

The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity

Zhanju Liu,^{*,1} Praveen K. Yadav,^{*} Xiaorong Xu,^{*} Jingling Su,^{*} Chi Chen,^{*} Maochun Tang,^{*} Hui Lin,^{*} Jifeng Yu,[†] Jiaming Qian,[‡] Ping-Chang Yang,[§] and Xingpeng Wang^{*}

^{*}Department of Gastroenterology, The Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, China; [†]Laboratory of Molecular Medicine, Department of Bioengineering, Zhengzhou University, Zhengzhou, China; [‡]Department of Gastroenterology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China; and [§]Department of Pathology and Molecular Medicine, McMaster Brain Body Institute, St. Joseph's Healthcare, McMaster University, Hamilton, Ontario, Canada

RECEIVED AUGUST 12, 2010; REVISED DECEMBER 12, 2010; ACCEPTED DECEMBER 14, 2010. DOI: 10.1189/jlb.0810456

ABSTRACT

This study analyzed IL-23p19 expression in inflamed mucosa of IBD and the role in the induction of IEL and NK cell activation as well as Th17 cell differentiation. Expression of IL-23p19 was performed by immunohistochemistry and quantitative real-time PCR. Expression of IL-23R was assessed by flow cytometry. Cytolytic activities of IEL and NK cells by IL-23 were determined by a standard ⁵¹Cr-release assay. Cytokine levels were analyzed by ELISA and quantitative real-time PCR. Expression of IL-23p19 was increased significantly in inflamed mucosa of CD compared with that in UC and healthy controls. Double-staining confirmed that IL-23p19⁺ cells were mainly CD68⁺ macrophages/DCs. IL-23R⁺ cells were increased significantly in PB- and LP-CD4⁺ and -CD8⁺ T and NK cells. IL-23 markedly promoted IBD IEL and NK cell activation and cytotoxicity and triggered IBD PB- and LP-T cells to secrete significantly higher levels of IFN- γ , TNF, IL-2, and IL-17A compared with controls. Importantly, IL-23 promoted IBD PB- or LP-CD4⁺ T cells to differentiate into Th17 cells, characterized by increased expression of IL-17A and RORC. Anti-TNF treatment could markedly reduce IL-23 expression and Th17 cell infiltration in inflamed mucosa of CD patients. These data indicate that IL-23 is highly expressed in inflamed mucosa of IBD and plays an important role in the induction of IEL, NK, and T

cell activation, proinflammatory cytokine secretion, and Th17 cell differentiation. Targeted therapy directed against IL-23p19 may have a therapeutic role in treatment of IBD. *J. Leukoc. Biol.* **89**: 597–606; 2011.

Introduction

Idiopathic IBD as well as CD and UC are chronic inflammatory disorders of the gastrointestinal tract. Although their exact etiology is still not understood completely, increasing data have demonstrated that dysregulation of the mucosal immune response toward the resident bacterial flora, together with genetic and environmental factors, plays an important role in the pathogenesis of human IBD [1, 2]. Alterations of the innate immune response contribute to disease development, as identified by the mutations in the Nod2 gene, which is considered a susceptible gene in some CD patients. Intestinal luminal bacterial antigens recognized by TLR and Nod are essential for the induction of mucosal inflammation, resulting in an exaggerated adaptive immune response, which leads to extensive bowel damage [3, 4]. Evidences have implicated that large numbers of leukocyte infiltrates are present in inflamed mucosa of IBD and that these leukocytes, as well as intestinal stromal cells, produce profound proinflammatory cytokines (e.g., IFN- γ , TNF, IL-1 β , IL-6, IL-12, IL-15) and express high levels of adhesion molecules (e.g., CD54, CD62 ligand) and chemokine receptors (e.g., CCR5, CCR9) [1–4].

IL-23 and IL-12 are members of a small family of proinflammatory heterodimer cytokines, sharing a common p40 subunit covalently linked to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23 [5]. IL-12R is comprised of an IL-12R β 1

Abbreviations: ABI=Applied Biosystems, Inc., CD=Crohn's disease, EAE=experimental allergic encephalomyelitis, IBD=inflammatory bowel disease, IEL=intraepithelial lymphocyte, LP=lamina propria, LPMC=lamina propria mononuclear cells, Nod=nucleotide-binding oligomerization domain, PB=peripheral blood, ROR=retinoic orphan receptor, UC=ulcerative colitis

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. Correspondence: Department of Gastroenterology, The Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China. E-mail: zhanjuliu@yahoo.com

and IL-12R β 2 subunit, whereas the receptor for IL-23 consists of the IL-12R β 1 subunit and a novel component termed IL-23R [6], which is expressed predominantly on T, NK, and NKT cells and to a smaller extent, on monocytes, macrophages, and DCs. After binding to the IL-23R, IL-23 preferentially induces memory T cell activation. Evidence has demonstrated that IL-23 exhibits some similar biological activities to IL-12. However, in comparison with IL-12 with profound induction of the Th1 immune response, as well as promotion of cytotoxic, antimicrobial, and anti-tumor responses, IL-23 is found to play a critical role in the maintenance of immune response by controlling T cell memory function and by influencing the proliferation and survival of IL-17-producing Th17 cells [7, 8]. Moreover, recent work has also shown that IL-23 could induce naïve CD4⁺ T cells to secrete IL-22, indicating that IL-23 is also associated with the differentiation of naïve CD4⁺ T cells [9].

Accumulating data have demonstrated that IL-23 plays a crucial role in the pathogenesis of a number of immune-mediated inflammatory diseases by recruitment of several inflammatory cells and Th17 cells [10, 11]. Aberrant expression of IL-23p19 leads to fatal multiorgan inflammation [12], and deregulation of IL-23 production by basal keratinocytes results in the appearance of eczematous skin diseases [13]. IL-23 could induce pathogenic, IL-17-secreting T cells in EAE and collagen-induced arthritis [10, 14, 15]. In contrast, IL-23p19-deficient mice are resistant to the development of EAE and have reduced T cell-mediated DTH responses [14, 16]. These findings indicate that the IL-23/Th17 axis plays a prominent, proinflammatory role in the pathogenesis of EAE and chronic joint inflammation [17]. In addition, IL-23 has been found to promote tumor incidence and growth through up-regulation of inflammatory responses in the tumor environments (e.g., increase of the matrix metalloproteinase 9, IL-17-producing lymphocytes, and angiogenesis but decrease of cytotoxic activity of CD8⁺ T cells), suggesting that IL-23 is an important molecule link between tumor-promoting, proinflammatory processes and the failure of the adaptive immune surveillance to infiltrate the tumor [18].

Despite the widely recognized importance of IL-23 in the pathogenesis of autoimmune diseases, the precise mechanisms by which IL-23 is involved in the pathogenesis of IBD are still elusive. Previous studies with murine models have demonstrated a requirement for IL-23 in the development of intestinal mucosal inflammation [19]. IL-23 has been found to play a key role in *Helicobacter hepaticus*-induced murine colitis through an increase of IFN- γ and IL-17 secretion by mucosal T cells. Consistent with this, IL-23p19 transgenic mice develop severe systemic inflammation, including the terminal ileum and colon [12]. In contrast, knocking out the IL-23p19 subunit demonstrates a decrease of proinflammatory cytokines (e.g., TNF, IFN- γ , IL-6, and IL-17) and the presence of less-intestinal mucosal inflammation [19, 20]. Moreover, blockade of IL-23 in vivo using anti-IL-23p19 mAb or its inhibitor STA-5326 could inhibit chronic intestinal inflammation in a colitis model and down-regulate a Th1-mediated immune response [20–22]. Elevation of IL-23p19 transcript levels has been observed in inflamed mucosa of IBD, and its expression is corre-

lated with the severity of endoscopic lesions [23]. Recent work has demonstrated that myeloid DCs from mesenteric LNs of CD patients, when stimulated with exogenous microbial antigens in vitro, produce higher levels of IL-23p19 and lower levels of IL-10 than UC and healthy controls [24]. Using a whole genome association study or transmission disequilibrium test implies that IL-23R gene variants are also associated with CD [25, 26]. These evidences suggest that IL-23 may be involved in the intestinal mucosal immune response [27].

In this study, the pathogenic role of IL-23 in the induction of mucosal inflammation in IBD was investigated. We found that in situ expression of IL-23p19 was increased significantly in inflamed mucosa of IBD, especially in CD, and that IL-23p19 was mainly expressed in the CD68⁺ macrophage and/or DC. IL-23R expression was markedly increased in CD4⁺, CD8⁺ T cell, and NK cells from IBD patients. Importantly, IL-23 markedly induced IEL and NK cell activation and immune response, triggered mucosal CD4⁺ T cell activation to secrete proinflammatory cytokines (e.g., TNF, IFN- γ , IL-2, IL-17A), and particularly promoted the Th17 cell differentiation, as characterized by IL-17 and RORC expression. Anti-TNF treatment could markedly down-regulate IL-23 expression and Th17 cell infiltration in inflamed mucosa of CD patients.

