IMMUNE MODULATORY EFFECTS OF *PEDIOCOCCUS PENTOSACEUS* DERIVED MEMBRANE VESICLES: MECHANISM OF ACTION AND THERAPEUTIC APPLICATIONS

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ABSTRACT

IMMUNE MODULATORY EFFECTS OF *PEDIOCOCCUS PENTOSACEUS* DERIVED MEMBRANE VESICLES: MECHANISM OF ACTION AND THERAPEUTIC APPLICATIONS

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In our previous studies, we characterized 5 different human gram positive commensal bacteria derived membrane vesicles (MVs) and compared their activity with non-pathogenic E.coli derived membrane vesicles. Results showed that commensal bacteria derived MVs had immunomodulatory properties whereas non-pathogenic E.coli derived membrane vesicles had immune stimulatory properties. In this thesis, we aimed to focus our attention to *Pediococcus pentosaceus*-derived MVs that displayed the highest immunomodulatory activity. In an immunization model, *Pediococcus pentosaceus*-derived MVs supressed anti-OVA specific IgG1 and IgG2c and CTL responses. Analysis of MV effect on different cell types showed that MVs exerted an immunomodulatory response by generating M2 macrophages and myeloid derived suppressor cells (MDSCs) but not regulatory T cells. MVs' anti-inflammatory effects were also tested in acute inflammation models established in mice. In zymosan induced peritonitis model, MVs ameliorated excessive inflammation by reducing neutrophil recruitment to peritoneal cavity and inhibiting macrophage loss caused by inflammation. In dextran sodium sulphate (DSS) induced acute colitis model, post-treatment with MVs (Day 0 and 3) prevented colon shortening and loss of crypt architecture. In an excisional wound healing model, intraperitoneal MV administration accelerated wound closure through recruitement

of PD-L1 expressing myeloid cells to the wound site. Collectively, these results indicate that *Pediococcus pentosaceus* derived membrane vesicles activates suppressor – regulatory cell types and can be used as potent anti-inflammatory agents for the treatment of inflammatory or autoimmune diseases.

Keywords: Membrane vesicles, commensal bacteria, immunomodulatory response, anti-inflammatory agent, M2 macrophages, MDSCs, peritonitis, DSS-induced colitis, wound healing

ÖZ

PEDIOCOCCUS PENTOSACEUS KÖKENLİ MEMBRAN KESECİKLERİNİN İMMÜN MODÜLATÖR ETKİLERİ: ETKİ MEKANİZMASI VE TERAPÖTİK UYGULAMALAR

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Önceki çalışmalarımızda 5 farklı insan kommensal bakteri izolatından salgılanan membran keseciklerini (MV) karakterize edip etkinliklerini patojenik olmayan E.coli bakteri izolatından salgılanan membran kesecikleriyle karşılaştırdık. Sonuçlar, kommensal bakteri kökenli MV'lerin immün düzenleyici etkilerinin olduğunu, buna karşın patojenik olmayan E.coli bakteri izolatından salgılanan MV'lerin ise immün uyarıcı özellikleri olduğunu gösterdi. Bu tez çalışmasında, en yüksek immün düzenleyici etki gösteren *Pediococcus pentosaceus* izolatından salgılanan MV'lerin etkileri ayrıntılı bir şekilde incelenmiştir. OVA model antijeni immünizasyon modelinde *Pediococcus pentosaceus* kökenli MV'ler anti-OVA spesifik IgG1 ve IgG2c ile CTL yanıtlarını baskılamıştır. MV'lerin farklı hücre tipleri üzerindeki etkileri incelendiğinde immün düzenleyici aktivitenin M2 makrofajlarından ve myeloid kökenli baskılayıcı hücrelerden kaynaklandığı, ödüzenleyici T hücrelerin bir rolü olmadığı gözlemlendi. MV'lerin antienflamatuar etkileri farede farklı akut enflamasyon modelleri oluşturularak test edildi. Zymosan aracılı peritonit modelinde MV'ler şiddetli enflammasyon oluşumunu, nötrofillerin periton boşluğuna toplanmasını ve makrofajların enflamasyon yanıtından dolayı ölümlerini engellemiştir. Dextran sodyum sülfat aracılı akut kolit modelinde MV'lerle yapılan geç tedavinin (0. ve 3. günlerde) anti-enflamatuar koruyucu etkileri olduğu, kolon

kısalmasını ve kript yapı bozulmasını engellediği gözlemlendi. Eksizyonel yara iyileşmesi modelinde, intraperitoneal MV uygulamasının yara bölgesine PD-L1 ifade eden miyeliod kökenli hücre toplanması aracılığıyla yara iyileşmesi sürecini hızlandırdığı bulunmuştur. Bütün bu sonuçlar *Pediococcus pentosaceus* izolatından salgılanan membran keseciklerinin baskılayıcı – düzenleyici hücrelerin aktivasyonu üzerinde etkileri olduğunu ve potansiyel antienflamatuar ajanlar olarak enflamatuar hastalıkların ya da otoimmün hastalıkların tedavisinde kullanılabileceklerini göstermektedir.

Anahtar Kelimeler: Membran kesecikleri, kommensal bakteriler, immün düzenleyici yanıt, antienflamatuar ajan M2 makrofajlar, MDSCs, peritonii, DSS-aracılı kolit, yara iyileşmesi

To my precious family…

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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
CBA	Cytometric bead array
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CpG	Unmethylated cytosine-phosphate-guaniosine 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
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
MDSC	Myeloid derived suppressive cells
Mf	Macrophage
MHC	Major histocompatibility complex
MV	Membrane vesicle
MyD88	Myeloid differentiation factor-88
NF-κB	Nuclear factor- kappa B
NK	Natural killer
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
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
T_{REG}	Regulatory T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1. The Immune System

The immune system and its components constitute defense mechanisms that protect the host against pathogenic organisms. It consists of a network of physical barriers, cells and soluble factors. Depending on the type of protective response, immune system can be sub-divided into two arms known as innate immune system (general defense) and adaptive immune system (specific defense).

Neutrophils, monocytes, macrophages, natural killer cells (NK cells), innate lymphoid cells, NK-T cells, dendritic cells (DCs), mast cells, basophils and eosinophils are members of innate immune cells and constitute the first line of defense against pathogenic microorganisms. The mucous membrane and the skin serve as chemical and physical barriers of the body which prevent pathogen entry (Lievin-Le Moal and Servin, 2006). Should a pathogen breach these barriers, the innate immune system generates a rapid inflammatory response. Unlike the innate immune system, the adaptive immune response is delayed and pathogen specific. Furthermore, adaptive immune response has the ability to develop immunological memory (Kumar *et al*., 2011). Cells of the innate immune system express germ line encoded receptors which are called as pattern recognition receptors (PRRs). These receptors recognize pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). Recognition of any PAMP and/or DAMP by PRRs initiates an immune response characterized by upregulation in expression of several effector molecules. In contrast, the adaptive immune system is antigen specific. T and B lymphocytes recognize specific antigens by distinct antigen recognition receptors generated by somatic gene rearrangements (Kawai and Akira,

2009). Antigen presentation to T lymphocytes is maintained by antigen presenting cells (APCs) such as macrophages, dendritic cells, and B lymphocytes. Activated T or B lymphocytes differentiate into effector cells. T cells support cell-mediated immune responses while B cells differentiate to antigen specific antibody secreting plasma cells and generate humoral immune response.

1.2. Innate Immune System

The primary line of defense against pathogens is generated through the cooperation of many components and factors of the innate immune system. These include chemical and physical barriers that prevent pathogen entry into the organism (Medzhitov, 2007). Specialized group of receptors expressed on various immune cells are able to detect markers of microbial infection (Janeway, 1989). These specialized receptors are collectively referred to as pattern recognition receptors (PRRs). PRRs are able to distinguish between self and non-self through the detection of pathogen associated molecular patterns (PAMPs) such as cell wall components of bacteria that are absent in the host (Kawai and Akira, 2009). Furthermore, these receptors can also sense damage to tissues by binding to danger associated molecular patterns (DAMPs) that are released into the extracellular environment as a result of mechanical, chemical or microbial damage. DAMPs bind to PRRs and trigger inflammation at the injury site (Seong and Matzinger, 2004). Recognition of DAMPs or PAMPs activates intracellular signaling pathways, generating an inflammatory response which is primarily maintained by the secretion of certain cytokines and chemokines (Table 1.1). The complement system is another member of the innate immune system proteins and aids in the opsonization and killing of infectious agents (Degn *et al*., 2007).

Table 1.1 Cytokines and their major functions (Adapted from Turner *et al.*, 2014)

	Cytokine	Main sources	Target cell	Major function
Interleukins	**IL-1**	Macrophages, B cells, DCs	B cells, NK cells, T-cells	Pyrogenic, pro-inflammatory, proliferation and differentiation, BM cell proliferation
	IL-2	T cells	Activated T and B cells, NK cells	Proliferation and activation
	IL-3	T cells, NK cells	Stem cells	Hematopoietic precursor proliferation and differentiation
	IL-4	Th cells	B cells, T cells, macrophages	Proliferation of B and cytotoxic T cells, enhances MHC class II expression, stimulates IgG and IgE production
	IL-5	Th cells	Eosinophils, B-cells	Proliferation and maturation, stimulates IgA and IgM production
	IL-6	Th cells, macrophages, fibroblasts	Activated B-cells, plasma cells	Differentiation into plasma cells, IgG production
	IL-7	BM stromal cells, epithelial cells	Stem cells	B and T cell growth factor
	IL-8	Macrophages	Neutrophils	Chemotaxis, pro-inflammatory
	IL-9	T cell	T cell	Growth and proliferation
	IL-10	T cell	B cells, macrophages	Inhibits cytokine production and mononuclear cell function, anti-inflammatory
	IL-11	BM stromal cells	B cells	Differentiation, induces acute phase proteins
	IL-12	T cells	NK cells	Activates NK cells
Tumour necrosis factors	**TNF-α**	Macrophages	Macrophages	Phagocyte cell activation, endotoxic shock
		Monocytes	Tumour cells	Tumour cytotoxicity, cachexia
	TNF-β	T-cells	Phagocytes, tumour cells	Chemotactic, phagocytosis, oncostatic, induces other cytokines
Interferons	**IFN-α**	Leukocytes	Various	Anti-viral
	IFN-β	Fibroblasts	Various	Anti-viral, anti-proliferative
	IFN-γ	T-cells	Various	Anti-viral, macrophage activation, increases neutrophil and monocyte function, MHC-I and -II expression on cells
Colony stimulating factors	**G-CSF**	Fibroblasts, endothelium	Stem cells in BM	Granulocyte production
	GM-CSF	T cells, macrophages, fibroblasts	Stem cells	Granulocyte, monocyte, eosinophil production
	M-CSF	Fibroblast, endothelium	Stem cells	Monocyte production and activation
	Erythropoietin	Endothelium	Stem cells	Red blood cell production
Others	**TGF-β**	T cells and B cells	Activated T and B cells	Inhibit T and B cell proliferation, inhibit haematopoiesis, promote wound healing

Abbreviations: BM, bone marrow; DCs, dendritic cells; G-CSF, granulocyte-colony stimulating factors; M-CSF, macrophage colony stimulating factor; Th, T helper cells.

Antigen presenting cells (APCs), such as dendritic cells, macrophages and B cells, are crucial components of the innate immune system as they are able to process cytosolic or extracellular antigens and present them to the members of the adaptive immune system. APCs degrade cytosolic antigens to small peptides and form complexes with their major histocompatibility complex (MHC) class I molecules. These are then translocated onto the cell surface to be engaged by CD8+ cytotoxic T cells. Divergently, exogenous antigens, are taken up by APCs and loaded onto MHC class II molecules that interact with CD4+ helper T cells. Dendritic cells (DC) represent the most potent APCs with the ability to prime naïve T cells. For this to occur, DC activation and maturation through PRR/PAMP interactions is a prerequisite which is followed by the expression of co-stimulatory molecules such as CD80 and CD86. Following maturation, DCs migrate to the nearest lymph node and activate naïve T lymphocytes. This process leads to the priming and maturation of T

lymphocytes. Moreover, diverse cytokines secreted from mature DCs provide the third signal to differentiate helper T cells into various effector subgroups such as T_H1, T_H2, T_H17 or regulatory T cells. These effector T cell types enable the immune system to give a proper response depending on the properties of the invader. For instance, T_H1 cells provide help for the removal of intracellular pathogens, whereas T_H2 cells support anti-parasite responses.

1.2.1. Pattern Recognition Receptors (PRRs)

Pattern recognition receptors are specialized germline encoded receptors capable of recognizing DAMPs and/or PAMPs. Typical PAMPs include peptidoglycan (PGN), lipopolysaccharide (LPS), fungal cell wall component β-glucan, bacterial cell wall component lipoteichoic acid like molecules, viral single or double stranded RNA (ssRNA or dsRNA), unmethylated cytosine-phosphate-guanine (CpG) motifs in the bacterial genome (Akira, 2006). Upon stimulation of PRRs by corresponding inducers, cells initiate certain signaling pathways leading to the release of inflammatory cytokines, chemokines, and anti-microbial peptides. PRRs are classified into distinct families of receptors: toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleic acid sensors (Table 1.2).

Table1.2: Pattern recognition receptors and their ligands (Adapted from Takeuchi and Akira, 2010)

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

1.2.1.1. Toll-like Receptors (TLRs)

Several TLRs have been identified in mice (12 types) and humans (10 types) (Kumar et al., 2011). Both species share the same TLRs from 1 to 9. Due to a stop codon expressed in mice, TLR10 is only present in human. In contrast, the human genome does not contain TLR11, TLR12, and TLR13 (Kawai, 2009). Structurally, TLRs are type I transmembrane receptors and are made up of three main domains: leucine-rich repeats present in the ectodomain, transmembrane domain, and Toll-interleukin 1 (IL-1) receptor (TIR) signaling domain. Signal transduction upon PAMP recognition by the ectodomain travels to the TIR signaling domain through the transmembrane region. PAMPs of bacterial, fungal, and protozoan origin are detected by TLR1, 2, 4, 5, and 6 which are present on the cell membrane. Nucleic acids of bacterial and viral

origin, on the other hand, are recognized by TLRs 3, 7, 8, and 9 that are expressed inside of endosomal vesicles (Kumar *et al*., 2011) (Table 1.3). The compartmentalization of TLRs has a critical role in appropriate ligand accessibility and discrimination of non-self from self molecules (Kumar et al., 2009).

Table 1.3. Toll like receptor family members (Adapted from Kumar, Kawai & Akira, 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, glycosylphosphophatidyl inositol mucin, zymosan
TLR3	Endosome	ssRNA virus, dsRNA virus, respiratory syncytial virus, murine cytomegalovirus
TLR4 (MD2, CD14, LBP)	Cell surface	Lipopolysaccride, 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 *streptococcus pneumonia*, 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, profillin-like molecule from *Toxoplasma gondii*

TLRs 1, 2, 4, 5, 6, and 11 are membrane bound TLRs that enable the detection of microbial cell membrane/wall motifs (Kaisho, 2001). TLR2 is commonly present as a heterodimer with TLR1 or TLR6. The TLR2/TLR1 heterodimer enables the recognition of triacylated lipopeptides from mycoplasma and gram-negative bacteria. The TLR2/TLR6 heterodimer, on the other hand, detects diacylated lipopeptides from mycoplasma and peptidoglycan (PGN) from gram-positive bacteria (Kawai, 2010). Moreover; TLR4 together with the adaptor MD2 and the cell-surface molecule CD14 recognize lipopolysaccharide (LPS) which is a major component of

the gram-negative bacterial outer membrane (Kim et al., 2007). TLR5 has a critical role in recognizing the bacterial flagellin. Intestinal epithelial cells express TLR5 on their basolateral surface which indicates the importance of TLR5 in detection of gut habitant flagellated bacteria (Kawai and Akira, 2009). Furthermore, TLR11 that is present in mice enables the recognition of uropathogenic bacteria. In addition, it mediates the detection of the parasitic component profilin-like molecule originating from *Toxoplasma gondii*.

Endosomal TLRs, which include TLR3, TLR7, TLR8, and TLR9, are evolved to detect nucleic acids and are localized in endosomal, lysosomal, and endolysosomal compartments in the cell. They initially reside in the ER but are translocated to endosomal compartments after PAMP exposure and initiate the signaling pathway for the production of type I interferons and inflammatory cytokines upon binding to foreign nucleic acids (Kawai and Akira, 2009). Figure 1.1 summarizes individual TLR molecules, their cognate ligands and signal transduction pathways activated as a result of recognition.

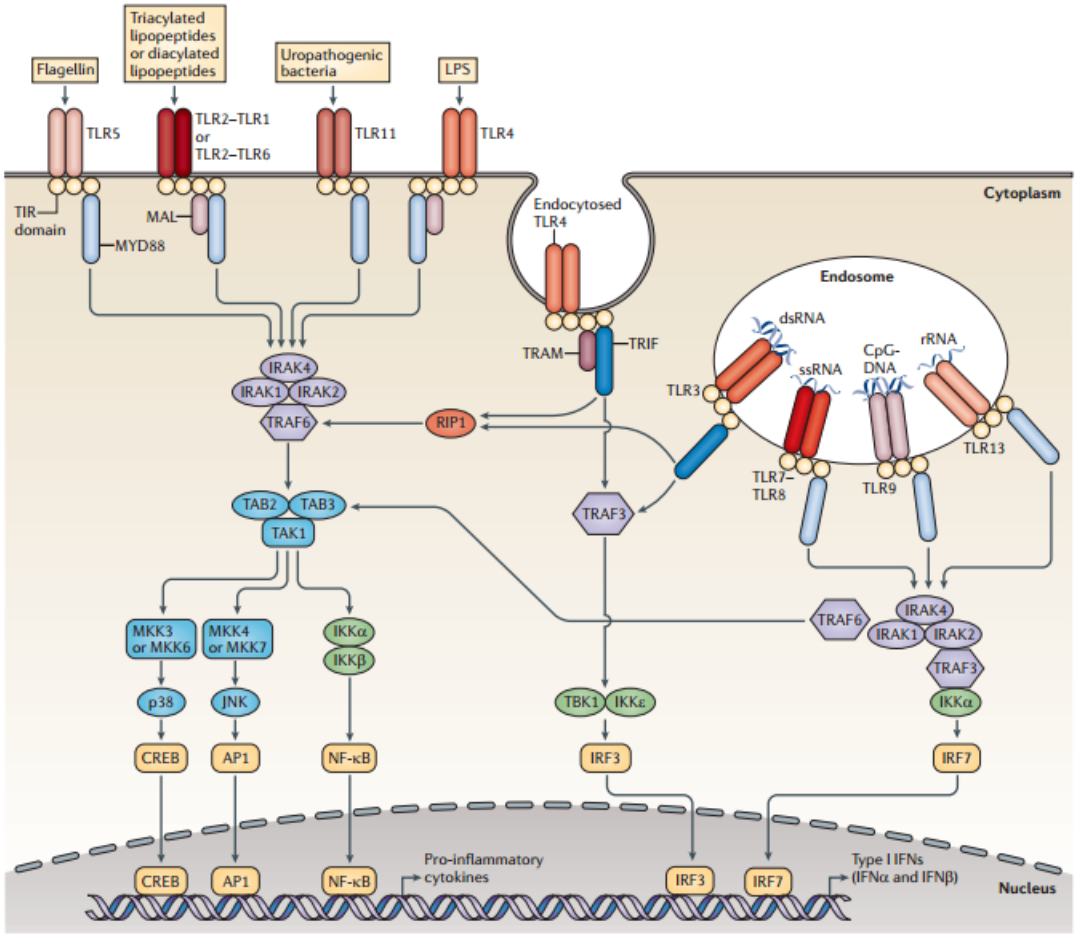

Figure 1.1. Toll like receptor signaling pathways (Adapted from O'Neill, Golenbock and Bowie, 2013)

1.2.1.2. Nucleic Acid Sensors

TLR3 is a member of the endosomal TLR family and recognizes dsRNA of ds RNA viruses or dsRNA that is produced during the replication of ssRNA viruses (Wang et al., 2004; Alexopoulou et al., 2001). This interaction initiates the production of type I interferon family of cytokines which is characteristic of an anti-viral immune response. TLR7 and TLR8 functionally share the ability of detecting ssRNA (Jurk et al., 2002). The signaling pathways of TLR3 and TLR7/TLR8 include the utilization of the adaptor molecules TRIF (TIR domain containing adaptor inducing IFN-β and MyD88 (myeloid differentiation primary response protein 88)). Another endosomal

TLR family member is TLR9 which recognizes unmethylated CpG motifs that have a 20X higher frequency in viral and bacterial DNA compared to mammalian DNA (Krieg et al.,1995). This mechanism enables the system to distinguish between prokaryotic and mammalian DNA for proper ligand detection.

Other than TLRs, cytosolic nucleic acid sensors can be extended to members of the RLR family RIG-I recognizing short dsRNA with 5'triphosphate caps and MDA5 detecting long genomic dsRNA. Cytosolic DNA, on the other hand, is recognized by the PYHIN family members AIM2 (absent in melanoma 2) and IFI16 (IFN-γ inducible protein16) (Reikine, Nguyen, and Modis, 2014). Another major cytosolic DNA sensing pathway includes the cyclicGMP-AMP synthase (cGAS) that acts through the stimulation of the adaptor molecule STING (stimulator of interferon genes) initiating type I interferon production (Hornung et al., 2016). Table 1.4 summarizes subcellular localizations of major nucleic acid sensors and their ligands.

Table 1.4. Localization of Nucleic acid sensors and their natural agonists (Adapted from Desmet and Ishii, 2012)

PRR	Localization	Sensed pathogens	Natural agonists	Synthetic agonists
TLR3	Endolysosomal compartment	dsRNA viruses, ssRNA viruses, dsDNA viruses	dsRNA	Polyl:C, polyU
TLR7	Endolysosomal compartment	ssRNA viruses, bacteria, fungi, protozoan parasites	GU-rich ssRNA	Imidazoquinolines (R848, imiquimod, 3M001), guanosine analogues
TLR8	Endolysosomal compartment	ssRNA viruses, bacteria, fungi, protozoan parasites	GU-rich ssRNA	Imidazoquinolines (R848, 3M002), guanosine analogues
TLR9	Endolysosomal compartment	dsDNA viruses, bacteria, protozoan parasites	DNA	CpG ODNs
RIG-I	Cytoplasm	ssRNA viruses, DNA viruses, *Flaviviridae*, reovirus, bacteria	Short RNA with 5′ppp and/or base pairing	Short polyI:C
MDA5	Cytoplasm	*Picornaviridae*, vaccinia virus, *Flaviviridae*, reovirus, bacteria	Long dsRNA	PolyI:C
NOD2	Cytoplasm	RNA viruses	ssRNA	–
DDX3	Cytoplasm	RNA viruses	RNA	–
DDX1–DDX21–DHX36	Cytoplasm	RNA viruses	dsRNA	PolyI:C
DDX60	Cytoplasm	RNA viruses, DNA viruses	ssRNA, dsRNA, dsDNA	–
DHX9	Cytoplasm	DNA viruses, RNA viruses	dsDNA, dsRNA	CpG-B ODNs
DHX36	Cytoplasm	DNA viruses	dsDNA	CpG-A ODNs
DDX41	Cytoplasm	DNA viruses, bacteria	DNA	–
AIM2	Cytoplasm	DNA viruses, bacteria	DNA	–
IFI16	Cytoplasm and nucleus	DNA viruses	dsDNA	–
ZBP1	Cytoplasm	DNA viruses, bacteria	dsDNA	–
LRRFIP1	Cytoplasm	DNA viruses, bacteria	dsDNA, dsRNA	–
STING	Cytoplasm	Bacteria	Cyclic di-GMP	–

5′ppp, 5′ triphosphate end; AIM2, absent in melanoma 2; dsRNA, double-stranded RNA; IFI16, IFNγ-inducible protein 16; LRRFIP1, leucine-rich repeat flightless-interacting protein 1; MDA5, melanoma differentiation-associated protein 5; NOD2, nucleotide-binding oligomerization domain protein 2; ODN, oligodeoxynucleotide; polyI:C, polyinosinic–polycytidylic acid; PRR, pattern-recognition receptor; RIG-I, retinoic acid-inducible gene I; ssRNA, single-stranded RNA; STING, stimulator of IFN genes; TLR, Toll-like receptor; ZBP1, Z-DNA-binding protein 1.

1.3. Bacteria derived Membrane Vesicles as a source of PRR ligands

1.3.1. Membrane Vesicle Formation

All eukaryotes and prokaryotes are known to produce and release vesicles to the environment. Such vesicles are called as exosomes or microparticles in eukaryotes and outer membrane vesicles or extracellular vesiclesin prokaryotes. Existence of outer membrane vesicles (OMV) produced by pathogenic and gram negative bacteria have been shown decades ago (Birdsell and Cota-Robles, 1967 and Knox et al., 1966). OMVs are bilayered lipid membrane vesicles and their size range from 20 nm – 250nm. They are secreted through all stages of growth. Gram negative bacteria derived membrane vesicles are composed of outer membrane and periplasmic components and contain lipopolysaccharide (LPS), DNA, RNA, periplasmic and membrane bound proteins, enzymes, toxins and peptidoglycan. Molecules like LPS, PGN, DNA and RNA are considered as microorganism associated molecular patterns (MAMPs). OMVs include multiple PRR ligands (MAMPs) and initiate proinflammatory immune responses through stimulating the production of cytokines, chemokines and antimicrobial peptides (Kaparakis-Liaskos and Ferrero, 2015). OMVs have been associated with production of biofilms, transfer of toxins and virulence factors, invasion of host cells and cytotoxicity which makes OMVs important in microbial pathogenesis.

The process of MV secretion in Gram positive bacteria is a more recently discovered phenomenon (Lee et al., 2009, Rivera et al.,2010, Deatherage and Cookson,2012, Macdonald and Kuehn,2012). Stapylococcus aureus derived MVs were the first gram positive bacteria derived membrane vesicles described in literature (Lee et al.,2009). Since gram positive bacteria do not possess an outer membrane, gram positive bacteria derived MVs consist of cytoplasmic membrane and cytosolic components. Gram positive bacteria derived vesicles also express multiple TLR ligands. One human commensal bacteria (Bacteroides fragilis) were shown to secrete polysaccharide A capsular antigen (PSA) containing membrane vesicles. PSA-OMVs were shown to trigger TLR2 mediated signaling in DC and produced

immunoregulatory cytokine IL-10, promoting maturation of regulatory T cells (Shen et al., 2012). Given that members of the microbiome impact the immune system, surprisingly few studies focused on the role of commensal-derived MVs in shaping the host immune responses (Shen et al., 2012, Fábrega et al., 2016, Kang et al., 2013).

