. non-inflamed tissues within the same patient, and a lower bacterial load in inflamed regions in CD patients (192). Also, serum reactivity against selected components of the gut microbiota is common, even in healthy individuals, and some CD associated serological markers against microbial antigens are present years before clinical manifestations in patients with CD, as well as in healthy individuals (193).

Various microorganisms that supposedly exert aggressive or protective functions relevant to Crohn's disease, such as adherent-invasive *Escherichia coli* and *Faecalibacterium prausnitzii*, respectively have been identified (194, 195). Furthermore, it is known that *Helicobacter pilori* has developed different mechanisms to disrupt the intracellular adhesions of the intestinal barrier (196), suggesting that other bacteria could act similarly. Yet, *E. coli* Nissle 1917 or ECOR63 enhance the epithelial barrier by up-regulating ZO-1 and claudin-14 and by downregulating claudin-2 (197). Also, Chelakkot et al. have demonstrated recently, that *Akkermancia muciniphila*, a known beneficial bacteria that reduces gut barrier disruption, upregulates occludin-2, decreasing the permeability of lipopolysaccharide-treated Caco-2 cells (198); a similar effect is seen when treating T84 monolayers with metabolites from the probiotic *Bifidobacterium infantis* Y1, which leads to an increase of ZO-1 while reducing claudin-2 (199). Treating Caco-2 cells with another probiotic, *Lactobacillus plantarum* MB452, also increased the transcription of occludins (200) and, *in vivo,* it increased occludin and ZO-1 (201). Some probiotics and commensals have also been shown to prevent, and even reverse, the adverse effects of pathogens on intestinal barrier function. For instance, when *L. plantarum* is co-cultured with enteroinvasive or enteropathogenic *E. coli,* it prevents the loss of permeability induced by those strains (202, 203). This data suggest that bacteria can directly regulate gut permeability by modulating cell-to-cell junctions. Thus, although it is believed that an inappropriate response against commensal gut microbiota occurs in IBD, it has been difficult to determine whether or not this process is secondary to an altered microbiota, a defective immune response, or a change in gut permeability (204), and whether these microbiome changes are primary or secondary to the disease. Of interest, some of the genes related to IBD were shown to control the bacterial microbiome and gut permeability, modulating cell-to-cell junctions. For instance, Nod2 prevents inflammation of the small intestine by restricting the expansion of the commensal *bacteroides vulgatus* (205).

Fungi is also a constituent of gut microbiota, however it just accounts for <0.1% of the total microbes (206). Antibiotic treatment increases fungi while decreasing bacteria populations, showing a competition between both kingdoms (207, 208). Alterations of GI bacterial populations and increased yeast can drive the development of a CD4 T-cell-mediated allergic airway response to subsequent mold spore exposure, suggesting a role for fungal microbiota in promoting immune-mediated diseases (208). In IBD patients, *Basidiomycota*, *Ascomycota*, and *C. albicans* are significantly elevated, whereas Saccharomyces, Candida, and Cladosporium are predominant in healthy individuals (209–211). Different components of the fungal cell wall such as chitin, β-glucans, and mannans can trigger the innate immune response, so it is not surprising that intestinal fungal invasion exacerbates colitis in mice (212).

Although virology focuses on pathogenic strains, most viruses are bacteriophages or endogenous retroviral elements. In fact, 99% of the annotated DNA viruses are bacteriophages (213). There are approximately $10^8$-$10^9$ virus-like particles (VLP) per gram of human stool, suggesting that viruses could play an important role in the bacterial community. The human gut bacteriophage varies intensively between subjects. However, they are temporally stable within individuals with dsDNA *Caudovirales* and ssDNA *Microviridae,* the two predominant viruses in healthy humans (214, 215). In IBD, virome richness

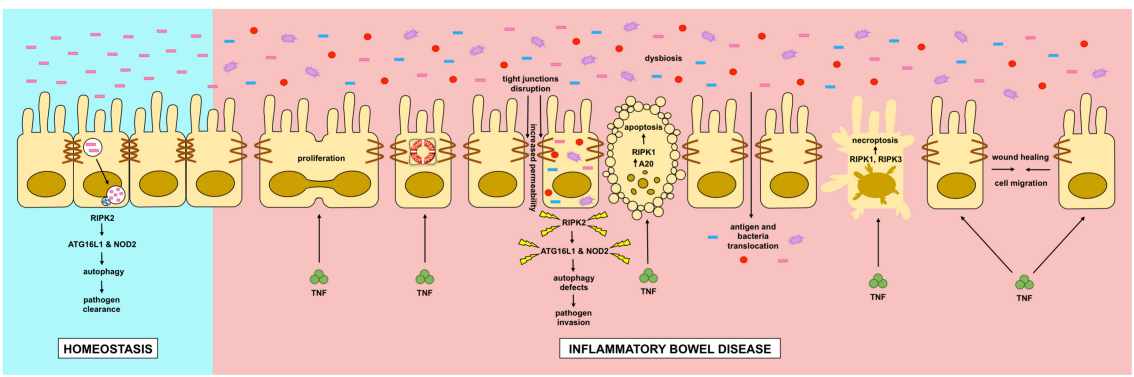


**FIGURE 4 |** Dysregulation of intestinal epithelial cell RIPK pathways promotes cell death in IBD. Dysregulation of RIPK (receptor interacting protein kinase) pathways play a key role in the inflammatory processes occurring in IBD (inflammatory bowel disease). TNF (tumor necrosis factor) has pleyotropic roles in intestinal epithelial cells. In homeostasis, TNF through the activation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, where RIPK1 has a scaffold function, it promotes cell proliferation, migration and survival, helping to a proper intestinal barrier regeneration. In IBD, TNF can induce apoptosis or necroptosis in a kinase dependent function of RIPK1 and RIPK1/3 respectively. Cell-to-cell adhesions are also loosened in IBD, allowing the translocation of food antigens and bacteria from the gut lumen. Another feature of IBD is microbial dysbiosis. Genetic mutations in RIPK2, NOD2 (nucleotide-binding oligomerization domain-containing protein 2) or ATG16L1 (autophagy-related protein 16 like 1) can impair a proper autophagy response allowing the proliferation and invasion of the host by pathogenic bacteria.

is increased with expansion from the order *Caudovirales* (216–218). This could be explained as a result of commensal microbes entering lytic cycles, or from new viruses introduction from new bacteria. In either case, bacteriophages can shape the gut microbiome, affecting bacterial fitness, diversity, and perhaps aiding in horizontal gene transfer (219, 220). Furthermore, viruses can translocate into the host, inducing immune responses (221–223). It is not surprising that mice with a genetic predisposition for CD (a mutation in the ATG16L1 gene) manifest the disease when infected with a gut norovirus while wild type mice controls did not (224). Another study showed that mice that were administered a cocktail of antiviral drugs had more severe colitis in the dextran sulfate sodium (DSS) model than ones treated with DSS alone. Overall, these results suggest a role for the virome in IBD, and new research will be needed to further understand its impact.

## CONCLUSIONS

In this review we discussed the role of RIPK and autophagy in relation to IBD (**Figure 4**). RIPK proteins seem to be plausible candidates for new drugs to treat inflammatory flares of IBD, preventing breakdown of the intestinal epithelial barrier. Additionally, autophagy seems to be a protective pathway, mainly by regulating intestinal homeostasis and pathogen protection, especially through paneth cells. Although further research is required to completely understand the pathophysiology of IBD, great advances in the field have improved the wellbeing of patients with the disease.

## AUTHOR CONTRIBUTIONS

RG-C, S-JY, SD, and MG contributed to the literature review. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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# Regulation of Immune Responses and Chronic Inflammation by Fibroblast-Like Synoviocytes

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Synovial tissue is a membranous non-immune organ lining joint cavities where it supports local immune responses, and functions directly and indirectly in joint destruction due to chronic inflammatory diseases such as rheumatoid arthritis (RA). Fibroblast-like synoviocytes (FLS), the dominant non-immune cells of synovial tissues, mainly contribute to joint destruction via multiple mechanisms. In RA, FLS respond to endogenous ligands of pattern recognition receptors (PRRs) and inflammatory cytokines as non-immune cells. In addition, FLS aid in the activation of immune responses by interacting with immune cells and by supporting ectopic lymphoid-like structure (ELS) formation in synovial tissues. Moreover, FLS directly cause the pathogenicity of RA i.e., joint deformities. Here, we describe new findings and review the mechanisms underlying the regulation of immune reactions by non-immune FLS and their roles in inflammatory diseases such as RA.

**Keywords: fibroblast-like synoviocytes (FLSs), synovial tissue, rheumatoid arthritis, non-immune cells, immune cells, autoantibodies, ectopic lymphoid-like structures (ELSs)**

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## INTRODUCTION

Non-immune cells of target organs play essential roles in the pathogenesis of chronic inflammatory and autoimmune diseases, forming the basis of the unique features of each disease (1). Fibroblast-like synoviocytes (FLS) are non-immune cells found in synovial tissues. FLS function in the pathogenesis of rheumatoid arthritis (RA), a type of chronic systemic arthritis. Autoantibodies, such as rheumatoid factor (RF), and anti-citrullinated peptide antibodies (ACPAs) are unique features of RA, and their presence indicates strong involvement of $CD4^+$ T cells and B cells in the RA pathogenesis (2). Therefore, cellular communication between FLS and hematopoietic immune cells may play a large role in the RA pathogenesis, including local autoantibody production in the RA synovium.

The synovium is a membranous organ lining the joint cavity. In normal physiological conditions within the joint cavity, the synovium supplies nutrients and the extracellular matrix (ECM) components of cartilage (3). FLS also strongly participate in the pathogenesis of RA. FLS support the development of the hyperplastic RA synovium as tertiary lymphoid organs (TLOs) by interacting with immune cells and organizing ectopic (tertiary) lymphoid-like structures (ELSs). Furthermore, FLS directly exert RA effector functions, which lead to joint deformity through osteoclastogenesis and the production of extracellular protease enzymes.

In this review, we describe new findings and examine the role of FLS in the RA pathogenesis.

## FLS RESPONSES VIA PRRS

Like stromal cells in other organs, such as the skin, gingiva, and lymph nodes (LNs), FLS play a role as innate immune cells by recognizing invading pathogens via PRRs such as Toll-like receptors (TLRs). Among the human TLR family, TLR1-7 is expressed by FLS (4). TLRs can recognize components of both pathogens and endogenous factors. Double-stranded and single-stranded RNA are recognized by TLR3 and TLR7, respectively. Necrotic cells in inflamed joints may be a source of endogenous ligands for these receptors (5). Endogenous ligands, such as heat-shock proteins and low-molecular-weight hyaluronan, were initially reported to be directly recognized by a heterodimer of TLR2/TLR4. However, highly pure ligands do not activate these receptors (6).

Of note, citrullination of endogenous ligands, such as fibrinogen and histones, stimulates the TLR4-mediated pathway (7, 8). Anti-TLR4 antibody significantly blocks the activation of monocytes by synovial fluid from RA patients exhibiting ACPAs (9), suggesting involvement of the TLR4-mediated pathway in the pathogenesis of RA. FLS are not activated by TLR9 plus CpG DNA (10). However, neutrophil extracellular traps (NETs) are internalized via the receptor for advanced glycosylation end products (RAGE)–TLR9 pathway, followed by promotion of the FLS inflammatory phenotype and human leukocyte antigen (HLA) class II upregulation (11). Thus, FLS recognize both pathogens and endogenous ligands through the PRRs, and these interactions lead to the pathogenesis of RA.

## FLS INTERACTIONS WITH IMMUNE CELLS

Autoantibodies, such as RF and ACPAs, are an important feature of RA, and their presence provides evidence of the involvement of $CD4^+$ helper T cells and B cells in the RA pathogenesis (2). The RA synovium frequently (40%) exhibits ELSs, which are discrete clusters of T cells, B cells, and macrophages (12). Consistent with the activated immune responses of these ELSs (1), B cells clonally expand in the RA synovium, presumably due to autoantigens, rather than in peripheral blood (13). During these activated responses, communication between FLS—a type of stromal cell—and immune cells may lead to the signature RA phenotype. In this section, we review the interactions of FLS with each cell type.

### Interactions With Macrophages

Under healthy conditions, resident monocytes are found in the intimal lining and sublining of synovial tissues (3, 14). Upon activation of synovial tissues, neoangiogenesis and chemokine recruitment function in the influx of peripheral monocytes into the synovium (3). In response to proinflammatory cytokines, FLS secrete chemoattractants, such as CCL2, CCL5, CCL8, CXCL5, and CXCL10, which leads to the recruitment of monocytes and macrophages (3). Cytokine networks at inflammatory sites contribute largely to the RA pathogenesis and the perpetuation of inflammation. Detailed analysis of the cytokine milieu of RA synovitis revealed that macrophages and fibroblasts are the major sources of proinflammatory cytokines (15). Anti-cytokine therapies, including anti-TNF and anti-IL-6, markedly improve the clinical results after RA treatment (16, 17). These cytokines form a vicious inflammatory cycle leading to synovial hyperplasia, influx of lymphocytes, and the production of effector proteins. Macrophages are the major source of IL-1β and TNF, and FLS in the intimal lining are the main source of IL-6 (15). Colony-stimulating factors, such as GM-CSF and M-CSF, are also produced primarily by FLS in the intimal lining (18). Upregulated GM-CSF production by IL-1β/TNF-stimulated FLS is involved in the local expansion of macrophages. GM-CSF rather than IFN-γ plays an important role in the induction of HLA class II expression on macrophages in the RA synovium (**Figure 1**) (15). Indeed, anti-CXCL10 treatment and anti-GM-CSF receptor treatment are clinically effective for RA (19, 20).

Another aspect of the interaction between macrophages and FLS is the induction of osteoclasts, which are specialized bone-absorbing cells that differentiate from macrophages. Actively transformed RA synovium, the so-called pannus, destroys the cartilage matrix and can invade bone. At the tip of the pannus, multinuclear cell osteoclasts greatly absorb adjacent bone. RANKL has been identified as the factor responsible for the differentiation of osteoclasts from macrophages (21–23). Activated FLS produce large amounts of RANKL and another essential factor, M-CSF. Clinically, anti-RANKL antibody significantly attenuates the bone destruction of RA (24).

### Interactions With T Cells

$CD4^+$ helper T cells are another important player in the RA pathogenesis. Genetic studies of RA-related genes revealed that T-cell-related genes, including *HLA-DR*, *PTPN22*, and *CTLA4*, are involved in RA (25), and that treatment that targets T cells is as effective as anti-TNF therapy (26). $CD4^+$ T cells differentiate into several types of subsets depending on the differentiation environment. IL-17-producing helper T (Th17) cells, follicular helper T (Tfh) cells, and PD-$1^{hi}$CXCR$5^-$ peripheral helper T (Tph) cells are thought to be involved in RA (27–29). Th17 cells function in the activation of FLS, macrophages, endothelial cells, and chondrocytes mainly via the biological effects of IL-17A (30). However, clinical trials of neutralizing anti-IL-17 antibodies demonstrated that Th17 cells play a role in the pathogenesis of psoriatic arthritis but less in that of RA (31, 32). The strong involvement of autoantibodies, such as RF and ACPAs, in RA suggests that B-helper activity is a key function of $CD4^+$ helper T cells. In LNs or tonsils, Tfh cells exert B-helper activity, and aid in class switching and affinity maturation of antibodies via the activity of the master transcription factor BCL-6 (33). However, the BCL-6 expression level is not increased in RA synovial $CD4^+$ T cells (34, 35) despite production of autoantibodies in the RA synovium (13). Recently identified by comprehensive analysis of clinical samples as a pathogenic $CD4^+$ subset in RA patients (36), Tph cells also play a role in B-helper activity and ELS formation at inflammatory sites (34, 35, 37).

Although not primary immune cells, FLS express immune-related genes, including HLA Class II, the gene required for presenting antigens to $CD4^+$ helper T cells, during the

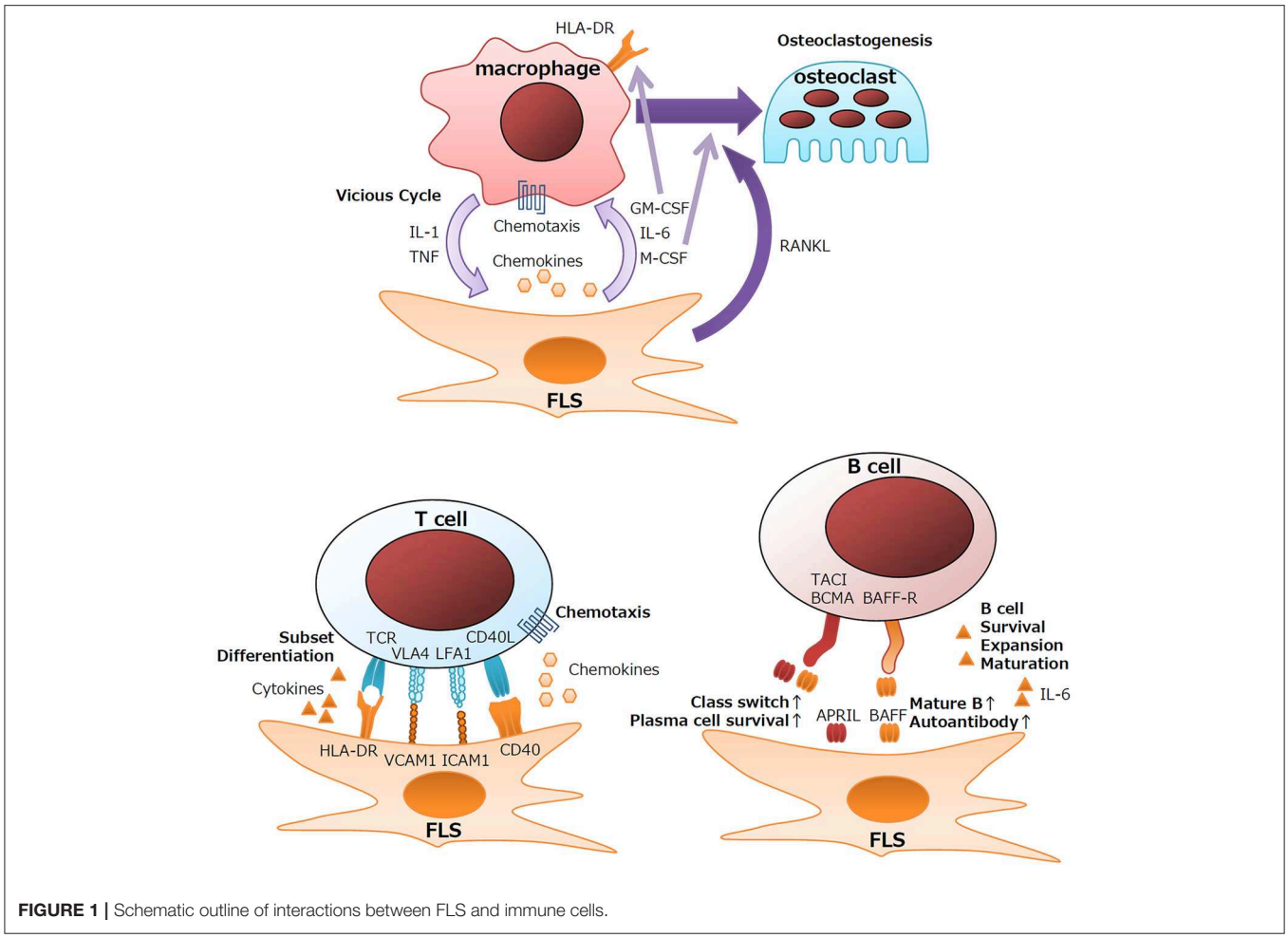


**FIGURE 1 |** Schematic outline of interactions between FLS and immune cells.

development of RA. FLS also function in the differentiation of T cells via cytokine production. TGF-β is known to be involved in the differentiation of several types of T-cell subsets, such as inducible T reg (iTreg), Th17, and Tph cells (38), by inducing the transcription factors FoxP3 (39), RORC (40), and Sox4/Maf (37). Chemokines from FLS also help recruit T cells. CXCL9/10/11, CCL20, and CCL2 recruit Th1, Th17, and Tph cells via the cytokine receptors CXCR3, CCR6, and CCR2, respectively (36, 41–43). RA FLS also significantly express higher amounts of CX3CL1 (fractalkine), and the expression of its sole receptor, CX3CR1, is upregulated in $CD4^+$ and $CD8^+$ T cells of patients with RA, suggesting the involvement of the CX3CL1/CX3CR1 axis in the pathogenesis of RA (44). Consistent with this, anti-CX3CL1 treatment has significant clinical effects for RA (45). Membrane proteins and adhesion molecules also lead to the activation of T cells and FLS. CD40L produced by $CD4^+$ T cells stimulates B-cell activity by stimulating CD40 signaling in B cells. Similarly, CD40L produced by T cells also stimulates FLS to release chemotactic molecules (46). LFA-3 on FLS and LFA-2 (CD2) on T cells are important for strengthening the adhesion between T cells and FLS (47). ICAM-1 and VCAM-1 expressed on FLS regulate the development of T cells by interacting with the integrins LFA-1 and VLA-4, respectively (48). Thus, FLS support the immunological functions of T cells via pleiotropic mechanisms (**Figure 1**).

