Interleukin-31 stimulates production of inflammatory mediators from human colonic subepithelial myofibroblasts

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Abstract. Interleukin (IL)-31 is mainly produced by $CD4^+$ T cells, in particular T cells skewed toward a Th2 phenotype. Here we report for the first time that IL-31 stimulates secretion of proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) from human colonic subepithelial myofibroblasts (SEMFs). The effects of IL-31 were investigated by cDNA microarrays, enzyme-linked immunosorbent assay, and real-time PCR. IL-31 effectively induced chemokines [IL-8, GRO-α (growth-related oncogene-α), MCP-3 (monocyte chemoattractant protein-3), CXCL3, CCL13 and CCL15], proinflammatory cytokines (IL-6, IL-16 and IL-32) and matrix metalloproteinases (MMP-1, MMP-3, MMP-25 and MMP-7). IL-31 dose-dependently induced secretion of IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3. The effects of IL-31 were comparable to the effects of IL-17A. IL-31 and IL-17A showed additive effects on IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3 secretion. In conclusion, we demonstrated that IL-31 is a potent inducer of proinflammatory mediators in human colonic SEMFs. IL-31 may function as a proinflammatory cytokine derived from Th2 cells.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tracts. Although the precise etiology of IBD remains unclear, a widely accepted hypothesis is that ubiquitous, commensal intestinal bacteria trigger an inappropriate, overactive, and ongoing mucosal immune response that mediates intestinal tissue damage in genetically susceptible individuals (1-3).

Recently, a novel cytokine, interleukin (IL)-31, was cloned and found to be produced mainly by $CD4^+$ T cells (4), in particular by skin-homing $CD45RO^+$ (memory) T cells. Transgenic mice overexpressing IL-31 either with a lymphocyte-specific promoter or a ubiquitous promoter experienced a skin phenotype closely resembling atopic dermatitis in human subjects (4). Notably, in mice IL-31 seems to be preferably produced by T cells skewed toward a Th2 phenotype; however, Th1-skewed T cells also produce substantial amounts of IL-31 (4). IL-31 mRNA expression is widely detectable in various organs, including the gastrointestinal tracts (4).

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 (5). However, IL-31 is clearly distinct from IL-6-type cytokines because it does not signal through glycoprotein-130 (GP-130), the common signaling receptor subunit. IL-31 uses a previously described orphan receptor, the glycoprotein 130-like monocyte receptor or glycoprotein 130-like receptor (GPL) (6), in combination with the oncostatin M receptor (OSMR) (4). Nevertheless, cellular responses induced by IL-31 remain unclear in all cell types.

Human colonic subepithelial myofibroblasts (SEMFs) are present immediately subjacent to the basement membrane in normal intestinal mucosa, juxtaposed against the bottom of epithelial cells (7,8). Colonic SEMFs play a role in inflammation and wound healing in the intestine (7-10). Here we report for the first time that IL-31 stimulates secretion of proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) in human colonic SEMFs, suggesting a role of IL-31 in the pathophysiology of IBD.

Materials and methods

Reagents. Recombinant human IL-31 and IL-17A were obtained from Pepro Tech (Rocky Hill, NJ).

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Abbreviations: IBD, inflammatory bowel disease; IL, interleukin; GRO, growth-related oncogene; MCP, monocyte chemoattractant protein; MMP, matrix-metalloproteinase; SEMF, subepithelial myofibroblast; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse-transcription polymerase chain reaction

Key words: interleukin-31, inflammatory mediators, colonic myofibroblasts

The isolation and culturing of human colonic SEMFs. Primary human colonic SEMFs were prepared according to a method described in our previous report (11). Cells were cultured in DMEM containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. Studies were performed on passage 3-6 SEMFs isolated from four surgically resected samples.

Quantification of human cytokines, chemokines and MMPs. Amounts of antigenic IL-6 and IL-8 in the samples were determined by sandwich ELISA kits purchased from Bio-Source (Camarillo, CA). The ELISA kit for matrix metalloproteinase (MMP)-1 was purchased from Amersham (Piscataway, NJ), MMP-3 was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan), and that for GRO-α and MCP-3 was purchased from R&D systems (Minneapolis, MN).

