IFN-γ synergizes with TNF-α but not with viable *H. pylori* in up-regulating CXC chemokine secretion in gastric epithelial cells

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SUMMARY

Helicobacter pylori colonizes the gastric epithelial surface and induces epithelial cells to increase production of the neutrophil attractant IL-8. Little is known about the role of the gastric epithelium in regulating mucosal T cell trafficking. We therefore characterized constitutive and regulated epithelial expression of the CXC chemokines IP-10, I-TAC and Mig, which specifically attract CXCR3 expressing CD4^{+} T cells. Human gastric epithelial cell lines (AGS, Kato III, NCI) were used to characterize the constitutive and regulated expression of three CXC chemokines in response to IFN-γ, TNF-α and different *H. pylori* preparations. Chemokine mRNA and protein production were measured by RT-PCR and ELISA. Gastric epithelial cells constitutively expressed mRNA for IP-10, Mig and I-TAC. IFN-γ in combination with TNF-α strongly induced secretion of those chemokines. Soluble or membranous fractions of *H. pylori* significantly inhibited IFN-γ/TNF-α induced epithelial cell IP-10 and Mig production. Gastric epithelial cells may contribute to mucosal T cell trafficking. The capacity of *H. pylori* products to inhibit IP-10 and Mig secretion may explain, at least in part, the failure to induce protective immunity against this bacterium and the ability of *H. pylori* to affect the presentation of the local inflammation.

Keywords gastric epithelial cells chemokines *H. pylori* IP-10 Mig I-TAC

INTRODUCTION

Helicobacter pylori infection is an important risk factor for chronic gastritis, peptic ulcer, gastric carcinoma and gastric B-cell lymphoma [1,2]. Although the pathological mechanisms involved in *H. pylori*-induced inflammation are not yet completely defined, there is accumulating evidence that activated neutrophils play an important role in the pathogenesis [1,2]. Gastric epithelial cells produce and secrete IL-8, a potent chemotactic and activating factor for leucocytes, in response to *H. pylori* infection both *in vivo* and *in vitro* [3]. Indeed, gastric mucosal levels of IL-8 are increased, and its activity correlates with histological severity in patients with *H. pylori*-induced gastritis [4] as well as cagA status [5]. Prolonged IL-8 production by gastric epithelial cells during *H. pylori* infection might result in the recruitment of leucocytes to infected tissues and therefore may be important in the regulation of inflammatory and immune processes in response to this bacterium [6].

Despite increasing evidence for involvement of T lymphocytes in *H. pylori*-associated gastritis, little is known about the signals that regulate T lymphocyte recruitment within the gastric lesions. Lymphocyte recruitment into tissues is a multistep process involving adhesion molecules and chemokines [7]. Chemokines

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are secreted basic proteins (6–14 kDa) subdivided into four families based on the relative position of their cysteine residues (CC, CXC, C, CXC3). CXC chemokines further fall into two classes based on the presence or absence of a NH$_2$-terminal sequence Glutamic acid–Leucine–Arginine (ERL). The ERL-containing CXC chemokines, such as IL-8, chemoattract neutrophils, whereas for the non-ELR CXC chemokines, three of them – IP-10 (IFN-inducible protein 10), Mig (monokine induced by IFN-γ) and I-TAC (IFN-inducible T cell α chemoattractant) – are IFN-γ inducible and potently chemoattract activated T lymphocytes [8]. All three chemokines signal through a common receptor, CXCR3, expressed by memory (CD45R0^{+}) T cells [9], preferentially of the Th1 subset, and by natural killer cells, but not by monocytes or neutrophils [10,11]. Recent studies have colocalized CD4^{+} T lymphocytes and IFN-γ in animal models and *H. pylori*-infected patients with chronic gastritis and MALT-lymphoma, indicating the dominance of a Th1-type response [12,13]. Moreover, IFN-γ has been shown to induce expression of CXC3 receptors on lymphocytes [10].

Since gastric epithelial cells have receptors for TNF-α and IFN-γ, and those cytokines are known to be potent inducers of IP-10, Mig and I-TAC production in several other cell types [14–16], we investigated whether gastric epithelial cells may produce IFN-γ-inducible chemokines that chemoattract T cell populations known to be important for mediating pathologic inflammation in the stomach. We further characterized the influence of *H. pylori*

bacterial lysates in terms of expression of the CXC chemokines IP-10 and Mig in gastric epithelial cells.

