Title: Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells.

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**Running title**  NOD1 detects bacterial vesicles
Summary

Gram negative bacterial peptidoglycan is specifically recognized by the host intracellular sensor NOD1, resulting in the generation of innate immune responses. Although epithelial cells are normally refractory to external stimulation with peptidoglycan, these cells have been shown to respond in a NOD1 dependent manner to Gram negative pathogens that can either invade or secrete factors into host cells. In the present work, we report that Gram negative bacteria can deliver peptidoglycan to cytosolic NOD1 in host cells via a novel mechanism involving outer membrane vesicles (OMVs). We purified OMVs from the Gram negative mucosal pathogens: *Helicobacter pylori, Pseudomonas aeruginosa* and *Neisseria gonorrhoea* and demonstrated that these peptidoglycan containing OMVs upregulated NF-κB and NOD1 dependent responses *in vitro*. These OMVs entered epithelial cells through lipid rafts thereby inducing NOD1 dependent responses *in vitro*. Moreover, OMVs delivered intragastrically to mice induced innate and adaptive immune responses via a NOD1 dependent but TLR independent mechanism. Collectively, our findings identify OMVs as a generalized mechanism whereby Gram-negative bacteria deliver peptidoglycan to cytosolic NOD1. We propose that OMVs released by bacteria *in vivo* may promote inflammation and pathology in infected hosts.
Introduction

The cytosolic host protein nucleotide binding oligomerization domain 1 (NOD1) has emerged as a key pathogen recognition molecule (PRM) for innate immune responses in epithelial cells (Fritz et al., 2006). This protein acts as an intracellular “sensor” of bacterial pathogens through its recognition of cell wall peptidoglycan (PG). As a result of detailed molecular studies, human NOD1 was shown to exhibit exquisite specificity for a diaminopimelate containing GlcNAc-MurNAc tripeptide (GM-TriDAP) motif that is almost exclusively found in Gram negative bacterial PG (Magalhaes et al., 2005). Although the specificity of the NOD1 ligand has been determined, the mechanisms whereby PG enters host cells and induces innate immune signaling during physiological conditions remain poorly understood.

Epithelial cells are generally refractory to external stimulation with microbial products, such as PG and lipopolysaccharide (LPS). The addition of synthetic or natural NOD1 agonists to the exterior of non-phagocytic epithelial cells in culture has no effect on initiating NOD1 signaling in these cells (Girardin et al., 2003). Certain bacterial pathogens, however, are able to trigger cytosolic NOD1 signaling in epithelial cells by either cell invasion (Girardin et al., 2001), or via the actions of a bacterial secretion system (Viala et al., 2004), suggesting that the intracellular presentation of PG may be required for signaling in these cells.

We previously reported that *H. pylori* utilizes the Type IV secretion system (T4SS) encoded by the “cag” pathogenicity island (cagPAI), to deliver PG to host cytosolic NOD1. The process of *H. pylori* transferring its PG into the host epithelial cell, and its subsequent recognition by NOD1, results in the activation of NF-κB and the production of interleukin-8 (IL-8) (Viala et al., 2004). However, findings of that study also indicated that another mechanism independent of the T4SS may exist
which is utilized by bacteria to transport PG into non-phagocytic host epithelial cells (Viala et al., 2004). Specifically, we reported that \textit{H. pylori} 251 harboring a non-functional \textit{cag} PAI, were still able to deliver radiolabeled PG to epithelial cells albeit with lower efficiency to bacteria with a functional T4SS (Viala et al., 2004). This finding would be consistent with the ability of \textit{H. pylori} bacteria without a functional T4SS to induce weak NOD1 responses in epithelial cells (Chaouche-Drider et al., 2009). Moreover, the existence of a T4SS independent mechanism for NOD1 activation may provide and explanation for the as yet unresolved question of how \textit{cag} PAI negative \textit{H. pylori} are able to initiate inflammation and pathology \textit{in vivo} (Backert et al., 2004; Yamaoka et al., 1997).

Previous studies showed that the microinjection of bacteria free supernatants of \textit{Shigella flexneri} induced pro-inflammatory responses in epithelial cells (Girardin et al., 2003; Philpott et al., 2000), suggesting that bacteria may secrete PG in a form that is suitable for NOD1 recognition. Although the supernatant associated material responsible for this activity was not identified, it was hypothesized that these bacteria free supernatants may contain outer membrane vesicles (OMVs) (Philpott et al., 2000).

OMVs, or “blebs,” are shed by Gram negative bacteria during normal growth, and have been reported to enter and transport virulence factors into host cells (Kuehn and Kesty, 2005). Given that OMVs contain numerous components of the bacterial cell wall (Kuehn and Kesty, 2005; Keenan et al., 2000), including PG hydrolyzing enzymes (Li et al., 1996), we speculated that OMVs may be involved in the intracellular delivery of PG to NOD1. In this study, we identify OMVs as a novel mechanism whereby all \textit{H. pylori} irrespective of their \textit{cag} PAI status, and indeed all Gram negative bacteria, may transport PG intracellularly so as to initiate NOD1
dependent NF-κB responses in non-phagocytic epithelial cells. We report that OMVs enter host epithelial cells via lipid rafts to transport their PG to NOD1 and induce pro-inflammatory responses. Using MyD88 and MAL knockout animals we determined that TLRs do not play a role in OMV induced innate immune responses in vivo. Furthermore, we identified NOD1 as being essential for the development of innate and adaptive immune responses to bacterial OMVs in vivo. Therefore, we propose that OMVs are a novel mechanism whereby Gram negative bacteria can transport their PG into the cytoplasm of non-phagocytic epithelial cells and initiate NOD1 dependent innate and adaptive immune responses in vivo.

Results

OMVs activate NF-κB dependent responses in non-phagocytic cells.

To determine whether OMVs can initiate NOD1 signaling in non-phagocytic cells, we purified OMVs from clinical and laboratory isolates of the Gram negative bacterium, *H. pylori* (Fig. S1A). This pathogen was previously shown to induce NOD1 signaling in epithelial cells via a bacterial T4SS encoded by the *cag*PAI (Viala et al., 2004). As we wished to identify a potentially T4SS independent mechanism for NOD1 signaling, we purified OMVs from *cag*PAI positive and *cag*PAI negative *H. pylori* strains, as well as from a *cag*PAI isogenic mutant (see below). The OMVs purified from *H. pylori* bacteria displayed a spherical, bi-layered morphology. All *H. pylori* OMVs separated by SDS-PAGE and subjected to Western blot analysis displayed a similar protein content and were largely devoid of the abundant cytoplasmic protein, urease, which can induce pro-inflammatory effects on gastric epithelial cells (Beswick et al., 2006) (Fig. S1B and C).
Next, the pro-inflammatory activities of *H. pylori* OMVs were determined by measuring nuclear factor-κB (NF-κB) dependent responses in AGS and HEK239 epithelial cell lines with functional NOD1 signaling (Girardin *et al.*, 2001; Girardin *et al.*, 2003). As a control, AGS cells were stimulated with live cagPAI positive *H. pylori* 251 bacteria, which were shown previously to activate NF-κB responses via a NOD1 dependent mechanism (Viala *et al.*, 2004) (Fig. 1). The external application of *H. pylori* OMVs, from cagPAI positive and negative strains, to epithelial cells induced significant NF-κB reporter activity compared to non-stimulated control cells (Fig. 1A). Therefore, OMVs purified from all *H. pylori* strains, irrespective of their cagPAI status, were capable of inducing NF-κB reporter activity. The variability in NF-κB reporter activity induced by OMVs from different strains correlated with previous findings describing the ability of these individual isolates to induce varying levels of NF-κB reporter activity and IL-8 production (Philpott *et al.*, 2002).

