The immunomodulatory roles of *Helicobacter pylori* outer membrane vesicles

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List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
BHI	Brain heart infusion
BSA	Bovine serum albumin
CBA	Cytometric bead array
CDI	Cell division index
CFSE	5(6)-CFDA, SE; CFSE (5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester)
DC	Dendritic cell
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonecleic acid
DNAse	Deoxyribonuclease
DOMVs	Detergent-derived outer membrane vesicles
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assat
ETEC	Enterotoxigenic *Escherichia coli*
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GFP	Green fluorescent protein
GGT	γ-glutamyl transpeptidase
GM_1	monosialotetrahexosylganglioside
GPI	glycosylphosphatidylinositol
HBA	Horse blood agar
HILIC	Hydrophilic interaction liquid chromatography
HLA	Human leukocyte antigen
HLB	Hydrophilic-lipophilic balance
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSP60	Heat shock protein 60
IAA	Iodacetamide
ICAM-1	Intracellular adhesion molecule-1
IEC	Intestinal epithelial cell
Ig	Immunoglobulin
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-2	Interleukin-2

IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
ILV	Intraluminal vesicles
IM	Inner membrane
IU	International units
LAM	lipoarabinomannan
Lpp	Lipoprotein
LPS	Lipopolysaccharide
LT	Heat labile enterotoxin
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatability complex
MIIC	MHC II-enriched compartment
MS/MS	Tandem mass spectrometry
MV	Membrane vesicles
MVB	Multivesicular bodies
NFκB	Nuclear-factor κB
NOD1	Nucleotide binding oligomerization domain 1
NOG	Octyl-β-D-glucopyranoside
NS	Non-stimulated
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
OVA	Ovalbumin
Pal	Peptiodoglycan associated lipoprotein
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PG	Peptidoglycan
PhoA	periplasmic alkaline phosphatase
PHS	Pooled human serum
PI	Propidium iodide
PIM	Phosphatidyl-myo-inositol mannosides
PQS	*Pseudomonas* quinolone signal
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time – polymerase chain reaction
RPMI	Roswell Park Memorial Institute
(RPTPα).	Protein-tyrosine phosphatase α
RPTPβ	Receptor-like protein tyrosine phosphatases-β
SD	Standard deviation
SSC	Side scatter
T4SS	Type 4 secretion system
TEER	Trans-epithelial electrical resistance
TFA	Trifluoroacetic acid
TLR	Toll like receptor
TLR-2	Toll like receptor-2

TLR-4	Toll like receptor-4
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells
VacA	Vacuolating cytotoxin A

Summary

During normal growth, Gram-negative bacteria shed nano-sized outer membrane vesicles (OMVs), ranging from 50-250 nm in diameter. OMVs have been studied in many Gram-negative bacterial species, yet little is still known about the biology of OMV release, nor how these vesicles enter host cells or how they induce immune responses.

To investigate OMV production by the gastric pathogen, *Helicobacter pylori*, we inactivated the *H. pylori* orthologues of *Escherichia coli tolB* and *pal*, encoding two of the critical components of the Tol-Pal system known to help maintain bacterial membrane integrity. We demonstrated that *H. pylori* mutant strains lacking an intact copy of *tolB* exhibited distinct outer membrane abnormalities, accompanied by defects in flagella formation. Importantly, *H. pylori* lacking functional TolB or Pal proteins exhibited extensive OMV production and release. The OMVs from these mutant strains induced high levels of pro-inflammatory cytokine (interleukin-8, IL-8) production. This work describes, for the first time, the importance of the TolB and Pal proteins in cell membrane integrity and OMV formation by *H. pylori*.

Work performed previously in our laboratory reported that Gram-negative OMVs enter epithelial cells *in vitro*, resulting in the generation of pro-inflammatory and adaptive immune responses. However, to date the precise mechanism of entry has not been determined. Studies have reported that not all OMVs enter cells using the same endocytic pathways. Using a panel of chemical inhibitors of key endocytosis pathways, we found that heterogeneously sized populations of OMVs were internalised by epithelial cells via macropinocytosis, clathrin and caveolin dependant endocytosis. Interestingly, however, when we fractionated OMVs into either small (< 50 nm) or

large (> 50 nm) sizes, we found that small OMVs entered host cells by each of the aforementioned pathways. In contrast, cell entry of large OMVs involved macropinocytosis and clathrin but not caveolin dependent endocytosis. Proteomic analyses of different sized OMVs revealed both shared and unique sets of proteins, indicating that particle size may also determine protein content.

Following internalisation by polarised epithelial cells, we found that *H. pylori* OMVs induce the basolateral secretion of the pro-inflammatory chemokine, IL-8. Interestingly, we found that internalised OMVs up-regulated the expression of HLA Class I and II molecules in epithelial cells. Moreover, stimulation of polarised epithelial cells with *H. pylori* OMVs resulted in the production of exosomes secreted at their basolateral surface containing OMV proteins. Importantly, we demonstrated that exosomes containing OMV proteins induced proliferation of human T cells in an antigen dependent manner, indicating that exosomes could function to present OMV antigens to mucosal T cells.

Collectively, our data suggest that proteins derived from internalised OMVs are packaged into secreted exosomes for presentation to immune cells. We speculate that through interactions with antigen presenting cells, these exosomes are capable of activating antigen specific T cells located beneath the epithelial cell layer, thereby providing a link between the generation of innate and adaptive immune responses to *H. pylori* OMVs at the mucosal epithelium.

Thesis Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:
I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.
This thesis includes no original papers published in peer reviewed journals and 2 unpublished publications. The core theme of the thesis is host immune responses to bacterial OMVs. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Monash Institute of Medical Research under the supervision of Associate Professor Richard Ferrero and Dr Maria Kaparakis-Liaskos.
The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.
In the case of chapters 2 and 3 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Increased outer membrane vesicle formation in *Helicobacter pylori tolB* and *pal* mutants	Submitted	My contribution included project conceptualisation and experimental design. In addition I performed all experiments, interpreted data and undertook the majority of the writing. 90%
3	Bacterial outer membrane vesicle size determines their mechanisms of host cell entry and protein content	Submitted	My contribution included project conceptualisation and experimental design. In addition I performed all experiments, interpreted data and undertook the majority of the writing.

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted]

Date: **10/02/2014**

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Chapter 1. Introduction

1.1 *Helicobacter pylori* and outer membrane vesicles

In 1940 it was noted by Freedburg and Barron that spirochetes were often present in gastric biopsy specimens (Freedburg and Barron 1940), yet the initial isolation of the spiral bacterium did not occur until 1982 (Warren and Marshall 1983). The bacteria were identified as curved or spiral Gram-negative bacilli that were motile and microaerophilic. Suspected to be a new species related to the *Campylobacter* genus, the bacteria were subsequently designated *Campylobacter pyloridis* (Marshall and Warren 1984), later to become known as *Campylobacter pylori,* and finally *Helicobacter pylori* (Dunn, Cohen et al. 1997). It is now known that around half of the population worldwide is infected with *H. pylori* and that this bacterium is usually acquired during childhood, persisting for life if left untreated (Dunn, Cohen et al. 1997). Infection with *H. pylori* results in the development of chronic gastric inflammation, which is always present but often asymptomatic (Blaser 1990), and may progress to more severe diseases such as gastro-duodenal ulcers, gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Nomura 1991, Parsonett 1991, Peek and Blaser 2002).

Structural analysis of gastric biopsies colonised by *H. pylori* revealed that the bacteria were often surrounded by small spherical vesicles that resembled the bacterial outer membrane (Fiocca, Necchi et al. 1999). These were subsequently identified as outer membrane vesicles (OMVs), nano-sized spherical structures derived from the Gram-negative bacterial membrane (Fiocca, Necchi et al. 1999). It has been known for over 40 years that Gram-negative bacteria produce OMVs (Mashburn and Whiteley 2006), however, it is only recently that these vesicles have become the focus of intense

research. The shedding of vesicles is common to both Gram-negative and Gram-positive (Lee, Choi et al. 2009) bacteria and occurs during active growth in almost any condition (Beveridge 1999, Peek and Blaser 2002). The biological roles of OMVs are many and varied, including toxin delivery (Kesty, Mason et al. 2004), transfer of virulence genes between bacteria (Yaron, Kolling et al. 2000) and modulation of the host response (reviewed in (Kuehn and Kesty 2005, Mashburn and Whiteley 2006)).

1.2 OMVs: Bacterial membrane vesicles

1.2.1 The bacterial cell envelope.

In order to understand how OMVs are formed and released, we must understand the structure of the bacterial cell envelope. The Gram-negative cell envelope consists of an inner (IM) and an outer membrane (OM), separated by an aqueous layer of periplasm where the peptidoglycan (PG) layer resides (reviewed in (Bos, Robert et al. 2007)). The IM, which is in contact with both the cytoplasm and the periplasm, is composed predominantly of phospholipids and proteins. The OM separates the periplasm from the external environment and is composed of phospholipids, lipopolysaccharide (LPS), lipoproteins and outer membrane proteins (OMPs) (reviewed in (Ruiz, Kahne et al. 2006)). Sandwiched between the IM and the OM is the periplasm, a viscous compartment containing soluble proteins and the PG layer. The PG layer, comprising glycan chains and oligopeptides, is in turn covalently linked to the OM via many associated proteins, including the highly abundant Braun's lipoprotein (Braun 1975). OMVs represent one mechanism whereby Gram-negative bacteria release OM, OMPs, soluble proteins and periplasm into the external environment (Beveridge 1999).

1.2.2 Mechanisms of outer membrane vesicle biogenesis

Interestingly, neither a naturally occurring, nor a mutant strain of any Gram negative bacterium has been found that does not produce OMVs (Kulp and Kuehn 2010), suggesting that vesicle formation is an essential and possibly conserved process. The precise mechanism by which Gram-negative bacteria form and release OMVs has yet to be defined, however, there are currently several hypotheses to explain the mechanism of vesicle biogenesis (reviewed in (Mashburn and Whiteley 2006)). (Figure. 1.1)

Membrane bulging (Model 1)

The model of OMV formation proposed by Wensink and Witholt in 1981 suggests that during normal growth, the OM of the bacterial cell wall may expand faster than the PG layer below. This subsequently may cause PG to detach from the OM at areas where the numbers of lipoprotein (Lpp) linkages are low. The membrane 'bulges' under pressure where it has detached from the PG, resulting in the eventual release of a spherical membrane vesicle (Wensink and Witholt 1981). This model was initially based on the observation that *Escherichia coli* OMVs contain only small amounts of Lpp, despite its high abundance in the bacterial outer membrane (Hoekstra, van der Laan et al. 1976, Wensink and Witholt 1981). In addition, many of the proteins that commonly associate with Lpp are absent or lacking in OMVs (Mashburn and Whiteley 2006). The authors therefore reasoned that OMVs may arise at areas of the membrane where Lpp is low or absent (Wensink and Witholt 1981). Further supporting this model is the observation that *E. coli* mutants lacking the outer membrane Lpp exhibit extensive vesicle formation (Sonntag, Schwarz et al. 1978).

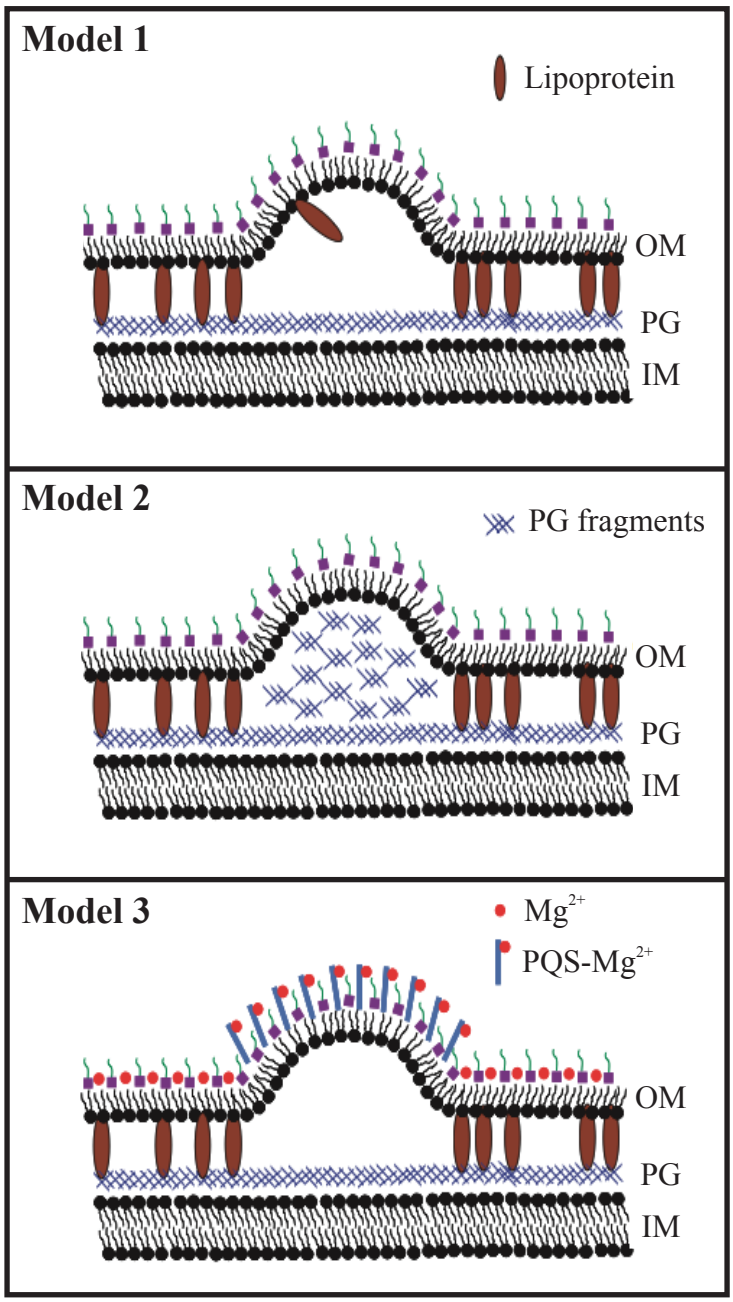

Figure 1.1 Proposed models of outer membrane vesicle biogenesis. Model 1) The outer membrane (OM) expands faster than the inner membrane (IM), bulging at regions of fewer peptidoglycan (PG) to lipoprotein (Lpp) linkages, resulting in the release of outer membrane vesicles (OMVs). Model 2) PG fragments derived from cell wall turnover build up, exerting a turgor pressure on the OM leading to blebbing of the OM and release of OMVs. Model 3) The ionic interactions between PQS and Mg^{2+} in the OM enhance the anionic repulsion between the lipopolysaccharide (LPS) molecules in the membrane resulting in blebbing and OMV release (Mashburn and Whiteley 2006).

Bacterial cell wall turnover (Model 2)

During normal growth many Gram-negative bacteria undergo cell wall turnover (Doyle, Chaloupka et al. 1988), wherein PG is excised from the cell wall and returned to the cytoplasm as muramyl peptides together with other cell wall turnover products (Goodell and Higgins 1987, Zhou, Srisatjaluk et al. 1998). A second model of vesicle biogenesis suggests that these cell wall turnover products are not effectively internalised by the cell, causing a build up at the surface that exerts a turgor pressure on the OM (Zhou, Srisatjaluk et al. 1998). If not alleviated, the membrane "bulges", forming blebs that continue to grow until they detach and are released as OMVs (Zhou, Srisatjaluk et al. 1998). Similar to the model proposed by Wensink and Witholt, the build up of PG is thought to occur in regions of the OM where there are fewer PG-Lpp linkages (Mashburn and Whiteley 2006). Supporting this theory is the observation that a component of PG and cell wall turnover product, muramic acid, is present within OMVs (Zhou, Srisatjaluk et al. 1998). Furthermore, over-expressed and misfolded proteins can be enriched in OMVs (McBroom and Kuehn 2007). These proteins could potentially gather under the surface of the OM, exerting pressure and causing membrane bulging and OMV release (Kadurugamuwa and Beveridge 1995, Horstman and Kuehn 2000, McBroom and Kuehn 2007). Many studies have been performed whereby the PG-OM links are disrupted through genetic mutation, resulting in mutant strains of bacteria that exhibit increased vesiculation (Sonntag, Schwarz et al. 1978, Bernadac, Gavioli et al. 1998, Llamas, Ramos et al. 2000, Cascales, Bernadac et al. 2002, Wessel, Liew et al. 2012). However, fewer PG-OM links do not fully explain OMV biogenesis, as vesiculation can be increased without affecting membrane integrity (McBroom, Johnson et al. 2006).

LPS selection (Model 3)

A third model of vesicle biogenesis relates more specifically to the formation of *Pseudomonas aeruginosa* OMVs. Most strains of *P. aeruginosa* possess two different forms of LPS which are chemically and immunologically distinct from one another; neutral charged A band LPS and negatively charged B band LPS (Rivera, Bruyan et al. 1988). This third model of OMV production came about following the observation that OMVs from *P. aeruginosa* comprise predominantly negatively charged B-band LPS rather than the more neutral A-band LPS (Kadurugamuwa and Beveridge 1995). This led to the proposal that the electronegative charge of the B-band LPS causes an anionic repulsion resulting in membrane instability. Furthermore, Mg^{2+} and $Ca2^{+}$ salt bridges exist in the OM that are important for neutralising the anionic repulsion between the two kinds of LPS molecules. Quinolone signal molecules, such as the *Pseudomonas* quinolone signal (PQS) released by *P. aeruginosa,* disrupt the Mg^{2+} and Ca^{2+} salt bridges in the OM thereby enhancing the anionic repulsion between the LPS and causing the membrane to curve outwards (Mashburn and Whiteley 2006, Mashburn-Warren, Howe et al. 2008). This destabilisation of the membrane results in membrane blebbing and OMV production (Mashburn and Whiteley 2005). It is likely that OMVs formed in this manner also arise from regions of the membrane that have fewer PG-Lpp linkages (reviewed in (Mashburn and Whiteley 2006)).

These three models are not mutually exclusive and each may contribute to vesicle biogenesis (Lee, Choi et al. 2008). Indeed, each model suggests that OMVs form at regions of the membrane where there are fewer PG-Lpp and OM linkages causing the integrity of the membrane to become weakened. A feature common to each of these models is that OMVs are formed when the pressure builds up forcing vesicle release. However, these models do not take into account the fact that OMVs are formed in a

variety of sizes, suggesting that there is no critical threshold of pressure forcing release (reviewed in (Kulp and Kuehn 2010)). Thus, the mechanisms of OMV biogenesis are likely to comprise a combination of many factors and remain a highly debated research topic.

1.2.3 Composition of bacterial OMVs

Bacterial OMVs are known to contain many of the proteins and lipids present in the outer membrane of the host bacterium. Indeed, a growing body of evidence suggests deoxyribonucleic acid (DNA), periplasmic and possibly cytoplasmic proteins, porins, phospholipids and a variety of toxins are also present within OMVs (Dorward, Garon et al. 1989, Horstman and Kuehn 2000, Kuehn and Kesty 2005, Lee, Choi et al. 2008). However, comparison of the bacterial OM and the OMV protein content of various strains of *E. coli* revealed that whilst the protein contents are similar, they are not identical (Horstman and Kuehn 2000). For example, the *E. coli* Lpp which is highly abundant in the OM, is found only in small proportions in secreted OMVs (Wensink and Witholt 1981). Similarly, whilst the outer membrane of *P. aeruginosa* contains primarily A-band LPS with smaller amounts of B-band LPS, OMVs contain predominantly B-band LPS (Kadurugamuwa and Beveridge 1995). Pathogenic bacteria also commonly incorporate virulence factors into their OMVs, such as the vacuolating cytotoxin A (VacA) of *H. pylori* (Fiocca, Necchi et al. 1999), the LPS-bound heat labile enterotoxin (LT) of enterotoxigenic *E. coli* (ETEC) (Horstman and Kuehn 2000) as well as a variety of adhesins, invasins and other enzymes (Lee, Choi et al. 2008). Indeed, Haurat *et al.* found that the human pathogen *Porphorymonas gingivalis* possesses a mechanism to selectively sort proteins into OMVs, providing an explanation for the relative abundance of virulence factors in OMVs when compared with the bacterial OM

(Haurat, Aduse-Opoku et al. 2011). Taken together, these studies suggest that OMV production is a regulated process rather than simply an artifact of bacterial growth and cell wall turnover.

1.2.4 Factors affecting vesicle production and composition

In addition to intrinsic bacterial factors, environmental and host factors can also influence vesicle production and composition (reviewed in (Kuehn and Kesty 2005)). Certain growth conditions, such as increased temperature (Katsui, Tsuchido et al. 1982), are known to have a major effect on vesiculation resulting in greater numbers of OMVs produced by the bacteria. An early study performed by Katsui *et al.* (Katsui, Tsuchido et al. 1982) involved heating the bacteria to 55°C and measuring the subsequent increase in OMV release. On the other hand, the formation of OMVs by the opportunistic pathogen *Serratia marcescens,* which is commonly found in soil and water, is significantly higher at lower environmental temperatures of 22 or 30°C compared to the physiological temperature of 37°C (McMahon, Castelli et al. 2012). Similarly, nutrient availability can affect bacterial vesiculation in different ways. In response to low nutrition, *Lysobacter spp.* up-regulates OMV production (Vasilyeva, Tsfasman et al. 2009), whereas *Pseudomonas fragi* produces more OMVs when nutrients become readily available (Thomson, Naidu et al. 1985).

Growth conditions can drastically alter not only the amount of OMVs produced, but also their composition. For example, when grown in iron limiting medium, *H. pylori* OMVs contain less VacA but more proteases than OMVs grown in standard media (Keenan and Allardyce 2000). When grown in the presence of the antibiotic gentamicin, OMV production by *P. aeruginosa* is increased three-fold. Moreover, the composition and characteristics of the OMVs produced are drastically altered compared with those

produced when the bacteria are grown in standard media (Kadurugamuwa and Beveridge 1995).

Interestingly, no known alterations in growth conditions nor genetic manipulation result in an absence of OMV production, indicating that vesiculation is an essential process for bacteria (McBroom, Johnson et al. 2006). OMVs are naturally produced in small amounts (Henry, Pommier et al. 2004) and are therefore difficult to culture in large quantities for research purposes, such as vaccine development. One method of OMV purification commonly used for the production of OMV based vaccines involves treatment of the bacteria with a detergent, resulting in an increased number of vesicles (Ferrari, Garaguso et al. 2006). The increase in vesiculation is a result of bacterial lysis and whilst these DOMVs are immunogenic and are safe for use in vaccination, their protein content differs dramatically from naturally produced OMVs (Ferrari, Garaguso et al. 2006). Thus, the development of hyper-vesiculating bacteria through the use of genetic manipulation has been widely studied in several bacteria (Bernadac, Gavioli et al. 1998, Llamas, Ramos et al. 2000, Dubuisson, Vianney et al. 2005, Scorza, Doro et al. 2008, Yeh, Comolli et al. 2010), as detailed below.

1.2.5 The role of the Tol-Pal system in OMV production

Genetic manipulation has been used to examine the regulation of OMV production in several bacteria using different gene expression systems that result in increased vesiculation (Henry, Pommier et al. 2004). A feature common to the majority of these is the disruption of membrane integrity. The integrity of the Gram-negative cell wall relies on a structural link between the shape determining PG layer and the OM. The major lipoprotein Lpp, a particularly abundant OMP that is covalently bound to the PG layer (Cascales, Bernadac et al. 2002), provides such a link. When Lpp is disrupted or absent,

membrane integrity is deregulated resulting in increased vesiculation (Cascales, Bernadac et al. 2002). Similarly, a mutation in any of the genes that make up the Tol-Pal system disrupts membrane integrity, increasing vesiculation by *E. coli* (Bernadac, Gavioli et al. 1998), *Pseudomonas putida* (Llamas, Ramos et al. 2000), *Erwinia chrysanthemi* (Dubuisson, Vianney et al. 2005) and *Caulobacter crescentus* (Yeh, Comolli et al. 2010). The Tol-Pal system (Figure 1.2) comprises five proteins: the IM proteins TolA, TolQ and TolR, a periplasmic protein TolB, and the PG associated lipoprotein (Pal), an OMP (Mizuno 1979, Cascales, Bernadac et al. 2002). The TolA-Q-R proteins form a complex at the IM, with TolA and TolR anchored by a single transmembrane domain, and TolQ by three transmembrane segments (Bouveret, Derouiche et al. 1995, Henry, Pommier et al. 2004). The periplasmic TolB interacts with Pal, which is anchored to the OM and interacts directly with PG. The two complexes, TolA-Q-R and TolB-Pal, are linked through TolA-Pal and TolA-TolB interactions (Henry, Pommier et al. 2004). The precise function of each protein in the Tol-Pal system has not yet been fully elucidated, however, it is known that each plays a role in maintaining OM integrity, as a mutation in any of the *tol-pal* genes results in a loss of integrity and increase in OMV production (Bernadac, Gavioli et al. 1998).

The *tol-pal* operon has been described in most Gram-negative bacteria and appears to be well conserved with some notable exceptions (Sturgis 2001). Interestingly bacteria that do not naturally encode the *tol-pal* genes: *Treponema pallidum* (Fraser, Norris et al. 1998), *Borrelia burgdorferi* (Fraser, Casjens et al. 1997) and *Rickettsia prowazekii* (Andersson, Zomorodipour et al. 1998), produce OMVs and are obligate intracellular parasites. Their inherent membrane instability restricts their niche to osmotically stable environments, such as within host cells (Rooney 2003). Furthermore, those bacteria that encode only the *tolQ* and *tolR* genes, but none of the other genes within the cluster such

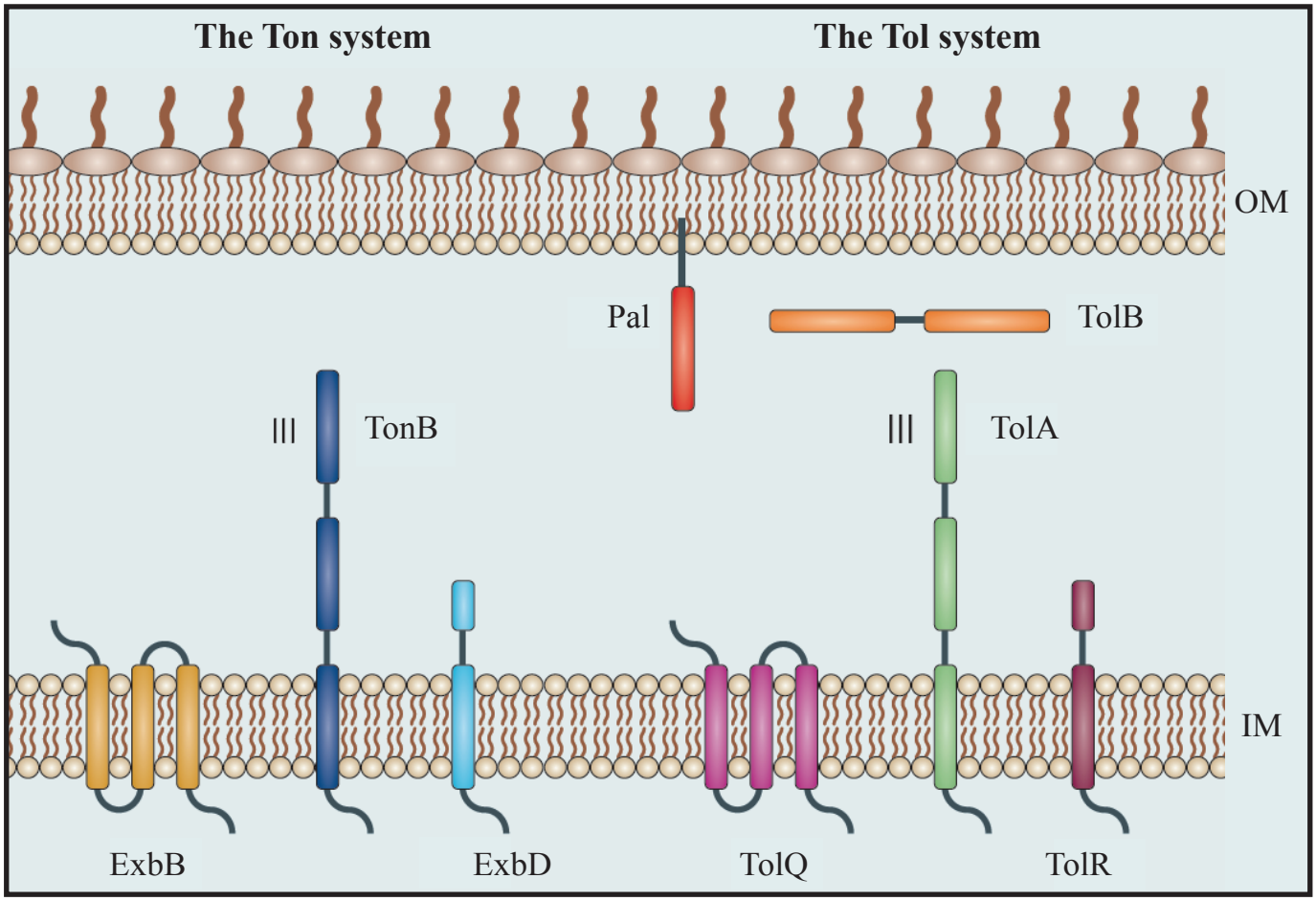

Figure 1.2: The Tol system of Gram-negative bacteria. The Tol system comprises five proteins: TolA, TolQ, TolR, TolB and Pal. TolA, Q and R form a protein complex at the inner membrane (IM). The periplasmic protein TolB interacts with Pal, which is anchored to the outer membrane (OM) and associated with the peptidoglycan layer. The two complexes are linked via interactions between TolA and Pal, and TolA and TolB. The Ton system comprises three proteins: ExbB, ExbD and TonB. The Tol and Ton systems share structural and functional similarities. (Adapted from (Gorringe and Pajón 2012))

as *Neisseria* spp. (Tettelin, Saunders et al. 2000) and *Porphorymonas* spp. (Sturgis 2001), naturally produce large quantities of OMVs due to their innate membrane instability (Dorward, Garon et al. 1989, Zhou, Srisatjaluk et al. 1998). Recently, *pal* and *tolB* homologues have been annotated in the genome sequence of *H. pylori* 26695 (Tomb, White et al. 1997, Angelini, Cendron et al. 2008). Drawing on previous observations that mutations in the *tol-pal* genes of *E. coli* result in loss of membrane integrity and overproduction of OMVs, it is possible that similar mutations or loss of function of homologous genes in *H. pylori* will bring about a similar phenotype.

1.2.6 Functions of bacterial OMVs

Many independent studies have revealed that OMVs are utilised by bacteria for diverse functional roles, including DNA transfer (Dorward, Garon et al. 1989), establishment of a colonisation niche (reviewed in (Ünal, Schaar et al. 2010)), modulation of the host response (Srisatjaluk, Doyle et al. 1999, Srisatjaluk, Kotwal et al. 2002, Chatterjee and Chaudhuri 2013), transmission of virulence factors (Fiocca, Necchi et al. 1999, Lindmark, Rompikuntal et al. 2009, Kaparakis, Turnbull et al. 2010, Chatterjee and Chaudhuri 2011), as well as playing a direct role in bacterial competition and survival (Amano, Takeuchi et al. 2010, Manning and Kuehn 2011).

Bacterial Survival – offence and defence

Many bacteria have developed a diverse range of characteristics to help out compete other species for nutrients and space within a niche, thereby aiding survival. OMVs secreted by certain species of bacteria harbour antimicrobial activity, such as those from *P. aeruginosa* that contain a small protein with murein hydrolase activity that cleaves the covalent bonds of PG in other bacteria, causing bacterial lysis (Li, Clarke et al.

1998). This bactericidal activity has also been demonstrated for a variety of other bacterial OMVs, including those from *Klebsiella, Proteus, Escherichia* and *Salmonella spp.* (Li, Clarke et al. 1998). More recently, *Myxococcus xanthus* were also shown to secrete OMVs with bacteriolytic activity (Evans, Davey et al. 2012). The antimicrobial activity of OMVs is likely to aid pathogenic bacteria in out competing commensal bacteria in the local environment, as well as providing an immediate source of nutrients from the surrounding lysed bacteria (Mashburn and Whiteley 2006).

OMVs have also been found to promote bacterial survival by providing a mechanism whereby damaged and unwanted proteins can be removed from the bacteria (McBroom and Kuehn 2007). One study demonstrated that when subject to OM damaging agents, a range of hyper-vesiculating *E. coli* mutants, including those lacking functional TolB and Pal proteins, demonstrated increased survival rates due to their ability to package misfolded proteins into OMVs, thereby reducing their damaging effects (McBroom and Kuehn 2007). Building on this, Manning and Kuehn found that hyper-vesiculating *E. coli* mutants were better able to survive treatment with anti-microbial peptides, or the T4 bacteriophage (Manning and Kuehn 2011). Furthermore, they found that even wild type *E. coli* strains exhibited increased survival in response to antimicrobial peptides or T4 phage when OMVs were added in conjunction. They postulated that OMVs contribute to bacterial defence and survival by adsorbing and neutralising environmental agents that act on the OM of bacteria (Manning and Kuehn 2011).

Transfer of proteins, toxins and whole OMVs to host cells

OMVs are proposed to act as intercellular 'communicasomes', mediating the transfer of a diverse range of molecules such as proteins, toxins and even whole vesicles to other bacteria or eukaryotic cells (reviewed in (Mashburn and Whiteley 2006)). Many Gram-

negative bacteria express surface adhesion molecules that are also present within the membrane of the OMVs, allowing adhesion to host cells, thereby aiding in toxin and protein delivery. The LT of ETEC (Kuehn and Kesty 2005) is one such protein that acts not only as a toxin that is delivered to the host cell, but also as an adhesin (Horstman and Kuehn 2000, Kuehn and Kesty 2005). Internalisation of ETEC OMVs by epithelial cells depends on specific interactions between LT and its host cell surface receptor monosialotetrahexosylganglioside (GM1), leading to internalisation of the OMVs by cholesterol-dependent, lipid raft-mediated endocytosis (Kesty, Mason et al. 2004). Similarly, Fiocca *et al.* showed that *H. pylori* OMVs are internalised by gastric epithelial cells into cytoplasmic vacuoles that are part of the endosomal compartment of the cell, thereby delivering the VacA toxin to host cells (Sommi, Ricci et al. 1998). Diverse Gram-negative bacteria exploit OMVs for the delivery of toxins, some of which are particularly abundant in OMVs and may be more active than the native toxin (reviewed in (Kuehn and Kesty 2005)). More recently, Gram-positive bacteria have been found to secrete MVs, and not surprisingly, MVs derived from *Staphylococcus aureus* (Gurung, Moon et al. 2011) and *Bacillus anthracis* (Rivera, Cordero et al. 2010) were found to deliver toxins to macrophages, resulting in cell death.

1.2.7 OMVS and host cell entry

The delivery of toxins and bacterial components to host cells via OMVs has been well researched, however, the precise mechanisms whereby OMVs deliver these components is not well understood. There are numerous pathways by which extracellular particles can gain entry to non phagocytic epithelial cells, the most common of which are macropinocytosis (Lewis 1931), clathrin-dependent and caveolin-dependent endocytosis (reviewed in (Mayor and Pagano 2007)) (Figure 1.3). Macropinocytosis is an actin-

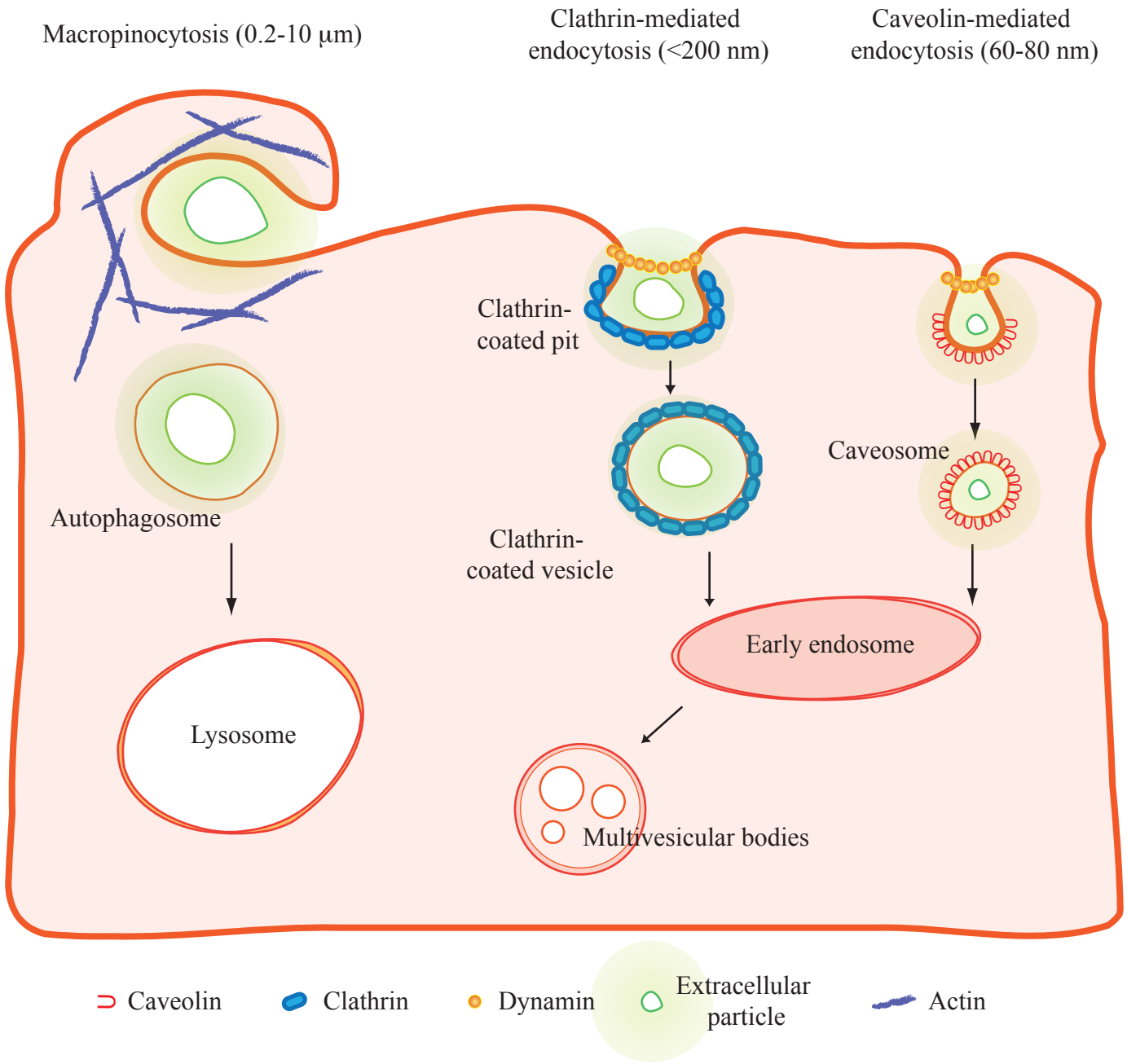

Figure 1.3: Pathways of entry at the cell surface. There are multiple pathways by which extracellular particles can gain entry into host cells. The three main mechanisms are macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis. Macropinocytosis is actin dependent and pinocytic vesicles can be up to 10 mm in diameter. Clathrin-dependent endocytosis involves the internalisation of cargo within clathrin-coated pits, which may be up to 200 nm in diameter. Caveolin dependent endocytosis occurs via the internalisation of 60-80 nm caveolae pits, however larger particles may enter via this mechanism.

mediated process involving cytoskeletal rearrangement resulting in membrane ruffling. These membrane ruffles extend outwards from the cell and join at their distal margins, engulfing extracellular fluid (Hewlett, Prescott et al. 1994). Macropinocytic vesicles are > 0.2 μm in diameter and can be as large as 10 μm (Hewlett, Prescott et al. 1994). Their relatively large size allows cells to non selectively internalise large particles and large quantities of extracellular material (Watts and Marsh 1992). Indeed, numerous pathogens, including bacteria, viruses and even protozoa, are known to gain entry into host cells via macropinocytosis (summarised in (Lim and Gleeson 2011)). Clathrin-dependent endocytosis occurs via the uptake into the cell of extracellular material within clathrin coated vesicles (Pearse 1976), with internalised material delivered to early endosomes for sorting (reviewed in (McMahon and Boucrot 2011)). Clathrin coated vesicles differ vastly in size, depending upon the material being endocytosed (Ehrlich, Boll et al. 2004). The observed upper limit in size is approximately 200 nm and larger cargoes are not taken up by this mechanism (Cureton, Massol et al. 2009). Caveolin-dependent endocytosis occurs through internalisation of 60-80 nm flask shaped caveolae pits at the cell surface (Palade 1953, Yamada 1955, Stan 2005). Despite the small size of caveolae pits, bacterial pathogens much larger than 80 nm can enter cells via caveolin dependent endocytosis (Shin, Gao et al. 2000). Notably, the small GTPase dynamin, plays an essential role in both clathrin and caveolin dependent endocytosis (Hinshaw and Schmid 1995, Henley, Krueger et al. 1998, Oh, McIntosh et al. 1998, Sweitzer and Hinshaw 1998). The internalisation of OMVs by host cells has been demonstrated for OMVs from a wide range of pathogens, however the reported mechanisms vary and include: fusion with the host cell plasma membrane, macropinocytosis, clathrin- and caveolin-dependent endocytosis. Given the discrepancy in the reported mechanisms of OMV cellular entry (summarised in Table 1.1), and their

Table 1.1 Proposed mechanisms of host cell entry of outer membrane vesicles derived from various bacteria

	Proposed mechanisms	Cells used	Ref
E. coli	Lipid raft-dependent; co-localise with caveolin; GM1 receptor mediated	Human colorectal epithelial cells	Kesty et al, 2004
H. pylori	Lipid raft-dependent	Human gastric epithelial cells	Kaparakis et al, 2010
	No requirement for cholesterol; VacA toxin enhances cellular uptake; Clathrin-dependent endocytosis important for uptake of VacA- OMVs	Human gastric epithelial cells	Parker et al, 2010
	OMVs demonstrated within cells but no mechanism reported	Human gastric epithelial cells	Fiocca et al, 1999
P. aeruginosa	Lipid raft-dependent	Human gastric epithelial cells	Kaparakis et al, 2010
	Fusion with lipid rafts	Human bronchial, human airway epithelial cells	Bomberger et al, 2009
	OMVs demonstrated within cells; LPS required for cell surface binding; recognition of OMV protein required for internalisation	Mouse macrophage	Ellis et al, 2010
N. gonorrhoeae	Lipid raft-dependent	Human gastric epithelial cells	Kaparakis et al, 2010
P. gingivalis	Lipid raft-dependent; clathrin-, caveolin- and dynamin-independent	Human cervical and gingival epithelial cells	Furuta et al, 2009
	Dynamin-dependent; clathrin-independent; actin mediated; phosphoinositide (PI) 3-kinase dependent; OMVs colocalise with GM1 and Cav-1	Human cervical epithelial cells	Tsuda et al, 2005
M. catarrhalis	Lipid raft-dependent; TLR-2 involved	Mouse macrophages	Schaar et al, 2010
	Lipid raft-dependent receptor mediated endocytosis	Primary human tonsillar B cells	Vidakovics et al, 2010
Vibrio cholerae	Demonstrate OMV entry but do not propose a mechanism	Human cervical epithelial cells	Bielig et al, 2011
	GM1 Receptor mediated; cholera toxin essential	HeLa derivative	Chatterjee & Chaudhuri, 2013
A. actinomycetemcomitans	Fusion	Human promyelocytic leukaemia cells	Demuth et al, 2003
	Lipid raft-dependent	Human cervical epithelial cells	Rompikuntal et al, 2011
Legionella pneumophila	Possible fusion	Human alveolar basal epithelial cells	Galka et al, 2008
Shigella flexneri	Demonstrate OMV entry but do not propose a mechanism	Human intestinal epithelial cells	Kadurugamuwa & Beveridge, 1996
Brucella abortus	OMV entry into phagocytic and non-phagocytic cells; clathrin-dependent endocytosis in THP1 cells; actin polymerisation involved	Human cervical epithelial cells, human monocytic cells	Pollack et al, 2012
Haemophilus influenzae	Lipid raft-dependent; caveolin-dependent endocytosis	Human pharynx epithelial cells	Sharpe et al, 2011

interest and current usage in OMV based vaccines, there exists a need to understand precisely how OMVs gain entry into host cells. Demuth *et al.* labelled OMVs from *Aggregatibacter actinomycetemcomitans* with a lipid tracking dye to demonstrate that upon interaction with host cells, the dye was transferred to the cytoplasmic membrane of the host cells, indicating that the vesicles were either associating with, or fusing with the membrane (Demuth, James et al. 2003). Similarly, *P. aeruginosa* OMVs have been suggested to fuse with host cell membranes via lipid rafts to deliver multiple virulence factors to the host cell cytoplasm (Kadurugamuwa and Beveridge 1996, Kadurugamuwa and Beveridge 1999, Bomberger, Maceachran et al. 2009). In fact, cholesterol rich lipid rafts appear to be essential for the uptake of OMVs from many bacteria, including *E. coli* (Kesty, Mason et al. 2004), *Neisseria gonorrhoeae, H. pylori* (Kaparakis, Turnbull et al. 2010), *P. gingivalis* (Furuta, Tsuda et al. 2009) and *Moraxella. catarrhalis* (Schaar, de Vries et al. 2010). However, the reported mechanisms differ, with some OMVs appearing to enter via receptor mediated endocytosis (Kesty, Mason et al. 2004, Furuta, Takeuchi et al. 2009, Furuta, Tsuda et al. 2009, Schaar, de Vries et al. 2010, Chatterjee and Chaudhuri 2011), whereas others enter via fusion (Garcia-del Portillo, Stein et al. 1997, Demuth, James et al. 2003, Galka, Wai et al. 2008, Bomberger, Maceachran et al. 2009) or lipid raft dependent (Kaparakis, Turnbull et al. 2010) or independent mechanisms (Parker, Chitcholtan et al. 2010). In fact, confusing the area further are reports proposing conflicting modes of entry for OMVs derived from the same bacteria. These include: *H. pylori* OMVs, for which two independent studies reported lipid raft dependency (Kaparakis, Turnbull et al. 2010) and independency (Parker, Chitcholtan et al. 2010), respectively; and *P. gingivalis*, for which one study reported clathrin and caveolin independent entry (Furuta, Tsuda et al. 2009), whereas another reported and caveolin-dependent

endocytosis (Tsuda, Amano et al. 2005). Interestingly, it has been proposed that vesicle size plays a crucial role in directing the mechanism of host cell entry and therefore, the intracellular trafficking and fate of internalised particles (Brewer, Pollock et al. 2004). As OMVs are produced in a wide range of sizes, it is possible that particle size plays a role in directing the preferential mode of host cell entry. The contrasting mechanisms of entry reported in these diverse studies (summarised in Table 1.1) highlight the need for further research if we are to understand their roles in host cell interactions and their contribution to bacterial pathogenesis.

1.2.8 OMVs and modulation of the host immune response

OMVs from several pathogenic bacteria are known to contain immunogenic proteins which are recognised by host immune cells (Hynes, Keenan et al. 2005, Roier, Leitner et al. 2012, Chatterjee and Chaudhuri 2013) and may act in immunomodulatory or stimulatory manners. OMVs derived from *P. aeruginosa* and *H. pylori* are able to induce production of the chemokine interleukin-8 (IL-8) by lung and gastric epithelial cells, respectively (Ismail, Hampton et al. 2003, Bauman and Kuehn 2006). Similarly, the endotoxin LPS, a major component of many OMVs (Hynes, Keenan et al. 2005, Lee, Choi et al. 2008, Roier, Leitner et al. 2012), is recognised by Toll-like receptor-4 (TLR-4) leading to the production of pro inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Zhao, Deng et al. 2013). OMVs may also aid in bacterial colonisation by helping the bacteria to escape host immune surveillance. For example, *N. gonorrhoeae* OMVs have been proposed to act as decoys, attracting immune cells that would otherwise recognise the whole bacteria, thereby avoiding detection themselves (Pettit and Judd 1992).

Certain bacteria have also been shown to package proteins within their OMVs that mediate immune suppression. To this end, *M. catarrhalis* incorporates the UspA1/A2 virulence factors into its OMVs. These proteins are able to bind the complement protein C3, thereby counteracting the complement cascade (Tan, Morgelin et al. 2007). Interestingly, *M. catarrhalis* and *H. infuenzae* are commonly co-isolated from patients with respiratory tract infections and Tan *et al*. found that the *M. catarrhalis* OMVs containing UspA1/A2 promoted the survival of both *M. catarrhalis* and *H. influenzae*, demonstrating that pathogens can co-operate to enhance survival (Tan, Morgelin et al. 2007). *P. gingivalis* OMVs are highly pro-inflammatory, inducing the expression of the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and E-selectin, thereby facilitating the migration of immune cells, such as neutrophils, into the affected area (Srisatjaluk, Doyle et al. 1999, Srisatjaluk, Kotwal et al. 2002). Concurrently, they are able to significantly inhibit interferon-γ (IFN-γ) dependent synthesis of major histocompatability complex (MHC) Class II molecules, which play a crucial role in the activation of the adaptive immune system (Srisatjaluk, Doyle et al. 1999). Conversely, infection with *H. pylori* is known to induce the expression of human leukocyte antigen (HLA) Class II molecules (the human equivalent of MHC), but not ICAM-1, in gastric epithelium (Engstrand, Scheynius et al. 1989). Therefore OMVs from a range of bacteria are able to act in both immunomodulatory and stimulatory manners.

1.2.9 OMVs and vaccination

Bacterial OMVs have long been recognised for their potential as vaccine candidates, particularly as the composition of OMVs largely reflects that of the bacterium and therefore tend to be enriched in many immunogenic OMPs (Schild, Nelson et al. 2008). A large number of independent studies have shown that immunisation with OMVs from

a wide range of bacteria induce protective immune responses (Keenan, Day et al. 2000, Alaniz, Deatherage et al. 2007). One such study by Schild *et al.* investigated the potential of *V. cholerae* OMVs as a vaccine candidate, concluding that in a murine model, immunisation with OMVs resulted in long-term protection against *V. cholerae* (Schild, Nelson et al. 2008). Furthermore, by incorporating the *E. coli* periplasmic alkaline phosphatase (PhoA) protein into *V. cholerae* OMVs, they showed that an immune response could be generated against PhoA following vaccination with these OMVs (Schild, Nelson et al. 2008). In a similar study, Chen and colleagues engineered *E. coli* OMVs to express green fluorescent protein (GFP) and demonstrated a strong anti-GFP antibody response from mice immunised with these OMVs (Chen, Osterrieder et al. 2010). Currently, there are several *N. meningitidis* OMV-based vaccines licensed for use in humans (Haneberg, Dalseg et al. 1998, Saunders, Shoemaker et al. 1999). Very recently, a new multicomponent OMV-based vaccine designed to provide protection against *N. meningitidis* has been licensed for use (Gorringe and Pajón 2012).

OMVs derived from *H. pylori* are known to contain several immunogenic proteins, including pro-inflammatory porin proteins (Keenan, Day et al. 2000), Lpp20 (Keenan, Oliaro et al. 2000) and VacA (Fiocca, Necchi et al. 1999). Intragastric immunisation of mice with *H. pylori* OMVs in the presence of an adjuvant, the cholera holotoxin, showed promising results with the induction of a protective immunoglobulin (Ig) G response (Keenan, Oliaro et al. 2000). Furthermore, the conserved and immunodominant antigen Lpp20 was identified as the specific target of these protective antibodies (Keenan, Oliaro et al. 2000), suggesting that this antigen may be a good candidate for use in vaccination. Moreover, work previously performed in our laboratory demonstrated that *H. pylori* OMVs were capable of inducing pro-inflammatory cytokines and bacterial specific antibodies, when delivered to mice

(Kaparakis, Turnbull et al. 2010). These studies highlight the potential use of OMVs in vaccination, not only for the bacteria from which the OMVs are derived, but also as engineered delivery systems for foreign antigens.

1.3 Exosomes: Eukaryotic cell membrane vesicles

The secretion of vesicles is a conserved cellular process that occurs not only in prokaryotes, but also in eukaryotes (Lee, Choi et al. 2008). One family of extracellular membrane vesicles released by host cells are known as exosomes, small vesicles of endocytic origin that are secreted by almost all cell types (Johnstone, Adam et al. 1987). Originally discovered in reticulocytes (Johnstone, Adam et al. 1987), exosomes have since been shown to be released by a variety of cell types of both hematopoietic and non-hematopoietic origins, including B cells, dendritic cells (DCs) and intestinal epithelial cells (IECs) (Raposo, Nijman et al. 1996, Zitvogel, Regnault et al. 1998, van Niel, Raposo et al. 2001). Recent studies suggest that exosomes may play a role in immune regulation (Thery, Duban et al. 2002), protein secretion (Johnstone, Mathew et al. 1991) and the intracellular transfer of molecules, such as RNA (Valadi, Ekstrom et al. 2007) and infectious agents (Izquierdo-Useros, Naranjo-Gómez et al. 2009). Of particular interest are the immune regulatory and antigen presenting capabilities of exosomes. Thus their potential applications in cancer immunotherapy (Zitvogel, Regnault et al. 1998, Escudier, Dorval et al. 2005) and vaccination (Beauvillain, Juste et al. 2009, Schnitzer, Berzel et al. 2010).

1.3.1 Protein and lipid composition of exosomes

Playing an important role in the formation and release of exosomes are multivesicular bodies (MVB), membrane enclosed organelles within eukaryotic cells that are part of the endocytic pathway (van Deurs, Holm et al. 1993). MVBs are important for the degradation of damaged and unwanted proteins, which are specifically sorted into MVBs for delivery to lysosomes where they are degraded. Alternatively, the inward budding of MVBs results in the formation of intraluminal vesicles (ILV) that are released into the extracellular environment as exosomes following fusion of the MVB membrane with the host cell plasma membrane (reviewed in (Piper and Katzmann 2007)) (Figure 1.4). During the formation of ILVs a variety of membrane proteins and lipids are incorporated into the membrane, maintaining similar protein and lipid profiles to those of the host cell (van Niel, Porto-Carreiro et al. 2006, Schorey and Bhatnagar 2008). Some protein classes common to most exosomes of different cellular origins are: adhesion proteins, such as integrins and ICAM-1 (Thery, Boussac et al. 2001, Mears, Craven et al. 2004); cytoskeletal components, such as actin and the annexin proteins (Thery, Boussac et al. 2001); and the heat shock proteins Hsp70 and Hsp90, which facilitate peptide loading onto MHC Class I and II molecules (Gastpar, Gehrmann et al. 2005). Exosomes also possess proteins and lipids that are specific to the cell type from which they were derived. For example, exosomes released from intestinal epithelial cells contain both MHC Class I and II molecules, or HLA Class I and II molecules (from mouse or human origin respectively) and the epithelial specific marker A33 (van Niel, Raposo et al. 2001, Buning, von Smolinski et al. 2008) (Figure 1.5).

