The Cag pathogenicity island and interaction between TLR2/NOD2 and NLRP3 regulate IL-1β production in *Helicobacter pylori* infected dendritic cells

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Helicobacter pylori **colonization of the stomach affects about half of the world population and is associated with the development of gastritis, ulcers, and cancer. Polymorphisms in the *IL1B* gene are linked to an increased risk of *H. pylori* associated cancer, but the bacterial and host factors that regulate interleukin (IL)-1β production in response to *H. pylori* infection remain unknown. Using murine BM-derived DCs, we show that the bacterial virulence factors cytotoxin-associated genes pathogenicity island and CagL, but not vacuolating cytotoxin A or CagA, regulate the induction of pro-IL-1β and the production of mature IL-1β in response to *H. pylori* infection. We further show that the host receptors, Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain 2 (NOD2), but not NOD1, are required for induction of pro-IL-1β and NOD-like receptor pyrin domain containing 3 (NLRP3) in *H. pylori* infected DCs. In contrast, NLRP3 and the adaptor ASC were essential for the activation of caspase-1, processing of pro-IL-1β into IL-1β, and IL-1β secretion. Finally, we show that mice deficient in caspase-1, IL-1β, and IL-1 receptor, but not NLRP3, are impaired in the clearance of CagA-positive *H. pylori* from the stomach when compared with WT mice. These studies identify bacterial *cag* pathogenicity island and the cooperative interaction among host innate receptors TLR2, NOD2, and NLRP3 as important regulators of IL-1β production in *H. pylori* infected DCs.**

Keywords: *Helicobacter pylori* · IL-1β · Inflammasome · NLRP3 · NOD2

Introduction

Detection of microbes by the immune system is mediated by the activation of host soluble factors and germline-encoded pattern-recognition receptors (PRRs) by microbial moieties or endogenous molecules generated in the setting of infection [1]. PRRs including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLRs), and retinoic acid-Inducible

Gene (RIG)-like helicases are activated by conserved and unique microbial structures [1, 2]. TLRs mediate recognition of several molecules including LPS and lipopeptides at the cell surface as well as microbial nucleic acids in endosomes [1]. In contrast, NLRs and RIG-like helicases induce innate immune responses through cytosolic sensing of bacterial and viral components [1,2]. Two NLR family members, NOD1 and NOD2, are activated by molecules produced during the synthesis and/or degradation of bacterial

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peptidoglycan [3–6]. Nod2 is activated by muramyl dipeptide, which is present in all Gram-negative and -positive bacteria [3–6]. In response to infection, TLRs and NOD2 induce transcription of immune response genes through the transcription factor NF-κB and MAPKs that ultimately culminate in host defense responses to eliminate microbial invasion.

A major inflammatory pathway induced in response to microbial infection is the inflammasome, a multiprotein platform that activates caspase-1 in phagocytes and mast cells [7, 8]. Once activated, caspase-1 cleaves pro-interleukin-1β (IL-1β) and pro-IL-18 into their biologically active secreted forms [7]. To date, several inflammasomes have been identified including those triggered by the activation of NLR family members, NLR caspase domain containing 4 (NLRC4), and NLR pyrin domain containing 3 (NLRP3) [7, 8]. Activation of the NLR caspase domain containing 4 is induced by the release of bacterial flagellin or PrgJ-like rod proteins into the host cell cytosol in response to infection with several bacterial pathogens including *Salmonella enterica* serovar Typhimurium, *Legionella pneumophila*, and *Pseudomonas aeruginosa* [9]. In mouse macrophages, activation of NLRP3 requires two signals. The first signal, referred to as priming, is NF-κB-dependent transcription of *pro-IL-1β* and *Nlrp3*, through the stimulation of PRRs by microbial products or certain cytokines such as tumor necrosis factor-α (TNF-α) or IL-1β [10, 11]. The second signal activates NLRP3 and is induced by ATP, certain bacterial toxins, or particulate matter [7–9]. In response to activating stimuli, NLRP3 recruits the adapter protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment and activation domain) to drive the activation of caspase-1 [7].

Helicobacter pylori chronically colonizes the gastric mucosa of more than 50% of the world population and can persist for life [12]. *Helicobacter pylori* infection can induce chronic gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [12–15]. *Helicobacter pylori* expresses virulence factors that include vacuolating cytotoxin A (VacA) and cytotoxin-associated genes pathogenicity island (*cag*PAI) [16, 17]. The *cag*PAI encodes components of a type IV secretion system (T4SS) capable of injecting into the host cell the CagA protein and other factors that are associated with more severe inflammatory disease [17]. A major cytokine induced in response to gastric *H. pylori* infection is IL-1β [18]. The importance of IL-1β in *H. pylori* infection is underscored by the observation that polymorphisms in the *IL1B* gene are associated with an increased predisposition to gastric cancers in infected individuals [18, 19]. Furthermore, transgenic mice overproducing IL-1β in the stomach develop gastric inflammation and carcinoma [20]. In dendritic cells (DCs), TLR2 is the major TLR that regulates cytokine responses to *H. pylori* infection [21] whereas in epithelial cell, NOD1 recognizes *H. pylori* peptidoglycan resulting in NF-κB activation and subsequent IL-8 production [22]. *Helicobacter pylori* induces the activation of caspase-1 in DCs [23]. However, little is known about the microbial molecules and host PRRs that mediate the production of IL-1β in response to *H. pylori* infection. In this study, we demonstrate that secretion of IL-1β in

DCs infected with *H. pylori* is regulated by *cag*PAI and requires host TLR2, NOD2, and the NLRP3 inflammasome. Specifically, *H. pylori* stimulation via TLR2 and NOD2 in DCs primes the NLRP3 inflammasome by inducing pro-IL-1β and NLRP3, which enables the activation of caspase-1 via NLRP3 and production of mature IL-1β. Finally, we provide evidence that IL-1β signaling regulates the colonization of *H. pylori* in vivo.

