

IN VITRO AND IN VIVO IMMUNOMODULATORY EFFECTS OF EXTRACELLULAR VESICLES

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By

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December, 2013

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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M.S. in Molecular Biology and Genetics

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The major theme of this thesis was to characterize and understand the immunomodulatory potential of extracellular vesicles (EVs) isolated from different mouse and human cell lines on immune cells. To this end, we first purified and characterized the particle nature of exosomes and microparticles (MPs) and exosomes by AFM and DLS analyses. Next, we documented their *in vitro* or *in vivo* differential uptake/internalization kinetics by immune cells. Finally, we tested the potential application of EVs as a drug delivery system. The physicochemical characterization studies confirmed that EVs were in vesicular form and upon reconstitution followed by lyophilization they retained their original sizes (i.e. size ranges were 100-150 nm for exosomes, and 250-500 nm for MPs, respectively). MPs derived from mouse cell lines (macrophage, T-cell and fibroblast) were stained and administered *in vitro* and *in vivo* to track their internalization by immune cells. When incubated in culture, origins of MPs greatly affected uptake rate and ratio by different immune cells. This differential internalization pattern of distinct MPs was reproduced when they were injected i.p. to mice. When incubated in culture B-cells took up the most MPs from fibroblast origin cells. Macrophages of peritoneal exudate cells took up RAW264.7 derived MPs at the highest level upon 24h post-ip injection. We tested whether MPs have a role in transportation or presentation of bacterial products during an ongoing infection by adhering circulating pathogenic by-products and carrying them to distant immune cells. Our data suggest that MPs could contribute to the severity of ongoing infection *in vivo*, since MPs from different cells can adhere distinct ligands such as LPS or DNA or even RNA and transmit these ligands to naive innate immune cells augmenting the immunostimulatory response raised against these ligands. Lastly, the immunotherapeutic potential of EVs harboring immunosuppressive synthetic oligodeoxynucleotide sequence namely, A151 was tested following dehydration-rehydration method on mouse splenocytes. EV-associated A151 displayed improved inhibition of immune activation triggered by TLR7/8 and TLR9 ligands on spleen cells. When taken together, this study established that various types of EVs derived from different cells induces plethora of activities on immune cells. Furthermore, EVs are potential drug carrier systems when loaded externally with suitable agents and can be harnessed in immunotherapy of diseases.

Keywords: Exosome, microparticle, TLR, immunosuppressive DNA, immunomodulation .

ÖZET

HÜCRE DIŐI KESECİKLERİN İN VİTRO VE İN VİVO İMMÜNMODÜLATÖR ETKİLERİ

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Moleküler Biyoloji ve Genetik, Yüksek Lisans

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Bu tezin ana konusu, değişik fare ve insan hücre hatlarından elde edilen, hücreler arası iletişimi sağlayan partiküllerin bağışıklık sistemi hücreleri üzerindeki olası etkilerini anlamak ve araştırmaktır. Bu amaçla öncelikle hücre dışı kesecikleri izole edip partiküler yapılarını atomik güç mikroskopu (AFM) ve dinamik ışık saçılması (DLS) metodları kullanılarak analiz ettik. Ek olarak bu partiküllerin in vitro ve in vivo olarak bağışık sistemi hücreleri tarafından hücre içine alınma kinetiğini de belgelemeye çalıştık. Son olarak da ilaç taşıma sistemi olarak olası kullanımlarını tanımladık. Keseciklerin fiziko-kimyasal karakterizasyonları DLS ve AFM ile yapılarak, bunların liyofilizasyon sonucu kesecik yapılarını korudukları ve boyutlarının değişmediği gösterildi (Ekzosomlar 100-150 nm boyutlarında, mikropartiküller ise 250-500 nm boyutlarında). Fare hücre hatlarından elde edilen mikropartiküller (Makrofaj, T hücresi ve Fibroblast) boyanarak in vitro ve in vivo olarak bağışıklık sistemi hücrelerince alımları takip edildi. Hücre kültürü çalışmalarında mikropartiküllerin kaynağının değişik bağışıklık sistemi hücrelerince alım oranını önemli derecede etkilediği gözlemlendi. Intraperitoneal olarak da farelere enjekte edilen mikropartiküllerin hücre içine alım farkları gösterilmiştir. Kültürde dalak hücreleri ile inkübe edildiğinde fibroblastlar tarafından üretilmiş vesiküllerin çoğunluğu B hücreleri tarafından alınırken, Ip enjeksiyonundan 24 saat sonra peritoneal sıvı hücrelerinin RAW 264.7 tarafından sentezlenen mikropartikülleri en yüksek düzeyde aldığı gözlemlenmiştir. Daha sonra mikropartiküllerin enfeksiyon sırasında patojenlere bağlanarak bakteri ürünlerinin taşınmasında ve uzak yerlerdeki bağışıklık sistemi hücrelerine sunulmasında rol alıp almadığı sorgulandı. Farklı mikropartiküllerin LPS, DNA ya da RNA gibi farklı ligandlara bağlanarak bunları uyarılmamış bağışıklık sistemi hücrelerine ilettiğini ve immün aktivasyonu artırdığı gözlemlenmiştir. Bu bulgular mikropartiküllerin in vivo enfeksiyonun düzeyine katkı sağladığını işaret etmektedir. Son olarak, bu keseciklerin potansiyel terapötik özellikleri sentetik bağışık sistemi baskılayıcı bir oligodeoksinükleotid olan A151 dehidrasyon-rehidrasyon yöntemi ile dalak hücrelerinde test edilmiştir. A151 içeren kesecikler TLR7/8 ve TLR9 ligandları ile uyarılan dalak hücreleri üzerinde daha etkin bir baskılama göstermiştir. Sonuç olarak bu çalışma değişik hücreler tarafından üretilen keseciklerin bağışık sistemi hücreleri üzerinde bir çok etkisi olduğunu göstermiştir. Ek olarak keseciklerin potansiyel bir ilaç taşıma sistemi olarak dışarıdan yüklenebilen uygun ajanlar olduğu ve hastalıkların tedavisinde kullanılabileceği anlaşılmıştır.

Anahtar sözcükler: Ekzosom, mikrokesecek, TLR, immunbaskılayıcı DNA.

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ABBREVIATIONS

A.Bleb	Apoptotic Bleb
A.Exo	Apoptotic Exosome
Ab	Antibody
AFM	Atomic force microscopy
APC	Antigen presenting cell
AIDS	acquired immunodeficiency syndrome
AO	Acridine orange
bp	Base pairs
BCR	B-cell receptor
Cag	Cytotoxin associated gene
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CpG	Unmethylated cytosine-phosphate-guanosine motifs
CFSE	Carboxyfluorescein succinimidyl ester
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DSL	Dynamic light scattering
DTP	Delayed type hypersensitivity
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunesorbent Assay
ESCRT	Endosomal sorting complex responsible for transport
EV	Extracellular vesicle
Exo	Exosome
FasL	Fas ligand
FBS	Fetal Bovine Serum
GPI	Glycosylphosphatidylinositol
HIV	Human Immunodeficiency Virus
HSC	Heat shock cognate
HSP	Heat shock protein
ICAM	Intercellular Adhesin Molecule
Ig	Immunoglobulin

I κ K	Inhibitor kappa B kinase
IL	Interleukin
ILV	Intraluminal vesicle
IFN	Interferon
IP	Interferon induced protein
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LBP	LPS-binding protein
LN	Lymph Node
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic Acid
MALP	Mycoplasmal lipopeptide
MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
miRNA	Micro RNA
MP	Microparticle
MSR	Macrophage scavenger receptor
mtDNA	Mitochondrial DNA
MV	Microvesicle
MVE	Multivesicular Body
MSC	Mesenchymal stem cell
MyD88	Myeloid Differentiation Primary Response gene 88
NF- κ K	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular patterns

PBS	Phosphate buffered saline
PEC	Peritoneal exudate cells
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
poly(I:C)	polyinosinic acid:cytidylic acid
PNPP	Para-nitrophenyl pyro phosphate
PRR	Pattern recognition receptors
PS	Phosphatidylserine
RIG	Retinoic acid inducible gene
RIP	Receptor-interacting protein
R848	Resiquimod
RT	Reverse transcriptase
SA-AKP	Streptavidin Alkaline-phosphatase
SP-DiOC	3,3'-Dioctadecyl-5,5'-Di(4-Sulfophenyl)Oxacarbocyanine
SSCL	Sterically stabilized cationic liposomes
ssRNA	Single-stranded RNA
STAT	Signal Transducer and Activator of Transcription
TB	Trypan Blue
TCR	T-cell receptor
TF	Transcription factor
Th	T-helper
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated factor
TRAM	TRIF-related adaptor molecules
Treg	Regulatory T-cell
TRIF	TIR domain containing adaptor inducing IFN- γ

