DC-derived IL-18 drives Treg differentiation, murine Helicobacter pylori-specific immune tolerance, and asthma protection

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Abstract: Persistent colonization with the gastric bacterial pathogen Helicobacter pylori causes gastritis and predisposes infected individuals to gastric cancer. Conversely, it is also linked to protection from allergic, chronic inflammatory, and autoimmune diseases. We demonstrate here that H. pylori inhibits LPS-induced maturation of DCs and reprograms DCs toward a tolerance-promoting phenotype. Our results showed that DCs exposed to H. pylori in vitro or in vivo failed to induce T cell effector functions. Instead, they efficiently induced expression of the forkhead transcription factor FoxP3, the master regulator of Tregs, in naive T cells. Depletion of DCs in mice infected with H. pylori during the neonatal period was sufficient to break H. pylori-specific tolerance. DC depletion resulted in improved control of the infection but also aggravated T cell-driven immunopathology. Consistent with the mouse data, DCs infiltrating the gastric mucosa of human H. pylori carriers exhibited a semimature DC-SIGN+HLA-DRhiCD80loCD86lo phenotype. Mechanistically, the tolerogenic activity of H. pylori-experienced DCs was shown to require IL-18 in vitro and in vivo; DC-derived IL-18 acted directly on T cells to drive their conversion to Tregs. CD4+CD25+ Tregs from infected wild-type mice but not Il18-/- or Il18r1-/- mice prevented airway inflammation and hyperresponsiveness in an experimental model of asthma. Taken together, our results indicate that tolerogenic reprogramming of DCs ensures the persistence of H. pylori and protects against allergic asthma in a process that requires IL-18.

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Persistent colonization with the gastric bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes infected individuals to gastric cancer. Conversely, it is also linked to protection from allergic, chronic inflammatory, and autoimmune diseases. We demonstrate here that *H. pylori* inhibits LPS-induced maturation of DCs and reprograms DCs toward a tolerance-promoting phenotype. Our results showed that DCs exposed to *H. pylori* in vitro or in vivo failed to induce T cell effector functions. Instead, they efficiently induced expression of the forkhead transcription factor FoxP3, the master regulator of Tregs, in naive T cells. Depletion of DCs in mice infected with *H. pylori* during the neonatal period was sufficient to break *H. pylori*–specific tolerance. DC deletion resulted in improved control of the infection but also aggravated T cell–driven immunopathology. Consistent with the mouse data, DCs infiltrating the gastric mucosa of human *H. pylori* carriers exhibited a semimature DC-SIGN′HLA-DRhiCD80hiCD86lo phenotype. Mechanistically, the tolerogenic activity of *H. pylori*–experienced DCs was to require IL-18 in vitro and in vivo; DC-derived IL-18 directly acted on T cells to drive their conversion to Tregs. CD4+CD25hi Tregs from infected wild-type mice but not *Il18r1−/− or Il18r1−/− mice prevented airway inflammation and hyperresponsiveness in an experimental model of asthma. Taken together, our results indicate that tolerogenic reprogramming of DCs ensures the persistence of *H. pylori* and protects against allergic asthma in a process that requires IL-18.

### Introduction

DCs are predominantly known for their function as potent inducers of anergy, deletion of autoreactive T cells, and the instruction and differentiation of Tregs (1). Under steady-state conditions, tissue-resident immature DCs express low levels of MHC class II (MHCII), costimulatory molecules, and proinflammatory cytokines but may transform to fully mature DCs upon antigen uptake, accompanied by the concomitant sensing of pathogen-associated molecular patterns (PAMPs) or of “danger” signals released by tissues in distress (2). Such PAMPs or danger-associated molecular patterns (DAMPs) are detected by membrane-bound TLRs or cytoplasmic Nod-like receptors (NLRs) (3, 4) and induce the expression of MHCII, CD40, CD80, and CD86 as well as a number of proinflammatory and T cell–activating cytokines, including IL-1β, IL-6, and IL-12 (2). Immature DCs that have taken up antigen, but have not simultaneously been exposed to TLR or NLR ligands, are thought to acquire a semimature state, characterized by high levels of MHCII but low or no expression of costimulatory molecules or proinflammatory cytokines; such semimature CD11c+MHCIIhiC D80hiCD86lo DCs are believed to exhibit tolerogenic (as opposed to stimulatory or immunogenic) properties (1). Tolerogenic DCs function by converting naive T cells into FoxP3+ Tregs with suppressive activity; Treg induction is achieved through antigen presentation in the absence of costimulatory signals or cytokines, either alone or in combination with the production of soluble and membrane-bound tolerogenic factors, such as IL-10, TGF-β, retinoic acid, and programmed death ligands (1, 5).