Previously we have shown that human Gram positive commensal bacteria also secrete membrane vesicles (MSc thesis by Esin Alpdundar, 2013). We have characterized these vesicles, including their size and morphology as shown in Figure 1.2 (atomic force microscopy images of human commensal bacteria derived membrane vesicle (Lactobacillus salivarius)).

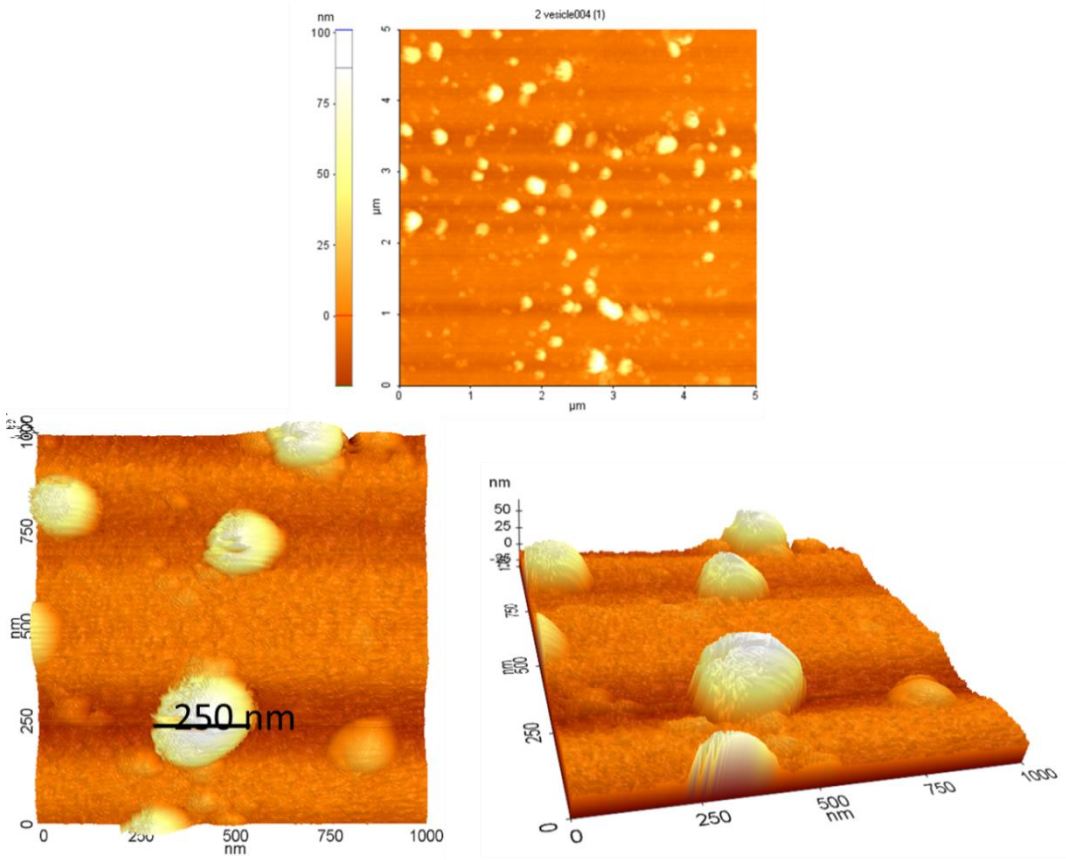

Figure 1.2 Atomic Force Microscopy images of Human Commensal Bacteria derived Membrane Vesicles (adopted from the MSc thesis of Esin Alpdündar, 2013).

1.3.2. Functions of Membrane Vesicles

Bacterial membrane vesicles have several different functions in mediating molecular transport of toxins and virulence factors, biofilm formation, modulating immune and stress responses. Present knowledge about bacteria derived membrane vesicles were generally based on pathogenic gram negative bacteria. However, more recently, several studies about gram positive bacteria derived membrane vesicles have also been reported. Membrane vesicles play important roles in quorum sensing and intraspecies cell to cell communication (Yanez-Mo *et al*, 2015). It is shown that MVs transfer resistance proteins for antibiotic resistance between same and different bacterial species (Ciofu *et al.*, 2000, Mashburn-warren and Whiteley 2006). Membrane vesicles also modify secretion of polysaccharide and virulence factor secretion into the environment (Deatherage and Cookson, 2012). One of the gram positive bacteria *Bacillus anthracis* is known to produce membrane vesicles containing anthrax toxin (Rivera *et al*., 2010). In the presence of environmental stress, membrane vesicle production is pivotal for bacterial survival. Membrane vesicles have important roles in formation of biofilms to support bacterial survival by enabling protection of bacterial community.

Bacterial member of human microbiota colonizes different anatomical locations and affect several host functions by interaction with different cell types. Pathogenic and symbiotic bacteria are known to interact with human cells through membrane vesicles (Yanez-Mo *et al*, 2015). Membrane vesicles are known to include multiple TLR ligands such as LPS, nucleic acids, lipoprotein, peptidoglycan. Collectively,hese ligands stimulate innate immune responses through recognition by TLRs and NLRs (Deatherage and Cookson, 2012). Pathogenic bacteria derived membrane vesicles may also contain antigens which activates adaptive immune responses (Alaniz *et al*., 2007, Bergman *et al*., 2005).

Since membrane vesicles have multiple TLR ligands, using them as immunotherapeutic agents will be more effective than using single TLR ligands to induce immune response. Given the importance of commensal bacteria in the

regulation of immune response, we further analyzed commensal bacteria derived membrane vesicles' immune modulatory effects.

1.4. Myeloid Cell Phenotypes Associated with Immune Regulation

1.4.1. Macrophages

Macrophages are myeloid cells of the innate immune system and play important roles in immune protection, tissue homeostasis and resolution of inflammation in response to injury or infection. Macrophages are divided into different subpopulations according to their anatomical location such as Kupffer cells in the liver and osteoclasts in the bone (Murray and Wynn, 2011). Since macrophages are important immunomodulators and effector cells, their activation determines and shapes the adaptive immune response.

Macrophages differentiate into two main subtypes according to the stimuli they encounter: classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (Martinez and Gordon, 2014) (Figure 1.4). M1 macrophages function in defense against pathogens such as bacteria, protozoa and viruses and promote anti-tumor immunity. In contrast, M2 macrophages are known to have anti-inflammatory functions and regulate wound healing process.

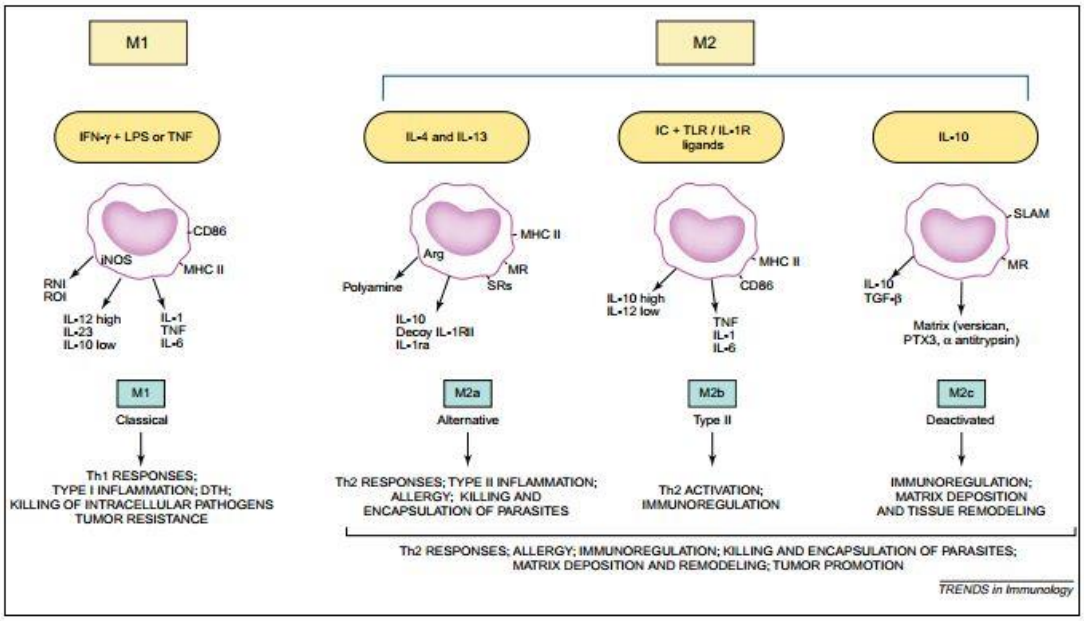

Figure 1.3. M1 and M2 macrophage polarization (Adopted from Mantovani et al., 2004)

Classically activated macrophages are effector macrophages activated during cell mediated immune responses. IFN-γ is one of the main cytokines that activate classically activated macrophages. This cytokine is generally produced by Th1 cells, natural killer (NK) cells and macrophages. Besides IFN-γ, LPS and TNF-α also activates classically activated macrophages. Classically activated macrophages initiate inflammatory responses for eliminating pathogens or the ensuing stimulus.

Alternatively activated macrophages are sub-grouped according to the stimuli that lead to their activation. M2 macrophages are characterized by secretion of large amounts of IL-10 in response to Fc receptor γ activation (Murray and Wynn, 2011). IL-4 activates M2a macrophages which support T helper-2 cells (Th2) mediated responses against parasites (Martinez and Gordon,2014). Wound healing macrophages can develop in response to both innate or adaptive signals. Glucocorticoids and IL-10 activates regulatory macrophages that limits inflammatory responses. Macrophages retain their plasticity and they can respond to environmental signals and change their phenotype (Mosser and Edwards,2008).

1.4.2. Myeloid derived Suppressor Cells

Myeloid derived suppressor cells are a heterogenous population of immature myeloid cells (IMC) generated from bone marrow precursors. Under normal circumstances, in healthy individuals, immature myeloid cells differentiate into immature granulocytes, dendritic cells and macrophages. In the steady state IMCs do not have suppressive properties and they are present in bone marrow. However, in pathological conditions such as inflammation, cancer and infections, differentiation of IMCs to mature cells is partially blocked, leading to the expansion of MDSCs (Gabrilovich and Nagaraj, 2009). MDSCs are known to suppress various T cell functions through expression of molecules like arginase-1 (ARG1), nitric oxide (NO) and reactive oxygen species (ROS). In mice, MDSCs are defined by the co-expression of CD11b and Gr1(Ly-6G/Ly-6C) and 20 -30% of the cells in bone marrow, 2 -4% of the cells in spleen have MDSC phenotype.

MDSCs have two major subtypes classified either as monocytic MDSC (M-MDSC) or granulocytic MDSCs (G-MDSC). These two subtypes of MDSCs have different suppressive mechanisms in various diseases such as cancer, infectious disease and autoimmune diseases (Mohavedi et al., 2008) Granulocytic MDSCs (G-MDSC or polymorphonuclear-MDSC (PMN-MDSC)) are characterized by the expression of $CD11b^{+}Ly6G^{+}Ly6C^{low}$ markers and express ARG-1, whereas monocytic MDSCs are characterized by the expression of $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ and iNOS (NOS2) (Youn et al., 2008). G-MDSC were shown to express high levels of reactive oxygen species (ROS) and low levels of nitric oxide (NO), whereas M-MDSCs were shown to express low levels of ROS and high levels of NO. Figure1.5 summarizes the suppressive mechanisms mediated by MDSC subtypes.

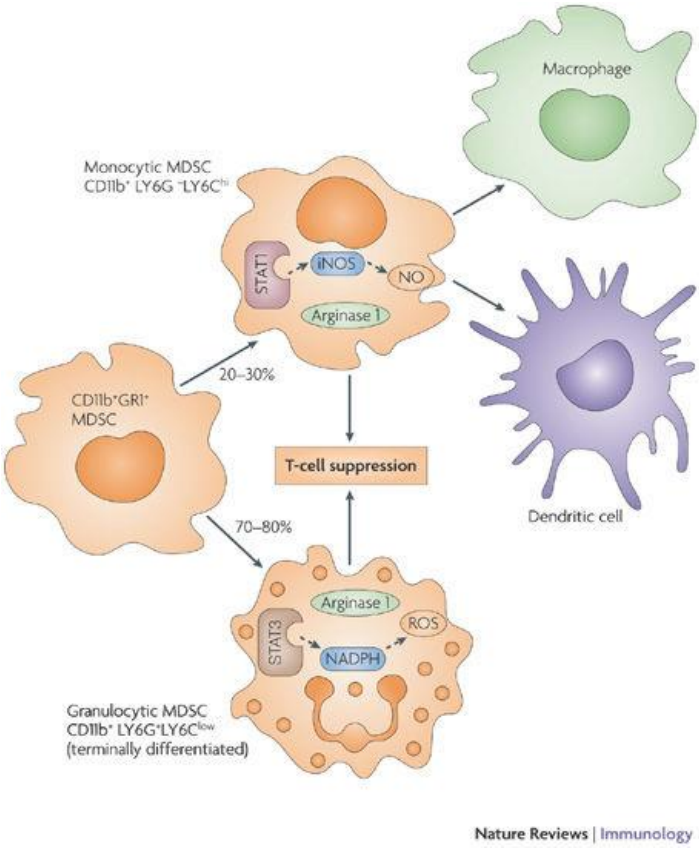

Figure 1.4. Suppressive mechanisms mediated by different subsets of MDSCs. (Adapted from Gabrilovich and Nagaraj, 2009).

1.5. Adaptive Immune System

Innate immunity initiates a generalized and rapid response to invading microorganisms. In contrast, adaptive immunity provides more extensive and finely tuned immune response against specific antigens. Adaptive or acquired immune system is associated with interactions between antigen presenting cells and T lymphocytes. Naïve T cells that have not encountered with a specific antigen circulate among blood and secondary lymphoid organs such as lymph nodes and spleen. Naïve T lymphocytes are activated through the engagement with antigen presenting cells that express MHC molecules loaded with specific antigens. Following this process, naïve T cells differentiate into effector T cells.

CD8 positive T lymphocytes recognize peptides presented on MHC class I molecules, whereas CD4 positive T lymphocytes recognize peptides presented on MHC class II molecules. CD4 positive T cells constitute a great majority of T cells which differentiate into different effector subtypes (T_H1, T_H2, T_{Reg}, T_H17 cells) depending on the cytokine profile in the environment (Figure 1.5). There are also additional helper T cell subsets such as T_H9, T_H22, T_{FH} (T follicular helper cells) which have various functions in adaptive immune response (Hirahara and Nakayama, 2016).

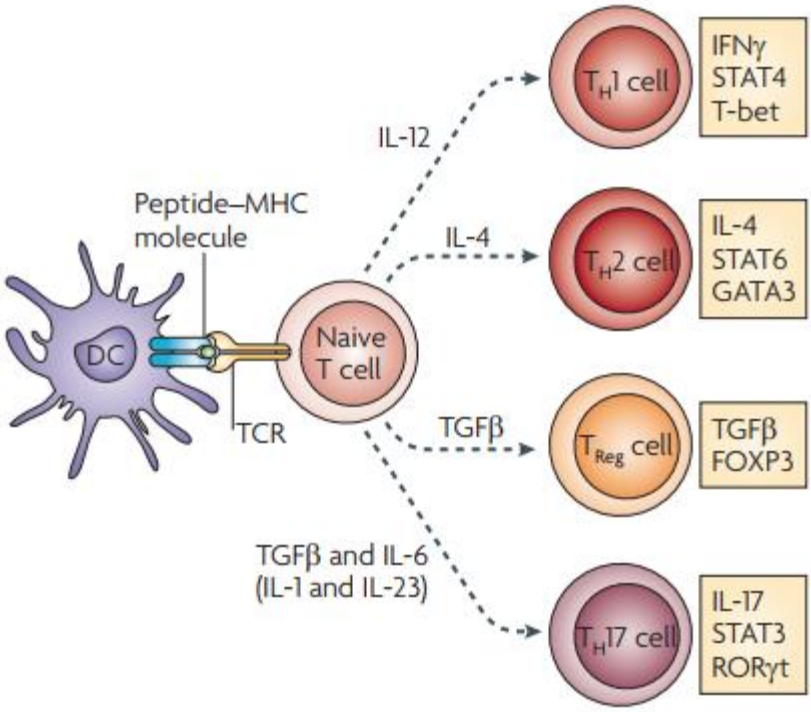

Figure 1.5. Helper T cell subsets (Adapted from Zou and Restifo, 2010)

B lymphocytes mediate humoral immune response by secreting antibodies after differentiation into effector plasma cells. Naïve B cells differentiate into plasma cells with the help of the signals received from the antigen and factors secreted by helper T cells. B cells are also able to differentiate independently from T cells by signals from B cell receptors (BCRs) and TLRs by generating IgM antibodies (Iwasaki and

Medzhitov, 2015). T cell dependent differentiation leads to secretion of IgG, IgA or IgE antibodies from plasma cells (Bonilla and Oettgen, 2010).

1.6. Microbiota

The microbiota is the population of microorganisms composed of commensal bacteria and other microorganisms (fungi, archaea, protozoa and viruses) which mostly colonize the epithelial surfaces of the host. The microbiota impacts various systems in the host and modifies innate and adaptive immune responses. The microbiota differ between all individuals and is shaped by the individual's lifestyle, genetic background, type of the birth delivery, colonization at the time of birth, disease incidence and antibiotic usage (Roy and Trinchieri, 2017). Microbial colonization starts after birth and evolves in the first years of human life (Maynard *et al*., 2012). In mature individuals, composition of microbiota remains fairly constant but there can be some changes in composition according to changes in lifestyle, diet or disease progression.

In the human body, microbial community colonizes anatomical locations such as skin, hair, nostrils, oral cavity, gastrointestinal tract, mouth etc. (Figure 1.6). The highest density of microbiota is found in the gastrointestinal tract with approximately $3x\ 10^{13}$ bacterial cells that generally exhibit commensalism with host (Sender *et al*., 2016). It is also known that gut microbiota exhibits mutualism with host by promoting bone marrow haematopoiesis, modulating immunity and regulating maturation and function of tissue resident cells (Erny D. *et al*., 2015). Normally, immune system maintains tolerance against microbiota. Microbiota is able to control many aspects of innate and adaptive immune responses (Molloy *et al*., 2012).

In healthy individuals, microbiota associated with epithelial barriers maintains protection against pathogens. Changes induced by diet change, antibiotic treatment or exposure to pathogens can lead to perturbations in the microbiota. Massive perturbations in gut microbiota cause dysbiosis which is characterized by imbalance

in the normally found microbial species (Rooks and Garrett, 2016). Dysbiosis is associated with susceptibility to several pathologies such as inflammatory diseases, metabolic disorders and allergies.

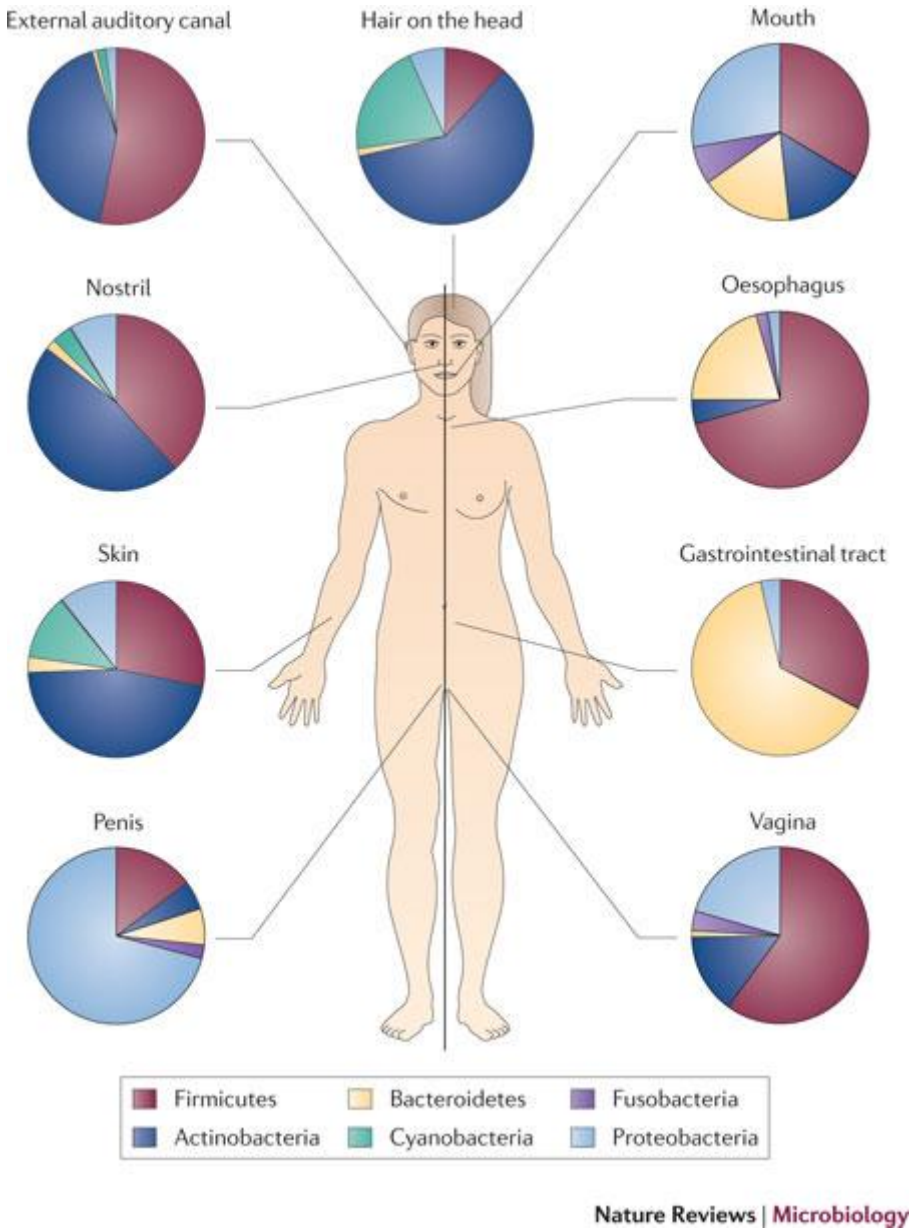

Figure 1.6 Microbiota composition in different body compartments (Adapted from Spor and Koren *et al.*, 2011)

Commensal bacteria play important roles in the regulation of immune responses. They promote both protective immunity and down modulate inflammation by activating IL-10 secreting regulatory T cells (Ichinohe et al., 2011, Ochoa-Repáraz et al., 2010). *Streptococcus, Lactococcus* and *Streptomyces spp.* which are members of gram positive bacteria are known to produce bacteriocins to prevent other bacterial strains' growth (Gallo *et al*., 2012). Commensal bacteria also reside on the skin where their products regulate the process of wound healing and restrain harmful inflammatory responses in case of tissue damage (Belkaid et al., 2013).

Considering the importance of commensal bacteria in immune regulation, we have focused on *Pediococcus pentosaceus* which is a member of Lactobacillaceae family. Lactobacilli strains are found in genitourinal tract and gut where they participate in tissue homeostasis by the secretion of antimicrobial factors (Spurbeck et al., 2011). Therefore, we isolated membrane vesicles from *Pediococcus pentosaceus* and further analyzed their immune modulatory properties.

1.7. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an uncontrolled chronic inflammatory disorder of gastrointestinal tract and comprises two disorders: ulcerative colitis (UC) and Crohn's disease (CD). IBD onset generally occurs at the age of 20 to 30 and majority of affected individuals progress to relapsing and chronic disease (Xavier and Podolsky, 2007).

In the ulcerative colitis, mucosal inflammation is restricted to the colon, whereas in Crohn's disease any site of the gastrointestinal tract (GI) can be affected. Histological properties of ulcerative colitis show, presence of significant numbers of neutrophils within lamina propria and crypts which causes abscess. In Crohn's disease any site of GI can be affected but commonly the terminal ileum, cecum, peri anal area and colon is involved. Inflammation can be patchy and segmental which is called as skip lesions whereas in ulcerative colitis inflammation is continuous (Bouma and Strober, 2003).

For the treatment of inflammatory bowel disease generally anti inflammatory agents and TNF- α blockers are used. In more severe conditions, surgical removal of obstructing segments and colectomy are performed. Dextran sulfate induced colitis is widely used as a model of ulcerative colitis in mice, mimicking the pathological changes that occur in humans.

1.8. Wound Healing

Wound healing is a complex biological process essential to maintain tissue homeostasis. The inflammatory response to wounding starts immediately through migration of circulating leukocytes (mostly neutrophils) from blood vessels to the wound area. Figure 1.7 summarizes the inflammatory cells that accumulate during different phases of wound repair. The phasesare divided to the following 4 groups: homeostasis, early inflammation, late inflammation and resolution/remodeling (Koh and DiPietro, 2011).

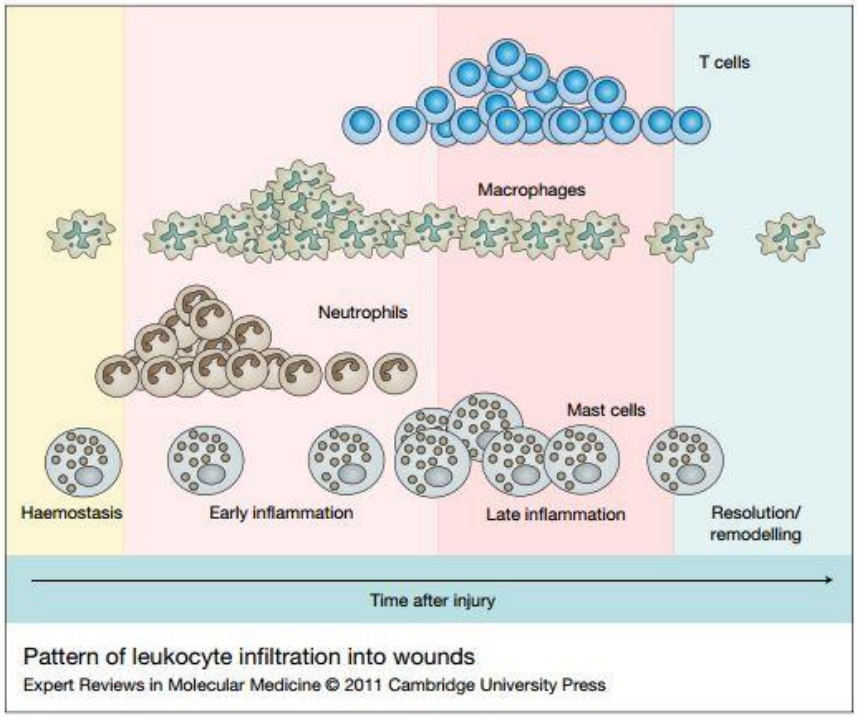

Figure 1.7. Pattern of leukocyte infiltration into wounds (Adapted from Koh and DiPietro 2011).

