A Functional Role for Interleukin-21 in Promoting the Synthesis of the T-Cell Chemoattractant, MIP-3α, by Gut Epithelial Cells

ROBERTA CARUSO,* DANIELE FINA,* ILARIA PELUSO,* CARMINE STOLFI,* MASSIMO CLAUDIO FANTINI,* VALENTINA GIOIA,* FLAVIO CAPRIOLI,* GIOVANNA DEL VECCHIO BLANCO,* OMERO ALESSANDRO PAOLUZI,* THOMAS T. MACDONALD,[†] FRANCESCO PALLONE,* and GIOVANNI MONTELEONE*

*Dipartimento di Medicina Interna e Centro di Eccellenza per lo Studio delle Malattie Complesse e Multifattoriali, Università Tor Vergata, Rome, Italy; [†]Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, London, United Kingdom

Background & Aims: **Interleukin (IL)-21, a T-cell–derived cytokine, is produced in excess in inflammatory bowel diseases (IBD). The IL-21 receptor (IL-21R) is expressed by immune and nonimmune cells, raising the possibility that IL-21 has broad effects in gut inflammation. In this study we examined whether intestinal epithelial cells express IL-21R and respond to IL-21 in IBD.** ***Methods:*** **IL-21R was evaluated in intestinal samples of IBD patients and controls by immunohistochemistry and Western blotting. Intestinal epithelial cells were stimulated with IL-21, and cell-free supernatants were evaluated by a protein array and enzyme-linked immunosorbent assay. The effect of IL-21–treated epithelial cell supernatants on blood lymphocyte migration was assessed using a chemotaxis assay. Finally, we evaluated the effect of a neutralizing IL-21 antibody on MIP-3α synthesis in ex vivo organ cultures of IBD mucosal explants.** ***Results:*** **Constitutive expression of IL-21R was seen in intestinal epithelial cells, but was higher in IBD patients than in controls. Stimulation of intestinal epithelial cells with IL-21 resulted in enhanced phosphorylation of ERK1/2 and p38 and increased synthesis of macrophage inflammatory protein-3 alpha (MIP-3α), a T-cell chemoattractant. Inhibition of ERK1/2 but not p38 suppressed IL-21–induced MIP-3α production. IL-21–treated cell culture supernatants enhanced in vitro lymphocyte migration, and this effect was inhibited by anti-MIP-3α antibody. Treatment of IBD explants with anti–IL-21 reduced MIP-3α production.** ***Conclusions:*** **These data show that intestinal epithelial cells are a target of IL-21 and that IL-21 is involved in the cross-talk between epithelial and immune cells in the gut.**

Crohn's disease (CD) and ulcerative colitis (UC) are the 2 major chronic inflammatory bowel diseases (IBD) in human beings. The cause of both IBDs is unknown, but evidence has been accumulated to show that the liability to develop CD or UC is influenced by a wide range of genetic and environmental factors.[1] It also is thought that IBDs are caused by excessive immune reactivity in the gut wall, most likely directed against constituents of the luminal flora. Indeed, the inflamed tissue in IBD patients is heavily infiltrated with activated inflammatory cells, mostly T cells, continuously recruited from the circulation.[1] The influx of immunocytes into the gut tissue is tightly regulated and is at least partly dependent on the release of chemokines, small inducible proteins that have the ability to induce migration of different cell types into tissues.[2] In the gut, epithelial cells are a major source of chemokines, and there is evidence that chemokine production can be regulated by T-cell– and macrophage-derived cytokines.[2]

Interleukin (IL)-21 is a newly described T-cell–derived cytokine that signals through the common γ-chain of the IL-2 receptor and its own unique receptor (designated IL-21R).[3] By using Northern blot analysis to characterize RNA expression levels, IL-21R initially was shown to have a lymphoid-restricted pattern of expression, with the strongest signals in the thymus, spleen, and lymph nodes. Cell-surface IL-21R also has been detected on B lymphocytes and CD56+ natural killer cells, and on activated T cells.[3] Activation of IL-21R on binding of IL-21 enhances the proliferation of anti–CD3-prestimulated T cells and promotes interferon-γ synthesis.[4] IL-21 also regulates B and natural killer cell proliferation, as well as natural killer cell cytotoxicity and interferon-γ production.[3] In line with this, we have shown previously that IL-21 is produced in excess in the inflamed mucosa of patients with IBD, and that IL-21 contributes to sustain the ongoing T helper type 1 (Th1) cell immunity in CD.[5] More recent studies have shown that, during inflammatory processes, IL-21R also can be expressed by nonimmune cells, such as fibroblasts, keratinocytes, and endothelial cells,[6,7] thus raising the possibility that IL-21 can integrate the functions of nonlymphoid cells with the immune system. This property could be particularly rele-

Abbreviations used in this paper: **ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IL-21R, interleukin-21 receptor; MAP, mitogen-activated protein; PBMC, peripheral blood mononuclear cell.**

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vant in the gut, where cross-talk between nonlymphoid cells and immune cells is thought to play a decisive role both in the control of mucosal homeostasis and the pathogenesis of IBD-related tissue damage.