Chapter 1

Introduction

1.1 Immune System

The immune system is an organization of cells and molecules which protects the body against infections. Because pathogens can rapidly evolve they can parry the host defense by developing resistance or being not detected. However, various immune mechanism have also evolved in order to recognize and eliminate the new pathogens. Even very simple organisms such as bacteria have a defense system consists of enzymes that protects against phage infections [1]. Yet, the mammals and other higher organism have a much more complicated mechanism called immune system which involves various cells, compounds and interactions. In mammals, the immune system protects the body from surface to interior as layers of protection. The first layer, skin forms a physical barrier to prevent bacteria and viruses from entering the body. The mucosal immunity is also belonged to first barrier of immune system. Mucus covers the largest area of the epithelial surface of body and eliminates gut or respiratory pathogens [2]. If pathogens survive the first barrier, the innate immune system detects and forms a quick response in order to activate mechanism to kill and digest pathogens. Innate immunity is evolutionarily one of the oldest defense mechanism found in all classes of animal and plant life. Innate immune system can distinguish self and non-self cells by recognizing specific pathogenic compounds. A baby's and an old man's innate immunity can recognize same range of foreign molecules. [3]. When pathogen is not eliminated by innate immune system adaptive immunity step in and activates various mechanism for recognition and clearance of pathogens. Only vertebrates have adaptive immune system. Unlike

innate immunity, adaptive immunity cannot form a quick response. It takes 2-3 days for immune cells to produce specific antibodies against pathogens. Adaptive immunity is also called as acquired immune system. Immune system gains ability to detect new pathogens by encountering with them. When, body is infected with the same pathogen again, immune system forms a quick response because it remembers the pathogen specific antigens. A newborn baby acquires adaptive immunity months after birth. [4] However, breast milk contains ready-made antibodies which provide a passive immunity for infant. Passive immunity can also occur when maternal antibodies are transferred to the fetus through placenta during pregnancy [5,6]. For many years, innate and adaptive immunity has been considered as two distinct mechanisms but recent studies of host defense against pathogens have demonstrated that the generated adaptive response is dependent on innate responses. Especially, Toll-like receptors(TLRs), the key elements of innate immunity have been shown to have an essential role in activation of adaptive immunity [7–10].

1.2 Innate Immunity

Innate immunity is the first line of defense mechanism that protect the host from infections. It is also known as non-specific immune system because it does not develop immunity against specific pathogens. Unlike adaptive immune system it does not provide long-lasting immunity to the host and provides immediate response to the infection [10].

Innate immunity in vertebrates consist of anatomical barriers, secretory molecular and cellular components. The epithelial surfaces, mucus and saliva form a physical barrier against microorganism. Fluids secreted from our body generally contains antimicrobial chemicals. For instance, fatty acids in sweat inhibit the bacterial growth. In tears, saliva and nasal secretions there are lysozyme and phospholipase which can disrupt the cell wall and membrane of the bacteria. Defensins and surfactants found in the lung track also have antimicrobial activity. If microorganisms pass the anatomical barriers infection occurs and humoral factors step in. Humoral factors play important role in the recognition of pathogen, inflammation, migration of immune cells, killing pathogen and clearing pathogenic waste. Complement system is one the major humoral factors. It may lead to increased vascular permeability for recruitment of phagocytic cells, and lysis and opsonization of bacteria. Inflammation in the infection site also activates coagulation system which can trigger clotting, increased vascular

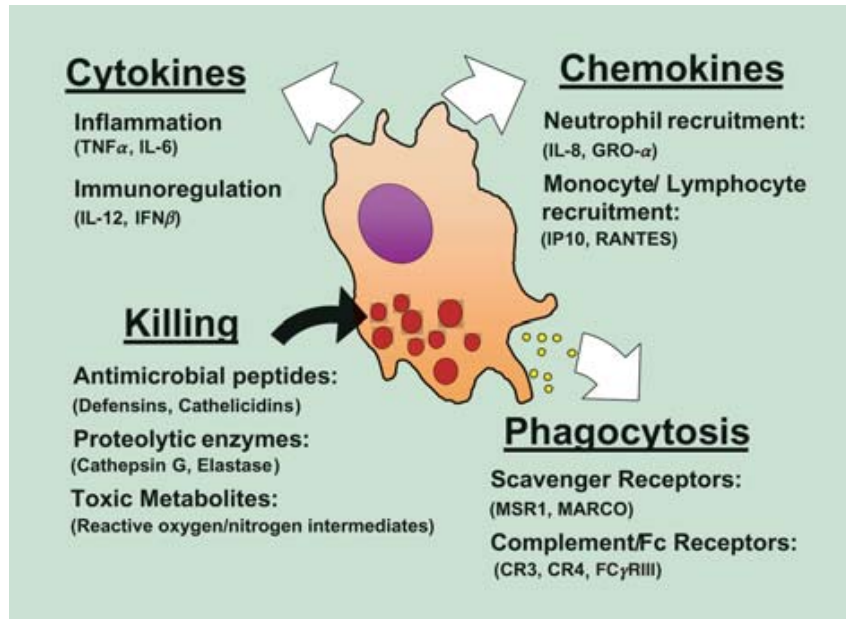


Figure 1.1: Overview of the diverse mechanisms activated during innate immune response to pathogens. Image adapted from [11].

permeability, secretion of chemotactic agents for phagocytic cells and antimicrobial molecules. Lactoferrin, transferrin, lysozyme are other antimicrobial humoral factors secreted by immune cells. The most important factors during infections are cytokines secreted by immune cells, such as interferons and interleukins which help signaling between immune cells. Also, interferons can limit virus replication by increasing p53 activity. Cellular barrier in the innate immune system consist of neutrophils, monocytes, macrophages, mast cells, natural killer (NK) cells and eosinophils. Neutrophils and macrophages can phagocytose invading organisms and kill them intracellularly. NK cells kills pathogens by secreting cytotoxic molecules. Eosinophils secrete chemical mediators which are effective in killing certain parasites. Multiple mechanisms used by innate immune system are depicted in Figure 1.1. Inflammation on the infection site is very important for the migration and activation of immune cells. It is initiated by pro-inflammatory cytokines released from macrophages after recognition of pathogen. These cells present certain receptors termed pattern recognition receptors (PRRs) on their surface, which can recognize common pathogenic molecules that are distinguishable from host molecules. Those non-self molecules are known as pathogen-associated molecular patterns (PAMPs). When PRRs on the cell surfaces interact with PAMPs inflammatory pathways are activated [1, 10].

1.3 Adaptive Immunity

Adaptive immunity is a highly sophisticated defensive system against pathogens and found in vertebrates. It is also known as acquired immune system because it develops new strategies during infections. Unlike innate immune system, adaptive immune response forms very slow (4-7 days), but it provides very long protection. Adaptive immunity is composed of highly specialized processes initiated with the activation of innate immune cells by pathogen recognition and presentation of digested pathogenic antigens by APCs. Three main classes of APCs are DCs, macrophages and B-cells. APCs begin processing pathogenic antigen by either phagocytosis or receptor mediated internalization of pathogen. After degradation of foreign structures, small fragments of antigens presented on cell surface as complexed with MHC class I and II molecules. Only professional APCs and certain activated epithelial cells have express MHC molecules but many infected cells can present pathogenic antigens. Secreted cytokines from innate immune cells and interaction from APCs initiate proliferation of naive T-cells (see Figure 1.2). Activated T-cells either kill recognized pathogens or help other cells to proliferate or activate them. T helper cells activates B-cells with matching antigen by interaction and $\text{IFN-}\gamma$ secretion. Activated B-cells multiply and mature into antibody producing plasma cells. Then, released antibodies attach to the pathogenic antigens and mark them. Last, those marked pathogens or infected cells are cleared killer cells such as cytotoxic T-cell, macrophages and NK-cells. Activated B-cells during primary immune response, can form memory B cells which are able to live for a long time and respond quickly in the case of infection with same pathogen [10,11].

1.4 Cells of the Immune System

Immune response against pathogens is prevailed complex interactions and activities of the diverse cells types. Primary lymphoid organs like the bone marrow and the thymus are the sites of lymphocyte production and maturation. Immune cells circulate through the blood and move to secondary lymphoid organs like the spleen and lymph nodes, where APCs present antigens to lymphocytes [10].