To confirm the role of a cagPAI independent mechanism for OMV induced responses in cells, we prepared OMVs from an isogenic cagPAI deletion mutant of the *H. pylori* 251 clinical isolate. *H. pylori* 251 cagPAI OMVs induced the up-regulation of several NF-κB dependent pro-inflammatory responses, as measured by human-β-defensin 2 (hBD2) and hBD3 reporter activity, as well as IL-8 production (Fig. 1B and 1C). In contrast, spent OMV free culture media or killed *H. pylori* 251 bacteria added directly to cells had no effect on IL-8 production (Fig. 1C). Furthermore, the microinjection of *H. pylori* 251 cagPAI OMVs, but not the NOD1 active motif of PG (GM-TriDAP) or culture medium alone, induced the nuclear translocation of the p65 subunit of NF-κB in epithelial cells (Fig. S2). These data demonstrated the ability of internalized OMVs to initiate NF-κB-dependent pro-inflammatory responses in epithelial cells.
OMVs specifically induce NOD1 dependent responses in non-phagocytic cells.

As the epithelial cell lines in the preceding studies express functional NOD1, and that OMVs were postulated to contain PG, we next sought to investigate the role of this PRM in OMV induced responses in epithelial cells and mouse embryonic fibroblasts (MEFs), with normal or impaired NOD1 signaling. NOD1 functionality was altered in these cells by either: expression of a dominant negative NOD1 construct (Viala et al., 2004) (Fig. 2A); stable knock-down of NOD1 expression (unpublished data) (Fig. 2B and C); or by gene disruption (Fig. 2D-F). In all instances, NF-κB dependent responses to H. pylori OMVs were significantly reduced in cells with impaired NOD1 signaling, when compared with wild type cells (P<0.05; Fig. 2A-F). These responses were shown to be independent of a cagPAI encoded secretion system (Fig. 2A-F). NOD1 dependency was also observed for AGS and MEF cell responses to OMVs from the Gram negative bacteria Neisseria gonorrhoea and Pseudomonas aeruginosa, which harbor PG with NOD1 agonist activity (Girardin et al., 2003; Travassos et al., 2005) (Fig. S3A and B respectively; P<0.05, Fig. 2B and 2D-F). Taken together, the data highlight the broad relevance of Gram negative bacterial OMVs as mediators of NOD1 dependent NF-κB responses in non-phagocytic cells. Furthermore, the findings reveal for the first time a potential mechanism by which H. pylori may drive the gastritis observed in cagPAI negative infections (Backert et al., 2004; Yamaoka et al., 1997).

Gram negative bacterial PG associated with OMVs is responsible for the NOD1 dependent NF-κB response induced in epithelial cells.
Consistent with previous reports (Kuehn and Kesty, 2005; Keenan et al., 2000) OMVs from *H. pylori*, *P. aeruginosa* and *N. gonorrhoea* contained proteins, whereas those from the latter two bacteria also contained DNA (Fig. S3E and F). To investigate the potential role of these OMV associated molecules in NF-κB activation, OMVs were subjected to heat, Proteinase K, or DNase treatment prior to stimulation of HEK293 cells (Fig. 3A and B). The effects of these treatments were assessed by electron microscopy, Western blot and agarose gel electrophoresis, respectively (Fig. S3C-F). Heat treatment did not significantly alter the morphology of OMVs (Fig. S3C and D), nor the ability of *H. pylori* and *N. gonorrhoea* OMVs to induce NF-κB activity. In contrast, heat treated *P. aeruginosa* OMVs displayed a small but significant reduction in immunostimulatory ability (Fig. 3A, \( P<0.05 \)). This may be consistent with the reported redundancy in PRM signaling to *P. aeruginosa* infection (Skerrett et al., 2007).

OMVs were also subjected to a continuous sucrose gradient, to remove any bacterial contaminants contained within the preparation. After separation by ultracentrifugation, fractions were analyzed by Western blotting (Figure S4A). Standardized amounts of each fraction (corresponding to approximately 10 µg protein) were tested for their ability to induce NF-κB-luciferase activity in HEK293 cells (Figure S4B). The fraction containing the peak NF-κB-inducing activity (Fraction 6) corresponded to that in which purified OMVs are normally isolated (at a density of 1.15 g/ml, 35% w/w sucrose) (Shang et al., 1998). The presence of OMVs within this fraction was confirmed by electron microscopy (Figure S4C). Relatively few proteins were found in this fraction, whereas a gradual increase in protein concentration was evident within heavier and less immunogenic fractions, indicating that many proteins associated with OMVs were removed during this purification.
process (Figure S4A). Collectively, these data suggest that proteins are unlikely to play a major role in the NOD1-dependent activity of *H. pylori* OMVs.

Nevertheless, to eliminate DNA and/or protein as mediators of *H. pylori* OMV activity, Proteinase K and DNase treated OMVs were added to HEK293 cells stably expressing Toll like receptor 2 (TLR2) or TLR9. These PRMs respond to bacterial lipoproteins and unmethylated DNA, respectively. The responsiveness of these cells was confirmed using *H. pylori* LPS, which atypically signals via TLR2 (Yokota et al., 2007; Lepper et al., 2005; Mandell et al., 2004) and CpG DNA, respectively (Fig. S5). Treated OMVs did not exhibit significantly altered NF-κB inducing activity in these cells (Fig. 3B). Furthermore, as neither HEK293 (Girardin et al., 2003) nor AGS cells (Kurt-Jones et al., 2004) possess a functional form of TLR2, and AGS cells cannot produce IL-8 in response to LPS stimulation (Backhed et al., 2003), we can conclude that *H. pylori* LPS was not responsible for activation of NF-κB by *H. pylori* OMVs. Collectively, these findings indicated that neither lipoproteins, DNA nor LPS were the prime agonists for NF-κB activation in epithelial cells stimulated by *H. pylori* OMVs.

