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LETTER TO THE EDITOR

HELICOBACTER PYLORI HP0175 PROMOTES THE PRODUCTION OF IL-23, IL-6, IL-1β AND TGF-β

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***Helicobacter pylori* infection induces a chronic gastric inflammatory infiltrate. This study was undertaken to evaluate the type of the innate immune responses elicited by the secreted peptidyl-prolyl cis-trans isomerase of *H. pylori* (HP0175). The cytokine production induced by HP0175 in neutrophils, and monocytes was evaluated. HP0175 was able to induce the expression of IL-23 in neutrophils, and monocytes, and IL-6, IL-1beta and TGF-beta in monocytes. These findings indicate that HP0175 is able to promote the activation of innate cells and the production of a cytokine milieu that may favour the development of Th17 response.**

Helicobacter pylori (*H. pylori)* is a spiral-shaped Gram-negative bacterium that chronically infects the stomach of more than 50% of the human population and represents the major cause of gastric cancer, gastric lymphoma, gastric autoimmunity and peptic ulcer diseases (1-5). The World Health Organization classifies *H. pylori* as a type I carcinogen for distal gastric cancer. Eradicating the bacterium, in high risk populations, reduces incidence of gastric cancer (6). *H. pylori* induces an inflammatory response in the gastric mucosa characterized by polymorphonuclear and mononuclear cell infiltration. The host immune response to *H. pylori* influences the clinical outcome of the infection. Following *H. pylori* infection, innate and acquired immune responses take place in the stomach and are characterized by activation of macrophages and dendritic cells, production of antibodies, and by differentiation and activation of T helper 1 (Th1) cells (7-9). However, emerging experimental evidence indicates that *H. pylori* promotes also the development of the Th17 subset (10-12). Interleukin (IL)-1b, IL-6, transforming growth factor-b (TGF-b), and IL-23 are key cytokines in the differentiation and expansion of Th17 cells (13, 14). IL-17, IL-21 and IL-23 are up-regulated in *H. pylori*-infected gastric mucosa (10, 15).

Key words: Helicobacter pylori, cytokines, IL-23, IL-1b, IL-6, TGF-b

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

A major unanswered question remains that of the chemical nature of the *H. pylori* factors responsible for the promotion of IL-23, IL-1b, IL-6, TGF-b. Bacterial products are known to possess immunomodulatory properties and to drive different types of innate and adaptive responses (16). The secreted peptidyl prolyl cis-trans isomerase of *H. pylori* is a protein termed HP0175 that has been shown to trans-activate epidermal growth factor receptor through TLR4 in gastric epithelial cells (17, 18).

Here, we report that HP0175 is able to stimulate the expression of different key cytokines for Th17 differentiation, such as IL-23, IL-1b and TGF-b by both monocytes and neutrophils, as well as IL-6 by monocytes. Taken together, these results suggest that HP0175 is an important factor involved in the genesis of Th17 response in human *H. pylori* infection.

MATERIALS AND METHODS

Reagents

The monoclonal blocking TLR2.1 antibody against TLR2 and the monoclonal blocking TLR4.1 antibody against TLR4 were from eBioscience. The HP0175 was expressed, and purified, as described earlier (19).

Preparation of neutrophils and monocytes

Human neutrophils and monocytes were prepared from healthy donors as described (9). Briefly, human neutrophils were prepared using dextran sedimentation, centrifugation through Ficoll-Paque (Amersham Pharmacia Biotech), and hypotonic lysis of contaminating erythrocytes. For monocytes separation, first PBMCs were isolated by centrifugation on Ficoll-Paque solution and laid on Percoll 46% vol/vol solution (Amersham Biosciences) in RPMI 1640–10% FCS and 4 mM HEPES. Monocytes were harvested, resuspended in medium–2% FCS, and separated from contaminating lymphocytes by adherence (1 h at 37°C) to plastic. Adherent monocytes were extensively washed with medium to remove residual non-adherent cells. The percentages of CD14+ cells were greater than 98%. Neutrophils and monocytes were cultured in RPMI-1640 10% FCS, in the presence of 5 μg/ml HP0175, or PBS, as control. When required, cells were preincubated 2 h with 20 μg/ml of anti-TLR2 blocking mAb or anti-TLR4 blocking mAb before HP0175 exposure.