MATERIALS AND METHODS

Reagents list

The recombinant human (rh) IFN-γ and TNF-α, anti-IP-10 (1 μg/μl), anti-Mig (1 μg/μl), as well as biotinylated antibodies against IP-10 and Mig used in the ELISA protocol and rh IP-10 (20 ng/μl) and rh Mig (20 ng/μl) were obtained from R & D Systems (Wiesbaden, Germany). Streptavidine horse radish peroxidase conjugate was obtained from Amersham Pharmacia Biotech (Freiburg, Germany). Annexin-V FITC was purchased from Bender Medsystems (Vienna, Austria), Ig isotype and propidium-iodide (PI) were puchased from BD PharMingen (La Jolla, San Diego, CA, USA)

Bacterial strain, epithelial cell lines and culture conditions

For *in vitro* experiments we used the cagA-positive *H. pylori* strain NTCT 11637 (DMSZ, Braunschweig, Germany). Bacteria were grown on columbia base agar containing 4% defibrinated horse blood (Oxoid, Wesel, Germany) and one tube of *H. pylori* selective supplement (Oxoid, Wesel, Germany) in 500 ml media. Supplement contains vancomycine, trimethoprime, cefusoldin and amphotericine B. Plates were freshly prepared and cooled down before use. The plates were incubated in a microaerophilic atmosphere containing 80% N_2, 15% CO_2, 5% O_2 at 37°C. Culture conditions were achieved by using an Anoxomat WS8080-System (MART, Lichtenvoorde, The Netherlands). The plates were incubated by use of a pressure resistant incubation vessel system. Cells were harvested after 5 d of culture and subsequently resuspended in Brucella Broth (BB) Bouillion (B & D, Heidelberg, Germany) containing 8% FCS and *H. pylori* selective supplement. Quantification was performed by optical density measurements on a spectralphotometer (Ultraspect III, Pharmacia Biotech, Freiburg, Germany). Bacteria were diluted in BB-FCS to an $OD_{550} = 1$ and used for further investigations.

The human gastric carcinoma cell lines AGS, Kato III and NCI were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line AGS was cultured in HAM's F12 media (Gibco, Deisenhofen, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Kato III and NCI were cultured in RPMI (Gibco) containing 20% FBS and 1% antibiotics. Cells were cultured at 37°C in 5% CO_2 saturated atmosphere.

Preparing soluble and membranous fractions of H. pylori

For the production of soluble and membranous fractions of *H. pylori* a cell suspension in PBS was sonicated for 20 min and 50% intensity (Sonorex Super RK255, Bandelin) and centrifuged for 30 min at 4°C. The supernatant was characterized as soluble fraction and the pellet dissolved in PBS as membranous fraction. Protein concentration was measured with the Biuret method (Bio-Rad Protein-Assay, Bio-Rad Laboratories GmbH, München, Germany).

Stimulation protocol

Confluent monolayers of human gastric cell lines were stimulated with TNF-α and IFN-γ (50 ng/ml each) alone or in combination. Negative controls were performed in each experimental setting without stimulation. Supernatants were harvested, centrifuged and collected for chemokine measurements by ELISA. For bacterial infection confluent epithelial monolayers were stimulated with IFN-γ and TNF-α alone or in combination for 12 h. Supernatants were removed and cells washed four times in PBS. The membranous and soluble fractions of *H. pylori* cells were used at a protein concentration of 50 μg/ml, if nothing else is stated. After 12 and 24 h of incubation time, supernatants were collected, centrifuged and analysed for Mig, IP-10 and IL-8 production. For each experiment duplicates were assessed.

ELISA

ELISA measurements for Mig and IP-10 were performed as described before [17]. Briefly, polysterol 96-well plates (Greiner, Solingen, Germany) were coated with monoclonal antibodies to IP-10 (1 : 250) and Mig (1 : 500) and incubated for 4 h at 37°C. Antibodies were blocked by an overnight incubation at 4°C with blocking solution (PBS, 5% dry milk as well as 0·1 Tween-20). As detection antibodies biotinylated goat anti-IP-10 (R & D systems) and anti-Mig (R & D systems) were diluted 1 : 500 and 1 : 800 in dilution buffer (PBS, 1% BSA and 0·1% Tween-20). Second step reagents were HRP-conjugated streptavidin (ECL, Amersham Pharmacia Biotech, Freiburg, Germany) diluted 1 : 4000 in dilution buffer. Bound HRP was visualized with TMB and H_2O_2 diluted in sodium acetate buffer, pH 6·0. The colour reaction was stopped by adding 50 μl of stop solution (1·2 M H_2SO4) and absorbency was measured at 450 nm. Concentration of the chemokines was calculated from the standard curves using rh IP-10 and Mig. The specific sensitivity for both ELISA's was 50 pg/ml. Supernatant concentrations of IL-8 were assessed by a specific sandwich ELISA (Biosource, Camarillo, USA), according to the manufacture's protocol. All samples were analysed in duplicates.

