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"INTERLEUKIN-21 CONTROLS INFLAMMATORY PATHWAYS IN IBD"

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Index:

3. CONTROL OF MATRIX METALLOPROTEINASE PRODUCTION IN HUMAN INTESTINAL FIBROBLASTS BY INTERLEUKIN 21

3.1. INTRODUCTION

3.2. MATERIALS AND METHODS

3.2.1. Patients and samples

3.2.2. Isolation and culture of intestinal fibroblasts

3.2.3. Effect of Crohn's disease lamina propria mononuclear cell derived IL-21 on MMP production

3.2.4. Western blotting

3.2.5. Gelatin zymography

3.2.6. RNA extraction, complementary DNA preparation and reverse transcriptase-polymerase chain reaction

3.2.7. Statistical analysis

3.3. RESULTS

3.3.1. IL21R is expressed by gut fibroblasts

3.3.2. IL21 promotes MMP synthesis by intestinal fibroblasts

3.3.3. IL21 does not enhance MMP RNA expression and new protein synthesis

3.3.4. Blocking IL21 reduces fibroblast MMP secretion induced by Crohn's disease LPMC supernatants

3.4. DISCUSSION

3.5. REFERENCES

4. A FUNCTIONAL ROLE FOR INTERLEUKIN-21 IN PROMOTING THE SYNTHESIS OF THE T-CELL CHEMOATTRACTANT, MIP-3A, BY GUT EPITHELIAL CELLS

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS

4.2.1. Patients and Samples

4.2.2. Isolation of Primary Gut Epithelial Cells

4.2.3. Intestinal Epithelial Cell Lines

4.2.4. Western Blotting

4.2.5. Analysis of IL-21R, Cell Growth, and Death by Flow Cytometry

4.2.6. Production of the Neutralizing IL-21 Antibody

4.2.7. Organ Culture

4.2.8. Human Cytokine Expression Array Assay

4.2.9. Determination of MIP-3α by ELISA

4.2.10. Chemotaxis Assay

4.2.11. Immunohistochemistry

4.2.12. Statistical Analysis

4.3. RESULTS

4.3.1. IL-21R Expression Is Increased on Gut Epithelial Cells of IBD Patients

4.3.2. IL-21 Enhances the Synthesis of MIP-3α by Colon Epithelial Cells

4.3.3. Induction of MIP-3α by IL-21 Is Dependent on ERK1/2 Activation

4.3.4. IL-21–Induced Colon Epithelial Cell–Derived MIP-3α Promotes Lymphocyte Migration In Vitro

1. INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of inflammatory bowel diseases (IBD) in humans. Although the aetiology of both CD and UC remains unknown, a large body of evidence suggests that IBD result from the interplay of environmental, genetic and immune factors [1, 2]. Studies in experimental models of IBD also indicate that the pathologic process consists of an aberrant immune response that is directed against components of the normal bacterial microflora [1]. IBD is characterized by a marked inflammatory infiltrate of the gut, and various degrees of tissue damage and remodelling, that profoundly affect the intestinal functions. $CD4^+$ T lymphocytes are important mediators of this pathologic process. Indeed, administration of drugs that target CD4+ T cell activation and/or activity is useful for inducing and maintaining clinical remission in IBD patients. Moreover, studies in animal models of IBD have shown that either targeted inhibition or over-expression of $CD4^+$ T cell gene products can profoundly alter the magnitude and outcome of the tissue-damaging inflammatory responses [3].

A Th1-mediated immune response governs the gut inflammation in Crohn' s disease

$CD4^+$ T lymphocytes can differentiate into distinct lineages of highly polarised cells that synthesize specific profiles of cytokines. The rapid advancement of molecular technologies and the relative case of access to the diseased gut tissues has boosted an active research over the past two decades, thus contributing to define the pattern of cytokines produced by $CD4^+$ T cells in IBD. It is now well-accepted that CD is characterised, at least in the late, chronic phase of the inflammatory process, by a marked infiltration of the affected wall with Th1-type lymphocytes, that synthesize high levels of interferon (IFN)-γ [1]. This is in accordance with the demonstration that in the gut of CD patients there is enhanced production of interleukin (IL)-12, a heterodimeric cytokine formed by two subunits (p40 and p35) and able to drive Th1 cell polarization [4]. Moreover, CD mucosal

$CD4^+$ T cells express high levels of IL-12Rβ2 chain, that is necessary for IL-12 signalling, and active STAT4, a transcription factor that is required for inducing IFN-γ gene promoter activity [5]. Moreover, CD lamina propria T lymphocytes (T-LPL) over-express T-bet, a member of the T-box family of transcription factors that enhances IFN-γ gene expression [6]. In line with these observations, studies in murine models of CD-like colitis have mechanistically shown that exaggerated and poorly controlled Th1 cell activity can trigger intestinal tissue damage [1]. On the other hand, blockade of IL-12 activity by a neutralising IL-12/p40 antibody prevents and/or ameliorates Th1-driven colitis [7]. Similarly, a recent study has shown that administration of an anti-IL-12/p40 antibody facilitates the induction and the maintenance of remission in patients with active CD [8]. In this context, it is however noteworthy that p40 is shared with IL-23, a cytokine produced in excess in CD patients, and able to expand both IFN-γ and IL-17-mediated inflammatory responses [9]. Therefore, it is plausible that the clinical benefit of the anti-IL-12/p40 antibody can be in part due to the neutralization of IL-23.

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2. INTERLEUKIN-21 ENHANCES T-HELPER CELL TYPE I SIGNALING AND INTERFERON-γ PRODUCTION IN CROHN'S DISEASE

2.1. INTRODUCTION

Activated T-helper (Th)1 cells play a central role in the pathogenesis of tissue damage in Crohn's disease (CD).[1] There is strong evidence that interleukin (IL)-12, the major Th1-inducing factor in humans, is up-regulated in CD tissue and that IL-12 contributes to the preferential expansion of interferon (IFN)-γ–secreting cells in this condition.[2,3] Studies in murine models also have shown that the IL-12/STAT4 signaling pathway is important in the development of Th1-mediated colitis resembling CD.[4,5]

Both in vitro and in vivo observations indicate that although IL-12 is dominant in directing Th1 development, the biological activity of additional cytokines is required for optimal differentiation of Th1 cells and production of IFN-γ.[6] For example, in CD mucosa there is abundant IL-18, and mucosal IL-12–induced IFN-γ production also is enhanced by IL-7 and IL-15, 2 cytokines that signal through the common γ-chain receptor subunit.[7–9] These findings also fit with the notion that stabilization of the polarized Th1 cell phenotype requires activation through multiple signaling pathways.[10,11]

IL-21 is a newly described cytokine produced by activated T cells that profoundly affects the growth and functional activity of T cells, B cells, and natural killer lymphocytes, in concert with other cytokines or activating stimuli.[12–14] The cytokine itself is related to IL-2, IL-4, and IL-15, and its cellular effects are mediated through a class I cytokine family receptor, IL-21R. IL-21R has homology to the shared α chain of the IL-2 and IL-15 receptors, interacts with the common γ-cytokine receptor chain, and appears to signal by association with Jak1 and Jak3, and phosphorylation of STAT1 and STAT3 molecules.[15] Thus, on the basis of biological activity and

receptor composition, it is highly likely that IL-21 helps sustain the ongoing Th1 mucosal response in CD, functioning as an autocrine growth and survival signal. We therefore examined the expression and functional role of IL-21 in CD.

2.2. MATERIALS AND METHODS

2.2.1. Patients and samples

Mucosal samples were taken from freshly obtained intestinal resection specimens of 29 patients with active CD. In 18 patients the primary site of disease was the terminal ileum and in 11 patients disease was present in both the ileum and colon. Thirteen patients were receiving corticosteroids, 10 were taking corticosteroids plus azathioprine, and 6 were taking mesalazine plus antibiotics. Eighteen CD patients had a fibrostenosing disease. The indication for surgery was chronic disease, unresponsive to medical treatment. From patients with ileocolonic involvement, mucosal samples were taken from involved (gross lesions) and uninvolved ileal and colonic areas. Additional mucosal samples were taken during endoscopy from 4 CD patients. In these patients the primary site of involvement was ileocolonic in 1, and colonic in the remaining patients. All of these patients had active disease and were receiving mesalazine.

Colonic mucosal samples also were taken from involved areas of 26 patients with active ulcerative colitis (UC) who were undergoing endoscopy and 4 patients who were undergoing colectomy for a chronic disease that was poorly responsive to medical treatment. Disease extent was substantial in 13 and distal in 17 patients. Ten patients were taking corticosteroids, 12 patients were taking mesalazine, and the remaining 8 patients were receiving no treatment.

Additional samples were taken from 6 patients with diverticular disease. As normal controls, samples were taken from 10 patients with irritable bowel syndrome undergoing endoscopy for

recurrent abdominal pain, and from macroscopically and microscopically unaffected colonic areas of 26 patients undergoing colectomy for colon cancer.

2.2.2. Lamina propria mononuclear cell isolation and culture Lamina propria mononuclear cells (LPMCs) were isolated by dithiothreitol–ethylenediaminetetraacetic acid and collagenase as previously described[2] and an aliquot was used immediately for extracting total proteins. The remaining LPMCs either were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (complete medium) (Sigma Chemical Aldrich, Milan, Italy) and cultured for the indicated times, or used to purify CD3+ T lamina propria lymphocytes (T-LPL). For this purpose, macrophage-depleted LPMCs were incubated for 30 minutes at 4°C with magnetically labeled CD14, CD19, and CD56 antibodies (Miltenyi Biotec, Calderara di Reno, Italy); T-LPL then were collected by negative selection using the magnetic cell sorting system (Miltenyi Biotec S.r.l.). CD4+, CD8+, and CD45RO+ LPL were purified from the T-LPL by positive selection using magnetic-labeled human CD4+, CD8+, or CD45RO+ antibodies (Miltenyi Biotec S.r.l.). To analyze mechanisms underlying IL-21 induction, normal T-LPL were treated with anti-CD3 (1% final dilution)[16] overnight, then extensively washed and cultured in complete medium with or without the addition of 10 ng/mL IL-12 (Peprotech EC LTD, London, UK) in the presence or absence of a neutralizing IFN-γ (10 μg/mL, R&D Systems, Abingdon, UK) or control isotype immunoglobulin (Ig)G antibody for a further 24 hours. In addition, CD LPMCs were cultured with or without a neutralizing IL-12 (10 μg/mL, R&D Systems) or control antibody for 12 hours and then stimulated with anti-CD3 for a further 24 hours. At the end, cell protein extracts were prepared and used for IL-21 analysis. To determine if IL-21 regulates mucosal Th1 cell responses, CD LPMCs were left untreated or treated with a recombinant human IL-21 receptor/Fc IgG fusion protein (20 μg/mL, R&D Systems) or control IgG for 12 hours and then stimulated with anti-CD3 or anti-CD3+CD28 (10 μg/mL, R&D Systems) for a further 24 hours. Cell protein extracts then were prepared and used for analysis of T-bet and STAT4, whereas cell culture supernatants were used for analysis of IFN-γ

and IL-4. IL-21 also was analyzed in autologous peripheral blood mononuclear cells (PBMC) obtained from 7 CD, 7 UC, and 8 control patients.

2.2.3. Western blotting

Western blot analysis for IL-21 was performed using total proteins extracted from intestinal samples from 18 CD, 17 UC, 6 diverticular disease patients, and 18 normal controls. In addition, total proteins were prepared from unfractioned LPMCs; purified CD3+, CD4+, CD8+, and CD45RO+ LPL; and PBMCs. For the detection of IL-21, 120-μg/sample total proteins from whole tissues or 20–60 μg from cells were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. IL-21 was detected by using a rabbit anti-human IL-21 (.5 μg/mL) (ProSci Incorporated, Poway, CA) followed by a horseradish peroxidase–conjugated goat anti-rabbit IgG monoclonal antibody (Dako, Milan, Italy; final dilution 1:20,000). The reaction was detected with a sensitive enhanced chemiluminescence kit (West DURA; Pierce, Rockford, IL). After the analysis of IL-21, blots were stripped and then incubated with a mouse anti-human β-actin antibody (final dilution, 1:5000; Sigma-Aldrich), as internal loading control, followed by a goat anti-mouse antibody conjugated to horseradish peroxidase (1:30,000 dilution) and detection by chemiluminescence.

T-bet was analyzed using specific mouse anti-human T-bet antibody (1:500 final dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:20,000 dilution) was used to detect primary antibody binding and immunoreactivity was visualized as indicated earlier.

To investigate STAT4, total proteins were separated on an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed for phosphorylated STAT4 (p-STAT4) content using a rabbit anti-human p-STAT4 (1.5 μg/mL) antibody (Histo-Line Laboratories, Milan, Italy). Goat anti-rabbit antibody conjugated to horseradish peroxidase (1:20,000 dilution; Dako) was used as a secondary antibody, and the reaction was developed as indicated earlier. After detection of p-

STAT4, blots were stripped and subsequently incubated with a rabbit anti-human STAT4 polyclonal antibody (1:600 final dilution, Santa Cruz Biotechnology), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:20,000 dilution; Dako). Computer-assisted scanning densitometry (Total Lab; AB.EL Sience-Ware Srl, Rome, Italy) was used to analyze the intensity of the immunoreactive bands.

2.2.4. Enzyme-linked immunosorbent assay

Cell culture supernatants were collected and used for analysis of IFN-γ (Peprotech) or IL-4 (R&D Systems) by enzyme-linked immunosorbent assay, according to the manufacturer's instructions.

2.2.5. Statistical analysis

Differences between groups were compared using either the Mann–Whitney U test, if the data were not distributed normally, or the Student *t* test, if the observations were consistent with a sample from a normally distributed population.

2.3. RESULTS

2.3.1. IL-21 expression is enhanced at the site of disease in CD

IL-21 was detected in tissue homogenates from all samples regardless of whether mucosal specimens were taken from inflammatory bowel disease patients or controls. However, there was a clear increase in the intensity of the IL-21 bands in CD compared with UC patients, diverticular disease patients, and normal controls (Figure 1*A*). In addition, IL-21 levels were higher in UC patients than in diverticular disease and normal control patients ($P < .01$), whereas no difference was seen between diverticular disease and normal controls (Figure 1*B*). Enhanced IL-21 was seen in involved but not uninvolved CD, and was not associated with any CD phenotype, such as fibrostenosing disease (Figure 2).

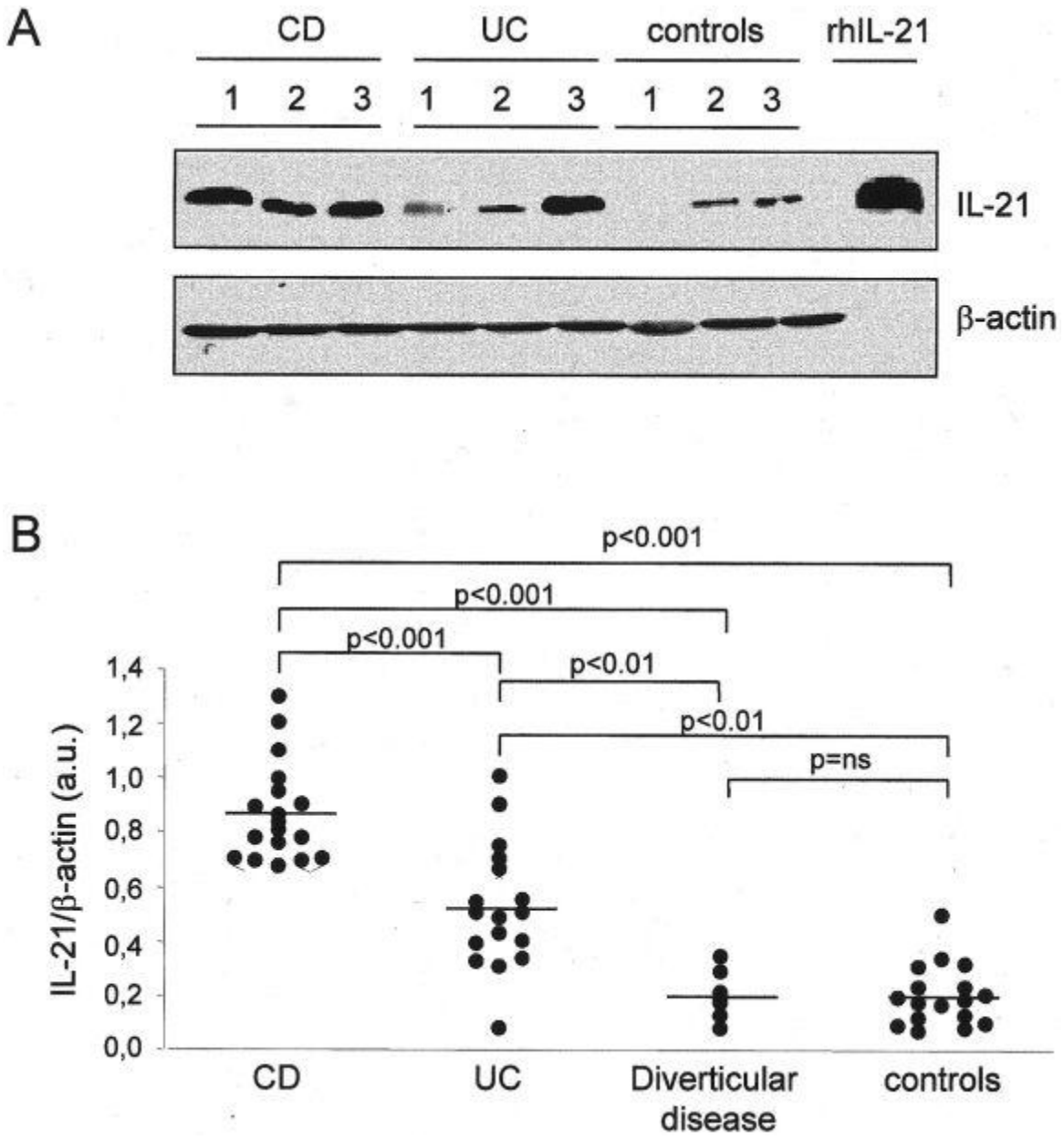

Figure 1. Enhanced IL-21 protein in CD. (*A*) Representative expression of IL-21 (upper blot) and β-actin (lower blot) protein in mucosal samples taken from 3 patients with CD, 3 patients with UC, and 3 normal controls. The example is representative of 7 separate experiments analyzing mucosal samples from 18 CD patients, 17 UC patients, and 18 normal controls. In the last lane, recombinant human IL-21 (50 ng/mL) was used as a positive control. (*B*) Quantitative analysis of IL-21/β-actin protein ratio in mucosal samples from 18 patients with CD, 17 patients with UC, 6 patients with diverticular disease, and 18 normal controls, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Each point represents the value (a.u.) of the IL-21/β-actin protein ratio in mucosal samples taken from a single subject. Horizontal bars indicate the median.

