Immunoregulation of antigen presenting and secretory functions of monocytic cells by Helicobacter pylori antigens in relation to impairment of lymphocyte expansion

Eliza Mnich¹, Adrian Gajewski¹, Karolina Rudnicka¹, Weronika Gonciarz¹, Paweł Stawerski², Krzysztof Hinc³, Michał Obuchowski³ and Magdalena Chmiela¹

¹Division of Gastroimmunology, Department of Immunology and Infectious Biology, Faculty of Biology and Environmental Protection, University of Łódź, Łódź, Poland; ²Department of Pathomorphology, Medical University of Łódź, Łódź, Poland; ³Intercollegiate Faculty of Bacteriology UG&MUG, Department of Molecular Bacteriology, Gdańsk, Poland

The role of Helicobacter pylori (H. pylori) antigens in driving a specific immune response against the bacteria causing gastroduodenal disorders is poorly understood. Using a guinea pig model mimicking the natural history of H. pylori infection, we evaluated the effectiveness of immature and mature macrophages in promoting the blastogenesis of splenocytes from H. pylori infected and uninfected animals, in response to H. pylori antigens: glycine acid extract (GE), cytotoxin associated gene A protein (CagA), urease A (UreA) and lipopolysaccharide (LPS). Lymphocyte expansion was assessed in 72 h cell cultures, containing: immature or mature macrophages derived from bone marrow monocytes, unstimulated or stimulated with H. pylori antigens for 2 h. The proliferation was expressed as a ratio of [³H]-thymidine incorporation into DNA of antigen-stimulated to unstimulated cells and the DNA damage was determined by DAPI cell staining. TGF-β and IFN-γ were assessed immunoenzymatically in cell culture supernatants. Lymphocytes of control and H. pylori-infected animals proliferated intensively in response to phytohaemagglutinin (PHA) and in cocultures with immature or mature macrophages treated with CagA or UreA (significantly) and GE (slightly) excluding the cultures containing H. pylori or E. coli LPS. This lymphocyte growth inhibition was related to DNA damage of monocytic cells in response to H. pylori or E. coli LPS and secretion of regulatory TGF-β, but not proinflammatory IFN-γ. Impaired homeostasis of monocytic cell function related to DNA damage and TGF-β release, in response to H. pylori LPS may lead to the suppression of adaptive immune response against the bacteria and development of chronic infection.

Key words: H. pylori, antigen presenting cells, lymphocyte expansion

INTRODUCTION

Helicobacter pylori bacteria belong to the Gram-negative rods exhibiting affinity to gastric epithelial cells and adapted to life in acidic environment of human stomach (Marshall, 2001). A characteristic symptom of H. pylori infection is an excessive inflammatory response, which results in the development of pathological processes in the gastric epithelium such as erosions, ulcers, variation in the cell phenotype, excessive proliferation of these cells as well as secretion of proinflammatory cytokines. These bacteria have been classified as carcinogens (Israel et al., 2001; Versalovic et al., 2003; Chmiela & Michetti, 2006). Changes occurring in the gastric epithelium colonized by H. pylori lead to the penetration of H. pylori antigens into the basal membrane, where they interact with extracellular matrix proteins (ECM). The bacteria also interact with infiltrating immune cells via Pathogen Recognition Receptors (PRR) stimulating them to cytokine secretion or can even enter the bloodstream (Dubreuil et al., 2002, Wessler & Backert, 2008). In most cases H. pylori colonizes the stomach for a long time before symptoms appear. It distinguishes these bacteria from other bacterial pathogens that cause acute infections. However, even in asymptomatic subjects, H. pylori induces histological gastritis, which is characterized by infiltration of gastric mucosa with the immune cells (Portal-Celhai & Perez-Perez, 2006). In Payer’s patches H. pylori antigens if translocated to a deeper parts of the gastrointestinal tract (Wisniewska et al., 2002), induce the processes of specific adaptive immune response. This response is conditioned by the ability of antigen presenting cells (APC), such as monocytes, macrophages and dendritic cells to present antigens to antigen specific T cells stimulating them to clonal expansion.