Results

*Helicobacter pylori cag*PAI and CagL but not VacA or CagA enhance IL-1β production in DCs

Helicobacter pylori has two major virulence factors, VacA and *cag*PAI [16, 17]. The *cag*PAI encodes components of the T4SS [17], so we determined whether the T4SS is involved in the regulation of IL-1β. We compared the ability of WT *H. pylori* and an isogenic mutant deficient in CagL, a critical component of the T4SS and *cag*PAI-associated pili [17], to induce IL-1 β secretion. To determine whether VacA, *cag*PAI, and/or CagL regulate IL-1β production in *H. pylori* infected DCs, we infected DCs with WT *H. pylori*, and isogenic mutants deficient in VacA, *cag*PAI, or CagL and found that IL-1β secretion elicited in DCs infected with either WT or the *H. pylori* VacA mutant were comparable (Fig. 1A). However, DCs infected with *cag*PAI or CagL mutants had reduced IL-1β secretion when compared with that of cells infected with WT *H. pylori* (Fig. 1A). The reduction in IL-1β secretion was not explained by impaired uptake of the mutant strains by DCs (Fig. 1C). The role of *cag*PAI in eliciting IL-1β production was confirmed in a second *H. pylori* strain (Fig. 1C). Likewise, impaired IL-1β release was also observed in another *H. pylori* strain deficient in CagL (Fig. 1D). In contrast, a CagA mutant induced comparable IL-1β production to its isogenic WT strain (Fig. 1E). Consistently, the induction of IL-1β mRNA by the *cag*PAI and CagL mutants was impaired compared with that by WT bacteria (Fig. 1F). In contrast, the induction of Nlrp3 mRNA by the *cag*PAI and CagL mutants was comparable with that of the WT bacterium (data not shown). Consistent with a role of *cag*PAI and CagL in the induction of IL-1β, the defective ability of the mutants to induce *Il1b* mRNA and to elicit IL-1β secretion was rescued by pretreatment of DCs with LPS (Fig. 1F). These results indicate that *cag*PAI and CagL, but not VacA or CagA, regulate the induction of pro-IL-1β and secondarily the production of IL-1β in *H. pylori* infected DCs.

TLR2 and NOD2 regulate pro-IL-1β expression and IL-1β release upon *H. pylori* infection in DCs

We next evaluated host factors required for IL-1β secretion in response to *H. pylori* infection. Specifically, we tested the ability of BM-derived DCs (BMDCs) from WT, *Tlr2⁻/⁻*, *Nod1⁻/⁻*, and *Nod2⁻/⁻* mice to secrete IL-1β in response to infection with *H. pylori* 26695. IL-1β secretion was reduced in DCs from *Nod2⁻/⁻* and *Tlr2⁻/⁻*, but not *Nod1⁻/⁻* mice, when compared with that in
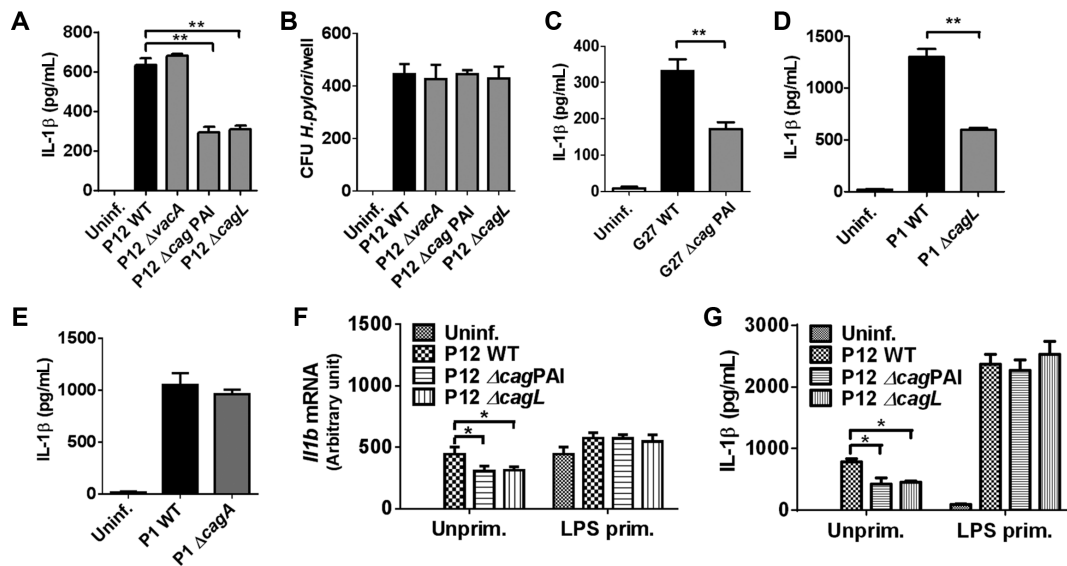
Figure 1. *Helicobacter pylori cag*PAI and CagL, but not VacA, enhances IL-1β induction in DCs. (A) DCs were infected with WT P12 *H. pylori* and isogenic mutants deficient in VacA, *cag*PAI, or CagL at a multiplicity of infection (MOI) of 20 overnight. IL-1β production was determined by ELISA. (B) Uptake of WT P12 *H. pylori* and isogenic mutants by DCs was measured. (C–E) DCs were infected with indicated WT *H. pylori* and isogenic mutants in (C) *cag*PAI, (D) CagL, and (E) CagA at an MOI of 20 overnight and IL-1β production was determined by ELISA. (F, G) DCs were infected with WT *H. pylori* and indicated isogenic mutant strains with or without LPS priming (6 h). (F) The mRNA expression of *Il1b* was evaluated by real-time PCR at 6 h after infection and fold increase (arbitrary unit) was obtained by comparison to the level of uninfected DCs. (G) IL-1β production was determined by ELISA. Data are shown as mean + SD of triplicate samples from one experiment representative of three independent experiments performed. **$p < 0.01$, two-tailed Student's *t*-test.