Real-time PCR analysis

Total RNA was isolated from 2 × 10^6 monocytes or neutrophils using TriZOL solution (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed and amplified with the following primers: 5'-AGCAACAGGGTGGTGGAC-3' and 5'-GTGTGGTGGGGGACTGAG-3' for GAPDH; 5'-TCCACCAGGGTCTGATTTTT-3' and 5'-TTGAAGCGGAGAAGGAGACG-3' for IL-23p19; 5'-ACAAAGGAGGCGAGGTTCTAA-3' and 5'-CCCTTGGGGGTCAGAAGAG-3' for IL-12p40; 5'-ATGGCCCTGTGCCTTAGTAGT-3' and 5'-CGGTTCTTCAAGGGAGGATTTT-3' for IL-12p35; 5'-AACCTGAACCTTCCAAAGATGG-3' and 5'-TCTGGCTTGTTCCTCACTACT-3' for IL-6; 5'-AGTGGTTGAGCCGTGGAG-3' and 5'-CCATGAGAAGCAGGAAAGG-3' for TGF-b; 5'- CTGTCCTGCGTGTTGAAAGA-3' and 5'-TTGGGTAATTTTTGGGATCTACA-3' for IL-1b. After the amplification, data analysis was performed using the second derivative method algorithm. For each sample, the amount of cytokine mRNA (IL-23p19, IL-12p40, IL-6, TGF-b and IL-1b) was expressed as *n*-fold of the normalized amount of mRNA from untreated cells (1 AU = mRNA cytokine concentration [fmol/μl]/mRNA GAPDH [fmol/μl]).

Detection of IL-23, IL-6, TGF-β, IL-1b in culture supernatants

Culture supernatants of neutrophils and monocytes, harvested for mRNA quantification, were collected at the same time points, and the amount of IL-23 for both cell type and IL-6, TGF-b and IL-1b for monocytes was quantified by ELISA (BioSource International).

Engagement of Toll-like receptor 4 (TLR-4) by HP0175 and its involvement in the production of interleukin-23 (IL-23)

To this aim, HEK 293 cells expressing either TLR-2 or TLR-4 were incubated with HP0175 and the NFkB activation was evaluated. As positive controls, *Listeria monocytogenes* was used for TLR-2-expressing cells and lipopolysaccharide (LPS) was used for TLR-4–expressing cells. Mock cells represented the negative control. Parallel culture samples of hTLR4-transfected HEK 293 cells were stimulated with graded concentrations of HP0175, or LPS, or *L. monocytogenes*. The NF-kB activation in each sample was quantified as OD values after 24 h of stimulation. We found a dose-dependent effect on TLR4, but not on TLR2, after stimulation with HP0175 (18). Furthermore, monocytes were either not preincubated or were preincubated for 2 h with 20 μg/ml of an anti-TLR-4 blocking antibody and then exposed to HP0175. After 6 h or 24 h, cells were harvested, and levels of mRNA for IL-23p19 were quantified by real-time polymerase chain

reaction analysis. Results were expressed in arbitrary units (AU). The 24-h culture supernatants from the same cells used for mRNA analysis were collected and analyzed by enzyme-linked immunosorbent assay for levels of the secreted IL-23 cytokine.

Monocyte flow cytometry analysis

Monocytes (2×10^6 per well) were seeded with HP0175 (5 μg/ml) in 24-well plates in RPMI 1640–10% FCS. At 7 days, cells were harvested and labeled with specific antibodies after saturation with human serum. All samples were analyzed by a BD FACSCalibur. Log MFI values were obtained by subtraction of the MFI of the isotype control from the MFI of the positively stained sample. To evaluate whether the differences between the peaks of cells were statistically significant with respect to control, the Kolmogorov-Smirnov test for analysis of histograms was used, according to the CellQuest software guide (BD). Flow cytometry data were expressed as means ± SEM. Values were compared by ANOVA test, with *P* values less than 0.05 considered significant.

Statistical analyses

Data are means ± SD. Statistical comparisons were performed using Student's *t*-test, Mann-Whitney test or the Wilcoxon test, as appropriate. Differences were considered as statistically significant when $P < 0.05$.