Persistent infection with the gram-negative gastric bacterial pathogen *Helicobacter pylori* results in chronic gastritis (6) and predisposes carriers to a high risk of developing gastric and duodenal ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma (7–9). We and others have shown previously that MHCII-restricted T cells are required for the control of this extracellular pathogen under conditions of experimental infection in naive mice (10) and for the development of vaccine-induced protective immunity (11, 12). Th1-polarized, pathogenic CD4+ T cells further represent the driving force behind the infection-associated gastric preneoplastic immunopathology that manifests histologically as atrophic gastritis, epithelial hyperplasia, and intestinal metaplasia in infected rodents and in a subset of chronically infected humans (10, 13–15). Consequently, targeting T cells pharmacologically prevents and even reverses the gastric immunopathology associated with chronic *Helicobacter* infection (16, 17). Interestingly, the outcome of the *Helicobacter*/host interaction varies dramatically depending on the age at the time of infection. Whereas mice infected as fully immunocompetent adults develop preneoplastic lesions within 2–4 months of infection with virulent *Helicobacter* strains, mice infected during the neonatal period are protected against gastric immunopathology due to their development of immune tolerance to the pathogen (18).
The depletion of Tregs breaks neonatally acquired tolerance and results in a dramatic reduction of bacterial loads and the development of Th1-associated immunopathology (18). Tregs induced during neonatal infection are further both required and sufficient to mediate the H. pylori–induced, but antigen-independent, cross-protection against allergic asthma that is evident in human carriers of H. pylori (19–22) and in experimentally infected mice (23). In an ovalbumin sensitization/challenge model of allergic asthma that is a hallmark of H. pylori infection, as assessed by staining for CD80 (Figure 1D) and by ELISA for IL-12p40 (Figure 1E). In contrast, direct contact between wild-type bacteria and the cells was required for this effect, as a transwell filter that prevented attachment of the bacteria also impaired the inhibition of LPS-induced DC maturation (Figure 1, F and G). The observed consequences of H. pylori infection were not due to cytotoxic effects of the bacteria on cultured DCs and were at least partially phenocopied by treatment with H. pylori extract (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI61029DS1). We further alternatively used the TLR2 and TLR9 ligands PAM3Cys and CpG to induce DC maturation; H. pylori infection also efficiently prevented CD80 expression and IL-12 secretion induced by these stimuli (Figure 1, H and I, and data not shown). Interestingly, we could not attribute the effects of H. pylori on DCs to the C-type lectin receptor DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), which was previously demonstrated to mediate immune escape by H. pylori (24). DCs transgenically expressing human DC-SIGN (hDC-SIGN) under the control of the cd11c promoter did not differ from wild-type DCs, with respect to the inhibitory effects of H. pylori on LPS-induced DC maturation; mice expressing hDC-SIGN (25) further did not differ from wild-type littermates in terms of H. pylori colonization levels (Supplemental Figure 1, D–F). Taken together, the results indicate that H. pylori has evolved a mechanism of impairing DC function in a manner that depends on direct contact but is not dependent of the type IV secretion system.

H. pylori exposure generates tolerogenic DCs with the ability to convert naive T cells to FoxP3+ Tregs in a contact- and TGFB-β–dependent manner. One of the inherent characteristics of semimature DCs is their ability to convert naive T cells into FoxP3+ Tregs with suppressive activity. To assess whether the exposure to H. pylori induces such tolerogenic DCs, we compared the ability of untreated and H. pylori–infected BM-DCs to convert naive T cells to FoxP3+ Tregs (Figure 2A). In line with their semimature phenotype (MHCII−/CD80−/CD86−/CD40−; Figure 1, A and B; data not shown for MHCII), BM-DCs that had been infected with H. pylori induced FoxP3+ Tregs significantly more efficiently than naive BM-DCs, when cultured in the presence of anti-CD3 cross-linking antibody and TGFB-β (Figure 2, A and B). Similarly, H. pylori–infected BM-DCs loaded with ovalbumin were better able than their naive counterparts to trigger the conversion of ovalbumin-specific (OTII) T cells to FoxP3+ Tregs (Figure 2, C and D). Direct contact between the infected BM-DCs and T cells was required for their efficient conversion to FoxP3+ Tregs, as the separation of both cell populations by a transwell filter abrogated the effect (Figure 2E). Interestingly, DCs isolated from the bone marrow of gene-targeted mice lacking MyD88 or TLR2, an important pattern recognition receptor for H. pylori (26, 27), were as capable of Treg conversion upon H. pylori exposure as wild-type BM-DCs (Figure 2F). We next immunomagnetically purified CD11c+ DCs from the mesenteric lymph nodes (MLNs) of C57BL/6 mice to more than 80% purity and infected them with live H. pylori prior to coculture with naive T cells. Infected MLN-derived DCs (MLN-DCs) were significantly more potent inducers of FoxP3 expression in T cells than naive BM-DCs in the presence of anti-CD3 cross-linking antibody and TGFB-β (Figure 2, and B). H. pylori–infected BM-DCs loaded with ovalbumin were better able than their naive counterparts to trigger the conversion of ovalbumin-specific (OTII) T cells to FoxP3+ Tregs (Figure 2A). In line with their semimature phenotype (MHCII−/CD80−/CD86−/CD40−; Figure 1, A and B; data not shown for MHCII), BM-DCs that had been infected with H. pylori induced FoxP3+ Tregs significantly more efficiently than naive BM-DCs, when cultured in the presence of anti-CD3 cross-linking antibody and TGFB-β (Figure 2, A and B). Similarly, H. pylori–infected BM-DCs loaded with ovalbumin were better able than their naive counterparts to trigger the conversion of ovalbumin-specific (OTII) T cells to FoxP3+ Tregs (Figure 2, C and D). Direct contact between the infected BM-DCs and T cells was required for their efficient conversion to FoxP3+ Tregs, as the separation of both cell populations by a transwell filter abrogated the effect (Figure 2E). Interestingly, DCs isolated from the bone marrow of gene-targeted mice lacking MyD88 or TLR2, an important pattern recognition receptor for H. pylori (26, 27), were as capable of Treg conversion upon H. pylori exposure as wild-type BM-DCs (Figure 2F). We next immunomagnetically purified CD11c+ DCs from the mesenteric lymph nodes (MLNs) of C57BL/6 mice to more than 80% purity and infected them with live H. pylori prior to coculture with naive T cells. Infected MLN-derived DCs (MLN-DCs) were significantly more potent inducers of FoxP3 expression in T cells than naive BM-DCs in the presence of anti-CD3 cross-linking antibody (Figure 2, G and H) or upon ovalbumin-specific stimulation of OTII T cells (Figure 2, I and J), indicating that the tolerizing effects of H. pylori exposure are common to both BM-DC and MLN-DC populations.