Some of the tissue resident cells (mast cells, γδ T cells and Langerhans cells) produce cytokines and chemokines to recruit inflammatory cells to wound area (Noli and Miolo, 2001, Jameson et al., 2004, Cumberbatch et al., 2000, Shaw and Martin, 2009). At the same time, inflammatory mediators released and antifibrinolytic coagulation cascade have been started to activate clotting. Platelet activation help the recruitment of inflammatory monocytes to the site of tissue injury. Recruited monocytes differentiate to M1 macrophages under the influence of proinflammatory cytokines. Figure 1.8.summarizes the role of macrophage subsets in wound healing. M1 macrophages secrete IL-6, IL-12, IL-1, TNF-α and inducible nitric oxide synthetase (iNOS) in early wound healing phase to support inflammatory antimicrobial response. M1 macrophages secrete matrix metalloproteases (MMPs) MMP2 and MMP9 to degrade the extracellular matrix and help in recruitment of inflammatory cells to the site of injury (Murray and Wynn, 2011). After the inflammatory stimulus or pathogen is eliminated, M1 macrophage activation is diminished and cytokines such as IL-4 and IL-13 produced by Th2 cells, mast cells and basophils promote accumulation of M2 macrophages. M2 macrophages regulate wound healing and fibrosis by secreting MMPS, growth factors and the regulatory cytokine TGF-β. During the last stage of wound healing, M2 macrophages express arginase 1 (Arg1), resistin like molecule-α (RELMα), IL-10 and programmed death ligand -2 (PD-L2) which leads to suppressive – regulatory response. M2 macrophages play an important role in facilitating resolution of wound healing and restore homeostasis by limiting fibrosis and collagen synthesis.

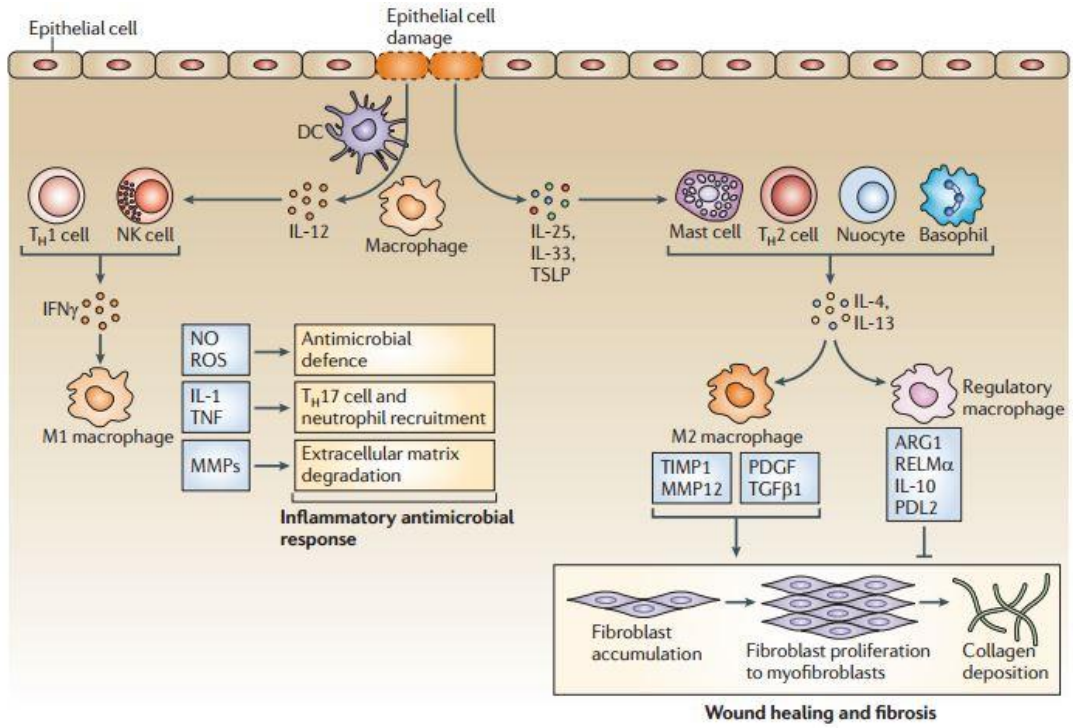

Figure 1.8 Role of macrophage subsets in regulation of inflammation and wound healing. (Adapted from Murray and Wynn,2011).

1.9. Aim of the study

This thesis aims to test the immunomodulatory potential of membrane vesicles (MVs) secreted from the human commensal bacteria *Pediococcus pentosaceus*. In our previous studies (MSc thesis by Esin Alpdundar, 2013), we worked with 5 different commensal bacteria derived membrane vesicles and compared their effects on immune cells with respect to E.coli derived outer membrane vesicles. After characterization of in vivo and in vitro immunomodulatory properties of commensal derived MVs, we have decided to continue our studies with the most potent MV that displayed the highest level of immunomodulatory activity.

To further asses the immunomodulatory features of MVs, we first tested how MVs modified the immune response generated against a protein antigen. After confirming that commensal bacteria derived MVs suppressed Th-1 dominated IgG2a production and cytotoxic T-cell responses, we aimed to deliniate the mechanism through which

MVs exerted their modulatory functions by analyzing possible suppressor cell types that differentiated in response to MV exposure. Since our findings supported the hypothesis that MVs led to generation and activation of immunosuppressor myeloid derived cell types, we also explored the potential anti-inflammatory effects of membrane vesicles in different acute inflammation models (peritonitis and DSS induced colitis). Finally, we aimed to assess the effect of MVs in a wound healing model.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell culture media and standard solutions

RPMI1640 media were purchased from Gibco and Biological Industries (BI). DNAse/RNAse free water, HEPES, L-glutamine, penicillin-streptomycin, non-essential amino acids and Fetal bovine serum (FBS) were purchased from Lonza and BI. Components of various culture media and buffers were given in detail in Appendix A.

2.1.2. Reagents

Antibodies used in flow cytometry were listed in the following table.

Table 2.1. Antibodies used in Flow Cytometry Assays

Product Name	Catalog Number	Company
APC anti-mouse CD274 (B7-H1, PD-L1)	124311	Biolegend/USA
APC anti-mouse CD273 (B7-DC, PD-L2)	107210	Biolegend/USA
FITC anti-mouse F4/80	123107	Biolegend/USA
PE anti-mouse F4/80	123110	Biolegend/USA
PE anti-mouse Ly-6C	128008	Biolegend/USA
FITC anti-mouse Ly-6G	127606	Biolegend/USA
PE/Cy7 anti-mouse Ly-6G	127618	Biolegend/USA
PE anti-mouse/human CD11b	101208	Biolegend/USA
Brilliant Violet 605 anti-mouse CD45	103155	Biolegend/USA
PE anti-mouse/rat/human FoxP3	320008	Biolegend/USA

Antibodies used in cytokine ELISA are listed in Table 2.2. For IgG ELISA, anti-mouse IgG1, IgG2c and total IgG were purchased from Southern Biotech (USA). P-nitrophenyl phosphate (PNPP) substrate were purchased from Thermo scientific and TMB substrate solution were purchased from Biolegend, USA.

Table 2.2. Antibodies used in cytokine ELISA

Product Name	Catalog Number	Company
Mouse IL-10	431401	Biolegend
Mouse IL-10	3431-1A-20	Mabtech
Mouse IFN-γ	430803	Biolegend
Mouse IFN-γ	3321-1A-20	Mabtech
Mouse IL-6	431303	Biolegend
Mouse TNF-α	439903	Biolegend

Recombinant cytokines and growth factors used in this study were listed in Table 2.3.

Table 2.3. Recombinant cytokines and growth factors

Product Name	Catalog Number	Company
Recombinant IFN-γ	21-8311-020	Tonbo Biosciences
Recombinant M-CSF	576404	Biolegend
Recombinant M-CSF	21-8983-U010	Tonbo Biosciences

2.1.3. Ligands and Antigens

PRR ligands were used in both in vitro and in vivo studies. Lipopolysaccharide (LPS) was obtained from Sigma, USA. Peptidoglycan and zymosan were purchased from Invivogen, USA. Chicken ovalbumin (OVA) antigen was obtained from

Sigma,USA. OVA MHC class I epitope SIINFEKL peptide was obtained from Anaspec,USA.

2.1.4. Bacterial Strains

Table 2.4.shows the bacterial strains used in this thesis.

Table 2.4. Bacterial Strains

Strains	Source	Gram Staining
Pediococcus pentosaceus	Infant Feces	+
Escherichia coli (DH5α)	ATCC 67877	-

2.1.5. Bacterial Culture Media and growth conditions

MRS medium and MRS agar were purchased from CONDA, Spain and prepared according to the manufacturer's protocol. Pediococcus pentosaceus was cultured at 37°C overnight in MRS agar plates or broth medium. Escherichia coli (DH5α) was grown at 37°C, at 150 rpm overnight in Luria Broth (LB) agar or medium (Appendix A). Agar plates were stored 1-2 months at +4°C; however for long term storage, a single colony was picked from the plate using a sterile wire loop and incubated overnight in growth medium. Following overnight incubation, the bacterial suspension was mixed in 1:1 V/V ratio with %40 glycerol solution and stored at -80°C.

2.2. Methods

2.2.1. Isolation of membrane vesicles

Bacterial growth curves and conditions were established previously as described in the MSc thesis (Esin Alpdundar, 2013). Briefly, *Streptococcus pentosaceus* broth culture was adjusted to 0.01 OD at 600nm and incubated overnight until 1 OD was achieved (stationary phase). Samples were then centrifuged at 6000 rpm for 20 minutes and cell-free supernatants were collected. To eliminate the possibility of

residual bacterial contamination, supernatants were filtered through a 0.2 μm filter. Filtered supernatants were then centrifuged twice (Hitachi, Himac ultracentrifuge) at 100,000g for 70 minutes. followed by an additional washing with PBS at 100,000g for 70 minutes. Finally, the membrane vesicle pellets were resuspended in PBS (500-1000 μl) and stored at -20°C until further use. Figure 2.1. summarizes the differential centrifugation protocol used in MV isolation.

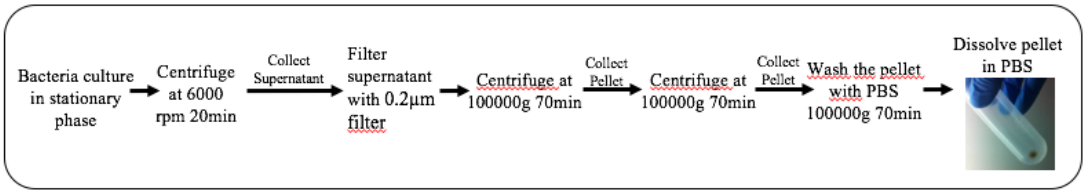

Figure 2.1. Membrane vesicle isolation protocol

2.2.1.1. Encapsulation of c-di-GMP and OVA into the Membrane Vesicles

Membrane vesicles (10μg/mouse) were mixed with c-di-GMP (15μg/mouse) or OVA (7.5μg/mouse) in PBS for encapsulation protocol. Encapsulation was performed by rehydration – dehydration method. Firstly, MV + c-di-GMP and MV + OVA were snap-frozen by using liquid nitrogen and freeze-dried overnight by using VirTis Benchtop K. Next day, DNase/RNase free dH_2O (1/10 amount of initial volume of mixture) was added to freeze dried samples to start encapsulation by rehydration method. Mixture was vortexed vigorously 6 times at 5 minutes intervals at room temperature. PBS was added to the mixture (4.5/10 amount of initial volume of mixture) and incubated for 10 minutes at room temperature. Finally, 4.5/10 volume of PBS was added to obtain the initial volume of the mixture. MVs encapsulated with OVA and c-di-GMP were stored at +4°C until further use.

2.2.2. Membrane Vesicles Characterization

2.2.2.1. Protein Quantification

Purified membrane vesicles were quantified by measuring protein concentration at 280 nm of absorbance by using a Nanodrop (BioDrop DUO).

2.2.2.2. Membrane vesicles analysis by polyacrylamid gel electrophoresis

To analyze membrane vesicles and whole bacterial lysates protein content, SDS-PAGE electrophoresis was used. Gels were prepared by using TGX Stain-free FastCast Acrylamide kit (10%; Bio-Rad, United States) according to the manufacturer's protocol. Briefly, resolving gel was prepared by mixing resolver A (3ml), resolver B (3ml), 10% APS (3 µl) and TEMED (3µl).. Stacking gel was prepared by mixing stacker A (1ml), stacker B (1ml), 10% APS (10µl) and TEMED (2µl). Following polymerization of the resolving gel, stacking gel solution was added on top and the plastic comb was inserted between the glass plates containing the gel. 25µg/well MVs (20µl/well) were mixed with 3X loading buffer (New England BioLabs) (10µl/well). Heat killed bacteria was prepared from steady-state bacterial culture (1ml) by heating to 70°C for 1 hour. Killed bacteria was washed and resuspended in 1 ml PBS. 20 µl of this suspension was mixed with 10 µl of 3X loading buffer. All samples were denatured for 5 minutes at 70°C. Samples were then loaded into wells and electrophoresis was conducted for 60 minutes at 185 V in running buffer (Appendix A). The gel was washed with dH_2O 3 times for 5 minutes to get rid of the running buffer and detergent. Gel was fixed with destaining solution which contains methanol and acetic acid (Appendix A) for 30 minutes to 1 hour. Staining was conducted with coomassie brilliant blue dye for 1 hour followed by destaining for an additional 1 hour to minimize background. Protein bands were visualized using the Bio-rad ChemiDoc MP Imaging System.

2.2.3. Cell Culture Conditions

2.2.3.1. Cell Lines

2.2.3.1.1. E.G7-OVA

E.G7-OVA cell line is a mouse lymphoma cell line and derived from C57BL/6 (H-2 b) EL4 cells (ATCC CRL-2113). This cell line is transfected with complete copy of chicken ovalbumin (pAc-meo-OVA plasmid) and neomycin (G-418) resistance gene and is used as a cell line constitutively expressing OVA as a model tumor antigen E.G7-OVA cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 mg/ml neomycin. Cell cultures were passaged every 2-3 days until they reached 80% confluency.

2.2.3.2. Preparation of Single Cell Suspensions from Mice

2.2.3.2.1. Maintenance of Animals

Animal studies were conducted at Bilkent University, Animal Housing Facility of the Department of Molecular Biology and Genetics. The ethical committee of Bilkent University approved all animal research experiments. *In vivo* disease and/or vaccination studies as well as *in vitro* experiments were conducted using BALB/c or C57BL/6 mice. Animal housing conditions were regulated using 12 hours dark/light cycles with steady temperatures (22°C ±2) and ad libitum food - water sources.

2.2.3.2.2. Preparation of Single Cell Suspensions from Spleens

Female or male C57BL/6 or BALB/c mice were sacrificed by cervical dislocation and spleens were removed and placed into 2% FBS containing RPMI 1640 wash buffer. Single cell suspensions were prepared by mashing spleen by using the back of the sterile syringe plunger in wash buffer. Cells were washed twice with wash buffer and centrifuged at 300 g for 5 minutes. After the second wash, cells were resuspended in 10% FBS supplemented RPMI and prepared for cell counting. Cell counting was performed by flow cytometer as described in section 2.2.3.3.

2.2.3.2.3. Bone Marrow Derived Macrophage (BMDM) Generation

C57BL/6 or BALB/c mice were euthanized by cervical dislocation and bones (femurs and tibias) were removed to collect the marrow. First, bones were washed with 70% ethanol to eliminate possible contaminants. To collect the bone marrows, both ends of bone were cut and bone marrow was extruded by flushing the cavity with 2% RPMI medium and a 21G syringe. Collected cells were passed through a 40 μm cell strainer (Falcon,USA) to obtain single cell suspension. Cells were washed in 2% FBS supplemented RPMI. Red blood cells were lysed using 2ml of ACK Lysis Buffer and incubation for 2 minutes at room temperature. Cells were washed for the last time and prepared for cell counting by re-suspension in 20% FBS supplemented RPMI. Cells were layered to petri dishes ($1x10^6$ cells/ml) or 48 well plates (600,000 cells/ml) and incubated in the presence of 20 ng/ml M-CSF for 6 days. At day 3, fresh 20 ng/ml M-CSF containing medium was added to the petri dishes (2 ml) or plates (200 μl) 6 days after the initiation of culture, cells were collected after incubation on ice for 5 minutes and extensive gentle pipetting. Following washing, cells were stimulated with MVs and/or other ligands.

2.2.3.2.4. Differentiation of Bone Marrow Cells by Membrane Vesicles

Bone marrow progenitor cells were isolated from C57BL/6 or BALB/c mice bones (Section 2.2.3.2.3) and layered to petri dishes or plates. Cells were incubated with 10 μg/ml MV in 20 % FBS supplemented RPMI medium. Additional MV containing media were introduced to cultures on day 3. After 6 days, cells were collected to assess the phenotype of differentiated cells by staining for specific cell-surface markers and imaging the shape of cellular nuclei. NucBlue Live Cell Stain (Molecular probes) was used to stain nuclei (10μl/well) by incubation for 15 – 30 minutes at room temperature. Cells were visualized using EVOS Floid cell imaging system (ThermoFisher Scientific).

2.2.3.2.5. Preparation of Single Cell Suspension from Wound with Liberase DL solution

8-12 weeks old BALB/c mice with excisional wounds were sacrificed at the end of one week and wounds were removed gently using surgical scissors. Wound tissue was cut into small pieces to improve the cell yield. Liberase DL solution (Roche) was diluted 1:5 with 2% FBS containing RPMI 1640 wash buffer to obtain 2.6 Wünsch units/ml working concentration. 100 µl/tube liberase solution was added to samples with the wound tissue and incubated at 37°C for 150 minutes. Tubes were vortexed vigorously every 20 minutes for 8 times. At the end of the incubation period, 100 µl 0.5% trypsin/EDTA was added and incubated for an additional 15 minutes at 37°C. After a final vortex, collected cells were passed through a 35µm cell strainer and washed with PBS. Cells were centrifuged at 600 xg for 5 minutes and fixed as described in Section 2.2.4.4.1.

2.2.3.3 Cell Counting

Single cells suspensions were pelleted and resuspended in 1 ml of 10% FBS supplemented RPMI or 10% FBS supplemented DMEM media. 20 µl of cells were transferred into 10 ml of Isoton II diluent buffer or PBS containing 3 drops of Zap-oglobin II Lytic Reagent to lyse red blood cells. Cell debris and apoptotic cells were excluded by gating live cells and particles within this gate was counted by using a flow cytometer (BD Accuri C6 or ACEA Novocyte). Final cell count was calculated by multiplying the obtained count with dilution factor (x 25,000).

2.2.4. Determination of Immunomodulatory Effects of Membrane Vesicles

2.2.4.1. In Vitro Stimulation with MVs

Immunomodulatory effects of membrane vesicles were determined in stimulation assays. Mouse splenocytes (400,000 cells/well), peritoneal exudate cells (100,000 cells/well) or BMDMs (200,000 cells/well) were stimulated in a total volume of 200 µl in 96-well flat-bottom plates with 3 different concentration of MVs (0.2 µg/ml, 1

μg/ml, 5 μg/ml). PGN (5 μg/ml), zymosan (10 μg/ml) or LPS (1 – 10 μg/ml) was used as positive controls in stimulations. Cells were incubated at 37°C for 24 hours, supernatants were collected and cytokine levels in culture supernatants were determined with cytokine ELISA. In some experiments, cells were collected from plates and were either fixed with 4 % paraformaldehyde and stained or stained without fixation on ice with specific surface markers, followed by flow cytometric analysis.

2.2.4.2. ELISA (Enzyme Linked Immunosorbent Assay)

Supernatants were collected and stored at -20°C after stimulation of cells. 96 well Immulon 2HB (Thermo Scientific, USA) or ELISA Immuno plates (SPL) were coated with specific anti-cytokine antibodies (50 μl/well) at different concentrations as described in Table 2.6. After overnight incubation at 4°C, plates were blocked with 200 μl/well blocking buffer (Appendix A) at room temperature for 3 hours. Following blocking, plates were washed with wash buffer (Appendix A) for 5 times. Supernatants and serially diluted recombinant cytokines were added into wells (50 μl/well) and incubated for 3 hours at RT. Following incubation, plates were washed as described before and biotinylated anti-cytokine detection antibodies in T cell buffer (Appendix A) were added to the wells (50 μl/well) and incubated overnight at 4°C. Plates were washed and previously prepared streptavidin-alkaline phosphatase solution (SA-AP) (1:1000 dilution in T cell buffer; 50 μl/well) or Avidin-horseradish peroxidase (Avidin-HRP) (1:1000 dilution in T cell buffer, 50 μl/well) were added to wells and incubated 1 hour at room temperature. For development of streptavidin-alkaline phosphatase containing plates, 50 μl/well of PNPP solution (1 tablet, 4ml ddH_2O, 1ml PNPP buffer) were added. Color development was followed at 405 nm using an ELISA plate reader (Multiskan FC Microplate Photometer, Thermo Scientific). For development of Avidin-horseradish peroxidase conjugate containing plates, 50μl of TMB substrate was added to wells and incubated for color development. Color development was followed by checking standards and blank

wells, and the reaction was stopped using sulfuric acid containing stop solution (30 µl/well). Color development was measured at 450 nm on an ELISA plate reader (Multiskan FC Microplate Photometer, Thermo Scientific). To minimize background, color development was also measured at 570 nm and substracted from values at 450 nm.

Table 2.5 ELISA antibody working concentrations and substrate conditions

Product Name & Company	Coating Concentration	Detection Concentration	Recombinant Standard Concentration	SA-AP or HRP	Substrate Type
mIL-10 (Biolegend)	1:200	1:200	2ng/ml	HRP 1:1000	TMB
mIL-10 (Mabtech)	2µg/ml	0,1µg/ml	2ng/ml	SA-AP 1:1000	PNPP
mIFNg (Mabtech)	1µg/ml	0,5µg/ml	10ng/ml	SA-AP 1:1000	PNPP
mIL-6 (Biolegend)	1:200	1:200	5ng/ml	HRP 1:1000	TMB
mTNF-α (Biolegend)	1:200	1:200	5ng/ml	HRP 1:1000	TMB

2.2.4.3. Determination of Gene Expression

2.2.4.3.1. Total RNA Isolation

Stimulated or unstimulated cells ($1 – 5 \times 10^6$) were collected into Eppendorf tubes and centrifuged at 300 g for 10 minutes to obtain pellets. For lysing the pellet, 1 ml of Trizol Reagent (Life Technologies) was added and mixed by pipetting. 200 µl of chloroform was added onto cell lysates and either shaken vigorously or vortexed for 15 seconds and then incubated at RT for 3 minutes. After incubation, samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. Following this, the aqueous phase was collected (generally 60% of the total volume) and transferred to a new Eppendorf tube. 500 µl of cold isopropanol was added and mixed gently. Samples

were incubated for 10 minutes at room temperature and then centrifuged at 13,200 rpm for 12 minutes at 4°C. Supernatants were discarded carefully. Pellets were washed twice with 75% EtOH and 100% EtOH, respectively. Pellets were centrifuged at 8000 rpm for 8 minutes at 4°C in each washing step. After discarding EtOH with pipette, cell pellets were left in a tilted position on a petri dish to allow air drying inside a laminar flow hood cabinet. Dried RNA pellets were resuspended in 20 µl of RNase/DNase free ddH_2O. RNA purity and concentration was determined by measuring sample OD values by using Nanodrop (BioDrop DUO). A260/A280 ratio between 1.8 – 2.2 was considered as of sufficient purity. Samples were stored at -80°C until further use.

2.2.4.3.2. cDNA Synthesis

cDNAs were synthesized from total RNA samples by using the cDNA synthesis kit (New England BioLabs) according to the manufacturer's instructions. All procedures were carried out on ice. Briefly, 6 µl final volume of 500 ng - 1µg total RNA was mixed with 2 µl Oligo(dT) and samples were denatured for 5 minutes at 70°C in Runik Thermal Cycler. At the end of denaturation, tubes were spinned down and 10 µl reaction mix and 2 µl enzyme mix were added. Negative control without enzyme mix was also prepared. This 20 µl final volume cDNA synthesis reaction mixture was incubated at 42°C for 1 hour and then incubated at 80°C for 5 minutes. cDNA samples were diluted to 50 µl by adding 30 µl of RNase/DNase free ddH_2O and stored at -20°C for further use.

2.2.4.3.3. Taqman Gene Expression Assay

Taqman gene expression assay was used to detect two macrophage markers: Arg1 (Mm00475988_m1) and NOS2 (Mm00440502_m1) (Applied Biosystems). 18S ribosomal RNA with reporter VIC/MGB was used as endogenous control and Taqman Universal master mix II was used as the master mix. Briefly, 0,5 µl of

primer, 0,5 µl of probe and 4,5 µl of master mix was prepared for each reaction and 4,5 µl of sample was added to each well. Thermal cycling conditions were summarized at Table 2.7. For RT-PCR, Bio-Rad CFX Connect Real-time system was used. Expression levels were determined by normalization to 18S rRNA.

Table 2.6. Thermal cycling conditions of Taqman gene expression assay

Thermal Cycling Conditions			
Parameter	**Polymerase Activation**	**PCR (40 Cycles)**	
		Denature	**Anneal/Extend**
Temperature (°C)	**95**	**95**	**60**
Time (mm:ss)	**10:00**	**0:15**	**1:00**

2.2.4.4. Flow Cytometry Analysis

2.2.4.4.1. Fixation of Cells

Cells were collected to Eppendorf tubes and centrifuged at 300g for 5 minutes. Supernatants were collected for ELISA and cell pellets were fixed in 4% paraformaldehyde containing Fixation medium A (100 µl/tube) at room temperature for 15 minutes. After fixation, cells were washed twice by adding 1 ml FACS buffer (Appendix A) and stored at 4°C for surface marker staining upto a week.