MATERIALS AND METHODS

Patients and samples

Surgical specimens were obtained from the inflamed ileum ($n=5$) and inflamed colon ($n=6$) of 11 patients with active CD (seven male and four female; mean 28 years) and inflamed colon of 12 patients with active UC (five male and seven female; mean 38 years). Endoscopic biopsies were also taken from macroscopically inflamed ileum and colon of eight patients with active CD (four male and four female; mean 29 years) and 15 patients with active UC (nine male and six female; mean 40 years). IBD patients were diagnosed on the basis of clinical, radiological, endoscopic, and histological manifestations by established criteria. Of all IBD patients, eight were receiving sulfasalazine or an oral mesalazine (Dr. Falk Pharma GmbH, Freiburg, Germany) at the time of operation and endoscopic examination. None of them received any immunosuppressants and biological agents (e.g., Infliximab). In addition, nine ileal samples and nine colonic samples were collected from surgical specimens of 16 patients (seven men and nine women; mean 48 years) undergoing right hemicolectomy for carcinoma, which were macroscopically normal and confirmed by histology. Biopsy samples for immunohistochemical staining were embedded immediately in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA), snap-frozen in liquid nitrogen, and stored at -80°C until processed. Tissue samples for isolation of IEL and LPMC were transferred immediately to the lab in ice-chilled RPMI-1640 medium supplemented with 10% heat-inactivated FCS and antibiotics.

Blood samples were also obtained from outpatients with active CD and UC, including nine cases with CD (six men and three women; mean 30 years) and 16 cases with UC (nine men and seven women; mean 36 years). Only six patients were treated with sulfasalazine, and four patients received mesalazine. None received any immunosuppressants and biological agents. In addition, blood samples from 16 healthy donors (10 men and six women; mean 32 years) were taken for comparison. The project was approved by the University Hospital Review Committees for Clinical Research (Tongji University, Shanghai, China), and written, informed consent was obtained from all patients and volunteers.

Reagents

Rabbit anti-human IL-23p19 polyclonal antibody was a kind gift of Dr. Sampsa Matikainen (Department of Microbiology, National Public Health Institute, Helsinki, Finland) [28]. Biotinylated anti-human IL-23R antibody (goat IgG) and human rIL-23 were purchased from R&D Systems (Minneapolis, MN, USA). Mouse anti-human anti-CD3 mAb (OKT3, mouse IgG2a) was obtained from eBiosciences (San Diego, CA, USA). FITC- or PE-conjugated anti-CD3 (clone UCHT1, mouse IgG1), PE-conjugated anti-CD4 (clone RPA-T4, mouse IgG1), PE-conjugated anti-CD8 (clone RPA-T8, mouse IgG1), PE-conjugated anti-CD20 (clone 2H7, mouse IgG2b), PE-conjugated anti-CD14 (clone M5E2, mouse IgG2a), PE-conjugated anti-CD56 (clone B159, mouse IgG1), FITC-conjugated goat anti-mouse IgG mAb, and FITC- or PE-conjugated isotype-matched control mouse IgG mAb were purchased from BD Pharmingen (San Diego, CA, USA). FITC-conjugated streptavidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). OptEIA ELISA kits of human TNF, IFN- γ , IL-2, IL-10, and IL-17A were purchased from BD Pharmingen. Cell cultures were performed in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 50 μ M 2-ME (all purchased from Invitrogen, Carlsbad, CA, USA).

Lymphocyte isolation and cell culture

IEL, PBMC, PB-CD4⁺ T cells, NK cells, and LP-CD4⁺ T cells were isolated as described in detail in our previous work [29–31]. All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 50 μ M 2-ME.

Immunohistochemistry

Expression of IL-23p19 in intestinal mucosa was performed using an avidin-biotin-peroxidase technique as described previously [30, 31]. Briefly, cryostat sections (5 μ m) of colonic biopsies were fixed in ice-cold acetone for 10 min, and endogenous peroxidase activity was blocked for 30 min at room temperature with 0.3% H₂O₂ in PBS containing 3% skim milk. Serial sections were first incubated with rabbit anti-human IL-23p19 polyclonal antibody (1:100 dilution) for 30 min. After washing in PBS, the sections were incubated for 30 min with biotin-labeled goat anti-rabbit IgG (Dako, Glostrup, Denmark) at 1:400. After additional washes in PBS, the sections were incubated for 30 min with avidin-biotin-peroxidase complex (Dako). The color reaction was developed with 3,3'-diaminobenzidine. The slides were counterstained with hematoxylin. Negative control experiments were performed by incubating sections with irrelevant isotype-matched mouse IgG or by omitting the primary antibody. To determine the frequency of IL-23-positive cells, positively stained cells were counted by examining at least 10 random fields (magnification, $\times$ 200) in each group and expressed as the percentage of positive cells over total cell number.

PCR analysis

The analyses of IL-23p19 gene expression were performed using RT-PCR and real-time quantitative PCR as described previously [30, 32]. For RT-PCR analysis, total RNA was extracted from intestinal mucosal biopsies using TRIzol reagent (Invitrogen), and RNA concentration and the purity were measured by a spectrophotometer according to the manufacturer's instructions. A constant amount of 1 μ g target RNA was used for cDNA synthesis. The cDNA reaction was conducted at 42°C for 80 min, using 100 U Superscript II RT and 5 mM oligo(dT)₁₆. PCR amplification of cDNA was performed by adding 2.5 mM dNTP, 2.5 U TaqDNA polymerase, and 0.25 μ M sense and antisense primer pairs specific for human IL-23p19 (sense, 5'-GGA CAA CAG TCA GTT CTG CTT GC-3'; antisense, 5'-GGA GGC TGC GAA GGA TTT TG-3') and human β -actin as a control (sense, 5'-ATG GGT CAG AAG GAT TCC-3'; antisense, 5'-ATG TCA CGC ACG ATT TCC CGC-3'; Beijing AuGCT Biotechnology Co., Ltd., Beijing, China). The reactions were processed in a DNA thermal cycler (PerkinEl-

mer Cetus, Wellesley, MA, USA) through 30 cycles of 94°C for 40 s, 58.5°C for 30 s, and 72°C for 60 s. The predicted products for IL-23p19 and β -actin were 260 and 500 bp, respectively. The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

For quantitative real-time PCR analysis, total cellular RNA from intestinal mucosa or from PB- or LP-CD4⁺ T cells stimulated with immobilized anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml) in the absence or presence of IL-23 (20 ng/ml) for 2 days was extracted with TRIzol reagents. After RNA preparation, the cDNA was synthesized with a RT kit, according to the manufacturer's instruction (Invitrogen). Quantitative PCR primers and probes for IL-17A, IL-23p19, RORC, and β -actin were designed with Primer Express software from ABI (Foster City, CA, USA), and real-time quantitative PCR was performed in the ABI Prism 7700 sequence detector (ABI) as described previously [30].

IL-23 triggers IBD NK cell activation

IL-23 has been reported to induce NK cell activation to produce IFN- γ [33, 34]. NK cells are unique in that they costimulatory express only the activating, low-affinity receptor Fc γ RIII (CD16). We have found that cross-linking of Fc γ RIII on NK cells by immobilized IgG synergizes with IL-21 to up-regulate NK cell cytokine secretion and lytic activity [30]. Therefore, we tested the ability of IL-23 in the induction of cytokine secretion by Fc γ R-activated IBD NK cells. To this end, a 96-well flat-bottom plate was immobilized with human IgG (100 μ g/ml) in cold PBS at 4°C overnight. Before being used, wells were washed three times with 200 μ l PBS to remove unbound human IgG. NK cells (2.0 $\times$ 10⁵/ml) were seeded in culture wells in triplicate in a final volume of 200 μ l/well in the absence or presence of IL-23 (20 ng/ml). After 48 h of culture, supernatants were harvested and analyzed by ELISA for IFN- γ and TNF production.