Given the demonstrated role for NOD1 in OMV induced responses (Fig. 2), it seemed likely that PG should be present within OMVs. To confirm this hypothesis, we prepared OMVs from an *H. pylori* strain (251 lysA) in which tritiated meso-diaminopimelic substrate is specifically incorporated into the GM-TriDAP motif of Gram negative PG (Viala et al., 2004). Silver deposits, corresponding to tritiated PG, were associated with AGS cells that had been co-cultured with OMVs from either *H. pylori* 251 lysA or isogenic cagPAI mutant (251lysAcagM) bacteria (Fig. 3C). These deposits were absent from non-treated cells. In agreement with the radiolabeling data, OMV preparations were found to contain approximately 0.3 to 0.5
ng of the muramic acid moiety of PG, per µg OMV protein (0.45 ng ± 0.053 of muramic acid per µg of OMVs, mean ± SEM; n=3 independent samples).

**OMVs enter non-phagocytic cells via lipid rafts to induce NOD1 dependent responses.**

The requirement for PG entry in cytosolic NOD1 signaling (Girardin *et al.*, 2001; Inohara *et al.*, 2001) suggested that OMVs must enter the intracellular compartment of cells. To investigate this question, AGS cells were co-cultured with Alexa Fluor 568 labeled OMVs and then permeabilized or not with Triton X-100. *H. pylori* OMVs were detected using anti-*H. pylori* OMVs and Alexa Fluor 488 antibodies. Confocal microscopy revealed the co-localization of Alexa Fluor 488 and 568 fluorochromes only within permeabilized AGS cells, thus indicating the intracellular location of OMVs (Fig. 4A and C. Movie S1 and S2).

As bacterial OMVs have been reported to deliver virulence factors into host cells via lipid rafts (Kesty *et al.*, 2004), we next examined whether these cholesterol enriched domains may similarly be involved in OMV mediated NOD1 signaling. For this, lipid rafts from the cell membranes of AGS cells were disrupted by treatment with Fumonisins B₁ (FB₁), an inhibitor of sphingomyelin incorporation into lipid rafts, or methyl-β-cyclodextrin (MβC), a cholesterol depleting agent. A lipid raft stain was used to confirm the reduction of lipid rafts on the surface of FB₁ and MβC treated AGS cells (Fig. S6). After incubation of cells with Alexa Fluor 568 labeled OMVs, FB₁ treated AGS cells exhibited very low levels of red fluorescence, when compared to non-treated cells, and less intracellular co-localization when permeabilized and stained with Alexa Fluor 488 labeled anti-*H. pylori* OMV antibodies (confocal; Fig. S7, epifluorescence; Fig. S8). These results strongly suggest that sequestration of
sphingomyelin from lipid rafts abrogates OMV internalization. In concordance with FB1 treated AGS cells, MβC treated cells exhibited significantly reduced amounts of Alexa Fluor labeled OMVs within their intracellular compartment, compared to non-treated cells, further suggesting that the disruption of lipid rafts abrogates OMV internalization (confocal; Fig. 4A-D, epifluorescence; Fig. S9).

MβC, but not FB1 (Gopee and Sharma, 2004), has no effect on NF-κB signaling in cells, thus allowing us to also determine the role of lipid rafts in NOD1 responses to OMVs. First, we established that MβC treatment did not affect cell viability in AGS and HEK293 cells (AGS; \(P = 0.1835\), HEK293 cells; \(P = 0.096\)), nor NOD1 independent signaling with phorbol myristate acetate (PMA) (Fig. S10). Next, we determined the role of lipid rafts in NF-κB responses by treating HEK293 and AGS cells with MβC prior to co-culture with \(H. pylori\) 251 cagPAI OMVs. MβC treatment of HEK293 and AGS cells abrogated OMV induced NF-κB activity and IL-8 production, respectively, when compared to non-treated controls (Fig. 4E; Fig. S10 respectively). Moreover, replenishment of cholesterol on MβC treated HEK293 cells completely restored the immunostimulatory capacity of OMVs (Fig. 4F), thereby excluding pleiotropic effects of MβC. Taken together, the data show that pharmacological disruption of lipid rafts prevents both OMV entry and the induction of innate immune signaling in host cells.

**NOD1 is essential for innate and adaptive immune responses against \(H. pylori\) OMVs in vivo.**

Having demonstrated the ability of OMVs to induce NOD1 dependent signaling in vitro, we next examined the effect of these structures on host immune responses in vivo. For this, we established a model to determined the gastric
expression levels of the NOD1 responsive chemokine gene Cxcl2 (Viala et al., 2004) in C57BL/6 mice that were intragastrically fed either a single dose of H. pylori 251 cagPAI OMVs or H. pylori SS1 bacteria, as a positive control. Gastric Cxcl2 mRNA was detected as early as 1 day post feeding, in both H. pylori OMV or H. pylori SS1 fed animals, compared to PBS controls (Fig. S11). These responses were maintained in H. pylori infected animals during the period of infection, whereas OMV induced gastric Cxcl2 responses declined to basal levels by day 7 post feeding (Fig. S11).

To determine the potential role of TLR ligands in the up-regulation of gastric Cxcl2 mRNA expression in vivo by H. pylori OMVs, we fed H. pylori 251 cagPAI OMVs to Mal (MyD88 adaptor like/TIRAP; TIR domain containing adaptor protein) and MyD88 knockout mice (KO) (Fig. 5A). Mal is an adaptor molecule required for TLR2 and TLR4 signaling (Kenny and O'Neill, 2008; Fitzgerald et al., 2001), whereas MyD88 is the key adaptor protein required for signaling by all TLRs, excluding TLR3 (Rakoff-Nahoum and Medzhitov, 2009). Oral administration of OMVs to Mal and MyD88 KO mice resulted in the up-regulation of gastric Cxcl2 mRNA to similar levels as those detected in wild type OMV fed mice, at 1 day post feeding (Fig. 5A) (P=0.52 and P=0.75, respectively, when compared to OMV fed WT mice). These data suggested that TLRs were not required for the observed gastric responses to H. pylori OMVs. Next, we examined the requirement of NOD1 for OMV induction of innate and adaptive immune responses in NOD1 wild type (WT; Card4+/+) and NOD1 KO (Card4−−) mice. Gastric Cxcl2 expression levels were significantly increased in WT animals that had been intragastrically fed H. pylori 251 cagPAI OMVs, when compared to those of both PBS fed WT and OMV fed KO animals, at one day post feeding (P<0.05; Fig. 5B). Furthermore, to examine the requirement of NOD1 for the development of an OMV specific humoral immune
response, mice were fed a second dose of OMVs at day 28 and antibody responses were measured 3 weeks later at day 49. The WT but not KO mice, displayed significant anti-

\textit{H. pylori} OMV IgG responses in their sera in response to mucosally administered \textit{H. pylori} OMVs, when compared to PBS fed animals 49 days post feeding (\(P=0.0311\) and \(P=0.696\), respectively; Fig. 5C). Collectively, these data definitively prove that while there may be other bacterial products associated with \textit{H. pylori} OMVs, TLR recognition of these bacterial components is not responsible for the initiation of inflammatory responses observed \textit{in vivo}. Therefore, these findings clearly identify that NOD1 is essential for the generation of OMV dependent innate immune responses in the mucosal compartment \textit{in vivo}, as well as the development of systemic OMV specific adaptive immune responses.