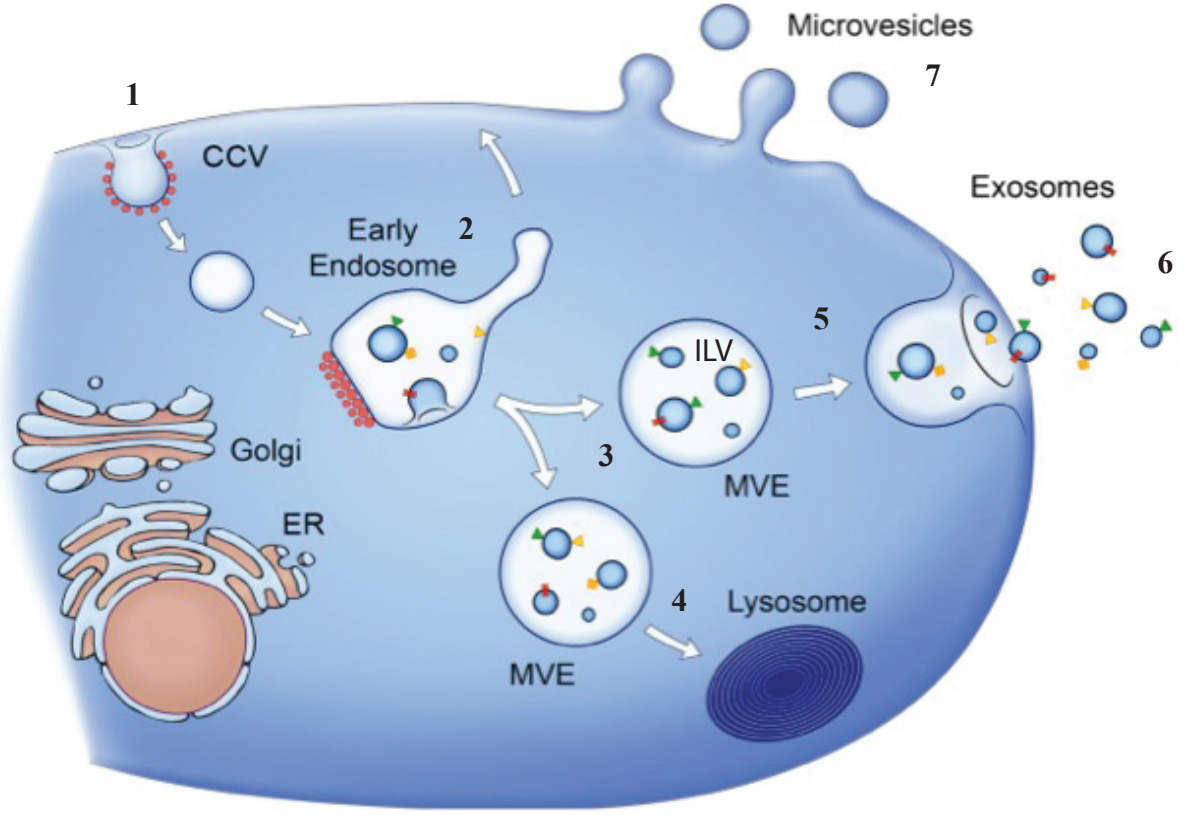

Figure 1.4: The release of exosomes from eukaryotic cells. Antigens are internalised (1) at the cell surface, for example within clathrin coated vesicles (CCV), and delivered to early endosomes (2). Early endosomes mature into multivesicular endosomes (MVE) containing intraluminal vesicles (ILV) that are formed by inward invagination of the membrane of MVB (3). In the degradation pathway MVBs fuse with lysosomes (4). Alternatively, MVBs fuse with the plasma membrane (5) and ILVs are released into the extracellular environment as exosomes (6). Microvesicles are similar in appearance to exosomes however they bud directly from the plasma membrane (7), (Adapted from (Raposo and Stoorvogel 2013)).

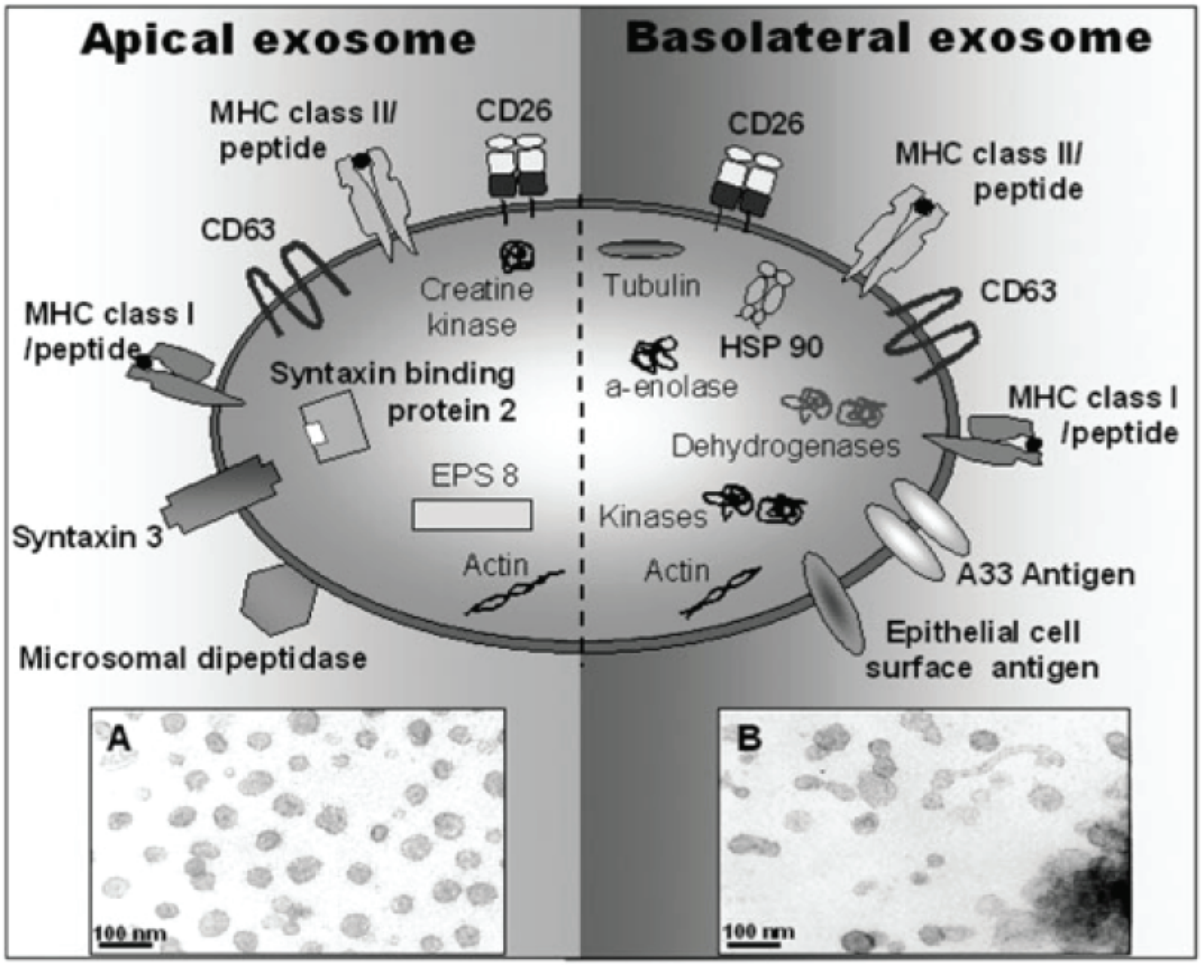

Figure 1.5: Proteins and molecules associated with epithelial cell derived exosomes. Some molecules such as MHC (or HLA) Class I and Class II, CD63 and cytoskeletal proteins are ubiquitously expressed in both apical and basolateral exosomes. Basolateral specific molecules include co-stimulatory molecules for antigen presentation, whereas apical exosomes carry molecules such as syntaxin 3. Electron micrograph of apical (a) and basolateral (b) exosomes derived from human T84 intestinal epithelial cells. (van Niel and Heyman 2002).

1.3.2 Biological functions of exosomes

Since their discovery, exosomes have been recognised for their clinical importance in disease and their potential in immunotherapy (Thery, Duban et al. 2002). Moreover, secreted exosomes are biologically active and are important for a variety of cellular pathways. Some of these are listed below.

Protein secretion

Exosomes were initially identified as a means to remove misfolded, overproduced or obsolete proteins from the cell (Johnstone, Adam et al. 1987), similar to the release of OMVs containing misfolded proteins by Gram-negative bacteria (McBroom and Kuehn 2007). This was first noticed in maturing reticulocytes that utilised exosomes for clearance of proteins, such as acetylcholinesterase and transferrin receptors that are no longer required (Johnstone, Adam et al. 1987)

Immune surveillance

Pathogens that are located primarily in an intracellular niche, such as *Chlamydia trachomatis*, *Mycobacterium spp.* and viruses, have a limited exposure of surface antigens to host effector cells compared to extracellular pathogens, such as *H. pylori* (Giles and Wyrick 2008, Schorey and Bhatnagar 2008). Work by Beatty *et al.* regarding *Mycobacterium bovis* infected macrophages led to the hypothesis that exosomes released by infected cells contain pathogen associated molecular patterns (PAMPs). Furthermore, mycobacterial PAMPs, such as lipoarabinomannan (LAM) and phosphatidyl-myo-inositol mannosides (PIM), were associated with early and late endosomes and MVB within mycobacterial-infected macrophages (Beatty, Rhoades et al. 2000). Subsequent studies confirmed that mycobacterial components were indeed released from mycobacterial-infected cells via exosomes (Rhoades, Hsu et al. 2003,

Bhatnagar and Schorey 2007). In addition, these exosomes were shown to have a pro-inflammatory effect on resting uninfected macrophages via interactions with TLR-2 and TLR-4, thereby assisting in the activation of innate immune responses (Bhatnagar and Schorey 2007). This antigen presenting function of exosomes is not limited to exosomes originating from Mycobacterial-infected cells. Indeed, macrophages infected with *Salmonella typhimurium* or *Toxoplama gondii* were found to release exosomes that contained PAMPs (Bhatnagar, Shinagawa et al. 2007). These exosomes were capable of stimulating TLR dependent pro-inflammatory responses from uninfected macrophages (Bhatnagar, Shinagawa et al. 2007). Collectively, these results demonstrate the ability of exosomes to participate in immune surveillance and induce pro-inflammatory host immune responses. Internalised *H. pylori* OMVs are thought to enter the endocytic pathway and it was suggested by Fiocca *et al.* that antigens derived from these internalised OMVs may move through the gastric epithelial monolayer to the lamina propria (Fiocca, Necchi et al. 1999). It is therefore possible that exosomes containing OMV-derived proteins may be released from gastric epithelial cells that have internalised OMVs, in a similar manner to release of antigen bearing exosomes from *M. bovis* infected macrophages. (Figure 1.6).

Antigen Presentation

Exosomes released from a variety of cell types are capable of antigen presentation (Schorey and Bhatnagar 2008). Proteins such as MHC Class II accumulate in the MVBs of antigen presenting cells (APCs), such as B cells and DCs, giving rise to a specialised subset of MVBs known as MHC II-enriched compartments (MIICs) (Piper and Katzmann 2007, Buning, von Smolinski et al. 2008). Antigen processing and subsequent peptide loading into MHC Class II molecules occurs in MIICs and similar compartments (Harding and Geuze 1993). A study by Raposo *et al.* revealed that in B

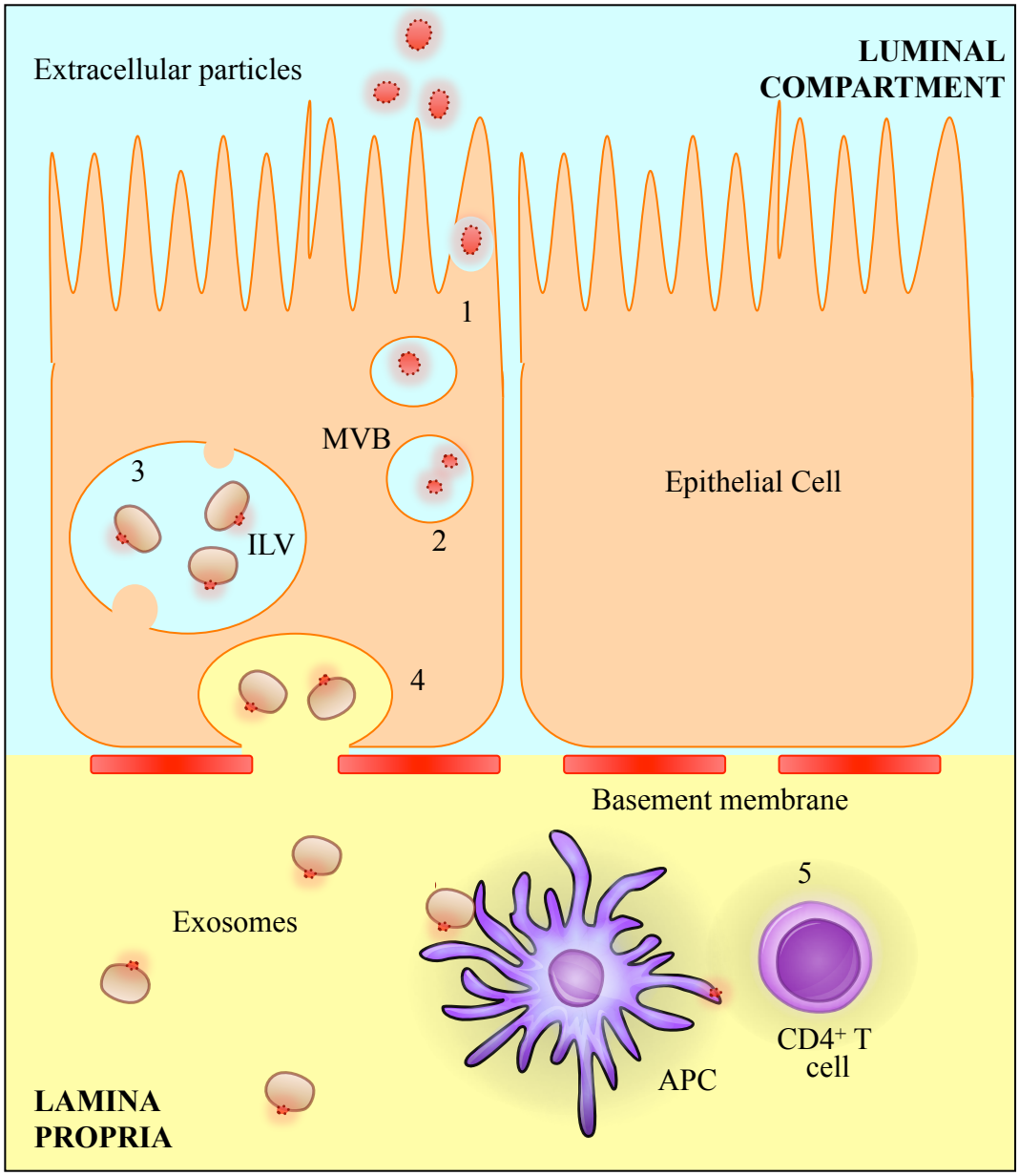

Figure 1.6: The production and release of exosomes containing extracellular peptides. Antigens are internalised at the apical surface of IECs and processed into early endosomes (1). These mature into multivesicular bodies (MVB) (2). Intraluminal vesicles (ILV) are formed by inward invagination of the membrane of MVBs (3). The MVB membrane fuses with the cell membrane releasing the ILVs into the extracellular environment where they are known as exosomes (4). Exosomes containing extracellular peptides may then then interact with antigen presenting cells (APCs) to activate $CD4^+$ T cells (5).

cells, the membrane of MIICs can fuse with the plasma membrane to release peptide bound MHC Class II enriched exosomes. Furthermore, B cell derived exosomes were able to present peptides to $CD4^+$ T cells resulting in peptide specific MHC Class II restricted responses (Raposo, Nijman et al. 1996). Since this pioneering study, a vast array of data has emerged supporting the hypothesis that exosomes can act as antigen presenting vesicles. One such study showed that DC derived exosomes display both MHC class I and MHC class II molecules, as well as the co-stimulatory molecules B7-1 and B7-2 required for T cell activation. Furthermore, these exosomes were capable of activating naïve $CD4^+$ T cells in an antigen specific manner (Thery, Duban et al. 2002). Human gastric epithelial cells are also known to express both HLA lass I and Class II, as well the co-stimulatory molecules required for T cell activation (Ye, Barrera et al. 1997), indicating a possible role in antigen presentation for exosomes released by these cells.

1.3.3 Epithelial cell derived exosomes and the immune system

IECs are known as non-professional APCs, however, they do express the key molecules required for antigen processing and presentation, including MHC Class I and II, invariant chain, HLA-DM, B7.1 and B7.2 (van Niel and Heyman 2002, Lin, Almqvist et al. 2005, Hundorfean, Zimmer et al. 2007). Interestingly, both HLA Class I and Class II molecules in IEC are located predominantly within late endosomes and on the basolateral membrane of IEC in human biopsy specimens (Hundorfean, Zimmer et al. 2007). IECs have been shown to secrete exosomes from both the apical and basolateral surfaces. However, basolateral exosomes tend to display a higher percentage of HLA Class II molecules than do apical exosomes, most likely due to the distribution of these molecules in IECs (Mallegol, van Niel et al. 2005, Hundorfean, Zimmer et al. 2007). In

addition, the surface proteins expressed by IEC derived exosomes differ depending on the site of release. Surface molecules such as the A33 antigen are found predominantly on exosomes released from the basolateral surface of IECs, whereas exosomes released from the apical surface are rich in syntaxin 3 and CD26, which are expressed on the apical surface of IECs (Mallegol, van Niel et al. 2005).

IECs serve as a physical barrier between the host and the external environment and are perfectly situated to sample luminal antigens (Buning, von Smolinski et al. 2008). However, they have a limited interaction with $CD4^+$ T cells in the intestinal lumen due to the presence of the basement membrane (van Niel and Heyman 2002). It has been hypothesised that exosomes released by IECs function as an antigen delivery system to the underlying immune cells. It is interesting to note that the expression of HLA Class II molecules, as well as the co-stimulatory molecule B7-2, are up regulated in IECs during infection with *H. pylori* (Engstrand, Scheynius et al. 1989, Krauss-Etschmann, Gruber et al. 2005). Moreover, it is known that HLA Class II expression is increased in response to IFN-γ (Ye, Barrera et al. 1997, Sommer, Faller et al. 1998), which is present in abundance during infection with *H. pylori* (Sommer, Faller et al. 1998). Importantly, exosomes derived from IECs grown in the presence of IFN-γ show a marked increase in peptide loaded MHC Class II expression, allowing them to activate $CD4^+$ T cells (van Niel, Mallegol et al. 2003).

These data, when coupled with the knowledge that *H. pylori* OMVs are internalised by epithelial cells (Fiocca, Necchi et al. 1999, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010), have led us to hypothesise that epithelial cells may present antigens derived from internalised OMVs in complex with HLA Class II to $CD4^+$ T cells. Furthermore, as exosomes have antigen-presenting capabilities, it is reasonable to

hypothesise that epithelial cell derived exosomes may also present *H. pylori* OMV/ HLA Class II peptide complexes to $CD4^+$ T cells.

Given that *H. pylori* OMVs resemble the bacterial outer membrane, studies on the host immune responses to these particles will help us understand the immune response to *H. pylori*.

1.4 *H. pylori* and the host immune response

H. pylori infection is usually acquired during childhood and despite active innate and adaptive immune responses, the bacterium usually persists in the gastric mucosa (reviewed in (Salama, Hartung et al. 2013)). It is therefore believed that *H. pylori* bacteria are somehow able to dampen the host immune response. Initial colonisation of the gastric mucosa by *H. pylori* requires the activity of a variety of virulence factors, including urease activity (Tsuda, Karita et al. 1994) and motility (Ottemann and Lowenthal 2002), which allow the bacteria to rapidly escape the acid environment of the stomach and penetrate the mucus layer to reach the epithelial cell surface (reviewed in (Salama, Hartung et al. 2013)). Having penetrated the mucus layer, the outer membrane proteins BabA and BabB (Guruge, Falk et al. 1998, Ilver, Arnqvist et al. 1998), together with other adhesins, mediate adhesion to the gastric epithelium, allowing the bacteria to come into contact with host gastric epithelial cells.

1.4.1 Innate immune defence and cytokine production

The first line of defence against *H. pylori* infection are the gastric epithelial cells and the innate immune cells that reside nearby. The innate detection of pathogens by these cells occurs via the detection of PAMPs by pattern recognition receptors (PRRs). TLRs are some of the most widely understood PRRs and detect diverse PAMPs, such as LPS

(via TLR4), lipoproteins (via TLR2) and flagellin (via TLR5) (reviewed in (Akira, Takeda et al. 2001)). *H. pylori* has evolved to evade detection by TLR4/5 through modifications to its Lipid A (Pérez-Pérez, Shepherd et al. 1995, Moran, Lindner et al. 1997, Cullen, Giles et al. 2011) and flagellin subunits, respectively (Gewirtz, Yu et al. 2004). TLRs are not the only PRRs known to play a role in *H. pylori* innate immune recognition and defence. *H. pylori* PG, which can be delivered into the host cell by either the type 4 secretion system (T4SS) (Viala, Chaput et al. 2004) or via OMVs (Kaparakis, Turnbull et al. 2010), is recognised intracellularly by the PRR Nucleotide-binding Oligomerization Domain 1 (NOD1). This recognition results in Nuclear Factor-κB (NF-κB) translocation and the subsequent production and secretion of the chemo attractant IL-8 (Viala, Chaput et al. 2004) which causes the influx of neutrophils and other immune mediators. The production of certain cytokines is strongly increased during *H. pylori* infection; in particular, increased levels of IFN-γ, IL-6, tumour necrosis factor-α (TNF-α), IL-1β and IL-10 are found in the gastric mucosa of infected individuals (Yamaoka, Kita et al. 1997, Harris, Smythies et al. 2000). Interestingly, IL-6 acts as a link between the innate and adaptive immune systems, by promoting neutrophil activation (Borish, Rosenbaum et al. 1989), stimulating antibody production by B cells (Yoshizaki, Nakagawa et al. 1984) and modulating T cell differentiation (reviewed in (Dienz and Rincon 2009)).

1.4.2 Adaptive Immune defence

H. pylori is also known to manipulate the host adaptive immune response, particularly through the actions of the VacA toxin. A seminal study performed by Haas and colleagues revealed that VacA was able to inhibit T cell proliferation, via the induction of cell cycle arrest (Gebert, Fischer et al. 2003). Not long after this discovery, Cover *et*

al. expanded on this work to show that VacA in fact suppressed interleukin-2 (IL-2) dependent cell cycle progression in a manner that did not affect IL-2 secretion nor IL-2 dependant cell survival (Sundrud, Torres et al. 2004). *H. pylori* can also inhibit T cell proliferation through the secretion of γ-glutamyl transpeptidase (GGT), which similarly arrests cell cycle progression, independent of VacA (Gerhard, Schmees et al. 2005, Schmees, Prinz et al. 2007). Interestingly, *H. pylori* is able to promote the differentiation of naïve T cells into regulatory T (Treg) cells (Oertli, Noben et al. 2013). This process is mediated by DCs that have been exposed to *H. pylori* and have thereby been reprogrammed to induce tolerance through the actions of VacA and GGT (Oertli, Noben et al. 2013). Treg cells accrue in the gastric mucosa of *H. pylori* infected individuals where they are able to suppress *H. pylori* specific memory T cell responses (Lundgren, Suri-Payer et al. 2003, Lundgren, Trollmo et al. 2005) (Figure 1.7)

1.5 Aims of this study

H. pylori is one of many Gram-negative pathogens that utilise naturally produced OMVs to deliver virulence factors to host cells. Immunisation studies with OMVs have yielded some positive results, indicating that OMVs may function as a useful tool for the generation of vaccines. However, OMVs are produced only in small amounts during normal growth and thus their purification is laborious. Therefore, the development of a method to obtain large volumes of OMVs for research purposes is highly desirable. The first aim of this study was to generate and characterise hyper-vesiculating strains of *H. pylori* that could then be used for further studies, thereby also aiding our understanding of OMV biogenesis in *H. pylori*.

In order to develop OMVs as vaccine carriers, we also need to understand more about the basic aspects of their biology; specifically, their mode of cellular entry and

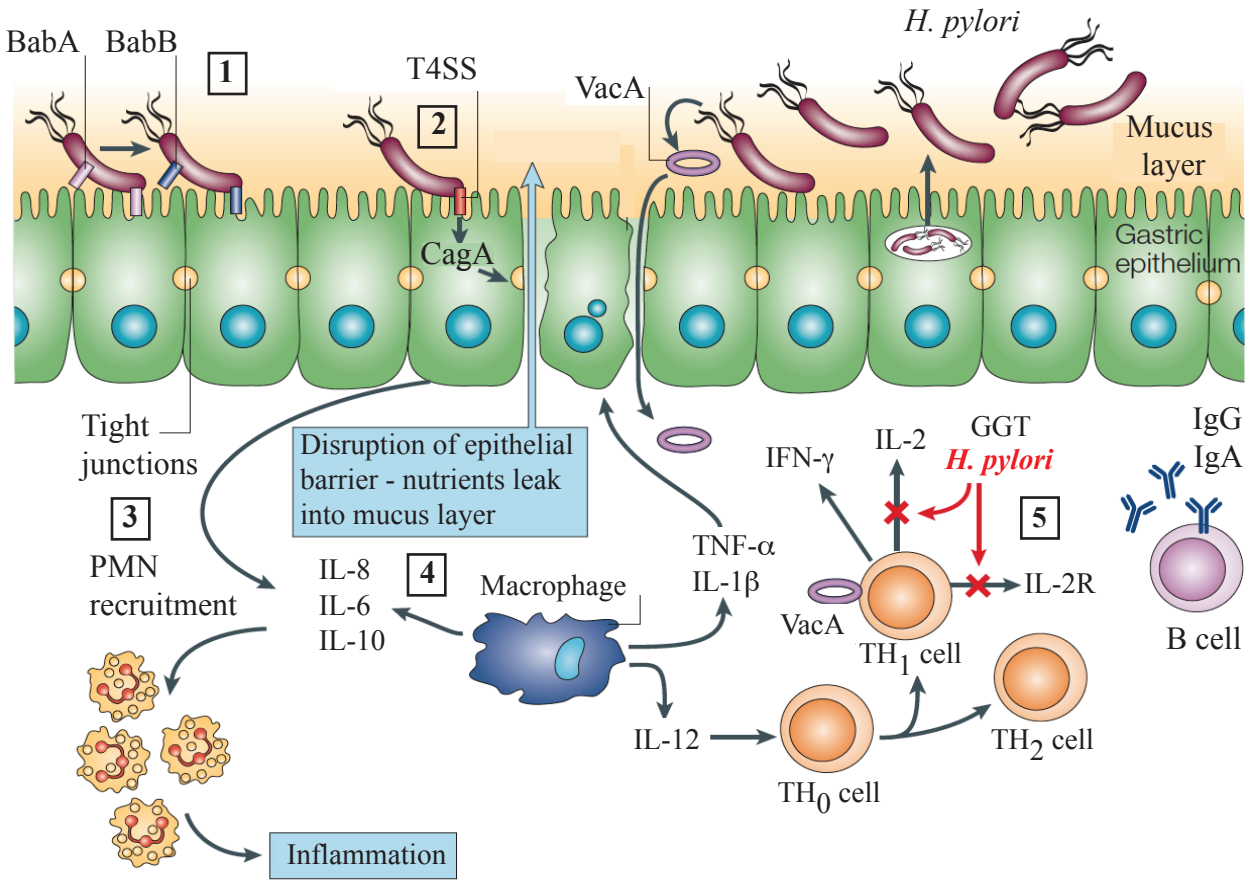

Figure 1.7: Interplay between *Helicobacter pylori* and the host immune response. *H. pylori* binds to gastric epithelial cells through BabA, BabB and other adhesins (1). Strains that carry the *cag*PAI can deliver CagA and PG into the host cell via the T4SS (2). PG is recognised by intracellular NOD1 leading to the secretion of IL-8 and subsequent PMN infiltration into the gastric mucosa (3). Pro-inflammatory cytokines IL-6, TNF-α, IL-12 and IL-1β, and the anti-inflammatory cytokine IL-10 are secreted into the gastric mucosa (4). *H. pylori* inhibits T cell activation and IL-2 signalling through the actions of VacA and GGT (5). (Adapted from (Monack, Mueller et al. 2004)).

intracellular fate. Many studies exist regarding the mode of OMV cellular entry, however, these studies are contradictory and to date the precise mechanism(s) of entry are still unknown. The second aim of the current study was to determine the precise mode of OMV internalisation. Moreover, we wished to determine the role of inherent factors, such as size and protein composition of OMVs, in directing the mechanism of OMV cellular entry. Internalised *H. pylori* OMVs are thought to enter the endocytic pathway, where they may be processed within the cell and packaged into epithelial cell derived exosomes. Interestingly, IECs have been shown to secrete exosomes that participate in antigen presentation and immune cell signalling via the presentation of peptide loaded MHC Class II molecules. Given this evidence, the third aim of this study was to determine whether *H. pylori* OMVs were processed intracellularly into secreted exosomes. Furthermore we wished to determine if exosomes containing OMV proteins could interact with immune cells, thereby potentiating presentation of OMV proteins to T cells.

Collectively, these studies will enhance our knowledge of *H. pylori* OMV production, internalisation by host cells and the development of adaptive immune responses. Further expanding our understanding of the precise mechanisms whereby OMVs enter host cells to induce the development of an immune response will greatly aid in OMV based vaccine design and provide us with a greater understanding of the contribution of OMVs to *H. pylori* pathogenesis.

Chapter 2: Increased outer membrane vesicle formation in *Helicobacter pylori tolB* and *pal* mutants.

2.1 Summary

OMVs released by Gram-negative bacteria are highly immunogenic (Ismail, Hampton et al. 2003, Kaparakis, Turnbull et al. 2010), inexpensive to produce and easy to transport. For these reasons, they are attractive candidates in the development of acellular vaccines. However, OMVs are naturally produced in small quantities (Henry, Pommier et al. 2004) and the culture of sufficient volumes for research purposes is laborious.

Previous work performed in our laboratory demonstrated that OMVs from *H. pylori* are potent inducers of both innate and adaptive immune responses (Kaparakis, Turnbull et al. 2010). Interestingly, several proteins found within *H. pylori* OMVs have been identified as potential vaccine candidates (Keenan, Oliaro et al. 2000, Godlewska, Pawlowski et al. 2008). Despite the potent effects of *H. pylori* OMVs on the host immune system, little is currently known regarding OMV formation in this pathogen.

Although the precise mechanism of OMV formation in Gram-negative bacteria has yet to be defined, the disruption of bacterial membrane integrity through the mutation of certain genes results in a marked increase in vesiculation (Bernadac, Gavioli et al. 1998). The best characterised of these are the genes within the *tol-pal* cluster. This cluster contains 5-7 genes, encoding proteins that are important for maintaining cell membrane integrity in bacteria (Sturgis 2001). Importantly, the *tol-pal* cluster is conserved across most Gram-negative organisms, including *H. pylori*, which was

reported to share homologues of *E. coli tolB* and *pal* (Sturgis 2001). Nothing is known, however, regarding the roles of TolB and Pal in this bacterial pathogen.

The following experiments were designed to investigate the functions of the TolB and Pal proteins in *H. pylori* membrane integrity and OMV formation. A secondary aim of the work was to generate hyper-vesiculating *H. pylori* strains that could ultimately be used in the development of a vaccine against this bacterial pathogen.

2.2 Declaration for thesis chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
My contribution included project conceptualisation and experimental design. In addition I performed all experiments, interpreted data and undertook the majority of the writing.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Judyta Praszkier	Assisted with molecular biology techniques, experimental design and manuscript editing	
Melanie L Hutton	Assisted molecular biology experiments	
David Steer	Performed Proteomic analysis	
Georg Ramm	Assisted with electron microscopy experiments	
Maria Kaparakis-Liaskos	Project conceptualisation, data interpretation and manuscript editing	
Richard L Ferrero	Project conceptualisation, data interpretation and manuscript editing	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date 10/02/2104**
Main Supervisor's Signature	[redacted]	**Date 10/02/2014**

2.2 Manuscript: Increased outer membrane vesicle formation in *H. pylori*

TITLE: **Increased outer membrane vesicle formation in *Helicobacter pylori tolB* and *pal* mutants.**

RUNNING TITLE: Increased OMV release in *H. pylori tolB-pal* mutants.

AUTHORS:

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[redacted]

Abstract

The *tol-pal* gene cluster in *Escherichia coli* encodes proteins playing important roles in the maintenance of bacterial cell membrane integrity and the formation of outer membrane vesicles (OMVs). Although the functions of the Tol-Pal system have been extensively studied in non-pathogenic Gram-negative bacteria, much less is known about analogous systems in pathogenic organisms. This study investigated the role of two key components of the Tol-Pal system (Tol, Pal) in cell morphology and OMV formation in the gastric pathogen, *Helicobacter pylori*. *H. pylori* Δ*tolB* and Δ*pal* bacteria exhibited aberrant cell morphology and/or flagella biosynthesis, consistent with perturbations in cell wall integrity. Importantly, we found that the disruption of either *H. pylori tolB* or *pal*, but not both genes, resulted in >600- and 22-fold increases, respectively, in the amounts of OMVs released ($P < 0.001$ and $P < 0.005$, respectively). The massively increased levels of OMV production by the *tolB* mutant were consistent with its extensive blebbing, as observed by scanning electron microscopy. OMVs from *H. pylori* Δ*tolB* and Δ*pal* bacteria harbored many of the major outer membrane and virulence proteins observed in wild-type OMVs. Interestingly, OMVs purified from Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants induced significantly higher levels of IL-8 production in gastric epithelial cells, when compared with wild-type OMVs ($P < 0.05$). Complementation of the mutants resulted in bacteria producing OMVs with similar IL-8-inducing activities to those of wild-type OMVs. Together, this work identifies the important roles of TolB and Pal in *H. pylori* membrane integrity and OMV formation.

Introduction

Outer membrane vesicles (OMVs) are nanosized lipid vesicles secreted by a diverse range of Gram-negative bacteria. These vesicles have been shown to contain DNA, proteins, lipids, peptidoglycan and toxins (reviewed in (1)). Secreted OMVs play many roles during infection, including helping to establish a colonization niche (2) and transmission of toxins and virulence factors to host cells (3). Importantly, OMVs have been implicated as mediators of various types of responses by the host immune system, including the production of pro-inflammatory cytokines (4), activation of antigen presenting cells (APCs) and the induction of B and T cell responses (5). OMVs are highly immunogenic (4), inexpensive to produce, very stable and easy to transport. For these reasons, OMVs are attractive candidates for vaccine study and development. Indeed, OMVs form the basis of several licensed vaccines, as well as other vaccines currently under development (6-8). However, only small quantities of OMVs are produced naturally during normal bacterial growth and the culture of sufficiently large volumes is labor intensive. Therefore, the development of a method to isolate large quantities of immunogenic OMVs with ease is highly desirable.

The precise mechanism of OMV formation has yet to be defined and is likely to differ depending on the bacterium. Nevertheless, it is commonly thought that vesicles form when the outer membrane of the bacterium bulges and pinches off (reviewed in (1)). The structure and integrity of the cell membrane is known to be an important factor in the release of OMVs (9). Indeed, it is well established that defects in a range of proteins involved in maintaining the structural integrity of the membrane result in increased vesiculation (10-12). The best characterized of these proteins are encoded by a gene cluster, known as *tol-pal*, comprising 5-7 genes. This gene cluster is conserved across most Gram-negative organisms (13). In *Escherichia coli*, the five proteins of the Tol-Pal system comprise three inner membrane proteins (TolA, TolQ and TolR) and a periplasmic protein (TolB), which interacts with an

outer membrane protein, peptidoglycan associated lipoprotein (Pal) (14). The Tol-Pal proteins are linked via TolA-Pal (15) and TolA-TolB (16) interactions. It was reported that the disruption of any of the genes within the *tol-pal* cluster resulted in increased vesiculation in *E coli* (9, 10), *Caulobacter crescentus (17)*, *Erwinia chrysanthemi* (18) and *Pseudomonas putida* (19). In addition, the disruption of these genes was shown to result in other abnormalities in the bacterium, including a reduction in the kinetics of bacterial growth (18, 20), changes in motility (18), decreased virulence (18, 20), as well as altered cell morphology (17, 18) and division (17, 18, 21). The functions of the Tol-Pal system have been studied in many non-pathogenic bacteria, yet the roles of these proteins in pathogenic organisms are less clear.

Although homologs of the *E. coli tolB* (HP1126) and *pal* (HP1125) genes were annotated in the genome of the gastric pathogen, *Helicobacter pylori* (22), no homologs for *tolR*, *tolQ* or *tolA* were found (22), suggesting that there may be some differences with respect to the classical functions of the proteins encoded by the *tol-pal* cluster in *E. coli* (Fig.1). Moreover, the functional roles of TolB and Pal proteins in *H. pylori* cell wall biology are unknown. Therefore, the major aim of the current study was to investigate the roles of the TolB and Pal proteins in OMV formation in *H. pylori*, a bacterium lacking a conventional Tol-Pal system. A secondary aim of the work was to generate hyper-vesiculating *H. pylori* strains. As *H. pylori* OMVs were shown to be highly immunogenic (4, 23), it may be possible to use such strains to develop an OMV-based vaccine against infections caused by this bacterial pathogen.

Here we demonstrate that genetic disruption of either the *H. pylori tolB* or *pal* genes resulted in significant morphological changes in the mutant bacteria. Importantly, we found that the disruption of either of the *H. pylori tolB* or *pal* genes severely affected membrane integrity, as evidenced by >600- and 22-fold increases, respectively, in the amounts of OMVs released by the respective mutants. This work establishes for the first time the importance of

the Tol-Pal cluster in cell membrane integrity and OMV formation in the gastric pathogen, *H. pylori*.

Materials and Methods

Bacterial strains, plasmids, culture media and growth conditions

Bacterial strains used and generated in this study are listed in Table S1 in the supplemental material. *H. pylori* strains were routinely cultured on horse blood agar (HBA) base No. 2 (Oxoid, Hampshire, UK), supplemented with 8% horse blood, or in Brain Heart Infusion broth (BHI, Bacto™ Brain Heart Infusion (Becton Dickinson and Company, Sparks, USA) supplemented with 10% (v/v) fetal calf serum (FCS). Skirrow's selective supplement was added to both media, as described previously (24). Mutant *H. pylori* strains were cultured on HBA supplemented with apramycin (30 μg/ml; Sigma) or chloramphenicol (25 μg/ml; Sigma), as appropriate. *E. coli* strains were cultured on Luria-Bertani agar or in Luria-Bertani broth, supplemented with apramycin (30 μg/ml) or chloramphenicol (25 μg/ml) as necessary. *H. pylori* strains were passaged twice on HBA plates prior to inoculation into BHI liquid broth for 16 hours. Cells were harvested into BHI liquid broth and inocula standardized to an A_{600} of 0.05. To monitor bacterial growth over time, cultures were dispensed into individual flasks for incubation for 8 or 16 hours in a microaerobic environment at 37 °C with shaking at 120 rpm. Bacterial growth was followed by measuring absorbance at A_{600} at 0, 8 and 16 hours. Viable counts (CFU per ml) were determined on HBA plates after 3-5 days of incubation, as described previously (25). The average generation time over a 16 hour time period was calculated as described by Willey et al. (26).

Molecular Biology Techniques

Genomic and plasmid DNA were purified using the Purelink™ Genomic DNA mini kit (Life Technologies, Ca, USA) and Purelink™ HiPure Plasmid Filter Midiprep kit (Life Technologies, Germany), respectively. PCR products were directly purified using the Purelink® PCR Purification kit (Invitrogen™). Alternatively, PCR products were

electrophoresed using 1.2% (w/v) agarose (Amresco) gels and then purified using the PureLink® Quick Gel Extraction Kit (Invitrogen™). Sequences of the oligonucleotides used in the study are listed in Table S2 in the supplemental material. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as per standard procedures described by Sambrook *et al*. (27) using reagents purchased from New England Biolabs.

***H. pylori* mutagenesis**

DNA fragments were amplified from *H. pylori* 26695 genomic DNA using GoTaq® Flexi DNA polymerase (Promega, WI. USA) with the primers LT1 and LT2 for *tolB*, and LT1 and LT4 for *tolBpal*. PCR products were purified and ligated into pGEM® T-Easy (Promega) as per the manufacturer's instructions. DNA was amplified from the appropriate pGEM® T-Easy constructs by inverse PCR (Failsafe™ PCR System; Epicentre) using primers LT7 and LT8 for *tolB* and LT7 and LT10 for *tolBpal*, resulting in insertion of KpnI and BamHI restriction endonuclease sites at position 1191346 and 1191273, respectively, of the *tolB* sequence and position 1191346 and 1190439, respectively, of the *tolBpal* sequence. The gentamicin resistance cassette (Gm^R) was excised from the pUC1813*apra* vector (28) by restriction digestion using BamHI and KpnI (New England Biolabs). Purified Failsafe PCR products were digested with BamHI and KpnI and ligated to the Gm^R cassette. Plasmid DNA was transformed into rubidium chloride competent *E. coli* DH5α, generating plasmids pLTtolB and pLTtolBpal. The correct integration of the GmR cassette into the plasmids was confirmed by PCR and sequence analysis using the following primer pairs: for pLTtolB, LT2/LT6 and LT1/LT5; for pLTtolBpal, LT2/LT6 and LT3/LT5.

Isogenic *H. pylori* Δ*tolB* and Δ*tolBpal* mutants were generated by natural transformation (29) and selected on HBA plates containing apramycin (30 μg/ml) and glycine betaine (10mM; Sigma). Chromosomal integration of the cassette was confirmed by PCR and sequence analysis

of genomic DNA using the following primer pairs: (for *H. pylori* Δ*tolB*) LT2/LT6 and LT1/LT5; (for *H. pylori* Δ*tolBpal*) LT2/LT6 and LT3/LT5.

H. pylori Δ*pal* mutants were constructed using the Gateway® Cloning System (Invitrogen, CA, USA). The 5' (1190712-1190452) and 3' (1190440-1190173) regions of HP1125 were amplified from *H. pylori* 26695 using primer pairs LT11/LT12 and LT13/LT14, respectively. Primers GmB4rF and GmB3rR were used to amplify the Gm^R cassette from the pUC1813*apra* vector. Using BP recombination, the 5' region of HP1125, Gm^R cassette and 3' region of HP1125 were inserted into the entry clones pDONR (P1, P4), pDONR (P4r, P3r) and pDONR (P3, P2), respectively, and transformed into OneShot®Mach1™$T1^R$ cells. The correct integration of PCR amplicons into each of the plasmids was confirmed by sequence analysis using the M13F and M13R primer pair. Insert DNA from all three entry clones were recombined into the pDEST17 destination vector using LR Clonase® II enzyme mix and transformed into OneShot®Mach1™$T1^R$ cells. The plasmid was designated pDESTPal and correct insertion confirmed by sequence analysis using the T7 promoter and T7 terminator primer pair. All reagents, plasmids and bacterial strains were purchased from Life Technologies. pDESTPal plasmid was transformed into *E. coli* DH5α, plasmid DNA was purified and used to transform *H. pylori*. *H. pylori* Δ*pal* mutants were generated by natural transformation and selected on HBA plates containing apramycin (30 μg/ml) and 10 mM glycine betaine. Chromosomal integration of the cassette was confirmed by PCR and sequencing using primers LT3/LT4.

Construction of pJPF3

The 5' (L) and 3' (R) regions of *rdxA* were amplified from *H. pylori* SS1 using primers rdxA.1F/rdxa.1R and rdxa.rF/rdxArR, respectively. The CATgc cassette was amplified from pHel2 (30), using primers CATgc.F and CATgc.R. All three PCR reactions were performed

using Phusion® High-Fidelity DNA Polymerase (New England biolabs). Purified PCR amplicons and an oligonucleotide containing multiple cloning site (rdxA-MCS-CATgc) were joined by Splicing Overlap Extension (SOE)-PCR, using primers rdxA.1F and rdxA.rR and Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies™). The resulting rdxA(L)-MCS- CATgc-rdxA(R) fragment was purified by agarose gel electrophoresis and cloned into pGEMT-easy. This pGEMT-easy recombinant plasmid was designated pJPF3.

Complementation of *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants

Gene complementation was initially attempted using the strong *ureA H. pylori* promoter to drive gene expression (31). Despite numerous attempts, however, no constructs were obtained. We therefore altered our strategy by using the putative native promoters for both *tolB* and *pal* (Fig. 1), identified from the transcriptome studies of Sharma *et al.* (32). Thus, a region of HP1130 that was postulated to contain the native promoter for the *exbD*-HP1128-HP1127-*tolB* operon (32) was amplified from *H. pylori* 26695 using primers HP1130.F/HP1130.R. The *tolB* and *pal* genes, including the ribosome binding site of *tolB,* were amplified from *H. pylori* 26695 in a single fragment using primers HP1126.F/LT13R. PCR amplicons were joined by SOE-PCR, digested with BamHI and KpnI and ligated to BamHI/KpnI- digested pJPF3 vector to give rise to plasmid pJPFTolBPal+, which was used to complement *H. pylori* Δ*tolBpal.* pJPFTolBPal+ was used as a template to amplify the DNA fragment containing the *exbD* promoter region and *tolB*, using primers HP1130F/139.TolBR. The PCR amplicon was digested with BamHI and KpnI and ligated to the pJPF3 vector to generate pJPFTolB+. All the above PCRs were performed using Phusion® High-Fidelity DNA Polymerase.

Construction of a plasmid to complement *pal* involved the use of SOE-PCR to join a DNA fragment containing the *exbD* promoter region, amplified from *H. pylori* 26695 using primers HP1130F/141.HP1130R, to a DNA fragment carrying the intact *pal* gene, including its predicted promoter (32), amplified using primers 141.PalF/LT13R. This *HP1130-pal* DNA

fragment was digested with BamHI and KpnI, and inserted into the BamHI/KpnI site of pJPF3, to produce pJPFPal+.

H. pylori Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants were complemented by electroporation of the appropriate pJPF3 derivative, followed by selection on HBA plates containing chloramphenicol (25 μg/ml). The chloramphenicol resistant transformants were screened by PCR and sequence analysis to confirm the insertion of the *tol/pal*-Cm^R fragment into the *rdxA* locus (33). The following primer pairs were used: for Δ*tolB* (*tolB*+), LT1/LT15 and LT2/HP0953.R; for Δ*pal* (*pal*+), LT15/LT3 and LT4/HP0953.R; for Δ*tolBpal* (*tolBpal*+), LT1/LT15 and LT4/HP0953.R.

Electroporation of *H. pylori*.

Briefly, *H. pylori* bacteria cultured for 24 hours on HBA plates were washed twice in ice-cold 15% (v/v) glycerol/9% (v/v) sucrose solution and resuspended in the glycerol/sucrose solution. 500ng plasmid DNA, freshly dialyzed against H_2O, was added to 50 μl aliquots of the *H. pylori* cell suspensions. DNA/cell mixtures were transferred to pre-chilled 0.2 cm cuvettes (BioRad, Australia) and pulsed in a Gene Pulser Xcell™ (BioRad, Australia) (34) set at 25 μF, 2.5 kV and 200 Ω with a pulse time of 6-7 milliseconds. Transformants were recovered on HBA for 16 hours at 37 °C and then selected using HBA supplemented with chloramphenicol (25 μg/ml) and incubated a further 5 days at 37 °C.

RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR)

H. pylori 251 and isogenic mutants were grown until log phase of growth (16 hours) and RNA was purified from bacteria using the Purelink RNA mini kit (Invitrogen), as per manufacturer's instructions for bacterial RNA isolation. To remove contaminating DNA, RNA was Deoxyribonuclease (DNAse) treated using the Turbo DNA free kit (Ambion, VIC, Australia). RNA (500 ng) was reverse transcribed using SuperScript III and random hexamers

(Invitrogen). Primers for the genes encoding the proteins Pal (qHP1125 F5 and qHP1125 R5), TolB (qHP1126 F3 and qHP1126 R3) and UvrC (uvrC F and uvrcR) are listed in table S2. Reaction mixes (final volume 10 μl) consisted of 20 ng cDNA, 5 μl SYBR Green buffer and 0.1 μM each primer. qRT-PCR gene expression analyses were performed in triplicate using a 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative changes in gene expression were determined using the ΔΔCt method (35) and normalised to the *uvrC* gene (36). Data acquisition and analyses were performed using the Sequence Detection System v2.3 software (Applied Biosystems).

Production of OMVs

Bacteria were cultured in BHI supplemented with 0.6 % (w/v) β-cyclodextrin (Sigma-Aldrich, MO, USA) with shaking at 120 rpm for 17 hours at 37°C under micro aerobic conditions. OMVs were obtained from cultures as described previously (4).

Cell co-culture assays

Human gastric adenocarcinoma (AGS) cells were routinely cultured using RPMI supplemented with 10% (v/v) FCS. Cells were seeded at a density of 1 x 10^5 cells per ml in 12 well plates (Becton Dickinson Labware, NY, USA) (37). Media was replaced with serum free media for 12 hours prior to stimulation with either *H. pylori* (multiplicity of infection (MOI) = 10:1) or OMVs (50 μg per ml), for 24 hours. IL-8 levels in the supernatants were determined using the BD OptEIA human IL-8 ELISA kit according to the manufacturer's instructions.

Quantification of OMV production

H. pylori mutant and wild-type strains were grown in 10 ml cultures of BHI supplemented with 10% FCS, with shaking at 120 rpm for 17 hours at 37°C under micro aerobic conditions. Bacterial free supernatants were filtered using 0.22 μm PES filters (Corning Incorporated, NY, USA). OMVs were washed with 30 mL PBS and concentrated to a final volume of 800 μl

using Amicon YM-10 columns (Millipore Ireland Ltd., Cork, Ireland). Ninety-six well plates (Nunc Maxisorb) were coated overnight at 4°C with either OMVs that had been serially diluted in coating buffer (0.1M $NaHCO_3$, 0.1M Na_2CO_3 (BDH Chemicals Australia)), or a standard curve of known concentrations of *H. pylori* 251 OMVs. Plates were blocked using 5% skim milk (w/v) in PBS and OMVs were detected using rabbit anti-*H. pylori* OMV antibodies (diluted 1/8000) (4), goat anti-Rabbit IgG (H&L) (1/2000; Chemicon International, CA, USA) and streptavidin HRP conjugate (1/2000; BD Biosciences, CA, USA). ELISAs were developed using BD OptEIA TMB substrate reagent (BD Biosciences) and read on a FLUOstar Optima micro plate reader (BMG Labtech).

Scanning Electron Microscopy (SEM)

H. pylori strains grown overnight in BHI broth were pelleted at 1000 x g for 10 minutes at 4°C. Bacteria were resuspended in 100 μl 4% glutaraldehyde and 8 % paraformaldehyde in 0.1 M sodium cacodylate buffer using wide bore tips. Bacteria were allowed to adhere to Therminox 12mm cover slips for 2 hours at room temperature and then 4°C overnight. Samples were washed twice with 0.1 M sodium cacodylate and dehydrated with 50%, 70%, 90%, 95% and 100% ethanol for 5 minutes each. Samples were then dehydrated 3 times with 100% absolute ethanol for 10 minutes. Samples were further dehydrated with Ethanol: HMDS solution at 2:1, 1:1 and 1:2 for 5 minutes each, prior to two washes with undiluted HMDS. After drying, samples were mounted in stubs and spatter coated with gold palladium. All reagents were purchased from Positech (USA). Images were taken with a Hitachi S-570 with a lab6 filament and images captured using Spectrum software.

Transmission electron microscopy (TEM)

OMVs from each strain were mixed 1:1 with 4% (w/v) paraformaldehyde, adhered to Formvar coated carbon grids and contrasted with 4% (w/v) uranyl-acetate pH 4.0 in 0.1 M oxalic acid. Samples were embedded in a solution of 9 parts 4% (v/v) uranyl acetate and 1 part 2 % (v/v)

methylcellulose. All reagents were from Positech (USA). Samples were viewed using a Hitachi H.7-7500 transmission electron microscope at 70K x view and images captured using Digital micrograph™ 1.71.38 (Gatan Inc.). Image analysis was performed using ImageJ v1.47k (National Institutes of Health, USA).

Proteomic analyses

OMV (20 μg) samples prepared in PBS with protease inhibitors (Complete Protease Inhibitor cocktail tablets; Roche) were reduced in 2.5 nM DTT at 60°C for 30 minutes. This was followed by alkylation with 10 mM iodacetamide for 30 minutes and digestion with 0.5 μg trypsin (Promega corp., Madison, WI, USA) in 20 mM ammonium bicarbonate at 37°C overnight. Tryptic digests were analyzed by LC-MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with an RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Samples were injected onto a PepMap100 2cm trap column with loading buffer (2% acetonitrile, 0.1% formic acid) at a flow rate of 15μl per minute. Peptides were eluted and separated with a Thermo RSLC PepMap100, 75μm id, 100Å pore size, reverse phased nano column with a 30 minute gradient of 90% buffer A (0.01% formic acid) to 30% buffer B (80% acetonitrile, 0.1% formic acid) in 20 minutes and then to 40% buffer B, to a complete 30 minute gradient, at a flow rate of 300 nl/minute. The eluant was nebulized and ionized using a Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900V. Peptides were selected for MS/MS analysis in Full MS/dd-MS_2 (TopN) mode with the following parameter settings: TopN 10, resolution 17500, MSMS AGC target 1e5, 60ms Max IT, NCE 27 and 3 m/z isolation window. Under fill ratio was at 10% and dynamic exclusion was set to 15 seconds. Data was processed using Proteome Discoverer V1.4 (Thermo Fisher Scientific) and searched against a customized database downloaded from the National Centre for Biotechnology Information (NCBI) ftp site using the MS Amanda search engine. The

following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 15 ppm; peptide fragment tolerance, ± 0.2 Da; peptide charge, 2+, 3+ and 4+; static modifications, carbamidomethyl; Dynamic modification, oxidation (Met). Low and medium confidence peptides were filtered with at least 0.02 FDR (high confidence).

