DIRECT EFFECTS OF HELICOBACTER PYLORI ON HUMAN CD4+ T-CELLS HAMPER DISENTANGLING A POSSIBLE BACTERIAL-MEDIATED INTERFERENCE WITH MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II-DEPENDENT ANTIGEN PRESENTATION TO THESE CELLS. TO OVERCOME THIS LIMITATION, WE EMPLOYED A PREVIOUSLY DESCRIBED ASSAY, WHICH ENABLES ASSESSING HUMAN ANTIGEN-PROCESSING CELL FUNCTION BY USING MURINE T-CELL HYBRIDOMA CELLS RESTRICTED BY HUMAN LEUKOCYTE ANTIGEN (HLA) ALLELES. HLA-DR1+ MONOCYTE-DERIVED DENDRITIC CELLS WERE EXPOSED TO H. PYLORI AND PULSED WITH THE ANTIGEN 85B FROM MYCOBACTERIUM TUBERCULOSIS (M. TUBERCULOSIS). INTERLEUKIN-2 (IL-2) SECRETION BY AG85Baa97-112-SPECIFIC HYBRIDOMA CELLS WAS THEN EVALUATED AS AN INTEGRAL REPORTER OF COGNATE ANTIGEN PRESENTATION. THIS METHODOLOGY ENABLED REVEALING OF INTERFERENCE OF H. PYLORI WITH THE ANTIGEN-PRESENTING CAPACITY OF HUMAN DENDRITIC CELLS.

**Keywords:** Helicobacter pylori, dendritic cells, antigen presentation, T-cells, hybridoma cells

**Introduction**

Helicobacter pylori (H. pylori) is considered as type I carcinogen by the World Health Organization (WHO) since 1994. In most cases, H. pylori-infected individuals remain asymptomatic. Chronic infection, however, is a major risk factor for the development of gastric cancer or lymphoma. Major histocompatibility complex class II (MHC-II)-restricted antigen presentation to CD4+ T-cells is a prerequisite for protection against H. pylori [1, 2], and persistent colonization might result from deficient antigen presentation and CD4+ T-cell activation. The induction of human T-cell immunity by antigen-presenting cells (APCs) involving H. pylori stimulation has been studied before [3–5]. Bacterial virulence factors, however, were shown to negatively affect human CD4+ T-cell activation by exerting direct anti-proliferative effects as well as by inhibiting T-cell cytokine production, such as interleukin-2 (IL-2) or interferon-γ (IFN-γ) [6–9]. In addition, the bacteria may directly modulate functions of human APCs [10]. To study the functional consequences of a possible modulation of antigen-processing and presentation in professional APCs by H. pylori, confounders such as direct bacterial effects on T-cell functions have to be eliminated. Here, we investigated antigen presentation by human monocyte-derived dendritic cells (DCs) to a murine CD4+ T-cell hybridoma. Murine T-cell functions are not affected by H. pylori stimulation and CD4+ T-cell-derived hybridoma cells respond to cognate presentation of T-cell epitopes relatively independent of additional costimulatory interactions by APCs.
The use of T-cell hybridoma cells from human leukocyte antigen (HLA)-transgenic mice as responder cells has previously been shown to enable quantitative detection of antigen processing by human APCs [11]. By applying this methodology to the \emph{H. pylori} system, we show that \emph{H. pylori} exposure hampers MHC-II-restricted antigen presentation by human DCs. This effect may rely on bacterial factors shared at least within additional Gram-negative bacteria as it can partly be mimicked when substituting \emph{H. pylori} with lipopolysaccharide (LPS) from \emph{Escherichia coli} (\emph{E. coli}).

Materials and methods

Preparation of \emph{H. pylori}

\emph{H. pylori} P12 wild type strain was grown and prepared as described elsewhere [10]. For stimulation, \emph{H. pylori} was adjusted to a multiplicity of infection (MOI) of 10.

\emph{T-cell hybridoma cell culturing}

AG85B\textsubscript{\textemdash}a	extsubscript{97-112} specific, HLA-DR1-restricted F9A6 cells [11] were grown in 75 cm\textsuperscript{2} flasks (TPP, Trasadingen, Switzerland) in DMEM medium (Gibco/Invitrogen, Life Technologies, Darmstadt, Germany), supplemented with 2 mM L-glutamine (Gibco), penicillin (100 U/ml), streptomycin (100 \mu g/ml), gentamicin (30 \mu g/ml), ciprofloxacin (10 \mu g/ml), 0.05 mM 2-mercaptoethanol (all from Sigma, Taufkirchen, Germany), and 10% fetal calf serum (Biochrom, Berlin, Germany) at 37 °C and 5% CO\textsubscript{2}. Cells were passaged every second day and harvested from day 3 to day 10.