Cytotoxic assay

The cytotoxicity of IEL and NK cells was assessed using HT-29 cells or K562 cells, respectively, as described previously [30]. To this end, the HT-29 or K562 cells (1 $\times$ 10⁶) were labeled with 100 μ Ci ⁵¹Cr for 1 h at 37°C, washed, and resuspended at a concentration of 1 $\times$ 10⁵ cells/ml. IEL or NK cells were used immediately after fresh isolation or stimulated in the presence or absence of IL-23 (20 ng/ml) for 24 h. IEL or NK cells (100 μ l) were incubated with ⁵¹Cr-labeled HT-29 cells or K562 target cells (100 μ l) at the indicated E:T ratios in triplicate cultures in 96-well round-bottom plates for a total volume of 200 μ l/well. After 4 h incubation at 37°C, 100 μ l supernatant was harvested, and release of ⁵¹Cr into the supernatant was quantified with an automated γ -counter. Spontaneous and maximum release of ⁵¹Cr was determined by incubating target cells in medium alone or with 1% Triton X-100. The percentage of cytotoxicity was calculated as the percent ⁵¹Cr release using the formula: (cpm experimental release–cpm spontaneous release)/(cpm maximum release–cpm spontaneous release) $\times$ 100.

IL-23 induces IBD IEL and T cell cytokine production

The cytokine production by IEL or PB-CD4⁺ T or LP-CD4⁺ T cells from IBD patients was determined after activation with IL-23. To this end, 24-well culture wells were precoated with anti-CD3 (5 μ g/ml) in 200 μ l PBS for 4 h at 37°C. Before being used, wells were washed three times with 200 μ l PBS to remove unbound anti-CD3 mAb. IEL or PB-CD4⁺ T or LP-CD4⁺ T cells (1 $\times$ 10⁶/ml) were cultured in a 1-ml vol in 24-well plastic plates in the absence or presence of IL-23 (20 ng/ml). After 48 h of culture, supernatants were harvested and assayed for IFN- γ , TNF, IL-2, IL-10, and IL-17A by sandwich ELISA using OptEIA ELISA kits (BD Pharmingen), according to the manufacturer's instructions [30]. The sensitivity of each assay was 10 pg/ml.

Flow cytometric analysis

The cells were resuspended in PBS, supplemented with 0.2% BSA and 0.1% Na₂S₂O₃ at a concentration of 1 $\times$ 10⁶ cells/ml, and blocked with puri-

fied CD16/CD32 antibody (2.4G.2, BD PharMingen) for 20 min on ice. A total of 2×10^4 – 1×10^5 cells was stained directly using PE-conjugated mAb to CD4, CD8, CD56, or isotype-matched control IgGs for 30 min at 4°C. Biotinylated anti-IL-23R was revealed by FITC-conjugated streptavidin. Cells were then washed twice with PBS, fixed in 0.5 ml 3% paraformaldehyde buffer, and analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA), using CellQuest software, according to our previous report [29–32].

Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical analysis was performed using the Wilcoxon test for paired samples and Mann-Whitney *U* test for unpaired data. A value of $P < 0.05$ was considered statistically significant.

RESULTS

IL-23p19 is highly expressed in inflamed mucosa of IBD

Immunohistochemical staining demonstrated that IL-23p19-positive cells were increased significantly in inflamed mucosa of IBD patients compared with healthy controls (Fig. 1). The percentage of IL-23p19-positive cells in the LP of inflamed ileum/colon from CD (20.2 ± 4.4) and of inflamed colon from UC (8.6 ± 2.8) was significantly higher than that in the ileum and colon from healthy controls (0.3 ± 0.1 ; $P < 0.05$). They were also markedly higher in inflamed mucosa of CD patients than in UC ($P < 0.05$). However, only a few positive cells were pres-

ent in sections from normal ileum and colon. Phenotypic analysis was performed in the sections from three patients with active CD, showing high scores for the expression of IL-23p19, and revealed that the majority of IL-23p19-positive cells was CD68⁺ macrophages/DCs (Fig. 1D). Intestinal epithelial cells were negative in all sections. Staining with an isotype-matched control antibody was negative (Fig. 1E).

IL-23p19 mRNA is increased expression in inflamed mucosa of IBD

Next, we analyzed mRNA expression levels of IL-23p19 in intestinal mucosa from IBD patients and healthy controls. RT-PCR was performed to determine levels of IL-23p19 mRNA expression in relation to that of the housekeeping gene β -actin. Fig. 2A illustrates the relative amounts of IL-23p19 and β -actin mRNA, as reflected by the intensity of the 260- and 500-bp PCR products, respectively. Moreover, real-time PCR analysis further reveals that the level of IL-23p19 mRNA was significantly higher in inflamed ileum/colon of IBD patients than that in healthy controls ($P < 0.001$; Fig. 2B).

IL-23R is highly expressed in PBMC and LPMC in IBD

After confirming *in situ* expression of IL-23p19 in inflamed mucosa of IBD patients, we wanted to address the frequencies of IL-23R expression in PBMC and LPMC in IBD patients us-

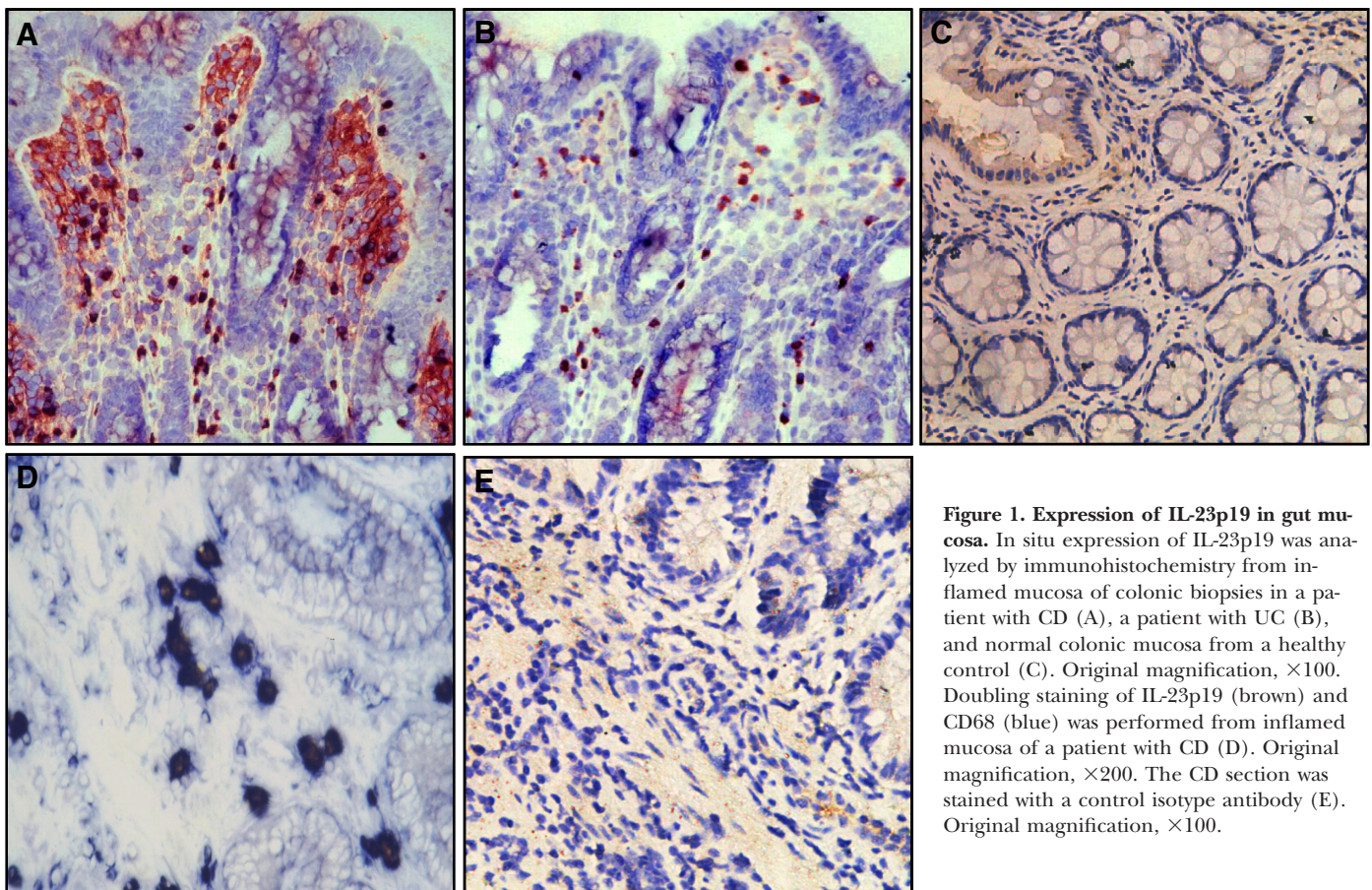


Figure 1. Expression of IL-23p19 in gut mucosa. *In situ* expression of IL-23p19 was analyzed by immunohistochemistry from inflamed mucosa of colonic biopsies in a patient with CD (A), a patient with UC (B), and normal colonic mucosa from a healthy control (C). Original magnification, $\times 100$. Doubling staining of IL-23p19 (brown) and CD68 (blue) was performed from inflamed mucosa of a patient with CD (D). Original magnification, $\times 200$. The CD section was stained with a control isotype antibody (E). Original magnification, $\times 100$.

