ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 4/2015
641–650
http://dx.doi.org/10.18388/abp.2015_1045

Regular paper

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

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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 [^{3}H]-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 co-cultures 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

Received: 29 April, 2015; revised: 16 September, 2015; accepted: 05 October, 2015; available on-line: 03 November, 2015

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 (Wiśniewska *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. py-*

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*The results were presented at the 6th International Weigl Conference on Microbiology, Gdańsk, Poland (8–10 July, 2015).

Abbreviations: APC, antigen presenting cells; CFU, colony forming units; cpm, counts per minute; cRPMI, complete RPMI-1640 culture medium; CagA, cytotoxin associated gene A antigen; COX, cyclooxygenase; dGE, denatureted glycine acid extract; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; dNTP, deoxynucleoside triphosphates; ECM, extracellular matrix; EDTA, ethylenediaminetetraacteic acid; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GE, glycine acid extract; *H. pylori*, *Helicobacter pylori*; HLO, *Helicobacter*-like organisms; HSP, heat shock protein; IFN-γ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NaCl, sodium chloride; NEC, necrotizing enterocolitis; NK, natural killer cells; Omp, outer membrane protein; PGE, prostaglandin; PRR, Pathogen Recognition Receptors; PBS, phosphate buffered saline; PHA, phytohaemagglutinin; PCR, Polymerase Chain Reaction; S.D., standard deviation; SI, stimulation index; TBS, Tris-buffered saline; TGF-β, transforming growth factor beta; UreA, subunit A of urease; UreC, subunit C of urease; VacA, vacuolating cytotoxin

lori 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; Xiuying, 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 [^{3}H]-thymidine incorporation assay. The lymphocyte proliferating activity was referred to the cell DNA damage, which was visualized by 4',6-diamidino-2-phenylindole (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 Commitee LKE9 (Decision ŁB 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 1×10^{10} 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 (Rechciński *et al*., 1997). The protein content in GE was 98.4% (NanoDrop 2000c Spectrophotometer, ThermoScientific, Wlatman, WY, 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. acynonychis* UreA subunit was amplified by a polymerase chain reaction (PCR) as previously described (Hinc *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 purrified 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* (10^{10} CFU/ml). Before administration of NaCl or *H. pylori*, the animals obtained orally 1 ml of 0.2 M $NaHCO_3$ 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 *Helicobacter*-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

Table 1. The sets of primers and polymerase chain reaction (PCR) conditions used for *ureC* and *cagA* amplification, including products length.

Target	Sequences (5′-3′) (F: forward, R: reverse)	Annealing temperature & number of PCR cycles	Product length
ureC	F, 5 ′AAAGCTTTTAGGGGTGTTAGGGGTTT 3′ R, 5 ′AAGCTTACTTTCTAACACTAACGC 3′	55ºC 40	294 bp
cagA	F, 5′ ATAATGCTAAATTAGACAACTTGAGCGA 3′ R, 5′ TTAGAATAATCAACAAACATCACGCCAT 3′	60ºC 40	298 bp

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 samples were amplified through 40 consecutive cycles (Covacci *et al*., 1993; Bickley *et al*., 1993).

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 10^4 bacteria. The visualization of *ureC* and *cagA* amplification products from the gastric tissues of *H. pylori* 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% CO_2. Non-adherent cells were washed out, and immature macrophages were detached for 10 min, 37°C, 5% CO_2, with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, containing 0.5 mM EDTA. After washing twice, fresh macrophages were adjusted to the density of 5×10^5 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