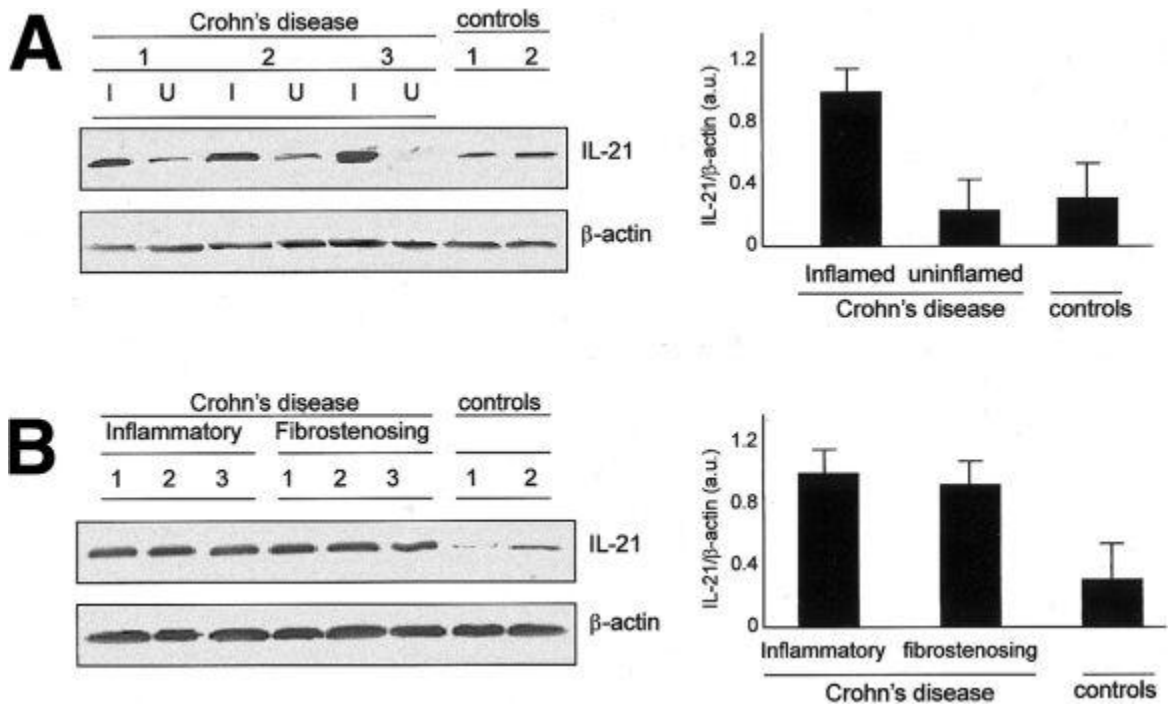

Figure 2. (*A*) Enhanced expression of IL-21 occurs at the site of disease in CD. Representative blot of IL-21 (upper panel) and β-actin (lower panel) in proteins extracted from both inflamed (I) and uninflamed (U) mucosal areas of 3 patients with CD and 2 normal controls. The example is representative of 3 separate experiments analyzing mucosal samples from 6 patients with CD and 8 normal controls. Right inset shows the quantitative analysis of IL-21/β-actin protein ratio in mucosal samples from inflamed and uninflamed areas of 8 patients with CD and 8 normal controls, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Data are expressed as mean ± SD. (*B*) Expression of IL-21 is not influenced by the CD phenotype. Representative blot of IL-21 (upper panel) and β-actin (lower panel) in proteins extracted from both mucosal areas of 4 patients with inflammatory CD, 4 patients with fibrostenosing CD, and 2 normal controls. The example is representative of 3 separate experiments analyzing mucosal samples from 8 patients with inflammatory CD, 9 patients with fibrostenosing CD, and 8 normal controls. Right inset shows the quantitative analysis of the IL-21/β-actin protein ratio in mucosal samples from 8 patients with inflammatory CD, 9 patients with fibrostenosing CD, and 8 normal controls, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Data are expressed as mean ± SD.

Increased expression of IL-21 also was seen in unfractioned LPMCs from CD patients in comparison with UC and control patients (Figure 3*A*). To exclude that differences reflected the higher number of mucosal T lymphocytes in CD patients, IL-21 expression also was assessed in purified CD3+ T-LPL from inflammatory bowel disease and normal control patients. As shown in Figure 3*B*, CD T-LPL contained higher amounts of IL-21 than that found in cells isolated from UC and control patients. This finding was not dependent on the state of T-LPL activation because IL-21 expression was more pronounced in CD45RO + T-LPL from CD patients in comparison with UC and control patients (Figure 3*B*, right inset). According to previously published reports,[12–14] IL-21

was detected in CD4+ but not CD8+ LPL (Figure 3*B*, left inset). In contrast, IL-21 was expressed at the same level in PBMCs from inflammatory bowel disease and control patients (Figure 3*C*).

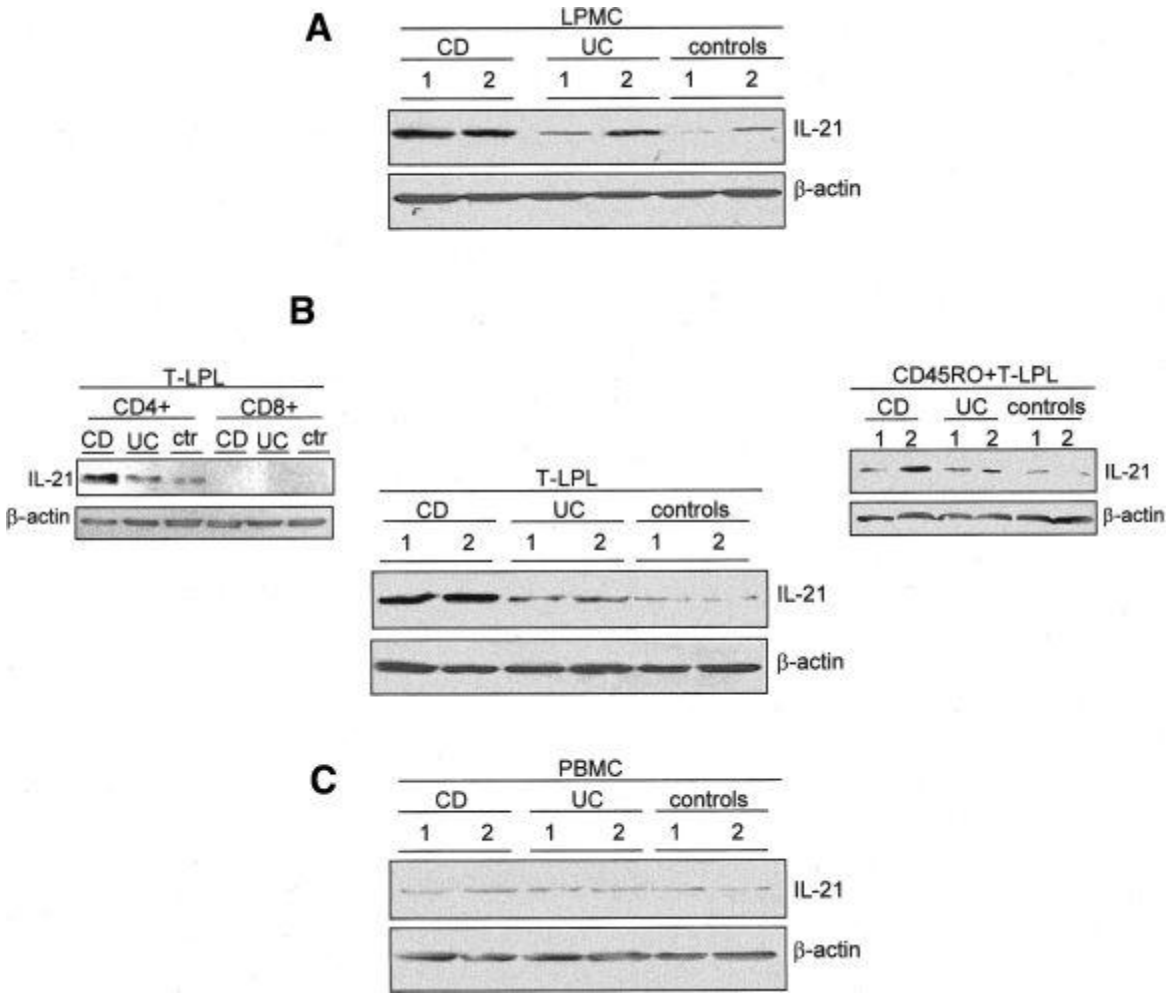

Figure 3. (*A*) Western blot showing IL-21 and β-actin protein in intestinal LPMCs, (*B*) CD3+ T-LPLs, and (*C*) PBMCs isolated from 2 patients with CD, 2 patients with UC, and 2 normal controls. The example is representative of 3 separate experiments analyzing LPMC and T-LPL samples from 6 CD patients, 5 UC patients, and 7 normal controls, and PBMCs from 7 CD patients, 7 UC patients, and 8 normal controls. Right inset shows a representative Western blot of IL-21 and β-actin protein in CD45RO+ intestinal LPLs isolated from 2 patients with CD, 2 patients with UC, and 2 normal controls. The example is representative of 4 separate experiments analyzing CD45RO+LPL samples from 5 CD patients, 3 UC patients, and 5 normal controls. Left inset shows a representative Western blot of IL-21 and β-actin protein in both CD4+ and CD8+ intestinal LPLs isolated from 1 patient with CD, 1 patient with UC, and 1 normal control (CTR). The example is representative of 3 separate experiments analyzing CD4+ and CD8+ LPL samples from 3 CD patients, 3 UC patients, and 3 normal controls.

2.3.2. IL-12 promotes IL-21 induction in the human gut

IL-21 is produced by activated T cells, but the exact mechanism underlying IL-21 induction remains unclear. The demonstration that IL-21 is expressed at higher levels in CD than control patients prompted us to explore the possibility that IL-21 synthesis could be sustained by cytokines, which are produced in excess in CD mucosa. To address this issue, we first cultured normal T-LPL with anti-CD3 to up-regulate the IL-12Rβ2 subunit and then stimulated them with exogenous IL-12. As expected, stimulation of T-LPL with anti-CD3 resulted in increased IL-21 levels (Figure 4*A*). Moreover, treatment of T-LPL with IL-12 increased the anti-CD3–stimulated IL-21 expression (Figure 4*A*). Because IL-12 is a potent inducer of IFN-γ,[6] we next examined whether the effect of IL-12 on IL-21 induction was mediated by IFN-γ. For this purpose, normal LPL were pretreated with a neutralizing IFN-γ antibody and then stimulated with IL-12; at the end the expression of IL-21 was examined by Western blotting. Interestingly, the anti–IFN-γ did not prevent the IL-12–induced IL-21 expression (Figure 4*A*). Of note, the same concentration of anti–IFN-γ abrogated the activation of STAT1 by IFN-γ in normal LPMCs, thus confirming its biological activity (not shown). To investigate further whether IL-12 regulates the induction of mucosal IL-21, CD LPMCs were pretreated with a neutralizing IL-12 antibody and then stimulated with anti-CD3. Treatment of CD LPMCs with anti-CD3 enhanced IL-21 expression, and this effect was decreased dramatically by anti–IL-12 (Figure 4*B*).

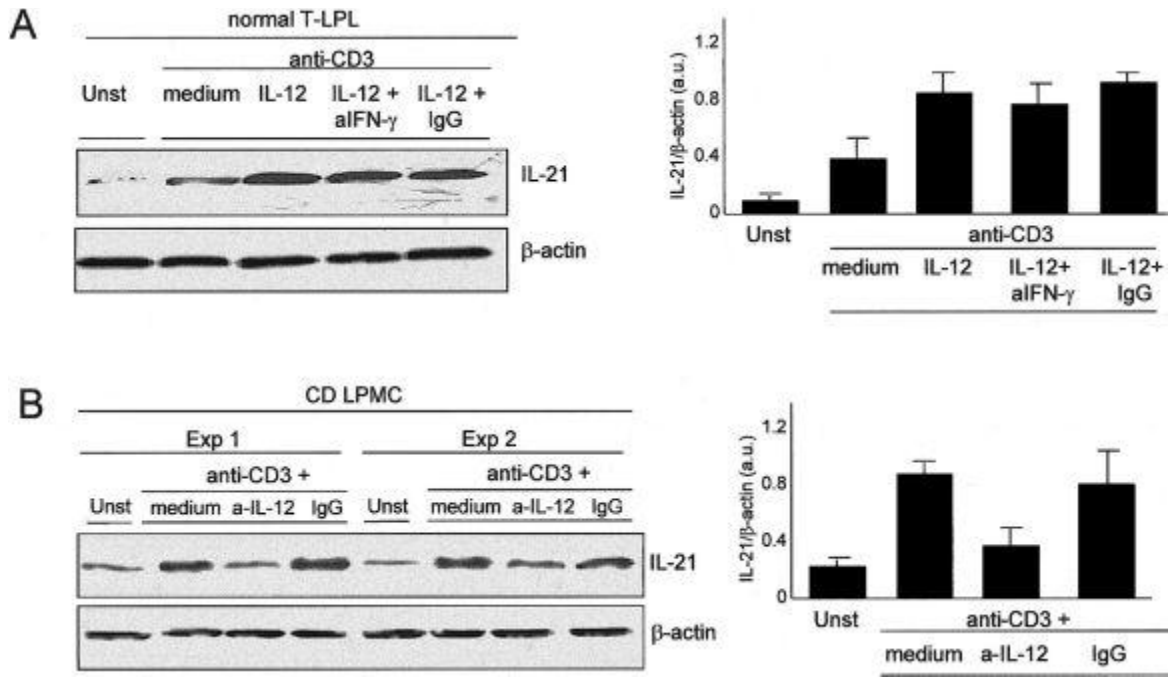

Figure 4. IL-12 enhances mucosal IL-21 expression. (*A*) Normal CD3+ T-LPL were left unstimulated (UNST) or treated with anti-CD3 antibody overnight, then washed and cultured with medium, IL-12, IL-12 + a neutralizing IFN-γ antibody (aIFN), or IL-12 + a control isotype antibody. Both antibodies were preincubated for 1 hour before adding IL-12. Cells were cultured for 24 hours and at the end protein was extracted and analyzed by Western blotting for IL-21 and β-actin. A representative Western blot is shown. Right inset shows the quantitative analysis of IL-21/β-actin protein ratio in T-LPL samples as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Data are expressed as mean ± SD of 4 separate experiments. (*B*) CD LPMCs were left unstimulated (UNST) or pretreated with medium, a neutralizing IL-12 (a-IL-12), or control isotype antibody overnight, then stimulated with anti-CD3 for 24 hours. At the end, proteins were extracted and analyzed by Western blotting for IL-21 and β-actin. Two representative Western blots are shown. Right inset shows the quantitative analysis of IL-21/β-actin protein ratio in CD LPMC samples as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (a.u.). Data are expressed as mean ± SD of 4 separate experiments.

