

## TOPIC HIGHLIGHT

Emiko Mizoguchi, MD, PhD, Series Editor

# Mucosal cytokine network in inflammatory bowel disease

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## Abstract

Inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are characterized by ongoing mucosal inflammation in which dysfunction of the host immunologic response against dietary factors and commensal bacteria is involved. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. This permits easy access for the luminal microbiota and dietary antigens to cells resident in the lamina propria, and stimulates further pathological immune cell responses. Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells. The clinical efficacy of targeting TNF- $\alpha$  clearly indicates that cytokines are the therapeutic targets in IBD patients. In this manuscript, we focus on the biological activities of recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32], which might play a role through interaction with TNF- $\alpha$  in the pathophysiology of IBD.

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## INTRODUCTION

Inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are chronic intestinal disorders of unknown etiology<sup>[1,2]</sup>. The most widely accepted hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria in genetically susceptible individuals<sup>[1,3,4]</sup>. Environmental factors may also precipitate the onset or reactivation of this disease<sup>[1,3,4]</sup>. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. Abnormal bacterial killing based on genetic factors such as the *NOD2* gene mutation also induces mucosal damage. The easy access for the luminal microbiota and dietary antigens into cells resident in the lamina propria thus stimulates pathological immune cell responses.

Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial and mesenchymal cells<sup>[5]</sup>. The clinical efficacy of targeting TNF- $\alpha$  clearly indicates that cytokines are one of the therapeutic targets of chronic inflammatory disorders such as IBD. Infliximab, a mouse/human chimeric mAb that binds to TNF- $\alpha$ , has shown efficacy in the treatment of moderate-to severe CD and a number of CD-related complications<sup>[6-8]</sup>. Although the precise mechanism of the action of infliximab is not clear, it neutralizes soluble TNF- $\alpha$ , alters cell signaling, and induces the apoptosis of activated inflammatory cells expressing surface TNF- $\alpha$  *via* multiple pathways. On the other hand, some patients are resistant to anti-TNF- $\alpha$  therapy. In such patients, the disease might be driven by molecular mechanisms dependent on other cytokines distinct from TNF- $\alpha$ . In this manuscript, we focused on recently-reported cytokines [Interleukin (IL)-17 cytokine family, IL-31 and IL-32] which might play a role through interactions with TNF- $\alpha$  in the pathophysiology of IBD.

## IL-17 CYTOKINE FAMILY

### IL-17 family and Th17 cells

The interleukin (IL)-17 cytokine family is a group of T cell derived cytokines. IL-17A was originally cloned by Rouvier *et al.*<sup>[9]</sup> and named CTLA8. It was subsequently renamed IL-17, and was more recently termed IL-17A. IL-17A stimulates various cell types to secrete various cytokines and chemokines, resulting in the induction of inflammation<sup>[10-15]</sup>. The IL-17 family may play a role in a number of diseases mediated by abnormal immune responses, such as rheumatoid arthritis<sup>[16,17]</sup>, pulmonary disease<sup>[15,18]</sup>, lupus<sup>[19]</sup>, multiple sclerosis<sup>[20]</sup>, and IBD<sup>[21]</sup>. Homology-based cloning recently revealed five additional members of the IL-17 family, termed IL-17B to IL-17F<sup>[10]</sup>. Among the IL-17 family members, IL-17F has the highest degree of homology with IL-17A (40% to 55%), followed by IL-17B (29%), IL-17D (25%), IL-17C (23%), and IL-17E (also named IL-25) is the most distantly related (17%)<sup>[10]</sup>. The major cellular source of IL-17A was initially described as activated CD4+ memory T lymphocytes. But, it was subsequently demonstrated that CD8+ memory T lymphocytes, eosinophils, neutrophils and monocytes can also produce IL-17A<sup>[10,22]</sup>. The cellular sources of IL-17B and IL-17C have not been identified. IL-17D is derived from resting CD4+ T cells and CD19+ B cells<sup>[15]</sup>. But, IL-17E (IL-25) expression is restricted to Th2 cells and mast cells<sup>[15]</sup>. The cellular source of the last member, IL-17F, has been reported to be activated CD4+ T cells, basophils and mast cells<sup>[15]</sup>.