## Interactions With B Cells

The clinical relevance of autoantibodies in RA supports the important roles of B cells in the RA pathogenesis. Indeed, administration of the B-cell-depleting anti-CD20 antibody, rituximab, produces good clinical results for RA (49). Autoantibodies develop initially in the synovium rather than in peripheral blood and are class-switched during the development of RA (13), which indicates that the local synovial environment is a main contributor to the development and maturation of autoantibody-producing B cells.

Upon TLR3 stimulation, FLS produce large quantities of B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) (50). Although both BAFF and APRIL bind to the receptor's B-cell maturation antigen (BCMA), transmembrane activator, and cyclophilin ligand interactor (TACI), only BAFF can bind to the BAFF receptor (BAFF-R) (51). Therefore, BAFF and APRIL exert different biological effects on B cells. BAFF is important for the maturation and survival of B cells; upregulated BAFF expression leads to an increase in the number of mature B cells and autoantibody production in mice (52). In contrast,

APRIL plays essential roles in class-switching of antibodies and the survival of plasma cells (51). However, the roles of BAFF and APRIL in RA remain to be determined. Anti-BAFF treatment for RA downregulates RF but has little effect on the clinical course of disease activity (53).

IL-6 is another key factor secreted by FLS that can affect B cell functions. In addition to its pleiotropic effects on multiple cell lineages, IL-6 plays key roles in the development of B cells. IL-6 is involved in the survival, expansion, and maturation of B cells (54), and it functions in the commitment to the Tfh cell subset via the induction of the transcription factor BCL-6 (55). Therefore, FLS support the function of B cells, including autoantibody production, and lead to the pathology of RA (**Figure 1**).

### FLS Support ELS Formation

Frequent ELS formation is an important feature of the RA synovium (12). In ELS, generally upregulated immune responses lead to autoantibody production in rheumatic diseases, or to anti-viral immunity or anti-tumor immunity depending on the features of diseases (1, 56–58). FLS play essential roles in ELS formation and in the regulation of ELS immune responses. During the developmental process of secondary lymphoid organs, such as LNs and tonsils, interactions between lymphoid tissue inducer (LTi) cells and lymphoid tissue organizer (LTo) cells coordinate organ formation. Similarly, FLS express signature genes of LTo cells and their derivative fibroblastic reticular cells (FRC) such as LTβR, IL-7, RANKL, CXCL13, CXCL12, CXCL21, CXCL19, VCAM-1, ICAM-1, and gp38 (12, 34, 48, 59–64). In addition, FLS also exert nurse-like activity by supporting lymphocyte pseudoemperipolesis (active migration of lymphocytes into the cytoplasm of nurse-like cells). FLS under RA or of non-RA conditions support the pseudoemperipolesis of T cells, B cells, and NK cells, aiding in their survival, activation, or functions such as IgG production (60, 65, 66). Due to such activity of FLS, TLO cells are (i) induced via initiators, such as LTαβ, IL-7, and RANKL (67), (ii) expanded via propagators, such as CXCL13/12/21/19, and (iii) maintained via adhesion molecules and nurse-like activity (**Figure 2**).

### Involvement in Organ-Specific Immune Responses

Although ELS exhibit an overlapping structure in several inflammatory diseases, the target autoantigens depend on the diseases. In the salivary gland ELSs of patients with Sjögren syndrome, B cells and plasma cells are frequently reactive against the ribonucleoproteins Ro/SSA and La/SSB, whereas autoantibodies specific for RA are RF and ACPA. These differences may be partly attributed to the differences in non-immune cells of target organs. One notable feature of FLS is their contribution to the joint architecture via formation of synovial anatomical components: the intimal lining and sublining. Indeed, FLS grown in three-dimensional culture self-direct their architecture to be very similar to that of the intimal lining (14), which borders between joint spaces and synovial tissues. This signature architecture of joints may function in the enrichment of disease-specific antigens, such as synovial fluid NETs, whose citrullinated histones are major targets of ACPA (12, 68, 69). Alternatively, a protein of FLS, citrullinated calreticulin of FLS, or citrullinated aggrecan from cartilage are other targets for RA autoreactive B cells or T cells, respectively (70, 71). These findings suggest a connection between autoantibodies and organ-specific antigens depending its structure and components.

## FLS EXERT EFFECTOR FUNCTIONS OF RA

As a consequence of activated immune responses, hyperplastic synovial tissues of RA (pannus) aggressively invade adjacent cartilage, tendon, and bones, leading to the destruction of multiple joints. Clinical studies demonstrated that the vasculature of the RA synovium reflects joint inflammation and correlates with future joint deformities. In this joint destruction, FLS directly exert effector functions. In this section, we discuss FLS effector functions and their regulation.

### Synovial Hyperplasia

As mentioned above, FLS are highly involved in the formation of the intimal lining of synovial tissues (14). The intimal lining comprises 1–3 cell layers in normal physiological conditions, but its thickness increases to 10–15 cell layers in the activated RA synovium (3, 46). Upregulated expression of effector factors in the hyperplastic intimal lining suggests the importance of the dysregulation of the intimal lining in the RA pathogenesis (15, 18). Dysregulation of apoptosis and proliferation of FLS via multiple genes may play a role in this hyperplasia (72–74). In three-dimensional culture conditions, the combination of growth factors (e.g., PDGF and TGF-β) with inflammatory cytokines (e.g., TNF) strongly induces hyperplasia of the synovial lining via activation of the PI3K–Akt pathway (75, 76). Adhesion molecules, such as cadherins and integrins, play essential roles in the formation and maintenance of the synovial lining. A deficiency in cadherin 11 leads to the disappearance of the intimal lining in mice (77). Integrin α9β1 is also preferentially expressed by FLS. Neutralization of integrin α9β1 or knockdown of its ligand tenascin-C abrogates the formation of the synovial lining (78). Based on these findings, in addition to integrin α9β1 and cadherin 11, FLS may play a role in the formation of the synovial lining. These adhesion molecules also have effector functions in RA. RA FLS cultured in a three-dimensional manner secrete greater amounts of effector factors, such as MMP1, MMP3, IL-6, or RANKL, than monolayer FLS.

The protein gp38, a FRC signature gene, may also function in the regulation of TLO size. Regarding LNs, gp38 is involved in the FRC regulation of LN size. Interaction of gp38 with its ligand CLEC2, which is preferentially expressed by dendritic cells in LNs, reduces the tension of fibroblastic reticular cells. Enlargement of LNs upon inflammation is significantly disturbed in gp38-deficient mice (79). Of note, RA FLS of the intimal lining express more gp38 than OA FLS (62), and platelets in the synovium preferentially express CLEC2 (80). However, it remains to be investigated whether gp38 is also involved in hypertrophy of the RA synovium. Treatments targeting integrins or other adhesion molecules may be candidate alternatives for patients with refractory synovial hyperplasia.

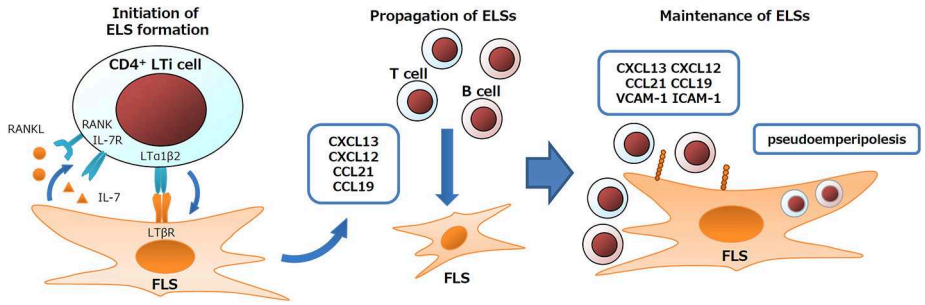


**FIGURE 2 |** Schematic outline of FLS contribution to TLO formation.

## Neoangiogenesis in the RA Synovium

The transitory pre-vascular inflammatory stage of the RA synovium is followed by a prominent vascular stage (46, 81), which is clinically detectable by power Doppler (PD) sonography as a reliable sign of active synovitis, and is significantly correlated with the poor prognosis of RA (82). The clinical connection between PD-positive synovial hyperplasia and the poor prognosis of RA strongly suggests the importance of the synovial vasculature in the RA pathogenesis. Hyperplasia of the intimal lining and infiltration of T cells, B cells, and macrophages into the sublining increase the metabolite demand and hypoxia, which induces marked new vessel formation (81). In particular, hypoxia accompanied by synovial hyperplasia drives the production of VEGF, the most important factor for neoangiogenesis, via the hypoxia-inducible transcription factor (HIF)-1α, whereas HIF-2α is involved in FLS functions of intimal lining (83–86). Subsequently, upregulated VEGF leads to the activation of the angiopoietin (Ang)/Tie-2 system. Although Ang1 is constitutively expressed by quiescent vasculature, the expression of Ang2 depends on endothelial cell activation (87). The activation of Tie-2 signaling via the Akt pathway is required for the proliferation and survival of endothelial cells (81). Observation of Tie-2 activation in synovial tissues from some unestablished RA patients might imply the involvement of angiogenesis process in the development of RA (88). Of note, RA FLS under hypoxic conditions are sufficient for angiogenesis employing multiple factors such VEGF, bFGF, TGF-β, IL-6, IL-8, CXCL12, ICAM-1, VCAM-1, and matrix metalloproteinases (84, 89). In the context of the clinical relevance of PD for the development of joint destruction, treatments targeting HIF-1α or angiogenic factors have been discussed as alternative treatments for RA (81, 90).

## Direct Effector Functions of FLS

One important feature of RA is the direct contribution of FLS to the degeneration of joints. Models of FLS transplanted together with cartilage into immunodeficient mice demonstrated that once activated, RA FLS acquire an aggressive phenotype that invades adjacent cartilage (91). RA FLS secrete multiple species of extracellular protease enzymes such as matrix metalloproteinases (MMPs). MMPs can be subdivided according to their substrates into collagenases, stromelysins, gelatinases, and membrane-type MMPs. The collagenases MMP-1 and MMP-13, and the stromelysin MMP-3 are the most important MMPs in the RA pathogenesis (3). FLS also produce tissue inhibitors of metalloproteinases (TIMPs). Cartilage destruction depends on the balance between MMPs and TIMPs. When the balance favors MMPs, cartilage degradation proceeds. The expression of MMPs, but not TIMPS, is upregulated by inflammatory cytokines (IL-1β, TNF, and IL-17) (3), which is consistent with the correlation between inflammation and cartilage degradation.

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) comprise another family of extracellular proteases. ADAMTS4 and ADAMTS5 produced by FLS lead to cartilage damage in RA (92). Adhesion molecules, such as integrins and cadherins, are also involved in cartilage degeneration. As described above, FLS in the intimal lining increase the expression of MMPs via signaling of integrin α9 and cadherin 11 (77, 78). RA FLS also express higher amounts of integrin α5β1, which plays an important role together with syndecan 4 in the adhesion of cells to, and destruction of, the cartilage matrix (93). Another essential function of FLS is osteoclastogenesis, which is also involved in joint destruction, via RANKL and M-CSF secretion. Although activated T cells also produce RANKL, conditional knockout *in vivo* experiments revealed that FLS contribute to bone destruction more than T cells (63).

# CONCLUSION

Several studies on RA have confirmed that FLS—non-immune cells found in target organs—play several roles in disease development. These findings have increased our understanding of the immune responses of ELSs at local inflammatory sites. However, many questions remain to be answered about the immune responses at local inflammatory sites, including autoantibody development in ELSs and the complex roles of helper T cells. Recent single-cell analysis has demonstrated that FLS can be classified into several subsets (94). Further investigation of the interactions between FLS and immune cells will improve our understanding of human immunology and aid in the development of new RA treatments.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Intrinsic Control of Surface Immune and Epithelial Homeostasis by Tissue-Resident Gut Stromal Cells

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The epithelial layer creates a chemical and physical barrier at the forefront of intestinal mucosa, and immune cells beneath the surface epithelium are poised to react to extrinsic factors, to maintain tissue homeostasis. Importantly, the nexus of epithelial–immune responses at mucosal surfaces is dexterously modulated by intrinsic stromal–mesenchymal cells. First, organogenesis of lymphoid tissues, including Peyer's patches, requires dynamic interplay between lymphoid cells and stromal cells, which have become known as "lymphoid organizers." Second, correct spatiotemporal interaction between these cell populations is essential to generate the infrastructure for gut immune responses. Moreover, immune cells at the intestinal barrier are functionally modulated by stromal cells; one such example is the stromal cell–mediated differentiation of innate immune cells, including innate lymphoid cells and mast cells. Ultimately, mucosal stromal cells orchestrate the destinations of epithelial and immune cells to maintain intestinal immune homeostasis.

**Keywords: mucosal immunology, mesenchymal cells, fibroblasts, intestinal stem cells (ISCs), Peyer's patches**

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## INTRODUCTION

The single layer of epithelial cells at the intestinal mucosa creates both a chemical and physical barrier to protect the body. Overlying the epithelial surface of the digestive tract are mucin-containing layers, which play an important role in preventing commensal bacteria from attaching directly to gut epithelium. A single layer of mucus covers the small intestinal epithelium, whereas two layers (inner and outer) overlay the epithelium of the stomach and colon (1). Mucus is net-like in structure and forms a gel due to its glycoprotein components, MUC2 (secreted by goblet cells in the intestine and colon) and MUC5AC (produced by gastric epithelial glands in the stomach) (2, 3). In addition, the mucus layer in the intestinal compartment contains anti-microbial peptides, including α-defensin, which is produced by Paneth cells in the intestinal crypts located at the base of villi (4), and secretory IgA molecules, which derive from the intestinal mucosa or

bile duct (5, 6). A lack of either of these secretory components leads to spontaneous intestinal inflammation or colitis, with insufficient segregation of pathologic and commensal bacteria from the intestinal epithelial layer (7, 8). Therefore, the chemical barriers mounted by mucus layers—the components of which originate from epithelial cells and are mediated by secretory systems—are required for both the maintenance of intestinal homeostasis and protection from infection.

To efficiently absorb nutrients from the ingested diet, the intestine contains a huge number of villi. The gut epithelial cells overlying these villi create a physical barrier by sealing the spaces together with other epithelial cells [reviewed in (9)]. These seals are the so-called cellular junctions, comprising both adherent and tight junctions. Various molecules within cellular junctions, such as E-cadherin, regulate epithelial permeability, such that disruption or genetic polymorphism of these molecules increases the susceptibility to intestinal inflammation (10). Stromal cell populations are located beneath these epithelial cells and the lamina propria (**Figure 1A**). One key stromal cell population called ISEMFs (intestinal sub-epithelial myofibroblasts) is located subjacent to the basement membranes of the intestinal epithelial cells (**Figure 1A**) (11). ISEMFs express Acta2 (α-smooth muscle actin); are phenotypically similar to smooth muscle; initiate villous contractions; express various receptors for cytokines, including TNFα and IL-1β; and sense epithelial barrier damage and inflammation (11, 12). Consequently, ISEMFs are involved in several steps of wound healing, including epithelial restitution and epithelial stem-cell proliferation (13, 14). During the process of epithelial restitution, epithelial cells from intestinal crypts rapidly migrate to cover and seal epithelium-denuded areas of intestine in the absence of epithelial proliferation [reviewed in (15, 16)]. In addition, ISEMF-like stromal cells are located beneath the basal membrane of the epithelial layer covering Peyer's patches (PPs), which is known as FAE (follicular associated epithelium) (**Figure 1B**). FAE includes a unique cell population harboring short microvilli, named microfold (M) cells [reviewed in (17, 18)]. These short microvilli are readily accessible to luminal bacteria, which express ligand molecules (e.g., on fimbriae) for attaching to the apical surface of M cells through specific receptors (e.g., glycoprotein 2 [GP2]). Stromal cells are involved in the development and maintenance of M cells and in intestinal mucosal immune responses (19). Through these functions, stromal cells act not only as a second barrier at the intestinal surface but also as determinants of the destinations of diverse immune cells to maintain immune homeostasis (20).

In this review, we highlight current knowledge regarding the crucial and complex roles of stromal cells from intestinal epithelial homeostasis to surface immune responses.

## SPATIOTEMPORAL MUCOSAL REGULATION BY INTESTINAL STROMAL CELLS

As indicated earlier, the stromal cells called ISEMFs are located beneath intestinal epithelial cells (11). ISEMFs express vimentin and Acta2 and contract after epithelial damage to limit the exposed wound area [reviewed in (12, 20)]. ISEMFs produce several growth factors, TGF-β1 and amphiregulin, and support epithelial proliferation by sensing cytokines associated with intestinal damage (e.g., TNFα and IL-1β) (21). In fact, *in vitro* co-culture of ISEMFs and epithelial cells or intestinal organoids (i.e., mini-gut) composed of epithelial cells shows that ISEMFs are critical for epithelial proliferation (13, 22). Furthermore, ISEMFs support the morphology of epithelial cells and the intestinal epithelial lining, because they produce and deposit various types of collagen, including types I, III, IV, V, and VI (23). Collagen types I and III are ubiquitous interstitial collagens and enhance epithelial cell growth (23), whereas type IV contributes to the formation of epithelial basement membranes, and type V is a pericellular collagen for thickening of the intestine wall (24). In addition, loss of collagen VI alters epithelial cell morphology (25). These cytokine-mediated biologic effects on and collagen-mediated physical support of epithelial cells by ISEMFs lead us to consider ISEMFs as a secondary barrier that harmoniously interacts with and promotes the epithelial cell defense function in the mucosal surface.

Stromal cell function is precisely regulated by the local tissue environment. In fact, the genes expressed differ among stromal cells according to their tissue location (26, 27). This remarkable difference in gene expression is particularly evident when comparing stem cell–related molecules (26). Expression levels of cytokines responsible for maintaining intestinal stem cell niches—that is, those involved in Wnt signaling (e.g., WNTs 2b and 5a and WNT agonists [e.g., R-spondins 1 and 3]) and BMP (bone morphogenetic protein) antagonists (e.g., Noggin, Gremlins [GREM] 1 and 2)—differ significantly among various villous regions (e.g., from tip to crypt) (26). Gene analysis of dissected human colonic tips and crypt compartments reveals that genes highly expressed in tips typically are induced by interruption of Wnt signaling through genes induced by dominant-negative transcription factor (TCF) 4 (e.g., p21, a gene that inhibits cell proliferation) and BMP2 (26). Furthermore, genes highly expressed in colonic crypts usually are repressed by dominant-negative TCF4 (e.g., MYC and Cell Division Cycle Associated 7, two genes involved in cell-cycle regulation) and the BMP antagonists GREM1 and GREM2 (26). Therefore, in small intestine, Paneth cells primarily and mesenchymal cells secondarily secrete niche factors (e.g., EGF, WNT3, and the Notch ligand Dll4); in contrast, mesenchymal cells are predominantly responsible for maintaining the stem cell niche in colon, which is devoid of Paneth cells (28, 29). These findings demonstrate the spatiotemporal regulatory mechanisms of stromal cells in creating intestinal stem cell niches.

