UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI MEDICINA E CHIRURGIA

DOTTORATO DI RICERCA IN FISIOPATOLOGIA SPERIMENTALE

XX CICLO

ESPRESSIONE E RUOLO FUNZIONALE DEI LINFOCITI Th-17 NELLA GASTRITE CORRELATA ALL'INFEZIONE DA HELICOBACTER PYLORI

Roberta Caruso

Docente Guida/Tutor: Prof. Giovanni Monteleone

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Introduction

Hp infection: mechanism and clinical manifestations.

Helicobacter pylori (Hp) is a spiral-shaped Gram-negative flagellate bacterium that colonizes the human gastric mucosa and chronically infects more than half of the human population. Infection is inversely correlated with socioeconomic conditions (1). The natural course of infection with H. pylori is extremely variable. Most infected individuals remain asymptomatic, but Hp-driven gastritis can lead to the development of gastroduodenal ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma (2-3). Most new Hp infections occur in children, but the lack of specific Hp-related clinical signs makes difficult to define the mode of transmission. Hp survives within the gastric mucus layer despite the acidic microenvironment, that limits the growth of most bacteria. This primarily relies upon the ability of Hp to secrete a large amount of urease that breaks down urea into carbon dioxide and ammonia, the latter buffering its environment. Most Hp organisms remain in the mucus layer, even though a small proportion adheres to the mucosal epithelial cells and rarely invades the mucosa (3). Moreover, Hp can inject into the epithelial cells bacterial products that modify epithelial cell functions (4).

The location and severity of gastritis also determine the risk of progression to gastric cancer. Therefore, the level of inflammation increases the risk of disease, but it does not seem to influence which disease develops. In contrast, this is thought to be largely influenced by the pattern of gastric inflammation. In particular, antral gastritis is associated with increased stimulated acid production and predisposes to duodenal ulceration, while corpus-predominant or pan-gastritis is associated with reduced acid production and predisposes to gastric ulcer and gastric adenocarcinoma (5). There is

also evidence that the degree of gastric infiltration by neutrophils correlates with the development of gastro-duodenal ulcerations, and this is in part dependent on the release of damaging inflammatory mediators such as reactive oxygen species (6-7).

Hp-mediated immune-inflammatory response.

Hp infection causes a marked infiltration of the gastric mucosa with neutrophils, macrophages, and lymphocytes. Because neutrophils are short-lived, they must be constantly recruited into the infected mucosa from circulation. However, 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 chemoattractants (8-10). 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 (11-13). Consistent with this, Hp infection elicits a marked mucosal accumulation of T lymphocytes which contribute to gastric pathology by synthesizing T helper (Th) 1-type cytokines, such interferon (IFN)-γ and tumor necrosis factor (TNF)-α (2,14). Moreover, Hp-infected gastric mucosa displays high levels of interleukin (IL)-12, the major Th1 inducing cytokine in man. Additionally, the number of cells producing interferon (IFN)-γ in the Hp-infected human gastric mucosa correlates with the severity of gastritis (15). Animal models also suggest that the extent of Th1 differentiation is important in pathogenesis. Mice with a predominant Th1 response develop more gastric inflammation during Hp colonization

than those with a Th2 response (16-17). Gastric inflammation and atrophic changes are abrogated in the absence of IFN-γ (18), while IFN-γ infusion into mice, even in the absence of Hp infection, induces pre-cancerous gastric atrophy, metaplasia and dysplasia (19). IL-12-deficient mice have also reduced gastric inflammatory infiltration and are unable to clear Hp infection (20).

IL-17 is over-produced in Hp-colonized gastric mucosa

Despite the marked Th1 cell response, Hp-colonized gastric mucosa also contains high levels of IL-17 (23-24). IL-17 positively regulates the synthesis of IL-8, a chemoattractant of neutrophils, by gastric mononuclear and epithelial cells, thus emphasizing the potential role of IL-17 in the Hp-driven inflammation (23). In this context, several studies have recently shown that IL-17, a key regulator of neutrophil chemotaxis, is produced in excess in Hp-infected stomach (23-24). By real-time PCR and Western blotting it was shown that IL-17 up-regulation occurs at both RNA and protein level in Hp-infected biopsies in comparison to uninfected biopsies either with or without gastritis (23). Notably, among Hp-positive patients, the gastric mucosa at the site of ulcers contains more IL-17 than the non-ulcerated mucosa of the antrum (24). Several observations suggest that IL-17 plays a decisive role in the neutrophil recruitment to the Hp-infected gastric mucosa. First, IL-17 levels correlate with the number of neutrophils infiltrating the Hp-infected mucosa (24). Second, both gastric LPMC and epithelial cells express IL-17 receptor and are functionally capable of responding to IL-17 by secreting IL-8 (23-25). Consistently, conditioned media of gastric epithelial cells stimulated with IL-17 enhance the migration of peripheral blood neutrophils, and this effect is inhibitable by a blocking anti-IL-8 but not anti-IL-17

antibody (23). Functional analysis of intracellular pathways involved in the induction of IL-8 synthesis by IL-17 revealed that IL-17 activates ERK1/2 MAP kinases in gastric epithelial cells, and that pharmacologic blockade of this pathway significantly inhibits IL-8 secretion (25). These findings are in line with the demonstration that activated ERK1/2 and IL-8 are more pronounced in gastric epithelial cells isolated from Hp-infected biopsies in comparison to uninfected controls, and that neutralization of endogenous IL-17 in ex vivo cultures of Hp-infected gastric biopsies down-regulates the expression of activated ERK1/2 and IL-8 (25). Finally, IL-17 expression positively correlates with IL-8 content in Hp-colonized biopsies (24).

Besides its effects on IL-8 synthesis, IL-17 exerts additional immune-regulatory functions that could influence the magnitude and/or severity of Hp-related gastritis. For example, IL-17 stimulates the production of IL-1, IL-6, and TNF-α by both immune and non-immune cells (26), and induces fibroblasts to make matrix metalloproteinases (MMPs) (27). MMPs are a family of proteases that can cleave multiple components of the extracellular matrix, thereby contributing to the mucosal damage (27). However, the factors involved in the control of IL-17 production in Hp-associated gastritis remain unclear.

The effector Th17 cells.

More recently, a novel pathway of inflammation characterized by excessive production of IL-17 has been described in a variety of immune-inflammatory diseases, both in humans and in mouse models, such as extrinsic allergic alveolitis, experimental autoimmune encephalomyelitis, collagen-induced arthritis, inflammatory bowel

diseases, and psoriasis (29-30). IL-17 was originally named cytotoxic T lymphocyte-associated-8 (CTLA-8), subsequently IL-17, and more recently IL-17A, since it is one of six related members belonging to the IL-17 family (IL-17A-F) (29). IL-17 was initially described at the message level as a product of human blood activated CD4+ memory T cells. Subsequent studies have shown that IL-17 can be also made by activated CD8+ T cells, TCRγδ+ T cells, and neutrophils (29). More recently, it was shown that IL-17 is produced by a specific subset of CD4+ T cells, termed T helper (Th) 17-cells, that is distinct from, and antagonized by the classical Th1 or Th2 cells (30). Th17-cells produce also, but to a lesser extent, TNF-α, IL-6, IL-17F, IL-21, IL-22, and granulocyte macrophage-colony stimulating factor (31-33).

In murine systems, the differentiation of IL-17-producing cells is dependent on the presence, during antigen stimulation, of IL-6 and TGF-β1, while the development of Th-17 cells, in humans, seems to be driven by IL-1β and IL-6 and not TGF-β. (34-36).There is also evidence that expansion and survival of Th17-cells require additional factors, such as IL-23 (35). IL-23 is a heterodimeric protein that is composed by the p40 subunit of IL-12 and a specific subunit, termed IL-23/p19. The functional IL-23 heterodimer is produced by activated dendritic cells (DC), monocytes and macrophages (37). IL-23 signals through a heterodimeric receptor complex consisting of IL-12Rβ1 and a second subunit designed IL-23R, expressed on T cells, natural killer cells, monocytes, and dendritic cells (38). Notably, mice deficient for IL-23/p19 show reduced numbers of Th-17 cells, thus confirming the role of IL-23 in enhancing IL-17 production (39). While previous studies have characterized the expression of TGF-β1 and IL-6 in Hp-infected stomach (40), no study has yet examined the involvement of IL-23 in Hp-related gastritis.

More recently studies in mice have shown that Th17 cells are the major producers of IL-21 (33), a cytokine that is capable of regulating the functional activity of both immune and non-immune cells(41). Its biological activity is mediated through a class I cytokine family receptor composed of a specific IL-21 receptor (IL-21R) and the common γ-chain receptor, that is also an essential component of IL-2, IL-4, IL-7, IL-9 and IL-15 receptors (42). Originally described on lymphoid cells, IL-21R has recently been documented on non-immune cells, such as fibroblasts and keratinocytes (43). 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 immune-mediated diseases (44-46). 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.

Matrix metalloproteinases (MMPs) are a family of neutral endopeptidases capable of degrading all extracellular matrix proteins and remodelling connective tissue (47). 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 (48). In Hp-infected mucosa, epithelial cells appear to be one of the major sources of MMPs (49-50). Although Hp by itself can induce gastric epithelial cells to release MMPs (51-52), accumulating evidence indicates that cytokines produced by mucosal T cells and macrophages are also major stimuli for MMP production (53-54).

In this study we first have investigate the role of IL-23 in Hp-infected gastric mucosa, second we examined IL-21 and IL-21R in the gastric mucosa of patients with Hp infection. finally, using the gastric cell lines, AGS and MKN28, we examined whether IL-21 controls the production of MMPs

Materials and Methods

Patients and Samples

Seventy-nine Hp-infected and 101 Hp-uninfected patients 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 biopsies taken from the corpus and antrum, and [13C] urea breath test. Patients were classified as Hp-infected or not infected only if the three tests were positive or negative respectively. In the Hp-negative group, 35 patients had gastritis and 44 subjects had no evidence of inflammation at histology. In the group of Hp-uninfected patients with gastritis, 14 were taking non-steroidal anti-inflammatory drugs, and 13 had a duodenal-gastric biliary reflux at the endoscopy, and 1 had a systemic vasculitis. In the remaining 7 Hp-uninfected patients, the cause of gastritis was unknown. Five Hp-infected patients were re-endoscoped 2 months after cessation of 1-week 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. Six biopsies were collected from 49 Hp-infected and 62 Hp-uninfected patients , and used for rapid urease test, histological examination, assessment of CagA status, cytokine RNA and/or protein analysis. Eight biopsies were available from the remaining 30 Hp-positive and 39 Hp-negative patients and used for rapid urease test, histological examination, and for isolating LPMC. Experiments for human studies received approval by the local Ethical Committee.

