Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells

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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 signalling 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 signalling in these cells (Girardin et al., 2003). Certain
bacterial pathogens, however, are able to trigger cytosolic NOD1 signalling 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 signalling in these cells.

We previously reported that Helicobacter 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 nuclear factor-κB (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 H. pylori 251 harbouring a non-functional cagPAI were still able to deliver radiolabelled 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 H. pylori bacteria without a functional T4SS to induce weak NOD1 responses in epithelial cells (Chauouche-Drider et al., 2009). Moreover, the existence of a T4SS-independent mechanism for NOD1 activation may provide an explanation for the as yet unresolved question of how cagPAI negative H. pylori are able to initiate inflammation and pathology in vivo (Yamaoka et al., 1997; Backert et al., 2004).

Previous studies showed that the microinjection of bacteria-free supernatants of Shigella flexneri-induced pro-inflammatory responses in epithelial cells (Philpott et al., 2000; Girardin et al., 2003), 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).

Outer membrane vesicles, 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 (Keenan et al., 2000; Kuehn and Kesty, 2005), including PG hydrolysing 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 H. pylori irrespective of their cagPAI 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 signalling 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 signalling in epithelial cells via a bacterial T4SS encoded by the cagPAI (Viala et al., 2004). As we wished to identify a potentially T4SS-independent mechanism for NOD1 signalling, we purified OMVs from cagPAI positive and cagPAI negative H. pylori strains, as well as from a cagPAI isogenic mutant (see below). The OMVs purified from H. pylori bacteria displayed a spherical, bilayered 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 NF-κB-dependent responses in AGS and HEK239 epithelial cell lines with functional NOD1 signalling (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 with 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 upregulation 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 C). 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-Triflam) 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 signalling. NOD1 functionality was altered in these cells by either: expression of a dominant negative NOD1 construct (Viala et al., 2004) (Fig. 2A); stable knockdown of NOD1 expression (Alexandra Grubman, Maria Kaparakis, Richard L. Ferrero et al.) (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 signalling, 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 harbour PG with NOD1 agonist activity (Girardin et al., 2003; Travas-sos et al., 2005) (Fig. S3A and B respectively; P < 0.05, Fig. 2B and D–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 (Yamaoka et al., 1997; Backert et al., 2004).

Gram-negative bacterial PG associated with OMVs is responsible for the NOD1-dependent NF-κB response induced in epithelial cells

Consistent with previous reports (Keenan et al., 2000; Kuehn and Kesty, 2005), OMVs from H. pylori, P. aerugi-nosa 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 signalling to *P. aeruginosa* infection (Skerrett et al., 2007).

Outer membrane vesicles were also subjected to a continuous sucrose gradient, to remove any bacterial contaminants contained within the preparation. After separation by ultracentrifugation, fractions were analysed by Western blotting (Fig. 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 (Fig. 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⁻¹, 35% w/w sucrose) (Shang et al., 1998). The presence of OMVs within this fraction was confirmed by electron microscopy (Fig. 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 (Fig. 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 (Mandell et al., 2004; Lepper et al., 2005; Yokota et al., 2007) 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-Tri DAP 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 (251*lysAcagM*) bacteria (Fig. 3C). These deposits were absent from non-treated cells. In agreement with the radiolabelling data, OMV preparations were found to contain approximately 0.3–0.5 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 signalling (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 labelled 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 colocalization of Alexa Fluor 488 and 568 fluorochromes only within permeabilized AGS cells stimulated by *H. pylori* OMVs (Fig. 4A and C; Movies 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...
Fig. 4. OMVs enter epithelial cells via lipid rafts. AGS cells were co-cultured with 10 μg Alexa 568 labelled *H. pylori* cagPAI OMVs (red). Lipid raft intact (A and C), or MβC-treated (B and D) cells were permeabilized with Triton X-100 (C and D), or left as controls (A and 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 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 pretreated 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 of ≥3 independent experiments. *P < 0.05 versus MβC-untreated cells.
enriched domains may similarly be involved in OMV-mediated NOD1 signalling. 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 labelled OMVs, FB1-treated AGS cells exhibited very low levels of red fluorescence, when compared with non-treated cells, and less intracellular colocalization when permeabilized and stained with Alexa Fluor 488 labelled anti-H. pylori 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 labelled OMVs within their intracellular compartment, compared with 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 signalling 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 signalling 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 with non-treated controls (Fig. 4E and 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 signalling 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 signalling in vitro, we next examined the effect of these structures on host immune responses in vivo. For this, we established a model to determine the gastric expression levels of the NOD1-responsive chemokine gene Cxcl2 (Via! 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 with 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 upregulation 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 signalling (Fitzgerald et al., 2001; Kenny and O’Neill, 2008), whereas MyD88 is the key adaptor protein required for signalling by all TLRs, excluding TLR3 (Rakoff-Nahoum and Medzhitov, 2009). Oral administration of OMVs to Mal and MyD88 KO mice resulted in the upregulation 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 with 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 with those of both PBS fed WT and OMV fed KO animals, at 1 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-H. pylori OMV IgG responses in their sera in response to mucosally administered H. pylori OMVs, when compared with 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 H. pylori OMVs, TLR recognition of these bacterial components is not responsible for the initiation of inflammatory responses observed in vivo. Therefore, these findings clearly identify that NOD1 is essential for the generation of OMV-dependent innate immune responses in the mucosal compartment in vivo, as well as the development of systemic OMV-specific adaptive immune responses.