Statistical analyses

All analyses were performed using Graphpad Prism version 6.0c. ELISA experiments and OMV size experiments were analyzed by Student's t-test. Error bars indicate the means $\pm$ standard deviation (SD). Differences were considered significant when *P<0.05.

Results

The *tol-pal* cluster in *H. pylori*

The *tol-pal* system in *E. coli* is comprised of two operons, *orf1 tolQRA* and *orf2 tolB pal* (13, 38) (Fig. 1A). A putative *tol-pal* cluster has been identified in *H. pylori* (13, 22, 39), however, it seems to be organized differently to that of *E. coli* and other Gram-negative bacteria (Fig. 1B). Indeed, although *H. pylori* homologs of *E. coli tolB* and *pal* were identified (*HP1126* and *HP1125*, respectively), an *H. pylori* homolog of *tolA* was not found (13, 22, 39). Furthermore, in place of *tolA*, *H. pylori* possesses two coding regions (*HP1127* and *HP1128*), with no known homologs in the databases. The *H. pylori tol-pal* cluster also contains two coding sequences (*HP1130* and *HP1129*) that were originally annotated as *exbB* and *exbD*, but which have since been suggested to be homologs of *tolQ* and *tolR*, respectively (13, 22, 39) (Fig. 1B). The *H. pylori tol-pal* gene cluster is also very different to that of *E. coli* at the transcriptional level (40), comprising three transcriptional units: *HP1137-HP1130*, containing *exbB* (*HP1130*); *HP1129–HP1126*, containing *exbD* (*HP1129*) and *tolB* (*HP1126*); HP1125-HP1123, containing *pal* (*HP1125*) (Fig. 1B). The presence of three transcriptional units is based on the data from a genome-wide transcriptomic analysis of *H. pylori* by Sharma *et al.* (32).

At the protein level, BLAST analyses revealed the deduced sequences of *H. pylori* TolB (HP1126) and Pal (HP1125) proteins to share 19% and 38% identity, respectively, with *E. coli* TolB and Pal (Fig. 1C). *H. pylori* ExbB and ExbD share 43% and 29% identity with *E. coli* TolQ and TolR, respectively. Finally, *H. pylori* HP1124 was shown to share 28% identity with another component of the *E. coli* Tol-Pal system, YbgF, which is known to interact with TolA in the bacterial periplasm (41, 42) (Fig. 1C). Taken together, the *H. pylori tol-pal* cluster differs from the canonical clusters found in the Gram-negative bacteria belonging to the gamma class of proteobacteria, such as *E. coli* and *Pseudomonas aeruginosa*. In order to gain

the first information on the Tol-Pal system of *H. pylori, an epsilonproteobacteria,,* we chose to focus our study on HP1126 and HP1125. These proteins were unambiguously annotated as counterparts of *E. coli* TolB and Pal, respectively, two proteins shown to be important for *E. coli* membrane integrity and OMV formation (10-12). To this end, we constructed *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants, as well as strains in which each of the defects had been repaired by complementation.

***H. pylori tolB-pal* mutants exhibit similar growth to the wild-type strain**

Given that *tolB-pal* mutations have been shown to result in lower growth rates in some Gram-negative bacteria (18), we first examined the growth in broth culture medium of the *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants compared with the isogenic wild-type strain. The *H. pylori* wild-type, Δ*tolB* and Δ*pal* mutants all grew at similar rates (Fig. 2A). *H. pylori* Δ*tolB* and Δ*tolB* (*tolB*+) bacteria started at lower bacterial densities than the other strains, but exhibited similar increases in growth and doubling times to those of the wild-type strain (data not shown). This is likely due to the extreme clumping nature of the Δ*tolB* and Δ*tolB* (*tolB*+) strains (data not shown), making the correct enumeration of cells within the starting inoculum very difficult. Nevertheless, the mean generation time measured over 16 hours was similar for all strains, and no significant differences were observed, indicating that increases in measured growth over 16 hours are similar for all strains (Fig. 2B). In contrast to other Gram-negative bacteria, the loss of functional TolB and/or Pal does not significantly affect the growth of *H. pylori.*

***H. pylori* Δ*tolB-pal* mutants exhibit morphological defects**

Previous work has demonstrated that the mutation of Tol-Pal proteins in Gram-negative bacteria results in profound morphological alterations (17, 18). Consistent with this observation, the *H. pylori* Δ*tol-pal* mutants each displayed morphological defects when

compared with the characteristic spiral morphology and flagellated forms of wild-type *H. pylori* bacteria (Fig. 3A). Specifically, the Δ*tolB* mutant (Fig. 3B) was slightly shorter than the wild-type and lacked fully formed flagella. In addition, the membrane integrity was severely disrupted, with the cells exhibiting extensive blebbing (Fig. 3). The Δ*pal* mutant (Fig. 3D) similarly appeared shorter and more rod-like in shape, when compared with the wild-type, but unlike Δ*tolB* bacteria, had multiple flagella. Interestingly, the Δ*tolBpal* double mutant (Fig. 3F) appeared similar in morphology to the wild-type, showing the characteristic spiral shape, however, no flagella were apparent and the bacteria appeared to have a cell division defect (Fig. 3). Δ*tolB*(*tolB*+), Δ*pal*(*pal*+), and Δ*tolBpal*(*tolBpal*+) complemented mutants displayed a spiral morphology, similar to that of the wild-type strain, however, all appeared to be lacking flagella (Fig. 3C, E, G). Thus, disruption of *tolB* and/or *pal* in *H. pylori* was shown to result in aberrant cell morphology and/or flagella biosynthesis consistent with perturbations in cell wall integrity.

***H. pylori* Δ*tolB* and Δ*pal* bacteria produce more OMVs than the wild-type strain**

We next determined whether the morphological changes observed in the *H. pylori* Δ*tolB-pal* mutants resulted in increased outer membrane vesiculation, by assessing the levels of OMV production in each strain using an ELISA-based method (Fig. 4). In agreement with the SEM data (Fig. 3B. C), the Δ*tolB* and Δ*pal* mutants produced > 600-fold (317 pg/CFU) and 22-fold (11 pg/CFU) more OMVs, respectively, than the wild-type strain (0.5 pg/CFU) (Fig. 4; $P < 0.001$ and $P < 0.005$, respectively). In contrast, the amounts of OMVs produced by the Δ*tolBpal* mutant were not significantly different to those of the wild-type bacteria, a finding also consistent with the more normal looking morphology of the mutant (Fig. 3G). Complementation of the Δ*pal,* but not the Δ*tolB* mutant, restored OMV production to wild-type

levels (Fig. 4). Taken together, these data demonstrate that disruption of *H. pylori tolB* or *pal* results in hyper-vesiculating bacteria.

The protein content of *H. pylori* Δ*tolB-pal* OMVs is very similar to that of wild-type bacteria.

In order to gain more information regarding the OMVs from *H. pylori* Δ*tolB-pal* mutants, we performed LC-MS/MS analyses on the OMVs derived from wild-type and **Δ***tol-pal* mutant strains. From this work, it was possible to unambiguously identify 47 proteins as being present in the OMVs purified from the wild-type and each of the mutant strains (Table 1; see also Table S3 in the supplemental material). Many of these proteins were either outer-membrane or hypothetical proteins. Amongst this group of proteins were the BabA/B and SabA adhesins, an OMV-associated vaccine candidate (Lpp20 (23)), virulence factors (HpaA, carbonic anhydrase, HtrA and CagD), as well as enzymes involved in disulfide bond breakage or formation (disulfide isomerase, DsbC) .

TolB was only detected within OMVs produced by the wild-type strain, Δ*pal* (*pal*+) and Δ*tolB* (*tolB*+) bacteria (Table S3 in the supplemental material). The absence of TolB from Δ*pal* OMVs is consistent with the fact that the interaction of this periplasmic protein with the outer membrane is dependent upon interactions with Pal (43). Pal was only detected in the *Δpal* (*pal*+) OMVs, but not in the OMVs produced by any of the other strains, including wild-type bacteria (Table S3 in the supplemental material). We speculate that Pal may be present in small amounts and thus could only be detected when over-expressed in the *H. pylori pal* (*pal*+) bacteria.

Interestingly, eight proteins were identified as being absent from the OMVs purified from *H. pylori* **Δ***pal* and **Δ***tolBpal* bacteria, and three of these proteins were also absent in OMVs of **Δ***tolB* mutant (Table 2). Of particular note, cytochrome c551 peroxidase was absent from

OMVs purified from all mutants lacking a functional Pal, whereas a plasminogen-binding protein (PgbB) was absent from those isolated from bacteria lacking both Tol and Pal. Both of these proteins were detected in the OMVs from the Δ*tolBpal* (*tolBpal*+) bacteria. As *H. pylori* was previously shown to release OMVs of varying sizes that also differed in their protein compositions (4, 44), we examined by TEM the OMVs purified from the wild-type and mutant strains. These were found to display similar sizes, with the majority (60% and 70%, respectively) of the OMVs being in the size range of 50-75 nm (Supplemental Fig. 1). Taken together, the data show that despite obvious defects in the membrane integrity of the *H. pylori* Δ*tol-pal* mutants, the OMVs derived from these bacteria varied only slightly in their protein composition, with respect to those of wild-type bacteria.

OMVs derived from *H. pylori* Δ*tolB* and Δ*pal* mutants induce higher levels of IL-8 production from host cells than wild-type OMVs.

Finally, as *H. pylori* OMVs are known to induce the production of IL-8 from human gastric epithelial cells (4, 45), we examined the immunostimulatory capabilities of OMVs derived from Δ*tol-pal* mutants. Interestingly, we found that OMVs derived from each of the mutant strains induced significantly higher IL-8 production from epithelial cells than the wild-type OMVs (Fig. 5. * $P < 0.05$), indicating that OMVs produced by the mutant strains are more potent in inducing pro-inflammatory signaling in epithelial cells than those produced by wild-type bacteria. OMVs produced by each of the complemented mutant strains induced similar levels of IL-8 as did the wild-type, confirming that the OMVs of Δ*tol-pal* mutants have more potent immunostimulatory properties than those of wild-type bacteria.

Discussion

The *tol-pal* gene cluster has been thoroughly characterized in *E. coli* (10, 11, 15, 38, 46-49), with loss of function shown to result in hyper-vesiculating bacteria. Furthermore, *tol-pal* genes have been demonstrated to play important roles in the maintenance of membrane integrity for a range of other Gram-negative bacteria (17, 18, 50). To date, however, there have been no functional studies regarding the role of *tol-pal* genes in *H. pylori*. As *tolB* and *pal* mutations in *E. coli* are known to alter cell wall integrity (10), we sought to disrupt the *tolB* and *pal* homologs in *H. pylori*, and in so doing, create hyper-vesiculating mutants of this bacterial pathogen.

H. pylori Δ*tolB* mutants displayed extensive "blebbing", had very few flagella, which were markedly shorter in length (Fig. 3B), and appeared to form clumps (data not shown), suggesting a division defect. Similar phenotypes have been observed in Δ*tolB-pal* mutants in the bacterial species, *Er. chrysanthemi* (18) and *C. crescentus* (17). In contrast to the Δ*tolB* bacteria, the *H. pylori* Δ*pal* mutants appeared slightly less spiral than their wild-type counterparts, with smooth membranes and multiple flagella (Fig. 3D). Surprisingly, the morphology of *H. pylori* Δ*tolBpal* mutant was very similar to that of wild-type bacteria, the only detectable difference being a complete lack of flagella (Fig. 3F). Although these findings cannot be fully explained, it is possible that the loss of both proteins in the Δ*tolBpal* mutant strain caused compensatory mutations in genes encoding factors involved in bacterial cell wall integrity.

Gene complementation of the *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants restored wild-type morphology, except for the lack of flagella. Peptidoglycan-associated outer membrane proteins, including those of the Pal family, share sequence homology in the C-terminal region with the *E. coli* motility protein, MotB (51). This region of MotB is important in anchoring the flagella structure to the peptidoglycan. It is therefore possible that any disruption of either the

TolB or Pal proteins may affect the functioning of the Tol-Pal complex as a whole, thereby altering the synthesis of the flagella or hindering the anchoring of the flagella to the peptidoglycan layer. As the stoichiometry of the Tol-Pal complex is essential for its proper functioning (10), it was suggested that even minor over-expression of the Tol-Pal proteins could be deleterious to the bacteria (50). Gene complementation of Δ*tolB* did not restore OMV production back to wild type levels. Analysis of gene expression of *pal* and *tolB* by this strain revealed that whilst expression of *tolB* is restored following gene complementation (Supplemental Fig 2A, expression of pal is not (Supplemental Fig 2B). This data further supports the notion that the stoichiometry of the TolBPal complex is essential for functional activity.

Importantly, we demonstrated that the *H. pylori* Δ*tolB* and Δ*pal* mutants produced >600-fold and 22-fold more OMVs than wild-type bacteria (Fig. 4), respectively, thus demonstrating a significant increase in vesiculation in these mutant strains. The very large increase in vesiculation for the *H. pylori* Δ*tolB* mutant is consistent with its rough membrane appearance (Fig. 3). Conversely, the *H. pylori* Δ*tolBpal* mutant strain produced similar quantities of OMVs as wild-type organisms, a finding in line with its normal morphology (Fig. 3B).

Proteomic analyses of the OMVs produced by each of the Δ*tolB-pal* mutant strains revealed only minor differences in the proteomic make-up of the OMVs produced by each strain (Table 1). Indeed, a large number of outer membrane and hypothetical proteins were common to all OMVs analyzed. Importantly, a known OMV-associated protein, Lpp20, which had previously been identified as a vaccine candidate (23, 52), was detected in OMVs from all strains tested. Interestingly, eight proteins were detected in the OMVs from wild-type bacteria but not in those from one or more of the *H. pylori* mutant strains, and only some of these proteins were detected in the complemented mutants (Table 2). It is possible that notwithstanding the genetic reconstitution of *tolB* and *pal* genes, the altered expression levels of *tolB* and *pal* in the

complemented mutants may have resulted in functional TolB-Pal complexes not being completely restored in the cell membranes of these strains.

Importantly, despite the similarities in protein composition, the OMVs from *H. pylori ΔtolB-pal* mutant strains induced significantly greater IL-8 responses in gastric epithelial cells than wild-type OMVs (Fig. 5). This was not entirely surprising, as our previous work showed that cell wall peptidoglycan, and not proteins, is important for the ability of *H. pylori* OMVs to induce IL-8 responses in these cells (4). Indeed, we previously demonstrated that OMVs trigger signaling through the cytoplasmic pathogen-recognition molecule, NOD1, which specifically senses Gram-negative peptidoglycan and not proteins (4). It is therefore plausible that disruption of membrane integrity in the *ΔtolB-pal* mutant strains results in OMVs in which peptidoglycan is rendered more accessible to detection by NOD1.

In conclusion, this work is the first to functionally characterize the Tol-Pal system in *H. pylori*. Specifically, we showed that *tolB* and *pal* mutations in *H. pylori* conferred profound morphological changes in cell shape and membrane integrity, resulting in hyper-vesiculating bacteria. Moreover, despite minor differences in protein content, the OMVs from these mutants had greater immunostimulatory properties than those produced by wild-type bacteria. Thus, we suggest that the OMVs from *H. pylori ΔtolB-pal* mutants may serve as useful tools for the development of vaccine candidates against *H. pylori* infection.

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Figure Legends

Fig. 1. The *H. pylori tol-pal* gene cluster. (A) Organization of the *E. coli* K12 subst. MG1655 *tol-pal* gene cluster. The two operons are shown in white and grey. (B) Predicted organization of the *H. pylori* strain 26695 *tol-pal* gene cluster and surrounding genes. The three predicted transcription units are shown in black, white and grey. Arrows indicate the direction of transcription and predicted putative transcription start sites. (C) Protein length (amino acids) and % identity shared between the *E. coli* and putative *H. pylori* Tol-Pal proteins.

Fig. 2: Growth of wild type *H. pylori* 251 and isogenic mutants. *H. pylori* from overnight cultures were inoculated into liquid culture and the starting OD adjusted to an A_{600} of 0.05. Values are the means pooled from three independent experiments and error bars represent the standard deviation (SD).

Fig. 3. *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants exhibit distinct morphologies compared with the isogenic wild-type bacteria. Bacteria were grown for 16 hours and their morphology examined by SEM.

Fig. 4. *H. pylori* Δ*tolB* and Δ*pal* mutants produce significantly more OMVs than the isogenic wild-type strain. The amounts of OMV production (in pg per CFU) by wild-type (WT), mutant (open bars) and complemented (striped bars) strains were determined by ELISA. Values are the means pooled from three independent experiments (determined in triplicate) and error bars indicate the standard deviation (SD). ***, $P<0.005$, **, $P<0.001$ compared with the wild-type.

Fig. 5. OMVs produced by *H. pylori* Δ*tolB-pal* mutants induced higher levels of IL-8 production in host cells than wild-type OMVs. AGS epithelial cells were co-cultured with OMVs derived from wild-type *H. pylori* (grey bar), Δ*tolB*, Δ*pal* or Δ*tolBpal* mutants (white bars) or complemented strains (striped bars). Values are representative of the means of three

individual experiments (determined in triplicate) and error bars indicate the standard deviation (SD). *, $P<0.05$ compared with the wild type

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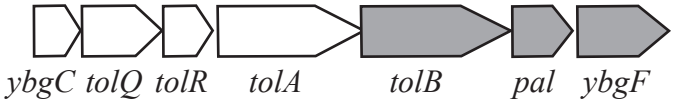
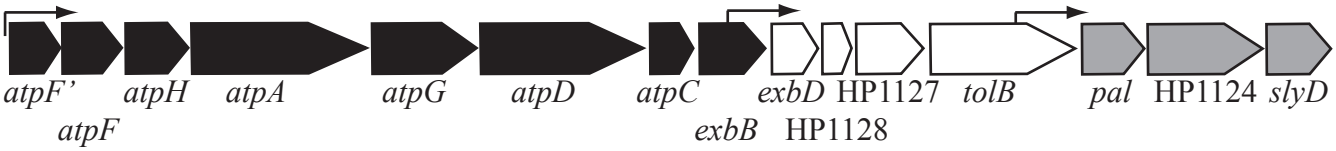
A



B

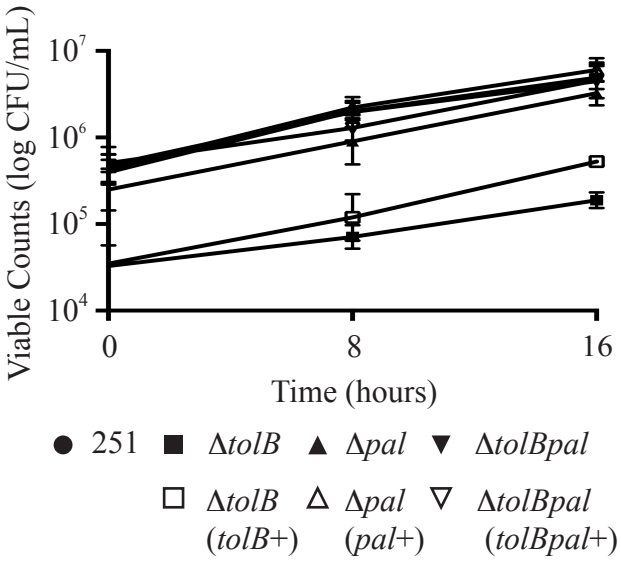


C

	TolQ	TolR	TolA	TolB	Pal	YbgF
E. coli K12 (no. amino acids)	230	142	421	430	173	263
H. pylori 26695 (no. amino acids)	189	133	-	417	179	331
% Identity	43	29	-	19	38	28

FIG 1. The *H. pylori tol-pal* gene cluster. (A) Organization of the *E. coli* K12 subst. MG1655 *tol-pal* gene cluster. The two operons are shown in white and grey. (B) Predicted organization of the *H. pylori* strain 26695 tol-pal gene cluster and surrounding genes. The three predicted transcription units are shown in black, white and grey. Arrows indicate the direction of transcription and predicted putative transcription start sites. (C) Protein length (amino acids) and % identity shared between the *E. coli* and putative *H. pylori* Tol-Pal proteins.

A



B

Strain	251	Δ*tolB*	Δ*tolB* (*tolB*+)	Δ*pal*	Δ*pal* (*pal*+)	Δ*tolBpal*	Δ*tolBpal* (*tolBpal*+)
G (hours)	2.35 +/- 0.08	2.28 +/- 0.07	2.35 +/- 0.18	2.38 +/- 0.17	2.35 +/- 0.09	2.52 +/- 0.25	2.46 +/- 0.26

FIG 2. Growth of wild type *H. pylori* 251 and isogenic mutants. *H. pylori* from overnight cultures were inoculated into liquid culture and the starting OD adjusted to an A_{600} of 0.05 (A). Values are the means pooled from three independent experiments and error bars represent the standard deviation (SD). Generation times (G, hours) of wild type *H. pylori* 251 and isogenic mutants was calculated at intervals of 8 hours and the results from 3 independent experiments pooled to give the mean generation time +/- the SD (B).

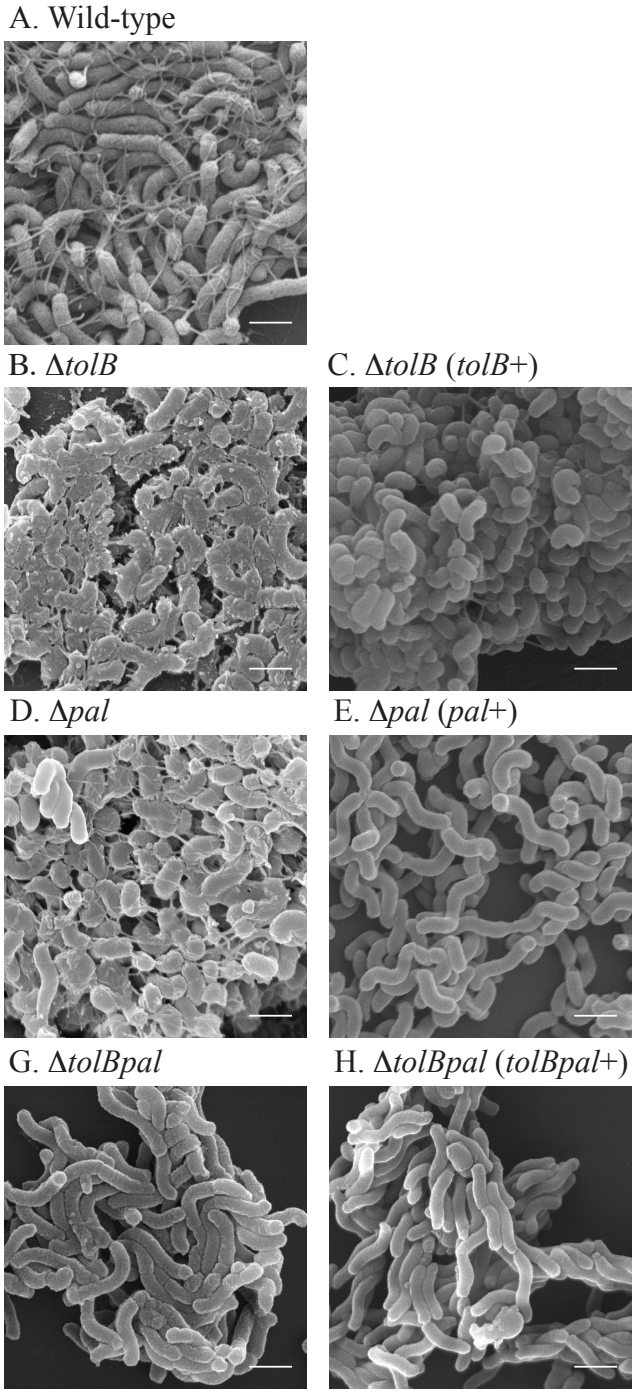


FIG 3. *H. pylori* Δ*tolB*, Δ*pal* and Δ*tolBpal* mutants exhibit distinct morpholgies compared with the isogenic wild-type bacteria. Bacteria were grown for 16 hours and their morphology examined by scanning electron microscopy. Images are representative of 3 independent experiments. Scale bar represents 1 μm.

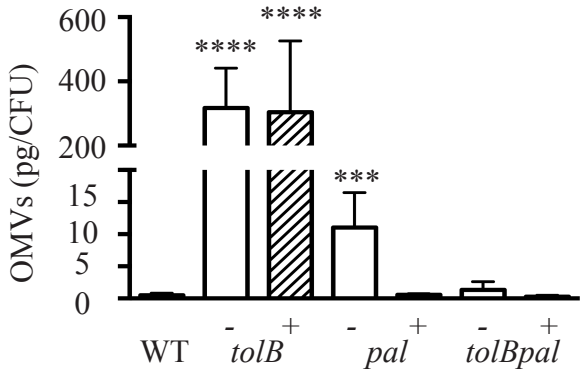


FIG 4. *H. pylori* Δ*tolB* and Δ*pal* mutants produce significantly more OMVs than the isogenic wild-type strain. The amounts of OMV production (in pg per CFU) by wild-type (WT), mutant (open bars; -) and complemented (striped bars; +) strains were determined by ELISA. Values are the means pooled from three independent experiments (determined in triplicate) and error bars indicate the standard deviation (SD). ****, P<0.0001, ***, P<0.001 compared with the wild-type.

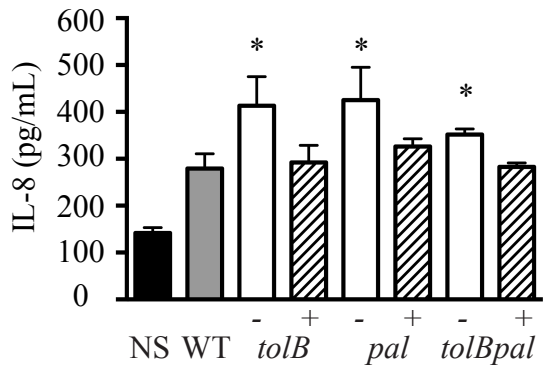


FIG 5. OMVs produced by *H. pylori* Δ*tolB-pal* mutants induced higher levels of IL-8 production in host cells than wild-type OMVs. AGS epithelial cells were co-cultured with OMVs derived from wild-type *H. pylori* (grey bar), Δ*tolB*, Δ*pal* or Δ*tolBpal* mutants (open bars; -) or complemented strains (striped bars; +). Values are representative of the means of three individual experiments (determined in triplicate) and error bars indicate the standard deviation (SD).
*, $P<0.05$ compared with the wild type

Table 1. Proteins common to wild type and isogenic mutant strains

Protein name	Gene no.
Outer membrane protein HopM (omp5)	HP0227
Outer membrane protein HopA (omp6)	HP0229
Outer membrane protein HopO (omp16)	HP0722
Outer membrane protein SabA, HopP (omp17)	HP0725
Outer membrane protein BabB, HopT (omp19)	HP0896
Outer membrane protein AlpA, HopC (omp20)	HP0912
Outer membrane protein AlpB, HopB (omp21)	HP0913
Outer membrane protein HopQ (omp27)	HP1177
Outer membrane protein BabA, HopS (omp28)	HP1243
Outer membrane protein HorK (omp32)	HP1501
Outer membrane protein HomA	HP0710
Outer membrane protein HomD	HP1453
Membrane-associated lipoprotein (lpp20)	HP1456
Membrane fusion protein of the HefABC efflux system (mtrC)	HP0606
Putative beta-lactamase HcpC	JHP1024
Flagellar sheath adhesin hpaA	HP0797
Neuraminyllactose-binding hemagglutinin homolog (hpaA)	HP0410
Carbonic anhydrase	HP1186
Catalase-like protein	HP0485
Iron(III) ABC transporter periplasmic iron-binding protein (ceuE)	HP1561
Disulphide isomerase	HP0231
Bifunctional methionine sulfoxide reductase A/B protein	HP0224
Thiol:disulfide interchange protein (dsbC),	HP0377
Serine protease (htrA)	HP1019
Thioredoxin	HP1458
Processing protease (ymxG)	HP0657
Protease (pqqE)	HP1012
Tumor necrosis factor alpha-inducing protein	HP0596
Conserved Hypothetical secreted protein	HP1286
Cag pathogenicity island protein CagD (cag24)	HP0545
Put. neuraminyllactose-binding hemagglutinin homolog	HP0492
Plasminogen binding protein PgbA	HP0508
Plasminogen-binding protein (PgbA)	JHP0458
Hypothetical protein	HP1454
Hypothetical protein	HP0408
Hypothetical protein	HP1173
Hypothetical protein	HP0129
Hypothetical protein	HP0721
Hypothetical protein	HP0953
Hypothetical protein	HP0203
Hypothetical protein	HP0719
Hypothetical protein	HP0232
Hypothetical protein	HP1524
Hypothetical protein	HP0130
Hypothetical protein	HP1098
Hypothetical protein	HP0783
Hypothetical protein	HP1285

Table 2. Proteins detected in OMVs derived from the wild type strain but missing from OMVs derived from at least one isogenic mutant strain.

Description		251	*tolB*	*tolB* (*tolB*+)	*pal*	*pal* (*pal*+)	*tolBpal*	*tolBpal* (*tolBpal*+)
outer membrane protein HopZ (omp1)	HP0009							
outer membrane protein HopE (omp15)	HP0706							
outer membrane protein HofC	HP0486							
outer membrane protein HofG	HP0914							
outer-membrane protein of the HefABC efflux system	HP0605							
iron-regulated outer membrane protein (frpB)	HP1512							
beta-lactamase HcpA	HP0211							
rare lipoprotein A (rlpA)	HP1571							
flagellar hook-basal body protein FliE	HP1557							
flagellin B	HP0115							
flagellar capping protein (FliD)	HP0752							
flagellar hook-associated protein FlgL	HP0295							
flagellar protein FlaG	HP0751							
flagellar basal body rod modification protein	HP0907							
secreted protein involved in flagellar motility	HP1462							
flagellar hook-associated protein FlgK	HP1119							
flavodoxin FldA	HP1161							
oligopeptide ABC transporter periplasmic oligopeptide-binding protein (oppA)	HP1252							
urease subunit alpha (UreA)	HP0073							
amino acid ABC transporter periplasmic binding protein (yckK)	HP0940							
L-asparaginase II	HP0723							

Description		251	*tolB*	*tolB* (*tolB*+)	*pal*	*pal* (*pal*+)	*tolBpal*	*tolBpal* (*tolBpal*+)
cytochrome c551 peroxidase	HP1461							
ATP-dependent nuclease (addB)	HP0275							
alkyl hydroperoxide reductase (tsaA)	HP1563							
vacuolating cytotoxin	HP0887							
elongation factor Tu	HP1205							
translocation protein TolB	HP1126							
comH	HP1527							
thioredoxin (trxA)	HP0824							
hypothetical secreted protein	HP1117							
plasminogen-binding protein (PgbB)	JHP079							
hypothetical protein	HP0135							
hypothetical protein	HP0367							
hypothetical protein	HP0204							
hypothetical protein	HP0720							
hypothetical protein	HP0057							
hypothetical protein	HP0973							
hypothetical protein	HP0906							
hypothetical protein	HP1233							
hypothetical protein	HP1463							
hypothetical protein	HP1455							
hypothetical protein	HP0211							
hypothetical protein	HP0836							
hypothetical protein	HP0863							

* gray shading indicates protein not detected

Table S1 Bacterial strains and plasmids used in this study

H. pylori strain	Antibiotic resistance	Reference
251	Metronidazole	(33)
251 Δ *tolB*	Gentamicin, metronidazole	This study
251 Δ *pal*	Gentamicin, metronidazole	This study
251 Δ *tolBpal*	Gentamicin, metronidazole	This study
251 *tolB* (*tolB*+)	Metronidazole, gentamicin, chloramphenicol	This study
251 *pal* (*pal*+)	Metronidazole, gentamicin, chloramphenicol	This study
251 *tolBpal* (*tolBpal*+)	Metronidazole, gentamicin, chloramphenicol	This study

Plasmids	Description	Reference
pGEM T-Easy	Cloning vector	Promega
pUC1813*apra*	Contains GmR cassette	(26)
pLTtolB	TolB iterrupted by GmR	This study
pLTtolBpal	TolBPal interupted by GmR	This study
pDONR (P1, P4)	Gateway cloning vector	Life Technologies
pDONR (P4r, P3r)	Gateway cloning vector	Life Technologies
pDONR (P3, P2)	Gateway cloning vector	Life Technologies
pDEST17	Gateway cloning vector	Life Technologies
pDESTPal	Pal interrupted by GmR	This study
pHel2	Source of CmR	(28)
pJPF3	pGEM T-Easy, CmR, N-terminal and C-terminal regions of *H. pylori* RdxA. MCS	This study
pJPFTolBPal+	CmR, HP1130 promoter, TolBpal	This study
pJPFTolB+	CmR, HP1130 promoter, TolB	This study
pJPFPal+	CmR, HP1120 promoter, HP1126 promoter, Pal	This study

TABLE S2 PCR Primers used in this study

Primer	Primer Sequence 5' - 3'
LT1	AGCCCCATGGCGTATTCTTG
LT2	ATCTCGTAGCGCTTGTGAGC
LT3	GCGCATTTGGGTTTGGTTTC
LT4	CGATGTGAGTGCTAAAACGG
LT5	GACACGATGCCAACACGACG
LT6	AGGGCCTCGATCAGTCCAAG
LT7	CGGGATCCACCGGATGTGTATTTGTATG
LT8	GAGGTACCCTTGAGAGCTAGCGATATTC
LT9	CGGGATCCAGCTTTAGATGAGATCGTGC
LT10	GAUCGGATTCTTTGATTTCATAC
LT11	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGAGATCTTCTGTATTTAG
LT12	GGGGACAACTTTGTATAGAAAAGTTGGGTGTTCTTTGATTTCATACTTG
LT13	GGGGACAACTTTGTATAATAAAGTTGGACTTTAGATGAGATCGTG
LT14	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTTCATTAATTTGACATC
LT15	TAAATTTGAGCATGGGGCAG
GmB4rF	GGGGACAACTTTTCTATACAAAGTTGCTGGTACCCGGGTGACTAAC
GmB3rR	GGGGACAACTTTATTATACAAAGTTGTGGATCCCCGTGTCATTATTCC
M13F	TGTAAAACGACGGCCAGT
M13R	TCACACAGGAAACAGCTATGAC
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	GCTAGTTATTGCTCAGCGG
LT15	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAATTTTTGGATCAAG
LT16	GGGGACAACTTTTGTATACAAAGTTGTATTTGAGCAAAAGAGGGGATC
LT17	GGGGACAACTTTGTATACAAAAGTTGCATTAGTTAATGAACGCTTCTG
LT18	GGGGACAACTTTGTATAGAAAAGTTGGGTGTTTTGGGGTGAGTTTCATCTC
LT19	GGGGACAACTTTTCTATACAAAGTTGCAATGAAGAGATCTTCTGTATTTAG

LT20	GGGGaCAACTTTATTATACAAAGTTGTTTACTTCATTAATTTGACATC
LT21	GGGGACAACTTTTCTATACAAAGTTGCAATGAGGTATTTATGGCTTTTTTTAATAC
LT22	GGGGACAACTTTATTATACAAAGTTGTTTACCAATCAAAGGCTTGTATTTTC
LT23	GGGGACAACTTTGTATAATAAAGTTGCAGAGATTCAACCACAGCATGC
LT24	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACAACCAAGTAATCGCATC
rdxA.lF	CGCCATTCTTGCAAGATGTTTGATAG
rdxA.lR	AATCTCACGCCAAGCATTTGAGC
rdxA.rF	CAACCACAGCATGCAAAAATTAGAAAGC
rdxA.rR	TCACAACCAAGTAATCGCATCAACTTTTG
rdxA-MCS-CATgc	GCTCAAATGCTTGGCGTGAGATTGGATCCTGGAGTCAAGCTTACCCGGGAGATCTGATATCGGTACCCC GAGATTTTCAGGAGCTAAGG
CATgc.F	CCGAGATTTTCAGGAGCTAAGGAAGC
CATgc.R	CTTTCTAATTTTTGCATGCTGTGGTTGTTACGCCCCGCCCTGCCACTC
HP1130.F	AATGGATCCGGTGCTTTATGGGCATAGCAAC
HP1130.R	TACCTCATTGTTCTTCCTTAGTGAAACGATTGAATCTAACATGATTATCCC
HP1126.F	CACTAAGGAAGAACAATGAGGTATTTATGG
LT13R	ATATGGTACCTTACTTCATTAATTTGACATCACTCTTC
139.TolBR	ATATGGTACCTTACCAATCAAAGGCTTGTATTTTC
141.HP1130R	GGTGATCAAATTCAAATTAAACGATTGAATCTAACATGATTATCCC
141.PalF	GTTTAATTTGAATTTGATCACCTTAAATAGC
HP0953.R	GACTCTTAGCGCTTAATGAAACGCTTG

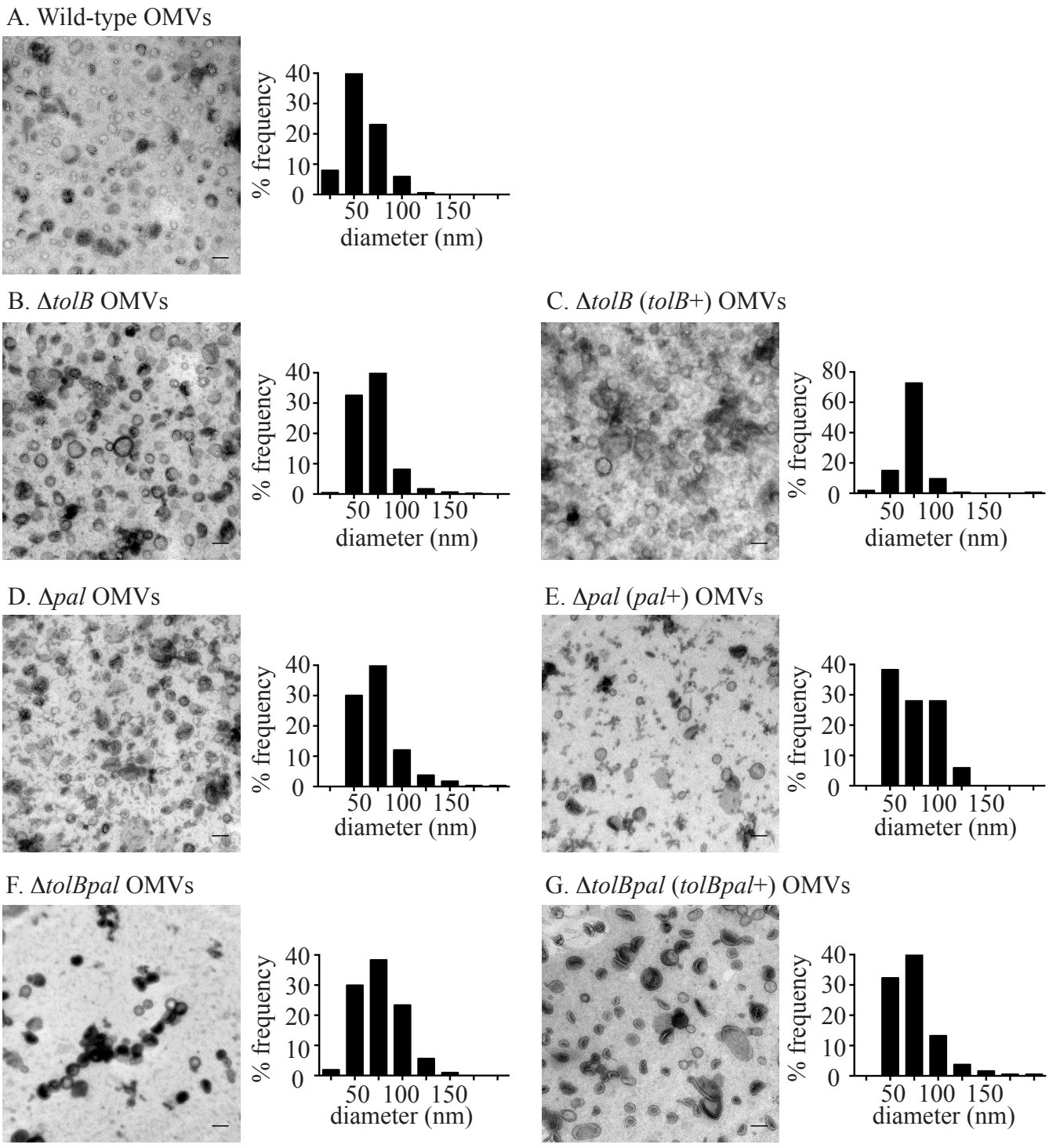


FIG S1. Size frequency and distribution of OMVs isolated from Δ*tolB-pal* mutants. OMVs were purified from the wild-type strain and each of the Δ*tolB-pal* mutants and visualized by TEM. The diameters of the OMVs were determined and used to plot the % frequency at which different sizes of OMVs were observed. Images are representative across at least 3 fields of view, comprising > 100 OMVs measured per sample. Scale bar is 100nm.

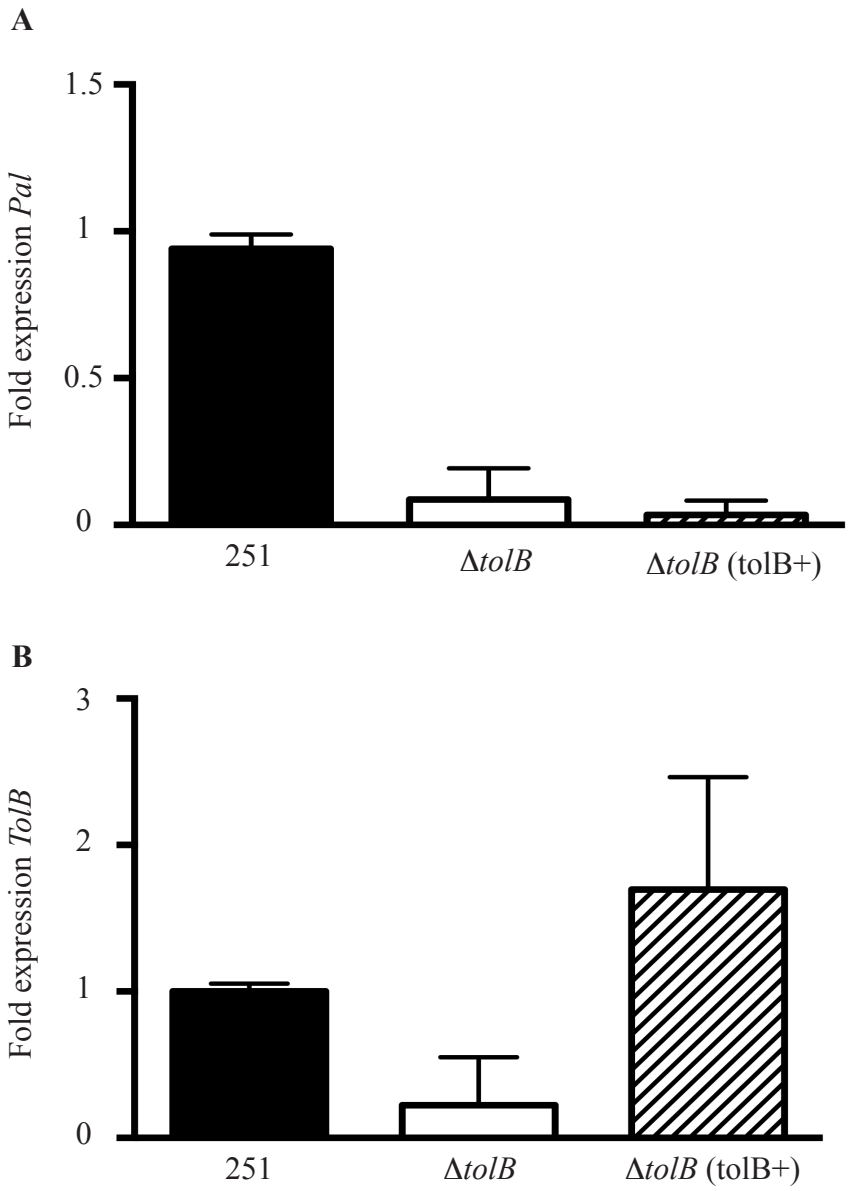


FIG S2. Expression of Pal and TolB by *H. pylori* wild type 251 and isogenic mutants Δ*tolB* and Δ*tolB* (*tolB*+). Expression of Pal (A) and TolB (B) by wild type *H. pylori* 251 (black bars), Δ*tolB* (open bars) or Δ*tolB* (*tolB*+) grown to log phase of growth by qRT-PCR. Data are from a single experiment performed in triplicate and gene expression values are plotted relative to gene expression by *H. pylori* 251.

Appendix 2.1. Generation of the plasmids pLTtolB and pLTTolBPal

Step 1: *tolB* and *tolBpal* were amplified from *H. pylori* 26695 genomic DNA by PCR, yielding 928bp and 1546bp DNA fragments, respectively.

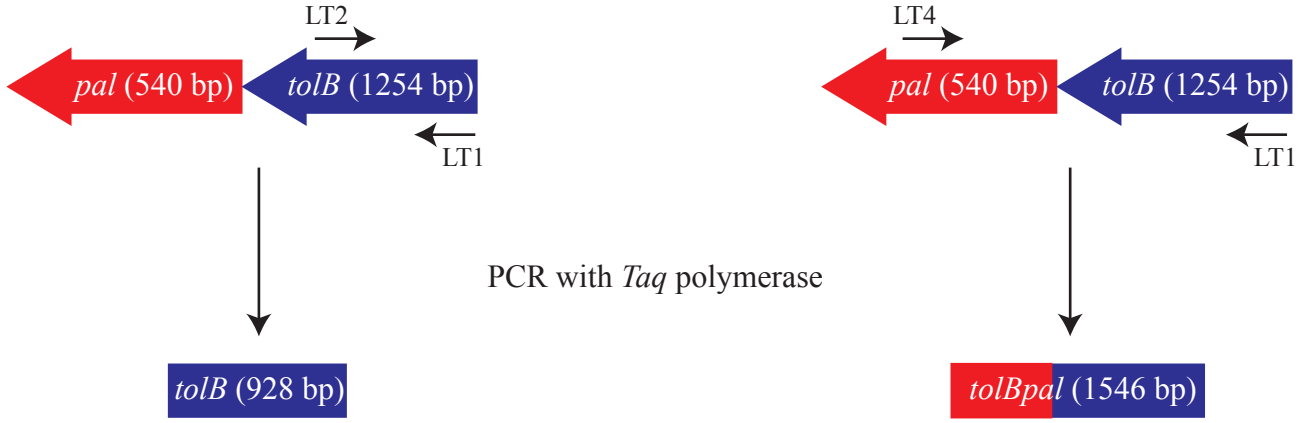
Step 2: DNA fragments were ligated to the pGEM T-Easy vector to give rise to the plasmids pGEM T-Easy tolB, and pGEM T-Easy tolBpal.

Step 3: Inverse Failsafe PCR was performed using primers to introduce the restriction enzyme sites BamHI and KpnI within the DNA sequence of either *tolB* or *tolBpal*.

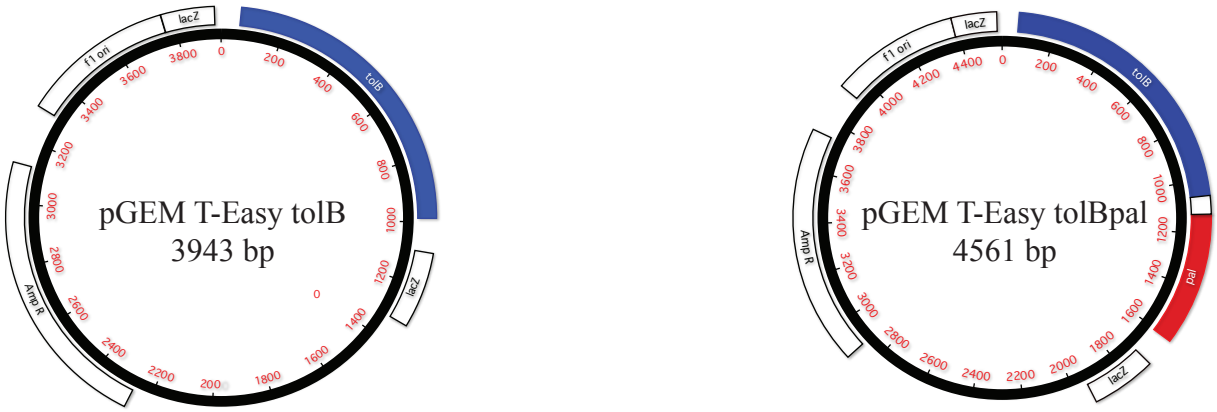
Step 4: The purified failsafe PCR products were digested with BamHI and KpnI. The 842 bp gentamicin resistance cassette (GmR) excised from the puc1813 vector by restriction digestion with BamHI and KpnI.

Step 5: Digested Failsafe PCR products were ligaed to the GmR cassette using T4 DNA ligase, generating plasmids pLTtolB and pLT TolBPal

1


LT2
pal (540 bp)
tolB (1254 bp)
LT1
LT4
pal (540 bp)
tolB (1254 bp)
LT1
PCR with Taq polymerase
tolB (928 bp)
tolBpal (1546 bp)

2


pGEM T-Easy tolB
3943 bp
pGEM T-Easy tolBpal
4561 bp

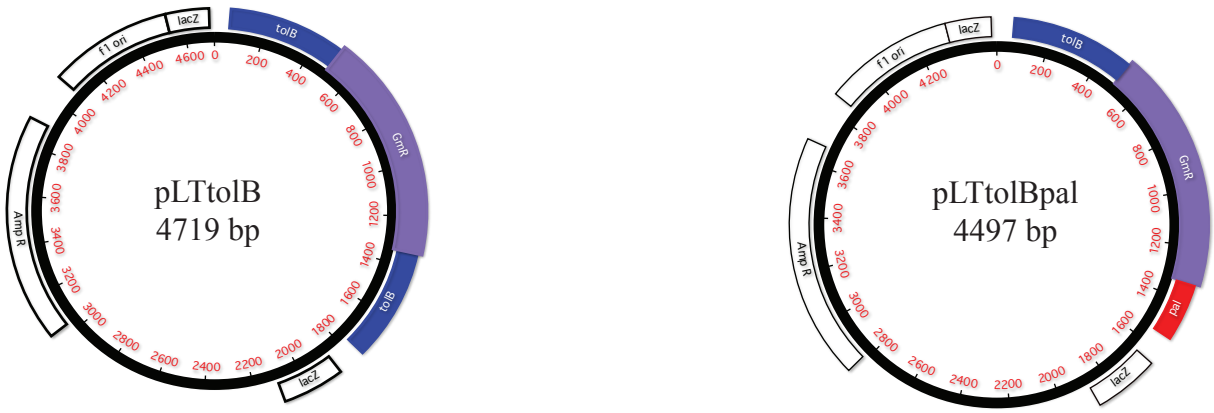
3


KpnI
tolB
3810 bp
tolB
BamHI
BamHI
pal
3668 bp
tolB
KpnI

4


KpnI
GmR (842 bp)
BamHI

5


pLTtolB
4719 bp
pLTtolBpal
4497 bp

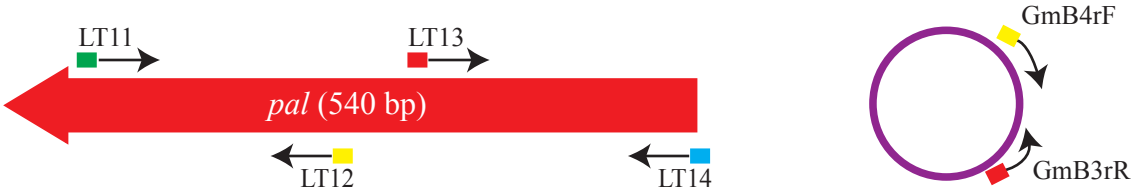
Appendix 2.2. Generation of pDESTPal using Gateway cloning

Step 1: The 5' and 4' regions of *pal* were amplified from *H. pylori* 26695 genomic DNA with primers designed to add attB1 and attB4, and attB2 and attB3 sites, respectively, to the resulting DNA fragments. The gentamicin resistance cassette (GmR) was amplified from the puc1813 vector with primers designed to add attB4r and attB3rt sites to the resulting DNA fragment.

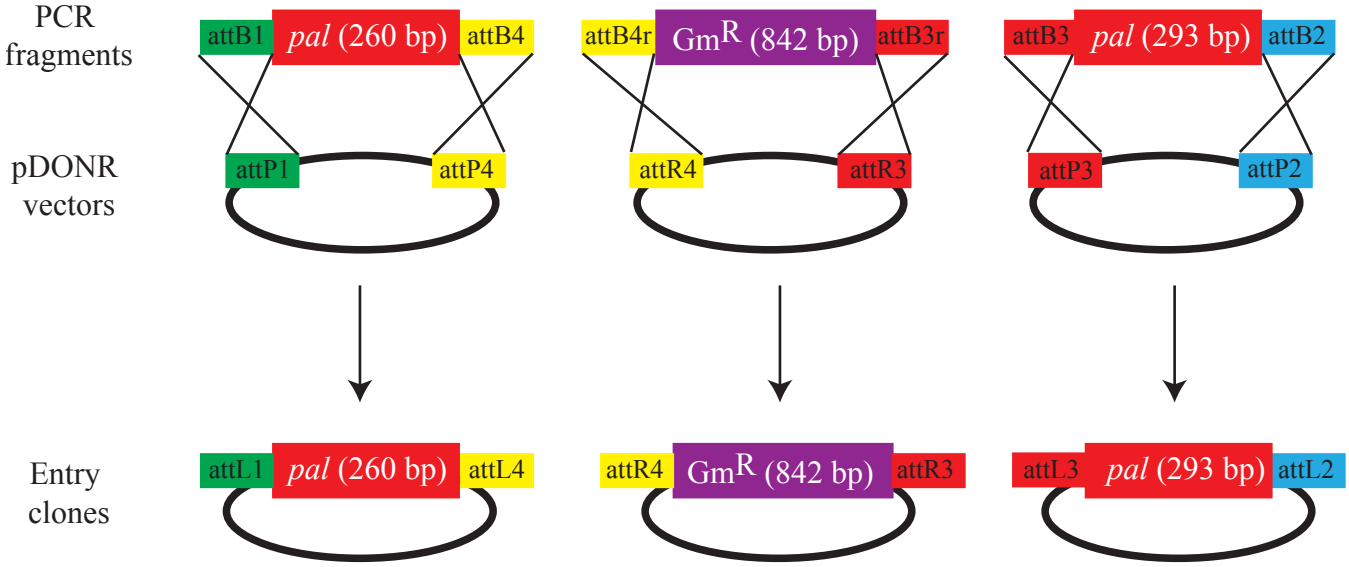
Step 2: DNA fragments containing att sites were inserted into pDONR vectors using the BP recombination reaction, to generate entry clones.