Directly underneath LGR5-expressing intestinal stem cells lie myofibroblasts and pericryptal stromal populations, which lack Acta2 expression but express CD34, podoplanin, and PDGF (platelet-derived growth factor) receptor α, and the WNT agonist R-spondin 3 (30). These cell populations also produce the winged-helix transcription factor named Foxl1 (forkhead box l1) (30), and a deficiency of Foxl1-expressing stromal cell populations leads to reduced production of niche factors (e.g., R-spondin 3, GREM1, WNT2b, WNT5a) in the crypt compartment. Importantly, Foxl1-deficient mice showed

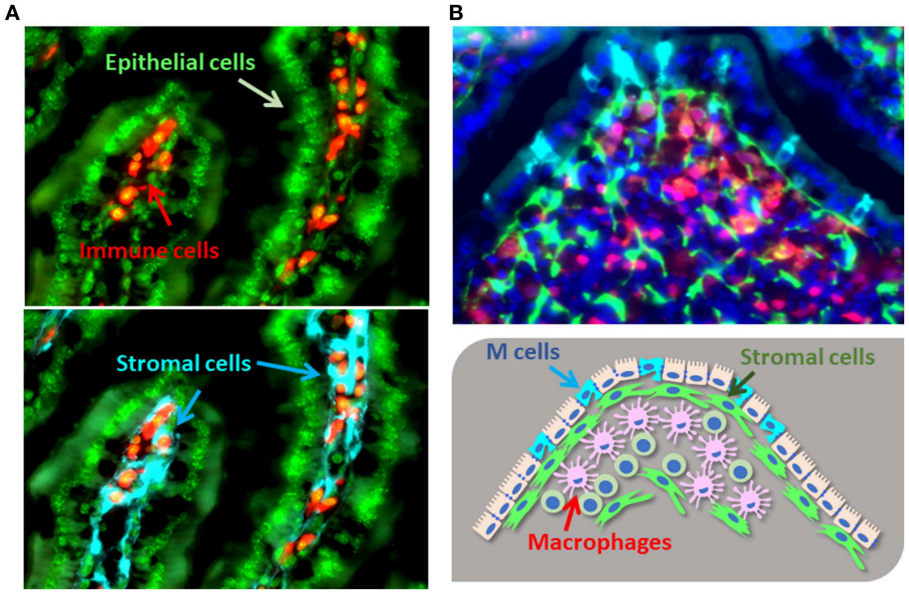


**FIGURE 1 |** Cellular localization of gut stromal cells. **(A)**. The colocalization of gut immune cells [e.g., B cells (CD19), red] and epithelial cells (DAPI, green) with stromal cells (type I collagen, light blue) in murine intestinal villi. **(B)**. Immune cells [e.g., macrophages (lysozyme), red] and stromal cells (type I collagen, green) reside under the M cells located in the FAE of PPs (GP2, light blue; DAPI, dark blue).

aberrant crypt structure, with ectopic and increased expression of Ephrin-B2 and Ephrin-B3 in epithelial cells (31). These factors are important for epithelial cell positioning along the crypt–villus axis, and their deficiency leads to intermingling of the proliferative and differentiated epithelial cell populations (32). These findings indicate various components of the spatiotemporal regulatory mechanism for stromal cells that ensures adequate stem cell niches and the maintenance of epithelial organization and integrity.

Recently identified additions to the stromal cell populations surrounding intestinal crypts are Foxl1-expressing telocytes (33). Telocytes are a unique type of interstitial cells, which also are found in reproductive tissues, including uterus and placenta [reviewed in (34, 35)]. Telocytes are characterized as having several very thin and long projections, called telopodes. In addition, like other stromal cells, telocytes express CD34, PDGF receptor α, and (typically) c-kit; however, gut telocytes do not express c-kit, unlike the interstitial cells of Cajal (36). Recent evidence reveals the importance of telocytes as a key producer of Wnt ligands in the intestinal crypt (33). Conditional deletion of porcupine, which shows homology to a family of o-acyl transferases that are involved in lipid modification and are required for Wnt production, from Foxl1$^{+}$ cell populations, including telocytes, abolishes the proliferation of stem and transit amplifying cells (33). Indeed, telocytes are absent during the active stage of Crohn's disease, and this lack is correlated with subsequent architectural disruption (37). In addition to those cellular populations, GLI family zinc finger 1 (Gli1) – and Acta2-expressing stromal cells similar to myofibroblasts are involved in maintaining stem cell niches through their production of WNT2b (38, 39). Disruption of Wnt in stromal populations that express Gli1 ameliorates colonic stem cell renewal as well as colonic epithelium integrity (39).

Recently, precise anatomical and immunohistochemical analyses performed in rat intestinal tissue have confirmed the presence of CD34$^{+}$Acta2$^{-}$ populations at the crypt base, PDGFR$^{high}$Acta2$^{+}$ cells laterally along the crypt, and PDGFR$^{high}$Acta2$^{-}$ populations at the tips of villi (40). Due to targeting of porcupine, aberrant secretion of Wnt from PDGFR-expressing pericryptal myofibroblasts ameliorates crypt formation in the neonatal mouse gut (41). However, this experimental system does not exclude the important contributions of other stromal cell populations (e.g., telocytes), thus implying that multiple stromal subsets are involved in the complex and compensative machinery for maintaining both the stem cell niche in the crypt region and epithelial integrity (**Figure 1**).

In addition to cell-targeting experiments, recent single-cell analysis has revealed precise stromal cell populations in the colons of humans and mice (27). In addition to myofibroblasts, at least 4 newly identified subsets of fibroblasts present in both mice and human colon have been characterized: SOX6$^{-}$CCL8$^{+}$FABP5$^{high}$; BMP2$^{high}$WNT5A$^{high}$; BCL2$^{high}$ OGN$^{high}$; and CD74$^{high}$CCL19$^{high}$ (27). SOX6$^{-}$CCL8$^{+}$FABP5$^{high}$ fibroblasts produce elastic fibers and fibrillar collagen and distribute throughout lamina propria, indicating the involvement of this population in the maintenance of structural and mechanical properties of colon tissue (27). Among those newly identified fibroblasts, BMP2$^{high}$WNT5A$^{high}$ fibroblasts express collagen for epithelial basement membrane (e.g. COL4A5 and COL4A6), predicted as its involvement in epithelial barrier formation (27). Indeed, those populations

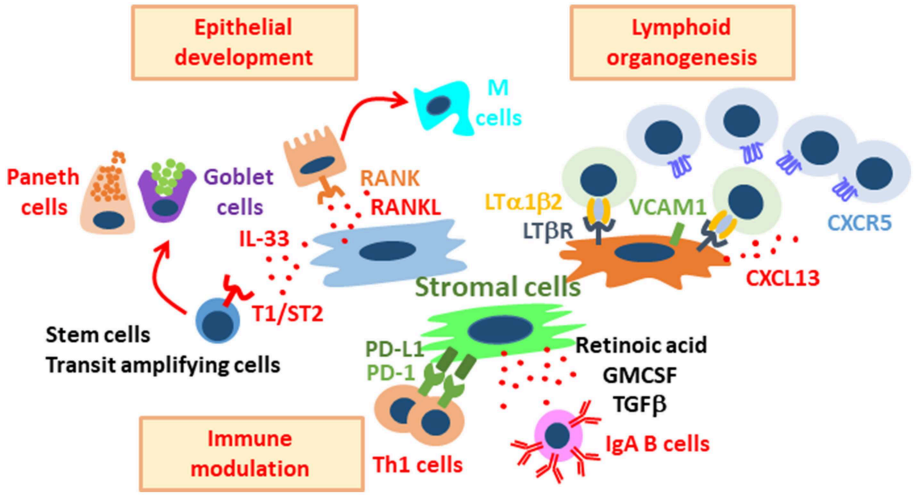


**FIGURE 2 |** Diverse roles of stromal cells in the regulation of intestinal immunity and physiology. Stromal cells support intestinal epithelial development, lymphoid organogenesis, and immune modulation. These stromal cell populations are distinct, and each population plays important roles in the maintenance of gut homeostasis through their interaction with stem, epithelial, and immune cells.

located near intestinal epithelium. Importantly, this population reduces in the UC patients, implicating that reduction of WNT5a production worsen crypt reconstitution and epithelial integrity (26).

On the contrary, $CD74^{high}CCL19^{high}$ fibroblasts expand in the UC patients (27). This population upregulates expression of TNF superfamily member 14 (LIGHT) and release IL-6 and IL-33 during colonic inflammation (27). IL-6 and LIGHT stimulation to the intestinal organoid under the condition mimicking inflammation (lacking Wnt) results in the reduction of epithelial proliferation and upregulation of stem cell maker gene expressions (e.g., LGR5) in organoid. Those results imply that the activation of $CD74^{high}CCL19^{high}$ fibroblasts lead to the production of quiescent label-retaining cells, precursor of secretory-type enterocytes (e.g., goblet cells and Paneth cells) (27).

Furthermore, IL-33 production from fibroblasts is mediated by IL-1β, IL-6, TNF-α, and bacterial cell components (e.g., lipopolysaccharide) (42). IL-33, in turn, stimulates ST2-expressing cryptal epithelial cells and the subsequent development of secretory-type enterocytes, including Paneth cells and goblet cells (42) (**Figure 2**). Collectively, during inflammation and pathogenic infection, the production of both anti-microbial peptides and mucus is enhanced by fibroblasts that sense "danger signals" (e.g., IL-33). In addition, Paneth cells in the small intestine and a $c\text{-}kit^{+}$ subset of colonic goblet cells support intestinal epithelial stem cells via secretion of Wnt, indicating that activation of cryptal stromal cells due to danger signaling leads to both protection and maintenance of the stem cell niche through dual pathways (directly and indirectly through secretory cell–derived Wnt) (43).

However, increase of IL-6 in colonic tissues, produced by continuously activated $CD74^{high}CCL19^{high}$ fibroblasts, may strongly influence on the function of macrophages and Th17 cells leading chronic inflammation. So that, regulation of the function of this fibroblast population will be useful therapy for IBD.

Stromal cells located the sub-epithelial compartments of PPs are required for the development of specific epithelial cells, including antigen-sampling M cells. M cells are considered to be "gatekeepers" of the mucosal immune system and serve as entry sites for luminal antigens and bacteria (**Figure 1B**). M cells have short microvilli and lack the ability to produce anti-microbial peptides and mucus, thus facilitating the attachment of bacteria to their apical surfaces (18). Indeed, M cells express various receptors and guidance molecules for both commensal and pathogenic bacteria (9). For example, GP2 is preferentially expressed on M cells and interacts with the fimbriae of bacteria (44, 45); antigen-presenting cells (e.g., dendritic cells) beneath M cells further capture and process bacterial antigens for the initiation of antigen-specific immune responses in PPs. Therefore, GP2-mediated translocation of bacteria leads to efficient induction of antigen-specific mucosal immune responses (e.g., IgA) (45). The receptor activator of NF-kB (RANK)–RANKL pathway is critically important for the development and differentiation of M cells (46). In particular, stimulation of gut organoids with RANKL revealed the essential roles of the RANK–RANKL pathway for the development of M cells from intestinal epithelial stem cells (47). Whereas, RANK is expressed on all intestinal epithelial cells, RANKL-expressing cells accumulate in the FAE region, and mice lacking either of those molecules showed decreased numbers of M cells in the FAE of PPs (46). Furthermore, RANKL deficiency in type VI collagen–expressing (Col6a1–Cre) cell populations, including M cell–inducing populations, led to the disappearance of M cells (19) (**Figure 2**). Importantly, those gene-modified mice showed decreased luminal IgA against commensal bacteria (19); therefore, M cell development, as orchestrated through stromal signaling, is a plausible initial

pathway through which PPs become able to mediate acquired mucosal immunity.

Overall, current knowledge supports that—as determinants of epithelial integrity and sentinels of mucosal immune responses—sub-epithelial and cryptal stromal cells play indispensable roles in the barrier function of the intestinal mucosa.

## STROMAL NETWORK FOR MUCOSAL LYMPHOID ORGANOGENESIS

In addition to epithelial regulation, another important role of stromal cells is their biologic contribution to lymphoid organogenesis [reviewed in (48)]. The organization of lymphoid structures is precisely programmed through molecular and cellular components that are achieved through a stromal network that orchestrates lymphocyte entry into and migration within tissues (49); reviewed extensively in Chang and Turley (50), van de Pavert and Mebius (51), Roozendaal and Mebius (52).

Lymphoid tissue contains 3 types of stromal cells: fibroblastic reticular cells, follicular dendritic cells, and marginal reticular cells (52). Analysis of collagen VI reporter mice revealed collagen VI expression by marginal reticular cells and follicular dendritic cells located in PPs but not in other secondary lymphoid organs, thereby indicating the existence of intestine-specific stromal precursors (53). In addition, M cell–inducing stromal cell populations are positive for collagen IV, again supporting the presence of unique stromal cells in mucosa-associated lymphoid organs (19). Interaction between hematopoietic lymphoid tissue inducer cells (LTi) and stromal lymphoid tissue organizer cells (LTo) establishes a microenvironmental milieu that supports the construction of lymphoid structures through integrin- and chemokine-dependent recruitment of lymphocytes (54) (**Figure 2**). In fact, organogenesis of PPs is widely accepted to be mediated through interaction between LTi (or PP inducer cells [PPi]) and stromal LTo cells [reviewed in (55)]. At embryonic day 16.5 in mice, aggregates of PPi cells are present at the site of $VCAM1^+$ LTo cells in gut (55). The LTi in PPs (i.e., PPi cells) are characterized as $CD3^-CD4^+IL\text{-}7R\alpha^+CD44^+CD90^+c\text{-}kit^+$, and they produce lymphotoxin. Importantly, another cell population—which are RET (rearranged during transfection)$^+CD3^-CD4^-IL\text{-}7R\alpha^-CD11c^+$ cells (so-called PP "initiator" cells)—must be present before clustering of and interaction between LTo and PPi cells can occur at the site of PP organogenesis (55–57). RET is an essential tyrosine kinase receptor for generation of the enteric nervous system; deficiency of RET led to an absence of enteric ganglia and subsequent development of Hirschsprung's disease (56, 58). In addition, the RET ligand artemin (also known as enovin or neublastin; a member of the Glial cell-derived neurotrophic factor ligand family) is produced from LTo cells and recruits PP "initiator" to the anlagen protein via the GFRα3–RET receptor complex (56). Importantly, reduction of $CD11c^+$ PP "initiator" cells decreases PP numbers in the CD11c–DTR mouse model, indicating the existence of PP "initiator"-dependent and -independent PP. Whether dependency on $CD11c^+$ PP "initiator" signaling, or the machinery of PP organogenesis differs according to the anatomic location of PP (e.g., duodenum, ileum) needs to be investigated.

LTo cells express various adhesion molecules (e.g., VCAM-1 and ICAM-1) in response to lymphotoxin and its receptor, both of which are expressed by LTo cells [(54); reviewed in (55)]. PP LTo cells are considered to be heterogeneous populations, according to their VCAM-1 and ICAM-1 expression levels, and various chemokines, including CXCL13 and CCL21, are abundantly released from VCAM-$1^{high}$ and ICAM-$1^{high}$ stromal cells [reviewed by (59, 60)]. In addition, LTo cells in PP release CCL19/21 and CXCL13, whereas those in the mediastinal lymph nodes release CCL7/11 and CXCL1 (61). Furthermore, mice lacking transcription factors; Id2 and Rorc, which are involved in the development of LTi cells, fail to organize PP (55).

Taking these findings together, stromal cells are involved in and are necessary for mucosa-associated lymphoid organogenesis. However, the nature and fate of various organizer cells (e.g., LTo cells) have not yet been elucidated and merit further research. The possible heterogeneity of LTo cells particularly ought to be investigated, given that the local tissue microenvironment (e.g., stromal cells) may influence this characteristic.

## STROMAL–IMMUNE INTERACTION FOR STROMAL CELL–MEDIATED PERIPHERAL DIFFERENTIATION AND FUNCTIONAL MODULATION OF THE INTESTINAL MUCOSAL IMMUNE SYSTEM

The seminal influence of stromal cells on the immune system occurs not only in mucosa-associated inductive or organized sites (e.g., gut-associated lymphoid tissue) but also at effector or diffused sites (e.g., intestinal lamina propria). Precise investigations of the immune cells in peripheral tissues revealed the direct interaction between stromal and immune cells (**Figure 1**). In this regard, activated immune cells stimulate fibroblasts to express Acta2; these induced fibroblasts—due to signaling through TGF-β1—subsequently develop into myofibroblasts [reviewed in (62)].

Several cascades of stromal cell-mediated immune regulation have been reported but remain poorly characterized as yet (63). For example, IgA production, which is critically involved in both innate and acquired mucosal protection (6), is regulated through stromal cell–derived retinoic acid and TGF-β1; these two factors directly and indirectly stimulate mucosal B cells and subsequently lead to the development of IgA-producing plasma cells (64). Another direct pathway involves the secretion of APRIL (a proliferation-inducing ligand) and BAFF (B-cell activating factor) from gut stromal cells; these factors then induce T cells and CD40-independent IgA class switching (65). In addition, an indirect pathway has been reported in which stromal cell-derived type I interferon stimulates plasmacytoid dendritic cells to produce APRIL and BAFF and subsequently enhance IgA production (66). Similarly, retinoic acid and GM-CSF from stromal cells condition dendritic cells to enhance the IgA

production pathway (64). Intriguingly, comparison of stromal cell gene expression among tissues reveals that the capability to produce retinoic acid differs among tissues and organs (e.g., colon, small intestine, lung, skin, liver, kidney) (67). Among stromal cells, preferential producers of retinoic acid reside in the small intestine and colon, whereas retinoic acid inactivators or stromal cells that express the enzyme Cyp26b1 occur solely in the cutaneous compartment (67). These data indicate the opposite roles of stromal cells in terms of retinoic acid metabolism in the intestinal mucosa compared with skin (67). Indeed, deficiency of Cyp26b1 or its inhibition by treatment with liarozole induces the expression of retinoic acid–dependent molecules (e.g., P2X7, an ATP receptor) in skin-resident mast cells and T cells, leading to their undesired activation and consequently inflammation (67, 68). Furthermore, whereas ectopic increases in retinoic acid in the cutaneous microenvironment induce chronic inflammation through P2X7 signaling (67), steady-state levels of mucosal P2X7 support the regulation of commensal dysbiosis to maintain homeostasis (69). These findings reveal the absolute requirement of stromal cells for maintenance of tissue-specific homeostasis and their highly variable roles which reflect the local and unique microenvironment of the individual tissue.