One recent topic in immunology is the newly identified Th17 lineage of CD4+ T cells<sup>[23]</sup>. Th17 cells are characterized by the production of a distinct profile of effector cytokines, including IL-17A, IL-17F, IL-6, IL-22 and IL-26, and have probably evolved to enhance immune and host defense responses distinct from those targeted by Th1 and Th2 cells<sup>[24-26]</sup>. Th17 cells develop from naïve CD4 T cell precursors in the presence of IL-6 and TGF- $\beta$ , and full differentiation to Th17 cells is dependent on IL-23<sup>[23]</sup>. Recent studies demonstrated a role for IL-21 in Th17 development<sup>[24,27]</sup>. IL-21 serves as an autocrine factor secreted by Th17 cells that promotes or sustains the Th17 lineage commitment. On the other hand, Th1 cells develop from naïve CD4 T cell precursors in the presence of IFN- $\gamma$ , whereas Th2 cells develop under the control of IL-4. Both IFN- $\gamma$  and IL-4 inhibit Th17 cell proliferation<sup>[28]</sup>. A recent study showed that the proliferation of Th17 cells is also inhibited by IL-27, an IL-12-related cytokine frequently present at sites of inflammation<sup>[29]</sup>. Th17 cells are characterized as a source of IL-17A and IL-17F, and much attention has been focused on their functions in normal and pathological immune responses.

We previously demonstrated that IL-17A-positive cells were increased in the inflamed mucosa of IBD patients<sup>[21]</sup>, and a recent study showed that IL-17F mRNA expression in the mucosa was elevated in CD patients<sup>[30]</sup>. These observations suggest that IL-17F as well as IL-17A might play a role in the inflammatory responses involved in the pathophysiology of IBD.

Table 1 IL-17A-induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL1 (Gro- $\alpha$ )	NM_001511.1	× 26.11
IL-20 receptor, alpha	NM_014432.1	× 15.22
CCR5	NM_000579.1	× 12.75
CXCL6 (GCP-2)	NM_002993.1	× 12.45
CXCL3 (Gro- $\gamma$ )	NM_002090.1	× 11.70
MMP-16	NM_005941.2	× 10.41
CXCL2 (Gro- $\beta$ )	NM_002089.1	× 9.75
IRAK3	NM_007199.1	× 9.06
IL-8	NM_000584.2	× 8.91
IL-22 receptor 1	NM_021258.1	× 8.22
MMP-1	NM_004142.1	× 6.35
Complement component 3	NM_000064.1	× 5.56
IL-6	NM_000600.1	× 4.63
Carbonic anhydrase XII	NM_001218.1	× 4.42
Superoxide dismutase 2	NM_000636.1	× 4.25
CCL19 (MIP-3 $\beta$ )	NM_006274.2	× 3.74
CCL7 (MCP-3)	NM_006273.2	× 2.77
CCL2 (MCP-1)	NM_002982.2	× 2.24

Human colonic subepithelial myofibroblasts were stimulated with IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip (Takara-Bio, Kyoto, Japan). The fold change values were determined as a ratio of Cy5 signal intensity (IL-17A stimulated values)/Cy3 signal intensity (non-stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

### Inflammatory responses induced by IL-17A and IL-17F

IL-17A promotes the expansion and recruitment of innate immune cells such as neutrophils, and also cooperates with TLR ligands, IL-1 $\beta$ , and TNF- $\alpha$  to enhance inflammatory reactions, and to stimulate the production of beta-defensins and other antimicrobial peptides<sup>[25,27]</sup>. Its receptor, IL-17RA, is ubiquitously expressed and shares many features with classical innate immune receptors such as shared intracellular tail motifs and convergence onto common inflammatory transcription pathways<sup>[31]</sup>. To investigate the genes altered in response to an IL-17A stimulus, we performed a cDNA microarray analysis in human colonic subepithelial myofibroblasts (SEMFs)<sup>[32]</sup>. Human colonic SEMFs are located immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed against the bottom of the epithelial cells<sup>[33,34]</sup>, and play a role in inflammation and wound healing in the intestine<sup>[33-36]</sup>. As shown in Table 1, IL-17A up-regulated several genes which have been reported to exert pro-inflammatory actions in the pathophysiology of acute and/or chronic inflammation. In particular, the induction of CXC-chemokines mRNA expression suggests that IL-17A is a potent inducer of innate immune responses *via* the accumulation and activation of neutrophils in the mucosa.