2.3.3. IL-21 enhances mucosal Th1 cell responses

We have shown previously that mucosal IL-12–induced Th1 cell differentiation is enhanced by IL-7 and IL-15,[9] 2 cytokines that signal through the common γ-chain receptor.[17,18] Because the common γ-chain receptor also is used by IL-21,[14,15] we explored the possibility that IL-21 could contribute to the ongoing Th1 cell response in CD. To this end, CD LPMCs were preincubated with or without a recombinant human IL-21R/Fc chimera and then stimulated with anti-CD3. After culture in medium alone, IFN-γ was measurable in the culture supernatants and both p-Stat4 and T-bet clearly were present in LPMCs (Figure 5). Stimulation of cells with anti-CD3 increased expression of p-Stat4 and T-bet, and secretion of IFN-γ. Importantly, neutralization of IL-21 activity inhibited the anti-CD3–stimulated p-Stat4 and T-bet induction, and this was associated with a significant down-

regulation in IFN-γ secretion (*P* = .02) (Figure 5). IL-4 barely was detectable in all samples, regardless of treatment with the IL-21R/Fc chimera (not shown). To confirm the role of IL-21 in regulating Th1 cell responses further, CD LPMCs were left either untreated or treated with the recombinant human IL-21R/Fc chimera and then stimulated with anti-CD3+anti-CD28. As expected, expression of T-bet and p-Stat4 as well as IFN-γ secretion (15,045 ± 4320 pg/mL) were markedly enhanced by anti-CD3+anti-CD28 stimulation. Importantly, neutralization of IL-21 in anti-CD3+CD28–stimulated CD LPMC cultures decreased both T-bet and p-Stat4, and this was associated with a significant inhibition of IFN-γ secretion (10,945 ± 1420 pg/mL, *P* = .037) (Figure 6).

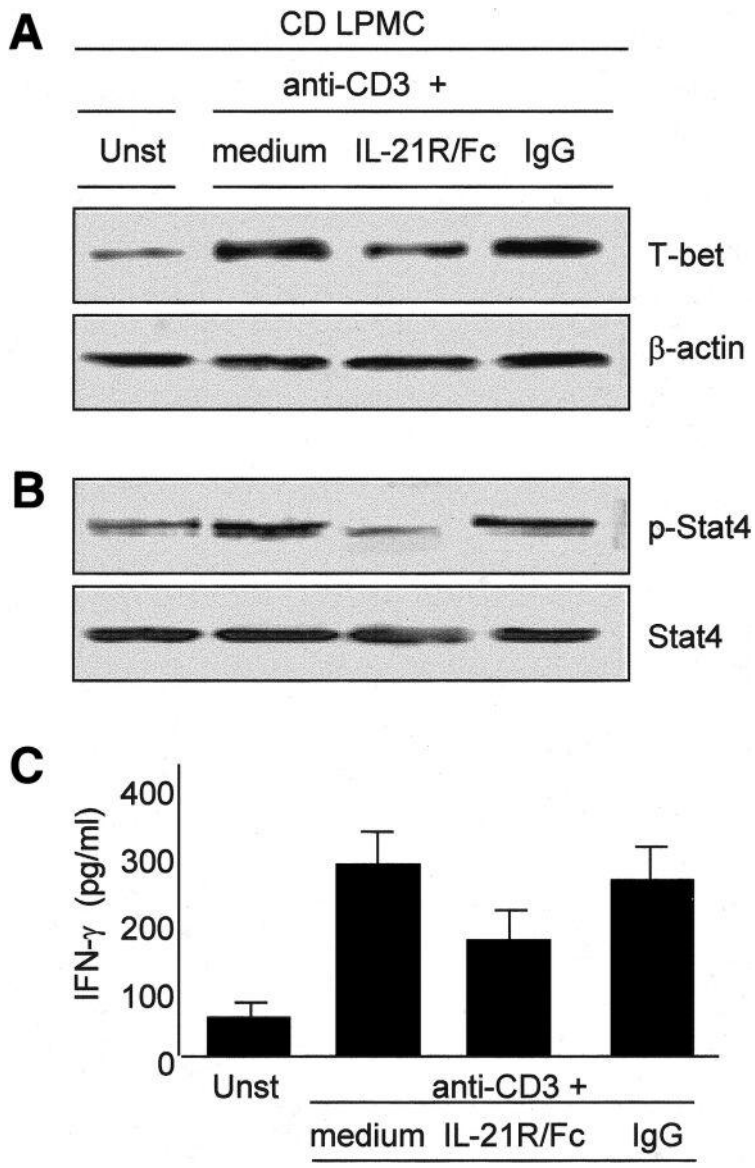

Figure 5. Neutralization of IL-21 activity decreases Th1 cell response in CD. CD LPMCs were left unstimulated (UNST) or pretreated with medium or a chimeric fusion protein blocking human IL-21 activity (IL-21R/Fc) or control IgG overnight, then stimulated with anti-CD3 for 24 hours. At the end, proteins were extracted and analyzed by Western blotting for (*A*) T-bet and β-actin or (*B*) phosphorylated and total Stat4. One representative of 5 separate experiments is shown. Cell culture supernatants also were collected and analyzed for the content of IFN-γ by enzyme-linked immunosorbent assay. (*C*) Data are expressed as pg/mL and indicate the mean ± SD of 5 separate experiments. Neutralization of IL-21 activity significantly decreases anti-CD3–stimulated CD LPMC IFN-γ secretion (*P* = .02).

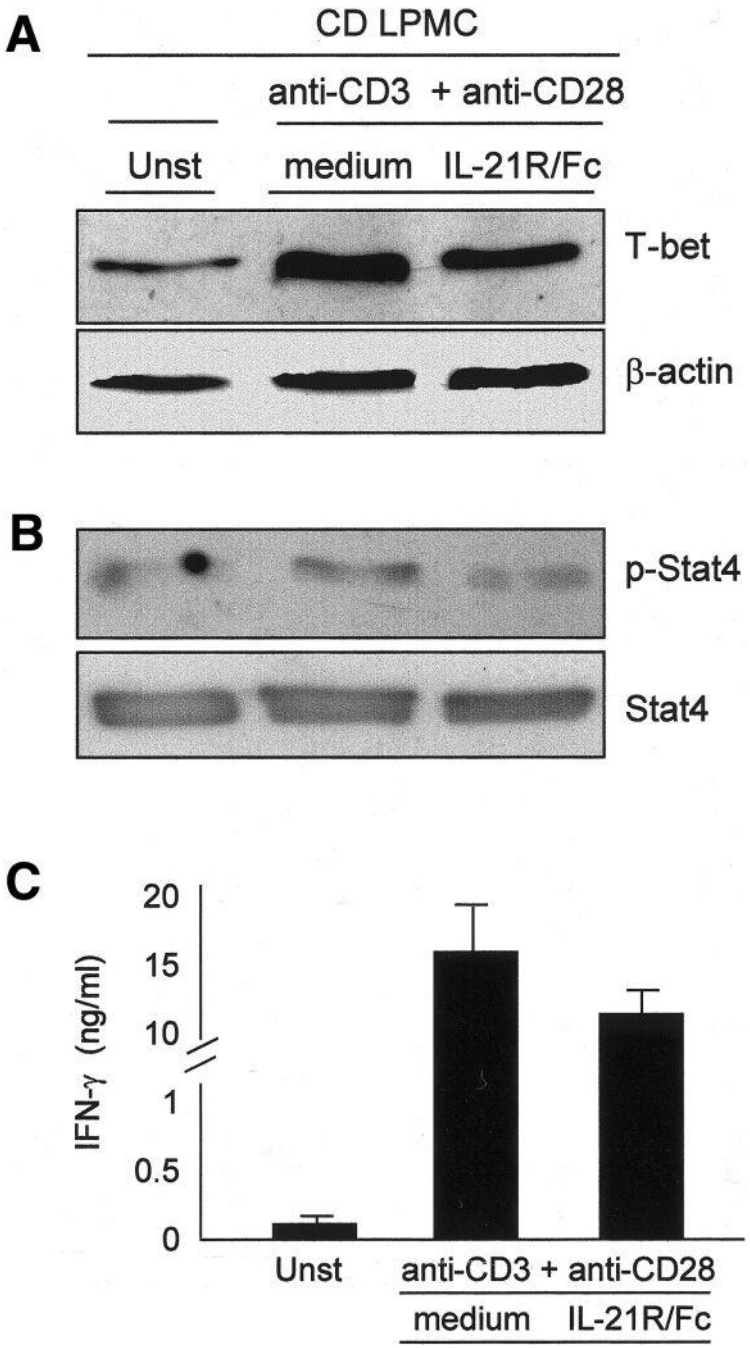

Figure 6. Neutralization of IL-21 activity decreases Th1 cell transcription factors and IFN-γ in CD LPMCs costimulated with anti-CD3+anti-CD28. LPMCs were left unstimulated (UNST) or pretreated with either medium or a chimeric fusion protein blocking human IL-21 activity (IL-21R/Fc) overnight, then stimulated with anti-CD3+anti-CD28 for 24 hours. At the end, proteins were extracted and analyzed by Western blotting for (*A*) T-bet and β-actin or (*B*) phosphorylated and total Stat4. One representative of 4 separate experiments is shown. Cell culture supernatants also were collected and analyzed for the content of IFN-γ by enzyme-linked immunosorbent assay. (*C*) Data are expressed as ng/mL and indicate the mean ± SD of 4 separate experiments. Neutralization of IL-21 activity significantly decreases anti-CD3–stimulated CD LPMC IFN-γ secretion (P = .037).

2.4. DISCUSSION

This study was performed to analyze the expression and role of IL-21 in CD. We show that IL-21 is produced at a higher level in CD patients than in UC and control patients. Enhanced expression of IL-21 is seen in areas with active inflammation, with no difference between ileal and colonic samples. IL-21 was analyzed by Western blotting because no commercial kit capable of quantitatively measuring IL-21 is available yet. Western blotting is not a quantitative technique and the arbitrary units we measured may not directly reflect the biological quantities of IL-21 protein within the intestinal mucosa. The fact that immunoreactivity for IL-21 was consistently higher in CD patients than in control patients, however, indicates that CD inflammatory response associates with a preferential induction of IL-21.

All of the evidence to date indicates that IL-21 is produced by activated CD4+ T cells, and analysis of RNA expression has established definitively that conditions inducing high levels of IL-21 message are those that best mimic T-cell activation.[12–14] This fits well with our demonstration that IL-21 is expressed constitutively in T-LPL samples regardless of their mucosal source, given that the vast majority of intestinal LPL bear the phenotype of memory activated T cells.[19] We also show that CD4+ but not CD8+ T-LPL express IL-21. In this context, IL-21 expression was assessed by a semiquantitative method (ie, Western blotting). Therefore, these data do not imply that intestinal CD8+ LPL are not able to produce IL-21, but they only indicate that IL-21 is made preferentially by CD4+ cells in the human gut. IL-21 is more pronounced in CD45RO+ LPL from CD patients than control patients, clearly indicating that the up-regulation of IL-21 in CD patients does not simply reflect the increased infiltration of the mucosa with activated T lymphocytes. Thus, it is highly likely that production of IL-21 by activated CD4+ T cells can be regulated further within the mucosal microenvironment. In this context, we report that stimulation of normal activated T-LPL with exogenous IL-12, a cytokine produced in excess in CD, enhances IL-21, whereas blocking IL-12 in CD LPMC cultures decreases IL-21 expression. The molecular mechanism by which IL-12 regulates IL-21

synthesis as yet remains unknown, but it is unlikely that it is dependent on the secretion of IFN-γ because the IL-12–stimulated LPL-derived IL-21 is not revertible by a neutralizing IFN-γ antibody.

CD is characterized by a massive infiltration of the inflamed regions with CD4+ T cells and macrophages. These cells are activated functionally and produce high levels of IFN-γ and tumor necrosis factor-α, indicative of a Th1 cytokine profile.[1] In line with this, we and other investigators recently have shown that in CD mucosa there are high levels of IL-12, the major Th1 inducing factor, and that IL-12 signaling promotes Th1 cell–mediated tissue damage.[2,3,16] In vivo, however, multiple cytokines are present simultaneously in sites of inflammation and they might have synergistic effects in controlling immune responses. Here we provide evidence that IL-21 mucosa contributes to stabilize the Th1 phenotype at the site of inflammation in CD. Indeed, blocking IL-21 activity in CD LPMC cultures decreases anti-CD3+anti-CD28–stimulated IFN-γ secretion and this effect mirrors the inhibition of active Stat4 and T-bet expression, 2 transcription factors that drive Th1 cell polarization and stabilization.[11] While this project was in progress, independent groups have documented the ability of IL-21 to modulate the induction of distinct cytokine patterns and promote specific immune responses. Strengell et al[20] showed that IL-21 enhances IFN-γ gene expression in activated human T cells and promotes the transcription of Th1-associated genes. The same group also documented a synergistic effect of IL-21 and IL-15 or IL-21 and IL-18 on IFN-γ messenger RNA synthesis and production, whereas Kasaian et al[22] showed that IL-21 cooperates with IL-2 and IL-15 in promoting IFN-γ synthesis.[21] Collectively, these data implicate IL-21 as a potent regulator of T-cell functions and establish a role for this cytokine in the maintenance of Th1 cell response. However, Wurster et al[23] reported that IL-21 is expressed preferentially in murine Th2 cells generated in vitro and in vivo and that it amplifies Th2 cell responses. The reason for this apparent discrepancy remains unknown. However, it is conceivable that it relies on the different cell contexts studied, and that

IL-21 can expand either Th1 or Th2 cell responses, similar to other cytokines that signal through the γ-chain receptor.[24–26]

Our data also show enhanced IL-21 production in UC in comparison with normal colonic mucosa. It is unlikely, however, that in UC, IL-21 activity is required for enhancing the local IFN-γ synthesis because the UC-associated mucosal inflammation is not characterized by a predominant Th1 cytokine response.[1] Thus, it is conceivable that IL-21 helps maintain distinct ongoing immune responses. For example, IL-21 might participate in the development of antigen-specific B-cell responses and contribute to the aberrant synthesis of auto-antibodies produced in UC mucosa, consistent with its ability to influence humoral immunity, and particularly the production of IgG1.[13,27]

We also feel that the demonstration that IL-21 production occurs in the normal intestine is consistent with the fact that CD4+ T cells from the normal intestinal lamina propria are activated and produce cytokines.[19,28] Studies performed in other systems suggest the possibility that IL-21 might affect the response of several mucosal cell types. Indeed, in addition to the indicated effects on T and B lymphocytes, IL-21 modulates the maturation and cytolytic activity of mature human natural killer cells.[13,14] Given its ability to activate JAK and Stat molecules in lymphoid cells, IL-21 also could regulate, in concert with other cytokines or costimuli, the survival and expansion of antigen-specific memory LPL, which have been primed in the gut-associated lymphoid tissue.[15,29,30]

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3. CONTROL OF MATRIX METALLOPROTEINASE PRODUCTION IN HUMAN INTESTINAL FIBROBLASTS BY INTERLEUKIN 21

3.1. INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of enzymes capable of degrading all components of the extracellular matrix.[1,2] Excess MMP activity is involved in many human diseases such as rheumatoid arthritis, osteoarthritis, periodontal diseases, and tumour invasion and progression.[1] MMPs also play an important part in the tissue degradation in Crohn's disease and ulcerative colitis, the two major forms of inflammatory bowel disease (IBD) in humans.[3–6] Moreover, we have shown that lamina propria T cell activation in human fetal gut explants increases MMP production, followed by matrix degradation.[7,8,9,10] Fibroblasts are the major source of MMPs in the human gut.[9–11]

Interleukin 21 (IL21) is a T cell-derived cytokine whose effects are mediated through a class I cytokine family receptor, IL21R,[12] that interacts with the common γchain receptor.[12–15] Consistent with the distribution of its receptor on immune cells, IL21 has been shown to affect the growth and functional activity of T, B and natural killer lymphocytes.[12,13] More recently, however, IL21R has been described in synovial macrophages and fibroblasts of patients with rheumatoid arthritis,[16] raising the possibility that IL21 may have additional cell targets in vivo.