Disease development depends on multiple factors: bacterial virulence components, host susceptibility and environmental conditions (Suzuki et al., 2012). It is known that H. pylori is a genetically diverse species (Israel et al., 2001; Chmiela & Michetti, 2006; Blaser & Berg, 2001). H. pylori...
H. pylori strains isolated from different hosts possess various virulence capacities, and their genetic diversity may appear also within the gastric niche of a single human host. Severity of diseases depends on specific H. pylori protein virulence factors such as CagA and vacuolating cytotoxin – VacA (Blaser & Berg, 2001). Other well-characterized H. pylori virulence factors include: urease (Dunn et al., 1990; Labigne et al., 1991), several outer membrane proteins (Omp), for instance Hop proteins (Hsp70-Hsp90 Organizing HSP) (Evans et al., 2000) and blood antigen binding adhesins (Boren et al., 1993). However, on the basis of a proteomics study it was calculated that more than 1200 proteins could be involved in the disease development (Rain et al., 2001; Xiyuing, 2013). The role of most H. pylori compounds during the course of H. pylori related diseases still needs to be clarified especially due to the fact that different H. pylori proteins may interact with a variety of host proteins and be involved in a number of pathogenic signalling pathways. It is supposed that the chronic nature of H. pylori infections may result from the dominance of antigens negatively modulating the course of immune processes.

The aim of this study was to evaluate the effectiveness of antigen presenting cells of monocytic lineage, including immature and mature macrophages, in promoting the blastogenic response of lymphocytes in response to well-characterized H. pylori antigens such as: glycine acid extract containing surface compounds, CagA protein, UreA and LPS. We used a guinea pig model, which allows following the H. pylori infection induced by the oral administration of these bacteria. We measured the blastogenic response of spleen derived T lymphocytes in 72 h cell cultures containing immature or mature macrophages derived from bone marrow monocytes, pulsed for 2 h with H. pylori antigens, using the [H]-thymidine incorporation assay. The lymphocyte proliferating activity was referred to the cell DNA damage, which was visualized by 4',6-diamidino-2-phenyldole (DAPI) staining, and to the concentration of pro-inflammatory and anti-inflammatory cytokines such as interferon gamma (IFN-γ) and transforming growth factor beta (TGF-β), respectively, in the cell culture supernatants.

MATERIALS AND METHODS

Animals. For the experiments, adult, three-month-old, 400–600 g of weight male Himalayan guinea pigs were used. Animals were bred in the Animal House at the Faculty of Biology and Environmental Protection, University of Łódź (Poland), kept in cages with free access to drinking water and fed with standard chow. All animal experiments were approved by the Local Ethics Committee LKE9 (Decision LB 646/2012).

Bacteria. Helicobacter pylori reference strain CCUG 17874, positive for VacA and CagA was from the Culture Collection, University of Gothenburg (Gothenburg, Sweden). H. pylori bacteria were stored at –80°C in Tris-buffered saline (TBS) containing 10% glycerol. Before being used in the experiments H. pylori bacteria were grown for 5 days on modified Helicobacter agar (Becton Dickinson, Heidelberg, Germany) in microaerophilic conditions (Gas Pak, Becton Dickinson, Heidelberg, Germany), at 37°C. The bacteria were harvested by scraping from agar plates, suspended in 0.85% sodium chloride (NaCl), pelleted by centrifugation (4000×g, for 15 min), and then washed twice under the same conditions. The pellet of bacteria was suspended in 0.85% NaCl to obtain the inoculum containing 1×10⁹ colony forming units – CFU/ml according to the McFarland scale.

H. pylori antigens. Surface H. pylori antigens included into the GE were extracted from the reference H. pylori strain CCUG 17874 using 0.2 M glycine buffer, pH 2.2, as previously described (Rečchiński et al., 1997). The protein content in GE was 98.4% (NanoDrop 2000c Spectrophotometer, ThermoScientific, Waltham, MA, USA). The GE preparations contained <0.001 EU/ml of LPS, as shown by the chromogenic Limulus amebocyte lysate test (Lonza, Braine-Alleud, Belgium). GE antigen was included in a further study at protein concentration of 10 µg/ml. Recombinant CagA protein (rCagA) from IRIS, Siena, Italy was used at the concentration of 1 µg/ml. The H. pylori UreA subunit was amplified by a polymerase chain reaction (PCR) as previously described (Hnc et al., 2010), and used as a homologue of H. pylori UreA protein at the concentration of 5 µg/ml. The antigen concentrations were adjusted experimentally (UreA) or adopted from previously performed experiments (Miszczyk et al., 2014). The LPS from the reference strain of H. pylori CCUG 17874 (courtesy of AP. Moran) was prepared by hot phenol-water extraction after pretreatment of the bacterial biomass with protease. Then, the crude LPS preparation was purified by RNase, DNase and proteinase K treatment and by ultracentrifugation, as previously described (Moran et al., 1992). The E. coli LPS derived from O55:B5 strain (Sigma, St. Louis, MI, USA) was used as control.

