

IMMUNOMODULATORY EFFECTS OF COMMENSAL BACTERIA-DERIVED
MEMBRANE VESICLES

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MEMBRANE VESICLES**

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ABSTRACT

IMMUNOMODULATORY EFFECTS OF COMMENSAL BACTERIA-DERIVED MEMBRANE VESICLES

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Constitutive secretion of extracellular membrane vesicles is a common feature of cells from all domains of life including Archaea, Bacteria, and Eukarya. Although the contribution of gram negative bacterial outer membrane vesicles in disease pathogenesis has been extensively studied, whether commensal bacteria constitutively secrete such vesicles is still unknown. Given the importance of microbiota as regulators of immune homeostasis, we aimed to assess the immunomodulatory properties of extracellular vesicles secreted from 5 different human commensal bacteria isolates in comparison to E.coli derived outer membrane vesicles (MVs). AFM microscopy, dynamic light scattering and zeta potential measurements revealed that commensal-derived membrane vesicles (MVs) were ~50- 300 nm in diameter and had high negative charge densities (-40 mV). Mouse spleen cells stimulated with commensal derived MVs secreted lower levels of pro-inflammatory cytokines (IFN γ and TNF α) and higher levels of IL-10 when compared to E.coli derived MVs. Similarly, commensal derived MVs failed to stimulate the maturation of antigen presenting cells. Mice immunized with an inactivated viral vaccine against the foot and mouth disease virus showed suppressed FMD-specific IgG2a response when the vaccine contained MVs derived from commensals but not from E.coli. These results indicate that human commensal bacteria-derived membrane vesicles can have powerful immunomodulatory effects and can have potential therapeutic applications as novel anti-inflammatory agents.

Keywords: Membrane vesicles, commensal bacteria, immune modulation, vaccine adjuvant

ÖZ

KOMMENSAL BAKTERİ KÖKENLİ MEMBRAN KESECİKLERİNİN İMMÜNOMODÜLATOR ETKİLERİ

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Hücre-dışı membran keseciği salgılanma olgusu, arkeler, bakteriler ve ökaryotlar dahil olmak üzere tüm hücrel yaşam türlerinde gözlenen ortak bir özelliktir. Gram negatif bakteri kökenli dış membran keseciklerinin hastalık patogeneze katkısına dair çok sayıda çalışma olmasına rağmen kommensal bakterilerin bu tür kesecikler salgılayıp salgılamadıkları henüz bilinmemektedir. Mikrobiyotanın immün homeostaz düzenlemesindeki önemi bilindiğinden bu çalışmada 5 farklı insan kommensal bakteri izolatının salgıladığı keseciklerin immüdüzenleyici etkilerini E.coli kökenli dış membran kesecikleriyle karşılaştırmalı olarak incelemeyi hedefledik. AKM mikroskopisi, dinamik ışık saçınımı ve zeta potansiyeli ölçümleri kommensal kökenli membran keseciklerinin (MV'ler) yaklaşık 50-300 nm ebatlarında ve yüksek eksi yük yoğunluğuna (-40 mV) sahip olduklarını gösterdi. Kommensal MV'lerle uyarılan fare dalak hücrelerinin E.coli keseciklerinin aksine daha az miktarda pro-enflamatuar sitokin (IFN γ ve TNF α) ve daha yüksek miktarda IL-10 salgıladıkları bulundu. Aynı şekilde kommensal kökenli MV'ler antijen sunum hücresi olgunlaşmasında etkisiz kaldılar. E.coli keseciklerinin aksine kommensal kökenli keseciklerin varlığında inaktive şap aşısı ile aşılanmış farelerdeki şapa özgü IgG2a yanıtlarında baskılanma olduğu gösterildi. Bu sonuçlar, insan kommensal kökenli membran keseciklerinin güçlü immün düzenleyici etkilere sahip olduklarını ve yeni tip anti-enflamatuar ajanlar olarak terapiye yönelik potansiyel uygulamalarının olabileceğini göstermektedir.

Anahtar Kelimeler: Membran kesecikleri, kommensal bakteriler, immün düzenleme, aşı adjuvanı

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
APC	Antigen presenting cell
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BDCA-2	Blood dendritic cell antigen 2
Bp	Base pairs
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CpG	Unmethylated cytosine-phosphate-guanosine motifs
CXCL	CXC-chemokine ligand
DAMP	Danger/damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
ELISpot	Enzyme Linked-Immunosorbent Spot
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus

hPBMC	Human peripheral blood mononuclear cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP 10	Interferon gamma-induced protein 10
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
Mf	Macrophage
MHC	Major histocompatibility complex
MV	Membrane vesicle
MyD88	Myeloid differentiation factor-88
NF- κ B	Nuclear factor- kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PdI	Polydispersity index
PGN	Peptidoglycan
PNPP	Para-nitrophenyl pyro phosphate
poly I:C	Polyriboinosinic polyribocytidylic acid
RPMI	Roswell Park Memorial Institute
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene-I

RLR	Retinoic acid-inducible gene-I like receptor
RNA	Ribonucleic acid
R848	Resiquimod
SA-AKP	Streptavidin-alkaline phosphatase
TcR	T cell receptor
T _{H1}	T helper type 1
T _{H2}	T helper type 2
T _{H17}	T helper type 17
T _{REG}	Regulatory T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1. Immune System

Invasion of a host by a pathogen triggers an immune response through interaction between pathogen derived virulence factors and host immune surveillance mechanisms (Kumar *et al.*, 2011). Immune system can discriminate between self and non-self foreign agents. Moreover, immune system has the ability to eliminate altered-self such as seen in the case of cancer cells (Sun, 2008). Immune system of vertebrates include two relevant types of immunity: the innate immune system and the adaptive immune system. The innate immune system is the primary line of defense against pathogens and is characterized by a rapid broadly-defined response. In contrast, the adaptive immune response is delayed and highly specific to the pathogen. Moreover, it can be long-lasting and is unique in developing immunological memory (Kumar *et al.*, 2011, Litman, 2005). The innate immune system detects microbial pathogens through specialized cells (such as macrophages, dendritic cells and NK cells) capable of recognizing general categories of “danger molecules” expressed by pathogens. Conversely, adaptive immune system is specific to an antigen recognized by unique antigen-recognition receptors expressed on T and B lymphocytes generated by somatic gene rearrangements during their development (Kawai and Akira, 2009). Antigen activated T or B cells undergo clonal expansion and differentiate into effector cells such as the antibody secreting plasma cells that help clear that specific antigen.

The skin and the mucosal epithelial layers serve as physical and chemical barriers of the body, and constitute the initial defense barrier against pathogen entry (Lievins-Le Moal, 2006). Following invasion of these barriers by pathogens, cells of the innate immune system respond to the insult by producing large amounts of proinflammatory molecules that would limit the spread of infection and help its clearance. Of the cells of the innate immune system, Dendritic cells (DCs), which are members of the so-called professional antigen presenting cells (APCs), hold special importance since they are vital in activating adaptive immune responses (Tel, 2012).

1.2. Innate Immune System

The innate immune system is the first line of defense against pathogens with many different units and subsystems. The skin and mucosal epithelial layer are both physical and chemical barriers in which the epidermal cells contribute to protection by producing antimicrobial peptides (Medzhitov, 2007). One of the major goals of the innate immune system is to sense microbial infections through a family of receptors that are mainly expressed on tissue resident specialized cells of innate immunity (Janeway, 1989). These receptors are collectively known as pattern recognition receptors (PRRs) and they recognize pathogen

associated molecular patterns (PAMPs) expressed by a variety of microorganisms. Since pathogens express PAMPs (such as bacterial cell-wall components) that are absent in the host, PRRs are able to discriminate between self and non-self (Kawai and Akira, 2009). However, in some circumstances innate immune cells also recognize self molecules and induce inflammation. In this case, presence of danger/damage associated molecular patterns (DAMPs) such as, heat shock proteins or ATP released from injured cells can act as “danger signals” and induce inflammation at the site of damage. Such DAMPs are also recognized by PRRs (Seong and Matzinger, 2004). Recognition of PAMPs or DAMPs initiates an inflammatory response which is mainly mediated by chemokines and cytokines secreted from activated cells (please see Table 1.1. and Table 1.2.). Epithelial cells, phagocytic cells and DC subsets express specific combinations of PRRs enabling them to generate tissue specific responses to microbial stimulation (Trinchieri and Sher, 2007). Another family of innate immune system proteins, the complement system, functions in opsonization/killing of bacteria and facilitates clearance of the infectious agent (Degn, 2007).

Table 1.1: Cytokines affect on target cells (Adapted from Murphy, 2008)

Cytokine	Produced by	Function
IL-1 β	Mf, keratinocytes	Fever, induction of acute-phase protein secretion, T cell and Mf activation
TNF α	Mf, DC, NK and T cells	Local inflammation and endothelial activation
IL6	Mf, DC	Fever, T and B cell growth and differentiation
IL-12	Mf, DC	Activation of NK cells, induction of CD4 T-cells to differentiate into T _H 1
IL-15	Many non-T cells	CD8 memory T cell survival, stimulation of NK and T cell growth
IL-18	Activated Mf	Induction of IFN γ secretion via NK and T cells, favors T _H 1 immunity
IFN α	DC	Anti-viral immunity, induction of MHC I expression
IFN γ	T cells, NK cells	Suppression of T _H 2 immunity, Mf activation, increased expression of antigen processing components

Table 1.2: Chemokines recruit target cells to sites of infection. (Adapted from Murphy, 2008)

Chemokines	Produced by	Attracted cells	Major effect
CXCL8 (IL-8)	Monocytes, Mf, DC	Neutrophils, naïve T cells	Mobilization, activation and degranulation of neutrophils
CCL3 (MIP-1 α)	Monocytes, T and Mast cells, fibroblasts	Monocyte, NK, T cells basophil, DC	Promotes T _H 1, antiviral defense, competes with HIV-1
CCL4 (MIP-1 β)	Monocytes, Mf, Neutrophils, endothelium	Monocyte, NK, T, DC	Competes with HIV-1
CCL2 (MCP-1)	Monocyte, Mf, fibroblast, keratinocyte	Monocyte, NK, T, DC basophil	Promotes T _H 2, activate Mf, histamine release from basophils
CXCL10 (IP10)	T, fibroblast, endothelial, monocyte, keratinocyte	Resting T cells, NK, monocytes	Promotes T _H 1, antiangiogenic, immunostimulant

Innate immunity and its antigen presenting cells are vital in processing of extracellular or cytosolic antigens and presentation of antigen-derived peptides to T-cells (Watts, 2010). In general, cytosolic antigen-derived peptides are complexed with Major Histocompatibility Complex (MHC) Class I molecules expressed by all nucleated cells and are presented to CD8+ cytotoxic T cells, whereas exogenous antigen-derived peptides are complexed with MHC Class II molecules expressed by APCs and are presented to CD4+ helper T cells. Immature DCs expressing various PRRs become activated and mature following PRR engagement by PAMPs. Mature DCs express co-stimulatory molecules (such as CD80 and CD86) which are essential for priming of naive T lymphocytes. Mature DCs migrate to the closest draining lymph nodes and initiate antigen presentation to naive T lymphocytes. Additionally, depending on PAMPs they are exposed to, DCs secrete various cytokines that shape differentiation of helper T cells into various classes of effector cells (Th1, Th2, Th17 or induced-regulatory T cells). These T cells then help in shaping an appropriate response suitable to the invading pathogen (for example, Th1 cells are effective in eliminating intracellular pathogens whereas Th2 cell help is essential to eliminate certain parasitic infections). The set of PRRs activated by a pathogen determines which cytokines/chemokines and adhesion molecules will be induced. Thus PRR expressing APCs and in particular DCs serve to link innate and adaptive immunity (Walport, 2008).

1.2.1. Pattern Recognition Receptors (PRRs)

Pattern recognition receptors are specialized to detect DAMPs or PAMPs and they are germline encoded receptors. Classical PAMPs include β -glucan of fungal cell wall, lipopolysaccharide (LPS), lipoteichoic acid like cell-wall components of bacteria, peptidoglycan, DNA containing unmethylated cytosine-phosphate-guanine (CpG) motifs in bacterial genome, viral single stranded RNA (ssRNA) or double stranded RNA (dsRNA),

and double stranded DNA (dsDNA) (Akira, 2006) (Table1.3). There are three main PRR families and these receptors participate in pattern recognition through specialized protein domains such as scavenger receptor cysteine-rich domain, the C-type lectin domain or the leucine-rich repeat (LRR) domain (Hollmig, 2009). Following ligand binding to PRRs, cells expressing these receptors activate various signaling pathways that trigger the release of chemokines, antimicrobial peptides or inflammatory cytokines (Medzhitov, 2000).

PRRs divided into three main families which are toll-like receptors (TLRs), the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).

Table1.3: PRRs and PAMPs (Adapted from Kawai and Akira,2009)

	PRRs (structure)	Adapters (structure)	PAMPs/Activators	Species	
TLR	TLR1-TLR2 (LRR-TIR) TLR2-TLR6 (LRR-TIR)	MyD88 (TIR-DD), TIRAP (TIR) MyD88 TIRAP	Triacyl lipopeptides Diacyl lipopeptides LTA Zymosan PGN Lipoarabinomannan Porins	Bacteria Mycoplasma Bacteria Fungus Bacteria Mycobacteria Bacteria (<i>Neisseria</i>) Parasites (<i>Trypanosoma</i>) Virus (Measles virus)	
	TLR2 (LRR-TIR)	MyD88, TIRAP	tGPI-mucin HA protein dsRNA	Virus Bacteria LPS	
	TLR3 (LRR-TIR) TLR4 (LRR-TIR)	TRIF (TIR) MyD88, TIRAP, TRIF, TRAM (TIR)	Envelope proteins Flagellin	Virus (RSV, MMTV) Bacteria	
	TLR5 (LRR-TIR) TLR7 (LRR-TIR) hTLR8 (LRR-TIR) TLR9 (LRR-TIR)	MyD88 MyD88 MyD88 MyD88	ssRNA ssRNA CpG DNA DNA	RNA virus RNA virus Bacteria DNA virus	
	mTLR11 (LRR-TIR)	MyD88	Malaria hemozoin Not determined	Parasites Bacteria (uropathogenic bacteria) Parasites (<i>Toxoplasma gondii</i>)	
			Profilin-like molecule	Virus	
	RLR	RIG-I (CARDx2-helicase)	IPS-1 (CARD)	RNA (5'-PPP ssRNA, short dsRNA)	Virus
		MDA5 (CARDx2-helicase) LGP2 (helicase)	IPS-1	RNA (poly IC, long dsRNA) RNA	Virus Virus
	NLR	NOD1/NLRC1 (CARD-NBD-LRR)	RICK (CARD), CARD9 (CARD)	IE-DAP	Bacteria
		NOD2/NLRC2 (CARDx2-NBD-LRR) NALP3/NLRP3 (PYD-NBD-LRR)	RICK, CARD9 ASC (PYD-CARD) CARDINAL (PYD-FIND)	MDP MDP RNA ATP Toxins	Bacteria Bacteria Bacteria, Virus Bacteria? Host? Bacteria
NALP1/NLRP1 (CARD-FIND-NBD-LRR-PYD) IPAF/NLRC4 (CARD-NBD-LRR) NAIP5 (BIRx3-NBD-LRR)		ASC	Uric acid, CPPD, amyloid- β Anthrax lethal toxin	Host Bacteria	
NAIP5 (BIRx3-NBD-LRR)			Flagellin Flagellin	Bacteria Bacteria	
CLR	Dectin-1 (lectin-ITAM)		β -Glucan	Fungi	

1.2.1.1. Toll-like Receptors (TLRs)

TLRs were among the first characterized family of PRRs. The Toll protein was originally identified in *Drosophila melanogaster* as a sensor initiating anti-fungal immunity (Yoneyama and Fujita, 2010). Mammalian equivalents of this protein was later named as Toll-like. Multiple TLR family members have been identified in humans (10 members) and mice (12 members) (Kumar *et al.*, 2011). TLR1 to 9 are similar in both human and mice. TLR10 is only expressed in humans (a stop codone prevents murine TLR10 gene expression). TLR13, TLR12 and TLR11 are lost in human genome (Kawai, 2009) but are expressed in mice. All TLRs are type I transmembrane proteins consisting of three major

domains: The leucine-rich repeats of the ectodomain mediate the recognition of PAMPs and is connected to the intracellular Toll–interleukin 1 (IL-1) receptor (TIR) signaling domain via a transmembrane domain. TLR 1, 2, 4, 5 and 6 are expressed on the cell surface and recognize bacteria, fungi and protozoa derived PAMPs, whereas TLRs 3, 7, 8 and 9 are expressed within endocytic compartments and recognize virus and bacteria derived nucleic acids (Kumar *et al.*, 2011). Table 1.4 and Figure 1.1 summarizes the major TLR family members, their cellular localization and their ligands. The cellular localization of TLRs (i.e, plasma membrane versus endosomal localization) determines ligand accessibility, and is important for the discrimination of microbial from self (Kumar, 2009).