Chip hybridization. The total cellular RNA (4 μg) extracted from human colonic SEMFs was converted to double-strand cDNA with a double-strand cDNA synthesis kit (Invitrogen) and oligo(dT) primers containing a T7 RNA polymerase promoter (Takara-Bio, Kyoto, Japan). Cy3- and Cy5-labeled cRNAs were generated from cDNA samples by transcription with T7 RNA polymerase (Takara-Bio). Samples obtained from non-stimulated SEMFs were labeled with Cy3, and samples from cells stimulated with IL-31 for 6 h were labeled with Cy5.

We employed an IntelliGene HS human expression chip (Takara-Bio), which includes ~16,000 characterized sequences based on the human NCBI reference data. Cy3- and Cy5-labeled cRNA probes were mixed and hybridized under the following conditions: (6X SSC, 0.2% SDS, 5X Denhardt's solution, 50% formamide) at 70˚C for 14 h. Chips were scanned with an Affymetrix 428 array scanner (Santa Clara, CA). Data analyses were performed using BioDiscovery ImaGene ver. 4.2 (El Segundo, CA). For data analyses, globallowess normalizations were performed (12).

Reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (13). RT-PCR analyses were performed according to a method described in our previous report (14). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. Primers specific for human cytokines, chemokines and MMPs were as follows: human IL-6, 5'TGAGAAAGGAGACA TGTAAC (nucleotides 262-282, Gene bank accession no. 000600) and 3'AGTGTCCTAACGCTCATACT (824-803); human IL-8, 5'ACATGACTTCCAAGCTGGCC (nucleotides 101-121, Gene bank accession no. 000584) (15) and 3'TTT TATGAATTCTCAGCCCT (404-385); human MMP-1, 5'A GATTTGCCAAGAGCAGATGT (nucleotides 437-458, Gene bank accession no. 002421) and 3'TCCATATATGGC TTGGATGCC (852-831); human MMP-3, 5'AACCTTTCC TGGCATCCCGAA (nucleotides 303-323, Gene bank accession no. 002422) and 3'CAGCCAACTGTGATC CTGCTT (880-860); human GRO-α, 5'CTCTTCCGCTCCT CTCACAG (nucleotides 35-54, Gene bank accession no. 001511) and 3'CACCAGTGAGCTTCCTCCTC (431-412); and human MCP-3, 5'ACCACCAGTAGCCACTGTCC (nucleotides 230-249, Gene bank accession no. 006273) and 3'TTCAAAACCCACCAAAATCC (581-562). Real-time PCR was performed using LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan) with specific primers as described above. PCR was conducted using a SYBR-Green PCR Master mix (Applied Biosystems, Foster city, CA). Data were normalized versus ß-actin for human IL-31.

Statistical analyses. Data are expressed as means ± SD. Statistical significance of changes was determined by the Mann-Whitney U test. Differences resulting in P values <0.05 were considered to be statistically significant.

Results

Microarray analyses of colonic SEMFs with IL-31. To define the role of IL-31 in the intestinal mucosa, we investigated how IL-31 modulates mRNA expression in human colonic subepithelial myofibroblasts (SEMFs). For this purpose, we engaged cDNA microarrays. Judging by the criteria of a >3-fold increase, the expression of 20 genes was altered by IL-31 stimulation (500 ng/ml, 12 h) (Table I). Each of three independent examinations demonstrated that IL-31 effectively induced chemokines [CXCL8 (IL-8), CXCL1 (growth-related oncogene; GRO-α), CCL7 (monocyte chemo-

Table I. IL-31-induced genes in human colonic myofibroblasts.