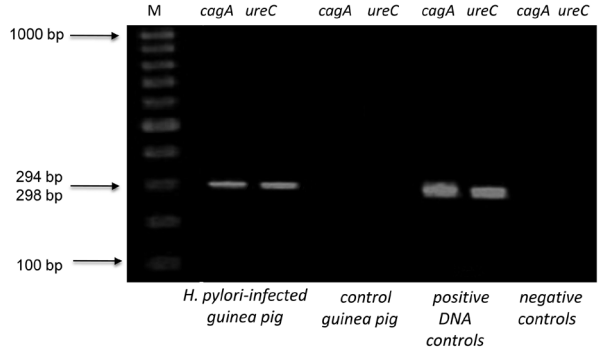

Figure 1. Example visualization of *ureC* and *cagA* PCR amplification products from the gastric tissue of guinea pig infected with *H. pylori* CCUG 17874 and control animal inoculated with 0.85% NaCl, separated by gel electrophoresis.
M — molecular standard, bp — base pair size, 294 bp — amplification product of *ureC* gene, 298 bp — amplification product of *cagA* gene, positive DNA control — DNA isolated from the reference *H. pylori* strain CCUG 17874 *ure*/*cagA* positive, negative control — sample lacking DNA.

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 ($300\times g$, 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 1×10^8 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 (1×10^8 cells/ml), over nylon wool fiber columns, 45 min, at 37°C, 5% CO_2. 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 2.5×10^6 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 2.5×10^6 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% CO_2) and autologous T lymphocytes were prepared according to the following procedure: the immature or mature macrophages (5×10^5 cells/ml), which were distributed into 96 well plates (100 µl/well) were stimulated for 2 h (37°C, 5% CO_2) 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 (2.5×10^6 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% CO_2. At 18 h before the end of cultivation, 1 µCi of [^{3}H]-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/