Recently, we found that among the IL-17 family members, IL-17F also strongly induced the secretion of inflammatory cytokines (IL-6, IL-8 and LIF) and matrix metalloproteinases (MMP-1 and MMP-3) in human colonic SEMFs<sup>[32]</sup>. Like IL-17A<sup>[37]</sup>, IL-17F stimulated IL-6, IL-8 and MCP-1 secretion *via* NF- $\kappa$ B and MAP kinase activation in human colonic SEMFs. The IL-6,

**Table 2** IL-17A augmented TNF- $\alpha$ -induced genes in human colonic myofibroblasts

Gene name	Ref seq ID.	Fold increase
CXCL2 (Gro- $\beta$ )	NM_00002089.1	× 34.42
CXCL1 (Gro- $\alpha$ )	NM_001511.1	× 23.69
IL-6	NM_000600.1	× 16.92
CXCL3 (Gro- $\gamma$ )	NM_002090.1	× 15.20
IL-1 $\beta$	NM_000576.2	× 11.59
GM-CSF	NM_000758.2	× 9.11
IL-1 $\alpha$	NM_000575.3	× 8.45
Amphiregulin	NM_001657.2	× 6.61
CCL20	NM_004591.1	× 5.21
MMP-3	NM_002422.2	× 4.12
CCL7 (MCP-3)	NM_006273.2	× 3.96
IL-13R $\alpha$ 2	NM_000640.2	× 3.76
CXCL6 (GCP-2)	NM_002993.1	× 3.74
IL-11	NM_000641.2	× 3.70
IL-8 (CXCL8)	NM_000584.2	× 3.65
FGF2	NM_002006.2	× 3.12
MMP-1	NM_004142.1	× 2.49
CCL5 (RANTES)	NM_002985.2	× 0.43

Human colonic subepithelial myofibroblasts were stimulated with TNF- $\alpha$  (50 ng/mL) or TNF- $\alpha$  (50 ng/mL) plus IL-17A (200 ng/mL) for 12 h, and the changes in gene expression were assessed by IntelliGene HS Human Expression Chip. The fold change values were determined as a ratio of Cy5 signal intensity (TNF- $\alpha$  plus IL-17A stimulated values)/Cy3 signal intensity (TNF- $\alpha$  stimulated values). The data were average of three independent analysis. The NCBI reference sequence code was presented following the gene name.

IL-8 and LIF secretion by human SEMFs in response to IL-17F as well as IL-17A emphasizes the importance of Th17 products in the induction of intestinal inflammation. Furthermore, the induction of IL-6 secretion from colonic SEMFs has a particular significance in the regulation of immune responses, and in the pathophysiology of IBD; IL-6 has recently been identified as an indispensable factor for the development of Th17 cells<sup>[23]</sup>. IL-6 commits naïve CD4 T cell precursors to differentiate into Th17 cells<sup>[23]</sup>. IL-17A and IL-17F might function as potent stimulators for IL-6 production, suggesting an amplification loop for the local development and maturation of Th17 cells. Therefore, the IL-17-colonic SEMFs-IL-6 axis may be important for local Th17 development in the intestinal mucosa.

### IL-17A/IL-17F augments TNF- $\alpha$ -induced inflammatory responses

As more important observations, IL-17A and IL-17F augment the TNF- $\alpha$ -induced IL-6 secretion in human colonic SEMFs<sup>[32,37]</sup>. This augmentation of TNF- $\alpha$ -induced IL-6 production by IL-17A is mediated by enhanced stability of the IL-6 mRNA<sup>[37,38]</sup>. A similar molecular mechanism can be postulated for the augmentation by IL-17F. IL-17A/IL-17F also augments the TNF- $\alpha$ -induced expression of granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage (GM)-CSF in human colonic SEMFs<sup>[39]</sup>.