\emph{Generation of human HLA-DR\textsuperscript{1} DCs}

Healthy blood donors that volunteered were HLA-typed by the tissue typing laboratory of the Charité, Campus Virchow Klinikum (Berlin, Germany). DCs were generated as described previously [10]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll density gradient centrifugation. CD14\textsuperscript{+} monocytes were enriched by using magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), and 3 \times 10\textsuperscript{6} cells/well were cultured in six-well culture plates (TPP) in complete RPMI medium, containing 2 mM L-glutamine, 10 mM HEPES (Gibco), penicillin (100 U/ml), streptomycin (100 \mu g/ml), 10% FCS, 1000 U/ml GM-CSF, and 100 U/ml recombinant IL-4 for 1 h at 37 °C in the presence of \emph{H. pylori}. LPS derived from \emph{E. coli} (Invivogen, Toulouse, France) (100 ng/ml) was used as a control stimulus while cells maintained in medium alone served as negative control. After 1 h of incubation, cells were washed twice with medium and resuspended in RPMI medium supplemented with 10% FCS, 1000 U/ml GM-CSF, 100 U/ml recombinant IL-4, penicillin (100 U/ml), streptomycin (100 \mu g/ml), and 100 \mu g/ml gentamicin (Sigma) for subsequent F9A6 cell activation.

\emph{T-cell hybridoma cell activation by DCs}

After stimulation, DCs were incubated in the presence or absence of 12 \mu g/ml recombinant antigen 85B from \emph{Mycobacterium tuberculosis} (\emph{M. tuberculosis}) (AG85B; Abcam, Cambridge) for 6 h at 37 °C. A total of 8 \times 10\textsuperscript{4} cells/well of a 96-well flat-bottom plate (TPP) in 100 \mu l of this suspension were incubated with 1 \times 10\textsuperscript{5} F9A6 cells in 100 \mu l of RPMI medium containing 10% FCS, 1000 U/ml GM-CSF, 100 U/ml IL-4, penicillin (100 U/ml), streptomycin (100 \mu g/ml), and gentamicin (100 \mu g/ml) for a further 12 h at 37 °C. For kinetic experiments, 12 \mu l/ml AG85B was added to the DCs 24 and 48 h prior to or at the time of exposure to \emph{H. pylori}. Incubation with F9A6 cells followed immediately or either 24 or 48 h later.

\emph{Analysis of IL-2 secretion by T-cell hybridoma cells}

Twelve hours after the addition of the F9A6 cells, cell-free supernatants were collected and stored at −80 °C until monitoring for IL-2 by sandwich ELISA (R&D) according to the manufacturer’s instructions.

\emph{Cell viability assay}

After 12 h of incubation, cells were tested for viability by using a colorimetric cell viability assay according to the manufacturer’s protocol (Colorimetric Cell Viability Kit I; PromoKine, Heidelberg, Germany).
Statistics

Statistical analyses were performed using the nonparametric Wilcoxon matched-pairs signed rank test. Differences were considered statistically significant for \( p < 0.05 \).

Ethics statement

The study was approved by the ethical committee of the Charité and informed consent was obtained from all donors.

Results

*H. pylori* has been shown to inhibit human but not murine CD4\(^+\) T-cell proliferation through direct interaction [6, 7, 9]. We first confirmed that *H. pylori* did not affect relevant murine F9A6 T-cell properties, such as viability or IL-2 secretion, by investigating F9A6 T-cell responses using anti-CD3 stimulation. F9A6 cells were incubated with *H. pylori* for 1 h, the bacteria were washed out, and the cells were transferred to plates coated with anti-CD3 mAbs. Control cells were not exposed to *H. pylori*. Twelve hours later, supernatants were harvested and IL-2 concentrations were determined by ELISA as an integral surrogate of cognate AG85B presentation by DCs. Supernatants from AG85B-exposed, uninfected DCs cocultured with F9A6 cells contained elevated concentrations of IL-2. In contrast, prior exposure of DCs to *H. pylori* significantly impaired IL-2 production by F9A6 cells (Fig. 2). This effect was not due to reduced viability of the antigen-presenting cells owing to the bacteria since we did not detect cell death in the DC populations in response to *H. pylori* stimulation (data not shown). Thus, incubation of immature DCs with *H. pylori* prior to AG85B addiction reduces IL-2 secretion of F9A6 cells incubated together with such DCs.