Table1.4: TLR family members, their subcellular localization and their specific ligands (Adapted from Kumar *et al.*, 2011)

TLR and (co-receptors)	Cellular localization	TLR ligands
TLR1/2	Cell surface	Triacyl lipopeptides
TLR2 (Dectin-1, C-type lectin)	Cell surface	Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphatidyl inositol mucin, zymosan
TLR3	Endosome	ssRNA virus, dsRNA virus, respiratory syncytial virus, murine cytomegalovirus
TLR4 (MD2, CD14, LBP)	Cell surface	Lipopolysaccharide, mannan, glycoinositolphospholipids, envelope and fusion proteins from mammary tumor virus and respiratory syncytial virus, respectively, endogenous oxidized phospholipids produced after H5N1 avian influenza virus infection, pneumolysin from <i>streptococcus pneumoniae</i> , paclitaxel.
TLR5	Cell surface	Flagellin from flagellated bacteria
TLR6/2 (CD36)	Cell surface	Diacyl lipopeptides from mycoplasma, lipoteichoic acid
TLR7	Endolysosome	ssRNA viruses, purine analog compounds (imidazoquinolines). RNA from bacteria from group B streptococcus
TLR8 (only in human)	Endolysosome	ssRNA from RNA virus, purine analog compounds (imidazoquinolines).
TLR9	Endolysosome	dsDNA viruses herpes simplex virus and murine cytomegalovirus, CpG motifs from bacteria and viruses, hemozoin malaria parasite
TLR11 (only in mouse)	Cell surface	Uropathogenic bacteria, profilin-like molecule from <i>Toxoplasma gondii</i>

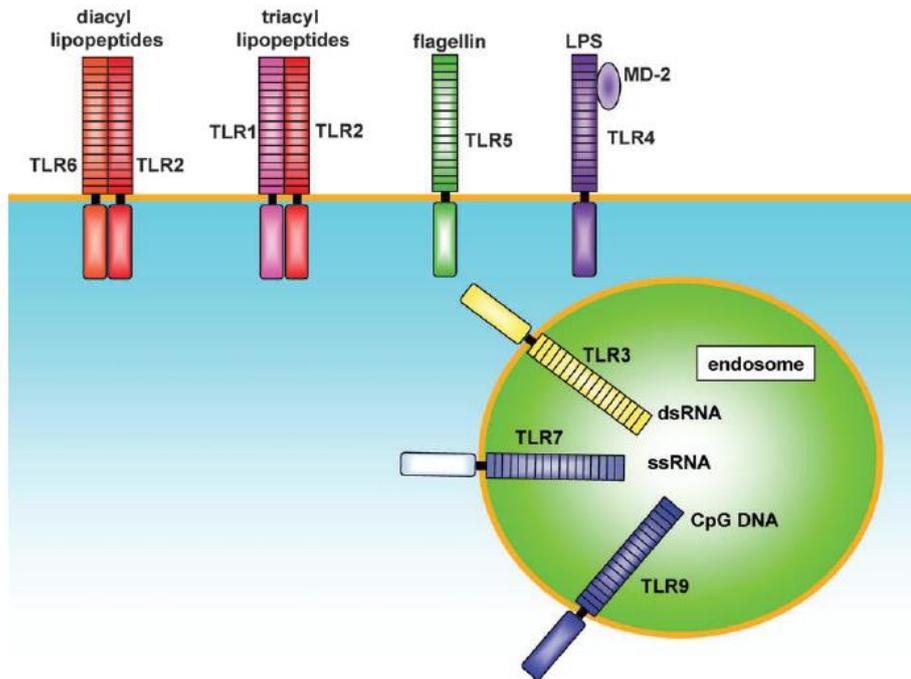


Figure1.1 Cellular localization of TLR family members (Adapted from Takeda and Akira, 2005)

1.2.1.1.1. Extracellular TLRs

Extracellular TLRs (TLR11, TLR6, TLR5, TLR4, TLR2 and TLR1) generally recognize microbial cell wall/membrane components (Kaisho, 2001). TLR2 recognizes zymosan from fungi, lipopeptides from bacteria, Gram-positive bacteria derived peptidoglycan and lipoteichoic acid, the hemagglutinin protein of the measles virus and lipoarabinomannan from mycobacteria (Schwandner *et al.*, 1999, Underhill *et al.*, 1999). TLR2 generally found in heterodimer form with TLR6 or TLR1. The TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from mycoplasma and Gram positive bacteria. The TLR1-TLR2 heterodimer recognizes triacylated lipopeptides from mycoplasma and Gram negative bacteria. (Kawai, 2010).

Outer membrane of gram negative bacteria contains lipopolysaccharide (LPS) which is recognized by TLR4. TLR4 is associated with a small adaptor, MD2 and together with the cell-surface expressed CD14 molecule, recognizes. Role of CD14 in LPS recognition is done by binding to the LPS binding protein (LBP) and delivering the LPS-LBP to the TLR4-MD2 complex (Kim *et al.*, 2007). The TLR4-MD2-LPS complex's two copies initiates signal transduction via recruiting intracellular adaptor molecules. TLR4 was also reported to recognize *Streptococcus pneumoniae* pneumolysin, respiratory syncytial virus fusion proteins and mouse mammary tumor virus envelope proteins. (Luxamechanporn, 2005).

TLR5 recognizes a protein component of bacterial flagella which is known as flagellin (Hayashi *et al.*,2001). TLR5 recognizes a central site of flagellin which has a role in

protofilament formation and bacterial motility. TLR5 is expressed on the basolateral surface of intestinal epithelial cells but not on macrophages or splenic DCs. This shows that TLR5 has a role in detection of invasive flagellated bacteria in the gut (Kawai and Akira, 2009).

TLR11 is only functional in mice and is similar to TLR5. This receptor is highly expressed in the bladder and kidney of mice and has a role in detection of uropathogenic bacterial components. It is known that TLR11 also recognizes a parasite component (profilin-like molecule) derived from *Toxoplasma gondii*. This molecule function as an actin-binding protein and is responsible from parasite motility and invasion. Recognition of the profilin-like molecule generates a robust NF- κ B- dependent inflammatory response and IL-12 production (Kucera, 2010).

1.2.1.1.2. Intracellular TLRs

TLR9, TLR8, TLR7 and TLR3 are intracellular TLRs, that are specialized in sensing nucleic acids. Intracellular TLRs are localized within intracellular compartments such as endosomes, lysosomes, in the endoplasmic reticulum and endolysosomes. Intracellular TLRs serve as sensors of foreign nucleic acids and trigger anti-viral innate immune responses by producing type I interferons and inflammatory cytokines (Kawai and Akira, 2009). The intracellular TLRs originally reside in the ER but are localized to the endosomes following PAMP engagement. In the case of TLR9, the N-terminal region is processed by lysosomal proteases such as asparagine endopeptidases and cathepsins in the endosomes and become functional receptors (Blasius and Beutler, 2010).

TLR3 recognizes double-stranded RNA, so this receptor recognize dsRNA viruses and dsRNA produced during the course of replication of ssRNA viruses (Wang *et al.*, 2004, Alexopoulou *et al.*, 2001) . Upon recognition of dsRNA, TLR3-mediated signaling triggers an anti-viral immune response characterized by type I interferon inflammatory cytokine production. TLR3 recognizes polyriboinosinic polyribocytidylic acid (poly I:C), a synthetic analog of viral dsRNA, which has been used in experiments to mimic viral infections (Schröder, 2005).

TLR7 recognizes ssRNA, imidazoquinoline derivatives such as imidazoquinoline, imiquimod, resiquimod (R848) and guanine analogs such as loxoribine (Hemmi *et al.*, 2002). TLR8 is very similar to TLR7 and detect ssRNA in humans (Jurk, 2002).

TLR9 recognizes unmethylated CpG motifs that present in viral and bacterial DNA (Klinmann and Kreig, 1995). The prokaryotic DNA has 20 X higher frequency of CpG motifs when it is compared to mammalian DNA. Moreover, in mammals the CpG motifs are mostly methylated. This difference between the prokaryotic versus the mammalian DNA forms the basis of ligand recognition by TLR9 (Hemmi, 2000).

1.3. Adaptive Immune System

Adaptive immune response occurs when an infection overcomes the innate defense mechanisms. In the absence of specific antigen, naive T cells circulate between blood-secondary lymphoid organs and lymphatics. Following encounter with a specific antigen, T

cells start to proliferate and differentiate into effector cells. Naive CD8⁺ T cells differentiate into cytotoxic effector T cells that recognize pathogen-derived peptides presented with MHC class I molecules on the surface of infected cells and kill these targets. Conversely, CD4⁺ T cells differentiate into a variety of subsets following recognition of pathogen-derived peptides presented by MHC class II expressing APCs. Depending on which cytokines are present in the immediate vicinity, these antigen-experienced helper T cells can differentiate into Th1, Th2 and Th17 or induced-Tregs (regulatory T cells) (Walport, 2008).

B cells are other important cells in adaptive immunity. Activation of naive B cells takes place after direct recognition of the antigen through the surface immunoglobulin (B cell receptor). However, in the absence of T cell help, such antigen-stimulated B cells differentiate into IgM secreting B cells (Boehm, 2011). Differentiation into plasma cells capable of secreting other antibody classes and subclasses (IgG, IgE or IgA) requires class switching and depends on specific T cell help (for example Th1 versus Th2) (Kaiser, 2010). For both B and T cells, a small proportion of antigen-experienced cells differentiate into memory cells that are important in rapid induction of re-call responses (secondary response).

This thesis investigates immunostimulatory/immunomodulatory roles of PRR ligand-rich membrane vesicles secreted from bacteria. The following sections will summarize some of the properties of these interesting structures.

1.4. Bacteria derived membrane vesicles as a source of TLR ligands

1.4.1. Membrane Vesicle Formation

Formation of spherical, membrane vesicles from cell surface is a common feature of organisms including both prokaryotes and eukaryotes. Vesicle secretion appears to be an universal phenomenon and is presented in gram positive and gram negative bacteria, archaea, fungi and parasites (Deatherage and Cookson, 2012). Membrane vesicle (MV) formation in gram negative bacteria (also known as outer membrane vesicles) was initially observed more than 50 years ago. Vesicle production is a continuous process and takes place at all stages of growth (Ellis and Kuehn, 2010). For pathogens, MV secretion aids to disperse virulence factors such as toxins, antigens and degradative enzymes into environment, facilitating host colonization (Deatherage and Cookson, 2012). Gram negative bacterial MVs have a size range of 10 – 300 nm and the vesicles contain outer membrane and periplasmic components such as proteins, lipoproteins, phospholipids, lipopolysaccharide (LPS) and DNA (Kulp and Kuehn, 2010, McBroom and Kuehn 2006). Gram positive bacteria also produce membrane vesicles (Lee *et al.*, 2009, Rivera *et al.*, 2010, Macdonald and Kuehn, 2012, Deatherage and Cookson, 2012). However, this is a newly discovered phenomenon and therefore the process is not well understood. In the case of gram negative bacteria, vesicles bud from the outer membrane (Figure 1.2). In contrast, since gram positive bacteria lack an outer membrane, their MVs originate from the cytoplasmic membrane and contain cytosolic components. Nevertheless, they appear to function in processes similar to their OMV counterparts (Lee *et al.*, 2009). Some gram positive bacterial membrane vesicles were

reportedly enriched in minor lipid components such as myristic and palmitic acids (Rivera *et al.*, 2010) and may also include toxins (Deatherage and Cookson, 2012).

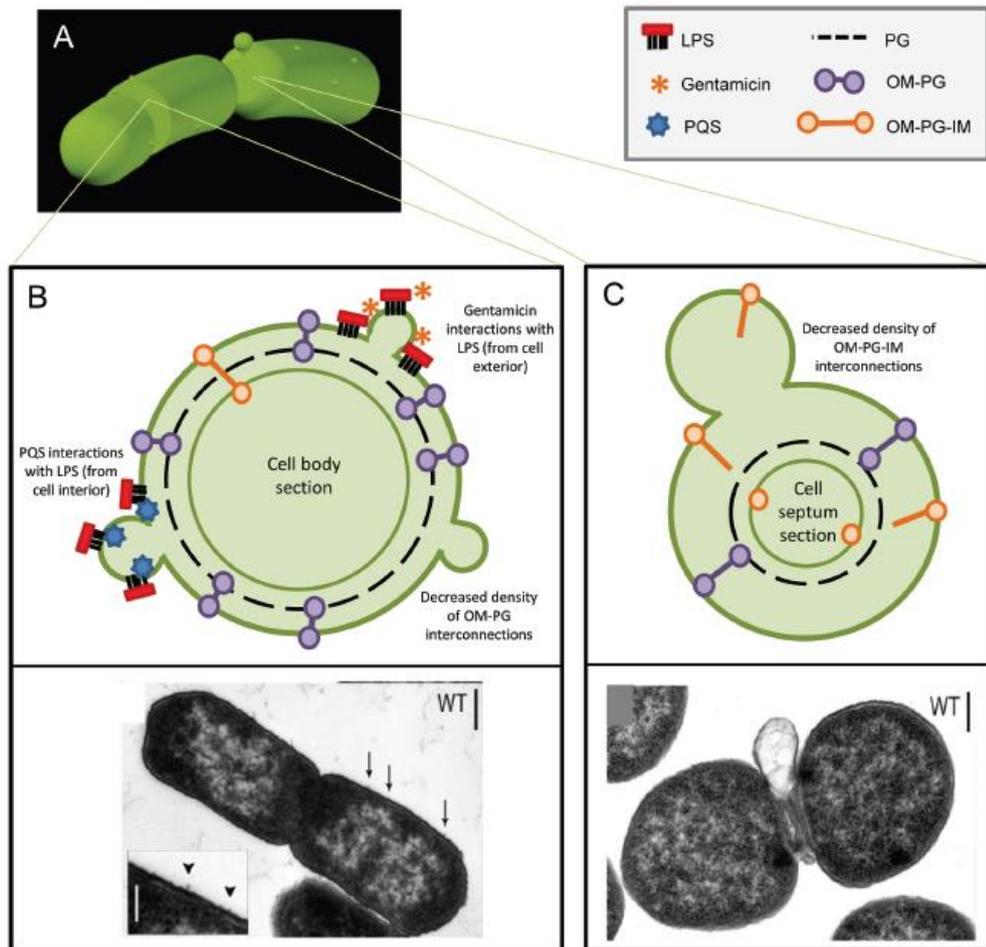


Figure 1.2 Suggested mechanism of gram negative bacteria derived membrane vesicle formation (Adapted from Deatherage and Cookson, 2012)

1.4.2. Biological functions of membrane vesicles

Membrane vesicles secreted from bacteria can mediate two opposing functions: offense versus defense. MVs transport virulence factors and toxins, modulate the immune response and aid colonization. Conversely, they function defensively by creating a decoy target for the immune system and hence increase the chance of survival of the organism in a hostile environment (Macdonald and Kuehn, 2012). Figure 1.2 summarizes the biological functions of bacteria-derived membrane vesicles. These vesicles provide a means of polysaccharide and virulence factor secretion to the environment, aid cell to cell communication, and stimulate the innate and adaptive immune responses through the TLR ligands present in their structure.

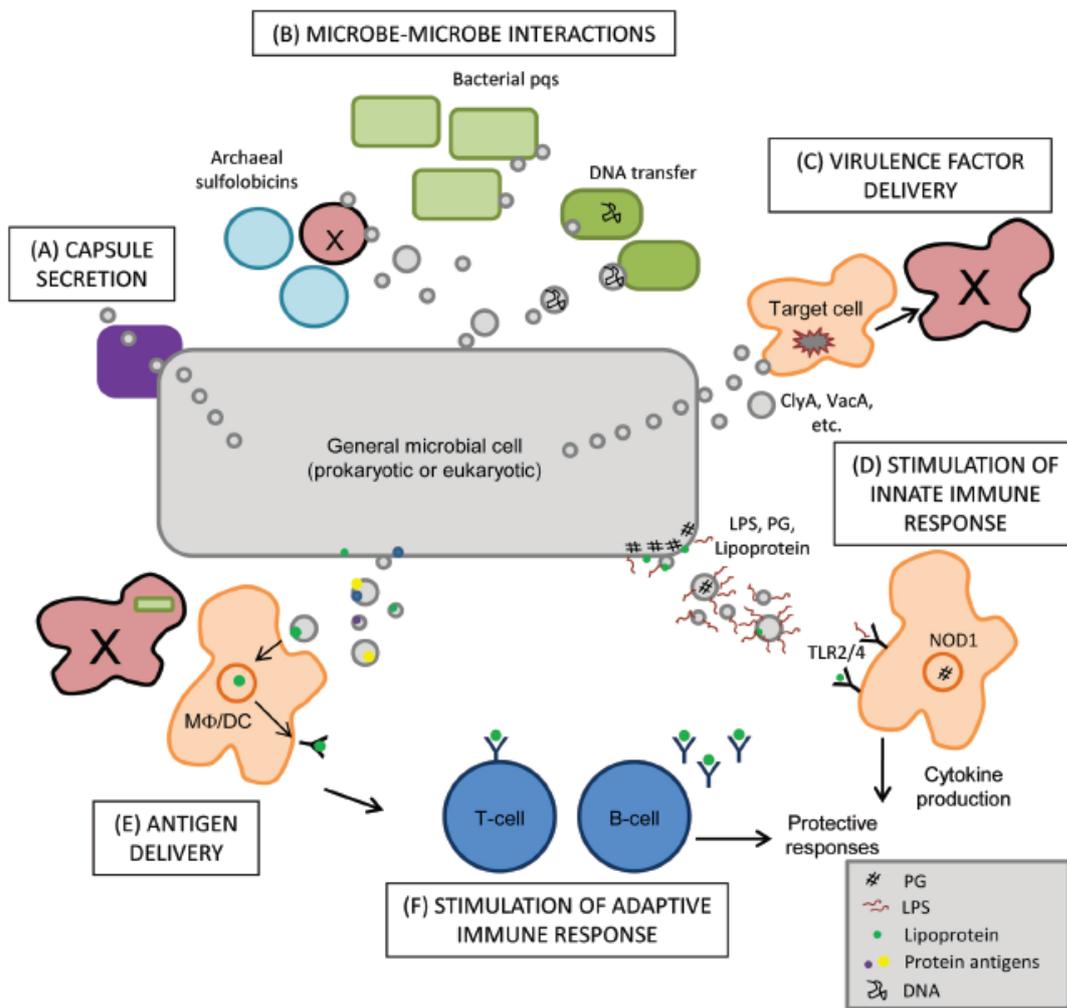


Figure 1.3 Biological functions of secreted membrane vesicle (Adapted from Deatherage and Cookson, 2012)

Recent evidence suggests that use of multiple TLR ligands induce a robust immune response (Kasturi *et al.*, 2012, Nourizadeh *et al.*, 2012). In this context, membrane vesicles are of interest since they are enriched in TLR ligands (such as peptidoglycans, LPS and nucleic acids) and could be of value as novel vaccine adjuvants/immunotherapeutic agents. The vesicular form of these ligands is more stable than their free counterparts. For example, Park *et al.* (2010) compared the role of *E.coli* derived membrane vesicles to free LPS and demonstrated a robust immune response when MVs were used as opposed to the non-vesicular ligand. Evidence for activation of host immune responses using gram negative bacteria-derived membrane vesicles is ample (Lee *et al.*, 2012, Kuehn *et al.*, 2005, Yoon *et al.*, 2011, Ellis *et al.*, 2010).