In addition to homeostatic regulation through the retinoic acid–dependent pathway, colon stromal cells directly influence T cell activity during inflammation through the programmed death ligand 1 (PD-L1)–programmed cell death protein 1 (PD-1) pathway (70). PD-1–mediated T cell inhibition is an important mechanism for preventing cancer (71, 72). In colon, stromal cells are the predominant PD-L1–expressing cells (73), and co-culture of PD-L1–expressing stromal cells and activated $CD4^+$ T cells reduced the production of IFNγ by this T cell population (74). In addition, the expression of PD-L1 at inflammatory sites was increased during ulcerative colitis but reduced during Crohn's disease; these findings indicate that stromal cell–associated suppression (or downregulation) of Th1 responses is diminished in Crohn's disease (74). Stromal PD-L1 expression is increased through TLR signaling (TLR1, 2, 4, and 5) (74), suggesting that exposure to commensal bacterial components during inflammation enhances anti-inflammatory properties of stromal cells-mediated by PD-L1. Importantly, bacterial stimulation is required for stromal cells to acquire IgA-inductive properties (66), thus germ-free conditions reduce the capability of stromal cells to produce IgA-enhancing cytokines (e.g., BAFF, a B-cell activating factor belonging to the TNF family) (66). Together, these findings indicate that the unique properties of intestinal stromal cells depend on input from the commensal microbiota.

During recent years, the importance of stromal cells for the maturation of immune cells in peripheral sites has emerged (20). For example, the maturation of mast cells (i.e., acquisition of granules) requires direct intercellular communication with stromal cells (20). The interaction of c-kit, expressed on mast cells, and stem cell factor on stromal cells is essential for mast cell maturation (75), and a deficiency in c-kit results in a lack of mast cells (75). In addition, group III phospholipase A2 from mast cells reportedly stimulates stromal cells (e.g., fibroblasts), which then produce prostaglandin D2 and bind to DP1 receptors on mast cells for their further activation (76). These two pathways collectively induce granule formation in mast cells. The contents of granules are regulated by stromal cells as well. Co-culture of mast cells with gut-derived stromal cells induces chondroitin sulfate and proteases (e.g., mast cell proteases 1 and 2) (67), all of which are involved in the clearance of parasites (77). Those properties are not induced through co-culture with skin-derived mast cells, indicating that the terminal differentiation and peripheral education of mast cells are tightly regulated by tissue- or organ-derived stromal cells. In addition to their role in mast cell development, stromal cells support the maturation of type 2 innate lymphoid cells (78). Single-cell RNA-Seq analysis revealed that the development and maturation of type 2 innate lymphoid cells are regulated by both $PDGFR^-$ and $PDGFR^+$ stromal cells through IL-33 and as yet unknown mediators, respectively (78). Furthermore, cooperation between these two stromal cell populations is important to the maintenance of type 2 innate lymphoid cells in the periphery.

Overall, it has become apparent that gut stromal cells act on diverse immune cell populations to support their differentiation and function. Additional studies that explore the crucial roles of the stromal–immune cell axis are essential for understanding how the local microenvironment are regulated. This line of the study will lead to the creation of new research and development platforms for the generation of novel preventive and therapeutic drugs and vaccines that target the stromal–immune cell axis to control inflammation, hypersensitivity, and infection.

## CONCLUSION AND PROSPECTIVES

Due to their multifaceted abilities (e.g., lymphoid genesis, peripheral education), stromal cells are important for maintaining the integrity of the intestinal mucosal barrier. In contrast, during inflammation, stromal cells exacerbate the pathologic conditions in fibrosis and carcinoma [reviewed in (79, 80)]. It is important to elucidate the "functional transition" of each stromal cell population from physiologic to pathologic states in the intestinal mucosa. In addition to those in the gut mucosa, stromal cells in other mucosal tissues, such as lung, have important and unique characteristics; the underlying functional mechanisms are gradually being uncovered and are reflecting the anatomic and functional individuality of these cells. For example, detailed analyses in lung stromal cell populations are revealing the genetic (e.g., $LGR5^+$, $LGR6^+$ populations), anatomic (e.g., bronchiolar, alveolar), and functional (e.g., epithelial differentiation) characteristics of each stromal cell subset (81, 82). However, in gut, even though the functional importance of intestinal stromal cells has gradually been uncovered, the stromal sub-populations are not yet clearly defined. In addition, epithelial–mesenchymal and endothelial–mesenchymal transitions are important pathways that reinforce or compensate for stromal cell populations; these cascades complicate efforts to clarify the fate and origin of stromal cells (83, 84). Further analysis to verify the functional modulations of stromal cells in mucosal health and disease states are required.

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# Type1 Interferons Potential Initiating Factors Linking Skin Wounds With Psoriasis Pathogenesis

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Psoriasis is a chronic autoimmune skin disease that can often be triggered upon skin injury, known as Koebner phenomenon. Type 1 interferons (IFNα and IFNβ), key cytokines that activate autoimmunity during viral infection, have been suggested to play an indispensable role in initiating psoriasis during skin injury. Type 1 IFN-inducible gene signature has been identified as one of the major upregulated gene signatures in psoriatic skin. Type 1 IFNs treatments often directly induce or exacerbate psoriasis, whereas blocking type 1 IFNs signaling pathway in animal models effectively inhibits the development of T cell-mediated skin inflammation and psoriasis-like inflammatory diseases. Epidermal keratinocytes (KCs) occupy the outermost position in the skin and are the first responder to skin injury. Skin injury rapidly induces IFNβ from KCs and IFNα from dermal plasmacytoid dendritic cells (pDCs) through distinct mechanisms. Host antimicrobial peptide LL37 potentiates double-stranded RNA (dsRNA) immune pathways in keratinocytes and single-stranded RNA or DNA pathways in pDCs, leading to production of distinct type 1 IFN genes. IFNβ from KC promotes dendritic cell maturation and the subsequent T cell proliferation, contributing to autoimmune activation during skin injury and psoriasis pathogenesis. Accumulating evidences have indicated an important role of this dsRNA immune pathway in psoriasis pathogenesis. Together, this review describes how skin injury induces type 1 IFNs from skin cells and how this may initiate autoimmune cascades that trigger psoriasis. Targeting keratinocytes or type 1 IFNs in combination with T cell therapy may result in more sustainable effect to treat auto-inflammatory skin diseases such as psoriasis.

**Keywords: type 1 interferons, interferon beta, innate immunity, keratinocytes, inflammation, skin wounds, psoriasis**

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## INTRODUCTION

Skin, the largest organ of human body, functions as a physical and immunological barrier to protect our bodies from external threats. The epidermis, positioned at the front line of defense, has evolved to provide rapid and specific innate immune response that shapes the adaptive immune response, leading to immediate as well as long term protection against physical dangers (1, 2). The barrier function of epidermis is primarily provided by keratinocytes (KCs), the pre-dominant epidermal cell type. While the physical barrier of the skin is maintained by a tightly controlled balance between proliferation and differentiation of KCs (3, 4), the immunological barrier function of epidermis

relies on rapid, precise and situation-specific innate immune responses of KCs to insults. Psoriasis is considered as a T cell-mediated autoimmune skin disease, whereas the role of KCs in initiating the early upstream events in psoriasis has been under-appreciated. In this review, we review current understanding of immunopathology of psoriasis, the role of keratinocytes in psoriasis initiation, and then emphasize on the role of type 1 IFNs in linking innate immune activation upon skin injury and the subsequent autoimmune amplification that leads to psoriasis.

## PSORIASIS: ETIOLOGY, IMMUNOPATHOGENESIS AND THERAPIES

Psoriasis is a chronic autoimmune skin disorder characterized by well-demarcated, raised areas of erythematous plaques, often covered by silvery scaling (5). It is estimated that psoriasis affects 125 million people worldwide (∼1.7% of the world population), including 2∼4% of the US or European populations and ∼0.5% of the Asians (6). The three principal histological features of psoriasis are hyperplastic/thickened epidermis, elongated and increased vascularity in the dermis, and inflammatory leukocyte infiltration (5). Beyond the lesions, psoriasis is also associated with several comorbidities, such as rheumatoid arthritis, atherosclerosis, cardiovascular diseases, obesity, type 2 diabetics, Alzheimer's disease, depression, and non-melanoma skin cancer (7, 8). Increased disease burden greatly impairs the quality of life in psoriasis patients. A better understanding of psoriasis pathophysiology will help to decipher the molecular alliance of psoriasis with its comorbidities.

The etiology of psoriasis remains obscure. As shown in **Figure 1**, besides genetic factors, several triggering factors have been linked with an exacerbation of psoriasis, such as infection, wound, obesity, stress, and drugs including beta-blockers, lithium, interferons, and imiquimod (9–11). Innate immune responses of resident keratinocytes or infiltrated plasmacytoid dendritic cells (pDCs) are believed to play a critical role in initiating the subsequent adaptive immune events including dendritic cell (DC) maturation and T cell activation (2, 12, 13). It has been shown that keratinocyte or pDCs derived cytokines, such as IL1β and type 1 IFNs, activate DC, which then stay local or travel to lymph nodes and secrete cytokines TNFα, interleukin (IL)-12 and IL-23, leading to type 1 T helper (Th1), and type 17 T helper (Th17) cell activation, respectively (2, 14). Activated T cells accumulate at the affected skin area, releasing additional cytokines, such as IFNγ, TNFα, IL-22, and IL-17A (15). These T cell derived cytokines recruit additional immune cells and boost keratinocyte activation, leading to the initiation of a self-propelled cycle of auto-inflammation and the ultimately uncontrolled keratinocyte hyperproliferation and psoriatic plaque formation (16, 17).

Preventing immune activation is the key to treat psoriasis. Conventional psoriasis therapies, including general immunosuppressive therapies (such as topical corticosteroids and systemic methotrexate or cyclosporin therapies), vitamin D analogs, topical retinoids, and UVB phototherapy, are associated with broadband immunosuppression and/or organ toxicities that can be problematic when used long term (18). New biological drugs against specific immunological elements have gained popularity as safe and effective alternatives to treat moderate to severe plaque psoriasis. These biological drugs including monoclonal antibodies against TNFα (such as etanercept and infliximab), IL12 (such as Ustekinumab), IL23 (such as Guselkumab and Tildrakizumab), IL-17A (such as Secukinumab and Ixekizumab), or IL17AR (such as Brodalumab) have shown clinical efficacy in improving skin conditions in clinical trials and most of these drug are already on the market (9, 19–21). However, relapse of the disease shortly after drug withdrawal is one of the major obstacles for these DC or T-cell targeted therapies in clinical trials (22–25), suggesting that preventing adaptive immune activation alone is not sufficient to treat psoriasis. A better understanding of the innate immune mechanisms initiating psoriasis is urgently needed to develop novel therapeutic approach to treat psoriasis.

## KOEBNER PHENOMENON, FROM SKIN WOUND TO PSORIASIS

The initial onset of psoriasis is often followed by chronic relapses of the disease triggered by wounding, infections and mechanical stress (5). In 1876, Hinrich Koebner, MD, first described the development of psoriatic lesion after physical trauma such as tattoos, horse bites, and wounds. Later, "Koebner phenomenon" has been used to describe the formation of isomorphic lesions on healthy skin following a cutaneous trauma (e.g., wounds, burns, or surgical incisions) and it is now not restricted to psoriasis but applies to many other skin conditions, such as lichen planus, vitiligo, and lupus erythematosus. Dermal pDCs, a rare population of circulating cells specialized in the production of type 1 IFNs, is thought to be one of the earliest events in psoriasis pathogenesis and subsequently primes the innate and adaptive immune system (12, 26). While dermal pDC activation in wounded skin may partially explain Koebner phenomenon, it is still unclear why even superficial tattoos can trigger the pathogenesis of psoriasis. An epidermal mechanism is likely to play a role in the development of a Koebner reaction in psoriatic patient.

## KERATINOCYTES UNDER FIRE OF PROINFLAMMATORY CYTOKINES

Keratinocytes, constituting ∼90% of the epidermal cells, are poised directly at the interface with the external environment, depositing them as the first responder to skin injury. Recent studies have established the essential role of keratinocytes in psoriasis initiation. Rapid innate immune responses of keratinocytes, to a variety of external stimuli, leads to production of an array of pro-inflammatory

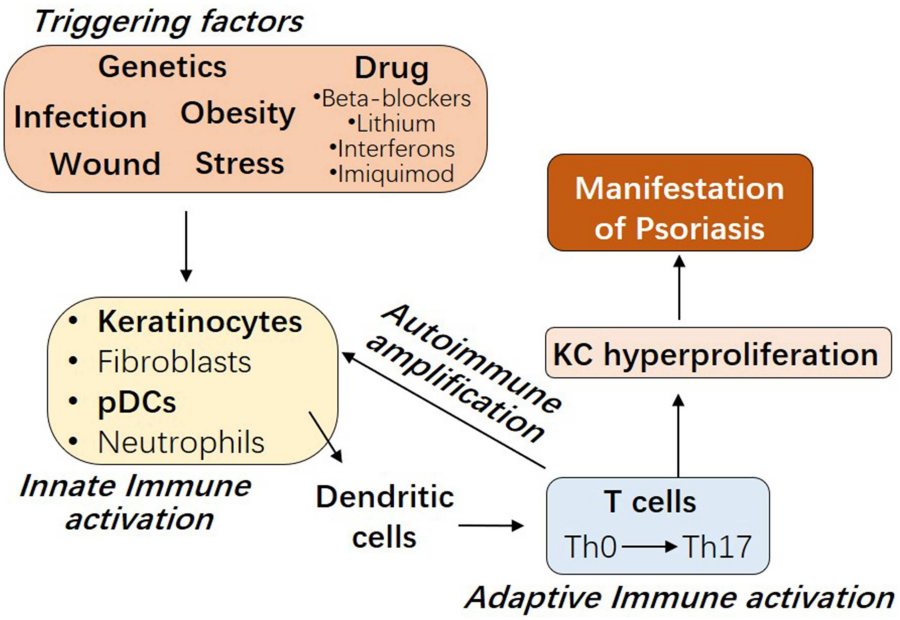


**FIGURE 1 |** The interplay between innate and adaptive immune cells in the pathogenesis of Psoriasis. Psoriasis can be triggered by several factors, including genetic variants, infection, wound, obesity, stress, and drugs. Early upstream events in psoriasis involve the innate immune activation of skin resident keratinocytes or fibroblasts or recruited plasmacytoid dendritic cells (pDCs) or neutrophils. Cytokines derived from these innate immune cells promote myeloid dendritic cell maturation, with consequent Th17 T cell development and the beginning of the adaptive immune phase. T cell infiltrate promotes inflammatory amplification of innate immune cells, leading to the formation of an autoimmune self-amplifying loop that drives pathogenic hyperproliferation of keratinocytes and manifestations of psoriasis.

cytokines or chemokines such as IFNβ, IL1β, IL36, TNF, IL6, IL8, IL25, and CXCL10 (2, 27–29). These keratinocyte-derived cytokines prime epidermal innate immune signals with dermal adaptive immune system, contributing to autoimmune activation and psoriasis pathogenesis (2, 3, 27, 28, 30, 31).

The inflammatory T cell phenotype of psoriasis can be initiated by altering innate immune system of keratinocytes in mice. For example, epidermal specific deletion of IKK2 (inhibitor of nuclear factor κB) (32) or c-JUN (33) or epidermal specific overexpression of Tie2 (34) or IL17C (35) or the active form of STAT3 (36) or IL25 (29) lead to spontaneous keratinocyte activation and cytokine release followed by the development of psoriasis-like skin inflammation. Recent study from Kabashima's group shows that conditional deletion of TRAF6 in keratinocytes abrogates DC activation, IL-23 production, and the subsequent IL-17 mediated psoriatic inflammation in the imiquimod psoriasis mouse model (37). Our group have found that induction of IFNβ from KC is one of the earliest innate immune events during skin injury (2). Keratinocyte-derived IFNβ promotes dendritic cell maturation and the subsequent T cell proliferation, leading to psoriatic inflammation development (2). These studies suggest that innate immune responses of keratinocytes are essential to initiate the autoimmune cascade and drive psoriasis pathogenesis, and type 1 IFNs may function as an early initiating factor linking skin wounds with adaptive immune activation that drives psoriasis.

## TYPE 1 IFNS AND AUTOIMMUNE DISEASES

Type 1 interferons (IFNs) belong to the class II family of cytokines, which is composed of 16 members, including 13 IFNα subtypes, IFNβ, IFNε, IFNκ, and IFNω (38). Among these type 1 IFNs, IFNα, and IFNβ are the most extensively studied. Type1 IFNs were first discovered more than 60 years ago as the key factors induced upon viral infection, owing to their ability to limit viral replication and promote immune activation (39–41). And now type 1 IFNs have been recognized as the central cytokines that link innate immunity with autoimmune activation during pathogenesis of several systemic autoimmune diseases and several organ-targeted inflammatory diseases (42, 43). Systemic lupus erythematosus (SLE) is the most well-studied autoimmune disease driven by type1 IFNs (42), and recent studies have also demonstrated pathogenic role of type 1 IFN in psoriasis, rheumatoid arthritis, diabetes mellitus, Sjogrens syndrome, dermatomyositis (DM), and systemic sclerosis (38, 44–46). SLE and psoriasis share many similar clinical features and can both be triggered by infection, wounding or type1 IFNs, and Th17 T cells contribute to pathogenesis of both diseases. However, SLE is characterized by overproduction of a wide array of autoantibodies and is therefore traditionally classified as a "B-cell disease" (47), whereas B cell contribution to psoriasis remains unclear. Distinct susceptible genes have been identified for SLE and psoriasis (6, 48), suggesting that genetic factors may contribute differential adaptive immune development in

response to type 1 IFNs, leading to distinct disease manifestation in SLE and psoriasis.

## TYPE 1 IFNS AND PSORIASIS PATHOGENESIS

Type 1 IFNs are associated with psoriasis. Psoriasis is often triggered by chronic viral infections or wounding, and type 1 IFNs as the key cytokines induced upon these conditions (49, 50). Clinically, type 1 IFNs treatments in patients with viral infection or multiple sclerosis (MS) often directly induce or exacerbate psoriasis or psoriasis arthritis (50–53). Furthermore, transcriptome analyses have identified type 1 IFN pathway genes as one of the top upregulated gene signatures in psoriatic skin, outscoring the upregulation of TNFα pathway gene signature (54–56). Immunostaining analyses have shown that while IFNα is pre-dominantly produced by dermal infiltrated pDCs, IFNβ expression is rapidly induced in epidermal keratinocytes as early as 1 day post-wounding and in psoriatic epidermis compared to normal human skin (2, 12, 13).

In line with these clinical observations, type 1 IFN pathway is also necessary for the development of T cell-mediated skin inflammation and psoriasis-like inflammatory diseases in mice. Mice treated with IFNα or IFNβ neutralizing antibodies, or mice lacking IFNAR (receptor for IFNα/β) failed to develop Th17 cell-mediated skin inflammation (12, 13). Fuchs's group shows that UV alleviates the imiquimod (IMQ)-induced psoriatic inflammation by downregulating IFNAR1 expression in keratinocytes (57). IMQ-induced psoriatic inflammation was blocked in *Ifnar* deficient ($Ifnar^{-/-}$) mice, and in contrast $Ifnar^{SA}$ mice (in which *Ifnar* ubiquitination and degradation was blocked) exhibited exacerbated inflammatory response to IMQ compared to wildtype controls (57), demonstrating that IFNAR plays a critical role in promoting the development of psoriasis like inflammation in mice. In addition, mice deficient for interferon regulatory factor 2 (*Irf 2*), the transcriptional attenuator of IFNα/β signaling, developed spontaneous psoriasis-like inflammatory disease (58). Type 1 IFNs upregulates the expression of IL22 receptor in keratinocytes, leading to an increase in keratinocytes' responsiveness to IL22, which drives Stat3 phosphorylation and keratinocyte hyperproliferation (15). Our group has also shown that IFNβ from activated human or mouse keratinocytes can directly promote cDC (conventional dendritic cell) maturation and the subsequent T cell proliferation *in vitro* (2). These collective evidences support that type 1 IFNs play an essential role in initiating skin inflammation during psoriasis pathogenesis.

