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IL-21 Is Highly Produced in *Helicobacter pylori*-Infected Gastric Mucosa and Promotes Gelatinases Synthesis

Robert Caruso,* Daniele Fina,* Ilaria Peluso,* Massimo Claudio Fantini,* Claudio Tosti,* Giovanna Del Vecchio Blanco,* Omero Alessandro Paoluzzi,* Flavio Caprioli,* Fabio Andrei,* Carmine Stolfi,* Marco Romano,* Vittorio Ricci,* Thomas T. MacDonald,* Francesco Pallone,* and Giovanni Monteleone2*

*Helicobacter pylori* (Hp) infection is associated with gastric inflammation and ulceration. The pathways of tissue damage in Hp-infected subjects are complex, but evidence indicates that T cell-derived cytokines enhance the synthesis of matrix metalloproteinases (MMP) that contribute to mucosal ulceration and epithelial damage. In this study, we have examined the role of the T cell cytokine IL-21 in Hp-infected gastric mucosa and evaluated whether IL-21 regulates MMP production by gastric epithelial cells. We show that IL-21 is constitutively expressed in gastric mucosa and is more abundant in biopsy specimens and purified mucosal CD3+ T cells from Hp-infected patients compared with normal patients and disease controls. We also demonstrate that IL-21R is expressed by primary gastric epithelial cells, as well as by the gastric epithelial cell lines AGS and MKN28. Consistently, AGS cells respond to IL-21 by increasing production of MMP-2 and MMP-9, but not MMP-1, MMP-3, MMP-7, or tissue inhibitors of MMP. Analysis of signaling pathways leading to MMP production reveals that IL-21 enhances NF-κB but not MAPK activation, and inhibition of NF-κB activation reduces IL-21-induced MMP-2 and MMP-9 production. Finally, we show that treatment of Hp-infected gastric explants with anti-IL-21 reduces epithelial cell-derived MMP-2 and MMP-9 production. These data indicate that IL-21 is overexpressed in Hp-infected gastric mucosa where it could contribute to increased epithelial gelatinase production. *The Journal of Immunology*, 2007, 178: 5957–5965.

Infection by *Helicobacter pylori* (Hp) bacteria affects more than half of the world’s population causing acute, chronic inflammation of the gastric mucosa. In the majority of cases, chronic gastritis remains asymptomatic, but it can lead to gastroduodenal ulceration. Eradication of Hp bacteria results in resolution of gastric inflammation and reduces the risk of recurrence of gastroduodenal ulceration (1, 2). The mechanisms by which Hp causes gastritis and mucosal damage are complex. In vitro studies suggest that Hp per se induces apoptosis of gastric epithelial cells and stimulates epithelial cells to secrete several chemokattractants (3–5). In contrast, infection of lymphocyte-deficient mice with various *Helicobacter* species fails to induce gastric inflammation and ulceration. However, transfer of T cells into these animals then results in severe gastritis, implying that host T cell-mediated immune responses to Hp infection are a major determinant of mucosal damage (6–8). Consistent with this outcome, there is a marked increase in Th1-type cytokines, including IFN-γ, IL-12, and TNF-α in Hp-infected mucosa, all of which have been reported to be involved in tissue degradation in other systems (1, 9–12).

Matrix metalloproteinases (MMPs) are a family of neutral endopeptidases capable of degrading all extracellular matrix proteins and remodeling connective tissue (13). Beyond these destructive properties, MMPs also cleave and activate several chemokines, thus contributing to the recruitment of circulating cells into inflamed tissues (14). Consistent with their proteolytic properties, increased production of MMPs has been documented in several human diseases characterized by tissue degradation, including Hp-associated gastritis and gastrointestinal ulcers (15–17). MMPs are produced by many cell types and their synthesis occurs in response to regulated signals provided by various soluble factors or cell-matrix interactions (18). In Hp-infected mucosa, epithelial cells appear to be one of the major sources of MMPs (19, 20). Although Hp by itself can induce gastric epithelial cells to release MMPs (21, 22), accumulating evidence indicates that cytokines produced by mucosal T cells and macrophages are also major stimuli for MMP production (16, 23, 24).

IL-21 is a newly described cytokine produced by activated CD4+ T cells, which regulates the growth and functional properties of T cells, B cells, NK cells, and dendritic cells (25). Its biological activity is mediated through a class I cytokine family receptor composed of a specific IL-21R and the common γ-chain (γc) receptor, that is also an essential component of IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (26). Originally described on lymphoid cells, IL-21R has recently been documented on nonimmune

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*Abbreviations used in this paper: Hp, *Helicobacter pylori*; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of MMP; γc, common γ-chain; T-PLP, T lamina propria lymphocyte; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; a.u., arbitrary unit.

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cells, such as fibroblasts and keratinocytes (27). High IL-21 and/or IL-21R expression occurs in human Th1-associated diseases, and administration of IL-21 to mice enhances the influx of immune cells into inflamed tissues and the severity of infection-mediated processes (28–30). In light of these findings, it is conceivable that IL-21 may play an important role in the initiation and perpetuation of chronic inflammatory processes.