RESULTS

HP0175 activates neutrophils and monocytes to release cytokines, such as IL-23, which are essential for the differentiation of Th17 cells

To evaluate the role of HP0175 in human immune response, we firstly evaluated which cytokines it was able to induce in cells of innate immunity. IL-23 is a heterodimeric cytokine sharing the p40 subunit with IL-12, and having a distinct p19 subunit. IL-23 is an essential survival factor for Th17 cells, as these cells are not present in mice lacking IL-23 (20). Fig. 1 shows that HP0175 was able to induce monocytes and neutrophils to express both IL-12p40 and IL-23p19 (Fig. 1, A-C, B-D). The kinetics of expression of the two subunits were consistent with those of protein accumulation in culture supernatants (Fig. 1, E and F). Furthermore, IL-12p35 mRNA (73±22AU) was also expressed by monocytes after 6-h stimulus with HP0175.

HP0175 promotes IL-23 via TLR4 signalling

To investigate the mechanism of the HP0175 activation of monocytes, we evaluated the possibility that the effects of HP0175 on innate cells was mediated by TLR activation. The IL-23 production induced by HP0175 on monocytes was not influenced by treatment with an anti-TLR-2 blocking mAb while it was abrogated by an anti-TLR-4 blocking mAb, thus demonstrating that IL-23 secretion followed binding to TLR-4 (Fig. 2, A and B).

HP0175 promotes the production of IL-6, IL-1b and TGF-b

Recent findings suggest that IL-23 is not required during the early differentiation of Th17 cells, whereas IL-6 is the crucial factor together with TGF-b and IL-1b; IL-23 would serve to expand previously differentiated Th17 cells (14). Accordingly, the mRNA and protein amounts of these cytokines were determined after HP0175 stimulation of monocytes and neutrophils. HP0175 elicited a 700-fold induction of IL-6 mRNA after 12 h of activation and high levels of IL-6 were released in culture supernatants of both monocytes and neutrophils (Fig. 3, C and D). HP0175 also induced a high expression of IL-1b m-RNA in both monocytes and neutrophils. Induction was observed as early as 0.5 h and peaked after 24 h, leading to accumulation of IL-1b protein in the supernatant (Fig. 3, A and B). Furthermore, TGF-b was found to significantly increase in both monocytes and neutrophils following HP0175 stimulus (Fig. 3, E and F).

These findings indicate that HP0175, acting on both monocytes and neutrophils, contributes to create a cytokine milieu enriched in IL-23, IL-6, TGF-b, and IL-1b with a strong potential of driving the differentiation of T cells towards the Th17 subset.

HP0175 promotes MHC class II upregulation in monocytes

While control monocytes incubated with medium progressively died within 36 hours of culture, monocytes incubated with HP0175 survived longer. In addition, HP0175–treated monocytes revealed a progressive shape modification and a tendency to cluster and to detach from the substrate. In order to better characterize these observations, the expression of maturation markers in HP0175–stimulated monocytes was evaluated. Compared with the corresponding baseline values, the expression of

