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IL-18, a Novel Immunoregulatory Cytokine, Is Up-Regulated in Crohn’s Disease: Expression and Localization in Intestinal Mucosal Cells

Theresa T. Pizarro, Miette H. Michie, Marcia Bentz, Jan Woraratanadharm, Michael F. Smith Jr., Eugene Foley, Christopher A. Moskaluk, Stephen J. Bickston, and Fabio Cominelli

IL-18, a novel immunoregulatory cytokine with potent IFN-γ-inducing activities, may play an important role in Th1-mediated chronic inflammatory disorders. The aim of the present study was to characterize the expression and localization of IL-18 in colonic specimens and isolated mucosal cell populations from patients with Crohn’s disease (CD), a prototypic Th1-mediated disorder. Using a semiquantitative RT-PCR protocol, IL-18 mRNA transcripts were found to be increased in freshly isolated intestinal epithelial cells (IEC) and lamina propria mononuclear cells (LPMC) from CD compared with ulcerative colitis (UC) and noninflamed control (cont) patients, and were more abundant in IEC compared with LPMC. Immunohistochemical analysis of surgically resected colonic tissues localized IL-18 to both LPMC (specifically, macrophages and dendritic cells) as well as IEC. Staining was more intense in CD compared with UC and cont, and in involved (inv) vs noninvolved (n inv) areas. Western blot analysis revealed that an 18.3-kDa band, consistent with both recombinant and mature human IL-18 protein, was found predominantly in CD vs UC intestinal mucosal biopsies; a second band of 24 kDa, consistent with the inactive IL-18 precursor, was detected in n inv areas from both CD and UC biopsies and was the sole form found in noninflamed cont. To our knowledge, this report is the first describing increased expression of IL-18 in a human Th1-mediated chronic inflammatory disease. In addition, our studies further support the concept that IEC and dendritic cells may possess important immunoregulatory functions in both normal, as well as pathological, mucosal immunity. The Journal of Immunology, 1999, 162: 6829–6835.
colonoscopy for diagnostic or surveillance purposes (n = 6/group) were used for Western blot analysis. All diagnoses were confirmed by clinical, macroscopic, and histologic criteria. In addition, medical records of all patients were reviewed and their histories and treatment modalities noted. None of the patients were on immunomodulators (6-mercaptopurine, azathioprine, and/or methotrexate) at the time of their treatment. Their treatment regimens included 5-aminosalicylic acid alone, steroids alone, 5-aminosalicylic acid and steroids, or no therapy at all. Informed consent was obtained from each patient, and the project was approved by the Internal Review Board of the University of Virginia Health Sciences Center.

Isolation of colonic mucosal cell populations and PBMC cell culture

All colonic surgical specimens were collected immediately following resection, opened longitudinally, rinsed, examined for gross morphological changes, and a representative full-thickness sample obtained. IEC and LPMC were isolated and purified (separately) as previously reported in detail (15). Briefly, for LPMC isolation, dissected intestinal mucosa was freed of mucus and epithelial cells in sequential steps using DTT (Sigma, St. Louis, MO) and EDTA, and subsequently digested with collagenase and deoxyribonuclease (both from Worthington Biochemical, Freehold, NJ). The resulting crude cell suspension was purified using a Ficoll-Hypaque gradient (lymphocyte separation medium (LSM); Organon Teknika, Durham, NC) following manufacturer’s protocol, and the preparations preferentially enriched for intestinal LPMC were subsequently washed twice with PBS and counted. Macrophages averaged 11% of LPMC, with no significant differences between control and IBD cells, as previously reported (15). IEC were isolated from mucosal strips by repeated incubations in a dispase solution (Boehringer Mannheim, Indianapolis, IN), followed by gentle vortexing and filtration through a nylon column. Mononuclear, red blood, and dead cells were removed using a 40% Percoll gradient (Pharmacia LKB Biotechnology, Piscataway, NJ), where the IEC equilibrated at the interface; resulting preparations preferentially enriched for IEC were collected, washed twice in PBS, and subsequently counted. Using an immunoperoxidase method, all cells with epithelial morphology were stained with the anti-keratin mAb, AE1/AE3 (Boehringer Mannheim), with <2% contaminating LPMC, as assessed by staining with a mAb directed against a leukocyte common Ag (CD45RB). PBMC, obtained from a normal volunteer and used as a positive control, were isolated through a Ficoll-Hypaque gradient (LSM; Organon Teknika) and cultured (5.0 × 10⁶ cells/ml) at 37°C in a 5% CO₂ atmosphere in the presence or absence of LPS (10 μg/ml; Sigma) for 6 h. For experiments analyzing IL-18 mRNA expression, total cellular RNA was prepared from freshly isolated IEC and LPMC populations, as well as cultured PBMC, using Trizol reagent (Life Technologies, Grand Island, NY) (10⁶ cells/ml) according to the manufacturer’s instructions, and processed by RT-PCR methodologies as later described.