H. pylori infection in guinea pigs. The animals (8) were divided into two groups. Group 1 consisted of 4 guinea pigs, which were inoculated per os three times (at two-day intervals) with 1 ml of sterile 0.85% NaCl, using a feeding needle (control group). Group 2 consisted of 4 animals, which were orally inoculated, three times (at two-day intervals), with 1 ml of freshly prepared suspension of H. pylori (10⁹ CFU/ml). Before administration of NaCl or H. pylori, the animals obtained orally 1 ml of 0.2 M NaHCO₃ to quickly neutralize the acidic pH of the stomach. Guinea pigs of both groups were euthanized 28 days after the last H. pylori challenge, on the basis of the Local Ethics Committee consent. Spleens were collected for isolation of splenocytes and enrichment of cell suspension with T lymphocytes.

Assessment of the H. pylori status. The H. pylori infection in guinea pigs was confirmed by the detection of Helioabacter-like organisms (HLO) in thin layer sections of the stomach tissue, which were stained by routine histological procedure with the Giemsa stain solution and analysed according to the Sydney scale using a light microscope and immunofluorescence staining of mucus smears with rabbit anti-H. pylori antibodies conjugated with fluorescein isothiocyanate (FITC) as previously described (Miszczyk et al., 2014). The cell imaging was performed using a JuLi Smart fluorescent cell analyzer (Digital Biotechnology, Boston, USA). The occurrence of H. pylori in the gastric mucosa was also confirmed by the PCR amplification of DNA using primers for the genes encoding CagA protein and UreC subunit of H. pylori urease. The gastric tissues from antrum area (200 mg) of H. pylori infected animals, 7 days and 28 days post inoculation, were homogenized and used for the extraction of total DNA (DNA-GenomicMini, A&A Biotechnology, Gdansk, Poland). The samples were used for the amplification of specific H. pylori ureC and cagA genes by a PCR. The reaction mixture contained equivalent amounts of DNA sample, sets of primers derived...
from the nucleotide sequence of *H. pylori* ureC gene and *cagA* (Oligopl, IBB PAN, Warsaw, Poland; Table 1), the DNA Taq polymerase and deoxynucleoside triphosphates — dNTP (Promega, Madison, USA).

The PCR amplified products: 294 bp (nucleotides 1–294) and 298 bp (nucleotides 1751–2048) of the *ureC* gene and *cagA* gene, respectively, were electrophoresed on ethidium bromide-supplemented gels and visualized by UV illumination. DNA isolated from the reference *H. pylori* strain CCUG 17874 was used as positive control. The assay was calibrated by the addition of a set number of bacterial cells to gastric tissue samples from *H. pylori* uninfected animals. The PCR detection limit was 10⁸ bacteria. The visualization of *ureC* and *cagA* amplification products from the gastric tissues of infected guinea pigs is shown in Fig. 1.

**Bone marrow derived immature and mature macrophages.** Bone marrow macrophages were isolated from tibias and femurs using complete RPMI-1640 culture medium (cRPMI, containing 10% FCS, and standard antibiotics), washed twice, and pelleted by centrifugation. The cells were suspended in 1 ml of cRPMI and attached to cell culture Petri dishes for 1 h, 37°C, 5% CO₂. Non-adherent cells were washed out, and immature macrophages were detached for 10 min, 37°C, 5% CO₂, with phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺, containing 0.5 mM EDTA. After washing twice, fresh macrophages were adjusted to the density of 5 × 10⁶ cells/ml in cRPMI and used for further experiments. For some experiments, adherent fresh immature macrophages, in 200 µl of cRPMI/well, were allowed to mature for 96 hours with half a volume of cRPMI exchange on the 3rd day of the incubation time.