RT-PCR analysis

RNA isolation was performed using standard methods (Stratagene, Amsterdam, The Netherlands), and total RNA was determined by measuring absorbency on a spectrophotometer (Eppendorf, Hamburg, Germany). Three μg of total RNA was reverse transcribed by MMLV reverse transcriptase (Gibco Life Technologies, Karlsruhe, Germany), and cDNA was amplified as already described [17]. The primers for I-TAC, Mig and IP-10 were as follows: I-TAC (sense 5′-GCT ATA GCC TTG GCT GTG ATA TTG TG-3′ and antisense 5′-CTG CCA CTT TCA CTG CTT TTA CC-3′) and for Mig (sense 5′-GTA TCT GAG GCA CAT GCT AG-3′ and antisense 5′-AAA GGC ACT GCA TTG TGG TAG G-3′) and IP-10 (sense 5′-AGT GGC ATT CAA GGA GTA CC-3′ and antisense 5′-ATC CTT GGA AGC ACT GCA TC-3′). The amplification profile for IP-10, Mig and I-TAC consisted of 27–35 cycles of a 45 s denaturation step at 95°C followed by 2·5 min annealing and extension at 60°C as described before [17]. Primers for IP-10, Mig and I-TAC did not amplify either murine genomic DNA or splenic cDNA. Negative control reactions had no added RNA in the RT and subsequent PCR amplification. PBMC isolated from the buffy coat of a healthy donor and stimulated overnight with 10 μg/ml PHA and 2 μg/ml of bacterial LPS were used as a source of cDNA for positive controls. Amplification products were run on 4% polyacrylamide gel electrophoresis and visualized by silver-staining.

Flow cytometric analysis

Necrotic membrane damage was excluded by trypan blue exclusion.
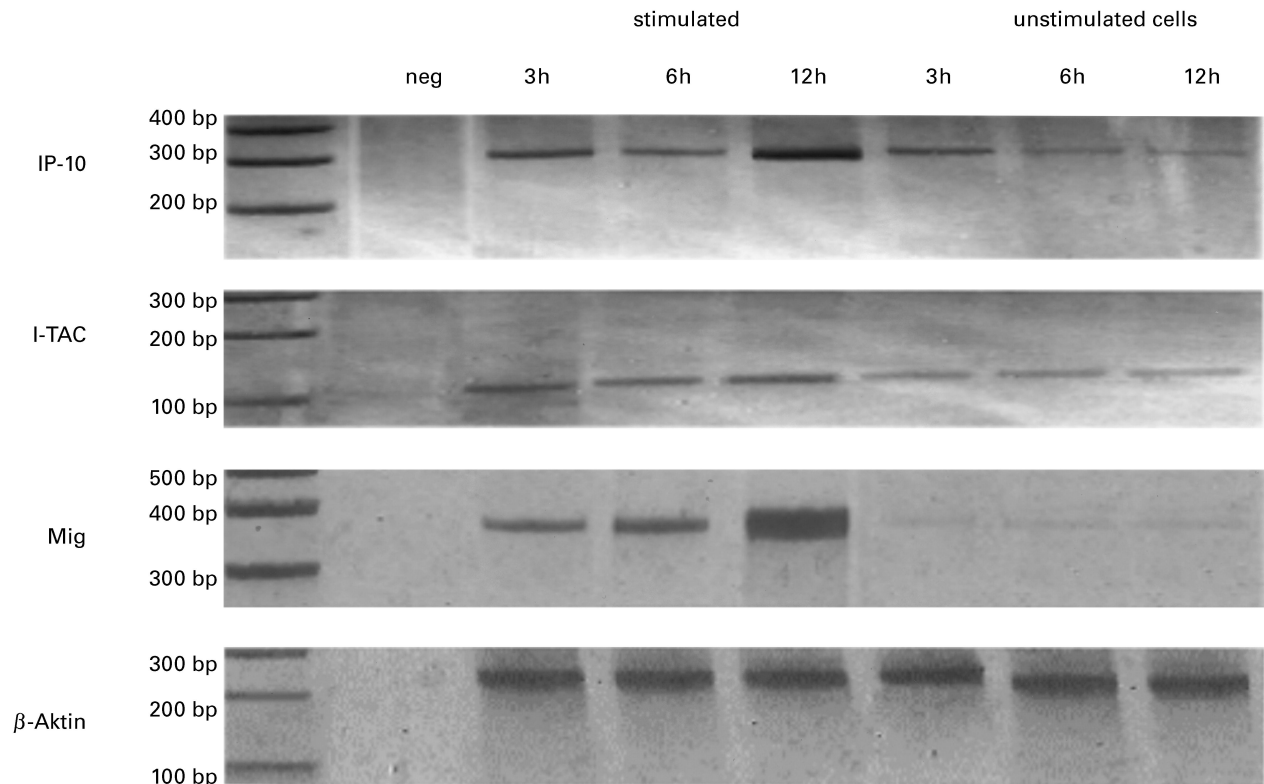
Fig. 1. Constitutive and regulated expression of IP-10, I-TAC and Mig in the gastric epithelial cell lines Kato III. Total RNA was isolated from stimulated and unstimulated confluent monolayers of cell lines after 3, 6 and 12 h as indicated. RT-PCR cDNA transcripts were generated and amplified using oligonucleotide primers specific for IP-10, Mig and I-TAC. RT-PCR reactions were also performed in the absence of RNA as a negative control. Amplification products were run on 4% polyacrylamide-gel electrophoresis, visualized by silver-staining and photographed. Representative results from one of three experiments are shown.