Processing of mucosal biopsies

Colonic mucosal biopsies were obtained and processed as previously described (16). IBD specimens were derived from areas of active disease (involved (inv)) as well as macroscopically noninvolved (n inv) areas from the same patient. In brief, tissue samples were rinsed twice in saline solution, placed in Eppendorf tubes containing 500 μl of RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 50 mM Tris (pH 8.0) containing aprotinin (1.0 μg/ml), 0.5 mM PMSF, 0.1 mM sodium orthovanadate; all from Sigma) and homogenized for 30 s using a Brinkmann (Westbury, NY) hand-held polytron. Tissue homogenates were briefly centrifuged and immediately frozen at −80°C for later assays.

Semi-quantitative RT-PCR for human (h)IL-18

cDNA was synthesized by RT from 0.50 μg total cellular RNA using the GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ), according to manufacturer’s protocol. The aforementioned reaction in the absence of murine leukemia virus RT served as a negative RT control. Resulting cDNA (1.0 μl of RT reaction) was amplified in a 25-μl reaction volume containing 0.4 μM primer (0.4 μM primer) of both the target gene (hIL-18) and housekeeping gene (human GAPDH), according to manufacturer’s protocol (GeneAmp RNA PCR kit) using AmpliTaq Gold DNA polymerase (both from Perkin-Elmer). PCR reaction mixture was overlaid with 50 μl mineral oil, and multiplex PCR amplification was conducted in a DNA thermal cycler (OmniGene Thermocycler; Hybaid, Teddington, U.K.) under the following conditions: 1 cycle of 94°C, 12 min; 30 cycles of 96°C, 30 s; 55°C, 1 min; 72°C, 30 s; and 1 cycle of 72°C, 10 min. The same RT sample derived from PBMC stimulated with LPS was used as a positive PCR control/internal standard, and RT negative plus PCR mixture, mixture only, and sterile double distilled H₂O only, served as negative PCR controls. Singleplex PCR, using primer pairs specific for either hIL-18 or hGAPDH only, was conducted in identical conditions as detailed above to verify multiplex PCR results and rule out the possibility of primer pair primer pair interactions. Resulting amplified fragments were resolved on 3% NuSieve GTG (FMC BioProducts, Rockland, ME): 1.5% agarose gels stained with ethidium bromide and visualized through a UV light digital imaging system. Negatives of direct positive images were measured using an analytical software (Scion Image 1.59; Scion, Frederick, MD), and relative quantitation of ethidium-stained bands from multiplex RT-PCR, representing integrated area under curve of densitometric tracing, were reported as the ratio of target gene (hIL-18) to housekeeping gene (GAPDH). All samples were normalized to an internal standard (PBMC + LPS) processed with each experiment to account for interassay variability. Each bar represents the mean ± SEM. Asterisks indicate significant differences between experimental groups (*, p < 0.02; **, p < 0.03). Statistical analysis was performed using the Mann-Whitney two-sample test.

![Expression of hIL-18 mRNA transcripts in freshly isolated intestinal mucosal cells. A. Representative Southern blot of multiplex RT-PCR hybridized with a human full-length IL-18 cDNA probe confirmed IL-18-specificity of amplified PCR products and showed detectable IL-18 mRNA transcripts in both intestinal EC and LPMC from cont and IBD patients. Increased IL-18 mRNA steady-state levels were observed in intestinal EC compared with LPMC and in mucosal cells from CD compared with both cont and UC patients. B. Ratio of IL-18 to control GAPDH mRNA levels demonstrated a significant increase in both intestinal EC (n = 6/group) and LPMC (n = 6/group) from CD, compared with cont and UC patients. Negatives of direct positive images were measured on gels, and relative quantitation of ethidium-stained bands from multiplex RT-PCR, representing integrated area under curve of densitometric tracing, were reported as the ratio of target gene (hIL-18) to housekeeping gene (GAPDH). All samples were normalized to an internal standard (PBMC + LPS) processed with each experiment to account for interassay variability. Each bar represents the mean ± SEM. Asterisks indicate significant differences between experimental groups (*, p < 0.02; **, p < 0.03). Statistical analysis was performed using the Mann-Whitney two-sample test. 279 bp, hIL-18.](http://www.jimmunol.org/content/177/10/6830/F1)