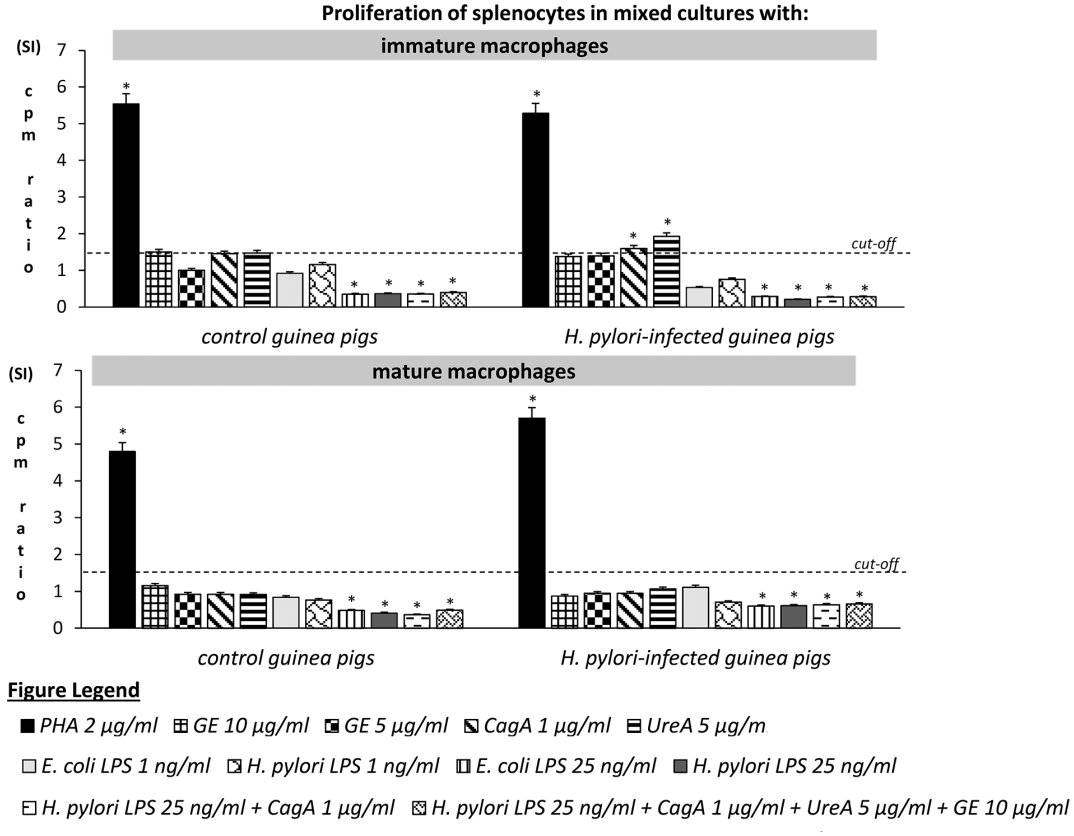

Figure 2. *H. pylori* antigen — driven proliferative response of lymphocytes in co-cultures of spleen lymphocytes T and immature or mature macrophages.
The proliferative activity of T lymphocytes isolated from uninfected and *H. pylori* infected guinea pigs was estimated in co-cultures with immature or mature macrophages, non-stimulated or stimulated for 2 h with *H. pylori* glycine acid extract (GE), UreA, CagA, LPS, the mixture of *H. pylori* LPS (25 ng/ml) and CagA or *H. pylori* LPS (25 ng/ml), CagA, UreA and GE. Control stimulators such as *E. coli* LPS and phytohaemagglutinin (PHA) were used. The proliferating activity of lymphocytes was evaluated on the basis of [^{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 *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.

RESULTS

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

Data in Fig. 2 show the proliferative activity of T lymphocytes isolated from *H. pylori* uninfected and *H. pylori* infected guinea pigs in co-cultures with immature or mature macrophages, non-stimulated or stimulated with *H. pylori* and control antigens. Experiments were performed using four animals per group. The lymphocytes isolated from uninfected and *H. pylori* infected animals proliferated intensively in response to PHA, in the cell cultures containing both immature and mature macrophages. The SI values depicting the cell proliferative activity were within the range 0.2–5.7.

Only the lymphocytes of *H. pylori* infected guinea pigs showed increased blastogenic response in the co-cultures with immature, but not mature macrophages, pulsed with *H. pylori* CagA (1 μg/ml) and UreA (5 μg/ml), ($p < 0.05$). In this group the responsiveness of lymphocytes in the presence of immature macrophages pulsed with GE was only slightly increased. The blastogenic response of lymphocytes from *H. pylori* infected animals was not increased in the cell cultures containing mature macrophages pulsed with CagA, UreA as well as GE. In the cell cultures containing lymphocytes and immature

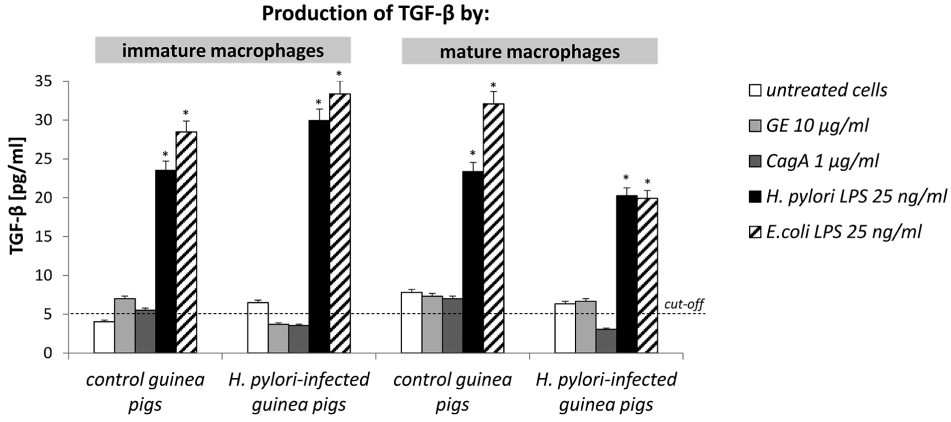

Figure 3. The concentration of TGF-β in the cell culture supernatants of immature or mature macrophages alone pulsed with *H. pylori* antigens.
Guinea pig macrophages, uninfected or infected with *H. pylori*, were pulsed for 2 h with *H. pylori* glycine acid extract (GE), CagA, UreA, *H. pylori* LPS, *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).