2.2.4.4.2. Cell Surface Marker Staining

Fixed cells were centrifuged at 300g for 5 minutes and resuspended with 100 µl FACS buffer containing 1 µg/ml of fluorochrome conjugated antibodies (Table 2.1). Cells were incubated for 30 minutes in dark at room temperature. After the incubation, 1 ml FACS buffer was added to the cells and centrifuged. Cells were washed with FACS buffer for the last time and resuspended in 200 µl PBS and analyzed on a BD Accuri C6 flow cytometer or NovoCyte flow cytometry (ACEA Biosciences).

2.2.4.4.3. Detection of Cytokine Levels from Blood Sera by Cytometric Bead Array (CBA)

Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences, USA) was used according to the manufacturer's protocol. The kit contains beads that are coated with specific capture antibodies for TNF, IL-17A, IL-4, IL-6, IL-2 and IL-10 which leads to the detection of cytokine content of samples. Briefly, lyophilized standards were two fold serially diluted with assay diluent from 1:2 to 1:256 (20 – 625 pg/ml). 50 µl of standards or samples and 50 µl of capture beads were added into 96-well plates and incubated at room temperature for 2 hours. After incubation, 100 µl wash buffer was added into the wells and centrifuged at 300 g for 5 minutes. Following addition of PE-labeled detection reagent, second washing step was performed by adding 200 µl wash buffer and centrifuged at 300g for 5 minutes. Samples were resuspended with 200 µl wash buffer and analysis was done by using ACEA Novocyte. Samples were analyzed by using FCAP array software (BD Biosciences).

2.2.5. In Vivo Experiments

2.2.5.1. Immunization of Mice with OVA model antigen

6–8 weeks old C57BL/6 mice were immunized with OVA model antigen (7.5 µg/mouse) in the absence or presence of MVs (10 µg/mouse) intraperitoneally on days 0 and 14. Primary and secondary bleeding were done on days 15 and 33, respectively. Blood samples were collected from tail veins and incubated at 37°C for 2 hours at incubator. Following clot formation, sera were collected to new tubes and centrifuged at 8000 rpm for 10 minutes. Finally, sera were transferred to 96 well plates and stored at -20°C until use.

2.2.5.1.1. Tumor challenge with EG.7 cell line

Animals immunized as described in Section 2.2.5.1 were challenged with E.G7-OVA tumorigenic cell line on day 51. For this, E.G7 cells were cultured and expanded as

described in Section 2.2.3.1.1. Mice were injected subcutaneously with $4x10^6$ E.G7-OVA cells into their right dorsal flanks. Tumor development was measured daily by a caliper and calculated as (length) x (width) x (height) and recorded as mm^3. Experiment design is summarized in Figure 2.2.

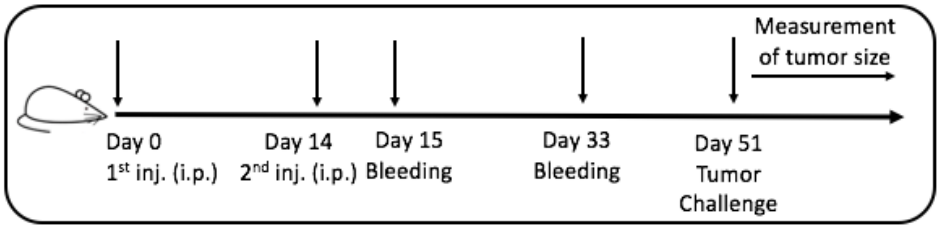

Figure 2.2. Experimental design summarizing OVA immunization and tumor challenge model

2.2.5.1.2. Measurement of OVA-specific IgG by ELISA

Immulon 1B microtiter plates were coated with 7.5 μg/ml (50 μl/well) OVA protein and incubated overnight at 4°C. Next day, plates were blocked with 200 μl/well blocking buffer and incubated at room temperature for 2 hours. After the washing step, 16X diluted sera were added to the first row of the plate and 4-fold serially diluted 8 times. Plates were incubated overnight at 4°C and washed. Following the washing step, alkaline phosphatase conjugated anti-immunoglobulin antibodies (1000 X diluted in T cell buffer; total IgG, IgG1, IgG2c) were added and incubated for 3 hours at RT. Finally, plates were washed and PNPP substrate was added (50 μl/well). OD values were detected at 405 nm using an ELISA plate reader (Thermo Scientific, USA).

2.2.5.1.3. IFN-γ ELISPOT

To determine OVA specific CD8+ T cell response, immunized mice splenocytes were stimulated with OVA MHC Class I specific epitope SIINFEKL peptide (257-264 peptide, Anaspec) and IFN-γ production was determined using ELISPOT. Two days before the SIINFEKL peptide stimulation of splenocytes, Immulon 2 HB plates were coated with 5 μg/ml anti-mouse IFN-γ antibody and incubated overnight at 4°C. Next day, plates were incubated at room temperature for 4 hours and then blocked with 200 μl of blocking buffer and incubated overnight at 4°C. At the day of experiment, blocked plates were washed for 5 times with wash buffer and 3 times with dH_2O for 5 minute intervals and kept at room temperature in laminar flow hood for further use. Splenocytes were prepared in a separate 96-well plate as $20x10^6$cells/ml and 4 fold serially diluted for 4 times. 150 μl of 10% Regular RPMI medium with SIINFEKL peptide were added on first 4 rows of coated plates and 150 μl of 10% Regular medium without SIINFEKL were added on last 4 rows as control of OVA specific IFN-γ production. 50 μl of serial diluted splenocytes were added to the top and bottom 4 rows. Plates were incubated overnight at 37°C in a CO2 incubator with special attention to not to move the plates during the incubation period. After incubation, plates were washed with wash buffer specific to ELISPOT assay (ddH_2O, Tween20) to disrupt any remaining cells and 50 μl of biotinylated anti mouse IFN-γ (1 μg/ml) was added for detection. Following 2 hours incubation at room temperature, Streptavidin-alkaline phosphatase (1:1000 dilution, 50 μl/well) was added to the wells and incubated for 1 hour at RT. After washing with wash buffer (Appendix A), Low melting agarose – BCIP substrate (4 ml 45°C heated BCIP solution + 1 ml low melting agarose (0.03gr)) was added (70 μl/well) to develop the spots. Next day, a dissecting microscope was used to count spots.

2.2.5.2. Determination of phenotype of cells generated following intraperotoneal injection of MVs

8 – 12 weeks old C57BL/6 mice (5 mice/group) were intraperitoneal (i.p.) injected with MVs and PBS as control at day -4 and -1. At day 0, mice were sacrificed by cervical dislocation. PEC and spleen cells were collected. Spleens were collected and single cell suspension were obtained as described in 2.2.3.2.2. Both PECs and splenocytes were fixed as described in section 2.2.4.4.1. Cells were stained with different cell surface markers to determine the phenotype of cells generated following MV injection.

2.2.5.3. Zymosan induced Peritonitis Model

8 – 12 weeks old male C57BL/6 mice were immunized with MVs (10 μg/mouse) and saline on day -3 and -1. At day 0, zymosan (100 μg/mouse) was administered to mice to induce peritonitis. 6 hours later, mice were sacrificed and peritoneal exudate cells (PEC) were collected by injecting 10 ml PBS to peritoneal cavity and collecting resident cells. Collected cells were fixed and surface stained as described previously. Blood samples were collected from heart and sera were prepared as described before in Section 2.2.5.1. Sera were used for detection of cytokines by using ELISA.

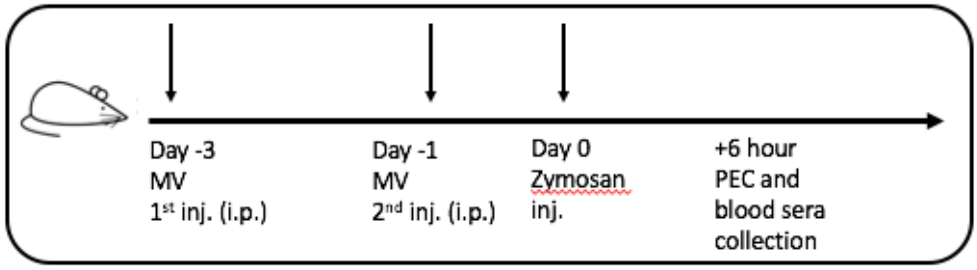

Figure 2.3. Experimental design of zymosan induced peritonitis model

2.2.5.4. Dextran Sulfate Sodium (DSS) Induced Acute Colitis Model

8 weeks old C57BL/6 mice were administered 3% Dextran Sulfate Sodium (36,000-50,000 molecular weight – Colitis Grade) (MP Biologicals) in drinking water for 4 days. MVs (10 μg/mouse) were injected i.p. on days -3 and 0 (pretreatment group) or on days 0 and +3(post-treatment group). Development of colitis was followed by measuring body weight and examination of blood in stools on a daily basis. After 4 days, drinking water without DSS was provided for an additional 3 days to allow colon epithelial cell recovery. At day 7, mice were sacrificed to analyze disease progression. Colons were dissected from surrounding mesentery and removed carefully. Colons were transected from both ends very carefully taking care not to damage their integrity. Isolated colons were then cleaned of stool by using a 10 ml syringe and gavage needle and flushing PBS through. Colon lengths were measured using a caliper and each colon was cut longitudinally by scissors. Flattened colon was rolled with the help of a toothpick and at the end fixed with 27G1/2 needle. The "Swiss-rolled" colons were placed in 10% buffered formalin solution for further histological analysis. Blood samples were collected from heart and used for detection of cytokine levels.

2.2.5.5. Wound Healing in Excisional Wound Model

8 – 12 weeks old BALB/c mice were used for wound healing experiments. Briefly, hair at the back of mice were shaved and prepared for excisional wound. Mice were anesthetized by using xylazine and ketamine mixture. Circular wounds were introduced using a 6 mm biopsy punch. To prevent contraction, a silicone ring splint (Invitrogen, USA) was incorporated around the wound using a tissue adhesive and then stabilized with sutures. Mice were observed daily and MV treatments were given every other day. MVs (10 μg/mouse) were administered either topically on the wound or by i.p. injections. Wound area (length and width) measurements were conducted every other day. 1 week later, mice were sacrificed and wound area was removed for histological analysis. PEC were collected and $4x10^6$ cells were fixed as

described in 2.2.4.4.1. and stained for cell surface markers. PEC was also stimulated with various PRR ligands and cytokine responses were analyzed by ELISA.

2.2.6. Statistical Analysis

For Statistical analysis, Kruskal-Wallis test was used to test difference among groups, Mann-Whitney U test was used to compare control vs. treatment groups. For the indicated in vivo studies, one-way ANOVA following Dunnett's multiple comparisons test was used. All analyses were done with the GraphPad Prism 6 (GraphPad Software Inc.). $p<0.05$ were considered as significant.

CHAPTER 3

RESULTS & DISCUSSIONS

3.1. Determination of Immunomodulatory properties of Commensal Bacteria derived Membrane Vesicles

All cellular life forms, including eukaryotes and prokaryotes are known to secrete vesicles into the environment. These extracellular vesicles are called as exosomes or microparticles in the case of eukaryotes and named as membrane vesicles (MVs) in the case of prokaryotes. Although the MV secretion phenomenon was initially thought to occur only in gram negative bacteria, it is now known that gram positive bacteria are also capable of secreting MVs (Yanez-Mo et al., 2015). MVs are enriched of several bacterial components including, membrane and cytosolic proteins, DNA, RNA, lipopolysaccharide (LPS), peptidoglycan and enzymes (Kaparakis-liaskos and Ferrero, 2015). Although bacterial MV studies mostly focused on their role in modifying host-pathogen interactions (Prados-Rosales et al., 2014 and Choi et al., 2015), physiological role of commensal-derived MVs remained largely ignored (Yanez-Mo et al., 2015). Available evidence suggests that commensal derived MVs might exert immunomodulatory functions (Shen et al., 2012; Zakharzhevskaya et al., 2017).

In our previous studies (MSc thesis by Esin Alpdundar, 2013), we worked with 5 different commensal bacteria derived membrane vesicles and compared their immune activity with E.coli derived outer membrane vesicles. All commensal bacteria strains (*Pediococcus pentosaceus, Lactobacillus salivarius, Lactobacillus fermentum, Enterococcus faecium*) were isolated from human sources (infant feces, breast milk) and E.coli DH5α strain was purchased from ATCC. Membrane vesicles

isolated from all strains were extensively characterized to confirm their vesicular nature. Atomic force microscopy (AFM) images of commensal MVs showed that these vesicles were spherical in shape with a size of 250 – 300 nm. Dynamic light scattering measurements confirmed our AFM results and zeta potential analysis showed that MVs had a high negative charge density. We also showed that MVs contained proteins and nucleic acids.

After these characterization experiments, we next analyzed MVs' immunomodulatory activities. We found that commensal bacteria derived MVs triggered higher levels of IL-10 secretion and lower amounts of IFN-γ secretion from mouse splenocytes when compared to non-commensal vesicles. We also showed that commensal bacteria derived MVs failed to induce antigen presenting cell (APC) maturation. In vivo activity of membrane vesicles were tested in immunization studies using the foot and mouse disease (FMD) vaccine as a model. Results revealed that FMD specific IgG responses were elevated in the presence of non-commensal E.coli derived membrane vesicles, whereas commensal bacteria derived MVs suppressed Th-1 dominated IgG2a production. Based on these results, we decided to further explore the immunomodulatory potential of commensal-derived MVs using the bacterial strain that demonstrated highest level of immunomodulatory activity (*Pediococcus pentosaceus*).

3.1.1. Determination of Protein Contents of Whole Bacteria and Membrane Vesicles by SDS-PAGE Gel Electrophoresis

To confirm that *Pediococcus pentosa*ceus derived MVs could be reproducibly purified with no major alterations in their content, 3 separate Pediococcus pentosaceus cultures were initiated, followed by MV purification. The protein content of these 3 separate batches were compared to the protein content of whole bacterial lysate using SDS-PAGE. As shown in the Figure 3.1, MVs share some but not all of the proteins expressed in whole bacteria. Membrane vesicle isolated from different cultures of Pediococcus pentosaceus were also analyzed to check protein

content similarity between different batches. All batches of MVs contained identical proteins of similar concentrations, confirming the consistency of MV production and isolation procedures when identical growth conditions were employed. Alterations in growth conditions may cause stress and affect MVs content and properties.

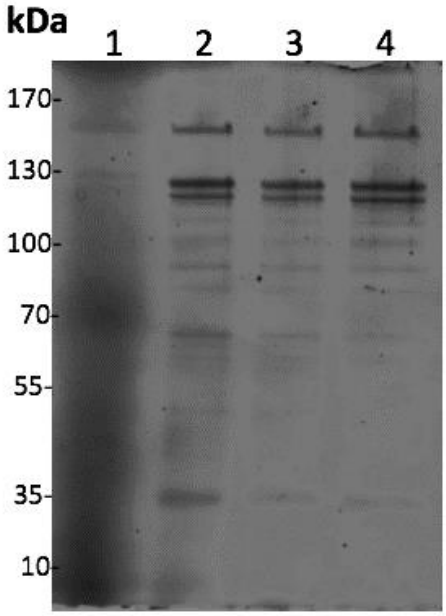

Figure 3.1. SDS-PAGE of whole bacteria and membrane vesicles. Pediococcus pentosaceus (20 µl/well) and Pediococcus pentosaceus derived MVs (25 µg/ml) were loaded onto a 10% polyacrylamide gel and electrophoresis was conducted for 60 min at 185 V. The gel was stained with Coomassie Blue and imaged under the Bio-rad ChemiDoc MP Imaging System. Pediococcus pentosaceus whole cell extract 2,3,4 Pediococcus pentosaceus MVs from different batches. Image is representative of 2 independent experiments.

3.2. In vivo Effects of Pediococcus pentosaceus derived Membrane Vesicles in an Immunization Experiment using Ovalbumin (OVA) as a Model Antigen

To determine the potential immunomodulatory effects of MVs and investigate how this activity changed when immunostimulatory agents were also included in the vaccine, mice were immunized with the following groups:

i)OVA alone, ii)OVA mixed with MV (OVA+MV), iii)OVA encapsulated in MV using the dehydration-rehydration method (OVA in MV), iv)OVA mixed with the

immunostimulatory cyclic-di-GMP (OVA+c-di-GMP) and v)OVA and c-di-GMP co-encapsulated in MV using the dehydration-rehydration method (OVA+c-di-GMP in MV). Bacterial second-messenger c-di-GMP was chosen as a potent immuno-stimulatory agent. C-di-GMP has been previously reported as a potential vaccine adjuvant in different formulations (Karaolis et al.,2007, Ogunniyi et al. 2008, Yildiz et al., 2015) and was used here as a positive control of vaccination. C57BL6 mice (5 mice/group) were immunized with the abovementioned formulations on day 0 and day 14. 2 weeks after the final vaccination, mice were bled and anti-OVA specific antibody responses (Total IgG, IgG1 and IgG2c) were determined from sera (Figure 3.2, Appendix). Following vaccination, when an adaptive immune response develops against an antigen, T helper cell subsets mold antigen specific antibody secretion from B cells. Th1 and Th2 cells modify the antibody subclasses produced from B cells through their characteristic cytokine-mediated help (IFNγ from Th1 and IL-4 from TH2), thereby supporting either IgG2c or IgG1 production, respectively. Results in Figure 3.3 show that both OVA & MV groups (OVA in MV & OVA + MV) generated similar levels of OVA-specific antibody secretion. In contrast, OVA in MV group suppressed IgG2c production when compared to OVA alone control group. However, when MVs were mixed with the immune stimulatory molecule c-di-GMP, MVs' immune suppressive activity were reversed. Figure 3.3 also shows anti-OVA specific IgG1 and IgG2c responses of individual mice at 1/32,768 serum dilution, demonstrating that MVs had no adjuvant activity on their own. These results suggest that, MVs when administered alone support a Th1-suppressing immuno modulatory activity but when MVs are mixed with a potent immune stimulatory molecule capable of acting as a danger molecule, their immunosuppressive activities are lost.

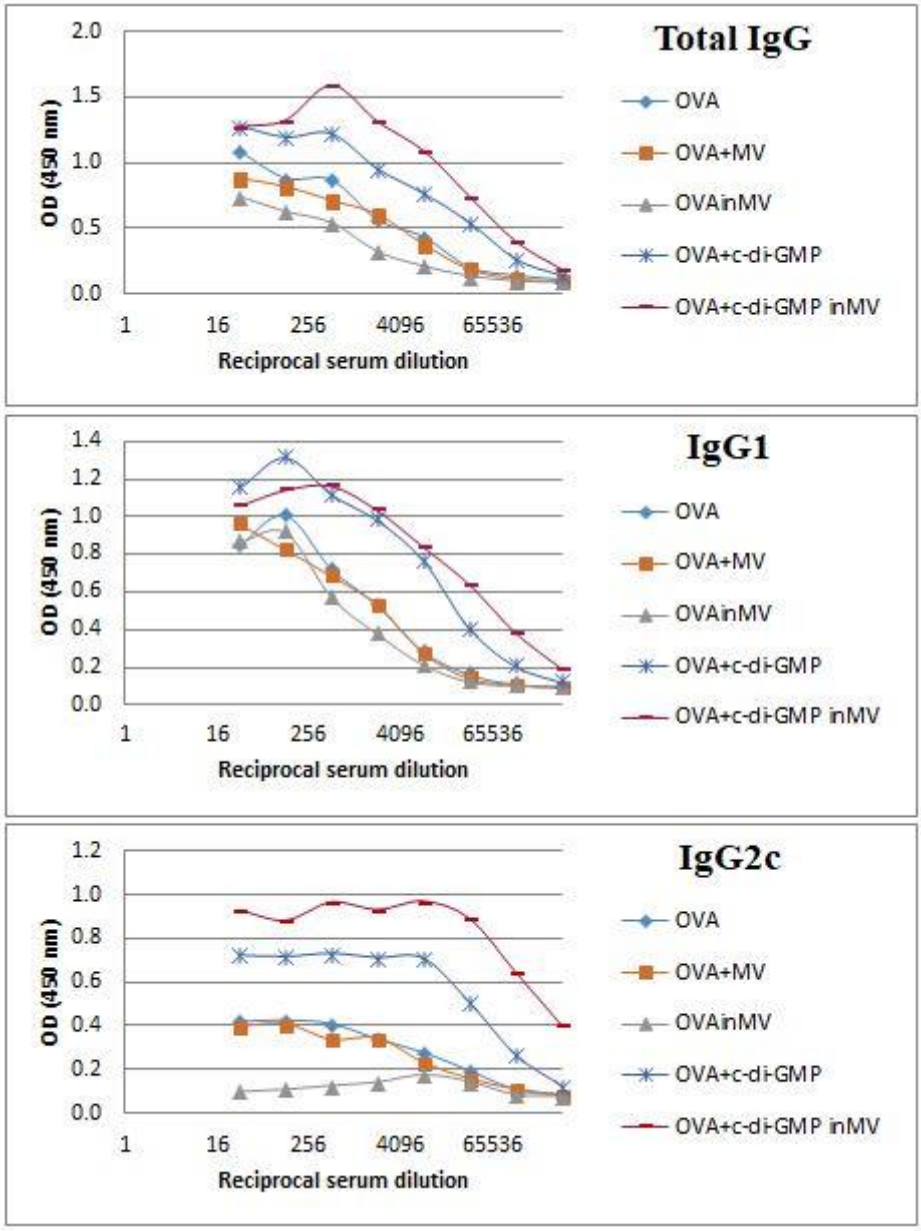

Figure 3.2. Anti-OVA specific total IgG, IgG1 and IgG2c response to different MV formulations. C57BL/6 mice were immunized on day 0 and 14 with OVA (7.5μg/mouse), MV(10 μg/mouse) and/or c-di-GMP(15 μg/mouse) and their different formulations. After secondary bleeding, OVA specific total IgG, IgG1, IgG2c levels were detected via ELISA. Results show average of 5 mice/group.

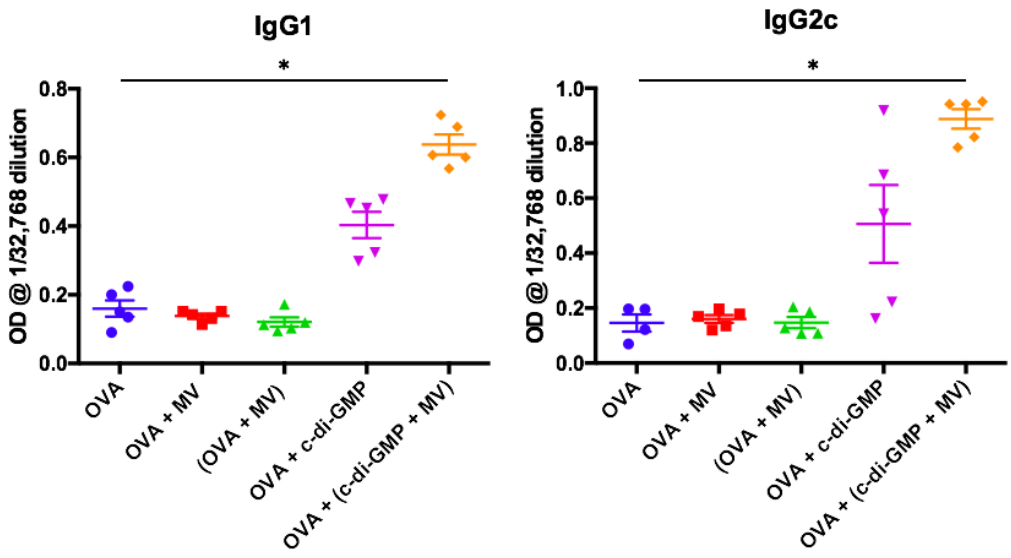

Figure 3.3. Anti-OVA Specific IgG1 and IgG2c antibody titers of individual mice immunized with MV formulations. C57BL/6 mice were immunized on day 0 and 14 with OVA (7.5 μg/mouse), MV (10 μg/mouse) and c-di-GMP (15 μg/mouse) and their formulation. After secondary bleeding, OVA specific total IgG, IgG1, IgG2c levels were detected via ELISA. Measurements were done at 1/32,768 serum dilution by ELISA. Symbols represent individual mice. Statistical significance was determined by Kruskal-Wallis test. * indicates $p<0.01$

3.2.1. Effect of Pediococcus pentosaceus derived Membrane Vesicles in EG.7 Thymoma Tumor Challenge

Since OVA encapsulated in MVs generated lower levels of anti-OVA IgG2c than the OVA alone group, to test whether there was indeed suppression of cell-mediated immunity mice were challenged with subcutaneous (s.c.) injection of OVA expressing E.G7 thymoma tumorigenic cell line on day 51. Tumor growth was observed daily and when tumors reached a palpable size, measurements were taken every second day. Representative tumor images and tumor sizes on day 14 are shown in figure 3.4. MV adjuvanted OVA groups (OVA + MV & OVA in MV) exacerbated tumor formation whereas c-di-GMP addition or encapsulation into MVs led to significant protective anti-tumor immunity.

To measure cytotoxic T cell mediated- anti-tumor activity generated in immunized mice, splenocytes were each mice were incubated with the MHC Class I epitope of OVA (SIINFEKL peptide) and IFN-γ secreting OVA-specific $CD8^+$ T cells were quantitified by ELISPOT. Samples without peptide pulsing served as negative controls. Results (Figure 3.5) show that, MV adjuvanted OVA groups led to lower IFN-γ production and the tumors generated in these animals had necrotic regions (Figure 3.4) implicating that anti-tumor cellular immunity was suppressed. In contrast, c-di-GMP adjuvanted groups had high levels of SIINFEKL-inducible IFN-γ when compared to OVA alone.

Collectively, these results show that, immunization with antigen plus MV alone did not generate protective anti-tumor immunity but exacerbated tumor development. However, when a powerful danger associated molecule was included in MVs (c-di-GMP), the observed immunosuppressive effect was switched from suppression to activation. In conclusion, *Pediococcus pentosaceus* derived MVs cannot be used on their own as protective agents in diseases that requires generation of protective antigen-specific CTL responses. On the other hand, we showed that, MVs improve c-di-GMPs effect and encapsulation into MVs make them more potent protective agent by providing c-di-GMP delivery to cytosol. These findings indicate that, MVs can be used as delivery agents to activate immune response.