\textbf{Discussion}

Since the discovery of NOD1 as the receptor responsible for the recognition of Gram negative PG (Girardin \textit{et al.}, 2003), numerous studies have examined the role of NOD1 in the initiation of innate immune responses. Most of these studies have used purified PG or NOD1 agonists that were introduced into epithelial cells by cell permeabilization, (Girardin \textit{et al.}, 2003) or by direct incubation with phagocytic cells, such as macrophages (Magalhaes \textit{et al.}, 2005). There have been limited examples, however, describing the physiological mechanisms by which Gram negative bacterial pathogens may transport their PG to cytosolic NOD1 within host cells, particularly non-phagocytic cells. To date, the two known mechanisms whereby Gram negative bacteria can deliver PG to cytosolic host NOD1 involve either cellular invasion (Girardin \textit{et al.}, 2001) or delivery via a bacterial secretion system (Viala \textit{et al.}, 2004).
However, PG molecules from almost all Gram negative pathogens, irrespective of their ability to invade host cells or to express a bacterial secretion system, can be detected by NOD1 and are able to initiate NOD1 dependent pro-inflammatory responses in host cells (reviewed in (Kaparakis et al., 2007; Fritz et al., 2006; Sansonetti, 2006)). Furthermore, it has been previously reported that in the absence of a T4SS, \textit{H. pylori} is still able to transfer PG into host epithelial cells albeit via a less efficient, unknown mechanism (Viala et al., 2004). Hence, a fundamental question in the area of NOD1 research has remained unanswered: how might all Gram negative pathogens, irrespective of their mode of infection, initiate NOD1 signaling in non-phagocytic epithelial cells? In this study, we have identified OMVs released by Gram negative bacteria as a generalized mechanism for the delivery of PG to host cytosolic NOD1.

In all multicellular organisms, membrane vesicles seem to function as a mechanism for intercellular communication and transportation of virulence determinants between cells during normal and stressed conditions (reviewed in (Stoorvogel et al., 2002)). We have now identified for the first time a novel physiological role for bacterial OMVs as initiators of immune responses in host cells. We suggest that membrane vesicles represent a new virulence mechanism by which both non-invasive and invasive bacteria initiate inflammatory processes in host cells.

Stimulation of cells with altered NOD1 functionality identified that OMV induced immune responses were initiated via NOD1. We found that OMVs containing PG enter host cells and initiate NOD1 dependent inflammatory responses. Furthermore, the introduction of PG or GM-Tri_{DAP} into the cytoplasm of NOD1 expressing cells, via microinjection, does not result in NOD1 dependent signaling (this study and SEG, unpublished data). Hence, administration of PG directly into the host cytosol is not
sufficient for NOD1 recognition. Our data suggest that OMVs, which are comprised of a bi-layered lipid membrane, facilitate the intracellular trafficking of PG in an appropriate form to cytosolic NOD1 and thus, the initiation of an immune response. Indeed, a previous study has identified that lipophilic acryl residues associated with iE-DAP, the core immunostimulatory component of NOD1, enhanced the NOD1 stimulatory activity of iE-DAP (Hasegawa et al., 2007), further supporting our hypothesis that lipid associated with PG can facilitate NOD1 signaling. The intracellular trafficking and interaction of NOD1 with OMV associated PG forms the basis of future research.

In this study, we report that lipid rafts located on the cell membrane, are utilized by OMVs as portals of entry into host cells. Indeed, depletion of lipid rafts on the surface of epithelial cells reduced OMV entry and NOD1 dependent responses. Conversely, cholesterol replenishment of the cells restored both the entry and immunostimulatory capacities of OMVs, thereby confirming the requirement of lipid rafts for OMV induced NF-κB responses. A possible explanation for the inability of microinjected NOD1 agonists to initiate signaling is due to the lipid membrane association of NOD1 and its potential inability to access its ligand. We speculated that OMV entry via lipid rafts may render PG accessible to membrane associated NOD1.

Although bacterial OMVs have been reported to deliver toxins via lipid rafts (Kesty et al., 2004), this is the first report to our knowledge in which lipid rafts have been shown to be critical for OMV induced innate immune responses in host cells. Interestingly, however, a recent report described the association of NOD1 and its downstream signaling molecule NEMO with the plasma membrane at the apical surface of human epithelial cells (Kufer et al., 2008). Moreover, the addition of invasive Shigella to cells provoked the further recruitment of membrane associated
NOD1 to the focal points of bacterial entry i.e. lipid raft domains (Kufer et al., 2008). These data are therefore consistent with our findings suggesting that lipid rafts are essential for OMV entry and NOD1 signaling in non-phagocytic epithelial cells.

We performed various studies to exclude the role of OMV associated TLR agonist in the induction of NF-κB immune responses. HEK293 and AGS cells are devoid of a functional form of TLR2 (Girardin et al., 2003; Kurt-Jones et al., 2004) and AGS cells do not produce IL-8 in response to LPS stimulation (Nilsson et al., 2008; Backhed et al., 2003). Therefore, we postulated that *H. pylori* LPS and lipoproteins associated with OMVs were not responsible for activation of NF-κB or NF-κB induced responses, such as hBDs and IL-8. Furthermore, by a process of exclusion, using either Proteinase K or DNAse treated OMVs, we established that lipoproteins and bacterial DNA were not required for OMV induced NF-κB responses in TLR2 and TLR9 expressing HEK293 cells.