**Discussion**

Since the discovery of NOD1 as the receptor responsible for the recognition of Gram-negative PG (Girardin et al.,
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 et al., 2003) or by direct incubation with phagocytic cells, such as macrophages (Magalhaes 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 et al., 2001) or delivery via a bacterial secretion system (Viala 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 Fritz et al., 2006; Sansonetti, 2006; Kaparakis et al., 2007). Furthermore, it has been previously reported that in the absence of a T4SS, 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 signalling 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-TriDAP into the cytoplasm of NOD1 expressing cells, via microinjection, does not result in NOD1-dependent signalling (this study and S.E.G., unpubl. 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 bilayered lipid membrane, facilitate the intracellular trafficking of PG in an appropriate form to cytosolic NOD1 and thus, the initiation

Figure 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 H. pylori 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 H. pylori 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. *P < 0.05 versus WT OMV mice.
C. H. pylori 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. *P < 0.05 versus WT PBS mice.
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 signalling. 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 signalling 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 signalling 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 signalling 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 (Backhed et al., 2003; Nilsson et al., 2008). 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 signalling 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-signalling pathogen, N. meningitidis (Saunders et al., 1999; Antignac et al., 2003). 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., 1999). This would explain how H. pylori cagPAI negative strains, which lack a functional T4SS, are still able to induce inflammation in vivo (Lee et al., 1997; Crabtree et al., 2002; Ohnita et al., 2005).

Experimental procedures

Bacteria

Helicobacter 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) (Phlipott 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). Neisseria gonorrhoeae MS11 and P. aeruginosa PA103pilA were cultured as described previously (Whitchurch et al., 2005; Gunsekere et al., 2006). P. aeruginosa PA103pilA does not produce many of the known virulence factors (e.g. pilin and flagellin).

Bacterial OMVs

Outer membrane vesicles were purified from log phase bacterial cultures (Keenan et al., 2000) and protein concentrations were determined by Bradford assay (Bio-Rad). 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 min. DNA and proteins were removed using 10 units ml⁻¹ DNase (Promega) or 100 μg ml⁻¹ proteinase K (Epicentre) respectively. The enzymes were inactivated at 75°C for 20 min. 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**

Outer membrane vesicle 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 h 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 knock-down and control cell lines were generated by integration of an expression vector containing siRNA directed to either NOD1 or EGFP respectively (R.L.F., manuscript in preparation). Reporter assays were performed with Igκ luciferase (Viala et al., 2004), hBD2 or hBD3, dTκ Renilla (Promega) or αCARD NOD1 (Viala et al., 2004) constructs. Cells transfected with luciferase constructs were stimulated for 8 h with OMVs (10 µg protein) or H. pylori 251 bacteria at an MOI of 10:1 (Philpott et al., 2002; Viala et al., 2004), 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 h. AGS culture supernatants were analysed 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) fluorescent 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 h, fixed and NF-κB was detected using anti-p65 NF-κB antibody (Cell Signaling Technologies) and anti-rabbit Alexa-568 labelled secondary antibody (Molecular Probes).