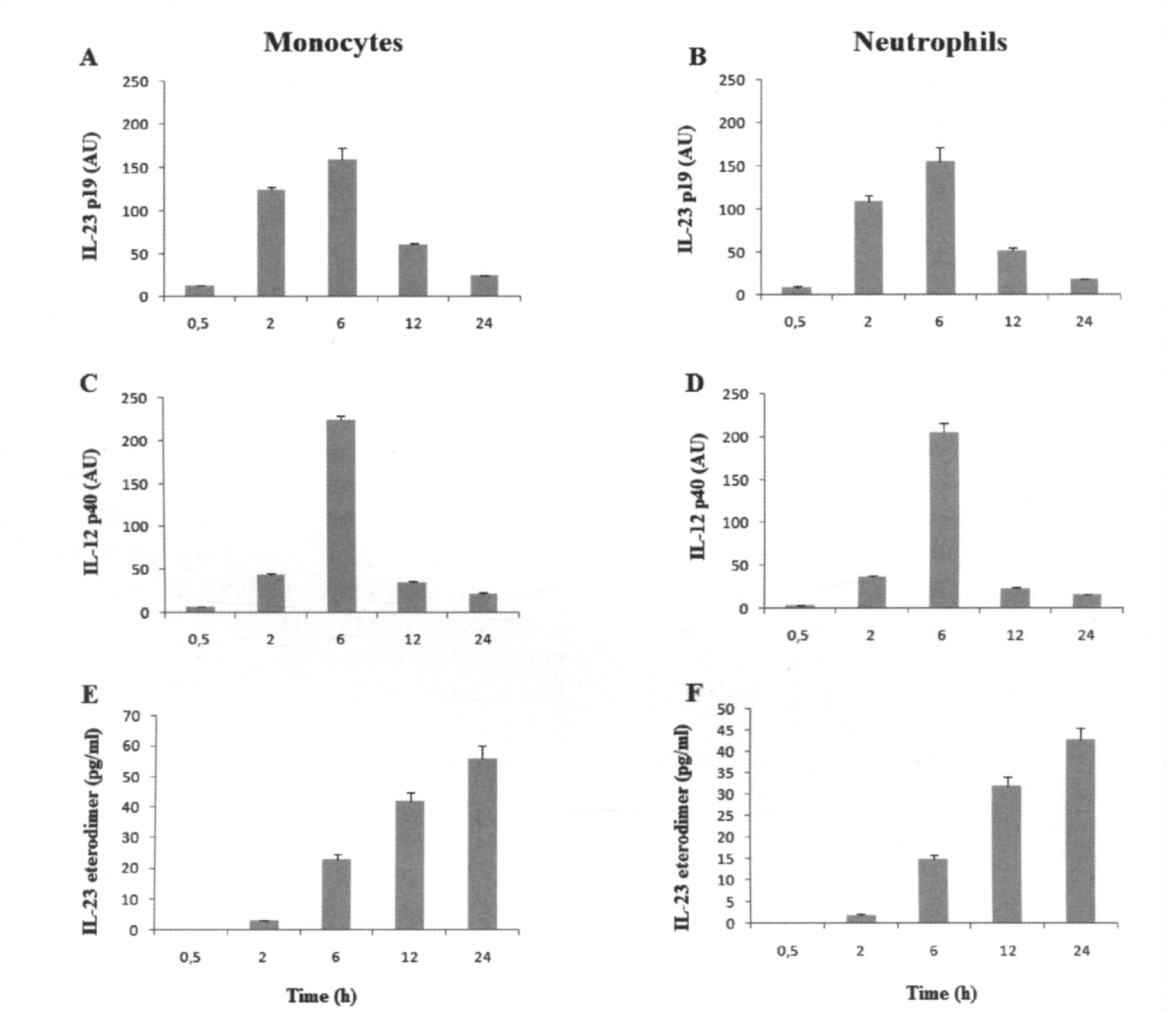

Fig. 1. *Kinetics of the synthesis and production of interleukin-23 (IL-23) by monocytes and neutrophils stimulated with HP0175. Levels of IL-12p40 (**A** and **B**) and IL-23p19 (**C** and **D**) cytokine mRNA in monocytes and neutrophils were determined at the indicated time points after HP0175 stimulation, by quantitative real-time polymerase chain reaction analysis. Representative results are shown from 1 of 4 experiments conducted with different cell preparations, after the subtraction of basal mRNA and protein levels of cytokines at the different time points. Values are the mean and SD arbitrary units (AU). Levels of IL-23 protein in monocytes (**E**) and neutrohils (**F**) from healthy donors were determined at the indicated time points after HP0175 stimulation by ELISA of culture supernatants from the same cells that had been harvested for mRNA evaluation. The kinetics of production were comparable among the different experiments, whereas the amounts produced varied among the different donors. Values are the mean and SD of 4 independent experiments using triplicate samples.*

HLA-DR increased markedly [mean fluorescence intensity (MFI) 782±34 *vs* 58±4.6] at day 7 of incubation with HP0175 (Fig. 4). Likewise, also the expression of B7-RP1, B7-H1, CD80, CD83 and CD 86 was up-regulated by incubation with HP0175 for 7 days, compared to untreated cells (Fig. 4).