DCs from WT mice (Fig. 2A). To determine whether TLR2 and NOD2 were redundant in the secretion of IL-1β, we generated mice doubly deficient in TLR2 and Nod2 and infected DCs from the mutant mice with *H. pylori*. The release of IL-1β in response to *H. pylori* was much lower in DCs from $Nod2^{-/-}Tlr2^{-/-}$ mice than in DCs from either $Tlr2^{-/-}$ or $Nod2^{-/-}$ mice (Fig. 2B). To determine whether TLR2 and Nod2 regulate the induction of pro-IL-1β, DCs from WT, $Tlr2^{-/-}$, $Nod1^{-/-}$, $Nod2^{-/-}$, and $Nod2^{-/-}Tlr2^{-/-}$ mice were infected with *H. pylori* and levels of pro-IL-1β induction were assessed by immunoblotting. We found that pro-IL-1β was induced upon infection in WT DCs. However, the levels of induction were reduced to a greater extent in doubly deficient $Nod2^{-/-}Tlr2^{-/-}$ DCs compared with those in single deficient DCs (Fig. 2C). To determine whether TLR2 and Nod2 regulate the transcriptional induction of pro-IL-1β, we prepared mRNA from WT and $Nod2^{-/-}Tlr2^{-/-}$ DCs before and after *H. pylori* infection and measured *Il1b* mRNA by quantitative real-time PCR. Consistent with the results shown in Figure 2C, the *Il1b* mRNA levels were significantly reduced in $Nod2^{-/-}$, $Tlr2^{-/-}$, and $Nod2^{-/-}Tlr2^{-/-}$ DCs (Fig. 2D) after *H. pylori* infection. These results indicate that NOD2 and TLR2 have a redundant role in *H. pylori* induced IL-1β secretion such that pro-IL-1β induction is significantly impaired in the absence of both receptors in DCs.

Processing of IL-1β upon H. pylori infection depends on caspase-1

IL-1β is synthesized as an inactive precursor and can be proteolytically cleaved by several proteases including caspase-1 into

its biologically active mature form [24]. To determine whether *H. pylori* induced IL-1β processing is dependent on caspase-1, we first evaluated the secretion of IL-1β in *H. pylori* infected DCs from WT or $Casp1^{-/-}$ mice. The release of IL-1β, but not TNF-α, was impaired in DCs from $Casp1^{-/-}$ mice (Fig. 3A and B). As a control for pro-IL-1β processing, we also stimulated DCs with LPS and ATP, a stimulus that potently induces cleavage of pro-IL-1β into its mature (p17) form via NLRP3 and caspase-1 [11]. Although pro-IL-1β induction was comparable in *H. pylori* infected DCs from WT and $Casp1^{-/-}$ mice, the processing of pro-IL-1β into IL-1β (p17) was impaired in DCs from $Casp1^{-/-}$ mice (Fig. 3C). In addition, infection of DCs with another *H. pylori* strain SPM326 that can colonize mice showed the same dependency on caspase-1 for pro-IL-1β and IL-1β secretion (Fig. 3D–F). These results indicate that processing and secretion of IL-1β in *H. pylori* infected DCs requires caspase-1.