In addition to its proinflammatory role, type 1 IFNs may also regulate keratinocyte differentiation. In both normal and psoriatic skin epidermis, IFNβ expression is restricted to KCs at the differentiated cell layers (2, 59, 60), and IFNβ is expressed by growth arrested or differentiated KCs but not by dividing KCs *in vitro* (59). Neutralizing IFNβ in culture medium inhibited differentiation, but addition of exogenous IFNβ did not stimulate differentiation or alter proliferation (59). Future studies are still needed to determine whether IFNβ expression in differentiated KCs is a consequence or the cause of KC cell arrest and/or differentiation, and to determine whether IFNβ contributes to the aberrant proliferation and differentiation in psoriasis.

## DISTINCT CELLULAR SOURCE OF IFNα AND IFNβ IN PSORIASIS

IFNα and IFNβ are produced by distinct cellular sources in wounded and/or psoriatic skin. Upon skin injury, pDCs rapidly infiltrate the skin, sense nucleic acid released from damaged cells, then produce large quantity of IFNα, which then initiates the autoimmune cascade (12, 13) (**Figure 2**). Activation of pDC precedes cDC or T cells activation (12), suggesting IFNα from pDC may play a role during early phase of disease progression. Our group has shown that while IFNα is primarily produced by pDC in the dermis, IFNβ is pre-dominantly produced by epidermal keratinocytes in skin wounds or psoriasis (2). Direct comparison of *in vitro* activated KCs and pDCs revealed that while KCs lack the ability to produce IFNα upon activation, activated KCs produce higher amount of IFNβ compared to activated pDCs (2). Secretion of IFNβ from keratinocytes promotes activation and maturation of classical dendritic cells, leading to the subsequent T cell proliferation and autoimmune amplification (2). Furthermore, keratinocyte-derived IFNβ can also promote pDC maturation and activation (2), suggesting that keratinocytes might also contribute to pDC activation during early phase of skin injury. Together, these findings suggest that KCs are an active source of IFNβ and can participate with pDCs to prime the adaptive immune system during psoriasis pathogenesis (**Figure 2**).

## REGULATION OF TYPE1 IFNS EXPRESSION BY PATTERN RECOGNITION RECEPTORS

Type 1 IFNs are known to be induced by a variety of DAMPs (damage associated molecular patterns) or PAMPs (pathogen associated molecular patterns) in either through Toll-like receptor (TLR)-dependent or TLR-independent pathways (38, 61) in a cell type specific manner. Type1 IFNs can be induced upon activation of endosomal TLR7 and 9 or cytosolic cGAS-STING (cyclic GMP-AMP synthase-stimulator of interferon genes) by host or viral or bacterial DNA, endosomal TLR8 by ssRNA, endosomal TLR3 or mitochondrial RIG1 (retinoic acid-inducible gene 1) -MAVS (mitochondrial antiviral-signaling protein) by host or viral dsRNA, or plasma membrane TLR4 by bacterial LPS (38, 61). The cell responsiveness to various DAMPs or PAMPs relies on the expression of pattern recognition receptors (PRRs). pDCs express high levels of TLR7 and TLR9, therefore pDC can rapidly sense self-DNA released upon injury then produce IFNα (13, 26, 39). While TLR4 and TLR8 are usually not expressed in pDC, these PRRs are highly expressed in classical DC or monocytes (62), making these cells highly responsive to bacterial LPS or self-RNA. In contrast to these myeloid derived immune cells, keratinocytes express high levels of TLR3 and MAVS, but not TLR4, 7, 8, or 9 (2, 30, 31).

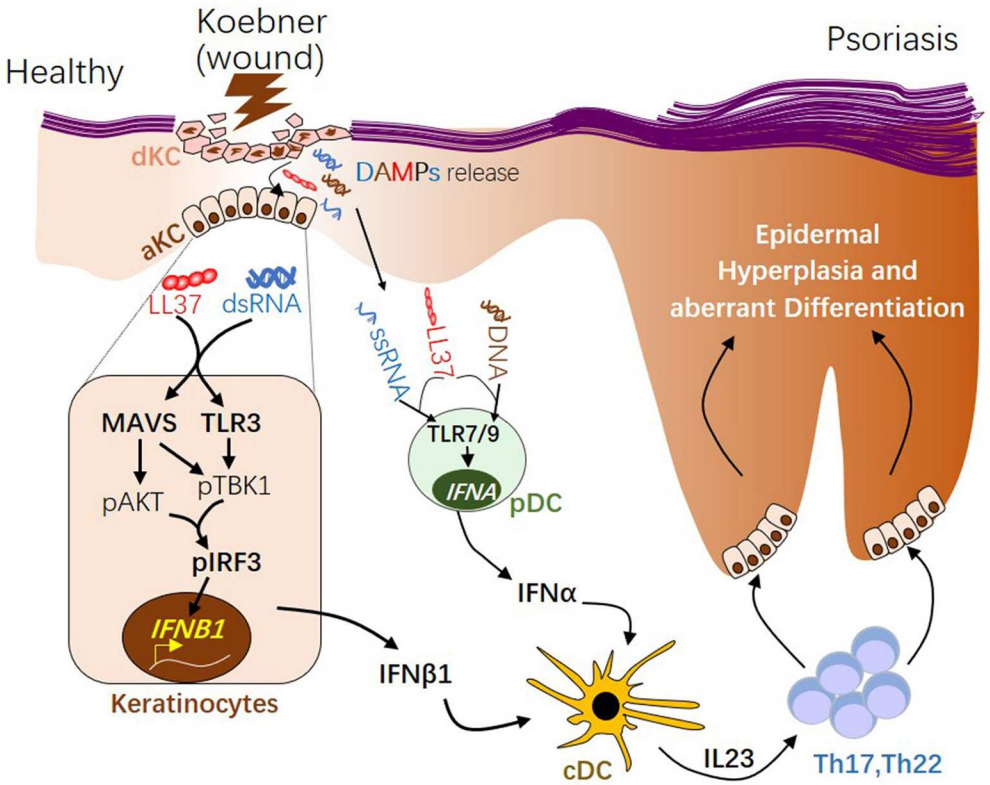


**FIGURE 2 |** The role of type 1 interferons in initiating psoriatic inflammation during skin injury. During skin injury (known as Koebner phenomenon), damaged keratinocytes (dKC) release self-nucleic acids, including dsRNA, ssRNA, and DNA. Antimicrobial peptide LL37, which is transiently induced in KC upon wounding enables dsRNA recognition by MAVS and TLR3 in KC, leading to activation of the pAKT-pTBK1-pIRF3 signaling cascade and the subsequent transcription initiation of the *IFNB1* gene from activated KCs (aKC). LL37 can also enables ssRNA or DNA recognition by TLR7 or 9 in pDCs, leading to transcription of *IFNA* family genes. Type 1 IFNs, including IFNβ from KC and IFNα from pDCs, promote maturation of conventional DCs (cDCs). Activated DCs produce IL23, promoting the development of Th17 and Th22 autoimmune T cells, which in turn act on keratinocytes, leading to epidermal hyperplasia and psoriasis pathogenesis. dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MAVS, Mitochondrial Antiviral Signaling Protein; TBK1, TANK-Binding Kinase 1; IRF3, interferon regulatory factor 3.

Therefore, keratinocytes rapidly produce IFNβ in response to dsRNA but not to TLR4, 7, 8, or 9 ligands (2). We have showed that, wounded keratinocytes upregulate the expression of antimicrobial peptide LL37, which then enables MAVS and TLR3 in keratinocytes to recognize dsRNA released from dying cells (2). By MAVS-dependent activation of TBK1 (TANK-Binding Kinase 1)-AKT (AKT serine/threonine kinase 1)-IRF3 (interferon regulatory factor 3) signaling pathway, keratinocytes produce and secrete IFNβ (2). Keratinocyte-derived IFNβ then promotes DC maturation and the subsequent T cell activation to facilitate the development of an autoimmune cutaneous inflammatory response (2). These results show that the cell type specific expression of pattern recognition receptors shape the unique and situation specific innate immune response of these cells.

## ROLE OF DSRNA SIGNALING IN PSORIASIS PATHOGENESIS

Recent studies have suggested an important role for dsRNA in autoimmune initiation during psoriasis pathogenesis. Extracellular RNA complexes have been found in psoriatic skin (62), and dsRNA is also detected in the cytosol of wounded or psoriatic KCs while it is normally localized in the nucleus of KCs in normal skin (2). Recently, it has been shown that the dsRNA accumulation in psoriatic keratinocytes is associated with impaired A-to-I RNA editing activity, which is essential for post-translational modification of dsRNA and unwinding of dsRNA structures (63). In line with these observations, functional analysis of the psoriasis susceptible gene implicates the involvement of innate immune responses to dsRNA in disease progression (64).

Accumulating evidences have demonstrated that dsRNA can be released as a DAMP by damaged cells or a PAMP by invading viruses to initiate host immune activation. We and others have shown that dsRNA released by tissue damage activates and TLR3 and it is required for normal inflammation or skin regeneration (30, 65). Viral dsRNA or endogenous dsRNA (such as U1 RNA) released upon injury acts on keratinocytes through the mitochondrial MAVS pathway to produce IFNβ, leading to adaptive immune activation (2, 31, 66). Skin injury or infection strongly induces the expression of cathelicidin antimicrobial peptide expression in KCs (2, 67), and LL37 (the α-helical

polypeptide derived from cathelicidin) forms pro-inflammatory nanocrystalline complex with dsRNA that potentiates pattern recognition receptor clustering and immune amplification (2, 68). Together these recent evidences have shown that dsRNA released upon infection or injury may synergize with the host antimicrobial peptide to initiate the autoimmune activation in psoriasis, and this dsRNA immune response in keratinocytes may explain the Koebner phenomenon and why viral infection and wounding triggers psoriasis.

## TARGETED THERAPIES FOR PSORIASIS: CURRENT STATUS AND FUTURE DEVELOPMENTS

Psoriasis is not just a T cell mediated disease. New biological drugs targeting the TNF/IL-23/IL-17 pathways have shown to be safe and efficacious in recent psoriasis clinical trials (9, 19, 20). However, potential problems including lack of long term efficacy and rapid regain of psoriasis upon drug removal (22, 23, 25) suggest that inhibiting T cell activation is only effective to alleviate the disease symptoms but it cannot cure the disease. If the initiating factors such as type 1 IFNs are still active, and these factors can quickly re-initiate the inflammatory cascade and reactivate pathogenic T cells upon withdrawal of the T cell targeted therapy. In addition, studies has suggested that cytokines including type 1 IFNs, TNF, and IL17, are interwoven, and each of these cytokines is the cornerstones of an inflammatory triangle that drives the development and maintenance of psoriasis (69). Targeting one of these cytokines may affect the others. For example, patents treated with TNF blocking agents sometimes develop paradoxical psoriasis and this is resulted from an overproduction of IFNα from pDCs upon TNF inhibition (70–72). Together, these clinical observations indicate that inhibiting activation of the innate immune system of KCs or pDCs that initiates the autoimmune cascade may be needed in addition to targeted T cell therapy to prevent reoccurrence of the disease upon drug withdrawal. Targeting MAVS orTLR3 in KC to prevent IFNβ production from the skin epidermis is of great potential for future targeted therapy development to treat psoriasis.

## CONCLUSION

Located at the skin surface, keratinocytes are constantly exposed to a variety of stimuli, and can secrete an array of cytokines, which function as secondary messengers connecting the innate immune with adaptive immune system. Type 1 IFNs, which are the essential mediator of autoimmunity and antivirus host defense, have emerged as an important initiating element in the immunopathology of autoimmune cascade and psoriasis. In contrast to dermal pDC which produce IFNα in response to ssRNA or DNA, keratinocytes express high levels of MAVS and TLR3 and therefore can sense self-dsRNA in the presence of antimicrobial peptide and to produce IFNβ. Production of Type 1 IFNs from these innate immune cells upon skin injury may explain the Koebner phenomenon, and this may also explain psoriasis triggered by other factors such as bacterial or viral infections as RNA or DNA released from pathogens will activate similar innate immune response. Future studies will be needed to develop targeted therapy to block the innate immune activation of these PRRs in keratinocytes, which may result in more sustainable interventions to treat psoriasis in addition to current T cell targeted therapies.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# An Update on the Role of Adipose Tissues in Psoriasis

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Psoriasis is a common chronic inflammatory skin disease that is increasingly being recognized as a disease that not only affects the skin but also has multi-systemic implications. The pathophysiological link between psoriasis and obesity is becoming increasingly elucidated by recent studies. The cross-talk between adipocytes and the immune system via various mediators such as adipokines could explain how obesity contributes to psoriasis. The effects of obesity on adipocytes include upregulation of pro-inflammatory adipokines such as leptin and resistin, downregulation of anti-inflammatory adipokine, and also the stimulation of pro-inflammatory cytokine production by macrophages. This article provides an update on the role of adipose tissues in psoriasis.

**Keywords: obesity, psoriasis, adipose tissue—obesity, adipokine cytokines, adipocyte, macrophage—cell, leptin**

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## INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease with an estimated worldwide prevalence of 0.5–11.4% in adults, and 0–1.4% in children (1). It is a hyperproliferative skin disorder with a complex immune-mediated etiology involving an interplay amongst T lymphocytes, dendritic cells, and keratinocytes via various cytokines (2–4). Scientific advancements over the past decade have empowered us with a greater understanding of the genetics, pathophysiology, co-morbidities, and treatment of psoriasis. Psoriasis is increasingly being recognized as a disease that not only affects the skin but also has multi-systemic implications. One of the comorbidities associated with psoriasis that has been rigorously studied in recent years is obesity (5). Typically defined as a body mass index (BMI) $\geq$30 kg/m$^2$, obesity has been described as an escalating global epidemic and a serious public health concern, especially in developed nations (6). Obesity has been well-known to cause significant excess in mortality and morbidity, being associated with a myriad of obesity-related complications. Obesity increases the risks of type 2 diabetes and cardiovascular diseases. Interestingly, obesity has also been found to be an independent risk factor for the development of psoriasis (7). The pathophysiological link between psoriasis and obesity is becoming increasingly elucidated. There have been mounting evidence to show that both psoriasis and obesity represent a pro-inflammatory state, and that immunological mechanisms in both conditions have significant overlap. Contrary to prior belief that the adipose tissue plays a role only in energy storage, it is in fact a large endocrine and secretory organ that produces a multitude of pro-inflammatory cytokines and adipokines, resulting in various downstream effects (8). Adipocytes are also known to regulate inflammation even locally in the skin (9). In this mini review article, we aim to shed some light on the role of adipose tissues in psoriasis.

## EPIDEMIOLOGY ON THE RELATIONSHIP BETWEEN OBESITY AND PSORIASIS

The association between obesity and psoriasis was consolidated by a systematic review with meta-analysis of observational studies between 1980 and 2012 (5). A total of 16 observational studies with 201,831 psoriasis patients were included in this meta-analysis. Compared with the general population, psoriasis patients have higher prevalence and incidence of obesity. The pooled odds ratio (OR) for obesity among patients with psoriasis was 1.66 (95% confidence interval (CI) 1.46–1.89) compared with those without psoriasis. Another more recent systematic review confirmed that different adiposity measures such as BMI, waist circumference, waist-to-hip ratio, and weight gain positively correlated with increased risk of psoriasis (10). The summary of relative risk (RR) for a 5-unit increment in BMI was 1.19 (95% CI 1.10–1.28). The summary of RR was 1.24 (95% CI 1.17–1.31) per 10 cm increase in waist circumference, 1.37 (95% CI 1.23–1.53) per 0.1 unit increase in waist-to-hip ratio, and 1.11 (95% CI 1.07–1.16) per 5 kg of weight gain.

Studies have also shown that there is a strong correlation between severity of psoriasis and obesity (11). Patients suffering from severe psoriasis were found to have greater odds of obesity than those with mild psoriasis. A population-based study conducted in the United Kingdom showed that there was a "dose-dependent" relationship between disease severity and obesity. Among the study population, those with mild, moderate, and severe psoriasis (based on body surface area involved) had the prevalence of obesity compared with controls increased by 14, 34, and 66%, respectively (12).

Obese children are also at higher risk of developing psoriasis. In an international cross-sectional study of 409 children with psoriasis, children with psoriasis were significantly more likely to be obese than controls (OR 4.29, 95% CI 1.96–9.39) (13). In a retrospective cohort study over a 10-year period, nearly 30,000 children with psoriasis were compared with an age-, sex-, and race-matched comparator cohort without psoriasis (14). In this study, it was found that children with psoriasis had higher rates of not just obesity, but also other components of metabolic syndrome such as hyperlipidemia, hypertriglyceridemia, hypertension, and diabetes compared to children who did not have psoriasis.

Initially, the higher prevalence of obesity in psoriasis patients was thought to be solely due to the negative psychosocial aspects of having psoriasis. Psoriasis patients compared to patients without psoriasis appear to have higher rates of unhealthy behavior such as overeating, sedentary lifestyle and smoking (15). However, over the years, it has been increasingly recognized that weight gain and increased adiposity may increase the risk of developing psoriasis. In the Nurses' Health Study II, the multivariate relative risk for developing psoriasis in obese women with a BMI $\geq$30 kg/m$^2$ was 1.73 (95% CI, 1.24–2.41) compared to only 0.76 (95% CI, 0.65–0.90) for women with a BMI $<$21 kg/m$^2$ (7). To support the notion that obesity plays a role in development of psoriasis, a retrospective study on the pediatric population showed that being overweight or obese preceded psoriasis by at least 2 years in 93% of children with psoriasis (16).

Studies have also shown that weight loss in patients suffering from psoriasis resulted in improved severity of disease. Case reports of patients with severe psoriasis who achieved remission after successful weight loss by post-bariatric surgery alluded to the fact that weight loss could potentially be an adjunctive treatment for psoriasis (17, 18). This was supported by a later retrospective study of 34 psoriasis patients who underwent weight loss surgery, which showed that almost two-third of them experienced improvement in the severity of psoriasis after surgery (19). Moreover, weight loss seemed to have an impact on the response to treatment with systemic therapies. A randomized controlled trial of 61 patients with moderate-to-severe psoriasis who are also obese showed that there was a better treatment response to cyclosporine when it was combined with a low-calorie diet when compared with cyclosporine alone (20). It has also been shown in a large multi-center longitudinal study that in psoriasis patients on biologic therapies, higher weight was associated with reduced odds of achieving $\geq$90% improvement in Psoriasis Area and Severity Index (PASI 90) at 6 months post-therapy (21).

## ADIPOCYTES IN THE SKIN AND THEIR ROLE IN IMMUNE RESPONSES

In humans, the subcutaneous tissue forms an uninterrupted layer throughout the body with exceptions of the hands and feet, accounting for a significant 8–18% of body weight in males and for 14–28% in females, and even more in obese adults (22). The largest adipose depots are abdominal white adipose tissue. However, studies have suggested that adipose tissues also exist within the skin dermis (**Figure 1**) (23). In humans, it was found that there are two histologically, anatomically and metabolically distinct layers of adipose tissues beneath the reticular dermis. The nomenclature of this layer of adipose tissues between the reticular dermis and deep layers of subcutaneous tissues still remains contentious. Some groups describe it as the "superficial subcutaneous adipose tissue" whilst others are calling it "dermal white adipose tissue" (24). No matter what the nomenclature is, these adipocytes mostly reside around skin appendages, especially around hair follicles (25). Apart from mature adipocytes, adipose tissues also consist of a variety of other cell types, collectively termed the stromal vascular fraction (SVF). These include mesenchymal stem cells, vascular endothelial cells, nerve cells, macrophages, T-cells, and B-cells.