H. pylori–experienced DCs are incapable of activating T cell effector responses. To examine whether the exposure of DCs to H. pylori not only renders them tolerogenic, but at the same time impairs their ability to activate T cell effector functions and to induce Th1 differentiation, we cocultured H. pylori–infected BM-DCs with naive OTII T cells under conditions of anti-CD3 cross-linking or ovalbumin-specific priming. Infected BM-DCs were significantly less able than naive BM-DCs to induce IFN-γ expression and secretion in T cells activated by CD3 cross-linking or ovalbumin priming, as determined by intracellular cytokine staining and ELISA...
T cell expansion, as assessed by \[^{3}H\] thymidine incorporation, was impaired as well (Figure 3C). To confirm these results with MLN-DCs, immunomagnetically isolated CD11c\(^{+}\) DCs from the MLNs of naive mice were infected ex vivo and cocultured with OTII-transgenic T cells under conditions of CD3 cross-linking or ovalbumin-specific priming. As observed with the BM-DCs, infected MLN-DCs were significantly less capable of inducing IFN-\(\gamma\) production upon CD3 cross-linking or ovalbumin-specific priming than uninfected MLN-DCs (Figure 3, D–F). The combined results suggest that \(H. pylori\)-experienced DCs are better inducers of Tregs, but worse inducers of effector T cells, than naive DCs without prior exposure to the bacteria.\n
\(H. pylori\)-experienced DCs fail to induce asthma in a DC-mediated ovalbumin sensitization and challenge model. We and others have shown recently that infection with \(H. pylori\) protects against allergic asthma in humans (19–22) and in mouse models of the disease (23). A hallmark of infected mice protected against asthma was their pulmonary infiltration with immature, CD11c\(^{+}\) MHCII\(^{lo}\) DCs, which were much less abundant in asthmatic mice (23). To determine whether exposure to \(H. pylori\) indeed generates DCs that are unable to promote asthma, we established a model in which 1 intranasal dose of ovalbumin-loaded DCs was used to sensitize mice to ovalbumin, followed by challenge with aerosolized ovalbumin 2 weeks later. Uninfected, ovalbumin-loaded DCs were compared

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**Figure 1**
The TLR ligand-induced maturation of DCs is impaired by \(H. pylori\) infection. (A–C) BM-DCs were infected with \(H. pylori\) (Hp) strain PMSS1 at a MOI of 50 and/or treated with 0.5 \(\mu\)g/ml \(E. coli\) LPS for 16 hours prior to (A and B) the flow cytometric analysis of CD80, CD86, and CD40 expression and (C) the quantification of IL-12p40, IL-6, and IL-10 secretion by ELISA. Representative FACS plots are shown for CD80 in A, and the average MFI of CD80, CD86, and CD40 expression of all CD11c\(^{+}\) cells is shown in B. (D and E) BM-DCs were infected with \(H. pylori\) strain PMSS1 or its isogenic mutant, PMSS1\(\Delta\)CagE (\(\Delta\)E), and/or treated with LPS for 16 hours and (D) assessed for CD80 expression and (E) IL-12p40 secretion. (F and G) BM-DCs were infected with \(H. pylori\) strain PMSS1 and/or treated with LPS for 16 hours and (F) assessed for CD80 expression and (G) IL-12p40 secretion; bacteria were separated from the cells by a transwell (tw) filter where indicated. (H and I) BM-DCs were infected with \(H. pylori\) strain PMSS1 and/or treated with 0.5 \(\mu\)g/ml LPS or 5 \(\mu\)g/ml Pam3Cys (Pam) for 16 hours and assessed for (H) CD80 expression and (I) IL-12p40 secretion. Data are representative of (D–I) at least 3 and (A–C) up to 8 independent experiments and are represented as mean ± SEM of triplicate cultures. \(P\) values were calculated using Student’s \(t\) test.
with *H. pylori*-infected, ovalbumin-loaded DCs, which had been subjected to antibiotic therapy to kill the bacteria prior to intranasal administration. Additional control groups received DCs that had not been pulsed with antigen. Mice that had received ovalbumin-loaded DCs developed characteristic airway hyperresponsiveness, as assessed upon methacholine inhalation (Figure 4, A and B), and exhibited high levels of peribronchiolar and perivascular inflammation and extensive goblet cell metaplasia (Figure 4, C–E). They further showed high levels of bronchoalveolar infiltration, as assessed by quantification of total cells in the bronchoalveolar lavage fluid (BALF) (Figure 4F). Whereas the BALF from non-asthmatic mice predominantly contained alveolar macrophages, the fraction of macrophages decreased in the BALF of asthmatic recipients of ovalbumin-loaded DCs (Figure 4G). The predomi-
nant populations of infiltrating cells in asthmatic mice were lymphocytes, neutrophils, and eosinophils (Figure 4, H–J). None of the described symptoms of asthma were elicited by ovalbumin-loaded DCs that had previously been exposed to *H. pylori* or by DCs not loaded with allergen (Figure 4). In conclusion, the ovalbumin/DC-induced asthma model confirms the inability of infected DCs to prime antigen-specific effector T cell responses.

Hypothesizing that *H. pylori* infection of mice should generate tolerogenic rather than immunogenic DCs in light of our in vitro findings, we infected mice with *H. pylori* PMSS1, a virulent patient isolate that harbors a functional Cag pathogenicity island, establishes persistent infection in mice, and induces the full range of gastric inflammation and preneoplastic pathology that characterizes the *H. pylori*-infected gastric mucosa of susceptible, chronically infected patients (18). In addition to mice infected at 6 weeks of age (i.e., as adults), we included mice that had been infected with *H. pylori* PMSS1 during the neonatal period (i.e., at 7 days of age). Neonatally infected mice develop tolerance rather than immunity to *H. pylori* infection (18); the neonatal infection model is therefore of particular interest in the context of elucidating the tolerogenic properties of *H. pylori*-infected DCs. As the MLNs represent 1 out of 2 documented sites of *H. pylori*–specific T cell priming along side the Peyer’s patches (16, 17, 28, 29), we immunomagnetically isolated MLN-derived CD11c+ DCs were infected and/or loaded with 20 μg/ml ovalbumin prior to coculturing with CD4+CD25− T cells in the presence of rIL-2 and anti-CD3ε mAb. (D) Representative FACS plots demonstrating intracellular IFN-γ are shown, (E) along with mean ± SEM of triplicate cocultures and (F) IFN-γ secretion into the supernatant as determined by ELISA. Numbers indicate the percentage of IFN-γ+ cells of the CD4+ gate. All data are representative of at least 3 independent experiments.