To further investigate the effects of IL-17A/IL-17F on TNF- $\alpha$ -induced genes, the alterations in gene expression were analyzed by cDNA microarrays in hu-

man colonic myofibroblasts. As shown in Table 2, IL-17A further enhanced the expression of various TNF- $\alpha$ -induced genes, such as IL-6, CXC-chemokines and CSFs. These observations suggest that the interactions between TNF- $\alpha$  and IL-17A/IL-17F potentially mobilized neutrophils, partially through granulopoiesis and CXC chemokine induction, as well as through increased survival locally. This interaction also potentially stimulated Th17 development through the stimulation of IL-6 secretion. Thus, a modulation of the immunological functions of colonic SEMFs by Th17-derived cytokines may be critical for the development of Th17 cells and the mucosal innate immune responses (Figure 1).

### Interactions between IL-17A and IL-22

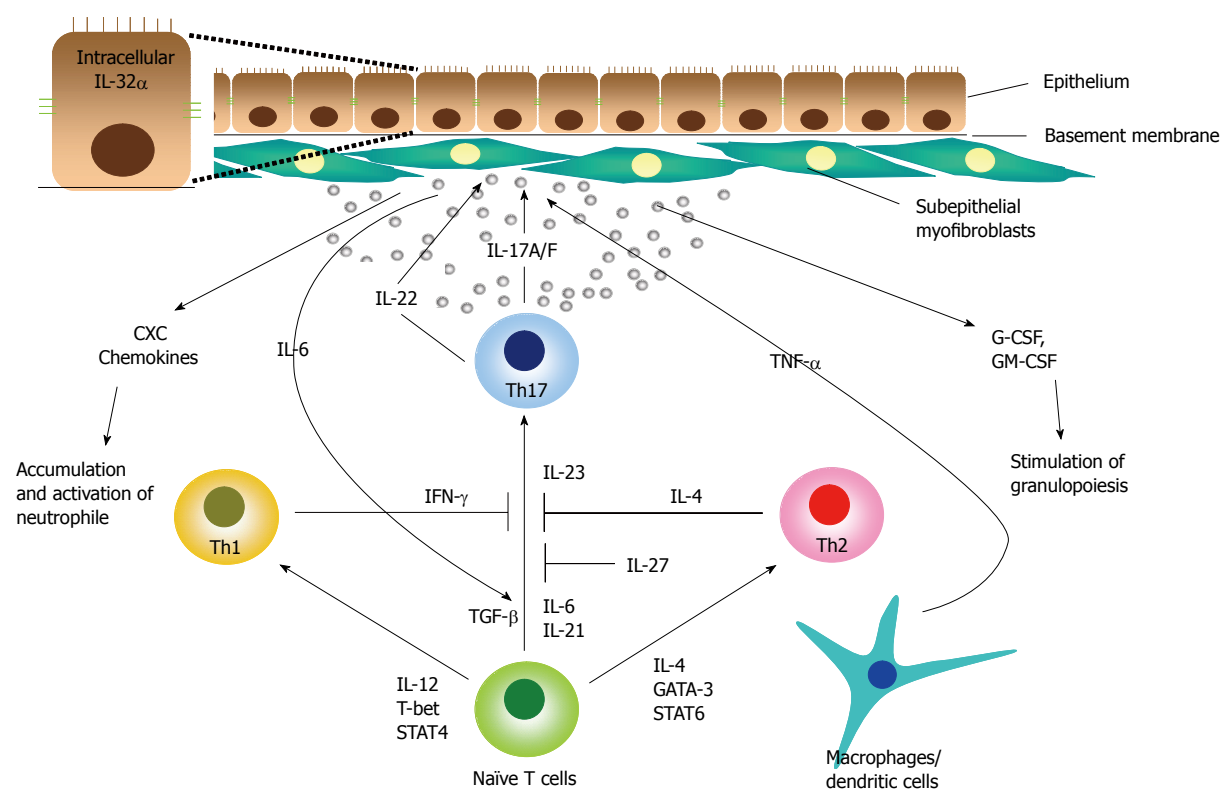
IL-22 was originally described as an IL-9-induced gene, and was termed "IL-10-related T cell-derived-inducible factor" (IL-TIF)<sup>[40,41]</sup>. IL-22 has 22% amino acid identity with IL-10, and belongs to a family of cytokines with limited homology to IL-10, namely IL-19, IL-20, IL-22, IL-24 and IL-26. The major sources of IL-22 are activated T cells, and IL-22 expression in other leukocyte populations such as monocytes, dendritic cells, NK cells and neutrophils is negligible. Recent studies have shown that Th17 cells are a source of IL-22<sup>[42,43]</sup>. We recently found that IL-22 expressing cells were increased in the inflamed mucosa of IBD patients<sup>[44]</sup>. In SEMFs, IL-22 upregulates the expression of inflammatory genes such as IL-6, IL-8, IL-11 and LIF *via* NF- $\kappa$ B, AP-1 and MAP-kinase dependent pathways<sup>[44]</sup>. Furthermore, the combination of IL-17A plus IL-22 showed an additive effect on transcription factor activation. These concerted responses were also observed as additive effects on cytokine mRNA expression and protein secretion. Thus, the cooperation between Th17 derived cytokines such as IL-17A and IL-22 may play an important role in the pathophysiology of IBD.