Obesity is a state of increased adiposity, resulting in changes in cell composition in adipose tissues. It is associated with increased numbers of macrophages in the SVF of both visceral and subcutaneous adipose tissues. This is supported by mouse studies which showed that that macrophages accounted for approximately 40% of the SVF in obese rodents, compared to only 10% in lean littermates. Macrophage-related genes are also found to be upregulated in these obese animals (26). Recruitment

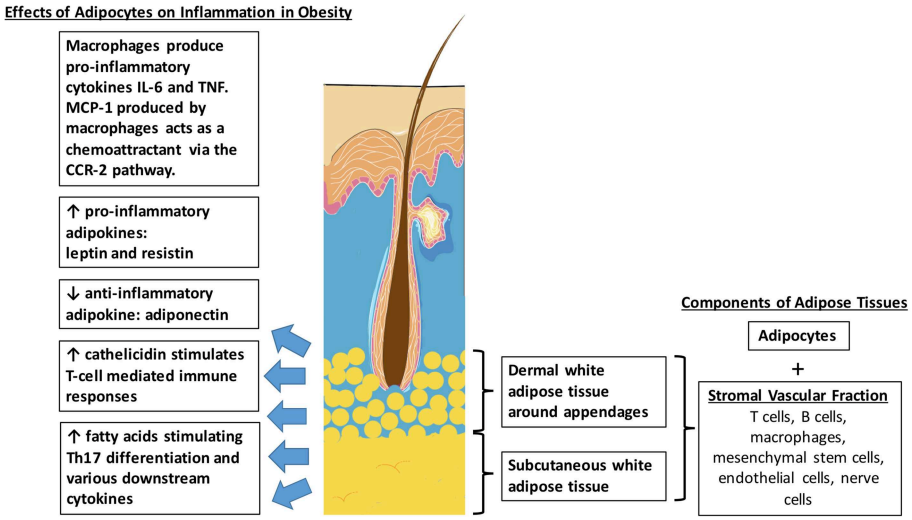


**FIGURE 1 |** Distribution of intradermal and subcutaneous white adipose tissue. Effects of adipocytes in obesity which potentially contributes to the pathophysiological link between obesity and psoriasis.

of macrophages into adipose tissues is an early event in obesity-induced adipose inflammation. The monocyte chemoattractant protein-1 (MCP-1), one of the main chemoattractants for macrophages via the C-C chemokine receptor 2 (CCR2) pathway, is secreted primarily by macrophages, vascular endothelial cells and also adipocytes (27). Adipose tissue macrophages express CCR2 and recruit additional monocytes and macrophages, promoting a feed-forward process. Activated macrophages then produce inflammatory cytokines such as IL (interleukin)-6 and TNF (tumor necrosis factor) which are known to exacerbate the symptoms of psoriasis as well (28).

Intradermal adipocytes also release high levels of antimicrobial peptides such as cathelicidin during early adipogenesis (29). Cathelicidin is found to be increased in lesional skin of psoriasis and plays an active role in inflammation. They form complexes with human self-DNA and in turn activate dermal plasmacytoid dendritic cells via toll-like receptor 9 (TLR9) to produce interferon-α (IFN-α) to stimulate T-cell-mediated immune responses (30). Serum cathelicidin protein levels were found to be significantly increased in obese, non-diabetic and pre-diabetic patients, compared with non-obese and non-diabetic patients (31). Therefore, the increased production of cathelicidin by intradermal adipocytes in obese patients could also contribute to the pathophysiology of psoriasis.

Another pathway in which adipocytes mediate immune responses is via lymphocytes. It has been shown that white adipose tissue acts as a reservoir for memory T-cells, a major component of the adaptive immune system (32). Lymphoid clusters were also found to be present in both human and mouse mesentery. Cells in these clusters proliferate in response to IL-2 and produce large amounts of cytokines such as IL-5, IL-6 and IL-13, modulating inflammation (33). These lymphocytes found in adipocytes can thus represent another factor that triggers the inflammatory response in psoriasis.

## ADIPOKINES AS A PATHOPHYSIOLOGICAL LINK BETWEEN PSORIASIS AND OBESITY

Besides serving the role as an effective lipid storage organ and a structure for insulation and mechanical support, it has been recognized that adipose tissues also act as an active secretory endocrine organ. Adipokines are a collective term used to describe bioactive proteins produced by adipose tissues which regulate various metabolic functions including lipid and glucose metabolism, inflammation, vascular homeostasis, and coagulation (34). Obesity leads to an overproduction of pro-inflammatory adipokines and the simultaneous reduction in the production of adipokines with anti-inflammatory properties. This creates a dysregulation in the function of adipokines, which influences local and systemic inflammation (35). In obesity, there is an expansion of white adipose tissue which is the primary site that produces adipokines such as leptin, resistin, and adiponectin. These represent the three most studied adipokines. Leptin and resistin are pro-inflammatory adipokines, whilst adiponectin has anti-inflammatory effects via inhibition of TNF-α (36) (**Figure 2**).

First discovered in 1994, leptin is the first adipokine identified and serves as a satiety factor, regulating food intake and energy expenditure, thus coordinating changes in energy balance and whole body nutritional status (37). Leptin is a product of obese gene and is produced mainly by adipocytes. It acts on leptin receptors and exerts a multitude of effects. Apart from its primary role in regulating satiety, leptin plays a part in bone metabolism and immune functions (38). Large adipocytes produce more leptin than small ones, and it is well-established that serum leptin concentrations are strongly correlated with overall body fat content (39). Leptin is not only increased in obesity, but also found to be increased in patients with psoriasis,

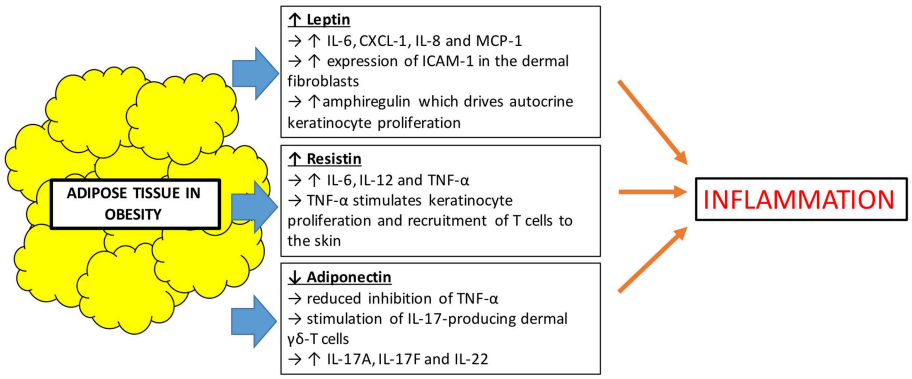


**FIGURE 2 |** Effects of adipokines in obesity leading to inflammation.

positively correlating with increasing severity of disease (40, 41). Understanding leptin's immunomodulatory function may help to explain its link to psoriasis. Leptin induces the production of IL-6, chemokine (C-X-C motif) ligand-1 (CXCL-1), IL-8, and monocyte chemoattractant protein-1 (MCP-1) and also the increased expression of intercellular adhesion molecule-1 (ICAM-1) in the dermal fibroblasts (42). IL-6, CXCL-1, and IL-8 may contribute to the hyperproliferative state of the epidermis in psoriatic skin. It has also been postulated that leptin induces psoriasis skin to produce amphiregulin, which is known to drive autocrine keratinocyte proliferation in culture (43). Although there has been contradicting studies showing leptin levels that are both increased or decreased in patients with psoriasis, a recent meta-analysis of 26 studies concluded that patients with psoriasis had higher leptin concentrations compared to the control population (44).

Resistin is an adipose-derived cysteine rich adipokine which not only helps in the regulation of glucose metabolism, but also has a role in inflammation. Like leptin, resistin is positively correlated with obesity. Resistin influences inflammation by inducing the release of pro-inflammatory cytokines (45). Studies have shown that resistin was also found to be present in higher concentrations in patients with psoriasis compared to control population. At the same time, there were also suggestions that increased levels of resistin were associated with increased severity of psoriasis (46). However, published studies on the association of resistin and psoriasis also had contradictory results. A recent meta-analysis attempted to demystify these inconsistencies and showed that indeed, higher serum concentrations of resistin level positively correlated with psoriasis disease progression (47). Resistin plays a pro-inflammatory role by stimulating the production of cytokines such as IL-6, IL-12, and TNF-α through the nuclear factor-kB signal pathway in human macrophages and peripheral mononuclear cells (48, 49). TNF-α is known to be able to stimulate keratinocyte proliferation and also recruitment of T-cells to the skin, therefore propagating the inflammatory pathway of psoriasis.

On the other hand, adiponectin is an anti-inflammatory adipokine which is secreted exclusively by adipose tissues. It enhances lipid metabolism by increasing lipid clearance from plasma and also helps in improving glycemic control (50). Unlike leptin and resistin, adiponectin is decreased in obese individuals compared to lean individuals (51). It is also found to be decreased in psoriasis patients when compared with control populations (44, 52). It has been shown that adiponectin has anti-inflammatory properties in keratinocytes *in vitro*, resulting from the inhibition of TNF-α. It is shown in a mouse study that lack of adiponectin exacerbates psoriasis-like skin inflammation with excessive infiltration of IL-17-producing dermal γδ-T cells (53). The inflamed skin of these adiponectin-deficient mice also expressed upregulation of Th17-related cytokines, IL-17A, IL-17F, and IL-22. Since IL-17 also plays a crucial role in the pathogenesis of psoriasis, it can be postulated that low levels of adiponectin, such as in obese individuals, could drive the inflammation in psoriasis.

Apart from the major adipokines discussed above, other adipokines that had been studied in psoriasis include the pro-inflammatory adipokines chemerin, lipocalin-2 and visfatin, as well as the anti-inflammatory adipokine omentin. A recent meta-analysis by Bai et al. (54) showed that serum levels of chemerin and lipocalin-2, like resistin, were also increased in psoriasis patients compared to healthy controls. However, differences in pooled serum levels of visfatin and omentin in psoriasis patients were not found to be significantly increased or decreased, respectively.

## ROLE OF FATTY ACIDS ON PSORIASIS

Another possible explanation for the link between obesity and psoriasis would be fatty acids (55). Fatty acids found in high fat diet, such as saturated fatty acids and trans fatty acids, are predominantly derived from adipose tissues and provide an important source of energy for metabolically active tissues in the body. In recent years, studies have shown that fatty acids also have the ability to influence inflammation in the body via various mechanisms. Fatty acid metabolism is intimately connected to T-helper cell 17 (Th17) function, which plays a pivotal role in psoriasis as well (56). High fat diet induced expression of enzymes involved in fatty acid metabolism such

as acetyl-CoA carboxylase 1 (ACC1), which therefore augments Th17 differentiation. Inhibition of fatty acid synthesis conversely reversed the obesity-induced increase of Th17 differentiation (57). Th17 then interacts with keratinocytes, endothelial cells and various immune cells including dendritic cells and neutrophils, driving the pathogenesis of psoriasis. Reactivation of memory Th17 cells is apparently responsible for chronic course of psoriasis (58). Various other studies support the fact that fatty acids play a role in psoriasis. Mouse psoriasis models induced by imiquimod demonstrated that the severity of disease was strongly correlated with free fatty acids concentration (59). Moreover, these obese mice, when compared to their lean counterparts, have an increased expression of IL-17A and IL-22 in the skin (60). Zhang et al. (61) reported that mice fed with high fat diet for 6 months spontaneously developed skin lesions. Inflammasome-related cytokines IL-1β and IL-18 were significantly upregulated in lesional skin compared to normal skin tissue, alluding to the fact that inflammasome signaling is somehow involved in the development of these skin lesions. Interestingly, the same study also showed that mice deficient in fatty acid binding protein, a transporter of fatty acids, were resistant to high fat diet induced skin lesions. A recent study also showed that resolvin E1 (RvE1), an omega-3 poly-unsaturated fatty acid (PUFA)-derived metabolite, potently suppressed the inflammatory cell infiltration and epidermal hyperplasia in an imiquimod-induced mouse psoriasis model. The study showed that RvE1 inhibited IL-23 production by dendritic cells and exerted inhibitory effects on migration of cutaneous dendritic cells and γδ T cells (62). Therefore, omega-3 PUFA derivatives may potentially be developed as a novel therapeutic agent for psoriasis.

## CONCLUSION

The relationship between psoriasis and obesity is a complex one and recent studies described above have enabled us to better understand their pathophysiological link. The cross-talk between adipocytes and the immune system via various mediators such as adipokines could explain how obesity contributes to psoriasis.

## AUTHOR CONTRIBUTIONS

YW wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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# Epithelial Cells as a Transmitter of Signals From Commensal Bacteria and Host Immune Cells

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Intestinal epithelial cells (IECs) are non-hematopoietic cells that form a physical barrier against external antigens. Recent studies indicate that IECs have pleiotropic functions in the regulation of luminal microbiota and the host immune system. IECs produce various immune modulatory cytokines and chemokines in response to commensal bacteria and contribute to developing the intestinal immune system. In contrast, IECs receive cytokine signals from immune cells and produce various immunological factors against luminal bacteria. This bidirectional function of IECs is critical to regulate homeostasis of microbiota and the host immune system. Disruption of the epithelial barrier leads to detrimental host diseases such as inflammatory bowel disease, colonic cancer, and pathogenic infection. This review provides an overview of the functions and physiology of IECs and highlights their bidirectional functions against luminal bacteria and immune cells, which contribute to maintaining gut homeostasis.

**Keywords: commensal bacteria, intestinal epithelial cells, immune system, out-side in signal, inside-out signal**

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## INTRODUCTION

The gastrointestinal (GI) tract is derived from progenitors in the foregut endoderm. It is a mucosal tissue covered by mucus and the major site for digestion and absorption of nutrients and water. The GI tract is therefore a unique organ that is constitutively exposed to various foreign antigens. Intestinal epithelial cells (IECs) covering the GI tract are non-hematopoietic cells that have a role in anatomical segregation of these innumerable luminal antigens such as food-derived materials and commensal microorganisms, as well as pathogens from host intestinal tissues (1). Because IECs form a protective wall against luminal antigens, the epithelial barrier is critical to maintain homeostasis of the GI tract. Indeed, disruption of the epithelial barrier by genotoxic irritants and endogenous genetic dysfunction leads to abnormal infiltration of luminal antigens and the development of inflammatory bowel diseases (IBDs) and infectious diseases (2).

In addition to the physical barrier function, IECs produce immunological molecules, such as mucus, antimicrobial molecules, and carbohydrate moieties, as well as secretory immunoglobulin A (SIgA) derived from plasma cells into the lumen (3). These chemical modulators prevent aberrant attachment and infiltration of luminal antigens into intestinal tissues. IECs also produce cytokines and chemokines as well as hormones that serve as modulators to fine tune the immune and nervous systems in the gut (3, 4). Therefore, IECs function as a bidirectional modulator of luminal antigens and the host.

Important environmental factors that modulate host metabolic and immune homeostasis are commensal microorganisms including bacteria, fungi, parasites, and viruses (5). In particular,

commensal bacteria are well-recognized as a booster of mucosal immune responses such as IgA production, T cell development and activation, and production of antimicrobial peptides by IECs (6, 7). Perturbation of commensal microbiota, which is termed as dysbiosis, and subsequent abnormal immune responses are pre-dispositions for the development of a range of local and systemic diseases such as IBDs, obesity, diabetes, cancer, and even autism (6). Recent studies have shown that IECs are important to recognize stimulation by commensal bacteria and direct induction and regulation of immune responses in the intestines. In contrast, immune cells underlining IECs direct production of immunological factors that influence luminal microbiota (8, 9). In this context, IECs coordinate crosstalk between luminal commensal bacteria and gut immune cells. This review highlights recent advances in understanding the unique features of IECs accompanied by luminal microbes and adjacent immune cells, and discusses unique functions of IECs as a bidirectional modulator of luminal bacteria and the immune system to maintain gut homeostasis.

## EPITHELIAL SUBSETS AND PHENOTYPES IN THE INTESTINES

The small intestine has unique protrusion structures called villi that contribute to extending the mucosal surface area to absorbing nutrients. In contrast, colon defects of villi have a relatively flat structure to prevent potential damage caused by intestinal contents in the event of transition from the upper to lower portion. IECs covering the intestinal tract are morphologically and functionally heterogeneous and have pleiotropic roles as a surface barrier system. Each epithelial cell subset, including enterocytes, goblet cells, Paneth cells, tuft cells, enteroendocrine cells, microfold (M) cells, and epithelial stem cells, have unique and specialized gene expression and functions, which cooperatively form a sophisticated epithelial layer against numerous antigens in the lumen. Disruption of the epithelial barrier allows abnormal intrusion of luminal antigens, including commensal bacteria, which causes detrimental inflammatory diseases such as IBDs, infection, and sepsis.

Epithelial cells (ECs) in the intestine are categorized into various subsets. The most prominent type of ECs are enterocytes that constitutively form tight junction structures and create the first line of the physical barrier against luminal antigens at the mucosal surface (1). In addition to physical barrier formation, enterocytes have the potential to produce antimicrobial molecules, such as regenerating islet-derived 3 (RegIII) γ and Ly6/PLAUR domain containing 8 (Lypd8), and generate various carbohydrate chains (8, 10, 11). In the context of immunological functions, enterocytes express polymeric immunoglobulin receptor (pIgR) at the basolateral side of the cell membrane. After binding to pIgR, dimeric IgA produced by lamina propria plasma cells transcytoses to the apical surface and is subsequently released as SIgA into the lumen.

Goblet cells are well-characterized as mucus-producing cells. The epithelial layer is covered by two mucus layers in the intestines, specifically thick inner and thin outer mucus layers (12). Mucin encoded by the Muc2 gene is a major component of intestinal mucus. Because deletion of the Muc2 gene leads to a defect of the inner mucus layer, commensal microbes can access the epithelial surface. The constitutive and abnormal bacterial stimulation observed in Muc2-deficient mice induces pathological inflammation and tumorigenesis in the colon (13, 14). Recently, sentinel goblet cells have been characterized in the colon. These cells produce mucin in response to bacterial signals, and NOD-like family pyrin domain containing 6 (NLRP6)/inflammasomes govern exocytosis of mucin into the lumen (15). Goblet cells also produce anti-inflammatory molecules such as trefoil factor 3, a tissue-protective factor that repairs the epithelial layer by inhibiting apoptosis, and resistin-like molecule (RELM) β (16, 17).

Paneth cells reside in the crypt basal region and intercalate with epithelial stem cells. They are specialized to produce antimicrobial molecules such as lysozyme, α-defensins, secretory phospholipase A2 (sPLA2), and RegIIIγ (11, 18). Paneth cells also express epidermal growth factor (EGF), Wnt3, and the Notch ligand delta-like 4 (Dll4) to maintain homeostasis of epithelial stem cells (19). Therefore, disruption of Paneth cell functions caused by aberrant endoplasmic reticulum stress and autophagy signals leads to pathogenic infection and IBDs (20, 21). Because Paneth cells are observed in the small intestine, but not in the colon, the anatomical location of typical epithelial cell subsets governs intestinal functions. Antimicrobial peptides specifically expressed in the colon may compensate for the function of Paneth cells in the small intestine (10). A recent single cell study of intestinal epithelial cell subsets provided evidence that each subset is functionally heterogeneous and ECs isolated from distinct intestinal regions have different phenotypes even in the same subsets (22). For example, Paneth cells, a secretor of antibacterial molecules, can be classified into two types: Paneth-1 and-2. Paneth-1 cells, which highly express α-defensin, are abundant in the ileum. In contrast, Paneth-2 cells, which preferentially express RNase1, are enriched in the duodenum. Although it is unclear why there is such a regional distribution of the subsets of each IEC type, several reports suggest that region-specific environmental stimuli may affect epithelial physiology in the gut. In particular, specific commensal bacteria colonizing a specific region of the intestinal tract direct IECs to express specific antibacterial peptides, carbohydrate moieties, and immune modulatory molecules that induce immune responses *in situ*.