Exposure of DCs to *H. pylori* induces the expression of molecules associated with DC activation, such as CD25,
Fig. 3. *H. pylori* but not LPS suppressed antigen presentation by human DCs for up to 48 h. Immature HLA-DR1+ DCs were incubated in the presence or absence of recombinant antigen 85B from *M. tuberculosis* (AG85B, 12 μg/ml) (A) 24 or 48 h prior to or (B, C) at the time of stimulation for 1 h with *H. pylori* (MOI of 10). Cells incubated in the presence of LPS (100 ng/ml) or maintained in medium alone served as controls. F9A6 cells were added (A, B) immediately after stimulation or (C) 24 or 48 h later. Twelve hours after the addition of F9A6 cells, cell-free supernatants were collected and concentrations of murine IL-2 were determined by ELISA. Each symbol per condition represents the data obtained from one independent experiment (one donor). Horizontal lines show the median values of six experiments (donors). *: $p < 0.05$ (Wilcoxon matched-pairs signed rank test).
CD80, and CD83, and results in a reduced capability of the cells to take up exogenous particles [10]. Hence, we were interested in how the kinetics of the H. pylori addition may influence the inhibitory effect of the bacteria on antigen presentation by DCs. We therefore altered the time interval between addition of antigen and infection by adding AG85B to immature HLA-DR1+ DCs 24 and 48 h prior to or at the time of H. pylori stimulation. Bacteria were washed out after 1 h and F9A6 cells were added to the DCs immediately. In similar experiments, DCs were exposed to antigen and simultaneously stimulated with H. pylori, but the time of F9A6 cell addition varied, i.e., they were added immediately or either 24 or 48 hours later. As controls we used LPS-stimulated DCs and cells kept in medium alone. The latter therefore did not receive a maturation stimulus. Supernatants were always collected 12 h after the addition of F9A6 cells and the concentrations of murine IL-2 secretion were measured.

In contrast to DCs kept in medium alone, we detected substantial amounts of IL-2 when DCs were incubated with H. pylori or LPS 24 or 48 h after AG85B addition (Fig. 3A). Comparable to our previous observation, IL-2 production by F9A6 cells was significantly reduced when AG85B was added at the time of H. pylori or LPS stimulation (Fig. 3B).

Similarly, we detected low levels of IL-2 in supernatants of H. pylori-treated DCs when F9A6 cells were added 24 or 48 h after stimulation (Fig. 3C). Notably, in comparison to H. pylori-stimulated DCs, IL-2 levels were significantly increased in supernatants of LPS-treated DCs, when F9A6 cells were added 24 or 48 h after stimulation (Fig. 3C). Thus, both LPS and H. pylori interfere with antigen presentation by DCs when the antigen is added around the time of stimulation. A substantial time span between antigen uptake and stimulation, in our experiments 24–48 h, is required for optimal antigen presentation. In contrast to LPS activation of DCs, however, H. pylori interferes for a prolonged period of time with presentation of exogenously added antigens by human DCs.

Discussion

Our approach offers a method to study antigen presentation to CD4+ helper type T cells by H. pylori-stimulated human DCs, irrespectively of immunomodulatory effects of bacterial virulence factors that directly affect human T-cell activation. We have previously shown that incubation of DCs with H. pylori or LPS does not affect the expression of HLA-DR but reduces the capability of engulfing exogenous particles following incubation of the cells for 1 h with either stimulus [10]. By adding antigen to cells simultaneously with bacterial stimulation, we excluded this maturation/activation-induced incapacity of the cells to pick up further antigen. The fact that LPS-treatment induced a similar although more transient inhibition of antigen presentation indicates that at least Gram-negative bacteria associated Toll-like receptor (TLR) ligands may be involved. Indeed, this offers a parsimonious explanation of similar observations that were reported for Salmonella enterica serovar Typhimurium-stimulated murine DCs where stimulation with Salmonella resulted in a reduced MHC-II presentation of antigenic peptides [12]. At first glance, it appears paradoxical and counter-intuitive that antigens in the course of DC activation by multicomponent-immunostimulatory agents are not efficiently presented. The reported observation, however, may highlight a physiologically important aspect of antigen-processing and presentation by DCs, i.e., focusing T-cell responses to antigens of the stimulating pathogen while limiting presentation of other exogenous or – physiologically more relevant – autoantigens that may inevitably be sampled by the DC at the same time. These effects may also be relevant for subunit vaccine formulation strategies since vaccines that physically link vaccine antigens to APC stimulating molecules seem to be more effective [13, 14].