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 and in their co-cultures with lymphocytes in relation to lymphocyte blastogenic response

The lymphocyte proliferative response depends on different signals provided during the formation of the immune synapse. The sources of these signals are direct macrophage-lymphocyte interactions and cytokines, which modulate positively or negatively the lymphocyte blastogenic response. We examined the macrophages alone as a source of TGF-β as well as mixed cultures of macrophages and lymphocytes as a source of both TGF-β and IFN-γ. The concentration of TGF-β significantly increased in the cell cultures of immature and mature macrophages from control and *H. pylori* infected guinea pigs, pulsed with *H. pylori* LPS and *E. coli* LPS at a concentration of 25 ng/ml (Fig. 3). TGF-β also increased in the mixed cultures of immature and mature macrophages primarily stimulated with *H. pylori* LPS or *E. coli* LPS and then co-cultured with T lymphocytes of *H. pylori* uninfected or infected animals (Fig. 4A, B). The increased concentration of TGF-β in these cell cultures was linked to the diminished lymphocyte blastogenic response as compared to the control. By comparison, the concentration of IFN-γ significantly increased in the cell cultures containing only immature macrophages of both control and *H. pylori* infected animals, pulsed with *H. pylori* GE and CagA, which were then co-cultured with lymphocytes (Fig. 4A, B). The enhanced production of IFN-γ in these cell cultures was related to significantly increased (CagA) or slightly increased (GE) lymphocyte proliferation.

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.