Epithelial cell isolation

Freshly obtained gastric biopsies of 8 Hp-infected patients and 12 Hp-uninfected patients (6 with gastritis) were used to isolate epithelial cells. Biopsies were incubated in dithiothreitol (DTT, 0.1 mM, Sigma-Aldrich, Milan, Italy) for ten minutes, then extensively washed in RPMI 1640 (Sigma-Aldrich) and finally incubated in 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 30 minutes 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% trypan blue (viability ranged from 80–85%). Both epithelial cells and the remaining epithelial cell-depleted mucosa were then used for extracting total proteins.

Lamina propria mononuclear cell isolation and culture

Freshly obtained gastric biopsies of 15 Hp-infected patients and 30 Hp-uninfected patients without gastritis were used to isolate LPMC. Biopsies were incubated in dithiothreitol (DTT, 0.1 mM, Sigma-Aldrich) for 10 minutes, then extensively washed in RPMI 1640 (Sigma-Aldrich) and incubated in 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 45 minutes at 37° C in the presence of 5% CO2. Finally, the biopsies were washed and incubated in collagenase (type D collagenase, 0.75 mg/ml, Roche Diagnostic, Monza, Italy) for 1 hour at 37° C. The viability of isolated cells ranged from 79 to 86% as assessed by trypan blue staining. LPMC isolated from 5 Hp-infected patients and 5 Hp-uninfected controls were used for assessing IL-1β RNA by real-time PCR.

To analyze the effects of IL-23 on IL-17, LPMC isolated from 10 Hp-negative biopsies were stimulated with activating anti-CD3 beads (Miltenyi Biotec, Calderara di Reno, Italy) in the presence or absence of IL-23 (50 ng/ml, Biosource, Camarillo, CA, USA). After 48 hours, supernatants were analyzed for the content of IL-17 by ELISA, and cells were used for IL-17 RNA analysis by real-time PCR. The same cell culture supernatants were also assessed for INF-γ and TNF-α by ELISA. To evaluate whether the effect of IL-23 on IL-17 relies on Stat3 activation, LPMC isolated from additional 9 Hp-negative biopsies were cultured with or without IL-23 (50 ng/ml) in the presence or absence of AG490, a Jak2/Stat inhibitor (100 μM, Inalco, Milan, Italy) or DMSO (vehicle) for 30 minutes. AG490 was pre-incubated for 30 minutes prior to adding IL-23. LPMC extracted from 3 further Hp-negative biopsies were cultured in the presence of activating CD3 beads and IL-23 (50 ng/ml) with or without AG490 or DMSO. After 48 hours, supernatants were analyzed for the content of IL-17 by ELISA. Additionally, to evaluate whether Hp can regulate IL-23 expression, gastric LPMC isolated from 3 Hp-negative patients were cultured with or without the addition of a broth culture filtrate derived from a Hp 60190 strain or a control Brucella strain (both used at a final concentration of 0.5 mg/ml). After 24 hours, cells were analyzed for IL-23/p19 RNA.

Gastric LPMC from 7 Hp-infected biopsies were stimulated with activating anti-CD3 beads (Miltenyi Biotec) in presence of a neutralizing anti-IL-23/p19 (5 μg/ml, R&D Systems, Inc. Minneapolis, MN, USA) or control antibody (5 μg/ml, Sigma-Aldrich) for 48 hours. Supernatants were examined for the content of IL-17, INF-γ and TNF-α by ELISA, and cells were used to analyze IL-17 RNA. To determine whether blockade of endogenous IL-23 reduced Stat1, Stat3, and Stat4 activation, gastric LPMC from 5 Hp-infected patients were incubated with the anti-IL23/p19 or control antibody for 24 hours and then stimulated with activating anti-CD3 beads for further 24 hours.

Additionally, LPMC were isolated from gastric biopsies of 5 Hp-infected patients and 6 Hp-uninfected patients (4 with gastritis) by DTT-EDTA-collagenase procedure and used for purifying CD3+ T lamina propria lymphocytes (T-LPL). LPMCs were incubated for 30 minutes at 4°C with magnetically labelled CD3 antibody (Miltenyi Biotec). T-LPL then were collected by positive selection using the magnetic cell sorting 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 Dulbecco's modified Eagle's or RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). To investigate factors involved in the induction of IL-21R in gastric epithelial cells, AGS were stimulated with 60190 and CCUG17874, two CagA-positive Hp strains, and G21, a CagA-negative Hp strain (final concentration: 5 x 107 CFU/ml). After 24 hour culture, cells were washed with 1x PBS, and then used for extracting total proteins. In parallel, AGS were cultured in the presence or absence of TNF-α (20 ng/ml, R&D Systems), IL-1β (20 ng/ml), and IFN-γ (200 ng/ml) (both from Peprotech EC LTD, London, UK) for 40 hours. To examine whether IL-21 regulates MMPs production, confluent AGS or MKN 28 cells were serum-starved for 24 hours and then stimulated with recombinant human IL-21 (10-50 ng/ml, Biosource) for the indicated time points.

RNA extraction, cDNA preparation and Real-time PCR

Analysis of IL-17, IL-23/p40, IL-23/p19, and IL-12/p35 RNA transcripts was performed by real-time PCR. Total RNA was extracted from gastric biopsies of 13 Hp-infected patients, 13 Hp-negative patients with gastritis, and 13 normal controls by TRIzol reagent according to the manufacturer's instructions (Invitrogen, Milan, Italy). Additionally, IL-1β transcripts were evaluated in freshly isolated LPMC. Quantitative analysis of IL-21 was performed by Real-time PCR using total RNA extracted from gastric biopsies of 10 Hp-infected patients, 5 Hp-negative patients with gastritis, 6 normal controls. To analyze the effect of IL-21 on MMP-2 and MMP-9 RNA expression, AGS were starved overnight and cultured in serum-free conditions in presence or absence of IL-21 (50ng/ml) for 90 minutes to 8 hours.

A constant amount of RNA (1 μg/sample) was retro-transcribed into complementary DNA (cDNA), and 1 μl of cDNA/sample was then amplified using the following conditions: denaturation 1 minute at 95°C, annealing 30 seconds at 58°C for IL-23/p19 and MMP-9, at 62°C for IL-17 and for β-actin, at 55°C for MMP-2, at 60°C for IL-21, followed by 30 seconds of extension at 72°C. Primers sequence was as follows: IL-23/p19, FWD:5′-GGGACACATGGATCTAAGAG-3′ and REV: 5′-GCAAGCAGAACTGACTGTTG-3′; IL-17A; FWD: 5′-ACTACAACCGATCCACCTCAC-3′ and REV: 5′-ACTTTGCCTCCCAGATCACAG-3′; MMP-2, FWD: 5'-TGACGGAAAGATGTGGTGTG-3' and REV: 5'-GGTGTAGGTGTAAATGGGTG-3'; MMP-9, FWD: 5'CGTCTTCCAGTACCGAGAGA-3' and REV: 5' GCAGGATGTCATAGGTCACG-3'; IL-21, FWD: 5'-GGAGAGGATTGTCATCTGTC-3' and REV: 5'-

CACAGTTTGTCTCTACATCTTC-3'. IL-23/p40, IL-12/p35 and IL-1β were evaluated using a commercially available TaqMan probe (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the IQ SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy). β-actin (FWD: 5'-AAGATGACCCAGATCATGTTTGAGACC-3' and REV:5'-AGCCAGTCCAGACGCAGGAT-3) was used as an internal control.

Analysis of intracellular IL-17 and IL-21R by flow cytometry

IL-17 and/or IFN-γ-producing cells were evaluated in LPMC isolated from 5 Hp-infected patients and 5 Hp-uninfected patients without gastritis. LPMC were stimulated with 4α-phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (1 μg/ml) (both from Sigma-Aldrich), brefeldin A (3 μg/ml) and monesin (2 μM) (both from eBioscience, San Diego, CA, USA) for 5 hours. Cells were stained with a mouse anti-human CD3 PerCP (1:50 final dilution, Becton Dickinson, Milan, Italy) and a mouse anti-human CD4 PE (1:50 final dilution, Miltenyi Biotec) at 4°C for 30 minutes, fixed and permeabilized. Cells were then stained with a mouse anti-human IL-17 APC (0,3 μg/ml, eBioscience) and a mouse anti-human IFN-γ FITC (0,5 μg/ml) or isotype control (both from Becton Dickinson), incubations were carried out at 4°C for 30 minutes. Finally, cells were washed, resuspended in PBS and analyzed by flow-cytometry. CD3+ T cells that did not express CD4 were considered as CD8+ T cells.

IL-21R was examined by incubating AGS and MKN28 cells with a phycoerythrin (PE)-labeled mouse anti-IL-21R (1:10 final dilution, R&D Systems) or isotype control antibody (1:20 dilution, Becton Dickinson). Moreover, IL-21R was assessed in freshly isolated gastric epithelial cells of 4 patients (2 with Hp infection). In these experiments,

cells were stained with the IL-21R PE, anti-CD3 PerCP (Becton Dickinson) or isotype control antibody. Incubations were carried out at 4°C for 30 minutes. Cells were then washed, resuspended in PBS and analyzed by flow-cytometry.

Protein extraction and Western blotting

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 P40, supplemented with 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM Na3VO4, and 1 mM NaF (all reagents were from Sigma-Aldrich). Lysates were clarified by centrifugation at 4 °C for 30 minutes at 12,000xg and then fractionated on SDS-polyacrylamide gels. The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk and then incubated with a mouse anti-human p-Stat1, or a mouse anti-human p-Stat3, or a mouse anti-human p-Stat4 (1:500 final dilution, all from Santa Cruz Biotechnology, CA, USA) followed by a horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1:20000 final dilution, Dako, Milan, Italy). The reaction was detected with a chemiluminescence kit (West DURA; Pierce, Rockford, IL). After detection of p-Stats, blots were stripped and subsequently incubated with a mouse anti-human total Stat1, Stat3, and Stat4 (1:500 final dilution, Santa Cruz Biotechnology), followed by a rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:20000 dilution, Dako). For assessing CagA status, total proteins extracted from Hp-infected patients were analyzed by using a mouse anti-CagA (Hp) monoclonal antibody (Santa Cruz Biotechnology).