We have therefore sought to determine the role of IL-21 in Hp-associated gastritis. We first examined IL-21 and IL-21R in the gastric mucosa of patients with Hp infection. Second, using the gastric cell lines AGS and MKN28, we examined whether IL-21 controls the production of MMPs. Finally, we dissected the intracellular cytokine signaling pathways underlying the IL-21-driven effects on MMP production.

Materials and Methods

Patients and samples

The 40 Hp-positive patients and 49 Hp-negative subjects undergoing upper esophagogastroduodenoscopy for dyspeptic symptoms were consecutively enrolled for this study. Hp infection was determined by the rapid urease test and histological examination of biopsy specimens taken from the corpus and antrum, and by a [13C]urea breath test. Patients were classified as Hp-infected or uninfected only if the three tests were positive or negative, respectively. In the Hp-negative group, 23 patients had gastritis and 26 patients had no evidence of inflammation at histology. In the group of 23 Hp-negative patients who had gastritis, 6 were on nonsteroidal anti-inflammatory drugs, 6 had a duodenal-gastric biliary reflux at endoscopy, and 1 had systemic vasculitis. In the remaining 10 Hp-negative patients who had gastritis, the cause was unknown. In the group of Hp-positive patients, 5 were re-endoscoped 2 mo after cessation of 1-wk treatment with esomeprazole (20 mg twice daily), amoxicillin (1000 mg twice daily), and clarithromycin (500 mg twice daily). All patients had successful Hp eradication as confirmed by [13C]urea breath test and histology. Four to 12 biopsy specimens were collected from each subject. Four biopsy samples were available from 27 Hp-positive and 31 Hp-negative patients. These samples were used for rapid urease test, histological examination, and IL-21 protein/RNA expression analysis. Eight biopsy results were available from the remaining 13 Hp-positive and 18 Hp-negative patients, and were used for isolating epithelial or lamina propria mononuclear cells or for performing the organ cultures.

Epithelial and lamina propria mononuclear cell isolation

Freshly obtained gastric biopsy specimens of 8 Hp-positive patients and 12 Hp-negative patients (6 with gastritis) were used to isolate epithelial cells. Biopsy specimens were incubated in DTT (0.1 mM; Sigma-Aldrich) for 10 min, then extensively washed in RPMI 1640 (Sigma-Aldrich) and finally incubated in 1 mM EDTA (Sigma-Aldrich) for 30 min at 37°C in the presence of 5% CO2. The resulting cell preparations contained 95% epithelial cells and <5% contaminating lymphocytes as assessed by flow cytometry. The isolated cells were checked for viability using 0.1% trypsin blue (viability ranged from 80 to 85%). Both epithelial cells and the re-remaining epithelial cell-depleted mucosa were then used for extracting total proteins. Additionally, lamina propria mononuclear cells were isolated from gastric biopsy of 5 Hp-positive patients and 6 Hp-negative patients (4 with gastritis) by DTT-EDTA-collagenase procedure (31) and used for purifying CD3+ T lamina propria lymphocytes (T-LPL). Lamina propria mononuclear cells were incubated for 30 min at 4°C with magnetically labeled CD3 Ab (Miltenyi Biotec). T-LPL then were collected by positive selection using the MACS system (Miltenyi Biotec). Purity of all cell populations used for this study was >92%.

Cell culture

The gastric epithelial cell lines AGS and MKN28 were cultured in DMEM or RPMI 1640 medium supplemented with 10% heat-inactivated FBS. To investigate factors involved in the induction of IL-21-21R in gastric epithelial cells, AGS were stimulated with Hp strains 60190 and CCUG 17874, both purchased from PeproTech. At the end, cells were analyzed for MAPKs and NF-κB activation, or vehicle (ethanol or DMSO) for 60 min before adding IL-21 (50 ng/ml) for further 48 h. Supernatants were used for analysis of MMPs and tissue inhibitors of MMPs (TIMPs) by Western blotting.

Protein extraction and Western blotting

All primary Abs were from Santa Cruz Biotechnology unless specified, whereas secondary Abs were from DakoCytomation. IL-21 was analyzed using total proteins extracted from biopsy of 17 Hp-positive and 20 Hp-negative patients (8 with gastritis) who were consecutively enrolled, and from purified CD3+ T-LPL of 5 Hp-positive and 6 Hp-negative patients. IL-21 was also analyzed in gastric biopsy specimens taken from 5 Hp-infected patients before and after the eradicating therapy. Samples were lysed for 60 min on ice in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, and 0.5% Nonidet P-40, supplemented with 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM Na3VO4, and 1 mM NaF (all reagents were from Sigma-Aldrich). Lysates were then centrifuged at 4°C for 30 min at 12,000 × g. Equal amounts of total proteins were fractionated on SDS-polyacrylamide gels. The membranes were blocked with TBS containing 0.05% Tween 20 and 5% nonfat dry milk and then incubated with a rabbit anti-human IL-21 (0.5 μg/ml; ProSci) followed by a HRP-conjugated goat anti-rabbit IgG Ab (1/20,000 final dilution). The reaction was detected with a chemiluminescence kit (West Dura; Pierce). Cell-assisted scanning densitometry (Total Lab; Abel) was used to analyze the density of the immunoreactive bands.