Although most research focused on the effects of pathogen-derived MVs, to date, there are no published results analysing whether commensal bacteria are capable of secreting MVs and/or how these MVs impact the immune system.

1.5. Microbiota

All mammals enter the world without microbial colonization because of the sterile environment of the womb. This process ceases to exist immediately after birth where the new-born is first exposed to the mother's microbiota that initiates microbial colonization in the digestive, respiratory and urogenital tracks. Skin also has a diverse range of microbiome that coexists throughout life (Maynard *et al.*, 2012). Figure 1.4 summarizes host- commensal interactions at distinct barrier sites. In total, there are 27 different body sites (skin, nostrils, hair, oral cavity etc.) that house unique communities of bacteria. Bacterial community composition is determined by the ecology of each body site (Costello *et al.*, 2009). Although microbiota generally involves different species of bacteria, archaea, viruses and eukaryotic microorganisms are also present in various tissues. The greatest density of microbiota is found in the gut, specifically in the ileum and colon. It is estimated that, microbiota numbers exceed the total number of cells in the body by 10-fold (~100 trillion organisms). Moreover, microbiota has 100 fold more unique genes than their hosts genome (Ley *et al.*, 2006).

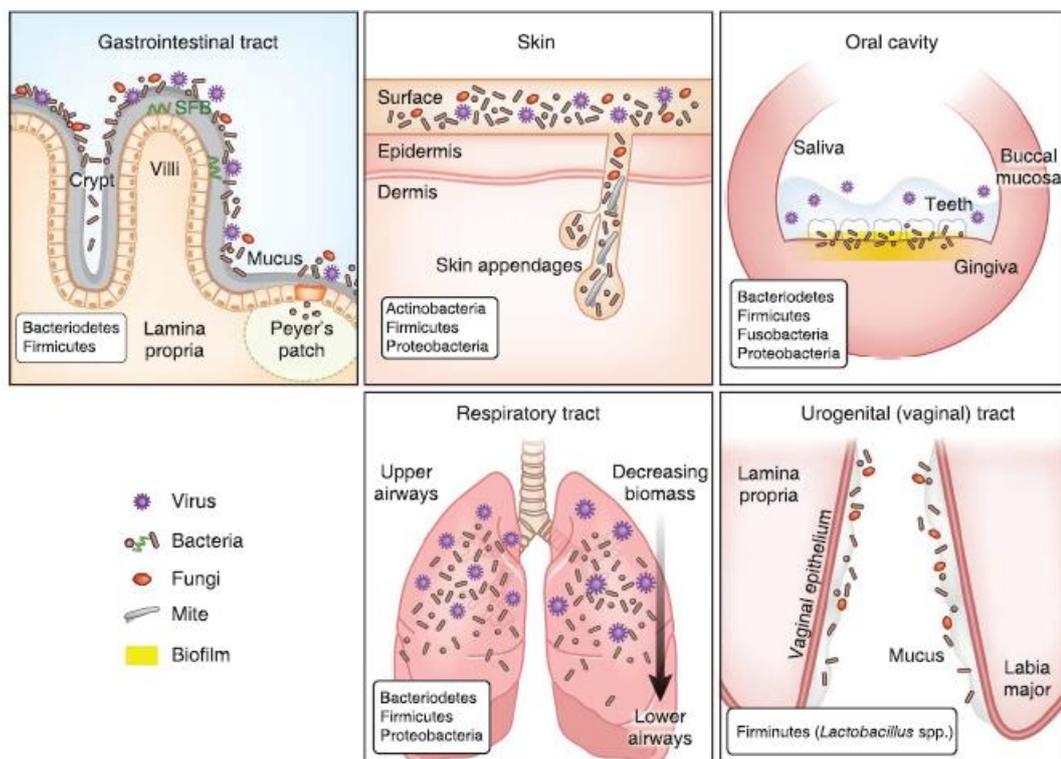


Figure 1.4 Tissue specific modes of host – commensal interactions at distinct barrier sites. (Adapted from Belkaid *et al.*, 2013)

The relationship between intestinal microbiota and its host provides mutual benefits. The microbiota benefits from the warm and nutrient rich environment of the gut and the host benefits from provided essential non-nutrient factors derived from microbial metabolites (Maynard *et al.*, 2012).

Mammals have formed an evolutionary partnership with commensals and they maintain tolerance against them. In the gastrointestinal tract, local immune responses maintain a

peaceful coexistence with the resident microbiota. Microbiota has the ability to control many aspects of innate and adaptive immune responses (Hooper *et al.*, 2010, Molloy *et al.*, 2012).

Intestinal microbial composition can change following antibiotic treatment (Willing *et al.*, 2011), dietary changes (Maslowski *et al.*, 2011) and exposure to gastrointestinal pathogens (Gill *et al.*, 2011). Large perturbations in gut microbiota lead to community imbalance which causes dysbiosis. Dysbiosis of gut microbiota is associated with severe pathologies such as inflammatory bowel disease and malnutrition (Belkaid *et al.*, 2013). Unique groups of commensals have role in the control of mucosal immune response (Molloy *et al.*, 2012). Commensal bacteria have the ability to promote protective immunity by inducing inflammasome mediated induction of IL-1 β and IL-18 (Ichinohe *et al.*, 2011). Besides, they can also influence autoimmune and allergic conditions (Belkaid *et al.*, 2012).

Commensal microbiota also has the ability to downmodulate inflammation by expansion of IL – 10 producing regulatory T cells (Ochoa-Repáraz *et al.*, 2010). Some gram positive bacteria (*Lactococcus*, *Streptococcus*, *Streptomyces spp.*) produce bacteriocins which inhibit the growth of other bacterial strains (Gallo *et al.*, 2012). In the case of tissue injury, skin commensal bacteria-derived products limit deleterious inflammatory responses and contribute to wound healing (Belkaid *et al.*, 2013). These examples emphasize the important immune regulatory roles of commensal bacteria.

As members of commensal microbiota, *Lactobacilli* strains are important residents of the gut and the genitourinal tract. It is known that *Lactobacilli* strains contribute to homeostasis by producing antimicrobial factors (Spurbeck *et al.*, 2011). Several *Lactobacilli* strains have been shown to suppress the epithelial cells to respond to a diverse range of TLR ligands (Rose WA 2nd *et al.*, 2012). Therefore, in this thesis we have focused on the immunomodulatory activities of MVs derived mostly from various *Lactobacillus* isolates.

1.6. Foot and Mouth Disease Vaccine

1.6.1. Foot Mouth Disease (FMD)

Foot-and-mouth disease is a viral disease that affects hoofed animals such as goats, pigs, sheep, cattle and deer. The disease causes high fever and is characterized by lameness and vesicular lesions on the snout, teats, feet and tongue, with high morbidity but low mortality (Grubman and Baxt, 2004). Foot and mouth disease is a severe and infectious disease with frequent outbreaks around the world (Zhang, 2011). FMD causes significant decrease in infected animal's weight and milk output and thus is considered as one of the most economically devastating diseases of livestock (Mort, 2005).

1.6.2. Foot and mouth disease virus (FMDV)

FMD virus (FMDV) is a member of the family Picornaviridae that is an RNA virus with single stranded RNA. FMDV's size ranges between 25-30 nm and it has an icosahedral capsid made of proteins, without an envelope (Carrillo, 2005). Virus mostly infect host via the respiratory tract following contact with or inhalation of airborne FMD (Rodriguez *et al.*, 2010). There are seven serotypes of the virus: A, O, C, Asia 1, Southern African Territories (SAT) 1, SAT2 and SAT3. Serotype O is the most prevalent type of FMD virus and is

distributed throughout Southern America, Middle East and Asia (Domenech, 2010) (Table 1.5).

Table 1.5 Regional distribution and serotypes of FMDV (Adapted from Zhang, 2011).

Area	Main serotypes in FMDV distribution areas						
	Type O	Type A	Type C	Type Asia I	SAT-1	SAT-2	SAT-3
China	+	+	-	+	-	-	-
Southeast Asia	+	+	-	+	-	-	-
Africa	+	-	-	-	+	+	+
Middle East	+	+	+	+	-	-	-
United Kingdom	+	-	-	-	-	-	-
South America	+	+	+	-	-	-	-

+ means positive, and ** means negative.

Infection causes vesicular lesions in coronary bands of the hoof and epithelia of the mouth (Sellers, 2000). After initial exposure, FMDV firstly replicates in the pharynx. The virus replicates to very high titers at the lesion site. Within 24 – 48 hours the virus spreads to the bloodstream and soon lesions appear in the mouth and feet (Pacheco, 2008). The virus can generally persists in the pharyngeal region, so even if the animal were to be vaccinated, they may pose threat as long term carriers (Donaldson, 2002).

1.6.3. Vaccines for FMD

The first studies to prevent FMD have been initiated in the late 1800s. FMDV was the first described animal virus and the vaccine was the first of its kind developed for animal vaccination (Lombard, 2007). Main problems associated with the FMD vaccine development were the presence of various serotypes and unpredictable risks of viral virulence. In 1937, the first vaccine was developed by collecting virus from vesicular fluids of infected cattles. The virus was then inactivated with formaldehyde and given to hoofed animals (Waldmann *et al.*, 1955). Inactivation with formaldehyde is not effective because it does not inactivate completely. For this reason, today, binary ethyleneimine (BEI) is used for FMD inactivation. FMD vaccines are produced under bio-secure conditions by infecting BHK-21 cells with live velogenic FMDV and inactivation is carried out using a chemical such as binary ethyleneimine (Rodriguez, 2009).

In spite of these improvements in FMD vaccine development, inactivated vaccines have short shelf life and there is a need for vaccine cold chain. Also, some serotypes grow poorly in cell culture, preventing production of large doses (Hu, 2007). Moreover, the current FMD vaccines do not induce long-term protection and multiple vaccinations are required.

In our country, the commercially available inactivated FMD virus vaccine is not effective in providing long-lasting immunity. There is a need for development of better vaccines which may induce immunological memory. A crucial aspect of each vaccine is the adjuvant. Use of potent and cheap adjuvants would be of great interest in development of better FMD vaccines. Therefore, in this study, we aimed to assess the vaccine adjuvant properties of non-commensal non-pathogenic *E.coli*-derived MVs as an economically viable source of nature-made vesicular TLR-ligand carrier with a potential to improve FMD-specific long-term

immunity. We also included commensal bacteria derived membrane vesicles in this system to further analyze the impact of commensal-derived MVs on adaptive immunity.

1.7. Aim of the study

This thesis aims to test the immunomodulatory and vaccine adjuvant potential of small membrane vesicles (MVs) naturally secreted from bacteria. Such MVs of pathogenic bacterial origin were previously shown to be enriched in TLR ligands (such as peptidoglycans, LPS and nucleic acids) in a more stable form than their free counterparts owing to their vesicular protection. This thesis intends to analyze the potential vaccine adjuvant activity of non-pathogenic E.coli-derived MVs as an economically viable source that may improve the immunogenicity of FMD vaccine currently used in our Country. Another aim of this thesis is to study the immunomodulatory activities of human commensal-derived MVs. MV production in Gram-positive bacteria has only recently been studied. To date, there are no studies conducted with commensal-derived MVs. Therefore, this thesis intends to analyze the immunomodulatory effects of MVs that will be isolated from 5 different Gram positive human commensal isolates. Following physical (nanoparticle size determination using atomic force microscopy and dynamic light scattering, net charge analysis through zeta potential measurement) and biochemical (protein and nucleic acid contents) characterization of MVs, their immune stimulatory activities and cellular internalization patterns will be assessed in various *in vitro* assays. These findings may be of interest since the immunomodulatory properties of MVs secreted from human commensal bacteria are currently unknown. Finally, the vaccine adjuvant potentials of MVs will be assessed using an *in vivo* murine vaccination model. Results from these studies could have enormous impact, leading to the general use of MVs as vaccine adjuvants and/or tolerance-inducing anti-inflammatory agents.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents

Fluorescence labeled antibodies were obtained from BioLegend (USA). 3.14 – 100 kDa protein ladder was from BioRad and 100-1000 bp DNA ladder was from Fermentas (USA),. Cytokine ELISA kits which includes monoclonal unlabeled antibodies and biotinylated antibodies, recombinant cytokines, SA-APs were from Mabtech (Sweden). *p*-nitrophenyl phosphate disodium salt (PNPP) which is substrate for alkaline phosphatase was obtained from Thermo Scientific, (USA). For IgG ELISA goat anti-mouse total IgG, IgG2a, IgG1 monoclonal antibodies conjugated with alkaline phosphatase (AP) were from Southern Biotech (USA).

2.1.2. TLR Ligands, Peptides and ODNs

TLR ligands for stimulations were as follows: lipopolysaccharide (LPS) (isolated from *E.coli*; Sigma, USA), peptidoglycan (PGN) isolated from *B.subtilis*; (Fluka, Switzerland), poly inosinic acid: cytidylic acid (pIC) (Amersham, UK) CpG ODNs were synthesized by IDT (Leuven, Belgium).

2.1.3. Buffers, Cell Culture Media and Other Standard Solutions

RPMI1640 media, DNase/RNase free water, sodium pyruvate, HEPES, low glucose DMEM media, penicillin/streptomycin, non-essential amino acid solution, L-glutamine, FBS were from Thermo Scientific (USA). Components of various culture media and different buffers such as 6X Loading Dye, PBS, FACS Buffer, T-cell buffer, Blocking Buffer, ELISA Wash buffer are given in detail in Appendix A.

2.1.4. Bacterial Strains

Bacterial strains used in this study and their sources are described in Table 2.1. Bacterial strains used in this study (Table 2.1) was a kind gift from Assoc Prof. Ihsan Gursel (Bilkent Univ. MBG Dept.)

Table 2.1 Bacterial Strains

	Strains	Source	Gram staining
#1	<i>Pediococcus pentosaceus</i>	Infant feces	+
#2	<i>Lactobacillus salivarius</i>	Infant feces	+
#3	<i>Lactobacillus fermentum</i>	Infant feces	+
#6	<i>Enterococcus faecium</i>	Infant feces	+
#7	<i>Pediococcus pentosaceus</i>	Human milk	+
	<i>Escherichia coli</i> (DH5 α)	ATCC 67877	-

2.1.4.1. Culture media and growth conditions

Commensal bacterial strains used in this study (see Table 2.1) were grown in MRS broth medium (Appendix A) or MRS agar plates (Conda, S.A.) (Appendix A) at 37°C overnight. *E. coli* was grown in luria broth (LB) or luria broth agar (Appendix A) at 37°C at 200 rpm. Short term maintenance of bacteria was achieved by storing the agar plates at 4°C for 1-2 months. For long term maintenance of bacteria, a single colony was picked, transferred to broth medium and incubated overnight. The suspension was then mixed with %40 glycerol solution (1:1 ratio).

2.2. Methods

2.2.1. Establishment of Bacterial Growth Curves

Bacteria secrete membrane vesicles into the environment at all phases of growth. However, isolation of such vesicles have been shown to be optimum from cultures that have just entered the steady-state phase of growth. Therefore, to determine the time point where each bacterial strain entered the steady-state, growth curve of individual strains were studied. For this, each strain of bacteria was transferred to appropriate liquid broth cultures and their concentrations were adjusted to an OD (600 nm) of 0.01. Growth was monitored by recording the OD (600 nm) values on an hourly basis using a spectrometer (Thermo Scientific, USA).