**Separation of splenocytes and enrichment of cell suspensions with T lymphocytes.** Isolated spleens were homogenized and splenocytes were washed in cRPMI by centrifugation (300 × g, 10 min, 20°C). Erythrocytes were lysed using a lysis buffer, and the remaining leukocytes were recovered by centrifugation. The cell viability was assessed by trypan blue exclusion and the suspensions of splenocytes were adjusted to the density of 1 × 10⁶ cells/ml in RPMI/5%FCS. Cell suspensions enriched with T lymphocytes were prepared by passing of the splenocyte suspensions, in a volume of 1 ml (1 × 10⁶ cells/ml) over nylon wool fiber columns, 45 min, at 37°C, 5% CO₂. Nonadherent T lymphocytes were washed out of the column with RPMI/5%FCS, assessed for viability, which was > 90%, and adjusted to the density of 2.5 × 10⁸ cells/ml in cRPMI. T lymphocytes were frozen in FCS/10%DMSO for 96 h, at −80°C, for the cultures of spleen lymphocytes with mature bone marrow macrophages. Before being used in cell culture experiments, the splenic lymphocytes were thawed and adjusted to the density of 2.5 × 10⁸ viable cells/ml in cRPMI.

**Co-cultures of bone marrow derived immature or mature macrophages with autologous T lymphocytes.** Two-stage cultures containing immature or mature bone marrow macrophages (incubated for 96 hours in cRPMI supplemented with β-mercaptoethanol, 37°C, 5% CO₂) and autologous T lymphocytes were prepared according to the following procedure: the immature or mature macrophages (5 × 10⁶ cells/ml), which were distributed into 96 well plates (100 µl/well) were stimulated for 2 h (37°C, 5% CO₂) with 5 µg/ml or 10 µg/ml of GE, 1 µg/ml of CagA, 5 µg/ml of UreA, 1 ng/ml or 25 ng/ml of *H. pylori* LPS or standard E. coli LPS. For studying the synergistic effects the following combinations of antigens were used: 1) *H. pylori* LPS (25 ng/ml) + CagA; 2) *H. pylori* LPS (25 ng/ml) + CagA + UreA + GE. The control for a cell proliferation assay was untreated cells. Then, the macrophages were washed out of stimulators. The immature macrophages were supplemented with freshly isolated autologous spleen T lymphocytes (2.5 × 10⁶ cells/ml), in the proportion 1:5, and the mature macrophages with thawed autologous lymphocytes. The cultures were supplemented with 100 µl of cRPMI or 2 µg/ml PHA, and incubated for further 72 h, at 37°C, 5% CO₂. At 18 h before the end of cultivation, 1 µCi of [³H]-thymidine was added to each well to estimate cell proliferation. All cell cultures were settled in triplicate.

**Measurement of cell proliferation.** The incorporation of thymidine was measured using a MicroBeta 2 scintillation counter (Wallac, Oy, Turku, Finland) after harvesting the cells on fibre filters. All cultures were settled in 3 repeats. The results were expressed as mean cpm/culture ± standard deviation (S.D.). The stimulation index (SI), expressing the relative cpm ratio, was calculated by dividing the cpm counts/
RESULTS

Blastogenic response of guinea pig splenocytes in the co-cultures with immature or mature bone marrow derived macrophages pulsed with \textit{H. pylori} antigens

Data in Fig. 2 show the proliferative activity of T lymphocytes isolated from uninfected and \textit{H. pylori} infected guinea pigs in co-cultures with immature or mature macrophages, non-stimulated or stimulated for 2 h with \textit{H. pylori} glycine acid extract (GE), UreA, CagA, LPS, the mixture of \textit{H. pylori} LPS (25 ng/ml) and CagA or \textit{H. pylori} LPS (25 ng/ml), CagA, UreA and GE. Control stimulators such as \textit{E. coli} LPS and phytohaemagglutinin (PHA) were used. The proliferating activity of lymphocytes was evaluated on the basis of \textsuperscript{3}H-thymidine incorporation. The stimulating index (SI) was calculated by dividing the radioactivity counts (cpm) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 alone. Shown are SI ± S.D. * \( p=0.03 \) vs untreated cells.

**Figure 2.** \textit{H. pylori} antigen — driven proliferative response of lymphocytes in co-cultures of spleen lymphocytes T and immature or mature macrophages.