## IL-31

### IL-31 has a Th2 cell origin

IL-31 was cloned, and then found to be mainly produced by CD4+ T cells<sup>[45]</sup>, in particular by skin-homing CD45RO+ (memory) T cells. Transgenic mice overexpressing IL-31 either with a lymphocyte-specific promoter or a ubiquitous promoter exhibit a skin phenotype closely resembling atopic dermatitis in human subjects<sup>[45]</sup>. In these mice, IL-31 seems to be preferentially produced by T cells skewed towards a Th2 phenotype; however, these Th1-skewed T cells also produce substantial amounts of IL-31<sup>[45]</sup>. IL-31 mRNA expression is widely detected in various organs, including the gastrointestinal tract<sup>[45]</sup>.

IL-31 is most closely related to the family of IL-6-type cytokines known to be involved in many immunomodulatory functions, particularly the acute-phase response, but also in the proliferation of B and T cells<sup>[46]</sup>. A recent study indicated that IL-31 sustains the survival of hematopoietic stem cells, and contributes to effects on the cycling and numbers of hematopoietic stem cells



**Figure 1** Mucosal cytokine network mediated by IL-17A/F and TNF- $\alpha$ . TGF- $\beta$  induces the differentiation of Th17 cells from naïve CD4<sup>+</sup> T cells in the presence of IL-6, and full differentiation to Th17 cells is dependent on IL-23. The transcriptional factors required for the development of Th1 (STAT4 and T-bet) and Th2 (STAT6) cells are not required for the induction of Th17/Th17 cells. IFN- $\gamma$  also suppresses the differentiation of Th17 cells. IL-4 inhibits the development of Th17 cells. One of the important roles of IL-17A/F is the augmentation of the TNF- $\alpha$ -induced secretion of IL-6, a crucial factor for Th17 cell development.

*in vivo*<sup>[47]</sup>. However, IL-31 is clearly distinct from the IL-6-type cytokines because it does not signal through glycoprotein-130 (GP-130), the common signaling receptor subunit. IL-31 uses an earlier described orphan receptor, the glycoprotein 130-like monocyte receptor or glycoprotein 130-like receptor (GPL)<sup>[48]</sup>, in combination with the oncostatin M receptor (OSMR)<sup>[45]</sup>, which is expressed on epithelial cells and keratinocytes<sup>[45,49]</sup>.

IL-31 binds directly to the GPL, and OSMR mainly plays a role in delivering the signaling information into the cells. GPL and OSMR are widely expressed in various tissues, including the gastrointestinal tract. This suggests a role for IL-31 in the immune and inflammatory responses of the intestine. In response to IL-31, its receptor complex recruits the Jak1, Jak2, STAT-1, STAT-3 and STAT5 signaling pathways, as well as the PI3-kinase/AKT cascade<sup>[49]</sup>. SHP-2 and Shc adaptor molecules are also recruited, and contribute to an increased activation of the MAP kinase pathway in response to IL-31<sup>[49]</sup>. Despite the extensive study of intracellular signaling pathways activated by IL-31 stimulation, the cellular responses to IL-31 were barely investigated in any cell type.