2.2.2. Isolation of membrane vesicles

Membrane vesicles secreted from 5 different commensal bacteria and *E. coli* DH5 α (Table 1) were isolated using the following protocol: fresh liquid broth cultures (500 ml) of each strain was adjusted to an OD (600 nm) value of 0.01 OD and incubated until an OD value of 1 was achieved (this corresponded to an early steady state for all cultures). The cultures (6x30 ml) were then centrifuged for 15 minutes at 6000 rpm and the cell-free supernatants were collected (Sorvall, USA). To ensure removal of residual bacterial cells, supernatants were sequentially filtered using 0.45 μ m and 0.20 μ m filters, respectively. Filtered supernatants were centrifuged (Beckman Coulter) at 100,000xg for 1 h, and the pellets were resuspended

in 30 ml of PBS. Following a second round of centrifugation (90 min at 100,000xg), pellets were resuspended in PBS (500µl – 1.5 ml) and the MVs were stored at -20°C until further use. For subsequent experiments, stored MVs were allowed to thaw slowly on ice.

2.2.3. Characterization of Membrane vesicles

2.2.3.1. Protein quantitation

Purified MVs were quantified by protein concentration analysis using a Nanodrop spectrophotometer (NanoDrop Technologies, USA) at an absorbance of 280 nm.

2.2.3.2. Analysis of Membrane Vesicles by Polyacrylamid Gel Electrophoresis

The protein content of the membrane vesicles were analyzed using SDS-PAGE method. For this, MVs (10 µl) were mixed with 4 µl of sample buffer (Appendix A), denatured at 95°C for 5 min and were loaded onto wells of the %5 Stacking gel residing above the %10 Resolving Gel (for gel preparation recipe\ see Appendix A). The proteins were then separated in the gel according to their size (Hames, 1998) by running the gel initially for 1 h at 85 V and then 100 min at 120 V (Hofer, Inc.). Protein ladder (3.14 – 100 kDa, BioRad) was used as a marker (5µl/well). Following completion of electrophoresis, the gel was washed 3 times with dH₂O for 5 minutes. To visualize the protein bands, the gel was then stained for 1 h with Coomassie Brilliant Blue dye (Appendix A) and destained using the destaining solution (Appendix A).

2.2.3.3. Analysis of Membrane Vesicles by Agarose Gel Electrophoresis

Membrane vesicles secreted from bacteria are known to contain nucleic acids. To confirm that the isolated MVs were positive for nucleic acids, 20 µl of each vesicle was mixed with 20 µl of lysis buffer (Appendix A) or and equal amount of buffer without the detergent. All samples were then mixed with 4 µl of 6X loading dye (Appendix A) and loaded onto a 1% agarose gel containing 0.2 µg/ml ethidium bromide. DNA ladder (100-1000 bp range; Fermentas) was used as a marker (3 µg/well). After loading samples and DNA ladder running was done by using 1X TAE buffer (Fischer Scientific) at 70 V for 60 minutes. The gels were visualized with UV transilluminator (Vilber Lourmat, France).

2.2.3.4. Acridine Orange staining and Flow Cytometric Analysis of MVs for Characterization of Nucleic Acid Content

Acridine orange (AO) is a cell permeable nucleic acid selective fluorescent cationic dye that interacts with DNA and RNA by intercalation or electrostatic attraction, respectively. The dye has an excitation maximum of 488 nm and emission maxima of 525 nm (green fluorescence) and 650 nm (red fluorescence) upon binding to DNA and RNA, respectively. To distinguish between MV-associated dsDNA and ssDNA/RNA, 50µl of vesicles (X µg/ml protein content) were stained with 13 µM acridine orange in a final volume of 500 µl in PBS. Following dye addition, vesicles were incubated for 15 min at room temperature and

flow cytometric analysis was conducted on a BD™ Accuri C6 Flow Cytometer. For all MV analyses, Forward Scatter threshold of the instrument was lowered to 10,000. Following data acquisition, all samples were treated with 10 % SDS (1:1 ratio; V/V) and incubated at 60°C for 10 minutes. A second set of data acquisition was conducted using these SDS-lysed samples.

2.2.3.5. Average Particle Size Analysis and Zeta Potential Measurements

MVs (1 µg/ml) were diluted 100X with DNase/RNase free H₂O and the final volume was adjusted to 1ml in a polystyrene cuvette suitable for dynamic light scattering analysis. For Zeta potential measurements, a disposable capillary cell was used (Nano ZS, Malvern, UK). All measurements were done by using the following parameters: dielectric constant 78.54, medium viscosity 0.88 mPa s, temperature 25°C, medium refractive index 1.330. Measurements were in duplicate, and the results were expressed as the average of two measurements ± S.D.

2.2.3.6. Atomic Force Microscopy (AFM)

AFM studies were performed to obtain information about the size and morphology of MVs. Membrane vesicles were diluted (100X) in DNase/RNase free H₂O prior to deposition on to silicon wafers (5µl of vesicle/wafer). Samples were air-dried for 30 min at room temperature. Non-contact mode images were taken using a PSIA XE-100E model AFM. Multi75Al model tips obtained from Budget Sensors. Tips' resonance frequency was 75 kHz and force constant was 3 N/m. Scan rate was kept at 0.73-0.79 Hz. Images were analyzed using XEI 1.6 software.

2.2.4. Cells and Culture Conditions

2.2.4.1. Preparation of Single Cell Suspensions from Spleen

2.2.4.1.1. Maintenance of Animals

All *in vitro* stimulation and *in vivo* immunization experiments involved the use of adult male or female BALB/c (8-12 weeks old). The animals were kept in the animal housing facility at the Department of Molecular Biology and Genetics at Bilkent University under controlled environment conditions (22 °C ±2) regulated with 12 hour light and 12 hour dark cycles. Animals were provided with unlimited access to food and water. All experimental procedures have been approved by the ethical committee of Bilkent University (Bil-AEC).

2.2.4.1.2. Preparation of Single Cell Suspensions from Spleen

Mice were sacrificed via cervical dislocation and spleens were removed and placed into 35 cm petri dishes. Spleens were mashed in 2 % FBS supplemented regular RPMI medium with the aid of a sterile syringe plunger to obtain single cell suspensions. Using a sterile plastic pasteur pipette, as such prepared splenocytes were transferred into 15 ml Falcon tubes and were washed 2 times in regular RPMI (2 % FBS; centrifugation at 1700 rpm for 10 min).

The final cell pellet was resuspended in regular RPMI (5 % FBS), and then counted as described below.

2.2.4.2. Cell Counting

Following preparation of splenocytes, pellets were resuspended in 1 ml of complete RPMI 1640 medium (5 % FBS). 10 μ l of this cell solution was diluted 10X, mixed with Trypan Blue solution (0.4 %) at 1:1 ratio and then applied to a hemacytometer by capillary action. Hemacytometer composed of 16 small squares at 4 corners with 1 mm² area. Counting was done under a light microscope and the cell number was calculated by the formula:

$$\frac{\text{Total cell number in 4 big squares} \times 10^4 \times 10}{4} = \text{number of cells per ml}$$

Cell concentration was adjusted to 2x10⁶ cells/ml for FACS analysis and 4x10⁶ cells/ml for ELISA, unless otherwise stated.

2.2.5. Determination of Immunostimulatory Activity of Membrane Vesicles

2.2.5.1. In vitro Stimulation of Cells with Membrane Vesicles

For stimulation of cells with MVs, 100 μ l of 4x10⁶ cells/ml were transferred to 96-well flat bottom tissue culture plates (400,000 cells/well). Final volumes were adjusted to 200 μ l following addition of MVs in 100 μ l 5% FBS supplemented complete medium. Three different MV concentrations were used in all *in vitro* experiments (0.2 μ g/ml, 1 μ g/ml, 5 μ g/ml). Stimulations were done duplicate for all treatments. For stimulation of cells in 15 ml tubes, cells were adjusted to 1,000,000 cells/tube and 100 μ l of stimulant were added to 1ml final volume with 5% FBS supplemented complete medium. Depending on the cytokine analyzed cell culture supernatants were collected after 24-48h of incubation.

2.2.5.2. Enzyme Linked-ImmunoSorbent Assay (ELISA)

Following *in vitro* stimulation of cells, supernatants were collected. Immulon 2B plates were coated with 50 μ l of monoclonal antibodies against mouse cytokines in PBS and incubated overnight. Table 2.2 summarized working concentrations of coating antibodies. After incubation, plates were blocked by using 200 μ l blocking buffer (Appendix A) for 2 h at RT. Later, plates were washed with ELISA washing buffer (Appendix A) and were dried by tapping. Supernatants and recombinant cytokine standards were added into the wells (50 μ l/well). Starting concentrations for recombinant cytokines were given in Table 2.2. Two-fold serial diluted recombinant cytokines were added into the blocked plates. Plates were incubated for 2 hours at room temperature and then washed as previously described. Biotinylated-secondary antibody solution which were 1:1000 diluted in T cell buffer (Appendix A) added to plates (50 μ l/well). Plates were incubated overnight at 4°C and then washed again. Following washing 1:1000 diluted streptavidin-alkaline phosphatase (SA-AP) added (50 μ l/well). Streptavidin-alkaline phosphatase solution in T cell buffer was

prepared at least 2 h prior to use and plates were incubated for 1 h at room temperature. After incubation plates were washed as before. Following washing step, plates were developed by using 50 μ l of PNPP substrate solution. Color development was followed at 405 nm by using Multiskan FC Microplate reader (Thermo Scientific, USA) at 30 min intervals.

Table 2.2 Antibodies and recombinants used in ELISA

Coating Antibodies	Working Concentration	Recombinants	Starting Concentration
Ab-mIFN δ (MabTech, Sweden)	1 μ g/ml	rec-mIFN δ (MabTech, Sweden)	10 ng/ml
Ab-mIL-10 (MabTech, Sweden)	2 μ g/ml	rec-mIL-10 (MabTech, Sweden)	16 ng/ml

2.2.5.3. Fluorescence Activated Cell Sorting (FACS)

2.2.5.3.1. Fixation of Cells

Cells were centrifuged at 1,600 rpm 10 min and the pellets were fixed by the addition of 4% paraformaldehyde (Fixation Medium A, Invitrogen, USA) while vortexing. Cells are incubated for 15 min at RT and then washed by using 1 ml FACS buffer (Appendix a). Cells were then transferred to 1.5 ml eppendorf tubes centrifuged at 1600 rpm 10 min, aspirated and resulting pellets were resuspended in FACS buffer. Fixed cells were kept at 4°C for a maximum of 7 days.

2.2.5.3.2. Cell Surface Marker Staining

Live or fixed cells were centrifuged and supernatants were discarded. For live cells all incubation and washing steps were done at 4°C whereas fixed cells were incubated at RT. After centrifuge, pellets were resuspended in 100 μ l FACS buffer containing 1 μ g/ml of fluorochrome conjugated antibody and incubated in dark for 30 min. Table 2.3 summarized cell surface markers used throughout this thesis. After staining, cells were washed with FACS Buffer for two times and resuspended in 400 μ l of PBS. Results were analyzed by using BDTM Accuri C6 Flow Cytometer.

Table 2.3 Fluorochrome conjugated antibodies used as cell surface markers

Ab- m CD11b- FITC	(Biolegend,USA)
Ab- m TNF- α – PE	(Biolegend,USA)
Ab- m CD11c – PE/Cy5	(Biolegend,USA)
Ab- m B220 – Alexa Fluor 647	(Biolegend,USA)

2.2.5.3.3. Intracellular Cytokine Staining

For intracellular TNF α staining, all stimulations were done as described before (Section 2.2.5.1) in the presence of 10 μ g/ml Brefeldin A (Biolegend). Brefeldin A used for preventing cytokine secretion into the medium. Cells incubated for 5 hours at 37°C then centrifuged and fixed as described in Section 2.2.5.3.1. Staining for specific cell-surface markers was achieved as described before (Section 2.2.5.3.2 and Table 3). Next, cells were permeabilized and stained for 30 min in the dark by using 100 μ l permeabilization medium (Invitrogen, USA) containing 0.5 μ g of TNF α -PE. Samples were washed twice more and analysed on a BDTM Accuri C6 Flow Cytometer.

2.2.6. Determination of Cell-surface Binding and Internalization of Membrane Vesicles

Membrane vesicles were labeled with 20 μ M of the green fluorescent lipophilic carbocyanine dye SP-DiOC18(3) for 30 min at 37°C. Splenocytes (400,000 cells/well) were incubated with the labeled MVs (0.2, 1 and 5 μ g) in a 96 well cell culture plate for 2 h at 37°C temperature. Cells were then washed, labeled with fluorochrome conjugated antibodies specific to dendritic cells (CD11c) and B-cells (B220), washed and analyzed on a BDTM Accuri C6 Flow Cytometer. After first acquisition for the detection of total SP-DiOC signal, cells transferred to a new plate and same volume of trypan blue (1:10 diluted, HyClone) was added to all samples. Analysis were done by using BDTM Accuri C6 Flow Cytometer to determine internalized membrane vesicles amount. Trypan Blue addition provided to quantitate the internalized signal by eliminating the cell-surface bound signal.

2.2.7. Immunization Studies

2.2.7.1. Foot and Mouth Disease (FMD) Vaccine

The FMD Institute (Ankara, Turkey) prepared and provided FMD Vaccine. The monovalent vaccine contained FMDV O/TUR/07 inactivated antigen only.

2.2.7.2. Immunization with FMDV Antigen in the absence or presence of Membrane Vesicles

6-8 week old female BALB/c mice (5/group) were immunized twice (intraperitoneal (ip), days 0 and 17) using the FMDV antigen (3 µg/mouse) alone or its combination with i) E.coli vesicle, ii) MV1 iii) MV2 or iv) MV3. All bacteria-derived vesicles were used at a concentration of 10 µg/mouse. Blood was collected from the tail veins 2 weeks after each immunization. Following clot formation, sera were transferred into eppendorf tubes, centrifuged at 8000 rpm for 5 min and stored at -20°C until use. IgG1, IgG2a and total IgG levels were detected by using ELISA, two weeks after each injection.

2.2.7.3. IgG ELISA

FMD antigen specific IgG1, IgG2a and total IgG were detected using ELISA. For this, Immulon 1B plates were coated with rabbit anti-Ser-O antibody (1:2000 diluted, 50 µl/well) in PBS and incubated overnight at 4°C. Blocking and washing steps were carried out as described for cytokine ELISA (Section 2.2.5.2.). As the source of antigen, 1/20 diluted supernatants of FMDV-infected Baby Hamster Kidney (BHK) cells (50µl/well in blocking buffer) were added and incubated for 2 h at room temperature. Following washing (see Section 2.2.5.2.), 80X diluted mouse sera were introduced to the first row of wells and serially diluted two-fold. All dilutions were conducted using PBS containing 1:500 diluted rabbit serum to block interference caused by heterophylic antibodies. Plates were incubated overnight at 4°C and washed as before. Goat anti-mouse IgG1/AP, IgG2a/AP or IgG total/AP (Southern Biotech) were 1:3000 diluted in T-cell buffer and were added to plates (50 µl/well). After incubation (2 h at room temperature) and washing step, PNPP substrate was added (see Section 2.2.5.2.) and formation of yellow color was followed at OD 405 nm using an a microplate reader (Thermo Scientific, USA).

2.2.7.4. Determination of FMD specific memory B cell formation by ELISPOT

To quantitate the number of FMD-specific memory B cells generated in vaccinated mice, ELISPOT assay was performed. Four months after the booster injection, mice were sacrificed and spleens were removed. Single cell suspensions were prepared as described in Section 2.2.4.1.2. . Cells were counted on a flow cytometer and the cell numbers were adjusted to be 10×10^6 cells/ml. All samples were stimulated in 6-well plates (10×10^6 cells in 4 ml complete RPMI supplemented with 10 % FBS) for 6 days with a combination of LPS (2µg/ml) and CpG ODN (0.5µg/ml) to allow for memory B cell expansion and differentiation into antibody secreting plasma cells. Four days after the initial stimulation, cultures were replenished with 1 ml of fresh medium addition. On day 5 of stimulation, Immulon 1B plates (Thermo Labsystems, USA) were coated with rabbit anti-Ser-O antibody (1:2000 diluted) and washed as described for the IgG ELISA. On day 6 of stimulation, cells were collected into 15 ml falcon tubes, washed two times as before (1700rpm for 10 min) and cells were resuspended to a concentration of 20×10^6 cells/ml. To prepare the template plates, 200µl of cells were added to the first row and then serially diluted ¼-fold (3 such dilutions were made to yield 4 different cell concentrations). 50µl of cells were transferred

from the template plates to the rabbit anti-Ser-O antibody coated plates. Total Volume was adjusted to 200 μ l and the plates were incubated in a CO₂ incubator at 37 °C overnight. After incubation, plates were washed using wash buffer without PBS (water + 0.25% tween20), followed by 50 μ l/well goat anti-mouse IgG1/AP or IgG2a/AP (Southern Biotech) addition (1:3000 diluted in T-cell buffer). Plates were washed for the final time after 2 hours of incubation and were developed using 70 μ l of BCIP-low melting agarose substrate solution (Appendix A). The substrate solution was kept at 45°C to prevent agarose solidification before addition to wells. After agarose solidification, plates were sealed. The next day spots were counted using a dissecting microscope.