TLR2 and NOD2 induce NLRP3 expression and pro-IL-1β processing upon H. pylori infection

We showed in Fig. 2 that TLR2 and NOD2 regulate the induction of pro-IL-1β and IL-1β secretion in *H. pylori* infected DCs. To determine whether TLR2 and NOD2 also regulate caspase-1 activation, WT and $Nod2^{-/-}Tlr2^{-/-}$ DCs were infected with *H. pylori* and caspase-1 activation was assessed by immunoblotting. The production of the p20 subunit of caspase-1 was induced by *H. pylori* infection in WT DCs, but was greatly impaired in $Nod2^{-/-}Tlr2^{-/-}$ DCs (Fig. 4A). Similarly, the amount of
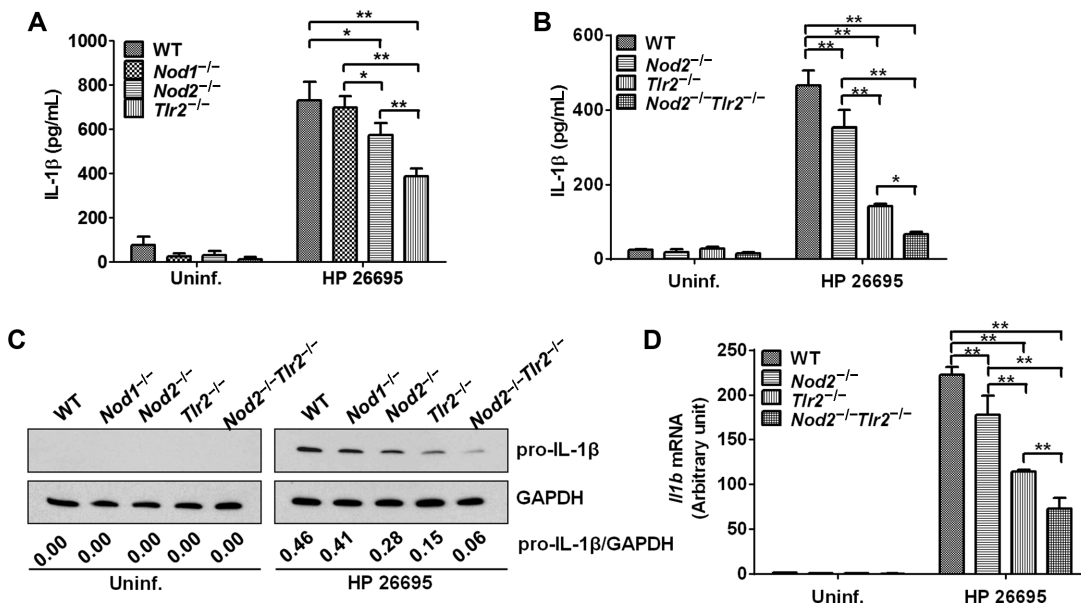
Figure 2. TLR2 and NOD2 induce pro-IL-1β expression and IL-1β release upon *H. pylori* infection in DCs. DCs from WT, *Tlr2⁻/⁻*, *Nod1⁻/⁻*, *Nod2⁻/⁻*, and *Nod2⁻/⁻Tlr2⁻/⁻* mice were infected with *H. pylori* at an MOI of 20 either (A, B) overnight or (C, D) for 6 h. (A, B) IL-1β secretion was determined by ELISA and (C) induction of pro-IL-1β was analyzed by immunoblotting. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D) mRNA expression of *Il1b* was evaluated by real-time PCR and fold increase (arbitrary units) was obtained by comparison to the level of uninfected DCs. (A, B, D) Data are shown as mean + SD of triplicate samples from one experiment representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, two-tailed Student's *t*-test.

processed IL-1β (p17) was reduced in *Nod2⁻/⁻Tlr2⁻/⁻* DCs when compared to WT cells (Fig. 4A). Activation of caspase-1 via the NLRP3 inflammasome is regulated, in part, by a priming step that involves the induction of *Nlrp3* [21]. Notably, infection of DCs by *H. pylori* increased the level of *Nlrp3* mRNA, which was reduced in *Nod2⁻/⁻*, *Tlr2⁻/⁻*, and *Nod2⁻/⁻Tlr2⁻/⁻* DCs (Fig. 4B). To further assess a role for NOD2/TLR2 in inflammasome priming (signal 1),

we pretreated DCs with LPS, a stimulus that primes the NLRP3 inflammasome, prior to *H. pylori* infection. Pretreatment of DCs with LPS enhanced the production of IL-1β in *H. pylori* infected cells and rescued the defective ability of DCs to secrete IL-1β in response to *H. pylori* infection (Fig. 4C). These results indicate that NOD2 and TLR2 contribute to caspase-1 activation via priming of the inflammasome.
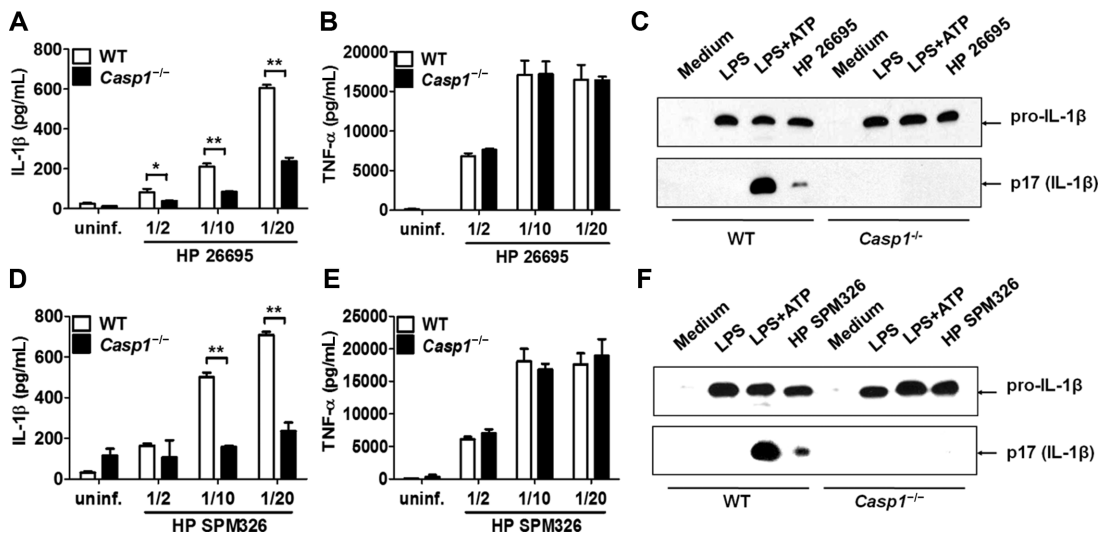