**Figure 3**

*H. pylori* infection impairs the ability of DCs to activate T cell effector functions. (A–C) BM-DCs were infected as described in Figure 2, A and B, and/or loaded with 20 μg/ml ovalbumin prior to coculturing with immunomagnetically isolated, splenic OTII CD4+CD25− T cells for 3 days in the presence of rIL-2. Anti-CD3ε mAb was added where indicated. (A) IFN-γ–producing CD4+ T cells were quantified by intracellular cytokine staining, and (B) IFN-γ secretion into the supernatant was measured by ELISA. (C) Proliferation of parallel cocultures was determined by [H] thymidine incorporation. T cells cultured without DCs served as controls (−). (D–F) Immunomagnetically isolated, MLN-derived CD11c+ DCs were infected and/or loaded with 20 μg/ml ovalbumin prior to coculturing with CD4+CD25− T cells in the presence of rIL-2 and anti-CD3ε mAb. (D) Representative FACS plots demonstrating intracellular IFN-γ are shown, (E) along with mean ± SEM of triplicate cocultures and (F) IFN-γ secretion into the supernatant as determined by ELISA. Numbers indicate the percentage of IFN-γ+ cells of the CD4+ gate. All data are representative of at least 3 independent experiments.

Neonatally induced *H. pylori*–specific immune tolerance requires DCs. *H. pylori* infection of mice should generate tolerogenic rather than immunogenic DCs in light of our in vitro findings, we infected mice with *H. pylori* PMSS1, a virulent patient isolate that harbors a functional Cag pathogenicity island, establishes persistent infection in mice, and induces the full range of gastric inflammation and preneoplastic pathology that characterizes the *H. pylori*-infected gastric mucosa of susceptible, chronically infected patients (18). In addition to mice infected at 6 weeks of age (i.e., as adults), we included mice that had been infected with *H. pylori* PMSS1 during the neonatal period (i.e., at 7 days of age). Neonatally infected mice develop tolerance rather than immunity to *H. pylori* infection (18); the neonatal infection model is therefore of particular interest in the context of elucidating the tolerogenic properties of *H. pylori*-infected DCs. As the MLNs represent 1 out of 2 documented sites of *H. pylori*–specific T cell priming along side the Peyer’s patches (16, 17, 28, 29), we immunomagnetically isolated MLN-derived CD11c+ DCs were infected and/or loaded with 20 μg/ml ovalbumin prior to coculturing with CD4+CD25− T cells in the presence of rIL-2 and anti-CD3ε mAb. (D) Representative FACS plots demonstrating intracellular IFN-γ are shown, (E) along with mean ± SEM of triplicate cocultures and (F) IFN-γ secretion into the supernatant as determined by ELISA. Numbers indicate the percentage of IFN-γ+ cells of the CD4+ gate. All data are representative of at least 3 independent experiments.
Figure 4

\(H.\) pylori–experienced DCs fail to induce asthma-like symptoms in an adoptive transfer model of DC-driven airway hyperresponsiveness. BM-DCs were infected and treated as described in Figure 2, A and B, and/or loaded with either 20 \(\mu\)g/ml ovalbumin (OVA-DC ± Hp [either infected or not infected with \(H.\) pylori]) or PBS only (PBS-DC ± Hp). 1 \(\times\) 10^6 BM-DCs per mouse were administered intranasally for the purpose of ovalbumin-specific sensitization; all mice were challenged 2 weeks later with aerosolized ovalbumin and assessed for the development of airway hyperresponsiveness and tissue inflammation. (A and B) Airway hyperresponsiveness, (A) as assessed by challenge with increasing doses of methacholine and (B) the highest dose of 100 mg/ml, respectively. (C–E) Tissue inflammation and goblet cell metaplasia, as assessed on H&E- and PAS-stained tissue sections. Representative micrographs are shown in D. Original magnification, \(\times\)100 (H&E); \(\times\)400 (PAS). Inflammation and PAS scores are shown in C and E. (F) Total cells contained in 1 ml BALF. Percentages of (G) macrophages, (H) lymphocytes, (I) neutrophils, and (J) eosinophils in 1 ml BALF, as determined by differential staining. In the scatter plots shown in C, E, and F–J, each data point represents an individual mouse. Data are representative of 3 independent experiments. Horizontal lines represent the median.
**Figure 5**

H. pylori-exposed DCs exhibit tolerogenic properties and are required for tolerance in vivo. (A–E) C57BL/6 mice were infected with H. pylori at 7 days (iN) or 6 weeks (iA) of age. Upon sacrifice, CD11c⁺ MLN-DCs were immunomagnetically isolated; cocultured for 3 days with splenic CD4⁺CD25⁻ T cells, rTGF-β, rIL-2, and anti-CD3ε mAb; and subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression. (A) CD25 and FoxP3 staining of the CD4⁺ gate is shown for representative donors and (B) quantified for all donors. Numbers indicate the percentage of FoxP3⁺CD25⁺ cells. (C) H. pylori colonization of mice analyzed in B. (D) CD4⁺ T cell infiltration into the gastric mucosa of mice shown in B and C. (E) DCs prepared, as described in A, were cultured with CD4⁺CD25⁻ T cells, rIL-2, and anti-CD3ε mAb and subjected to intracellular IFN-γ staining. Each symbol represents an individual donor, and data are pooled from 2 experiments in B–E. (F–L) cd11c-DTR tg mice and their wild-type littermates were infected at 1 week of age with H. pylori strain PMSS1 or remained uninfected. (F) All mice received diphtheria toxin during the final 2 weeks of the experiment and were sacrificed 8 weeks after infection. (G) Gastric H. pylori colonization. (H and I) Gastric CD45⁺ leukocyte and CD4⁺ T cell infiltration. (J) Intracellular IFN-γ expression by gastric CD4⁺ T cells. (K and L) Gastric histopathology, as assessed on Giemsa-stained sections. Representative micrographs are shown in L. Original magnification, ×100 (top); ×200 (bottom). Inflammation scores are shown in K. Data in F–L are pooled from 3 experiments. Horizontal lines indicate the medians.
mutual exclusion of tolerogenic and immunogenic properties in a given DC population. To obtain functional evidence that DCs are required for the development and maintenance of neonatally acquired immunological tolerance to *H. pylori* infection, we infected mice that transgenically express the diphtheria toxin receptor under the control of the CD11c promoter (*cd11c*-DTR tg mice) at 1 week of age. At 6 weeks after infection, the *cd11c*-DTR tg mice as well as their nontransgenic, infected littermates and respective uninfected controls of both genotypes received 3 weekly doses of diphtheria toxin for 2 weeks (Figure 5F), resulting in the depletion of approximately 40%-50% of DCs in the gastric mucosa, MLNs, and spleen (Supplemental Figure 2 and data not shown). DC-depleted mice were colonized at significantly lower levels (Figure 5G) and exhibited higher levels of leukocyte and CD4+ T cell infiltration into the gastric mucosa than their DC-replete, infected littermates and uninfected controls (Figure 5, H and I). A higher proportion of CD4+ gastric mucosal T cells expressed IFN-γ in the DC-depleted, infected mice than in the other 3 groups (Figure 5J). The infiltration of leukocytes into the gastric mucosa of DC-depleted, infected mice was histologically evident as mild to moderate gastritis, a feature that is never observed in neonatally infected, fully tolerant mice at such early time points after infection (Figure 5, K and L). In summary, the depletion of CD11c+ DCs in the *cd11c*-DTR tg model reveals that the maintenance of *H. pylori*–specific immunological tolerance requires DCs, a finding that may be explained by the predominantly tolerogenic properties of *H. pylori*–experienced DCs in vitro and in vivo.