Gene name	NCBI reference sequence ID	Fold change value
CXCL8 (IL-8)	NM_000584.2	24.67
CXCL1 (GRO-α)	NM_001511.1	21.39
CCL7 (MCP-3)	NM_006273.2	18.15
MMP-3	NM_002422.2	14.63
MMP-1	NM_004142.1	12.92
Toll-like receptor 8	NM_016610.2	10.78
CXCL3 (GRO-γ)	NM_002090.1	8.67
IL-6	NM_000600.1	8.63
Toll-like receptor 10	NM_030956.1	7.78
Caveolin 2	NM_001233.2	6.61
CCL13 (MCP-4)	NM_005408.2	4.85
MMP-25	NM_022468.3	4.51
Caveolin-1	NM_001753.3	4.42
CCL15 (Leukotactin-1)	NM_004167.2	4.32
TIMP-3	NM_000362.3	3.99
CCL2 (MCP-1)	NM_002982.2	3.80
IL-16	NM_172217.1	3.35
MMP-7	NM_002423.2	3.35
IL-32	NM_004221.2	3.02
SOD2	NM_000636.1	3.02

Human colonic subepithelial myofibroblasts were stimulated with IL-31 (500 ng/ml) for 12 h, and the changes in gene expression were assessed by IntelliGene HS human expression chip (Takara-Bio). The fold change values were determined as a ratio of Cy5 signal intensity (IL-31-stimulated values)/Cy3 signal intensity (non-stimulated values). The data were the averages of three independent analyses.

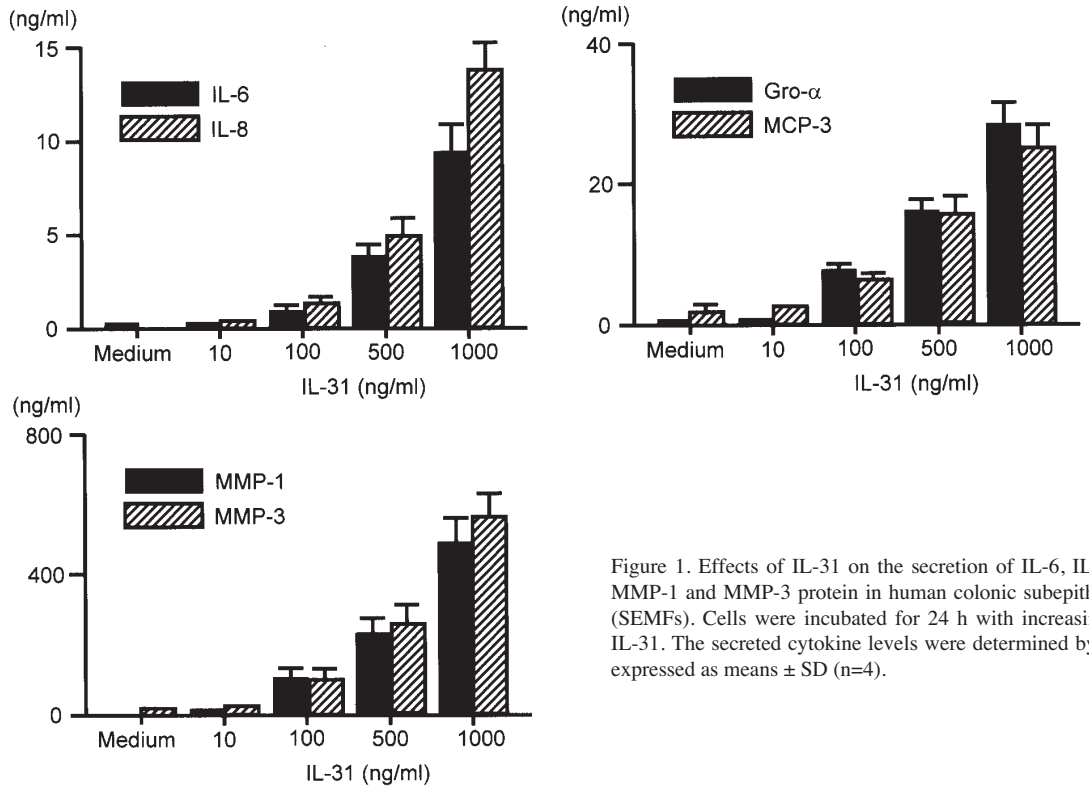

Figure 1. Effects of IL-31 on the secretion of IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3 protein in human colonic subepithelial myofibroblasts (SEMFs). Cells were incubated for 24 h with increasing concentrations of IL-31. The secreted cytokine levels were determined by ELISA. Values are expressed as means ± SD (n=4).