IL-21R and the γc subunit were analyzed in total proteins prepared from epithelial cells and epithelial cell-depleted mucosa, AGS and MKN28 cells, using the following Abs: anti-IL-21R (1 μg/ml; R&D Systems), anti-γc, (1/500 final dilution), anti-cytokeratin-18 (1/400; Sigma-Aldrich), and anti-β-actin (1/5000; Sigma-Aldrich). Appropriate HRP-conjugated secondary Abs were then used and bound Abs visualized using ECL (West Dura; Pierce).

To analyze the effect of IL-21 on MAPK and NF-κB activation, AGS cells were stimulated for the indicated times and cytoplasmic proteins were then prepared by incubating the cells for 1 min with the buffer described. After removing the pellets containing intact cells and nuclei, the remaining proteins were collected and stored at −80°C. For the detection of MMPs and TIMPs in the AGS and MKN cell culture supernatants, 100 μg of proteins was loaded per lane and separated on SDS-PAGE, and the proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% milk in PBS for 1 h, then incubated with the following Abs: anti-MMP-1, anti-MMP-2, anti-MMP-3, anti-MMP-7, anti-TIMP-1, and anti-TIMP-2 (all 1 μg/ml; R&D systems) followed by a HRP-conjugated goat anti-mouse Ab conjugated to HRP (1/20,000 final dilution).

IL-21 (10–50 ng/ml; BioSource International) for the indicated time points. Moreover, to examine whether the effect of IL-21 on MMPs relies on MAPKs or NF-κB activity, confluent AGS or MKN28 cells were starved in serum-free medium for 24 h and then stimulated with IL-21 (50 ng/ml) for 5–60 min. As controls, cells were stimulated with epidermal growth factor (200 ng/ml) or IL-1β (10 ng/ml), both purchased from PeproTech. At the end, cells were analyzed for MAPKs and NF-κB activation. In parallel, serum-starved cells were preincubated with PD98059, an inhibitor of ERK1/2 (50 μM), SB203580, an inhibitor of p38 (10 μM), or a JNK inhibitor 420116 (5 μM) (all from Inalco) or with N-tosyl-l-phenylalanine chloromethyl ketone (TPCK (10 μM); Sigma-Aldrich), an inhibitor of NF-κB activation, or vehicle (ethanol or DMSO) for 60 min before adding IL-21 (50 ng/ml) for further 48 h. Supernatants were used for analysis of MMPs and tissue inhibitors of MMPs (TIMPs) by Western blotting.
Additionally, MMP-2 and MMP-9 were evaluated in epithelial cell extracts of gastric mucosal explants cultured for 24 h with the anti-IL-21 Ab or control IgG using the same Abs.

For assessing CagA status, total proteins extracted from Hp-infected patients were analyzed by using a mouse anti-CagA (Hp) mAb (Santa Cruz Biotechnology).

Gelatin zymography

The 20 µl of concentrated AGS cell culture supernatants was electrophoresed under nonreducing conditions in a 8% acrylamide gel containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were washed at room temperature for 30 min in 2.5% Triton X-100 (Sigma-Aldrich), then equilibrated at room temperature with gentle agitation in developing buffer (50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij35) (Sigma-Aldrich). 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.

EMSA analysis

Nuclear protein-DNA binding assay was conducted for 20 min at room temperature in 20 µl of binding buffer containing 1 µg of poly(dI:dC), 2.5% glycerol, 5 mM MgCl2 (all the reagents were from Pierce), 10 fmol biotin-labeled NF-κB oligonucleotides, and 10 µg of nuclear proteins. The DNA probe was prepared by annealing the two consensuses NF-κB oligonucleotides, which were labeled at the 3′ end with biotin using a commercially available kit (Pierce). The binding specificity was confirmed by incubating the nuclear extracts with unlabeled-specific NF-κB oligonucleotide or unrelated IFN-stimulated gene (ISG)-stimulated response element oligonucleotide in 4-fold molar excess. For Ab blocking assays, a goat anti-human p65 or control isotype IgG (2 µg/sample) was incubated with the nuclear proteins for 40 min before adding the DNA probe. After blotting the membrane labeled oligonucleotides were detected with a chemiluminescence EMSA kit (Pierce).