DCs infiltrating the gastric mucosa of *H. pylori*–infected patients express HLA-DR but lack costimulatory molecules. To examine the recruitment and localization of DCs to the gastric mucosa of *H. pylori*–infected individuals, we examined antral biopsies collected at endoscopy from *H. pylori*–infected and uninfected volunteers. Cryosections from antral biopsies were immunostained for DC-SIGN, a marker for human DCs (30). The large majority of DC-SIGN+ DCs were...
Figure 7
Treg induction by tolerogenic DCs requires IL-18. (A) Wild-type BM-DCs were infected with *H. pylori* PMSS1 (MOI 50), and IL-18 secretion was assessed by ELISA. (B and C) Wild-type and *Il18*–/– BM-DCs were infected as described in A, and cocultured at a 1:2 ratio with immunomagnetically isolated, splenic OTII CD4+CD25– T cells for 3 days in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb prior to the flow cytometric analysis of CD4, CD25, and FoxP3 expression. (B) Representative FACS plots of the CD4+ gate are shown, (C) along with mean ± SEM of triplicate cocultures. (D–G) C57BL/6 and BL/6.*Il18*–/– mice were infected at 7 days (iN) of age with 1 orogastric dose of *H. pylori* or remained uninfected. Upon sacrifice after 4 weeks after infection, CD11c+ DCs were immunomagnetically isolated from single cell MLN suspensions of individual mice and cocultured for 3 days with splenic CD4+CD25– T cells at a 1:2 ratio in the presence of rIL-2 and anti-CD3ε mAb and (D and E) with or (F and G) without rTGF-β. (D and E) Cocultures were subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression (representative mice are shown in E, and the quantification of all mice is shown in D) or (F and G) CD4 and IFN-γ or IL-17 expression. Data shown are representative of at least 3 independent (A–C) in vitro and (D–G) in vivo experiments. Horizontal lines represent the median. (B and E) Numbers indicate the percentage of FoxP3+CD25+ cells.
found scattered in the lamina propria of both uninfected and
*H. pylori*-infected individuals (Figure 6A), and some DCs were
positioned just underneath the epithelium. The frequency of DC-
SIGN+DCs was significantly higher in *H. pylori*-infected
individuals (Figure 6B). Interestingly, whereas *Treg* differentiation
induced by MLN-DCs from neonatally infected wild-type mice
were better able to control naive *OTII* T cells more efficiently
than control DCs, independent of whether the T cells were
activated by anti-CD3 cross-linking (Figure 7, D and E). In line
with a reported role for IL-18 in *Th1* differentiation in vitro,
both uninfected and infected *Il18r1*−/−BM-DCs failed to induce
IFN-γ expression in cocultured naive T cells (Supplemental
Figure 4, A–D). We then compared the efficiency of FoxP3
induction by *H. pylori*-infected wild-type and *Il18r1*−/−BM-DCs
under conditions of anti-CD3 cross-linking and ovalbumin-
specific priming. As demonstrated above (Figure 2),
wild-type BM-DCs that had been infected with *H. pylori*
induced FoxP3 expression in cocultured naive *OTII* T cells
more efficiently than control BM-DCs, independent of whether the T cells were activated by antici-CD3 cross-linking (Figure 7, B and C) or ovalbumin (Supplemental
Figure 3, A and C). Interestingly, infected *Il18r1*−/−BM-DCs were
better able to control naive T cells in vitro. To test this notion, we first examined whether IL-18 secretion was induced upon *H. pylori*
infec-
tion of BM-DCs, which was indeed the case (Figure 7A). We
then compared the efficiency of FoxP3 induction by *H. pylori-
infected wild-type and *Il18r1*−/−BM-DCs under conditions of
anti-CD3 cross-linking and ovalbumin-specific priming. As
demonstrated above (Figure 2), wild-type BM-DCs that had
been infected with *H. pylori* induced FoxP3 expression in
cocultured naive T cells more efficiently than control