2.3. Statistical Analysis

Data were statistically analyzed using the IBM SPSS v17.0 software. Student T-test (one tailed unpaired comparison) was conducted between untreated and treated groups. A P value of < 0.05 was considered as significant.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Determination of Growth Curve of Bacterial Isolates

Bacteria secrete membrane vesicles (MVs) to mediate interactions with their environment. Until recently, the study of MV formation has largely focused on Gram-negative bacteria. This process was ignored in gram-positive bacteria due to a lack of a second lipid bilayer which is the site of outer membrane vesicle formation in Gram-negative bacteria. Despite differences in cell wall and membrane structure, such vesicles were shown to be released by Gram-positive bacteria and archaea (Deatherage and Cookson, 2012; Ellen et al., 2010; Lee et al., 2009; Rivera et al., 2010; Soler et al., 2008). However, these reports are restricted to MV secretion from Gram-positive pathogens and there have been no published results on Gram-positive commensal-derived MV production. Therefore, to analyze the immunoregulatory roles of MVs that might be secreted from commensals, we first studied the growth curves of individual bacteria to determine the steady-state for isolation of membrane vesicles. All commensal strains and the E.coli DH5 α demonstrated typical growth curve characteristic (lag phase, exponential phase and steady state; Figure 3.1.). Based on this data and for practical purposes, it was decided that for all bacterial strains, MV isolation would be initiated following 12-18 h of growth.

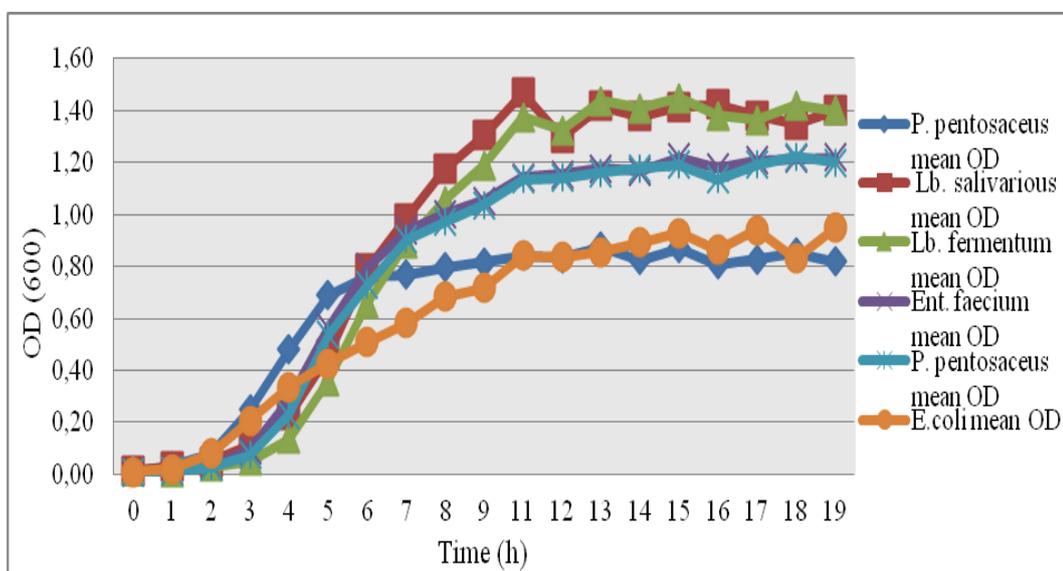


Figure 3.1 Growth curves of bacterial strains used in this study. Each bacterial strain was transferred to liquid broth culture (500 ml) and initial cell concentrations were adjusted to an OD (600 nm) of 0.01. Growth was monitored at 37°C by recording the OD (600 nm) values on an hourly basis.

3.2. Protein Contents of Membrane Vesicles Based on SDS-PAGE Gel Electrophoresis

Identification of membrane vesicles from bacteria relies on several different criteria, one of which is the presence of multiple proteins. SDS-PAGE gel electrophoretic analysis of MVs for the presence of proteins revealed the presence of common and unique proteins, depending on the bacterial species analyzed (Figure 3.2). Of note, the negative controls (MRS and LB media) showed no such bands, indicating that bacteria-free supernatants contained active secreted products that could be pelleted by ultracentrifugation.

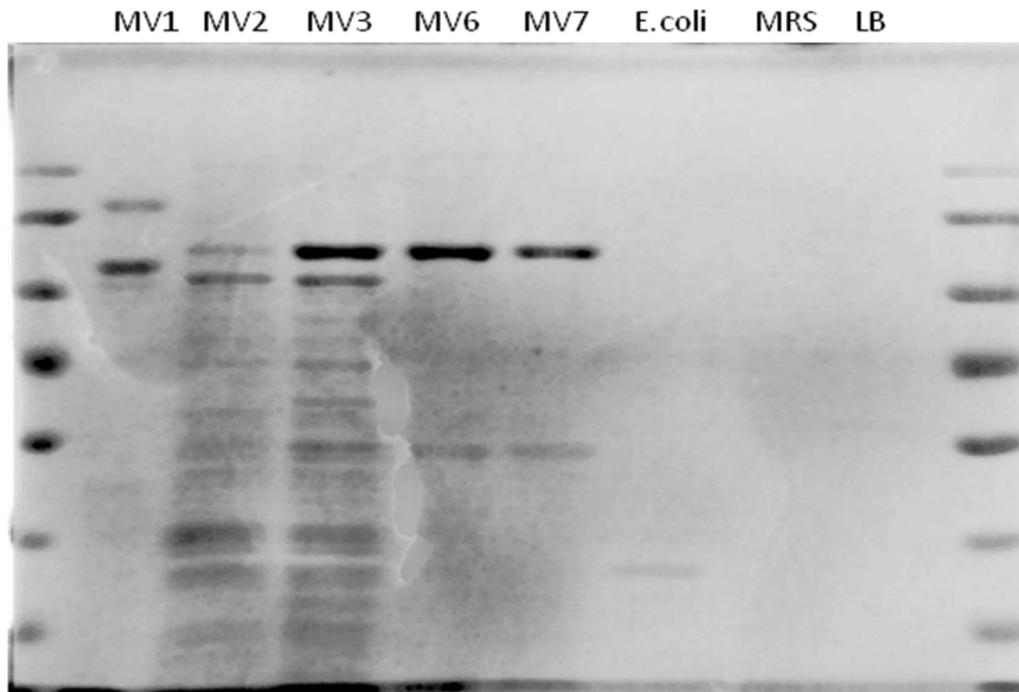


Figure 3.2 SDS-PAGE Gel Electrophoresis of Membrane Vesicles. MVs (5 $\mu\text{g}/\text{well}$) were loaded into wells of a 5-10 % gradient gel containing. Protein ladder of 3.14 – 100 kDa

MW range was used as a marker (5 $\mu\text{l}/\text{well}$). The gel image is representative of 3 independent experiments, each giving similar results.

3.3. Nucleic acid Contents of Membrane Vesicles Based on Agarose Gel Electrophoresis

Bacteria derived vesicles were also shown to contain nucleic acids. For example, in *Acinetobacter baumannii*, *E. coli* and *Neisseria gonorrhoeae*, outer membrane vesicle associated-DNA was involved in lateral gene transfer (Dorward *et al.*, 1989; Rumbo *et al.*, 2011; Yaron *et al.*, 2000). Therefore, to determine whether purified commensal-derived MVs also contained nucleic acids, the vesicles were applied onto an agarose gel prior to or after detergent lysis (Figure 3.3). Results show that, before lysis all MVs yielded a single band of nucleic acids that were all < 0.5 bp in length. Nucleic acid staining was also visible inside the wells suggesting that some of the DNA is encapsulated inside the MVs and thus remain in the wells whereas vesicle-surface associated nucleic acids freely move in the electrical field. This is supported by the finding that lysed vesicles yield 2 separate bands: one associated with surface-adsorbed DNA (lower bands that are < 0.5 kb) and one that was released

following lysis (> 0.5 kb). In contrast to vesicle-surface associated DNA, length of the intravesicular DNA ranged between 0.5-2 kb, depending on the bacterial species. These results are consistent with published findings demonstrating gram negative bacteria-derived vesicles carry both luminal as well as vesicle-surface associated DNA (Kuehn and Kesty, 2005). The identity of DNA in such vesicles were reported to include chromosomal, plasmid, and phage DNA (Dorward et al. 1989; Kolling and Matthews 1999; Yaron et al. 2000), and the source depended on the bacterial species or strain. Thus, in summary, these results show that similar to Gram negative bacteria derived outer membrane vesicles, all Gram positive commensals tested here also produce MVs associated with DNA.

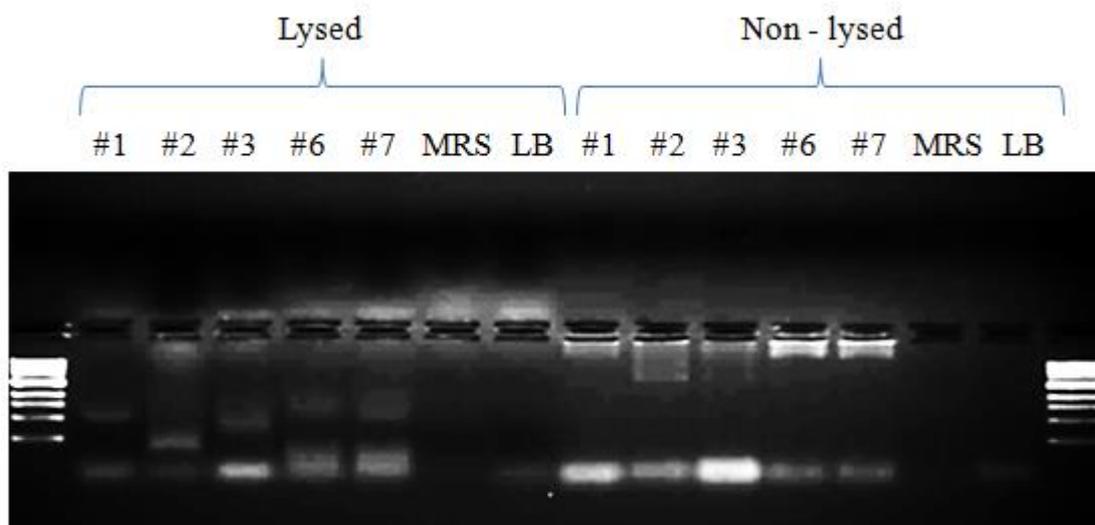


Figure 3.3 Agarose Gel Electrophoresis of Membrane Vesicles. MVs (detergent lysed or intact) were loaded into wells of a 1% agarose gel containing 0.2 $\mu\text{g/ml}$ ethidium bromide. DNA ladder of 0.5-10 kb range was used as a marker (5 $\mu\text{g/well}$). The gel image is representative 3 independent experiments, each giving similar results.

3.4. Acridine Orange staining and Flow Cytometric Analysis of MVs for Characterization of Nucleic Acid Content

As stated before, the commensal-derived MVs are associated with nucleic acids. To understand what type of nucleic acids were involved, MVs were stained with the membrane-permeant dye acridine orange. The dye emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. Figure 3.4 (A) shows the dot plots of acridine orange stained MVs (upper panels). Plots indicate that dsDNA and ssDNA/RNA content of vesicles differ between bacterial isolates. Nucleic acid-related fluorescence signal was lost following lysis with SDS (Figure 3.4 A, lower panels). The staining assay was repeated with similar results as shown in Figure 3.4 (B). Overall results indicate that MV nucleic acid contents show large variability between species.

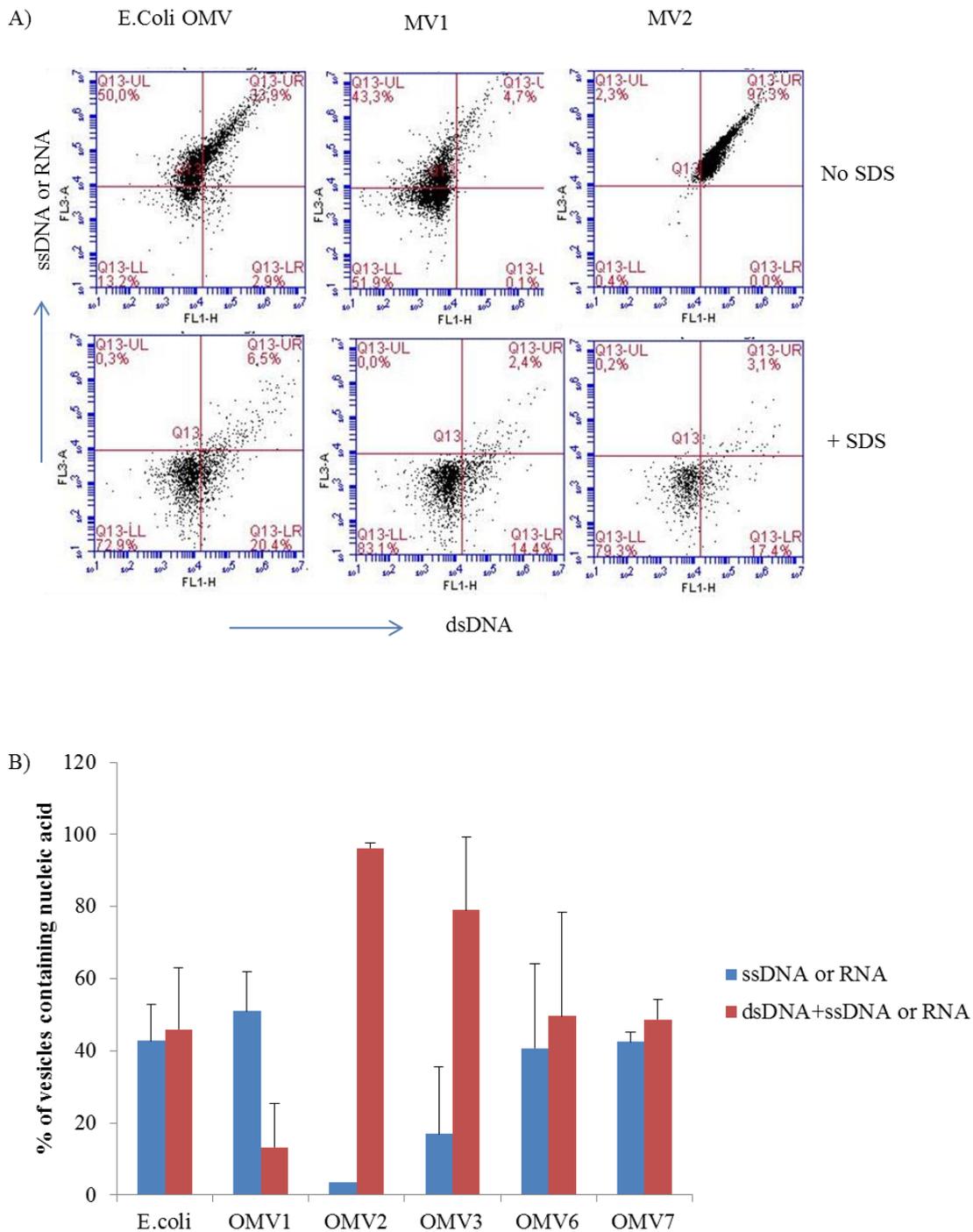


Figure 3.4 Acridine orange staining of vesicles. 50 μ l of vesicles were stained with 13 μ M acridine orange and then analyzed by flow cytometry. (A) upper panels show dot plots of acridine orange stained vesicles, lower panels show loss of nucleic acid signal following SDS treatment. (B) Nucleic acid content of E.coli OMVs versus commensal MVs. Results are the average of two independent MV staining experiments, using two different batches of isolated vesicles (mean \pm S.D).

3.5. Zeta Potential and Size Analysis of Membrane Vesicles

Zeta Potential analysis is a technique for determining the surface charge of nanoparticles such as vesicles in solution. It is considered as an important indicator of stability. The MVs used in this study had high negative zeta potentials and ranged between -35mV to -45mV (Figure 3.5 A). Such high zeta potentials suggest that the vesicles remain in solution without aggregation and remain stable. Dynamic light scattering measurements show that MVs have different sizes that ranged from 190nm to 400nm (Figure 3.5 B). These results show that bacterial membrane vesicles have higher surface negative charges than mammalian exosomes (~ -10 mV) and are larger in size.