FIGURE 1.
beled with synthesized from a full length 481-bp hIL-18 cDNA sequence and radiola-
citrate phosphate/EDTA, and 5% BSA/nonfat milk; an IL-18 probe, syn-
3
lution containing 7% SDS, 1% polyethylene glycol, 2
standard saline
membranes. Resulting filters were prehybridized for 4 ha t 65°C in a so-
UV-cross-linked (Fb-UVXL-1000 X-linker; Stratagene, La Jolla, CA) to
filters hybridized overnight at 65°C. Hybridized filters were washed (2
3
GAGTC-3' (upstream) and 5'-AAGGTGAGGAGTGGGTGTC-3' (downstream), resulting in an 880-bp amplified fragment.

Southern blot analysis
Southern blot analysis was performed on resolved multiplex PCR products to verify the specificity of hIL-18 PCR-amplified cDNA fragments. Specifically, resulting gels were denatured using a 1.5 M NaCl/0.5 M NaOH solution (45 min), rinsed briefly with double distilled H2O, and neutralized with a 1.0 M Tris/1.5 M NaCl solution (2 × 30 min). Gels were then transferred to Magnagraph nylon membranes (Schleicher & Schuell, Keene, NH) using 20× SSC buffer, and transferred DNA fragments were UV-cross-linked (Fb-UVXL-1000 X-linker; Stratagene, La Jolla, CA) to membranes. Resulting filters were prehybridized for 4 h at 65°C in a so-

Immunohistochemical studies
As mentioned earlier, all colonic surgical specimens were collected imme-
diately following resection, opened longitudinally, rinsed, examined for
gross morphological changes, and a representative full-thickness sample
obtained. IBD specimens were derived from areas of active disease (inv) as
well as macroscopically n inv areas. Tissue samples were fixed by immer-
sion in 10% buffered formalin phosphate (Fisher Scientific, Pittsburgh, PA)
for 2–7 days at 4°C, sequentially dehydrated in 50%, 70%, 95%, and ab-
solute ethanol (30 min/each) with agitation, and finally cleared in xylene
(2 × 30 min). Samples were then embedded in Paraplast® embedding
media (Oxford Labware, St. Louis, MO), and resulting blocks were stored
at room temperature for later tissue sectioning. Five-micron-thick serial
sections were obtained, mounted on poly-L-lysine-coated Superfrost/Plus
glass slides (Fischer Scientific), deparaffinized in xylene (2 × 10 min), and
dehydrated in absolute ethanol (2 × 2 min). Colonic tissue sections were
then blocked with normal rabbit serum (Vectastain Elite ABC kit; Vector
Laboratories, Burlingame, CA) for 20 min, permeabilized for 15 min with
saponin buffer (1% FCS, 0.1% sodium azide, 0.1% saponin in PBS; all
from Sigma), and blocked again for endogenous peroxidase using 1% H2O2
dehydrated in absolute ethanol (2 × 2 min). Colonic tissue sections were

FIGURE 2. Immunohistochemical staining for hIL-18 in paraffin-embedded sections from noninflamed control and UC intestinal tissue resections. A, Using an affinity-
purified goat anti-human IL-18 polyclonal Ab, cont colonic mucosa obtained from a
resection specimen without evidence of IBD revealed weak to moderate staining of
superficial IEC, as well as scattered inflammatory cells within the lamina propria. C, In colonic mucosa not involved by inflammatory changes from a UC patient (UC n
inv), more diffuse and qualitatively in-
creased staining of the epithelium was ob-
served when compared with cont. E, In in-
flamed and eroded mucosa from a UC
specimen (UC inv), increased epithelial
staining compared with cont was again ob-
served; in addition, granulation tissue ad-
jjacent to the epithelium demonstrated
weak to moderate staining of scattered in-
flammatory cells. B, D, and F, Control im-
munohistochemical reactions using an iso-
type goat IgG that correspond to specimens
in A, C, and E, respectively, are shown.
A–D, ×100 original magnification; E and
F, ×150 original magnification.
Western blot analysis