Step 3: Insert DNA from all three entry clones were recombined into the pDEST17 vector using LR Clonase II, to generate the final plasmid pDESTPal.

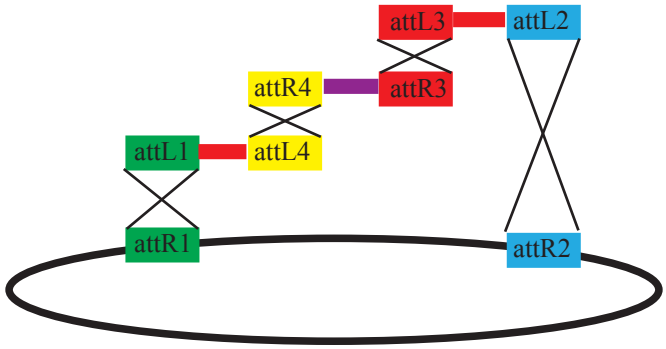
1

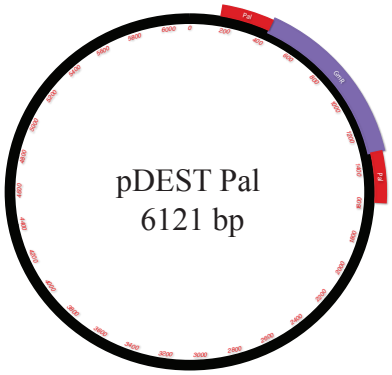

LT11
LT13
pal (540 bp)
LT12
LT14
GmB4rF
GmB3rR

2


PCR fragments
attB1
pal (260 bp)
attB4
attB4r
GmR (842 bp)
attB3r
attB3
pal (293 bp)
attB2
pDONR vectors
attP1
attP4
attR4
attR3
attP3
attP2
Entry clones
attL1
pal (260 bp)
attL4
attR4
GmR (842 bp)
attR3
attL3
pal (293 bp)
attL2

3


attL3
attL2
attR4
attR3
attL1
attL4
attR1
attR2

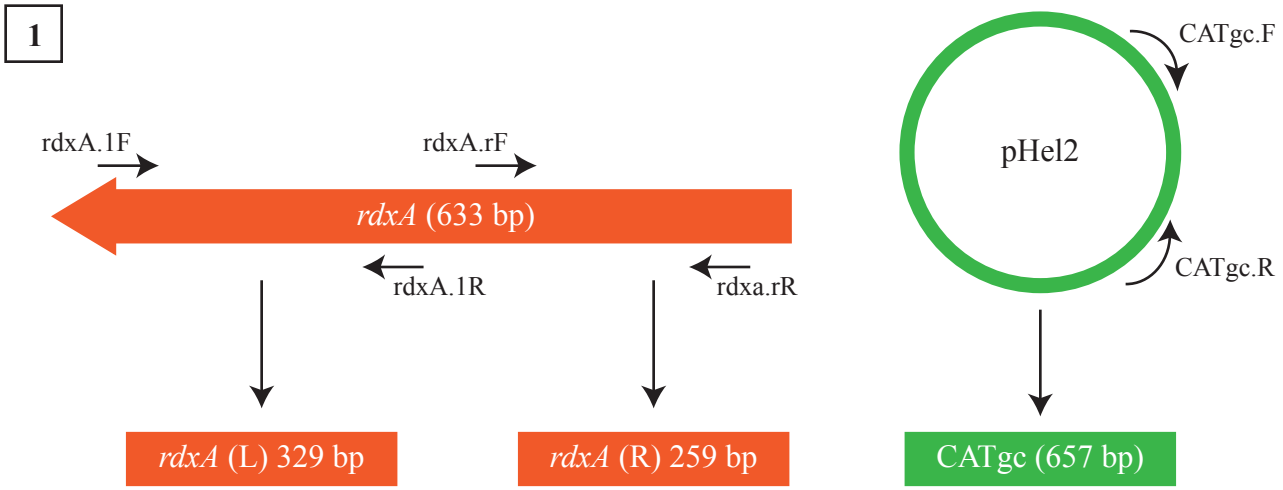

pDEST Pal
6121 bp

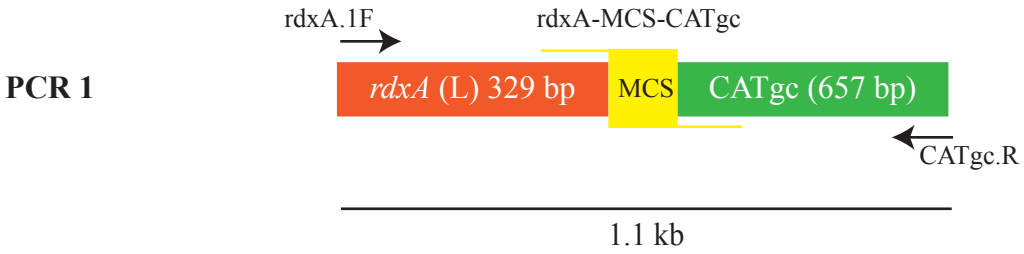
Appendix 2.3. Generation of pJPF3

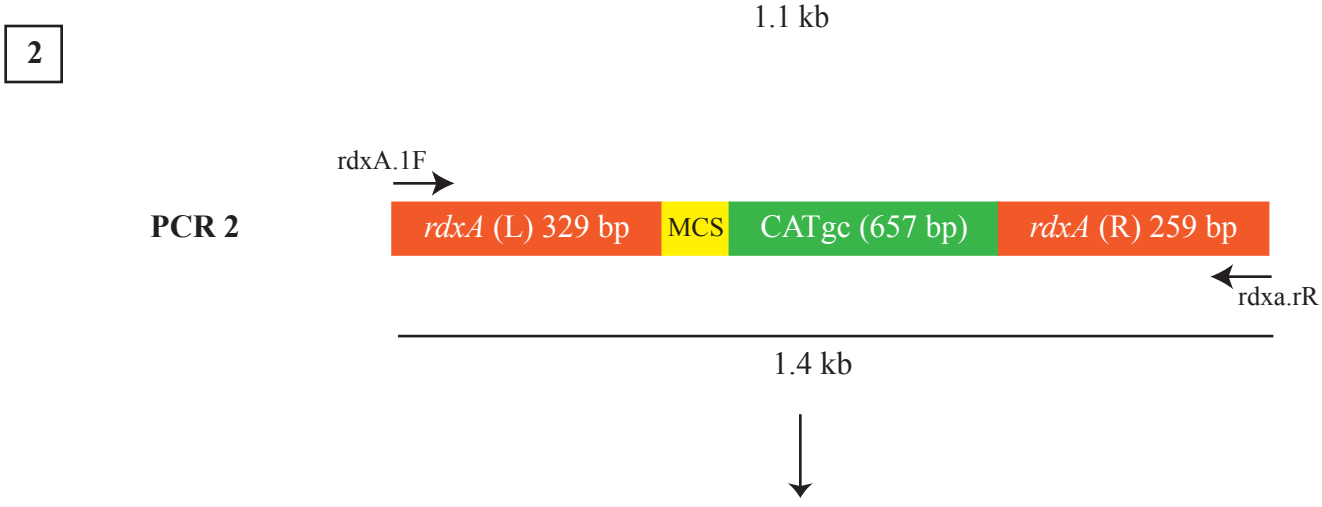
Step 1: The 5' and 3' ends of *rdxA* (designated *rdxA*(L) and *rdxA*(R), respectively) were amplified from *H. pylori* SS1 genomic DNA. The chloramphenicol resistance cassette (CATgc) was amplified from the plasmid pHEL2. All PCRs were performed with Phusion High-Fidelity DNA polymerase in order to generate blunt-ended DNA required for overlap extension PCR.

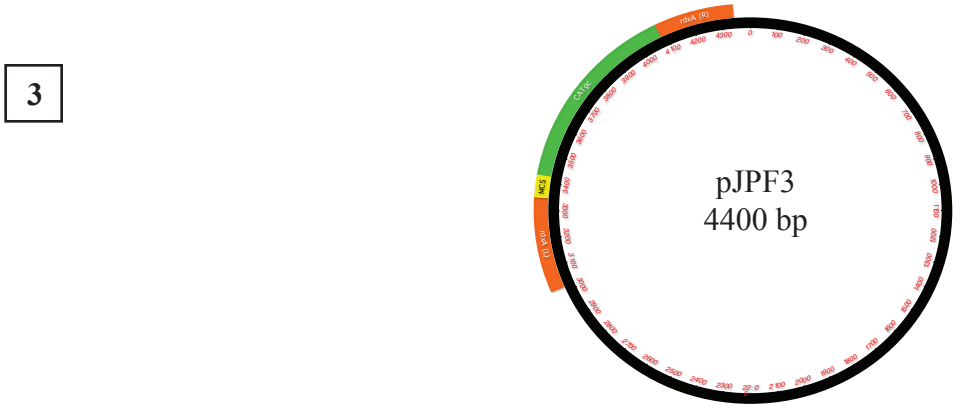
Step 2: Splicing overlap extension PCR (SOE-PCR) was performed to add a multiple cloning site (MCS) between the rdxA(L) and the CATgc, and then to join the rdxA(L)-MCS-CATgc to the rdxA(R). The rdxA-MCS-CATgc primer used to insert the MCS was designed to have regions complementary to rdxA(L) at the 3' end, and CATgc at the 5' end to allow the DNA fragments to join during SOE-PCR.

Step 3: The resulting rdxA(L)-MCS-CATgc-rdxA(R) DNA fragment was cloned into pGEM T-Easy, giving rise to the plasmid pJPF3.


1
rdxA.1F
rdxA.rF
rdxA (633 bp)
rdxA.1R
rdxa.rR
rdxA (L) 329 bp
rdxA (R) 259 bp
pHel2
CATgc.F
CATgc.R
CATgc (657 bp)


PCR 1
rdxA.1F
rdxA-MCS-CATgc
rdxA (L) 329 bp
MCS
CATgc (657 bp)
CATgc.R
1.1 kb


2
PCR 2
rdxA.1F
rdxA (L) 329 bp
MCS
CATgc (657 bp)
rdxA (R) 259 bp
rdxa.rR
1.4 kb


3
pJPF3
4400 bp

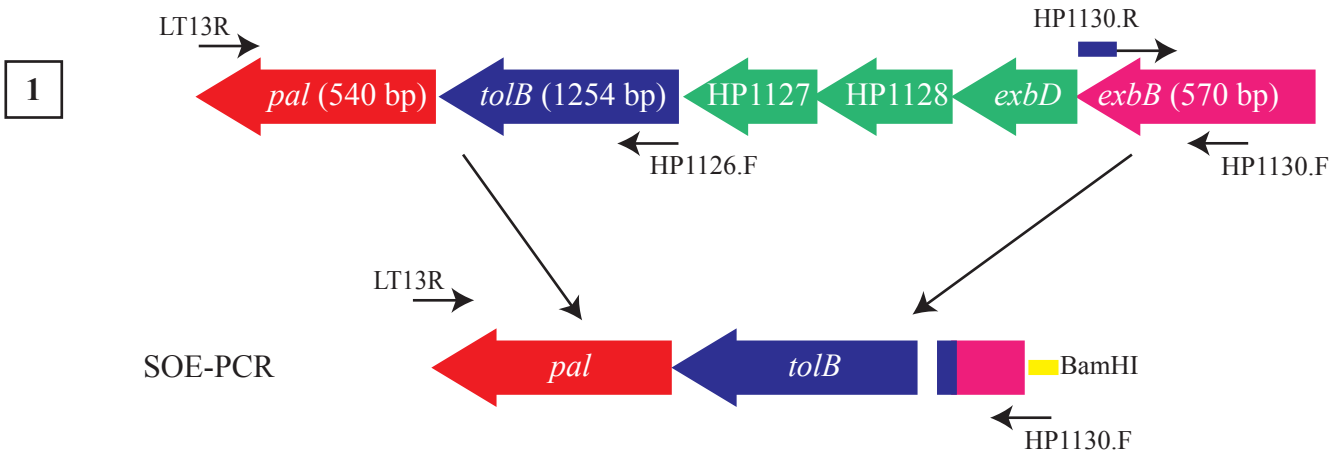
Appendix 2.4. Generation of plasmids pJPFTolBPal+ and pJPFTolBPal+

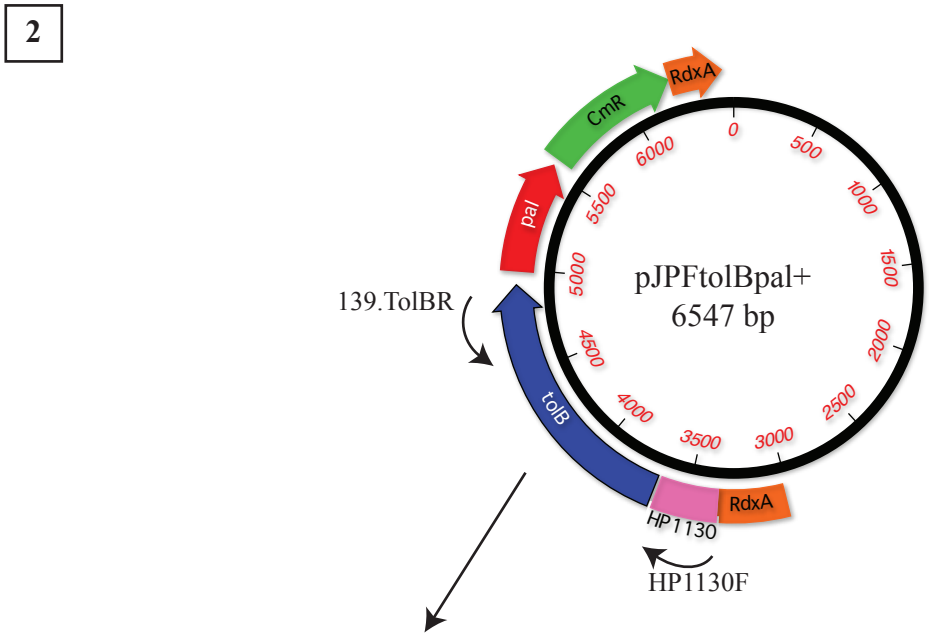
Step 1: the *tolB* and *pal* genes, as well as the putative ribosome binding site for *tolB*, were amplified from *H. pylori* 26695 genomic DNA by PCR. The primer LT13R contained a KpnI restriction enzyme site. The promoter region for the *exbD*-HP1128-HP1127-*tolB* operon (designated here as HP1130) was amplified from *H. pylori* 26695 genomic DNA. The 5' region of the primer HP1130.R used to amplify the promoter region contained a small section of DNA complementary to the 3' end of *tolB*, to allow the DNA fragments to join during SOE-PCR. The primer HP1130F contained a BamHI restriction enzyme site. All PCR reactions were performed using Phusion High Fidelity DNA polymerase.

Step 2: DNA fragments were joined by SOE-PCR. The resulting DNA fragment was digested with BamHI and KpnI and ligated to BamHI/KpnI digested pJPF3 vector, to give rise to the pJPFTolBPal+ plasmid.

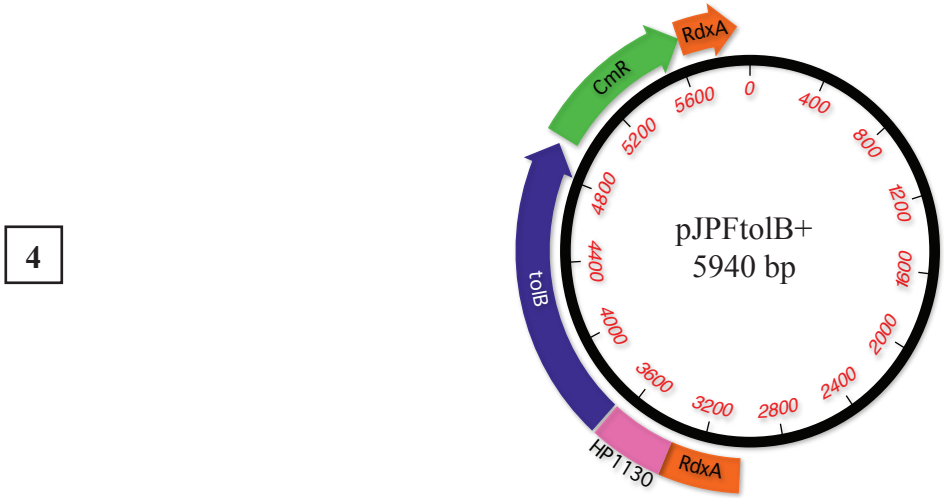
Step 3: pJPFTolBPal+ was used as a template to amplify the HP1130P together with tolB, using primers to add BamHI and KpnI restriction enzyme sites.

Step 4: The PCR product was digested with BamHI and KpnI and ligated to BamHI/KpnI digested pJPF3, giving rise to the plasmid pJPFtolB+.


1
LT13R
HP1130.R
pal (540 bp)
tolB (1254 bp)
HP1127
HP1128
exbD
exbB (570 bp)
HP1126.F
HP1130.F
LT13R
SOE-PCR
pal
tolB
BamHI
HP1130.F


2
RdxA
CmR
pal
pJPFtolBpal+
6547 bp
139.TolBR
tolB
HP1130
RdxA
HP1130F


3
KpnI
tolB
BamHI


4
RdxA
CmR
tolB
pJPFtolB+
5940 bp
HP1130
RdxA

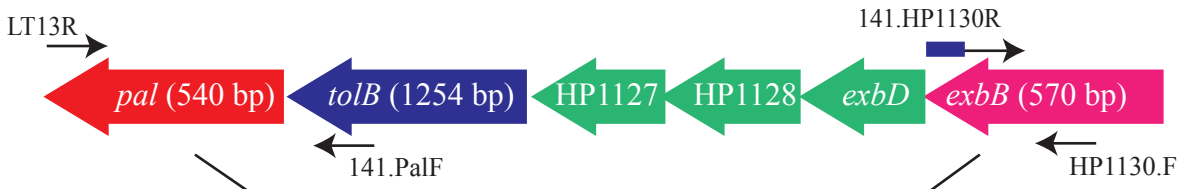
Appendix 2.5. Generation of pJPFPal+

Step 1: *pal*, together with the putative native promoter (contained in the 3' region of *tolB*) was amplified from *H. pylori* 26695 genomic DNA. The primer LT13R contained a KpnI restriction enzyme site. The promoter region within HP1130 was also amplified and a BamHI restriction enzyme site added to the 3' end. The primer HP1130.R contained a short region of DNA complementary to tolB, allowing the two DNA fragments to be joined during SOE-PCR.

Step 2: The two DNA fragments were joined by SOE-PCR.

Step 3: The resulting DNA fragment was digested with BamHI and KpnI and ligated to BamHI/KpnI digested pJPF3, giving rise to pJPFPal+.

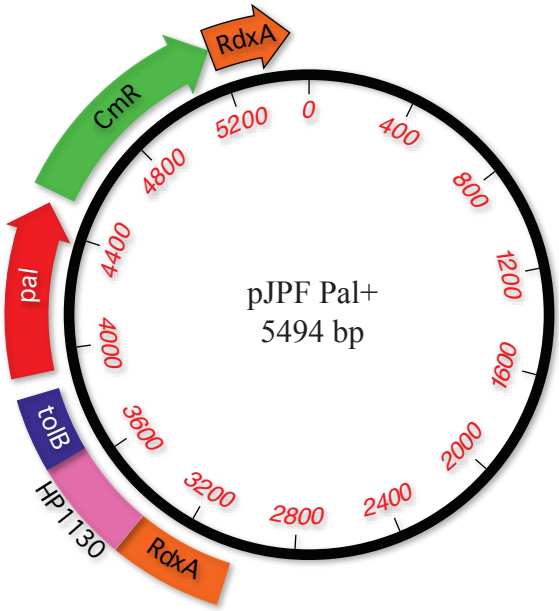
1


LT13R
141.HP1130R
pal (540 bp)
tolB (1254 bp)
HP1127
HP1128
exbD
exbB (570 bp)
141.PalF
HP1130.F

2


LT13R
KpnI
pal
BamHI
HP1130.F

3


RdxA
CmR
pal
tolB
HP1130
RdxA
pJPF Pal+
5494 bp
0
400
800
1200
1600
2000
2400
2800
3200
3600
4000
4400
4800
5200

2.4 Final Discussion

OMVs have long been recognised for their potential use in vaccination, yet are produced only in small amounts, therefore complicating their use in vaccines. *H. pylori* OMVs contain within them several known immunogenic proteins, including Lpp20 (Keenan, Oliaro et al. 2000), making them ideal vaccine candidates. As for other bacteria, however, the OMV yields from this bacterium are typically low (approximately 0.5 pg/CFU). The data provided in this chapter documents the generation of hyper-vesiculating strains of *H. pylori*, via the mutation of the genes encoding the conserved outer membrane proteins, TolB and Pal.

We demonstrated that mutations in either *H. pylori tolB* or *pal* had little effect on the growth rates of the bacteria. We next examined their morphology, as previous work performed by others has demonstrated that in other bacteria, strains lacking TolB or Pal exhibit morphological abnormalities (Dubuisson, Vianney et al. 2005). Similarly, we found that *H. pylori* Δ*tolB* bacteria exhibited profound morphological alterations, most notably the lack of fully formed flagella. Furthermore, visual examination indicated that Δ*tolB* bacteria demonstrated extensive blebbing at the cell surface. *H. pylori* Δ*pal* bacteria, however, retained flagella expression. Importantly, we determined that *H. pylori* Δ*tolB* or Δ*pal* demonstrated >600- and 22-fold increases in OMV production respectively. Further analysis of these OMVs revealed that they were capable of inducing pro-inflammatory cytokine responses from host cells. Furthermore, OMVs produced by the mutant strains contained similar protein contents, when compared with those produced by the wild type.

This work describes, for the first time, the importance of the TolB and Pal proteins in cell membrane integrity and OMV formation in the gastric pathogen *H. pylori*. These

data suggest that the OMVs produced by these hyper-vesiculating *tolB-pal* bacteria may be suitable for use in further studies, including vaccine development.

Chapter 3. Bacterial outer membrane vesicle size determines their mechanisms of host cell entry and protein content.

3.1 Summary

Gram-negative pathogens ubiquitously shed OMVs that deliver proteins and toxins to host cells. There exists a large body of evidence demonstrating OMVs within host cells (Kadurugamuwa and Beveridge 1995, Kadurugamuwa and Beveridge 1998, Fiocca, Necchi et al. 1999, Demuth, James et al. 2003, Kesty, Mason et al. 2004, Tsuda, Amano et al. 2005, Galka, Wai et al. 2008, Bomberger, Maceachran et al. 2009, Furuta, Takeuchi et al. 2009, Furuta, Tsuda et al. 2009, Ellis, Leiman et al. 2010, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010, Schaar, de Vries et al. 2010, Vidakovics, Jendholm et al. 2010, Bielig, Rompikuntal et al. 2011, Chatterjee and Chaudhuri 2011, Rompikuntal, Thay et al. 2011), however, the precise mechanism(s) of OMV cellular uptake have yet to be defined. Indeed, a search of the literature reveals numerous studies proposing various mechanisms of entry. These include, but are not limited to: lipid raft dependent (Kesty, Mason et al. 2004, Kaparakis, Turnbull et al. 2010) and independent (Parker, Chitcholtan et al. 2010) internalisation, endocytosis (Tsuda, Amano et al. 2005), receptor mediated endocytosis (Vidakovics, Jendholm et al. 2010) and host cell fusion (Demuth, James et al. 2003, Bomberger, Maceachran et al. 2009). In fact, many of these reported mechanisms are contradictory, even within OMVs derived from the same bacteria. One possible explanation is that vesicle size may be a factor determining the mode of OMV cell entry. Indeed, it has been hypothesised that particle size can determine not only the mechanism of host cell entry, but also the mode of intracellular trafficking and degradation (Brewer, Pollock et al.

2004). With regards to *H. pylori*, previous experiments performed within our laboratory have demonstrated lipid raft-dependent endocytosis (Kaparakis, Turnbull et al. 2010). However, other works have proposed that *H. pylori* OMVs do not require lipid rafts for internalisation (Parker, Chitcholtan et al. 2010).

The striking discrepancy in the reported mechanisms utilised by OMVs to enter host cells emphasises that further research is required in order to elucidate the precise mechanisms of OMV entry. Expanding our knowledge of the pathways utilised by OMVs to enter host cells will enable us to further understand their role in disease and advance the development of OMV-based vaccines.

The following series of experiments were thus designed to: firstly, characterise the precise mode of host cell internalisation of *H. pylori* OMVs; secondly, to determine the role, if any, of OMV size on the preferential mechanism of entry. Finally, we examined the protein content of OMVs of different sizes, in order to determine if OMV size correlated with differences in protein content.

3.2 Declaration for thesis chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
My contribution included project conceptualisation and experimental design. In addition I performed all experiments, interpreted data and undertook the majority of the writing.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
David L Steer	Performed proteomic analysis	
Camden Y-W Lo	Provided technical assistance with analysis of microscopy data	
Georg Ramm	Assisted with electron microscopy experiments	
Richard L Ferrero	Project conceptualisation, data interpretation and manuscript editing	
***Maria Kaparakis-Liaskos**	Project conceptualisation, data interpretation and manuscript editing	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date 10/02/2014**
Main Supervisor's Signature	[redacted]	**Date 10/02/2014**

3.3 Manuscript: Bacterial outer membrane size determines their mechanisms of host cell entry and protein content

Bacterial outer membrane vesicle size determines their mechanisms of host cell entry and protein content.

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Running Heading: OMV size defines protein content and cellular entry.

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Word Count Abstract: 250

Word count text: 34,883 characters excluding references

Abstract

Gram-negative pathogens ubiquitously shed outer membrane vesicles (OMVs) that contribute to pathogenesis via the delivery of toxins and virulence factors into host cells. OMVs can be readily detected within host epithelial cells, however, the precise mechanism of OMV cellular uptake has yet to be defined.

Bacterial OMVs are capable of inducing protective immune responses against bacterial infection and are therefore attractive vaccine candidates. In order to fully understand how OMVs initiate immune responses, we need to elucidate the mechanisms behind their entry and subsequent intracellular fate. Using *Helicobacter pylori* OMVs as a model to assess the uptake of OMVs by non-phagocytic cells, we demonstrate that a heterogeneous population of OMVs enter epithelial cells via the three main mechanisms of endocytosis; macropinocytosis, clathrin and caveolin dependent endocytosis, to varying extents. Moreover, we found that vesicle size has an important role in directing the route of entry, as small OMVs of approximately 20 nm enter host cells via macropinocytosis and all mechanisms of endocytosis, whereas the entry of OMVs greater than 50 nm did not involve clathrin mediated endocytosis. We suggest that small and large OMVs are processed within the cell via different intracellular degradation pathways. Importantly, proteomic analysis revealed that different sized OMVs contain both unique and shared proteins, with fewer proteins contained within small OMVs, indicating that OMV size plays a role in determining their protein composition. This is the first report demonstrating the importance of OMV size on their mechanisms of host cell entry, protein cargo and potentially intracellular fate.

INTRODUCTION

Gram-negative bacteria ubiquitously shed vesicles known as outer membrane vesicles (OMVs) during their normal growth (reviewed in (1)). OMVs are spherical, bi-layered membrane nanostructures ranging from 20 to 300 nm in size, and their release occurs naturally both *in vitro* and *in vivo* (reviewed in (1)). The importance of OMV production during the natural course of infection and in pathogenesis has been highlighted by the identification of OMVs within infected host tissues, including the gastric mucosa of *Helicobacter pylori* infected individuals (2), as well as in the cerebrospinal fluid and sera of patients with meningococcal infection (3). More recently, the ability of OMVs produced by commensal bacteria to prevent diseases such as experimental colitis has been reported (4), further broadening the role of OMVs in disease and gut homeostasis.

OMVs from a range of bacteria have been analyzed and shown to have a similar protein (5-7) and lipid (8) composition to the outer membranes of the parent bacteria. Specifically, OMVs contain inner and outer membrane proteins, periplasmic proteins (9), lipopolysaccharide (LPS) (9), peptidoglycan (PG) (10), DNA (11-13) and toxins (2, 14-17). As the protein composition of OMVs are highly similar to that of their parent bacterium, their use as vaccine candidates is currently being extensively examined (18-25).

OMVs are extremely inflammatory in nature. We, and others have shown that OMVs from the Gram negative pathogens *H. pylori*, *Neisseria*, *Pseudomonas*, *Campylobacter* and *Vibrio* are capable of inducing IL-8 responses by non-phagocytic epithelial cells (10, 26-28). All of these studies reported that OMV entry into non-phagocytic epithelial cells was essential for the induction of pro-inflammatory responses. However, to date, the exact mechanisms whereby OMVs enter non-phagocytic cells, resulting in the development of an inflammatory response, have not

been fully elucidated. Indeed, the literature contains numerous studies with contradictory findings regarding the mechanisms utilized by OMVs to enter host cells. We and others have demonstrated a role for cholesterol-rich lipid rafts in OMV entry into non-phagocytic host cells (1, 10, 29-31), whereas other reports described lipid raft independent mechanisms of OMV entry (26). Furthermore, the requirement for endocytosis (1, 30, 32-34) or macropinocytosis (29), requiring actin and cytoskeletal rearrangements, remains unclear. The striking divergence in the reported mechanisms utilized by OMVs to enter host cells emphasizes that further research is warranted to elucidate the exact mechanisms of OMV entry. Expanding our knowledge of the pathways utilized by OMVs to enter host cells will enable us to further understand their role in pathogenesis and advance the development of OMV-based vaccines.

In this study, we characterized the exact mechanisms whereby OMVs enter non-phagocytic epithelial cells using a panel of chemical inhibitors to specifically block non-phagocytic cell entry pathways, notably: macropinocytosis, clathrin and caveolin dependent endocytosis. Our findings revealed that a heterogeneous sized population of OMVs entered human epithelial cells via macropinocytosis, caveolin and clathrin dependent endocytosis to varying extents, and that inhibition of each of the three pathways resulted in a significant reduction in OMV-induced IL-8 responses. Furthermore, we have demonstrated for the first time that OMV size predetermines the mechanism of entry into host cells. Specifically, we found that OMVs of approximately 20 nm entered host cells via clathrin and caveolin dependent endocytosis, in addition to macropinocytosis, whereas larger OMVs did not utilize clathrin for entry into host cells. Furthermore, we found that the intracellular processing of different sized OMVs appears to differ, indicating that OMVs entering host cells via different pathways undergo distinct intracellular processing. Finally, we

determined that OMV size predetermines the protein composition of OMVs, as smaller OMVs contain fewer proteins within them. Collectively these findings are the first to report that OMV size plays a crucial role in the mechanisms of host cell entry and intracellular processing, their protein content and, we propose, their ability to activate pro-inflammatory immune responses. These findings have major implications for understanding the role of OMVs in bacterial pathogenesis and facilitating their development as vaccines.

MATERIALS AND METHODS

Bacterial strains and OMV purification

The *H. pylori* 251 *cag*PAI strain (10) was routinely cultured on Horse Blood Agar medium (Blood Agar Base No2, Oxoid, Hampshire, England) or in Brain Heart Infusion broth (BHI, Bacto™ Brain Heart Infusion, Becton Dickinson and Company, Sparks, USA), supplemented with 0.6 % (w/v) β-cyclodextrin (Sigma-Aldrich, MO, USA) with shaking at 120 rpm. Cultures were grown at 37°C under microaerobic conditions (35). *H. pylori* OMVs were purified from log phase cultures as described previously (10). In brief, bacteria were pelleted from overnight cultures by centrifugation at 2,500 x g for 20 minutes at 4°C. Supernatants were subsequently filtered using a 0.22 μm PES filter (Corning Incorporated, NY USA) and OMVs were pelleted from these supernatants by ultracentrifugation (100,000 x g, 2 hours, 4°C). The resulting OMV pellets were resuspended in PBS and protein concentrations determined by the Bradford Protein Assay (Bio-Rad Laboratories Inc., CA, USA).

Sucrose gradient purification of OMVs

OMV preparations (6 ml) were layered onto discontinuous sucrose gradients, consisting of 12.5 ml 25 % (w/v) sucrose (Fluka Biochemika, Steinheim Switzerland), 15.5 ml 42 % (w/v) sucrose, and 5 ml 56 % (w/v) sucrose and subjected to ultracentrifugation (100,000 x g, 16 hours, 4°C) (10, 36). Thirteen fractions (3ml each) were collected, washed with PBS to remove any remaining sucrose and concentrated to a final volume of 500 μl using Amicon YM-10 columns (Millipore Ireland Ltd., Cork. Ireland).

Fluorescent labeling of OMVs

OMVs (2 mg/ml) were labeled with 1 % (v/v) 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes Eugene, OR, USA) for 20 minutes at 37 °C (26). Excess dye was removed by washing OMVs 3 times with PBS using Amicon Ultra 0.5 ml Ultracel 3K (Millipore Ireland, Cork, Ireland).

Cell culture

Human gastric adenocarcinoma (AGS) and human embryonic kidney (HEK293) cells were routinely cultured using RPMI or DMEM respectively, supplemented with 10 % (v/v) fetal calf serum (FCS) (Gibco, Invitrogen, NY). Cells were seeded at a density of 1 x 10^5 cells per ml in 12 or 24 well plates (Becton Dickinson Labware, NJ, USA) for 24 hours, after which media was replaced with media not containing FCS.

Cytotoxicity assay

AGS or HEK293 cells were treated with endocytosis inhibitors or 0.5 % (w/v) sodium azide (37) (Sigma-Aldrich, USA) for 30 minutes. Cells were then washed and media replaced for 4 hours. Cellular cytotoxicity was measured using the CellTiter-Glo® assay (Promega Corporation, WI, USA), according to the manufacturer's instructions. In brief, cells were allowed to equilibrate to room temperature for 30 minutes and CellTiter-Glo® reagent (Promega Corporation) was added to cells at a ratio of 1:1. Luminescence was measured using a FLUOstar OPTIMA (BMG Labtech, Australia).

Flow Cytometry

The effectiveness of trypan blue quenching of OMV associated DiO fluorescence was examined by flow cytometry. AGS cells were incubated with DiO labeled OMVs for 4 hours prior to permeabilization with 0.01 % (v/v) Triton-X for 10 minutes, or left non permeabilized. Fluorescence was quenched with trypan blue (0.025% final concentration). Cells were washed once and resuspended in DPBS (Gibco, Invitrogen, NY) containing 2 % (v/v) FCS and analyzed by flow cytometry using BD FACS CANTO II and BD FACS Diva software v 6.0. A total of 6 x 10^4 cells were counted for each condition. Data was analyzed using FlowJo version 7.6.

Inhibition of OMV entry into epithelial cells

Inhibition of OMV entry was performed using chemical inhibitors of endocytosis (all from Sigma-Aldrich, MO, USA) at the following concentrations, as described previously (38): bafilomycin A1 (10 nM), cytochalasin D (2 μM), dynasore

monohydrate (10 μM), nocodazole (3.3 μM) and valinomycin (10 μM). AGS or HEK293 cells were pre-treated with inhibitors for 30 minutes. The cells were subsequently washed twice and the media replaced prior to incubation with 50 μg/ml of OMVs for 4 or 16 hours for fluorescence analysis and 24 hours to quantify IL-8 production by ELISA.

Fluorescence microscopy

AGS cells were seeded onto glass coverslips in 12 well plates (Becton Dickinson Labware, NJ, USA) and cultured overnight. Cells were pre-treated with inhibitors for 30 minutes, washed twice and media replaced, prior to co-culture with 50 μg/ml of DiO labeled OMVs for 4 or 16 hours. Effectiveness of trypan blue quenching of DiO fluorescence was examined by permeabilization of cells with 0.01 % (v/v) Triton-X for 10 minutes, prior to addition of trypan blue. For all other experiments, extracellular fluorescence was quenched using 0.025 % (v/v) trypan blue (Sigma Chemical Co., MO, USA) (26), prior to washing three times with PBS and fixing with 4 % (v/v) formaldehyde (Merck, Darmstadt, Germany) for 20 minutes. Nuclei were stained with 4',6-diamidino-2-phenylindole, dilactate (DAPI; Molecular Probes, OR, USA), prior to mounting in Dako Fluorescent mounting medium (Dako North America Inc., CA, USA). Images were acquired on an Applied Precision Instruments DeltaVision deconvolution microscope using a 40 x 1.35NA oil objective at 512 x 512 x 14 bit per channel. Z-stacks (10-15 μm) at 0.2 μm per slice were acquired and deconvolved based on the point spread function (PSF) of the system. Images were analyzed by Imaris (v7.1.0 Bitplane AG), where the average intensity density of OMV fluorescence was derived by measuring the sum intensity of OMV fluorescence

divided by the OMV volume, then averaged across cells in the field of view. These arbitrary intensity density units were then normalized to OMV-alone groups and expressed as average signal density. The means of each condition were determined for 3 independent experiments and plotted as average signal density.

Transmission Electron Microscopy

OMV samples were resuspended in 4 % (v/v) formaldehyde in PBS. Samples were adhered to Formvar coated carbon grids and contrasted with equal volumes of 4 % (w/v) uranyl-acetate pH 4.0 and 0.1 M oxalic acid. Samples were embedded in a solution of 9 parts 4 % (v/v) uranyl acetate and 1 part 2 % (v/v) methyl cellulose. All reagents were purchased from Positech (USA). Grids were viewed using a Hitachi H.7-7500 transmission electron microscope at 70K x view and images captured using Digital micrograph™ 1.71.38 (Gatan Inc.). Image analysis was performed using ImageJ v1.47n (National Institutes of Health, USA).

Polyacrylamide gel electrophoresis of OMVs

Heterogeneous or fractionated OMV preparations (10 μg) were separated using Novex® 10-20 % Tris-Glycine gels (Life Technologies, CA, USA). Proteins contained within OMVs were visualized by staining in Instant Blue™ (Expedeon Ltd., Cambridgeshire, UK).

Proteomic analysis of OMVs

OMV preparations (6 μg) were reduced in 2.5 mM DTT at 60^0C for 30 minutes followed by alkylation with 10 mM Iodoacetamide for 30 minutes in the dark at room temperature. Following alkylation, 0.5 μg Trypsin (Promega corp., Madison, WI, USA) in 20mM Ammonium bicarbonate was added and the samples were incubated at 37^0C overnight. Tryptic digests were analyzed by LC-MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with an RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Samples were injected onto a PepMap100 2cm trap column with loading buffer (2 % Acetonitrile, 0.1 % Formic acid) at a flow rate of 15 μl/minute. The peptides were eluted and separated with a Thermo RSLC PepMap100, 75μm id, 100Å pore size, reversed phase nano column with a 30 minute gradient of 90% buffer A (0.1 % Formic acid) to 30 % buffer B (80 % Acetonitrile 0.1 % formic acid) in 20 minutes and then to 40% buffer B, to a complete 30 minute gradient, at a flow rate of 300 nl/minute. The eluant was nebulized and ionized using a Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900V. Peptides were selected for MS/MS analysis in Full MS/dd-MS^2 (TopN) mode with the following parameter settings: TopN 10, resolution 17500, MSMS AGC target 1e5, 60ms Max IT, NCE 27 and 3 m/z isolation window. Underfill ratio was at 10 % and dynamic exclusion was set to 15 seconds. Data was processed using Proteome Discoverer V1.4 (Thermo Fisher Scientific) and searched against a custom database downloaded from the National Centre for Biotechnology Information (NCBI) ftp site using the MS Amanda search engine. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 15 ppm; peptide fragment tolerance, ± 0.2 Da; peptide charge, 2+, 3+ and 4+; static

modifications, carbamidomethyl; Dynamic modification, oxidation (Met). Low and medium confidence peptides were filtered with at least 0.02 FDR (high confidence).

IL-8 ELISA

Inhibition of entry was indirectly determined by the measurement of IL-8 levels in the supernatants using the BD OptEIA human IL-8 ELISA kit as per the manufacturer's instructions (BD Biosciences, CA, USA).

Statistical analysis

Error bars indicate the mean $\pm$ standard error of the mean (SEM). Fluorescence microscopy experiments were analyzed by One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test. IL-8 responses were analyzed using the student's t-test. Statistical analyses were performed using Prism software. Differences were considered significant when $*P < 0.05$, $**P < 0.01$.

RESULTS

A heterogeneous population of OMVs enter host cells predominantly via macropinocytosis and caveolin dependent endocytosis.

In this study we sought to elucidate the precise molecular mechanism of OMV entry into human cells. To do this, we treated human gastric (AGS) and embryonic kidney (HEK293) cells using a panel of chemical inhibitors to selectively block specific endocytosis and intracellular degradation pathways, prior to the addition of

fluorescently labeled *H. pylori* OMVs. We initially confirmed the viability of AGS and HEK293 cells post treatment with pre-defined concentrations of inhibitors of the endocytosis pathways (Fig. S1 A, B respectively). Cells were treated for 30 minutes with either of the following inhibitors: cytochalasin D or nocodazole, to block macropinocytosis, dynasore monohydrate to inhibit dynamin dependent endocytosis, valinomycin to specifically block clathrin mediated endocytosis, or bafilomycin A1 to specifically inhibit phagolysosome degradation (38). Sodium azide was used as a positive control for cell death. Cell viability of inhibitor-treated and control AGS and HEK293 cells was determined using the CellTiter-Glo assay (Fig. S1). Our results demonstrated that both AGS and HEK293 cells remained viable 4 hours post treatment with specific inhibitors of the macropinocytosis or endocytosis pathways, compared to azide control cells (Fig. S1).

We next sought to elucidate the endocytic mechanisms utilized by fluorescently-labeled *H. pylori* OMV to enter host cells. Any fluorescence associated with extracellular OMVs was excluded from our analysis using the cell impermeant dye, trypan blue. The effectiveness of trypan blue quenching of fluorescence was confirmed in AGS cells that had been first co-cultured with DiO-fluorescently labeled *H. pylori* OMVs, then permeabilized using 0.01 % Triton-X and treated with trypan blue. Effective quenching of fluorescence was confirmed and quantified using both fluorescence microscopy and flow cytometry (Fig. S2). OMV-stimulated cells that were permeabilized and treated with trypan blue had negligible detectable fluorescence, compared with OMV-stimulated control cells, as determined by fluorescence microscopy (Fig. S2 A-D) and flow cytometry analysis (Fig. S2E).

The mechanisms of *H. pylori* OMV entry into epithelial cells was determined using cells that were pre-treated with chemical inhibitors followed by co-culture with

DiO-labeled OMVs for 4 hours. OMV internalization was visualized and quantified using fluorescence microscopy (Fig. 1A, B). Inhibition of macropinocytosis and dynamin dependent endocytosis, using cytochalasin D or dynasore monohydrate, respectively, resulted in significant reductions in OMV-associated intracellular fluorescence in AGS cells, compared with untreated cells (Fig. 1A and B, $P<0.05$). A similar trend was observed using the macropinocytosis inhibitor nocodazole, however, the reduction in OMV-associated fluorescence was not statistically significant. As the small GTPase dynamin is essential for both clathrin and caveolin mediated endocytosis, we used valinomycin, which specifically blocks clathrin mediated endocytosis to distinguish between these two mechanisms. A slight, albeit not significant, reduction of OMV-associated fluorescence was observed when cells were pre-treated with valinomycin (Fig. 1A, B). Similarly, treatment of cells with bafilomycin, which inhibits phagolysosomal degradation, reduced the levels of intracellular OMV-fluorescence although this reduction was not statistically significant. This finding shows that inhibition of phagolysosomal degradation does not prevent degradation of intracellular OMVs, suggesting that intracellular OMVs may undergo multiple pathways of intracellular degradation. Collectively these data suggest that a heterogeneous population of OMVs enter host cells predominantly via macropinocytosis and caveolin mediated endocytosis, with a minor role for clathrin.

We, and others, have previously demonstrated that OMV entry into non-phagocytic epithelial cells results in the production of the pro-inflammatory cytokine, IL-8 (10, 39). Therefore, we used IL-8 production as a measure of OMV-induced responses in AGS (Fig. 1C) and HEK293 cells (Fig. S3). Pre-treatment of both AGS and HEK293 cells with cytochalasin, nocodazole and dynasore significantly reduced IL-8 production in response to OMV stimulation (Fig. 1C, Fig. S3, respectively). In

addition, inhibition of clathrin mediated endocytosis with valinomycin resulted in a significant reduction of IL-8 in response to *H. pylori* OMV stimulation, indicating that a minor inhibition of OMV entry (Fig. 1B) may have profound effects on OMV-induced inflammation. (Fig. 1C, $P<0.01$).

Collectively, these findings demonstrated that a heterogeneous population of OMVs enter non-phagocytic host cells predominantly via macropinocytosis and dynamin dependent caveolin mediated endocytosis, and to a lesser extent via clathrin mediated endocytosis. Furthermore, inhibition of all pathways had a direct effect on OMV-induced inflammatory responses, indicating that a small alteration in the number of intracellular OMVs may have profound effects on the development of IL-8 responses in host cells.

OMVs of different sizes enter host cells via specific mechanisms.

Gram negative bacterial OMVs range in size from 20 to 300 nm (reviewed in (1)). We used sucrose gradient ultracentrifugation to separate a heterogeneous population of *H. pylori* OMVs into two main populations, differing in both size and density (10). Thirteen fractions were collected in total, of which fraction 6 contained a homogeneous population of small OMVs of approximately 20 nm in diameter, as determined by TEM (Fig. 2A, C). Fraction 12 contained two distinct populations of OMVs, being approximately 50 nm and 100 nm in diameter, respectively (Fig. 2B, 2C). Quantification of each distinct population of OMVs in fraction 12 revealed that 83% of OMVs were approximately 50 nm in diameter, whereas 17 % were approximately 100 nm in size. We used these fractionated OMVs to determine whether OMV size may define the mechanism of entry into non-phagocytic host cells.

Small OMVs of approximately 20 nm were fluorescently labeled with DiO and added to AGS cells that had been treated with inhibitors of endocytosis and macropinocytosis, or non-treated cells. Extracellular OMV-associated fluorescence was quenched using trypan blue and the number of internalized OMVs was quantified using fluorescence microscopy (Fig. 3A, B). Our findings revealed that the internalization of small OMVs occurred via macropinocytosis, clathrin and caveolin mediated endocytosis (Fig. 3A, B, $P<0.01$). Furthermore, bafilomycin treatment did not decrease the level of intracellular OMV-associated fluorescence, indicating that inhibition of the phagolysosomal pathway prevented the degradation of internalized small OMVs. These findings suggest that OMVs of approximately 20 nm enter host cells in a macropinocytosis, clathrin and caveolin dependent manner, and once internalized, these vesicles undergo phagolysosomal degradation.

Larger OMVs have a more restricted pathway of entry into host cells.

Analysis of the mechanisms of entry of larger OMVs contained in fraction 12 revealed that these OMVs use a more restricted route of entry into host cells, compared to smaller OMVs (Fig. 4). Fluorescence microscopy revealed that inhibition of macropinocytosis with cytochalasin or nocodazole significantly reduced entry of larger fluorescently-labeled OMVs (>50 nm) into host AGS cells (Fig. 4A, B, $P<0.01$). Furthermore, dynasore, and not valinomycin, reduced the number of intracellular larger OMVs, indicating that large OMVs enter AGS cells in a dynamin and caveolin dependent, but clathrin independent manner (Fig. 4A, B). In addition, bafilomycin treatment did not inhibit intracellular degradation of large OMVs, suggesting that large OMVs do not significantly enter the phagolysosomal

degradation pathway (Fig. 4). Therefore, we conclude that larger *H. pylori* OMVs have more restricted mechanisms of entry into host cells, involving both macropinocytosis and caveolin dependent pathways (Fig. 4 A, B).

OMV size predetermines their protein content

It has been reported that bacteria may selectively package protein cargo into OMVs (40). However, it is not known if OMV size predetermines their protein content or cargo. Therefore, we sought to determine if there was a size-dependent difference in protein composition of *H. pylori* OMVs. For this, we performed SDS-PAGE analysis of small, large and heterogeneous OMV populations. As shown in Figure 5A, the smaller *H. pylori* OMVs within fraction 6 contained fewer proteins, when compared with both large OMVs, contained within fraction 12, and a heterogeneous population of OMVs. Indeed, these findings corroborate our preliminary finding identifying that fewer proteins were contained within smaller OMVs (10). This finding was also confirmed by LC-MS/MS proteomic analysis, whereby a total of 28 *H. pylori* specific proteins were contained within small OMVs, compared with a total of 137 proteins contained within large OMVs (Fig. 5B; Table S1, Table S2). Of all of the proteins identified within both small and large OMVs, 113 were unique to large OMVs, 4 proteins were unique to small OMVs and 24 proteins were common to both sized OMV preparations (Fig. 5B). Interestingly, proteins associated with *H. pylori* survival or virulence were common to both small and large OMVs, including: urease A and B subunits, neutrophil activating protein (NAP), vacuolating cytotoxin (VacA) and the porin HopA (Table 1). Larger OMVs contained many of the known *H. pylori* adhesins, such as SabA, BabA, iron regulated proteins,

the Hop family of outer membrane proteins and numerous flagella basal and hook proteins (Table S2), which were absent from smaller OMVs (Table S1). The 4 proteins exclusively contained within small OMVs were predominately associated with metabolism, and not virulence or adhesion (Table S1). Collectively, this proteomic analysis confirmed that larger OMVs contain more proteins within them compared to smaller OMVs, and that most of the *H. pylori* adhesins are contained within these protein-rich large OMVs. However, both small and large populations of OMVs contained many known virulence determinants, suggesting that both sized OMVs play a role in pathogenesis.

DISCUSSION

OMVs are produced by all Gram negative bacteria as part of their normal growth and have been reported to play a role in pathogenesis, bacterial cell communication and biofilm formation (17, 41-43). The ability of OMVs from various bacteria to enter host cells has been well documented. However, the specific mechanisms of OMV entry into non-phagocytic host cells via various groups differ and as yet, the precise mode of entry remains unclear (1, 10, 26, 29-31, 33, 34, 44, 45). Recently, Gram positive bacteria including *Staphylococcus, Bacillus* and *Mycobacteria* species have also been reported to produce membrane vesicles that function to enter cells and induce host cell death (45-48), highlighting the likely importance of bacterial membrane vesicle production in essentially all types of bacterial infections. The implications of bacterial membrane vesicles in pathogenesis emphasises the need to elucidate the precise mechanism(s) whereby these vesicles

enter host cells, resulting in the initiation of bacterial specific immune responses in the host.

The aim of the present study was firstly, to characterize the specific mode of entry of *H. pylori* OMVs into host epithelial cells, and secondly, to determine if vesicle size played a role in determining the mode of entry and OMV protein composition. Using chemical inhibition of the three main pathways of endocytosis: macropinocytosis, clathrin and caveolin mediated endocytosis, we found that entry of a heterogeneous population of OMVs into non-phagocytic human epithelial cells was significantly decreased when macropinocytosis and caveolin mediated endocytosis were inhibited (Fig. 1). We also established a minor, albeit not statistically significant, role for clathrin in heterogeneous OMV entry into human AGS cells. However, when examining the effect of inhibition of macropinocytosis, clathrin and caveolin dependent endocytosis on OMV-induced IL-8 responses, we discovered that all 3 pathways were required for maximal induction of IL-8 production in response to OMV stimulation. This is the first report identifying that a small reduction in OMV entry may have a profound effect on the host inflammatory response. Indeed, this data was further validated by measurement of IL-8 production in HEK293 cells treated with inhibitors and subsequently stimulated with OMVs (Fig. S3). Collectively, our findings suggest that the contradictory findings of numerous studies describing various mechanisms of OMV entry into host cells may all be correct. We speculate that variations in OMV size may be a reason for the discrepancies in the mechanisms of OMV host cell entry reported by various groups. Therefore, we conclude that OMV entry into non-phagocytic cells can occur via multiple mechanisms.

Several studies indicate that particle size plays a crucial role in determining the pathways of entry utilized by OMVs, and subsequently their intracellular fate (49,

50). Indeed, in our previous study, we identified that OMVs less than 100 nm in diameter induced higher levels of NF-κB activity than larger OMVs, suggesting that these smaller OMVs may be more efficient at entering host epithelial cells and initiating pro-inflammatory responses (10). To determine the role of OMV size in host cell entry, we used a previously reported method to separate OMVs according to size and density (10). Using this method, we separated a heterogeneous population of *H. pylori* OMVs into two populations that were enriched for either small (approximately 20 nm) or large (>50 nm) OMVs. Using fluorescence microscopy, we found that a homogeneous population of small OMVs entered host cells via all three mechanisms of endocytosis (Fig. 3), whereas a population of large OMVs only entered via macropinocytosis and caveolin mediated endocytosis (Fig. 4). We previously identified that small OMVs were more immunogenic than larger OMVs, as indicated by a greater induction of NF-κB activity, suggesting that these OMVs may be more efficient at entering host cells (10). Indeed, we have shown that these small OMVs are able to more efficiently enter host cells via each of the three main mechanisms of endocytosis, potentially assisting their pro-inflammatory role, whereas larger OMVs have a more restricted route of entry into non-phagocytic host cells.

A recent study of OMV formation in *Acinetobacter baumannii* demonstrated that small OMVs were formed at the bacterial distal ends where the peptidoglycan layer of the bacteria was thicker, whereas large OMVs formed at sites of internal bacterial cell division (51). In addition the authors found that small OMVs formed primarily during early log phase cultures, whereas large OMVs were formed during later stages of *Acinetobacter baumannii* growth (51). This finding may explain the differences observed between various groups examining entry of OMVs into host cells. For example, Kesty *et al.,* showed that enterotoxigenic *Escherichia coli* OMVs

interacted with host cell caveolin, and that inhibition of clathrin mediated endocytosis had no effect on vesicle uptake (1). However, clathrin mediated endocytosis was reported by others to be required for internalization of *H. pylori* OMVs into host cells (26). We identified that a heterogeneous population of OMVs enter via a variety of mechanisms and that OMV size predetermines the route of OMV entry into host cells. Each of the aforementioned studies isolated OMVs from bacterial cultures at different growth stages, which may result in a difference in the sizes of OMVs contained within each heterogeneous OMV preparation used, and hence the discrepancy in the findings. Therefore, we suggest that determining the size of OMVs contained within an OMV preparation is vital and may account for the differences seen in the modes of OMV entry between these and other studies.