Furthermore apoptosis and necrosis were evaluated by double-labelling with annexin V-FITC as well as measurement of propidium iodide (PI) uptake into nonpermeabilized cells and subsequent flow cytometry. Briefly, confluent monolayers of gastric epithelial cell lines ($0.5-1 \times 10^7$ cells) were coincubated with different concentrations of living *H. pylori* or soluble and membranous fractions of *H. pylori* for 20 h. Cells were stained with annexin V-FITC and PI in PBS for 1 h at room temperature in the dark. FITC-conjugated murine MoAbs with unrelated specificity were always used as controls for unspecific binding of mAB. For positive controls, cells were permeabilized with Saponin 0·1% for 1 h. Two-colour FACS analysis was performed using a Becton Dickinson FACScan flow cytometer. Data acquisition and analysis were based on Lysis II software (Becton Dickinson, Heidelberg, Germany).

Analysis of data
In the statistical calculations the data were treated as being nonparametric, and results were analysed by the two-tailed Wilcoxon's signed rank test. Results are presented as mean ± 95% confidence interval, unless stated otherwise. *P*-values < 0·05 were considered significant.

RESULTS

Constitutive and regulated expression of Mig, IP-10 and I-TAC mRNA by human gastric epithelial cell lines
Non-stimulated gastric epithelial cell lines (Kato III, AGS, NCI)

constitutively expressed detectable levels of mRNA for IP-10, Mig and I-TAC (Fig. 1). Since gastric epithelial cells have receptors for IFN-γ and TNF-α and as these cytokines have been shown to up-regulate the expression of Mig, IP-10 and I-TAC mRNA by human monocytes, neutrophils [15], astrocytes [18] and bronchial epithelial cells [19], we next examined the regulated mRNA expression of these chemokines in human gastric epithelial cells in response to TNF-α and IFN-γ stimulation. We found an increased expression of I-TAC, Mig and IP-10 starting 2–3 h after stimulation with IFN-γ in combination with TNF-α, and reaching maximal levels by 8–12 h (Fig. 1). No significant effect was observed employing IFN-γ and TNF-α alone, not even with concentrations as high as 100 ng/ml.