Finally, we confirmed that TLRs are redundant for the induction of innate immune response against OMVs *in vivo* by administering *H. pylori* 251 cagPAI OMVs orally to WT and KO mice. We showed that MyD88 and Mal KO mice, which are defective in all of the known TLR pathways involved in signaling to bacterial pathogens, generated rapid inflammatory responses to OMVs, thus excluding a requirement for TLRs in these responses. In contrast, NOD1 KO mice did not produce any Cxcl2 mRNA responses, nor any OMV specific IgG antibodies, in response to oral administration of *H. pylori* OMVs. These findings provide an immunological basis for the known efficacy of OMV based vaccines, such as the one developed against the NOD1 signaling pathogen, *N. meningitidis* (Antignac et al., 2003; Saunders et al., 1999). We also propose that OMVs may play a role in the inflammatory conditions associated with persistently colonizing pathogens, such as *H. pylori* (Fiocca et al.,
This would explain how *H. pylori* cagPAI negative strains, which lack a functional T4SS, are still able to induce inflammation *in vivo* (Ohnita *et al.*, 2005; Crabtree *et al.*, 2002; Lee *et al.*, 1997).
Experimental procedures

**Bacteria** *H. pylori* 189, 249, 26695, 251 and 251 cagPAI isolates were cultured on blood agar medium or in Brain Heart Infusion (BHI) broth supplemented with 0.2 % (w/v) β-cyclodextrin (Sigma) (Philpott *et al.*, 2002). The *H. pylori* 251 cagPAI deletion mutant was constructed by natural transformation with pJP46 (Odenbreit *et al.*, 2001). The mouse adapted *H. pylori* Sydney Strain 1 (SS1) was cultured using blood agar (Ferrero *et al.*, 1998). *N. gonorrhoeae* MS11 and *P. aeruginosa* PA103pilA were cultured as described previously (Gunasekere *et al.*, 2006; Whitchurch *et al.*, 2005). *P. aeruginosa* PA103pilA does not produce many of the known virulence factors (*e.g.* pilin and flagellin).

**Bacterial OMVs** OMVs were purified from log phase bacterial cultures (Keenan *et al.*, 2000) and protein concentrations were determined by Bradford assay (BioRad). OMVs were stained using Alexa Fluor 488, 568 (Molecular Probes) or 0.5 % (w/v) uranyl acetate. Muramic acid contained within OMVs and standard solutions (MDP, Invivogen), was quantified (Hadzija, 1974). OMV associated PG was tritiated as previously described (Viala *et al.*, 2004). OMVs were heat treated by boiling at 100°C for 20 minutes. DNA and proteins were removed using 10 units/ml of DNase (Promega) or 100 µg/ml of Proteinase K (Epicentre), respectively. The enzymes were inactivated at 75°C for 20 minutes. Proteinase K was further inactivated using an inhibitor (Cocktail Set I, Calbiochem). The effectiveness of treatments was confirmed by Western blot or agarose gel electrophoresis, respectively. OMVs from *H. pylori* strains were probed with antibodies to either: in-house rabbit anti-*H. pylori*, or rabbit anti-*H. pylori* urease subunits A or B.
Sucrose gradient purification of OMVs

OMV preparations were washed 3 times with PBS using an Amicon YM-10 column (Millipore), prior to layering onto discontinuous sucrose gradients that were subjected to centrifugation at 100,000 g for 16 hours as previously described (Shang et al., 1998). Fractions (3 ml) were collected, washed 3 times with PBS using an Amicon YM-10 column and concentrated to a final volume of 500 µl. Each fraction was tested for their protein concentration, their protein profile by Western blot analysis, and their ability to induce NF-κB luciferase activity in HEK293 cells. The presence of OMVs within Fraction 6 was confirmed by electron microscopy (Jeol 200CX 200KV transmission electron microscope).

Epithelial cell culture assays

HeLa, HEK293 and AGS cells were cultured using standard techniques. Stable AGS NOD1 knockdown and control cell lines were generated by integration of an expression vector containing siRNA directed to either NOD1 or EGFP, respectively (manuscript in preparation, R.L.F). Reporter assays were performed with Igκ luciferase (Viala et al., 2004), hBD2 or hBD3, dTK Renilla (Promega) or ΔCARD NOD1 (Viala et al., 2004) constructs. Cells transfected with luciferase constructs were stimulated for 8 hours with OMVs (10 µg protein) or H. pylori 251 bacteria at a MOI of 10:1 (Viala et al., 2004; Philpott et al., 2002), then lysed (Reporter lysis buffer, Promega) and the luminescence measured using a FLUOstar Optima luminometer (BMG Labtech). H. pylori 251 bacteria were killed by fixing with 1% (w/v) formaldehyde, and subsequently plated on blood agar to confirm they were no longer viable (Huang et al., 1995). Digitonin permeabilized HEK293 cells were stimulated with equivalent amounts of phenol water extracted, bacteria free S. flexneri supernatants. AGS cells were stimulated with 160 nM phorbol
myristate acetate (PMA, Invitrogen). TLR2 expressing HEK293 cells were stimulated with 6.25 ng of *H. pylori* (Dr A. Moran, The National University of Ireland, Galway, Ireland) or 125 ng ultrapure *E. coli* LPS (Invivogen), and TLR9 expressing HEK293 cells were stimulated with 100 nM CpG DNA (a gift from Dr A. Mansell, Monash Institute for Medical Research, Monash University, Australia) for 8 hours. AGS culture supernatants were analyzed for IL-8 production (OptEIA™, BD Biosciences).

**Cell microinjection** HeLa cells, grown on 10 mm coverslips in serum free conditions, were microinjected using an approach adapted from our earlier studies (Philpott *et al.*, 2000; Coleman *et al.*, 2001). Coverslips were mounted in a recording chamber (Warner Instruments) on a Leica DMLFS microscope and cell filling was monitored using 0.2 % (w/v) Lucifer yellow and 0.05 % (w/v) fluorescein dextran (Molecular Probes, 3 kDa). Micropipettes were mounted on a micromanipulator (MP-285, Sutter), connected to a picospritzer (General Valve) to facilitate cell loading. After microinjection, cells were incubated at 37 °C for 2 hours, fixed and NF-κB was detected using anti-p65 NF-κB antibody (Cell Signaling Technologies) and anti-rabbit Alexa-568 labeled secondary antibody (Molecular Probes).

**Internalization of OMVs** AGS cells grown on coverslips were co-cultured with Alexa Fluor labeled OMVs, for 16 hours, fixed in 4 % (w/v) paraformaldehyde and permeabilized using 0.1 % (v/v) Triton-X 100 in PBS. Slides were blocked in a 5 % (v/v) FCS and 0.1 % (w/v) BSA solution, with or without the addition of 0.1 % (v/v) Triton-X-100, then incubated with a combination of: either in-house rabbit anti-*H. pylori* or rabbit anti-*H. pylori* OMV and goat anti-rabbit IgG Alexa-568 or Alexa 488 labeled antibodies (Invitrogen). Slides were visualized using AX70 or BX51
upright UV microscopes (Olympus). Confocal images were obtained using a Leica SP5 multiphoton confocal microscope. Images were prepared for publication using Analysis software (Olympus, Tokyo, Japan).