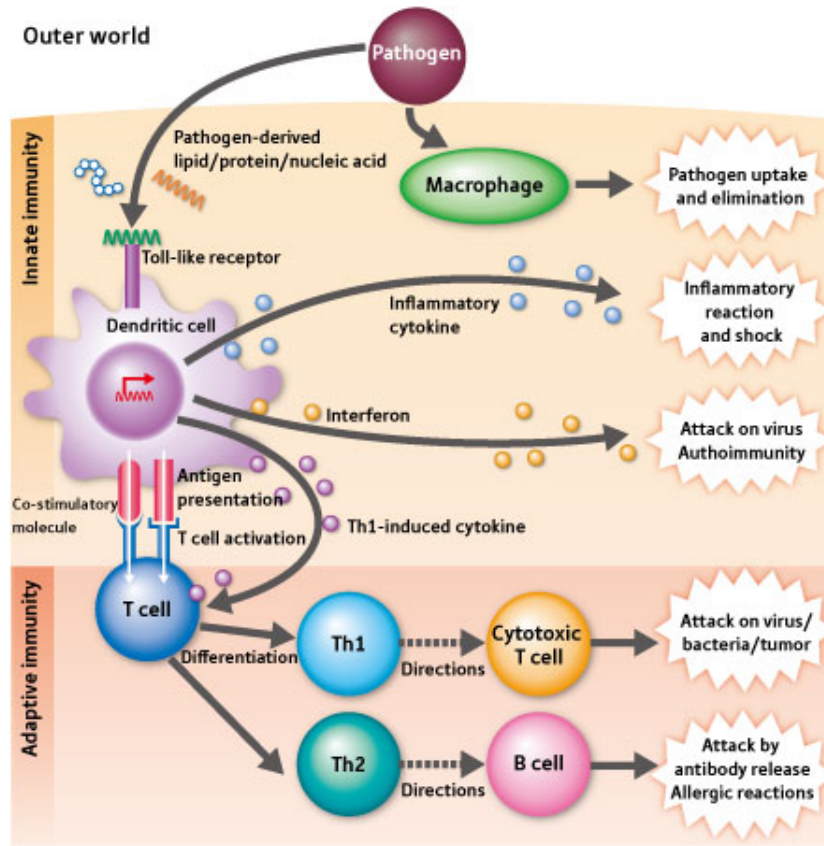


Figure 1.2: Innate response triggered by PRR-PAMP interactions induces and instruct adaptive immunity through proinflammatory cytokines and dendritic cell mediated antigen presentation to T-cells. Image adapted from [12].

1.4.1 Dendritic cells

Dendritic cells (DCs) are the most specialized antigen presenting cells, initiating both primary and secondary immune responses. The main function of DCs is to process antigens and transmit the information to the T cells of the immune system, acting as a messenger which links innate and adaptive immunity [10]. DCs are in an immature state with high phagocytic capacity when they originate from hematopoietic bone marrow progenitor cells. Once activated DCs migrate to lymph nodes where they interact with T-cells and B-cells to initiate adaptive immune response. DCs have PRRs such as TLRs which makes them detect danger signals [13] and become activated. During activation DCs leave their immature state; becoming less phagocytic, producing cytokines and upregulating co-stimulatory molecules such as CD86 which is required for T-cell activation [14].

DCs are mainly present in tissues but rarely found in the blood circulation where they constitute approximately 1 percent of the peripheral blood mononuclear cells (PBMC). Tissues in contact with external environment are more likely to present DCs, such as skin and the inner lining of the nose, lungs, stomachs and intestines. Humans have two subsets of DCs in peripheral blood called myeloid (m)DC and plasmacytoid (p)DC. Phenotypes and functions of mDCs and pDCs are incompletely described but some of their common and contrary characteristics have been determined. mDCs are CD11c+ and major stimulators of T-cells whereas pDCs are DC11c- and can produce large amounts of type I interferon upon activation [15].

1.4.2 T lymphocytes

T cells are the critical components of the adaptive immunity. T-cells are originated from haematopoietic stem cells in bone marrow but they complete their maturation in thymus. The T cell receptor (TCR) is a highly specific molecule found on the surface of T cells and responsible for recognizing antigen bound to MHC class proteins. TCR diversity is carried by a series of gene rearrangement events in the thymus. T cells expressing a wide repertoire of TCR undergo positive and negative thymic selection before completing their maturation [10]. In the positive selection, thymic stroma cells express MHC molecules and T-cells with TCR that are able to interact with those MHC molecules properly selected. MHC interaction provides survival signal during this step and T-cells with no survival signals die. Survived T-cells migrate from cortex to the medulla of the thymus. In medulla (negative selection), stroma cells express MHC/self-peptide complexes and T-cells which strongly interact with those molecules receive apoptotic signals and die. Survived ones leave thymus as matured naive T cells. When a naive T cell interacts with an activated APC it differentiates into effector cell of which there are several types. After a pathogen infection is cleared most of the effector T-cells undergo apoptosis, but some of them survive as long-lived memory cells which can form rapid immune response against same pathogen [10].

1.4.2.1 Cytotoxic T cells

The cytotoxic T-cells (also known as killer T cells) harbor pathogens and cancer cells. They have the CD8 co-receptor in association of TCR on their surface which can bind to MHC

class I:peptide complex. Upon activation they interact with target cells and release cytotoxic effector molecules like perforin and granzymes [10].

1.4.2.2 T helper cells

T helper cells express CD4 co-receptor which can recognize MHC class II:peptide complexes together with TCR. Upon TCR triggered activation, T helper cells differentiate into two major subtypes of cells known as Th1 and Th2 [16]. Each subtype produce different cytokines have distinct functions. Th1 helper cell facilitate eradication of intracellular bacteria and protozoa through activation of natural killer cells and macrophages by IFN- γ secretion whereas Th2 helper cells activate B-cells to produce immunoglobulins [17].

1.4.2.3 Regulatory T cells

During negative selection of in thymus, auto-reactive T-cells are not completely excluded and move into circulation. Regulatory T cells (T regs) suppress the effector functions of those auto-reactive T-cells through release of IL-10, and anti-inflammatory cytokine and TGF- β [18]. T regs divide into two subtypes, naturally occurring and adaptive [19]. Naturally occurring T regs are matured in the thymus with immunosuppressive properties whereas adaptive T regs differentiate from the naive peripheral T cells [18].

1.4.3 B lymphocytes

B cells are vital components of adaptive immune system. In mammals, they are formed in bone marrow and complete their maturation here. B cells which leave bone marrow recirculate and populate in the spleen and lymph nodes. They present the B cell receptors (BRC) on the surface, which can bind antigens. Self-reactive BCR carrying B cells are also eliminated in the bone marrow. BCR is a membrane bound antibody composed of a conserved shaft attached to cell membrane and a unique, randomly determined antigen-binding site (immunoglobulin). Each B cell express unique BCR which can bind to only one particular antigen. B cells become activated when two BCR are cross-linked by this particular antigen. Then, as an APC, B cell process antigen and present the peptides as attached to MHC class II molecules which attract a matching T helper cell. Cytokines secreted by T cell

triggers complete activation of B cells. Activated B cells begin to multiply and mature into soluble immunoglobulin (antibody) producing plasma cells. Released antibodies attach to the matching antigens. Cells or pathogens marked with antibodies then cleared by complement cascade or killer immune cells. TLR signaling or repetitive bacterial peptides may also trigger B cell activation without T cell interaction [10,20,21].

1.4.4 Macrophages

Macrophages are produced by differentiation of monocytes in tissues. They are the first cells which recognize pathogens and produce immune response. Macrophages are specialized phagocytic cells which can ingest pathogens or attack with killer molecules. Macrophages have role in both innate and adaptive immunity. When pathogenic compounds are recognized through PRRs in a non-specific manner, macrophages become activated, increasing surface area and becoming highly phagocytic. Macrophages present antigens of ingested pathogens on their surface and can activate T cells. Also, upon activation macrophages secrete chemokines and cytokines which can attract and activate other immune cells [10,22].

1.5 Cytokines

Cytokines are the main contributors of communication between immune cells. Various secreted cytokines have roles in triggering inflammation, activation and differentiation of immune cells as well as suppressing immune response. Some of the cytokines are proinflammatory which are necessary for the initiation of immune response to recruit granulocytes and lymphocytes. Other cytokines are anti-inflammatory and function in the inhibition of inflammation and promotion of healing once the infection is secured [23]. Over-exuberant immune responses may cause autoimmune and auto-inflammatory diseases and serious injury. Therefore, anti-inflammatory cytokines are essential for the recovery of the body after infections and injuries.

Tumor necrosis factor alpha (TNF- α) is mostly secreted by activated macrophages and triggers local containment of infection. TNF- α secretion by macrophages on the infection site induces increased release of plasma proteins into tissue, enhanced blood vessel permeability, increased phagocyte and lymphocyte migration into tissue and improved platelet adhesion

to blood vessel wall. Although TNF- α secretion is essential local containment of infection, in certain bacterial infections in bloodstream, systemic release of TNF- α into the circulation causes loss a significant volume of plasma and eventually to shock, known as septic shock [24].

Interleukins (ILs) are subgroup of cytokines which mediates activation, differentiation and development of immune cells. IL-1 β is mainly produced by blood monocytes and macrophages as a potent mediator to immune response to infections and injuries. IL-1 β is one of the early cytokines released during infection and induces expression of other proinflammatory cytokines and activation of T-cells and macrophages [24]. IL-6 is another proinflammatory cytokine mostly released by T-cells, macrophages and endothelial cells. It has a role in growth and differentiation of B-cells, acute phase protein production and fever [24]. Increased expression and secretion of IL-6 induces autoimmune and autoinflammatory conditions in various diseases such as atherosclerosis [25], diabetes [26], Alzheimer's Disease [27], systemic lupus erythematosus [28], multiple myeloma [29], Behçet's disease [30], rheumatoid arthritis [31] and prostate cancer [32].