Our data suggest that OMV size may also regulate the mechanism of intracellular degradation of OMVs. Bafilomycin A1 is a macrolide antibiotic that inhibits the fusion between autophagosomes and lysosomes, thereby preventing degradation of internalized particles (52). Surprisingly, we found that fewer large OMVs were detected in cells treated with bafilomycin A1 (Fig. 4). Whereas, small OMV-associated fluorescence was maintained following pre-treatment with bafilomycin A1, suggesting that small OMVs are intracellularly processed by the phagolysosomal degradation pathway. A similar phenomenon was observed by Lee *et al*., who suggested that bafilomycin treatment was required for processing of intracellular bacterial peptidoglycan (38), which we have reported to be present within OMVs (10). It is therefore possible that small OMVs enter host cells and are incorporated into autophagosomes, whereas large OMVs may be processed through a different pathway and exit early endosomes prior to formation of the autophagosomes.

The mechanisms of intracellular trafficking and fate of different sized OMVs form the basis of our future studies.

Another significant difference between small and large OMVs is their protein composition. Smaller OMVs of approximately 20 nm in size contained significantly fewer proteins within them, when compared to larger OMVs. In addition, we found that larger *H. pylori* OMVs contained a prevalence of adhesion proteins that were absent from smaller OMVs, which may facilitate their entry into host cells. We identified 24 proteins common to both small and large OMVs; these were mostly proteins associated with virulence, including the vacuolating toxin (VacA), demonstrating a pathogenic role for OMVs of various sizes. An earlier study reported that OMVs containing the vacuolating toxin (VacA) were less dependent on clathrin for entry, when compared with VacA- OMVs, indicating that toxin-containing OMVs may enter host cells by more than one mechanism (26). To our knowledge, no studies have been performed regarding the amount of VacA toxin associated with OMVs from different strains of *H. pylori*, or OMVs of different sizes, and it is plausible that different sized OMVs may contain varying amounts of toxin, which may also facilitate their entry via receptor mediated endocytosis.

In summary, we believe that these findings have for the first time clarified an important issue within field of OMV research, by defining the mechanisms of OMV entry into host cells and identifying a previously unknown dependency of OMV size on their intracellular degradation and protein composition. More specifically, we showed that a heterogeneous sized population of OMVs enter host cells via each of the three main mechanisms of endocytosis: macropinocytosis, caveolin and clathrin mediated endocytosis, to various extents. We demonstrated that a small reduction in OMV entry had a profound effect on reducing the pro-inflammatory responses

induced in epithelial cells. Further examination of OMVs that had been separated based on their size and density revealed a previously unknown dependency of OMV size on their mode of entry into host cells. We found that small OMVs enter host cells via all 3 mechanisms of endocytosis, however, large OMVs do not require clathrin for cellular entry. We believe that the discrepancies in the literature regarding the mechanisms of OMV entry into host cells are most likely due to the use of heterogeneous populations of OMVs, ranging in different sizes. Moreover, we suggest that larger OMVs are processed in a different manner to the population of small sized OMVs. Further examination of the impact of particle size on the mechanisms of OMV degradation forms the basis of our future research. Surprisingly, proteomic analysis of small and large OMV populations revealed that these OMVs differ in the quantity and types of proteins contained within them. Collectively, these findings have fundamental and significant implications that should be considered when examining the role of OMVs in pathogenesis, their protein content, and ultimately their use as vaccines against bacterial infections in humans.

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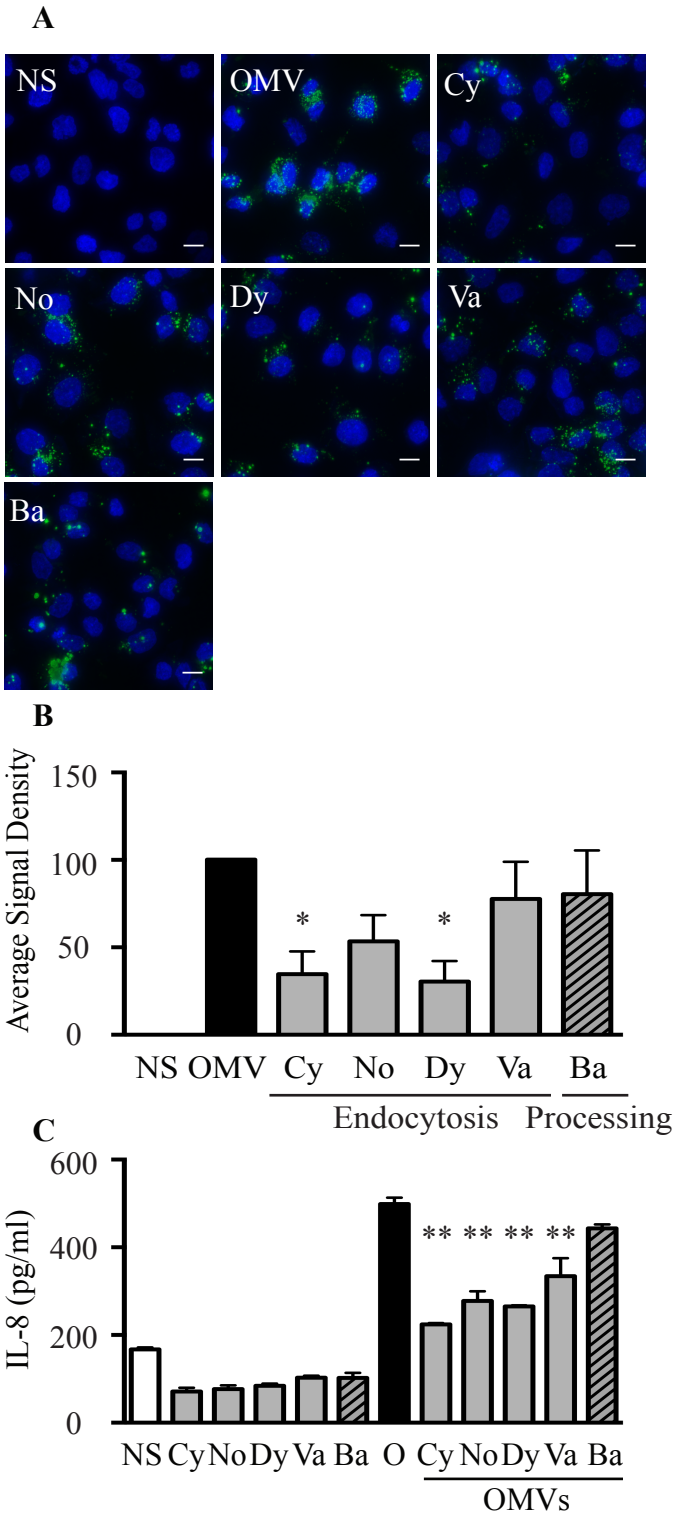

A
NS
OMV
Cy
No
Dy
Va
Ba
B
Average Signal Density
150
100
50
0
*
*
NS OMV Cy No Dy Va Ba
Endocytosis
Processing
C
IL-8 (pg/ml)
600
400
200
0
** ** ** **
NS Cy No Dy Va Ba O Cy No Dy Va Ba
OMVs

FIG 1. Heterogeneous populations of OMVs enter host cells via macropinocytosis and dynamin dependant caveolin mediated endocytosis. (A) AGS cells were either left non-stimulated (NS), co-cultured with DiO-labeled (green) heterogeneous sized OMVs (OMV), or pre-treated with one of the following inhibitors prior to co-culture with OMVs; cytochalasin D (Cy), nocodazole (No), dynasore (Dy), valinomycin (Va) or bafilomycin (Ba). Nuclear DNA was stained using DAPI (blue) to allow enumeration of cells. Extracellular fluorescence was quenched with trypan blue. Scale bar indicates 10 μm. Images are representative of 10 fields of view from 3 replicate experiments. (B) The average signal density of green fluorescence, indicating intracellular OMVs, in AGS cells was compared between cells cultured with OMVs alone (OMV), or cells treated with inhibitors and subsequently stimulated with fluorescently-labeled OMVs. Values were normalized to those of the OMV alone group (OMV). Data are means of 3 independent experiments in which >100 cells were counted per treatment within each experiment. Error bars indicate ± standard errors of the means. * $P< 0.05$ compared to OMV control group (ANOVA). (C) IL-8 production by AGS cells that were non-stimulated (NS), stimulated with OMVs alone as a control (O), pre-treated with chemical inhibitors alone (grey bars), or treated with chemical inhibitors prior to co-culture with OMVs was determined. IL-8 within the culture supernatants of cells 24 hours after stimulation with OMVs was determined by ELISA. Data are representative of three independent experiments Error bars indicate ± standard errors of the means ** $P<0.01$ (Students t test).

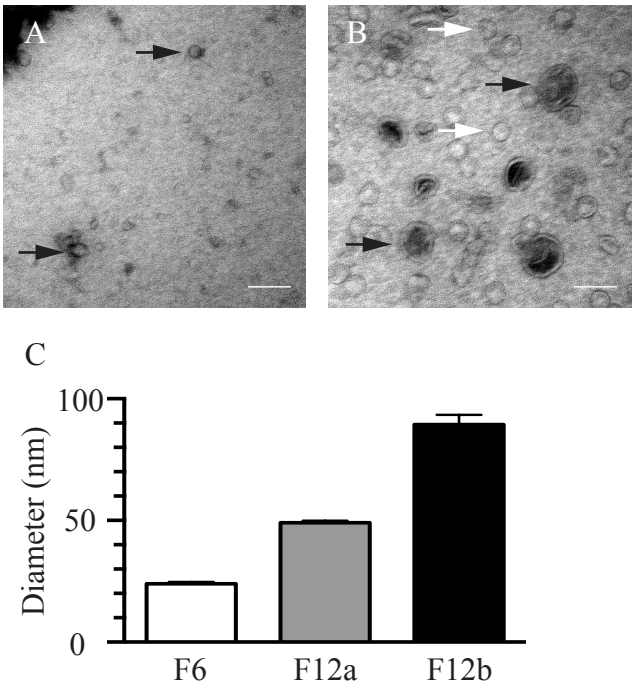


FIG 2 Heterogeneous sized populations of OMVs purified using sucrose gradient purification. (A) Transmission electron micrographs of small OMV present in fraction 6 and (B) larger OMVs present in fraction 12. (A) Fraction 6 contained a homogeneous population of small OMVs, approximately 20 nm in size, indicated by the black arrows. (B) Fraction 12 contained two distinct populations of OMVs ranging between 50 nm (white arrows) and 100 nm (black arrows) in diameter. Scale bar represents 100 nm. (C) The diameters (nm) of OMVs contained within each fraction were measured for >100 OMVs per fraction. OMVs contained within fraction 6 were approximately 20 nm in diameter. Whereas, two distinct populations were found in fraction 12 of approximately 50 nm (F12a, white arrows) and 100 nm (F12b, black arrows) in diameter. Error bars indicate ± standard errors of the means.

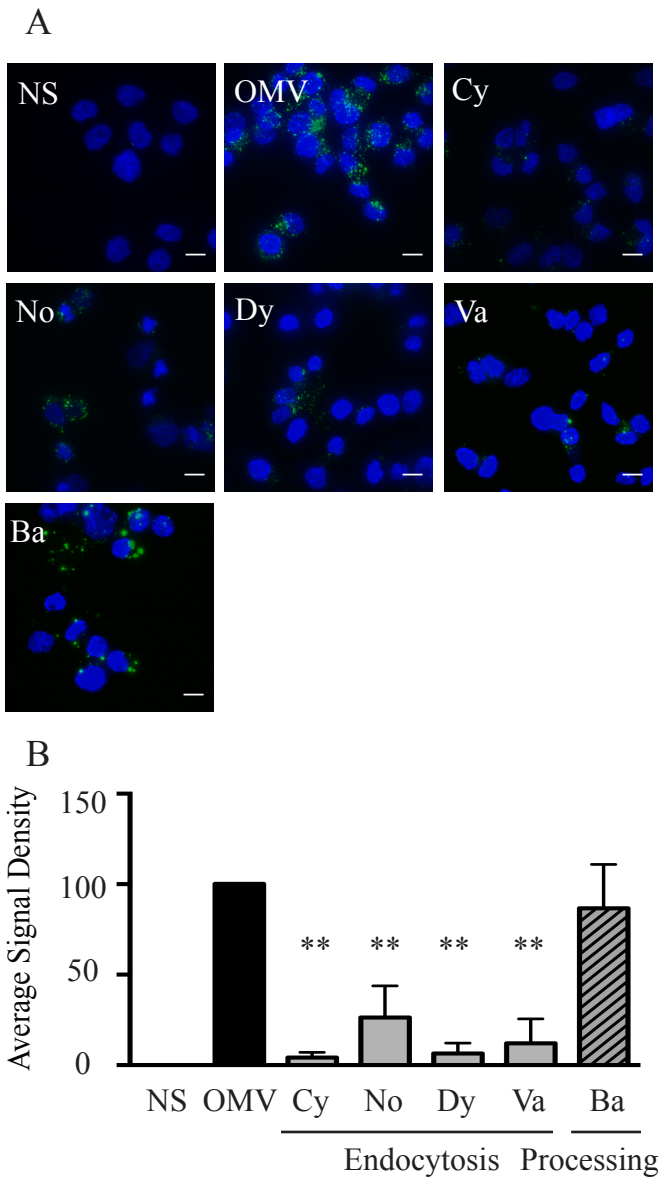


FIG 3 Small OMVs enter host cells via endocytosis and macropinocytosis. (A) AGS cells were either left non-stimulated (NS), co-cultured with DiO-labeled (green) homogeneous small sized OMVs (OMV), or pre-treated with one of the following inhibitors prior to co-culture with small OMVs; cytochalasin D (Cy), nocodazole (No), dynasore (Dy), valinomycin (Va) or bafilomycin (Ba). Nuclear DNA was stained using DAPI (blue) to allow enumeration of cells. Extracellular fluorescence was quenched with trypan blue. Scale bar indicates 10μm. Images are representative from 10 fields of view from 3 replicate experiments. (B) The average signal density of green fluorescence, indicating intracellular OMVs, in AGS cells was compared between cells cultured with OMVs alone (OMV), or cells treated with inhibitors and subsequently stimulated with fluorescently-labeled OMVs. Values were normalized to those of the OMV alone group (OMV). Data are means of 3 independent experiments in which >100 cells were counted per treatment within each experiment. Error bars indicate ± standard errors of the means. * *P< 0.01 compared to OMV control group (ANOVA).

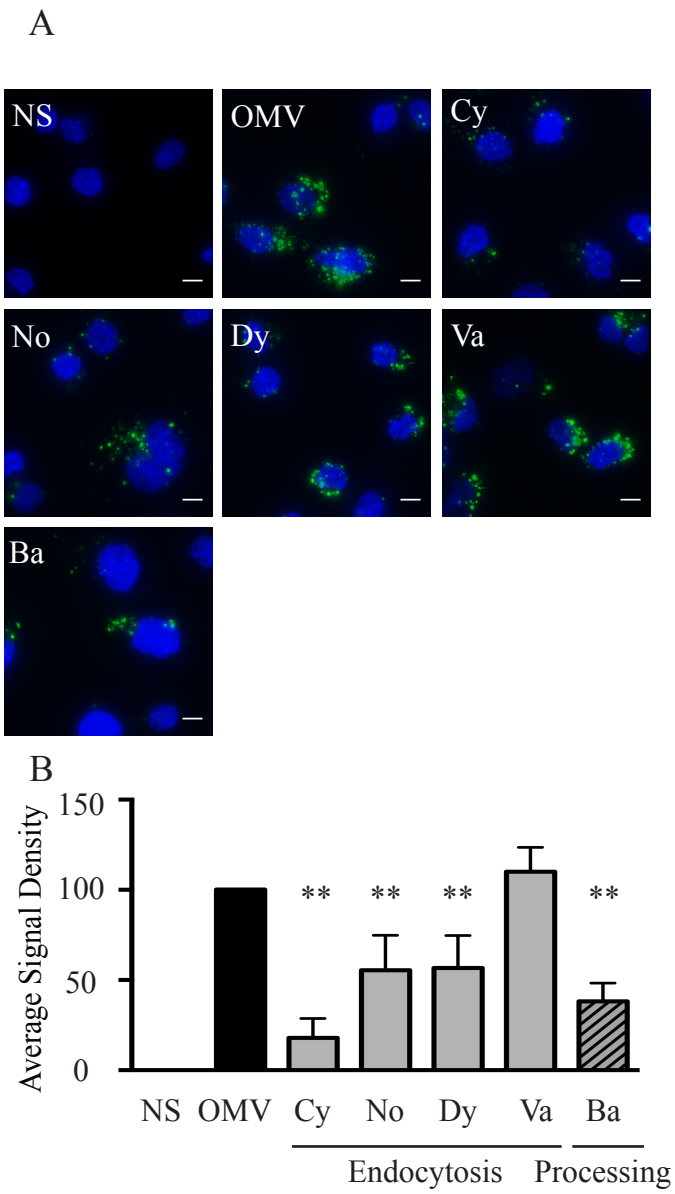


FIG 4. Large OMVs enter host cells via macropinocytosis and caveolin mediated endocytosis but not clathrin mediated endocytosis. (A) AGS cells were either left non-stimulated (NS), co-cultured with DiO-labeled (green) large sized OMVs (OMV), or pre-treated with one of the following inhibitors prior to co-culture with large OMVs; cytochalasin D (Cy), nocodazole (No), dynasore (Dy), valinomycin (Va) or bafilomycin (Ba). Nuclear DNA was stained using DAPI (blue) to allow enumeration of cells. Extracellular fluorescence was quenched with trypan blue. Scale bar indicates 10μm. Images are representative from 10 fields of view from 3 replicate experiments. (B) The average signal density of green fluorescence, indicating intracellular OMVs, in AGS cells was compared between cells cultured with OMVs alone (OMV), or cells treated with inhibitors and subsequently stimulated with fluorescently-labeled OMVs. Values were normalized to those of the OMV alone group (OMV). Data are means of 3 independent experiments in which >100 cells were counted per treatment within each experiment. Error bars indicate ± standard errors of the means. ** $P < 0.01$ compared to OMV control group (ANOVA).

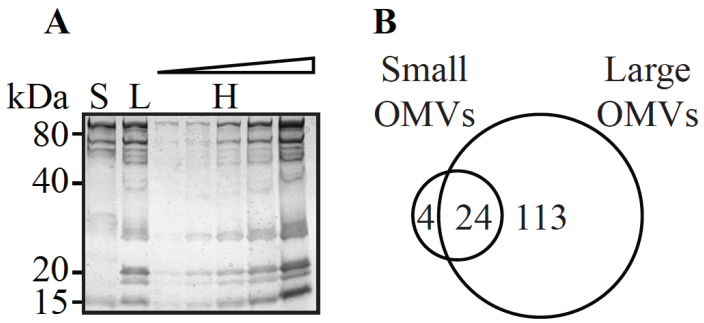


FIG 5 OMVs of different sizes vary in their protein content. (A) Instant-blue stained tris-glycine gel showing protein profiles of small (S), large (L) and heterogenous (H) OMVs, the latter in increasing concentrations. (B) Venn Diagram of proteins detected within small (approximately 20 nm) and large (> 50 nm) OMVs. LC MS/MS analysis determined that 137 proteins were exclusively contained within large OMVs, whereas 4 proteins were unique to small OMVs. A total of 24 proteins were found in both small and large OMVs.

TABLE 1 ***H. pylori*** **proteins common in both small and large OMVs (Fraction 6 and 12)**

Description	Gene No.	Score Small OMVs	Coverage Small OMVs	Score Large OMVs	Coverage Large OMVs
Outer Membrane Proteins					
outer membrane protein HopA (omp6)	HP0229	125.13	2.90	12690.51	17.18
thioredoxin	HP1548	166.95	14.42	1183.40	34.62
peptidoglycan associated lipoprotein precursor (omp18)	HP1125	205.39	10.61	1809.68	17.88
Metabolism					
urease subunit alpha	HP0073	13062.19	56.72	68.49	8.40
gamma-glutamyltranspeptidase (ggt)	HP1118	693.31	5.82	7237.48	16.93
urease subunit beta	HP0072	26367.78	52.55	2594.83	14.76
iron(III) ABC transporter periplasmic iron-binding protein (ceuE)	HP1562	363.55	7.21	6586.97	37.24
carbonic anhydrase	HP1186	1372.04	18.81	5111.16	34.65
catalase-like protein	HP0485	123.63	4.14	3216.80	30.89
iron(III) ABC transporter periplasmic iron-binding protein (ceuE)	HP1561	140.59	2.99	2559.96	18.21
catalase	HP0875	7584.68	47.13	26552.56	54.26
Post translational modification, protein turnover, chaperones					
chaperonin GroEL	HP0010	5672.37	35.35	373.71	4.58
bifunctional methionine sulfoxide reductase A/B protein	HP0224	1826.54	14.48	12539.26	31.20
serine protease (htrA)	HP1019	141.34	2.48	6565.50	25.51
alkyl hydroperoxide reductase (tsaA)	HP1563	503.19	5.56	413.07	14.65
Other					
neutrophil activating protein (napA) (bacterioferritin)	HP0243	4741.96	38.19	376.22	16.67
hypothetical protein HP0231	HP0231	400.83	3.77	8592.37	38.49
hypothetical protein HP0305	HP0305	142.05	5.98	2442.19	38.04
hypothetical protein HP1454	HP1454	234.35	4.95	9609.87	35.64
hypothetical protein HP0129	HP0129	293.54	7.09	3695.28	24.82
hypothetical protein HP0721	HP0721	683.38	18.42	6681.98	19.08
vacuolating cytotoxin	HP0887	1510.21	3.33	7883.58	15.89
neuraminyllactose-binding hemagglutinin homolog (hpaA)	HP0410	227.63	4.82	4096.93	14.86
hypothetical protein HP1286	HP1286	613.71	11.54	4171.54	17.58

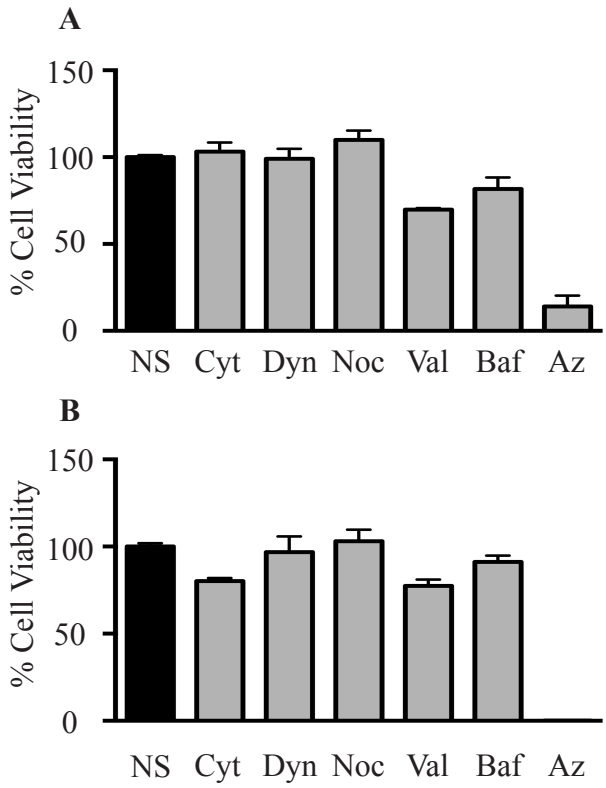


Figure S1: AGS and HEK cells treated with inhibitors of endocytosis remain viable. AGS (A) and HEK (B) cells were treated with either of the inhibitors cytochalasin D (Cyt), dynasore (Dyn), nocodazole, (N), valinomycin (Val), bafilomycin (Baf), or sodium azide (Az) as a positive control of cell death, for 30 minutes. Cells were subsequently washed and further incubated for 4 hours. Cell Viability was measured using the CellTiter-Glo assay. Percentage of cell viability was determined by comparing treated cells to non-treated control cells. Data are representative of two individual experiments. Data are represented as mean ± SEM.

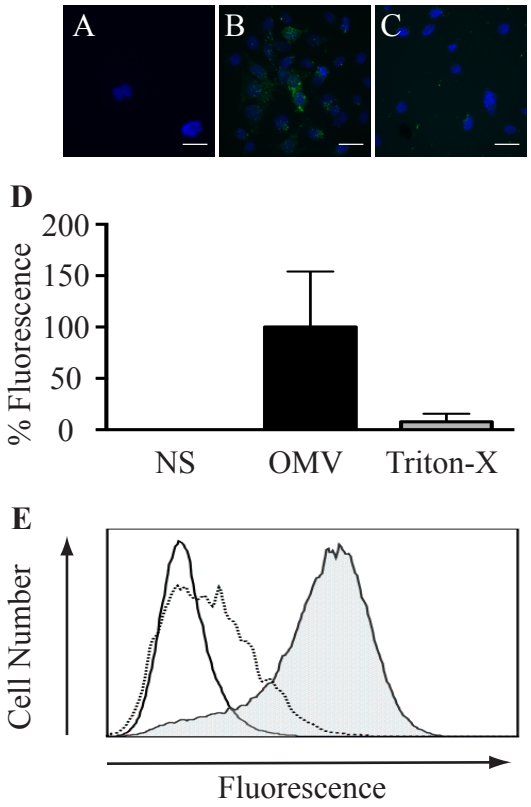


Figure S2: OMV-associated fluorescence is effectively quenched using trypan blue. AGS cells were incubated with 50 μg/ml of DiO fluorescently-labeled OMVs (B, C) or left non-stimulated (A). Four hours later, cells were permeabilized with Triton-X (B) or left untreated (A, C) and fluorescence was quenched using trypan blue (A, B, C). Cells were subsequently imaged using fluorescence microscopy (A-C). Scale bar indicates 10 μm. (D) The percentage of green fluorescence in samples A-C was quantified using confocal microscopy. The average signal density of DiO-OMV fluorescence (green) was determined. Values were normalized to those of the OMV group. Data are mean fluorescence in >100 cells per treatment. Error bars indicate ± standard errors of the means (SEM). (E) AGS Cells that were either non-stimulated (NS, black line), or

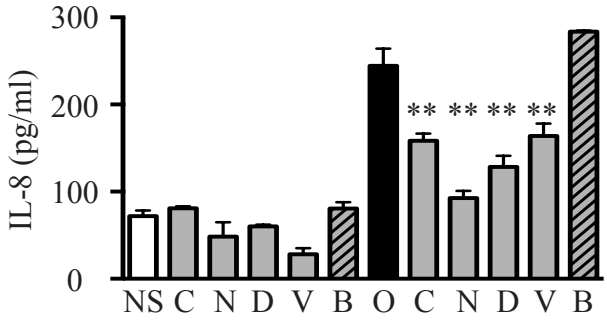


Figure S3. A heterogeneous population of OMVs enter host cells via macropinocytosis, clathrin and caveolin mediated endocytosis. HEK cells were left non-treated (NS; open bar), stimulated with OMVs (O; black bar) or pre-treated with inhibitors (grey bars) cytochalasin D (C), nocodazole (N), dynasore (D), valinomycin (V), bafilomycin (B) prior to co-culture with heterogeneous sized OMVs. IL-8 responses were determined 24 hours later by ELISA. Data are representative of three independent experiments performed in triplicate. Shown are the mean ± standard errors of the means (SEM) (determined in triplicate). ** $P<0.01$.

TABLE S1 *H. pylori* OMV proteins detected in fraction 6

Accession #	Description	Gene No.	Score	Coverage	# of peptides	kDa	Calculated pI
	Metabolism						
NP_207952	flavodoxin (fldA)	HP1161	969.27	17.07	2	17.5	4.59
NP_207309	glutamine synthetase (glnA)	HP0512	1831.71	14.55	6	54.5	6.13
NP_207447	nonheme iron-containing ferritin (pfr)	HP0653	186.01	13.77	1	19.3	5.67
NP_206828	type II citrate synthase	HP0026	188.78	4.23	1	48.3	7.88

TABLE S2 ***H. pylori*** **OMV proteins detected in fraction 12**

Accession #	Description	Gene No.	Score	Coverage	# peptides	kDa	Calculated pI
Major outer membrane protein family							
NP_207500	outer membrane protein (omp15)	HP0706	2671.51	32.23	5	29.9	8.87
NP_207968	outer membrane protein (omp27)	HP1177	35410.35	30.11	13	69.6	9.11
NP_208292	outer membrane protein HorK (omp32)	HP1501	4858.85	24.74	6	42.9	9.41
NP_208260	outer membrane protein HorJ (omp31)	HP1469	3672.96	23.39	5	28.1	9.51
NP_207704	outer membrane protein AlpA, HopC (omp20)	HP0912	3424.75	22.14	5	55.9	9.10
NP_207025	outer membrane protein HopM (omp5)	HP0227	4680.15	19.83	8	75.7	8.40
NP_207516	outer membrane protein (omp16)	HP0722	10128.12	16.45	10	67.0	9.20
NP_207689	outer membrane protein BabB, HopT (omp19)	HP0896	7863.94	15.11	6	77.6	8.50
NP_207519	outer membrane protein SabA, HopP (omp17)	HP0725	3960.35	12.72	5	69.4	9.07
NP_207270	outer membrane protein HorE (omp11)	HP0472	402.79	12.37	2	20.9	9.13
NP_206927	outer membrane protein HorB (omp4)	HP0127	386.82	9.79	2	31.9	9.54
NP_207947	outer membrane protein HopI (omp25)	HP1156	3703.52	9.77	7	76.3	9.06
NP_207052	outer membrane protein HopG (omp8)	HP0254	1512.42	8.35	4	47.5	9.19
NP_207050	outer membrane protein HopF (omp7)	HP0252	494.92	8.21	2	53.3	9.26
NP_207465	outer membrane protein HorF (omp14)	HP0671	452.71	3.33	1	30.3	9.11
NP_207705	outer membrane protein AlpB, HopB (omp21)	HP0913	2869.39	2.65	1	57.0	9.17
NP_206827	outer membrane protein HopD (omp2)	HP0025	167.16	1.55	1	77.6	9.10
NP_207115	outer membrane protein HopU (omp9)	HP0317	5264.76	9.53	4	81.1	8.50
NP_206811	outer membrane protein HopZ	HP0009	3073.13	4.42	2	69.2	8.92
Iron-regulated outer membrane proteins							
NP_208303	iron-regulated outer membrane protein (frpB)	HP1512	5230.75	9.92	7	97.3	9.01
NP_207480	iron(III) dicitrate transport protein (fecA)	HP0686	409.44	4.30	2	87.6	9.47
NP_208191	iron(III) dicitrate transport protein (fecA)	HP1400	302.90	1.66	1	94.8	9.04
Efflux pump outer membrane proteins and other outer membrane proteins							
NP_207632	outer membrane protein P1 (ompP1)	HP0839	2280.71	11.75	6	63.6	9.47
NP_207449	protective surface antigen D15	HP0655	1821.64	6.33	5	102.6	9.00
NP_208247	membrane-associated lipoprotein (lpp20)	HP1456	9984.98	56.00	9	19.1	9.51
Cell wall/membrane biogenesis							

NP_207761	nickel-cobalt-cadmium resistance protein (nccB)	HP0970	341.93	6.41	2	39.6	8.56
NP_207401	membrane fusion protein (mtrC)	HP0606	3238.19	44.02	9	25.9	8.82
NP_208362	rare lipoprotein A (rlpA)	HP1571	1020.16	14.29	4	35.5	9.33
NP_208142	protease	HP1350	4719.19	26.80	13	50.5	9.38
Cell motility							
NP_207396	flagellin A FlaA	HP0601	761.89	8.04	3	53.3	6.43
NP_207699	flagellar basal body rod modification protein FlgD	HP0907	288.29	5.32	1	33.7	5.24
NP_208268	flagellar basal body P-ring biosynthesis protein FlgA	HP1477	155.72	3.21	1	24.3	8.97
NP_207664	flagellar hook protein FlgE	HP0870	4847.00	21.17	11	76.2	5.25
NP_208348	flagellar hook-basal body protein FliE	HP1557	722.41	20.18	2	12.2	8.40
Intracellular trafficking and secretion							
NP_206841	conjugal plasmid transfer system protein ComB9	HP0039	261.10	3.68	1	37.5	9.07
NP_207917	translocation protein TolB	HP1126	3380.35	25.18	8	47.8	9.14
Posttranslational modification, protein turnover, chaperones							
NP_208253	secreted protein involved in flagellar motility	HP1462	1744.55	30.23	4	20.5	7.03
NP_207175	thiol:disulfide interchange protein (dsbC)	HP0377	1743.55	15.84	3	25.3	8.12
NP_206974	cell binding factor 2	HP0175	6379.87	31.10	9	34.0	9.29
Metabolism							
NP_207732	amino acid ABC transporter periplasmic binding protein (yckK)	HP0940	1739.55	19.14	5	28.7	9.44
NP_207963	glutamine ABC transporter periplasmic glutamine-binding protein (glnH)	HP1172	1001.65	23.83	5	31.2	7.40
NP_207073	ATP-dependent nuclease (addB)	HP0275	2323.09	11.16	5	49.7	8.76
NP_208252	cytochrome c551 peroxidase	HP1461	789.36	12.29	3	38.8	9.03
NP_208355	predicted outer membrane lipoprotein plpA	HP1564	3116.99	26.57	6	30.1	9.10
NP_208019	cytochrome c553	HP1227	1869.16	31.25	3	10.3	9.48
NP_207121	nuclease NucT	HP0323	181.24	5.00	1	20.1	9.47
NP_206904	2',3'-cyclic-nucleotide 2'-phosphodiesterase	HP0104	169.13	1.72	1	65.8	8.68
Relaxes negatively supercoiled DNA							
NP_206916	DNA topoisomerase I	HP0116	104.24	1.90	1	83.1	8.90
Other							
NP_208058	NADH dehydrogenase subunit G	HP1266	195.29	1.18	1	94.2	5.44
NP_207617	thioredoxin (trxA)	HP0824	375.61	21.70	2	11.8	5.22
NP_207590	flagellar sheath adhesin hpaA	HP0797	12199.52	31.15	8	29.0	8.53

NP_207451	processing protease (ymxG)	HP0657	2028.30	20.83	9	48.8	9.10
NP_207802	protease (pqqE)	HP1012	4013.25	21.40	9	50.3	8.76
NP_207391	tumor necrosis factor alpha-inducing protein	HP0596	3433.69	44.27	6	21.9	8.51
NP_206820	hypothetical protein	HP0018	383.87	6.40	2	53.4	8.98
NP_207630	hypothetical protein HP0837	HP0837	1577.25	38.24	4	11.2	9.44
NP_207964	hypothetical protein HP1173	HP1173	3378.60	33.33	6	20.6	8.84
NP_206935	hypothetical protein HP0135	HP0135	940.29	31.82	1	5.0	7.18
NP_206897	hypothetical protein HP0097	HP0097	2268.08	28.69	5	26.1	8.47
NP_207283	hypothetical protein HP0486	HP0486	7848.20	27.27	12	59.4	9.33
NP_207002	hypothetical protein HP0203	HP0203	423.85	26.37	2	10.6	8.65
NP_206930	hypothetical protein HP0130	HP0130	1128.02	17.48	4	32.7	9.47
NP_207539	hypothetical protein HP0746	HP0746	1528.91	17.42	6	48.1	9.17
NP_208077	hypothetical protein HP1285	HP1285	1284.72	17.39	3	26.3	9.28
NP_207915	hypothetical protein HP1124	HP1124	987.19	16.92	4	38.4	9.36
NP_206930	hypothetical protein HP0130	HP0130	1128.02	17.48	4	32.7	9.47
NP_207539	hypothetical protein HP0746	HP0746	1528.91	17.42	6	48.1	9.17
NP_208077	hypothetical protein HP1285	HP1285	1284.72	17.39	3	26.3	9.28
NP_207915	hypothetical protein HP1124	HP1124	987.19	16.92	4	38.4	9.36
NP_208118	hypothetical protein HP1326	HP1326	357.66	15.20	2	13.8	9.63
NP_206887	hypothetical protein HP0087	HP0087	1291.71	14.00	6	52.3	9.23
NP_207033	hypothetical protein HP0235	HP0235	1597.57	13.80	4	39.4	7.53
NP_207453	hypothetical protein HP0659	HP0659	1280.98	13.77	5	47.6	8.94
NP_208248	hypothetical protein HP1457	HP1457	3099.03	12.86	3	23.2	8.59
NP_207513	hypothetical protein HP0719	HP0719	1613.86	12.84	1	12.2	9.99
NP_208314	hypothetical protein HP1524	HP1524	212.75	12.17	1	13.3	6.93
NP_207706	hypothetical protein HP0914	HP0914	1805.81	11.67	4	58.8	9.16
NP_207030	hypothetical protein HP0232	HP0232	1262.52	11.63	2	22.9	9.48
NP_207289	hypothetical protein HP0492	HP0492	1773.89	11.51	4	31.9	9.11
NP_206959	hypothetical protein HP0160	HP0160	1571.97	11.11	3	34.1	9.09
NP_208244	hypothetical protein HP1453	HP1453	3941.97	10.72	6	82.3	5.83
NP_208315	hypothetical protein HP1525	HP1525	937.75	10.43	2	24.8	9.48
NP_208246	hypothetical protein HP1455	HP1455	615.25	10.00	1	14.7	9.39
NP_207657	hypothetical protein HP0863	HP0863	1690.66	9.96	4	61.9	9.16

NP_208317	hypothetical protein HP1527	HP1527	1113.04	9.60	5	54.7	7.08
NP_207003	hypothetical protein HP0204	HP0204	305.30	9.45	1	14.5	9.11
NP_207874	hypothetical protein HP1083	HP1083	1992.68	9.39	4	53.1	9.47
NP_207206	hypothetical protein HP0408	HP0408	125.06	9.26	1	18.7	9.45
NP_207724	hypothetical protein HP0932	HP0932	172.54	9.00	1	11.9	8.79
NP_207908	hypothetical protein HP1117	HP1117	1543.22	8.98	2	29.0	8.50
NP_207305	hypothetical protein HP0508	HP0508	964.49	8.19	4	52.6	9.16
NP_206880	hypothetical protein HP0080	HP0080	1173.89	8.09	4	65.9	4.64
NP_207629	hypothetical protein HP0836	HP0836	665.53	7.56	1	13.2	10.17
NP_207400	hypothetical protein HP0605	HP0605	834.92	7.55	3	54.6	9.00
NP_207764	hypothetical protein HP0973	HP0973	1225.88	7.08	2	39.8	9.36
NP_207009	hypothetical protein HP0211	HP0211	97.57	6.80	1	27.3	8.31
NP_207872	hypothetical protein HP1081	HP1081	280.12	6.28	1	23.7	9.48
NP_207165	hypothetical protein HP0367	HP0367	416.30	5.94	1	23.2	8.22
NP_207504	hypothetical protein HP0710	HP0710	5878.12	5.91	4	73.3	7.56
NP_207889	hypothetical protein HP1098	HP1098	474.57	5.52	1	31.6	8.47
NP_207958	hypothetical protein HP1167	HP1167	725.63	5.52	2	52.6	9.50
NP_207574	hypothetical protein HP0781	HP0781	362.42	5.13	2	49.4	5.81
NP_207848	hypothetical protein HP1057	HP1057	315.39	5.00	1	27.4	9.35
NP_207007	hypothetical protein HP0209	HP0209	285.15	4.89	2	52.6	9.58
NP_208337	hypothetical protein HP1546	HP1546	142.61	4.71	1	19.6	8.50
NP_207846	hypothetical protein HP1055	HP1055	204.99	4.46	1	35.7	8.43
NP_207350	hypothetical protein HP0555	HP0555	156.67	3.30	1	31.4	9.25
NP_207847	hypothetical protein HP1056	HP1056	155.43	2.82	1	32.3	8.94
NP_207575	hypothetical protein HP0782	HP0782	204.75	2.20	1	52.0	9.31
NP_207897	hypothetical protein HP1106	HP1106	180.95	2.07	1	51.1	9.50
NP_207581	hypothetical protein HP0788	HP0788	148.52	1.80	1	56.8	8.95
NP_207745	hypothetical protein HP0953	HP0953	2854.88	46.81	8	21.2	8.60
NP_207520	hypothetical protein HP0726	HP0726	165.99	2.95	1	34.1	9.67

3.4 Final Discussion

The findings from this chapter demonstrate that *H. pylori* OMVs are internalised by host cells via diverse mechanisms, including macropinocytosis, clathrin- and caveolin-dependent endocytosis. This is not surprising, as other groups have attributed OMV internalisation to each of these mechanisms. However, the reported mechanisms of entry differ across the various bacteria and experimental systems. In this chapter we have provided evidence supporting our hypothesis that OMV size plays a role in directing the route of host cell entry. Indeed, we found that OMVs smaller than 50 nm were capable of entering host cells via each of the listed mechanisms of endocytosis, whereas OMVs larger than 50 nm could enter cells via macropinocytosis and caveolin-dependent endocytosis, but not clathrin-dependent endocytosis.

Interestingly, we found that small OMVs and large OMVs may be degraded within the cell via distinct pathways, as treatment of the cells with bafilomycin A1 hindered the intracellular degradation of small but not large OMVs. As bafilomycin A1 prevents the fusion of autophagosomes with lysosomes, thereby preventing lysosomal degradation, we surmised that the intracellular degradation pathways of OMVs may differ depending on their mode of internalisation, and therefore, their size.

The data reported here provide the first evidence that OMV size also predetermines protein content. Although it has been suggested that OMVs of different sizes may vary in their protein constituents (Elmi, Watson et al. 2012), this hypothesis has thus far not been investigated in any detail. Using LC-MS/MS analysis, we have demonstrated that small OMVs comprise fewer proteins than large OMVs and that these different populations of OMVs contain within them both unique and shared sets of proteins.

In summary, the data presented in this chapter demonstrate that OMV size plays a role in directing the mechanisms of host cell internalisation and also predetermines their protein content.

Chapter 4. Bacterial outer membrane vesicles are processed into antigen presenting exosomes by epithelial cells, which induce the proliferation of human T cells.

4.1 Introduction

The release of extracellular vesicles known as exosomes has been demonstrated for a wide range of eukaryotic cell types, including reticulocytes (Johnstone, Adam et al. 1987), B lymphocytes (Raposo, Nijman et al. 1996) and DCs (Zitvogel, Regnault et al. 1998). Since their discovery, exosomes have been found to represent an important mechanism of cell to cell communication, acting as 'communicasomes' for the transfer of DNA, protein, lipids, RNA between eukaryotic cells (reviewed in (Simons and Raposo 2009)).

Derived from MVB within the cell, exosomes contain not only cargo derived from the cell of origin, but may also contain extracellular contents that have entered the cell at the plasma membrane, such as viral RNA (Narayanan, Iordanskiy et al. 2013) and bacterial products (Bhatnagar and Schorey 2007). Indeed, studies by Bhatnager *et al.* reported that exosomes released from macrophages infected with *M. bovis* or *Mycobacterium avium* contained specific bacterial PAMPs such as LIM and glycopeptolipids, respectively (Bhatnagar and Schorey 2007, Bhatnagar, Shinagawa et al. 2007). Furthermore, it was shown that these exosomes containing bacterial PAMPs were capable of stimulating pro-inflammatory responses by macrophages *in vitro* and *in vivo* (Bhatnagar, Shinagawa et al. 2007).

In 2001, van Niel and colleagues demonstrated that polarised IECs were capable of secreting exosomes that were enriched in HLA Class I and II molecules, required for antigen presentation (van Niel, Raposo et al. 2001). Later work published by the same group demonstrated that exosomes secreted by IECs, which had been cultured with ovalbumin (OVA) peptide, were capable of inducing OVA-specific humoral immune responses following administration to mice (van Niel, Mallegol et al. 2003). Further work performed by van Niel and colleagues demonstrated that IEC exosomes expressing HLA Class II / human serum albumin (HSA) peptide complexes, targeted and were internalised by follicular DCs. These DCs were then able to present HSA peptide to T cells (Mallegol, van Niel et al. 2007), indicating that exosomes may potentiate peptide presentation to T cells via interactions with DCs.

Given that bacterial OMVs are known to enter host epithelial cells via the endocytic pathway (Kesty, Mason et al. 2004, Furuta, Tsuda et al. 2009, Parker, Chitcholtan et al. 2010) (Chapter 3), we propose that internalised *H. pylori* OMVs may be processed within the cell and packaged within exosomes. Furthermore we propose that these OMV-containing exosomes are capable of interacting with APCs to facilitate the presentation of *H. pylori* OMV proteins to the local immune system.

In this chapter, we demonstrate the internalisation of *H. pylori* OMVs by polarised IECs and the subsequent inclusion of OMV proteins within exosomes secreted basolaterally from these cells. Furthermore, we establish that these OMV containing exosomes are capable of presenting OMV derived proteins to T cells, via interactions with DCs, resulting in antigen specific T cell proliferation.

4.2 Methods

4.2.1 Growth of *H. pylori*

H. pylori 251 (Viala, Chaput et al. 2004) was routinely cultured on horse blood agar (HBA) plates [Blood agar base No.2 (Oxoid Ltd., Hampshire, England) containing 8% (v/v) horse blood (Bio-Lab, VIC, Australia) and Skirrow's selective supplement [155 μg/ml polymixin B (Sigma, Steinheim, Germany), 6.25 mg/ml vancomycin (Sigma), 3.125 mg/ml trimethoprim (Sigma), and 1.25 mg/ml amphotericin B (Amresco, OH, USA)]. *H. pylori* mutant strain 251 *cag*PAI (Kaparakis, Turnbull et al. 2010) was cultured on HBA supplemented with 20 μg/ml kanamycin (Sigma). Liquid broth cultures were incubated in 25 cm^2 or 75 cm^2 tissue culture flasks (BD Falcon™) in final volumes of 10 ml or 40 ml of brain heart infusion (BHI; Oxoid) containing 10% fetal calf serum (FCS; Thermo Electron, Melbourne, Australia). *H. pylori* were incubated under microaerobic conditions in anaerobic jars containing a Campygen gas pack (Oxoid) for 24-36 hours at 37˚C. Additionally, liquid cultures were shaken at 125 rpm. Viable counts were performed using 10 fold serial dilutions of bacteria in BHI and incubated for 3-5 days prior to enumeration. *H. pylori* was fixed by resuspension in 4% (v/v) formaldehyde in phosphate buffered saline (PBS) for 20 minutes on ice. Bacteria were subsequently washed 3 times by centrifugation in PBS at 2,500 x g for 10 minutes. The absence of viability of formaldehyde fixed bacteria was confirmed by bacterial culture, as described above.

4.2.2 Preparation of OMVs

For the production of *H. pylori* OMVs, bacteria were grown in BHI medium supplemented with 0.6% (w/v) β-cyclodextrin (Sigma) in place of FCS. Cultures were

incubated under microaerobic conditions for approximately 17 hours at 37˚C with shaking at 140 rpm. Bacteria were pelleted by centrifugation at room temperature at 3,220 x g for 20 and cell free supernatants filtered using Stericup-GP with 0.22 μm filters (Millipore, Ireland). Supernatants were ultracentrifuged in Ultra-Clear™ centrifuge tubes (Beckman Coulter) using a Sorvall RC100 ultracentrifuge (Thermo-Scientific) and an AH629 rotor (Thermo-Scientific). OMV pellets were resuspended in final volumes of 1 ml PBS. Samples were concentrated using Amicon Ultracel 10K filtration units (Millipore). Protein concentrations of OMV samples were determined using a modified method of the BioRad Protein Assay (BioRad Laboraties Inc., USA). Aliquots of OMV samples and samples of bovine serum albumin (BSA; Sigma) at a range of known concentrations, were incubated with octyl-β-D-glucopyranoside (NOG; Sigma-Aldrich), at a final concentration of 7.5% (w/v) for 10 minutes at room temperature. The addition of the detergent NOG solubilises membrane bound proteins allowing more accurate measurement of the protein content of membrane bound proteins (Fanger 1987), such as those in OMVs. Samples were mixed with the dye reagent from the Bradford protein assay kit at a ratio of 4:1 and incubated for 5 minutes at room temperature. The absorbance at 595 nm was measured using a Beckman DU® 530 Life Science UV/Vis Spectrophotometer and the protein concentrations determined by comparison against a standard curve of BSA samples.

4.2.3 Growth and maintenance of cell lines

T84 IECs were routinely cultured in Dulbecco's Modified Eagles Medium:Ham's-F12 nutrient mixture (DMEM:F12; Gibco) supplemented with 10% (v/v) FCS and 1 % (v/v) penicillin-streptomycin (complete DMEM:F12; Gibco). T84 cells were incubated at 37˚C in 5% CO_2 in 25 cm^2 or 75 cm^2 tissue culture flasks. T84 cells were detached from

flasks by incubation with TrypLE™ Express (Life Technologies) for 5-7 minutes at 37˚C in 5% CO_2. Detached cells were collected in DMEM:F12 and centrifuged at room temperature for 5 minutes at 300 x g, prior to resuspension in complete DMEM:F12. T84 cells were seeded at 1 x 10^5 cells per well in 24 well plates (BD Falcon™). Alternatively, to obtain a polarised monolayer of cells, T84 cells were seeded at 10^6 cells per cm^2 in Transwell® 24 mm diameter inserts, with 3 μm pore size (Corning Incorporated, NY, USA). The media in Transwell® dishes was replaced every second day. Cells were considered fully polarised when a stable Transepithelial electrical resistance (TEER) of 700Ω•cm^2 was reached (15-21 days of culture). TEER was measured using a Millicell Electrical Resistance System (Millipore).

4.2.4 Dextran-flux assay

Polarised T84 cells with a TEER of 700Ω•cm^2 were stimulated basolaterally with 100 international units (IU) IFN-γ (Gibco®) per ml for 48 hours, or OMVs (100 μg) apically for 24 hours. Alexa Fluor® 488 conjugated 3 kDa Dextran beads (Molecular Probes®; 500ng) were added apically to cells and 100 μl aliquots taken from the basolateral compartment after 3 hours. To allow measurement of the amount of beads that were able to pass through the cell monolayer, a standard curve was prepared (10-200 ng dextran). Fluorescence was measured at excitation 485, emission 520 using a FLUOstar Optima plate reader (BMG Labtech).

4.2.5 Alexa fluor® 568-labelling of OMVs

Aliquots (500 μl) of *H. pylori* 251 *cag*PAI OMVs (2 mg/ml), were fluorescently labelled with Alexa fluor® 568 using an Alexa Fluor® 568 Protein Labelling Kit

(Invitrogen Molecular Probes, Oregon, USA), as per the manufacturer's instructions. Briefly, OMVs were mixed with 1M sodium hydrogen carbonate (AJAX chemicals, Sydney, Australia) and added to a reactive dye, followed by a 1 hour incubation at room temperature in the dark with gentle agitation. Purification resin was added to a column, the buffer allowed to drain away and OMV mixtures added slowly and allowed to enter the resin. Elution buffer was added to the column and the fluorescent band containing labelled OMVs collected. Alexa fluor® 568-tagged OMVs were stored at -20˚C in the dark until required.

4.2.6 Confocal fluorescence microscopy to visualise OMV entry into polarised cells.

Alexa fluor® 568-labelled OMVs (50 μg per ml) were added apically to cells in 2 ml aliquots and left in contact with cells for 16 hours. Cells were then washed 3 times with sterile PBS and fixed with 4% (v/v) formaldehyde in PBS for 20 minutes. Cells were washed 3 times and blocked using blocking buffer (2% (w/v) BSA Fraction V (Roche Diagnostics) in PBS). Cells were washed 3 times with PBS and incubated with a polyclonal rabbit anti-*H. pylori* 251 *cag*PAI OMV (Kaparakis, Turnbull et al. 2010), diluted 1/500 in bocking buffer for 1 hour at room temperature. Alternatively, cells were incubated with blocking buffer alone. Cells were washed 3 times and incubated with Alexa Fluor® 488 goat anti-rabbit IgG antibody (Invitrogen Molecular Probes), diluted 1/1000 in blocking buffer for 40 minutes at room temperature. Cells that did not require antibody staining were incubated with blocking buffer alone. For samples requiring permeabilisation of cells, Triton-X (MERCK) was added to blocking buffer at a concentration of 0.1% (v/v) during each incubation. Following 3 washes with PBS the membranes were cut from inserts and placed on glass microscope slides (Sailbrad,

China) with cells facing up. A single drop of Prolong Gold (Invitrogen Molecular Probes) was added to cells and 0.17 mm cover slips placed on top. Slides were left to cure overnight at room temperature and then viewed using Nikon C1 upright microscope. Images were captured using NIS Elements AR.3.0 software (both Nikon Instruments Inc. NY) and analysed using ImageJ 1.42q (National Institutes of Health, USA).

4.2.7 Enzyme Linked Immunosorbent Assay (ELISA)

IL-8 production was measured using the BD OptEIA™ Human IL-8 ELISA kit (BD Pharmingen), as per the manufacturer's instructions. Briefly, wells of a 96 well Maxisorp plate (Nunc, Roskilde, Denmark) were coated with 100 µl purified mouse anti-human IL-8 diluted 1/500 in carbonate coating buffer [0.1M $NaHCO_3$, 0.1M Na_2CO_3 (both BDH Chemicals, Vic, Aus) pH 9.5] overnight at 4˚C. Plates were washed 3 times with 300 µl per well of wash buffer [0.05% Tween-20 in PBS], using a BIO-TEK plate washer (BIO-TEK, VT, USA), prior to blocking with 200 µl per well of assay diluent [10% (v/v) FCS in PBS] at room temperature for 1 hour. IL-8 standards (100 µl) were added to wells in duplicate. Sample aliquots (100 µl) were added to wells in triplicate and incubated for 2 hours at room temperature. IL-8 was detected by addition of 100 µl of biotin-labelled mouse anti-human IL-8 antibody and streptavidin-horseradish peroxidase (HRP) (both diluted 1/1000), for 1 hour each. Substrate solution (100 µl; BD Biosciences, San Diego, CA, USA) was added to each well and allowed to develop for 15 minutes in the dark. The reaction was then stopped with 100 µl per well of 0.5M H_2SO_4 and absorbance values measured at 450nm, using a FLUOstar Optima plate reader.

4.2.8 RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR)

Non-polarised T84 cells were either left non-stimulated or stimulated with 100 IU per ml of recombinant human IFN-γ for 48 hours (Ye, Barrera et al. 1997). Polarised T84 cells were basolaterally stimulated with 100 U per ml IFN-γ, or not, for 48 hours. Live *H. pylori* 251 (multiplicity of infection (MOI) = 10:1), or OMVs (50 or 200 μg/ml), were added apically to polarised T84 cells for 24 hours. RNA was purified from cells using the Purelink RNA mini kit (Invitrogen), as per manufacturer's instructions. To remove contaminating DNA, RNA was Deoxyribonuclease (DNAse) treated using the Turbo DNA free kit (Ambion, VIC, Australia). RNA (500 ng) was reverse transcribed using SuperScript III (Invitrogen). Taqman primers for the genes encoding 18s rRNA, HLA Class I and HLA Class II are listed in 4.1. Reaction mixes (final volume 10 μl) consisted of 20 ng cDNA, 2 μl 10xRT buffer and 0.5 μl primer. qRT-PCR gene expression analyses were performed in triplicate using a 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative changes in gene expression were determined using the ΔΔCt method (Kennedy, Najdovska et al. 2011) and normalised to the 18s rRNA gene. Data acquisition and analyses were performed using the Sequence Detection System v2.3 software (Applied Biosystems).