**Depletion of lipid rafts using methyl-β-cyclodextrin or Fumonisin B₁ and staining of lipid rafts.** Cells were cholesterol or sphingolipid depleted using methyl-β-cyclodextrin (MβC; Sigma), or Fumonisin B₁ (FB₁; Sigma), respectively. Cells were depleted of cholesterol by a 30 minute treatment with 4 nM MβC in serum free media. Cell viability after MβC treatment was confirmed using the luminescence based Cell Titer Glow assay (Promega). Cells were depleted of sphingomyelin by growing them in the presence of 100 µM FB1 for 2 days. After either treatment, the cells were subjected to OMV stimulation, or had their lipid rafts stained using the Vybrant® lipid raft labeling kit (Molecular probes). Cholesterol was added to cholesterol depleted cells by treating with 250 µM cholesterol (5-cholesten-3β-ol; Sigma) and 4 nM MβC for 30 minutes.

**Co-culture studies with tritiated OMV associated PG**

PG within OMVs from *H. pylori* lysA or lysAcagM bacteria was specifically tritiated using a previously described technique (Viala *et al.*, 2004). AGS cells were co-cultured overnight with tritiated OMVs (300 µg protein), and the presence of tritiated PG detected. The slides were counter stained with Giemsa stain.

**MEF isolation and cell culture** All NOD1 animal experimentation was performed at the University of Toronto (protocol number: 20006359). MEFs were isolated from C57BL/6 WT and NOD1 (*Card4*−/−) KO mice, which had been backcrossed more than
eight times onto a C57BL/6 background (Girardin et al., 2003). MEFs were cultured in 96 well plates (4 x 10^4 cells/ well), then stimulated for 24 hours with either: OMVs, highly purified *Escherichia coli* LPS (100 ng/ml; Lausen, Switzerland); Gram-positive PG from *Staphylococcus aureus* (100 ng/ml; Sigma) or Pam3Cys (100 ng/ml; Sigma). Chemokine and cytokine levels were determined by ELISA (DuoSet, R&D Systems).

**Mouse immunization and measurement of immune responses** Age matched male and female animals were used in all experiments. Immunization of NOD1, MyD88 and Mal knockout animals was performed at The University of Toronto, The University of Melbourne and Monash University, respectively. All animal experimentation was performed in accordance with the animal ethics approval obtained from the relevant institution. Animals received either a single intragastrically delivered 100 µl dose of 50 µg of *H. pylori* 251 cagPAI OMVs in PBS, 3x10^7 *H. pylori* SS1 bacteria in 100 µl of PBS or PBS alone (Ferrero et al., 1998). Their stomachs were removed 16 hours later, washed with PBS and stored in RNAlater (Ambion). In some experiments, stomachs from animals were analyzed at later timepoints, as indicated in the figure legends. Gastric RNA was purified using TRIzol reagent (Invitrogen). RNA was treated with RNase free DNAse (Promega), prior to generation of cDNA (SuperScript III, Invitrogen). The cDNA was amplified using primers for murine actin (forward primer: 5’ ACGGCCAGGTCATCACTATTG, reverse primer 5’CAAGAAGGAAGGCTGGAAAGA) and murine *Cxcl2* (forward primer: 5’ GGGCGGTCAAAAAGTTTGC, reverse primer 5’ ATTTGTTTCAGTATCTTTTGGATGATTTTC). Up-regulation of *Cxcl2* mRNA in the stomach tissue, normalized to actin mRNA, was detected using SYBR GREEN
PCR mastermix and ABI7300 (ABI) or Mastercycler ep realplex (Eppendorf) real time machines.

**Antibody production in response to OMV stimulation** OMV fed mice were gavaged at 28 days with a second 100 µl dose of 50 µg of *H. pylori* 251 cagPAI OMVs in PBS and antibody responses were determined 21 days later, at day 49. PBS control mice were gavaged with PBS at both timepoints. Mouse anti-*H. pylori* OMV IgG responses were detected by ELISA using a method adapted from our earlier studies (Kaparakis et al., 2006). Maxisorb 96 well plates were coated with 250 µg/ml of *H. pylori* 251 cagPAI OMVs. Sera were initially diluted 1:50 and serial dilutions of these were performed to determine the endpoint titre. OMV specific mouse anti-IgG antibodies were detected using anti-mouse IgG biotin-labeled antibody (Chemicon) and streptavidin-HRP (Chemicon). ELISAs were developed using BD OptEIA TMB substrate reagent (BD biosciences). Absorbances were read at OD$_{450nm}$ using a FLUOstar Optima (BMG Labtech). Serum endpoint titres were expressed as the reciprocal of the dilution of serum that gave an OD$_{450nm}$ value five times the value of the background. Mice with an antibody titre below the detection limit of the assay (log$_{10}$ 1.7) were assigned a titre of log$_{10}$ 1.7. Positive and negative control sera were included in all ELISA experiments.

**Statistical analysis** IL-8 responses were analyzed using the Student’s $t$-test. Luciferase activity, antibody titres and mRNA levels were analyzed using the Mann Whitney U-test. Differences were considered significant when $P<0.05$. 
References


Figure legends

Fig. 1. Bacterial OMVs induce NF-κB dependent responses in cells.

(A) NF-κB reporter activity in HEK293 cells stimulated with 10 µg OMVs from cagPAI positive (251, 26695) or negative (189, 249) *H. pylori* strains, *H. pylori* bacteria (251) or culture medium (control). (B) hBD2 and hBD3 reporter activities in HEK293 cells. (C) IL-8 production by AGS cells stimulated with viable or killed *H. pylori* 251 bacteria, 10 µg *H. pylori* 251 cagPAI OMVs or OMV control corresponding to spent media. Error bars indicate ± SEM between triplicates. All data are representative of ≥ 3 independent experiments.

Fig. 2. OMVs induce NOD1 dependent NF-κB responses in non-phagocytic cells.

(A) NF-κB activity in OMV stimulated (10 µg) HEK293 cells co-transfected with 20-100 ng of dominant negative NOD1 (∆CARD). (B) NF-κB activity and (C) IL-8 responses in AGS siRNA stable knockdown cells stimulated with 10 µg OMVs from *H. pylori* 251 cagPAI, *N. gonorrhoeae* or *P. aeruginosa*. OMV control corresponds to spent media (D) KC, (E) MIP-2 and (F) IL-6 production in wild-type and NOD1 knockout (NOD1 KO) MEFs stimulated with 100 ng *E. coli* LPS, Gram positive PG, Pam3Cys (P3Cys) or 10 µg OMVs from *H. pylori* 251 cagPAI (Hp), *N. gonorrhoeae* (Ng) or *P. aeruginosa* (Pa). Error bars indicate ± SEM between triplicates. All data are representative of ≥ 3 independent experiments. * denotes P<0.05 versus controls.

Fig. 3. DNase and protease treatment of OMVs does not abolish their immunostimulatory ability.