### **IL-31 stimulates inflammatory responses in colon myofibroblasts**

To define the role of IL-31 in the intestinal mucosa, we investigated how IL-31 modulates mRNA expression in human colonic SEMFs. An analysis of the cDNA

microarrays indicated that IL-31 effectively induced the secretion of chemokines [CXCL8 (IL-8), CXCL1 (growth-related oncogene; GRO- $\alpha$ ), CCL7 (monocyte chemoattractant protein-3; MCP-3), CXCL3, CCL13, CCL15], proinflammatory cytokines (IL-6, IL-16 and IL-32), and matrix metalloproteinases (MMP-1, MMP-3, MMP-25 and MMP-7). The stimulatory effects of IL-31 were comparable to the effects of IL-17A. Furthermore, simultaneous stimulation with IL-31 and IL-17A showed additive effects on IL-6, IL-8, GRO- $\alpha$ , MCP-3, MMP-1 and MMP-3 secretion. Similar effects for IL-31 have been reported in bronchial epithelial cells<sup>[50]</sup>. In bronchial epithelial cells, IL-31 could significantly elevate both gene and protein expressions of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1/CCL2). The combination of IL-31 with either IL-4 or IL-13 further enhanced VEGF and CCL2 production. In these cells, IL-31 could activate p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK).

As mentioned above, IL-31 is a Th2 type cytokine, and these findings indicate that Th2 cells may be involved in the immune and inflammatory responses of the intestinal mucosa through IL-31 secretion. Since IL-31 and IL-17A stimulate the secretion of proinflammatory mediators in an additive manner, Th2-derived IL-31 and Th17-derived IL-17A cooperate in the pathophysiology of IBD.

## IL-32

### IL-32 cytokine family

IL-32 is a recently described cytokine produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells<sup>[51,52]</sup>. IL-32 is a proinflammatory cytokine originally described as a transcript termed NK4, found in activated natural killer (NK) cells and T lymphocytes<sup>[53]</sup>. Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that the epithelial cells are the dominant and widespread source<sup>[54]</sup>. The gene encoding IL-32 is located on human chromosome 16p13.3, and is organized into eight exons<sup>[55]</sup>. There are four splice variants (IL-32 $\alpha$ , IL-32 $\beta$ , IL-32 $\delta$  and IL-32 $\gamma$ ), and IL-32 $\alpha$  is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)- $\gamma$  in lung epithelial cells and monocytes<sup>[51]</sup>. IL-32 stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8 by *via* the activation of NF- $\kappa$ B and p38 mitogen-activated protein kinases (MAPKs)<sup>[51,52]</sup>. IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis<sup>[54,56-58]</sup>, mycobacterium tuberculosis infection<sup>[59,60]</sup>, and IBD<sup>[61]</sup>.

### Intracellular accumulation of IL-32

The amino acid sequence derived from the initial NK4 cDNA contained a signal peptide without a transmembrane domain<sup>[53,54]</sup>. But, the transcript was never expressed as a recombinant protein, and was not sequenced. Activated human T cells generate IL-32 with a molecular weight of 25 kDa, which on Western blotting analysis is found in the lysates rather than the supernatants. Similar findings were reported for 293T cells transfected with either IL-32 $\gamma$  or IL-32 $\beta$ <sup>[62]</sup>. In human peripheral blood mononuclear cells stimulated with ConA, most of the IL-32 was found in the lysates<sup>[51]</sup>. On the other hand, the overexpression of IL-32 $\alpha$  or IL-32 $\beta$  in COS cells resulted in secreted IL-32<sup>[51]</sup>. It remains unclear which isoforms are secreted from which particular cell type. Activated T cells and NK cells do not secrete IL-32, or alternatively, the secreted IL-32 is derived from apoptotic cells due to the presence of GAPDH in the same supernatants. These observations suggest that IL-32 is secreted only as a result of cell death<sup>[63]</sup>. Recently, we observed that in colon cancer cell lines, proinflammatory cytokines induce the intracellular accumulation of IL-32 $\alpha$ , but does not induce secretion<sup>[61]</sup>. Similar results were also observed in myofibroblasts isolated from the normal human pancreas<sup>[64]</sup>. Goda *et al* suggest that there is a role for intracellular IL-32 with cell death, since attenuating intracellular IL-32 levels resulted in decreased cell death<sup>[62]</sup>. These results also support the concept that high levels of intracellular IL-32 $\beta$  may induce cell death. One hypothesis is that the proinflammatory activity of IL-32 may act upon its release through cell death (apoptosis).