Another unique epithelial subset—Tuft cells—sense luminal helminths, such as *Tritrichomonas muris*, through their GTP-binding protein, α-gustducin (23). Tuft cells produce IL-25 and thymic stromal lymphopoietin (TSLP) in response to helminths to induce Th2-type immune responses and protect against infection by a helminth (24).

M cells reside in the follicle-associated epithelium (FAE) covering secondary lymphoid tissues such as Peyer's patch and isolated lymphoid follicles. They have unique morphological characteristics such as irregular microvilli and pocket structures containing lymphocytes and dendritic cells (DCs). M cells take up antigens from the lumen as well as serve as an entrance for pathogenic and non-pathogenic microorganisms. A recent

report showed that allograft inflammatory factor 1 (Aif1) is a critical molecule for uptake of antigens such as those of *Salmonella typhimurium* (25). Receptor activator of nuclear factor-κB ligand (RANKL) and TNF receptor-associated factor 6 (TRAF6)-mediated NF-κB signaling regulate differentiation of M cells. Mesenchymal cells residing under the FAE produce RANKL that is critical for the differentiation of M cells via epithelial RANK (26, 27). All IECs derived from stem cells expressing leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) reside at the base of the crypt. These stem cells become transient proliferative cells that differentiate into each mature epithelial subset.

## MICROBIOTA DIRECTLY AFFECTS EPITHELIAL PHYSIOLOGY

More than $1 \times 10^{13}$ bacteria symbiotically colonize the human intestines (28). Each bacterial species adapts to the specific intestinal environment for colonization, such as oxygen concentration, pH, redox potential, nutrient supplies, host secretions, and intestinal motility. Therefore, each bacterial species colonize at different sections along the intestines (29, 30). Mice are usually maintained under specific pathogen-free (SPF) conditions with sterile chow and water. Even under this condition, inter-mouse variations are observed from the phylum to the operational taxonomic unit (OTU) levels, especially in gastric and small intestinal samples. Fecal microbiota has a relatively similar bacterial population as the large intestine (30). A community of bacteria resides in each GI tract region of wildtype (WT) mice. At the family level, anaerobes including *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were enriched in the large intestine and feces, while *Lactobacillaceae* predominantly colonized the small intestine and stomach in a murine model (30). At the genus level, the large intestine and feces had a higher percentage of *Bacteroides*, *Prevotella*, and *Alistipes*, while *Lactobacillus* had a higher proportion in the stomach and small intestine (30). In the stomach and upper part of the small intestine, the number of bacteria is low compared with the lower part of the GI tract (31). It is difficult to discriminate "transient" bacteria that pass through the intestines and "colonized" bacteria that proliferate and are stably observed in the intestines. Mice usually feed on feces that may pass through the stomach and small intestine, which may also affect the bacterial population.

Importantly, the functions of IECs are affected by stimulation of luminal antigens including commensal bacteria. As reported previously, commensal bacteria influence epithelial physiology and subsequent intestinal lymphoid structures and can cause abnormal villous morphology and epithelial cell proliferation (32). The mucus layer is thick in the distal colon in concert with the abundance of goblet cells. This gradient is parallel to the load of commensal bacteria. It has been reported that Toll-like receptor (TLR) and NLRP6 signaling in IECs control differentiation of goblet cells in response to microbial stimulation (33, 34). Therefore, germ-free (GF) mice and antibiotic-treated mice have a thin mucus layer. Production of antimicrobial peptides such as RegIIIγ in GF mice diminishes compared with WT mice (35). In addition, replication of epithelial stem cells is disrupted, and thus, antibiotic-treated mice are susceptible to colitis induced by physical and chemical disruption of IECs (36). TLR2, TLR4, and Myd88, a downstream signaling molecule of these receptors, are responsible for detection of commensal bacteria. Epithelial cell proliferation is abnormal in mice lacking TLR2, TLR4, and Myd88, and these mice are susceptible to dextran sulfate sodium (DSS)-induced colitis (36). Furthermore, TLR-commensal bacterium interactions are important to form an epithelial barrier by maintaining tight junction proteins in IECs (36, 37). Deficiency of such TLR signaling in IECs exacerbates colitis induced by pathogenic bacteria such as *Citrobacter rodentium* (38).

Intestinal bacteria synthesize a variety of materials derived from foods as well as carbohydrates secreted from host IECs as a consequence of their metabolism. These metabolites have been reported to stimulate IECs and modulate epithelial physiology. Important metabolites produced by commensal bacteria are short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. These SCFAs are usually generated from the process of fermentation of dietary fibers. *Bifidobacterium longum*, an obligate anaerobe, produces acetate that protects IECs against apoptosis induced by O157 toxin (39). Acetate also induces goblet cell differentiation, secretion of mucin, and decoration of mucin glycans with sialic acid (40). Because of their lack of commensal bacteria, GF mice have shorter Muc2 O-glycans and express several glycosyltransferases at a low level in IECs (40). Other anaerobic bacteria, such as *Clostridium* clusters IV and IXa, *Faecalibacterium prausnitzii*, and *Bacteroides thetaiotaomicron*, produce butyrate. Although butyrate is an important SCFA to establish the gut immune system, as discussed later, butyrate represses the proliferation of intestinal stem cells during DSS treatment (41). This may prevent abnormal transformation of IECs under exogenous stress from the luminal environment. Butyrate diminishes proliferative activity by inhibiting histone deacetylase (HDAC) enzymes and inducing Forkhead box O3 (Foxo3) expression in epithelial stem cells (41). The colonic crypt architecture is suggested to protect against exposure of a high concentration of butyrate to these epithelial stem cells (41). Furthermore, butyrate from commensal bacteria promotes colonic oxygen consumption by stabilizing the transcription factor Hypoxia-inducible factor (HIF), which augments epithelial barrier functions (42).

In addition to SCFAs, other metabolites from bacteria such as lactate induce hyperproliferation of colonic ECs (43). Because lactate is an energy source, epithelial stem cells use lactate produced by adjacent Paneth cells, which promotes differentiation and proliferation (44). Despite these accumulating data, it is still controversial whether lactate-producing bacteria such as *Lactobacillus* influence epithelial stem cell homeostasis *in vivo*, especially in humans. Taken together, commensal bacteria and commensal-derived metabolites are important to maintain homeostasis of the epithelial barrier. Dysbiosis observed in IBD patients and colitis mouse models, such as an expansion of *Enterobacteriaceae*, may presumably reduce these metabolites, leading to disruption of the epithelial barrier system (45, 46).

In the intestines, fungi and bacteria compete for niches, and these microbes influence each other. Although the number of fungi is relatively low compared with bacteria, fungi are a major microbial population in the gut. For example, *Candida*, *Saccharomyces*, *Aspergillus*, *Cryptococcus*, *Malassezia*, *Cladosporium*, *Galactomyces*, and *Trichosporon* have been reported to colonize human intestines (47–49). In contrast to humans, fungi colonization in the intestines of experimental mice is dependent on the animal facility. Whereas, fungi are observed in the gut of WT mice, termed as mycobiota, laboratory mice bred in some facilities are resistant to fungal colonization in the steady state. This could be because the composition of commensal bacteria colonizing mice is distinct between animal facilities. Indeed, depletion of gut microbiota by antibiotic treatment allows colonization of *Candida* and *Saccharomyces* in the gut (50, 51). Among commensal microbiota, *Blautia producta* and *B. thetaiotamicron* have been identified as the bacteria responsible for colonization resistance against *C. albicans*. These bacteria induce production of cathelicidin-related antimicrobial peptide (CRAMP) from IECs, which is mediated by the transcription factor hypoxia-inducible factor-1α, resulting in the inhibition of colonization of the gut by *C. albicans* (50). Interestingly, colonization of *Candida* and *Saccharomyces* ameliorates DSS-induced colitis, although the detailed mechanism is still unclear (51). Therefore, luminal microorganisms, including bacteria and fungi, are closely associated with the intestinal epithelial physiology.

## "OUTSIDE-IN SIGNALS" FROM MICROBIOTA MODULATE IMMUNE RESPONSES

A difficulty of research related to commensal bacteria is that the environment of the animal facility, especially food and water, affects commensal microbiota. For example, the numbers of segmented filamentous bacteria (SFB) and *Lactobacillus murinus* are dramatically reduced in C57BL/6 mice bred in Jackson laboratory, but not in Taconic farm, which is closely associated with the development of host mucosal immune systems such as the development of T helper 17 (Th17) cells (35). Antibiotic-treated and GF mice are widely used to investigate the effect of commensal bacteria on host physiological and pathological functions. In addition, researchers have established GF mice colonized with specific bacteria, termed gnotobiotic mice, using a vinyl isolator under sterile conditions to uncover the role of each bacterial species. It has been reported that colonization of specific bacteria triggers the development of immune cells in the gut (6). The intestinal immune system has tolerance for commensal bacteria. However, this tolerance does not mean no response, but a response to commensal bacteria without pathogenic symptoms, which is also considered as "physiological inflammation." Indeed, the numbers of T cells and $IgA^+$ cells are strikingly reduced and secondary lymphoid organs are immature in GF mice compared with WT mice. Whereas, commensal bacteria directly stimulate immune cells in lamina propria in some cases, IECs stimulated by commensal bacteria also initiate immune responses. This review terms this cascade from commensal bacteria to immune cells through IECs as "outside-in signals." IECs recognize signals from various commensal species and transmit these outside-in signals to immune cells. Therefore, IECs play a central role in the establishment of the gut immune system in response to commensal bacteria.

Intestinal epithelial cells (IECs) recognize luminal bacterial signals by a variety of pattern recognition receptors including TLRs and nod-like receptors (NLRs). Enterocytes express TLR2, TLR3, TLR4, TLR5, and TLR9 (52, 53). Unlike immune cells, IECs have the unique feature of cellular polarization that facilitates the anatomical distribution of TLRs. Although IECs usually express TLRs at the basolateral membrane to circumvent the induction of detrimental inflammatory responses, TLR2 and TLR9 are also expressed on the apical side of IECs (54). TLR signaling in IECs leads to the expression of inflammatory cytokines and chemokines such as IL1β, IL-6, IL-18, and CCL20. In contrast, apical stimulation of TLR9 induces an immune inhibitory effect through stabilization of IκB, demonstrating the unique ability of IECs to differentially respond to microbial signals using the same receptors expressed at apical and basolateral positions (55). In addition to TLRs, IECs express NLRs to detect bacterial components and danger signals. The expression and functions of NLR in IECs are summarized in another review (56).

Commensal bacteria induce development and maturation of secondary lymphoid organs such as Peyer's patch and isolated lymphoid follicles (ILFs). Indeed, Peyer's patches are hypoplastic and ILFs are hardly observed in GF and antibiotic-treated mice. The FAE covering Peyer's patch and ILFs recognizes luminal bacteria and initiates and organizes these secondary lymphoid organs. Antigen-presenting cells located under FAEs are mostly DCs. These DCs take up luminal antigens from M cells in FAEs and prime T cells and subsequent activation of B cells to initiate antigen-specific immune responses. FAE induces structural organization of Peyer's patch and ILFs by producing chemokines such as CCL20 and CCL9 in mice and CCL20 and CCL23 in humans (57, 58). DCs localized in the subepithelial dome region are recruited by chemokines such as macrophage inflammatory protein (MIP) 3α (59). CCL20 expressed by FAEs also recruits $CCR6^+$ B cells into ILFs and Peyer's patch follicles (60). Although the bacterial recognition system in FAE of Peyer's patch is still mostly unclear, a set of TLRs expressed in the FAE may affect such chemokine expression (61). In the context of ILF development, nucleotide-binding oligomerization domain containing 1 (NOD1) expressed in FAEs recognizes peptidoglycans (PGNs) derived from Gram-negative bacteria and induces CCL20 expression and subsequent recruitment of $CCR6^+$ DCs and group 3 innate lymphoid cells (ILC3) (62) (**Figure 1A**).

Intraepithelial lymphocytes (IELs) are a T cell subset spatially distributed between IECs. Homeostasis of IELs, especially TCRαβ, but not TCRγδ IELs, is maintained by signals from commensal bacteria. In GF mice, the number of IELs is dramatically reduced compared with WT mice (63). Several reports have shown that specific commensal bacterial species are involved in the development of IELs. For example, SFB and

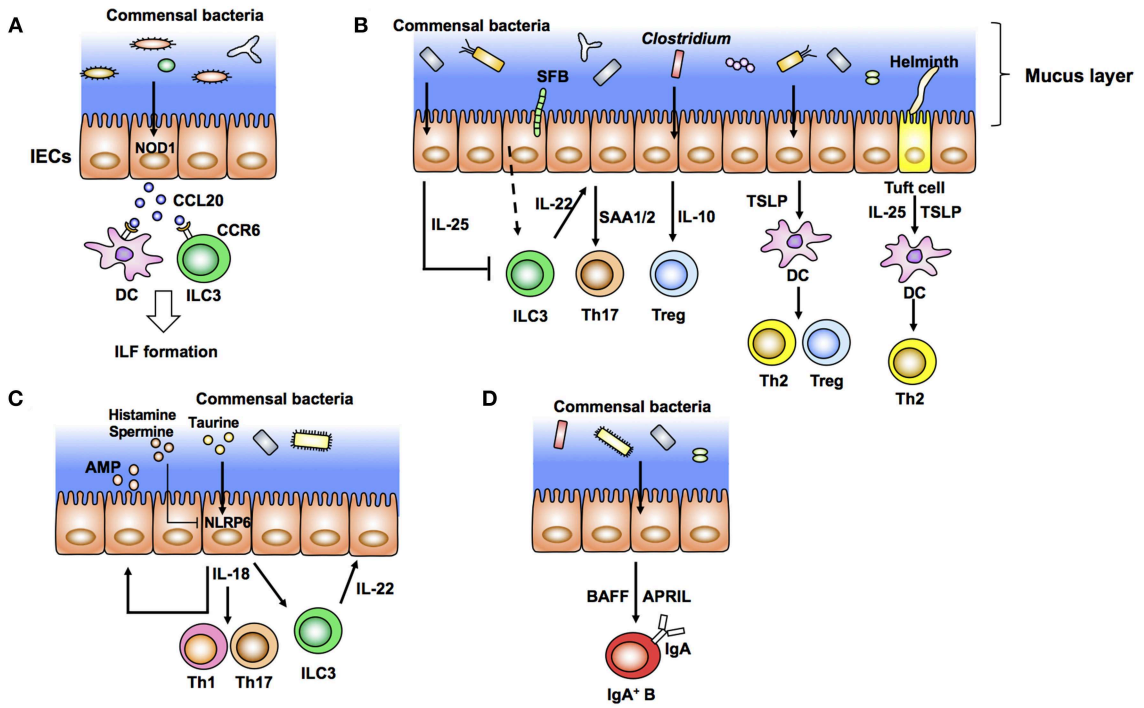


**FIGURE 1 |** Intestinal epithelial cells (IECs) modulate the gut immune system in response to commensal bacteria (outside-in signals). **(A)** Diverse microbiota provides the ligands of NOD1 expressed in IECs. These ligands induce production of epithelial CCL20 as well as recruitment of CCR6$^+$ dendritic cells (DCs) and group 3 innate lymphoid cells (ILC3) that initiate the development of isolated lymphoid follicles (ILFs). **(B)** Specific commensal bacteria, such as segmented filamentous bacteria (SFB) and *Clostridium*, induce differentiation of Th17 cells and Tregs, respectively. SFB induce production of IL-22 from ILC3. IL-22 elicits epithelial SAA1/2 and subsequent Th17 cell differentiation. IECs produce IL-10 in response to Clostridia and induce Treg differentiation. Epithelial IL-25 limits the production of IL-22 from ILC3. IECs condition dendritic cells (DCs) to a tolerogenic phenotype through the production of TSLP. Tuft cells recognize helminth signals and produce IL-25 and TSLP that skew Th2 immune responses. **(C)** Epithelial IL-18 production is mediated by activation of NLRP6 in response to taurine produced by commensal bacteria. Epithelial IL-18 induces the production of IL-22 from ILC3 and antimicrobial peptides (AMP) from epithelial cells in an autocrine manner. **(D)** Commensal bacteria elicit production of APRIL and BAFF by IECs and IgA class switching of B cells.

*Lactobacillus reuteri* induce IEL subsets such as CD4$^+$ CD8αα$^+$ IELs (63, 64). Although metabolites produced by commensal bacteria directly affect IEL development, IECs also mediate IEL development and functions. IL-15 expressed by IECs participates in IEL maintenance through trans-presentation of IL-15 to IL-15Ra expressed on IELs (65). Although the mechanism is still unknown, IL-15 is induced following exposure to commensal bacteria (66). In addition, secretion of IL-15 by IECs is dependent on MyD88. Therefore, the numbers of IELs are reduced in MyD88-deficient mice, which can be restored upon transgenic expression of IL-15 (67). This suggests that commensal signals regulate IEL numbers through induction of IL-15 production by IECs.

In the lamina propria region, differentiation of T helper cells, especially Th1, Th17, Tregs, and CD8 T cells, is controlled by various commensal microbes (35, 46, 68–72) (**Figure 1B**). Among these T cells, IL-17-producing Th17 cells positive for the transcription factor RORγt are induced by SFB and a mixture of 20 species of human commensal bacteria (35, 72) (**Figure 1A**). SFB specifically colonize at the epithelial layer in the ileum and are closely associated with IECs. This association between bacteria and IECs is critical to induce Th17 cells (71). Rat-derived SFB, which are not able to attach to murine IECs, do not induce Th17 cells in mice (71). In addition, *C. rodentium* expressing structural protein intimin interact with IECs and induce Th17 cells. However, Th17 cells are not induced in mice infected with an intimin-deficient strain (71). In addition to bacteria, pathogenic fungus *C. albicans* colonize the epithelial surface and induce Th17 cells (71). Epithelium-associated *Escherichia coli* and *Bifidobacteria adolescentis* isolated from humans also induce inflammatory Th17 cells (73). In addition to these pathogenic and non-pathogenic microorganisms, *Acinetobacter* spp., *Bacteroides fragilis*, and Proteobacteria have been reported as epithelium-associated bacteria (74). However, it is still unknown whether these bacteria have the potential to induce Th17 cells.

In the process of Th17 cell development, production of IL-22 by ILC3 in response to SFB attachment to microvilli of IECs elicits serum amyloid A proteins 1 and 2 (SAA1/2) from ileal ECs, which augment Th17 cell development (75) (**Figure 1A**). Importantly, IL-22 produced by ILC3 trigger activation of signal transducer and activator of transcription 3 (STAT3), a transcription factor downstream of the IL-22 receptor, in IECs. SAAs derived from ileal ECs are critical for the development of Th17 cells (75). Although the mechanism of how the

association of SFB with IECs induces IL-22 production by ILC3 is still unclear, reports suggest that IECs have a role in the initiation and enhancement of Th17 cell development. Th17 cells and IL-22 production by ILC3 are important to prevent infection by *C. rodentium* (35). In addition to bacteria, *T. musculis*, a previously unrecognized commensal protozoan, activates NLRP6 in IECs and subsequent IL-18 production. Microbiota-derived metabolites, such as taurine, histamine, and spermine, regulate secretion of IL-18 and anti-microbial peptides by modulating NLRP6 inflammasome signaling (15) (**Figure 1C**). Taurine derived from microbiota enhances IL-18 production in IECs (**Figure 1C**). In contrast, histamine and spermine inhibit this IL-18 production (15) (**Figure 1C**). Defects in epithelial NLRP6 reduce cleavage of caspase-1 and subsequent IL-18 secretion as well as the expression of antimicrobial peptides that predispose to the development of dysbiosis and colonic inflammation (15, 76). These microbiota-derived metabolites, epithelial IL-18 production, and the anti-microbial peptide production cascade are important to maintain microbiota diversity and colonic homeostasis. Indeed, NLRP6-deficient mice have spontaneous intestinal hyperplasia, inflammatory cell recruitment, and exacerbated experimental colitis caused by pathobiont colonization (76). In contrast to this report, other groups have recently shown that NLRP6 deficiency has no effect on the community of gut microbiota (77, 78). These reports indicate the importance of performing experiments with littermates when addressing the function of gut microbiota. Several factors including mouse facilities and experimental designs may also account for the discrepancies in the results. Therefore, the role of NLRP6 in IECs against microbiota is still controversial. Epithelial IL-18 also enhances the production of IL-22 from ILC3 in the steady state (79) (**Figure 1C**). Because IL-18 secreted from IECs augments inflammatory Th1 and Th17 cell differentiation, mice infected with *T. musculis* are susceptible to T cell-induced colitis and sporadic colorectal tumors compared with uninfected mice (80, 81). In contrast, *T. musculis*-infected mice are resistant to mucosal infection by *S. typhimurium* (80). These studies indicate that IECs modulate intestinal pathogenesis as well as homeostasis in response to commensal bacteria.