IL-21 was analyzed using total proteins extracted from biopsies of 17 Hp-positive and 20 Hp-negative (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 biopsies taken from 5 Hp-infected patients before and after the eradicating therapy. To evaluate IL-21, the membranes were incubated with a rabbit anti-human IL-21 (0.5 μg/ml, ProSci Incorporated, Poway, CA) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:20000 final dilution).

IL-21R and the common γ-chain subunit were analyzed in total proteins prepared from epithelial cells and epithelial cell-depleted mucosa, AGS and MKN28 cells using the following antibodies: anti-IL-21R (1 μg/ml, R&D Systems), anti-common γ-chain (1: 500 final dilution), anti-cytokeratin-18 (1:400, Sigma-Aldrich), and anti-β-actin (1: 5000, Sigma-Aldrich). Appropriate horse-radish peroxidase-conjugated secondary antibodies were then used and bound antibodies visualized using enhanced chemiluminescence (West DURA, Pierce).

For the detection of MMPs and TIMPs in the AGS and MKN cell culture, equivalent amounts of supernatants were loaded onto each lane of the 10% SDS-PAGE, and the membrane were then incubated with the following monoclonal mouse anti-human antibodies: MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, and TIMP-2 (1 μg/ml; R&D Systems) followed by a rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:20000 final dilution). Additionally, MMP-2 and MMP-9 were evaluated in epithelial cell extracts of gastric mucosal explants cultured for 24 hours with the anti-IL-21 antibody or control IgG using the same antibodies.

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

ELISA

IL-17, IFN-γ, and TNF-α were evaluated in supernatants of gastric LPMC using sensitive ELISA kits (Peprotech EC Ltd for IL-17 and IFN-γ, and R&D Systems Inc for TNF-α). IL-23 was evaluated in total extracts of biopsies taken from 12 Hp-infected and 23 Hp-unifected patients (11 with gastritis) using a sensitive ELISA kit (Bender Med Systems, Burlingame, CA, USA). IL-23 values were expressed as pg/100 µg of total proteins.

Gelatin zymography

Twenty µl of concentrated AGS cell culture supernatants were electrophoresed under non-reducing conditions in a 8% acrylamide gel containing 1 mg/ml gelatin (Sigma-Aldrich). After electrophoresis, the gels were washed at room temperature for 30 minutes in 2.5% Triton X-100 (Sigma-Aldrich), then equilibrated at room temperature with gentle agitation in developing buffer (50 mM Tris-HCl, pH7.4, 0.2 M NaCl; 5 mM CaCl2, 0.02% Brij35) (Sigma-Aldrich). After 30 minutes, the gels were incubated in fresh developing buffer and incubated overnight at 37°C, then stained with 0,25% Coomassie Blue for 1 hour and destained in 50% methanol, and 10% glacial acetic acid.

Production of the neutralizing IL-21 antibody

A human IL-21 peptide (NVSIKKLKRKPPSTN) was synthesized and used to immunize New Zealand White (NZW) rabbits by Washington Biotechnology Company (Washington, USA). The IL-21 peptide was injected subcutaneously with adjuvant into

specific-pathogen-free rabbits (NZW). Blood was taken 6 or 8 weeks following initial immunization, and antiserum titre was characterized by ELISA. As a control, serum was taken from the same rabbits prior their immunization. Purification of anti-IL-21 antibody (IgG) was carried out using the Melon Gel IgG Spin purification kit according to the manufacturer's instructions (Pierce).

The neutralizing activity of the anti-IL-21 antibody was confirmed by showing that it inhibited the effect of IL-21 on p-Stat3 induction in peripheral blood mononuclear cells.

Organ culture

Mucosal biopsies were taken from 5 Hp-infected patients and cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100U/ml), and streptomycin (100 μg/ml). Cultures were performed with or without the addition of the neutralizing IL-21 or control antibody (10 μg/ml) for 24 hours. 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

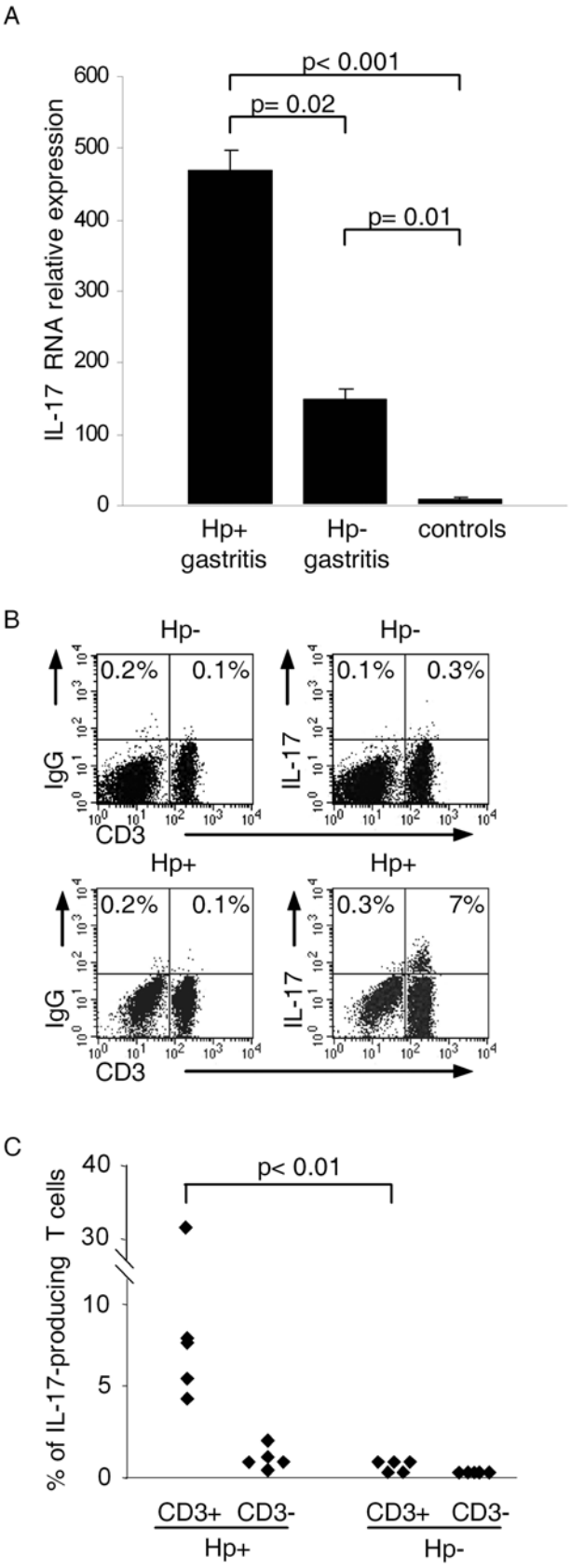
Differences between groups were compared using the Student's t test, or ANOVA and Wilcoxon tests. Paired t test was used for analysing changes of cytokines in cell cultures.

Results

IL-17 expression is enhanced in Hp-infected gastric mucosa

To confirm that IL-17 is produced in excess in Hp-infected gastric mucosa, we first analyzed IL-17 RNA transcripts in gastric biopsies of Hp-infected patients and controls by real-time PCR. IL-17 RNA was detectable in all samples regardless of whether biopsies were taken from Hp-infected or uninfected patients (Fig. 1A). However, IL-17 RNA expression was significantly increased in biopsies of Hp-infected patients compared to Hp-uninfected patients with gastritis or without gastritis. In Hp-uninfected patients, expression of IL-17 RNA transcripts were significantly increased in samples with gastritis in comparison to normal controls (Fig. 1A). We then determined if T cells contributed to the increase in IL-17 in Hp-infected mucosa. Accordingly, IL-17 and CD3 were simultaneously evaluated in freshly isolated gastric lamina propria mononuclear cells (LPMC) of 10 patients (5 with Hp infection) by flow cytometry. The percentage of IL-17-making CD3+ T cells ranged from 4.8 to 33.7% in Hp-infected samples while only few CD3+ T cells (from 0.3 to 0.6%) from Hp-uninfected samples were positive for IL-17 (Fig. 1B-C). The fraction of IL-17-producing CD3-negative LPMC ranged from 0.09 to 1.4% in Hp-infected samples and from 0.02 to 0.1% in Hp-uninfected samples (Fig. 1 B-C).

Figure 1


A
p< 0.001
p= 0.02
p= 0.01
IL-17 RNA relative expression
600
500
400
300
200
100
0
Hp+ gastritis
Hp- gastritis
controls
B
Hp-
Hp-
0.2%
0.1%
0.1%
0.3%
IgG
IL-17
CD3
Hp+
Hp+
0.2%
0.1%
0.3%
7%
IgG
IL-17
CD3
C
p< 0.01
% of IL-17-producing T cells
40
30
10
5
0
CD3+ CD3-
Hp+
CD3+ CD3-
Hp-