IL-12 (known as NK-cell stimulatory factor) is chiefly secreted by macrophages and DCs. It activates NK-cells and induces differentiation of T helper cells into Th1-like cells. Disrupted IL-12 production or functioning causes impaired IFN- γ production and Th1 responses [24]. Interferons (IFN) are large group of proteins which have various functions in metabolism, such as anti-viral activity, modulating immune responses against viruses, bacteria, parasites and tumor cells and promoting activation and differentiation of cells. Interferons (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells. They allow communication between cells to trigger the protective defenses of the immune system that eradicate pathogens or tumors. They are mainly divided into two subclasses according to the their roles: Type I IFN, Type II IFN. Type I interferons (IFN- α and IFN- β) are essential for protection against viral infections , whereas type II interferon (IFN- γ) has roles against bacterial and parasitic infections. IFN- α and IFN- β are secreted by many cell types especially pDCs and lymphocytes and stimulate macrophages and NK cells to elicit antiviral and anti-tumor responses. IFN- γ is secreted by activated T-cells and NK cells, and function in the regulation of the inflammatory responses in both innate and adaptive immunity. T helper cells produces IFN- γ to help macrophages kill the bacteria and to induce proliferation of B-cells and class switching [24]. Extensive production of IFN- γ is associated with various autoinflammatory and autoimmune diseases [33].

1.6 Pattern Recognition Receptors

PRRs are primitive part of the immune system, which are essential for detecting invading pathogens and initiating innate and adaptive immunity. PRRs are activated by PAMPs present in microbial structures or by damage-associated molecular patterns (DAMPs) exposed or released by damaged cells. PRRs are composed of multiple membrane-associated and cytosolic receptor families. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are membrane associated PRR families whereas NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors (ALRs) are cytosolic ones. Recognition of PAMPs by PRRs usually triggers the intracellular signal transduction cascades which initiate expression of pro-inflammatory cytokines, chemokines and antiviral molecules [34]. On the other hand, ligand binding to some NLRs or ALRs may trigger clustering of receptor monomers, inducing formation of inflammasome complexes. Inflammasomes activate Caspase-1 which induces expression of pro-inflammatory cytokines IL-1 β and IL-18 [35]. Because a pathogen contains various PAMPs such as lipopolysaccharides (LPS), ssRNA and ssDNA multiple PRRs can be activated simultaneously, causing crosstalk between different receptors and cascades which can also affect the direction of the immune response [36].

1.6.1 Toll-like receptors

TLRs are PRRs that have been conserved from insects to mammals and essential molecules that alerts the immune system against microbial infections. They are named for their similarity to *Toll* which was firstly discovered as in the fruit fly *Drosophila melanogaster* as a receptor required for dorsoventral axis formation in early development [37]. Later, *Toll* was shown to have essential roles in fly's immunity against fungal functions [38]. TLRs, the mammalian homologue of *Toll* was identified in 1997, which was shown to actively induce cytokine production and immune activation of T-cells [39]. Role of TLRs in recognition of PAMPs discovered in genetic experiments which demonstrated that a mouse strain with a missense mutation in the *Tlr4* gene was resistant to LPS induced endotoxic shock [40]. Subsequently, many other TLRs recognized which can recognize diverse variety of PAMPs derived from bacteria, viruses, protozoa and fungi. To date, 13 TLRs identified in mammals, each recognizing unique PAMPs- table. Each TLR is composed of three regions: a leucine-rich repeat (LRR) region mediating ligand-binding specificity, a transmembrane

Table 1.1: Ligands, adapter proteins and localization of TLRs. Table is modified from [11, 41–63].

TLRs	Ligand(s)	Ligand Source	Adapter(s)	Location		Cell Types	References
				Surface	Endosome		
1	Triacylated lipoproteins (with TLR2)	Bacteria	MyD88, MAL	+	-	Monocytes, Macrophages, B-cells	[45, 46]
2	Glycolipids, Lipoproteins, Peptidoglycan, Zymosan	Bacteria, Fungi	MyD88, MAL	+	-	Monocytes, Macrophages, mDCs, Mast Cells	[47, 48]
3	dsRNA, poly(I:C)	Viruses	TRIF	-	+	DCs, B-cells	[50, 51]
4	LPS, RSV-F protein	Bacteria	MyD88, MAL, TRIF, TRAM	+	-	Monocytes, Macrophages, mDCs, Mast cells, B-cells	[52, 53]
5	Flagellin	Bacteria	MyD88	+	-	Monocytes, Macrophages, Intestinal epithelium	[54, 55]
6	Diacylated lipoproteins (with TLR2)	Mycoplasma	MyD88, MAL	+	-	Monocytes, Macrophages, Mast cells, B-cells	[56]
7	Imidazoquinolines (3M, R848), Loxoribine, Bropiramine, ssRNA	RNA viruses	MyD88	-	+	Monocytes, Macrophages, pDCs, B-cells	[57]
8	Imidazoquinolines, ssRNA		MyD88	-	+	Monocytes, Macrophages, Mast Cells	[58]
9	Bacterial DNA, Unmethylated CpG motifs	Bacteria, DNA viruses	MyD88	-	+	Monocytes, Macrophages, Bcells, pDCs	[59, 60]
10	Unknown	Unknown	MyD88	+	-	pDCs, B-cells	[61, 62]
11	Profilin	<i>Toxoplasma gondii</i>	MyD88	-	+	Monocytes, Macrophages, Kidney cells, Liver Cells	[63]
12	Profilin	<i>Toxoplasma gondii</i>	MyD88	?	?	Neurons, pDCs, Macrophages	[64]
13	Bacterial ribosomal RNA	Bacteria, Virus	MyD88	-	+	Monocytes, Macrophages, mDCs	[65, 66]

domain and a Toll/Interleukin-1 receptor (TIR) domain initiating intracellular signaling pathways [64]. Most TLRs can recognize specific PAMPs by itself but there are certain co-receptors augmenting receptor-ligand affinity, such as CD14 which cooperates with TLR4 for detection of LPS in the presence of Lipopolysaccharide-binding protein (LBP), a soluble acute-phase protein that binds LPS [65].

TLRs are localized either on cell surface or on the membrane of cellular compartments. TLRs are differentially expressed most cell types, especially immune cells. The cell types which each TLR is expressed, and the cellular localization of them are summarized in Table 1.1.

Upon activation, the TIR domain of TLRs can interact different adapter molecules in cytoplasm such as Myeloid differentiation factor-88 (MyD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF), which may induce various signal pathways. TLR signaling is divided into two distinct adapter dependent pathways, the MyD88-dependent and TRIF-dependent (or MyD88-independent) pathway.

The MyD88-dependent signal pathway induced by ligand triggered dimerization of TLR receptors, except TLR3. Activation of NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and mitogen-activated protein (MAP) kinase pathways is primarily induced by MyD88. NFB is a very important transcription factor involved in control of diverse variety of cellular actions such as stress induced and immune responses, developmental processes, growth and apoptosis.

Activated MyD88 recruits kinases to trigger the signal pathway which eventually leads phosphorylation and degradation of I κ B (inhibitor of NF κ B), allowing NF κ B to diffuse into cell nucleus and induce transcription of inflammatory cytokines [66]. TRIF-dependent pathway is utilized by TLR3 and TLR4 upon recognition of dsRNA and LPS respectively. TRIF can activate certain kinases which initiate a branch of signal pathways which eventually lead activation of NFB or interferon regulatory factor (IRF3) [66,67]. IRF3 is a transcription factor which enables production of type I interferons [68]. Depending on the circumstances and the ligands, TLR activation can trigger signal pathways through different adapter proteins but TLR 4 is the only one which uses all known four adapters. TIR mediated simultaneous activation of different adapter signal pathways is necessary in certain conditions for production of inflammatory cytokines [66]. The known adapters used by TLRs are represented in Table 1.1 .

As the importance of innate immunity for generation of adaptive immune responses was indicated before [7–9], the key elements of innate immunity, TLRs are very important for activation adaptive immunity. Therefore, using TLR ligands as adjuvants within human vaccines may provide stronger and prolonged adaptive immunity [69]. Especially TLR7/8 and TLR9 ligands are very promising adjuvants due their presentation on immune-adaptive immunity linking pDC surfaces [70]. Although, TLR7/8 ligands demonstrated great success as vaccine adjuvants against pathogens [71, 72], TLR9 ligands (CpG ODNs) were reported to be more effective for augmenting both humoral and cell mediated immune response [73]. Also, extensive variety of CpG ODNs with diverse and potent effects on immune responses offers therapies against pathogens, autoinflammatory diseases and even cancer [74].

1.7 Immunomodulatory synthetic DNA motifs

TLR9 can discriminate self and non-self DNA and trigger immune response according to the circumstance. Even though the stimulatory effect of bacterial DNA has been reported, the DNA motifs in mammalian and adenoviral genomes have been shown to be immunosuppressive. Both stimulatory and suppressive synthetic DNA motifs are promising agents for vaccine development and treatment of inflammatory conditions, respectively.

1.7.1 Immune stimulatory synthetic DNA motifs

In 1995, the immune stimulatory effects of unmethylated CpG motifs in bacterial DNA on B-cells was demonstrated [75]. Later, it was discovered the recognition of bacterial CpG motifs was mediated by TLR9 [57]. Although, the recognition mechanism of CpG motifs by TLR9 is unclear, synthetic CpG ODN induced various responses depending on their sequence, length and backbone. The immunostimulatory strength of different CpG ODN may differ but they have been divided into three subclasses according to their structural characteristics and impact [76].