Secretion of Mig and IP-10 by human gastric epithelial cell lines
We turned our attention to the question whether Mig and IP-10 mRNA expression was accompanied by secretion of those chemokines. Gastric epithelial cells were stimulated with different concentrations of IFN-γ and TNF-α and protein secretion was measured in culture supernatants by ELISA. In all gastric cell lines, IFN-γ and TNF-α synergistically increased IP-10 and Mig secretion in a dose- (Fig. 2a) and time-dependent manner (Fig. 2b). In the absence of IFN-γ and TNF-α, gastric epithelial cells secreted < 50 pg/ml of IP-10 and Mig. In all cell lines investigated, a slight up-regulation of IP-10 secretion could be observed after stimulation with IFN-γ alone, while TNF-α had no effect on IP-10 secretion. Mig secretion could only be
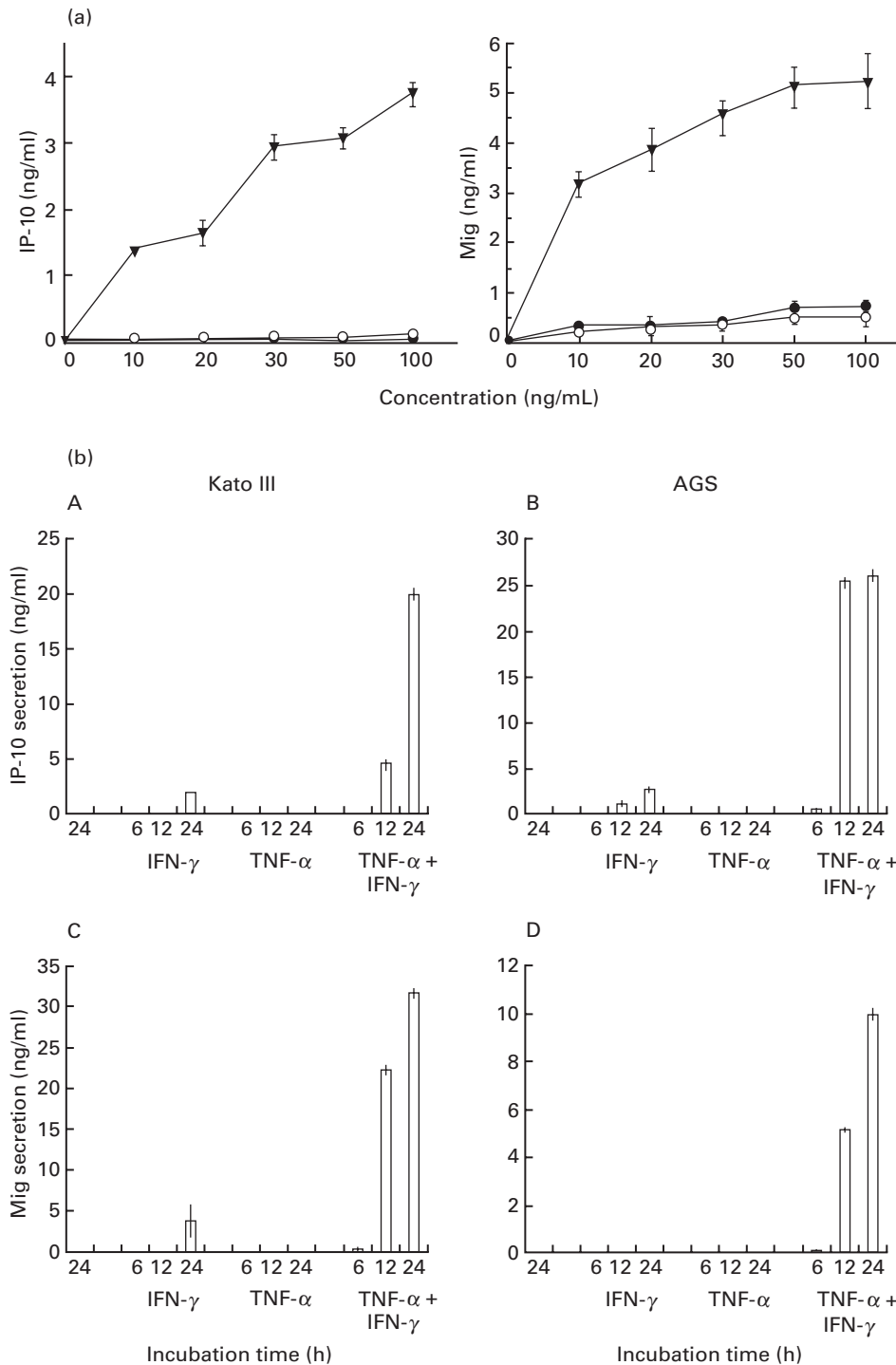
Fig. 2. (a) Dose-dependent secretion of IP-10 and Mig by gastric epithelial cells. Confluent monolayers of NCI cell lines were stimulated with increasing concentrations of either TNF-α or IFN-γ or both in combination (● IFN-γ; ○ TNF-α; ▼ TNF-α + IFN-γ). Supernatants were harvested after 18 h and chemokine-secretion was measured by ELISA. Similar results were obtained using AGS and NCI cell lines. Values are expressed as mean ± CI. Representative results from one of four independent experiments are shown. (b) Time-dependent secretion of IP-10 and Mig by gastric epithelial cells. Confluent monolayers of AGS and Kato III cells were stimulated for different times (as indicated) with either IFN-γ 50 ng/ml or TNF-α 50 ng/ml alone or both cytokines in combination. Supernatants were harvested after 6, 12 and 18 h and chemokine concentrations were measured by ELISA. Values are expressed as mean ± CI. Representative results from one of four experiments are shown.