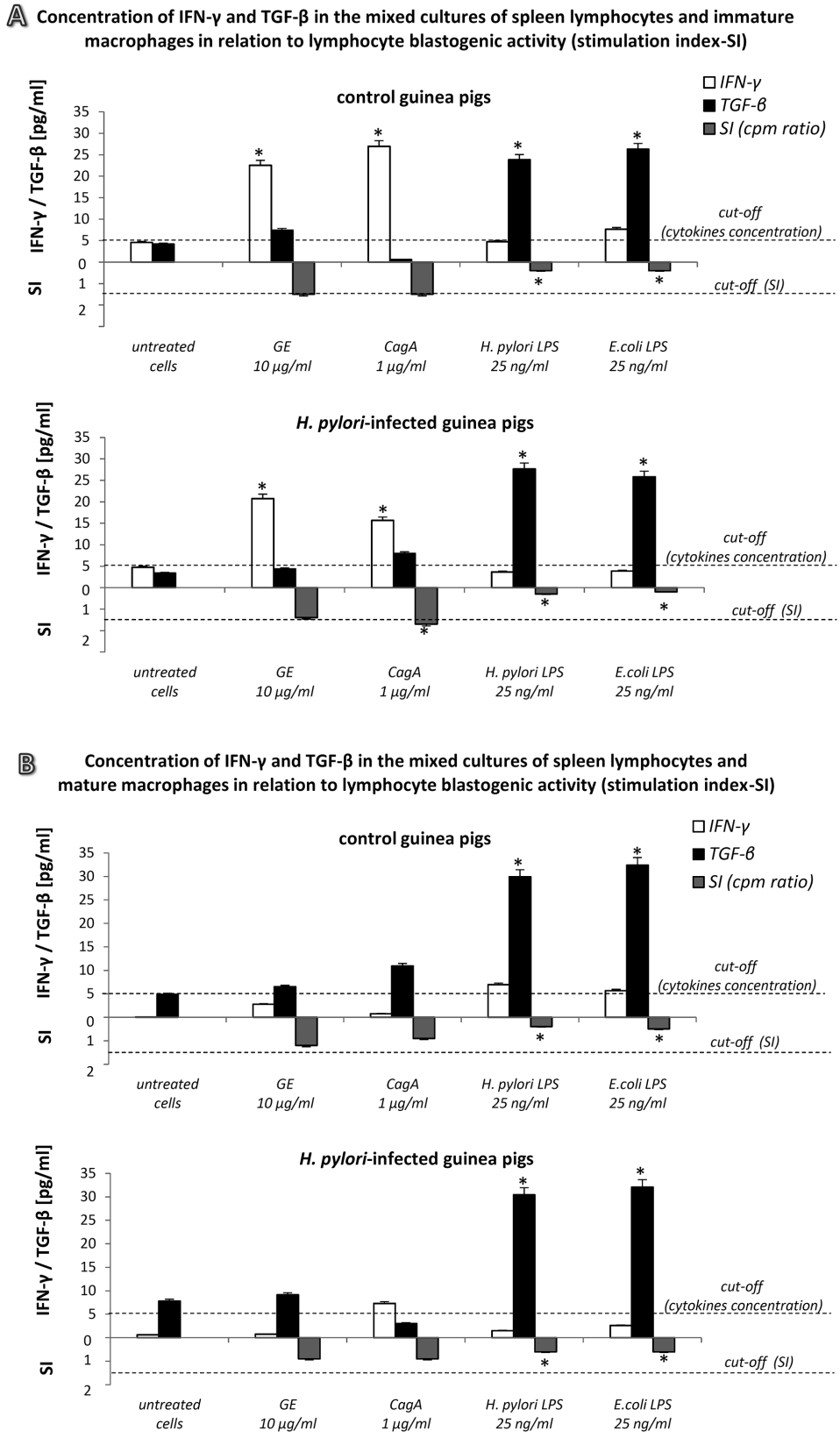

Figure 4. The concentration of IFN-γ and TGF-β in the cell culture supernatants from mixed cultures of immature (A) or mature (B) macrophages pulsed with *H. pylori* antigens with lymphocytes in relation to lymphocyte blastogenic response.
Macrophages isolated from guinea pigs uninfected or infected 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).