(A) NF-κB reporter activity in HEK293 cells stimulated with 10 µg of non-treated or heat treated OMVs from *H. pylori* 251 cagPAI (Hp), *N. gonorrhoeae* (Ng) or
P. aeruginosa (Pa). (B) NF-κB reporter activity in TLR2 and TLR9 expressing HEK293 cells stimulated with 10 µg of non-treated, heat, Proteinase K or DNase treated H. pylori 251 cagPAI OMVs. Percentage values reported with respect to cells stimulated with non-treated OMVs. Error bars indicate ± SEM between triplicates. Data are representative of ≥ 3 independent experiments. * denotes P<0.05 versus controls. (C) AGS cells co-cultured with 300 µg tritiated OMVs (observed as brown granules) from H. pylori lysA or lysAcagM strains (100 x magnification). Data are representative of three independent experiments.

Fig. 4. OMVs enter epithelial cells via lipid rafts.
AGS cells were co-cultured with 10 µg Alexa 568 labeled H. pylori cagPAI OMVs (red). Lipid raft intact (A, C), or MβC treated (B, D) cells were permeabilized with Triton X-100 (C-D), or left as controls (A-B). OMVs were detected using rabbit anti-H. pylori OMVs and anti-rabbit Alexa Fluor 488 antibodies, respectively (green). Cells were visualized by confocal microscopy. Labeled OMVs (red) were predominantly contained within the intracellular compartment of AGS cells whereas fewer were within MβC treated cells. Only permeabilized cells exhibited areas of intracellular dual fluorescence (yellow; indicated by the arrows). Scale bar represents 20 µm. (E) NF-κB activity in HEK293 cells pre-treated or not with MβC prior to stimulation with 10 µg OMVs from H. pylori 251 cagPAI (Hp), N. gonorrhoeae (Ng) or P. aeruginosa (Pa). (F) NF-κB activity in control, MβC treated, or MβC treated and cholesterol replenished HEK293 cells stimulated with 10 µg H. pylori 251 cagPAI OMVs. Error bars indicate ± SEM between triplicates. All data are representative ≥ 3 independent experiments. * denotes P<0.05 versus MβC untreated cells.
Fig. 5. NOD1 (Card4<sup>−/−</sup>) KO mice do not respond to OMV stimulation.

(A) Gastric Cxcl2 mRNA responses in C57BL/6 WT (WT), Mal KO (Mal) and MyD88 KO (MyD88) mice orally administered PBS (open symbols) or 50 µg <i>H. pylori</i> 251 cagPAI OMVs (filled symbols). Responses were measured 1 day post feeding. Horizontal lines indicate the mean ± SEM values corresponding to each group of animals. Data for Mal and MyD88 KO animals were pooled from two independent experiments, whereas the data for WT mice were pooled from four independent experiments. (B) Gastric Cxcl2 mRNA responses in C57BL/6 WT (WT) and NOD1 KO (NOD1) mice orally administered PBS (open symbols) or 50 µg <i>H. pylori</i> 251 cagPAI OMVs (filled symbols). Responses were measured 1 day post feeding. Line indicates average response of WT OMV fed animals. Results are from two experiments pooled, minimum n=3 mice per group in each experiment. * denotes <i>P</i>&lt;0.05 versus WT OMV mice. (C) <i>H. pylori</i> OMV specific IgG antibody titre of C57BL/6 WT (WT) and NOD1 KO (NOD1) mice. n=3 mice per PBS control groups. n=5 and n=4 mice per OMV fed WT and KO groups respectively. * denotes <i>P</i>&lt;0.05 versus WT PBS mice.
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Fig. S1. Purification of H. pylori OMVs and analysis of their protein content.

(A) Representative transmission electron micrograph (TEM) of H. pylori OMVs (indicated by arrows), which were purified from H. pylori (strain 189, 27,500x magnification). Scale bar indicates 500 nm. H. pylori OMVs were analyzed for
protein content by Western blotting. OMVs from *H. pylori* strains: 1) 26695, 2) 251, 3) 251 *cagPAI*, 4) 189 and 5) 249, were probed with antibodies to either: (B) *H. pylori* total extracts, or (C) *H. pylori* urease subunits A (UreA) or B (UreB). As a control, the antibodies were also reacted against whole bacteria of *H. pylori* 251 (lane 6). Molecular weight markers (Panel B) are indicated on the left hand side of the membrane. Arrows (Panel C) indicate the molecular weights and positions of the urease subunits on an SDS-PAGE gel.

**Fig. S2. Microinjection of HeLa cells with *H. pylori* OMVs.**

Representative images of HeLa cells co-microinjected with FITC-Dextran, Lucifer Yellow and either: BHI broth, GM-TriDAP (TriDap) or *H. pylori* OMVs. Lucifer Yellow and FITC-Dextran were used to identify microinjected cells both during microinjection and after immunofluorescence staining, respectively. NF-κB localization within microinjected cells was determined using rabbit anti-NF-κB and anti-rabbit Alexa Fluor 568 antibodies, respectively. As a control, HeLa cells that were not injected were reacted with an anti-rabbit Alexa 568 antibody to determine the level of non-specific binding. All microinjected cells (identified by the white arrows) displayed diffuse NF-κB staining, whereas only OMV injected cells had NF-κB staining localised in their nucleus. Images are representative of two independent experiments. (Scale bar indicates 50 µm.)

**Fig. S3. Purification of *N. gonorrhoeae* and *P. aeruginosa* OMVs and confirmation of the removal of proteins and DNA from OMV preparations.**

Representative TEMs of OMVs (indicated by the arrows) purified from (A) *N. gonorrhoeae* and (B) *P. aeruginosa*. Scale bar indicates 100 nm. Representative
TEMs of (C) control and (D) heat treated *H. pylori* OMVs (indicated by the arrows). Scale bar indicates 100 nm. (E) Western blot analysis of total proteins in *H. pylori* OMV preparations subjected to heat denaturation (lane 1), Proteinase K digestion (lane 2) or no treatment (lane 3). Treated and non-treated *H. pylori* OMVs were transferred to nitrocellulose membrane and probed with antisera against an *H. pylori* total extract. (F) OMV preparations from *N. gonorrhoeae* (lanes 1 and 2) and *P. aeruginosa* (lanes 3 and 4) were subjected to DNase treatment (lanes 2 and 4) or mock treated (lanes 1 and 3). OMVs was separated by agarose gel electrophoresis and stained with SYBR Green (Invitrogen).