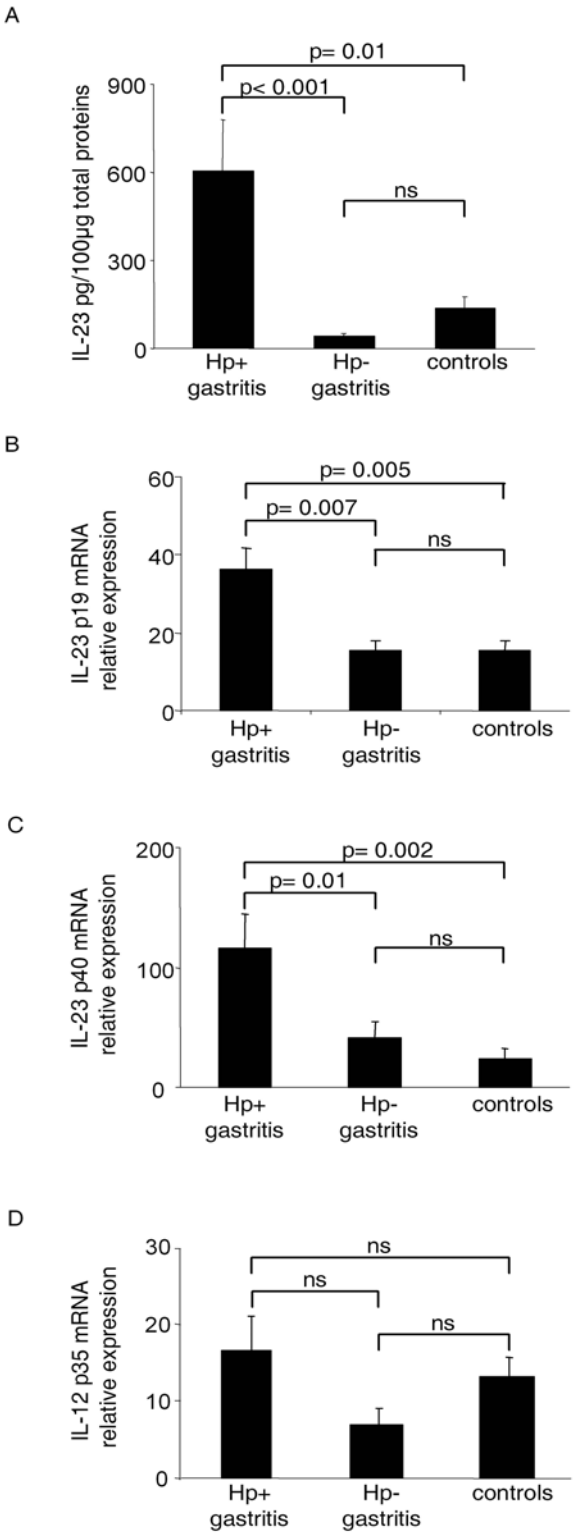
Elevated IL-23 is seen in Hp-related gastritis

The fact that IL-23 positively regulates IL-17 production by immune cells prompted us to examine whether IL-23 is involved in the control of IL-17 production in Hp-related gastritis (55-56). Initially, we examined IL-23 protein expression in biopsies taken from Hp-infected patients and controls by ELISA. IL-23 was constitutively produced in the human stomach, but its expression was significantly increased in biopsies of Hp-infected patients compared to Hp-uninfected patients with gastritis or without gastritis (Fig. 2A). Among Hp-infected patients, IL-23 protein expression did not differ between persons who were infected with CagA strain and those who were CagA negative. Similarly, there was no relation between IL-23 protein level and the degree of gastritis. No difference in IL-23 protein expression was seen between Hp-uninfected patients with gastritis and normal samples (Fig. 2A). In subsequent experiments, we showed that a broth culture filtrate derived from the Hp 60190 strain induced a two-fold increase in the content of IL-23/p19 RNA in LPMC isolated from Hp-uninfected subjects.

To analyze if IL-23 is regulated at the transcriptional level, biopsies from Hp-infected patients and controls were assessed for the content of IL-23/p40 and p19 subunit RNA by real-time PCR. Transcripts for IL-23/p19 were significantly higher in Hp-infected patients than Hp-uninfected patients either with or without gastritis (Fig 2B). Similarly, IL-23/p40 RNA was expressed at higher levels in Hp-infected biopsies than Hp-negative samples either with or without gastritis (Fig. 2C). By contrast, no difference was seen in terms of IL-23/p40 and p19 RNA transcripts between Hp-uninfected patients with gastritis and normal controls (Fig. 2, B and C). Expression of IL-12/p35 transcripts did not differ among groups (Fig. 2D). Since IL-17 production is

positively regulated by IL-1β (31-32), we also analysed transcripts for this cytokine in LPMC isolated from Hp-infected and uninfected patients by real-time PCR. IL-1β was more pronounced in samples from Hp-infected patients (32,16 ± 7 arbitrary units) than controls (10 ± 2,67 arbitrary units, P=0.02).

Figure 2


A
p= 0.01
p< 0.001
ns
IL-23 pg/100µg total proteins
900
600
300
0
Hp+ gastritis
Hp- gastritis
controls
B
p= 0.005
p= 0.007
ns
IL-23 p19 mRNA relative expression
60
40
20
0
Hp+ gastritis
Hp- gastritis
controls
C
p= 0.002
p= 0.01
ns
IL-23 p40 mRNA relative expression
200
100
0
Hp+ gastritis
Hp- gastritis
controls
D
ns
ns
ns
IL-12 p35 mRNA relative expression
30
20
10
0
Hp+ gastritis
Hp- gastritis
controls

IL-23 activates Stat3 and enhances IL-17 production by gastric lamina propria mononuclear cells

To determine if IL-23 controls IL-17 production in the gastric mucosa, normal LPMC were activated with anti-CD3 in the presence or absence of IL-23. IL-23 significantly enhanced IL-17 secretion (Fig 3A). Real-time PCR analysis confirmed that IL-23 elicited a robust IL-17 response (Fig. 3B).

Since studies in various cell systems have shown that IL-23 can transcriptionally enhance IL-17 via the Stat3 pathway (57), we then investigated the role of Stat3 in IL-23-driven IL-17 synthesis in gastric LPMC. As expected treatment of normal gastric LPMC with IL-23 increased p-Stat3 (Fig. 4A), and abrogation of Stat3 activation by AG490 resulted in a significant inhibition of IL-23-induced IL-17 secretion (Fig. 4B). To confirm these findings, we examined whether blocking endogenous IL-23 reduced Stat3 activation and IL-17 production in LPMC isolated from Hp-infected biopsies. Treatment of LPMC with anti-IL-23 inhibited p-Stat3 (Fig. 4C), and this was associated with a diminished production of IL-17. The inhibitory effect of anti-IL-23/p19 on IL-17 expression was seen at both RNA and protein level (Fig. 4D and E).

Figure 3

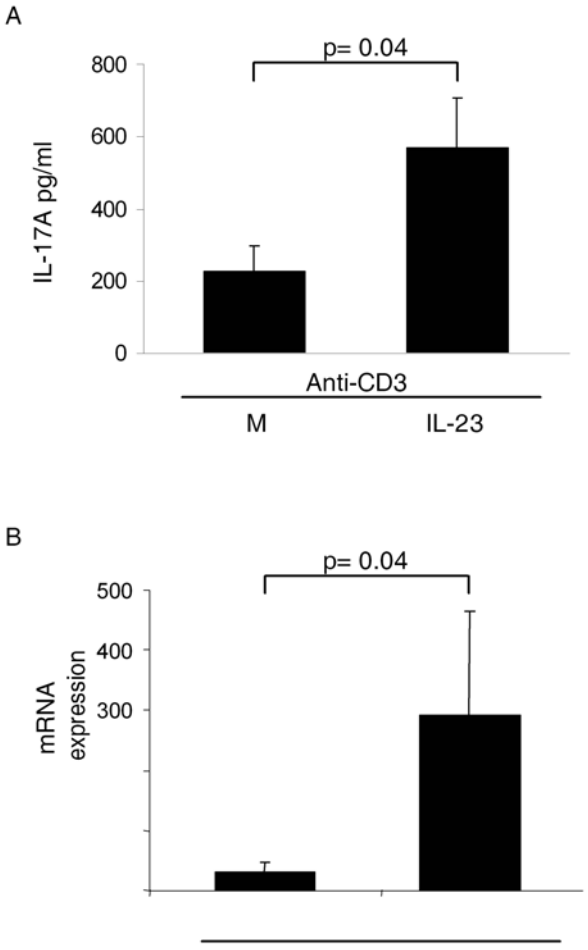
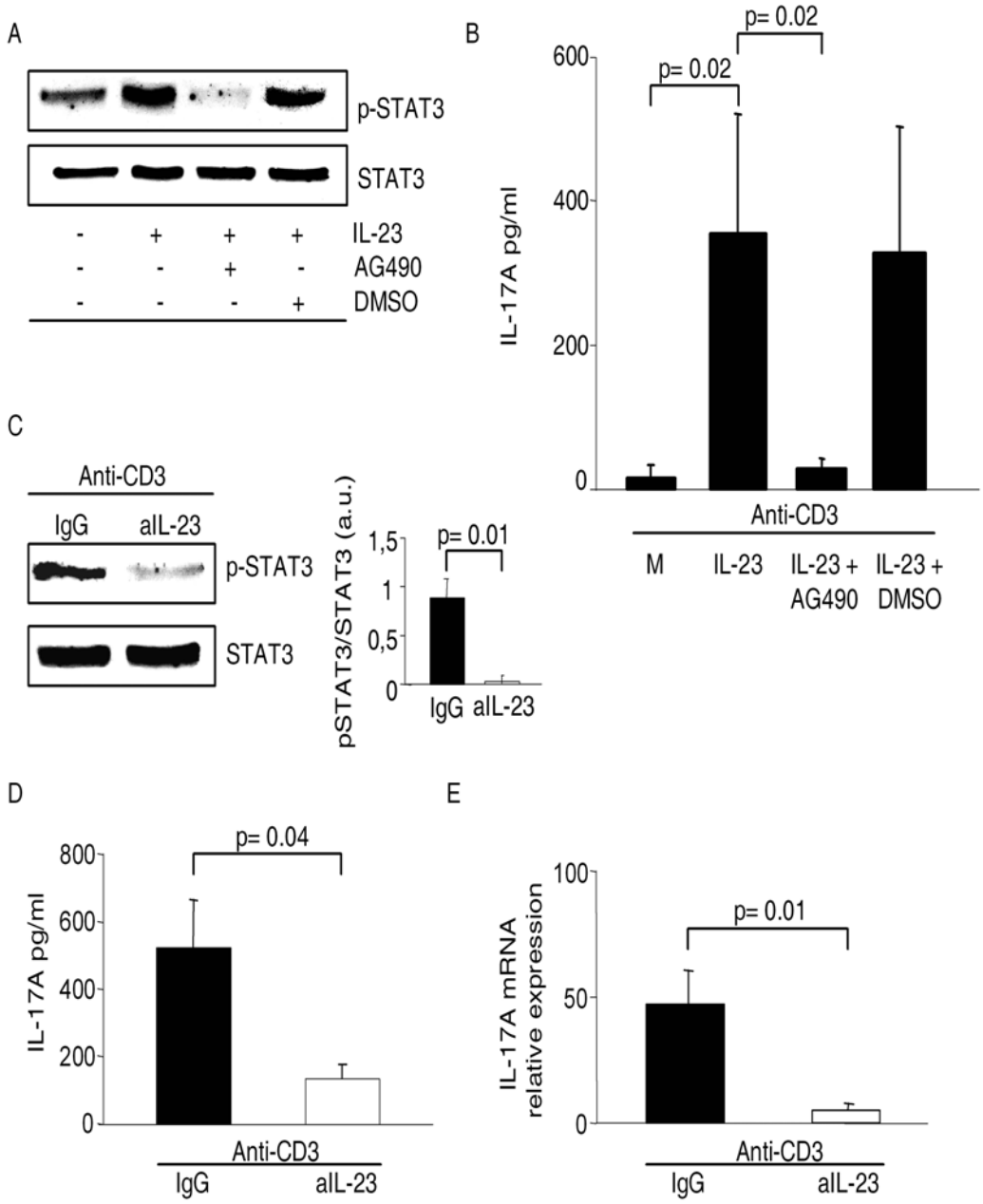