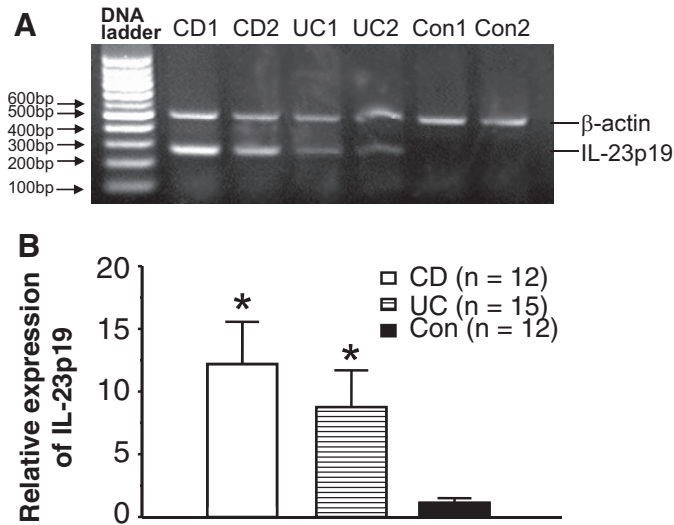


Figure 2. mRNA levels of IL-23p19 in intestinal mucosa. (A) Representative determination of mRNA levels of IL-23p19 from inflamed mucosa of two CD patients (CD1, CD2), two UC patients (UC1, UC2), and two healthy controls (Con1, Con2) by RT-PCR. β -Actin was used as an internal control. The gel is representative of five separate experiments from 10 CD patients, 10 UC patients, and 12 healthy controls and shows the IL-23p19 and β -actin PCR products. (B) Levels of mRNA for IL-23p19 were detected from inflamed mucosa of 10 CD patients, 12 UC patients, and 10 healthy controls by quantitative real-time PCR; gene expression was normalized relative to β -actin mRNA level in each sample. * $P < 0.05$ versus control.

ing a flow cytometry. As shown in **Fig. 3** and Supplemental Fig. 1, $CD4^+$, $CD8^+$ T cells, and NK cells in freshly isolated PBMC and LPMC from IBD patients expressed significantly higher levels of IL-23R compared with those in healthy controls ($P < 0.05$).

IL-23 promotes IBD IEL activation and cytotoxic activity

IEL are present between the epithelial cells of the intestinal mucosa close to gut lumen and have been considered as the sentinels to encounter luminal antigens. They display several phenotypic and functional characteristics distinct from peripheral T cells, contain predominant $TCR\gamma\delta^+$ $CD4^-CD8^+$ T cells, and have spontaneous cytotoxicity against epithelial targets [35]. We analyzed IL-23R expression in $CD8^+$ IEL and found that the percentage of IL-23R $^+$ $CD8^+$ IEL in CD patients (10.95 ± 2.36 ; $n=5$) and UC patients (8.24 ± 2.03 ; $n=5$) was increased significantly from that in healthy controls (2.45 ± 0.32 ; $n=5$; Supplemental Fig. 2). As recent work has demonstrated that IL-23 could trigger IEL activation to secrete IL-17A [36], we wanted to determine whether IL-23 could induce IBD IEL activation and cytolytic activities. To this end, IEL (1.0×10^6 /ml) from IBD patients and healthy controls were cultured with immobilized anti-CD3 ($5 \mu\text{g}/\text{ml}$) in the presence or absence of IL-23 ($20 \text{ ng}/\text{ml}$). As shown in **Fig. 4, A–D**, IEL from healthy controls produced low levels of IFN- γ , TNF, IL-2, and IL-17A when stimulated with anti-CD3 alone for 48 h, and IL-23 could trigger healthy IEL activation to produce more

IFN- γ , TNF, and IL-2 but not IL-17A. In contrast, IBD IEL, when stimulated with anti-CD3 alone, produced significantly more amounts of IFN- γ , TNF, IL-2, and IL-17A than healthy controls ($P < 0.05$). Interestingly, the inclusion of IL-23 induced significantly higher levels of IFN- γ , TNF, IL-2, and IL-17A as compared with IBD IEL stimulated with anti-CD3 alone or healthy controls cultured with anti-CD3 and IL-23 ($P < 0.05$). Relatively low levels of IL-4 and IL-10 were observed in the culture supernatants (data not shown). Additionally, IEL were also isolated from six patients with *Salmonella*- or *Listeria monocytogenes*-infected colitis, stimulated in vitro as described above, and produced the same levels of cytokines as healthy controls (data not shown). Therefore, our data suggest that IL-23 potently induces IBD IEL activation, resulting in secretion of proinflammatory cytokines (e.g., IFN- γ , TNF, IL-2, IL-17A) and involving a mucosal immune response.

To test whether IL-23 could promote IEL cytolytic activity, freshly isolated IEL from IBD patients and healthy controls were used immediately or cultured with anti-CD3 ($5 \mu\text{g}/\text{ml}$) and IL-23 ($20 \text{ ng}/\text{ml}$) for 24 h. The lytic activity of these IEL was then assessed in a standard 4-h chromium release. As shown in **Fig. 4E**, IL-23-preactivated IEL cells from healthy controls demonstrated a low level of cytotoxicity against the HT-29 intestinal epithelial cell line, whereas IL-23-preactivated

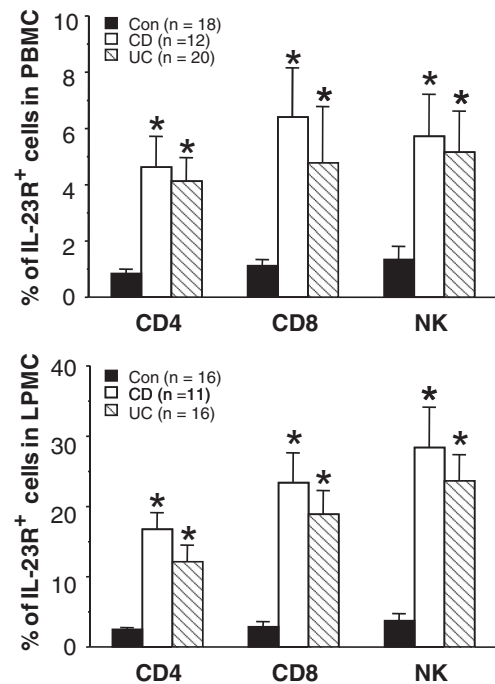


Figure 3. Expression of IL-23R in PBMC and LPMC. Freshly isolated PBMC (upper) and LPMC (lower) from IBD patients and healthy controls were stained with biotinylated anti-human IL-23R mAb or isotype-matched control mAb. FITC-conjugated streptavidin was used as a second staining for biotinylated antibodies. After two washes with PBS, the cells were then stained with PE-conjugated anti-CD4, anti-CD8, or anti-CD56 mAb, respectively. Expression of IL-23R in the indicated subsets was analyzed on a FACSCalibur. The percentage of IL-23R $^+$ $CD4^+$, $CD8^+$ T, and $CD56^+$ NK cells was then calculated. * $P < 0.05$ versus control.