DISCUSSION

The HP0175 is a peptidyl-prolyl cis-trans isomerase (PPIase) secreted protein of *H. pylori* (17). It is one of the bacterial antigens recognized by sera of *H. pylori*-infected patients (18). HP0175

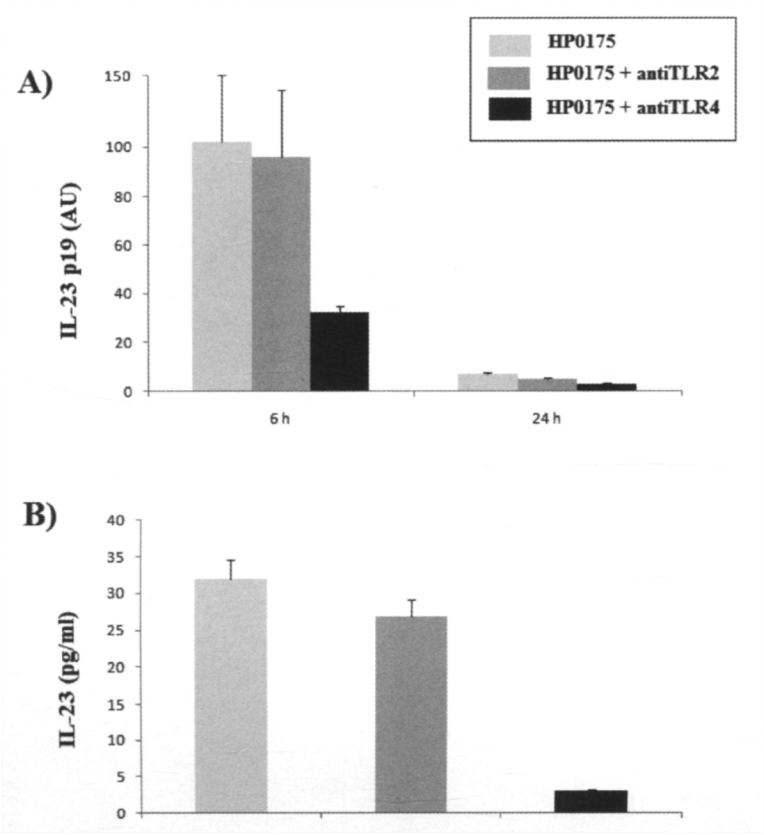

Fig. 2. *Involvement of Toll-like receptor 4 (TLR4) in the HP0175-induced monocyte production of interleukin-23 (IL-23). Monocytes were either not preincubated or were preincubated for 2 h with 20 μg/ml of an anti–TLR4 or anti-TLR2 blocking antibody and then exposed to HP0175. After 6 h or 24 h, cells were harvested, and levels of mRNA for IL-23p19 (A) were quantified by real-time polymerase chain reaction analysis. Results are expressed in arbitrary units (AU). The 24-h culture supernatants from the same cells used for mRNA analysis were collected and analyzed by enzyme-linked immunosorbent assay for levels of the secreted IL-23 cytokine (B). Values are the mean and SD of 4 independent experiments using triplicate samples.*

is able to activate both epidermal growth factor receptor and NF-kB via IkBa pathway. TLR4-dependent mitogen- and stress-activated protein kinase 1 (MSK1) activation by HP0175 resulted in chromatin modification and activation of several transcription factors that are likely to contribute to the ultimate outcome of the infection (18). The present work reports several major findings that are relevant to *H. pylori* infection: a) HP0175 induces IL-23 production in neutrophils and monocytes, and b) it promotes IL-23 production via TLR4 signalling; and c) it promotes IL-1b, TGF-b, and IL-6 in monocytes.

Here we report that HP0175 activates neutrophils and monocytes to increase mRNA expression and secretion of IL-23, IL-1b, TGF-b and IL-6, proteins, which are crucial for the induction of Th17 responses (14). These findings do not exclude the possibility that additional bacterial factors contribute to the generation of a pro-Th17 milieu in *H. pylori* infection. Within the inflammatory cytokines, IL-1b has been shown to exert important effects on gastric epithelial cells, that might lead to activation of oncogenes and finally to gastric cancer (21, 22). HP0175 is a TLR4 agonist able to activate NF-κB in HEK TLR4-transfected cells, and the involvement of TLR4 receptor in the activation of monocytes by HP0175 is also supported by the abrogation of IL-23 expression by a specific anti-TLR4 blocking antibody. Although IL-23 has been shown in the past to have anti-cancer properties (23, 24), emerging studies demonstrated that expression of IL-23 is increased in human tumours and foresaw a role