Figure 3. Processing of IL-1β upon *H. pylori* infection depends on caspase-1. DCs from WT and *Casp1⁻/⁻* mice were infected with *H. pylori* (A–C) HP 29965 or (D–F) HP SPM326 at the indicated MOI (A, B, D, E) overnight or (C, F) at an MOI of 100 for 6 h. (A, D) IL-1β and (B, E) TNF-α production was determined by ELISA and (C, F) induction of pro-IL-1β and p17 was analyzed by immunoblotting. (A, B, D, E) Data are shown as mean + SD of triplicate samples from one experiment representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, two-tailed Student's *t*-test.
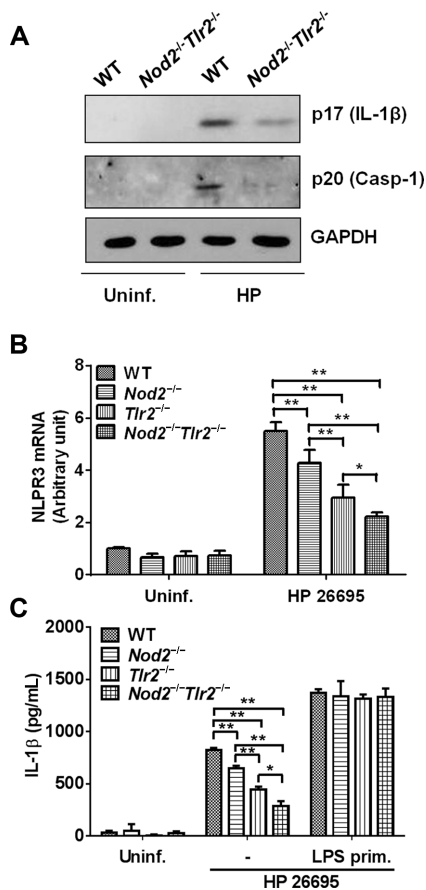
Figure 4. TLR2 and NOD2 induce *Nlrp3* expression and pro-IL-1β processing upon *H. pylori* infection. (A) DCs from WT and *Nod2⁻/⁻Tlr2⁻/⁻* mice were infected with *H. pylori* at an MOI of 100 for 6 h. The production of the p20 subunit of caspase-1 and processed IL-1β (p17) was analyzed by immunoblotting. GAPDH was used as a loading control. (B) DCs from WT and indicated WT and mutant DCs were infected with *H. pylori* at an MOI of 20 for 6 h. mRNA expression of *Nlrp3* was evaluated by real-time PCR and fold increase (arbitrary units) was obtained by comparison to the level of uninfected DCs. (C) DCs from WT and indicated mutant DCs were infected with *H. pylori* overnight with or without LPS priming (6 h). IL-1β production was determined by ELISA. (B, C) Data are shown as mean + SD of triplicate samples from one experiment representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, two-tailed Student's *t*-test.

Helicobacter pylori infection activates caspase-1 via the NLRP3 inflammasome in DCs

We next determined which inflammasome was involved in IL-1β secretion induced by *H. pylori* infection. DCs from WT and mice deficient in *Nlrp3*, *Nlrc4*, or the common adaptor *Asc* were infected with *H. pylori*. IL-1β secretion induced by infection was reduced in DC deficient in *Nlrp3* or *Asc*, but not in cells deficient in *Nlrc4* (Fig. 5A). The impairment in IL-1β secretion in *Nlrp3⁻/⁻* or *Asc⁻/⁻* DCs was specific in that the production of TNF-α in response to *H. pylori* infection was unimpaired (Fig. 5B). Consistently, activation of caspase-1 and production of mature IL-1β (p17) were abrogated in *Nlrp3⁻/⁻* and *Asc⁻/⁻* DCs, but not in *Nlrc4⁻/⁻* DCs (Fig. 5C). These results indicate that caspase-1-dependent IL-1β

processing and secretion requires the NLRP3 inflammasome in *H. pylori* infected DCs.