Analysis of IL-21R and cell growth and death by flow cytometry

IL-21R was examined by incubating AGS and MKN28 cells with a PE-labeled mouse anti-IL-21R (1/10 final dilution; R&D Systems) or isotype control Ab (1/20 dilution; BD Biosciences). Moreover, IL-21R was assessed in freshly isolated gastric epithelial cells of four patients (two with Hp infection). In these experiments, cells were stained with the IL-21R PE, anti-CD3 PerCP (BD Biosciences), or isotype control Ab. Incubations were conducted at 4°C for 30 min. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry.

To assess the effect of IL-21 on gastric epithelial cell growth and survival, serum-starved AGS cells were either left untreated or treated with 10–50 ng/ml IL-21 for 48 h. To track the proliferation, cells were incubated in 0.2 µM CFSE (Invitrogen Life Technologies) at 37°C for 30 min and extensively washed before culture. After 5 days culture, CFSE fluorescence was evaluated, thus determining the proportion of cells undergoing divisions. The fraction of annexin V- and propidium iodide-positive cells was evaluated using a commercially available kit (Beckman Coulter).

RNA extraction, cDNA preparation, and real-time PCR

Quantitative analysis of IL-21 was performed by real-time PCR using total RNA extracted from gastric biopsies of 10 Hp-positive patients, 5 Hp-negative patients with gastritis, and 6 normal controls. To analyze the effect of IL-21 on MMP-2 and MMP-9 RNA expression, AGS were starved over 6 h of culture in serum-free medium with presence or absence of IL-21 (50 ng/ml) for 90 min to 8 h. RNA was extracted by using TRIzol reagent according to the manufacturer’s instructions (Invitrogen Life Technologies). A constant amount of RNA (2 µg/sample) was retro-transcribed into cDNA, and 1 µl of cDNA/sample was then amplified using the following conditions: denaturation 1 min at 95°C, annealing 30 s at 55°C for MMP-2, at 58°C for MMP-9, at 60°C for IL-21, and at 62°C for β-actin, followed by 30 s of extension at 72°C. Primers sequence was as follows: MMP-2 (forward) 5′-TGACGGAAAGATTGTGTTG-3′ and (reverse) 5′-GTTGATTGGTAAATGGGTG-3′; MMP-9 (forward) 5′-GCTTCTCCAGTACCCAGAGA-3′ and (reverse) 5′-GGCGAGTTGATCTAGTGC-3′; and IL-21 (forward) 5′-GGAGAAGGTGATCTACGTCT-3′ and (reverse) 5′-CAGCAAGTGTTGCTCTACATCTC-3′. Real-time PCR was performed using the IQ SYBR Green Supermix (Bio-Rad). β-Actin (forward) 5′-AAGGATAGCAGCATGTGGGAAC-3′ and (reverse) 5′-AGCCAGTCCAGGCCAGGAT-3′ primer sequence was used as an internal control.

Production of the neutralizing IL-21 Ab

A human IL-21 peptide (NVSKKLRKPKSTN) was synthesized and used to immunize New Zealand White rabbits by Washington Biotechnologies. The IL-21 peptide was injected s.c. with adjuvant into specific pathogen-free rabbits (New Zealand White). Blood was taken 6 or 8 wk following initial immunization, and antiserum titer was characterized by ELISA. As a control, serum was taken from the same rabbits prior to their immunization. Purification of anti-IL-21 Ab (IgG) was conducted using the Melon Gel IgG Spin purification kit according to the manufacturer’s instructions (Pierce).

The neutralizing activity of the anti-IL-21 Ab was confirmed by showing that it inhibited the effect of IL-21 on phospho-Stat3 induction in PBMC.

Organic culture

Mucosal biopsy specimens were taken from five Hp-infected patients and cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) as previously indicated (31). Cultures were performed with or without the addition of the neutralizing IL-21 or control Ab (10 µg/ml) for 24 h. At the end, epithelial cells were isolated and then analyzed for the expression of MMP-2, MMP-9, and cytokeratin-18 by Western blotting.

Statistical analysis

Differences between groups were compared using the ANOVA and Wilcoxon tests.

Results

IL-21 expression is enhanced in Hp-infected gastric mucosa

IL-21 protein was detectable in all samples regardless of whether biopsies were taken from Hp-infected or uninfected patients (Fig. 1A). However, as shown in Fig. 1B, IL-21 expression was significantly increased in biopsy specimens of Hp-infected patients compared with uninfected patients who had gastritis (p = 0.01) or did not have gastritis (p < 0.001). Among Hp-infected patients, IL-21 protein expression did not differ between persons who were infected with CagA strain and those who were not CagA infected (data not shown). No difference in IL-21 protein expression was seen between uninfected patients who had gastritis and normal samples (Fig. 1B). High IL-21 was also seen in gastric CD3+ T-LPL isolated from Hp-infected patients compared with T cells isolated from uninfected patients (Fig. 1C).