Total protein levels of tissue homogenates derived from colonic mucosal biopsies (n = 6/experimental group) were quantitated using a modification of the Bradford colorimetric procedure (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). Biopsy homogenates (standardized to 20 μg of total protein/lane) were then resolved by 15% denaturing SDS-PAGE and transferred for 1 h at 100 V using transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris (pH 8.0); all from Sigma) onto polyvinylidene difluoride membrane (Bio-Rad) using an electrophoretic transfer unit (Mini Trans-Blot Electrophoretic transfer cell; Bio-Rad). Recombinant hIL-18 (35 ng) (a kind gift from Dr. Monica Tsang) was used as a positive control. Following transfer, membranes were blocked overnight at 4°C in 20 ml of 5% nonfat dry milk in PBS, washed in PBS containing 0.1% Tween (Sigma) (3 × 10 min), and incubated with an affinity-purified goat anti-human IL-18 polyclonal Ab (1 μg/ml) (a kind gift from M. Tsang) for 3 h at room temperature on an orbital shaker. Blots were subsequently washed (3 × 10 min), as above, and incubated with an anti-goat IgG conjugated HRP (1:100) (Sigma) for 1.5 h at room temperature, also on an orbital shaker. Finally, blots were washed (2 × 10 min), as above, and in PBS only (1 × 10 min), incubated with 6.5 ml/membrane of enhanced chemiluminescence detection reagent (Amersham Life Science, Arlington Heights, IL) for 1 min at room temperature, and exposed to X-OMAT autoradiography film with intensifying screens for 5–15 s.

Statistical analyses

For IL-18 mRNA levels, all samples were normalized to the internal standard and results (IL-18/GAPDH) are expressed as mean ± SEM. The data were analyzed using a statistical program (BMPD, Los Angeles, CA). The methods used included the Kruskal-Wallis test for nonparametric data and the Mann-Whitney two-sample test for parametric data. Differences were considered significant when p < 0.05.

Results

Expression of IL-18 mRNA transcripts in isolated mucosal cell populations

The expression of mRNA transcripts in freshly isolated CD, UC, and noninflamed controls is represented in Fig. 1. IL-18 mRNA transcripts were found to be more abundant in both IEC and LPMC obtained from CD compared with UC and controls. Statistical analyses (Fig. 1A) confirmed the specificity of the amplified products to hIL-18. Fig. 1B shows the relative levels of IL-18 mRNA transcripts in IEC and LPMC obtained from CD, UC, and noninflamed controls (n = 6/group). IL-18 mRNA transcripts were significantly increased in CD (2.68 ± 0.36 for IEC and 1.25 ± 0.17 for LPMC) compared with cont (1.29 ± 0.31 for IEC, p > 0.02 and 0.55 ± 0.19 for LPMC, p < 0.03) as well as UC (1.89 ± 0.26 for IEC, p < 0.04 and 0.72 ± 0.12 for LPMC, p < 0.04). Although a trend in increased IL-18 mRNA levels was detected in intestinal mucosal cell populations from UC compared with cont, these observed differences did not reach statistical significance.
Immuno-histochemical localization of IL-18 in surgically resected colonic specimens

In colonic mucosa obtained from surgical noninflamed controls, weak to moderate staining of superficial IEC and scattered inflammatory cells within the lamina propria was observed (Fig. 2A). By comparison, more diffuse and qualitatively increased IL-18 staining of the epithelium was observed in n inv colonic mucosa of UC patients (Fig. 2C). In severely inflamed UC tissues, increased epithelial staining, as well as weak to moderate staining of scattered inflammatory cells, was detected (Fig. 2E). Corresponding control sections using an isotype goat IgG are shown in Fig. 2, B, D, and F.

Western blot analysis of hIL-18 expression in colonic mucosal biopsies obtained from IBD and noninflamed control patients. An amount of 20 μg of total protein from tissue homogenates (n = 6/experimental group) was resolved by 15% denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat dry milk in PBS, subsequently washed in PBS containing 0.1% Tween, and incubated with an affinity-purified goat anti-human IL-18 polyclonal Ab (1.0 μg/ml). Blots were finally incubated with an anti-goat IgG conjugated HRP Ab (1:100), and hIL-18 was detected by enhanced chemiluminescence. Representative blot of six separate experiments is shown and demonstrates that the 18.3-kDa mature form of IL-18 (lower arrow) is more abundant in CD compared with UC intestinal mucosal biopsies and is absent in cont. The 24-kDa inactive precursor form (middle arrow) was detected in nonaffected areas from both CD and UC biopsies and was the sole form found in normal noninflamed controls. A third band of ~31 kDa was observed in n inv CD and UC biopsies and was the sole form found in normal noninflamed controls. A third band of ~31 kDa was observed in n inv CD and UC biopsies and was the sole form found in normal noninflamed controls.
UC colonic mucosal biopsies and was absent in noninflamed controls as well as inv areas of IBD specimens (Fig. 5). In addition, the control lane contains a band ~36 kDa in size, and most likely represents a dimer product of the recombinant hIL-18 protein.