IL-1β signaling regulates *H. pylori* colonization in vivo

We next determined whether IL-1β signaling regulates the extent of *H. pylori* colonization in mice. To assess this, WT and mice deficient in IL-1β, IL-1 receptor caspase-1, and NLRP3 were orally infected with *H. pylori* and bacterial loads were determined in gastric tissue after infection. The SS1 *H. pylori* strain is widely used for in vivo studies. However, the mouse-adapted SS1 strain lacks a functional Cag T4SS [25], which we have found to be important for IL-1β production (Fig. 1). Therefore, we used in these experiments SPM326, a CagA-positive *H. pylori* strain that induces robust IL-1β production (Fig. 3D), expresses a functional Cag T4SS, and colonizes mice at low levels [26]. Consistently, low or undetectable pathogen colonization was found in the stomach of WT mice 4 weeks after infection (Fig. 6). Notably, the bacterial loads in the stomach were increased in *Il1b⁻/⁻*, *Il1r⁻/⁻*, and *Casp1⁻/⁻* mice when compared with those in WT mice (Fig. 6A–C). In contrast, we found comparable levels of pathogen colonization in *Nlrp3⁻/⁻* and WT mice (Fig. 6D). These results indicate that IL-1β signaling can limit colonization of *H. pylori*, but this is NLRP3-independent, in vivo.

Discussion

It is known that the mucosal levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α are significantly higher in *H. pylori* positive than *H. pylori* negative gastric specimens [27, 28]. Furthermore, the production of pro-inflammatory cytokines is important in the pathogenesis of *H. pylori* infection and development of *H. pylori* associated complications such as cancer [13, 18, 27, 28]. Among these pro-inflammatory cytokines, IL-1β can increase the expression of other cytokines, such as IL-6 and TNF-α, and regulate the expression of adhesion molecules and influx of inflammatory cells [29]. However, the mechanism by which IL-1β is produced in response to *H. pylori* infection remains poorly understood. In the present study, we showed that IL-1β secretion in *H. pylori* infected DCs is mediated by cooperative interaction between TLR2/NOD2 and NLRP3. TLR2 and NOD2 are both required for the transcriptional induction of pro-IL-1β and the priming of the inflammasome that is mediated by the upregulation of NLRP3. Based on the analysis of single and double deficient cells, the results indicate that although TLR2 is more critical to the induction of pro-IL-1β and NLRP3, NOD2 also contributes to this process, which is consistent with the ability of these PRRs to induce gene expression via NF-κB and MAPK activation [1, 30]. In contrast, NLRP3 was required for caspase-1 activation, processing of pro-IL-1β, and the release of mature IL-1β. These results are in agreement with the current view of NLRP3 activation that relies on two signals for the assembly of the inflammasome. While TLR2 and NOD2 provide signal 1 through the induction of NLRP3, the identity of signal 2, which
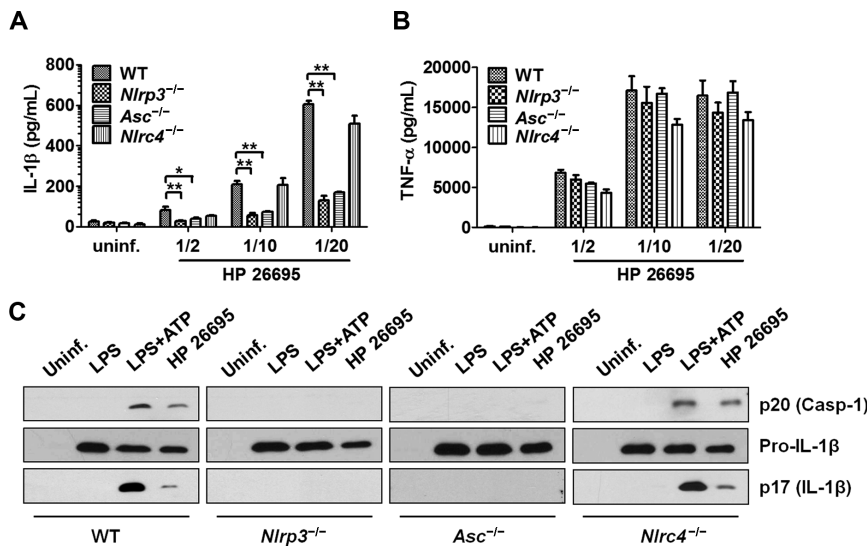
Figure 5. *Helicobacter pylori* infection activates caspase-1 via the NLRP3 inflammasome in DCs. DCs from WT and *Nlrp3*^{-/-}, *Nlrp4*^{-/-}, *Asc*^{-/-}, and *Nlrc4*^{-/-} mice were infected with *H. pylori* either (A, B) at the indicated MOI overnight or (C) at an MOI of 100 for 6 h. (A, B) IL-1β and TNF-α production was determined by ELISA and (C) activated caspase-1 (p20) and processed IL-1β (p17) was analyzed by immunoblotting. (A, B) Data are shown as mean + SD of triplicate samples from one experiment representative of three independent experiments. *p < 0.05, **p < 0.01, two-tailed Student's *t*-test.
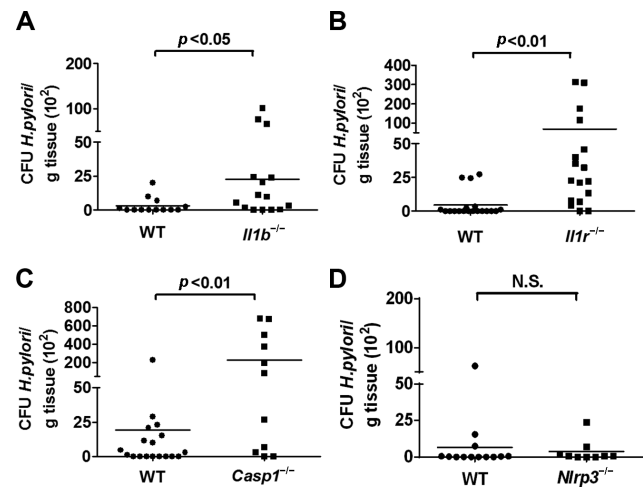