### Molecular mechanisms regulating IL-32 $\alpha$ induction

Shioya *et al* demonstrated that stimulation with IL-1 $\beta$ ,

IFN- $\gamma$  and TNF- $\alpha$  enhanced IL-32 $\alpha$  mRNA expression in three colon cancer cell lines<sup>[61]</sup>. TNF- $\alpha$  was the strongest among them. These factors also induced the intracellular accumulation of IL-32 $\alpha$ . Since transfection with the mutant form of I $\kappa$ B $\alpha$  inhibited the effects of both IL-1 $\beta$  and TNF- $\alpha$  on IL-32 $\alpha$  mRNA expression, NF- $\kappa$ B must play a role in IL-1 $\beta$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression.

Nishida *et al* analyzed IL-32 $\alpha$  expression in non-transformed myofibroblasts derived from the normal human pancreas<sup>[64]</sup>. IL-32 $\alpha$  mRNA was weakly expressed without any stimulus, and its expression was markedly enhanced by IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$ . IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  enhanced the intracellular accumulation of IL-32 $\alpha$  protein. But, IL-32 $\alpha$  was not detected in the supernatants. An inhibitor of phosphatidylinositol 3-kinase, (LY294002) significantly suppressed the IL-1 $\beta$ -, IFN- $\gamma$ - and TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blotting analysis. Furthermore, LY294002 suppressed both IL-1 $\beta$ - and TNF- $\alpha$ -induced NF- $\kappa$ B activation, as well as IL-1 $\beta$ -, TNF- $\alpha$ - and IFN- $\gamma$ -induced AP-1 activation. A blockade of NF- $\kappa$ B and AP-1 activation by an adenovirus expressing a stable mutant form of I $\kappa$ B $\alpha$  and a dominant negative mutant of c-Jun markedly suppressed the IL-1 $\beta$ -, IFN- $\gamma$ - and/or TNF- $\alpha$ -induced IL-32 $\alpha$  mRNA expression. Thus, they concluded that IL-32 $\alpha$  mRNA expression was dependent on interactions between the PI3K/Akt-pathway and the NF- $\kappa$ B/AP-1 system.

### IL-32 $\alpha$ and IBD

Shioya *et al* performed an immunohistochemical analysis to evaluate the expression of IL-32 $\alpha$  protein in the mucosa of IBD patients<sup>[61]</sup>. IL-32 $\alpha$  was weakly immunexpressed by epithelial cells in the normal colonic mucosa and samples of ischemic colitis. In contrast, the epithelial expression of IL-32 $\alpha$  was markedly enhanced in the inflamed region of active UC and CD patients<sup>[61]</sup>. In particular, IL-32 $\alpha$  expression tends to increase in samples from active CD patients. IL-32 $\alpha$  expression was barely detectable in leukocytes. Thus, these observations indicate that epithelial cells are the major expression site for IL-32 $\alpha$  in the intestinal mucosa, and that IL-32 $\alpha$  expression is enhanced in the IBD mucosa.