To confirm further that Hp-related gastritis is associated with enhanced synthesis of IL-21, we assessed IL-21 in gastric biopsies of five Hp-infected patients before and after successful eradication. As shown in Fig. 1D, eradication of Hp resulted in a reduced expression of IL-21. To also analyze whether IL-21 is regulated at the transcriptional level, mucosal samples from Hp-infected patients and controls were assessed for the content of IL-21 RNA transcripts by real-time PCR. Fig. 1E shows that IL-21 RNA expression was significantly higher in Hp-infected patients than uninfected patients either with or without gastritis (p = 0.04 or p = 0.02, respectively). No difference was seen in terms of IL-21 RNA transcripts between uninfected patients with gastritis and normal controls (Fig. 1E).

IL-21R is expressed by gastric epithelial cells

Our attempts to characterize IL-21R by immunohistochemistry, using commercially available Abs, were unsuccessful. Therefore, IL-21R was assessed by Western blotting using total proteins extracted from both epithelial cells and epithelial cell-depleted mucosa. Two or more distinct bands with a molecular mass ranging from 55 to 72 kDa and corresponding to glycosylated forms of IL-21R (32) were detected in epithelial extracts of patients and controls (Fig. 2A). In particular, two isoforms of IL-21R with a molecular mass of ~60–72 kDa, respectively, were seen in all
Increased IL-21 expression in gastric biopsies from patients with Hp infection. A. Representative Western blot showing IL-21 and β-actin protein in mucosal samples taken from three patients with Hp-related gastritis (Hp⁺), three Hp-negative patients with gastritis (Hp⁻ gastritis), and three patients without gastritis (controls). One of four separate experiments analyzing total samples of 17 Hp-infected patients, 8 uninfected patients with gastritis, and 12 subjects without gastritis is shown. B, Quantitative analysis of the ratio of IL-21 to β-actin protein in mucosal samples taken from 17 Hp-infected gastritis patients, 8 Hp-unrelated gastritis patients, and 12 controls as measured by densitometry scanning of Western blots. Data are expressed in arbitrary units (a.u.). Each symbol indicates the IL-21 to β-actin protein ratio in a single patient, and thick bar indicates median. C, Representative Western blotting showing IL-21 and β-actin protein in purified CD3⁺ lamina propria mononuclear cells (LPMC) isolated from three Hp-positive patients and three Hp-negative patients. One of two separate experiments in which similar results were obtained is shown. Note in the group of Hp-negative patients, the first two had gastritis. D, The quantitative analysis of the IL-21 to β-actin protein ratio in mucosal samples is shown taken from five Hp-infected patients before and after a successful eradicating therapy. E, Real-time PCR data for IL-21 RNA transcripts in gastric biopsies of 10 patients with Hp-related gastritis (Hp⁺), 5 uninfected patients with gastritis (Hp⁻ gastritis), and 6 normal controls (ctr). Levels are normalized to β-actin. Data are mean ± SD by error bars of all experiments.

To confirm that the IL-21R bands seen in epithelial cell extracts were not due to the small number of lymphocytes present in these specimens of Hp-infected patients or controls. Densitometric analysis of such bands showed no difference among groups (Fig. 2B). Two additional, and very faint bands, with a molecular mass of ~55–65 kDa, were variably expressed in samples from Hp-infected and uninfected patients (Fig. 2A), and the intensity of such bands did not differ among groups (data not shown). Extracts from epithelial cells of Hp-infected patients and both inflamed and normal controls also contained the γc subunit (Fig. 2A). Analysis of cytokeratin-18 confirmed the epithelial origin of such extracts (Fig. 2A).

The high expression of IL-21R in Hp-infected gastric epithelial cells prompted us to investigate whether IL-21 controls the function of these cells. Because primary gastric epithelial cells are difficult to grow for extended periods, we selected AGS and MKN28 cancer cells as in vitro model of gastric epithelial cells. In initial experiments we showed that IL-21 affected neither the growth nor the survival of both AGS and MKN28 cells (data not shown).

During Hp infection, gastric epithelial cells produce MMPs in response to a variety of inflammatory stimuli (19, 20, 23). Therefore, we assessed whether IL-21 can regulate MMP production by gastric epithelial cells. Serum-starved AGS cells were stimulated with IL-21 and MMP production was then evaluated by Western blotting. As shown in Fig. 3A, IL-21 dose dependently enhanced the secretion of MMP-2 and MMP-9 (gelatinases A and B), mediated with three different Hp strains or inflammatory cytokines, and then IL-21R was analyzed by Western blotting. As shown in Fig. 2F, the three Hp strains were able to enhance IL-21R protein expression. Similarly, IL-21R was enhanced by IFN-γ (Fig. 2G), clearly indicating that IL-21R can be induced by multiple inflammatory stimuli in gastric epithelial cells. Note that the different intensity of the immunoreactive bands for IL-21R in unstimulated AGS (Fig. 2D vs F and G) relies on the fact that different amounts of protein were analyzed in separate experiments.
Data are expressed in a.u. Each symbol indicates the IL-21R to subjects is shown, as measured by densitometry scanning of Western blots.