Figure 6. IL-1β signaling regulates *H. pylori* colonization in vivo. *Helicobacter pylori* colonization was determined in the stomach of WT, (A) *Il1b*^{-/-}, (B) *Il1r*^{-/-}, (C) *Casp1*^{-/-}, and (D) *Nlrp3*^{-/-} mice 4 weeks postinfection by quantitative culture. Values are expressed as colony-forming units (CFUs) per gram stomach tissue. Each symbol represents one animal and bars represent means. Data shown are pooled from two experiments. N.S., not significant. Statistical significance determined by one-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison test.

activates NLRP3 and is presumably provided by a molecule produced by *H. pylori*, remains to be determined. Although NLRP3 was required for IL-1β secretion in DCs infected with *H. pylori* in vitro, NLRP3 was not essential to clear the pathogen in the stomach. Because the clearance of *H. pylori* was impaired in mice deficient in IL-1β, IL-1R1, or caspase-1, the results suggest that the regulation of IL-1β in response to *H. pylori* infection in vivo is complex and may involve additional inflammasomes, which is not revealed in studies with DCs in vitro.

Helicobacter pylori possesses two major virulence factors, VacA and *cag*PAI. We provide evidence that *cag*PAI promotes IL-1β secretion by enhancing the transcriptional induction of IL-1β whereas VacA was dispensable. The *cag*PAI is a 40 kb stretch of DNA that encodes components of a T4SS that forms a pilus for the injection of virulence factors into host target cells [17]. The mechanism by which *cag*PAI regulates transcription of IL-1β is unclear. The observation that *H. pylori* deficient in CagL, an essential component of the Cag T4SS apparatus, is impaired in inducing IL-1β suggests that effector proteins or other bacterial molecules injected into the host cytosol via the T4SS might be involved in the regulation of IL-1β. Notably, CagA, a major effector that is translocated via the Cag T4SS, was not involved in the regulation of IL-1β. Previous studies showed that peptidoglycan fragments can be delivered via the Cag T4SS to the cytosol of epithelial cells eliciting NOD1 activation [22]. Because NOD2 is involved in the induction of IL-1β, it is possible that peptidoglycan molecules containing the muramyl dipeptide motif may be leaked into the cytosol of DCs, leading to NOD2 activation. However, we found that *Nod1*^{-/-}*Nod2*^{-/-} DCs infected with the *cag*PAI mutant still elicited reduced IL-1β secretion when compared with mutant DCs infected with the WT bacterium (results not shown). These results suggest that the mechanism by which *cag*PAI regulates transcription of IL-1β is not via translocation of Nod1/Nod2 microbial agonists into the host cytosol. Because CagL also binds to β1 integrins on the target cell surface, it is also possible that interactions between CagL and host receptors on DCs regulate IL-1β production.

Single-nucleotide polymorphisms of the *IL1B* gene are associated with an increased risk for the development of gastric cancer in the setting of *H. pylori* infection [18]. The mechanism by which IL-1β gene variants promote cancer is poorly understood and controversial. For example, some authors have reported that these genetic polymorphisms are associated with increased production of IL-1β, which has been suggested to induce hypochlorhydria, progressive gastric atrophy, and increased risk for gastric cancer [18]. Using a mouse model of IL-1β overexpression in the stomach, Tu et al. [20] provided evidence that IL-1β induces the recruitment of myeloid-derived suppressor cells to the stomach and the

activation of these cells may contribute to cancer development through the production of IL-6 and TNF-α. However, Sugimoto et al. [31] reported that IL-1β gene polymorphisms are linked to lower production of IL-1β in the gastric mucosa of individuals infected with *H. pylori* and lower pathogen eradication rate. Our results are consistent with the latter study in which we found that IL-1β signaling inhibits *H. pylori* colonization in mice. However, Hitzler et al. [23] reported that IL-1R-null mice had comparable colonization of the *H. pylori* SS1 strain. Unlike the *H. pylori* CagA-positive SPM326 strain used in our in vivo studies, the *H. pylori* SS1 strain lacks a functional Cag T4SS [25]. Thus, differential expression of *cag*PAI encoded factors that regulate IL-1β production may account, in part, for the difference in results. Thus, it is possible that increased *H. pylori* colonization as a result of deficient host IL-1β production promotes enhanced inflammatory responses to the pathogen and increased risk for cancer development.

Materials and methods

Ethics statement

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Michigan Committee on Use and Care of Animals (Approved Protocol Number: 09716).