In addition to the development of Th17 cells, IECs coordinate anti-inflammatory Th cell responses. *Clostridium* class IV, class XIVa, and cluster XVIII and *B. fragilis* have been reported to induce Tregs in the colon (68, 69, 71) (**Figure 1B**). In particular, colonization of *Clostridium* elicits production of IL-10, a potent anti-inflammatory cytokine, from IECs, which enhances differentiation of colonic Tregs (68) (**Figure 1B**). IECs also direct anti-inflammatory Treg and Th2 cell differentiation by producing TSLP after bacterial stimulation (**Figure 1B**). Expression of TSLP is elevated in IECs treated with non-invasive bacteria *in vitro* (82). TSLP from IECs conditions DCs to an anti-inflammatory status and promotes polarization of T cells toward Tregs and Th2 cells (82) (**Figure 1B**). TSLP derived from IECs prevents IL-12-stimulated induction of DCs and enhances the production of IL-6 and IL-10. Expression of IL-12, a Th1-prone cytokine, from DCs is therefore augmented under a TSLPR-deficient condition (83). IEC-intrinsic IKKβ controls TSLP induction. Thus, both IKKβ- and TSLPR-deficient mice have defects in Th2 responses and are susceptible to parasitic *Trichuris* infection (83). IECs also induce anti-inflammatory Th2 cytokine IL-25 (IL-17E) in response to commensal bacteria. Thus, IL-25 expression is defective in GF mice. IL-25 from IECs inhibits IL-22 production from ILC3 (84, 85) (**Figure 1B**). Because IL-22 from ILC3 is important for epithelial cell proliferation, IL-25 inhibits epithelial tissue repair and exaggerates DSS-induced colitis (84). Taken together, intestinal ECs transmit signals from luminal bacteria to immune cells and trigger positive and negative T cell responses to maintain gut homeostasis.

Commensal bacteria are also important to induce intestinal immunoglobulin A (IgA). IgA production is impaired in GF mice, which is correlated with immature secondary lymphoid tissues (32). The IgA induction by commensal bacteria may depend on anatomical colonization of specific commensal bacteria. For example, SFB induce IgA in the ileum, but a mixture of 46 *Clostridia* and *Bacteroides acidifaciens* induce IgA in the colon (86, 87). In addition to SFB, epithelium-associated commensal bacteria, such as *Mucispirillum*, activate T cell-dependent IgA production (88). In the lamina propria region, IgA is induced in a T cell-independent manner (89). In this context, IEC-derived cytokines, especially TNF superfamily members, B cell-activating factor of the tumor necrosis factor family (BAFF), and proliferation-inducing ligand (APRIL), induce class switching to IgA2, the main mucosal IgA class in humans (**Figure 1D**). TSLP produced from IECs triggers APRIL production by DCs in response to TLR-mediated signals from commensal bacteria (90). Ectopic expression of TLR4 in IECs augments the expression of CCL20, CCL28, and APRIL that recruit and activate lamina propria (LP) $CCR6^+$ B cells and IgA class switching (91). These data support a model in which IECs stimulated by commensal bacteria initiate T cell-independent IgA production.

## "INSIDE-OUT SIGNALS" MODULATE MICROBIOTA

Because of the development of experimental tools to analyze bacterial DNA, such as next-generation sequencing with bioinformatics and quantitative PCR, the number, diversity, population, and gene expression of the microbiota have been investigated comprehensively. Using these powerful tools, it is possible to analyze microbiota isolated from specific patients and genetically modified murine models. Based on the accumulated studies, it has been reported that intestinal immune cells together with IECs affect luminal microbiota. Because of the anatomical features of IECs exposed to the luminal environment, immunological factors are usually produced by IECs. However, intestinal immune cells express various bacterial recognition receptors. Therefore, immune cells underlying IECs detect bacterial signals and transfer the signal to IECs to produce immunological factors. The cascade from immune cells to commensal bacteria through IECs is termed as "inside-out signals" in this review. In addition to signals from the luminal environment, resident immune cells adjacent to IECs calibrate epithelial physiology and luminal microbiota.

Dimeric IgA produced by plasma cells in the LP binds to pIgR expressed on the basolateral side of IECs. After binding to pIgR, IgA is transcytosed to the apical surface and subsequently released as SIgA into the lumen (**Figure 2A**). SIgA is important to neutralize bacterial toxins, virus infection, and invasion of pathogenic and non-pathogenic bacteria into IECs. Recent elegant studies employing IgA-seq analysis have revealed that IgA-coated microbiota has the characteristic of immunogenic commensal bacteria (88, 92, 93). IgA-seq is a method that combines $IgA^+$ bacterial cell sorting and 16S rRNA gene sequencing of sorted bacteria to characterize intestinal microbiota coated with IgA. Using this method, SFB, *Bacteroidales*, *Lactobacillus*, and unclassified *Erysipelotrichaceae* were identified to be highly coated with IgA in SPF mice (**Figure 2A**). Under an inflammasome-deficient condition, mice have aberrant commensal microbiota with a colitogenic feature. In these mice, the *Prevotellaceae* family and *Helicobacter sp. flexispira*, in addition to *Lactobacillus* and SFB, are coated with IgA (93). Although it is still controversial whether SIgA regulates homeostasis of commensal bacteria, activation-induced cytidine deaminase (AID)-deficient mice with defects in immunoglobulin class switch recombination (CSR) and somatic hypermutation (SHM) have aberrant microbiota and hyperplasia of germinal center B cells (94). In particular, SHM rather than CSR is important to maintain homeostasis of gut microbiota (95). SIgA also affects bacterial gene expression. SIgA downregulates the expression of capsular polysaccharide synthesis 4 (CPS4) and enhances CPS5 expression, both of which are epitopes of *B. thetaiotaomicron* for adaptation to the intestinal environment (96).

As described above, bacterial components and metabolites directly stimulate IECs and maintain homeostasis of the epithelial barrier. However, disruption of epithelial homeostasis leads to pathogenic intestinal inflammation caused by the production of proinflammatory cytokines, such as TNF, IL-22, and IL17a, by innate and acquired immune cells. For example, IEC-specific deletion of components that activate nuclear factor-κB (NF-κB), especially the inhibitor of NF-κB (IκB) kinase (IKK) complex and NF-κB essential modulator (NEMO), results in spontaneous colitis caused by elevation of epithelial apoptosis and exaggerated DSS-induced colitis (97, 98). Commensal bacteria stimulate colonic DCs and/or macrophages through MyD88 signaling and induce aberrant TNF production, accelerating epithelial apoptosis, and inflammation (97) (**Figure 2B**). This inflammation is impaired in TNFRI-deficient IECs (97). These data indicate that epithelial NF-κB signals contribute to maintaining homeostasis of the epithelial barrier and inhibit excess production of inflammatory cytokines and pathogenic inflammation. In contrast to NEMO, deletion of IKKα in IECs results in TSLP overproduction and inhibition of IL-22 production by ILC3 (99). Because of the disruption of epithelial homeostasis, IKKα-deficient mice are susceptible to colitis and pathogenic infection (98).

An important and well-characterized cytokine from immune cells, which stimulates IECs is IL-22. IL-22 is a member of the IL-10 cytokine family usually produced by Th17/Th22 and ILC3 under stimulation by IL-23. Because IL-22R is constitutively and specifically expressed in IECs (100), IL-22 contributes to affecting epithelial cell proliferation, differentiation, glycosylation, and production of antimicrobial peptides. In particular, IL-22-mediated epithelial stem cell regeneration is critical to maintain gut homeostasis after genotoxic treatment and pathogenic infection (101, 102). Indeed, IL-22 induces expression of antimicrobial molecules RegIIIβ and RegIIIγ in IECs for exclusion of pathogenic bacteria such as *C. rodentium* (100) (**Figure 2C**). In addition to such immunological features, IL-22 production by ILC3 creates a host-commensal symbiotic platform in the gut. IECs express various carbohydrate moieties on the apical surface of their cell membrane. Fucosylated glycans are synthesized by addition of an L-fucose residue via an α1-2 linkage to the terminal β-D-galactose of glycan in a process catalyzed by fucosyltransferase (Fut), especially Fut2 expressed in IECs. This epithelial α1, 2-fucosylation is initiated by colonization of SFB and stimulation of DCs by lipopolysaccharide (LPS). After such stimulation, IL-22 from ILC3 induces Fut2 expression and α1, 2-fucosylation in IECs (8, 9) (**Figure 2C**). Importantly, epithelial α1, 2-fucosylation prevents infection and contributes to maintaining commensal microbiota and gut homeostasis (8, 9, 103). In contrast to the positive effect of ILC3, IL-10-producing T cells negatively regulate the induction of ectopic epithelial α1, 2-fucosylation (104). Interestingly, high numbers of α1, 2-$fucose^+$ IECs and Th17 cells are observed in the ileum, but not in the duodenum (8, 105). The regional gradients of epithelial α1, 2-fucose and the number of Th17 cells correspond to the colonization of SFB. Therefore, colonization of specific microbes affects both the localization and activation/differentiation state of immune and ECs. In addition to α1, 2-fucose, expression of RegIIIγ and nitric oxide synthase 2 (NOS2), and SAA production in ileal ECs is controlled by SFB colonization (35). Although the detailed mechanism is unclear, attachment of bacteria to intestinal ECs triggers the subsequent unique development of immune cells in the gut (72). Another report has shown that IL-23 produced by $CD103^+$ $CD11c^+$ $TLR5^+$ cells induces IL-22 from ILC3 in response to bacterial flagellin (106) (**Figure 2C**).

Among the members of the IL-17 cytokine family, IL-17a is mainly produced by γδ T cells and Th17 cells. IL-17a induces epithelial NF-kB signaling and maintains tight junctions (107, 108). IL17a-deficient mice are susceptible to DSS-induced colitis. Therefore, IL17a is also important to maintain the epithelial barrier function and homeostasis of the intestines (109). IL17a and IL-17R signaling in IECs elicits the expression of immunological factors, such as α-defensin, Nox1, and pIgR, which regulate SFB colonization in the intestines (110) (**Figure 2C**). As mentioned above, intestinal Th17 cells are induced by commensal bacteria, especially SFB, and mice colonized with SFB are resistant to infection by *C. rodentium* (35). Therefore, SFB colonization regulates homeostasis of itself and prevents infectious diseases mediated by IL-17a.

In the stomach, *Lactobacillus* produce the metabolite dietary tryptophan, a ligand of aryl hydrocarbon receptor (AHR), which also promotes differentiation of IL-22-producing ILC3. IL-22 induces secretion of antimicrobial peptides from gastric ECs, which prevent colonization by *C. albicans* (111). It has

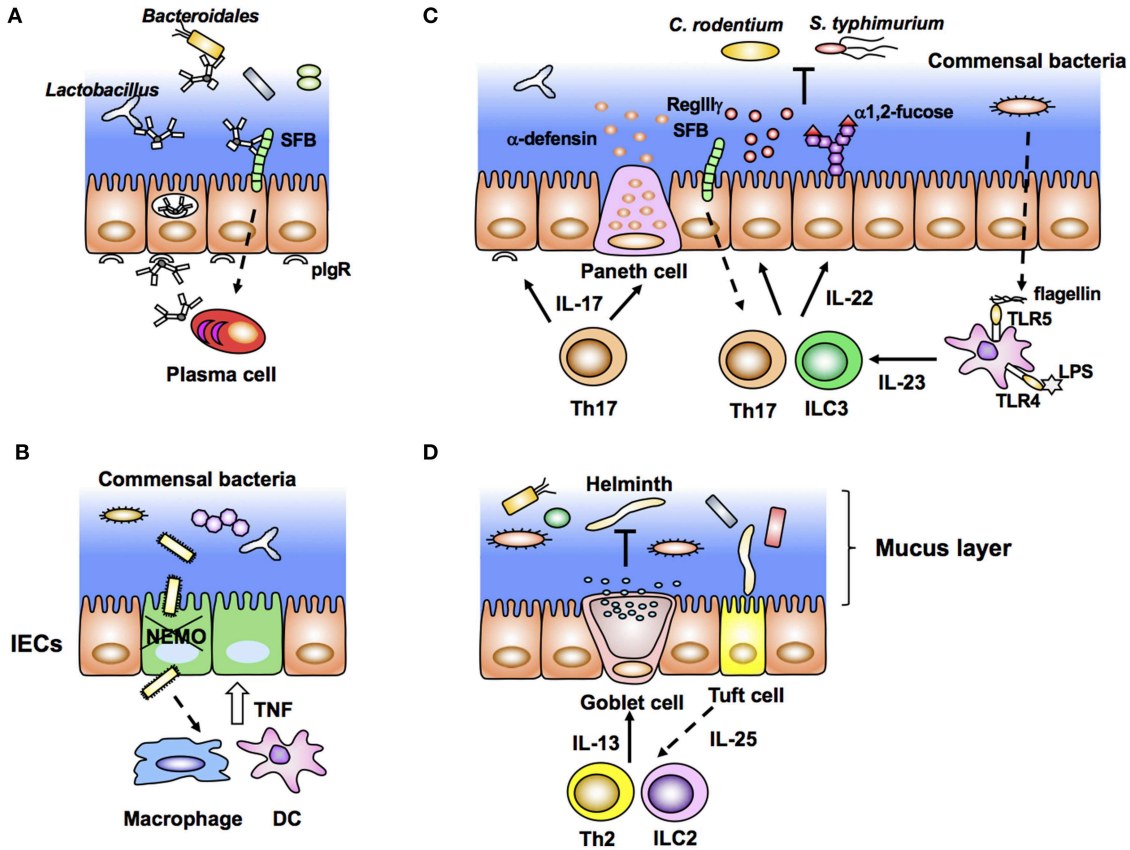


**FIGURE 2 |** Intestinal epithelial cells (IECs) stimulated by immune cells affect gut microbiota (inside-out signals). **(A)** Dimeric IgA antibodies produced by plasma cells in the lamina propria bind to pIgR expressed on the basolateral membrane of IECs, undergo transcytosis, and are secreted into the lumen as SIgA. SIgA binds to commensal bacteria and maintains their homeostasis. **(B)** NEMO deficiency in IECs allows bacterial infiltration that leads to aberrant production of TNF from macrophages/DCs and further apoptosis of IECs. **(C)** Lamina propria DCs produce IL-23 in response to bacterial flagellin and LPS. IL-23 induces production of IL-22 from group 3 innate lymphoid cells (ILC3) and Th17 cells, leading to expression of epithelial anti-microbial molecules, such as RegIIIγ and α1,2-fucose. RegIIIγ and α1,2-fucose, which regulate the luminal microbial population. **(D)** Tuft cells produce IL-25 in response to helminth infection. Epithelial IL-25 promotes IL-13 production from ILC2 and Th2 cells, and subsequent production of mucus from goblet cells.

been reported that dysbiosis, which is an altered *Lactobacillus* population, is observed in caspase-associated recruitment domain 9 (CARD9)-deficient mice (112). Dysbiosis observed in mice lacking Card9 affects tryptophan metabolism and impairs stimulation of AHR, leading to impairment of IL-22 production and expression of epithelial RegIIIβ and RegIIIγ (112). In addition to the induction of IL-22, AHR activation induces epithelial cytochrome P4501 (CYP1) enzymes that oxygenate AHR ligands. The numbers of Th17 cells and ILC3 are dramatically reduced in mice with IEC-specific depletion of Cyp1a1, resulting in increased susceptibility to infection by *C. rodentium* (113).

In the context of helminth infection, the aforementioned IL-25-secreting Tuft cells condition Th2 immune responses as described above. ILC2 and Th2 cells activated by IL-25 produce IL-13 and IL-33. Notably, IL-13 enhances the production of mucus and antimicrobial peptide resistin-like molecule β (RELMβ) by goblet cells, which mediate worm expulsion (**Figure 2D**). In this manner, commensal bacteria, IECs, and immune cells interact with each other and create a network system at the mucosal surface, and IECs serve as key players in this interplay. The triangular regulatory machinery consisting of commensal bacteria, IECs, and immune cells is an important and representative model for understanding intestinal homeostasis (**Figure 2**).

## CONCLUDING REMARKS

Commensal bacteria naturally co-inhabit the intestines of their host. Because IECs encounter luminal antigens including those of these symbionts, IECs produce symbiotic factors, such as carbohydrate moieties and mucus, as well as immunological mediators including antimicrobial peptides (11, 114). In addition to SIgA, immune cells direct IECs to produce symbiotic and immunological factors, and influence the microbiota and gut homeostasis.

As discussed above, immune cells produce cytokines in response to signals from commensal bacteria. How these immune

cells detect commensal bacteria is an important question that remains to be answered. One possibility is that CX3CR1$^+$ cells extending their dendrites into the lumen directly detect bacterial stimulation (115). A recent study showed that lactate from commensal bacteria induces the extension of dendrites from these cells (116) and another report has shown that metabolites produced by butyrate directly stimulate T cells in the lamina propria (117). Future studies are required to identify the mechanism by which metabolites produced by bacteria in the lumen reach immune cells through IECs. Reports have shown that commensal bacteria induce Th17 cell development and IL-22$^+$ ILC3 in the steady state (8, 35, 72, 75). DCs, which present bacterial antigens to T cells, are critical for the induction of Th17 cells (118, 119). In addition, IL-23 produced by TLR5$^+$ DCs, which detect bacterial flagellin, induce IL-22 expression in ILC3 (106). These data suggest that bacterial antigens reach and stimulate LP DCs. How these bacterial antigens and metabolites stimulate LP immune cells and the roles of IECs in this process are still unknown. IECs may fill such an anatomical gap between commensal bacteria and immune cells. For example, goblet cells take up antigens from the lumen into LP CD11c$^+$ DCs (120). Further analysis is needed to reveal the detailed mechanism by which luminal bacteria stimulate immune cells underneath IECs.

Recent single cell analysis of IECs has uncovered heterogeneous gene expression, even in each subset of IECs (22). As discussed in this review, IECs are located at the interface between the luminal environment and host immune cells, which are stimulated by both of them. In addition, IECs transmit inside-out signals to luminal bacteria and outside-in signals to immune cells. This bidirectional stimulation of IECs may be one reason that each subset of IECs displays complicated gene expression patterns. In particular, specific commensal bacteria colonize their appropriate areas of the intestines and modulate epithelial physiology and immune responses. The gnotobiote system *in vivo* and development of commensal bacteria and organoid coculture and/or organ culture systems *in vitro* may provide useful information regarding how IECs respond to these luminal bacteria. Identification of these mechanisms is essential to better understand the host-microbiota interface and functional diversity of intestinal ECs. Furthermore, understanding of the role of IECs as a transmitter of luminal and immune signals is important for development of strategies to prevent bowel diseases including IBD, colonic cancer, and infection.

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The reviewer KK is currently co-organizing a Research Topic with one of the authors YG, and confirms the absence of any other collaboration.

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