Figure 2. Expression of IL-21R by gastric epithelial cells. A, Representative Western blot showing IL-21R, γc, cytokeratin-18 (Cytok), and β-actin in primary gastric epithelial cells isolated from three patients with Hp-related gastritis (Hp+), three uninfected patients with gastritis (Hp–), and three subjects without gastritis (controls). One of three separate experiments analyzing total samples of eight Hp-infected patients, six uninfected patients with gastritis, and six subjects without gastritis is shown. The representative flow cytometry dot plots to the right show the expression of IL-21R and CD3 in epithelial cells isolated from gastric biopsy of one Hp-infected patient. The percentage of positive cells is indicated within the quadrants. One of four representative experiments is shown. B, Quantitative analysis of the IL-21R to β-actin protein ratio in primary gastric epithelial cells isolated from eight patients with Hp-related gastritis, six patients with Hp-unrelated gastritis, and six normal control subjects is shown, as measured by densitometry scanning of Western blots. Data are expressed in a.u. Each symbol indicates the IL-21R to β-actin protein ratio in a single sample, and the thick bar indicates median. For each sample, two isoforms of IL-21R with molecular mass of 62 and 72 kDa, respectively, were analyzed. C, Representative Western blot showing IL-21R, γc, cytokeratin-18 (Cytok), and β-actin in epithelial cell-depleted mucosa isolated from three patients with Hp-related gastritis (Hp+), three Hp-negative patients with gastritis (Hp– gastritis), and three normal control subjects. One of three separate experiments analyzing total samples of eight Hp-infected patients, six uninfected patients with gastritis, and six subjects without gastritis is shown. The representative flow cytometry dot plots to the right show the expression of IL-21R and CD3 in epithelial cells isolated from gastric biopsy of one Hp-infected patient. The percentage of positive cells is indicated within the quadrants. One of four representative experiments is shown. D, Representative Western blot showing IL-21R, γc, cytokeratin-18 (Cytok), and β-actin in total extracts of AGS and MKN28 cells. E, Representative flow cytometry showing the expression of IL-21R in AGS and MKN28 cells. Isotype control (dark gray histogram) and IL-21 (open histogram) Ab stainings are represented. Percentages shown are positive cells within the plot. F, Representative Western blots for IL-21R and β-actin in total extracts of AGS cells either left unstimulated or treated with three different Hp strains: 60190 (lane 1), G21 (lane 2), and CCUG 17874 (lane 3) for 24 h show the three Hp strains were able to enhance IL-21R protein expression. G, Representative Western blots for IL-21R and β-actin in total extracts of AGS cells left either unstimulated (−) or stimulated (+) with the specified cytokines for 40 h.

IL-21-induced gelatinase production does not rely on MAPK activation

Studies in various cell systems have shown that gelatinases can be transcriptionally regulated by multiple factors that activate MAPKs (13). Therefore, we examined whether IL-21 enhances the phosphorylation of MAPKs in AGS cells. As shown in Fig. 4A, phosphorylation of each MAPK was evident in untreated AGS cells and was not modified by IL-21. To prove further that MAPKs are not involved in the IL-21-induced gelatinase production, we tested the effect of specific MAPK inhibitors on the IL-21-mediated gelatinase secretion. In initial experiments, we showed that these compounds were active in our system as they markedly inhibited the phosphorylation of the corresponding MAPK induced in AGS cells by epidermal growth factor or IL-1β (Fig. 4B). However, treatment of AGS cells with these inhibitors did not affect the IL-21-induced MMP-2 and MMP-9 production (Fig. 4C).

IL-21 induces MMPs production through a NF-κB-dependent mechanism

MMP production is also regulated by NF-κB (13), so we then investigated the role of NF-κB in IL-21-driven gelatinase production. As activation of NF-κB requires ubiquitination and proteosome-mediated degradation of IκB-α (33), we first examined whether IL-21 altered the cellular content of IκB-α. Serum-starved AGS cells were stimulated with IL-21 for the indicated time points and then IκBα was evaluated by Western blotting. Stimulation of AGS cells with IL-21 caused a time-dependent reduction of IκB-α expression, which was evident at 5 min and returned at basal levels within 20 min of stimulation (Fig. 5A). As expected, the decreased expression of IκB-α in IL-21-treated cells was followed by enhanced NF-κB-DNA binding activity that was evident at
10 min and persisted during the time course (Fig. 5B). Assays with excess of unlabeled specific or unspecific probes and NF-κB/p65 Ab confirmed the specificity of NF-κB bands induced by IL-21 (Fig. 5C).

To confirm that the effect of IL-21 on gelatinase synthesis was dependent on NF-κB, AGS cells were preincubated with TPCK (an inhibitor of NF-κB activation) before adding IL-21. As expected, TPCK completely abrogated NF-κB binding activity in AGS cells (Fig. 6A). TPCK also reduced but did not abrogate the IL-21-induced production of MMP-2 and MMP-9 (Fig. 6B).