**Fig. S4. Sucrose gradient purified OMVs contain reduced amounts of bacterial proteins but retain their ability to induce NF-κB activity in HEK293 cells.**

OMVs were purified by continuous gradient sucrose ultracentrifugation. Fractions were collected (3 mls) and concentrated to 500 µl. (A) Equivalent amounts (25 µl) of each fraction were analyzed for their protein content by Western blotting. Fractions were numbered F1 to F13, with F1 being the lightest fraction. OMVs prior to sucrose gradient purification (O) were also analyzed for their protein profile. Molecular weight markers are indicated on the left-hand side of the membrane. (B) Standardized amounts of each fraction (approximately 10 µg protein) were tested for their ability to induce NF-κB-luciferase activity in HEK293 cells. Error bars indicate ± SEM between triplicates. Data is representative of two independent experiments. (C) Representative TEM of the highest NF-κB-luciferase inducing fraction (F6) containing sucrose purified *H. pylori* OMVs (indicated by the arrows). Scale bar indicates 100 nm.
**Figure S5. TLR2 and TLR9 expressing HEK293 cells respond to *H. pylori* LPS and CpG DNA stimulation.**

NF-κB reporter activity in TLR2 and TLR9 expressing HEK293 cells stimulated with 6.25 ng *H. pylori* LPS or 100 nM CpG DNA for 8 hours. Error bars indicate ± SEM between triplicates. Data are representative of 3 independent experiments.

**Fig. S6. FB1 and MβC treatments disrupt lipid raft domains on host cell membranes.**

Lipid rafts on AGS cell membranes of FB1 treated, MβC treated and control (non-treated) cells were examined using the Vybrant® lipid raft stain (Molecular Probes). Stained cells were examined by phase contrast and fluorescence microscopy (left and right panels, respectively; 100 x magnification). Lipid rafts are stained red. Images are representative of three independent experiments.

**Fig. S7. OMVs enter epithelial cells via lipid rafts and entry is hindered in FB1 treated AGS cells.**

AGS cells were co-cultured with 10 µg of Alexa 568 labeled *H. pylori* cagPAI OMVs (red). Lipid raft intact (A, C), or FB1 treated (B, D) AGS cells were permeabilized with Triton X-100 (C-D), or left as controls (A-B). OMVs were detected using rabbit anti-*H. pylori* OMVs and anti-rabbit Alexa Fluor 488 antibodies, respectively (green). Cells were visualized by confocal microscopy. Labelled OMVs (red) were predominantly contained within the intracellular compartment of AGS cells and fewer OMVs were located within FB1 treated cells, highlighting the requirement of lipid rafts for OMV entry. Only permeabilized cells exhibited areas of intracellular dual fluorescence (yellow; arrows). Scale bar represents 20 µm.
Fig. S8. FB1-treatment reduces the ability of OMVs to enter the intracellular compartment of epithelial cells.

Alexa Fluor 568 labeled *H. pylori* OMVs (10 µg) (red) were co-cultured with FB1-treated (B and D) or -untreated (A and C) AGS cells and analyzed by epifluorescence. Cells were permeabilized with Triton X-100 (C-D), or left as controls (A-B). OMVs were detected using rabbit anti-*H. pylori* OMVs and anti-rabbit Alexa Fluor 488 antibodies, respectively (green). Cells were visualized by epifluorescence. FB1 treatment significantly reduced the level of fluorescent OMVs associated with AGS cells. Co-localisation (yellow) is indicated by the arrows (100 x magnification). Data are representative of more than two independent experiments.

Fig. S9. MβC -treatment reduces the ability of OMVs to enter the intracellular compartment of epithelial cells via lipid rafts.

Alexa Fluor 488 labeled *H. pylori* OMVs (10 µg) (green) were co-cultured with MβC-treated (B and D) or -untreated (A and C) AGS cells and analyzed by epifluorescence. Cells were permeabilized with Triton X-100 (C-D), or left as controls (A-B). OMVs were detected using rabbit anti-*H. pylori* OMVs and anti-rabbit Alexa Fluor 568 antibodies (red), respectively. Cells were visualized by epifluorescence. MβC treatment significantly reduced the level of fluorescent OMVs associated with AGS cells. Co-localisation (yellow) is indicated by the arrows (100 x magnification). Data are representative of more than three independent experiments.

Fig. S10. MβC treatment of AGS cells reduces OMV-induced IL-8 responses but not NOD1 independent responses.
AGS cells were pre-treated or not with MβC prior to stimulation for 24 hours with either *H. pylori* 251 (Hp), OMVs (10 µg) isolated from *H. pylori* 251 cagPAI (OMVs), or 160 nM phorbol myristate acetate (PMA, Invitrogen). Cell culture supernatants were analyzed for IL-8 production by ELISA. Error bars indicate ± SEM between triplicates. Data are representative of two independent experiments. * denotes *P*=0.04, ** denotes *P*=0.001 versus controls. *P*=0.115 PMA versus MβC + PMA.

Fig. S11. *H. pylori* OMVs induce gastric Cxcl2 mRNA responses in WT C57BL/6 mice.

Gastric Cxcl2 mRNA responses in WT C57BL/6 female mice orally administered with A) 3x10⁷ *H. pylori* SS1 (filled symbols), B) 50 µg *H. pylori* cagPAI OMVs (filled symbols) or PBS (open symbols). Column indicates average response of animals. Error bars indicate ± SEM between samples from individual mice. n=3 mice per group at each timepoint.

Movie S1 Legend  Co-localization of internalized *H. pylori* cagPAI OMVs and anti-*H. pylori* antibody within permeabilized AGS cells.

AGS cells were co-cultured with 10 µg of Alexa 568 labeled *H. pylori* cagPAI OMVs (red). Cells were permeabilized with Triton X-100 and OMVs were detected using rabbit anti-*H. pylori* OMVs and anti-rabbit Alexa Fluor 488 antibodies, respectively (green). Cells were visualized by confocal microscopy. Labeled OMVs (red) were identified as being contained within the intracellular compartment of AGS cells by the presence of areas of intracellular dual fluorescence (yellow) in permeabilized cells.
Images were acquired using a 40 x objective and 4x zoom, rendered using Imaris x64 and a threshold applied to render isosurfaces.

**Movie S2 Legend**  
**Absence of co-localization of internalized* H. pylori cagPAI OMVs and anti-*H. pylori* antibody within intact AGS cells.**

AGS cells were co-cultured with 10 µg of Alexa 568 labeled *H. pylori cagPAI* OMVs (red). Cell membranes were left intact (not treated with Triton X-100) and extracellular OMVs were detected using rabbit anti-*H. pylori* OMVs and anti-rabbit Alexa Fluor 488 antibodies, respectively (green). Cells were visualized by confocal microscopy. Labeled OMVs (red) were identified as being contained within the intracellular compartment of AGS cells, as there is an absence of intracellular dual fluorescence (yellow) within cells. Images were acquired using a 40 x objective and 4x zoom, rendered using Imaris x64 and a threshold applied to render isosurfaces.
Figure 1
Figure 2
Figure 3
Figure 4
Supporting Information
Figure 1
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Figure 2
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Figure 6
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Figure 8
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