- Type D ODNs (also known as class A) are characterized by a central palindromic phosphodiester (PO) CpG sequence and PS-modified 3' end poly(G) track. The poly

G favors forms G-tetrad formation which provides higher molecular weight and subsequently high stability and increased endosomal uptake. D ODNs activates pDCs and triggers IFN- α expression. They weakly stimulate TLR9-dependent NF-B signaling and pro-inflammatory cytokine production [77–79].

- Type K ODNs (also known as class B) are 18-28mer(much larger than D-ODNs) motifs with a linear structure. They contain one or more 6mer CpG motifs with full phosphorothioate backbone. K ODNs strongly activate B-cells and TLR9-dependent NF-B signaling but weakly promote IFN- α expression. They are promising vaccine adjuvants [75, 79, 80].
- Class C ODNs have characteristics of both K and D ODNs. They are composed of a CpG-containing palindromic motif with full PS backbone. They can activate both pDC and B-cells, also stimulate production IFN- α and pro-inflammatory cytokine [81].

1.7.2 Immunosuppressive synthetic DNA motifs

There are also synthetic DNA motifs which have immunosuppressive effects on immune system in a TLR9-dependent or independent way. Inhibitory sequence motifs were firstly described in DNA of adenovirus serotype [82]. Later, G-rich or methylated mammalian DNA motifs suppressing CpG induced immune activation through NF-B and AP-1 induction were also reported [83–85]. Suppressive motifs were also determined in mammalian telomeric sequences (TTAGGG) [86] and DNA of commensal bacteria [87]. After the discovery of suppressive sequences, synthetic various ODNs were produced to reveal mechanism and therapeutic potential. When immunosuppressive motifs were initially identified, they were known to have ability to block CpG-induced immune activation. However, suppressive ODNs were not inhibitors of CpG uptake [85] and they were shown to hamper multiple forms of immune stimulation [88–91]. One of the most effective and studied suppressive ODN is A151 which express multiple TTAGGG motifs, mimicking the immunosuppressive activity of telomeric DNA [86]. The immunosuppressive effect of the A151 is connected to its G-tetrad structure formed by Hoogsteen binding of poly-G runs (Depicted in Figure 1.3). The action mechanism of A151 is still unclear but it can down-regulate the production of proinflammatory and Th1 cytokines by inhibiting phosphorylation of STAT1/STAT4 [86, 92]. *In vivo* administration of A151 demonstrated promising progresses against various pathogenic conditions like autoimmune and inflammatory diseases in murine. [92–95].

Disorders in immune system cause abnormal immune responses which give rise lethal autoimmune diseases. The immune system attacks the body's own cells and tissues, ending up with tissue, organ damage. There is no accurate treatment for autoimmune diseases but immunosuppressive are used to down-regulate immune response and avoid tissue damage. Systemic lupus erythematosus (SLE) is an autoimmune disease which mostly harms the heart, joints, lungs, blood vessels, liver, kidneys, skin and nervous system [96]. Intraperitoneal (IP) injection of A151 to female NZB/W mice (a useful model to study pathogenesis and treatment of human SLE [97]) significantly decreased the severity of the disease and prolonged the survival [95, 98]. Septic arthritis is another autoimmune disease caused by joint infections by certain bacteria. It was reported that, both IP and intra-articular administration of A151 significantly reduced inflammation and the swelling on the joint of the CpG-induced septic arthritis murine models [99, 100]. Uveitis is a autoimmune disease characterized by inflammation of uvea and IP or local administration of A151 successfully reduced ocular inflammation in three differently uveitis induced murine models [95, 101]. Protective effect of A151 against inflammatory conditions such as lethal endotoxic shock and acute slicosis was also demonstrated in mice [92, 95].

As a result, A151 has a great potential as a immunosuppressive drug against diverse pathological conditions , but its delivery in biological milieu is prone to digestion and serum adsorption, leading

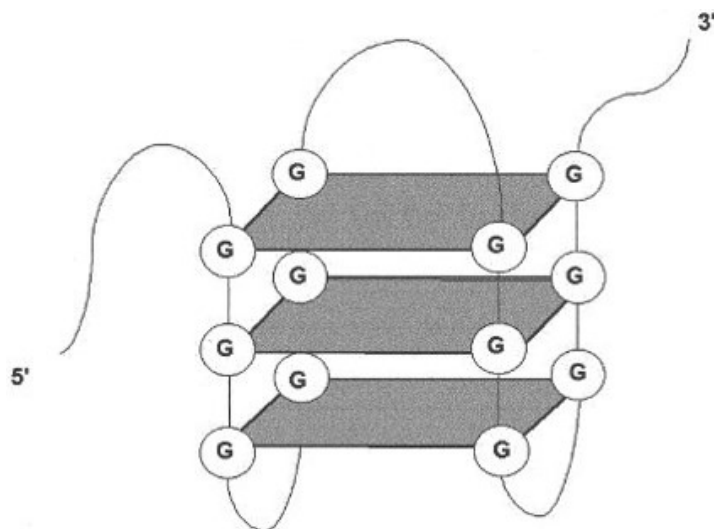


Figure 1.3: Representation of the G-tetrad structure of A151, formed through Hoogsteen hydrogen bonding among G bases.

to limited bioavailability, hampering its *in vivo* performance. Surely, phosphorothioate chemistry makes A151 more stable for *in vivo* use in experimental animal models of auto/inflammatory and auto-immune diseases, better delivery systems are required to unveil its full potential.

1.8 Extracellular Vesicles

Intercellular communication is essential for all life forms, especially multicellular organism. Communication between cells can be achieved by cell-to-cell contact, and transmission of specific molecules. In last two decades, a new mechanism for intercellular communication has been discovered that covers transfer of plasma membrane derived extracellular vesicles (EVs). The release of apoptotic blebs during apoptosis was known long ago but the secretion of membrane vesicles from healthy cells has recently demonstrated. Since then, EVs have been isolated from nearly all types of mammalian cells, including stem cells, immune cells, nerve cells and tumor cells. Intercellular communication through EVs were identified in not only mammalian cells but in lower eukaryotes and prokaryotes. Until 1996 these vesicles were initially regarded as membrane debris or waste containers with no real biological effect. However, Raposa et al. showed that extracellular vesicles could stimulate adaptive immune responses [102]. Since then, many studies have demonstrated the significance of EVs in intercellular communication via transfer of nucleic acids, lipids, cytokines and proteins. As EVs have been isolated from cell culture medium of cell lines and primary cells they have also been purified from most bodily fluids and the studies indicate that these vesicles have a key role in not only the maintenance of physiological processes stem cells but immune surveillance, tissue repair, blood coagulation, but also in the pathology causing several diseases [103]. When the cell derived vesicles were identified, they were known to be originated from cell membrane by direct shedding. Although, the term exosome was firstly used for vesicles ranging from 40 to 1,000 nm the subcellular origin of these vesicles was remained as secret until 1984 [104]. Then it has been determined that the release of many nanovesicles during reticulocyte differentiation were outcome of multivesicular endosome (MVE) fusion with the plasma membrane [105, 106]. Currenty, these multivesicular endosome derived nanovesicles(40-100 nm) are referred as exosomes, whereas the directly shedded vesicles from plasma membrane (40 to 1,000 nm) are referred as microvesicles, ectosomes, shedding vesicles or microparticles([104, 107]). Distinct mechanisms of exosome

and MP secretion are simply represented in Figure 1.4. The secretory mechanisms, function and therapeutic applications of exosomes and microparticles (MP) are growing research area in the field of physiology, immunology and genetics. However, there are controversial issues about distanciation of those vesicles from each other. Current methods for exosome and MP isolation (ultracentrifugation, filtering, sucrose gradient) are not able to separate MPs from exosomes completely. MPs with the sizes in the range between 50-100 nm are also isolated as mixed up with exosomes. Therefore, the function and actions of exosomes may not be accurate. On the other hand, most of the previous studies did not deal with exosomes and MPs separately, but experimented on mixed populations, spawning controversial data.