4.2.4.2.9 Detection of HLA Class I and II on T84 cells using flow cytometry

Non-polarised T84 cells were treated with 1000 IU/ml recombinant human IFN-γ, for 48 hours in 5% CO_2 at 37˚C. Following IFN-γ stimulation, media were replaced with DMEM:F12 and incubated a further 24 hours to allow up-regulation of HLA Class I and II molecules. Cells were co-cultured with *H. pylori* 251 (MOI = 10:1) or OMVs (50 or 200 μg per ml) for 24 hours. Cells were detached from tissue culture plates by

Table 4.1: Taqman gene expression assays used in this study

Gene	Reference	Amplicon length
18S5	Hs03928990_g1	61
HLA-B	Hs00818803_g1	189
HLA-DRB1, HLA-DRB4, HLA-DRB5	Hs04192463_mH	95

incubation with 1mM ethylenediaminetetraacetic acid (EDTA) disodium dihydrate (amresco) for 20-30 minutes and suspended in FACS buffer (Dulbecco's phosphate buffered saline (DPBS; Gibco) with 1% (v/v) FCS), prior to centrifugation at 1000 x g for 5 mins at 4˚C. Cells were incubated with fluorescein isothiocyanate (FITC) mouse anti-human HLA-DQR and phycoerythrin (PE) mouse anti-human HLA-B (both BD Pharmingen™) for 30 minutes on ice in the dark. Single stained and non-stained cells were included as controls. Cells were then washed using FACS buffer, pelleted by centrifugation and resuspended in FACS fixative [DPBS with 2% (v/v) formaldehyde]. Cells were acquired using BD FACS Canto II and BD FACS Diva software v 60 (both BD Biosciences, CA, USA). Non-viable cells were excluded from analysis based on forward scatter (FSC; granularity) and side scatter (SSC; size) profiles. Data were analysed using FlowJo version 7.2.2. Results are displayed as histograms, and expressed as the ratio of the mean fluorescence intensity (MFI) of HLA Class I or II from cells cultured with *H. pylori* or OMVs : cells cultured with medium alone (Ciantar and Mannering 2011).

4.2.10 Isolation and characterisation of exosomes

Polarised T84 cells were co-cultured with *H. pylori* 251 *cag*PAI OMVs (100μg), live *H. pylori* 251 (MOI = 10:1) or formaldehyde fixed *H. pylori* 251 (MOI = 10:1). OMVs were left in contact with cells for 24 hours. For bacterial stimulations, medium was replaced after 1 hour of incubation for a further 23 hours. Following 24 hours in culture, supernatant samples were collected for analysis using IL-8 ELISA, and the medium was replaced with exosome culture medium (DMEM:F12 supplemented with 1% (v/v) penicillin-streptomycin and 2% (w/v) BSA) for a further 48 hours. Isolation of exosomes was performed as follows, based on the method of Van Niel *et al*. (van Niel,

Raposo et al. 2001). Supernatants were collected from the apical and basolateral compartments and subjected to serial centrifugations at 300 x g for 10 minutes (to remove dead cells and cellular debris), 2000 x g for 20 minutes and 10,000 x g for 30 minutes (to remove any remaining contaminating cellular material) using a Heraeus Multifuge 3SR+ (Thermo Scientific). All centrifugations were performed at 4°C. The final supernatants were washed by ultracentrifugation 3 times in sterile PBS at 100,000 x g (4°C) for 1 hour to wash and concentrate the exosomes. Pellets were subsequently resuspended in PBS (100 μl). Basolateral exosomes purified from non stimulated cells or OMV-stimulated cells were designated "empty" exosomes or "OMV exosomes, respectively. Protein concentrations were determined using the Quant-iT™ protein assay kit and a Qubit fluorometer (both from Invitrogen), as per the manufacturer's instructions.

4.2.11 Transmission electron microscopy

Exosome and OMV pellets were resuspended in 2% (v/v) paraformaldehyde in PBS and added to Formvar coated carbon grids. Samples were contrasted with 2% (w/v) uranyl-acetate / 0.15M oxalate (pH 7). Samples were embedded in a solution of 1 part 4% (v/v) uranyl acetate and 9 parts 2% (v/v) methyl cellulose. All reagents were purchased from Prositech (USA). Samples were viewed using a Hitachi H.7-7500 transmission electron microscope at 70,000-fold magnification and images captured using Digital micrograph™ 1.71.38 software (Gatan Inc.). Image analysis was performed using ImageJ v1.47k (National Institutes of Health, USA).

4.2.12 Separation of IEC, exosomal and OMV proteins by gradient gel electrophoresis

Cell lysates, exosomes and OMV samples were resuspended in 4X NuPAGE® LDS sample buffer with 10X NuPAGE® Reducing agent (final volume 10 µl). Samples containing equal protein concentrations were heated for 10 minutes at 70°C and loaded onto NuPAGE® Novex® 4-12% Bis-Tris Gels (Life Technologies) with a Colour Plus pre-stained protein ladder (10-230 kDa; New England BioLabs). Gels were immersed in 1X NuPAGE® MOPS buffer with 1X NuPAGE® antioxidant in the upper chamber of XCell SureLock® Mini-Cells (Life Technologies). Samples were electrophoresed at 200V for 50 minutes.

4.2.13 Detection of IEC, exosomal and OMV proteins by Western blot analysis

Proteins were transferred to 0.2 µm pore size nitrocellulose membranes (iBlot® transfer stacks; Novex® Life Technologies) using the iBlot® Gel Transfer Device (Invitrogen™, Life Technologies). Membranes were blocked using 5% (w/v) skim milk (Diploma) in PBS and incubated for 2 hours at room temperature. Membranes were washed 3 times for 5 minutes each in PBS with 0.05% (v/v) Tween-20 (amresco). Membranes were incubated with rabbit anti-*H. pylori* 251 *cag*PAI OMV antibodies (diluted 1/2500 in 1% (w/v) skim milk) (Kaparakis, Turnbull et al. 2010) overnight at 4˚C. Membranes were washed and incubated with biotin conjugated goat anti-rabbit IgG (Chemicon; diluted 1/1000 in 1% skim milk) for 2 hours at room temperature. Membranes were subsequently washed and incubated with streptavidin-HRP (Millipore; diluted 1/1000 in 1% skim milk) for 1 hour. Proteins were detected using Electrochemiluminescence (ECL) Chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) and films developed using a Fuji FPM 100A film processor.

4.2.14 Protein sample preparation for mass spectrometry

Exosome samples (100 µg) were suspended in PBS with protease inhibitors (Complete Protease Inhibitor cocktail tablets; Roche). Associate Professor Stuart Cordwell performed sample preparation and analysis at the School of Molecular Bioscience, the University of Sydney.

Exosome preparations were resuspended in 100 µL 6M urea / 2M thiourea and bath sonicated for 5 minutes prior to 4 rounds of freeze-thaw. Proteins were reduced in 10 mM dithiothreitol (DTT) at 25°C for 1 hr, followed by alkylation in 20 mM iodoacetamide (IAA) at 25°C in the dark for 30 minutes. Proteins were diluted 1:8 in 40 mM ammonium bicarbonate and digested overnight in trypsin (1 µg) for 16 hours. Digestion was terminated by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1%. Peptides were concentrated and desalted using hydrophilic-lipophilic balance (HLB) solid phase extraction. Prior to mass spectrometric analysis, samples were fractionated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent 1200 series HPLC. Fractionation was performed using a 20 cm long, 320 µm I.D. column packed with TSK-Amide 80 HILIC resin (3 µm particle size). Samples were resuspended in 100% buffer B (90% MeCN, 0.1% TFA) and separated using a linear gradient as follows: sample loading for 10 minutes at 100% buffer B (12 µl/min); sample elution from 100% buffer B to 60% buffer B for 25 minutes (6 µl/min), ending in a column re-equilibration step for 20 minutes. Peptide elution was monitored by a multiwavelength detector at 280 ± 4 nm, 254 ± 4 nm, 260 ± 4 nm and 230 ± 4 nm. Following separation, all samples were lyophilised to complete dryness using a Speedvac and stored at -20°C until mass spectrometric analysis.

4.2.15 Mass Spectrometry

Purified peptides were resuspended in 100% Buffer A (2% MeCN, 0.1% FA, 98.9% H_2O) and analysed by tandem mass spectrometry (MS/MS), using an Eksigent Nano LC Ultra 2D Plus coupled to an AB SCIEX 5600 TripleTOF mass spectrometer. Liquid chromatography was performed using a two-column set up where peptides were loaded onto a trap column (0.5 cm, 150 µm I.D., C18) in 5% Buffer B (90% MeCN, 0.1% FA) for 8 mins, at a flow-rate of 5 µl/min. Following valve switching, peptides were then eluted onto an analytical column (15 cm, 75 µm I.D., C18Aq Reprosil) and separated on a linear of gradient of 5% Buffer B to 60% Buffer B over 90 minutes, at a flow-rate of 300 nl/min. Eluted peptides were sprayed by electrospray ionisation directly into the TripleTOF system at 150°C. The top 20 most abundant ions were selected for MS/MS by collision-induced dissociation, using the following parameters: full scan high resolution MS between 350 and 2000 m/z; MS/MS events between 100 – 2000 *m/z* using 'high sensitivity' mode, isolation width of 3.0 amu and a minimum charge state of +2. All samples were written to .wiff files and later converted to .mgf utilising the AB SCIEX file converter.

Mascot searching was performed using the publicly available *H. pylori* database 26695 (reverse concatenated), with the following parameters: trypsin cleavage with a maximum of two missed cleavages; 0.2 Da tolerance for both MS and MS/MS; variable oxidation of methionine; variable carbamidomethylation of cysteines and variable N-terminal pyroglutamation. Following database searching, results were compiled using Scaffold 3 (Proteome Software) and searched using X!Tandem with identical parameters as outlined for Mascot. Peptide and protein validation was performed with the inbuilt PeptideProphet and ProteinProphet algorithms provided by Scaffold to obtain peptides and proteins with 90% confidence and below 1% FDR. Single peptide

identifications for proteins were also manually validated for correct sequence assignment.

4.2.16 Isolation and separation of human PBMCs using CD3 MACS beads

Blood was obtained by venepuncture from healthy, *H. pylori* negative, volunteer donors. Peripheral blood mononuclear cells (PBMCs) were isolated over Ficoll-Paque PLUS (GE Healthcare, Australia) and washed twice in DPBS by centrifugation at 500 x g for 5 minutes at room temperature. Cells were cultured in Roswell Park Memorial Institute (RPMI; Gibco®) medium supplemented with 5% pooled human male AB serum (PHS; Lonza), 1% penicillin-streptomycin and 0.05 mM 2-mercaptoethanol (Gibco®; complete T cell media).

4.2.17 Separation of human PBMCs using CD3 MACS beads

PBMC suspensions were centrifuged at 300 x g for 10 minutes, supernatants were removed and cells were resuspended in 80 µl MACS buffer (DPBS with 0.5% BSA Fraction V and 3mM EDTA) per 10^7 cells containing CD3 micro beads (Miltenyi Biotec; 20 µl per 10^7 cells). Cells were subsequently incubated for 15 minutes on ice, washed using MACS buffer and resuspended in 500 µl MACS buffer. $CD3^+$ cells were separated from $CD3^-$ cells using a MidiMACS™ Separator and LS columns (both Miltenyi Biotec). Separation of $CD3^+$ and $CD3^-$ cell populations was confirmed by staining cells with brilliant violet mouse anti-human CD3 antibody (Clone UCHT1; BD Pharmingen) and analysis by flow cytometry. Cells were washed in DPBS and resuspended in 1 ml RPMI with 10% (v/v) PHS. To prepare cells for storage in liquid nitrogen, equal volumes of RPMI containing 20% (v/v) dimethyl sulfoxide (DMSO;

Sigma) were added drop wise to cell suspensions over 2 minutes, on ice. Cells were then stored in liquid nitrogen until required.

4.2.18 Determining *H. pylori* status of donors by Western blot analysis

Aliquots of *H. pylori* lysate (10 μg) were suspended in 4X NuPAGE® LDS sample buffer with 10X NuPAGE® Reducing agent (final volume 10 μl). Samples were prepared and electrophoresed as described in section 4.2.12. Proteins were transferred to nitrocellulose membranes as described in section 4.2.13. Blocked membranes were incubated with donor sera (diluted 1/100 in 1% skim milk), or rabbit anti-*H. pylori* sera (diluted 1/5000 in 1% skim milk) overnight at overnight at 4˚C. Membranes were washed and incubated with biotin conjugated goat anti-human IgG (H+L) (ZyMax) (Invitrogen; diluted 1/1000 in 1% skim milk) for 2 hours at room temperature. Membranes were washed and incubated with streptavidin-HRP (Millipore; diluted 1/1000 in 1% skim milk) for 1 hour. Proteins were detected as described in section 4.2.14.

4.2.19 T cell proliferation assay

T cell proliferation assays were performed as previously described (Mannering, Dromey et al. 2005). Briefly, PBMCs (1 x 10^7) were incubated with 0.1 μM 5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester (5(6)-CFDA, SE (CFSE; Molecular Probes®) for 5 minutes, at 37°C. Cells were washed once with DPBS containing 1% (v/v) PHS and resuspended at 1 x 10^6 per ml. *H. pylori* lysates were prepared by 5 freeze-thaw cycles, in DPBS containing complete mini protease inhibitor cocktail. The absence of bacterial viability was confirmed by culture on HBA as described above. CFSE stained cells were either left non-stimulated, or stimulated with *H. pylori* 251

lysate (10 μg/ml), "empty" OMVs (20 μg/ml), "OMV" exosomes (20 μg/ml). Inactivated influenza virus (2 μg/ml (Ciantar and Mannering 2011)), or CystoStim (as per manufacturers instructions; Miltenyi Biotec) were included as positive controls. Cells were cultured in 96 well-curved bottom plates (200 μl; BD Falcon™) in triplicate for 7 days. Following 7 days in culture, supernatants were collected and stored at -80°C for cytokine analyses. To measure the proliferation of $CD4^+$ and $CD8^+$ cells, cells were collected and stained with Alexa Fluor®647 anti-human CD4 (clone RPA-T4), PE anti-human CD8 (clone RPA-T8; both BD Pharmingen™) antibodies. Propidium-iodide (PI; 40 μg/ml; Molecular Probes®, Life Technologies™) was used to allow exclusion of non-viable cells for all flow cytometry experiments. Non-stained cells were included in all experiments to set the compensations on the flow cytometer. Optimal gain and compensation settings were determined for each individual experiment based on the non-stained and single stained samples. Samples were acquired using a FACS Canto II and BD FACS Diva software v.60. The cell division index (CDI) was calculated as follows. Gates were placed around PI negative (viable) lineage specific cells, either $CD4^+$ or $CD8^+$, which were either $CFSE^{bright}$ (non divided) or $CFSE^{dim}$ (divided). The average number of $CFSE^{dim}$ cells per 5000 $CFSE^{bright}$ cells was determined, and the CDI calculated using the following formula:

$$\text{CDI} = \frac{\text{Average number CFSE}^{dim}/5000\text{CFSE}^{bright}\text{ cells with antigen}}{\text{Average number CFSE}^{dim}/5000\text{CFSE}^{bright}\text{ cells without antigen}}$$

A CDI of >2 is considered to be a positive response (Mannering, Dromey et al. 2005).

4.2.20 Th1/Th2/Th17 cytokine analysis using cytometric bead array (CBA)

Cytokine secretion by human PBMCs was analysed using the BD CBA Human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA), as per the manufacturer's instructions. Briefly, supernatants (50 µl) from cell proliferation assays were added to the Th1/Th2/Th17 capture bead suspension and incubated for 3 hours at room temperature. Standards were prepared as per manufacturer's instructions. Samples were washed with the supplied wash buffer and analysed using a FACS Canto II flow cytometer and BD FACS Diva software v 60. Data were formatted and further analysed using FlowJo version 7.2.2

4.2.21 Isolation of *H. pylori* responsive T cells by cell sorting

For isolation of *H. pylori* reactive T cells by fluorescence activated cell sorting (FACS), CFSE-labelled cells (3 x 10^7) were stimulated with *H. pylori* lysate as above, and cultured for 7 days. Cells were collected in DPBS, centrifuged at 500 x g for 5 mins at room temperature and incubated with brilliant violet mouse anti-human CD3 antibody for 20 minutes on ice in the dark. Cells were washed once by centrifugation in DPBS and resuspended in DPBS with 1% PHS, and PI (40 µg/ml). Cells were sorted using a BD Influx™ cell sorter (BD Biosciences). Doublets (cell aggregates) were excluded based on forward and side scatter profiles and non-viable cells excluded based on PI staining. Single PI^-, $CD3^+$, $CFSE^{dim}$ cells were collected, washed once in DPBS and resuspended (1 x 10^6 per ml) in complete T cell media supplemented with recombinant human IL-2 (50 IU/ml; PeproTech), recombinant human IL-7 (5 ng/ml; Miltenyi Biotec) and Dynabeads® Human T-Activator CD3/CD28/CD137 (Gibco®, Life Technologies™) at a ratio of 1 bead per 10 cells. Media were replaced every 2-3 days with fresh media supplemented with cytokines. After 7 days in culture, the beads were

removed and cells were cultured in media with cytokines at the aforementioned concentrations.

4.2.22 Click-iT® EdU cell proliferation assay

Human $CD3^-$ cells were thawed from liquid nitrogen and allowed to recover for 1 day in complete T cell medium, followed by irradiation at 25 Gray. Irradiated cells were treated with 1000 IU/ml DNAse I (Sigma-Aldrich) for 30 minutes and then washed with DPBS. Human *H. pylori*-reactive $CD3^+$ T cells were seeded in 96 well curved bottom plates at 1 x 10^6 cells per ml, with 5 x 10^5 irradiated $CD3^-$ cells per ml in a final volume of 200 µl. Cells were left non-stimulated or co-cultured with either *H. pylori* lysate (10 µg/ml), CytoStim, "empty" exosomes (20 µg/ml) or "OMV" exosomes (20 µg/ml). T cell proliferation was measured using the Click-iT® EdU Alexa Fluor® Flow Cytometry Assay Kit (Molecular Probes®, Life Technologies™), performed as follows. After 6 days in culture, 5-ethynyl-2'-deoxyuridine (EdU) was added to a final concentration of 10 mM and incubated for a further 24 hours. Cells were collected and washed once with 1% BSA (Gibco) in DPBS and fixed with Click-iT® fixative (100 µl). Following another wash, cells were resuspended in Click-iT® saponin-based permeabilisation and wash reagent (100 µl) and labelled with the Click-iT® reaction cocktail, as per the manufacturer's instructions. Following a 30-minute incubation, cells were washed twice with Click-iT® saponin-based permeabilisation and wash reagent and analysed by flow cytometry.

4.2.23 Statistical analysis

All analyses were performed using Graphpad Prism version 6.0c. ELISA and qRT-PCR experiments were analysed by Student's t-test. Error bars indicate the means ± standard

deviation (SD). CBA experiments were analysed using analysis of variance (ANOVA) and the Friedman test, with Dunn's multiple comparisons test. For each of the above listed tests, differences were considered significant when *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

4.3 Results

4.3.1 *H. pylori* OMVs are internalised by polarised epithelial cells

There is now a vast body of evidence demonstrating that Gram-negative OMVs from a variety of bacteria are able to enter non-polarised epithelial cells (Horstman and Kuehn 2000, Bomberger, Maceachran et al. 2009, Furuta, Tsuda et al. 2009), including those from *H. pylori* (Fiocca, Necchi et al. 1999, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010). The cells used in the aforementioned studies do not form polarised monolayers or intact tight junctions and hence, do not reflect the physiological conditions of gastric epithelium encountered by *H. pylori* within the host (El-Etr, Mueller et al. 2004). We therefore wished to examine the ability of *H. pylori* OMVs to enter polarised epithelial cells. However, due to the absence of an appropriate gastric epithelial cell line that can form polarised monolayers, we used T84 IECs as a model to study OMV entry into polarised epithelial cells. T84 IECs have been widely used to study *H. pylori* interactions with host cells (Corthesy-Theulaz, Porta et al. 1996, Hofman, Ricci et al. 2000, Le'Negrate, Ricci et al. 2001, El-Etr, Mueller et al. 2004, Gewirtz, Yu et al. 2004) and are known to secrete exosomes (van Niel, Raposo et al. 2001), making them an appropriate cell line for our studies.

Initially, we sought to determine whether co-culture of polarised IECs with OMVs would affect the integrity of the monolayer. For this, cells were grown on Transwell® filters and the TEER was measured every 3 days until the cells were completely polarised (700 $\Omega \bullet cm^2$) (van Niel, Raposo et al. 2001). Once polarised (0 hours), cells were co-cultured with media alone or OMVs and the TEER measured 24 and 72 hours later (Figure 4.1A). There were no significant decreases in the TEER of either non-stimulated cell monolayers, or those that had been cultured with OMVs, after 24 hours. The TEER of both non-stimulated and OMV-stimulated cells did decrease after 72

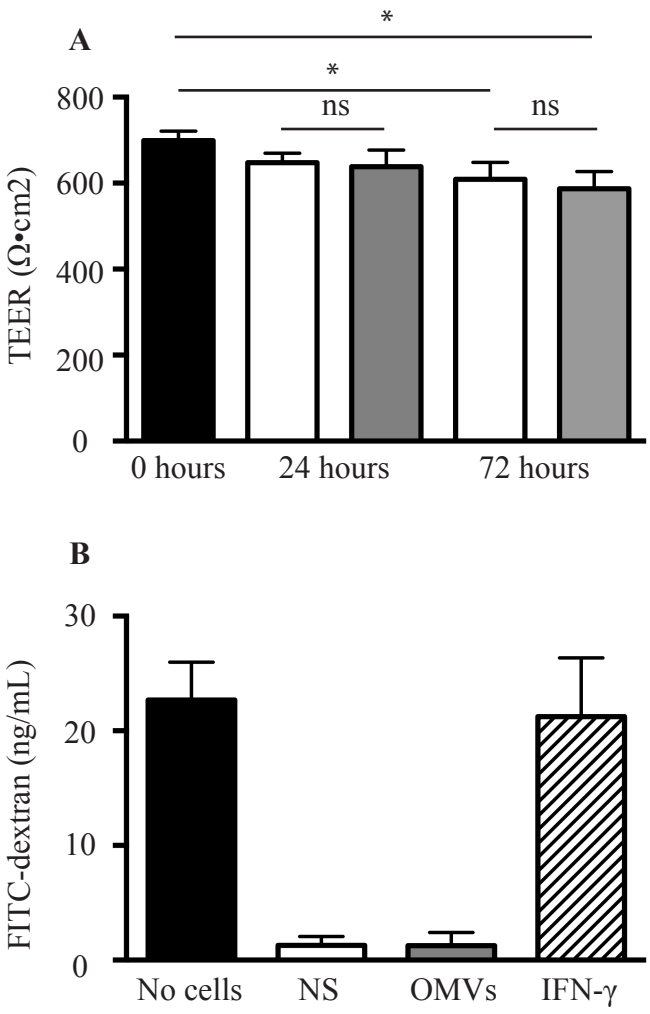


Figure 4.1. Treatment of polarised T84 intestinal epithelial cells with OMVs does not affect the integrity of the monolayer. T84 cells were grown for 15-21 days until polarised. Cells were considered polarised when they reached a stable TEER of 700Ω•cm2 (0 hours) (A). TEER was measured 24 and 72 hours after culture with medium alone (white bars) or stimulation with OMVs (grey bars) (B). The integrity of the monolayer was determined by the passage of FITC-dextran beads from the apical compartment to the basolateral compartment. Cells were grown until polarised and then cultured with medium alone (white bar), OMVs added apically (grey bar) or IFN-γ added basolaterally (striped bar) for 24 hours. Beads were added apically and the quantity of beads able to pass through the monolayer was determined after 3 hours. Data are represented as the means and standard deviation (SD) pooled from six experiments performed in triplicate (A) and one experiment performed in triplicate (B). *$P < 0.05$

hours (Figure 4.1A, *$P > 0.05$ for both non-stimulated and OMV-stimulated cells at 72 hours compared to cells at 0 hours). However, there were no significant decreases in the measured TEER between the non-stimulated and the OMV stimulated cells 24 or 72 hours after stimulation (Figure 4.1A). These data suggest that the decrease in measured TEER of the polarised cell monolayer after a stable TEER of 700Ω•cm^2 has been reached is not due to stimulation with *H. pylori* 251 OMVs.

To confirm the integrity of the monolayer, cells were grown until polarised and then treated apically with media alone or OMVs, or basolaterally with IFN-γ (Madara and Stafford 1989) for 24 hours. IFN-γ is known to increase the permeability of a polarised T84 cell monolayer when added basolaterally, as the IFN-γ receptor is present on the basolateral cell membrane (Boivin, Roy et al. 2009). Therefore, we used IFN-γ as a positive control to increase the permeability of T84 epithelial cell monolayers. FITC-conjugated 3kDa dextran beads were added to cells apically and the passage of beads through the monolayer was measured 3 hours later (Figure 4.1 B). Our data showed that beads were detected within the basolateral compartment of cells treated with IFN-γ (approximately 20-25 ng beads per ml) but not OMVs (approximately 2 ng beads per ml). Thus we concluded that treatment of polarised intestinal T84 epithelial cells with OMVs did not affect the integrity of the monolayer.

In order to determine if *H. pylori* OMVs were able to enter polarised epithelial cells, T84 cells were grown on Transwell® filters until a stable TEER of 700 Ω•cm^2 was reached and then co-cultured with OMVs. Intracellular OMVs were then detected using an anti-OMV specific antibody and visualised using confocal fluorescence microscopy.

To confirm that the cells and the membrane on which they were grown were not auto fluorescent, cells were cultured with medium alone (Figure 4.2 1A-C Incubation of permeabilised cells with the secondary antibodies; rabbit anti-*H. pylori* OMV and goat

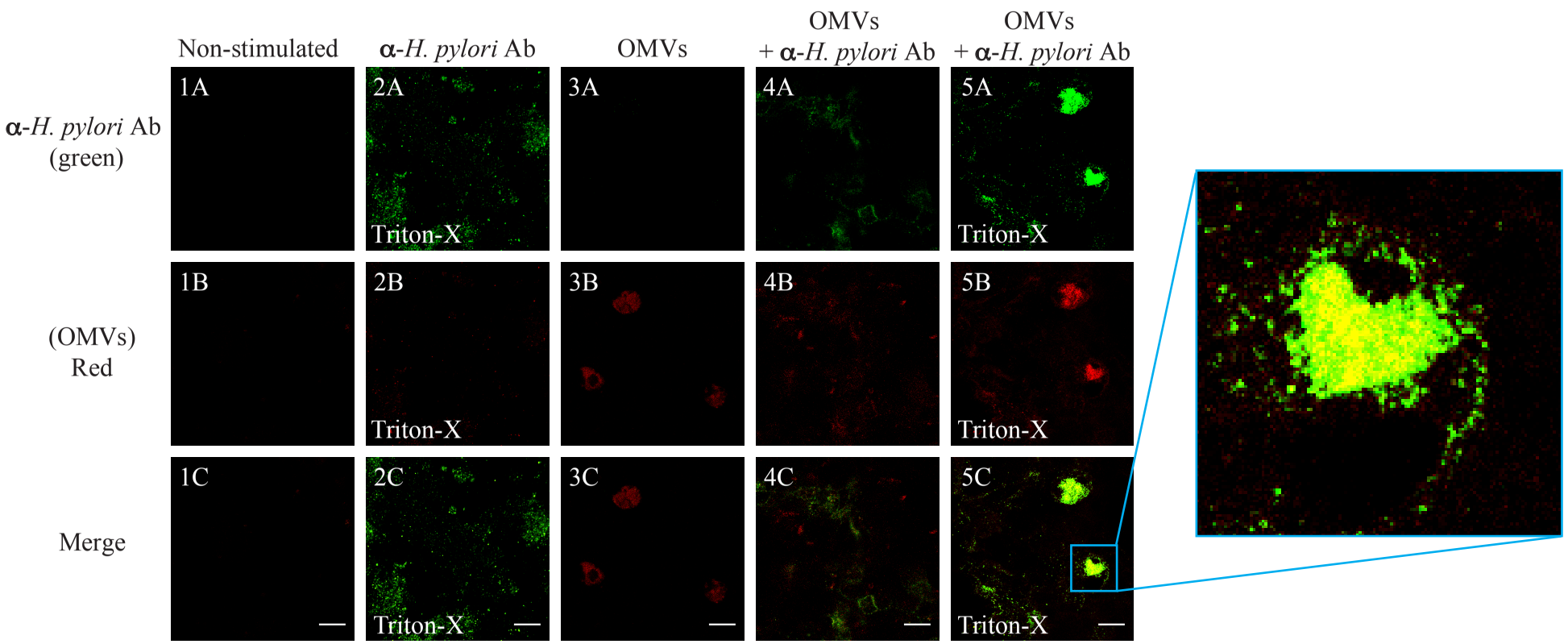


Figure 4.2. *H. pylori* OMVs enter polarised human T84 epithelial cells. Polarised T84 epithelial cells were co-cultured with AlexaFluor 568 labelled *H. pylori* OMVs (OMVs; red; 3, 4, 5) or medium alone (1, 2). Cells were permeabilised with Triton-X (2, 5) or not (1, 3, 4). OMVs were detected using rabbit anti-*H. pylori* OMV and goat anti-rabbit AlexaFluor 488 antibodies (anti-*H. pylori* Ab; green; 2, 4, 5). Images show green fluorescence (1-5B), red fluorescence (1-5B) and red and green images merged to show areas of co-localisation (yellow; 1-5C). Cells were visualised by confocal microscopy. OMVs (red) were contained within AGS cells and areas of dual fluorescence were only observed when cells were permeabilised with Triton-X (5C and zoom). Scale bar represents 10 mm. Zoomed image is 4x magnification. Data are representative of three independent experiments.

anti-rabbit IgG Alexa Fluor® 488, allowed us to demonstrate the ability of these antibodies to enter permeabilised cells (Figure 4.2 2A-C). Cells co-cultured with Alexa Fluor® 568-labelled OMVs alone were used to demonstrate that OMVs entered host cells without the need for permeabilisation (Figure 4.2 3A-C). Permeabilisation of the cells was required to allow antibodies to enter and co localise with intracellular OMVs (Figure 4.2 4A-C). Finally, permeabilisation of cells co-cultured with OMVs and subsequently incubated with secondary antibodies (Figure 4.2 5A-C), allowed us to visualise intracellular OMVs as areas of dual fluorescence (Figure 4.2 5C; yellow). This allowed us to show for the first time that *H. pylori* OMVs are internalised by polarised epithelial cells.

4.3.2 Stimulation of polarised epithelial cells with *H. pylori* OMVs induced the production and basolateral secretion of IL-8

IL-8 is an important chemo-attractant and activator of neutrophils and the infiltration of neutrophils into the gastric mucosa is a hallmark of *H. pylori* infection (Dixon 1991). Similarly, *H. pylori* OMVs induce IL-8 production by epithelial cells (Ismail, Hampton et al. 2003, Mullaney, Brown et al. 2009) and previous work performed in our laboratory demonstrated that the production of IL-8 is dependant upon OMV internalisation (Kaparakis, Turnbull et al. 2010). Thus, having shown that *H. pylori* OMVs are internalised by polarised T84 cells, we next wished to investigate the ability of internalised OMVs to induce IL-8 production by these cells.

Polarised T84 cells were co-cultured with either live *H. pylori*, formaldehyde fixed *H. pylori* or OMVs. The levels of IL-8 secreted into the apical and basolateral compartments were measured after 24 hours (Figure 4.3). There were no differences in the levels of IL-8 detected in either the apical or basolateral compartments of non-

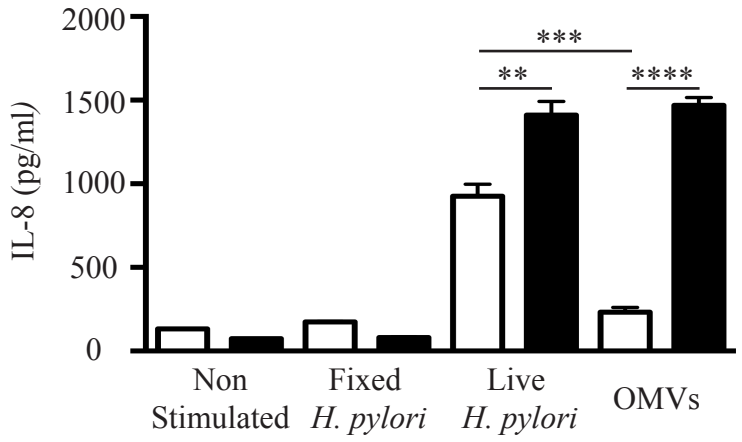


Figure 4.3. *H. pylori* and OMVs induce the basolateral secretion of IL-8 by polarised epithelial cells. Polarised T84 cells were co-cultured with media alone (non stimulated), formaldehyde fixed *H. pylori*, live *H. pylori* or OMVs. IL-8 production was measured from the apical (white) and basolateral (black) compartments, 24 hours post stimulation. Values represent the means and the SD of samples tested in triplicate and are representative of > 3 independent experiments. **P < 0.01, *** P <0.001, ****P < 0.0001.

stimulated cells compared with cells cultured with formaldehyde fixed *H. pylori* (Figure 4.3). In accordance with previously published studies (Hofman, Ricci et al. 2000), stimulation of polarised T84 epithelial cells with *H. pylori* resulted in significantly higher levels of IL-8 secreted into the basolateral compartment than the apical compartment (**$P < 0.01$). Similarly, polarised T84 cells co-cultured with OMVs secreted significantly higher levels of IL-8 into the basolateral compartment compared with the apical compartment (****$P < 0.0001$). Interestingly, apical secretion of IL-8 from polarised IECs was higher in response to *H. pylori* when compared with OMVs (***$P < 0.001$).

4.3.3 *H. pylori* OMVs up-regulate HLA Class II expression by epithelial cells

Engstrand and colleagues demonstrated that HLA Class II expression was up-regulated in gastric biopsy specimens taken from *H. pylori* infected patients compared to healthy donors (Engstrand, Scheynius et al. 1989, Wee, Teh et al. 1992). The up-regulation of HLA Class II by human epithelial cells (Fan, Crowe et al. 1998) and mouse gastric epithelial cells (Maekawa, Kinoshita et al. 1997) was subsequently demonstrated. However, to date there has been no published work regarding the effect of *H. pylori* OMVs on HLA Class I or Class II expression by human epithelial cells. This led us to query the expression levels of HLA Class I and II by epithelial cells in response to stimulation with *H. pylori* or *H. pylori* OMVs. As IFN-γ is known to increase HLA Class I and II expression by T84 cells (Colgan, Parkos et al. 1994), we included this as a positive control.

In our studies we found that non-polarised IECs demonstrated a 4-fold up-regulation of HLA Class I molecules (Figure 4.4 A, ***$P < 0.001$) and 1000-fold up-regulation of HLA Class II (Figure 4.4 B, ****$P < 0.0001$) in response to *H. pylori*. Interestingly, we

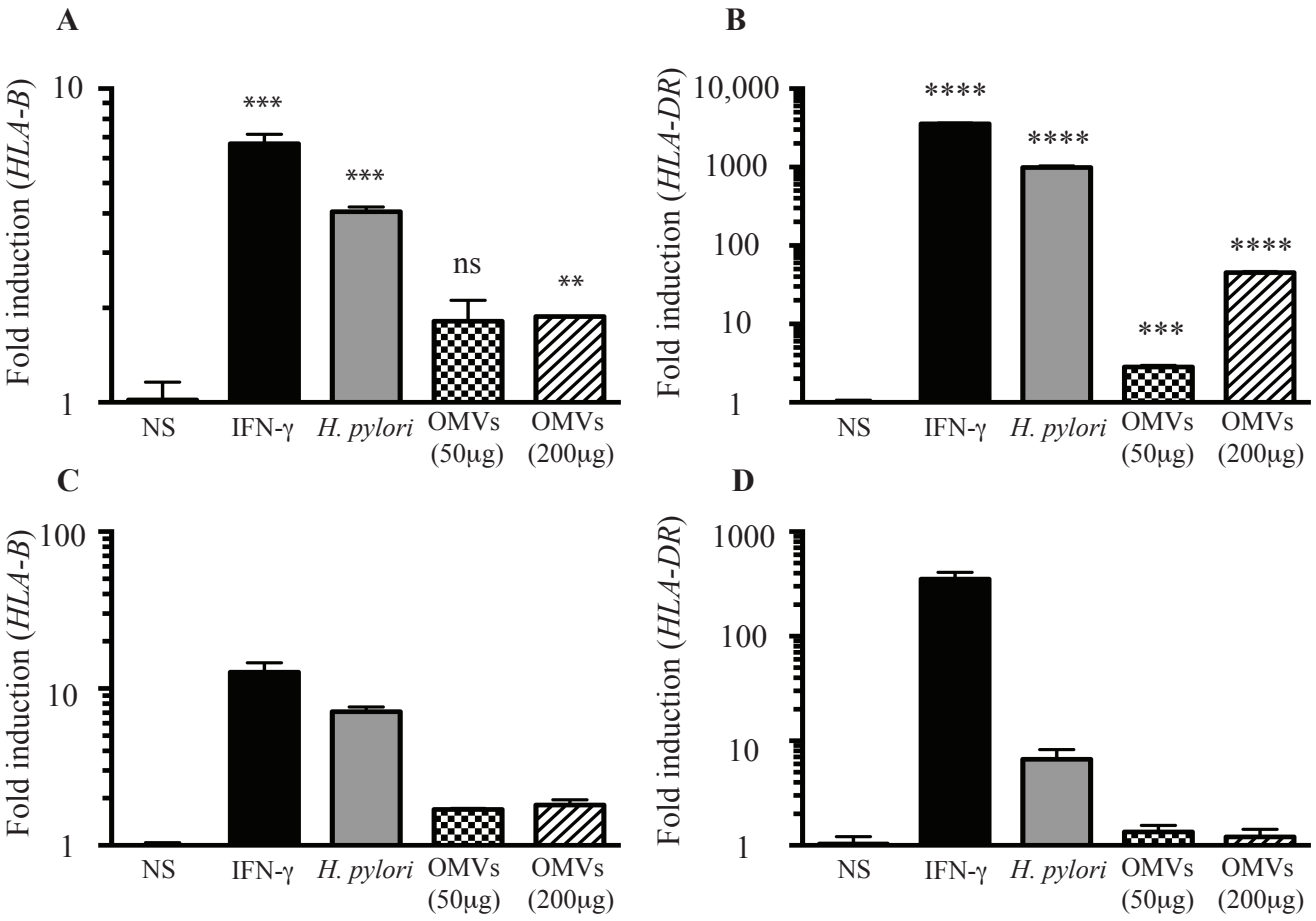
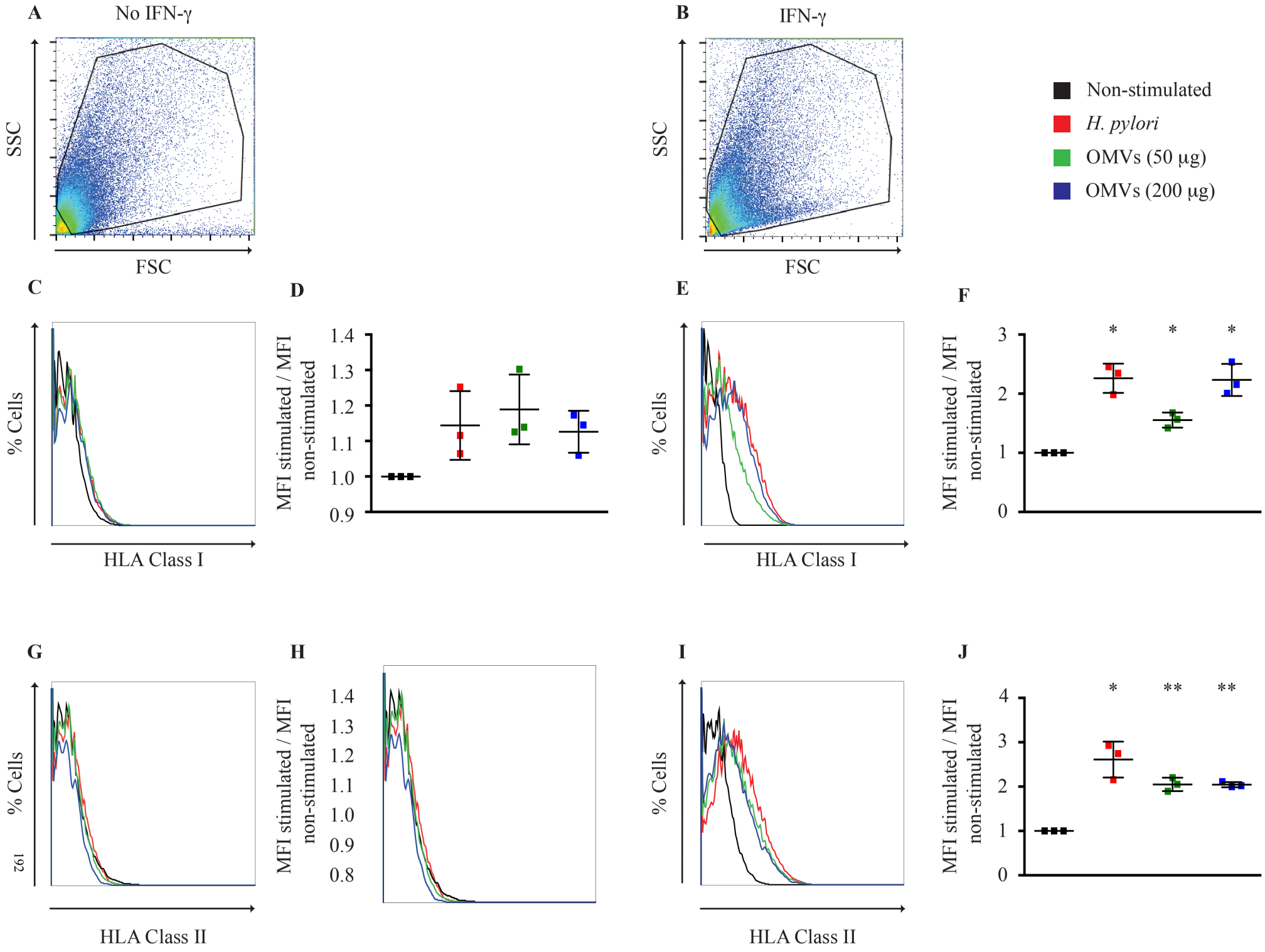


Figure 4.4: *H. pylori* and *H. pylori* OMVs up-regulate HLA Class I and II by non-polarised epithelial cells following pre-treatment with IFN-γ. Non-polarised (A, B) or polarised (C, D) T84 cells were left non-stimulated (NS) or treated with IFN-γ bsolaterally (black bars), *H. pylori* (grey bars) apically or OMVs (50μg; chequered bars or 200 μg; striped bars) apically. The expression of HLA Class I (A, C) or HLA Class II (B,D) was measured by qRT-PCR. Values are the means and standard deviation (SD) and are representative of three experiments performed in triplicate (A, B) and two experiments performed in triplicate (C, D). $^{**}P < 0.01$, $^{***}P<0.001$, $^{****}P<0.0001$)

found that non-polarised IECs demonstrated a 1.5-fold and 2-fold up-regulation of HLA Class I expression (Figure 4.4 A) in response to OMVs at 50 μg (ns) or 200 μg per ml (**$P < 0.01$), respectively. Similarly, we demonstrated 3-fold and 45-fold increases in HLA Class II (Figure 4.4 B) expression in response to OMVs at 50 μg (*** $P < 0.001$) or 200 μg per ml (****$P < 0.0001$), respectively. Similarly, polarised epithelial cells up-regulated HLA Class I (Figure 4.4 C) and Class II (Figure 4.4 D) expression in response to *H. pylori* (7-fold and 10-fold, respectively) or OMVs at 50 μg (1.6-fold and 1.6-fold, respectively) or 200 μg per ml (2-fold and 1.5-fold, respectively).

We next wished to confirm the ability of T84 epithelial cells to up-regulate HLA Class I and II in response to *H. pylori* or OMVs using flow cytometry. Non-polarised T84 cells were pre-treated, or not, with IFN-γ for 48 hours, prior to co-culture with *H. pylori* or OMVs for 24 hours. Interestingly, the up-regulation of HLA Class I and II was only detectable by cells that had been pre-treated with IFN-γ (Figure 4.5 B, E, F, I, J) and was not detectable in cells that were not pre-treated IFN-γ (Figure 4.5 A, C, D, G, H). HLA Class I expression by IECs pre-treated with IFN-γ was up-regulated in response to *H. pylori* or OMVs (Figure 4.5 E, F; both *$P < 0.05$). Furthermore, HLA Class II expression was significantly increased in response to *H. pylori* by non-polarised cells pre-treated with IFN-γ compared to cells stimulated with IFN-γ alone (Figure 4.5 I, J, *$P < 0.05$). HLA Class II expression was also up-regulated by co-culture of IFN-γ treated cells with *H. pylori* OMVs compared with cells stimulated with IFN-γ alone (Figure 4.5 I, J, **$P < 0.01$).

Figure 4.5: *H. pylori* and *H. pylori* OMVs up-regulate cell surface expression of HLA Class I and II by non-polarised epithelial cells following pre-treatment with IFN-γ. Cells were pre-treated for 24 hours with IFN-γ (A, C, D, G, H) or not (B, E, F, I, J) prior to co-culture with medium alone (black), *H. pylori* bacteria (red) or OMVs (200 μg, blue; 50μg, green). Live cells were selected based on their forward scatter (FSC) and side scatter (SSC) profiles (A, B), and were gated for further analysis of their cell surface expression of HLA Class I (C, E) and Class II (G, I) (plotted as histograms). The MFI of HLA Class I (D, F) or II (H, J) were determined. Results are expressed as the ratios of the MFIs of cells cultured with H. pylori or OMVs:cells cultured with medium alone. Histograms are representative of three individual experiments. For MFI graphs, values represent three individual experiments, error bars indicate the SD.


A
No IFN-γ
SSC
FSC
B
IFN-γ
SSC
FSC
Non-stimulated
H. pylori
OMVs (50 μg)
OMVs (200 μg)
C
% Cells
HLA Class I
D
MFI stimulated / MFI non-stimulated
1.4
1.3
1.2
1.1
1.0
0.9
E
% Cells
HLA Class I
F
MFI stimulated / MFI non-stimulated
3
2
1
0
*
*
*
G
% Cells
192
HLA Class II
H
MFI stimulated / MFI non-stimulated
1.4
1.3
1.2
1.1
1.0
0.9
0.8
I
% Cells
HLA Class II
J
MFI stimulated / MFI non-stimulated
4
3
2
1
0
*
**
**

4.3.4 OMV proteins are present within cell lysates and exosomes samples of IEC co-cultured with *H. pylori* OMVs

In the first study demonstrating intracellular *H. pylori* OMVs, Fiocca *et al.* proposed that internalised OMVs may enter the endocytic pathway from which exosomes are derived (Fiocca, Necchi et al. 1999). This led us to hypothesise that internalised OMVs may be processed intracellularly and subsequently packaged within exosomes that are released from the cells.

In order to test this hypothesis, we co-cultured *H. pylori* OMVs with polarised T84 cells, which secrete exosomes from the apical and basolateral surfaces (van Niel, Raposo et al. 2001). The resulting apical and basolateral exosomes were viewed by TEM (Figure 4.6) and subjected to SDS/PAGE and Western blot analysis with anti-*H. pylori* OMV antibodies (Figure 4.7). TEM analysis of exosomes secreted from the apical surface (apical exosomes) revealed a relatively homogeneous population of single vesicles ranging from approximately 50-100 nm in diameter (Figure 4.6A). The exosomes secreted from the basolateral surface (basolateral exosomes) (Figure 4.6B) exhibited the similar heterogeneous aggregate morphology described by Van Niel *et al* (van Niel, Raposo et al. 2001) (white arrowhead, Figure 4.6B), with their sizes ranging from approximately 50-180 nm in diameter. Both apical and basolateral exosomes exhibited the characteristic 'cup shape' morphology that resembles a flattened sphere with a bilipid layer (Chaput, Taieb et al. 2005) (indicated by black arrows, Figure 4.6).

Using Western blot analysis, we detected OMV proteins within the OMV stimulated cell lysate and exosome samples, indicating that proteins derived from intracellular OMVs are present within the cell lysates, as well as exosomes (Figure 4.7). Interestingly, the major protein present appears to be approximately 18-20 kDa (indicated by arrow) and is similar to the size of Lpp20, a known OMV associated

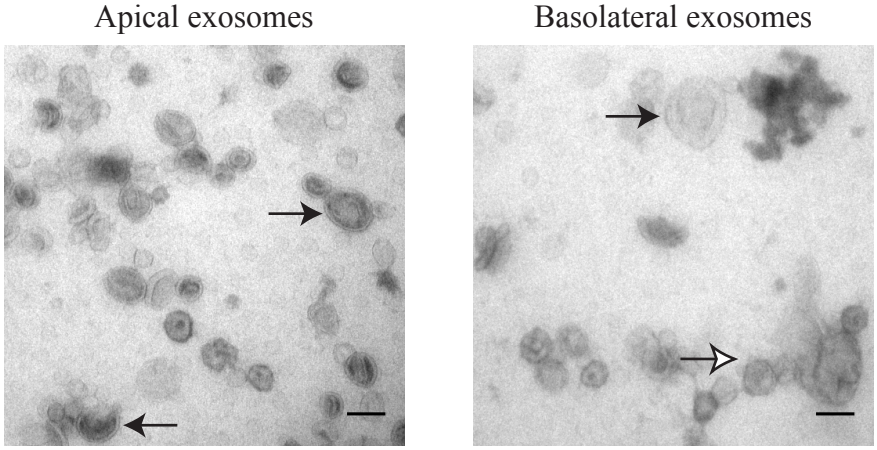


Figure 4.6: TEM analysis of exosomes. Exosomes obtained from apical cell culture conditioned media appeared as single vesicles, ranging from 50-100 nm in diameter. Exosomes obtained from basolateral cell culture conditioned media were more heterogeneous in nature, approximately 50-180 nm in diameter, and often appeared as aggregates (white arrowhead). Both apical and basolateral exosomes exhibited a double-layer membrane (black arrows). Scale bar indicates 100 nm.

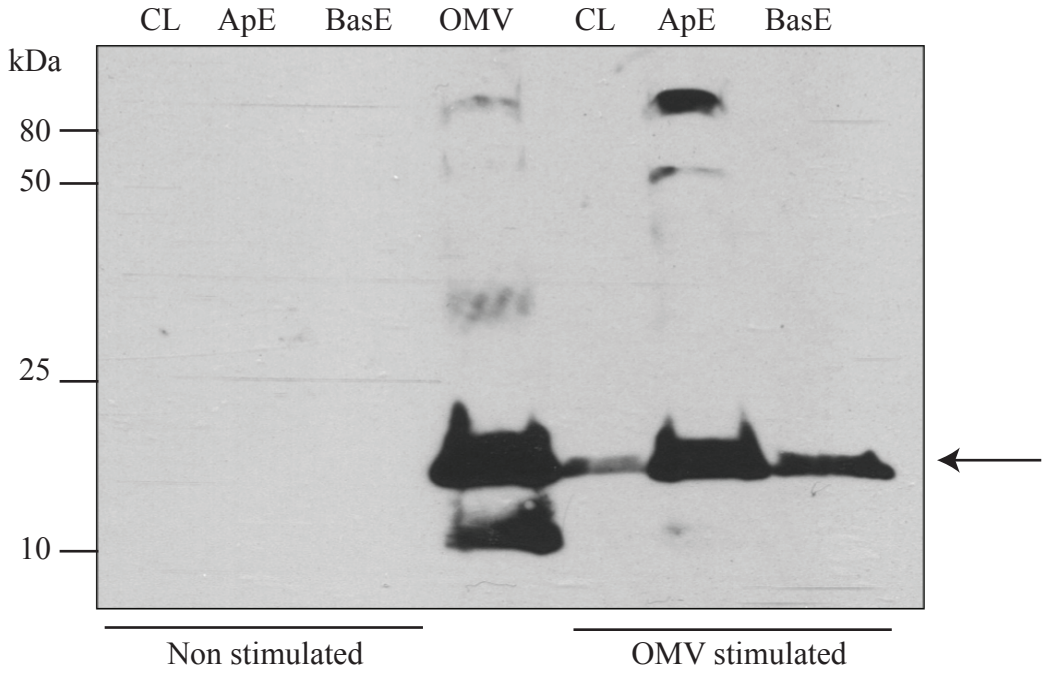


Figure 4.7: OMV proteins detected within exosomes derived from polarised T84 cells cultured with *H. pylori* OMVs. Western blot analysis, using an anti-*H. pylori* OMV antibody, of cell lysates (CL), apical exosomes (ApE), and basolateral exosomes (BasE) derived from either non stimulated or *H. pylori* OMV stimulated polarised T84 epithelial cells. An OMV (OMV) preparation was included as a positive control. Arrow indicates the main protein band of approximately 18-20 kDa. Representative of > 3 independent experiments.

vaccine candidate (Keenan, Oliaro et al. 2000). Relatively few bands greater then 20 kDa were present.

Having demonstrated the presence of OMV proteins within exosomes, we next used mass spectrometry to identify the proteins present within basolateral exosome samples (Table 4.2 and Table S1). We detected 8 *H. pylori*-specific proteins within basolateral exosomes derived from OMV stimulated cells ("OMV" exosomes) that were not detected within basolateral exosomes derived from non-stimulated cells ("empty" exosomes). Interestingly, these *H. pylori* proteins included the following known virulence factors: VacA, HopQ, catalase and Lpp20 (Keenan, Oliaro et al. 2000).

From the sequencing data presented in Chapter 3, we know that 6 of the 7 proteins identified in this study within exosomes are also found within large OMVs (Chapter 3 Table 1 and Table S2). The scores represent the overall abundance of peptides within the sample analysed. Of the proteins identified within large OMVs, the average score was 2865 and the median was 1536, with the lowest score being 68 and the highest being 34510. Therefore, the reported initial abundance within *H. pylori* OMVs of the proteins detected in "OMV" exosomes is not particularly low (Table 4.3).