### Blockade of IL-21 results in a reduced production of MMP-2 and MMP-9 in organ cultures of gastric mucosal explants of Hp-infected patients

We next conducted ex vivo organ cultures and examined whether blocking endogenous IL-21 reduced MMP-2 and MMP-9 in gastric mucosal explants of patients with Hp infection. We used a neutralizing IL-21 Ab we produced by immunizing rabbits with a specific human IL-21 peptide. Gastric biopsy specimens taken from five Hp-infected patients were cultured with the anti-IL-21 Ab or control Ab for 24 h. Epithelial cell extracts were then prepared and used to analyze MMP-2 and MMP-9 by Western blotting. Treatment of biopsy specimens with anti-IL-21 resulted in a reduced production of both MMP-2 and MMP-9 as compared with biopsy specimens cultured...
with the control IgG (Fig. 7A). Densitometry analysis of immunoreactive bands showed that the anti-IL-21 significantly reduced (p = 0.04) the synthesis of both MMP-2 and MMP-9 (Fig. 7B).

**FIGURE 5.** IL-21 enhances NF-kB activity in gastric epithelial cells. A, Representative Western blot showing IκB-α (upper) and β-actin (lower) in cytoplasmatic proteins extracted from AGS cells unstimulated (Unst) or stimulated with IL-21 (50 ng/ml) for the indicated time points. One of four separate experiments in which similar results were obtained is shown. B, Representative EMSA blot showing NF-kB-binding DNA complexes in AGS cells either left unstimulated (Unst) or stimulated with IL-21 (50 ng/ml) for the indicated time points. In the free lane, no nuclear extract was loaded. One of four separate experiments in which similar results were obtained is shown. C, Representative EMSA blot showing the specificity of NF-kB-binding DNA complex. Nuclear proteins extracted from IL-21-stimulated AGS cells were incubated with (+) or without (−) an excess of unlabeled specific NF-kB or unspecific IFN-stimulated genes/IFN-stimulated response element probe, or a goat anti-p65 or control Ab. No nuclear extract was loaded in the first lane. One of four separate experiments in which similar results were obtained is shown.

**FIGURE 6.** IL-21-induced synthesis of gelatinases in gastric epithelial cells partly depends on NF-kB activity. A, Representative EMSA blot showing NF-kB-binding DNA complex in AGS cells stimulated with IL-21 in the presence or absence of TPCK (10 μM) or DMSO (vehicle). No nuclear extract was loaded in the first lane. One of two separate experiments in which similar results were obtained is shown. B, Effect of graded doses of TPCK on the IL-21-mediated MMP-2 and MMP-9 production. AGS cells were preincubated with TPCK 1 h before adding IL-21 (50 ng/ml) for an additional 48 h. Cell-free supernatants were then collected and analyzed by Western blotting. One of three separate experiments in which similar results were obtained is shown.

**FIGURE 7.** Blocking endogenous IL-21 results in a significant decrease in the production of MMP-2 and MMP-9 in Hp-infected gastric mucosa. A, Mucosal samples were taken from Hp-infected patients and cultured in the presence of a neutralizing IL-21 or control Ab (10 μg/ml) for 24 h. Epithelial cells were then isolated from mucosal explants. MMP-2, MMP-9, and cytokeratin-18 (Cytok) were then evaluated by Western blotting. Two of five separate experiments are shown. B, Quantitative analysis of the MMP-2 to cytokeratin-18 and MMP-9 to cytokeratin-18 protein ratio in five Hp-infected gastric biopsy samples, cultured as indicated in A, and measured by densitometry scanning of Western blots. Data are expressed in a.u. and indicate mean ± SD by error bars. Biopsy specimens treated with anti-IL-21 show a significant reduction (p = 0.04) in the content of MMP-2 and MMP-9 in comparison to biopsy specimens treated with control IgG.

**Discussion**

In this study, we show that Hp-related gastritis is associated with a marked increase in IL-21, a T cell-derived cytokine. IL-21 protein was semiquantitatively analyzed by Western blotting because no commercially kit capable of quantitatively measuring IL-21 is available. However, although the densiometric units we measured might not directly reflect the biological quantities of IL-21 protein within the gastric mucosa, they allow the relative differences between different patient groups and types of cells to be determined. In Hp-negative patients with gastritis, IL-21 protein expression was not different from that found in normal patients, indicating that by itself, nonspecific gastric inflammation is not sufficient to enhance the production of IL-21. Therefore, it is likely that the Hp-driven inflammatory response leads to a marked induction of IL-21 in the stomach. This likelihood is also supported by the fact that eradication of Hp markedly reduced IL-21 expression. Moreover, high levels of IL-21 were also seen in purified CD3+ LPL from Hp-positive patients, clearly indicating that the marked expression of IL-21 in Hp-positive biopsy specimens was not simply a reflection of the accumulation of CD3+ T cells in these samples. Whether IL-21 up-regulation is directly driven by Hp components that penetrate into the lamina propria and/or molecules produced by host mucosal cells remains, however, to be ascertained.