In this study we have therefore extended our analysis on the role of IL-21 in IBD. In particular, we have examined whether intestinal epithelial cells express IL-21R and respond to IL-21.

Materials and Methods

Patients and Samples

Mucosal samples were taken from 20 patients with CD undergoing resection for chronic disease unresponsive to medical treatment. In 16 patients the primary site of disease was the terminal ileum and right colon, whereas in the remaining 4 patients the disease was located in the colon. Eleven patients were receiving corticosteroids, 6 were taking corticosteroids plus azathioprine, and 3 were taking antibiotics plus mesalazine. In addition, mucosal colonic biopsy specimens were available from 12 CD patients undergoing colonoscopy for active disease. Nine patients were taking mesalazine, and the remaining patients had discontinued therapy. Mucosal samples also were taken from 13 patients with active UC undergoing endoscopy and 5 patients undergoing colectomy for a chronic disease unresponsive to medical treatment. Disease extent was substantial in 9 and distal in 9 patients. Seven patients were taking corticosteroids, and 11 patients were taking mesalazine. Moreover, colonic mucosal samples were taken from 4 patients with diverticular disease. Normal controls included samples taken from 13 patients with irritable bowel syndrome, and from macroscopically and microscopically unaffected colonic areas of 15 patients undergoing colectomy for colon cancer.

Isolation of Primary Gut Epithelial Cells

Freshly obtained colonic biopsy specimens from 9 patients with CD, 8 patients with UC, 4 patients with diverticular disease, and 14 normal controls were used to isolate epithelial cells by dithiothreitol (.1 mmol/L) and ethylenediaminetetraacetic acid (1 mmol/L, both from Sigma-Aldrich, Milan, Italy). The resulting cell preparations contained less than 5% contaminating lymphocytes as assessed by flow cytometry. The isolated cells were checked for viability using 0.1% trypan blue (viability range, 81%–88%) and then used for extracting total proteins.

Intestinal Epithelial Cell Lines

The colon epithelial cell lines, DLD-1, HT-29, Caco-2, HT-115, Colo205, and T84 were cultured in 25-cm^2 plastic flasks and maintained at 37°C in a humidified atmosphere of 5% CO_2 in appropriate medium supplemented with 10% inactivated fetal bovine serum. To examine whether these cell lines respond to IL-21, 10^5 cells were plated into each well of a 24-well plate, and left to adhere for 24 hours. Cells then were starved for 24 hours and stimulated with recombinant human IL-21 (50–500 ng/mL, R&D Systems, Inc. Minneapolis, MN) for the indicated time points. In parallel experiments, cells were stimulated with tumor necrosis factor-α (20 ng/mL; R&D Systems). To examine the effect of IL-21 on mitogen-activated protein (MAP) kinases, serum-starved cells were stimulated with IL-21 (200 ng/mL) for 10–60 minutes, then lysed, and total extracts were analyzed for the content of MAP kinases by Western blotting. In parallel, cells were preincubated with PD98059, an inhibitor of extracellular signal-regulated kinases 1/2 (ERK1/2) (50 μmol/L), or SB202190, an inhibitor of p38 (10 μmol/L), or a c-Jun NH2-terminal kinase (JNK) inhibitor (5 μmol/L, 420116) (all from Inalco, Milan, Italy), or vehicle (ethanol or dimethyl sulfoxide) for 30 minutes before adding IL-21 (200 ng/mL) for a further 48 hours. The concentration of each inhibitor was selected on the basis of preliminary experiments showing that at the specified dose the compound inhibited only the corresponding MAP kinase. At the end of cell culture, MIP-3α was evaluated in the cell-free culture supernatants by enzyme-linked immunosorbent assay (ELISA). To examine whether IL-21R expression is modulated by inflammatory cytokines, serum-starved cells were either left untreated or treated with IL-1β (20 ng/mL; Peprotech EC Ltd., London, UK), IL-6 (20 ng/mL; Peprotech), IL-21 (200 ng/mL), TNF alpha (20 ng/mL, R&D Systems), or IFN-γ (200 ng/mL, Peprotech) for 24 hours, and then the fraction of IL-21R–positive cells was evaluated by flow cytometry.

Western Blotting

All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) unless specified, and secondary antibodies were from Dako (Milan, Italy). Both primary intestinal epithelial cells and cell lines were lysed for 60 minutes on ice in buffer containing 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid, and 0.2 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*,-tetraacetic acid, supplemented with 1 mmol/L dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (all reagents were from Sigma-Aldrich). Cell lysates were clarified by centrifugation at 4°C for 30 minutes at 12,000×*g*. Equal amounts of total proteins were fractionated on sodium dodecyl sulfate–polyacrylamide gels. The membranes were blocked with Tris-buffered saline containing .05% Tween 20 and 5% nonfat dry milk and then incubated, depending on the experiment, with the following antibodies: anti–IL-21R (1 μg/mL; R&D Systems), anti-common γ-chain (1:500 final dilution), anti–β-actin (1:5000; Sigma-Aldrich), anti-cytokeratin-18 (1:400; Sigma-Aldrich), anti–α-smooth

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