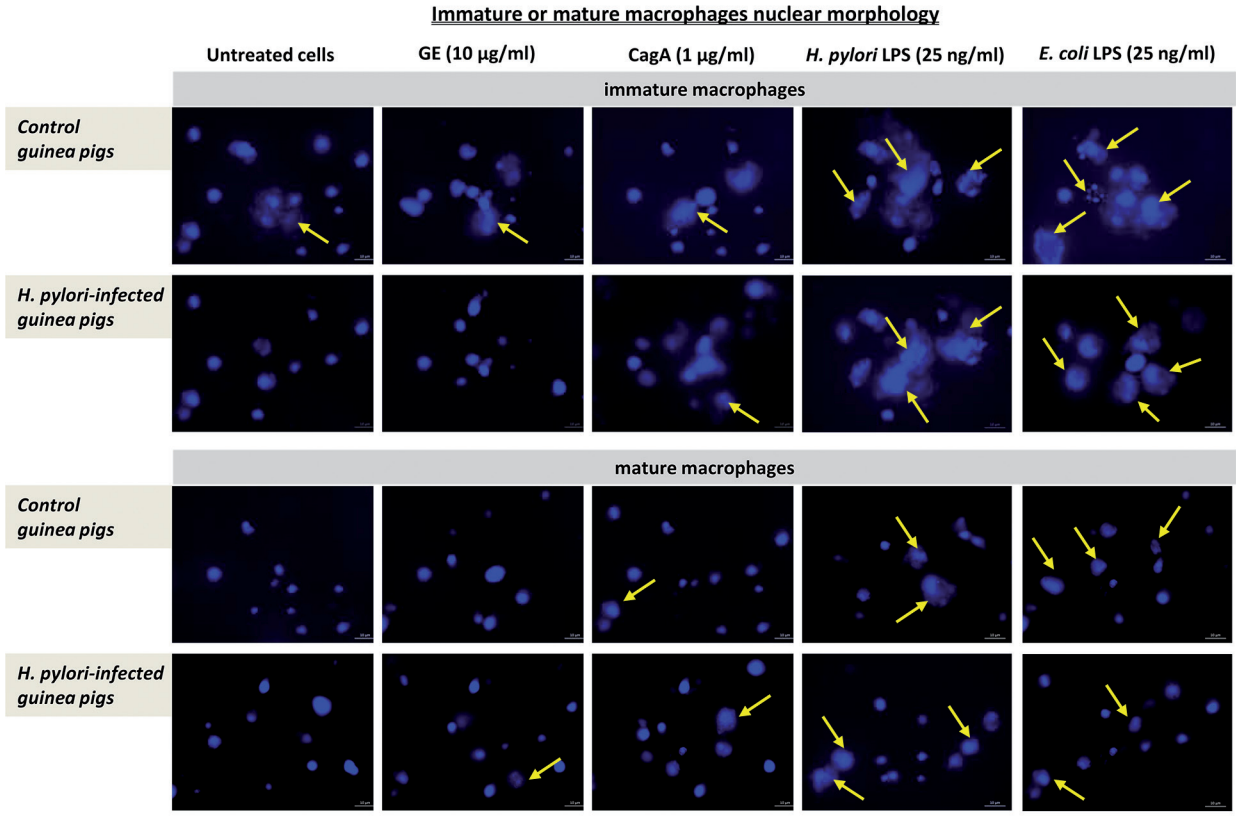

Figure 5. The nuclear morphology of immature or mature macrophages.
Guinea pig macrophages (immature or mature) were pulsed for 2 h with *H. pylori* glycine acid extract (GE), CagA, UreA, *H. pylori* LPS, *E. coli* LPS or left unstimulated (RPMI-1640 alone). The DAPI staining assay was used to detect cells with damaged DNA. The cell imaging was performed using the fluorescent microscope (Axio Scope A1, Zeiss). Arrows indicate the damaged nuclei of macrophages (magnification, x1000).

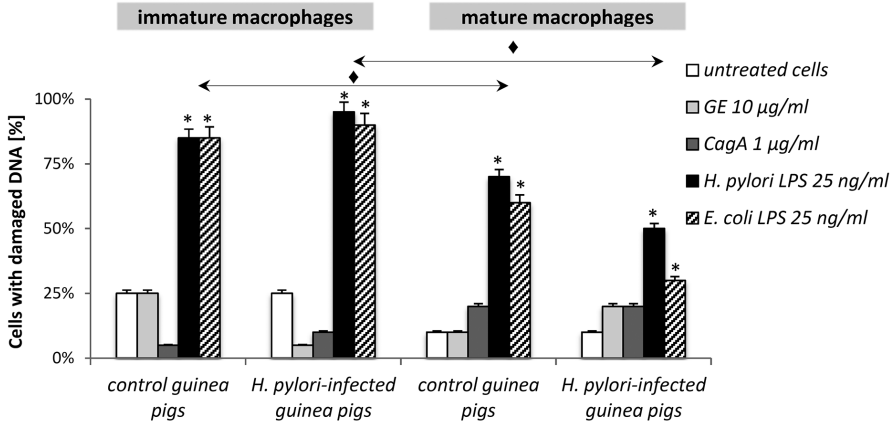

Figure 6. The percentage of immature or mature macrophages with damaged DNA.
Guinea pig macrophages (immature or mature) were pulsed for 2 h with *H. pylori* glycine acid extract (GE), CagA, UreA, *H. pylori* LPS, *E. coli* LPS or left unstimulated (RPMI-1640 alone). The DAPI staining assay was used to detect cells with damaged DNA (**p*<0.05 *vs* macrophages; ♦*p*<0,05 immature *vs* mature macrophages). The percentage of macrophages with damaged DNA was calculated.

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-

rophages 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 monocytic 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 common determinants of *H. pylori* antigens and other infectious agents colonizing guinea pigs. Crossreacting 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 monocytic 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 (Massague *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.*,

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 monocytic 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 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 monocytic 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 bacterial colonization and prolong infection (Rudnicka *et al*., 2003).

CONCLUSIONS

Using a guinea pig model mimicking the natural history of *H. pylori* infection we investigated the effectiveness of monocytes and macrophages in promoting the expansion of T lymphocytes derived from splenocytes of *H. pylori* infected and uninfected animals, in response to *H. pylori* antigens: glycine acid extract, CagA protein, urease A subunit and LPS. It was shown that impaired homeostasis of monocytic cell function in relation to DNA damage and TGF-β release, in response to *H. pylori* LPS, may lead to the suppression of adaptive immune response against these bacteria and development of chronic infection.

Acknowledgements

The project was financed from the means of National Science Centre of Poland, granted on the basis of decision number DEC-2013/09/N/NZ6/00805, the Ministry of Science and Higher Education grant (NN 303451738) and the Project entitled "PhD Students, Regional Investment in Young Scientists. D-RIM BIO acronym" co-financing from the European Union under the Operational Programme Human Capital Investment, sub-measure 8.2.1.

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