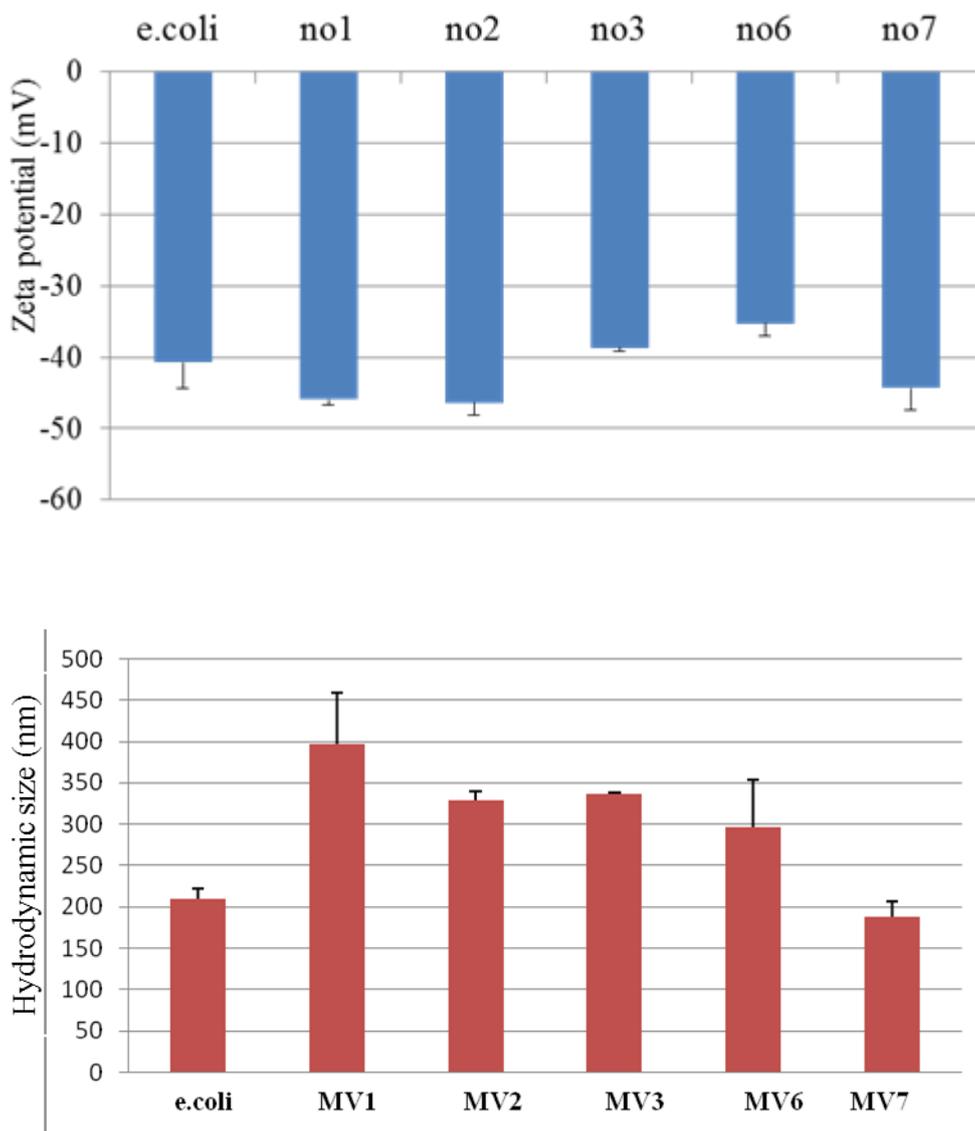


Figure 3.5 Zeta potentials (A) and hydrodynamic sizes (B) of MVs. Vesicles were diluted 50X with DNase/RNase free H₂O. Zeta potentials and average particle sizes were measured by using the following parameters: dielectric constant 78.54, medium refractive index 1.330,

temperature 25°C, medium viscosity 0.88 mPa s. The results are given as the average of two replicates \pm S.D.

3.6. Atomic Force Microscopy (AFM)

To analyze the size and morphology of MVs, samples were also investigated by atomic force microscopy. Representative images taken using non-contact mode and MV2 are presented in Figure 3.6. Upper panel shows topographic image of a 5x5 (μm) region containing numerous MVs. Lower panels show 3D image of MVs (1x1 μm region). The vesicles appear to have spherical shapes and one representative vesicle was calculated to have a size of approximately 250 nm. Dynamic light scattering results are in agreement with this finding. Thus, biochemical and physical characteristics of commensal-derived MVs demonstrate these vesicles to be negatively charged and spherical in nature, composed of proteins, lipids and nucleic acids similar to those described from Gram negative organisms.

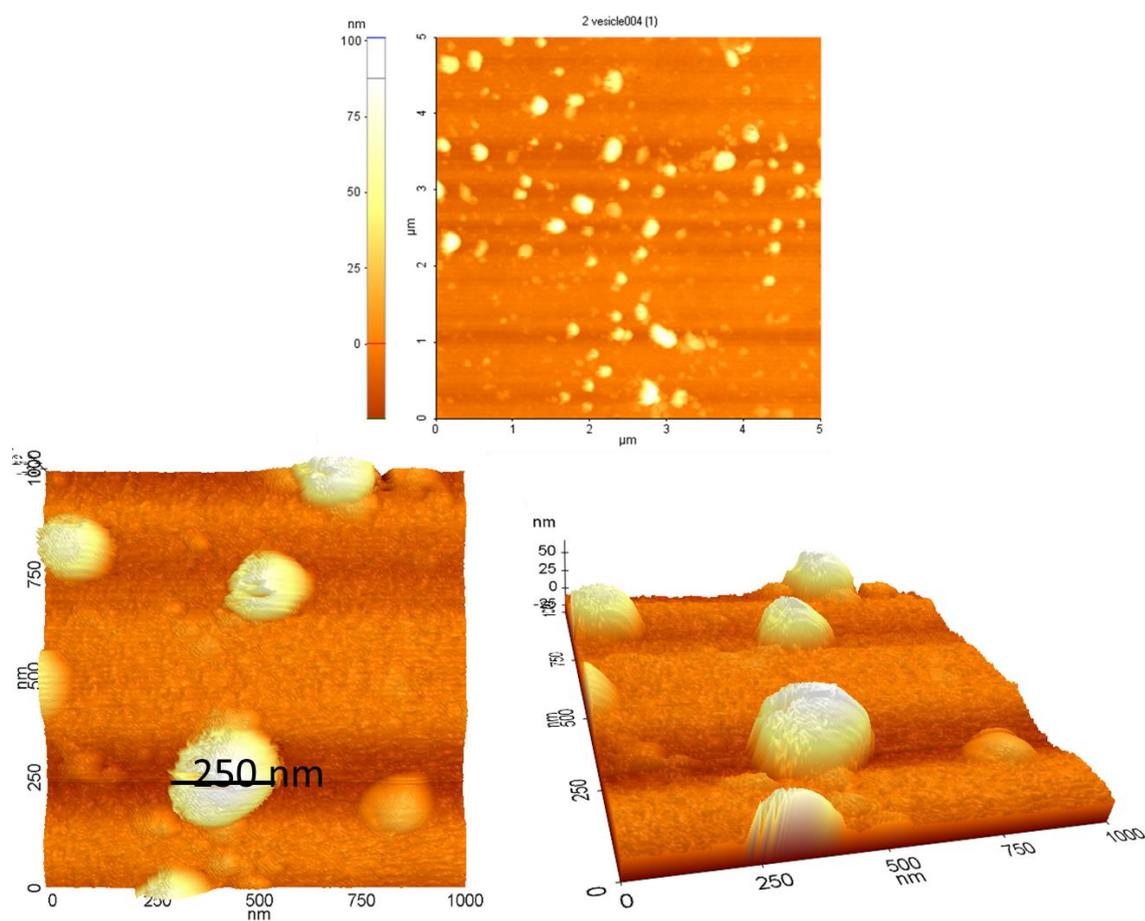


Figure 3.6 Atomic Force Microscopy images of MVs. 5 μl aliquot of MV2 from 1000X diluted solution was deposited onto silicon wafers and images were collected using non-contact mode.

3.7. Determination of *in vitro* Immunomodulatory activities of membrane vesicles

As previously described in Section 1.4.1, bacterial membrane vesicles contain nucleic acids, lipids, and proteins. Pathogen-derived MVs of Gram negative bacterial origin have been reported to contain multiple TLR ligands (Park *et al.*, 2010) and mediate immune cell activation and inflammation (Ellis and Kuehn, 2010). In contrast, whether commensals also produce MVs and if so how these secreted vesicles affect the immune system are currently unknown. Having established that several human commensal bacterial strains also secrete MVs, we next focused on *in vitro* immune stimulation experiments in order to compare the activities of gram positive commensal-derived versus gram negative non-commensal derived MVs. For this, mouse splenocytes were stimulated with various doses of MVs and immune responses were assessed by analyzing production of several different cytokines or cell-surface markers.

IL-10 and IFN- γ responses obtained following stimulation with MVs are shown in Figure 3.7 A and B, respectively. Membrane vesicles derived from *E.coli* induced highest levels of IFN- γ production even at the lowest concentration tested (9-fold increase). In contrast, commensal bacteria-derived vesicles induced very little IFN- γ production at this dose (0.2 $\mu\text{g/ml}$, 1-3 fold increase). Thus in general, IFN- γ production by *E.coli* vesicles were higher than those seen with commensal MVs.

It is known that *E.coli* MVs induce a Th1-dominated response that is associated with elevated IFN- γ production (Kim *et al.*, 2013). Conversely, it is thought that commensal bacteria and the mammalian immune system co-evolved to establish a symbiotic relationship where a Th-1 dominated response would have been deleterious to the existence of the colonized microorganisms. To counteract this, commensals may have evolved additional mechanisms to trigger a regulatory immune responses. Interestingly, commensal bacteria have been shown to possess immune suppressive DNA motifs which may contribute to immune homeostasis (Bouladoux *et al.*, 2012). To assess whether commensal-derived MVs could contribute to establishment of immune regulation, we also followed the production of the key regulatory cytokine IL-10. As seen in Figure 3.7 B, all MVs produced detectable levels of this cytokine when used at the highest dose (5 $\mu\text{g/ml}$). MV1 was the most effective commensal-derived vesicle for this response. Although *E.coli* vesicles also triggered production of this cytokine, it should be noted that this is coupled with secretion of high amounts of IFN γ . Thus, IL-10 production in the presence of Th-1 type of cytokines serves to normalize a potentially tissue damaging inflammatory response whereas IL-10 in the absence of a dominant Th1 response generates regulatory cells (Anderson *et al.*, 2007). Coupled with the results that will be shown in the following sections, one can conclude that in general, when compared to *E.coli* MVs, commensal vesicles do not induce high levels of IFN- γ but trigger IL-10 secretion and thus might contribute to generation of tolerogenic responses.

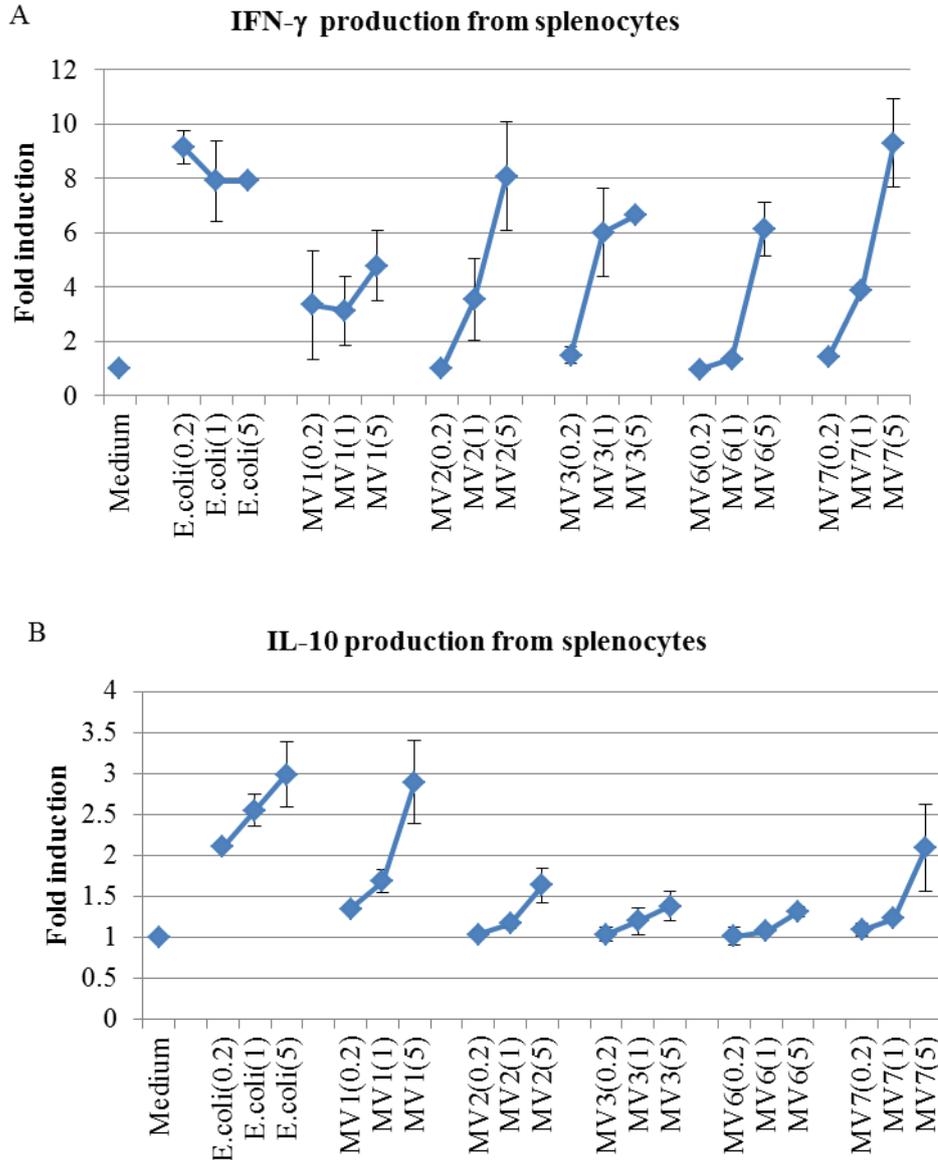


Figure 3.7 IFN- γ and IL-10 responses of splenocytes stimulated with various MV preparations. 4×10^6 /ml splenocytes from 3-5 mice were stimulated with 0.2 μ g/ml, 1 μ g/ml or 5 μ g/ml MVs for 24 h and cytokine production was assessed from culture supernatants by ELISA.

Next, in order to better understand how commensal vesicles affect antigen presenting cell (APC) function, mouse splenocytes were stimulated with E.coli versus commensal-derived MVs and expression of MHC Class II/CD86 maturation marker percentages were determined. APCs are vital for the initiation of adaptive immune responses since these cells provide the antigen-specific first signal to T cells through antigenic peptide/MHC complexes and the second signal through co-stimulatory molecule/CD28 interaction. Figure 3.8 shows that splenocytes stimulated with E.coli MVs upregulated cell-surface expression of MHC Class II and CD86 in a dose-dependent manner. Similar to lipopolysaccharide stimulated cultures, E.coli MVs (5 μ g/ml) triggered a 3.5-fold increase in the number of double positive

cells ($P < 0.05$), whereas commensal bacteria derived vesicles failed to induce significant activation even when used at the maximum dose. These results support our hypothesis that commensal bacteria-derived membrane vesicles may exert tolerogenic effects.

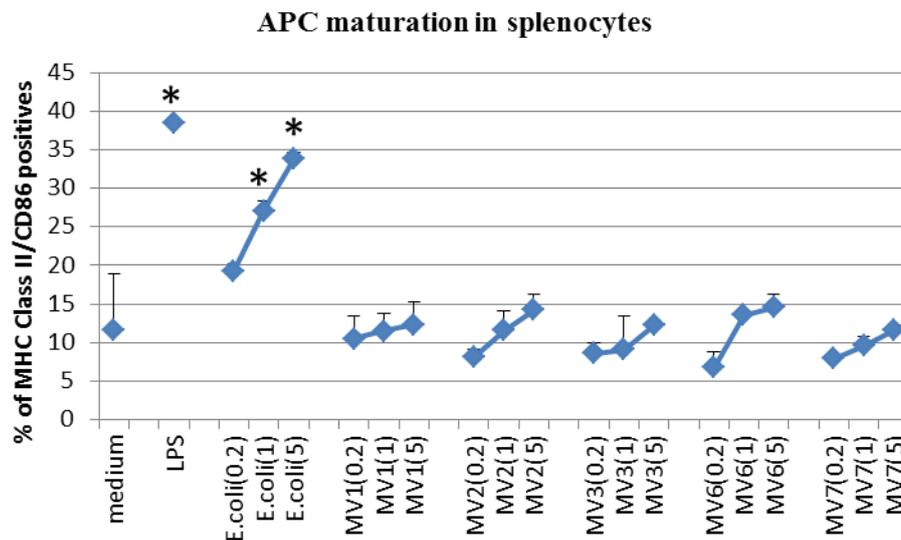


Figure 3.8 Maturation of antigen presenting cells following stimulation with MVs. Splenocytes ($4 \times 10^6/\text{ml}$) from 3-5 mice were stimulated with $0.2 \mu\text{g/ml}$, $1 \mu\text{g/ml}$ or $5 \mu\text{g/ml}$ MVs for 24 h and cells were then stained with fluorescently labeled anti-mouse I-A/I-E and anti-mouse CD86. Percent of double positive cells were assessed by flow cytometry. * indicates $P < 0.05$

Previous work had established that *E.coli* membrane vesicles induce systemic inflammatory response and trigger tumor necrosis factor α ($\text{TNF}\alpha$) secretion from APCs (Park et al., 2010). To examine whether commensal bacteria derived membrane vesicles also trigger $\text{TNF}\alpha$ induction, spleen cells were stimulated with *E.coli* versus two different commensal MVs (MV1 and MV2) and $\text{TNF}\alpha$ producing cells were determined using intracellular cytokine staining. Figures 3.9 A and B present percent of $\text{TNF}\alpha$ producing macrophages (CD11b^+) and dendritic cells (CD11c^+). Accordingly, whereas $\sim 5\%$ of macrophages spontaneously produced this cytokine, $\sim 33\%$ of *E.coli* MV-stimulated cells secreted $\text{TNF}\alpha$. Conversely, commensal-derived MVs triggered much less $\text{TNF}\alpha$ (19% with MV1 and 5% with MV2). Similarly, $\text{TNF}\alpha$ production from dendritic cells was stimulated more efficiently with *E.coli* MVs when compared to commensal MV stimulated samples.