The proliferative activity of T lymphocytes isolated from uninfected and \textit{H. pylori} infected guinea pigs was estimated in co-cultures with immature or mature macrophages, non-stimulated or stimulated for 2 h with \textit{H. pylori} glycine acid extract (GE), UreA, CagA, LPS, the mixture of \textit{H. pylori} LPS (25 ng/ml) and CagA or \textit{H. pylori} LPS (25 ng/ml), CagA, UreA and GE. Control stimulators such as \textit{E. coli} LPS and phytohaemagglutinin (PHA) were used. The proliferating activity of lymphocytes was evaluated on the basis of \textsuperscript{3}H-thymidine incorporation. The stimulating index (SI) was calculated by dividing the radioactivity counts (cpm) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 alone. Shown are SI ± S.D. * \( p=0.03 \) vs untreated cells.

min for the cell cultures with a stimulator by the cpm counts/min for the cell cultures without a stimulator. The SI values higher than or equal to 1.5 were considered as a positive result in the proliferation assay.

**DAPI staining of cell nuclei.** Damage to the cell nuclei was considered as sign of apoptosis, which was determined by staining the cells with DAPI (Sigma, St. Louis, MI, USA), a fluorescent dye which has a strong affinity to the AT base pair in DNA. The cells were fixed with 4% formaldehyde, and stained with DAPI solution (2.5 µg/ml) for 15 minutes at room temperature. Preparations were viewed under a fluorescent microscope at a wavelength of 358 nm (excitation) and 461 nm (emission). We evaluated the percentage of the cells with damaged nucleus.

**ELISA assays for the assessment of IFN-γ and TGF-β concentration.** IFN-γ and TGF-β levels were estimated in the cell culture supernatants of immature or mature macrophages stimulated for 2 h with \textit{H. pylori} antigens as well as in the cell culture supernatants from macrophage co-cultures with lymphocytes using commercially available ELISA assays (MyBioSource, San Diego, USA) with the sensitivity of 5 pg/ml for both cytokines.

**Statistical analysis.** The Statistica 10 PL program with non-parametric tests was used: the Mann-Whitney U test (for impaired data) to verify the hypothesis that two compared samples came from two statistically different populations.
or mature macrophages isolated from *H. pylori* infected animals, stimulated for 2 h before being used in the proliferation assay with *H. pylori* LPS or *E. coli* LPS at a concentration of 25 ng/ml, the lymphocyte blastogenic activity was significantly diminished as compared to the activity of lymphocytes in the cell cultures containing non-treated macrophages. Lymphocyte proliferation was also inhibited in the cell cultures containing T lymphocytes from *H. pylori* infected animals and immature or mature macrophages, which were pulsed with the composition of *H. pylori* antigens containing *H. pylori* LPS (25 ng/ml) and CagA (1 µg/ml) or *H. pylori* LPS (25 ng/ml), CagA (1 µg/ml), UreA (5 µg/ml) and GE (10 µg/ml).

In the cell cultures containing lymphocytes isolated from *H. pylori* uninfected guinea pigs and immature macrophages pretreated with GE, CagA or UreA the proliferative response was only slightly increased and sustained in the cell cultures containing macrophages pulsed with low dose of LPSs. There was no enhancement of blastogenic response of control guinea pig lymphocytes in their co-cultures with mature macrophages exposed to GE, CagA, UreA and LPSs used in a concentration of 1 µg/ml. However, similarly as in the case of lymphocytes derived from *H. pylori* infected guinea pigs, the proliferative response of lymphocytes isolated from control animals and co-cultured with both immature or mature macrophages pulsed with higher dose of LPSs alone or their mixture with CagA, UreA and GE was significantly inhibited.

The concentration of IFN-γ and TGF-β in the cell culture supernatants of immature or mature macrophages alone pulsed with *H. pylori* antigens.

Guinea pig macrophages, uninfected or infected with *H. pylori*, were pulsed for 2 h with *H. pylori* glycine acid extract (GE), CagA, UreA, *H. pylori* LPS, *E. coli* LPS or left unstimulated (RPMI-1640 alone). The commercial ELISA kit assay was used to detect the concentration of transforming growth factor beta (TGF-β), derived from immature or mature macrophages (*p*<0.05 vs untreated cells).