We have recently shown that IL21 is produced in excess in the gut of patients with Crohn's disease, and that IL21 helps sustain the Th1 mucosal response in this disease.[17] However, high IL21 was also seen in patients with ulcerative colitis,[17] a disease that is not associated with a predominant Th1 cell response.[18] This raises the possibility that, in the gut, IL21 can sustain additional inflammatory pathways apart from enhancing Th1 cell immunity. Therefore, we have examined whether intestinal fibroblasts express IL21R, and investigated the effect of IL21 on MMP production.

3.2. MATERIALS AND METHODS

3.2.1. Patients and samples

Mucosal samples were taken from surgical specimens of 12 patients with Crohn's disease. Eight patients were receiving corticosteroids, and four were taking corticosteroids and azathioprine. Mucosal samples were also taken from four patients with active ulcerative colitis undergoing endoscopy and five patients undergoing colectomy for a chronic disease unresponsive to medical treatment. Five patients were taking corticosteroids and four were taking mesalazine. Normal controls included samples taken from four patients with irritable bowel syndrome, and from macroscopically and microscopically unaffected areas of six patients undergoing colectomy for colon cancer.

3.2.2. Isolation and culture of intestinal fibroblasts

Intestinal fibroblasts were isolated and phenotypically characterised as described elsewhere.[19] Briefly, fresh colon was washed in Hank's balanced salt solution (Sigma-Aldrich, Milan, Italy), and strips of mucosa were cut into small fragments and placed on the bottom of tissue culture dishes with modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics and 1% non-essential amino acids (all from Sigma-Aldrich). Fibroblasts grew from the fragments within 3–4 days. Cells used for experiments were all between passages 3 and 8.

To examine whether IL21R expression is regulated by inflammatory stimuli, fibroblasts, isolated from six controls, were starved overnight and then stimulated with IL1ß (20 ng/ml; Peprotech EC, London, UK) or tumour necrosis factor (TNF) α(15 ng/ml, R&D Systems, Abingdon, UK) for 24–48 h.

To examine whether IL21 regulates production of MMP, confluent fibroblasts were starved overnight and then stimulated with recombinant human IL21 (10–50 ng/ml, R&D Systems) for 2–48 h. In parallel, fibroblasts were cultured with or without IL21 (25 ng/ml) or TNF α(15 ng/ml). In

experiments with inhibitors of gene transcription, protein synthesis and secretion, fibroblasts were incubated with or without actinomycin D (5 µg/ml) or cycloheximide (10 µg/ml) or brefeldin A (5 µg/ml; Sigma-Aldrich) for 2 h before adding IL21 or TNF α for a further 18 h.

To examine whether fibroblast growth was affected by IL21, fibroblasts were cultured in 96-well dishes ($2x10^3$ cells/well) and allowed to adhere overnight. The non-adherent cells were then removed and fresh media containing IL21 was added for a further 48 h. Bromodeoxyuridine was added to the cells during the last 6 h of incubation, and the level of bromodeoxyuridine-positive cells was assessed by a colorimetric kit (Roche Diagnostics, Monza, Italy).

3.2.3. Effect of Crohn's disease lamina propria mononuclear cell derived IL21 on MMP production

To examine whether Crohn's disease lamina propria mononuclear cell (LPMC)-derived IL21 enhances MMP synthesis by fibroblasts, Crohn's disease LPMC, isolated as described previously,[20] were cultured in modified Eagle's medium containing 1% nutridoma (Roche Diagnostics). After 48 h, supernatants were collected and used (1:20 final dilution) to stimulate confluent Crohn's disease intestinal fibroblasts, in the presence or absence of a recombinant human IL21 receptor/Fc fusion protein (IL21R/Fc; 20 µg/ml; R&D Systems) or control immunoglobulin (Ig)G. MMPs were analysed after 48 h by western blotting.

3.2.4. Western blotting

Western blotting for IL21R was carried out using total extracts of fibroblasts isolated from seven patients with Crohn's disease, seven patients with ulcerative colitis, and seven normal controls, of the fetal gut fibroblast cell line, CCD18CO (LGC Promochem, Sesto San Giovanni, Italy) and peripheral blood CD3+ lymphocytes (PBL). IL21R was also examined in total extracts of normal fibroblasts stimulated with TNF α or IL1ß. IL21R was detected using a monoclonal mouse anti-human IL21R (1 µg/ml; R&D Systems) followed by a horseradish peroxidase-conjugated rabbit

anti-mouse IgG (Dako SpA, Milan, Italy). The reaction was detected with a chemiluminescence kit (Pierce, Rockford, Illinois, USA). After IL21R analysis, blots were stripped and incubated with a monoclonal mouse anti-human common γ chain antibody (1:500 final dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) and finally with a mouse anti-human ß-actin antibody (Sigma-Aldrich) as internal loading control. Computer-assisted scanning densitometry was used to analyse the intensity of the immunoreactive bands.

For the detection of MMPs and tissue inhibitor of metalloproteinases (TIMPs), the membranes were incubated with the following monoclonal anti-human antibodies: MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 (1 µg/ml; R&D Systems).

3.2.5. Gelatin zymography

Fibroblast culture supernatants were electrophoresed under non-reducing conditions in an 8% acrylamide gel containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were washed for 30 min in 2.5% Triton X-100, then equilibrated in developing buffer (50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij35). After 30 min, the gels were incubated in fresh developing buffer and incubated overnight at 37°C, then stained with 0.25% Coomassie Blue for 1 h and destained in 50% methanol and 10% glacial acetic acid.

3.2.6. RNA extraction, complementary DNA preparation and reverse transcriptase-polymerase chain reaction

IL21R and common γchain receptor RNA were analysed in samples extracted from fibroblasts of five patients with Crohn's disease, five patients with ulcerative colitis, five controls, CCD18CO and PBL by reverse transcription-polymerase chain reaction (RT-PCR). RNA isolation, reverse transcription of the RNA and RT-PCR were carried out as described previously. [20] IL21R and common γ chain receptor primers and PCR conditions have been described elsewhere.[21,22] A constant amount of RNA (500 ng/sample) was retrotranscribed into complementary DNA (cDNA),

and 2 µl of cDNA/sample was amplified using the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 58°C for both MMP and ß actin, and extension for 1 min at 72°C using the following primers. MMP-1, FWD: 5'-TTG TCC TCA CTG AGG GAA AC-3', REV: 5'-AGG TTA GCT TAC TGT CAC AC-3'; MMP-2, FWD: 5'-CCT GTT TGT GCT GAA GGA CA-3', REV: 5'-GTA CTT GCC ATC CTT CTC AA-3'; MMP-3, FWD: 5'-GCC CCT GGG CCA GGG ATT AAT GGA GAT GC-3'; REV: 5'-ATC TTG AGA CAG GCG GAA CCG AGT CAG G-3'; MMP-9, FWD: 5'-GTC GAA ATC TCT GGG GCC TG-3', REV: 5'-AAA CCG GTC GTC GGT GTC GT-3'; ß-actin, FWD: 5':GGC ACC ACA CCT TCT ACA-3', REV: 5': CAGGTCTTTGCGGATGTC-3'. In preliminary experiments, we established the optimal number of cycles to obtain a PCR product within the linear phase of the amplification. Therefore, cDNA was amplified with ß actin primers for 22 cycles and MMP primers for 28 cycles. RT-PCR products were electrophoresed in 1% agarose gel, bands quantified by densitometry and values expressed as arbitrary units. RT-PCR product specificity was confirmed by restriction analysis.

3.2.7. Statistical analysis

Differences between groups were compared using Student's t test.

3.3. RESULTS

3.3.1. IL21R is expressed by gut fibroblasts

Constitutive expression of IL21R was seen in fibroblasts from patients with IBD and controls, and CCD18CO (fig 1A). Densitometric analysis of IL21R bands showed no significant difference between IBD and normal controls (fig 1A, right inset). Data were confirmed by showing that these cells contain RNA transcripts for IL21R (fig 1B). Intestinal fibroblasts also expressed the common γ-chain receptor (fig 1 A,B). Notably, both IL1ß and TNF α, two cytokines which are produced in excess in IBD,[23] significantly enhanced IL21R expression (fig 2; $p<0.02$).

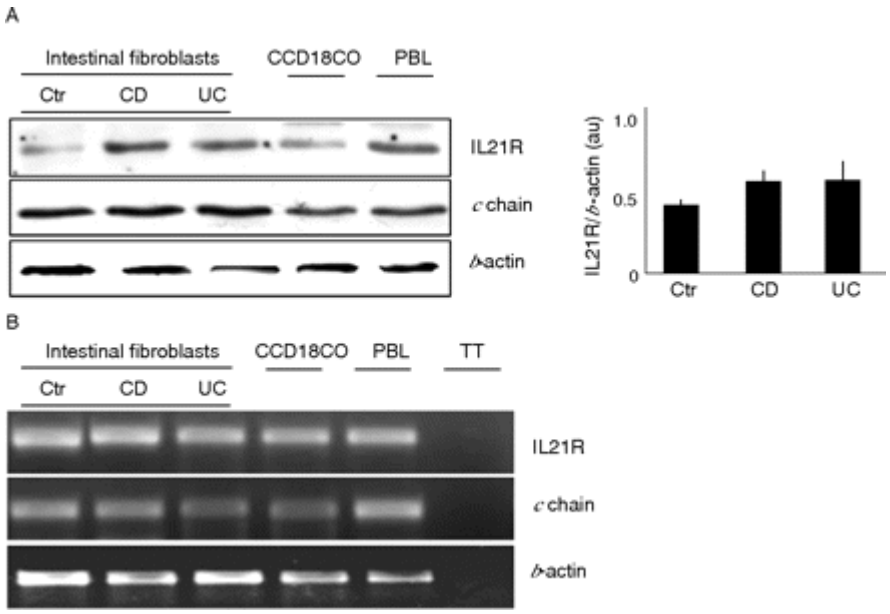

Figure 1 (A) Representative western blots showing interleukin 21 receptor (IL21R) (upper blot), common γchain (middle blot) and ß-actin (lower blot) in fibroblasts isolated from the colon of one control (Ctr), one patient with Crohn's disease (CD), one patient with ulcerative colitis (UC), from fetal gut fibroblast cell lines (CCD18CO) and from peripheral blood lymphocytes (PBL, used as a positive control). The right inset shows the quantitative analysis of IL21R/ß-actin protein ratio in fibroblasts isolated from seven controls, seven patients with Crohn's disease and seven patients with ulcerative colitis as measured by densitometry scanning of western blots. Values are expressed in arbitrary units (au) and indicate mean (standard deviation (SD)) of all experiments. (B) Representative electrophoretic gel showing reverse transcription-polymerase chain reaction products for IL21R, common γchain and ß-actin in RNA samples prepared from fibroblasts of one control, one patient with Crohn's disease, and one patient with ulcerative colitis, from CCD18CO and from PBL. TT, tube test, in which amplification was carried using RNA instead of cDNA. One of three separate experiments is shown.

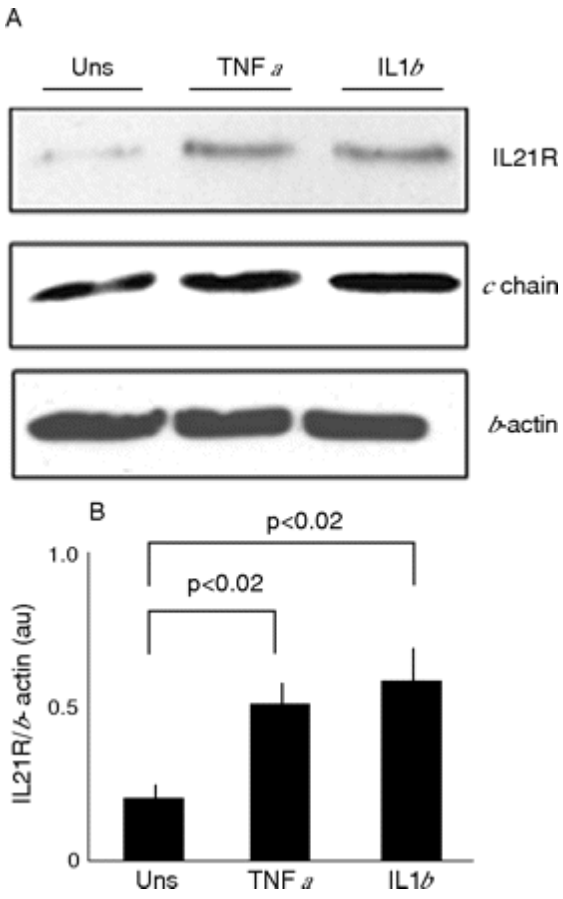

Figure 2 Stimulation of normal intestinal fibroblasts with tumour necrosis factor (TNF) αand interleukin (IL)1ß results in enhanced IL21 receptor (IL21R) expression ($p<0.02$). (A) Representative western blot showing IL21R (upper blot), common γchain (middle blot) and ß-actin (lower blot) in control fibroblasts either left unstimulated (Uns) or stimulated with TNF αand IL1ß for 24 h. (B) Quantitative analysis of IL21R/ß-actin protein ratio in control fibroblasts, cultured as indicated in (A), as measured by densitometry scanning of western blots. Values are expressed in arbitrary units (au) and indicate mean (standard deviation (SD)) of four separate experiments.

3.3.2. IL21 promotes MMP synthesis by intestinal fibroblasts

To examine whether fibroblasts respond to IL21, we evaluated the effect of IL21 stimulation on MMP synthesis. Figure 3 shows that IL21 dose dependently enhanced the secretion of distinct MMP isoforms, which correspond to the mature and active enzymes.[2,8,9] This effect was seen regardless of whether fibroblasts were isolated from normal patients or from patients with IBD, as well as in CCD18CO cells (not shown). The ability of IL21 to enhance the synthesis of gelatinases (MMP-2 and MMP-9) was confirmed by zymography (fig 3, inset). By contrast, no considerable change in the secretion of TIMP-1 and 2 was seen in IL21-stimulated fibroblasts. Importantly, IL21 did not increase fibroblast proliferation (0.45 (0.1) arbitrary units in untreated cells *v* 0.37 (0.23) in IL21-treated cells), excluding the possibility that differences in the release of MMPs were secondary to changes in cell growth.

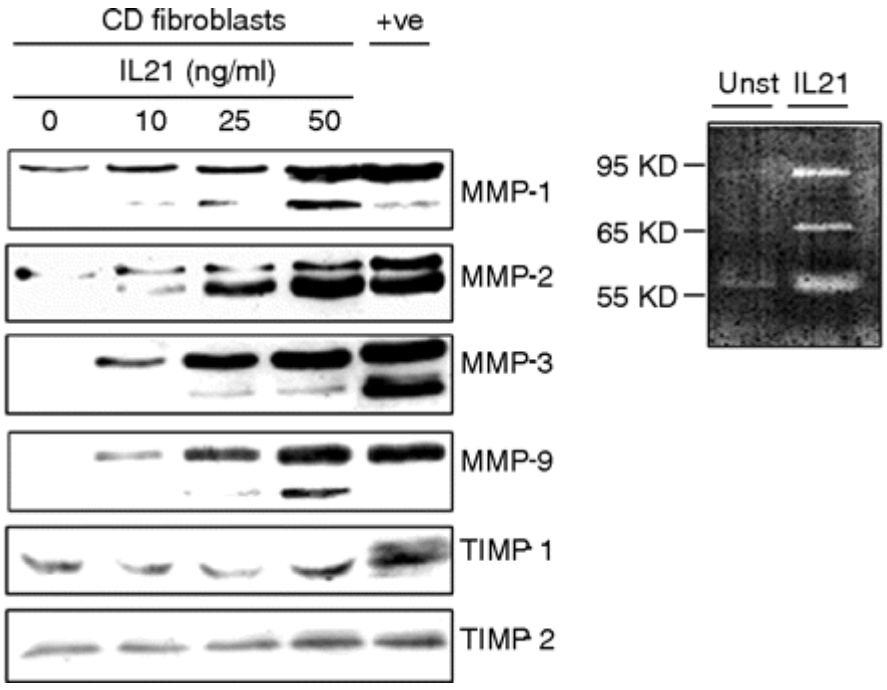

Figure 3 Representative western blots showing MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 in fibroblasts isolated from the colon of one patient with Crohn's disease (CD), and either left untreated or treated with graded doses of interleukin (IL) 21 for 48 h. One of 12 separate experiments is shown. In the last lane (+ve), total proteins extracted from the inflamed colon of a patient with Crohn's disease were used as a positive control. The right inset shows a representative zymogram of gelatinolytic activities in supernatants from Crohn's disease fibroblasts either left unstimulated (Unst) or stimulated with 50 ng/ml IL21 for 48 h. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

As TNF $\alpha$is known to upregulate MMP-1 and MMP-3 production by intestinal fibroblasts,[9,10] we examined whether IL21 cooperates with TNF αin inducing MMPs. Both IL21 and TNF αenhanced the secretion of MMP-1, MMP-2, MMP-3 and MMP-9. Cells stimulated with IL21 and TNF α together produced more MMPs than either IL21 or TNF αalone. Secretion of TIMP-1 and 2 remained unchanged (fig 4).