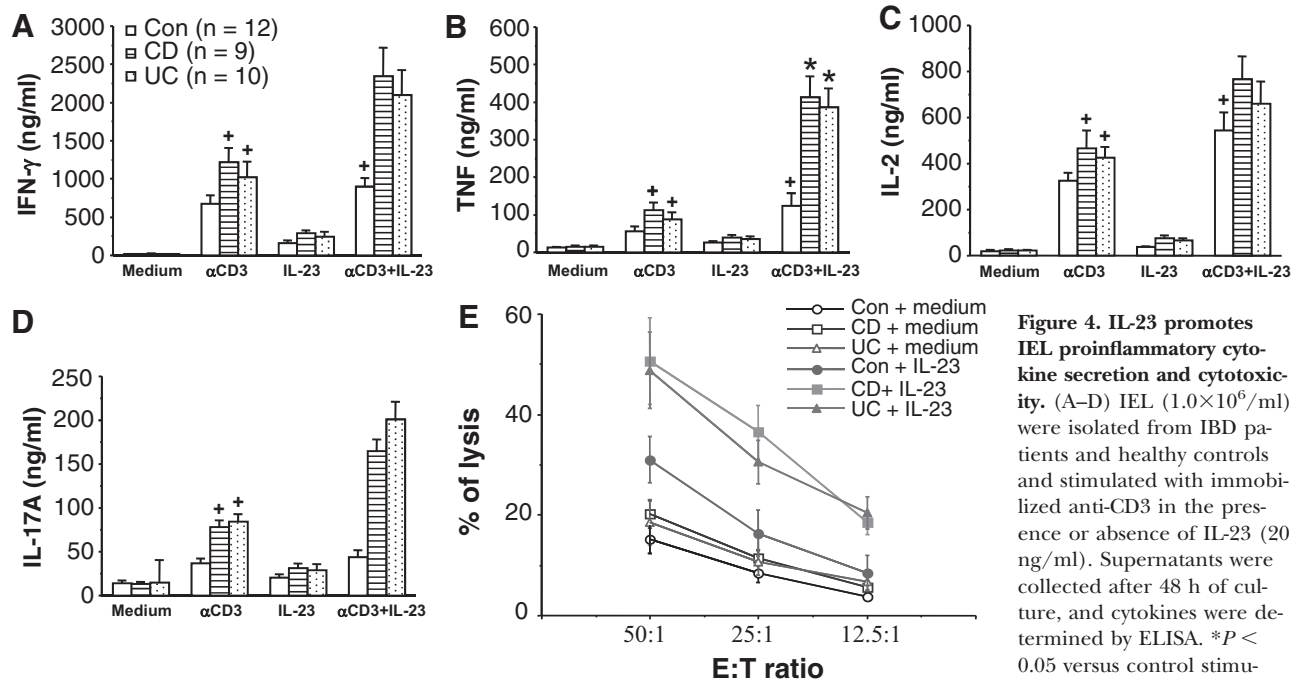


Figure 4. IL-23 promotes IEL proinflammatory cytokine secretion and cytotoxicity. (A–D) IEL (1.0×10^6 /ml) were isolated from IBD patients and healthy controls and stimulated with immobilized anti-CD3 in the presence or absence of IL-23 (20 ng/ml). Supernatants were collected after 48 h of culture, and cytokines were determined by ELISA. * $P < 0.05$ versus control stimulated with αCD3 alone.

lated with αCD3 + IL-23 or values stimulated with αCD3 alone from the same group; + $P < 0.05$ versus control stimulated with αCD3 alone. (E) IEL (1.0×10^6 /ml) from IBD patients and healthy controls were stimulated with immobilized anti-CD3 in the presence of IL-23 (20 ng/ml) for 24 h. These IL-23-primed IEL cells were incubated with ^{51}Cr -labeled HT-29 cells (1.0×10^5 /ml) at the indicated E:T ratios in triplicate cultures at 37°C for 4 h to determine the cytolytic activity of IEL. The results represent the means of three independent experiments. * $P < 0.05$ versus values cultured in medium alone or control stimulated with αCD3 + IL-23 at the same time-point.

IBD IEL showed a significant cytotoxicity against the target cells. In addition, IEL were isolated from five patients with *Salmonella*-infected colitis and studied after 24 h stimulation with anti-CD3 and IL-23 as indicated above. The cytolytic activity of these IEL was also tested and displayed little lytic activities against the epithelial-derived target cells, with no significant difference from healthy controls (data not shown).

IL-23 enhances IBD NK cell activation and cytotoxic activity

IL-23 has been shown to up-regulate NK cell activation and enhance the predominant biological activity through production of immunoregulatory cytokines, especially IFN-γ [33, 34]. Importantly, IL-23 signaling plays an important role in the maintenance of NK cell maturation and cytotoxicity potential [37]. After confirming IL-23R to increase expression in IBD NK cells, we then investigated whether IL-23 could trigger IBD NK cell cytokine secretion and cytolytic activities. To this end, purified NK cells (2.0×10^5 /ml) from PB of IBD patients and healthy controls were cultured in a 96-well flat-bottom plate immobilized with human IgG (100 μg/ml) in the presence or absence of IL-23 (20 ng/ml). IBD NK cells, when stimulated with IL-23 for 48 h, produced significant amounts of IFN-γ and TNF from healthy controls ($P < 0.05$; Fig. 5, A and B). Relatively low levels of IL-2 were observed in the culture supernatants, whereas IL-4 and IL-10 were almost undetectable in all experiments (data not shown). In contrast, PB-NK cells from IBD patients and healthy controls were observed not to pro-

duce IL-22 under stimulation with IL-23 in vitro (data not shown), consistent with a previous report [38]. Additionally, PB-NK cells were isolated from four patients with *Salmonella*-infected colitis and two patients with *L. monocytogenes*-infected colitis, stimulated in vitro as described above, and produced the same levels of cytokines as compared with healthy controls (data not shown). Taken together, our results suggest that IL-23 potently induces IBD NK cell differentiation, leading to secretion of proinflammatory cytokines such as TNF and IFN-γ.

To assess the cytotoxicity of IBD NK cells in response to IL-23 stimulation, freshly isolated NK cells from IBD patients and healthy controls were used immediately or cultured in the presence of IL-23 (20 ng/ml) for 24 h. The lytic activity of IL-23-activated NK cells was then assessed in a standard 4-h chromium release. As shown in Fig. 5C, IL-23-activated NK cells from healthy controls displayed a low level of cytotoxicity against the NK-sensitive line K562. In contrast, IL-23-activated IBD NK cells exhibited stronger cytotoxicity against the target cells. In addition, freshly isolated PB-NK cells from four patients with *Salmonella*-infected colitis exhibited a low level of lytic activities against the target cells, with no significant difference from healthy controls (data not shown).

IL-23 induces IBD CD4⁺ T cell proinflammatory cytokine secretion

A previous study [39] has demonstrated that IL-23 induces IBD T cell activation and proinflammatory cytokine secretion.

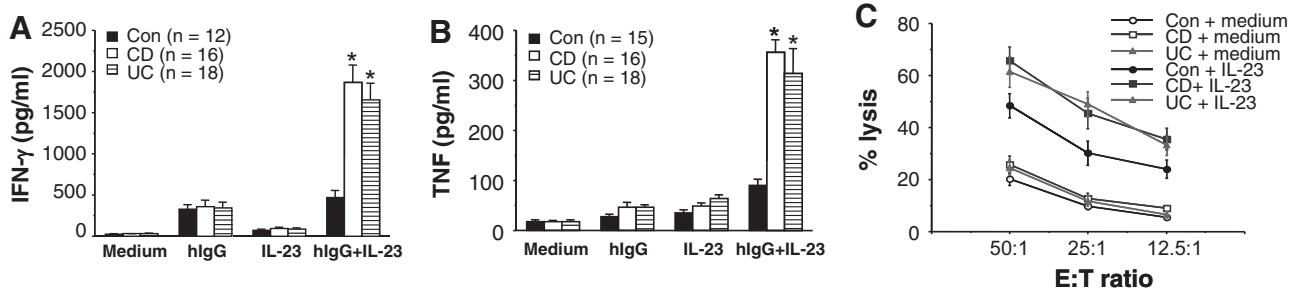


Figure 5. IL-23 enhances NK cell proinflammatory cytokine secretion and cytolytic activities. (A and B) Purified NK cells (2.0×10^5 /ml) from PB of IBD patients and healthy controls were cultured in a 96-well flat-bottom plate, immobilized with human IgG (hlgG; 100 μ g/ml) in the absence or presence of IL-23 (20 ng/ml). After 48 h of culture, the supernatants were harvested and analyzed for IFN- γ and TNF by ELISA. Results are representative of three independent experiments. * $P < 0.05$ versus control in the same culture condition. (C) Purified NK cells (1.0×10^6 /ml) from IBD patients and healthy controls were stimulated in the absence or presence of IL-23 (20 ng/ml) for 24 h. These IL-23-primed NK cells were incubated with 51 Cr-labeled K562 cells (1.0×10^5 /ml) at the indicated E:T ratios in triplicate cultures at 37°C for 4 h to determine the cytolytic activity of NK cells. The results represent the means of three independent experiments. * $P < 0.05$ versus values cultured in medium alone or control stimulated with IL-23 at the same time-point.