**Assessment of monocytic cell nuclei damage in the milieu of *H. pylori* antigens**

The cytotoxic activity of bacterial agents could be a possible reason for the dysfunction of monocytic cells as APCs. One of the cytotoxic effects may be damage to the cellular genetic material. We estimated the percentage of both immature and mature guinea pig macrophages with damaged cell nuclei in the cell cultures carried out in the presence or absence of *H. pylori* antigens using a DAPI staining assay. The images of control cells and the cells with defective cell nuclei are visualized in Fig. 5. As shown in Fig. 6 the percentage of both immature and mature macrophages from uninfected and *H. pylori* infected guinea pigs did not differ significantly as compared to the control (cell cultures in RPMI-1640 medium alone). By comparison, the percentage of cells with affected morphology of cell nuclei significantly increased in the cell cultures carried out in the presence of *H. pylori* LPS as well as *E. coli* LPS at a concentration of 25 ng/ml. Such changes were observed in cell cultures of immature and mature macrophages from both control and *H. pylori* infected animals. However, mature macrophages of control animals and in particular *H. pylori* infected guinea pigs, were less sensitive to the damaging effect of LPSs.
Figure 4. The concentration of IFN-γ and TGF-β in the mixed cultures of spleen lymphocytes and immature (A) or mature (B) macrophages pulsed with *H. pylori* antigens with lymphocytes in relation to lymphocyte blastogenic response.

Macrophages isolated from guinea pigs uninfected or infected with *H. pylori*, were pulsed for 2 h with *H. pylori* glycine acid extract (GE), CagA, UreA, *H. pylori* LPS, *E. coli* LPS or left unstimulated (RPMI-1640 alone), and then were co-cultured with T lymphocytes for 72 h. The commercial ELISA kit assay was used to detect the concentration of transforming growth factor beta (TGF-β) and interferon gamma (IFN-γ) derived from mixed cultures (*p* < 0.05 vs untreated cells).
DISCUSSION

The chronic nature of *H. pylori* infections makes it necessary to elucidate this condition. A possible reason for permanent *H. pylori* infections can be a modulation of the host immune cell function by various components of these bacteria. Previous studies showed that *H. pylori* surface antigens of the glycine acid extract of these bacteria potentiated the cytotoxic activity of human natural killer (NK) cells while LPS of *H. pylori* caused its inhibition (Rudnicka *et al*., 2012, 2015). Also, phagocytic properties of human peripheral blood polymorphonuclear leukocytes were reduced in the presence of *H. pylori* LPS (Grębowska *et al*., 2008). Phagocytes such as mac-
ropages not only eliminate infectious agents but also present their antigens to lymphocytes during the development of specific adaptive immunity, which is most effective in the elimination of pathogens. Guinea pigs are a convenient model for studying the development of specific adaptive immune response to *H. pylori* antigens. In our previous study we showed that lymphocytes isolated from mesenteric lymph nodes, particularly CD4^+^, but not lymphocytes obtained from peripheral blood responded by proliferation to *H. pylori* antigens included in GE (Miszczyk et al., 2014). D’elios *et al.*, showed that in humans with *H. pylori* related chronic gastritis or MALT lymphoma, CD4^+^ T-cell clones, but not CD8^+^, proliferated to *H. pylori* antigens (D’elios *et al.*, 1999). The blastogenic activity of T lymphocytes in mesenteric lymph nodes was shaped by their interaction with antigen presenting cells and the cytokine balance. However, the spleen lymphocytes of *H. pylori* infected animals did not proliferate in the presence of macrophages pulsed with *H. pylori* GE, either immature or mature. To understand the role of various *H. pylori* antigens in the impairment of proliferative activity of guinea pigs’ splenocytes in this study, in addition to GE, we used well defined *H. pylori* antigens including: CagA, UreA and LPS. We focused on the blastogenic response of T lymphocytes derived from splenocytes in the co-cultures with immature or mature bone marrow derived macrophages pulsed with *H. pylori* antigens in relation to the concentration of immunomodulatory cytokines such as IFN-γ and TGF-β as well as to apoptosis signs in monocyte cells. The blastogenic response of T lymphocytes from *H. pylori* infected animals increased significantly when these cells were co-cultured with immature macrophages exposed to CagA and UreA but not to LPS. In the presence of immature or mature macrophages pulsed with *H. pylori* LPS or with *E. coli* LPS, prior to the proliferation assay, the blastogenic response of lymphocytes was impaired as compared to their natural activity in the culture medium alone. These relations were observed in the cell cultures of both *H. pylori* infected and uninfected animals. The proliferative response of lymphocytes derived from both groups of animals co-cultured with immature macrophages pulsed with GE was slightly enhanced. The slightly enhanced proliferation of lymphocytes derived from control guinea pigs might reflect a cellular response to components of *H. pylori* antigens. Cross-reacting antigens were identified in *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Haemophilus influenzae* (Johansen *et al.*, 1995). Although guinea pigs do not get naturally infected with *H. pylori*, the knowledge on the colonization with other *Helicobacter* species is insufficient.