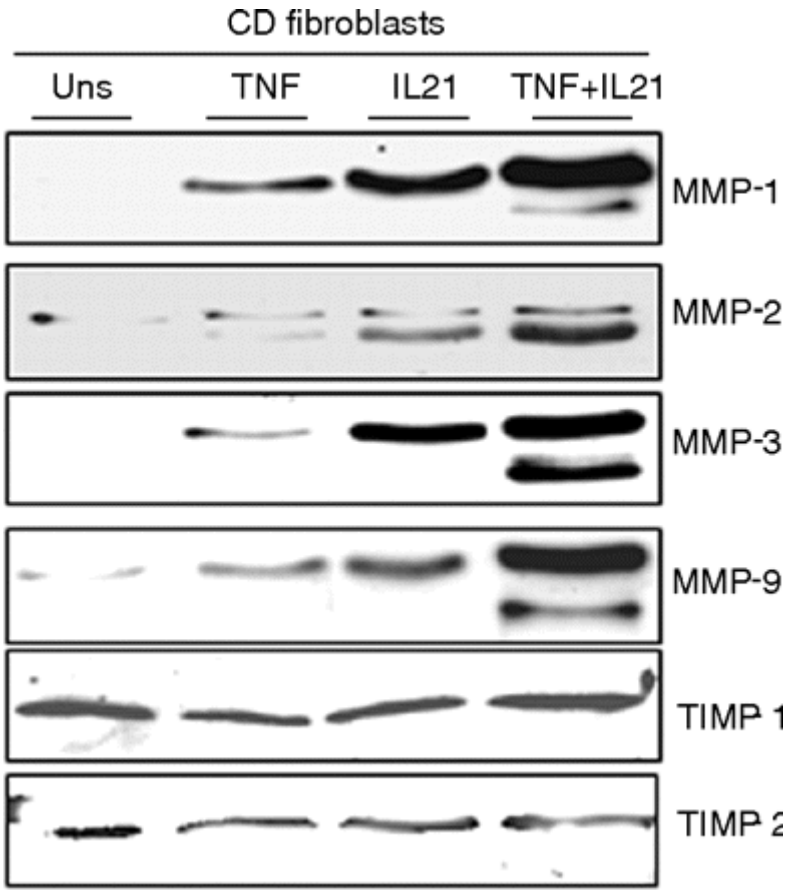

Figure 4 Representative western blots showing MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 in culture supernatants of fibroblasts, isolated from one patient with Crohn's disease, and either left untreated (Uns) or treated with interleukin (IL) 21 (25 ng/ml) or tumour necrosis factor α(TNF α) (15 ng/ml) for 48 h. One of five separate experiments is shown. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

3.3.3. IL21 does not enhance MMP RNA expression and new protein synthesis

To investigate increased transcription as a potential mechanism for the IL21-induced secretion of MMPs, MMP RNA expression was assessed by RT-PCR. MMP RNA transcripts were seen in untreated fibroblasts and were not increased by IL-21 (fig 5A). By contrast, TNF αenhanced MMP-1, MMP-3 and MMP-9, but not MMP-2 RNA (fig 5A, B).

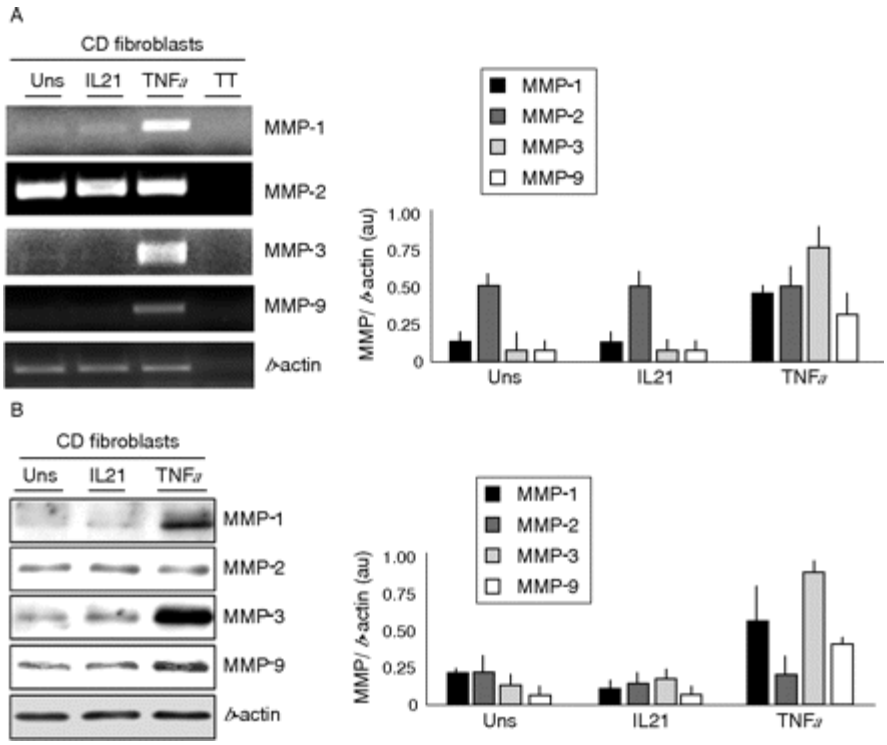

Figure 5 (A) Representative electrophoretic gel showing reverse transcriptase-polymerase chain raction products for matrix metallopsoteinase (MMP) -1, MMP-2, MMP-3, MMP-9 and ß-actin in RNA samples prepared from fibroblasts, isolated from the colon of one patient with Crohn's disease (CD), and either left untreated (Uns) or treated with interleukin (IL) 21 (50 ng/ml) or tumour necrosis factor (TNF) α(15 ng/ml) for 8 h. TT, tube test, in which amplification was carried out using RNA instead of cDNA. One of three separate experiments is shown. The right inset shows the quantitative analysis of MMP/ß-actin RNA transcripts in intestinal fibroblasts with Crohn's disease either left unstimulated or stimulated with IL21 (50 ng/ml) or TNF α(15 ng/ml) for 8 h. Values are expressed in arbitrary units (au) and indicate mean (standard deviation (SD)) of three separate experiments. (B) Representative western blots showing MMP-1, MMP-2, MMP-3, MMP-9 and ß-actin in total extracts prepared from fibroblasts isolated from the colon of one patient with Crohn's disease and either left unstimulated or stimulated with IL21 (50 ng/ml) or TNF α(15 ng/ml) for 48 h. One of four separate experiments is shown. The right inset shows the quantitative analysis of MMP/ß-actin protein ratio in intestinal fibroblasts with Crohn's disease either unstimulated or activated with IL21 (50 ng/ml) or TNF α(15 ng/ml) for 48 h. Values are expressed in arbitrary units (au) and indicate mean (SD) of four separate experiments.

To determine whether IL21 upregulates the level of MMPs before secretion, cell-associated MMPs were assayed by western blotting using total extracts. IL21 did not alter the cellular content of any MMP, whereas TNF αincreased the expression of MMP-1, MMP-3 and MMP-9, but not MMP-2 (fig 5B). Inhibitors of RNA and protein synthesis or secretion were then used to confirm our findings. The protein secretion inhibitor, brefeldin A, abrogated the effect of IL21 on MMP production (fig 6A). By contrast, neither actinomycin D nor cycloheximide inhibited the IL21-mediated MMP secretion (fig 6A). Both actinomycin D and cycloheximide inhibited the TNF α-

induced MMP-1 and MMP-3 secretion, indicating that these compounds were active in our system (fig 6B). Data suggest that IL21 affects neither gene transcription nor de novo protein synthesis, but it may enhance the secretion of preconstituted or newly synthesised MMPs. This hypothesis was also supported by the demonstration that IL21 induced a rapid secretion of MMPs, which was evident as early as 2 h after stimulation (fig 7).

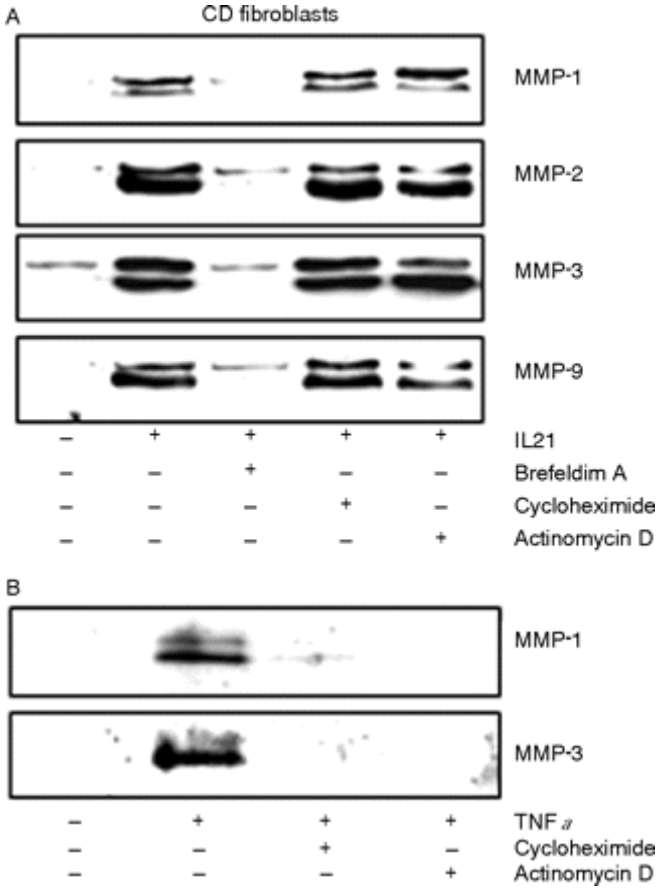

Figure 6 (A) Representative western blots showing matrix metalloproteinase (MMP)-1, MMP-2, MMP-3 and MMP-9 in culture supernatants of fibroblasts isolated from one patient with Crohn's disease (CD) and either untreated (Uns) or preincubated with medium alone, brefeldin A (5 µg/ml), actinomycin D (5 µg/ml) or cycloheximide (10 µg/ml) for 2 h, and then stimulated with interleukin (IL)21 (50 ng/ml) for a further 18 h. (B) Representative western blots showing MMP-1 and MMP-3 in culture supernatants of fibroblasts isolated from one patient with Crohn's disease and either untreated or preincubated with medium alone, actinomycin D (5 µg/ml) or cycloheximide (10 µg/ml) for 2 h, and then stimulated with TNF α(15 ng/ml) for a further 18 h. Two of four representative experiments are shown.

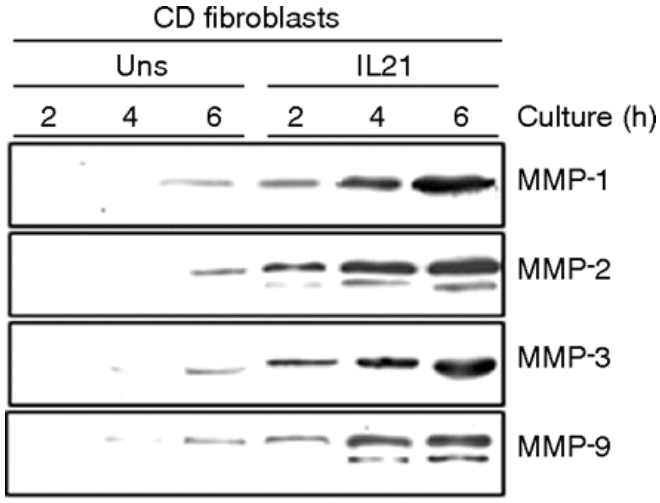

Figure 7 Representative western blots showing matrix metallo-proteinase (MMP)-1, MMP-2, MMP-3 and MMP-9 in culture supernatants of fibroblasts isolated from one patient with Crohn's disease (CD) and either left untreated (Uns) or treated with 50 ng/ml interleukin (IL)21 for the indicated time points. One of four representative experiments is shown.

3.3.4. Blocking IL21 reduces fibroblast MMP secretion induced by Crohn's disease LPMC supernatants

To confirm further that IL21 regulates matrix metalloproteinase (MMP) production, Crohn's disease fibroblasts were stimulated with Crohn's disease LPMC supernatants in the presence or absence of IL21R/Fc. Unstimulated fibroblasts released low levels of MMPs, but responded to Crohn's disease LPMC supernatant stimulation with enhanced synthesis of all MMPs (fig 8). The addition of IL21R/Fc to the fibroblast cultures decreased the production of MMPs induced by Crohn's disease LPMC supernatants (fig 8).

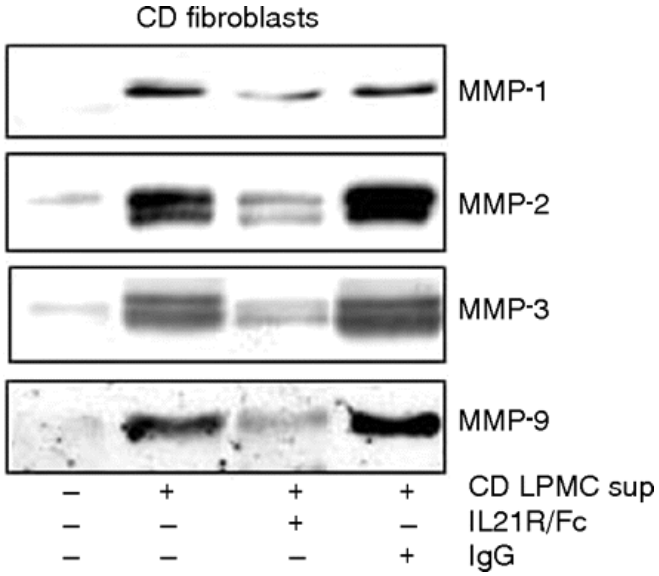

Figure 8 . Neutralisation of interleukin (IL)21 activity reduces fibroblast matrix metalloproteinase (MMP) secretion induced by Crohn's disease (CD) lamina propria mononuclear cell (LPMC) supernatants. Fibroblasts were incubated with or without Crohn's disease LPMC supernatants (1:20 final dilution) in the presence or absence of a blocking IL21R fusion protein (IL21R/Fc) or control immunoglobulin G (IgG) for 48 h. At the end, fibroblast-free supernatants were analysed by western blotting for MMP-1, MMP-2, MMP-3 and MMP-9. One representative of three separate experiments is shown.

3.4. DISCUSSION

In this study, we examined whether IL21 regulates the synthesis of MMPs by intestinal fibroblasts, given that fibroblasts are a major source of MMPs in the gut and that MMP production can be enhanced by T cell-produced cytokines.[3,4,5,6,7,8,9,10] Initially we showed that IL21R is constitutively expressed by intestinal fibroblasts, and that such expression can be enhanced by TNF $\alpha$and IL1ß. In this context, however, it is worth noting that IL21R protein expression was semiquantitatively assessed by western blotting, as our attempts to characterise IL21R by flow cytometry using commercially available antibodies were unsuccessful. We cannot therefore exclude the possibility

that the arbitrary units we measured may not exactly reflect the biological quantities of IL21R protein in those cells. Intestinal fibroblasts also express the common γchain receptor, suggesting that these cells may be potential targets of IL21 in vivo. Indeed, intestinal fibroblasts responded to IL21 by increasing the production of MMPs. The effects of IL21 on MMP synthesis were observed with concentrations of IL21 that are similar to those used by other authors to test the biological effects of this cytokine in vitro.[15] We do not know whether such concentrations reflect the amount of IL21 produced in the gut of patients with IBD, as no commercial kit is yet available to quantify human IL21. However, our previous western blotting analysis of IL21 showed that the intensity of immunoreactive bands in patients with IBD was not different from that obtained with 50 ng/ml rhIL-21[17]—that is, the maximal dose we used to stimulate fibroblasts. We restricted our analysis of the effect of IL21 on the synthesis of MMP-1, MMP-2, MMP-3 and MMP-9, as these proteases are produced in excess in the gut of patients with IBD.[3,4,6,11]

MMP production or activity can be regulated at multiple levels including gene activation and transcription, mRNA stability, secretion, proenzyme activation and inactivation by endogenous inhibitors.[1,2] We thus examined how IL21 regulates MMP synthesis in gut fibroblasts. Our data suggest that regulation of MMPs by IL21 does not occur at the transcriptional level. In fact, stimulation of fibroblasts with IL21 did not alter the expression of MMP RNA transcripts. Additionally, the intracellular level of MMP proteins was not increased by IL21, and the IL21-induced MMP synthesis was not affected by inhibitors of gene transcription and de novo protein synthesis. It is thus plausible that IL21 preferentially increases the secretion of either preconstituted or newly synthesised MMPs, as treatment of fibroblasts with brefeldin A inhibited IL21-induced MMP secretion, and increased secretion of MMP was seen after exposure to IL21 for a short time.