Competing interests

The authors declare no conflict of interest.

Funding sources

This study was supported by the DFG (SFB 633).

Acknowledgements

We thank David H. Canaday for providing the AG85B subgroup IIA, specific, HLA-DR1-restricted F9A6 cells.

References

1. Aebischer T, Burmann D, Eppler HJ, Schneider T, Cherepnev G, Walduck AK, Kunkel D, Moos V, Loddenkemper C, Jiadze I, Panasyuk M, Stolte M, Graham DY, Zeitz M, Meyer TF: Correlation of T cell response and bacterial clearance in human volunteers challenged with Helicobacter pylori revealed by randomised controlled vaccination with Ty21a-based Salmonella vaccines. Gut 57(8), 1065–1072 (2008)
2. Ermak TH, Giannasca PJ, Nichols R, Myers GA, Nedrud J, Weltzin R, Lee CK, Kleanthous H, Monath TP: Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses. J Exp Med 188(12), 2277–2288 (1998)
3. Bimczok D, Clements RH, Waite KB, Novak L, Eckhoff DE, Mannon PJ, Smith PD, Smythies LE: Human primary gastric dendritic cells induce a Th1 response to H. pylori. Mucosal Immunol 3(3), 260–269 (2010)
4. Khamri W, Walker MM, Clark P, Atherton J, Thursz MR, Bamford KB, Lechler RI, Lombardi G: Helicobacter pylori stimulates dendritic cells to induce interleukin-17 expression from CD4+ T lymphocytes. Infect Immun 78(2), 845–853 (2010)
5. Mitchell PJ, Afzali B, Fazekasova H, Chen D, Ali N, Powell N, Lord GM, Lechler RI, Lombardi G: \textit{Helicobacter pylori} induces \textit{in-vivo} expansion of human regulatory T cells through stimulating interleukin-1β production by dendritic cells. Clin Exp Immunol 170(3), 300–309 (2012)

6. Beigier-Bompadre M, Moos V, Belogolova E, Allers K, Schneider T, Churin Y, Ignatius R, Meyer TF, Aeberscher T: Modulation of the CD4+ T-cell response by \textit{Helicobacter pylori} depends on known virulence factors and bacterial cholesterol and cholesterol α-glucoside content. J Infect Dis 204(9), 1339–1348 (2011)

7. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R: \textit{Helicobacter pylori} vacuolating cytotoxin inhibits T lymphocyte activation. Science 301(5636), 1099–1102 (2003)

8. Schmees C, Prinz C, Treptau T, Rad R, Hengst L, Voland P, Bauer S, Brenner L, Schmid RM, Gerhard M: Inhibition of T-cell proliferation by \textit{Helicobacter pylori} gamma-glutamyl transpeptidase. Gastroenterology 132(5), 1820–1833 (2007)

9. Sundrud MS, Torres VJ, Unutmaz D, Cover TL: Inhibition of primary human T cell proliferation by \textit{Helicobacter pylori} vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. Proc Natl Acad Sci U S A 101(20), 7727–7732 (2004)

10. Fehlings M, Drobbe L, Moos V, Renner Viveros P, Hagen J, Beigier-Bompadre M, Pang E, Belogolova E, Churin Y, Schneider T, Meyer TF, Aeberscher T, Ignatius R: Comparative analysis of the interaction of \textit{Helicobacter pylori} with human dendritic cells, macrophages, and monocytes. Infect Immun 80(8), 2724–2734 (2010)

11. Canaday DH, Gehring A, Leonard EG, Eilertson B, Schreiber JR, Harding CV, Boom WH: T-cell hybridomas from HLA-transgenic mice as tools for analysis of human antigen processing. J Immunol Methods 281(1–2), 129–142 (2003)

12. Cheminay C, Möhlenbrink A, Hensel M: Intracellular Salmonella inhibit antigen presentation by dendritic cells. J Immunol 174(5), 2892–2899 (2005)

13. Husseiny MI, Hensel M: Evaluation of Salmonella live vaccines with chromosomal expression cassettes for translocated fusion proteins. Vaccine 27(28), 3780–3787 (2009)

14. Xu X, Husein MI, Goldwich A, Hensel M: Efficacy of intracellular activated promoters for generation of Salmonella-based vaccines. Infect Immun 78(11), 4828–4838 (2010)