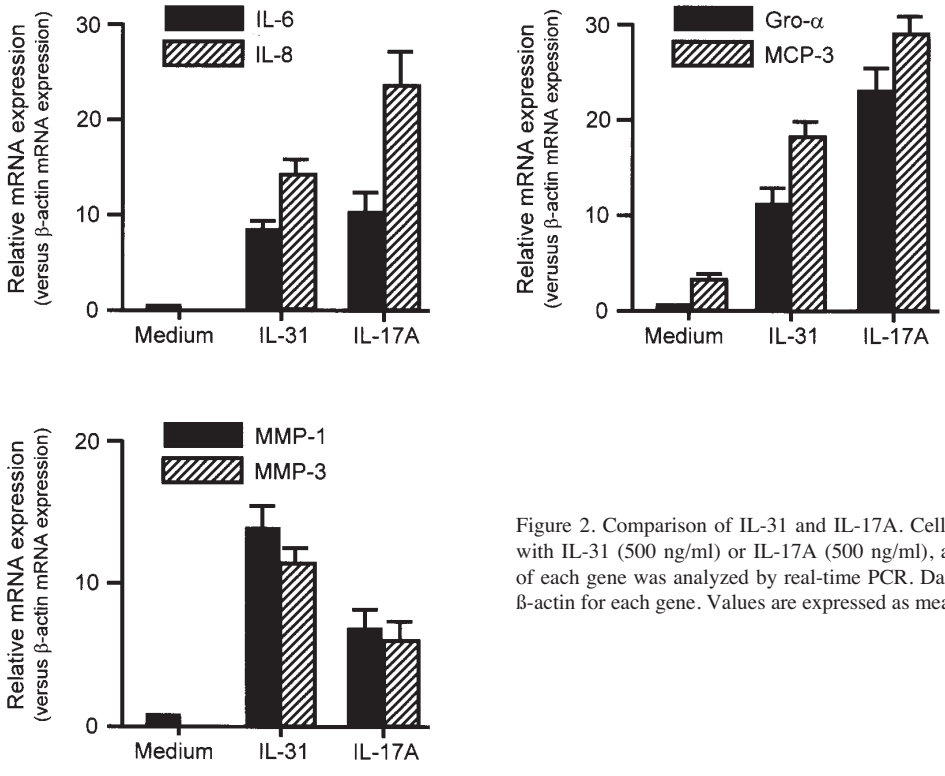

Figure 2. Comparison of IL-31 and IL-17A. Cells were incubated for 12 h with IL-31 (500 ng/ml) or IL-17A (500 ng/ml), and the mRNA expression of each gene was analyzed by real-time PCR. Data were normalized versus ß-actin for each gene. Values are expressed as means ± SD (n=4).

attractant protein-3; MCP-3), CXCL3, CCL13, and CCL15]; pro-inflammatory cytokines (IL-6, IL-16, and IL-32), and matrix metalloproteinases (MMP-1, MMP-3, MMP-25, and MMP-7).

Effects of IL-31 on secretion of cytokines, chemokines and MMPs. Among induced genes, we confirmed the effects of IL-31 on secretion of IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3. As shown in Fig. 1, IL-31 dose-dependently induced secretion of these factors. Stimulatory effects of IL-31 were detectable at 100 ng/ml.