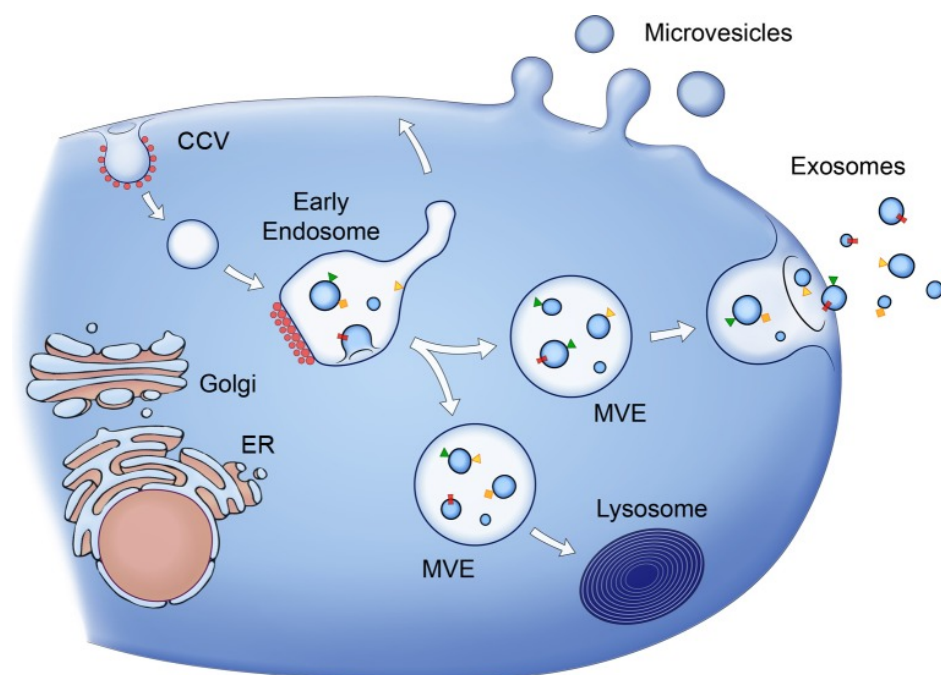


Figure 1.4: MPs are formed by directly shedding from the plasma membrane whereas, exosomes are constituted in MVE and released through fusion of cell membrane and MVE. Image adapted from [107].

1.9 Microparticles

Microparticles are small (40-1000 nm) vesicles shed from a variety of cells [108]. MPs were termed as platelet dust when they were first identified by Peter Wolf during an ongoing blood coagulation research in 1967 as a product of platelets [109]. Although MPs were

initially thought to be useless cellular waste, decades of research focused on the tiny vesicles have defined their roles as participators in thrombosis, cellular signaling, vascular injury, homeostasis, inflammation and messengers in the transport of bioactive lipids, cytokines, complement and immune signaling [108, 110–112]. Function of the MPs are dependent on the derived cell type. However, distinct phenotypic subsets of MPs may have different functions, even if derived from same cell type.

In circulation of healthy individuals, MPs were derived most abundantly from platelets followed by red blood cells, leukocyte and endothelial cells [111, 113]. MP amount, phenotype in circulation altered by various diseases, such as atherosclerosis, diabetes, cancer, sepsis, pulmonary hypertension and rheumatic diseases [114, 115]. For instance, platelet MPs are increased in myocardial infarction [116], hypertension [117], diabetes [118] and cancer [119], whereas endothelial MPs abundantly present in acute coronary syndromes [116] and Type 1 diabetes mellitus [120]. Diabetic patients have leukocyte originated MPs in their circulation approximately threefold higher than healthy individuals [120]. Under different pathological conditions, cellular origins and phenotypes of circulating MPs may shift (See Figure 1.5). Since diverse phenotypes MPs are associated with various homeostatic activities, microparticle research is an important emerging field focused on characterization of distinct phenotypes, investigation of their formation mechanism and factors triggering their release and determination of their effects on pathogenesis of cardiovascular diseases or distinct inflammatory conditions.

1.9.1 Microparticle formation

Unlike exosomes, MPs directly shed from the plasma membrane of the parent cell. The mechanism behind MP formation is unclear but studies have suggested some factors have role in MP formation, chop off mechanism.

Remodeling of the plasma membrane is essential for MP formation. Cell membrane bilayer has a specific lipid composition in inner and outer leaflets. Aminophospholipids (PS and phosphatidylethanolamine) are specifically found in the inner leaflet, whereas phosphatidylcholine and sphingomyelin are enriched in outer one. Localization and composition of those factors are essential for the preserving plasma membrane asymmetry. The control of lipid distribution on the transbilayer is carried by three transmembrane lipid transporters;

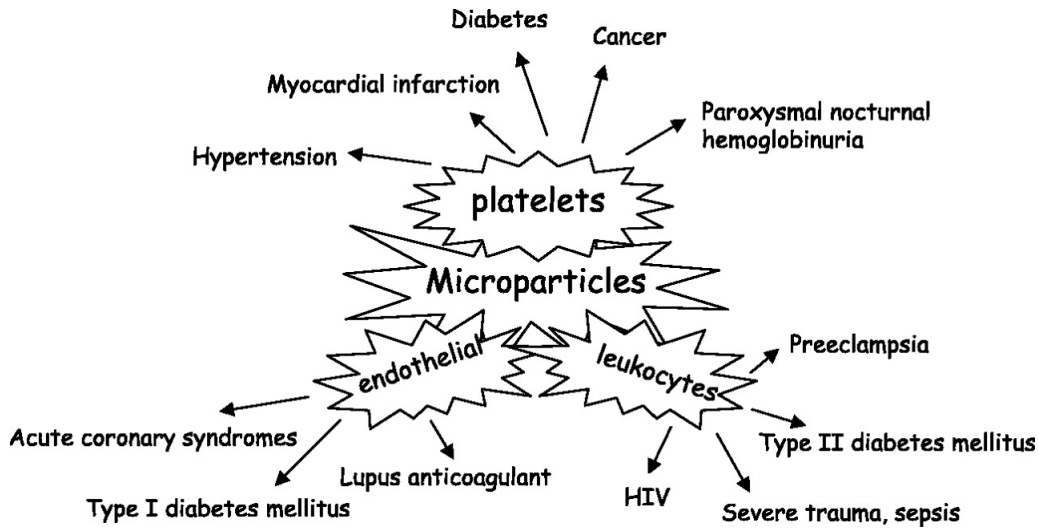


Figure 1.5: The cellular origins and phenotypes of circulated MPs are affected by pathological conditions. Image adapted from [121].

flippase, floppase and lipid scramblase [122]. Cell activation or apoptosis induction cause significant and sustained increase of cytosolic Ca^{2+} which lead to the disruption of the membrane asymmetry by enhancing scramblase and floppase activities and inhibiting PS specific flippase. Especially, flippase inhibition is a very essential stage which cause exposure of the PS, followed by cytoskeleton degradation by Ca^{2+} - dependent proteolysis and lastly MP release [123]. Because apoptotic or dying cells lose membrane asymmetry and secrete MPs it is a problematic to distinguish whether they are derived from healthy or unhealthy cells during *in vitro* culturing. In future studies, healthy cell MPs may be sorted out from the others and better understanding of function and composition of MP subpopulations.

1.9.2 Microparticle composition

In comparison with exosomes, less is known about the protein and lipid composition of MPs. Each MP has cytoplasmic and plasma membrane properties of the originated cell however it is not clear whether they carry enriched particular components. If their secretion mechanisms are clearly understood, more information about their content can be revealed.

Because composition of MPs is dependent on cell origin, any alteration on the cell effect the MP content. Any environmental stimuli which make cell produce different proteins,

mRNA, miRNAs also directly affect the MP composition. A general representation of MP composition is depicted in Figure 1.6.

Each MP present specific surface membrane proteins based on their origin. For instance, endothelial MPs have CD31 and CD146, whereas leukocyte MPs present CD4, CD3, or CD8 at their surface membrane [116,124]. Thus, source of the circulating MPs can be detected with cell marker Ab staining. Also, MP surface is phosphatidylserine(PS) enriched which makes them bind to labeled Annexin-V and consequently detectable by flow cytometry [125].

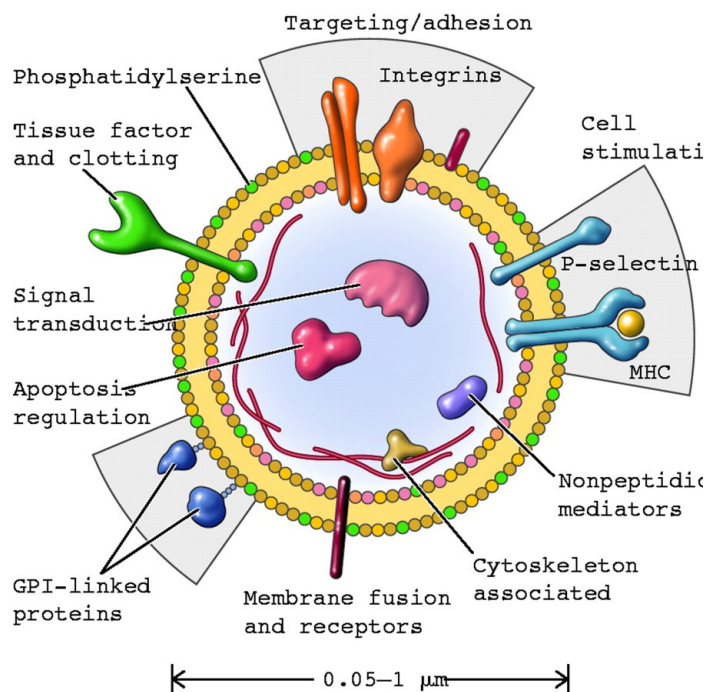


Figure 1.6: Representation of MP composition. Surface of a MP is consist of molecules from parental cell plasma membrane. Cellular origin and conditions affect the MP phenotype. This representation does not reflect exact and complete content of MPs. Image adapted from [123].