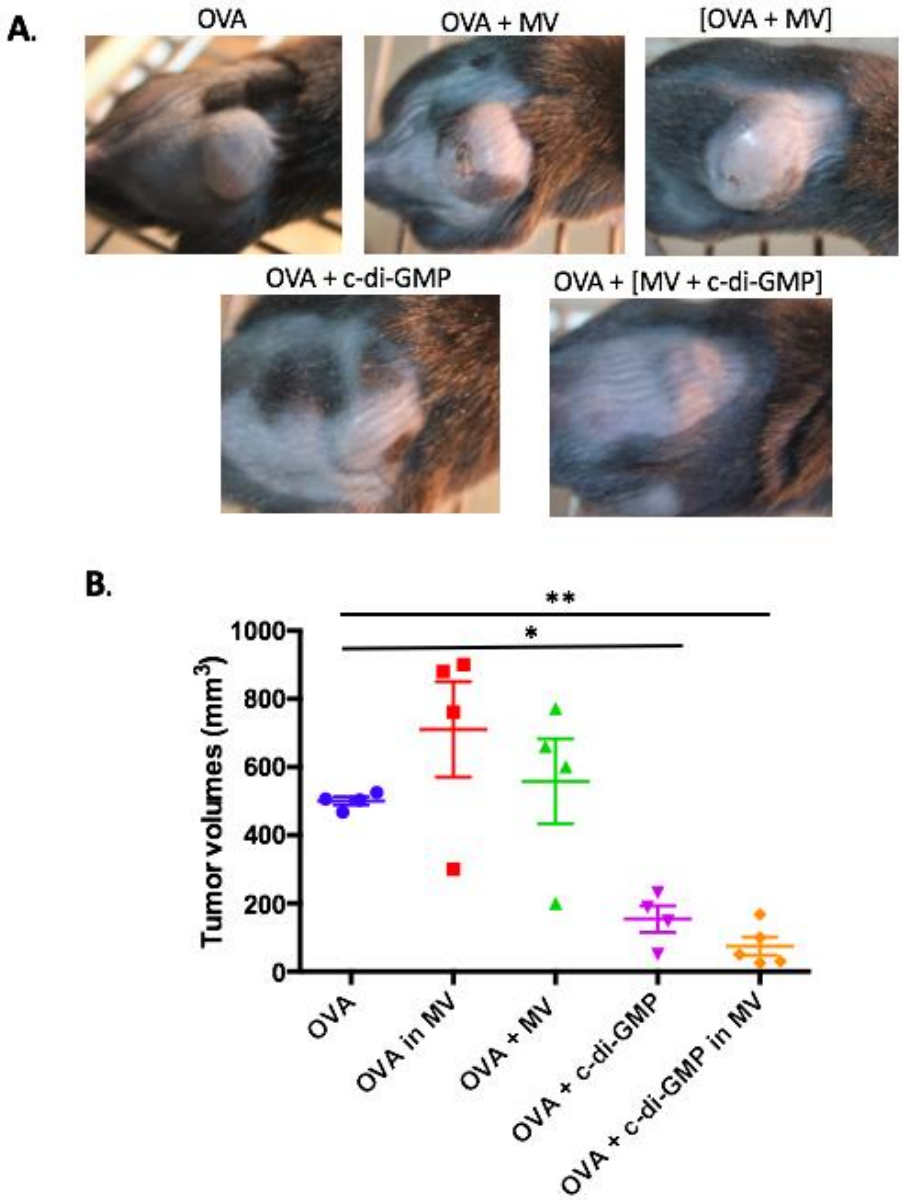

Figure 3.4. Tumor volume measurements 14 days after challenge with E.G7 thymoma tumor cells. C57BL/6 mice were immunized on day 0 and 14 with OVA (7.5 μg/mouse), MV (10 μg/mouse) and c-di-GMP (15 μg/mouse) and their formulation. On day 51, mice were inoculated with OVA specific E.G7 thymoma cells ($4x10^6$ cells/mouse). Tumor measurements were made daily and calculated as (length) x (width) x (height) and recorded as mm^3. Representative images of tumors (A) and tumor volumes (B) 14 days after inoculation. Statistical significance was tested by ordinary one-way ANOVA. * indicates $p<0.03$, ** indicates $p<0.005$.

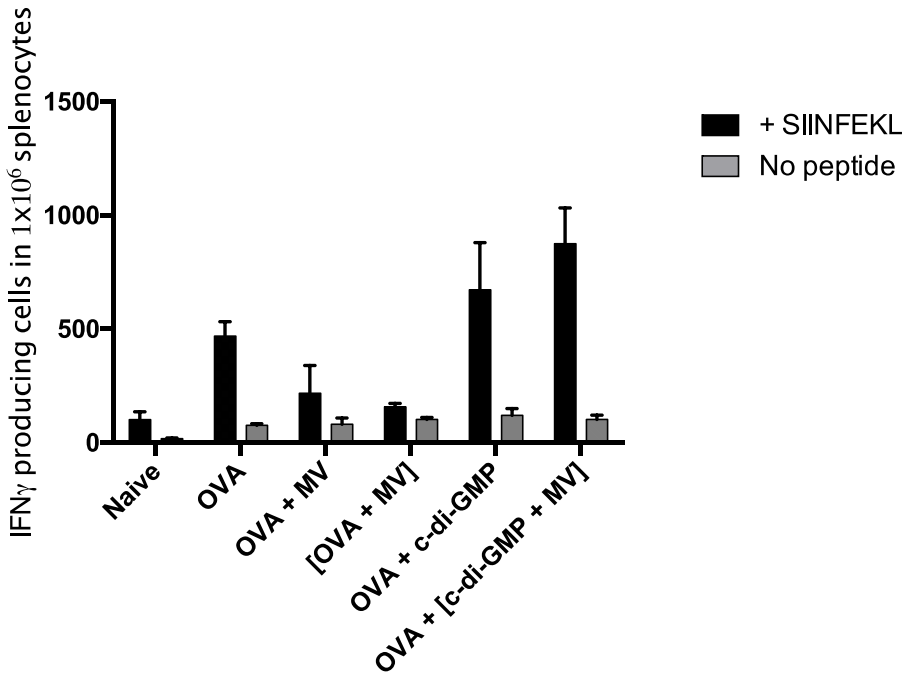

Figure 3.5. IFN-γ production as a result of ex vivo stimulation of splenocytes with SIINFEKL peptide. C57BL/6 mice were immunized on day 0 and 14 with OVA (7.5 μg/mouse), MV(10 μg/mouse) and c-di-GMP(15 μg/mouse) and their formulation. After mice were sacrificed, splenocytes were ex vivo stimulated with SIINFEKL peptide (MHC Class I epitope of OVA antigen) for 24 hours and IFNγ producing cells were analyzed by ELISPOT assay. Statistical significance was determined by Kruskal-Wallis test. Difference between groups was not significant.

3.3. Determination of in vitro Immunomodulatory Activity of Commensal Bacteria derived Membrane Vesicles

3.3.1. Determination of Activity of Membrane Vesicles on Bone Marrow derived Macrophages (BMDM)

Several TLR ligands, including TLR2/6 agonist Pam2CSK4, TLR4 agonist LPS and TLR5 ligand flagellin have been reported to enhance MDSC frequency and activity (Maruyama et al., 2015, Delano et al., 2007; Bunt et al., 2009, Rieber et al., 2013). Since MVs express multiple TLR ligands, we wanted to analyze the effect of commensal-derived MVs on Macrophage polarization and MDSC generation and activity.

To assess the activity of MVs on different immune modulatory cell types, we first focused on bone marrow derived macrophages (BMDM) and analyzed the effect of MVs on macrophage polarization. When mature macrophages (M0 macrophages) encounter various activators, they can differentiate into two distinct cell types: classical (M1) macrophages or alternative (M2) macrophages. M1 macrophages are known to induce inflammatory response whereas M2 macrophages support an immunomodulatory response. M1 macrophages are known to secrete Il-6, TNF-α and nitric oxide and upregulate iNOS (NOS2) expression. In contrast, M2 macrophages secrete IL-10 and express Arginase-1 (Arg-1) (Martinez and Gordon, 2014).

To test MV activity on macrophage polarization, bone marrow derived M0 macrophages were generated from bone marrow progenitors using M-CSF (20ng/ml) as described in Section 2.2.3.2.3. On day 6, matured macrophages were collected and transferred to wells of 96 well plates (200,000cells/well). M0 macrophages were then stimulated with 3 different concentrations of MVs or with the M1 polarizing ligand LPS or the M2 polarizing ligand PGN as positive controls of M1/M2 macrophage polarizing agents, respectively. 24 h after stimulation, culture supernatants were collected and assessed by ELISA for secreted cytokines and expression of Arg-1 and NOS2 was determined from the cells by qRT-PCR.
Figure 3.6 shows that, MV treatment of M0 macrophages triggered IL-10 and IL-6 but not TNFα secretion in a dose-dependent manner. IL-10 levels were significantly higher (3-fold) than the untreated and control groups when 5 μg/ml MV was employed. In contrast, MVs stimulated very low levels of pro-inflammatory IL-6 and TNF-α secretion. As expected, M1 polarizing ligand LPS potently upregulated IL-6 and TNFα production (Figure 3.6).

IL-10 secretion is an important indicator of M2 macrophage activation and our results suggested that MVs could polarize the macrophages towards the M2

phenotype. To confirm this, expression levels of specific M1 and M2 macrophage markers NOS2 and Arg-1were also determined. After 24 h incubation of mature macrophages with 3 different dose of MVs and controls, Arg-1 and iNOS expressions were analyzed via qRT-PCR. Expression levels were determined by normalization to endogenous control 18S RNA. Figure 3.7. shows that, similar to the M2 polarizing ligand PGN, MVs potently upregulated the expression of Arg-1. M1 marker NOS2 expression was strongly upregulated with the M1 polarizing ligand LPS, but not with PGN or MVs.

Collectively, these results indicate that MV treatment of M0 macrophages support alternative macrophage activation (M2 macrophages) with immunomodulatory activity. It is known that MVs contain multiple TLR ligands such as PGN and lipoteichoic acid, all of which can trigger signaling more effectively than PGN and therefore stimulate higher levels of IL-10 and Arg1 production.

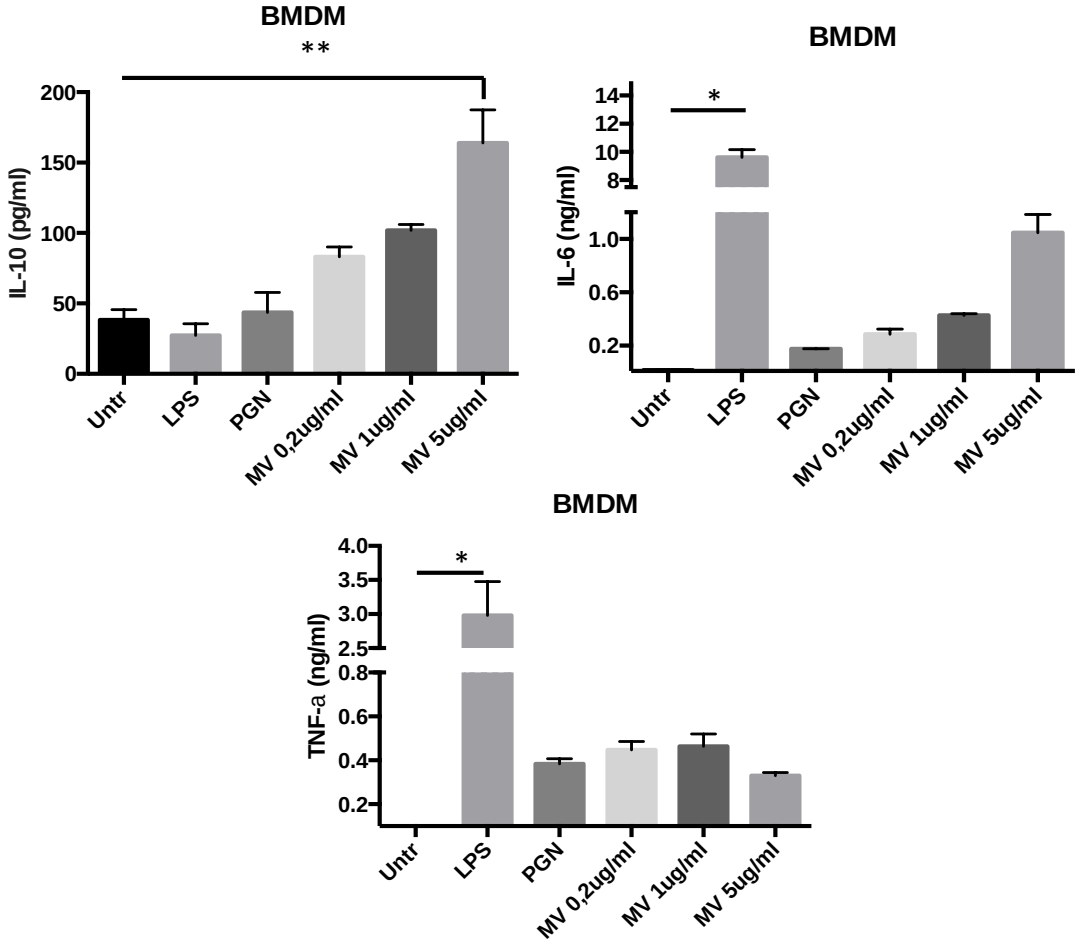

Figure 3.6. IL-10, IL-6 and TNF-α cytokine secretion profile of BMDMs stimulated with MVs or M1/M2 polarizing ligands. 600,000 BMDMs/ml were stimulated with 0.2μg/ml, 1 μg/ml or 5 μg/ml MVs or with PGN (5 μg/ml) or LPS (1 μg/ml) for 24 hours. Cytokine production was determined from culture supernatants by ELISA. Statistical significance was tested by Kruskal-Wallis test. ** indicates $p<0.0097$ and * indicates $p<0.0273$.

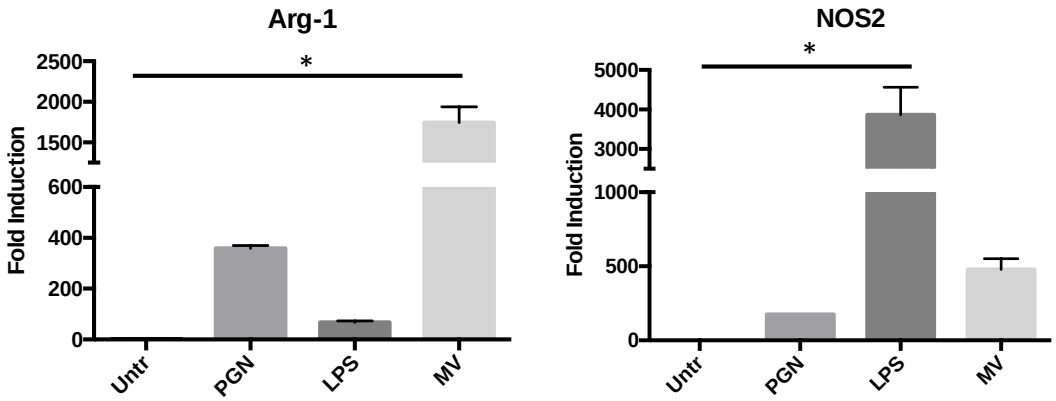

Figure 3.7. Expression levels of M1 and M2 macrophage markers in BMDMs following stimulation with MVs, PGN or LPS. BMDMs (600,000 cells/ml) were stimulated with MV (5 μg/ml), PGN (5 μg/ml) and LPS (1 μg/ml) for 24 hours. After stimulation, cells were collected, and RNA isolation and cDNA synthesis were performed. Gene expression for M1/M2 macrophage markers were measured by using Taqman gene expression assay. Expression levels were determined by normalization to 18S rRNA. Statistical significance was tested by Kruskal-Wallis test. * indicates $p<0.0429$.

Immune system has various checkpoints to regulate a healthy immune response. One such inhibitory checkpoint, the PD-L1/PD-1 interaction has been described as one of the major mechanisms of MDSC immunosuppressive function (Noman et.al, 2014). PD-L1 is one of the ligand of PD-1 checkpoint and it is known that PD-L1 binding to PD-1 inhibits T cell activation. PD-L1 is also overexpressed in tumor microenvironment and contributes to inhibition of cytotoxic T cells (Pardoll, 2012). Given the importance of PD-L1 expression in immune regulation, the effect of MVs on PD-L1 expression in BMDMs were also assessed. Following 24 h stimulation with MVs, BMDMs were collected and stained for F4/80 macrophage marker and PD-L1 (Figure 3.8). Results show that stimulation with MVs induced the expression of PD-L1 in BMDMs, suggesting that MV-activated macrophages acquired an immunosuppressive potential. In summary, our results demonstrate that MV-treated BMDMs produce high levels of IL-10, express Arginase-1 and PD-L1, all of which could contribute to the immunomodulatory phenotype.

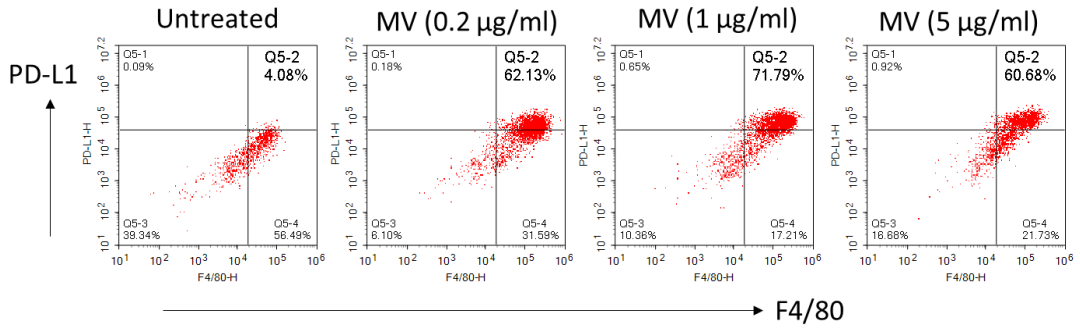

Figure 3.8. Upregulation of PD-L1 expression in BMDMs stimulated with MVs. BMDMs (200,000 cells/well) were stimulated with 3 different MV concentrations (0.2 µg/ml, 1µg/ml, 5µg/ml) for 24 hours. Following stimulation, cells were collected and stained for PD-L1 and F4/80 cell surface markers and expression levels were determined using flow cytometry.

3.3.2. Determination of Activity of Membrane Vesicles on Bone Marrow Progenitors

Previous work has shown that myeloid precursors differentiate into immune-suppressive MDSCs following stimulation with LPS and other TLR ligands (Arora et al., 2010). To understand whether MVs had a similar effect, bone marrow progenitor cells were incubated in the presence of MVs (10 µg/ml) without M-CSF for 6 days. As control, another batch of cells were incubated with M-CSF (20 ng/ml) to generate M0 macrophages. M0 macrophages were then differentiated into M1 or M2 macrophages following incubation in the presence of M-CSF (20 ng/ml) + LPS (1 µg/ml) or M-CSF (20 ng/ml) + PGN (5 µg/ml), respectively. M-CSF treated bone marrow precursors were also incubated with 2 different MV doses (5 µg/ml & 10 µg/ml) during the differentiation process. Culture supernatants from M-CSF differentiated, M1/M2 polarized or MV treated samples were collected and subjected to IL-10 ELISA as an indicator of immunomodulatory activity. As seen in Figure 3.9, MV alone and MV + M-CSF groups led to secretion of high levels of IL-10. These results suggest that MVs can directly induce MDSC differentiation from bone marrow precursors without the need for additional growth factors.

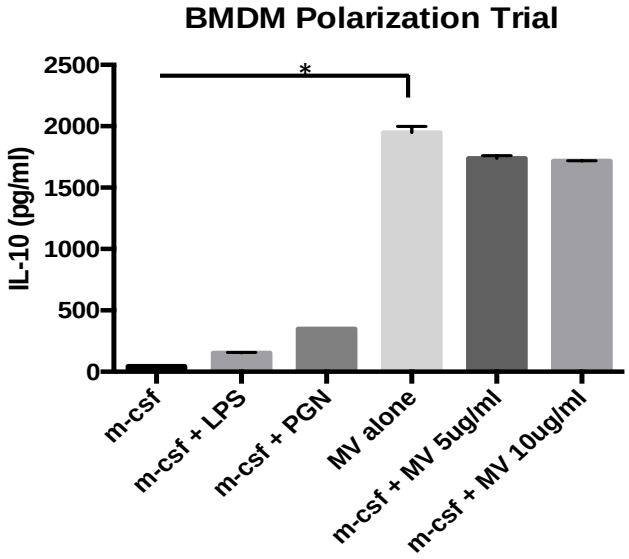

Figure 3.9. Differentiation of bone marrow progenitor cells incubated with MVs for 6 days. Bone marrow progenitor cells were incubated in the presence of M-CSF (20 ng/ml), M-CSF (20 ng/ml) + LPS (1 μg/ml), M-CSF (20 ng/ml) + PGN (5 μg/ml), MV (10 μg/ml), M-CSF (20 ng/ml) + MV (5μg/ml), M-CSF (20 ng/ml) + MV (10 μg/ml) for 6 days. At the end of the incubation, supernatants were collected and IL-10 levels were measured by ELISA. Statistical significance was tested by Kruskal Wallis test. * indicates $p<0.0277$.

Myeloid derived suppressor cells (MDSC) are a heterogeneous population of immature granulocytes, dendritic cells and macrophages and are known to suppress various T cell functions. MDSCs have two major subtypes classified either as monocytic MDSC (M-MDSC) or granulocytic MDSCs. Granulocytic MDSCs (G-MDSC) are characterized by the expression of $CD11b^{+}Ly6G^{+}Ly6C^{low}$ markers, whereas monocytic MDSCs are characterized by the expression of $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ (Gabrilovich and Nagaraj, 2009). Furthermore, granulocytic MDSCs have characteristic ring-shaped nuclei (Greifenberg et al., 2009). To assess whether MV-differentiated bone marrow precursors adopted M-MDSC or G-MDSC nuclear morphology, differentiated cells were stained with NucBlue Live Ready probe (ThermoFisher Scientific) and nuclei were visualized under Floid cell imaging station. Results revealed that cells that differentiated upon MV exposure displayed

ring shaped nuclei whereas M-CSF treated macrophages had central round nuclei, indicating that MVs were indeed driving the differentiation of bone marrow precursors into MDSCs which also include G-MDSCs with an immunomodulatory phenotype (Figure 3.10). High levels of IL-10 secretion and differentiation into cells with ring-shaped nuclei are important indicators of G-MDSC. Future experiments with M-MDSC and G-MDSC-specific markers are planned to determine the percentages of each population in detail.

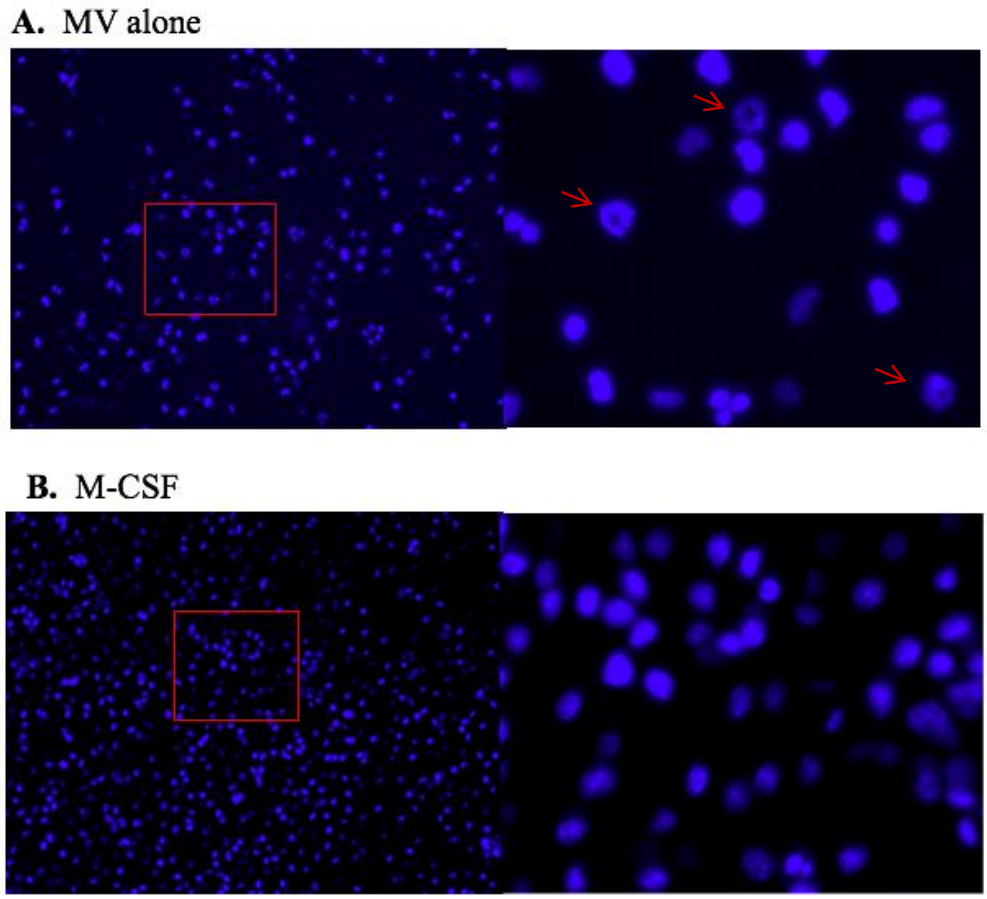

Figure 3.10. Analysis of differentiated bone marrow progenitor cells based on nuclear morphology. Bone marrow progenitor cells were incubated with MVs (10 μg/ml) or M-CSF (20 ng/ml) for 6 days and after the incubation period nuclei were stained with NucBlue live cell stain (10 μl/well). Cells were visualized using EVOS Floid cell imaging system (ThermoFisher Scientific).

3.4. Phenotype determination of cells generated following intraperotoneal injection of MVs

To determine the phenotype of cells differentiating in response to MV injection, 8 - 12 weeks old C57BL/6 mice (5/group) were i.p. injected with MVs (10 μg/mouse) or saline (Naïve group) on day -4 and -1 as described in Section 2.2.5.2. On day 0, mice were sacrificed, peritoneal exudate cells (PEC) and spleen cells were collected and cells were stained for M-MDSC or G-MDSC-specific cell surface markers CD11b, Ly6G and Ly6C. A gating strategy to include single cells (FSC-A/FSC-H plot) and CD11b positive cells (CD11b/FSC-H plot) was used as shown in Figure 3.11. Granulocytic MDSCs were Ly6G positive and Ly6C dim cells (Figure 3.11., R7 Gate) whereas monocytic MDSCs were gated as Ly6C positive and Ly6G negative cells (Figure 3.11., R6 Gate).