Although it was once considered that MMPs were transcriptionally regulated and rapidly secreted, recent evidence shows that MMPs are stored in secreted granules, ready for rapid release. For example, coculture of a monocytic cell line with metastatic colon cancer cells results in an increased production of MMP-2 without any increase in mRNA levels.[24] Subcellular compartmentalisation of

MMPs within caveolar structures has been described in endothelial cells, and activation of these cells by various stimuli results in enhanced MMP secretion, with no change in MMP RNA or de novo protein synthesis.[25–28] By contrast, TNF αaugmented the RNA expression of MMP-1, MMP-3 and MMP-9, but not MMP-2, supporting data of previous reports showing that MMP-2 can be regulated via a different pathway from that of other MMPs, including MMP-9.[1,2,29,30] These data suggest that inflammatory cytokines such as TNF α may transcriptionally increase MMP production, but this effect is amplified by IL21 at the post-transcriptional level.

The proteolytic activity of MMPs is tightly controlled by TIMPs.[1,2] One of the most striking features of this study is that TIMP-1 and TIMP-2 protein remained unchanged after IL21 stimulation. These findings are consistent with previous reports showing high MMP production without concomitant TIMP elevation in other systems. For example, in IBD tissue, increased expression of MMP-3 occurs with no change in TIMP-1 production.[5,6] Stimulation of scleral fibroblasts from patients with necrotising scleritis by TNF αresulted in a twofold increase in TIMP-1 mRNA compared with a sevenfold increase in stromelysin-1 mRNA.[31] There was no similar increase in TIMP-1 mRNA in the aqueous humour of patients with uveitis, although MMPs were increased.[32]

In conclusion, we have shown that IL21 increases MMP production by fibroblasts, thus confirming and expanding on our previous data showing that cytokines are important mediators of the cross talk between immune and non-immune cells in the gut.[5,9,18] The in vivo relevance of our findings relates to the fact that supernatants of Crohn's disease LPMC rapidly increase MMP production by fibroblasts and this is partially inhibited by IL21R/Fc. The fact that the IL21R/FC reduces but does not abrogate the effect of Crohn's disease LPMC supernatants on fibroblast MMP production indicates that Crohn's disease LPMC make additional MMP-inducing molecules other than IL21. Indeed, molecules such as IL1ß, IL6, IL17, IL22 and TNF α, all of which could regulate MMPs, are produced in excess in Crohn's disease.[2,5,10,18,33,34]

Although IL21 was originally described as an important regulator of T, B and NK cells, recent studies have shown that IL21 modulates the activity of multiple cell types and triggers several inflammatory pathways.[14,35,36] These observations, with the demonstration of upregulation of IL21 in IBD tissue and enhancement of MMPs in fibroblasts by IL21, suggest that blocking IL21 can help in limiting the tissue-damaging inflammatory response in IBD.

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4. A FUNCTIONAL ROLE FOR INTERLEUKIN-21 IN PROMOTING THE SYNTHESIS OF THE T-CELL CHEMOATTRACTANT, MIP-3A, BY GUT EPITHELIAL CELLS

4.1. INTRODUCTION

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 relevant 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.

4.2. MATERIALS AND METHODS

4.2.1. 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.

4.2.2. 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.

4.2.3. 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.

4.2.4. 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), anticytokeratin-18 (1:400; Sigma-Aldrich), anti–α-smooth muscle actin (2 μg/mL; Sigma-Aldrich), anti–MIP-3α (2 μg/mL; R&D Systems), anti-p signal transducers and activators of transcription (Stat)3, anti–total Stat3, anti–p-ERK1/2, anti–total ERK1/2, anti–p-p38, anti–total p38, anti–p-JNK and anti–total JNK (all used at 1:500 final dilution). Appropriate horseradish-peroxidase–conjugated secondary antibodies then were used and bound antibodies were visualized using enhanced chemiluminescence (Pierce, S.I.A.L., Rome, Italy).

Computer-assisted scanning densitometry (Total lab, AB.EL S.r.l., Rome, Italy) was used to analyze the intensity of the immunoreactive bands.

4.2.5. Analysis of IL-21R, Cell Growth, and Death by Flow Cytometry

IL-21R was examined by incubating cells with a phycoerythrin-labeled mouse anti-IL-21R (1:10 final dilution; R&D Systems) or isotype control antibody (1:20 dilution; Becton Dickinson, Milan, Italy) at 4°C for 30 minutes. Cells then were washed, resuspended in phosphate-buffered saline, and analyzed by flow cytometry.

To assess the effect of IL-21 on colon epithelial cell growth and survival, serum-starved cell lines were either left untreated or treated with 50–200 ng/mL IL-21 for 48 hours. To track the proliferation, cells were incubated in 0.2 μmol/L carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Milan, Italy) at 37°C for 30 minutes and extensively washed before culture. After 5 days of culture, carboxyfluorescein diacetate succinimidyl ester fluorescence was evaluated and the proportion of cells undergoing divisions was determined, thus allowing calculation of both precursor frequency and proliferative index. The fraction of Annexin V– and propidium iodide–positive cells was evaluated using a commercially available kit (Beckmann Coulter, Milan, Italy).

4.2.6. Production of the Neutralizing IL-21 Antibody

A human IL-21 peptide (NVSIKKLKRKPPSTN) was synthesized and used to immunize New Zealand White rabbits by Washington Biotechnology Company (Simpsonville, MD). The IL-21 peptide was injected subcutaneously with adjuvant into specific-pathogen–free rabbits (New Zealand White). Blood was taken 6 or 8 weeks after initial immunization, and the antiserum titer was characterized by ELISA. As a control, serum was taken from the same rabbits before their immunization. Purification of anti–IL-21 antibody (immunoglobulin [Ig]G) was performed using the Melon Gel IgG Spin purification kit according to the manufacturer's instructions (Pierce).

To test the neutralizing activity of the anti–IL-21 antibody, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll stratification using blood taken from healthy volunteers and cultured in RPMI containing 0.5% bovine serum albumin with or without the initial addition of human recombinant IL-21 (200 ng/mL) in the presence or absence of graded doses of the anti–IL-21 (1–10 μg/mL) or control antibody. After 30 minutes, total extracts were prepared and analyzed for the content of p-Stat3 by Western blotting.

4.2.7. Organ Culture

Mucosal biopsy specimens were taken from 4 patients with active CD and 4 patients with active UC and then placed on iron grids with the mucosal face upward in the central well of an organ culture dish containing RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) (Life Technologies-GibcoBRL, Milan, Italy). Cultures were performed with or without the addition of the neutralizing IL-21 or control antibody (10 μg/mL) for 24 hours and then the supernatants were examined for the content of MIP-3α by ELISA. To examine whether the anti–IL-21 reduced the enterocyte production of MIP-3α, additional biopsy specimens were taken from 3 patients with CD and 2 patients with UC were cultured as described earlier. At the end, epithelial cells were isolated and then analyzed for the expression of MIP-3α by Western blotting.

4.2.8. Human Cytokine Expression Array Assay

The human cytokine arrays 6.1 and 7.1 were purchased from Ray Biotech (Norcross, GA) and used following the manufacturer's instructions. Briefly, supernatants of DLD-1 cells either left untreated or treated with 50–200 ng/mL IL-21 were added to membranes, each immobilized with 60 different capture antibodies, and incubated at 4°C overnight. The membranes then were incubated with biotin-conjugated antibodies for 2 hours, and then with horseradish-peroxidase–conjugated streptavidin for 30 minutes. Unbound reagents were removed by washing, and the bound antibodies on the membranes were visualized using an enhanced chemiluminescence system.

4.2.9. Determination of MIP-3α by ELISA

MIP-3α was evaluated in supernatants of organ cultures of IBD biopsy specimens and in supernatants of colon epithelial cell lines using a sensitive ELISA kit (R&D Systems). In the case of organ culture supernatants, values of MIP-3α were expressed as pg/μg of total proteins.

4.2.10. Chemotaxis Assay

To examine the effect of IL-21–stimulated cell culture supernatants on the migration of lymphocytes, PBMCs were isolated from healthy volunteers and used to purify CD3+ T cells using a multisort magnetic microbeads kit (Miltenyi Biotec, Bologna, Italy). Cells were resuspended in RPMI 1640 containing no fetal bovine serum at a final concentration of 5×10^6/mL. The chemotaxis assay was performed in a 48-well microchemotaxis chamber (Corning-Costar, Cambridge, MA). The bottom wells of the chamber were filled with 600 μL of conditioned medium, consisting of 100 μL supernatants of DLD-1 or HT-29 cells either left untreated or treated with IL-21 and 500 μL of fresh RPMI without fetal bovine serum, or 600 μL of RPMI alone (control solution). In parallel, wells were filled with 600 μL RPMI 1640 containing 200 ng/mL of IL-21. To ascertain whether the chemotactic effects of the IL-21–treated cell culture supernatants were dependent on MIP-3α activity, either a neutralizing anti–human (h)MIP-3α (5 μg/mL) or control antibody was added to the conditioned medium 60 minutes before testing its effect on lymphocyte migration. The concentration of the anti–MIP-3α was selected on the basis of preliminary experiments showing that it was able to fully neutralize the chemotactic effect induced by 1 ng/mL recombinant human (rh)MIP-3α, a concentration that is higher than that measured in the conditioned medium. In additional experiments, wells were filled with 600 μL of medium consisting of 100 μL supernatants of organ cultures of IBD biopsy specimens either left untreated or treated with the anti–IL-21 or control antibody and 500 μL of fresh RPMI. A filter with a pore size of 5 μm was placed on the bottom wells, and 100 μL of cell suspension was pipetted into the upper wells as duplicate samples. The chamber was incubated in humidified air with 5% CO_2 at 37°C for

90 minutes. Cells that completely migrated through the filter were counted in 10 random high-power fields. The chemotactic response was defined as the mean number of migrating cells per high-power field and expressed as a percentage of control solution.

4.2.11. Immunohistochemistry

Tissue sections from 9 patients with CD, 9 patients with UC, and 10 normal controls were cut, deparaffinized, and dehydrated through xylene and ethanol. Incubation with a mouse anti-human IL-21R antibody (20 μg/mL; R&D Systems) was performed at room temperature for 1 hour. After rinsing in Tris-buffered saline (Sigma), slides were incubated with a secondary antibody conjugated to horseradish-peroxidase (1:50 dilution; Dako) for 30 minutes at room temperature. Immunoreactive cells were visualized by the addition of diaminobenzidine (Sigma-Aldrich) as substrate and lightly counterstained with hematoxylin. Isotype control sections were prepared under identical immunohistochemical conditions, as described earlier, replacing the primary antibody with a purified, normal mouse IgG control antibody (Dako). Tissue dehydration through graded alcohol and xylene was followed by mounting.

4.2.12. Statistical Analysis

Differences between groups were compared using the Student *t* test, analysis of variance, and Wilcoxon tests.

4.3. RESULTS

4.3.1. IL-21R Expression Is Increased on Gut Epithelial Cells of IBD Patients

To examine if intestinal epithelial cells express IL-21R, we performed an immunohistochemical analysis of IL-21R in colonic specimens taken from patients with IBD and normal controls. Colonic epithelial cells in control individuals constitutively expressed IL-21R (Figure 1*A*). In these sections,

IL-21R was mostly evident on surface epithelial cells, whereas there was little staining of the crypt epithelium. In addition, IL-21R was expressed by some elements of the lamina propria. Notably, a more pronounced expression of IL-21R was seen in colonic epithelial and lamina propria cells of patients with CD and patients with UC in comparison with normal controls (Figure 1*A*). These results were confirmed independently by Western blotting. Total extracts of freshly isolated colonic epithelial cells of patients with CD and patients with UC contained higher levels of IL-21R than those prepared from normal cells (Figure 1*B* and *C*). After analysis of IL-21R, Western blots were stripped and analyzed for the expression of cytokeratin-18 as an internal loading control for epithelial cells. After that, blots were stripped and incubated with an α-smooth muscle actin antibody to confirm that the epithelial cell extracts were not contaminated by fibroblasts (Figure 1*B*). IL-21R was expressed at the same level as controls in epithelial cells isolated from patients with diverticular disease (not shown). Western blotting analysis revealed that epithelial cells isolated from IBD patients and controls also expressed the common γ−chain receptor (Figure 1*B*), essential for IL-21–driven intracellular signals.[3] IL-21R also was expressed constitutively by colon epithelial cell lines (Figure 1*D*). The fact that IL-21R is up-regulated in the inflamed gut of patients with CD and patients with UC prompted us to explore whether cytokines that are produced in excess in these diseases may positively regulate IL-21R. To this end, serum-starved DLD-1 cells were treated with various inflammatory cytokines for 24 hours and then the expression of IL-21R was examined by flow cytometry. As shown in Figure 1*E*, IL-21 but not the other cytokines enhanced the expression of IL-21R ($P = .04$). Notably, at the specified concentrations, each of these cytokines was able to activate Stat or MAP kinase molecules in DLD-1 and HT-29 cells, thus indicating that they were active in our system.

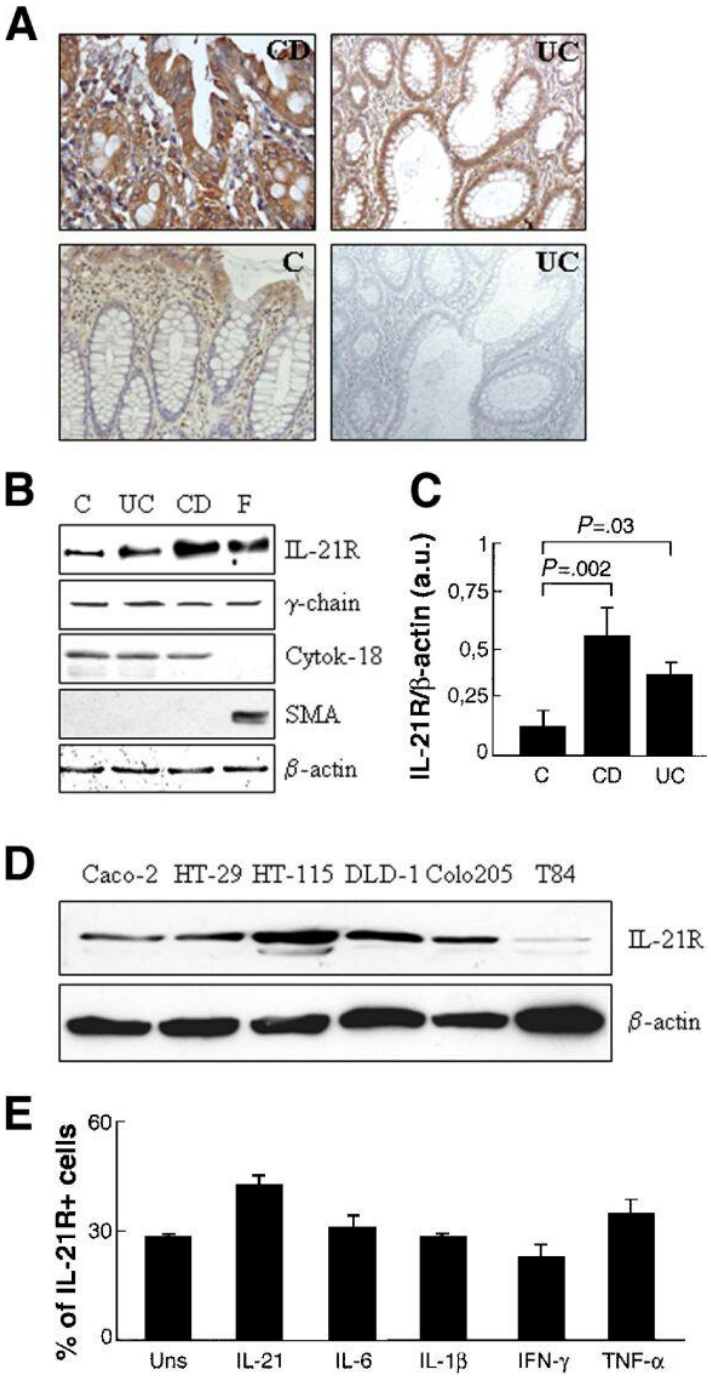
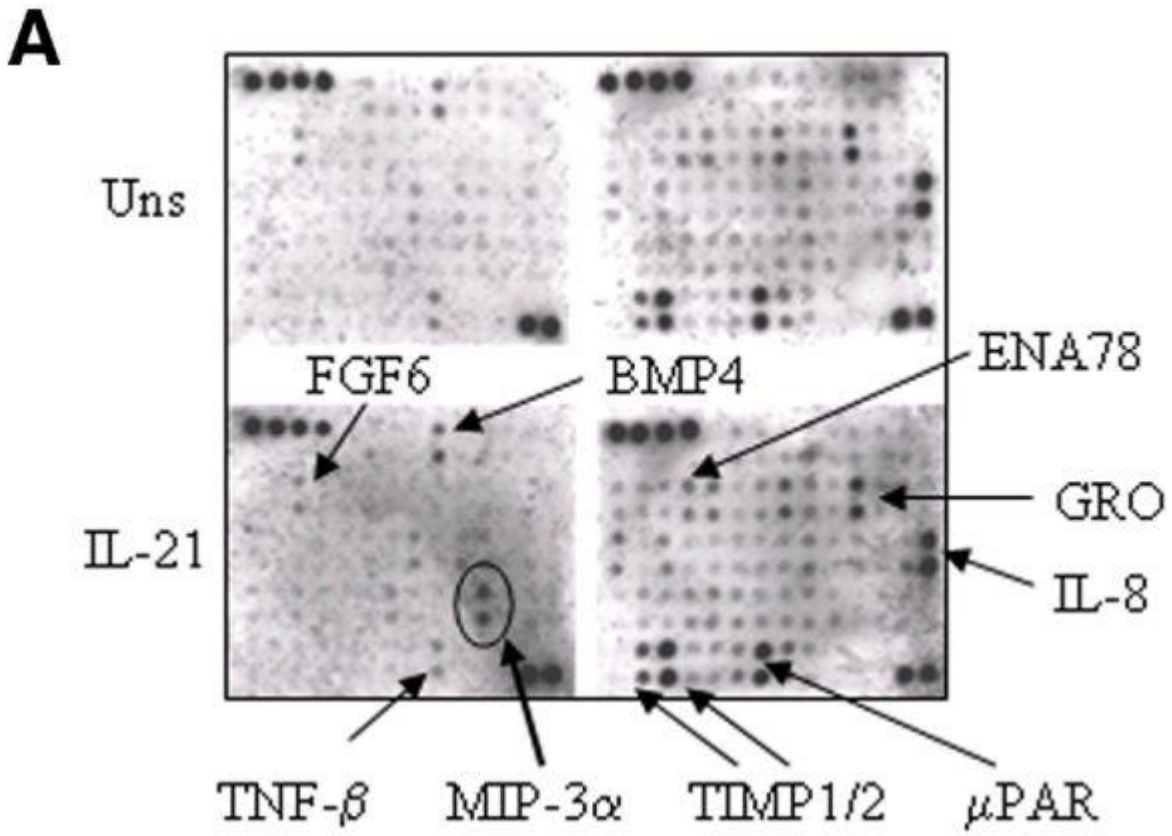