In addition to the cytoplasmic proteins, MPs also contains some mRNA, microRNA and proteases of the parental cell [126–129]. Also a very recent study has revealed the presence of genomic DNA fragments in MPs isolated from human plasma [130].

1.9.3 Microparticle function

MPs are secreted by normal or activated cells as a consequence of differential environmental effects. Therefore, MPs can be seen as pathways which exchange information between cells. Because MPs carry mRNA, miRNA, transcription factors of the parent cell, they can affect the transcription profile of the target cell [126, 127]. Microparticles can interact with target cell through different mechanisms in order to mediate cell-to-cell communication. They can activate intracellular signaling by interacting target cell membrane receptors, release their content into cytoplasm by fusing cell membrane, internalized through phagocytosis, macropinocytosis or receptor-mediated endocytosis. Additionally, membrane proteins presented on surface of microparticles can be cleaved by extracellular proteases and exposed ligands can bind to target cell receptors to induce signal pathways [127, 131, 132] (see Figure 1.7). However, the physiological and pathophysiological roles of MPs are unclear. Findings suggest that MPs have roles in inflammation, coagulation, vascular function, apoptosis and cell proliferation or differentiation [123, 133]. Especially, circulating MPs allows membrane interactions between distant vascular cells.

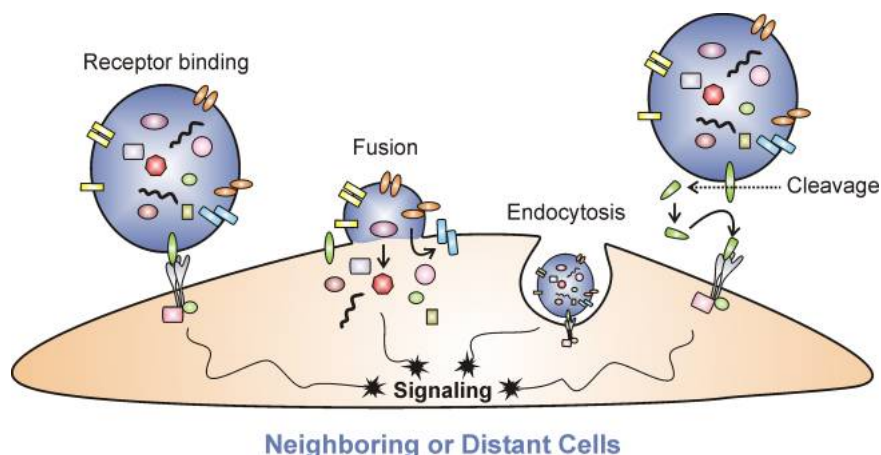


Figure 1.7: Extracellular vesicles can transmit signals through their inside content or surface proteins. The proposed mechanisms behind EV-mediated signal pathways are Receptor binding, direct fusion with target cell plasma membrane, endocytosis and receptor cleavage. Image adapted from [132].

MPs can be beneficial or deleterious depending on condition. Normally, MPs are transducers of biological information. MPs shed from activated macrophages can transmit inflammatory signals to foreign cells thus accelerate and strengthen immune responses [134] (also

see Fig 1.12). On the other hand, extensive secretion of MPs may cause significant procoagulation and inflammatory effects provoking adverse clinical symptoms in several vascular diseases [133,135]. Moreover, during some viral infections they may cause infection of viral genetic information to foreign cells easily because they are considered as "host" by other cells [136]. Also, tumor derived MPs can suppress anti-tumor responses in favor of tumor growth [137].

Role of the MPs in inflammation is one of the most popular research field currently. The implications of MPs in inflammation has been reported by many studies. The most apparent relationship is the elevated MP levels in various inflammatory conditions such as sepsis [138, 139], HIV and Ebola virus infection [140,141] and malaria [142,143]. Although, MP secretion is enhanced during infections, stimulation with inflammatory cytokines such as TNF, IL-1, IL-6 and type 1 interferons in the absence of infection also caused elevated MP secretion [144, 145]. As expected, both *in vitro* and *in vivo* administration of bacterial endotoxins induced MP secretion in various cell types. For instance, apoptosis related MP secretion was observed in LPS stimulated epithelial cells, whereas monocytes secrete MPs upon immune activation [146–149]. Moreover, increased MP levels were reported in the circulation Plasmodium-inoculated laboratory animals [150]. Especially platelet derived MPs in circulation have significant roles in inflammation. Activated platelets secrete proinflammatory IL-1 β -rich MPs which may favor atherosclerosis plaque formation [151,152]. Also, MPs secreted from *in vitro* activated platelets enhanced expression of cell adhesion molecules and proinflammatory cytokines in endothelial cells and THP-1 cell line [153]. On the line, leukocyte MPs in circulation induced secretion of cytokines and expression of surface coagulation factors in endothelial cells [154].

MPs have been suggested to have roles behind certain physiological immune escape mechanism. It was reported that, during pregnancy trophoblast release FasL bearing MPs to induce T-cell death and subsequently escape from immune system despite its semi-allograft properties [155]. Tumor cells such as ovarian cancer cells also may secrete FasL-bearing MPs into the extracellular medium and cause apoptosis of lymphoid cells. Considering the fact that normal ovarian epithelial cells do not secrete FasL bearing MPs, it is suggested that tumor cells developed a mechanism that neutralize Fas-bearing immune cells which facilitate their escape from immune system [156, 157]. Another implication of MPs in immune escape also reported in AIDS. CCR5 is chemokine receptor localized on the surface of white blood cells and this receptor is used by many HIV versions to enter and infect the cell [158].

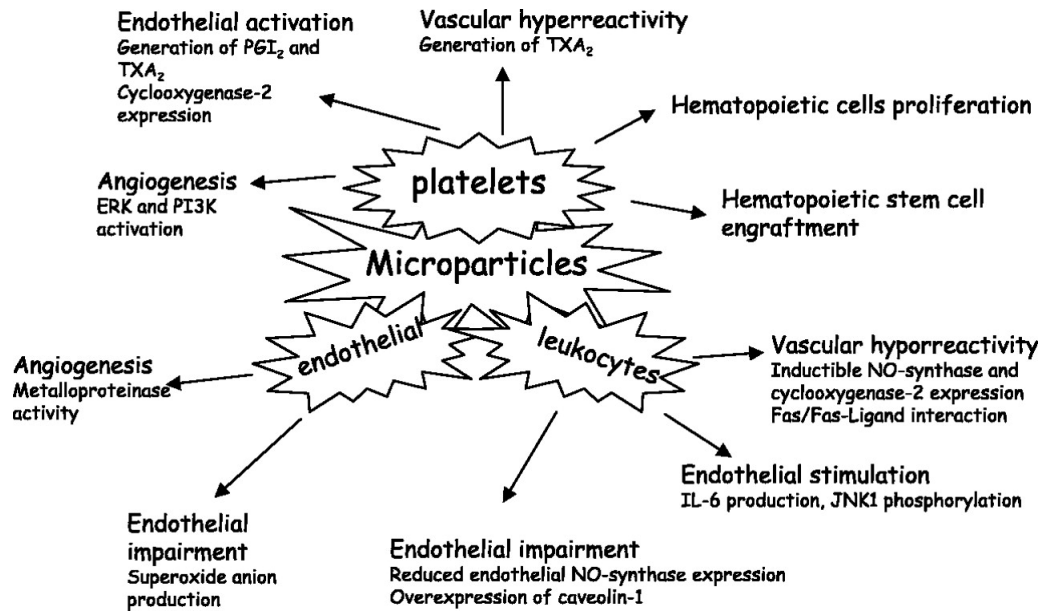


Figure 1.8: Physiological effects and actions of MPs from different cellular origin. Image adapted from [121].

MPs released from CCR5-positive cells rendered deficient peripheral blood mononuclear cells CCR positive and thus susceptible to HIV-1 infection [159]. Another study also confirmed the receptor transfer from CXCR4 positive platelets and megakaryocytes to CXCR4-null cells through MPs [160].

It was reported that neutrophil derived MPs, termed exosomes may have potential roles in opsonization due to their ability to efficiently bind opsonized bacteria and become adherent to monocytic and endothelial cells [161,162].

Among countless significant physiological activities of MPs in human body, their role in immune system is very diverse and essential. MP can remotely transport bacterial or viral antigens via blood stream to APCs such as macrophages and DCs in the spleen without the need for direct interaction with infected cells. Also, they can penetrate almost all tissues and physiological barriers which cells cannot pass through and convey the signals. Such remote interactions maintain a distance between site of infection and the site of immune induction, also enhance the probability of early detection of infection. In certain circumstances MPs can embrace parent cells assignment. For instance, TNF-stimulated, endothelial cell-derived MPs can stimulate activation and maturation of pDCs [163]. Moreover, MPs secreted from

Mycobacterium-infected macrophages were able to activate nave T cells by antigen presenting, thus rendering APC T-cell interactions unnecessary. Also, according to that study, MPs were more effective than exosomes at inducing T-cell proliferation [164,165].