Table 1. Inhibition of Mig and IP-10 but not IL-8 by membranous (MF) and soluble fractions (SF) of *H. pylori*

Stimulation with	Chemokine secretion (ng/ml)		
	IP-10	Mig	IL-8
TNF (50 ng/ml)	n.d.	n.d.	2·318 ± 0·264
IFN (50 ng/ml)	n.d.	n.d.	0·227 ± 0·06
IFN + TNF (50 ng/ml each)	25·46 ± 1·23	13·23 ± 0·87	2·362 ± 0·047
SF (500 μg/ml)	n.d.	n.d.	0·274 ± 0·15
MF (500 μg/ml)	n.d.	n.d.	0·132 ± 0·07
IFN + TNF + SF (50 μg/ml)	4·47 ± 0·33	4·195 ± 0·64	2·301 ± 0·033
IFN + TNF + SF (100 μg/ml)	3·009 ± 0·36	3·984 ± 0·45	2·524 ± 0·78
IFN + TNF + SF (500 μg/ml)	2·77 ± 0·42	3·307 ± 0·21	2·282 ± 0·195
IFN + TNF + MF (50 μg/ml)	9·62 ± 0·53	5·29 ± 0·34	2·535 ± 0·006
IFN + TNF + MF (100 μg/ml)	2·7 ± 0·41	2·23 ± 0·39	2·884 ± 0·124
IFN + TNF + MF (500 μg/ml)	1·54 ± 0·23	0·82 ± 0·32	3·002 ± 0·142

Stimulated gastric epithelial cells were coincubated with membranous and soluble fractions of *H. pylori* in concentrations of 50, 100 and 500 μg/ml. After 20 h of stimulation supernatants were harvested and centrifuged. ELISA measurements were performed as described in Materials and Methods. Values are expressed as mean ± CI. Representative results from one of four experiments are shown. n.d. = not determined.

detected in Kato III cells upon stimulation with IFN-γ alone and was not observed when cells were stimulated with TNF-α alone (Fig. 2b).

Effects of living H. pylori *on epithelial IL-8, Mig and IP-10 secretion*

To determine the effect of living bacteria on IP-10 and Mig secretion, different concentrations (1×10^7 and 1×10^8) were coincubated with gastric epithelial cells for 24 h. IL-8 was included as positive control as *H. pylori* is well known to induce IL-8 secretion in gastric epithelial cells. As Fig. 3 shows *H. pylori* dose-dependently induces IL-8 secretion in gastric epithelial cells, while IP-10 and Mig secretion remained unchanged. Longer
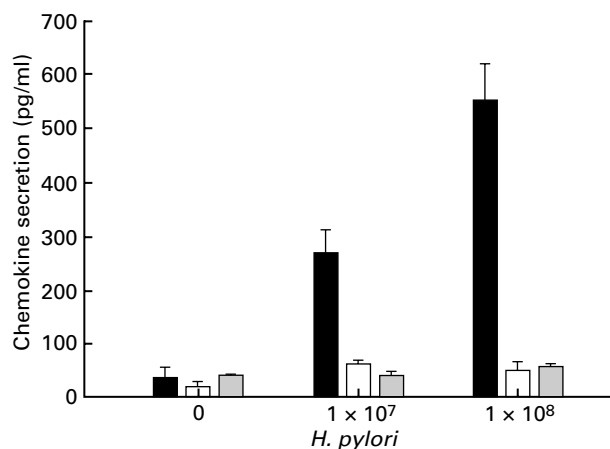
Fig. 3. Dose-dependent increase in IL-8 but neither IP-10 nor Mig secretion by living *H. pylori* (■ IL-8 secretion; □ IP-10 secretion; ▨ Mig secretion). Confluent monolayers of AGS cell line (2×10^6 cells) were coincubated with different concentrations of living *H. pylori* for 20 h. Supernatants were harvested, centrifuged and analysed for chemokine-secretion by ELISA. Values are expressed as mean ± CI. Representative results from one of four experiments are shown.

incubation periods had no further effect on chemokine secretion (data not shown).

Effects of soluble and membranous fractions of H. pylori *on epithelial Mig and IP-10 secretion*

Helicobacter pylori preparations have been shown to differ from living bacteria in their potential to modulate cytokine production [20–22]. We therefore performed further experiments to determine the impact of different *H. pylori* preparations on chemokine secretion by gastric epithelial cell lines. Soluble and membranous fractions of *H. pylori* were generated as described in Materials and Methods. Gastric epithelial cells were stimulated with INF-γ and TNF-α (50 ng/ml each) for 20 h and additionally with soluble or membranous fractions of *H. pylori* sonicates. Chemokine secretion was measured in supernatants by ELISA measurement. In agreement with previous studies [23] we found a strong IL-8 production by gastric epithelial cells in response to TNF-α. Fractions of *H. pylori* neither alone nor in combination with TNF-α and IFN-γ stimulated IL-8 secretion. In contrast, both membranous and soluble fractions of *H. pylori* induced a marked decrease of Mig and IP-10 concentration in supernatants of gastric epithelial cells, as measured by ELISA (Table 1). Apoptosis and necrosis were excluded by FACS-analysis (Fig. 4).