A
p= 0.04
800
600
400
200
0
IL-17A pg/ml
Anti-CD3
M
IL-23
B
p= 0.04
500
400
300
mRNA expression

Figure 4


A
p-STAT3
STAT3
- + + + IL-23
- - + - AG490
- - - + DMSO
B
p= 0.02
p= 0.02
600
400
200
0
IL-17A pg/ml
Anti-CD3
M
IL-23
IL-23 + AG490
IL-23 + DMSO
C
Anti-CD3
IgG
aIL-23
p-STAT3
STAT3
pSTAT3/STAT3 (a.u.)
1,5
1
0,5
0
p= 0.01
IgG
aIL-23
D
p= 0.04
800
600
400
200
0
IL-17A pg/ml
Anti-CD3
IgG
aIL-23
E
100
50
0
IL-17A mRNA relative expression
p= 0.01
Anti-CD3
IgG
aIL-23

IL-23 enhances IFN-γ but not TNF-α production in gastric LPMC

There is evidence that IL-23 can enhance Th1-type cytokine production under certain conditions (37). Therefore, we extended our analysis to the role of IL-23 in controlling IFN-γ and TNF-α production by gastric LPMC. IFN-γ was measurable in the culture supernatants of anti-CD3-activated normal gastric LPMC. Moreover, stimulation of anti-CD3-treated LPMC with IL-23 significantly increased IFN-γ secretion (Fig. 5A). By contrast, TNF-α secretion was not significantly modified by IL-23 in the same LPMC cultures (Fig. 5B). In line with these results, blockade of endogenous IL-23 by the anti-IL-23/p19 significantly reduced IFN-γ (Fig. 5C) but not TNF-α (Fig. 5D) secretion in cultures of LPMC isolated from Hp-infected biopsies. Since induction of IFN-γ is positively regulated by Stat1 and Stat4 activity (58-59), we also examined the effect of anti-IL-23 treatment on p-Stat1 and p-Stat4. As shown in figure 5E, anti-IL-23 reduced the expression of both p-Stat1 and p-Stat4.

It was recently shown that during human chronic intestinal inflammation, a subset of mucosal T cells can express both IL-17 and IFN-γ (60). Therefore, we simultaneously evaluated the expression of IL-17 and IFN-γ in gastric T cells. As shown in the representative flow-cytometry dot-plot in figure 6A, the fraction of CD3+ T cells expressing either IL-17 or IFN-γ was higher in Hp-infected samples than in controls. By contrast, the percentage of CD3+ T cells that expressed both IL-17 and IFN-γ was less than 1% independently of the Hp status. Similarly, the percentages of both CD4+ and CD8+ T cells making either IL-17 or IFN-γ were higher in Hp-infected biopsies than in controls, while less than 1% of these cells expressed both IL-17 and IFN-γ in Hp-infected and uninfected samples (Fig. 6B and C).

Figure 5

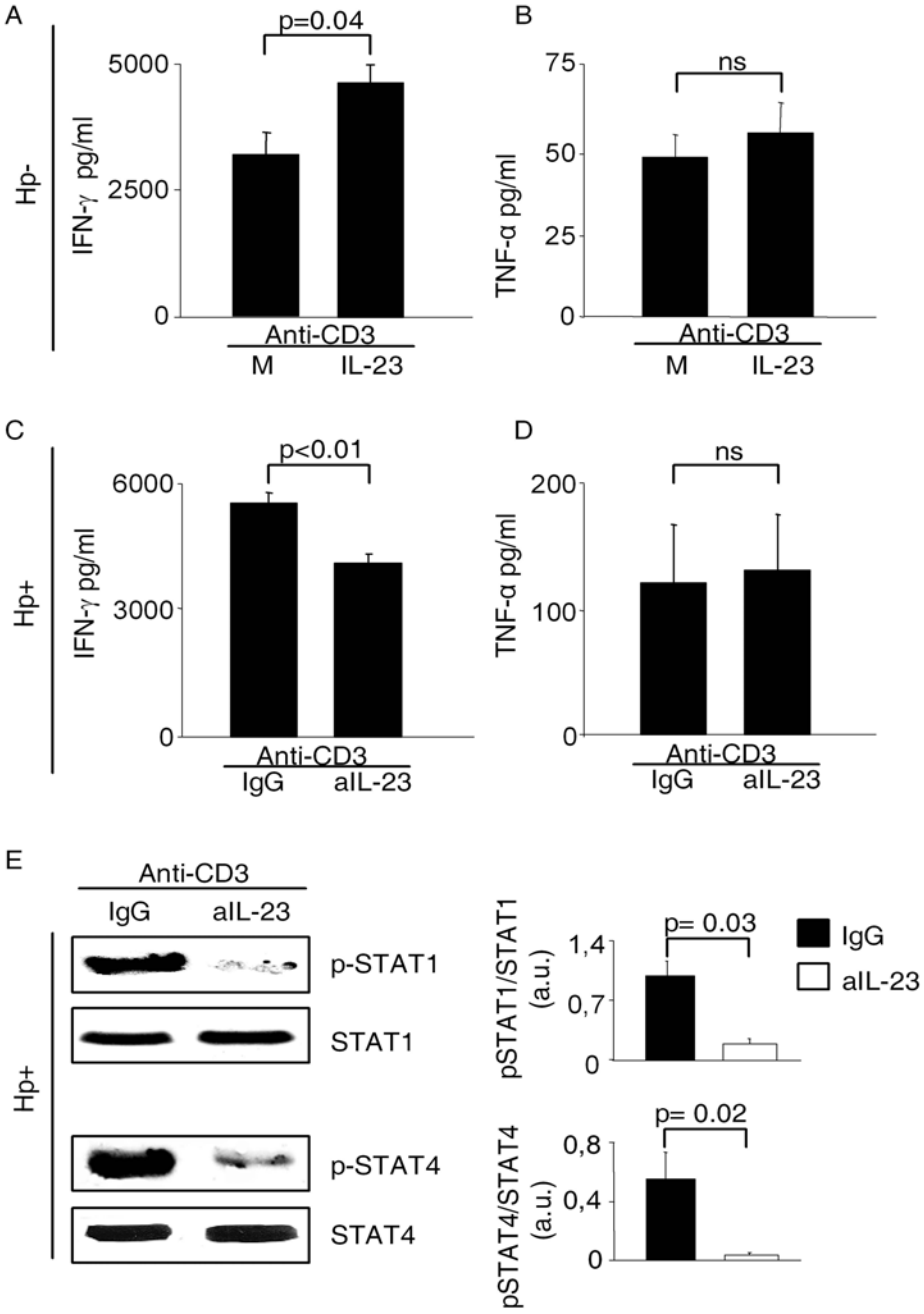
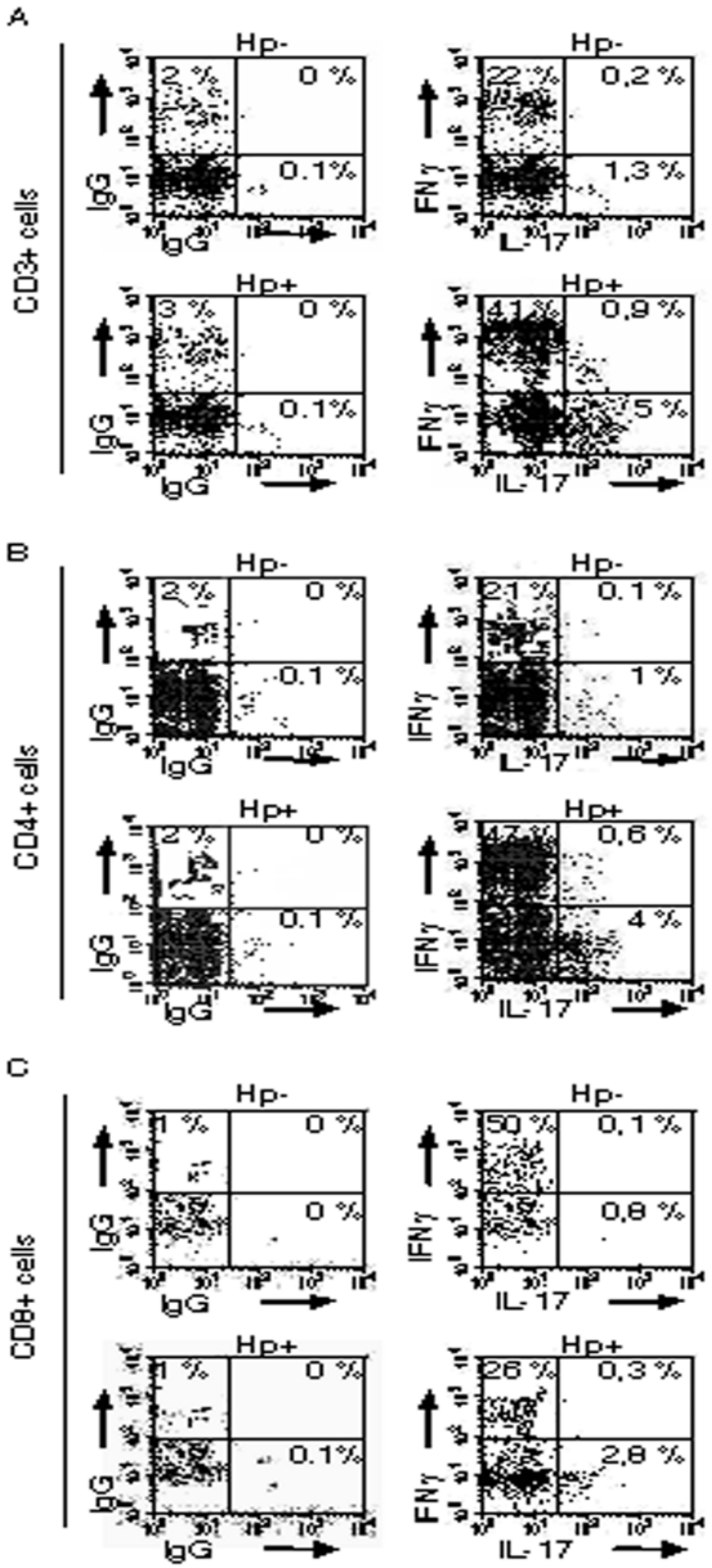