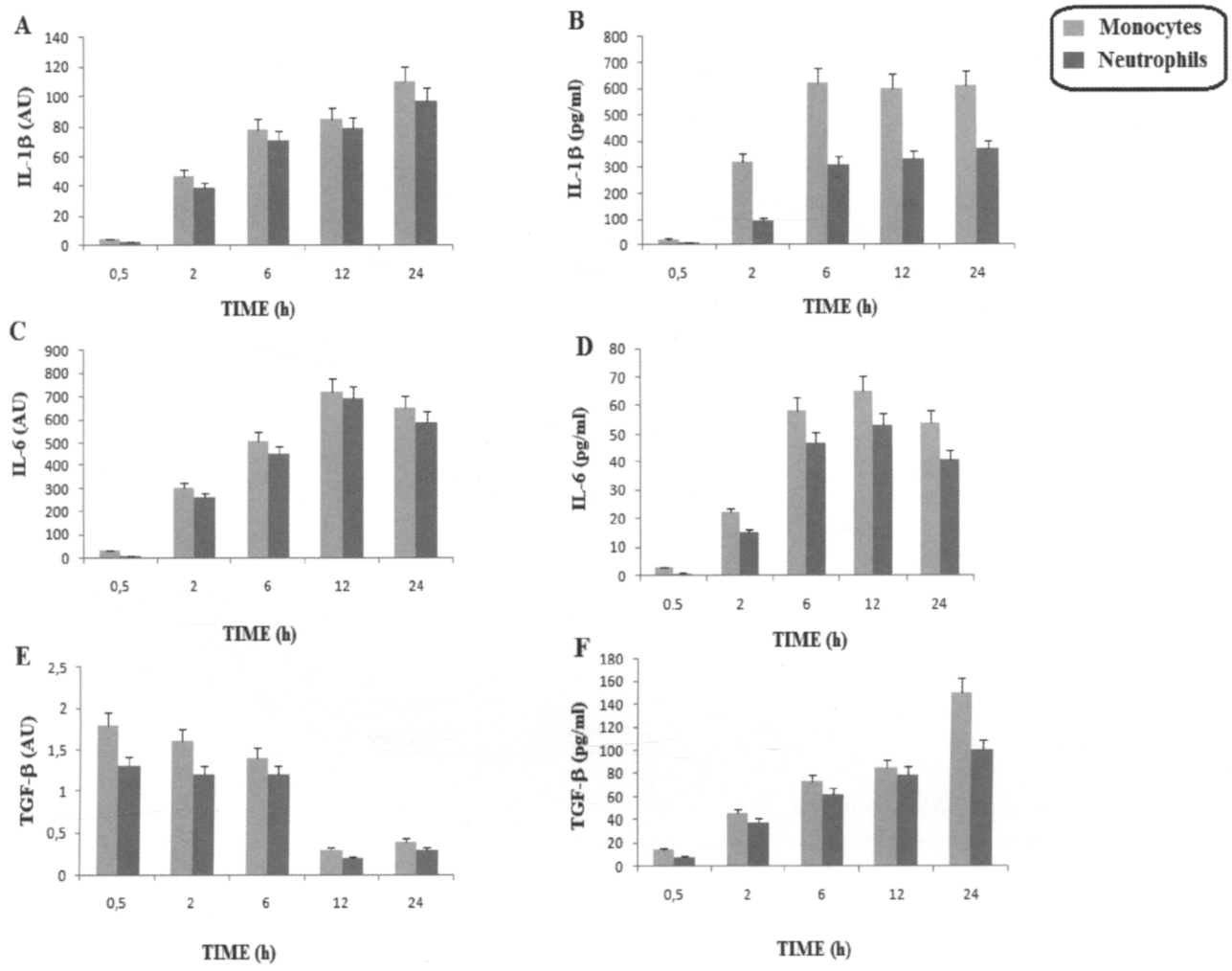

Fig. 3. *Kinetics of the synthesis and production of IL-1β, IL-6, and TGF-β, by monocytes and neutrophils stimulated with HP0175. Levels of IL-1β, IL-6, and TGF-β mRNAs (**A**, **C**, and **E**) were determined by quantitative real-time polymerase chain reaction at the indicated time points after HP0175 stimulation. AU = arbitrary units. Levels of IL-1β, IL-6, and TGF-β proteins (**B**, **D**, and **F**) were determined by ELISA at the indicated time points after HP0175 stimulation. Culture supernatants from the same monocytes and neutrophils that were harvested for mRNA evaluation were used for these analyses. Values are the mean and SD of 4 independent experiments using triplicate samples.*