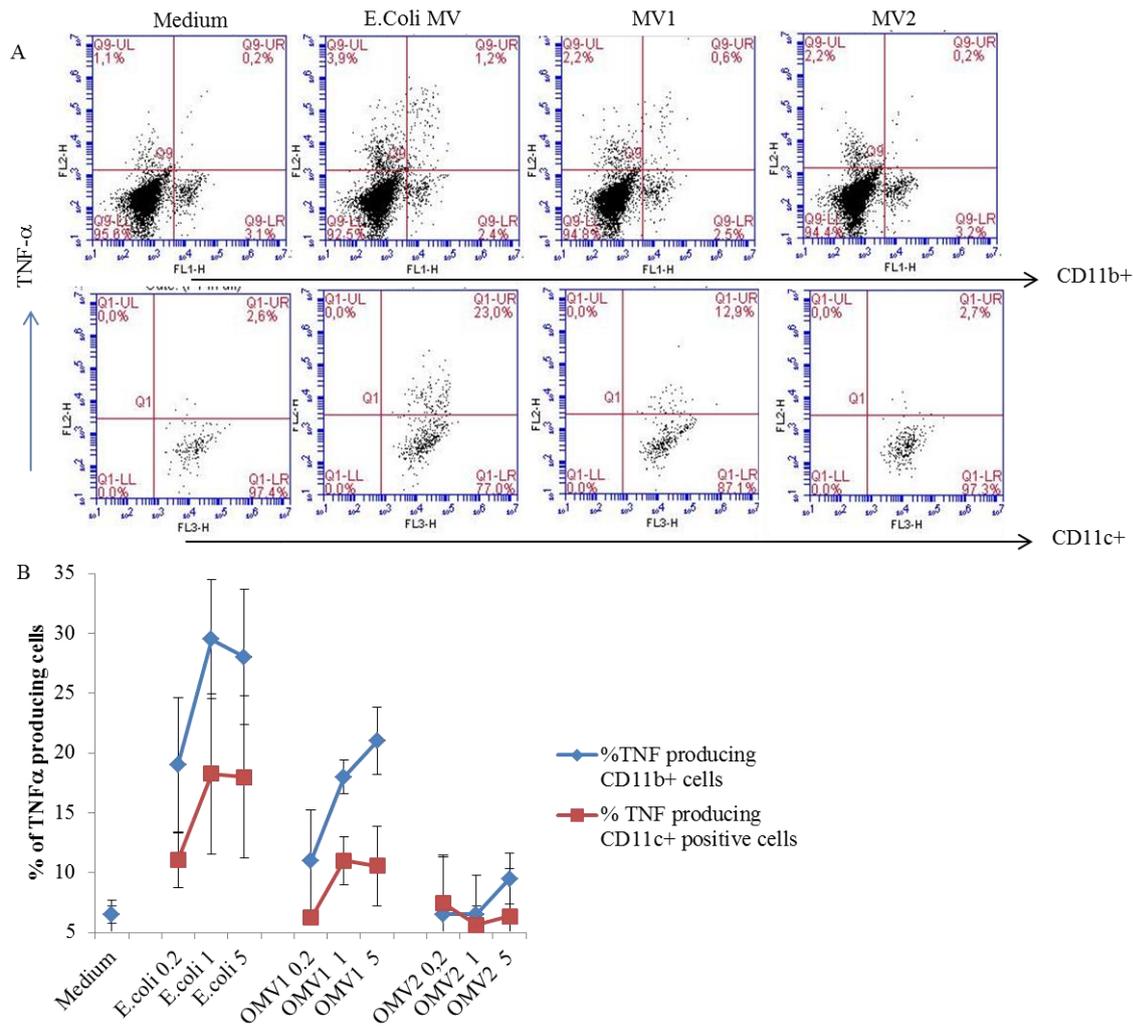


Figure 3.9 Intracellular cytokine staining of TNF α produced in macrophages (A, upper panels and B, blue lines) and DCs (A, lower panels and B, red lines) following stimulation with membrane vesicles. The results are the representative (A) and mean results (B) of 3 independent experiments.

In summary, results of the in vitro experiments suggest that in contrast to E.coli-derived MVs, commensal-derived MVs stimulate the innate immune response less effectively and may possess properties that might suppress over-exuberant immune reactivity (see IL-10 production data).

3.8. Analysis of Cell-Surface Binding and Internalization of Membrane Vesicles

To identify the cells that readily internalized MVs, splenocytes were incubated with SP-DiOC18(3) labeled membrane vesicles and those that were positive for the SP-DiOC18(3) signal were analyzed following cell-surface staining for specific leukocyte subsets. To discriminate between cell-surface bound versus internalized fluorescent signal, samples were acquired before and after trypan blue quenching. Interestingly, labeled vesicles were found to associate mostly with B220+ B cells (Figure 3.10 A) in a dose dependent manner. Following identification of this cell type as the major leukocyte population physically

interacting with MVs, magnitude of the SP-DiOC18(3) signal was analyzed using gated B cells. Data presented in Figures 3.10 (B) and (C) show that the vesicles were mostly internalized since trypan blue quenching could not reduce the amount of the fluorescent signal. In all groups, internalization and uptake increased in a concentration dependent manner. However, since labeling efficiencies of vesicles were not equal for all MVs and showed variation depending on the bacterial species used, it was not possible to make a comparison among MVs as to which vesicle was more avidly internalized.

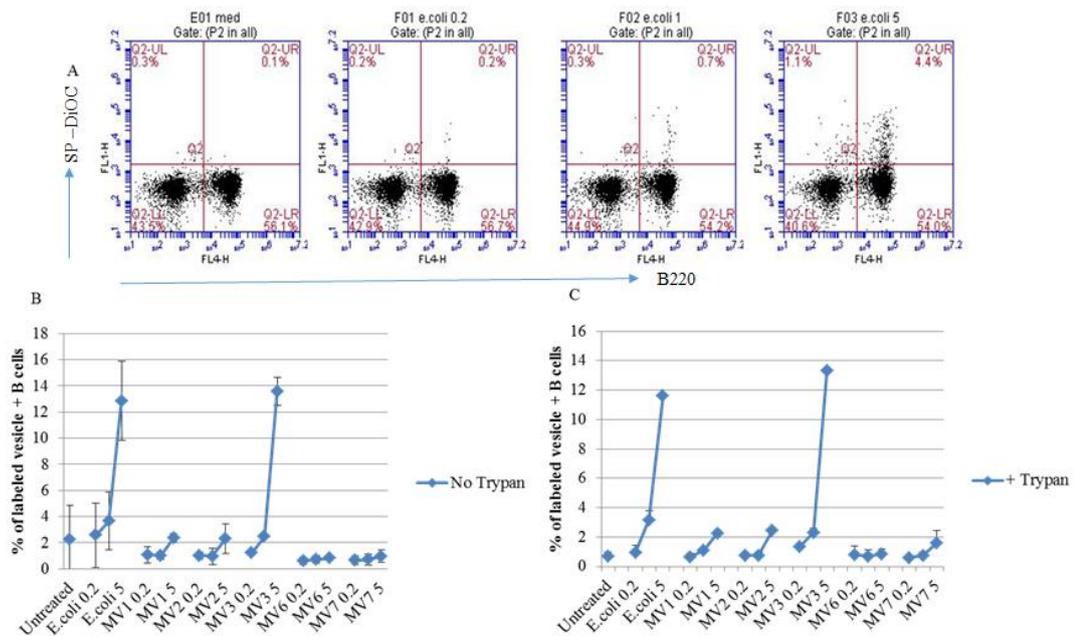


Figure 3.10 Internalization and uptake of membrane vesicles in mouse splenocytes. (A) % of SP-DiOC18(3) labeled vesicle positive cells in whole spleen stained with anti-B220 antibodies (B cell marker) was determined using flow cytometry. Percentages of SP-DiOC18(3) positive B cells before (B) and after (C) trypan blue quenching. Labeled membrane vesicles were incubated with splenocytes (400,000cells/well) in a total volume of 200µl for 4 hours at 37°C. Positive cells were detected by flow cytometry before and after trypan blue addition (1:10 diluted).

These results show that the major cell population recognizing and internalizing bacteria-derived MVs are B cells and hence MVs can directly modify the functional activity of this population.

3.9. Vaccination Study

The aim of the vaccination study was to investigate the immunostimulatory activity of E.coli-derived membrane vesicles and assess the immunomodulatory potential of commensal-derived MVs. For this, inactivated FMD virus vaccine without the adjuvant Montanide ISA 720 was used as the model vaccine. The commercially available vaccine, Montanide ISA206 adjuvanted inactivated FMD virus fails to provide long-lasting immunity,

necessitating development of better vaccines that can specifically induce immunological memory. Thus, using this model, we wanted to explore 2 opposing concepts: 1. whether E.coli-derived MVs could boost and extend the longevity of FMD-specific antibody responses and 2. whether commensal-derived vesicles could suppress antigen-specific immune responses and generate a more tolerogenic response.

3.9.1. Determination of Antigen specific total IgG, IgG1 and IgG2a antibody responses by ELISA

6-8 week old female BALB/c mice (5/group) were immunized on days 0 and 17 using the FMD antigen (3 μ g/mouse) and its combinations with membrane vesicles as described in Section 2.2.7.2. Blood was collected 2 weeks after each immunization. FMD-specific secondary IgG responses of all immunized groups have been summarized in Figures 3.11 A, B and C. Results indicate that E.coli derived MV adjuvanted groups generated higher levels of FMD-specific total IgG, IgG1 and IgG2a when compared to FMD vaccinated group alone. In contrast, MV1 and MV3 adjuvanted groups generated similar levels of antigen-specific total IgG and IgG1 when compared to FMD vaccine. MV2 was the only commensal-derived vesicle that elevated the FMD-specific IgG1 and total IgG responses to levels observed with the E.coli adjuvanted groups.

A)

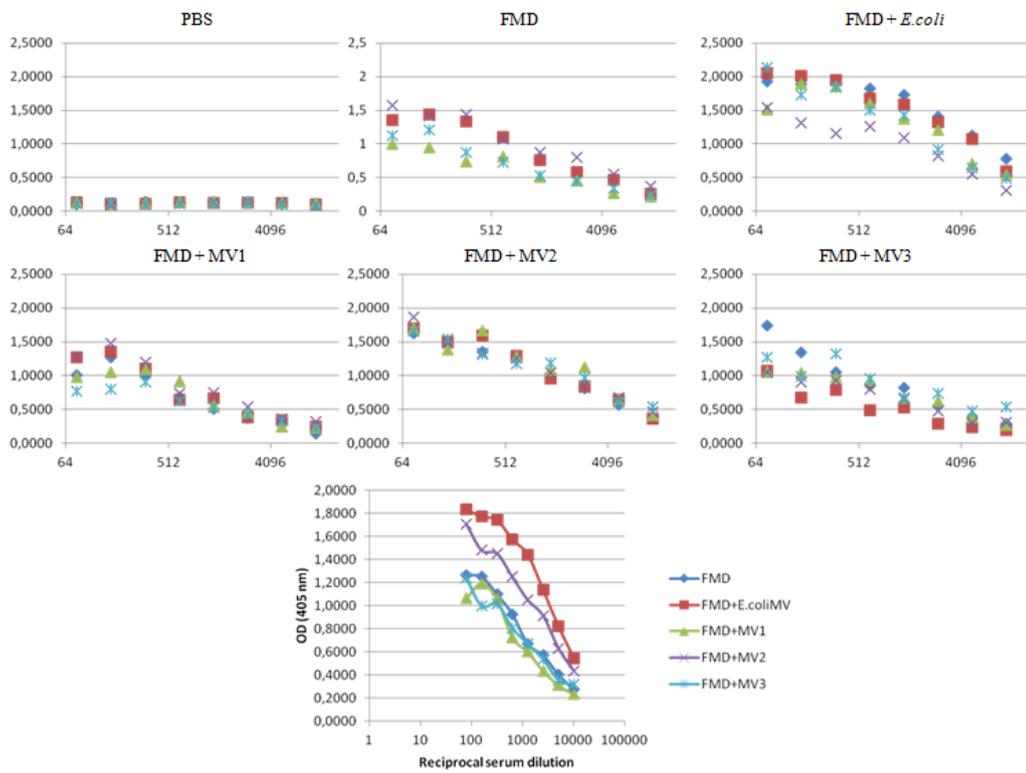


Figure 3.11 Anti -serotype-O-specific IgG responses of individual immunized mice (A)
Total IgG response

B)

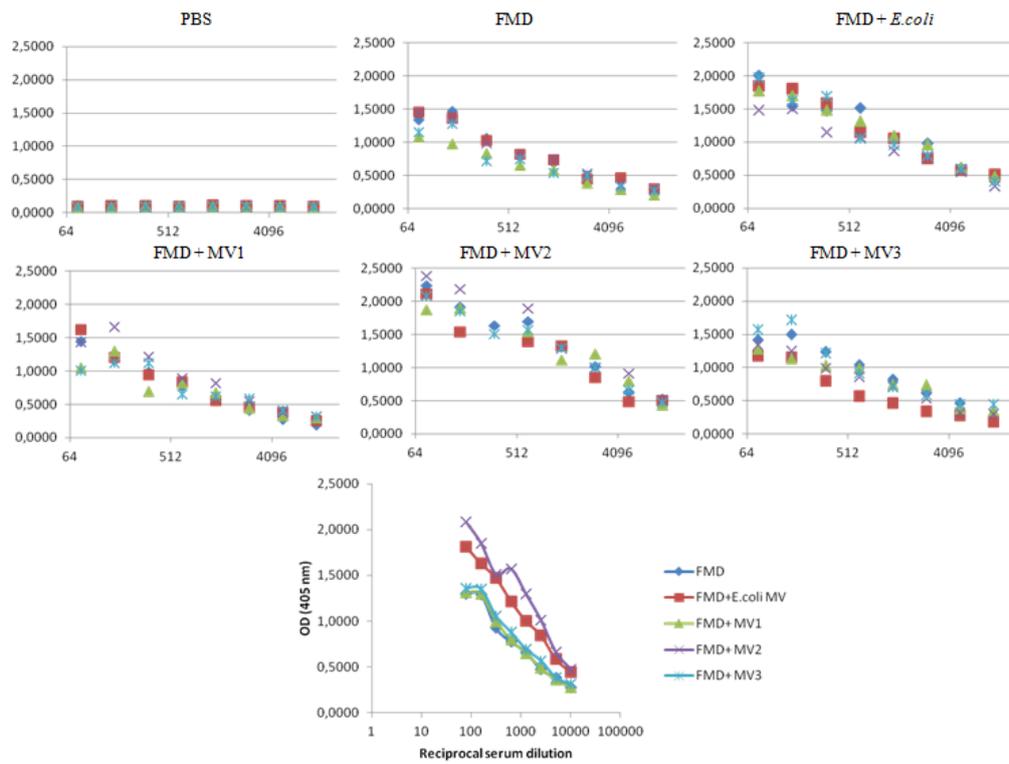


Figure 3.11 (continued) Anti-serotype-O-specific IgG responses of individual immunized mice (B) IgG1 response

C)

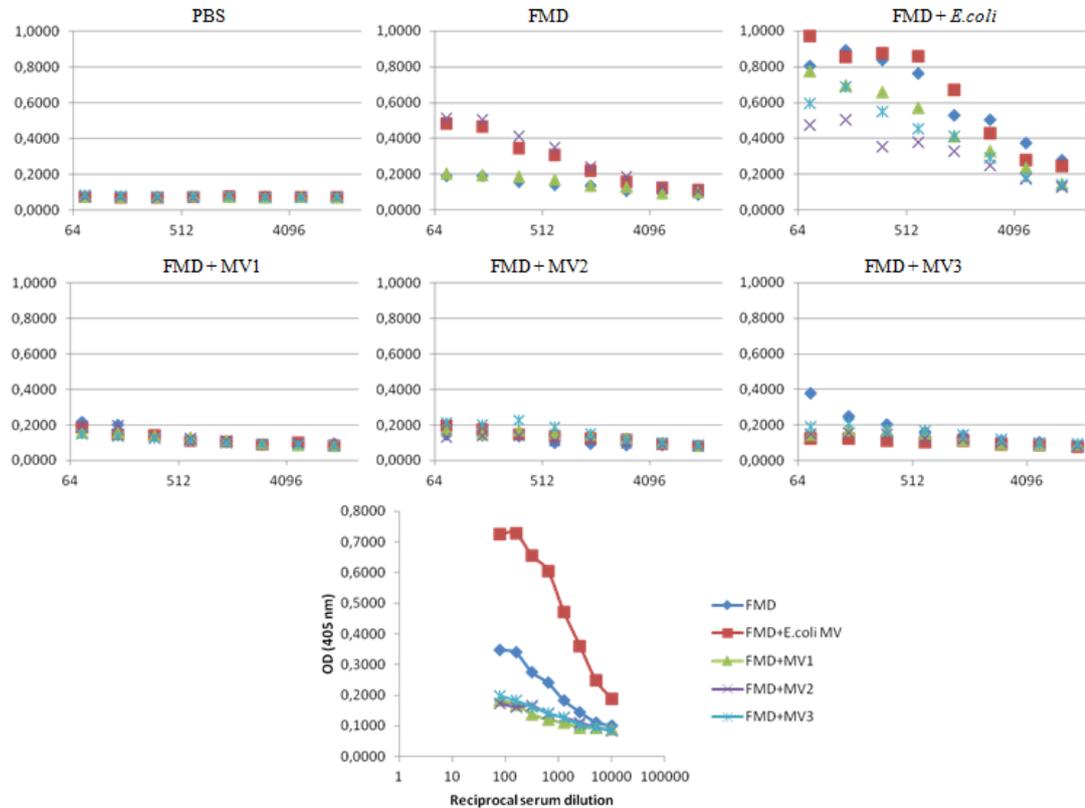


Figure 3.11 (continued) Anti-serotype-O-specific IgG responses of individual immunized mice (C) IgG2a response. 64X diluted sera were serially diluted $\frac{1}{2}$ -fold. Plots show the OD (405 nm) values for individual sera at several different dilutions.