Western blotting analysis also revealed constitutive expression of IL-21R in primary gastric epithelial cells from both Hp-positive and Hp-negative patients, as well as in gastric epithelial cancer cells, thus confirming and expanding on previous studies showing that IL-21R can be expressed on both immune and nonimmune cells (25–27). No apparent difference in IL-21R expression was, however, seen in epithelial cell extracts of Hp-infected and uninfected samples, thus suggesting that additional factors other than Hp-related inflammation can regulate IL-21R. Indeed, we provide...
evidence that IL-21R can be induced on AGS by different Hp strains as well as nonspecific inflammatory cytokines.

Importantly, primary gastric epithelial cells, AGS and MKN28 cells expressed also the γc subunit that is essential for IL-21-driven intracellular signaling (26). Overall, these data suggest that gastric epithelial cells have the potential to respond to IL-21, and indeed, treatment of both AGS and MKN28 cells with IL-21 resulted in enhanced synthesis of MMP-2 and MMP-9. The inducing effect of IL-21 on both gelatinases was evident at the RNA and protein level. However, the fold increase in IL-21-induced MMP-9 RNA expression over baseline was marginal. This finding does not appear surprising because MMP production can be regulated at various levels by transcriptional and posttranscriptional mechanisms (13). Therefore, it is possible that the increased secretion of MMP-9 in IL-21-stimulated AGS cell culture can rely on the ability of this cytokine to control both the MMP-9 promoter activity and protein synthesis/secretion.

Other MMPs reported to be increased in the Hp-infected mucosa, such as MMP-1, MMP-3, and MMP-7 (21, 22, 34), were not induced by IL-21. Similarly, IL-21 did not alter the production of TIMP-1 and TIMP-2. This fits well with previous studies showing that MMP synthesis can be differentially regulated by inflammatory stimuli, and that high MMP production can occur without concomitant TIMP production (13).

In subsequent experiments, we investigated putative mechanisms involved in the control of MMPs by IL-21. Although MMP gene transcription is positively regulated by MAPKs in various cell systems (13), and IL-21 is able to augment ERK1/2 phosphorylation in myeloma cells and immature promyelocytic HL-60 cells (29, 35), stimulation of AGS cells with IL-21 did not activate any MAPK in our hands. Preincubation of cells with specific inhibitors of MAPK also did not prevent the IL-21-mediated induction of MMP-2 and MMP-9. In contrast, the demonstration that IL-21 activates NF-κB and that pharmacologic inhibition of NF-κB largely reduces the IL-21-induced secretion of MMP-2 and MMP-9 implies that NF-κB is involved in the IL-21-driven gelatinases induction. This demonstration agrees with previous studies showing that NF-κB enhances the production of gelatinases both in epithelial and nonepithelial cells (13, 20). However, the demonstration that complete inhibition of NF-κB activity by TPCK reduced but did not abrogate the IL-21-induced secretion of MMP-2 and MMP-9 suggests that additional signaling pathways are involved in the control of gelatinase production by IL-21. In this context, it is noteworthy that studies in other systems have shown that gelatinase production can be regulated by Ets-1, and the transcription factor Y-box protein-1 (36–40). MMP-9 promoter region contains also a functional binding site for Sp-1 (41), thus implying a possible role for Sp-1 in the regulation of MMP-9 gene transcription. Studies are now in progress to assess this issue as well as the molecular mechanism by which IL-21 activates NF-κB in gastric epithelial cells.

The functional relevance of our data relates to the in vivo demonstration that both gelatinases A and B are produced in excess in the Hp-infected gastric mucosa by various cell types, including epithelial cells, and that this increase is not accompanied by changes in the levels of TIMPs (20, 34). Gelatinases can cleave various substrates, such as gelatins (types I and V), pericellular and minor collagens (types IV, V, VII, and X), or elastin and fibronectin (13), which are important components of the extracellular matrix of the gastric mucosa. Therefore, the imbalance of gelatinases and their inhibitors could contribute to the tissue remodeling, as well as mucosal damage, in patients with Hp infection. Gelatinase B has also the ability to cleave and activate various chemokines (42). Based upon these observations it is thus possible to speculate that the high levels of IL-21 seen in the mucosa of Hp-infected patients may both amplify the local inflammatory process and trigger molecular pathways that ultimately cause mucosal degradation and remodeling. This cytokine effect is also supported by the demonstration that neutralization of endogenous IL-21 in isolated cultures of Hp-infected gastric biopsies resulted in a diminished production of both MMP-2 and MMP-9 by epithelial cells. Therefore, these findings support further the notion that T cell-derived cytokines are important mediators in the cross-talk between immune and nonimmune cells during chronic inflammatory processes.

**Disclosures**

The authors have no financial conflict of interest.

**References**