It is interesting that only CagA and UreA, but not GE complex, were able to promote the lymphocyte proliferation. D’elios *et al.* showed that composition of intracellular and surface *H. pylori* antigens induce blastogenic response of T lymphocytes more effectively (D’elios *et al.*, 1999). Our results suggest that CagA and UreA are immunodominant antigens. It is also possible that in vivo exposure of animals to *H. pylori* antigens was too short or that the availability of *H. pylori* antigens for the stimulation of immune processes from the surface of the gastric mucosa was insufficient. Other than in the spleen may also be a location of sensitized lymphocytes capable of clonal expansion in response to *H. pylori* antigens. Previously it was shown that lymphocytes of guinea pigs infected with *H. pylori*, which proliferated in the milieu of GE antigens were deposited in the mesenteric lymph nodes (Miszczyk *et al.*, 2014). Another study showed that antigen-specific lymphocytes from the patients infected with *H. pylori* were accumulated in the basal membrane of the gastric mucosa (Quiding-Jarbrink *et al.*, 2001).

In this study we showed that only immature macrophages pulsed with CagA or UreA were able to promote the lymphocyte proliferation. It cannot be excluded that in the milieu of *H. pylori* antigens the maturation of macrophages and their ability to present antigens might be impaired. This may be due to the diminished expression of surface molecules involved in this process. In vitro priming of human peripheral blood mononuclear cells with LPS clearly down-regulated major histocompatibility complex class II (MHC) molecules and the costimulatory molecule CD86 (Wolk *et al.*, 2000). Our results indicate the ability of *H. pylori* LPS, similarly as *E. coli* LPS to affect the ability of both immature and mature macrophages to promote lymphocyte proliferation. Despite of LPS also VacA induces intracellular signaling and activates p38 in macrophages, which can play an important role in *H. pylori* pathogenesis (Boncristiano *et al.*, 2003).