**Figure 8**
Treg differentiation and the development of *H. pylori*-specific tolerance requires IL-18 signaling in vivo. (A) Single cell MLN preparations from individual C57BL/6, BL/6*I18−/−*, and BL/6*I18r1−/−* mice neonatally infected with *H. pylori* were subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression and compared with respective uninfected controls. Each data point represents an individual mouse; horizontal lines indicate medians. (B) Wild-type BM-DCs were infected with *H. pylori* PMSS1 (MOI 50) and cocultured at a 1:2 ratio with wild-type or *Il18r1−/−* CD4+CD25*−T cells for 3 days in the presence of rTGF-β, rIL-2, and anti-CD3e mAb prior to analysis of CD4, CD25 and FoxP3 expression. Mean ± SEM of triplicate cocultures are shown. (C–F) Wild-type C57BL/6 and BL/6*I18r1−/−* mice were neonatally infected with *H. pylori* and sacrificed 4 weeks after infection. (C) Gastric *H. pylori* colonization. (D and E) Gastric mucosal CD45+ leukocytes and CD4+ T cell infiltration. (F) IFN-γ expression by stomach-infiltrating CD4+ T cells, as determined by intracellular cytokine staining. Horizontal lines represent the median.
IL-18 signaling is required for Treg differentiation and H. pylori-specific tolerance in vivo. Hypothesizing based on the above results that Treg numbers should consequently differ between the MLNs of wild-type and II18−/− mice, we quantified the proportion of FoxP3+CD25+ cells in the MLN CD4+ T cell population of neonatally infected wild-type C57BL/6 or BL/6 II18−/− donors (4–6 per group) 1 day before the first challenge. Negative controls were challenged without prior sensitization. (A and B) Airway hyperresponsiveness, (A) as assessed by challenge with increasing doses of methacholine and (B) the highest dose of 50 mg/ml, respectively. (C–E) Tissue inflammation and goblet cell metaplasia, as assessed on H&E- and PAS-stained tissue sections. Representative micrographs are shown in E. Original magnification, ×100 (H&E); ×400 (PAS). Inflammation and PAS scores are shown in C and D, respectively. (F) Total cells contained in 1 ml BALF. (G) Eosinophils in 1 ml BALF. Horizontal lines indicate medians. s/c, sensitized/challenged.
IL-18 acts directly on T cells to facilitate their conversion to FoxP3+CD25+ Tregs, compared naïve wild-type and Il18r1−/− T cells, with respect to their ability to upregulate FoxP3 upon coculture with H. pylori–experienced BM-DCs. Indeed, Il18r1−/− T cells showed a strongly reduced ability to upregulate FoxP3, indicating that IL-18 signaling in T cells is required for efficient Treg conversion in vitro (Figure 8B). We next examined the ability of Il18r1−/− mice to develop tolerance to H. pylori as a consequence of experimental infection during the neonatal period. Neonatally infected Il18r1−/− mice exhibited significantly lower colonization levels than wild-type mice infected at the same age (Figure 8C), which was accompanied by higher gastric leukocyte and T cell infiltration (Figure 8, D and E) and stronger gastric production of IFN-γ and IL-17, as determined by intracellular cytokine staining and/or qPCR (Figure 8F and Supplemental Figure 5). We conclude from these results that T cell–intrinsic IL-18 signaling is essential for Treg differentiation in vitro and in vivo, and is also required for the generation of Treg-mediated, neonatally acquired tolerance to H. pylori infection.

CD4+CD25+ T cells isolated from neonatally infected wild-type mice but not Il18r1−/− or Il18r1−/− mice suppress allergen-induced asthma. Given the requirement for DC-derived IL-18 in Treg differentiation in vitro and in vivo, we hypothesized that CD4+CD25+ T cells immunomagnetically isolated from the MLNs of neonatally infected Il18r1−/− or Il18r1−/− mice should lack suppressive activity in a murine model of experimentally induced allergic airway disease. Having shown earlier that neonatal infection of wild-type mice with H. pylori generates Tregs with highly suppressive activity in the asthma model (23), we compared immunomagnetically isolated CD4+CD25+ T cells from neonatally infected wild-type, Il18r1−/−, or Il18r1−/− animals, with respect to their ability to inhibit experimentally induced asthma (Figure 9 and Supplemental Figure 6). Mice were sensitized i.p. with alum-adjuvanted ovalbumin and subsequently challenged by 3 consecutive daily exposures to aerosolized ovalbumin. Groups of mice were intravenously administered CD4+CD25+ T cells isolated from either neonatally infected wild-type, Il18r1−/−, or Il18r1−/− animals or from age-matched uninfected controls of each genotype 1 day prior to the first challenge. All mice were examined with respect to clinical and histopathological parameters of asthma development 2 days after the last challenge. Sensitized, challenged mice exhibited increased airway resistance compared with nonsensitized controls, as assessed by methacholine challenge, which could be prevented by CD4+CD25+ T cells from infected wild-type animals but not infected Il18r1−/− animals (Figure 9, A and B) or Il18r1−/− animals (Supplemental Figure 6, A and B). CD4+CD25+ T cells from uninfected control groups did not prevent allergen-induced airway resistance (Figure 9, A and B). Similarly, the lung inflammation and PAS–goblet cell metaplasia that are hallmarks of asthma in this model were prevented by CD4+CD25+ T cells from infected wild-type mice but not infected Il18r1−/−, Il18r1−/−, or uninfected mice (Figure 9, C–E, and Supplemental Figure 6, C and D). A quantitative analysis of allergen-induced bronchoalveolar inflammation and eosinophilia confirmed these findings (Figure 9, F and G, and Supplemental Figure 6, E and F). Interestingly, quantitative RT-PCR analysis of IL-10 expression performed on freshly purified CD4+CD25+ T cells revealed that T cells isolated from infected wild-type mice, but not infected Il18r1−/− mice, expressed copious amounts of IL-10 compared with CD4+CD25+ T cells from the corresponding uninfected donors (Supplemental Figure 6, G and H), implying that this cytokine may be key in asthma protection by H. pylori–induced Tregs. Overall, the results suggest that Treg differentiation and function is severely impaired under conditions of IL-18 or IL-18R deficiency, underscoring the importance of this cytokine in Treg biology.