To investigate the role of IL-23 in the induction of T cell activation, PB-CD4⁺ or LP-CD4⁺ T cells were isolated from IBD patients and healthy controls and stimulated with anti-CD3 (5 μ g/ml) + anti-CD28 (5 μ g/ml) in the absence or presence of IL-23 (20 ng/ml). Anti-CD28 mAb was used in this culture condition to stimulate CD4⁺ T cells in a manner that partially mimics stimulation by APCs and potently induces CD4⁺ T cell activation. After 48 h of culture, supernatants were harvested and assayed for IFN- γ , TNF, IL-2, IL-10, and IL-17A. The results demonstrated that IBD PB-CD4⁺ or LP-CD4⁺ T cells produced significantly higher levels of IFN- γ , TNF, IL-2, and IL-17 compared with controls ($P < 0.05$; Supplemental Tables 1 and 2). However, low levels of IL-10 were observed in all groups with no differences between IBD patients and healthy controls, suggesting that IL-23 could not induce T cells to produce IL-10. Therefore, these data indicate that IBD CD4⁺ T cells se-

crete elevated amounts of proinflammatory cytokines (i.e., IFN- γ , TNF, IL-2, IL-17A) in response to IL-23 stimulation.

IL-23 induces IBD CD4⁺ T cells to differentiate into Th17 cells

Given that IL-23R was also observed to have increased expression in IBD CD4⁺ T cells, we next examined whether IL-23 could itself promote Th17 differentiation in IBD under in vitro stimulatory conditions. PB- or LP-CD4⁺ T cells were cultured in stimulation with immobilized anti-CD3 and anti-CD28 in the absence or presence of IL-23 (20 ng/ml) for 48 h. Anti-CD28 mAb was also used in this culture condition to stimulate CD4⁺ T cells in a manner that partially mimics stimulation by APCs and potently induces CD4⁺ T cell activation. Levels of IL-17A and transcription factor RORC mRNA expression were analyzed by quantitative real-time PCR, and gene expression

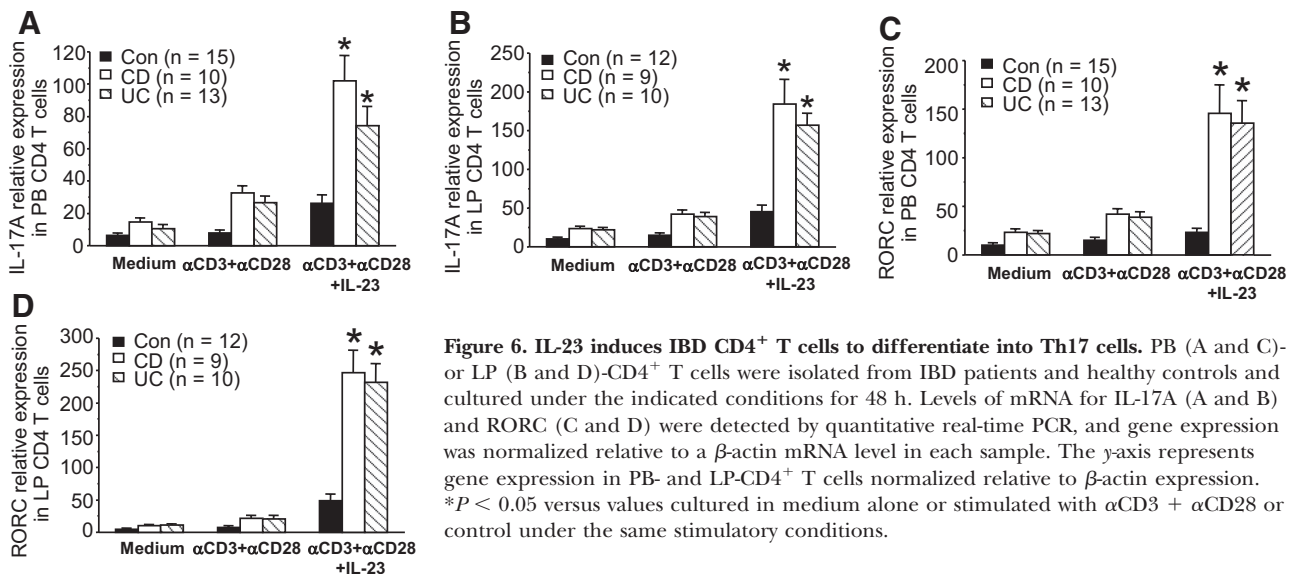


Figure 6. IL-23 induces IBD CD4⁺ T cells to differentiate into Th17 cells. PB (A and C)- or LP (B and D)-CD4⁺ T cells were isolated from IBD patients and healthy controls and cultured under the indicated conditions for 48 h. Levels of mRNA for IL-17A (A and B) and RORC (C and D) were detected by quantitative real-time PCR, and gene expression was normalized relative to a β -actin mRNA level in each sample. The y-axis represents gene expression in PB- and LP-CD4⁺ T cells normalized relative to β -actin expression. * $P < 0.05$ versus values cultured in medium alone or stimulated with α CD3 + α CD28 or control under the same stimulatory conditions.

was normalized relative to β -actin expression in each sample. As shown in **Fig. 6**, IL-23 induced high levels of IL-17A mRNA and RORC, which is an essential factor for Th17 differentiation [40]. These results indicate that IL-23 could facilitate mucosal Th17 cell development and markedly up-regulate IL-17A expression, leading to intestinal mucosal inflammation.

Anti-TNF treatment down-regulates mucosal IL-23p19 expression and Th17 cell infiltration in CD patients

Our previous data have demonstrated that anti-TNF treatment could prevent experimental colitis in SCID mice through down-regulation of IL-23 and IL-17 production [32]. To investigate the mechanism of anti-TNF mAb (e.g., Infliximab) in the treatment of CD, the levels of TNF, IL-23p19, IL-17A, and RORC were examined using quantitative real-time PCR in inflamed mucosa of CD patients after Infliximab treatment. Eight patients with active CD were treated with Infliximab (5 mg/kg) at Weeks 0, 2, and 6, according to protocols described previously [41]; colonoscopy examination was performed in these patients at Week 10, intestinal mucosal biopsies were harvested, and the levels of TNF, IL-23p19, IL-17A, and RORC mRNA were analyzed as described previously [30]. As shown in **Fig. 7**, the levels of TNF, IL-23p19, IL-17A, and RORC were markedly decreased after Infliximab treatment as compared with those at the beginning of Infliximab administration. Interestingly, RORC was also found to decrease significantly after Infliximab treatment compared with that before treatment ($P < 0.05$). These data indicate that anti-TNF treatment effectively inhibits intestinal mucosal inflammation in CD patients through down-regulation of IL-23 expression and Th17 cell infiltration.

DISCUSSION

The study presented here demonstrated that IL-23p19 was increased significantly at transcriptional and translational levels in inflamed areas of IBD, particularly in CD, as compared with that in UC and healthy controls. IL-23p19-positive cells were mainly CD68⁺ macrophages/DCs. IL-23R was mainly expressed in CD4⁺, CD8⁺ T, and NK cells. Importantly, IL-23 markedly enhanced IBD IEL, NK cell activation, and cytotoxic activities against targets and promoted IBD CD4⁺ T cells to secrete proinflammatory cytokines (e.g., TNF, IFN- γ , and IL-

17) and differentiate into Th17 cells. This work provides new access points to further dissect a potent role of IL-23 in the induction of an intestinal mucosal lymphocyte immune response, indicating that IL-23 is involved in the pathogenesis of IBD and importantly, that targeted therapy directed against IL-23 may be effective in the treatment of IBD.

IEL are a population of lymphocytes that reside within the intestinal epithelium and represent a large population of lymphocytes located at the interface between the body and the antigenic intestinal flora [35]. IEL are initially envisioned to be the first line of defense against mucosal pathogens, thus they are considered to play an important role in immune surveillance and the maintenance of mucosal homeostasis by actively or negatively regulating mucosal innate and acquired immunity. IEL are mainly composed of CD8⁺ T cells, but some CD4⁺ T cells and a few double-positive cells are also present [35]. The majorities of CD8⁺ IEL in human gut express the $\alpha\alpha$ homodimeric form of the CD8 molecule characteristic of TCR $\alpha\beta$ ⁺ or TCR $\gamma\delta$ ⁺ CD44⁺ CD45RO⁺ CD62^{low} memory phenotype and immunologically distinct from peripheral T cells, which express the CD8 $\alpha\beta$ heterodimer. Despite their varied function and phenotype, the great majority of IEL is characterized by a partial activation state and has spontaneous cytotoxic activity, contributing to the early host defense against dysplastic intestinal epithelial cells or cancer cells [35]. Evidences have proved that some γ c-related cytokines (e.g., IL-7, IL-15, IL-21) and IL-23 are involved in the IEL development, activation, and survival [35, 36], indicating that these proinflammatory cytokines may play an important role in regulating immune response of IEL under inflammatory conditions, particularly in IBD. In the current study, we found that freshly isolated IEL from IBD patients and healthy controls were demonstrated to be spontaneous cytolytic activities against target cells, consistent with a previous report showing that increased cytolytic activity of intestinal IEL against epithelial-derived target cells was present in patients with IBD [42]. Interestingly, we found that IL-23 could strongly augment IBD IEL cytolytic activities, suggesting that IEL localized between epithelial cells have profound cytotoxicity against intestinal epithelial cells under inflammatory conditions. Moreover, our data also demonstrated that IL-23 could potentially induce IBD IEL activation to produce large amounts of proinflammatory cytokines (e.g., IFN- γ , TNF, IL-2, IL-17A). Thus, our findings