Comparison of proinflammatory effects of IL-31 with effects of IL-17A. Previously, we demonstrated that IL-17A, a

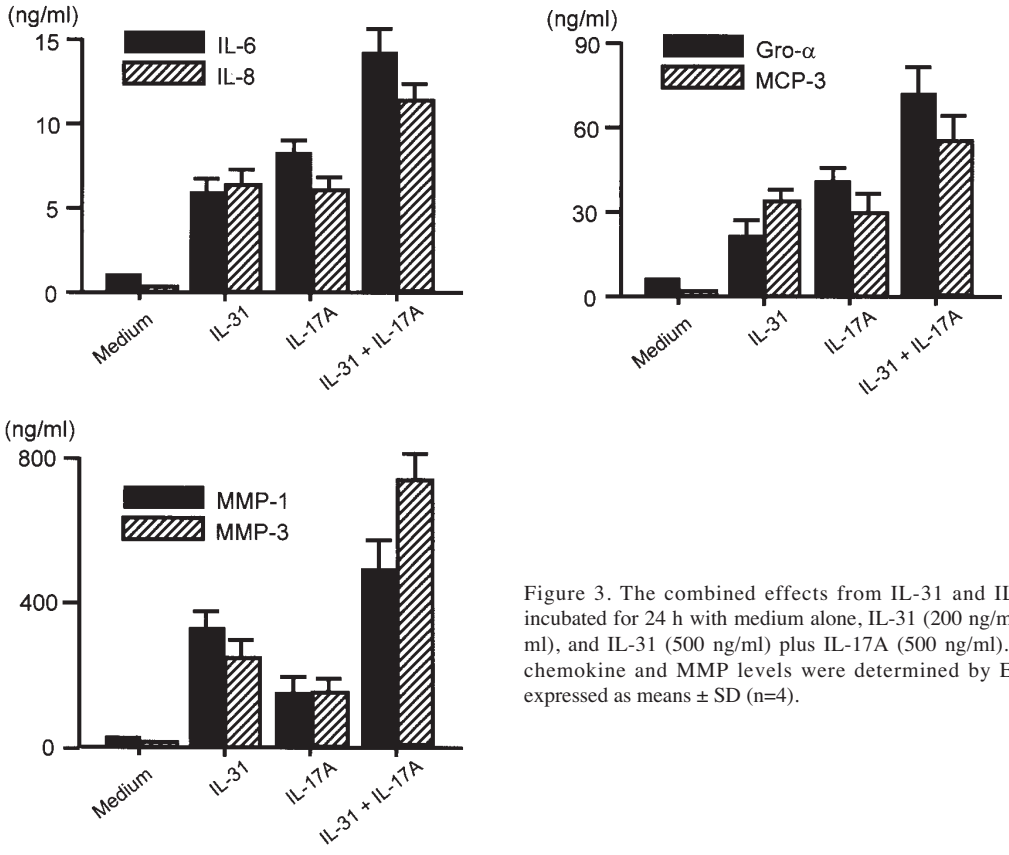

Figure 3. The combined effects from IL-31 and IL-17A. Cells were incubated for 24 h with medium alone, IL-31 (200 ng/ml), IL-17A (200 ng/ml), and IL-31 (500 ng/ml) plus IL-17A (500 ng/ml). Secreted cytokine, chemokine and MMP levels were determined by ELISA. Values are expressed as means ± SD (n=4).

proinflammatory cytokine produced by Th17 $CD4^+$ T cells (16), stimulated IL-6, IL-8 and MMP-3 secretion in human colonic SEMFs (17,18). To compare the effects of IL-31 with those of IL-17A, we used the real-time PCR method. As shown in Fig. 2, IL-17A also induced the mRNA expression of IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3 in these cells. Stimulatory effects of IL-31 were comparable to the effects of IL-17A (Fig. 2).

Combination of IL-31 plus IL-17A. Next, we tested the combined effects of IL-31 and IL-17A in human colonic SEMFs. As shown in Fig. 3, simultaneous stimulation with IL-31 and IL-17A showed additive effects on IL-6, IL-8, GRO-α, MCP-3, MMP-1 and MMP-3 secretion.

Discussion

Accumulating evidence indicates that IBD is a T-cell-mediated disease. For example, we previously reported an increase in the number of infiltrating T cells expressing IL-17 and/or IL-22 in IBD mucosa (19,20). Mucosa-infiltrating effector T cells represent histopathological hallmarks of IBD, and T cell-derived cytokines play a crucial role in the pathophysiology of IBD.