A
Hp-
IFN-γ pg/ml
5000
2500
0
p=0.04
Anti-CD3
M
IL-23
B
TNF-α pg/ml
75
50
25
0
ns
Anti-CD3
M
IL-23
C
Hp+
IFN-γ pg/ml
6000
3000
0
p<0.01
Anti-CD3
IgG
aIL-23
D
TNF-α pg/ml
200
100
0
ns
Anti-CD3
IgG
aIL-23
E
Anti-CD3
IgG
aIL-23
Hp+
p-STAT1
STAT1
p-STAT4
STAT4
pSTAT1/STAT1 (a.u.)
1,4
0,7
0
p= 0.03
IgG
aIL-23
pSTAT4/STAT4 (a.u.)
0,8
0,4
0
p= 0.02

Figure 6


A
CD3+ cells
Hp-
2 %
0 %
0.1%
IgG
IgG
22 %
0,2 %
1,3 %
FNγ
IL-17
Hp+
3 %
0 %
0.1%
41 %
0,9 %
5 %
B
CD4+ cells
2 %
0 %
0.1 %
2,1 %
0.1 %
1 %
IFNγ
4,7 %
0,6 %
4 %
C
CD8+ cells
1 %
0 %
0 %
50 %
0,1 %
0,8 %
26 %
0,3 %
2,8 %

IL-21, a product of Th17 cells, is highly expressed in Hp-infected mucosa and contributes to tissue damaging inflammatory response.

More recently studies in mice have shown that Th17 cells are the major producers of IL-21 (33), a cytokine that is capable of regulating the functional activity of both immune and non-immune cells (41). So, we have extended our observation by assessing the role of such a cytokine in the Hp-related pathology. To this end, biopsies were taken from Hp-positive and Hp-negative patients to evaluate the IL-21 content. IL-21 protein was detectable in all samples regardless of whether biopsies were taken from Hp-infected or uninfected patients (Fig. 7A). However, as shown in figure 7B, IL-21 expression was significantly increased in biopsies of Hp-infected patients compared to Hp-uninfected patients with gastritis ($P=0.01$) or without 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 CagA negative. No difference in IL-21 protein expression was seen between Hp-uninfected patients with gastritis and normal samples (Fig. 7B). High IL-21 was also seen in gastric CD3+ T-LPL isolated from Hp-infected patients compared to T cells isolated from Hp-uninfected patients (Fig. 7C).

To confirm further that Hp-related gastritis is associated with enhanced synthesis of IL-21, we assessed IL-21 in gastric biopsies of 5 Hp-infected patients before and after successful eradication. As shown in figure 7D, eradication of Hp resulted in a reduced expression of IL-21.

To also analyse if 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. Figure 7E shows that IL-21 RNA expression was significantly higher in Hp-infected patients than Hp-uninfected patients either with or

without gastritis (P=0.02 and P=0.001, respectively). No difference was seen in terms of IL-21 RNA transcripts between Hp-uninfected patients with gastritis and normal controls (Fig. 7E).

Additionally, we characterized the expression of the IL-21R in gastric epithelial cells. To this end we assessed IL-21R by Western blotting using total proteins extracted from both epithelial cells and epithelial cell-depleted mucosa. Two or more distinct bands with a molecular weight ranging from 55 to 72 KDa and corresponding to glycosylated forms of IL-21R (61) were detected in epithelial extracts of patients and controls (Fig. 8A). In particular, two isoforms of IL-21R with a molecular weight of approximately 60 and 72 KDa respectively were seen in all samples regardless of whether epithelial cells were isolated from biopsies of Hp-infected patients or controls. Densitometric analysis of such bands showed no difference among groups (Figure 8B). Two additional, and very faint bands, with a molecular weight of approximately 55 and 65 KDa, were variably expressed in samples from Hp-infected and uninfected patients (Fig. 8A), and the intensity of such bands did not differ among groups. Extracts from epithelial cells of Hp-infected patients and both inflamed and normal controls also contained the common γ-chain subunit (Fig. 8A). Analysis of cytokeratin-18 confirmed the epithelial origin of such extracts (Fig. 8A)

To confirm that the IL-21R bands seen in epithelial cell extracts were not due to the small numbers of lymphocytes present in these preparation, IL-21R and CD3 were simultaneously evaluated in freshly isolated gastric epithelial cells of 3 patients (2 with Hp infection) by flow cytometry. As shown in the representative dot plot in figure 8A, inset, only 0.6% of the IL-21R-positive cells were stained with the anti-CD3.

Both IL-21R and the common γ-chain subunit were also seen in all epithelial cell-depleted mucosal samples (Fig. 8C). Stripping and reprobing each of these blots with a

cytokeratin-18 antibody showed that samples were not contaminated with epithelial cell extracts. Consistent with the above data, both IL-21R and the common-γ chain subunit were detected in AGS and MKN 28 cells by Western blotting (Fig. 8D). Flow cytometry analysis confirmed that these two cell lines expressed surface IL-21R (Fig. 8E). To investigate factors involved in the induction of IL-21R, AGS were either left unstimulated or stimulated with 3 different Hp strains or inflammatory cytokines, and then IL-21R was analyzed by Western blotting. As shown in figure 8F, the 3 Hp strains were able to enhance IL-21R protein expression. Similarly, IL-21R was enhanced by IL-1β, TNF-α, and IFN-γ (Figure 8G), clearly indicating that IL-21R can be induced by multiple inflammatory stimuli in gastric epithelial cells.

The high expression of IL-21R in Hp-infected gastric epithelial cells prompted us to investigate whether IL-21 controls the function(s) of these cells. Since 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.

During Hp infection, gastric epithelial cells produce MMPs in response to a variety of inflammatory stimuli (48-49). 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 figure 9A, IL-21 dose-dependently enhanced the secretion of MMP-2 and -9 (gelatinases A and B), while the synthesis of MMP-1 remained unchanged. MMP-3, MMP-7, TIMP-1 and TIMP-2 were undetectable regardless of whether cells were either left untreated or treated with IL-21. The ability of IL-21 to enhance the production of both MMP-2 and MMP-9 was confirmed by gelatin zymography (Fig. 9A inset). In addition, real-time PCR assays showed that IL-21 augmented the expression of both MMP-2 and MMP-9 RNA transcripts (Fig. 9B and C). To confirm

that the effect of IL-21 on gelatinases synthesis, we carried out *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 antibody we produced by immunizing rabbits with a specific human IL-21 peptide. Gastric biopsies taken from 5 Hp-infected patients were cultured with the anti-IL-21 antibody or control antibody for 24 hours. Epithelial cell extracts were then prepared and used to analyze MMP-2 and MMP-9 by Western blotting. Treatment of biopsies with anti-IL-21 resulted in a reduced production of both MMP-2 and MMP-9 as compared with biopsies cultured with the control IgG (Fig. 10A). Densitometry analysis of immunoreactive bands showed that the anti-IL-21 significantly reduced the synthesis of both MMP-2 and MMP-9 (Fig. 10B, P=0.04).