Netea *et al* recently demonstrated that IL-32 augments the production of IL-1 $\beta$  and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism<sup>[52]</sup>. NODs are a family of intracytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF- $\kappa$ B activation<sup>[65]</sup>. Mutations in NOD2 have been implicated in the pathogenesis of CD<sup>[66,67]</sup>, and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in terms of cytokine production<sup>[68,69]</sup>. Recently, it has been shown that NOD2 mutations in CD patients potentiate NF- $\kappa$ B activity

and IL-1 $\beta$  processing<sup>[70]</sup>. Thus, these findings suggest a pivotal role for IL-32 in the pathophysiology of IBD, and in particular CD. Since IL-32 $\alpha$  is a proinflammatory cytokine characterized by NF- $\kappa$ B and p38 MAPK activating activities<sup>[51,57]</sup> and because IL-32 acts synergistically with NOD ligands to induce proinflammatory cytokines<sup>[52]</sup>, the overexpression of IL-32 $\alpha$  in the IBD mucosa strongly suggests that it plays an important role in the inflammatory and antibacterial responses involved in the pathogenesis of IBD.

Recent studies have focused on the role of innate immunity in the pathogenesis of IBD<sup>[4]</sup>. The initial step of innate immunity is mediated by the recognition of pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs) and NOD proteins (NODs)<sup>[71]</sup>. TLRs are located mainly on cell-surface membranes, but NODs function as intracellular recognition systems<sup>[65,66]</sup>. In human monocytes, IL-32 acts synergistically with NOD specific peptidoglycans for the release of IL-1 $\beta$  and IL-6<sup>[52]</sup>. The synergistic effects of IL-32 and the NOD ligands on cytokine production is abolished in cells from CD patients bearing the NOD2 frameshift mutation 3020insC, indicating that this synergism between IL-32 plus MDP depends on NOD2<sup>[52]</sup>. Interactions between NOD-1 and IL-32 also potentiate proinflammatory cytokine production<sup>[52]</sup>. Furthermore, Berrebi *et al* previously reported the overexpression of NOD2 in infiltrated monocytes and epithelial cells in the IBD mucosa<sup>[72]</sup>. These observations suggest that overexpressed IL-32 may cause a specific and excessive stimulation of the NOD pathways, which leads to a marked amplification in IL-1 $\beta$  and IL-6 production in the IBD mucosa.

IL-32 was initially characterized as an inducer of TNF- $\alpha$  in circulating monocytes<sup>[51]</sup>, and hence inflammatory responses in the affected mucosa of IBD patients may be amplified by the consecutive loop of IL-32-induced TNF- $\alpha$  secretion from monocytes and TNF- $\alpha$ -stimulated IL-32 secretion from epithelial cells. This loop may be further amplified by the Th1 cytokine IFN- $\gamma$ . Previously, it has been reported that TNF- $\alpha$  and IFN- $\gamma$  synergistically induced the release of NOD2<sup>[73]</sup>, which supports the coupled regulation of IL-32 $\alpha$  and NOD2. The coupled regulation of IL-32 $\alpha$  with NOD2 may account for the rapid and efficient induction of innate immune responses at the intestinal mucosa. Furthermore, these data suggest that an amelioration of IBD symptoms by TNF- $\alpha$ -targeting therapies may be partially dependent on interference in the TNF- $\alpha$ -IL-32 loop.

The apoptosis of IECs is considered a normal biological function to eliminate damaged epithelial cells, and to restore epithelial cell growth, regulation, and epithelial integrity<sup>[74]</sup>. An overexpression of cytoplasmic IL-32 $\alpha$  might account for the induction of apoptosis in damaged epithelial cells at the inflamed mucosa of IBD patients, leading to an efficient elimination and the rapid induction of mucosal repair. Apoptosis caused by accumulated IL-32 can be considered a host defense mechanism against invading microorganisms, in which

damaged epithelial cells are efficiently eliminated along with the invading microorganisms, and thus any further invasion of the microorganisms can be blocked.

## CONCLUSION

In this review, we have summarized the newly reported cytokines which may play significant roles in the pathophysiology of IBD. An augmentation of TNF- $\alpha$  effects by IL-17A/F and a possible amplifying cascade between TNF- $\alpha$  and epithelial-derived IL-32 are of particular interest. The clinical efficacy of TNF- $\alpha$  blocking may be associated with an interruption of these cascades. The discovery of new cytokines and the determination of their biological activities may support the development of a novel therapeutic strategy for the treatment of IBD patients.

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