DISCUSSION

Chronic *H. pylori* infection is characterized by a pleiomorphic inflammatory cell infiltrate, composed of large numbers of polymorphonuclear and mononuclear cells in the epithelium and underlying lamina propria [2]. Because in addition to neutrophils predominantly Th1 cells are thought to play a central role in the pathogenesis of gastric inflammation in *H. pylori* infection [24], this study focused on the mediators that are important for the selective recruitment of these effector cells. In the present study, we demonstrate that human gastric epithelial cells produce IP-10, Mig and I-TAC at the mRNA and protein levels. These
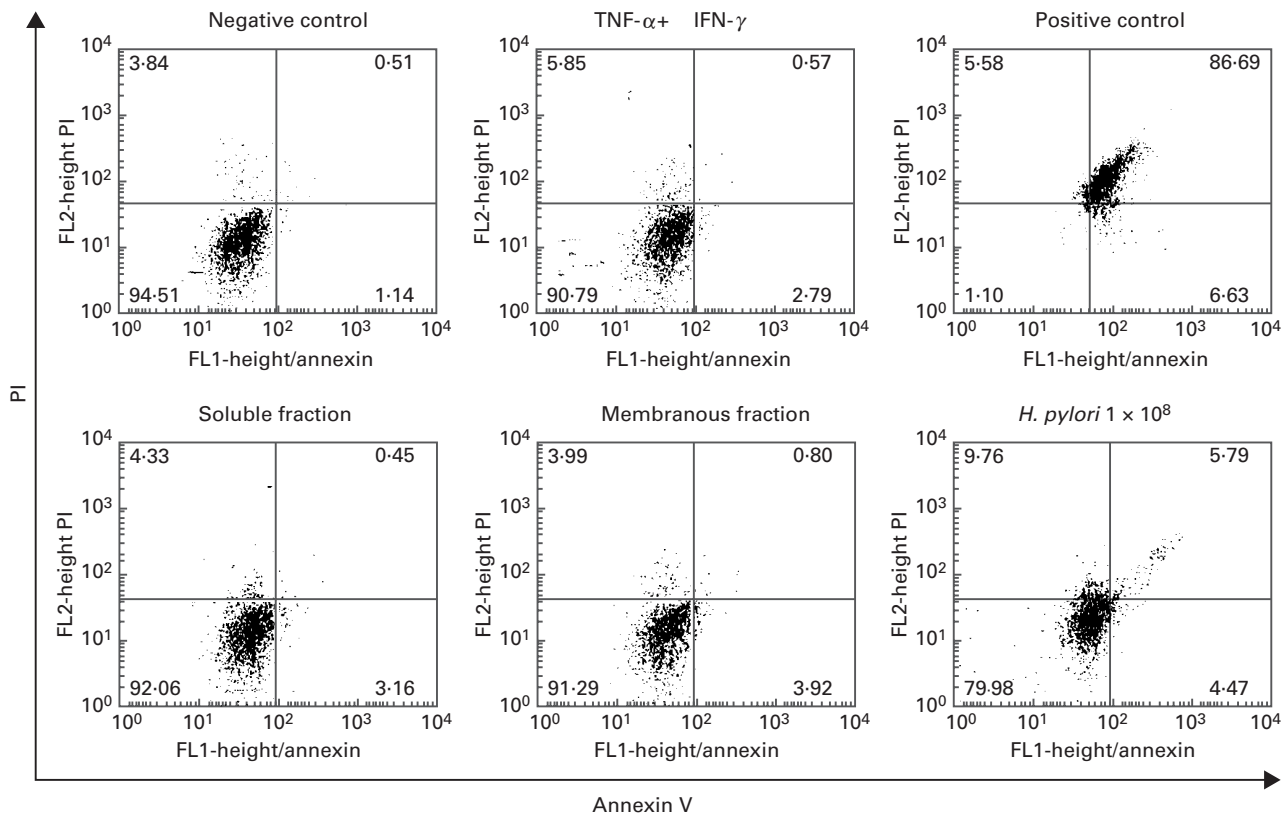
Fig. 4. Determination of necrosis and apoptosis by propidium iodide (PI) and annexin V staining. Necrotic membrane damage as well as apoptosis of gastric epithelial cells cultured for 20 h in medium or in the presence of TNF-α and IFN-γ alone or in combination with membranous or soluble fractions (50 μg/ml) as well as living *H. pylori* (1×10^8) was excluded by double staining with PI and annexin V. Gastric epithelial cells showed an increase in annexin V and PI staining after incubation with living *H. pylori* for 20 h, but remained negative for PI and annexin V concerning soluble or membranous fractions. Gastric epithelial cells permeabilized with 0·1% saponin served as positive control. Data are presented as percentage of gated cells.

chemokines, all induced by IFN-γ, are known to be strong ligands for the CXCR3 receptor on T cells. Because the CXCR3 receptor is predominantly expressed on Th1 cells, it seems likely that IP-10, Mig and I-TAC might contribute to the selective recruitment of Th1 cells in sites of inflammation with high IFN-γ production, e.g. *H. pylori*-positive gastritis [25].