Figure 1. Increased expression of IL-21R in gut epithelium of IBD patients. (*A*) IL-21R staining in colon specimens from 1 patient with CD, 1 patient with UC, and 1 normal control (C). In the lower right panel, the UC section was stained with a control isotype antibody. Figure is representative of 6 separate experiments in which sections of 9 patients with CD, 9 patients with UC, and 10 normal controls were analyzed. Original magnification, 40×. (*B*) Representative Western blot showing IL-21R, the common γ-chain, cytokeratin-18 (CYTOK18), α-smooth muscle actin (SMA), and β-actin in total proteins extracted from freshly isolated intestinal epithelial cells of 1 normal control (C), 1 patient with UC, and 1 patient with CD. F, IBD colonic fibroblasts. One of 5 separate experiments in which proteins extracted from 10 normal controls, 8 patients with CD, and 7 patients with UC were analyzed. (*C*) IL-21R/β-actin protein ratio in proteins prepared from epithelial cells isolated from the colon of 10 normal controls, 8 patients with CD, and 7 patients with UC. Values are expressed in arbitrary densitometric units (A.U.) and indicate the mean ± SD of all the experiments. (*D*) Representative Western blot showing IL-21R and β-actin in total protein extracted from colon cancer epithelial cell lines. (*E*) IL-21 enhances the expression of IL-21R in DLD-1 cells. Cells were either left unstimulated (UNS) or stimulated with the specified cytokines for 24 hours, and then the percentage of IL-21R–positive cells was evaluated by flow cytometry. Data indicate the mean ± SD of 3 separate experiments. IL-21 significantly enhances IL-21R expression (*P* = .04), whereas no significant change in IL-21R expression was seen in cells stimulated with the other cytokines.

4.3.2. IL-21 Enhances the Synthesis of MIP-3α by Colon Epithelial Cells

In initial experiments, we determined if IL-21 affected cell growth. To this end, colon cell lines were labeled with carboxyfluorescein diacetate succinimidyl ester and cultured in the presence or absence of graded doses of IL-21 for 48 hours, followed by flow-cytometry analysis. Carboxyfluorescein diacetate succinimidyl ester dilutions revealed that IL-21 did not affect the growth of DLD-1, HT-29, and Colo205 cells (not shown). We also examined the effect of IL-21 on colon epithelial cell death. The percentage of Annexin V–positive/propidium iodide–positive DLD-1 cells in IL-21–treated cultures (8% ± 3%) was the same as in untreated cell cultures (9% ± 5%). Similar results were obtained with HT-29 and Colo205 cells (not shown).

Several studies have shown that intestinal epithelial cells respond to inflammatory molecules by releasing increased amounts of chemokines and cytokines.[8–11] To evaluate whether IL-21 exerts similar effects on intestinal epithelial cells, DLD-1 cells were stimulated with IL-21 and the culture supernatants then were screened for the content of 120 different proteins using a human protein antibody array. As shown in Figure 2*A*, stimulation of cells with IL-21 enhanced the synthesis of MIP-3α, whereas the secretion of other chemokines and cytokines remained unchanged. Analysis of DLD-1 cell culture supernatants by ELISA confirmed the effect of IL-21 on the induction of MIP-3α protein synthesis. Indeed, IL-21 dose-dependently enhanced the secretion of MIP-3α (Figure 2*B*, $P < .02$). However, IL-21 was less effective than tumor necrosis factor-α in inducing MIP-3α synthesis by DLD-1 cells (219 ± 34 pg/mL and 520 ± 156 pg/mL MIP-3α after stimulation with 200 ng/mL IL-21 and 20 ng/mL tumor necrosis factor-α, respectively).



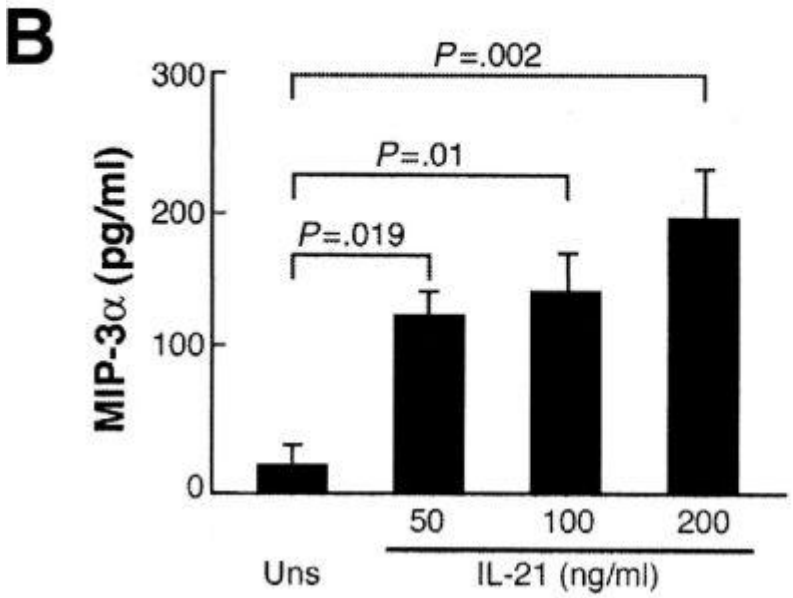


Figure 2. IL-21 enhances the secretion of MIP-3α in DLD-1 cells. (*A*) Serum-starved cells were either left unstimulated or stimulated with 50 ng/mL of IL-21 for 48 hours, and then the cell-free culture supernatants were analyzed by a commercial protein array kit that is able to evaluate 120 different proteins simultaneously. Enhanced MIP-3α secretion was seen in IL-21–stimulated cells (circle). For illustration, several other chemokines/molecules also are highlighted. Similar results were obtained when cells were stimulated with 200 ng/mL of IL-21 (not shown). (*B*) Analysis of MIP-3α in 48-hour culture supernatants of DLD-1 cells either unstimulated or cultured with graded doses of IL-21 by ELISA. IL-21 dose-dependently enhances MIP-3α ($P < .02$). Data indicate the mean ± SD of 5 separate experiments.

4.3.3. Induction of MIP-3α by IL-21 Is Dependent on ERK1/2 Activation

Studies in other systems have shown that MIP-3α production can be regulated by MAP kinases.12,13 Because there is evidence that IL-21 activates ERK1/2 in myeloma cells and immature promyelocytic HL-60 cells,14,15 we evaluated whether the induction of MIP-3α by IL-21 was dependent on this signaling pathway. We first assessed the effect of IL-21 on the activation of MAP kinases in DLD-1 cells. IL-21 rapidly enhanced the phosphorylation of both ERK1/2 and p38, whereas p-JNK remained unchanged (Figure 3A). We next assessed the effect of specific inhibitors of MAP kinases on IL-21–induced MIP-3α production. Treatment of cells with ERK1/2 inhibitor resulted in a complete suppression of the IL-21–induced MIP-3α secretion (P = .001, Figure 3B). In contrast, neither p38 nor JNK inhibition affected the IL-21–driven MIP-3α synthesis (Figure 3B).

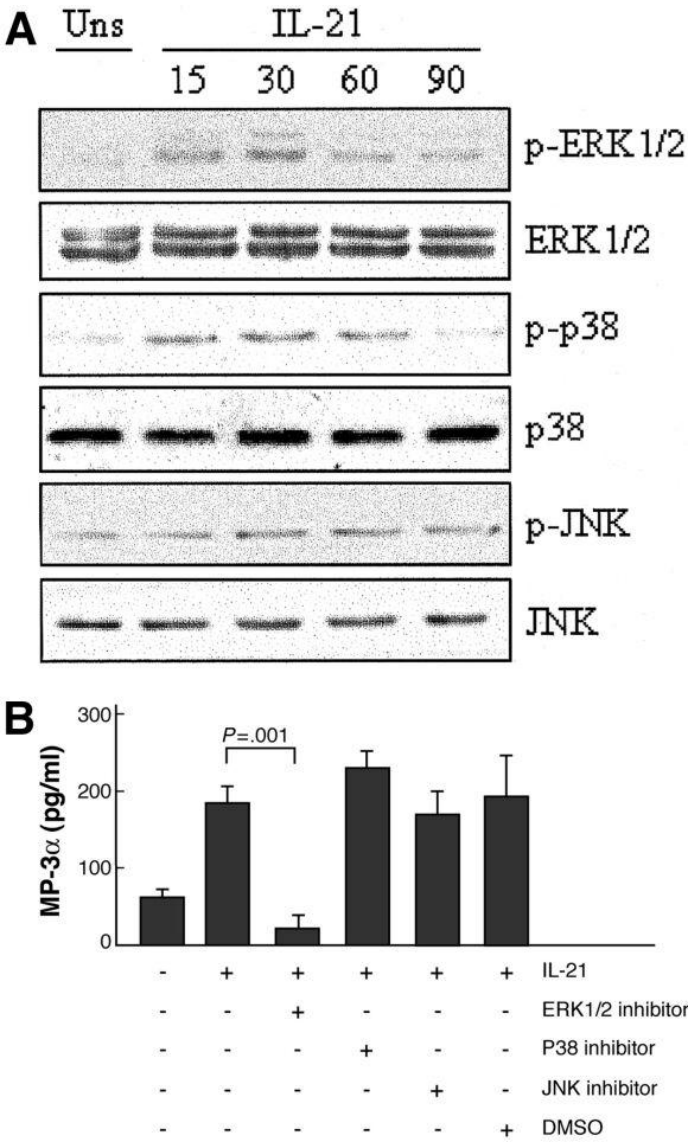


Figure 3. (*A*) IL-21 enhances phosphorylation of ERK1/2 and p38 but not JNK in DLD-1 cells. Serum-starved cells were either left untreated or treated with IL-21 (200 ng/mL) for the indicated time points, and then phosphorylated and total forms of MAP kinases were evaluated by Western blotting. One of 6 experiments in which similar results were obtained is shown. (*B*) Inhibition of ERK1/2 but not p38 or JNK significantly decreases IL-12–induced MIP-3α secretion by DLD-1 cells (*P* = .001). Serum-starved cells were preincubated with specific inhibitors of ERK1/2, p38, or JNK for 30 minutes as indicated in the Materials and Methods section and then stimulated with IL-21 for a further 48 hours. Dimethyl sulfoxide (DMSO) was used as a vehicle. At the end, cell-free supernatants were evaluated for MIP-3α content by ELISA.

4.3.4. IL-21–Induced Colon Epithelial Cell–Derived MIP-3α Promotes Lymphocyte Migration In Vitro

MIP-3α mobilizes T cells to areas of immune challenge.16,17 Therefore, we examined whether IL-21–stimulated epithelial cell–derived MIP-3α was capable of enhancing the migration of peripheral blood lymphocytes using an in vitro chemotaxis assay. The fraction of lymphocytes migrating in response to IL-21–treated cell culture supernatants (conditioned medium) was significantly higher than that observed in response to supernatants of untreated cells (Figure 4A,P = .02) and this effect was seen regardless of whether conditioned medium of DLD-1 or HT-29 cells was used (not shown). To determine whether the lymphocyte migration induced by the conditioned medium was mediated by MIP-3α, the medium was incubated with a neutralizing MIP-3α or control isotype antibody before testing its effect on lymphocyte migration. The anti–MIP-3α but not control antibody significantly reduced the conditioned medium–induced lymphocyte migration (Figure 4B, P = .03).

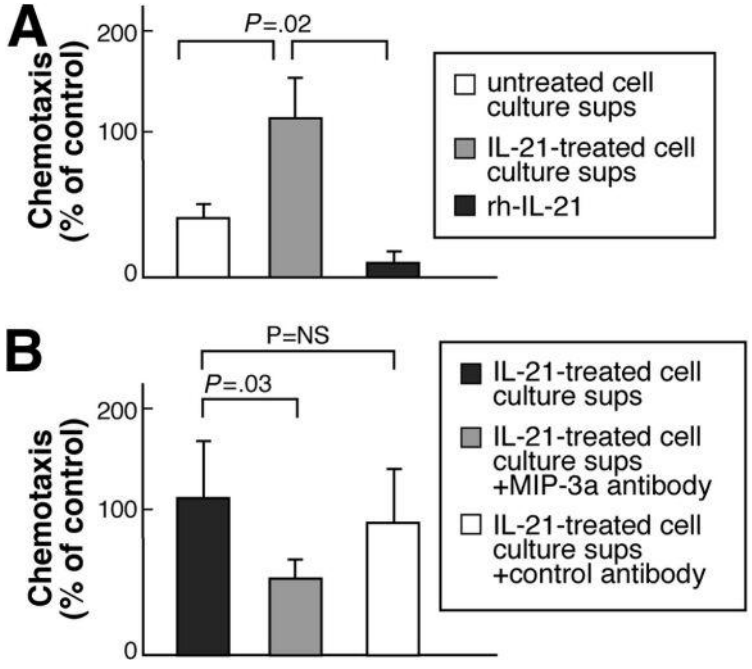


Figure 4. IL-21–stimulated DLD-1 cell culture supernatants (sups) enhance the migration of peripheral blood T lymphocytes. (*A*) Human peripheral blood CD3+ T lymphocyte migration in chemotaxis multiwell chamber induced by 120 minutes of stimulation with culture supernatants of either untreated or IL-21 (200 ng/mL)–treated DLD-1 cells (conditioned medium), or rh–IL-21 (200 ng/mL). At the end, cells migrating in the lower wells were counted as indicated in the Materials and Methods section. IL-21–stimulated cell culture supernatants caused a significant increase in lymphocyte migration in comparison with the supernatants of untreated cells or rh–IL-21 (*P* = .02). Data indicate the mean ± SD of 3 separate experiments and are expressed as a percentage of migration in comparison with controls (cells migrating in response to medium alone). □, Untreated cell culture sups; , IL-21–treated cell culture sups; ■, rh–IL-21. (*B*) Lymphocyte migration induced by IL-21–stimulated cell culture supernatants (conditioned medium) is inhibited by a neutralizing MIP-3α antibody (*P* = .03). The in vitro chemotaxis was performed as indicated in *A* with the exception that the IL-21–stimulated DLD-1 cell culture supernatants were used in the presence or absence of a neutralizing MIP-3α or control antibody (5 μg/mL). Data indicate the mean ± SD of 3 separate experiments and are expressed as the percentage of migration in comparison with controls (cells migrating in response to medium alone). ■, IL-21–treated cell culture sups; , IL-21–treated cell culture sups + MIP-3α antibody; □, IL-21–treated cell culture sups + control antibody.