**Internalization of OMVs**

AGS cells grown on coverslips were co-cultured with Alexa Fluor labelled OMVs, for 16 h, 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 labelled 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 Fumonisins B1 and staining of lipid rafts**

Cells were cholesterol or sphingolipid depleted using methyl-β-cyclodextrin (MβC; Sigma), or Fumonisins B1 (FB1; Sigma) respectively. Cells were depleted of cholesterol by a 30 min treatment with 4 mM MβC in serum-free media. Cell viability after MβC treatment was confirmed using the luminescence based Cell Titer Glo 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 labelling kit (Molecular probes). Cholesterol was added to cholesterol depleted cells by treating with 250 μM cholesterol (5-choleno-3β-ol; Sigma) and 4 mM MβC for 30 min.

**Co-cultured 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 back-crossed more than eight times onto a C57BL/6 background (Girardin et al., 2003). MEFs were cultured in 96-well plates (4 × 10^4 cells per well), then stimulated for 24 h with either: OMVs, highly purified Escherichia coli LPS (100 ng ml⁻¹; Lausen, Switzerland); Gram-positive PG from Staphylococcus aureus (100 ng ml⁻¹; Sigma) or Pam3Cys (100 ng ml⁻¹; 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, MyDB8 and Mal knockout animals was performed at The University of Toronto, The University of...
Antibody production in response to OMV stimulation

The 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 time points. 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⁻¹ of H. pylori 251 cagPAI OMVs. Sera were initially diluted 1:50 and serial dilutions of these were performed to determine the end-point titre. OMV-specific mouse anti-IgG antibodies were detected using anti-mouse IgG biotin-labelled antibody (Chemicon) and streptavidin-HRP (Chemicon). ELISAs were developed using BD OptEIA TMB substrate reagent (BD Biosciences). Absorbances were read at OD450 using a FLUOstar machines. Serum end-point titres were expressed as the reciprocal of the dilution of serum that gave an OD450 value five times the value of the background. Mice with an antibody titre below the detection limit of the assay (log10 1.7) were assigned a titre of log10 1.7. Positive and negative control sera were included in all ELISA experiments.

Statistical analysis

IL-8 responses were analysed using the Student’s t-test. Luciferase activity, antibody titres and mRNA levels were analysed using the Mann–Whitney U-test. Differences were considered significant when P < 0.05.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**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, 27 500× magnification). Scale bar indicates 500 nm. *H. pylori* OMVs were analysed for protein content by Western blotting. OMVs from *H. pylori* strains, (1) 251, (2) 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 (B) are indicated on the left hand side of the membrane. Arrows (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 micro-injected 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 localized in their nucleus. Images are representative of two independent experiments (Scale bar indicates 50 μm).
trols (A and 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 labelled *H. pylori* OMVs (10 μg) (red) were co-cultured with FB1-treated (B and D) or -untreated (A and C) AGS cells and analysed by epifluorescence. Cells were permeabilized with Triton X-100 (C and D), or left as controls (A and 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. Colocalization (yellow) is indicated by the arrows (100× magnification). Data are representative of more than two independent experiments.

**Fig. S9.** MjIC treatment reduces the ability of OMVs to enter the intracellular compartment of epithelial cells via lipid rafts. Alexa Fluor 488 labelled *H. pylori* OMVs (10 μg) (green) were co-cultured with MjIC-treated (B and D) or -untreated (A and C) AGS cells and analysed 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. MjIC treatment significantly reduced the level of fluorescent OMVs associated with AGS cells. Colocalization (yellow) is indicated by the arrows (100× magnification). Data are representative of more than three independent experiments.

**Fig. S10.** MjIC treatment of AGS cells reduces OMV-induced IL-8 responses but not NOD1-independent responses. AGS cells were pretreated or not with MjIC prior to stimulation for 24 h 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 analysed for IL-8 production by ELISA. Error bars indicate ±SEM between triplicates. Data are representative of two independent experiments. *P = 0.04, **P = 0.001 versus controls. P = 0.115 PMA versus MjIC + 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) 3 × 10^7 *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. Three mice per group at each time point.

**Movie S1.** Colocalization 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 labelled *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. Labelled 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× objective and 4× zoom, rendered using Imaris ×64 and a threshold applied to render isosurfaces.

**Movie S2.** Absence of colocalization 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 labelled *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. Labelled 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× objective and 4× zoom, rendered using Imaris ×64 and a threshold applied to render isosurfaces.

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