Mice

$Nod1^{-/-}$, $Nod2^{-/-}$, $Casp1^{-/-}$, $Asc^{-/-}$, $Nlrp3^{-/-}$, and $Nlrc4^{-/-}$ in C57BL/6J background have been previously described [32–34]. Mice deficient in TLR2 in C57BL/6J background were a gift from Dr. Shizuo Akira (Osaka University, Japan). C57BL/6J mice were originally purchased from The Jackson Laboratory and maintained in our laboratory. Mice deficient in both NOD2 and TLR2 were generated by crossing $Nod2^{-/-}$ and $Tlr2^{-/-}$ mice and intercrossing the F_1 generation. Mice were housed in a pathogen-free facility.

Reagents and bacterial culture

Ultrapure LPS from *Escherichia coli* O111:B4 was purchased from InvivoGen. *Helicobacter pylori* strain 26695, P1 WT, isogenic mutant P1 ΔcagL (CagL deficient), P12 WT, isogenic mutant P12 ΔvacA (VacA deficient), P12 ΔcagPAI (*cag*PAI deficient), and P12 ΔcagL have been described [35]. *Helicobacter pylori* strain G27 WT and isogenic mutant G27 ΔcagPAI were gifts from Dr. Scott Merrell (Uniformed Services University of the Health Sciences, Bethesda, MD, USA) and SPM326 from Dr. Lesley Smythies (University of Alabama, Birmingham, AL, USA). *Helicobacter pylori*

was routinely grown on Campylobacter agar plates or Brucella broth containing 10% of FBS, 10 μg/mL of vancomycin (Sigma), 5 μg/mL of trimethoprim (Sigma), and 1 μg/mL of nystatin (Sigma) at 37°C under microaerobic conditions. *Helicobacter pylori* was isolated from gastric homogenates cultured on plates contained 200 μg/mL of bacitracin (Sigma), 6 μg/mL of vancomycin (Sigma), 16 μg/mL of cefsulodin (Sigma), and 20 μg/mL of trimethoprim (Sigma) to inhibit the growth of normal gastric flora.

Preparation of BMDCs and infection with *H. pylori*

BMDCs were prepared as previously described [11]. Briefly, BM cells were cultured with GM-CSF (20 ng/mL), with fresh GM-CSF added on days 3 and 5. After 7 days, nonadherent cells were collected by vigorous aspiration. BMDCs were seeded in 48-well plates (2×10^5/well) for enzyme-linked immunosorbent assay (ELISA) or six-well plates (5×10^6/well) for immunoblotting and quantitative PCR and infected with *H. pylori* overnight or 6 h, respectively.

Bacterial invasion assay

The invasion efficiency of *H. pylori* strains was evaluated using a gentamicin protection assay. Briefly, BMDCs were infected for 20 min and then incubated for 20 min at 37°C in medium containing gentamicin (100 μg/mL) to kill extracellular bacteria. The infected cells were then washed in PBS, lysed in 0.5% TritonX-100/PBS, and the number of intracellular bacteria was determined by plating.

Quantitative real-time PCR

RNA was extracted using the RNeasy Mini kit (Qiagen) and cDNA was prepared from 0.1 μg of RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instruction. Quantitative real-time PCR was performed by the StepOne Real-Time PCR System using SYBR green buffer according the manufacturer's instruction (Applied Biosystems). β-Actin was used for normalization. The following primer sequences were used; IL-1β forward: 5′-GATCCACACTCTCCAGCTGCA-3′; IL-1 β reverse: 5′-CAACCAACAAGTGATATTCTCCATG-3′; *Nlrp3* forward: 5′-ATGGTATGCCAGGAGGACAG-3′; *Nlrp3* reverse: 5′-ATGCTCCTTGACCAGTTGGA-3′; *Actb* forward: 5′-CAATAGTGATGACCTGGCCGT-3′; *Actb* reverse: 5′-CAATAGTGATGACCTGGCCGT-3′.

Measurement of cytokines

Mouse cytokines were measured in culture supernatants using the ELISA kit from R&D systems.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% Nonidet P-40, complete protease inhibitor cocktail (Roche), and 2 mM dithiothreitol. After centrifugation at 20 000 $\times g$ for 15 min, the supernatant was mixed with $5\times$ SDS buffer and boiled for 10 min, and samples were separated by SDS-PAGE and transferred to polyvinyldifluoride membranes. Membranes were incubated with rabbit antibody to mouse caspase-1 (a gift from P. Vandenabeele, University of Ghent, Ghent, Belgium), goat antibody to mouse IL-1β (R&D systems), and mouse antibody to mouse GAPDH (Millipore). Proteins were detected by ECL kit.

Mouse infection

Mice were inoculated three times by oral gavage with 500 µL of *H. pylori* strain SPM326 ($2–8 \times 10^9$/mL) with 1 day separating each inoculation. After 4 weeks, mice were euthanized by CO_2 and stomachs were removed and washed with sterile water. Washed stomachs were homogenized, plated onto agar plates, and incubated under microaerobic conditions at 37°C for 5–7 days.

Statistical analysis

Statistical significance between groups was determined by the two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by post hoc analysis (Newman–Keuls multiple comparison test) (Graphpad Prism 5). Differences were considered significant at $p < 0.05$.

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