4.3.5. Blockade of IL-21 Results in a Reduced Production of MIP-3α in Organ Cultures of Mucosal Explants of IBD Patients

We next performed ex vivo organ cultures and examined whether blocking endogenous IL-21 reduced MIP-3α in mucosal explants of patients with IBD. We used a neutralizing IL-21 antibody we raised by immunizing rabbits with a specific human IL-21 peptide. To determine whether the anti–IL-21 was able to neutralize IL-21 activity, we first assessed the effect of the antibody on IL-21–stimulated p-Stat3 in human PBMCs. Treatment of PBMCs with IL-21 enhanced p-Stat3 (Figure 5A), and this effect was reduced markedly by anti–IL-21, but not control antibody, in a dose-dependent fashion. Subsequently, we treated IBD biopsy specimens with the anti–IL-21 antibody or control antibody for 24 hours and then analyzed the secretion of MIP-3α by ELISA. Anti–IL-21 but not control antibody significantly reduced the levels of MIP-3α, regardless of whether biopsy specimens were from CD patients or UC patients (Figure 5B and C, $P < .01$). Moreover, Western blotting of extracts of epithelial cells isolated from the mucosal explants showed that the anti–IL-21 reduced the colonocyte production of MIP-3α, and this was evident in samples from both CD and UC (Figure 5D).

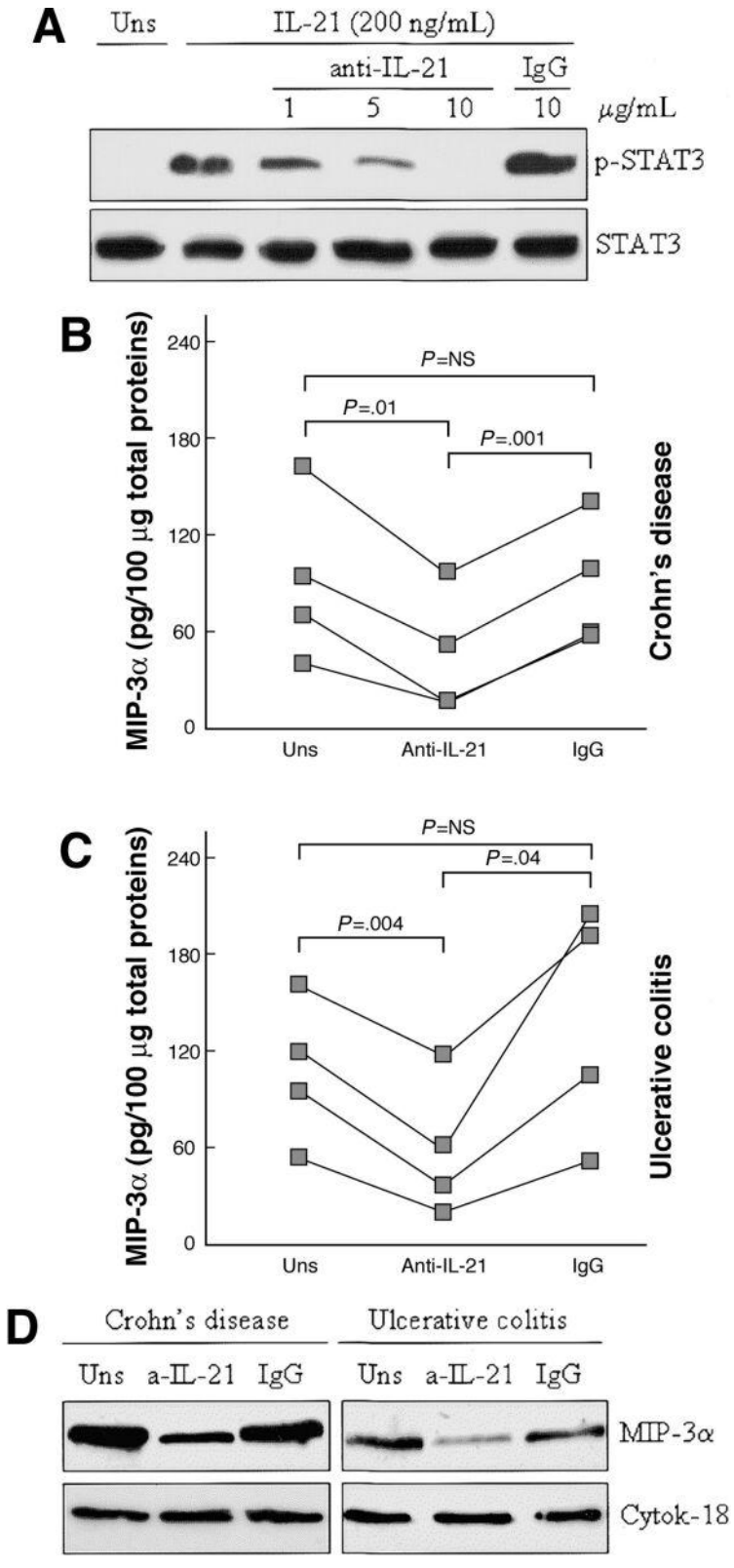


Figure 5. (*A*) Effect of a neutralizing IL-21 antibody on the induction of phosphorylated Stat3 (p-Stat3) by IL-21 in PBMCs. (*A*) PBMCs were isolated from a normal subject and cultured with or without (UNS) 200 ng/mL of IL-21 in the presence or absence of anti–IL-21 or control antibody at the indicated concentrations. After 30 minutes, PBMCs were collected and total proteins were extracted. Both phosphorylated and total Stat3 then were assessed by Western blotting. One of 3 separate experiments in which similar results were obtained is shown. (*B* and *C*) Blocking endogenous IL-21 results in a significant decrease in the production of MIP-3α in IBD mucosa. Mucosal samples were taken from (*B*) 4 patients with CD and (*C*) 4 patients with UC and cultured with or without the initial addition of a neutralizing IL-21 or control antibody (10 μg/mL) for 24 hours. At the end, organ culture supernatants were collected and analyzed for the content of MIP-3α by ELISA. The neutralizing IL-21 but not control antibody significantly decreased the production of MIP-3α and this is evident regardless of whether biopsy specimens were taken from patients with CD (P = .01) or UC (P = .004). (*D*) Representative Western blots for MIP-3α (upper blot) and cytokeratin-18 (lower blot) in epithelial cells isolated from mucosal explants cultured as indicated in *B* and *C*. The examples are representative of 2 separate experiments analyzing in total samples from 3 patients with CD and 2 patients with UC.

Anti–IL-21 also decreased the ability of organ culture supernatants to attract T cells. Indeed, the percentage of CD3+ lymphocytes that migrated in response to untreated organ culture supernatants was 43% ± 11% greater than that measured in response to medium alone. This percentage was significantly higher than the fraction of cells that migrated in response to anti–IL-21–treated organ culture supernatants (21% ± 7%, P = .04) but not different from that induced by control antibody-treated organ culture supernatants (51% ± 12%) (Figure 6).

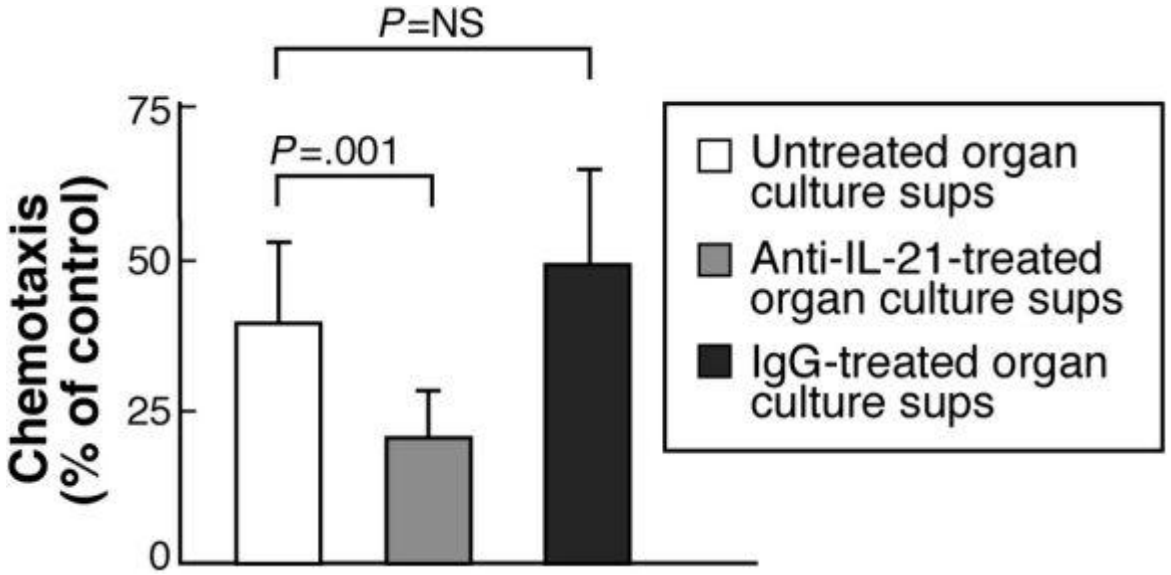


Figure 6. Lymphocyte migration induced by supernatants of mucosal explants cultured in the presence or absence of anti–IL-21 or control IgG antibody (10 μg/mL) for 24 hours. The in vitro chemotaxis was performed as indicated in Figure 4. The migration of lymphocytes induced by supernatants of IBD mucosal explants is reduced significantly by treatment with anti–IL-21 (*P* = .04) but not IgG. Data indicate the mean ± SD of 7 separate experiments in which supernatants of organ cultures of mucosal explants taken from 4 CD patients and 3 UC patients were used. Data are expressed as the percentage of migration in comparison with controls (cells migrating in response to medium alone). □, Untreated organ culture sups; , anti–IL-21–treated organ culture sups; ■, IgG-treated organ culture sups.

4.4. DISCUSSION

In vitro and in vivo studies have shown convincingly that intestinal epithelial cells play an active role in chronic inflammation. They are capable of synthesizing cytokines and chemokines that promote the recruitment of inflammatory cells into the gut tissue and/or modulate the functional activity of mucosal leukocytes.[8,18,19] Cytokines produced by resident leukocytes also regulate

intestinal epithelial cell function.[1,20] Thus, in IBD, active cross-talk between epithelial cells and leukocytes almost certainly amplifies and maintains ongoing mucosal inflammation.

In this report we show that human colon epithelial cell lines and primary intestinal epithelial cells constitutively express IL-21R, that IL-21R expression is up-regulated in the gut epithelium of patients with CD and patients with UC, and that IL-21 induces MIP-3α production by epithelial cells. These findings and the recent demonstration that IL-21 is produced in excess in these diseases[5] suggest that IL-21/IL-21R signals may play an important role in the pathogenesis of IBD.

Intestinal epithelial cells express the common γ-chain subunit that interacts with IL-21R and is essential for IL-21–driven intracellular signals.[3] It is thus highly plausible that intestinal epithelial cells are a potential target of IL-21 in vivo. IL-21 did not alter either the growth or the death of intestinal epithelial cells, thus excluding the possibility that the increased synthesis of MIP-3α was secondary to changes in cell proliferation and/or viability. We do not know if the IL-21 concentrations used to stimulate intestinal epithelial cells is comparable with that present in the intestinal lamina propria of IBD patients because no commercially available kit is yet available to measure human IL-21. However, our previous Western blotting analysis of IL-21 in the gut revealed that the intensity of immunoreactive bands in IBD patients was not different from that obtained with 50 and 200 ng/mL recombinant human (rh)IL-21, the doses we used to stimulate colon epithelial cells. The IL-21 doses we selected for our experiments are also similar to those used by other workers to test the biological effects of this cytokine in vitro.[15]

By using molecular and pharmacologic approaches, we also provide evidence that MAP kinase–mediated signaling pathways are involved in IL-21–induced MIP-3α synthesis. Indeed, we showed that IL-21 activates ERK1/2 and p38 but not JNK in colon cell lines, and that inhibition of ERK1/2 but not p38 abrogates IL-21–induced MIP-3α synthesis. This is consistent with the demonstration that the MIP-3α promoter region contains binding sites for activator protein-1, a transcription factor activated by MAP kinases.[21] Our findings also fit with data of previous studies showing that

flagellin, a specific ligand for Toll-like receptor 5, stimulates MIP-3α production by nontransformed human colonic cells, through an ERK-dependent mechanism.[22] In addition, induction of MIP-3α in epithelial cells of the respiratory tract by inflammatory cytokines, such as IL-17, is dependent on MAP kinase activity. However, in these cells, neither p38 nor JNK inhibition attenuated the IL-17–induced MIP-3α.[23]

Although there is considerable overlap and redundancy in the chemokine system, with most chemokines acting on a range of cell types, the known functional properties of some chemokines render them interesting targets to regulate the recruitment of T cells into the mucosa of IBD patients. MIP-3α is up-regulated in the inflamed gut of IBD patients, and it acts via CCR6 expressed on CD45RO+ T lymphocytes bearing the gut-homing integrin α4β7.[21,24,25] MIP-3α also promotes the recruitment of immature dendritic cells to mucosal surfaces, allowing for their antigen uptake and further migration toward secondary lymphoid organs where they could promote T-cell activation.[21] There also is evidence that high MIP-3α concentrations are found in the intestine of mice with dextran sulfate sodium (DSS) colitis, and the administration of an anti–MIP-3α antibody significantly attenuates the DSS-induced T- and B-cell intestinal accumulation.[16] We also show here that IL-21–treated colon cell supernatants enhance the in vitro migration of peripheral blood T cells, and that this effect can be inhibited by a neutralizing MIP-3α. However, the fact that migration of T cells induced by IL-21–treated colon cell supernatants was only partially inhibited but not completely suppressed by the neutralizing MIP-3α antibody suggests the possibility that IL-21 induces the synthesis of additional chemoattractants other than MIP-3α.

Because polarized primary gut epithelial cells are difficult to grow for extended periods, most of our studies used colon cell lines. However, to show the importance of IL-21R signaling in tissues, we showed that neutralization of IL-21 in ex vivo organ cultures of IBD explants reduced epithelial MIP-3α production.

Whatever the underlying mechanism, the ability of IL-21 to promote the mobilization of leukocytes from the circulation into the inflamed tissues is clearly generally important. For example, in the murine air pouch model, the administration of IL-21 was followed by a marked tissue accumulation of neutrophils and mononuclear cells.[15] Studies in experimental models of autoimmune diseases have shown that administration of IL-21 into mice enhances the recruitment of leukocytes into inflamed tissues and increases the severity of the inflammation.[26]

In conclusion, the present study shows that intestinal epithelial cells express IL-21R and respond to IL-21 with enhanced synthesis of MIP-3α, thus widening the spectrum of T-cell–derived cytokines that can regulate the production of chemoattractants in IBD mucosa. In addition, our data offer preliminary evidence for a novel mechanism of IL-21 in orchestrating the cross-talk between immune and epithelial cells in the gut. These findings and the demonstration that IL-21 is up-regulated in IBD suggest that blockade of IL-21 can be a new and promising way to limit the ongoing inflammation in patients with IBD.

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5. CONCLUSIONS

In recent years, the advancement of sophisticated biological technologies has facilitated the molecular characterization of IBD through the identification of specific cytokine patterns. There is no doubt that genetic predisposition to sustained inflammatory responses and hyper-responsiveness to components of the bacterial flora are major contributing factors in the induction of the Th1-driven tissue-damaging inflammation in patients with CD. It is also clear that the local immune response in UC patients is less characterized, even though the production of Th2-type cytokines, such as IL-4 and IL-13, seems to be predominant. Despite these differences, downstream inflammatory events are the same in CD and UC, and therefore strategies aimed at inhibiting these non-specific inflammatory processes may be therapeutic in both diseases. IL-21 could be an emerging target in both IBD. Although the full role of IL-21 in the IBD-related immune response remains to be clarified, evidence indicates that IL-21 may modulate the activity of multiple cell types in the gut, and play an important role in orchestrating the cross-talk between immune and non-immune cells that eventually leads to the tissue damage.