After 2 i.p. injections, the percent of G-MDSCs or M-MDSCs did not significantly increased in spleen cells when compared to control group (Figures 3.11 and 3.12). In contrast, significant numbers of both granulocytic and monocytic MDSCs accumulated in the peritoneal cavity of MV administered mice when compared to PBS treated mice (Figures 3.11 and 3.12). These results suggest that, MVs could potently generate M-MDSCs and G-MDSCs in vivo.

To test whether MVs also affected regulatory T cell development as another important immunomodulatory cell type, Treg percentages in spleen and PEC were also analyzed (Hori et al.,2003). Results demonstrated that MVs had no effect on Treg cell numbers (Appendix B). In conclusion, MVs contribute to immunomodulatory response by generating MDSCs and M2 macrophages but not regulatory T cells.

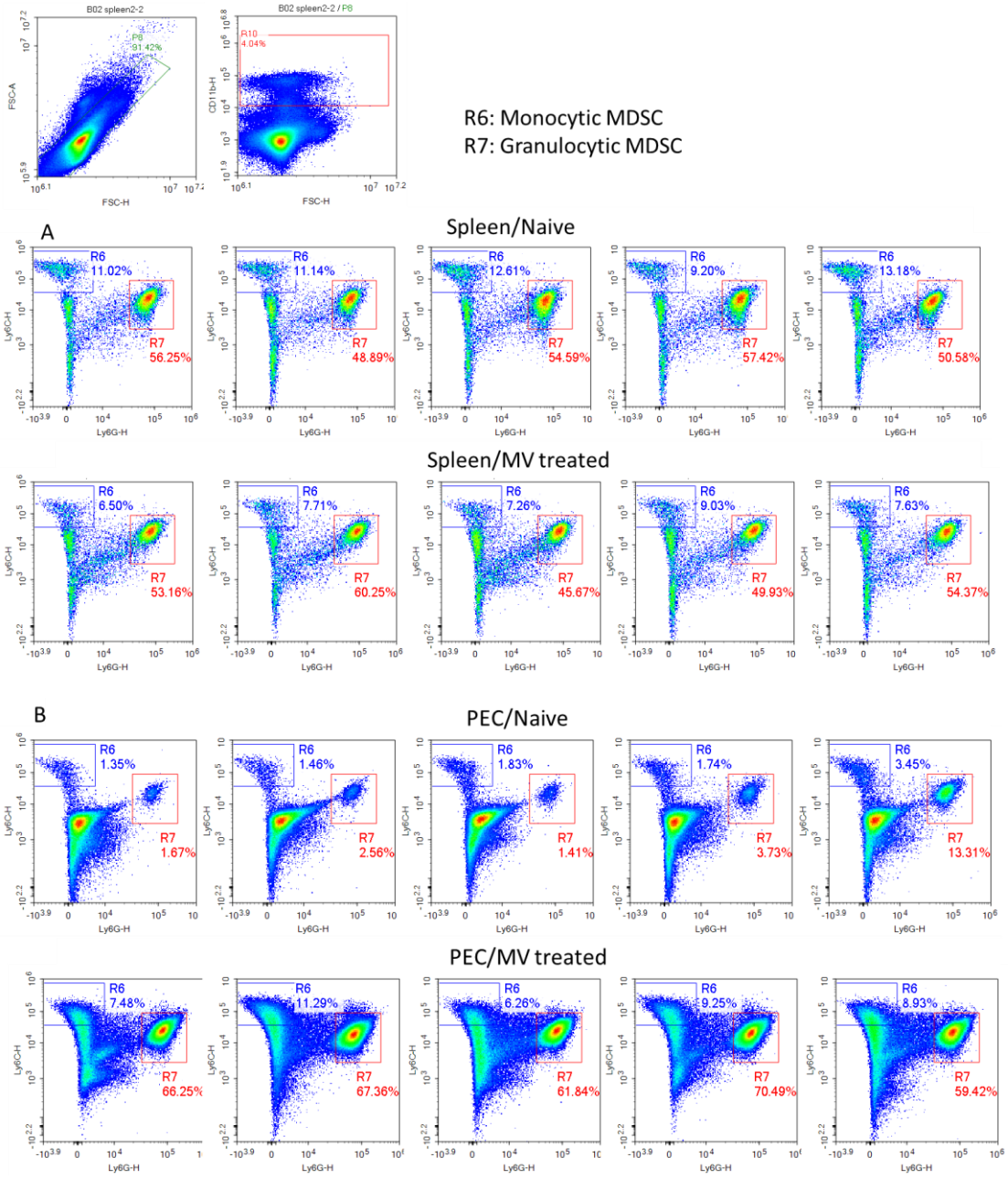

Figure 3.11. Determination of phenotype of cells generated following intraperitoneal injection of MVs. C57BL/6 mice were i.p. injected with MVs (10 μg/mouse) and saline on days -4 and -1. On day 0, mice were sacrificed and splenocytes (A) and PECs (B) were stained for CD11b, Ly6G and Ly6C expression. $CD11b^+$ cells were gated and analyzed for M-MDSC and G-MDSC percentages. R6 gates show monocytic MDSCs and R7 gate shows granulocytic MDSCs.

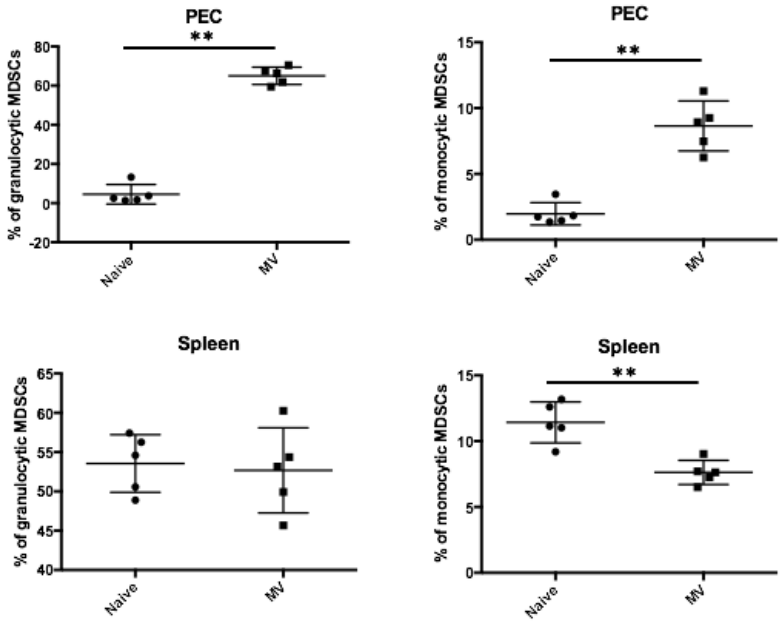

Figure 3.12. Determination of percentage of monocytic and granulocytic MDSCs generated following intraperitoneal injection of MVs. Statistical significance was tested by Mann-Whitney test ** indicates $p<0.0079$

3.5. In vivo Immunomodulatory Effects of MVs in a Zymosan induced Peritonitis Model

Acute inflammatory response caused by infection or injury stimulate white blood cells to migrate to the site of infection/injury. Acute inflammation is generally correlated with neutrophil rich cellular infiltrates and results with elimination of infectious agents together with resident or recruited macrophages (Medzhitov, 2008). To assess whether MVs could suppress/ameliorate excessive inflammation in acute peritonitis model, zymosan (an insoluble polysaccharide cell wall component of *Saccharomyces cerevisiae*), was administered to mice intraperitoneally. Previous publications have shown that zymosan administration induce all the hallmarks of acute inflammation (Cash et al.2009, Doherty et al.1985). Therefore, this model was chosen to evaluate the immunomodulatory potential of MVs. On days -3 and -1, 8 – 12 weeks old male C57BL/6 mice (4mice/group) were injected with 10 μg/mouse MVs or saline as control. On day 0, zymosan (100μg/mouse) was administered

intraperitoneally to induce acute inflammation. 6 hours after injection of zymosan, mice were sacrificed and neutrophil and macrophage accumulation in the peritoneal cavity was analyzed by flow cytometry. Naïve mice with no zymosan administration served as the negative control group. Ly6G positive F4/80 negative cells were defined as neutrophils, whereas F4/80 and CD45 positive cells were defined as macrophages.

Figure 3.13 A shows that, Naïve mice had low levels of neutrophils in the peritoneal cavity, whereas zymosan injected untreated peritonitis group had massive neutrophil recruitment when compared to other groups. Pre-administration of MVs significantly reduced neutrophil recruitment when compared with acute peritonitis group (Figure 3.13.B) and ameliorated the acute inflammation.

Macrophages represent another important cell type that are recruited to the site of inflammation to try to eliminate the inducers of inflammation. Figure 3.14. shows that macrophage percentages in the peritoneal cavity of zymosan induced peritonitis group had severely reduced numbers of macrophages when compared to the control group, probably due to activation-induced cell death. MV preadministration rescued this macrophage loss significantly. Furthermore, serum IL-6 levels were found to be elevated in acute peritonitis induced mice, whereas MV-pretreatment normalized serum IL-6 levels (Figure 3.14(C)), suggesting that the inflammatory response was suppressed in MV-pretreated peritonitis group. Collectively, these results show that, MVs exert an immunoprotective effect against zymosan induced peritonitis and inhibit exaggerated inflammation.

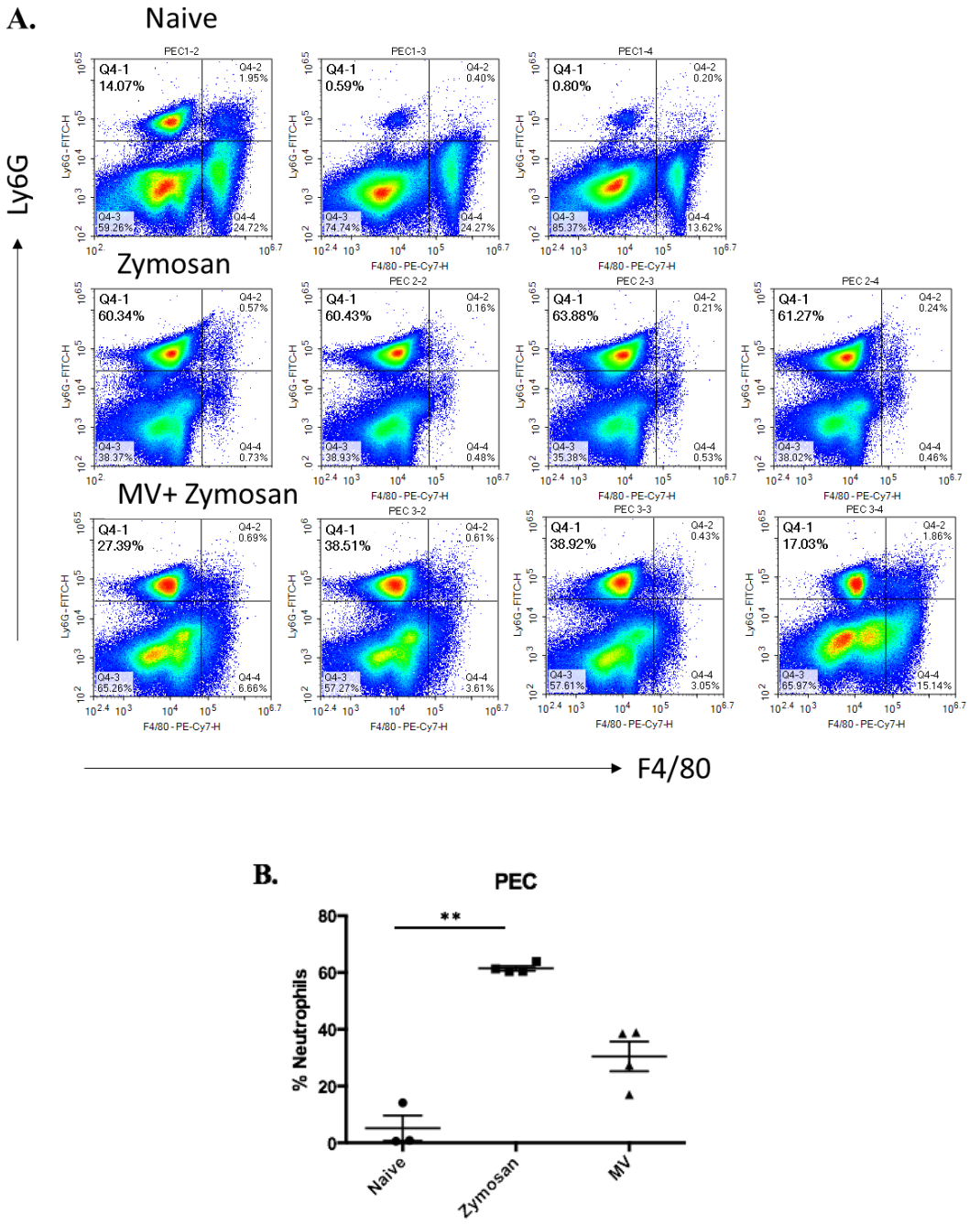

Figure 3.13. MVs pre-administration ameliorates neutrophils accumulation in zymosan induced peritonitis model. 8- 12 weeks of male C57BL/6 mice were pretreated with MVs or saline on days -3 and -1. On day 0, zymosan was injected intraperitoneally to induce peritonitis. 6 hours later, mice were sacrificed and PECs were stained for neutrophil or macrophage markers and analyzed via flow cytometer. Ly6G positive F4/80 negative cells were considered as neutrophils (Q4-1 gate) (A). Percentage of neutrophils are shown in (B). Statistical significance was tested by Kruskal-Wallis test. ** indicates $p<0.0092$

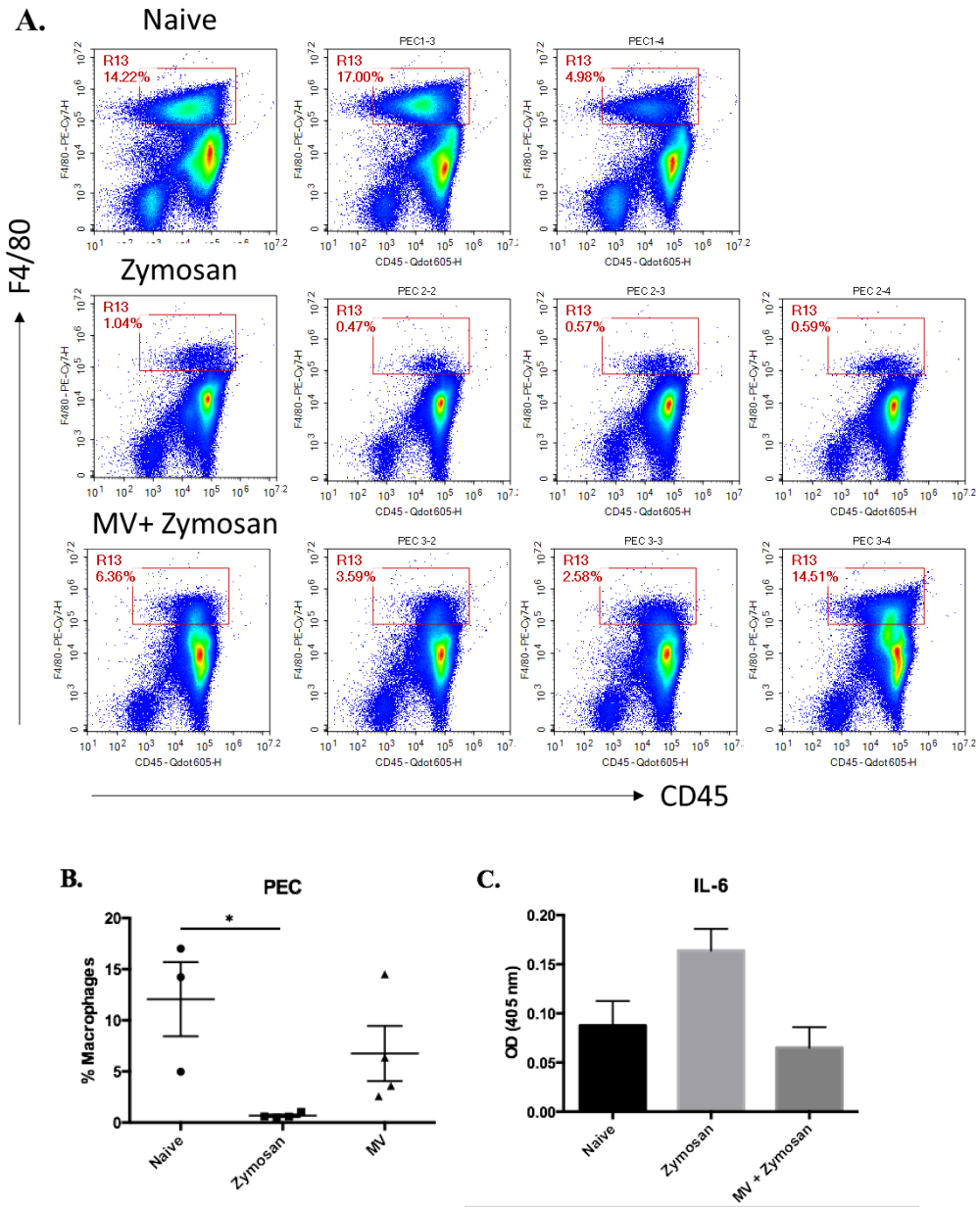

Figure 3.14. MV preadministration ameliorates macrophage death in zymosan induced peritonitis model. 8- 12 weeks of male C57BL/6 mice were pretreated with MVs or saline on days -3 and -1. On day 0, zymosan was injected intraperitoneally to induce peritonitis. 6 hours later, mice were sacrificed and PECs were stained for macrophage markers and analyzed via flow cytometer. CD45 and F4/80 positive cells were considered as macrophages (R13 gate) (A). Percentage of macrophages are shown in (B). Statistical significance was tested by Kruskal-Wallis test. Serum IL-6 concentrations were determined by ELISA (C). * indicates $p<0.0309$

3.6. In vivo Immunomodulatory Effects of MVs in Dextran Sulfate Sodium (DSS) Induced Colitis Model

Inflammatory bowel disease (IBD) is an inflammatory disorder of the gastrointestinal track. Crohn's disease (CD) and Ulcerative Colitis (UC) are considered as two major inflammatory bowel disease subtypes in humans. subtypes. In Crohn's disease, inflammation may occur at any site of the gastrointestinal tract whereas, ulcerative colitis is restricted to the colon (Xavier and Podolsky,2007, Bouma and Strober, 2003). To mimic ulcerative colitis in a mouse model, dexran sulphate sodium (DSS) induced colitis model is generally preferred because of its simplicity and similarities with human UC (Chassaing et al.,2015). To induce colitis, 8 weeks old C57BL/6 mice were administered with 3% DSS (36,000 – 50,000 molecular weight) in drinking water for 4 days, and then were supplemented with drinking water without DSS for 3 more days to allow epithelial recovery. Since MVs inhibited exaggerated inflammation in zymosan induced peritonitis model, they were considered as promising agents for IBD treatment. To test whether MVs had a protective effect in DSS-induced colitis, a pre-treatment and a post-treatment MV administration group was employed. In the pre-treatment group, MVs were administered on days -3 and 0 of DSS exposure and in the post-treatment group MVs were administered on days 0 and +3 intraperitoneally. All results were compared to naïve mice with no DSS administration as the negative control group. Mice were observed for presence or absence of blood in stools and for stool consistency on a daily basis as preliminary indicators of colitis. At the end of DSS administration (Day 4), mice in DSS induced colitis group had runny stools with traces of blood whereas MV treated groups had near-normal stools with little or no blood.

One of the most important hallmarks of colitis is the reduction of the colon length (Ishiguro et al., 2006). Colon lengths from control group, DSS induced colitis group and 2 different MV treatments (pre-treatment and post-treatment) were shown in Figure 3.15. Colons of mice in DSS induced colitis group had significant reduction in the colon length when compared to control group ($p<0.0012$, Figure 3.15.B), indicating inflammation-associated pathology. In contrast, MV treated groups had

reduced colon shortening which was statistically not different from the healthy control group. When percent colon length changes were compared, between DSS treated group vs MV treatments, there was a significant difference between the DSS induced colitis group and the post-treatment MV group only. Presence of blood in stool is another hallmark of colitis, to understand intestinal bleeding stool of the mice were also examined. At the end of DSS administration (Day 4), DSS induced colitis group mice have soft stools with traces of blood whereas MV treated groups have more near-normal type of stool.

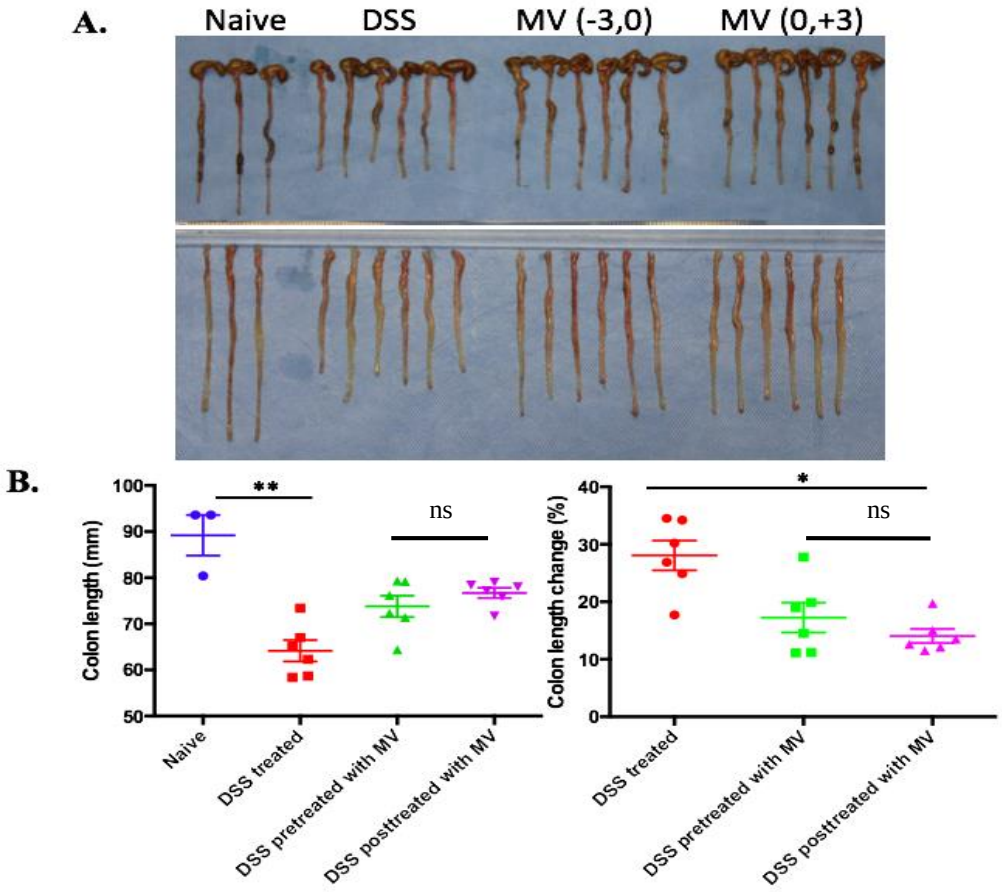

Figure 3.15. Immunomodulatory effects of MVs in dextran sulfate sodium (DSS) induced colitis model. 8 weeks old C57BL/6 mice were administered 3% DSS in drinking water for 4 days. MVs (10 μg/mouse) were injected i.p. on days -3 and 0 (pretreatment group) or on days 0 and +3(post-treatment group).. Colon lengths were measured and visualized (A). Groups are as follows: 1. Naïve, 2. DSS treated, 3. DSS with pretreated MV and 4. DSS with posttreated MV. Colon lengths were determined by measuring with a caliper and percent length change were also calculated (B). Statistical significance was tested by Kruskal Wallis test. ** indicates $p<0.0012$, * indicates $p<0.0137$, ns indicates not significant.

Several histological changes occur in DSS induced colitis model, including, epithelial degeneration, mucin depletion, disappearance of epithelial cells as a consequence of necrosis and loss of crypts. Neutrophil infiltration to lamina propria and submucosa results in an inflammatory response in the colon (Perse and Cerar, 2012), where neutrophils migrate into crypt lumen (crypt abscess) and initiate

cryptitis, resulting in destruction of crypt architecture (Chassaing et al.,2015). To analyze the histological changes that occurred in DSS treated versus DSS+MV treated groups, colons were transected, cut longitudinally and rolled. Swiss rolled colons were fixed and then stained with hematoxylin and eosin (H&E) for further analysis. Figure 3.16 shows that colon sections of naïve mice display intact colonic crypt organization. In contrast, in DSS treated mice, crypt architecture was disrupted and accompanied by inflammation and tissue damage. Both pretreatment and posttreatment with MVs ameliorated disease progression, however the crypt architecture was protected and remained nearly intact only in the post-treatment group (Figure 3.16).