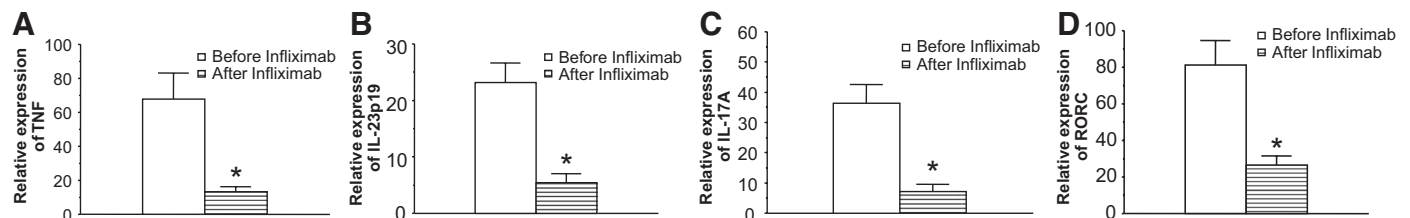


Figure 7. Anti-TNF treatment inhibits mucosal IL-23p19 expression and Th17 cell infiltrations in CD patients. Intestinal mucosal biopsies were harvested during colonoscopy from eight CD patients treated with Infliximab 4 weeks after the third injection, and mRNA levels of TNF, IL-23p19, IL-17A, and RORC were analyzed by quantitative real-time PCR as described above. Gene expression was normalized relative to β -actin mRNA level in each sample. * $P < 0.05$ versus values before Infliximab treatment.

imply that IEL under inflammatory conditions may be closely associated with intestinal mucosal injury in IBD.

NK cells are important effector cells of the innate immune system required for the first line of defense against transformed and infected cells and play an essential role in linking innate and adaptive immunity through their ability to secrete IFN- γ . At the early stage of infection, NK cells are considered as the primary source of IFN- γ , shaping the adaptive immunity through differentiation of CD4⁺ T cells to the Th1 subsets [43]. Previous work has proven that stromal cells and cytokine/growth factors play a critical role in the progression of NK cell development. IL-2 and IL-15 are reported to be required for maturation and development of NK cells [44]. Our recent work has proven that IL-21 could also enhance the activation and differentiation of NK cells of IBD patients [30]. In the current study, we found that IL-23 strongly induced IBD NK cell activation, showing increased secretion of proinflammatory cytokines (e.g., IFN- γ , TNF) and cytolytic activities. These findings suggest that IL-23 produced by intestinal mucosal macrophages/DCs in inflamed mucosa of IBD could promote a PB NK cell effector response. However, LP-resident NK cells have not been investigated thoroughly, and their biological function within this immune compartment is not well-known. Evidences have demonstrated that NK cell-mediated, natural cytotoxicity is present in the LP and that these NK cells express the NK cell lineage marker NKP46, NKG2D, NK1.1. Recent work has also demonstrated two distinct subsets of intestinal mucosal NKP46⁺ NK cells, according to the expression of ROR γ t. The ROR γ t⁻ subset functions as typical NK cells dependently on IL-15 but not on ROR γ t and displays NK cell activities (e.g., cytotoxicity, IFN- γ secretion), and the ROR γ t⁺ subpopulation develops independently of IL-15 but required ROR γ t [45, 46]. Most interestingly, these CD3⁻NKP46⁺ cells located in the intestinal mucosa express ROR γ t and IL-22 but not IL-17A in response to IL-23 stimulation, and these cells lack normal NK cell function, such as expression of perforin and IFN- γ , indicating that these NK cells play an important role in mucosal homeostasis and protectable immune response, particularly under microbial challenge [38]. Based on our current study, intestinal mucosal NK cells may also be associated with a proinflammatory response under inflammatory conditions and play an important role in mucosal homeostasis and defense against luminal microbial challenge, although the precise role of IL-23 in the induction of intestinal mucosal NK cell effect response needs to be investigated further.

IL-23p19 was found to increase expression in inflamed colon and sera of IBD patients [23]. Genome-wide association studies indicate that IL-23R involves the differentiation of Th17 cells and is associated with susceptibility to CD and also partly to UC [25, 26]. Further analysis confirmed that the polymorphism R381Q in the IL-23R chain confers protection against IBD. Th17 cells have been observed to be present in human PB and intestinal mucosa from patients with CD [47]. In agreement with the critical role of IL-23 in a mucosal inflammatory response, intestinal inflammation was observed to be highly attenuated in IL-23p19^{-/-} mice, and anti-IL-23p19 could ameliorate the parameters of colitis, including decreased

production of proinflammatory cytokines [19–21]. A clinical trial with anti-IL-12/IL-23p40 mAb, which blocks IL-12 and IL-23 biological activities, could induce clinical responses and remissions in patients with active CD, and clinical improvement was associated with a reduction in the production of IL-12 and IFN- γ at the inflamed mucosa [48]. Therefore, blockade of the IL-23p19 signaling pathway would be helpful in the treatment of IBD.

AUTHORSHIP

Z.L., P.-C.Y., X.W., and J.Q. conceived of and designed the research; Z.L., P.K.Y., J.S., and C.C. performed the majority of the experiments; X.X., M.T., H.L., and J.Y. collected the samples and performed the statistical analysis; Z.L. wrote the paper and obtained the funding; and all authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (Nos. 30770988 and 30971358). The authors are grateful to Dr. S. Matikainen (National Public Health Institute, Helsinki, Finland) for providing anti-human IL-23p19 antibody.

REFERENCES

- Podolsky, D. K. (2002) Inflammatory bowel disease. *N. Engl. J. Med.* **347**, 417–429.
- Xavier, R. J., Podolsky, D. K. (2007) Unraveling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427–434.
- Sartor, R. B. (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat. Clin. Pract. Gastroenterol. Hepatol.* **3**, 390–407.
- Cho, J. H. (2008) The genetics and immunopathogenesis of inflammatory bowel disease. *Nat. Rev. Immunol.* **8**, 458–466.
- Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., et al. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**, 715–725.
- Parham, C., Chirica, M., Timans, J., Vaisberg, E., Travis, M., Cheung, J., Pflanz, S., Zhang, R., Singh, K. P., Vega, F., et al. (2002) A receptor for the heterodimeric cytokine IL-23 is composed of IL-12R β 1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol.* **168**, 5699–5708.
- Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* **6**, 1123–1141.
- Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., Weaver, C. T. (2005) Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–1132.
- Zheng, Y., Danilenko, D. M., Valdez, P., Kasman, I., Eastham-Anderson, J., Wu, J., Ouyang, W. (2007) Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445**, 648–651.
- Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., Cua, D. J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233–240.
- Korn, T., Bettelli, E., Oukka, M., Kuchroo, V. K. (2009) IL-17 and Th17 cells. *Annu. Rev. Immunol.* **27**, 485–517.
- Wiekowski, M. T., Leach, M. W., Evans, E. W., Sullivan, L., Chen, S. C., Vassileva, G., Bazan, J. F., Gorman, D. M., Kastelein, R. A., Narula, S., Lira, S. A. (2001) Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J. Immunol.* **166**, 7563–7570.
- Kopp, T., Lenz, P., Bello-Fernandez, C., Kastelein, R. A., Kupper, T. S., Stingl, G. (2003) IL-23 production by cosecretion of endogenous p19 and transgenic p40 in keratin 14/p40 transgenic mice: evidence for enhanced cutaneous immunity. *J. Immunol.* **170**, 5438–5444.

14. Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A., Sedgwick, J. D. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744–748.
15. Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D., Cua, D. J. (2003) Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* **198**, 1951–1957.
16. Ghilardi, N., Klijavin, N., Chen, Q., Lucas, S., Gurney, A. L., Sauvage, F. J. (2004) Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. *J. Immunol.* **172**, 2827–2833.
17. Pernis, A. B. (2009) Th17 cells in rheumatoid arthritis and systemic lupus erythematosus. *J. Intern. Med.* **265**, 644–652.
18. Langowski, J. L., Zhang, X., Wu, L., Mattson, J. D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R. A., Oft, M. (2006) IL-23 promotes tumor incidence and growth. *Nature* **442**, 461–465.
19. Kullberg, M. C., Jankovic, D., Feng, C. G., Hue, S., Gorelick, P. L., McKenzie, B. S., Cua, D. J., Powrie, F., Cheever, A. W., Maloy, K. J., Sher, A. (2006) IL-23 plays a key role in *Helicobacter hepaticus*-induced T cell-dependent colitis. *J. Exp. Med.* **203**, 2485–2494.
20. Hue, S., Ahern, P., Buonocore, S., Kullberg, M. C., Cua, D. J., McKenzie, B. S., Powrie, F., Maloy, K. J. (2006) Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J. Exp. Med.* **203**, 2473–2483.
21. Elson, C. O., Cong, Y., Weaver, C. T., Schoeb, T. R., McClanahan, T. K., Fick, R. B., Kastelein, R. A. (2007) Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* **132**, 2359–2370.
22. Wada, Y., Lu, R., Zhou, D., Chu, J., Przewlaka, T., Zhang, S., Li, L., Wu, Y., Qin, J., Balasubramanyam, V., Barsoum, J., Ono, M. (2007) Selective abrogation of Th1 response by STA-5326, a potent IL-12/IL-23 inhibitor. *Blood* **109**, 1156–1164.
23. Schmidt, C., Giese, T., Ludwig, B., Mueller-Molaian, I., Marth, T., Zeugem, S., Meuer, S., Stallmach, A. (2005) Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm. Bowel Dis.* **11**, 16–23.
24. Sakuraba, A., Sato, T., Kamada, N., Kitazume, M., Sugita, A., Hibi, T. (2009) Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. *Gastroenterology* **137**, 1736–1745.
25. Duerr, R. H., Taylor, K. D., Brant, S. R., Rioux, J. D., Silverberg, M. S., Daly, M. J., Steinhardt, A. H., Abraham, C., Regueiro, M., Griffiths, A., Dasopoulou, T., Bitton, A., Yang, H., Targan, S., Datta, L. W., Kistner, E. O., Schumm, L. P., Lee, A. T., Gregersen, P. K., Barmada, M. M., Rotter, J. I., Nicolaie, D. L., Cho, J. H. (2006) A genome-wide association study identifies IL-23R as an inflammatory bowel disease gene. *Science* **314**, 1461–1463.
26. Dubinsky, M. C., Wang, D., Picornell, Y., Wrobel, I., Katzir, L., Quiros, A., Dutridge, D., Wabbeh, G., Silber, G., Bahar, R., Mengesha, E., Targan, S. R., Taylor, K. D., Rotter, J. I. (2007) IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. *Inflamm. Bowel Dis.* **13**, 511–515.
27. Liu, Z. J., Yadav, P. K., Su, J. L., Wang, J. S., Fei, K. (2009) Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. *World J. Gastroenterol.* **15**, 5784–5788.
28. Pirhonen, J., Matikainen, S., Julkunen, I. (2002) Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. *J. Immunol.* **169**, 5673–5678.
29. Liu, Z., Lefrançois, L. (2004) Intestinal epithelial antigen induces mucosal CD8 T cell tolerance, activation and inflammatory response. *J. Immunol.* **173**, 4324–4330.
30. Liu, Z., Yang, L., Cui, Y., Wang, X., Guo, C., Huang, Z., Kan, Q., Liu, Z., Liu, Y. (2009) IL-21 enhances NK cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease. *Inflamm. Bowel Dis.* **15**, 1133–1144.
31. Li, J., Kong, K., Shen, W., Liu, Z. (2006) IL-21 induces T cell activation and proinflammatory cytokine secretion in rheumatoid arthritis. *Scand. J. Immunol.* **64**, 515–522.
32. Liu, Z., Jiu, J., Liu, S., Fa, X., Li, F., Du, Y. (2007) Blockage of tumor necrosis factor prevents intestinal mucosal inflammation through down-regulation of interleukin-23 secretion. *J. Autoimmun.* **29**, 187–194.
33. Butchar, J. P., Rajaram, M. V., Ganesan, L. P., Parsa, K. V., Clay, C. D., Schlesinger, L. S., Tridandapani, S. (2007) *Francisella tularensis* induces IL-23 production in human monocytes. *J. Immunol.* **178**, 4445–4454.
34. Van de Wetering, D., de Paus, R. A., van Dissel, J. P., van den Vosse, E. (2009) *Salmonella* induced IL-23 and IL-1 β allow for IL-12 production by monocytes and M ϕ 1 through induction of IFN- γ in CD56⁺ NK/NK-like T cells. *PLoS ONE* **4**, e8396.
35. Kunisawa, J., Takahashi, I., Kiyono, H. (2007) Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine. *Immunol. Rev.* **215**, 136–153.
36. Schaefer, J. S., Montufar-Solis, D., Vigneswaran, N., Klein, J. R. (2010) ICOS promotes IL-17 synthesis in colonic intraepithelial lymphocytes in IL-10^{-/-} mice. *J. Leukoc. Biol.* **87**, 301–308.
37. Guia, S., Cognet, C., de Beaucoudrey, L., Tessmer, M. S., Jouanguy, E., Berger, C., Filipe-Santos, O., Feinberg, J., Camcioglu, Y., Levy, J., Al-Jumaa, S., Al-Hajjar, S., Stephan, J. L., Fieschi, C., Abel, L., Brossay, L., Casanova, J. L., Vivier, E. (2008) A role for interleukin-12/23 in the maturation of human natural killer and CD56⁺ T cells in vivo. *Blood* **111**, 5008–5016.
38. Cella, M., Fuchs, A., Vermi, W., Facchetti, F., Otero, K., Lennerz, J. K., Doherty, J. M., Mills, J. C., Colonna, M. (2009) A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* **457**, 722–725.
39. Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., Kitazume, M. T., Nakazawa, A., Sugita, A., Koganei, K., Isobe, K., Hibi, T. (2008) IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut* **57**, 1682–1689.
40. Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelletier, A., Lafaille, J. J., Cua, D. J., Littman, D. R. (2006) The orphan nuclear receptor ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* **126**, 1121–1133.
41. Schnitzler, F., Fidler, H., Ferrante, M., Noman, M., Arijis, I., Van Assche, G., Hoffman, I., Van Steen, K., Vermeire, S., Rutgeerts, P. (2009) Mucosal healing predicts long-term outcome of maintenance therapy with Infliximab in Crohn's disease. *Inflamm. Bowel Dis.* **15**, 1295–1301.
42. Nüssler, N. C., Stange, B., Hoffman, R., Schraut, W. H., Bauer, A. J., Neuhäus, P. (2000) Enhanced cytolytic activity of intestinal intraepithelial lymphocytes in patients with Crohn's disease. *Langenbecks Arch. Surg.* **385**, 218–224.
43. Martín-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F. (2004) Induced recruitment of NK cells to lymph nodes provides IFN- γ for T(H)1 priming. *Nat. Immunol.* **5**, 1260–1265.
44. Becknell, B., Caligiuri, M. A. (2005) Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Adv. Immunol.* **86**, 209–239.
45. Luci, C., Reynders, A., Ivanov, I. I., Cognet, C., Chiche, L., Chasson, L., Hardwigen, J., Anguiano, E., Banchereau, J., Chaussabel, D., Dalod, M., Littman, D. R., Vivier, E., Tomasello, E. (2009) Influence of the transcription factor ROR γ t on the development of NKp46⁺ cell population in gut and skin. *Nat. Immunol.* **10**, 75–82.
46. Sanos, S. L., Bui, V. L., Mortha, A., Oberle, K., Heners, C., Johnen, C., Diefenbach, A. (2009) ROR γ t and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46⁺ cells. *Nat. Immunol.* **10**, 83–91.
47. Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, B., Fili, L., Ferri, S., Frosali, F., Giddici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E., Romagnani, S. (2007) Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* **204**, 1849–1861.
48. Mannon, P. J., Fuss, I. J., Mayer, L., Elson, C. O., Sandborn, W. J., Present, D., Dolin, B., Goodman, N., Groden, C., Hornung, R. L., Quezada, M., Yang, Z., Neurath, M. F., Salfeld, J., Veldman, G. M., Schwertschlag, U., Strober, W. (2004) Anti-interleukin-12 antibody for active Crohn's disease. *N. Engl. J. Med.* **351**, 2069–2079.

KEY WORDS:

Crohn's disease · mononuclear cell · ulcerative colitis