Figure 7

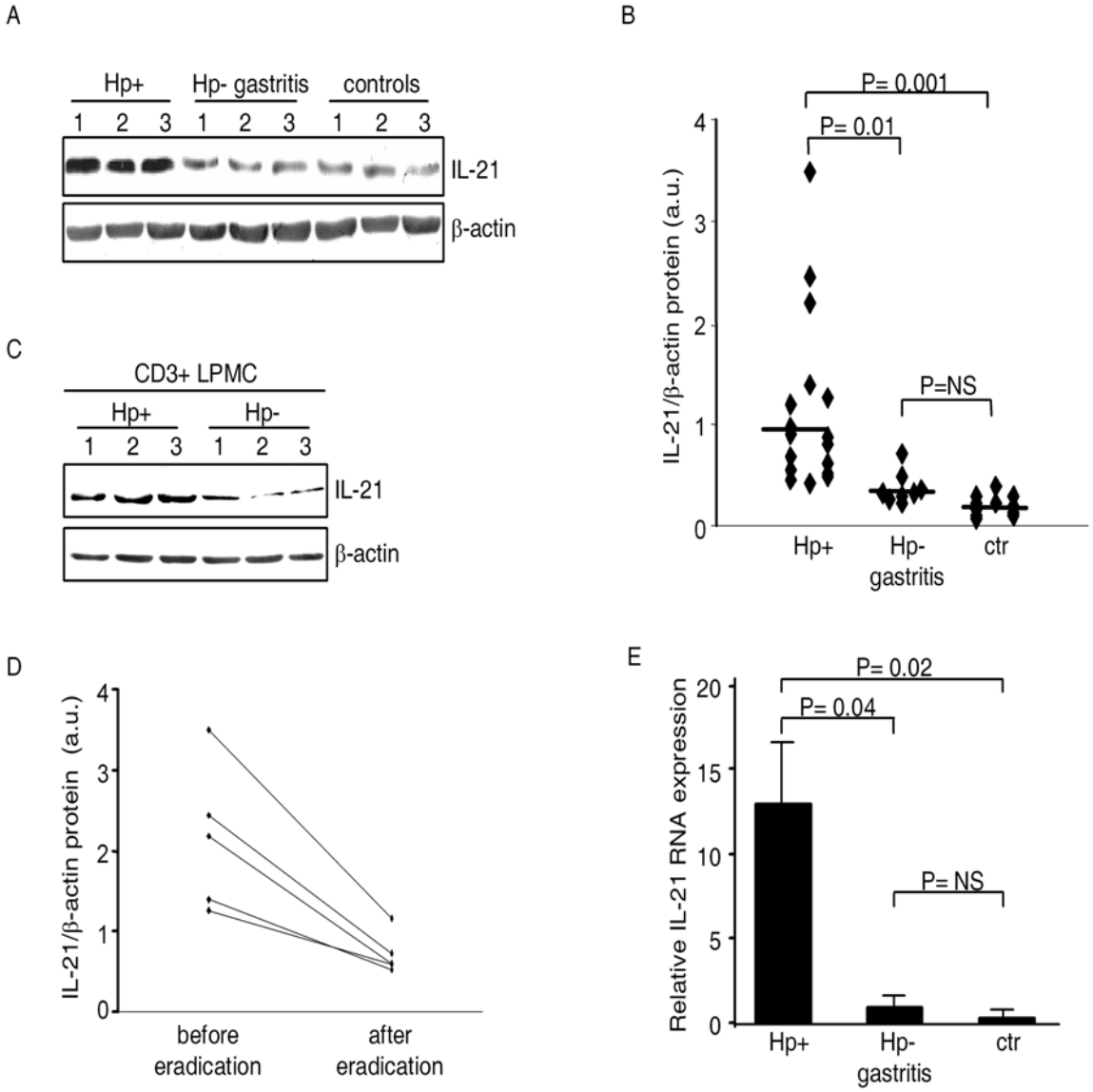

A
Hp+
Hp- gastritis
controls
1 2 3 1 2 3 1 2 3
IL-21
β-actin
B
P= 0.001
P= 0.01
P=NS
IL-21/β-actin protein (a.u.)
0
1
2
3
4
Hp+
Hp- gastritis
ctr
C
CD3+ LPMC
Hp+
Hp-
1 2 3 1 2 3
IL-21
β-actin
D
IL-21/β-actin protein (a.u.)
0
1
2
3
4
before eradication
after eradication
E
P= 0.02
P= 0.04
P= NS
Relative IL-21 RNA expression
0
5
10
15
20
Hp+
Hp- gastritis
ctr

Figure 8

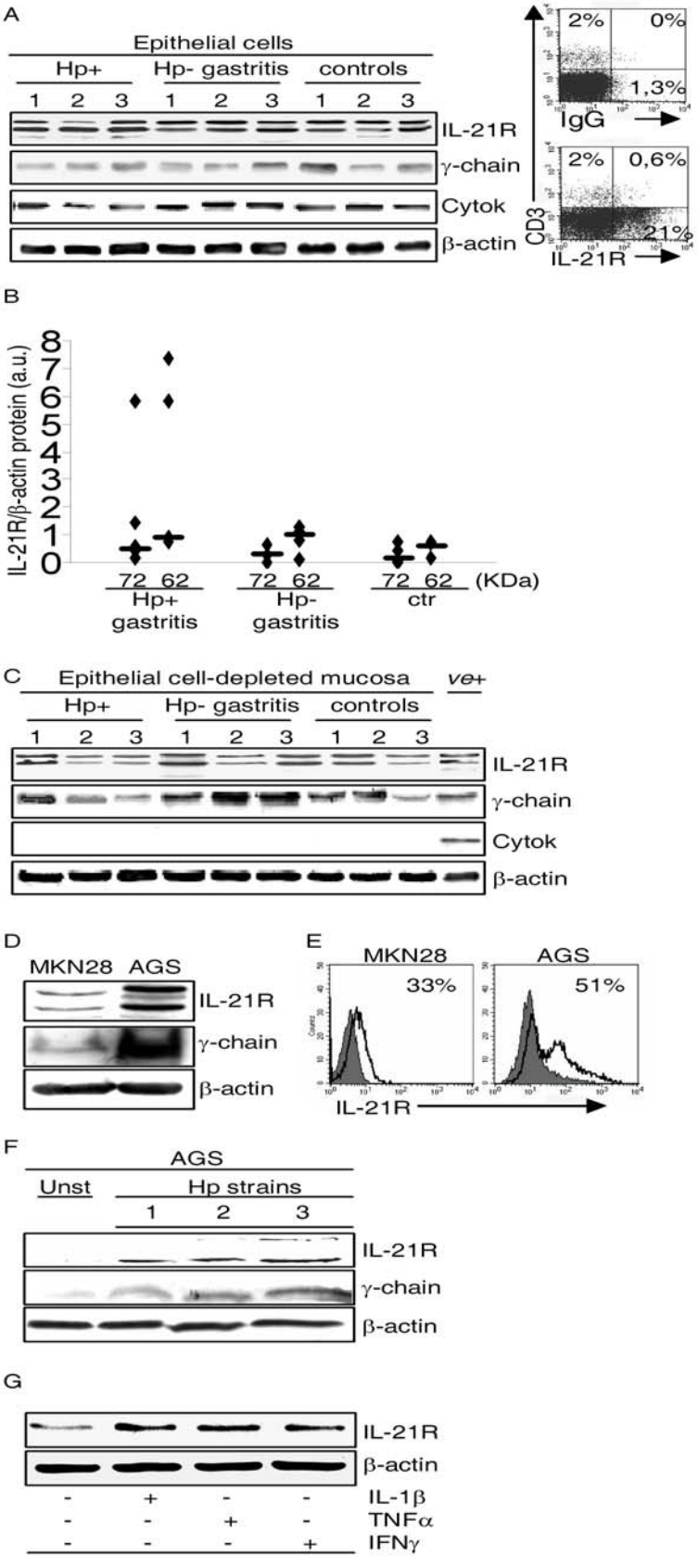

A
Epithelial cells
Hp+
Hp- gastritis
controls
1 2 3 1 2 3 1 2 3
IL-21R
γ-chain
Cytok
β-actin
2%
0%
1,3%
IgG
2%
0,6%
21%
CD3
IL-21R
B
IL-21R/β-actin protein (a.u.)
0 1 2 3 4 5 6 7 8
72 62
Hp+ gastritis
72 62
Hp- gastritis
72 62
ctr
(KDa)
C
Epithelial cell-depleted mucosa
ve+
Hp+
Hp- gastritis
controls
1 2 3 1 2 3 1 2 3
IL-21R
γ-chain
Cytok
β-actin
D
MKN28 AGS
IL-21R
γ-chain
β-actin
E
MKN28
AGS
33%
51%
IL-21R
F
AGS
Unst
Hp strains
1 2 3
IL-21R
γ-chain
β-actin
G
IL-21R
β-actin
- + - - IL-1β
- - + - TNFα
- - - + IFNγ

Figure 9

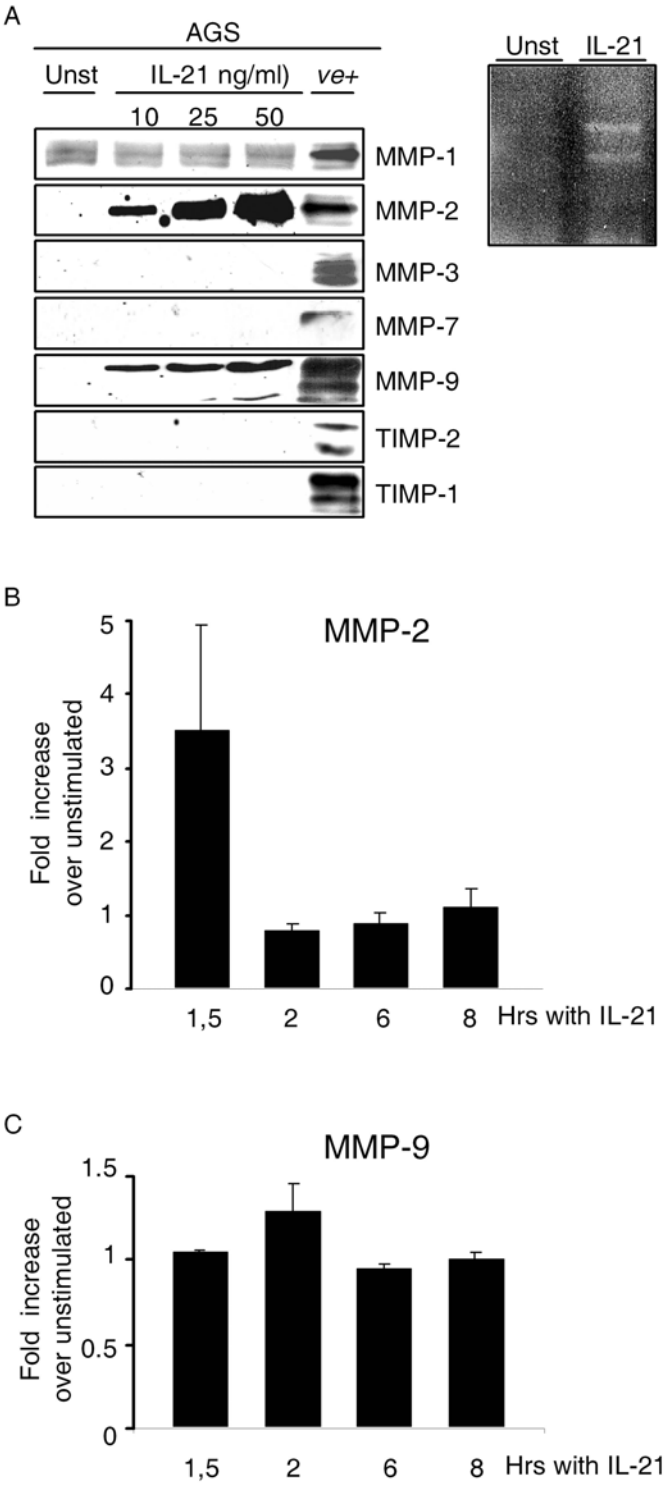
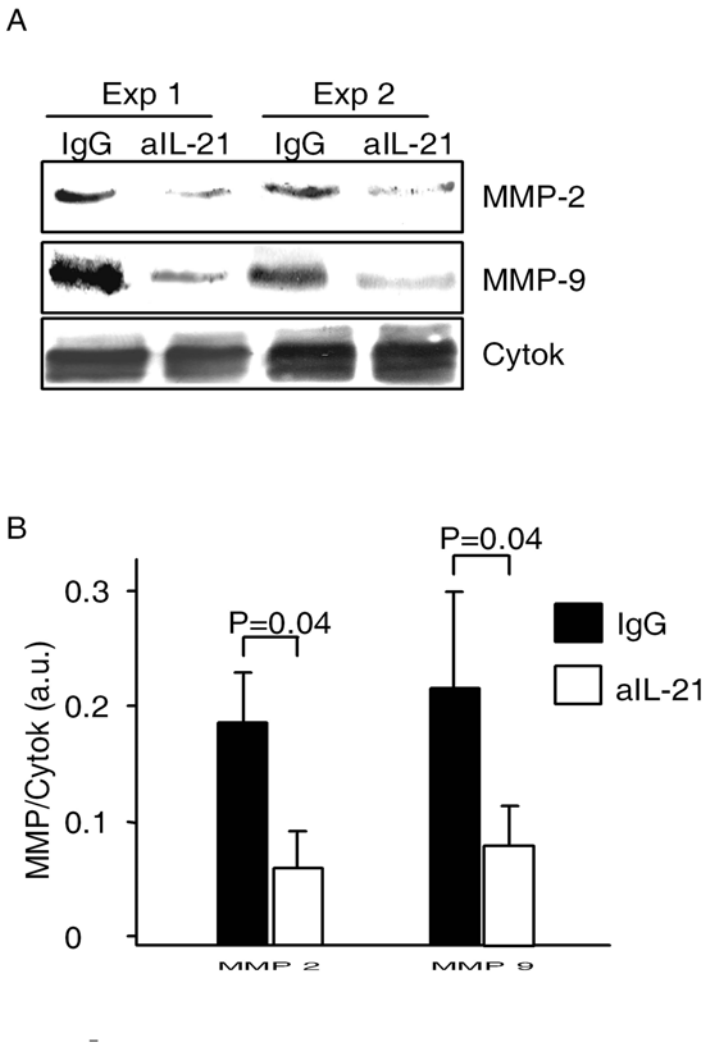