of IL-23 in the carcinogenesis of cancers (25, 26). Furthermore, it has been highlighted that IL-23 might represent an important molecular connection between the rise in tumor-associated inflammation and the lack of tumor surveillance by promoting angiogenesis and inflammatory responses, such as up-regulation of MMP9, but reducing cytotoxic T-cell infiltration (26). The transforming growth factor-β (TGF-β) and its signalling pathway play a critical role in the progression of human cancer and contribute to up-regulate VEGF production (27, 28, 29).

Taken together, the results obtained to date suggest that *H. pylori* HP0175 plays a major role in promoting a Th17 response in human *H. pylori* infection. Particularly, the present results demonstrate that the *H. pylori* HP0175 protein elicits a peculiar type of inflammation, rich in IL-23, IL-6, IL-1b and TGF-b, which, if long-lasting and unabated, may represent an immunopathological condition that

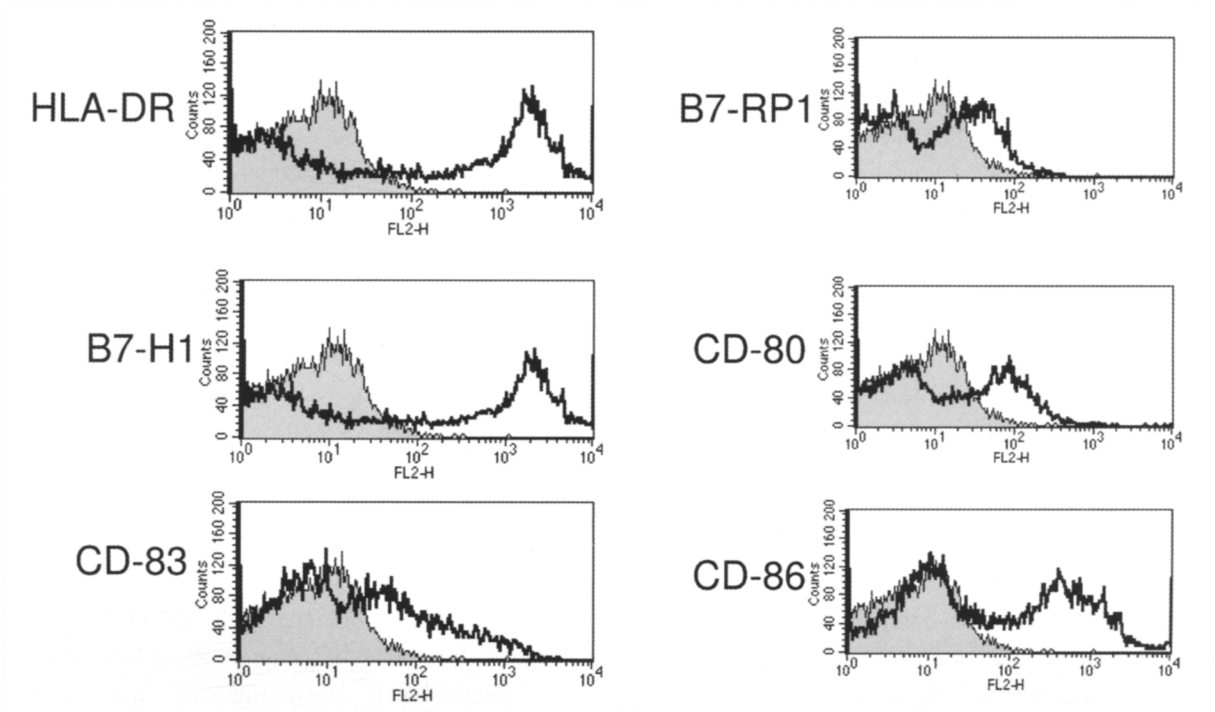

Fig. 4. *Flow cytometric analysis of HP0175–stimulated monocytes. Solid and dotted lines correspond to HP0175–treated monocytes and to isotype controls, respectively (representative result of 4 consecutive experiments).*

might pave the way to gastric cancer.

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