**Discussion**

*H. pylori* infection is acquired during early childhood and typically persists for life (31). Chronic infection with *H. pylori* causes gastritis and predisposes to gastric cancer but has also been associated epidemiologically with protection against asthma (19, 20, 22), multiple sclerosis (32), and inflammatory bowel disease (33). The beneficial effects of the infection are particularly evident in the pediatric population, with respect to both asthma and colitis incidence (21, 34). Experimental infection with *H. pylori* during the neonatal period recapitulates the protective effects against asthma in mouse models of the disease (23). We show here that the exposure of DCs to *H. pylori* had profound effects on the properties of these cells in vitro and in vivo. *H. pylori*–experienced BM-DCs or MLN-DCs lose their ability to mature into fully immunogenic, stimulatory antigen-presentation cells and to activate effector functions in naïve T cells. Instead, *H. pylori*-exposed Tregs show a strongly reduced ability to upregulate FoxP3, indicating that IL-18 signaling in T cells is required for efficient Treg differentiation in vitro and in vivo, and is also required for the generation of Treg-mediated, neonatally acquired tolerance to *H. pylori* infection.
tolerogenic activity of *H. pylori*-experienced DCs in vitro and in vivo. In contrast to wild-type cells, BM-DCs derived from *Il18r1−/−* mice failed to induce FoxP3 expression in naive T cells upon exposure to *H. pylori*; more importantly, *Il18r1−/−* MLN-DCs from neonatally infected mice exhibited the same defect in Treg induction relative to their wild-type counterparts. DC-derived IL-18 appears to act directly on T cells and to promote their conversion to Tregs: IL-18R−/− DCs failed to upregulate FoxP3 upon coculture with tolerogenic DCs, and both IL-18− and IL-18R−/− deficient mice exhibited strongly decreased overall Treg numbers in the draining MLNs. Furthermore, CD4+CD25+ MLN T cells isolated from both gene-targeted strains are less capable of suppressing allergic airway disease in an allergen-induced sensitization and challenge model. All available data thus suggest that IL-18 contributes to *H. pylori* persistence and fine-tunes the balance between infection control on the one hand and excessive immunopathological T cell responses on the other. The results are confirmed in vivo by our observation that *Il18r1−/−* mice failed to develop immune tolerance to neonatal infection with *H. pylori*. Our data are in line with recent studies demonstrating a protective role for IL-18, produced upon activation of the NLRP3 inflammasome, in DSS-induced colitis (39, 40). In this model, lack of expression of NLRP3, caspase-1, and ASC in intestinal epithelial cells rendered mice hypersusceptible to DSS-induced colitis (39, 40).

As mentioned earlier, the protection against asthma is mediated by *H. pylori*-induced Tregs in experimentally infected mice: asthma protection is abrogated by the systemic depletion of Tregs and can be adoptively transferred from infected donors to uninfected recipients via Tregs (23). An interesting characteristic of neonatally infected mice that have been subjected to an asthma-inducing protocol is their pulmonary infiltration by highly suppressive Tregs and by semimature DCs (23). Our finding that *H. pylori*-exposed DCs are incapable of sensitizing mice to ovalbumin when administered intranasally makes it seem likely that *H. pylori*-mediated asthma protection is a direct consequence of the reprogramming of DCs in vivo. However, as DCs are not known to migrate between the mucosal surfaces of the lung and gastrointestinal tract, it appears likely that highly mobile Tregs are induced in the MLNs and then migrate to the airways, in which they exert their suppressive activity and protect against allergen-induced asthma. DCs and Tregs efficiently regulate one another in a negative feedback loop, promoting a tolerogenic, immunosuppressive environment. For instance, Onishi et al. reported that FoxP3+ Tregs form aggregates on DCs, thereby actively downregulating their costimulatory molecules and maturation markers and impairing the ability of DCs to activate antigen-specific, naive T cells (41). In summary, we show here that *H. pylori* possesses the distinct ability to reprogram DCs toward a tolerogenic phenotype in vitro and in vivo, a process that ensures persistence of the bacteria in the host and may cross-protect against chronic inflammatory and autoimmune diseases. The semimature phenotype of lamina propria DCs that is a hallmark of the gastric mucosa of asymptomatic human *H. pylori* carriers likely represents the human cellular counterpart of the murine tolerogenic DC population.

**Methods**

Animal experimentation; bronchialalveolar lavage, lung, and gastric histopathology, and assessment of *H. pylori* colonization. CS7BL/6 wild-type, BL/6.Tb2−/−, BL/6.Myd88−/−, OT II TCR transgenic, and *cd11c*-DTR tg mice were originally purchased from Charles River Laboratories; BL/6.Il18−/−, BL/6.Il18r1−/−, and *cd11c*-hDC-SIGN transgenic mice were obtained from Wolf-Dietrich Hardt (Federal Institute of Technology, Zürich, Switzerland), Burkhard Becher (University of Zürich), and Tim Sparwasser (Medical School, University of Hannover, Hannover, Germany). All mice were bred at a University of Zurich specific pathogen–free facility. Mice were housed in individually ventilated cages. Mixed gender groups were infected at either 7 days or 6 weeks of age with 1 orogastric dose of approximately 2 × 10^7 CFUs *H. pylori* PMSS1 (18). In vivo depletion of DCs was achieved by i.p. injections of 4 ng diphteria toxin per g of body weight at 2-day intervals for 2 weeks. For asthma induction, mice were sensitized either by 2 i.p. injections of 20 μg ovalbumin (Sigma-Aldrich) emulsified in 2.25 mg aluminum hydroxide (Alum Injext; Pierce) with a 2-week interval or by intranasal delivery of 1 × 10^6 BM-DCs loaded overnight (o/n) with 20 μg/ml ovalbumin (Sigma-Aldrich). Irrespective of the mode of sensitization, mice were challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 minutes daily on days 14, 15, and 16 after (second) sensitization. Airway resistance measurements were performed on anesthetized, intubated, and mechanically ventilated mice (Flexivent, Scireq) in response to increasing doses of inhaled methacholine, as described previously (42). Lungs were lavaged via the trachea with 1 ml PBS. BALF cells were counted using trypan blue dye exclusion. Differential cell counts of lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with the Microscopy Hemacolor Set (Merck). Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Lung tissue sections were stained with H&E and PAS and were examined on a BX40 Olympus microscope in blinded fashion by 2 independent experimenters. Peribronchial inflammation was scored on a scale from 0 to 4 on 5 randomly chosen areas per slide, as described previously (43). PAS-positive goblet cells were quantified per 1 mm of basement membrane in the primary bronchus and several medium-sized bronchi using Soft Imaging Systems software. Stomachs were retrieved and dissected longitudinally into equally sized pieces. For the quantitative assessment of *H. pylori* colonization, 1 stomach section was homogenized in Brucella broth, and serial dilutions were plated on horse blood plates for colony counting as described previously (18). For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded stomach sections were scored on a scale from 0 to 6 for the parameters of chronic inflammation, atrophy, epithelial hyperplasia, and metaplasia, as described in detail previously (10). All gastric histopathology images were taken at ×100 or ×200 final magnification on a Leica Leitz DM RB microscope equipped with a DFC 420C camera. Images were acquired using Leica Application Suite 3.3.0 software.