In vivo, gastric infection with *H. pylori* induces the mucosal production of various cytokines in the host, including TNF-α and IFN-γ [26]. Recent reports have also shown that the number of IFN-γ-producing cells is increased in the stomach during chronic gastritis [2]. Our findings of high levels of IP-10, Mig and I-TAC production by gastric epithelial cells after stimulation with IFN-γ and TNF-α *in vitro* indicate that gastric epithelial cells have the potential to participate in mucosal T-cell recruitment during inflammatory responses. By chemoattracting IFN-γ-producing CD4$^+$ T cells in close proximity to the epithelium, epithelial-derived IP-10, Mig and I-TAC, which themselves are up-regulated by IFN-γ, may activate a positive feedback loop. The net effect of this would be amplification of the mucosal Th1 response seen in *H. pylori*-associated diseases [24]. Although gastric epithelial cells themselves do not produce IFN-γ, intraepithelial T cells and/ or IFN-γ-producing mucosal dendritic cells present may provide a regulatory signal to epithelial cells and thereby act as an initial stimulus for epithelial cell IP-10, Mig and I-TAC production.

In our experiments, we observed synergy between TNF-α and IFN-γ in the induction of epithelial Mig and IP-10 secretion, while TNF-α or IFN-γ alone had little or no effect. This data is consistent with a prior report on the transcriptional activation of the murine IP-10 gene by IFN-γ and TNF-α in mouse fibroblasts [27,28], but differs from studies in intestinal epithelium and normal bronchial epithelium, where IFN-γ alone was able to maximally induce IP-10, Mig and I-TAC mRNA expression [17,19].

In a recent study, IP-10 and Mig expression has been described in the gastric mucosa *in vivo* for the first time by employing *in situ* hybridization and immunohistochemistry [29]. Eck *et al.* showed in this study that IP-10 and Mig are expressed by endothelial cells of gastric mucosal vessels and by mononuclear cells at sites of T cell infiltration. In contrast to our work they could not detect any IP-10 and Mig expression by the gastric epithelium, while we found strong increase of IP-10 and Mig secretion by gastric epithelial cell lines in response to TNF-α and IFN-γ. Similar conflicting reports using *in situ* hybridization to localize the cellular origin of chemokines exist for IL-8. This cytokine was identified in macrophages, neutrophils and epithelial cells in active lesions of patients with inflammatory bowel disease, whereas other studies could not detect intestinal epithelial IL-8 mRNA expression [30–34].

Furthermore, in this study we were able to show that soluble and membranous fractions of *H. pylori* but not living bacteria dose-dependently inhibited IFN-γ/TNF-α induced epithelial cell IP-10 and Mig production without changing the epithelial cell

survival. Previous studies have already demonstrated that certain *H. pylori* preparations, e.g. urease, secreted proteins and bacterial DNA, can differ from living bacteria regarding their ability to modulate the local and/or systemic immune response [35–37]. Living *H. pylori* possess an immunosuppressive activity, either by the inhibition of antigen- or mitogen induced proliferative responses of PBMC and gastric epithelial cells [20] and/or by the inhibition of phagocytosis by monocytes and polymorphonuclear cells [38]. These inhibitory effects might be overcome by Th1-cytokines, which in turn are potent stimulators of the humoral immune response. Consequently, *H. pylori* infection and gastric inflammation might persist due to reduced influx of $CD4^+$ cells preferentially of the Th1 subset. For this reason, it seems to be rational that *H. pylori* might have evolved strategies to minimize the immigration of activated Th1 cells thus explaining – at least in part – the failure to induce protective immunity against this bacterium.

In conclusion, the herein demonstrated cytokine-induced IP-10, Mig and I-TAC secretion by the gastric epithelium may represent an important element in the local gastric inflammatory immune response. The novel finding that *H. pylori* fractions are capable of suppressing the IP-10 and Mig protein production by the gastric epithelium is likely to represent a selective advantage by which this pathogen evades the host's immune response.

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