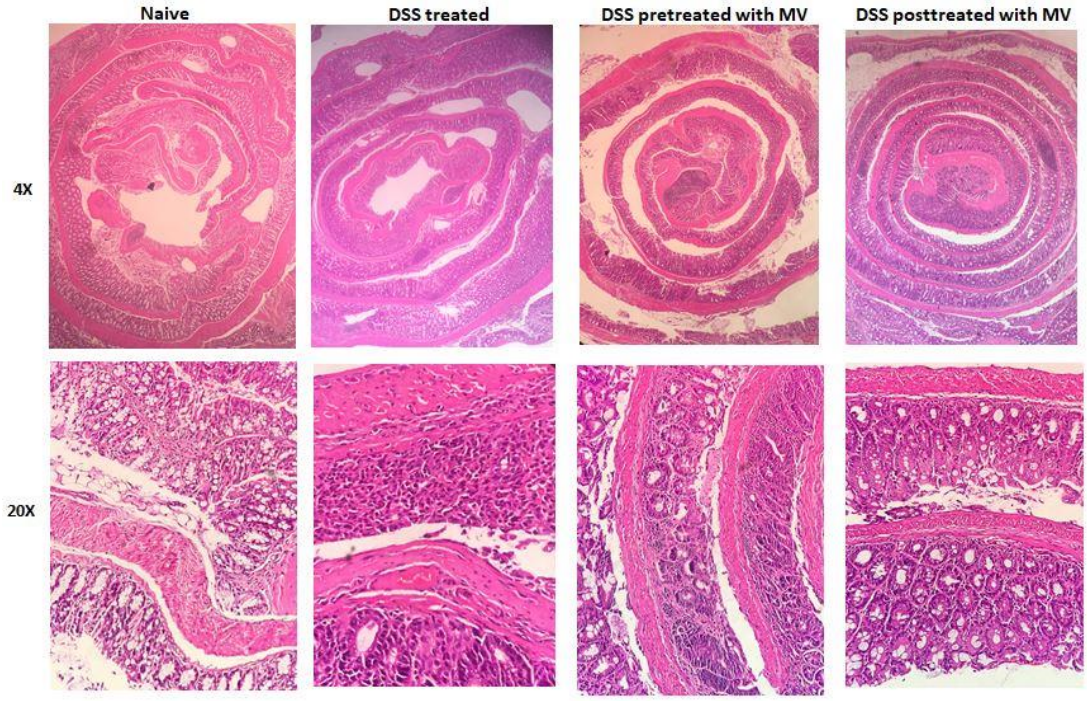

Figure 3.16. Histological analysis of immunomodulatory effects of MVs in dextran sulfate sodium (DSS) induced colitis model. 8 weeks old C57BL/6 mice were administered 3% DSS in drinking water for 4 days followed by normal water supplementation for 3 days. MVs were intraperitoneally administered either on days -3 and 0 (pre-treatment) or on days 0 and +3 (post-treatment). On day 7, mice were sacrificed and colons were removed, cut longitudinally and rolled. Swiss rolled colons were placed in 10% buffered formalin and prepared for hematoxylin & eosin (H&E) staining.

Inflammatory mediators such as IL-6, TNF-α and IFN-γ were previously shown to impact development of inflammatory bowel disease (Podolsky,2002 & Ishiguro et al.,2006). To test whether MVs altered the production of inflammatory mediators or exerted anti-inflammatory effects through IL-10 secretion, levels of IL-6 versus IL-10 was measured from sera of mice from each group using cytometric bead array (CBA).

CBA results revealed that serum IL-6 levels were elevated in both DSS induced colitis group and pre-treatment MV group whereas post-treatment of MV group had near-normal serum IL-6 levels (Figure 3.16.). IL-10 levels were increased in MV post-treatment group when compared to the control group. These results suggest that, MV post-treatment had a more prominent protective effect, possibly suppressing DSS-induced colitis throughout DSS-administration, suggesting that MVs could be of interest as novel immunomodulatory therapeutic agents in inflammatory diseases.

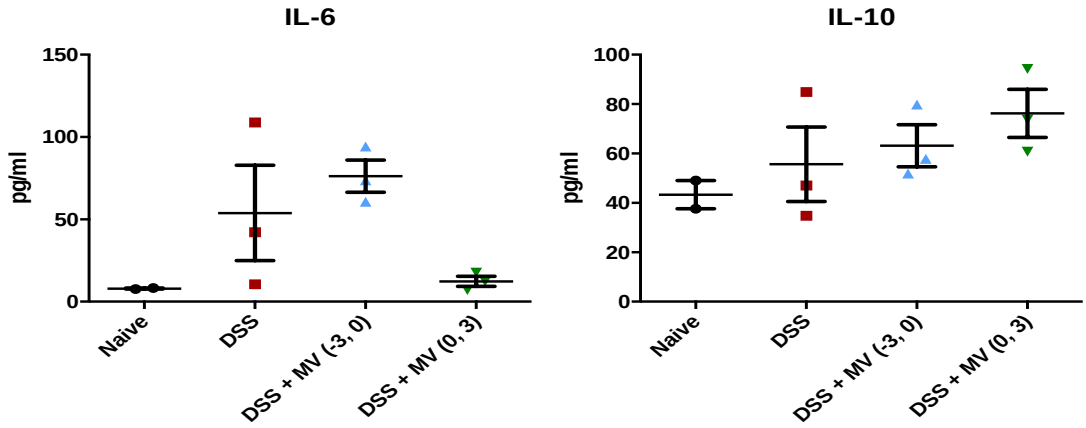

Figure 3.17. Serum IL-6 and IL-10 cytokine profiles in dextran sulfate sodium (DSS) induced colitis model groups. 8 weeks old C57BL/6 mice were administered 3% DSS in drinking water for 4 followed by normal water supplementation for 3 days. MVs (10 μg/mouse) were i.p. injected either on days -3 and 0 (pre-treatment) or on days 0 and +3 (post-treatment). After mice were sacrificed, blood samples were collected from heart and cytokine levels were determined by using cytometric bead array (CBA) and flow cytometry.

3.7. In vivo Immunomodulatory Effects of MVs on wound healing in an Excisional Wound Model

Wound healing is a complex biological process and important for tissue homeostasis. Immediately following injury, an inflammatory response is initiated, characterized by migration of circulating leukocytes, mostly neutrophils, to the wound area (Shaw and Martin, 2009). Later, macrophages migrate to the wound area and exhibit different functions: host defense, resolution of inflammation, removal of cell debris and/or tissue recovery following injury (Koh and DiPietro, 2011). In early stages of wound healing, macrophages produce high amounts of IL-6, IL-12, IL-1 and TNF-α and recruit and/or activate M1 macrophages. Although M1 macrophages have important roles in early phases of wound healing, M2 macrophages secrete factors which stimulate proliferation, angiogenesis, fibroblast differentiation, promoting wound healing (Murray et al.,2011).

Since our data indicated that MVs preferentially supported the differentiation of alternatively-activated wound healing M2 macrophages, we hypothesized that MV treatment would prove to be of benefit in a wound healing model. To test this hypothesis, cutaneous excisional wounds were introduced in 8 -12 weeks old female BALB/c mice by using a 6-mm biopsy punch. Wounds were stabilized through the use of silicone splints and healing process was evaluated in untreated versus topically or systemically MV treated animals. MVs were either directly applied to the wound area topically or were administered through i.p. injection every second day after wound excision. Wound area was measured every second day and compared with the untreated control group. Results revealed that on days 5 and 7, wound area in MV i.p. treated group were significantly decreased when compared with the control group (***$p<0.0002$, *****$p<0.0001$, Figure 3.18.B). Topical administration of MVs had no significant effect on wound healing. These results suggest that i.p. MV administration accelerates wound closure possibly through a MDSC and/or M2 macrophage-supportive process.

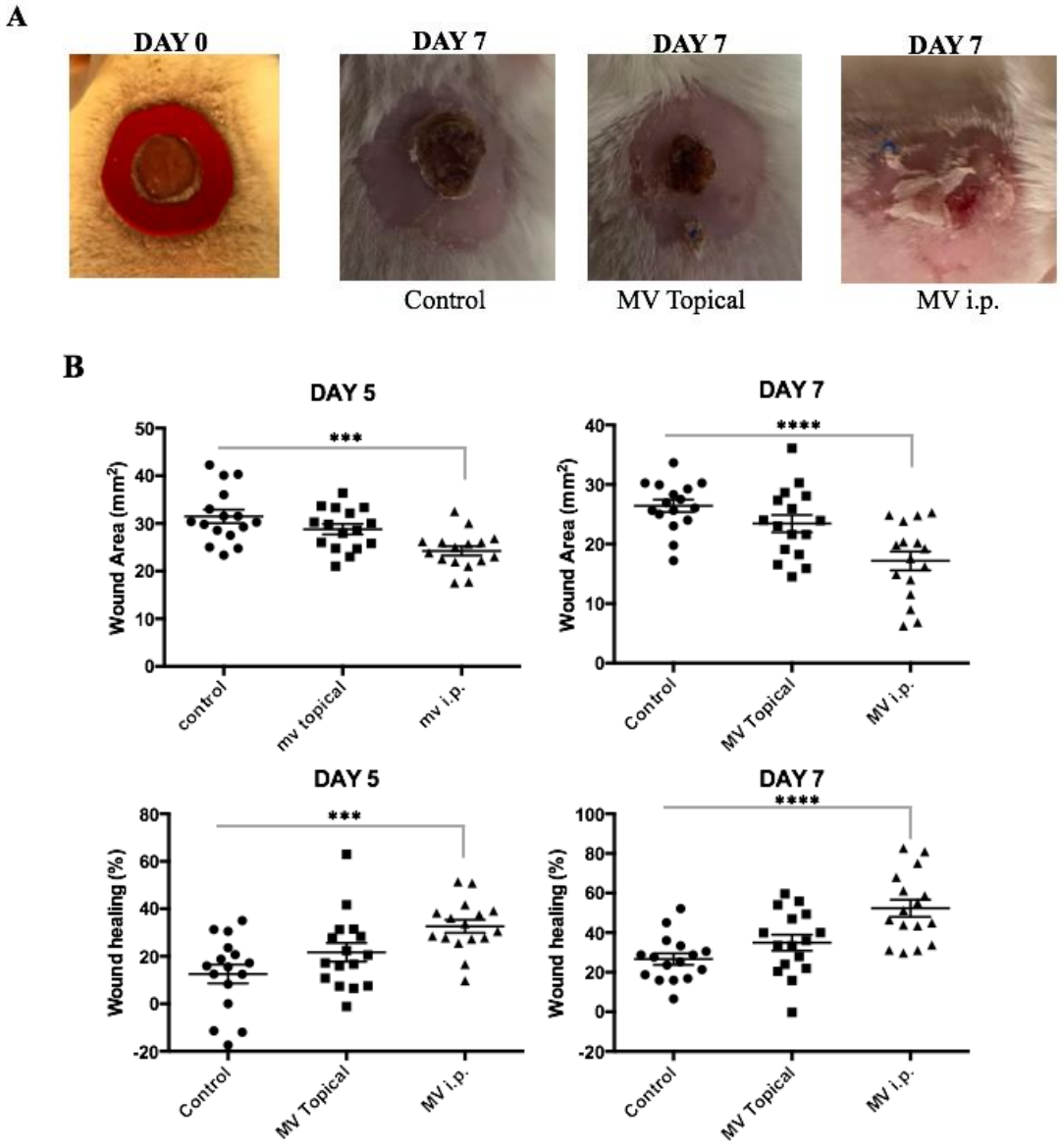

Figure 3.18. Immunomodulatory Effects of MVs in an excisional wound healing model. 8 – 12 weeks old BALB/c mice were used for wound healing experiments. Circular excisional wounds were introduced through the use of a 6-mm biopsy punch and wound closure was followed for 7 days. (A) shows representative images of the wound area from the test groups. MVs (10 μg/mouse) were administered either topically on the wound or by i.p. injections. Wound area (length and width) measurements were conducted every other day by caliper. Wound area and percentage of wound healing represent the average of 15 wounds for every group from 2 independent in vivo experiments (B). Statistical significance was tested by One-way ANOVA Dunnett's multiple comparisons test. *** indicates $p<0.0002$, **** indicates $p<0.0001$.

To analyze the cell types infiltrating the wounds after 7 days, wound area from each mouse were removed and single cell suspensions were obtained using liberase buffer containing collagenase I and collagenase II. Since previous data suggested that MV administration generated regulatory/suppressive cell types of myeloid origin,cells were stained for CD11b, Ly6G and the immunosuppressive PD-L1 marker expression. CD11b and Ly6G positive cells were gated to analyze granulocytic myeloid derived suppressor cells (G-MDSC). As mentioned previously, PD-L1 is an important cell-surface immunomodulatory molecule in MDSCs mediating immunosuppressive functions. As shown in the Figure 3.19, i.p. administration of MVs led to significantly higher numbers of MDSCs recruitment to the wound area when compared with the control group. These results suggest that, i.p. administration of MVs might accelerate the wound healing process by regulating inflammatory responses and recruiting suppressor cell types to wound area. To improve the effect of topical treatment, MVs can be formulated into a cream rather than the currently employed solution-based format to provide proper contact with the wound and ease of use.

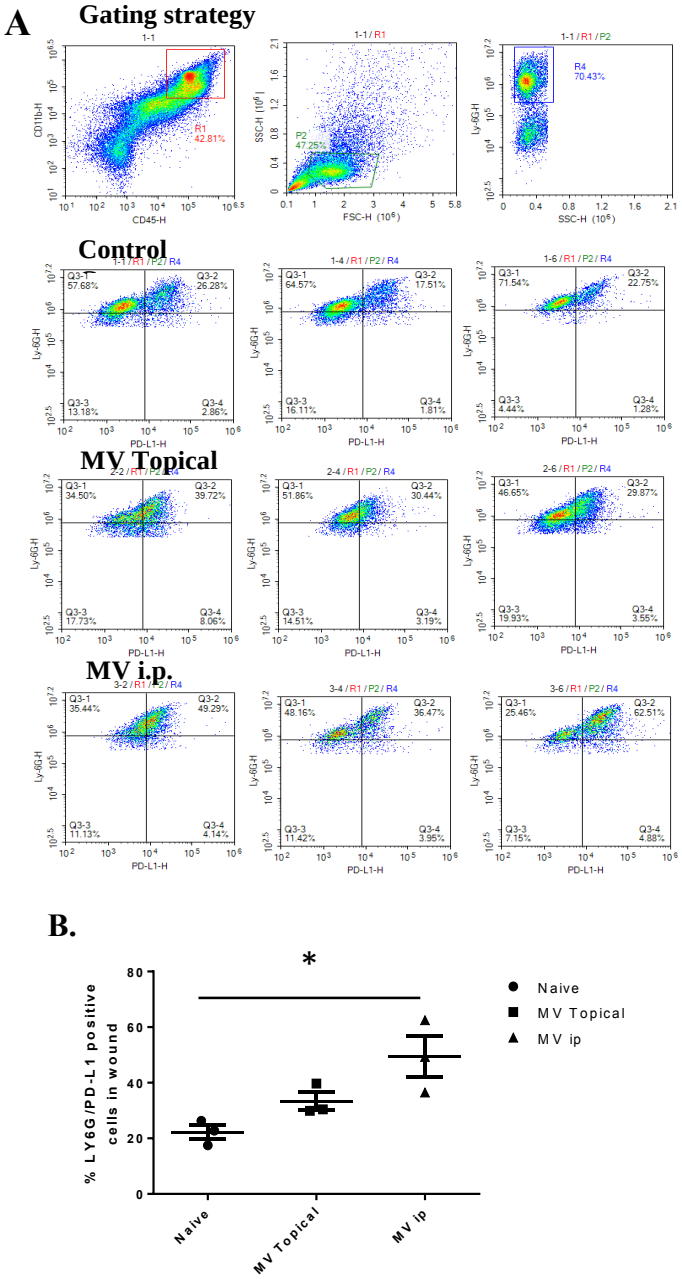

Figure 3.19. Analysis of immune suppressive cell types in the wound area. 8 – 12 weeks old BALB/c mice were used for wound healing experiments. Circular wounds were introduced by using a 6-mm biopsy punch. MVs (10 µg/mouse) were administered either topically on the wound or by i.p. injections. After mice were sacrificed, single cell suspensions were obtained by using Liberase and cells were stained for specific marker expression. CD11b and Ly6G positive cells were gated as indicated (A) and percent of cells expressing PD-L1 were analysed by flow cytometry. Statistical significance between groups (B) was tested by Kruskal-Wallis test. * indicates $p<0.01$.

Histological analysis of wound area was carried out by fixing wounds in 10% buffered formalin and staining with hematoxylin & eosin (H&E). Results show that, the MV treated group had accelerated epithelization when compared with the naïve group (Figure 3.20). In the naïve group, higher levels of inflammatory cell infiltration and cell debris are observed, indicative of ongoing inflammatory phase, whereas in MV treated groups, histological findings are consistent with the resolution/remodeling phase of wound healing. Collectively, these results show that, MV treatment supported differentiation of immunosuppressor cell types (M2 macrophages and/or G-MDSCs), thereby promoting wound healing process. Treatment with MVs in impaired healing situations such as diabetes and ageing might be of therapeutic value in recruitment and/or activation of regulatory cells in the healing process.

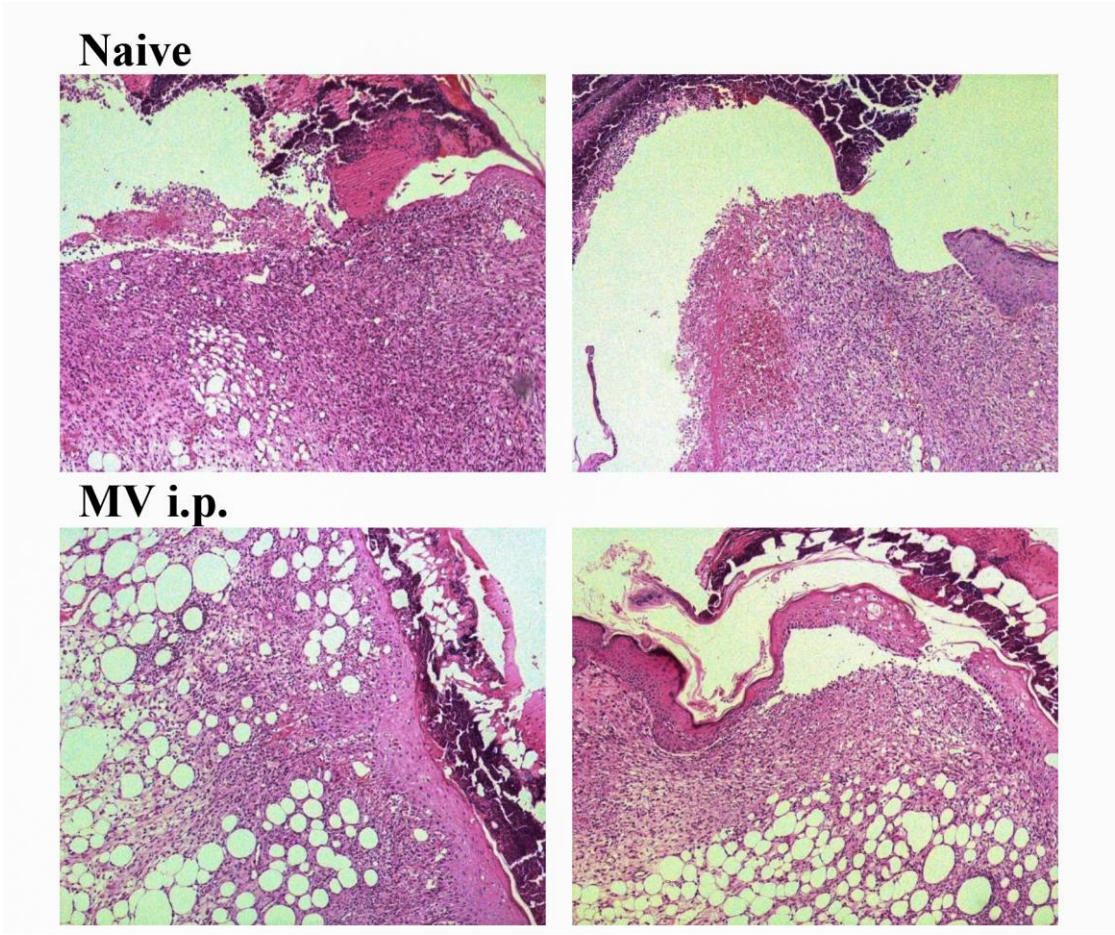

Figure 3.20. Histological analysis of immunomodulatory effects of MVs in an excisional wound healing model. 8 – 12 weeks old BALB/c mice were used for wound healing experiments. Circular wounds were introduced by using a 6-mm biopsy punch. MVs (10 μg/mouse) were administered by i.p. injections and compared with control mice without any treatment. On day 7, mice were sacrificed, half of the wound area were cut and fixed with 10% buffered formalin and prepared for hematoxylin & eosin (H&E) staining.

CHAPTER 4

CONCLUSIONS & FUTURE PERSPECTIVES

This thesis intended to determine the immunomodulatory potential of *Pediococcus pentosaceus* derived membrane vesicles and test their immunosuppressive and therapeutic activities in vitro and in vivo using cellular differentiation, immunization and inflammatory disease models. Membrane vesicles are produced by both Gram negative and Gram positive bacteria to mediate intercellular communication, antigen delivery, host evasion and immune response modification. MVs are composed of various membrane-associated, periplasmic and cytoplasmic proteins/enzymes/ antigens and can include several PRR ligands such as nucleic acids, peptidoglycan, lipotheicoic acid, and lipopolysaccharide, which makes them potential vaccine carriers and adjuvants (Kaparakis-liaskos and Ferrero, 2015). Present knowledge on bacteria derived membrane vesicles generally focused on pathogenic gram negative bacteria. Studies about two pathogenic gram positive bacteria derived membrane vesicles (M. tuberculosis and S. aureus) were shown to modulate host immune responses (Prados-Rosales et al., 2014, Choi et al., 2015). One human commensal bacteria (Bacteroides fragilis), was shown to secrete polysaccharide A capsular antigen (PSA) containing outer membrane vesicles. PSA-OMVs were shown to trigger TLR2 mediated signaling in DC, stimulating immunoregulatory cytokine IL-10 secretion, promoting maturation of regulatory T cells (Shen et al., 2012).

In our previous studies with MVs (MSc thesis by Esin Alpdundar, 2013), we characterized human gram positive commensal bacteria derived membrane vesicles and compared their activity with non-pathogenic E.coli derived membrane vesicles. We found that commensal bacteria derived MVs triggered higher amounts of IL-10 secretion and lower amounts of IFN-γ secretion from mouse splenocytes when compared to non-commensal derived vesicles. We also showed that commensal

bacteria derived MVs failed to induce antigen presenting cell (APC) maturation. In vivo activity of membrane vesicles was tested in immunization studies using the foot and mouse disease (FMD) vaccine as a model. Results revealed that FMD specific IgG responses were elevated in the presence of non-commensal E.coli derived membrane vesicles, whereas commensal bacteria derived MVs suppressed Th-1 dominated IgG2a production.

Based on these findings, we focused our attention to *Pediococcus pentosaceus*-derived MVs that displayed the highest immunomodulatory activity. Since we wanted to test the immunotherapeutic potential of these MVs, we first had to ensure that each MV batch we prepared had to be identical in composition. To confirm this, MVs isolated from parallel bacterial cultures were compared for their protein contents. Results showed that all batches of MVs contained identical protein bands of similar concentrations supportive of reproducible MV production and isolation protocol.

To confirm our earlier findings from the MSc study showing that commensal-derived MVs could suppress antigen-specific antibody production in vivo, MVs or MVs plus an immunostimulatory agent (cyclic-di-GMP) were administered to mice together with the model vaccine antigen OVA. Results of this immunization study showed that, MV administration alone suppressed anti-OVA specific IgG1 and IgG2c responses. In contrast, inclusion of a danger signal (cyclic-di-GMP) with MVs abrogated their immunosuppressive activity. These results suggest that, when MVs are administered alone, they support a Th1-suppressing immunomodulatory activity but when MVs are mixed with a potent immune stimulatory molecule, this activity is lost. Ensuing tumor challenge experiments with OVA expressing EG.7 thymoma cells provided evidence that MV adjuvanted groups exacerbated tumor formation, whereas MV enrichment with the immunostimulatory agent generated protective anti-tumor response. Therefore, we concluded that MVs could interfere with cellular immunity and suppressed cytotoxic T cell activity. SIINFEKL-stimulated IFN- γ production from cytotoxic T cells were found to be diminished in MV adjuvanted groups, corroborating this conclusion. These results were consistent with our

previous findings and showed that MVs specifically suppressed antigen-specific Th1 and CTL-mediated immune responses.

To understand the mechanism behind this immunosuppressive activity, we hypothesized that MVs could be exerting an immunomodulatory activity by supporting the generating of one of the known regulatory cell types (such as M2 macrophages, myeloid derived suppressor cells or regulatory T cells). Previous studies have shown that several TLR ligands could enhance MDSC frequency and activity (Maruyama et al., 2015, Delano et al., 2007; Bunt et al., 2009, Rieber et al., 2013). Since MVs express multiple TLR ligands, we wanted to analyze the effect of commensal-derived MVs on Macrophage polarization and MDSC generation and activity. BMDMs treated with MVs produced low levels of IL-6 and TNF-α, higher levels of IL-10 and upregulated the expression of Arginase-1 (Arg-1), supporting polarization towards the M2 macrophage phenotype. Furthermore, MVs induced the expression of PD-L1, which is important for inhibition of activated T cells. These results suggest that, BMDM stimulation with MVs lead to the activation of alternatively activated macrophages (M2 macrophages) with an immunomodulatory phenotype.

To assess how MVs impacted the differentiation of hematopoietic stem cells, bone marrow progenitor cells were treated with MVs and results were compared to G-CSF treated samples. MV treated bone marrow progenitors differentiated into cells with characteristic ring-shaped nuclei capable of secreting high levels of IL-10. This nuclear shape and the presence of the immunosuppressive cytokines are characteristic of granulocytic MDSCs. To analyze whether a similar phenomenon occurred in vivo, MVs were intraperitoneally injected and the phenotype of cells accumulating in the peritoneal cavity was determined. In this setting, MVs significantly increased the numbers of both granulocytic and monocytic MDSCs but had no effect on regulatory T cells. These results indicate that MVs generate an immunomodulatory response by generating M2 macrophages and MDSCs but not regulatory T cells.

Since we showed that MVs generated cells with potent immunomodulatory activities, we wanted to assess their anti-inflammatory effects in acute inflammation models. In an acute zymosan-induced peritonitis model, MVs ameliorated excessive inflammation by reducing neutrophil recruitment to peritoneal cavity and inhibiting macrophage loss caused by inflammation. Serum IL-6 levels were normalized in MV treated mice when compared to acute peritonitis induced group, suggesting the suppression of inflammation.

Next, we tested the therapeutic effect of MVs in ulcerative colitis (UC) model to determine whether MVs could inhibit colonic inflammation. In this mouse model of DSS-induced colitis, MV treatment reduced colon shortening which is an important hallmark of disease progression. According to histological analysis, MVs ameliorated disease progression especially in the post-treatment group. Post treatment with MVs protected the colon, wherein the crypt architecture remained nearly intact. Serum cytokine responses also showed that post treatment with MVs lowered circulating IL-6 levels whereas in colitis group and pre-treatment MV groups, IL-6 levels were elevated. Circulating levels of the anti-inflammatory cytokine IL-10 was higher in MV post-treatment group when compared to the DSS treated control group. These results suggest that, MV post-treatment had a more prominent anti-inflammatory protective effect, possibly suppressing DSS-induced colitis, suggesting that MVs could be of interest as novel immunomodulatory therapeutic agents in inflammatory diseases.