Volunteers and assessment of gastritis and *H. pylori* colonization. Volunteers were recruited among blood donors at Sahlgrenska University Hospital after serologic analysis, and *H. pylori* infection was subsequently confirmed or excluded by culture on Scirrow plates. Gastric antrum biopsies were collected from 8 *H. pylori*-infected volunteers (7 males and 1 female, aged between 25–57 years) and 10 uninfected volunteers (5 males and 5 females, aged between 23–62 years) by endoscopy, immediately frozen in OCT medium, and later used for immunohistochemical staining of DCs.

In addition, 1 biopsy from each volunteer was fixed in formalin, paraffin embedded, and examined by an experienced histopathologist for the grade of gastritis and the presence of Helicobacter-like organisms (HLOs) using the updated Sydney system (44). The serology, culture, and pathology results agreed in all cases, except those for 1 individual who did not have visible HLOs in the pathological examination but was culture and serology positive. Biopsy samples from 6 uninfected subjects were histologically normal without inflammation or HLOs, and biopsy samples from 4 uninfected subjects had mild chronic gastritis (score of 1) but no HLOs or active inflammation. In contrast, active chronic inflammation and HLOs were observed in biopsy samples from antrum of all *H. pylori*-infected subjects. The *H. pylori*—
infected individuals had a chronic inflammation score of 1.9 ± 0.4 (mean ± standard deviation), an active inflammation score of 1.4 ± 0.7, and an HLO score of 1.9 ± 0.8. Atrophy and metaplasia was seen in 1 of the *H. pylori*-infected subjects, and metaplasia alone was seen in 1 of the *H. pylori*-infected subjects.

**Immunohistochemical and flow cytometric analysis of human gastric DCs.** Antrum biopsies from *H. pylori*-infected and uninfected volunteers were cryosectioned into 8-μm thick sections, fixed for 10 minutes in ice-cold acetone, and endogenous peroxidase blocked with glucose-oxidase for 20 minutes. Sections were stained with mouse monoclonal antibodies to DC-SIGN (clone DCN46, BD Biosciences) in PBS containing 5% human and rabbit serum at room temperature for 30 minutes, followed by an HRP-conjugated rabbit antibody to mouse immunoglobulins (DakoCyto-mation). Isotype control antibodies were always run in parallel. The area stained with the respective antibodies was calculated relative to the total area of each tissue section, using Biopix image analysis software. For isotype control analysis 7-Aminoactinomycin D (7AAD, Sigma-Aldrich) was used to exclude dead cells. Samples were acquired on an LSR-II flow cytometer (BD Biosciences) and immunomagnetic isolation of DCs using mouse-specific CD11c microbeads (Miltenyi Biotec). DC cultures were infected o/n with wild-type *H. pylori* PMS11 or an isogenic mutant lacking the cagE gene (*ΔcagE*) described previously (18). Bacteria were killed with 200 U penicillin/0.2 mg streptomycin/ml for 6 hours prior to the addition of T cells.

CD4+CD25+ T cells were prepared from single cell suspensions of naïve C57BL/6 spleens by immunomagnetic sorting (R&D Systems). DCs were cocultured with CD4+CD25+ T cells at a ratio of 1.2 (0.5 × 10^6 DCs to 1 × 10^5 T cells) in RPMI containing 10% FCS, 10 ng/ml recombinant TGF-β (rTGF-β) (PeproTech), 10 ng/ml recombinant IL-2 (HL-2) (R&D Systems), and 1 μg/ml anti-CD3e (BD Bioscience). After 72 hours of coculture, the cells were stained first for CD4 and CD25 and then, after fixation and permeabilization, for FoxP3 (FoxP3-APC, eBioscience). The percentage of FoxP3+ T cells was assessed by FACS. IFN-γ was determined based on serology, culture, and pathol-
genus* C. trachomatis* (L243; all from BD Biosciences), and appropriate isotype controls were run in parallel. The area stained with the respective antibodies was calculated relative to the total area of each tissue section, using Biopix image analysis software. For isolation of MLN-DCs, MLNs of individual mice were digested in 1 mg/ml collagenase (Sigma-Aldrich) for 30 minutes at 37°C, with shaking prior to mechanical disruption between glass slides and filtering through a cell strainer (40 μm). Single cell suspensions were stained directly for FACS anal-
ysis. The following antibodies were used: CD11c-biotin, CD44-FTTC, CD4-APC, CD86-FTTC, and CD40-APC (all from BD Biosciences) as well as CD45-PE and CD80-APC (both from BioLegend). IFN-γ, IL-12 (rIL-12) (R&D Systems), and IL-18 (MBL) production by DCs was assessed by an HLO score of 1.9 ± 0.8. Atrophy and metaplasia was seen in 1 of 5 specimens. All human studies were approved by the Regional Ethics Committee of Western Sweden, and informed consent was obtained from all participants. All animal experimentation was reviewed and approved by the Zurich Cantonal veterinary office (63/2008 and 170/2009 to A. Müller).

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