Antigen presentation by monocyte cells and blastogenic response of lymphocytes are regulated by soluble immunomodulators such as IFN-γ and TGF-β regulating a diverse array of biological activities, including cell growth, the cell cycle, early development, differentiation, chemotaxis, hematopoiesis and immune functions (Massigne *et al.*, 2000). Hannon and Beach demonstrated that TGF-β1 exerts its growth inhibitory effects towards B lymphocytes through the downregulation of the activity of genes involved in cellular proliferation such as cyclin-dependent kinases (Hannon & Beach, 1994). The results of this study indicate the role of IFN-γ and TGF-β in the blastogenic response of lymphocytes to *H. pylori* antigens presented by both immature or mature macrophages. The elevation of IFN-γ in the mixed cultures of lymphocytes and immature macrophages pulsed with CagA or GE was linked with significantly or slightly increased lymphocyte proliferating activity, respectively. The presence of IFN-γ in these cell cultures may indicate the activation of both macrophages and antigen-specific lymphocytes. By comparison, the inhibition of lymphocytes proliferation in the cell cultures containing immature or mature macrophages pulsed with *H. pylori* or *E. coli* LPS was considered in relation to elevated levels of TGF-β with negative regulatory activity. Wallet *et al.* showed that TGF-β promotes the suppression of immature dendritic cell activation and maturation (Wallet *et al.*, 2005). One of the molecular targets of TGF-β-mediated suppression in dendritic cells appears to be the transcription factor RunX3 (Fainaru *et al.*, 2004). In our study this cytokine was produced by macrophages, as evidenced by the increase of TGF-β in the macrophage cultures in the presence of LPSs. However, this cytokine may also be delivered by regulatory lymphocytes in mixed cultures with macrophages. Such regulatory activity may result in downregulation of interleukin (IL)-2 production, which is an important lymphocyte growth factor. Previously we showed that exogenous IL-2 increased the prevalence of the proliferative response of primarily LPS non-responding lymphocytes from dyspeptic children infected with *H. pylori* although *H. pylori* LPS alone presented very weak if any capacity to stimulate human peripheral blood mononuclear cells (Rudnicka *et al.*, 2003). It was also showed that *H. pylori* LPS inhibited the cytotoxic activity of human NK cells to target HeLa cells, which was associated with increased cell population capable of producing regulatory IL-10, but not proinflammatory IFN-γ, promoting the cytotoxic activity of NK cells in the milieu of GE (Rudnicka *et al.*, 2003).
2015). Bryn and co-workers (Bryn et al., 2007) demonstrated that monocytes activated with E. coli LPS serotype O26:B6 suppressed T-cell immune responses and induced FOXP3+ T cells in a cyclooxygenase 2 (COX-2) — prostaglandin E2 (PGE2) — dependent manner and that PGE2 converted resting CD4+ T cells into CD25 FOXP3+ T cells with a suppressive phenotype. These processes were related to enhanced secretion of regulatory IL-10 but not TGF-β by monocytes in response to LPS. The differences in the cytokine secretion in response to LPSs observed in various models could be due to different sensitivity of monocyteic cells to LPSs as well as concentration and structure of LPS molecules. The complex strategy of H. pylori bacteria for the survival in the gastric mucosa of the host involves structural modifications of LPS lipid A to diminish its endotoxic properties and the expression and variation of Lewis determinants, arranged in O-specific chains of H. pylori LPS (Chmiela et al., 2014). Wolk et al., showed an impact of phenotypic alterations on the auxiliary function of monocytes, in the presence of bacterial LPS, which was linked to the diminished lymphocyte proliferation and IFN-γ production (Wolk et al., 2000). The importance of TGF-β as a modulator of epithelial cell homeostasis in the gastrointestinal tract exposed to LPS was suggested by Nguyen (Nguyen et al., 2015). Using a model of necrotizing enterocolitis (NEC) in young mice they demonstrated that TGF-β of dietary or endogenous origin may regulate the responses of immature intestinal epithelial cells against LPS stimuli, thereby supporting cellular homeostasis and innate immunity in response to bacterial colonization, and the first enteral feeding in early life. A similar mechanism may be activated in macrophages exposed to LPS in order to protect against inflammatory disorders.

We considered the cytotoxic effect of H. pylori antigens towards APCs as a possible reason for diminished lymphocyte proliferation. Damage to the cell structure or dysfunction of cell viability can occur due to cell lysis or apoptosis, which is associated with damage to the cell genetic material. By DAPI staining we showed an increased percentage of both immature and mature macrophages with altered morphology of the cell nuclei equal to the apoptosis symptoms as a result of the cell exposure to H. pylori LPS or standard E. coli LPS. Less sensitive to LPS were mature macrophages from normal animals inoculated with H. pylori. This may indicate a previous cell response in vivo, in terms of the intensification of repair processes. It is possible that impaired ability of T lymphocytes to multiply in the presence of macrophages pulsed with H. pylori LPS or E. coli LPS might be related to the intensity of TGF-β production and induction of apoptosis in monocyctic cells. It is possible that LPS may provide the apoptosis signal. However, since macrophages have receptors for TGF-β, this might promote an autocrine signaling. In the studies on the impact of TGF-β on lung fibrosis it was shown that TGF-β plays a pivotal role as a potent inducer of apoptosis in alveolar epithelial cells possibly due to the induction of reactive oxygen species as part of its signalling pathway (Fernandez & Eickelberg, 2012). It is worth mentioning that macrophages undergoing apoptosis in response to LPS may deliver a signal to viable cells to produce TGF-β for downregulation of inflammatory cytokine production (Monks et al., 2005). The negative correlation between the type B inflammation and responsiveness of lymphocytes to H. pylori LPS was observed. It might suggest that this molecule by modulation of immunocompetent cells may reduce gastric inflammation, decrease bate-
adhesins: review and colonization: mechanisms and clinical outcomes.