Mice modified to overexpress IL-31 developed severe pruritis, alopecia and skin lesions, indicating the important immune functions of IL-31 in skin diseases. IL-31 is expressed by Th2 cells, and it signals through a heterodimeric receptor composed of GP130-like receptor (GPL) and oncostatin M receptor (OSMR), which is expressed on epithelial cells and keratinocytes (4,21). IL-31 directly binds to GPL, and OSMR mainly plays a role in the delivering of signaling information into cells. In response to IL-31, its receptor complex recruits Jak1, Jak2, STAT-1, STAT-3, and STAT5 signaling pathways, as well as the PI3-kinase/AKT cascade (21). SHP-2 and Shc adapter molecules are also recruited and contribute to an increased activation of the MAP kinase pathway in response to IL-31 (21). Despite the extensive study of intracellular signaling pathways activated by IL-31 stimulation, cellular responses to IL-31 have been scarcely investigated in any cell type. This may be due to a limited number of published reports concerning IL-31.

Sonkoly *et al* (36) previously demonstrated that GPL and OSMR are widely expressed in various tissues including the gastrointestinal tract. This suggests a role of IL-31 in immune and inflammatory responses in the intestine. In this study, we investigated how IL-31 modulates gene expression in human colonic SEMFs using cDNA microarray analyses, and found that IL-31 induces various genes which play major roles in the pathophysiology of IBD. We confirmed some of these responses by real-time PCR and ELISAs. Given the data presented herein, one hypothesis is that IL-31 is a pro-inflammatory cytokine derived from Th2 T cells.

IL-31 stimulated IL-6 gene expression in human colonic SEMFs. IL-6 is a pleiotropic cytokine with many pathophysiologic roles in humans (22). Its role in the pathophysiology of IBD was shown by the effects of anti-IL-6R antibodies to eliminate clinical symptoms of CD patients (23-25). IL-31 also up-regulated chemokine (IL-8, GRO-α, GRO-γ, MCP-1, MCP-3, MCP-4, CCL-15) gene expression in colonic SEMFs. A histological feature of IBD is characterized by marked accumulation of inflammatory cells such as

granulocytes and monocytes. IL-31 may contribute to granulocyte and monocyte accumulation in the intestinal mucosa via stimulation of chemokine secretion. Furthermore, IL-31 induced MMP (MMP-1, MM-3, MMP-7 and MMP-25) mRNA expression in these cells. MMPs are a family of calcium-dependent neutral proteases which orchestrate the developmental and homeostatic remodelling of the extracellular matrix (ECM) (26). ECM degradation is mainly dependent on the local release of MMPs, and increased secretion of MMPs results in tissue destruction. Collectively, these responses induced by IL-31 in human colonic SEMFs indicate that Th2 T cells may be involved in immune and inflammatory responses in intestinal mucosa through IL-31 secretion.

The IL-17 cytokine family is a recently discovered group of cytokines. IL-17A was originally cloned by Rouvier *et al (*27) and named CTLA8, subsequently renamed IL-17, and more recently IL-17A. IL-17A stimulates various cell types to secrete various cytokines and chemokines, resulting in the induction of inflammation (28-33). The IL-17 family may play a role in a number of diseases mediated by abnormal immune responses, such as rheumatoid arthritis (34,35) and IBD (19). In this study, to characterize the inflammatory potential of IL-31, we compared proinflammatory effects of IL-31 with those of IL-17A. As shown in Fig. 2, IL-31 and IL-17A comparably induced proinflammatory genes in human colonic SEMFs. Furthermore, IL-31 and IL-17A additively stimulated secretion of proinflammatory mediators. Thus, Th2-derived IL-31 and Th17-derived IL-17A may cooperate in the pathophysiology of IBD.

In conclusion, we demonstrated that IL-31 is a potent inducer of proinflammatory mediators from human colonic SEMFs. To date, the pathological role of IL-31 was solely reported in the field of dermatology. However, our findings presented in this study suggest a role of IL-31 in the inflammatory responses of the intestine. Further investigations to analyze IL-31 functions are required to identify the role of IL-31 in the pathogenesis of IBD.

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