Were these proteins found at higher abundances within "OMV" exosomes, this would suggest that the OMVs were passing intact through the cell monolayers via the intracellular junctions. We believe that the low abundance of these proteins within "OMV" exosome preparations reflects a low abundance of *H. pylori* peptides in the exosomes, as OMVs are degraded within the cell.

Of the 239 human proteins identified in either of the exosomes samples, 55 were unique to "empty" exosomes, whereas no unique human proteins were contained within "OMV" exosomes (Tables S1 and S2). The A33 antigen, an intestinal epithelial cell marker known to be associated with epithelial cell derived exosomes (van Niel,

Table 4.2: *H. pylori* proteins in "OMV" exosomes

Gene name	Open reading frame no.	Protein name	Molecular Weight (kDa)	Identification probability	Sequence coverage	Number of peptides	Sequences (positions outside brackets)
lpp20	HP1456	Lpp20 lipoprotein	19	100%	11%	2	91(R)ANLAANLK(S)98 43(K)GAPDWVVGDLEK(V)54
flaA	HP0601	Flagellin A	53	100%	4.50%	2	41(K)AADDASGMTVADSLR(S)55 111(K)AIQSDIVR(L)118
vacA	HP0887	Vacuolating cytotoxin autotransporter	139	100%	1.40%	2	762(R)LALYNNNNR(M)770 620(R)NIKNVEITR(K)628
flgE	HP0870	Flagellar hook protein	76	99%	1.40%	1	685(R)SLTNLIVVQR(G)694
	HP1457	Putative uncharacterized protein	23	100%	12.00%	2	162(K)VSSIAASISSSR(Q)173 174(R)QRLDYDFTLSLTNR(K)187
katA	HP0875	Catalase	59	100%	4.40%	2	94(R)FSTVAGER(G)101 347(R)LGVNYPQIPVNKPR(C)360
metK	HP0197	S-adenosylmethionine synthase	42	100%	4.00%	1	231(K)FVIGGPQGDAGLTGRK(I)245
hopQ	HP1177	Outer membrane protein (Omp27)	70	100%	4.70%	2	50(K)LSDTYEQLSR(L)59 89(K)TLAGGTTNSPAYQATLLALR(S)108

Table 4.3. Abundance scores of proteins within “large” OMVs that were

Gene name	Open reading frame no.	Protein name	Score	# Peptides
lpp20	HP1456	membrane-associated lipoprotein	9984.98	9
katA	HP0875	catalase	26552.56	21
hopQ	HP1177	outer membrane protein	35410.35	13
flgE	HP0870	flagellar hook protein	4847	11
vacA	HP0887	vacuolating cytotoxin	7883.58	15
flaA	HP0601	flagellin A	761.89	3

Mallegol et al. 2003) was found to be present within both "empty" and "OMV" exosomes. The common exosome markers HSP60, and CD9 were also present in both samples. Furthermore, a variety of exosome associated proteins were identified, including a variety of Rabs, Raps, Annexins, integrins and clathrin heavy chain (Table S4.1) (Mathivanan, Lim et al. 2010). Interestingly, an HLA Class I protein, HLA-A, was detected in both exosomes samples, whereas no HLA Class II proteins were identified in either sample.

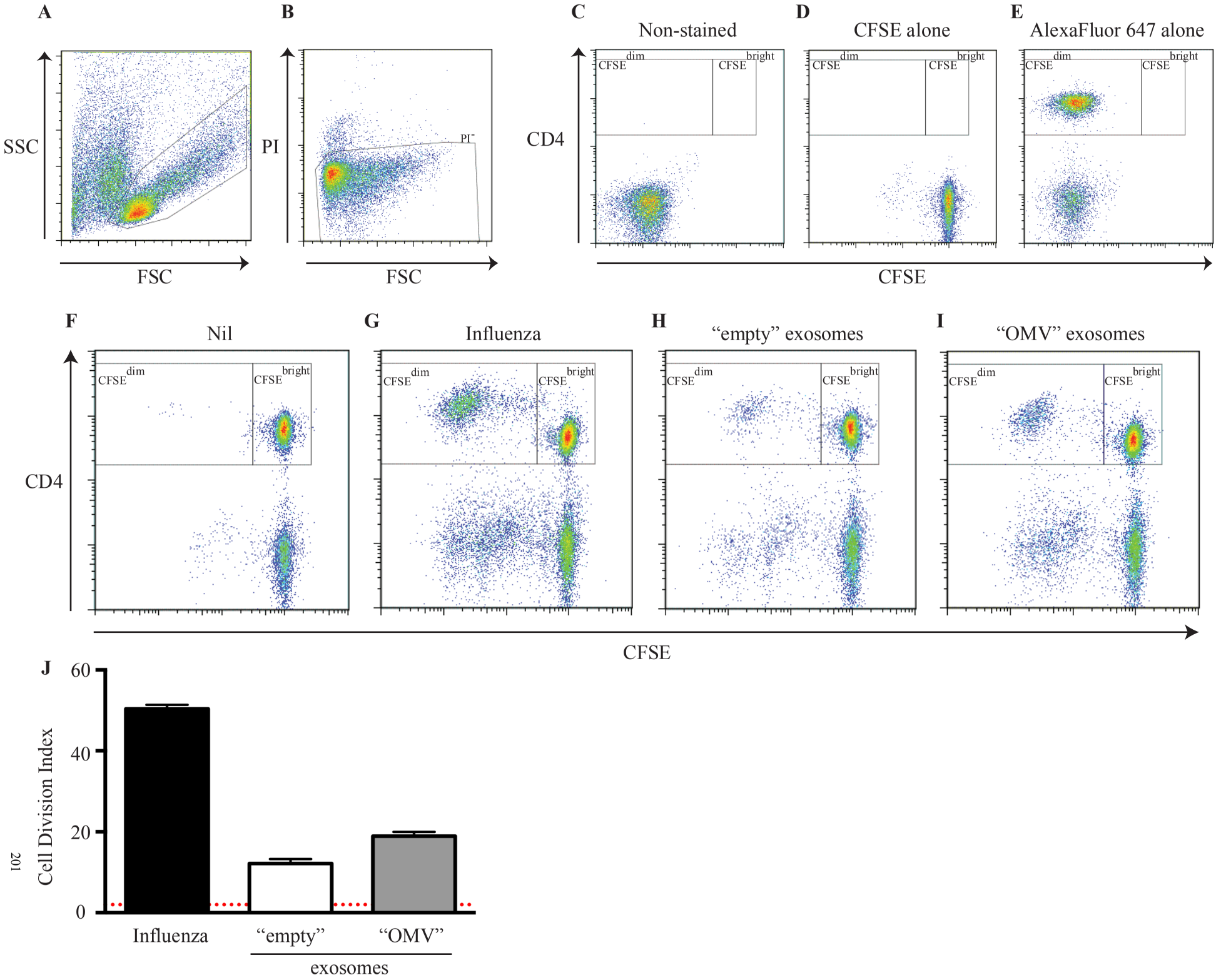
4.3.5 Human T cells proliferate in response to OMV-containing exosomes

Having determined that "OMV" exosomes contained within them specific *H. pylori* proteins, we next wished to investigate their ability to induce *H. pylori* specific T cell responses. For this, we used a method developed by Mannering *et al.* to measure the proliferation of $CD4^+$ T cells in response to antigen stimulation (Mannering, Morris et al. 2003). PBMCs were isolated from healthy, *H. pylori* negative human volunteers (Appendix 4.1) and labelled with CFSE prior to co-culture with media alone, influenza virus as a positive control (Ciantar and Mannering 2011), "empty" exosomes or "OMV" exosomes (Figure 4.8 F-I). We found that $CD4^+$ T cells proliferated in response to "OMV" exosomes, with a CDI of 22 (Figure 4.8 I, J). However we found that $CD4^+$ T cells also responded to "empty" exosomes, with a CDI of 13 (Figure 4.8 H, J), indicating an allogeneic T cell response to the human exosomal proteins present.

4.3.6 Human T cells proliferate in response to *H. pylori* lysate.

We hypothesised that the allogeneic response to the human proteins present in exosomes (Figure 4.8) would be reduced if we used a pool of *H. pylori* reactive T cells. Therefore, we sought to isolate a pool of T cells that proliferated in response to

Figure 4.8: Human $CD4^+$ T cells proliferate in response to exosomes. Human CFSE labelled PBMCs were incubated with medium alone (Nil), Influenza virus (Influenza), basolateral exosomes derived from non-stimulated cells ("empty" exosomes) or exosomes derived from OMV-stimulated cells ("OMV" exosomes). Lymphocytes were gated based on their forward scatter (FSC) and side scatter (SSC) profiles (A). Non-viable cells were excluded from analysis based on PI positive staining (B). Gates were placed around CFSEbright and CFSEdim $CD4^+$ cells based on the staining profiles for non-stained cells (C), CFSE alone stained cells (D) and AlexaFluor 647 (CD4) alone stained cells (E). The levels of proliferation of cells in response to media alone (F) Influenza (G), "empty" exosomes (H) or "OMV" exosomes (I) were determined by calculation of the cell division index (J). Values represent the means determined in triplicate from an individual donor. A CDI of >2 is considered a positive response (indicated by red dashed line).


A
SSC
FSC
B
PI
PI-
FSC
C
Non-stained
CD4
dim
bright
CFSE
CFSE
D
CFSE alone
E
AlexaFluor 647 alone
CFSE
F
Nil
G
Influenza
H
"empty" exosomes
I
"OMV" exosomes
CD4
CFSE
J
Cell Division Index
60
40
20
0
Influenza
"empty"
"OMV"
exosomes

H. pylori lysate, that we could then culture with "empty" or "OMV" exosomes to measure *H. pylori* antigen specific proliferative responses. In order to determine the correct concentration of *H. pylori* lysate to induce T cell proliferation, we stimulated CFSE labelled PBMCs with *H. pylori* lysate at a range of concentrations (1 – 100 μg per ml) and measured their proliferation using the CFSE proliferation assay (Figure 4.9 F). The highest levels of proliferation of $CD4^+$ (FACS plots not shown) and $CD8^+$ T cells were measured at lower concentrations of *H. pylori* stimulation (5 and 10μg per ml; Figure 4.9 G-H, respectively). As human T cells were previously found to respond to *H. pylori* lysate at 10 μg per ml (D'Elios, Manghetti et al. 1997), we used this concentration of *H. pylori* lysate for the remainder of our studies.

To isolate a population of *H. pylori* reactive T cells, CFSE labelled PBMCs were cultured with *H. pylori* lysate and then $CD3^+$ T cells that had divided were sorted from cells that had not divided based on their CFSE staining. In parallel, T cell proliferation assays were performed to investigate the $CD4^+$ and $CD8^+$ T cell proliferative responses to *H. pylori* lysate between the various donors. CytoStim (Campbell, Foerster et al. 2010) was used as a positive control for proliferation. We found that whilst T cells from all donors proliferated in response to *H. pylori* lysate (Figure 4.10 E, F), the CDI values differed vastly from donor to donor and between $CD4^+$ and $CD8^+$ T cells (Table 4.4). This was also reflected in the CDI of cells cultured with CytoStim, with donors who exhibited a lower CDI in response to *H. pylori* also demonstrating a lower CDI for CytoStim (Table 4.4). A CDI of >2 is considered positive, and each donor demonstrated a CDI of >2 in response to *H. pylori* lysate for both $CD4^+$ and $CD8^+$ cells (Table 4.4).

Table 4.4: Proliferative response (CDI) of human $CD4^+$ and $CD8^+$ T cells to

	CDI of $CD4^+$ T cells in response to:		CDI of $CD8^+$ T cells in response to:	
	CytoStim	*H. pylori* lysate	CytoStim	*H. pylori* lysate
Donor a	356.68	66.66	1299.86	125.77
Donor b	273.81	10.57	805.25	12.14
Donor c	57.59	2.08	76.60	2.06
Donor d	432.24	14.14	412.84	11.97
Donor e	216.55	16.75	207.99	10.95

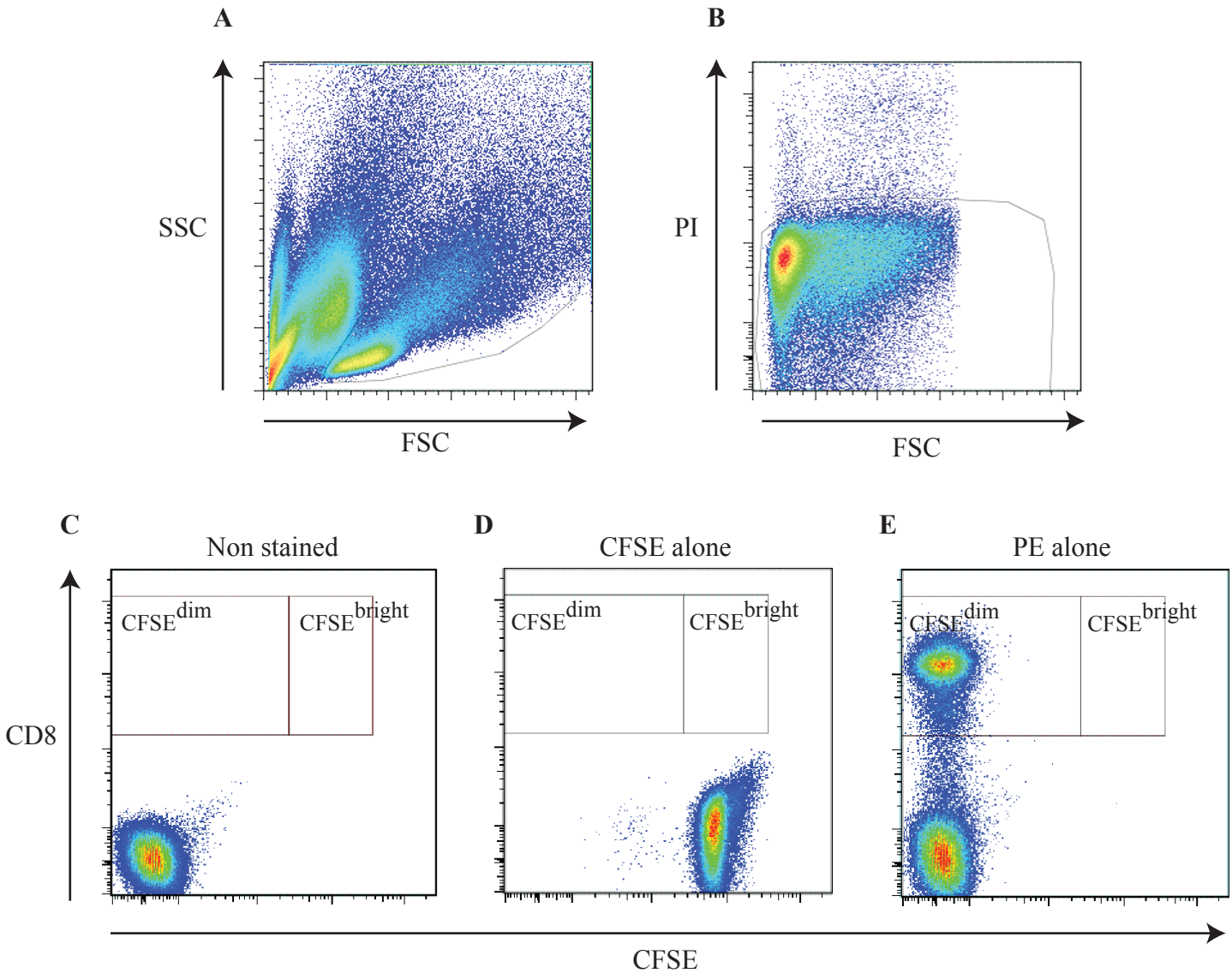


Figure 4.9: Proliferative responses of human CD4$^+$ and CD8$^+$ T cells when cultured with increasing doses of *H. pylori* lysate. Lymphocytes were gated on based on their forward scatter (FSC) and side scatter (SSC) profiles (A). Non-viable cells were excluded from analysis based on PI positive staining (B). Gates were placed around CFSE^{bright} and CFSE^{dim} CD4$^+$ (not shown) or CD8$^+$ cells based on the staining profiles for non-stained cells (C), CFSE alone stained cells (D) and PE (CD8) alone stained cells (E) or AlexaFluor 647 (CD4) alone stained cells (not shown). The levels of proliferation of CD4+ (G; FACS plots not shown) and CD8$^+$ (H) T cells in response to increasing doses (1 - 100 µg per ml) of *H. pylori* lysate (F) were determined by calculation of the cell division index. Values represent the CDI of a single donor. A CDI of >2 is considered a positive response (indicated with a red dashed line).

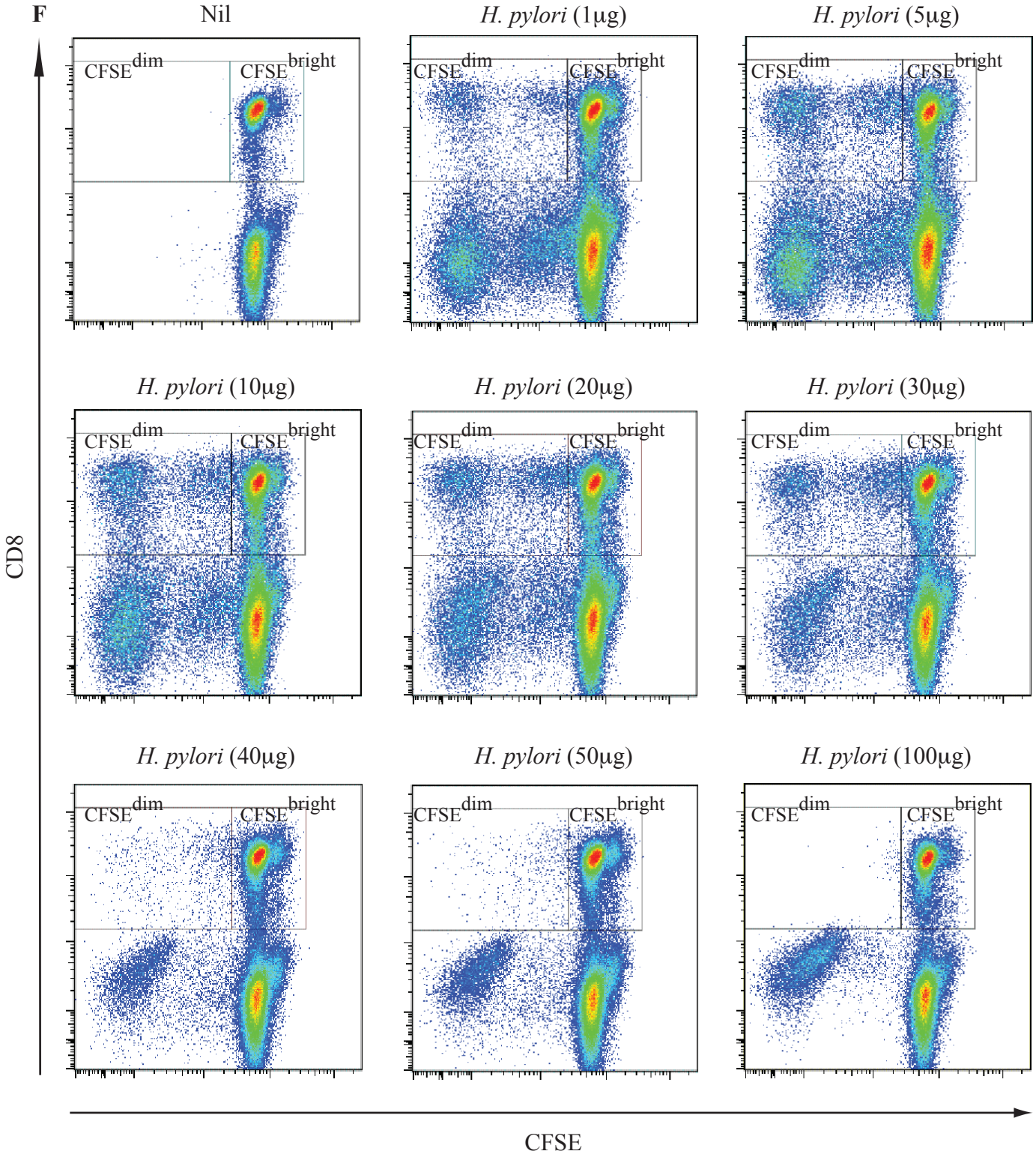
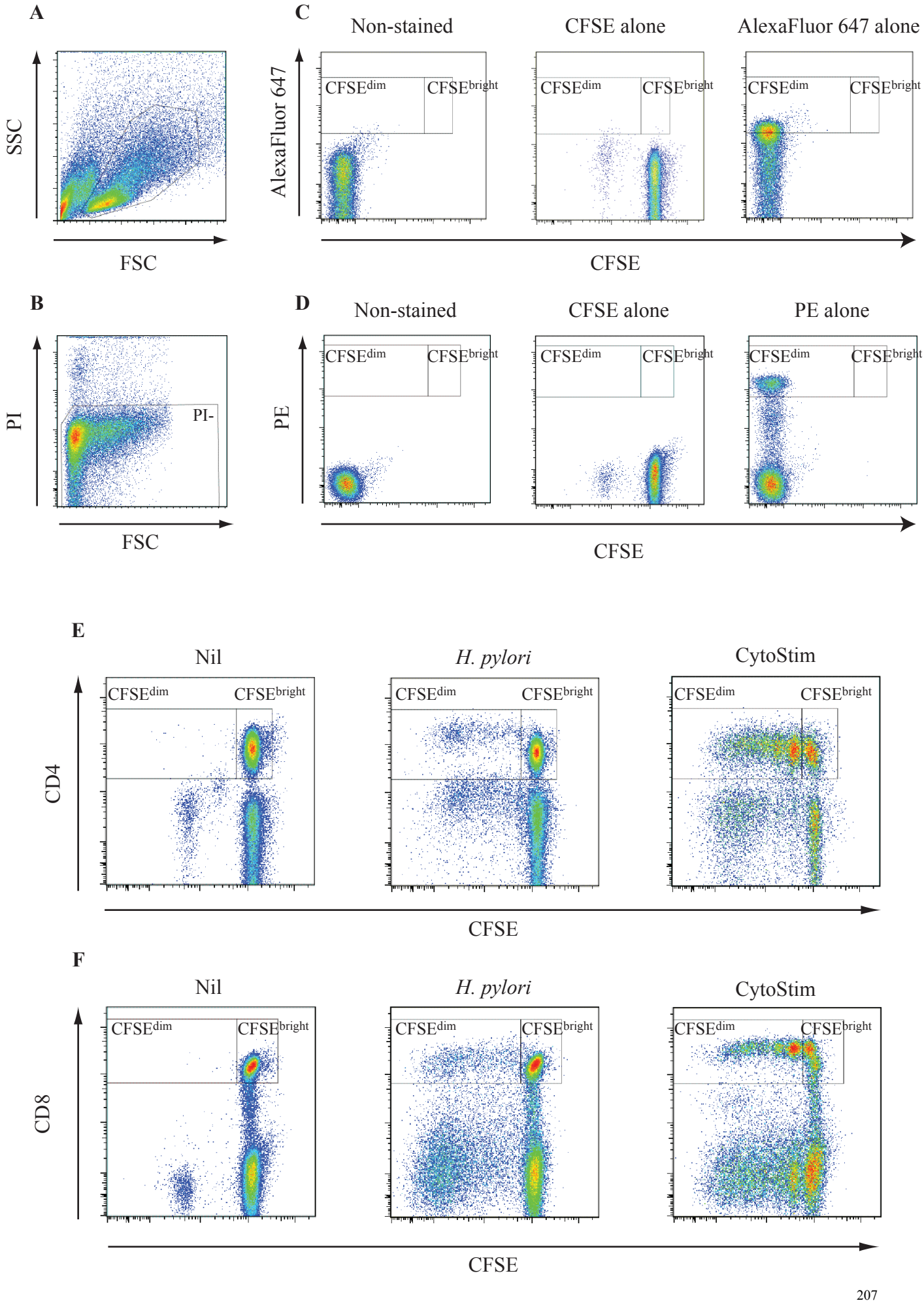

F
Nil
H. pylori (1μg)
H. pylori (5μg)
H. pylori (10μg)
H. pylori (20μg)
H. pylori (30μg)
H. pylori (40μg)
H. pylori (50μg)
H. pylori (100μg)
CFSE dim
CFSE bright
CD8
CFSE
G
CD4+
CDI
20
15
10
5
0
1 5 10 20 30 40 50 100
H. pylori lysate (μg)
H
CD8+
1500
1000
500
0
1 5 10 20 30 40 50 100
H. pylori lysate (μg)

Figure 4.10: Human PBMCs proliferate in response to *H. pylori* lysate. CFSE labelled PBMCs were cultured with medium alone (Nil), *H. pylori* lysate (*H. pylori*) or CytoStim (CytoStim) and the proliferative responses of $CD4^+$ and $CD8^+$ T cells measured after 7 days. Gates were placed around lymphocytes based on their FSC and SSC profiles (A). Non-viable cells were excluded from analysis based on PI positive staining (B). Gates were placed around $CFSE^{bright}$ and $CFSE^{dim}$ $CD4^+$ (C) or $CD8^+$ (D) cells, based on the single colour staining profiles to allow calculation of the cell division index of $CD4^+$ (E) or $CD8^+$ (F) cells. Scatter plots are representative of data collected from 5 individual donors performed in triplicate.


A
SSC
FSC
B
PI
PI-
FSC
C
Non-stained
CFSE alone
AlexaFluor 647 alone
AlexaFluor 647
CFSE^dim
CFSE^bright
CFSE
D
Non-stained
CFSE alone
PE alone
PE
CFSE^dim
CFSE^bright
CFSE
E
Nil
H. pylori
CytoStim
CD4
CFSE^dim
CFSE^bright
CFSE
F
Nil
H. pylori
CytoStim
CD8
CFSE^dim
CFSE^bright
CFSE

4.3.7 *H. pylori* lysate or OMVs induce Th1 and Th2 cytokine secretion by human PBMCs.

In order to examine the cytokine profiles of PBMCs cultured with *H. pylori* lysate or *H. pylori* OMVs, we collected supernatants 7 days post stimulation, during CFSE proliferation assays (as described in section 4.3.6). *H. pylori* lysate induced the secretion of IFN-γ, IL-6, IL-10, TNF-α and IL-2 (*****P*<0.0001, ***P*<0.01, *****P*<0.0001, *****P*<0.0001 and **P*<0.05, respectively, Figure 4.11) by human PBMCs. OMVs induced a similar cytokine profile by PBMCs as the *H. pylori* lysate, with induction of the cytokines IL-6 , IL-10 and TNF-α (*****P*<0.0001, **P*<0.05 and ***P*<0.01, respectively, Figure 4.11). There was a trend, albeit non-significant, towards higher IFN-γ production by cells in response to OMVs (average 75 pg per ml) compared with no stimulation (average 3.9 pg per ml). Interestingly, the induction of IFN-γ, IL-10 and TNF-α secretion from T cells in response to OMVs, was increased when compared with non-stimulated cells, but than that induced by the *H. pylori* lysate. Furthermore, the induction of IL-6 was comparable between *H. pylori* lysate and OMVs. Neither *H. pylori* lysate nor OMVs induced the secretion of IL-2, IL-4 or IL-17A by human PBMCs (Figure 4.11).

4.3.8 Human *H. pylori* reactive T cells proliferate in response to exosomes containing OMV proteins.

H. pylori reactive $CD3^+$ T cells (Section 4.3.6) were co-cultured with either *H. pylori* lysate as a positive control for antigen specific proliferation, “empty” exosomes or “OMV” exosomes. Proliferation was measured using a Click-IT EdU based proliferation assay (Figure 4.12). The Click-iT EdU proliferation assay was chosen in preference to the CFSE based T cell proliferation assay used previously in this study to

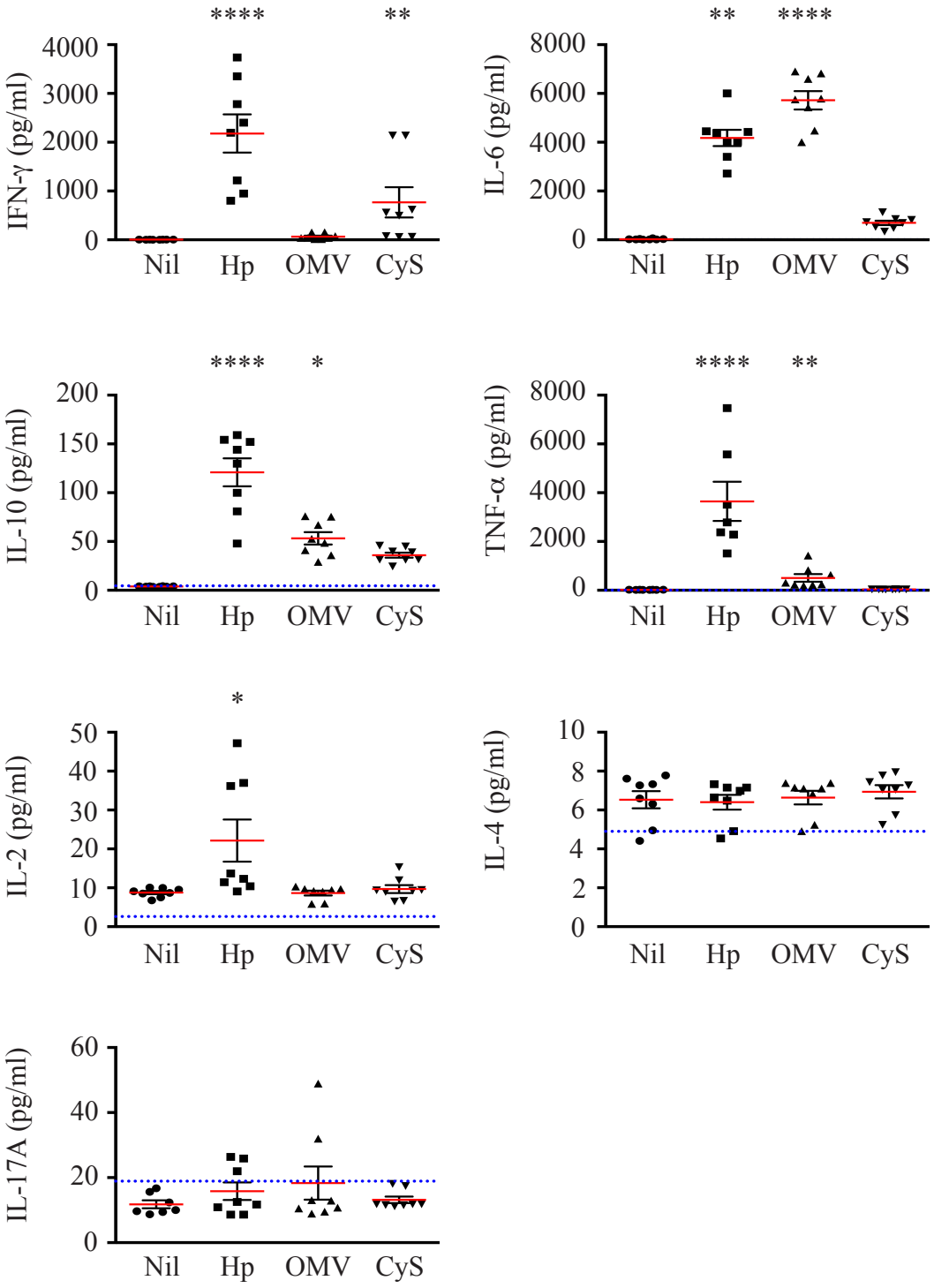
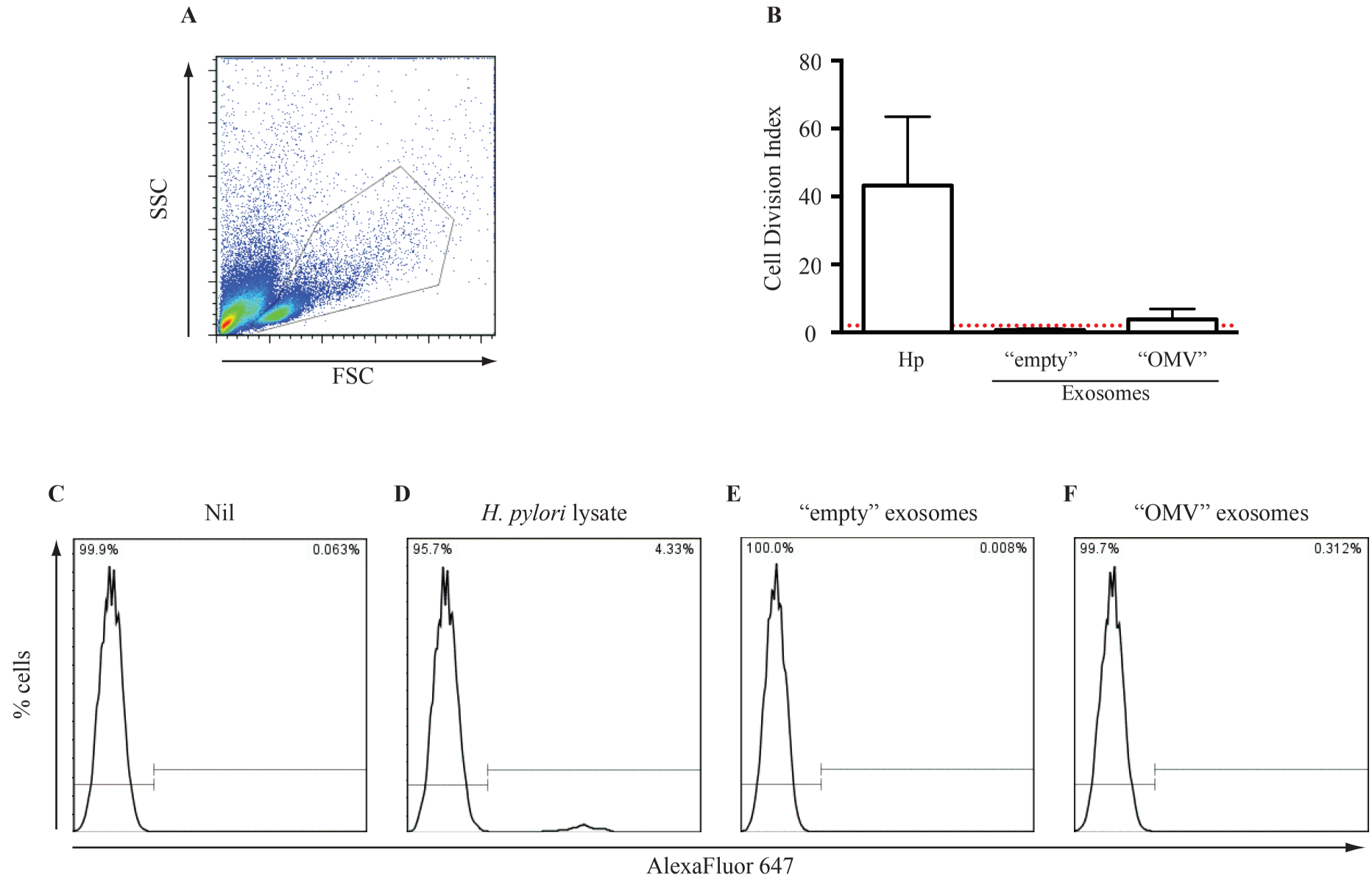


Figure 4.11: Human PBMCs produce pro- and anti-inflammatory cytokines in response to *H. pylori* lysate and OMVs. Human PBMCs were stimulated with *H. pylori* lysate (Hp; squares), OMVs (OMV; triangles), CytoStim (CyS; inverted triangles, positive control) or left non-stimulated (Nil; circles). Supernatants were collected 7 days later and analysed using by flow cytometry. The blue dashed lines indicate the detection limit for each cytokine where visible. Shown are the responses of 7 individual donors determined in duplicate. Error bars represent the means (red dash) and standard deviations (SD). **** P <0.0001, ** P <0.01, * P <0.05.

Figure 4.12: *H. pylori* reactive human T cells proliferate in response to exosomes derived from OMV stimulated cells ("OMV" exosomes). Human *H. pylori* reactive $CD3^+$ T cells were cultured with irradiated $CD3^-$ cells and were left non stimulated (C; Nil) or stimulated with either *H. pylori* lysate (D), "empty" exosomes (E) or "OMV" exosomes (F). Proliferation was measured using a Click-iT EdU proliferation assay. A gate was placed around live cells based on their FSC and SSC profiles (A). Representative histograms from an individual donor are shown. Values indicate the % of cells that had proliferated or not. The CDI of $CD3^+$ T cells in response to *H. pylori* lysate (Hp), "empty" exosomes or "OMV" exosomes was determined (B). Values are the means and stardard deviations determined from two individual donors determined in duplicate. A cell division index of >2 is considered a positive response (indicated by a red dashed line).


A
SSC
FSC
B
Cell Division Index
80
60
40
20
0
Hp
"empty"
"OMV"
Exosomes
C
Nil
99.9%
0.063%
D
H. pylori lysate
95.7%
4.33%
E
"empty" exosomes
100.0%
0.008%
F
"OMV" exosomes
99.7%
0.312%
% cells
AlexaFluor 647

avoid the possibility of residual fluorescence remaining from either CFSE or antibody staining during the initial cell sort. The CDI was determined for each population (Figure 4.12 B). *H. pylori* antigen specific proliferation was observed by cells co-cultured with *H. pylori* lysate (Figure 4.12 D, B; CDI of >40 for both donor 1 and donor 2) and for cells co-cultured with "OMV" exosomes (Figure 4.12 B, F; CDI>2 for both donors). No antigen specific proliferation was observed in response to CytoStim or "empty" exosomes for either donor.

4.4 Discussion

The binding and internalisation of *H. pylori* OMVs, both in cell culture and in gastric biopsy specimens, is well established (Fiocca, Necchi et al. 1999, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010). Recent studies, including those performed in our laboratory, utilised non-polarised gastric epithelial cell lines, which grow as a single monolayer lacking apical and basolateral surfaces, as well as proper cell junctions. *H. pylori* is known to preferentially attach to cells in the gastric mucosa near the intercellular junctions (Hazell, Lee et al. 1986, Amieva, Vogelmann et al. 2003) which are only present in polarised cell monolayers. The use of T84 intestinal epithelial cells, which form a polarised monolayer with a brush border, tight junctions and desmosomes (Madara, Stafford et al. 1987, El-Etr, Mueller et al. 2004) in a manner analogous to the mucosal epithelial cells found in the host, allowed us to perform our studies using an *in vitro* model that mimics the *in vivo* environment. Initially, we wished to demonstrate that in this model system of polarised T84 cells, co-culture with *H. pylori* OMVs would not affect the integrity of the cell monolayer. Using TEER measurements (Figure 4.1 A) and dextran flux studies (Figure 4.1B) we showed that the apical administration of OMVs to polarised T84 cells did not drastically affect the integrity of the epithelial cell monolayer. Having shown that the monolayer remains intact following OMV stimulation, we next examined the internalisation of *H. pylori* OMVs by these cells. Using confocal fluorescence microscopy we were able to demonstrate for the first time that *H. pylori* OMVs are internalised by polarised T84 epithelial cells (Figure 4.2).

It has been widely demonstrated that *H. pylori* and *H. pylori* OMVs induce the production and secretion of IL-8 from epithelial cells (Crabtree, Wyatt et al. 1994, Ismail, Hampton et al. 2003, Kaparakis, Turnbull et al. 2010). Therefore we wished to

examine the ability of internalised *H. pylori* OMVs to induce this important cytokine response from polarised IECs. Confirming the data of others, we demonstrated that live *H. pylori* induced the production and predominantly basolateral secretion of IL-8 from polarised T84 epithelial cells (Figure 4.3) (Hofman, Ricci et al. 2000). Furthermore, there was no increase in the secretion of IL-8 in response to formaldehyde fixed *H. pylori* (Crabtree, Farmery et al. 1994). Interestingly, we found that OMVs induced similar levels of basolateral IL-8 secretion from polarised epithelial cells compared to the levels induced by live *H. pylori* (Figure 4.3). Taken together, these data demonstrate for the first time that internalised *H. pylori* OMVs interact with polarised host epithelial cells to induce the production of pro-inflammatory mediators, without affecting the barrier function of the cells.

In addition to the production of IL-8, host cells have been shown to respond to *H. pylori* infection by up-regulating the expression of HLA Class II (Engstrand, Scheynius et al. 1989, Scheynius and Engstrand 1991, Wee, Teh et al. 1992, Fan, Long et al. 1996, Fan, Crowe et al. 1998, Archimandritis, Sougioultzis et al. 2000). Furthermore, the up-regulation of both HLA Class I and Class II by other cells, such as lymphocytes, in response to *H. pylori* has been demonstrated (Fan, Long et al. 1996, Suleymanov 2003, Lopes, Victorino et al. 2006). However, there is no data in the literature regarding the effect of *H. pylori* on the expression of HLA Class I by epithelial cells, nor have the effects of OMVs on HLA Class I or II expression by epithelial cells been investigated. In fact, the only data relating to OMVs and HLA Class II expression was performed by Srisatjaluk and colleagues, who found that OMVs derived from *Porphorymonas gingivalis* inhibited IFN-γ mediated up-regulation of HLA Class II by endothelial cells (Srisatjaluk, Doyle et al. 1999, Srisatjaluk, Kotwal et al. 2002). Therefore we examined the levels of HLA Class I and II expression by non-polarised and polarised epithelial

cells in response to *H. pylori* or OMVs using qRT-PCR analysis (Figure 4.4) and flow cytometry (Figure 4.5). Initial qRT-PCR experiments performed using non-polarised T84 cells showed the up-regulation of both HLA Class I (Figure 4.4 A) and Class II (Figure 4.4 B) in response to *H. pylori* and OMVs. Interestingly, the up-regulation of HLA Class II was much more apparent than Class I, with >1000-fold increase in HLA Class II expression by *H. pylori* treated cells, and > 45 by cells treated with OMVs at 200μg. Whereas the fold increase in HLA Class I in response to *H. pylori* or OMVs was only 4-fold and 2-fold, respectively. We then performed these experiments using polarised T84 cells and saw a similar trend, with the increase in expression of both HLA Class I and II, with Class II (Figure 4.4 D) being more apparent than Class I (Figure 4.4 C). Thus we have confirmed the data of others showing that HLA Class II gene expression is up regulated by epithelial cells in response to *H. pylori* infection. In addition, we have shown for the first time that *H. pylori* OMVs are able to induce the up-regulation of HLA Class II expression by these cells. Furthermore, we have demonstrated that *H. pylori* and OMVs augment the expression of HLA Class I by polarised and non-polarised epithelial cells. To the best of our knowledge this is the first time that this has been demonstrated.

Using flow cytometry, we were able to detect increases in HLA Class I and II cell surface expression by non-polarised cells pre-treated with IFN-γ, in response to *H. pylori* (Figure 4.5 E, F, I, J). Given that *H. pylori* is able to stimulate the secretion of IFN-γ from mononuclear cells, such as Th1 cells (Fan, Chua et al. 1994, D'Elios, Manghetti et al. 1997), which are increased in number during infection (Karttunen, Karttunen et al. 1995), it is not surprising that IFN-γ must be present for HLA up-regulation to occur. We were similarly able to demonstrate that non-polarised cells up-

regulate HLA Class I and II surface expression in response to *H. pylori* OMVs. A novel finding that has not previously been reported.

In the first report showing *H. pylori* OMV entry into epithelial cells, Fiocca *et al.* suggested that internalised OMVs entered into the endosomal network where they appeared to remain intact (Fiocca, Necchi et al. 1999). They also suggested that antigens derived from these internalised OMVs might then reach the lamina propria, although the mechanism by which this may occur was only speculated upon. Furthermore, bacterial PG, which is an important component of OMVs (Kaparakis, Turnbull et al. 2010), can be internalised by epithelial cells and packaged into exosomes which are secreted from the basolateral surface of the cells (Bu, Wang et al. 2010). Given that macrophages infected with *Mycobacterium* have been shown to secrete exosomes containing mycobacterial antigens (Bhatnagar and Schorey 2007), and that exosomes are derived from this endosomal network where internalised OMVs are proposed to reside, we hypothesised that internalised OMVs or their proteins may be packaged within exosomes and secreted by epithelial cells into the lamina propria.

To examine this, we isolated exosomes secreted either apically or basolaterally into the culture medium from cells cultured with OMVs. Purified apical and basolateral exosomes were found to display the characteristic exosomal morphology noted by others (van Niel, Raposo et al. 2001) (Figure 4.6). Western blot analysis using anti-*H. pylori* OMV specific antibodies revealed *H. pylori* OMV specific proteins within the cell lysates, apical exosomes and basolateral exosomes derived from OMV stimulated T84 cells (Figure 4.7). However, no OMV proteins were present in the cell lysates, or exosome samples isolated from non-stimulated cells (Figure 4.7). Interestingly, in the cell lysates and basolateral exosome samples from OMV stimulated cells, only protein(s) at approximately 18-20 kDa were detected, indicating low molecular weight

protein(s). Alternatively, these low molecular weight proteins may represent larger OMV-derived proteins that had been processed intracellularly into smaller, lower molecular weight proteins.

Using mass spectrometry, we identified 8 *H. pylori* specific proteins within "OMV" exosomes that were absent from "empty" exosomes (Table 4.2). These *H. pylori* proteins were Lpp20, flagellin A, VacA, flagella hook protein, catalase, S-adenosylmethionine synthase and HopQ. The presence of Lpp20 within "OMV" exosomes was particularly interesting, as this outer membrane protein has been recognised as a potential vaccine candidate. Most importantly, Lpp20 is approximately 18k Da (Keenan, Oliaro et al. 2000) and may potentially represent the low molecular weight protein present in our Western blot. In addition, we detected other known OMV-associated virulence factors (Mullaney, Brown et al. 2009, Olofsson, Vallström et al. 2010) including VacA, HopQ (Belogolova, Bauer et al. 2013) and catalase (Table 4.2) within "OMV" exosomes. Taken together, the Western blot and the proteomic analysis indicate that internalised OMVs are processed within the cell, and some of their proteins are packaged into exosomes that are released from the basolateral surface of epithelial cells.

Over the last decade, there has been a large volume of research regarding the antigen presenting capacity of exosomes, particularly those derived from APCs, which have been shown to release MHC expressing exosomes capable of stimulating T cell responses (Thery, Duban et al. 2002, Sprent 2005). These exosomes have been proposed as a source of "pre-processed" antigens, as they bear peptide loaded MHC complexes and are efficiently internalised by DCs that can then present antigens to naïve T cells (Morelli, Larregina et al. 2004). Although not professional APCs, IECs express many of the surface receptors required for antigen presentation, including HLA

Class I and II and the co-stimulatory molecule CD58 (Framson, Cho et al. 1999). Interestingly, IEC exosomes bearing MHC Class II/OVA complexes are capable of acting as antigen presenting vesicles, which when inoculated into mice resulted in OVA-specific humoral immune responses (van Niel, Mallegol et al. 2003). More important to host pathogen biology, however, was the discovery that macrophages infected with *Mycobacterium* release pro-inflammatory exosomes that contain within them mycobacterial PAMPS (Bhatnagar and Schorey 2007, Bhatnagar, Shinagawa et al. 2007). Furthermore, these exosomes have since been shown to bear MHC Class II/peptide complexes and are capable of presenting antigen directly to T cells to induce proliferation (Ramachandra, Qu et al. 2010). Given these data, we hypothesised that exosomes containing OMV derived proteins may be capable of interacting with T cells to induce OMV antigen specific proliferation. To test this hypothesis, we performed some preliminary experiments in an attempt to induce human PBMCs to proliferate in response to "OMV" exosomes. For this, proliferation was measured via the dilution of CFSE through successive rounds of T cell division, in response to "empty" exosomes or "OMV" exosomes. Using this method, we demonstrated significant T cell proliferation in response to "OMV" exosomes (Figure 8), with a CDI of 22. However, these T cells also proliferated in response to "empty" exosomes, with a CDI of 13, indicating an allogeneic T cell response to human proteins contained within the exosomes. The interaction of IEC derived exosomes with immune cells has been demonstrated by several groups. In each of these studies however, a defined system was utilised with either T cell hybridomas specific for a particular peptide (Mallegol, van Niel et al. 2007), or mice that were pre-sensitised to the peptide of interest (van Niel, Mallegol et al. 2003). One of these studies, performed by Mallegol *et al*. demonstrated that IEC derived exosomes were capable of presenting antigen to T cells, via specific interactions

with DCs. That study utilised an HLA-DR4 expressing T84 cell line, and loaded the exosomes with the HLA-DR4 specific peptide, ^{3}H-HAS 64-76. The authors then showed that these peptide loaded exosomes were capable of activating a HLA-DR4 restricted T cell hybridomas in the presence of DCs (Mallegol, van Niel et al. 2007). A similar study performed by the same group delivered OVA peptide loaded exosomes to OVA sensitised mice, thereby demonstrating an OVA-specific humoral response (van Niel, Mallegol et al. 2003). We therefore hypothesised that in order to remove the allogeneic T cells proliferative response to exosomal proteins, we would need to isolate a pool of *H. pylori* reactive T cells and then use these to measure the proliferative response to OMV proteins within "OMV" exosomes.

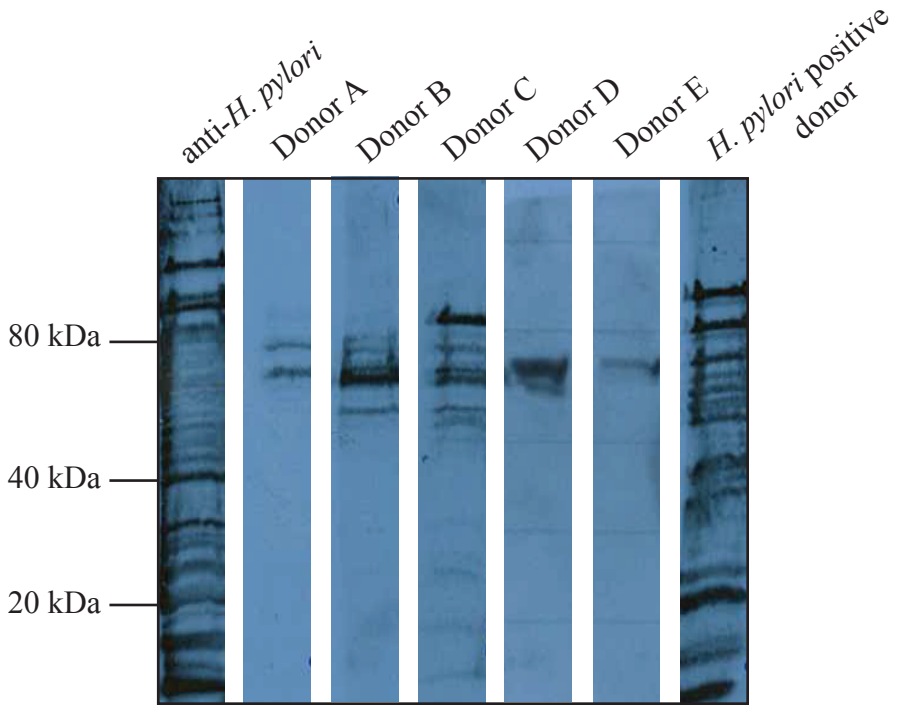
Initially, we characterised the proliferative responses of human T cells to *H. pylori* lysate and OMVs. We found that whilst PBMCs from all donors proliferated in response to both the positive control, CytoStim and *H. pylori*, the extent of proliferation differed vastly. Those that recorded a lower CDI for CytoStim had a similarly lower CDI for *H. pylori* (Figure 4.10, Table 4.4). Importantly, all donors tested demonstrated positive T cell proliferative responses to *H. pylori* lysate. Interestingly, we found that in response to stimulation with *H. pylori* lysate, PBMCs secreted significant levels of IFN-γ, TNF-α, IL-6, IL-2 and IL-10. Moreover, stimulation with *H. pylori* OMVs similarly induced the secretion of the cytokines TNF-α, IL-6 and IL-10. (Figure 4.11). In infected individuals, high levels of the pro-inflammatory cytokines IL-8, IL-1β, TNF-α and IL-6, as well as the Th1 associated cytokines, IFN-γ, IL-12 and IL-18, dominate the cytokine milieu in the gastric mucosa (Yamaoka, Kita et al. 1997, Sakai, Kita et al. 2008). Furthermore, the secretion of the anti-inflammatory and immunosuppressive cytokine IL-10 is increased in the gastric mucosa of *H. pylori* infected patients (Bodger, Wyatt et al. 1997). The production of pro-inflammatory cytokines, such as IFN-γ, IL-6

and TNF-α, are thought to help control infection whilst contributing to gastric pathology and the development of preneoplastic changes in the gastric mucosa (Sayi, Kohler et al. 2009). Moreover, despite robust pro-inflammatory cytokine responses and the infiltration of immune cells into the infected area, the host is more often than not unable to clear the infection. This, in part, may be due to the release of 'inhibitory" cytokines, such as IL-10, which has potent anti-inflammatory properties, and is capable of suppressing T cell cytokine production and proliferation (Del Prete, De Carli et al. 1993, Bodger, Bromelow et al. 2001). To the best of our knowledge, this is the first report demonstrating pro- and anti-inflammatory cytokine production by human T cells in response to OMVs.

Finally, we demonstrated that a pool of *H. pylori* reactive human T cells proliferated in response to re-stimulation with *H. pylori* lysate, indicating that the expansion of a clonal *H. pylori* reactive T cell pool had been successful. These *H. pylori* reactive T cells proliferated specifically in response to OMV proteins contained within "OMV" exosomes (Figure 4.12). Moreover, no proliferation was observed in response to "empty" exosomes or CytoStim, indicating that the proliferation in response to "OMV" exosomes was *H. pylori* antigen specific. These data indicate that exosomes are capable of presenting OMV derived proteins to T cells, in the presence of antigen presenting cells, thereby initiating T cell proliferative immune responses. In order to gain significance, PBMCs from a greater number of individuals will need to be tested for their proliferative responses to "empty" and "OMV" exosomes.

In summary, this study has demonstrated for the first time that internalised *H. pylori* OMVs up-regulate the expression of HLA Class I and II on the cell surface and induce the basolateral secretion of IL-8. Furthermore, stimulation of polarised epithelial cells

results in basolaterally produced exosomes that contain within them known immunogenic OMV proteins. Finally, these exosomes had immunostimulatory capacity, as they induced the antigen specific proliferation of human T cells. Collectively, our findings suggest that upon entry into epithelial cells, OMVs are processed and packaged into exosomes, which facilitate the generation of antigen specific T cell responses. We propose that these exosomes are important for presenting luminal antigens to immune cells, thereby providing a link between the generation of innate and adaptive immune responses at the mucosal epithelium.