Since MVs supported M2 macrophage polarization, we thought that such alternatively activated macrophages (M2 macrophages) could be beneficial in wound healing process because of the role of M2 macrophages in regulating this process and restoring homeostasis. In an excisional wound healing model, intraperitoneal MV administration accelerated wound closure, suggesting that M2 macrophages could be supported. MV administration (i.p.) also led to recruitment of significant numbers of MDSCs expressing PD-L1 to wound area. Histological analysis showed that, MVs accelerate epithelization and healing process when compared with untreated mice.

MV treatment supported differentiation of immunosuppressor cell types (M2 macrophages and/or G-MDSCs), thereby promoting wound healing process.

In conclusion, our studies on *Pediococcus pentosaceus* derived membrane vesicles have shown that MVs have potent immunomodulatory effects. MVs' capacity to mobilize and/or activate suppressive – regulatory cell types make them potent anti-inflammatory agents for the treatment of inflammatory disease or autoimmune diseases.

To better understand *Pediococcus pentosaceus* derived membrane vesicles' immunomodulatory properties and mechanism of action, we have planned for the following future experiments:

1. Using optimal growth conditions, we showed that MVs isolated from different batches had identical protein contents with similar immunomodulatory properties. Stress conditions such as, changes in the pH, increasing or decreasing growth temperature may cause a variation in MVs' constituents and hence impact immomodulatory activity. We are planning to isolate membrane vesicles from bacteria grown under various stress conditions and analyze their immunomodulatory properties.
2. To determine MV's effect on T cell polarization, we are planning to generate bone marrow derived macrophages and stimulate these macrophages with MVs. At the same time, we are planning to isolate naïve T cells from mouse spleens and perform co-culture with macrophages. After the incubation period, we will determine cytokine levels from culture supernatants for IL-10, IFN-ɣ, IL-4 and IL-17. To analyze IL-10 secretion from regulatory T cells we will perform intracellular cytokine staining by staining Foxp3 marker and IL-10 cytokine. Furthermore, MV treated macrophages will be co-cultured with CFSE-stained naïve T cells and reduction in a-CD3/a-CD28-mediated proliferation response will be assessed in comparison to naïve T-cells co-cultured with classical M0 macrophages.
3. Since MVs are enriched with TLR ligands, we think that peptidoglycan (PGN) which is recognized by TLR2 might be a dominant molecule through which MVs

exert their immunomodulatory function. To test for this assumption, we are planning to transfect TLR2 to HEK cells and stimulate these cells with MVs to analyze TLR2 specific response. Further with collaborations, TLR2 knockout mice could be used for analyzing MVs immunomodulatory effects in the absence of TLR2 pathway on various disease models.

4. MVs effect on differentiation of bone marrow progenitor cells to granulocytic myeloid derived suppressor cells can be further determined by analyzing Arginase-1 expression levels by qRT-PCR. Differentiated cells can also be stained for G-MDSC markers (CD11b, Ly6G and Ly6C) and analyzed by using flow cytometer.

5. Lastly, we are planning to extend our statistical analysis by using different methods to check significance (One-way ANOVA, two-way ANOVA and MANOVA) by using R and SPSS.

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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 ddH_2O
- 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 $Na_2HPO_4 . 2H_2O$
- 2 grams KH_2PO_4

Complete into 1 lt with ddH_2O (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 dH_2O

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 ddH_2O

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 dH_2O

Running Buffer [10x]

- 30 g Tris
- 144 g Glycine
- 10 g SDS

Complete 1 L with dH_2O

Use 1x diluted in dH_2O.

Staining Solution

- 0.1 g Coomasie Brilliant Blue G-250
- 100 ml methanol
- 20 ml acetic acid

Destaining Solution

- 500 ml methanol
- 100 ml acetic acid
- 400 ml water

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 ddH_2O

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)

APPENDIX B

PHENOTYPE DETERMINATION OF CELLS GENERATED FOLLOWING IP INJECTION OF MVS

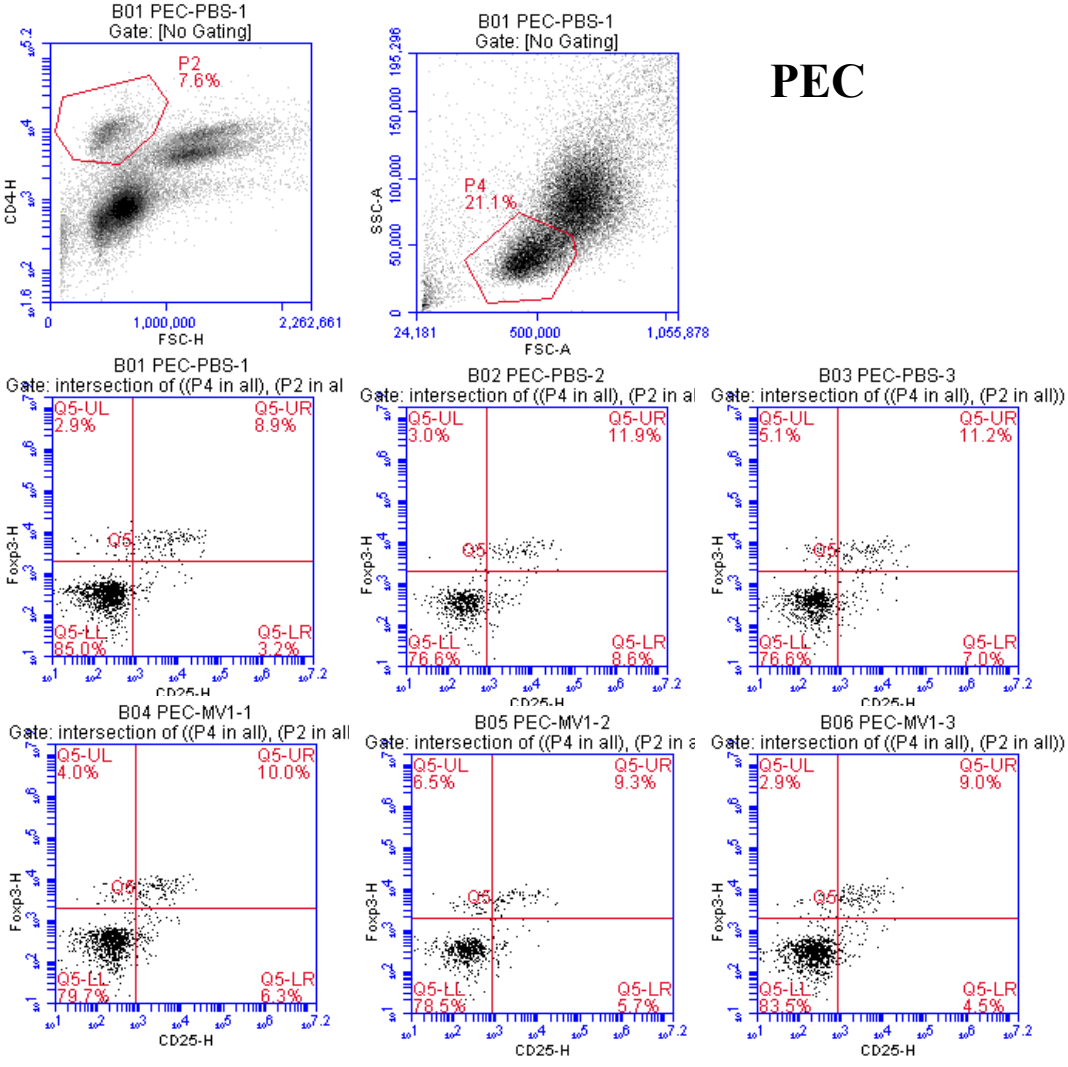

Figure B.1 Phenotype determination of cells generated following intraperitoneal injection of MVs.

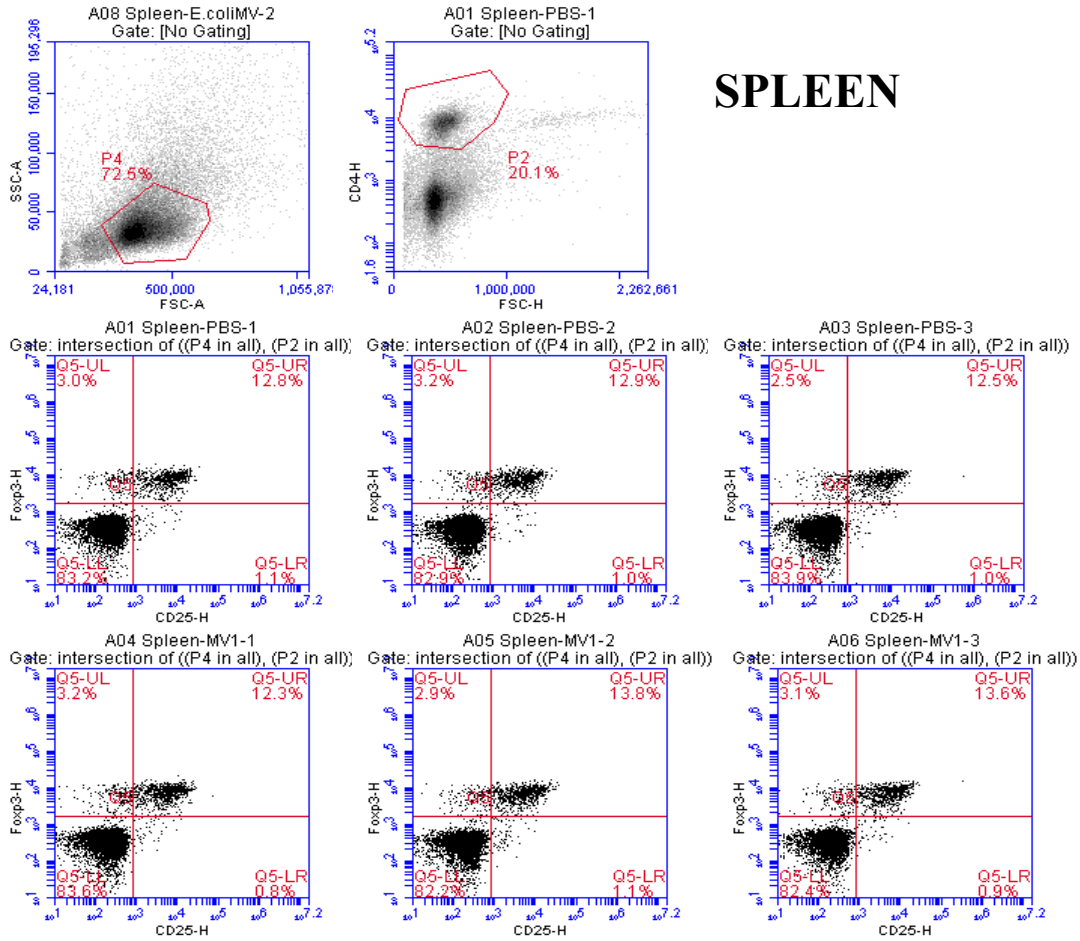

Figure B.2 Phenotype determination of cells generated following intraperitoneal injection of MVs. C57BL/6 mice were i.p. injected with MVs (10 μg/mouse) and saline on days -4 and -1. On day 0, mice were sacrificed and splenocytes (A) and PECs (B) were stained for CD4, CD25 and Foxp3 expression. CD4 cells were gated and analyzed for CD25 and Foxp3 percentages.

APPENDIX C

qRT-PCR AMPLIFICATION CURVE OF ARGINASE-1 AND NOS-2

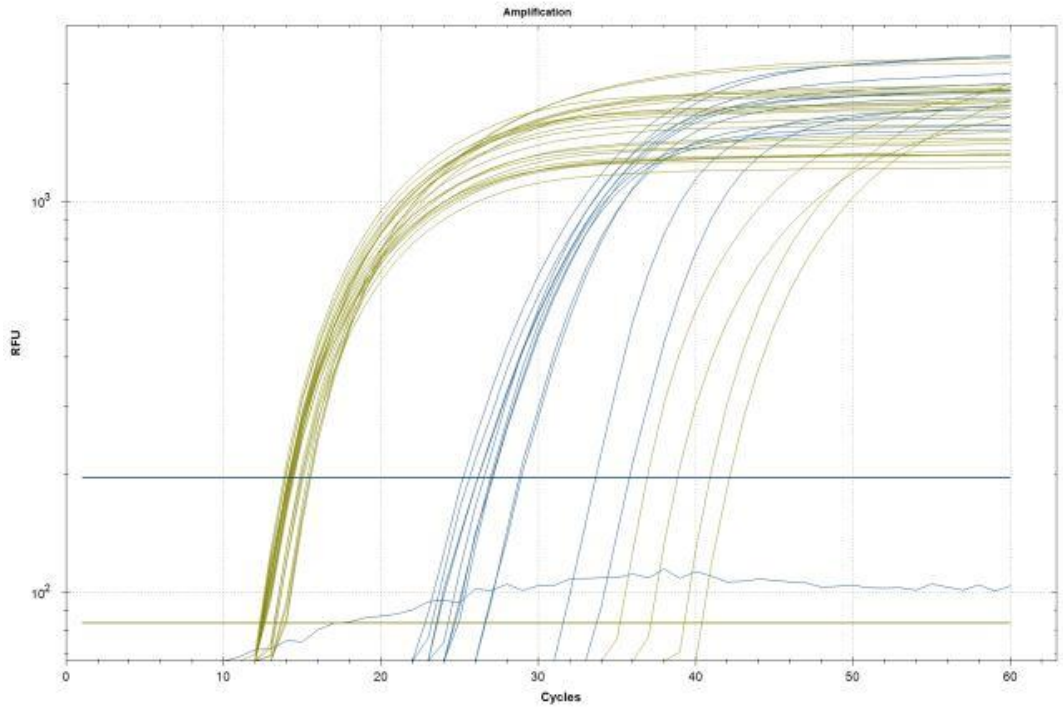

Figure C.1. qRT-PCR Amplification Curve of Arginase-1. Green lines indicate endogenous control 18S RNA, Blue lines indicate Arginase-1 (Arg-1)

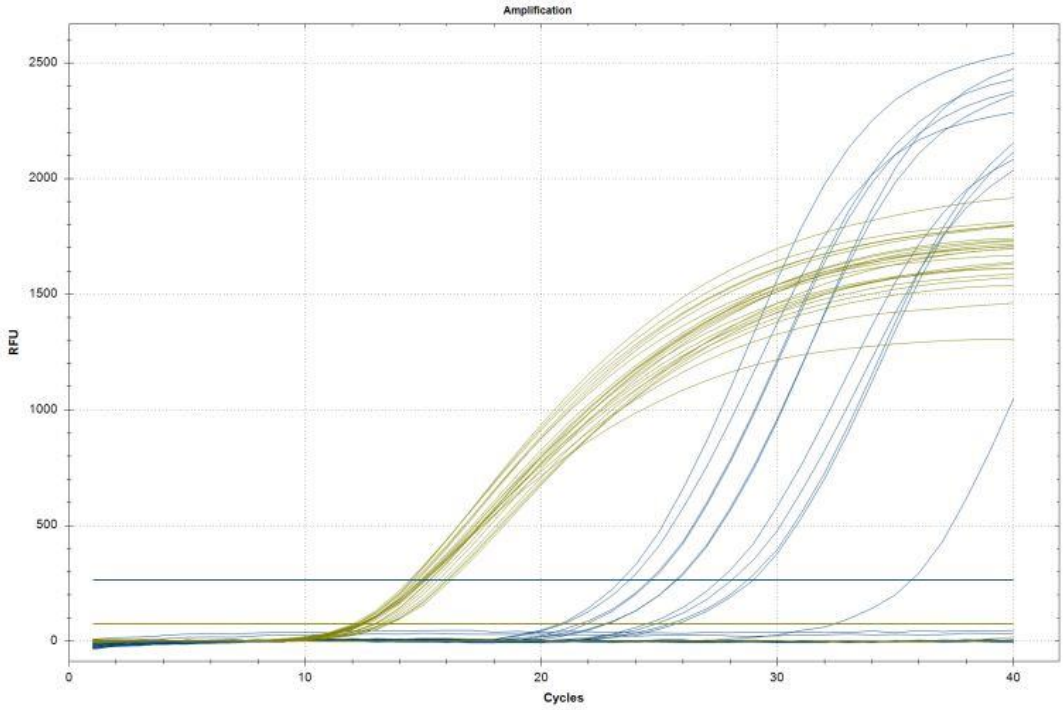

Figure C.2. qRT-PCR Amplification Curve of NOS2. Green lines indicate endogenous control 18S RNA, Blue lines indicate NOS2.

APPENDIX D

ANTI-OVA SPECIFIC IgG1 AND IgG2C ANTIBODY TITERS

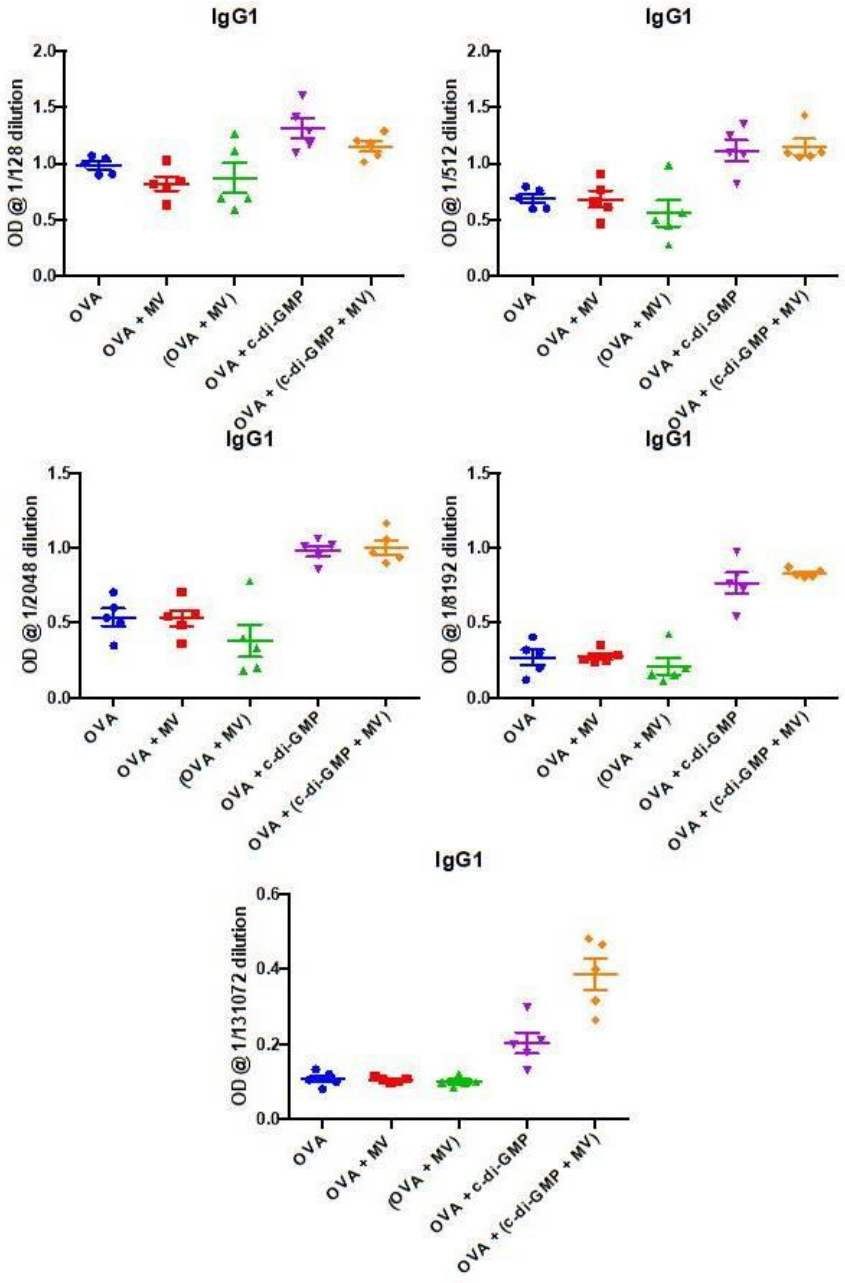

Figure D.1. Anti-OVA specific IgG2c antibody titers of individual mice immunized with MV formulations

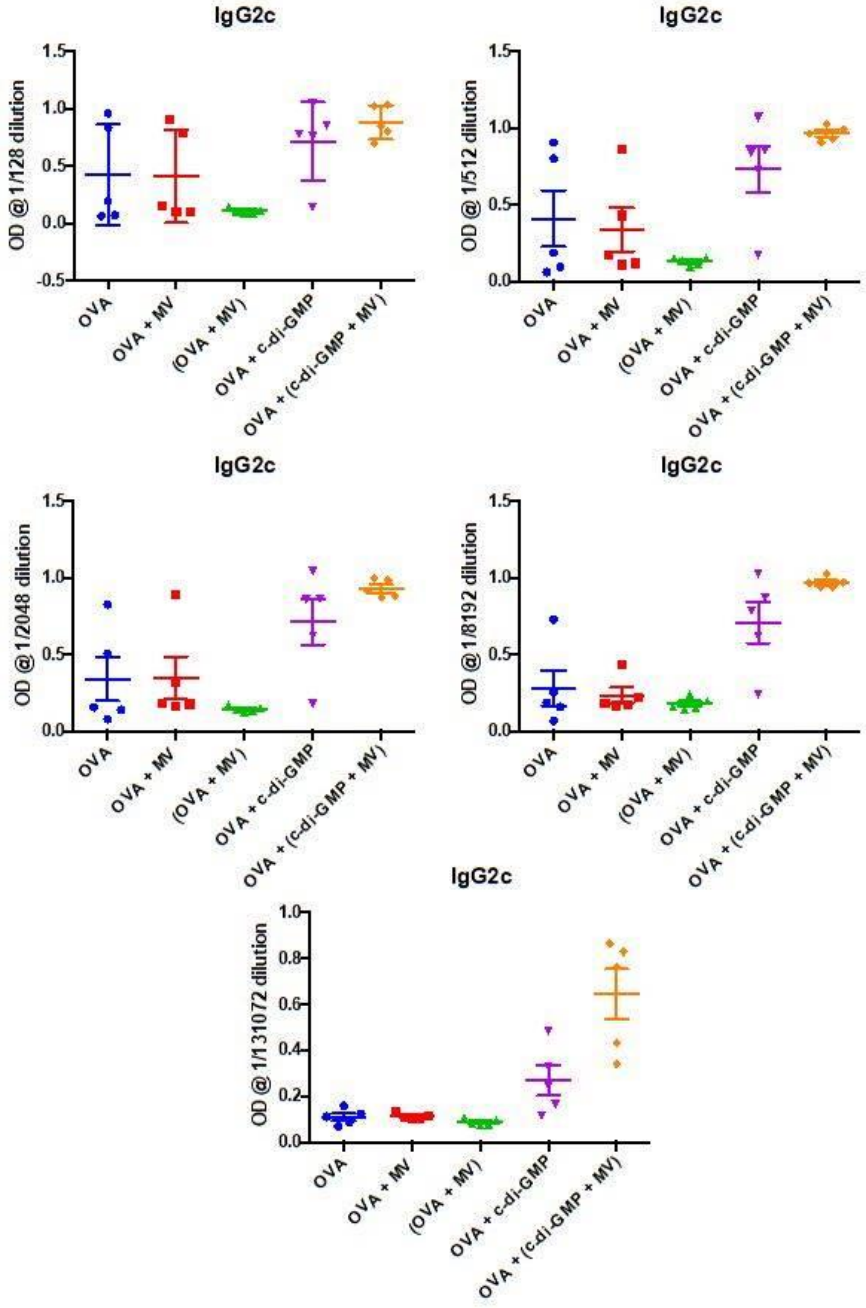

Figure D.2. Anti-OVA specific IgG2c antibody titers of individual mice immunized with MV formulations C57BL/6 mice were immunized on day 0 and 14 with OVA (7.5 μg/mouse), MV (10 μg/mouse) and c-di-GMP (15 μg/mouse) and their formulation. After secondary bleeding, OVA specific total IgG, IgG1, IgG2c levels were detected via ELISA. Measurements were done at 1/32,768 serum dilution by ELISA. Symbols represent individual mice.

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PUBLICATIONS:

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2. Bayyurt B, Tincer G, Almacioglu K, **Alpdundar E**, Gursel M, Gursel I. (2017) Encapsulation of two different TLR ligands into liposomes confer protective immunity and prevent tumor development. **J Control Release**. doi: 10.1016/j.jconrel.2017.01.004.
3. Yildiz S, **Alpdundar E**, Gungor B, Kahraman T, Bayyurt B, Gursel I, Gursel M. (2015) Enhanced immunostimulatory activity of cyclic dinucleotides on mouse cells when complexed with a cell-penetrating peptide or combined with CpG. **Eur J Immunol.** doi: 10.1002/eji.201445133
4. Gungor B, Yagci FC, Tincer G, Bayyurt B, **Alpdundar E**, Yildiz S, Ozcan M, Gursel I, Gursel M. (2014) CpG ODN nanorings induce IFNα from plasmacytoid dendritic cells and demonstrate potent vaccine adjuvant activity. **Sci Transl Med.** doi: 10.1126/scitranslmed.3007909

SCHOLARSHIPS & AWARDS:

1. Registration and accommodation award by EFIS (European Federation of Immunological Societies) (Molecular Immunology & Immunogenetics Congress 3, Antalya, Turkey, April 2016).
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CONGRESS & WORKSHOPS:

1. **Alpdundar E,** Bayyurt B, Aydin M, Akçalı C, Gursel I, Gursel M, "Kommensal Bakteri kökenli membran keseciklerin immün düzenleyici etkileri" 24th National Immunology Congress April 27-30th, İstanbul, Turkey, 2017 **(Oral Presentation)**.
2. **Alpdündar E.**, Yıldız S., Bayyurt B., Aydın M., Şanlı A., Akçalı C., Gürsel İ., Gürsel M., "Commensal Bacteria-derived Membrane Vesicles As Novel Immunomodulatory Therapeutic Agents", 3rd Molecular Immunology & Immunogenetics Congress (MIMIC 2016) April 27-30th, Antalya, Turkey, 2016 **(Poster Presentation)**.
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