For all vaccinated groups the most striking differences in antibody responses were observed when IgG2a titers were analyzed (Figure 3.9). Titers were calculated as the \log_2 reciprocal serum dilution that generated an OD value of + 5 standard deviations above the background OD observed with the PBS injected control group (cut-off OD of 0.1). Analysis of \log_2 IgG2a titers (Figure 3.11) revealed that E.coli MV adjuvanted groups significantly boosted the FMD-specific IgG2a response 4.7-fold when compared to FMD vaccine ($P < 0.05$). Conversely, commensal MV adjuvanted groups decreased IgG2a titers significantly (~ 10 -fold with MV1 and MV2 and 4.5-fold with MV3, $P < 0.05$).

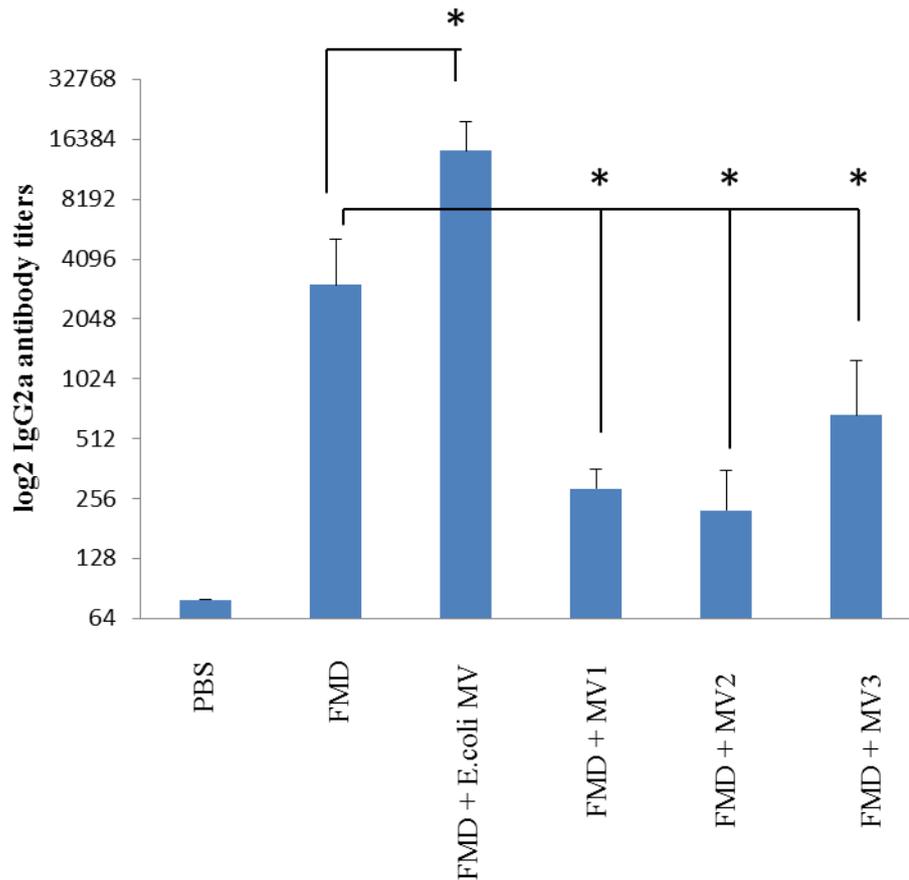


Figure 3.12 Average log₂ IgG2a antibody titers of groups of immunized mice (average of 5 mice \pm S.D). Titers were calculated as the log₂ reciprocal serum dilution that generated an OD value of + 5 standard deviations above the background OD observed with the PBS injected control group (cut-off OD of 0.1). *indicates $P < 0.05$.

B lymphocytes are specialized cells that function in antigen-specific antibody-mediated immune responses. The nature of the antibody subclass secreted (i.e from IgM to IgG, IgA or IgE) is heavily influenced by the type of T cell help provided to the antigen activated B cell. Mice have four different classes of IgG: IgG1, IgG2a, IgG2b and IgG3. Of these, production of IgG2a subclass depends on Th1 type of cytokines secreted by pro-inflammatory helper T cells (Th1 cells). In contrast, IgG1 subclass production is dependent on Th2 cells (Banerjee, 2010). A Th-1 dominated immune response is essential in clearance of intracellular pathogens. In this context, vaccine adjuvants are very important since they can influence the Th1-Th2 balance and thus can alter the antibody subclass generated (Morrow *et al.*, 2010). Combined with the above information, the results presented in this section suggest that E.coli-derived MVs can act as effective Th-1 promoting vaccine adjuvants. Conversely, commensal-derived MVs suppress Th-1 dominated responses and thus may be of benefit in treatment of certain autoimmune diseases such as type I diabetes mellitus that has a Th-1 associated pathology (Shimada *et al.*, 2009).

3.9.2. Determination of FMD specific memory B cell formation by ELISPOT

FMD vaccine suffers from generation of poor memory responses, necessitating vaccination of animals every 4-6 months to provide protection (Doel et al., 1996). In order to assess how memory B cell responses are affected in MV adjuvanted groups, splenocytes from vaccinated mice were incubated with LPS and CpG ODN combination for 5 days to allow for memory B cell expansion and differentiation into antibody secreting plasma cells. The expanded cells were then transferred onto anti-Ser-O antibody coated plates and antibody secreting cells were quantitated by ELISPOT as described in Section 2.2.7.4. Results of this study are presented in Figures 3.13 A and B. According to this, E.coli derived membrane vesicle adjuvanted group triggered significantly higher numbers of antibody secreting plasma cells than the FMD vaccine alone (~ 2.5-fold, Figure 3.11 B). In this assay, memory B cells activated by CpG ODN + LPS respond by rapid proliferation, differentiation into plasmablasts, and an increase in Ig secretion. Since CpG+LPS stimulation is used in this process, the isotype of the antibody secreted does not directly represent the isotype of antibodies present in vivo. However, the assay indirectly quantitates available memory B cells capable of responding to TLR ligand stimulation (Henn et al., 2009). Thus, these results suggest that E.coli derived MVs are effective adjuvants in inducing memory B cell responses. By contrast, MV1 and MV3 adjuvanted groups caused a significant decrease in the number of memory B cells generated when compared to FMD vaccine (~ 2.3-fold decrease, Figure 3.13 B). MV2 which was previously shown to trigger FMD-specific IgG1 responses (Figures 3.11 B and 3.12) showed no such effect. These results suggest that depending on the bacterial species in question, commensal bacteria derived vesicles may suppress memory B cell responses and might be of value as tolerance inducing adjuvants in treatment of autoimmune diseases.

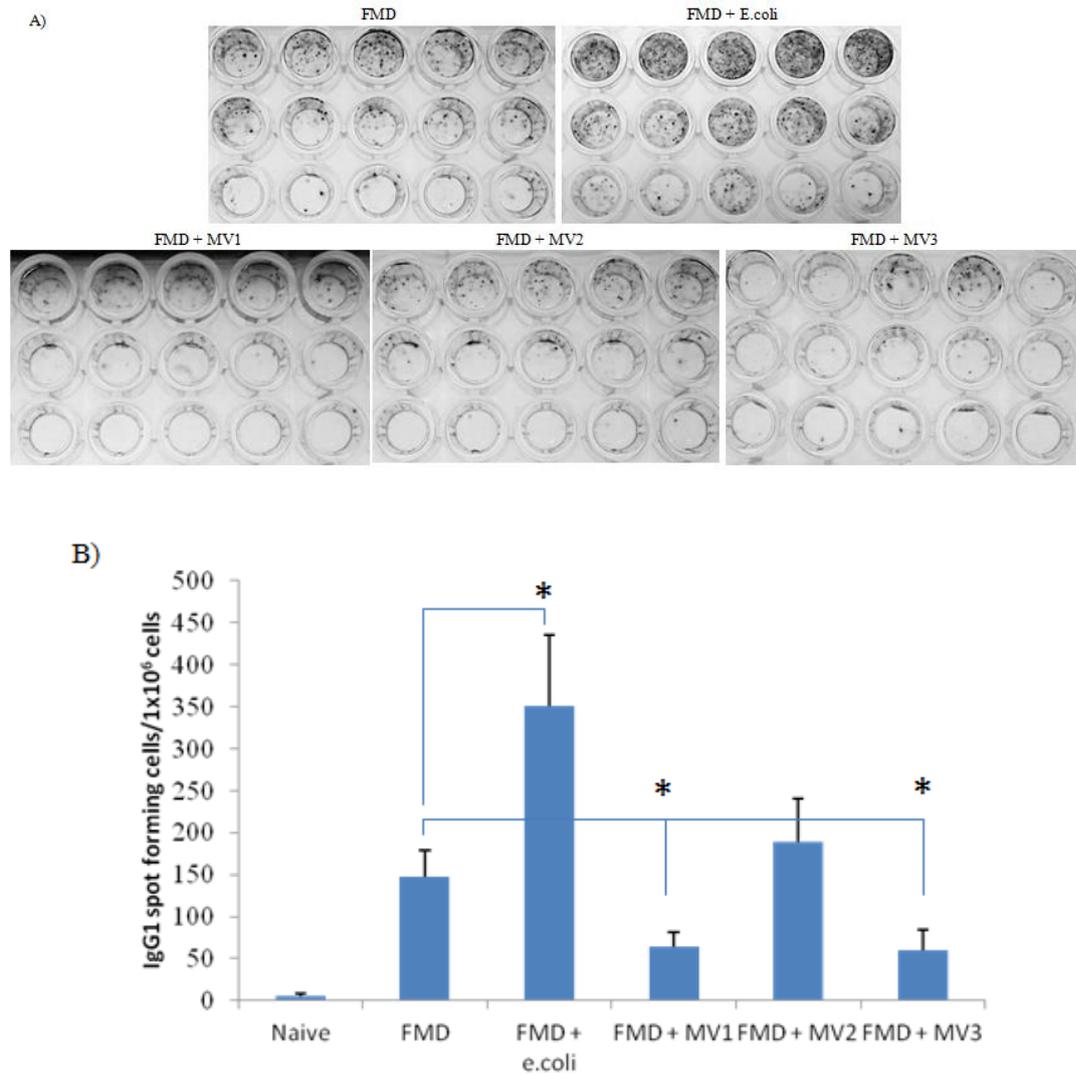


Figure 3.13 Ab secreting cells as determined by ELISPOT following in vitro expansion of memory B cells for 5 days (A) spot formation (B) IgG1 spot forming cells. E.coli membrane vesicles induce formation of memory B cells higher than FMD antigen alone. MV1 and MV3 suppress antigen effect and reduce formation of memory B cells.

CHAPTER 4

CONCLUSION

This thesis intended to test the immunomodulatory and/or the vaccine adjuvant potential of small membrane vesicles (MVs) naturally secreted from bacteria. For this, MVs were purified either from non-pathogenic *E. coli* or from 5 different Gram positive human commensal bacterial strains, followed by their characterization. SDS-PAGE gel electrophoretic analysis of MVs showed the presence of both common and unique proteins, depending on the bacterial species analyzed. All MVs incorporated nucleic acids and their nature (double stranded versus single stranded) and concentration showed variation between different isolates. The finding that MVs contain nucleic acids is an important one since this would have implications about their immunostimulatory activity. For example, in one study using *Moraxella catarrhalis* MVs, the presence of unmethylated CpG-DNA motifs were found to be critical for B cell activation (Vidakovics *et al.*, 2010). In contrast, there are reports suggesting that certain commensal bacteria strains may possess DNA enriched in immune suppressive sequences (Bouladoux *et al.*, 2012). Whether the nucleic acids associated with the MVs used in this thesis are immune suppressive or immune stimulatory remains to be determined and will be tested in future studies. In vitro stimulation of mouse splenocytes with the MVs revealed the following findings: 1. Commensal derived MVs trigger lower levels of IFN γ and higher levels of IL-10 secretion from mouse splenocytes when compared to *E. coli* MVs. 2. *E. coli* vesicles induced APC maturation whereas commensal derived MVs were ineffective in this assay. 3. TNF α inducing potential of *E. coli* derived MVs were higher in both CD11b and CD11c positive cells. Collectively these results suggest that commensal derived MVs may possess PAMPs that may show antagonistic effects leading to immune suppression rather than immune activation. In this context, evidence shows that when certain TLR ligands are used in combination, they negatively regulate the production of proinflammatory cytokines whereas increase the production of IL-10 (Trinchieri and Sher, 2007, Re, F., Strominger, J. L., 2004). The in vivo activity of bacterial MVs were also tested and the results were consistent with the in vitro findings. *E. coli* derived MV adjuvanted groups generated higher levels of FMD-specific total IgG, IgG1 and IgG2a when compared to FMD vaccinated group alone. In contrast, MV1 and MV3 adjuvanted groups generated similar levels of antigen-specific total IgG and IgG1 when compared to FMD vaccine. MV2 was the only commensal-derived vesicle that elevated the FMD-specific IgG1 and total IgG responses to levels observed with the *E. coli* adjuvanted groups. These results indicate that *E. coli*-derived MVs can act as effective Th-1 promoting vaccine adjuvants. Conversely, commensal-derived MVs suppress Th-1 dominated responses and thus may be of benefit in treatment of certain autoimmune diseases such as type I diabetes mellitus that has a Th-1 associated pathology. Elispot analysis show that commensal bacteria derived vesicles may suppress memory B cell responses and might be of value as tolerance inducing adjuvants in the treatment of autoimmune diseases.

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APPENDIX A

BUFFERS, SOLUTIONS AND CULTURE MEDIA

Blocking Buffer (ELISA)

- 500 ml 1x PBS
- 25 grams BSA (5%)
- 250 µl Tween20 (0,025%)

Crystal particles of BSA should be dissolved very well, with magnetic-heating stirrer for 20-30 min. The buffer should be stored at -20°C.

BCIP-low melting agarose substrate solution

- 4 ml BCIP
- 1 ml water
- 0,03 g low melting agarose

BCIP should be heated up to 56 degree in water bath, after warming water and agarose should be added and microwaved until agarose completely dissolved.

Loading Dye (Agarose gel)

- 0,009 grams Bromophenol blue
- 0,009 grams Xylen cyanol
- 2,8 ml ddH₂O
- 1,2 ml 0,5M EDTA
- 11 ml glycerol

After preparing, just vortex it.

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8,01 grams Na₂HPO₄ · 2H₂O
- 2 grams KH₂PO₄

Complete into 1 lt with ddH₂O (pH= 6,8).

For 1X PBS's pH should be ≈ 7,2-7,4 and should be autoclaved prior to use.

PBS-BSA-Na azide (FACs Buffer)

- 500 ml 1x PBS
- 5g BSA (1%)
- 125mg (0,25%)

Resolving Gel 10% (SDS-PAGE)

- 3,3ml 30% Acrylamide
- 2,5 ml 1,5 M Tris
- 100 µl 10% SDS
- 100 µl 10% APS
- 4 µl TEMED
- 4 ml dH₂O

SDS sample buffer (4x)

- 0,5 M Tris- HCl pH 6.8
 - 0,8 g SDS
 - 4 ml Glycerol
 - 40 mg Bromophenol blue
 - 800 µl 2-Mercaptoethanol
- Complete into 10 ml with ddH₂O

Stacking Gel 5% (SDS-PAGE)

- 1,7 ml 30% Acrylamide
- 1,25 ml 1 M Tris
- 100 µl 10% SDS
- 100 µl 10% APS
- 10 µl TEMED
- 6,8 ml dH₂O

T-cell Buffer [ELISA]

- 500 ml 1x PBS
- 25 ml FBS (5%)
- 250 µl Tween20 (0,025%)

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2,5 ml Tween20
- 4,5 lt ddH₂O

RPMI-1640 (Hyclone)

- 2 % : 10 ml FBS (FBS = inactivated at 55°C)
- 5 % : 25 ml FBS
- 10 % : 50 ml FBS
- 5 ml Penicillin/Streptomycin (50 µg/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES (Biological Industries), (10 mM final concentration from 1 M stock)
- 5 ml Na Pyruvate, (0,11 mg/ml final concentration from 100 mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)