Supplementary Figure 4.1: *H. pylori* status of peripheral blood donors. *H. pylori* 251 lysate (10 μg/ml) was seperated by electrophoresis and potential *H. pylori* infection status of donors was assessed by incubating donor serum with membranes. anti-*H. pylori* antibody, and a donor with a positive infection status were included as controls.

Appendix Table 4.1: Human proteins common to "empty" and "OMV" exosomes

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
ACTB	Actin, cytoplasmic 1	42 kDa	23	14	55%	36%	100%	100%
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	113 kDa	33	14	32%	13%	100%	100%
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	36 kDa	23	14	69%	45%	100%	100%
PGK1	Phosphoglycerate kinase 1	45 kDa	21	16	60%	41%	100%	100%
CKB	Creatine kinase B-type	43 kDa	21	13	61%	47%	100%	100%
PKM2	Pyruvate kinase isozymes M1/M2	58 kDa	23	12	50%	24%	100%	100%
HIST1H2BC	Histone H2B type 1-C/E/F/G/I	14 kDa	9	7	45%	41%	100%	100%
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	469 kDa	31	13	10%	3.30%	100%	100%
KRT1	Keratin, type II cytoskeletal 1	66 kDa	18	12	28%	22%	100%	100%
ENO1	Alpha-enolase	47 kDa	22	9	60%	23%	100%	100%
TUBB4B	Tubulin beta-4B chain	50 kDa	15	12	50%	33%	100%	100%
ANXA2	Annexin A2	39 kDa	20	8	58%	31%	100%	100%
HIST1H3A	Histone H3.1	15 kDa	6	7	33%	31%	100%	100%
ALDOA	Fructose-bisphosphate aldolase A	39 kDa	17	8	58%	27%	100%	100%
HIST1H4A	Histone H4	11 kDa	9	7	60%	56%	100%	100%
KRT9	Keratin, type I cytoskeletal 9	62 kDa	14	9	38%	25%	100%	100%
LDHA	L-lactate dehydrogenase A chain	37 kDa	13	8	33%	20%	100%	100%
KRT2	Keratin, type II cytoskeletal 2 epidermal	65 kDa	14	5	36%	16%	100%	100%
NDRG1	Protein NDRG1	43 kDa	11	5	43%	18%	100%	100%
HIST1H2AD	Histone H2A type 1-D	14 kDa	5	4	38%	25%	100%	100%
TPI1	Triosephosphate isomerase	31 kDa	12	5	45%	23%	100%	100%
LGALS3	Galectin-3	26 kDa	8	4	28%	18%	100%	100%
AGRN	Agrin	215 kDa	16	7	12%	4.60%	100%	100%
HSP90AB1	Heat shock protein HSP 90-beta	83 kDa	14	7	24%	11%	100%	100%
ANXA4	Annexin A4	36 kDa	13	7	41%	24%	100%	100%
GPA33	Cell surface A33 antigen	36 kDa	8	2	20%	11%	100%	100%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
ITGB1	Integrin beta-1	88 kDa	14	6	18%	7.80%	100%	100%
TUBA1B	Tubulin alpha-1B chain	50 kDa	11	6	34%	20%	100%	100%
HSPA8	Heat shock cognate 71 kDa protein	71 kDa	17	5	31%	9.90%	100%	100%
KRT10	Keratin, type I cytoskeletal 10	59 kDa	11	1	27%	3.30%	100%	100%
ACTN4	Alpha-actinin-4	105 kDa	15	2	23%	2.60%	100%	100%
SLC12A2	Solute carrier family 12 member 2	131 kDa	11	5	16%	6.60%	100%	100%
CTNNA1	Catenin alpha-1	100 kDa	16	5	24%	7.20%	100%	100%
JUP	Junction plakoglobin	82 kDa	12	6	22%	12%	100%	100%
ITGB4	Integrin beta-4	202 kDa	14	5	12%	3.30%	100%	100%
EEF1A1	Elongation factor 1-alpha 1	50 kDa	9	6	28%	20%	100%	100%
FABP1	Fatty acid-binding protein, liver	14 kDa	9	5	69%	41%	100%	100%
MYH9	Myosin-9	227 kDa	15	4	13%	2.90%	100%	100%
KRT18	Keratin, type I cytoskeletal 18	48 kDa	8	9	26%	27%	100%	100%
PPIA	Peptidyl-prolyl cis-trans isomerase A	18 kDa	5	6	41%	41%	100%	100%
EPB41L2	Band 4.1-like protein 2	113 kDa	17	1	25%	1.70%	100%	74%
VIL1	Villin-1	93 kDa	12	7	19%	9.90%	100%	100%
EZR	Ezrin	69 kDa	9	2	20%	5.50%	100%	100%
FLNB	Filamin-B	278 kDa	13	7	6.70%	3.70%	100%	100%
LGALS4	Galectin-4	36 kDa	9	8	33%	29%	100%	100%
KRT8	Keratin, type II cytoskeletal 8	54 kDa	7	9	22%	16%	100%	100%
ITGA6	Integrin alpha-6	127 kDa	7	4	8.10%	2.20%	100%	100%
UBA1	Ubiquitin-like modifier-activating enzyme 1	118 kDa	9	5	14%	7.60%	100%	100%
GPI	Glucose-6-phosphate isomerase	63 kDa	9	1	23%	1.40%	100%	99%
AHNAK	Neuroblast differentiation-associated protein	629 kDa	9	4	2.30%	0.71%	100%	100%
CLTC	Clathrin heavy chain 1	192 kDa	13	3	11%	1.60%	100%	100%
ALB	Serum albumin	69 kDa	2	3	4.90%	6.60%	100%	100%
HIST1H1C	Histone H1.2	21 kDa	6	6	29%	23%	100%	100%
EEF2	Elongation factor 2	95 kDa	8	5	13%	8.40%	100%	100%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
ITGA2	Integrin alpha-2	129 kDa	11	3	14%	4.70%	100%	100%
YWHAZ	14-3-3 protein zeta/delta	28 kDa	7	3	31%	17%	100%	100%
PGAM1	Phosphoglycerate mutase 1	29 kDa	10	3	45%	11%	100%	100%
MUC13	Mucin-13	55 kDa	6	2	13%	5.90%	100%	100%
CTNND1	Catenin delta-1	108 kDa	8	3	12%	3.00%	100%	100%
MVP	Major vault protein	99 kDa	10	3	22%	6.50%	100%	100%
SLC3A2	4F2 cell-surface antigen heavy chain	68 kDa	8	3	18%	7.50%	100%	100%
CTNNB1	Catenin beta-1	85 kDa	7	3	14%	4.90%	100%	100%
MIF	Macrophage migration inhibitory factor	12 kDa	2	1	32%	9.60%	100%	74%
PFKB	6-phosphofructokinase type C	86 kDa	7	4	15%	7.30%	100%	100%
PRDX2	Peroxiredoxin-2	22 kDa	5	4	22%	24%	100%	100%
PRDX5	Peroxiredoxin-5, mitochondrial	22 kDa	4	3	18%	13%	100%	100%
PDIA3	Protein disulfide-isomerase A3	57 kDa	6	5	18%	12%	100%	100%
CFL1	Cofilin-1	19 kDa	3	3	22%	22%	100%	100%
A2M	Alpha-2-macroglobulin	163 kDa	3	2	2.20%	1.60%	100%	100%
HSP90AA1	Heat shock protein HSP 90-alpha	85 kDa	6	1	19%	7.40%	100%	74%
FLNA	Filamin-A	281 kDa	8	4	4.80%	2.30%	100%	100%
SELENBP1	Selenium-binding protein 1	52 kDa	4	3	12%	8.90%	100%	100%
TUBB	Tubulin beta chain	50 kDa	4	2	46%	25%	100%	100%
PTGFRN	Prostaglandin F2 receptor negative regulator	99 kDa	8	1	14%	1.10%	100%	99%
TKT	Transketolase	68 kDa	8	4	17%	10.00%	100%	100%
SERPINB1	Leukocyte elastase inhibitor	43 kDa	6	2	23%	5.80%	100%	100%
PFN1	Profilin-1	15 kDa	6	2	46%	23%	100%	100%
CD44	CD44 antigen	82 kDa	6	3	9.60%	4.70%	100%	100%
RAC1	Ras-related C3 botulinum toxin substrate 1	21 kDa	3	3	14%	14%	100%	100%
CDC42	Cell division control protein 42 homolog	21 kDa	4	3	29%	18%	100%	100%
GNAS	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas	111 kDa	6	1	7.00%	1.50%	100%	74%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
ATP1B1	Sodium/potassium-transporting ATPase subunit beta-1	35 kDa	4	1	15%	4.60%	100%	74%
MYL6	Myosin light polypeptide 6	17 kDa	6	3	54%	28%	100%	100%
PKP3	Plakophilin-3	87 kDa	6	1	14%	3.00%	100%	100%
CLIC1	Chloride intracellular channel protein 1	27 kDa	7	2	34%	12%	100%	100%
HSPA1A	Heat shock 70 kDa protein 1A/1B	70 kDa	5	1	17%	2.50%	100%	74%
HSPD1	60 kDa heat shock protein, mitochondrial	61 kDa	4	5	11%	14%	100%	100%
ANXA3	Annexin A3	36 kDa	4	4	19%	15%	100%	100%
TAGLN2	Transgelin-2	22 kDa	3	3	18%	25%	100%	100%
UBA52	Ubiquitin-60S ribosomal protein L40	15 kDa	2	3	14%	38%	100%	100%
ALDOC	Fructose-bisphosphate aldolase C	39 kDa	7	2	33%	10%	100%	100%
KIAA1199	Protein KIAA1199	153 kDa	8	2	9.90%	1.70%	100%	100%
EHD4	EH domain-containing protein 4	61 kDa	6	1	19%	2.80%	100%	74%
GDI2	Rab GDP dissociation inhibitor beta	51 kDa	4	6	11%	21%	100%	100%
ATP5B	ATP synthase subunit beta, mitochondrial	57 kDa	3	7	10%	21%	100%	100%
CCT8	T-complex protein 1 subunit theta	60 kDa	6	2	15%	4.40%	100%	100%
CCT3	T-complex protein 1 subunit gamma	61 kDa	7	2	19%	7.70%	100%	100%
CCT2	T-complex protein 1 subunit beta	57 kDa	7	2	24%	7.70%	100%	100%
RALA	Ras-related protein Ral-A	24 kDa	5	2	26%	11%	100%	100%
PYGB	Glycogen phosphorylase, brain form	97 kDa	5	1	7.80%	1.80%	100%	99%
HSP90B1	Endoplasmin	92 kDa	4	1	8.20%	3.00%	100%	99%
SLC1A5	Neutral amino acid transporter B(0)	57 kDa	4	1	10.00%	3.70%	100%	74%
SPTBN1	Spectrin beta chain, brain 1	275 kDa	4	2	3.00%	0.97%	100%	100%
PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	46 kDa	5	2	14%	7.20%	100%	100%
WDR1	WD repeat-containing protein 1	66 kDa	6	1	20%	3.00%	100%	98%
ATP1B3	Sodium/potassium-transporting ATPase subunit beta-3	32 kDa	3	1	18%	3.90%	100%	74%
DSP	Desmoplakin	332 kDa	3	4	1.80%	2.70%	100%	100%
ANXA11	Annexin A11	54 kDa	4	2	14%	6.10%	100%	100%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
H2AFY	Core histone macro-H2A.1	40 kDa	5	1	24%	4.60%	100%	100%
DSG2	Desmoglein-2	122 kDa	6	1	11%	1.60%	100%	74%
EPCAM	Epithelial cell adhesion molecule	35 kDa	4	1	20%	3.20%	100%	74%
HSPB1	Heat shock protein beta-1	23 kDa	4	1	36%	7.80%	100%	74%
VASP	Vasodilator-stimulated phosphoprotein	40 kDa	3	3	11%	11%	100%	100%
HLA-A	HLA class I histocompatibility antigen, A-3 alpha chain	41 kDa	3	1	9.60%	3.00%	100%	100%
VTN	Vitronectin	54 kDa	2	2	3.80%	6.10%	100%	100%
ANXA1	Annexin A1	39 kDa	5	1	26%	4.00%	100%	99%
H1F0	Histone H1.0	21 kDa	3	1	18%	5.70%	100%	99%
PRDX1	Peroxiredoxin-1	22 kDa	4	1	25%	9.00%	100%	74%
PKP2	Plakophilin-2	97 kDa	4	1	10%	1.60%	100%	74%
EEF1G	Elongation factor 1-gamma	50 kDa	4	1	10%	2.30%	100%	74%
YWHAE	14-3-3 protein epsilon	29 kDa	3	1	15%	5.50%	100%	74%
HINT1	Histidine triad nucleotide-binding protein 1	14 kDa	2	1	30%	19%	100%	74%
DDAH1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	31 kDa	2	1	8.40%	4.20%	100%	74%
RAB11A	Ras-related protein Rab-11A	24 kDa	4	2	24%	13%	100%	100%
PLS3	Plastin-3	71 kDa	2	2	4.10%	4.30%	100%	100%
CCT6A	T-complex protein 1 subunit zeta	58 kDa	4	1	12%	3.60%	100%	99%
IGSF8	Immunoglobulin superfamily member 8	65 kDa	5	1	12%	2.60%	100%	98%
MFGE8	Lactadherin	43 kDa	4	1	17%	3.90%	100%	74%
ANXA5	Annexin A5	36 kDa	4	1	13%	5.00%	100%	74%
NQO1	NAD(P)H dehydrogenase [quinone] 1	31 kDa	4	1	12%	4.00%	100%	74%
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	77 kDa	3	1	7.30%	2.30%	100%	74%
CCT5	T-complex protein 1 subunit epsilon	60 kDa	3	3	8.10%	8.90%	100%	100%
RAP1B	Ras-related protein Rap-1b	21 kDa	2	1	13%	7.60%	100%	100%
LIN7C	Protein lin-7 homolog C	22 kDa	4	2	30%	12%	100%	100%
PDXK	Pyridoxal kinase	35 kDa	4	2	19%	9.90%	100%	100%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
ATP5A1	ATP synthase subunit alpha, mitochondrial	60 kDa	1	3	3.30%	5.60%	98%	100%
LLGL2	Lethal(2) giant larvae protein homolog 2	113 kDa	5	1	9.50%	1.10%	100%	74%
KRT16	Keratin, type I cytoskeletal 16	51 kDa	2	1	11%	3.00%	100%	74%
PLS1	Plastin-1	70 kDa	3	1	12%	2.20%	100%	74%
CD9	CD9 antigen	25 kDa	2	1	9.60%	4.40%	100%	74%
RAB7A	Ras-related protein Rab-7a	23 kDa	4	2	22%	12%	100%	100%
TCP1	T-complex protein 1 subunit alpha	60 kDa	3	2	7.60%	6.80%	100%	100%
RPS4X	40S ribosomal protein S4, X isoform	30 kDa	2	2	7.60%	9.90%	100%	100%
CAPG	Macrophage-capping protein	38 kDa	3	2	11%	8.30%	100%	100%
RAB5C	Ras-related protein Rab-5C	23 kDa	2	2	15%	20%	100%	100%
GANAB	Neutral alpha-glucosidase AB	107 kDa	2	2	3.30%	3.20%	100%	100%
TINAGL1	Tubulointerstitial nephritis antigen-like	52 kDa	3	1	7.70%	2.80%	100%	98%
GOT1	Aspartate aminotransferase, cytoplasmic	46 kDa	2	1	8.50%	5.10%	100%	98%
MYH14	Myosin-14	228 kDa	2	1	2.60%	1.50%	100%	98%
YWHAG	14-3-3 protein gamma	28 kDa	3	1	22%	10%	100%	74%
BSG	Basigin	42 kDa	3	1	11%	2.30%	100%	74%
SRI	Sorcin	22 kDa	2	1	6.10%	6.10%	100%	74%
ATIC	Bifunctional purine biosynthesis protein PURH	65 kDa	2	2	5.90%	6.40%	100%	100%
HSPA9	Stress-70 protein, mitochondrial	74 kDa	2	1	4.40%	1.80%	100%	74%
AARS	Alanine--tRNA ligase, cytoplasmic	107 kDa	3	1	5.20%	1.10%	100%	74%
CORO1B	Coronin-1B	54 kDa	3	1	14%	3.30%	100%	74%
PSMA6	Proteasome subunit alpha type-6	27 kDa	3	1	20%	7.30%	100%	74%
UGDH	UDP-glucose 6-dehydrogenase	55 kDa	2	1	4.00%	4.70%	100%	74%
EFNB1	Ephrin-B1	38 kDa	2	1	9.00%	3.80%	100%	74%
NME1	Nucleoside diphosphate kinase A	17 kDa	2	1	19%	7.90%	100%	74%
GDF15	Growth/differentiation factor 15	34 kDa	2	2	6.20%	6.20%	100%	100%
KRT5	Keratin, type II cytoskeletal 5	62 kDa	2	2	7.80%	4.20%	100%	100%
RAN	GTP-binding nuclear protein Ran	24 kDa	2	2	12%	12%	100%	100%

			Unique Peptides		Sequence		Protein	
Gene Symbol	Identified Proteins	Molecular Weight	"empty"	"OMV"	"empty"	"OMV"	"empty"	"OMV"
RRAS2	Ras-related protein R-Ras2	23 kDa	2	2	11%	11%	100%	100%
VDAC2	Voltage-dependent anion-selective channel protein 2	32 kDa	2	1	10%	4.10%	100%	99%
KRT19	Keratin, type I cytoskeletal 19	44 kDa	1	2	6.20%	9.30%	98%	100%
MDH2	Malate dehydrogenase, mitochondrial	36 kDa	1	2	5.60%	11%	98%	100%
PSMA3	Proteasome subunit alpha type-3	28 kDa	2	1	10%	7.80%	100%	74%
C9	Complement component C9	63 kDa	2	1	3.90%	1.60%	100%	74%
CORO1C	Coronin-1C	53 kDa	2	1	8.40%	3.40%	100%	74%
CSTB	Cystatin-B	11 kDa	2	1	34%	12%	100%	74%
EPB41L4B	Band 4.1-like protein 4B	100 kDa	2	1	2.90%	1.80%	100%	74%
CAPN5	Calpain-5	73 kDa	2	1	6.60%	3.00%	100%	99%
FAM129B	Niban-like protein 1	84 kDa	2	1	3.90%	2.90%	100%	74%
ANXA7	Annexin A7	53 kDa	2	1	4.70%	2.00%	100%	74%
LAMA3	Laminin subunit alpha-3	367 kDa	2	1	0.93%	0.51%	100%	74%
MYO1C	Unconventional myosin-Ic	122 kDa	2	1	2.10%	0.85%	100%	74%
PDIA6	Protein disulfide-isomerase A6	48 kDa	2	1	6.60%	5.70%	100%	74%
CAPZB	F-actin-capping protein subunit beta	31 kDa	2	1	8.70%	3.60%	100%	74%
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2	40 kDa	2	1	13%	9.00%	100%	74%
CKMT1A	Creatine kinase U-type, mitochondrial	47 kDa	2	1	8.90%	3.80%	100%	74%
ABCC3	Canalicular multispecific organic anion transporter 2	169 kDa	2	1	2.00%	1.40%	100%	74%
NT5E	5'-nucleotidase	63 kDa	1	2	3.10%	6.60%	50%	100%
PFKL	6-phosphofructokinase, liver type	85 kDa	1	2	5.10%	5.00%	50%	100%

Appendix Table 4.2: Human proteins found only in "empty" exosomes

Gene symbol	Identified Proteins	Molecular Weight	Unique Peptides	Sequence Coverage	Protein Identification Probability
PDCD6IP	Programmed cell death 6-interacting protein	96 kDa	10	15%	100%
FN1	Fibronectin	263 kDa	6	3.70%	100%
CASK	Peripheral plasma membrane protein CASK	105 kDa	6	8.50%	100%
IQGAP1	Ras GTPase-activating-like protein IQGAP1	189 kDa	8	8.80%	100%
CLDN3	Claudin-3	23 kDa	5	20%	100%
MYO1B	Unconventional myosin-Ib	132 kDa	5	7.50%	100%
ATP2B1	Plasma membrane calcium-transporting ATPase 1	139 kDa	6	7.50%	100%
IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	47 kDa	8	21%	100%
EPS8	Epidermal growth factor receptor kinase substrate 8	92 kDa	5	11%	100%
LSR	Lipolysis-stimulated lipoprotein receptor	71 kDa	5	13%	100%
ITGAV	Integrin alpha-V	116 kDa	4	7.00%	100%
CBR1	Carbonyl reductase [NADPH] 1	30 kDa	4	18%	100%
HSPA5	78 kDa glucose-regulated protein	72 kDa	4	11%	100%
KRAS	GTPase Kras	22 kDa	4	31%	100%
F11R	Junctional adhesion molecule A	33 kDa	2	12%	100%
MDH1	Malate dehydrogenase, cytoplasmic	36 kDa	2	6.30%	100%
EPS8L3	Epidermal growth factor receptor kinase substrate 8-like protein 3	67 kDa	3	7.60%	100%
CCT4	T-complex protein 1 subunit delta	58 kDa	4	14%	100%
FASN	Fatty acid synthase	273 kDa	3	2.00%	100%
HIST1H1B	Histone H1.5	23 kDa	3	30%	100%
PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	38 kDa	3	14%	100%
PSMA1	Proteasome subunit alpha type-1	30 kDa	2	14%	100%
C4A	Complement C4-A	193 kDa	2	1.50%	100%
EIF4A1	Eukaryotic initiation factor 4A-I	46 kDa	4	17%	100%
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	48 kDa	2	9.00%	100%
AHCY	Adenosylhomocysteinase	48 kDa	3	5.60%	100%
PEBP1	Phosphatidylethanolamine-binding protein 1	21 kDa	3	29%	100%
SERPINE2	Glia-derived nexin	44 kDa	3	14%	100%
RPL18	60S ribosomal protein L18	22 kDa	3	19%	100%

Gene symbol	Identified Proteins	Molecular Weight	Unique Peptides	Sequence Coverage	Protein Identification Probability
VCP	Transitional endoplasmic reticulum ATPase	89 kDa	2	5.70%	100%
RPL3	60S ribosomal protein L3	46 kDa	3	15%	100%
CAP1	Adenylyl cyclase-associated protein 1	52 kDa	3	12%	100%
PCBP1	Poly(rC)-binding protein 1	37 kDa	3	15%	100%
PSMB1	Proteasome subunit beta type-1	26 kDa	3	20%	100%
S100A11	Protein S100-A11	12 kDa	3	37%	100%
LAMC1	Laminin subunit gamma-1	178 kDa	2	2.10%	100%
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	54 kDa	2	5.50%	100%
LAMA5	Laminin subunit alpha-5	400 kDa	2	0.81%	100%
ARF1	ADP-ribosylation factor 1	21 kDa	2	17%	100%
BANF1	Barrier-to-autointegration factor	10 kDa	2	29%	100%
KPNB1	Importin subunit beta-1	97 kDa	2	3.40%	100%
PLXNB2	Plexin-B2	205 kDa	2	1.60%	100%
GBE1	1,4-alpha-glucan-branching enzyme	80 kDa	3	6.60%	100%
CDCP1	CUB domain-containing protein 1	93 kDa	2	2.30%	100%
PPP2R1A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	65 kDa	2	8.10%	100%
AKR1C1	Aldo-keto reductase family 1 member C1	37 kDa	2	8.40%	100%
COL17A1	Collagen alpha-1(XVII) chain	150 kDa	2	1.80%	100%
EHD1	EH domain-containing protein 1	61 kDa	2	8.80%	100%
FERMT1	Fermitin family homolog 1	77 kDa	2	4.70%	100%
RAB10	Ras-related protein Rab-10	23 kDa	2	17%	100%
S100A16	Protein S100-A16	12 kDa	2	27%	100%
ST14	Suppressor of tumorigenicity 14 protein	95 kDa	2	4.00%	100%
CHMP4B	Charged multivesicular body protein 4b	25 kDa	2	11%	100%
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	91 kDa	2	4.10%	100%
PHGDG	D-3-phosphoglycerate dehydrogenase	57 kDa	2	4.50%	100%

Chapter 5. Final Discussion and Future Directions

Like other Gram-negative bacteria, *H. pylori* sheds OMVs during normal growth, which are thought to deliver proteins, pro-inflammatory products and other virulence factors into host cells (Fiocca, Necchi et al. 1999, Ismail, Hampton et al. 2003, Kaparakis, Turnbull et al. 2010). OMVs have long been known to induce similar responses by the host immune system as their parent bacteria, owing to their similar but not identical protein content (Mullaney, Brown et al. 2009, Olofsson, Vallström et al. 2010). Furthermore, OMVs are often enriched in virulence factors (Sidhu, Vorholter et al. 2008, Bomberger, Maceachran et al. 2009, Lindmark, Rompikuntal et al. 2009, Rompikuntal, Thay et al. 2011, Avila-Calderon, Lopez-Merino et al. 2012, Chowdhury and Jagannadham 2013). For these reasons, the use of OMVs as acellular vaccines has been researched extensively (Haneberg, Dalseg et al. 1998, Davenport, Groves et al. 2008, Roberts, Moreno et al. 2008, Schild, Nelson et al. 2008, Holst, Martin et al. 2009, Chen, Osterrieder et al. 2010, Avila-Calderon, Lopez-Merino et al. 2012, Roier, Leitner et al. 2012, Mitra, Chakrabarti et al. 2013). The limited studies regarding *H. pylori* OMVs suggest that they are capable of interacting with host cells and inducing comparable responses as *H. pylori* (Ismail, Hampton et al. 2003, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010, Parker and Keenan 2012). However, little is known about how OMVs are produced, how they enter host epithelial cells and how they influence the host immune system to drive inflammation and adaptive immune responses.

It is difficult and labour intensive to culture sufficient volumes of OMVs for research purposes and vaccine development. The natural production of large amounts of OMVs has only been described for a limited number of bacterial species (Henry, Pommier et

al. 2004) but not for *H. pylori*. The first aim of this study was to generate hyper-vesiculating strains of *H. pylori*, allowing us to examine the role of membrane stability in OMV production by this bacterium. If OMVs are to be successfully developed as vaccines, we also need to understand more about their interactions with the host, specifically, how they enter host cells to induce anti-bacterial responses. Thus, we next wished to characterise the molecular mechanisms of OMV entry into host epithelial cells, as despite extensive evidence demonstrating OMV entry into host cells (Kadurugamuwa and Beveridge 1998, Sharma, Tummuru et al. 1998, Demuth, James et al. 2003, Kesty, Mason et al. 2004, Tsuda, Amano et al. 2005, Galka, Wai et al. 2008, Bomberger, Maceachran et al. 2009, Furuta, Takeuchi et al. 2009, Furuta, Tsuda et al. 2009, Ellis, Leiman et al. 2010, Kaparakis, Turnbull et al. 2010, Parker, Chitcholtan et al. 2010, Schaar, de Vries et al. 2010, Vidakovics, Jendholm et al. 2010, Bielig, Rompikuntal et al. 2011, Chatterjee and Chaudhuri 2011, Pollak, Delpino et al. 2012), the precise mechanism(s) remain(s) undetermined. Previous work from our laboratory has demonstrated that *H. pylori* OMVs are capable of inducing bacterial specific antibody responses when delivered orally to mice. Despite this evidence, the mechanisms whereby OMVs are able to generate bacterial specific immune responses also remain unknown. Interestingly, research performed by other groups has shown that pathogen infected cells can secrete exosomes containing within them proteins derived from the internalised pathogen (Bhatnagar and Schorey 2007, Bhatnagar, Shinagawa et al. 2007). Furthermore, exosomes containing PAMPS were shown to be capable of antigen presentation, ultimately resulting in the generation of T cell responses (Ramachandra, Qu et al. 2010). Thus, we hypothesised that antigens derived from internalised OMVs may be packaged within exosomes that are released from host epithelial cells

Therefore, the three aims of this study were as follows:

1. To generate hyper-vesiculating strains of *H. pylori*
2. To determine the molecular mechanisms of OMV cellular entry, and to define the role, if any, of size in directing the route of entry
3. To determine the fate of internalised *H. pylori* OMVs, and to characterise the human T cell proliferative response to OMV proteins contained within epithelial cell derived exosomes.

To address the first aim, we disrupted the membrane integrity of *H. pylori* using standard molecular biology techniques and then assessed the impact on OMV production. In *E. coli*, the disruption of membrane integrity through mutation of any of the *tol-pal* genes has been found to increase vesicle production (Sonntag, Schwarz et al. 1978, Bernadac, Gavioli et al. 1998, Llamas, Ramos et al. 2000, Dubuisson, Vianney et al. 2005, Yeh, Comolli et al. 2010). Although homologues of the *tolB* and *pal* genes have been annotated in the *H. pylori* genome (Sturgis 2001), to date there is no published work regarding the functional roles of TolB and Pal in *H. pylori*. We found that the disruption of *tolB* brought about profound morphological changes (Chapter 2). Notably, strains lacking TolB displayed extensive "blebbing" at the cell surface, together with fewer, shorter flagella. Interestingly, strains lacking Pal retained flagella expression, whereas those lacking both TolB and Pal lacked flagella entirely. Importantly, in addition to morphological changes, we demonstrated that in *H. pylori*, disruption of either the *tolB* or *pal* genes alone resulted in hyper-vesiculating bacteria displaying 600- and 22-fold increases in OMV production, respectively. However, disruption of both *tolB* and *pal* did not result in a hyper-vesiculating phenotype. Whilst the reasons for this remain unknown, it is possible that the loss of both proteins

together, rather than individual proteins, is so devastating to the bacterial OM that other OM proteins compensate for their loss. Indeed, other researchers have noted that when proteins involved in bacterial cell division are missing or mutated, other OM proteins such as Lpp, are able to compensate for the lack of critical division proteins (Bernard, Sadasivam et al. 2007, Gerding, Ogata et al. 2007). Interestingly, the proteins of the Tol-Pal complex are recruited to bacterial division sites where they are proposed to form a sub complex of the division apparatus, aiding in cell division (Gerding, Ogata et al. 2007). It is therefore possible that other OM proteins somehow compensate for the loss of both TolB and Pal together.

Previous work from our laboratory demonstrated that OMVs can deliver PG to the cytosol of host cells, resulting in the induction of pro-inflammatory responses characterised by IL-8 production (Kaparakis, Turnbull et al. 2010). Interestingly, OMVs produced by each of the Δ*tolB-pal* mutant strains generated in this study induced significantly higher levels of IL-8 production by host cells than did those produced by the wild type strain. Proteomic analysis of OMVs derived from the wild type and each of the mutant strains of *H. pylori* revealed subtle differences in the protein content of each. Present in all OMVs examined were a variety of virulence factors including: the adhesion proteins BabA and BabB, a known vaccine candidate Lpp20, the VacA cytotoxin, and TNF-α-inducing protein (Tip-α).

H. pylori is well known to induce NF-κB activity in host cells, resulting in the secretion of IL-8 (Sharma, Tummuru et al. 1995). Two major *H. pylori* products known to induce IL-8 secretion from host cells are CagA (Crabtree, Wyatt et al. 1994, Crabtree, Covacci et al. 1995) and PG (Viala, Chaput et al. 2004). Interestingly, heat shock protein 60 (HSP60) (Takenaka, Yokota et al. 2004), and more recently HP0986 (Devi, Ansari et al. 2014) have been found to induce NF-κB activity and IL-8 production from gastric

epithelial cells. Whilst CagA has been detected on the surface of *H. pylori* wild type OMVs (Olofsson, Vallström et al. 2010), we were only able to detect the protein in association with Δ*tolB* OMVs, and it is therefore unlikely to be responsible for any increased IL-8 production from host cells in response to Δ*tol-pal* OMVs. HSP60 was only detected in association with Δ*tolB* (+*tolB*), Δ*pal* and Δ*pal* (+*pal*) OMVs, and HP0986 was not detected at all, again suggesting that these proteins are unlikely to be the cause of the increased IL-8 production. Indeed, from previous work we know that proteinase K treated *H. pylori* OMVs induced similar levels of IL-8 secretion by host cells as non-treated OMVs (Kaparakis, Turnbull et al. 2010). Furthermore, OMVs smaller than 100 nm induce higher levels of NF-κB than OMVs larger than 100 nm, despite their lower protein content (Kaparakis, Turnbull et al. 2010). Taken together, these data indicated that the protein content of OMVs was not the main activator of NF-κB, rather the PG contained within OMVs was found to be the main agonist for NF-κB activity. Furthermore, it is possible that differences in PG content may help to explain the differences in immunogenicity of OMVs produced by Δ*tolB,* Δ*pal* and Δ*tolBpal* bacteria.

It is important to note however, that although the protein content is unlikely to be responsible for the increases in IL-8 inducing ability, the proteins present are likely to play an important role in the generation of *H. pylori* specific adaptive immune responses. Further work is required to determine the immunostimulatory capacity of OMVs produced by these mutant strains of *H. pylori*. Specifically, we would like to utilise an established mouse model (Kaparakis, Turnbull et al. 2010) to investigate the ability of these OMVs to induce humoral and innate immune responses *in vivo*. For this, mice would be fed intragastrically with OMVs from the various strains, and gastric *Cxcl2* responses would be measured 1 and 7 days post feeding. To investigate the

ability of these OMVs to induce humoral immune responses we would measure anti-*H. pylori* OMV IgG responses in the sera of mice that had been fed OMVs, compared with mice that had been fed PBS. Serum IgG specificity could then be determined (Keenan, Oliaro et al. 2000), allowing us to identify any *H. pylori* OMV proteins able to induce humoral immune responses in mice, thereby strengthening their suitability in vaccine development against this pathogen.

It would also be interesting to examine the contribution of OMVs in bacterial colonisation. Since *H. pylori* is known to require flagella for efficient colonisation of the stomach (Ottermann and Lowenthal 2002), it is likely that Δ*tolB and* Δ*tolBpal* strains would be unable to colonise mice. Therefore to examine this, we could infect mice with mouse colonising mutant strains of *H. pylori* Δ*pal,* which exhibits an increase in OMV production*,* and compare the colonisation levels between the mutant and the wild type.

In summary, the work performed in this study demonstrated that *H. pylori* bacteria lacking either TolB or Pal exhibited profound morphological abnormalities accompanied by an increase in vesiculation. Furthermore, the protein content of the OMVs produced by these mutant strains was similar to that of wild type OMVs, containing several known immunogenic proteins. Finally, these OMVs were capable of inducing significant levels of IL-8 production by host cells, indicating that whilst they differ slightly from those produced by wild type *H. pylori*, they are suitable for use in further studies, including those for an OMV based vaccine against infection by this pathogen.

Previous work from our laboratory demonstrated that Gram-negative OMVs deliver PG to the host cell cytosol following internalisation by non phagocytic epithelial cells (Kaparakis, Turnbull et al. 2010). Prior to beginning this study, the entry of OMVs into

epithelial cells had been shown for numerous pathogens including *V. cholerae* (Chatterjee and Chaudhuri 2011), *P. gingivalis* (Furuta, Takeuchi et al. 2009, Furuta, Tsuda et al. 2009), *E. coli* (Kesty, Mason et al. 2004), *P. aeruginosa* (Bomberger, Maceachran et al. 2009) and *H. pylori* (Parker, Hampton et al. 2005, Kaparakis, Turnbull et al. 2010). However, the mechanisms of entry reported differed according to the study. These conflicting reports regarding OMV entry led us to investigate the precise mechanisms of OMV cellular entry. Furthermore, as OMVs are produced in a range of sizes, we also wished to investigate the contribution of OMV size to their mode of entry, as a possible explanation for the conflicting reports.

In order to measure OMV entry into host cells we used two techniques: fluorescence microscopy and IL-8 production by host cells. Initially, all experiments were performed using a heterogeneous population of OMVs. Using a panel of chemical inhibitors to block the various pathways of endocytosis (macropinocytosis, clathrin- and caveolin-dependent endocytosis) we determined that heterogeneous populations of *H. pylori* OMVs enter host cells by each of these mechanisms. Furthermore, we found that even partial inhibition of OMV entry resulted in a significant decrease in IL-8 production by host cells (Chapter 3).

Studies have shown that particles of a particular size preferentially enter cells via specific endocytosis pathways (Mayor and Pagano 2007). Moreover, the size of the particles and therefore the mechanisms of entry may also define their intracellular degradation pathway (Brewer, Pollock et al. 2004). One such study performed by Rejman *et al.* demonstrated that the internalisation of smaller particles (< 200 nm) relied on clathrin-dependent endocytosis, whereas when the size of the particles increased (500 nm) the mode of entry became caveolin-dependent (16). OMVs smaller than 100 nm in diameter have been found to induce higher levels of NF-κB activity than larger

OMVs, suggesting that these smaller OMVs may be more efficient at entering host epithelial cells to initiate pro-inflammatory responses (Kaparakis, Turnbull et al. 2010). Given that OMVs produced by different bacteria exhibit wide ranges in size and that the method of isolation used may also affect particle size (van de Waterbeemd, Zomer et al. 2013), we surmised that size may direct the mechanism of entry and therefore account for the differing results found by other groups. To further investigate this theory, we compared the preferential modes of host cell entry by small (< 50nm) and large (>50nm) OMVs. Using chemical inhibition of endocytosis we found that small OMVs entered host epithelial cells via all mechanisms of endocytosis, whereas large OMVs entered by macropinocytosis and caveolin-dependent endocytosis, but not by clathrin-dependent endocytosis (Chapter 3). Further analysis of small and large OMVs revealed that they contained both unique and shared proteins. Taken together, these data indicate that OMV size predetermines not only the protein content but also the preferential mechanism of entry.

Despite having identified a role for OMV size in directing the mechanism of host cell entry, we did not investigate the potential role of host cell-surface receptors in this process. VacA, which we found to be present in both small and large populations of OMVs (Chapter 3), has been shown to bind to several host cell surface components, including sphingomyelin (Gupta, Patel et al. 2008), glycosylphosphatidylinositol (GPI)-anchored proteins (Ricci, Galmiche et al. 2000), protein-tyrosine phosphatase α (RPTPα) (Yahiro, Wada et al. 2003) and receptor-like protein tyrosine phosphatases-β (RPTPβ) (Nakayama, Hisatsune et al. 2006). Receptor-bound VacA subsequently clusters at the cell surface in association with cholesterol-rich lipid rafts (Nakayama, Hisatsune et al. 2006), which we have previously shown to be important for OMV internalisation (Kaparakis, Turnbull et al. 2010). Interestingly, Parker *et al*. found that

OMVs lacking VacA entered host cells via different mechanisms than OMVs containing VacA, indicating that VacA may play a role in directing entry (Parker, Chitcholtan et al. 2010). Furthermore, *H. pylori* has been proposed to interact with many host cell surface receptors that are located within lipid rafts, including α5β1 (Kwok, Zabler et al. 2007), and epidermal growth factor receptor (EGFR) (Tegtmeyer, Zabler et al. 2009). Therefore, the next step in determining the precise mode of OMV host cell entry would be to examine the role of host cell surface receptors. This could be done through the use of specific antibodies to block ligand binding. The effect of blocking the interaction of OMVs with these cell surface receptors could then be measured by fluorescent microscopy, IL-8 production (as described in chapter 3) and flow cytometry (Parker, Chitcholtan et al. 2010). Alternatively, we could investigate the host cell receptor(s) involved in OMV internalisation by transfecting gastric epithelial cells with an siRNA genome library. We can then monitor the levels of NF-κB activity in host cells by luciferase assay (Kaparakis, Turnbull et al. 2010) and IL-8 ELISA as described in chapter 3. As it is possible that the siRNAs in a library such as this could block other components of the NF-κB pathway, we could also visualise intracellular

Despite numerous studies regarding the entry of OMVs into host cells, the entry of OMVs into polarised cells has thus far not been investigated. Moreover, there have been limited studies regarding the intracellular fate of internalised OMVs. We therefore wished to investigate the entry and subsequent intracellular fate of OMVs within polarised epithelial cells. Certain epithelial cell lines can be cultured to form a polarised monolayer, with apical and basolateral membranes which are otherwise not found in non-polarised cells (Corthesy-Theulaz, Porta et al. 1996). Moreover, due to our interest in the potential for proteins derived from internalised OMVs to be packaged within exosomes, we chose to work with T84 IECs, a cell line that is able to both form

polarised monolayers, and to secrete apical and basolateral exosomes. Thus, we were able to translate our previous findings of OMV internalisation by non-phagocytic epithelial cells, into a model that more closely mimics the *in vivo* environment encountered in the host. Using the T84 model we showed for the first time that *H. pylori* OMVs enter polarised epithelial cells at the apical surface (Chapter 4). Interestingly, OMVs induced significantly higher levels of IL-8 from the basolateral but not the apical surface, when compared with non-stimulated cells (Chapter 4). It has previously been shown that T84 cells secrete significantly higher amounts of IL-8 from the basolateral surface than the apical surface of cells in response to stimulation by bacteria such as *H. pylori* (Hofman, Ricci et al. 2000) and *S. typhimurium* (McCormick, Hofman et al. 1995). This establishes a transepithelial cytokine gradient that is likely to promote the migration of neutrophils to the epithelium, such as occurs during *H. pylori* infection (Crabtree, Kersulyte et al. 1999). Although it has been previously shown that *H. pylori* OMVs are able to induce the production of IL-8 from non polarised AGS (Ismail, Hampton et al. 2003) and T84 cells (Mullaney, Brown et al. 2009), we have provided the first evidence that OMVs enter polarised epithelial cells and induce IL-8 production to similar levels as the *H. pylori* strain from which they were generated (Chapter 4). In addition to the production of IL-8, host epithelial cells respond to *H. pylori* infection by the up-regulation of HLA Class II expression (Engstrand, Scheynius et al. 1989, Maekawa, Kinoshita et al. 1997). This led us to postulate that epithelial cells may also up-regulate HLA Class II in response to *H. pylori* OMVs. Furthermore, as IEC-derived exosomes bearing HLA Class II/peptide complexes are capable of potentiating antigen presentation (Mallegol, van Niel et al. 2007), we reasoned that exosomes released from OMV-containing cells may express HLA Class II/OMV peptide complexes at their

surface. Using qRT-PCR and flow cytometry, we demonstrated that *H. pylori* and *H. pylori* OMVs up regulate HLA Class I and II by IECs.

It was known that exosomes released by IECs and bearing HLA Class II/peptide complexes are capable of interacting with DCs, thereby facilitating peptide presentation to T cells (Mallegol, van Niel et al. 2007). These data, together with our findings that *H. pylori* OMVs enter polarised IECs, led us to postulate that antigens derived from internalised OMVs may be processed within the cell and packaged within exosomes that are able to interact with immune cells such as T cells and DCs. Using Western blot and proteomic analysis we found that exosomes released from polarised human epithelial cells that had been co-cultured with OMVs ("OMV" exosomes) did indeed contain within them OMV-derived proteins. Interestingly, of the 8 OMV-associated proteins detected by LC-MS/MS protein analysis, 5 are known virulence factors; catalase, flagellin A, VacA, flagella hook protein E and HopQ. Furthermore, Lpp20, a known OMV-associated vaccine candidate (Keenan, Oliaro et al. 2000), was among the 8 proteins identified within "OMV" exosomes (Chapter 4).

Given that exosomes carrying bacterial PAMPs are capable of presenting bacterial antigens to T cells (Ramachandra, Qu et al. 2010), we hypothesised that exosomes carrying *H. pylori* OMV proteins may be able to present these antigens to immune cells. Initially, we measured the proliferative response of human PBMCs to either "OMV" exosomes or exosomes from cells not exposed to OMVs, referred to as "empty" exosomes. Whilst there was a significant increase in T cell proliferation in response to "OMV" exosomes, there was also a smaller, yet still significant, proliferative response to "empty" exosomes. This suggested that human T cells were mounting an allogeneic response to the human "non-self" proteins within the exosomes. In order to remove this background proliferation, we isolated and expanded a pool of polyclonal *H. pylori*

reactive T cells with which to repeat these experiments. Using cell proliferation assays and cytokine analysis, we demonstrated that both human polyclonal *H. pylori* reactive $CD4^+$ and $CD8^+$ T cells proliferated in response to *H. pylori* lysate and secreted the pro-inflammatory cytokines IFN-γ, IL-6 and TNF-α as well as the anti-inflammatory cytokine IL-10, all of which are hallmarks of *H. pylori* infection (Bodger, Wyatt et al. 1997, Yamaoka, Kita et al. 1997, Sayi, Kohler et al. 2009). Finally, we determined that exosomes containing OMV proteins were indeed able to elicit a proliferative response by these *H. pylori* reactive polyclonal T cells.

Further work is required to validate this finding, using PBMCs taken from at least 3 donors. Moreover, this study did not examine the proliferative response of *H. pylori* positive donors and how this may affect the outcomes of T cell proliferation in response to *H. pylori* antigen stimulation. Given that PBMC responses to *H. pylori* are lower in *H. pylori* positive patients than *H. pylori* negative patients (Fan, Chua et al. 1994), the proliferative response of T cells from *H. pylori* positive donors to "OMV" exosomes may be less pronounced than those from *H. pylori* negative donors. It would therefore be interesting to measure and compare the proliferation of T cells from *H. pylori* positive and negative donors in response to exosomes containing *H. pylori* OMV proteins. It would also be important to examine T cell cytokine responses following stimulation with either "empty" or "OMV" exosomes.

Whilst we did determine that *H. pylori* OMV proteins are contained within exosomes released from cells containing intracellular OMVs (Chapter 4), we did not investigate the possibility that these proteins exist as HLA Class II/OMV peptide complexes. Nor did we investigate in any detail the expression of HLA Class I or II by exosomes released from polarised T84 IECs. Although we did not identify any HLA Class II molecules present within our exosome samples using LC-MS/MS, it may be that these

molecules were present in lower amounts than were detectable with our method of analysis. As we have shown that HLA Class II is up regulated at the cell surface only by cells that have been pre-treated with IFN-γ, it is possible that we would only identify HLA Class II in exosomes derived from cells pre-treated with IFN-γ prior to co-culture with OMVs. In future experiments, T84 cells (both polarised and non-polarised) could be treated with IFN-γ, prior to co-culture with OMVs or *H. pylori*. Since exosomes are too small for direct FACS analysis, alternative methods have been developed utilising magnetic beads coated with antibodies of interest (Clayton, Court et al. 2001), some of which are now commercially available (Exo-Flow, System Biosciences). Using magnetic beads coated with antibodies against HLA Class II, we could investigate the exosomal surface expression of HLA Class II molecules of exosomes derived from cells cultured with IFN-γ and OMVs. Furthermore, other groups have demonstrated that exosomes bearing HLA Class II/peptide complexes are capable of presenting antigen to DCs, with subsequent induction of T cell responses (Mallegol, van Niel et al. 2007). In order to identify if the OMV derived proteins we detected within our exosome samples exist as HLA Class II/peptide complexes, we could purify the HLA Class II molecules by affinity chromatography using specific antibodies against HLA Class II (Fissolo, Haag et al. 2009). The peptides could then be separated by HILIC and analysed by LC-MS/MS as described in Chapter 4. This would allow us to further elucidate the antigen presenting capabilities of exosomes containing OMV derived proteins. Finally, in order to determine if exosomes are directly capable of presenting antigen, or if there is a requirement for APCs, it will be necessary to perform T cell proliferation assays in the presence or absence of APCs, as described in chapter 4. Given that IEC derived exosomes have been shown to be capable of presenting antigen only in the presence of DCs (Vincent-Schneider, Stumptner-Cuvelette et al. 2002, Mallegol, van Niel et al.

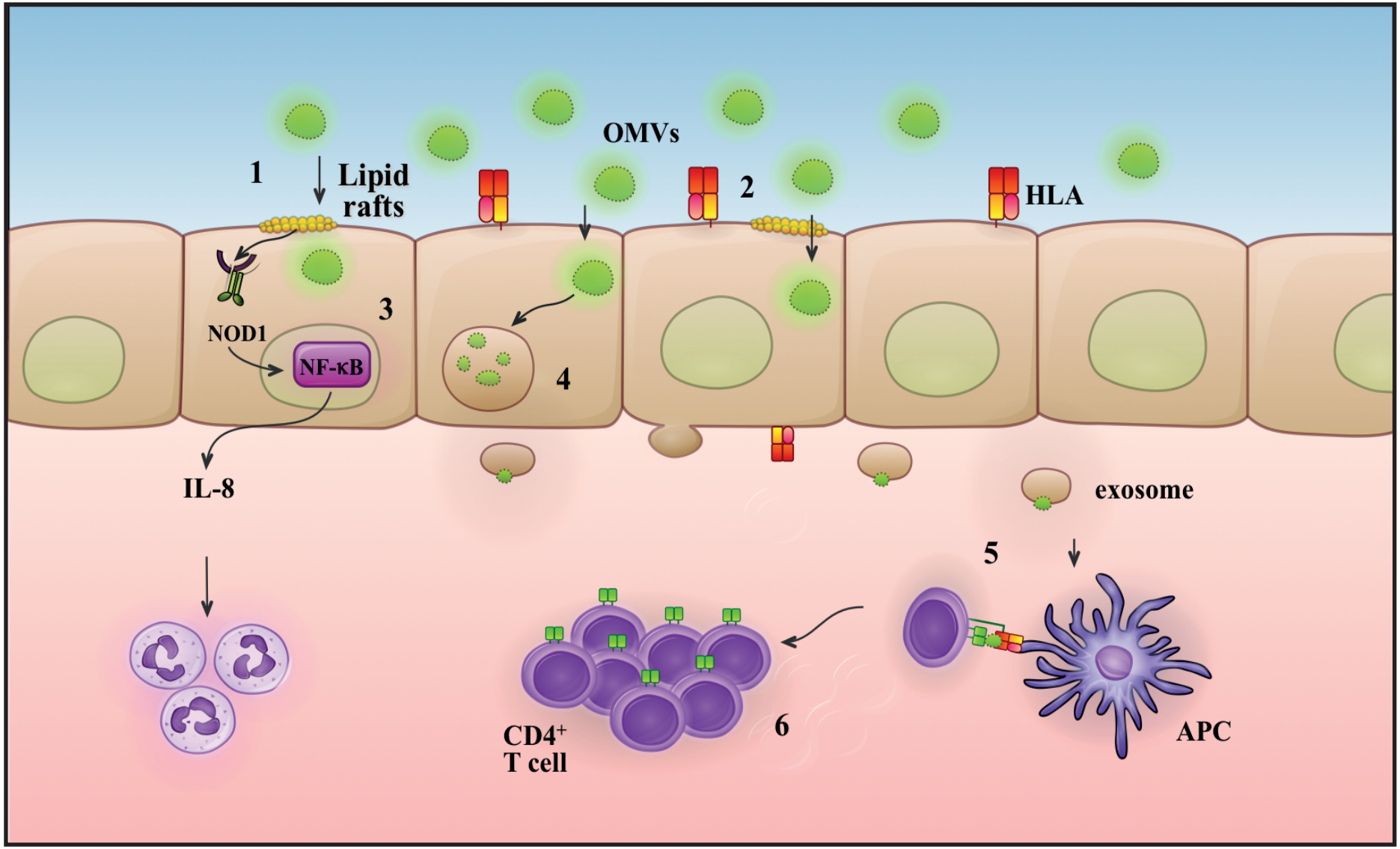
2007), we hypothesise that DCs are required for the generation of T cell responses. Interestingly, exosomes are internalised by phagocytic cells such as DCs (Morelli, Larregina et al. 2004, Feng, Zhao et al. 2010). Therefore, it is possible that IEC derived exosomes containing OMV-derived proteins (or bearing HLA Class II/OMV peptide complexes) are internalised by DCs, followed by presentation of OMV-derived proteins on the surface of DCs to mucosal T cells.

In summary, this work has demonstrated that the disruption of the proteins TolB and Pal in *H. pylori* results in distinct morphological alterations, accompanied by an increase in outer membrane OMV production. We demonstrated that the size of these OMV plays a critical role in directing the precise mode of entry into host cells. Moreover, polarised epithelial cells containing intracellular OMVs basolaterally secreted exosomes that contained OMV proteins within them. Importantly, we showed that exosomes containing OMV proteins induced proliferation of human T cells in the presence of APCs, indicating that exosomes could function to present OMV antigens to mucosal T cells. We propose that through interactions with APCs such as DCs, these exosomes are capable of inducing antigen specific proliferation of T cells located beneath the epithelial cell layer, thereby providing a link between the generation of innate and adaptive immune responses to *H. pylori* at the mucosal epithelium. Our model for *H. pylori* OMV induced innate and adaptive immune responses is as follows. *H. pylori* OMVs are internalised at the host cell surface by macropinocytosis, clathrin- and/or caveolin-dependent endocytosis, depending on the size of the OMVs. PG, delivered to the interior of the cell by OMVs, interacts with NOD1 to up regulate NFκB activation and IL-8 production. IL-8 is secreted predominantly from the basolateral surface of host cells, thereby helping to establish a transepithelial cytokine gradient that aids in

neutrophil recruitment to the affected area. Intracellular OMVs also induce the up regulation of HLA Class I and II by host epithelial cells. Intracellular OMVs are processed within the cell and packaged within exosomes, which are secreted into the extracellular environment. These OMV-containing exosomes are capable of inducing antigen specific T cell proliferation, through interactions with APCs (Figure 5.1).

Overall, this work has further expanded our knowledge of how OMVs are able to enter host epithelial cells. Importantly, we have provided the first evidence demonstrating that internalised OMVs are processed within the cell and packed within exosomes that capable of inducing bacterial specific T cell proliferation.

Figure 5.1. Proposed model of *H. pylori* OMV induced innate and adaptive immune responses. *H. pylori* OMVs enter host cells via lipid rafts (Kaparakis et al, 2010) by macropinocytosis, clathrin- and caveolin-dependent endocytosis (1). OMVs induce the upregulation of HLA Class I and Class II by epithelial cells (2). OMV-PG interacts with NOD1 to induce NF-κB induction and IL-8 production. IL-8 is secreted predominantly from the basolateral surface of cells, attracting neutrophils to the area (3). Intracellular OMVs are processed within the cells and packaged within exosomes that are released from the cells (4). Exosomes containing OMV proteins interact with APCs such as DCs, which present OMV-derived proteins to T cells, inducing antigen specific proliferation (5). Modified from a figure designed by M Kaparakis-Liaskos and compiled by P. Alwis.


1
Lipid rafts
OMVs
2
HLA
3
NOD1
NF-κB
4
IL-8
exosome
5
CD4+ T cell
6
APC

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