Discussion

There is increasing evidence that IL-18 may be a key proinflammatory cytokine as well as an important mediator of Th1 polarized immune responses (reviewed in Ref. 17). In the present study, we investigated the expression and cellular localization of IL-18 in CD, a prototypic Th1-mediated disease. Our results show that the mature form of IL-18 is indeed markedly overexpressed in intestinal lesions of CD patients, but not in UC, another form of IBD in which polarized Th2 responses are believed to play a dominant role (18). To our knowledge, this is the first report of a putative role of IL-18 in mediating human organ-specific autoimmunity.

IL-18 transcripts were detected in colonic mucosal cells from both CD and UC, as well as noninflamed control patients. However, IL-18 mRNA levels were significantly increased in both LPMC and IEC populations from CD patients only when compared with the aforementioned experimental groups. By comparison, although the inactive pro-form (24 kDa) was the sole form detected in nonaffected controls and in macroscopically uninv IBD colonic biopsies, the mature form was highly expressed in only affected CD tissues. The increased expression of mature IL-18 protein appears to be specific for CD, since UC tissues with comparable severity of inflammation displayed only minimally detectable levels of IL-18. Thus, the activation of IL-18 in CD is unlikely secondary to the inflammatory responses, but may represent a primary abnormality within this patient population. However, since the mature form of IL-18 is the result of IL-1β-converting enzyme (ICE or caspase-1) cleavage (19), it may be speculated that a dysregulation of ICE synthesis may also be involved in the pathogenesis of CD (20). In addition, the presence of a 31-kDa protein that cross-reacts with the hIL-18 Ab was detected in nonaffected intestinal diseased tissues from both CD and UC patients. Characterization and function of this protein as a possible IL-18 isoform or a variant of the IL-18 gene is currently under investigation in our laboratory.

Immunohistochemical analysis of full thickness colonic resections from the different experimental groups revealed constitutive levels of IL-18 in IEC. However, in moderately and severely affected tissues from CD, IL-18 staining in IEC appeared more intense and was also observed within the lamina propria. IL-18-positive staining cells within the lamina propria were found to be morphologically consistent with tissue histiocytes and dendritic cells. These results further support the concept that epithelial cells, as well as resident dendritic cells, are active participants in inflammatory responses within the local gut milieu by having the ability to produce an array of immunoregulatory cytokines (21). In particular, the ability to produce IL-18, a potent Th1 proinflammatory cytokine, is of particular relevance.

A model to explain the pathogenesis of CD has been recently proposed (13, 22). In this model, an initiating antigenic stimulus activates a dysregulated and exaggerated Th1 response driven by a putative Th1-inductive cytokine, yet to be identified. The role of IL-12 and/or TNF-α in mediating this process has been implicated. This is followed by increased IFN-γ and possibly TNF-α production by CD4+ T cells. These two cytokines induce macrophage gene expression with production of proinflammatory cytokines, such as IL-1, TNF, and IL-8, which are directly mediating the observed damage to IEC. The results presented in this report are consistent with this hypothesis, but point out the possible relevance of IL-18 to this pathogenic model. In fact, IL-18 is not only produced by APC (i.e., tissue histiocytes and dendritic cells) to mount a Th1 polarized response in synergy with, and independently of, IL-12 through IFN-γ production (Th1-inductive phase), but it also stimulates TNF-α gene expression and secretion from activated T cells (effector phase) (7). In addition, IL-18 is capable of stimulating IL-1β as well as IL-8 from activated macrophages, thus affecting the final common pathway of CD immunopathogenesis.

Therefore, it is conceivable that IL-18 may, in fact, fulfill the requisite as a primary initiating cytokine in Th1-mediated diseases, such as CD. Recent animal studies using mAb neutralization against IL-18 in organ-specific autoimmune diseases have supported this concept (17).

In summary, our studies provide conclusive evidence that IL-18 may play a key pathogenic role in Th1-mediated disorders, such as CD, and provide a rationale for anti-IL-18-based treatment in these conditions. Differently from current anti-cytokine strategies, such as blockade of TNF-α in CD and rheumatoid arthritis, which primarily target the Th1 effector phase, this approach would possess the theoretical advantage of affecting the inductive phase of Th1 T cell activation. Future animal, as well as human, studies will test the validity of this hypothesis.

Acknowledgments

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References