A
AGS
Unst
IL-21 ng/ml)
ve+
10
25
50
MMP-1
MMP-2
MMP-3
MMP-7
MMP-9
TIMP-2
TIMP-1
Unst
IL-21
B
MMP-2
Fold increase over unstimulated
0
1
2
3
4
5
1,5
2
6
8
Hrs with IL-21
C
MMP-9
Fold increase over unstimulated
0
0.5
1
1.5
1,5
2
6
8
Hrs with IL-21

Figure 10


A
Exp 1
Exp 2
IgG
aIL-21
IgG
aIL-21
MMP-2
MMP-9
Cytok
B
P=0.04
P=0.04
0.3
0.2
0.1
0
MMP/Cytok (a.u.)
IgG
aIL-21
MMP 2
MMP 9

Discussion

This study was undertaken to evaluate the role of IL-23/IL-17 pathway in Hp-related gastritis. We first show that IL-17 is over-expressed in Hp-colonized gastric biopsies, and that T cells infiltrating the gastric mucosa of Hp-infected patients are a cellular source of this cytokine. Flow-cytometry analysis showed that both CD4+ and CD8+ T cells were positive for IL-17. Whether additional cells, such as neutrophils, contribute to the increased production of IL-17 during Hp infection remains however to be ascertained. Second, we provide evidence that IL-23, a cytokine that is able to sustain IL-17 production, is produced in excess in Hp-colonized mucosa. IL-23 was also detected in all Hp-negative biopsies. However, in Hp-uninfected patients with gastritis, IL-23 production was not different from normal biopsies, indicating that by itself, non-specific gastric inflammation is not sufficient to enhance IL-23 expression. On the other hand, our data indicate that Hp by itself and/or Hp-driven inflammation can enhance IL-23 production in the stomach. This is also supported by the demonstration that Hp-derived products increased IL-23/p19 RNA expression in LPMC isolated from uninfected gastric samples. While this project was ongoing, it was shown that Hp enhances IL-23 secretion by monocyte-derived dendritic cells, and that Hp neutrophil-activating protein induces the expression of IL-23 in cultured neutrophils and monocytes (62-63).

Analysis of IL-23 RNA expression by real-time PCR revealed that both IL-23/p40 and p19 subunits are up-regulated in biopsies of Hp-infected patients, indicating that IL-23 is regulated at the transcriptional level in this condition. The exact molecular mechanism that leads to the overexpression of IL-23 during Hp infection remains

however unknown, and it remains to be ascertained which cells make the functional heterodimeric IL-23. One of the difficulties in studying the regulation of IL-23 in specific gastric cell types is that studies have to be carried out on pinch biopsies which restrict the amount of material available for analysis. Nonetheless, it is likely that IL-23 is mostly produced by antigen presenting cells (APC). Indeed, the data available so far indicate that only APC concomitantly express IL-23/p40 and p19 and are capable of making functional heterodimeric IL-23, while IL-23/p19 may be also expressed by T cells and endothelial cells (37,64,65).

In accordance with the emerging role of IL-23 in controlling the expansion/survival of Th17 cells (35,66), we here show that IL-23 enhances IL-17 production by normal gastric LPMC and that blockade of endogenous IL-23 down-regulates IL-17 in LPMC isolated from Hp-infected biopsies. We would like however to point out that additional cytokines other than IL-23 could regulate the maintenance of IL-17-produing cells in Hp-infected gastric mucosa. A likely candidate might be IL-1β, as this cytokine is up-regulated in Hp-colonized stomach, and IL-1R1-deficient mice fail to mount a robust Th17 response (67-68). Moreover, IL-1R1-deficient cells do not produce IL-17 in response to IL-23 (68). IL-17 is also positively regulated by IL-15 both in murine CD4+ T cells and human blood mononuclear cells (69). However, we previously showed that IL-15 is down-regulated at the transcriptional level in the Hp-infected mucosa (70), thus making unlikely the involvement of IL-15 in the control of IL-17 production during Hp-related gastritis.

Functional analysis of signaling pathway(s) underlying the regulation of IL-17 by IL-23 suggests the involvement of Stat3. Indeed, neutralization of endogenous IL-23 by anti-IL-23/p19 reduces Stat3 activation and inhibited IL-17 production in LPMC from Hp-infected biopsies. Moreover, IL-23 activates Stat3 in normal gastric LPMC

and pharmacologic inhibition of Stat3 by AG490 suppresses the IL-23-driven IL-17 production. AG490 also inhibits the activation of other Stat molecules, that could be activated by IL-23, such as Stat1 and Stat4. Nonetheless, studies in other systems have shown that cells deficient in Stat1 and Stat4 still retain the ability to differentiate into Th17 cells in vitro following TCR stimulation in the presence of IL-23 (71). By contrast, both in vitro and in vivo studies have shown that Stat3 is essential for the induction and expansion of IL-17-producing cells in response to cytokine stimulation (72). The mechanism by which Stat3 regulates IL-17 synthesis is not yet fully understood. One possibility is that Stat3 directly binds to the promoter of IL-17 gene and enhances its transcriptional activity (72). Another possibility is that Stat3 facilitates the induction of RORγt, a specific master regulator of Th17-cell differentiation (72-74). Finally, Stat3 could enhance the expression of IL-23R, thereby amplifying a positive feed-back loop that helps stabilize and/or maintain the IL-23/IL-17 pathway (72).

Beyond its ability to enhance IL-17 production, IL-23 also controls Th1 cell responses. Indeed, it is known that IL-23 activates Stat4 and enhances IFN-γ production in cultures of memory T cells, albeit to a lesser extent than IL-12 (37). Our data further support this notion. Indeed, we showed that IL-23 enhances IFN-γ synthesis in normal gastric LPMC, and the anti-IL-23/p19 diminishes the production of IFN-γ in LPMC of Hp-infected patients. IL-23 does not affect the production of TNF-α We cannot however exclude a possibility of an effect of IL-23 on TNF producing Th17 cells because it is probable that the majority of the TNF-α made by LPMC from Hp infected patients is from macrophages and not T cells. Overall however, in line with our results, Kullberg et al recently showed, in two models of H. hepaticus-

triggered T cell-dependent colitis, that IL-23 drives both IFN-γ and IL-17 responses that together synergize to trigger severe intestinal inflammation (59).

Colonization of gastric epithelial cells with Hp induces an inflammatory mucosal reaction characterized by a massive infiltration of neutrophils (2-3). Although, Hp by itself can stimulate cultured gastric epithelial cells to synthesize IL-8, the major neutrophil chemoattractant in humans, studies in immunodeficient mice have shown that infection with various Helicobacter species fails to induce gastric inflammation (11). However, transfer of T lymphocytes into these animals then results in a severe gastritis (11), thus suggesting that T cells play a major role in promoting the recruitment of inflammatory cells during Hp infection. Our data support further this concept, since IL-17 is able to activate ERK1/2 MAP kinase in gastric epithelial cells thereby promoting IL-8 secretion (25). We have recently shown that ERK1/2 activity and IL-8 are highly expressed in freshly isolated Hp-colonized gastric epithelial cells, and that neutralization of endogenous IL-17 in gastric biopsies of Hp-infected patients down-regulates ERK1/2 activation and IL-8 production (23,25). The involvement of IL-17 in Hp-related gastritis is also supported by the demonstration that this cytokine is able to stimulate both immune and non-immune cells to produce multiple inflammatory mediators, such as IL-1, IL-6, TNF-α, matrix metalloproteinases and especially IL-21 (27,29,33).

Additionally, we show that Hp-related gastritis is associated with a marked increase in IL-21, a Th-17 cell-derived cytokine. IL-21 protein was semiquantitatively analyzed by Western blotting, In Hp-uninfected patients with gastritis, IL-21 protein expression was not different from normal biopsies, indicating that by itself, non-specific 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 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-infected patients, clearly indicating that the marked expression of IL-21 in Hp-infected biopsies 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-infected and -uninfected 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 non-immune cells (41-43). 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 here evidence that IL-21R can be induced on AGS by different Hp strains as well as non-specific inflammatory cytokines. Importantly, primary gastric epithelial cells, AGS and MKN28 cells expressed also the common-γ chain subunit that is essential for IL-21-driven intracellular signalling (42). 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. Other MMPs reported to be increased in the Hp-infected mucosa, such as MMP-1, MMP-3, and MMP-7 (51, 52, 76), were not induced by IL-21. Similarly, IL-21 did not alter the production of TIMP-1 and TIMP-2. This well fits 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 (47).

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 is also supported by the demonstration that neutralization of endogenous IL-21 in organ 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 non-immune cells during chronic inflammatory processes.

To our knowledge, this report is the first in which IL-23 and IL-21 over-production is documented in gastric mucosa during Hp infection. This IL-23 is biologically active, since it contributes to enhance the production of both IL-17 and IFN-γ, two cytokines that are supposed to orchestrate the local tissue inflammation in this condition. Additionally, Il-21 is an important mediator in the cross-talk between immune and non-immune cells during chronic inflammatory processes.

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