Additionally, elevated levels of MPs presenting CD4 antigen in circulation of human immunodeficiency virus (HIV)-infected patients have been determined [140]. Enhanced levels of MPs derived from activated leukocytes were observed in circulation in case of severe trauma [166]. Also, in preeclampsia, granulocyte and lymphocyte originated MP subpopulations significantly increased [167]. Almost every mammalian cell types are known to be able to secrete MPs. Even smooth muscle cells release MPs which were suggested to have potential functions in atherosclerosis and thrombus formation [121,168,169]. MPs may have pleiotropic effects on the vascular wall in a consequence of their variety of subpopulations. MPs act as vectors transmitting specific messages to other cells in regulating vascular function and dysfunction depending on the donor cell type [121].

Mouse macrophage cell line secreted significantly higher amounts of MPs when activated by various TLR ligands [170]. In the same study, a relevance between MP production and nitric oxide (NO) release was reported and NO synthase inhibitors also greatly hampered MP secretion [170]. NO is most commonly observed in monocytes and macrophages. Therefore, it is plausible that each cell may have distinct mechanisms for MP production. Moreover, same cell types may have different mechanism for producing subpopulations of MPs under distinct conditions.

1.9.4 Microparticle immunotherapy

MPs are promising therapeutic tools for diverse applications such as vaccination, drug delivery, gene therapy due to their physiological structure. They can pass through every tissues and be accumulated by cells rapidly. Unlike artificial vesicles, MPs are completely natural and non-toxic. MPs from non-activated immune cells generally does not cause any immune response in the recipient circulation, thus minimize the side effects. There are many therapeutic agents which are effective *in vitro* studies but become impotent upon *in vivo* administration. Absorption by serum or other tissues and degradation before reaching target site hamper the functioning of those therapeutic agents. MPs are potential delivery systems for those agents because of their cell membrane derived lipid structure. Also, MPs can be

used for targeted therapies which also enhance the activity of the agents. MPs derived from different cell types generally have distinct affinities to other cells. Choosing right MP type may provide both protection and target specificity for therapeutic agents or drugs.

Because MPs convey immunological signals between site of infection and immune induction, they are suggested to be promising vaccination tools. Since, antigen carrying MPs can effectively stimulate both innate and humoral responses, they can be used to increase efficiency of adjuvants.

MPs may affect transcription profile of target cell by transporting mRNA, miRNA and TFs [126]. Non-coding small RNA based therapies have shown great promise for many diseases such as cancer [171]. However, their nucleic acid structure limits their bioavailability in biological fluids. MPs are great delivery vehicles for such nucleic acid based therapeutic agents.

Altering MP composition by genetically engineering of donor cell is another promising approach in MP based therapeutics [172]. It has been reported that, MPs derived from suicide gene mRNA transfected donor cells, were administered to schwannoma and successfully hampered tumor growth [173]. Several studies demonstrated that, mesenchymal stem cell (MSC) MPs have MSC mimicking properties such as apoptosis inhibition, induction of cell proliferation subsequent wound healing [174]. Thus MSC derived MPs are potential therapeutic tools in regenerative medicine.

MPs are potential markers for early diagnosis of cardiovascular and other diseases by their virtue of joining circulation from any site. Therefore, characterization of MPs from healthy and unhealthy cells is essential to exploit their such property in future. There are some studies carried out to use MPs as diagnostic tool for atherosclerosis vascular disease [175], cancer [176] and smoking dependent lung damage [177].

1.10 Exosomes

Exosomes are small membrane vesicles with the size 40-100 nm. It is secreted from multivesicular endosomes in most cell types including B cells [102], T cells, macrophages, DC [178], mast cells, epithelial cells, platelets, reticulocytes, tumor cells. Moreover, exosomes and

exosome-like vesicles have been isolated from various body fluids such as semen [179], blood serum [180], urine [181], saliva [182], breast milk [183], amniotic fluid [184], ascites fluid [185], cerebrospinal fluid [186] and bile [107, 187]. Exosomes were initially described in 1983 in the maturing reticulocytes [188, 189] and they become a significant target in immunology in 1996, when Raposo demonstrated the feature of B-cell isolated exosomes in stimulating T-cells [102]. Since the Raposo's milestone achievement in exosome history, the exosomes have become an significant subject in antigen presentation and immune regulation. Although their biological role has not been fully revealed yet, they have shown great success in therapeutic approaches.

1.10.1 Exosome formation

Exosomes are formed in MVBs/endosomes of the cell which is a membrane-bounded compartment functioning as an intermediate in the degradation pathway of proteins either internalized from plasma membrane or transported from Golgi network [190]. Interestingly, the secretion of exosome mechanism was demonstrated on a study about the iron-binding transferrin receptor by reticulocytes during maturation into erythrocytes in 1983 by Harding *et al.* [188]. The down-regulation of transferrin receptors was considered as related to endocytosis and lysosomal degradation and the visualization of colloidal gold-conjugated transferrin accumulated by reticulocytes by electron microscopy suddenly revealed the multivesicular bodies and their fusion with plasma membrane [188](see Figure 1.9).

Exosomes are formed by inward budding of the membrane of the MVB and released to extracellular space after fusion of MVB with cell membrane [191]. Although these compartments share many elements, MVBs either fuse with lysosomes in lysosomal pathway for degradation of their contents or fuse with plasma membrane for secretion of exosomes. These two distinct fates rely on cholesterol proportion on MVBs. When cholesterol-rich MVBs are in exosome secretory pathway, morphologically identical but cholesterol-poor ones are prone to fuse with lysosomes [192].

Generation of exosomes in MVBs involves the lateral segregation of cargo at the delimiting membrane of an endosome and inward budding and pinching of vesicles into the endosomal lumen. Two mechanisms for exosome production have been identified so far; endosomal sorting complex responsible for transport (ESCRT)-dependent and ESCRT-independent.

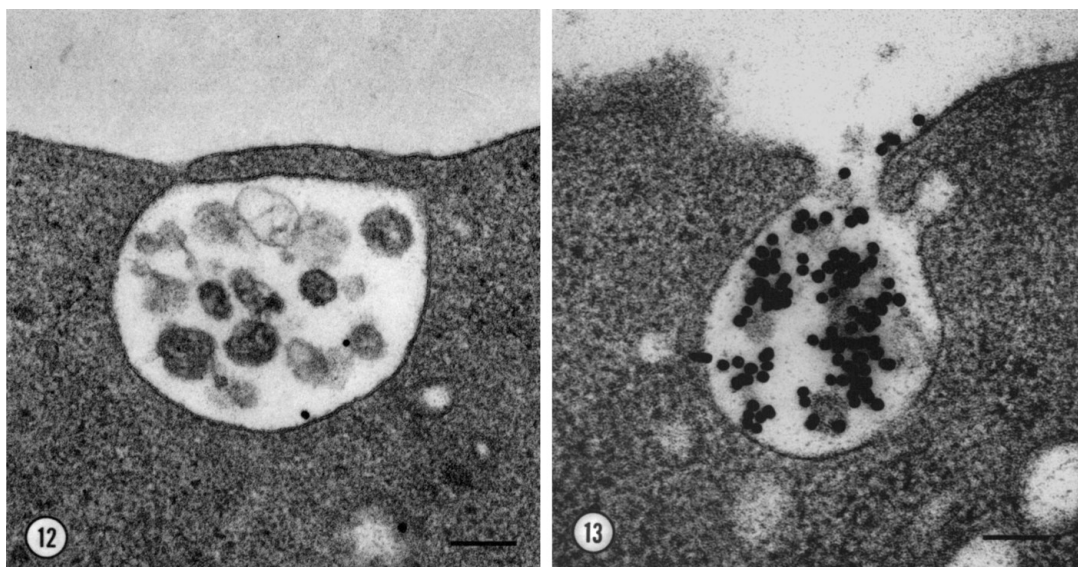


Figure 1.9: Electron microscopy image of exosomes released by exocytosis of MVEs. Colloidal gold-conjugated transferrin were internalized by reticulocyte transferrin receptors and enabled the visualization of such secretion mechanism by electron microscopy. On left, accumulated vesicle associated transferrins (Bar, 200 nm). Right, fusion of MVB with plasma membrane and secretion of internal vesicles (Bar, 100 nm). Image adapted from [104,188].

There are four multiprotein complexes (ESCRT)-0, -I, -II, -III, which have specific roles in the process of exosome formation; recognition and sequestration of ubiquitinated membrane proteins at the endosomal delimiting membrane, budding and fission of intraluminal vesicles (ILV) [194,195].(see Figure 1.10) ESCRT-independent exosome biogenesis and secretion has been observed in oligodendroglial cell lines, which secrete proteolipid protein associated exosomes. In these cell lines, ceramide-triggered budding take place, which is dependent on a ceramide producing enzyme called sphingomyelinase [196]. Also, both ceramide and ESCRT independent MVB formation was observed in pigment-producing melanocytes [197]. Specific membrane proteins including tetraspanins and CD63 (also a marker for exosomes) were shown to have a role in ILV formation [198,199].

1.10.2 Exosome composition

The molecular composition of exosomes is dependent on the cell type of origin but most exosomes contain highly concentrated certain molecules in common. Tetraspanins are the

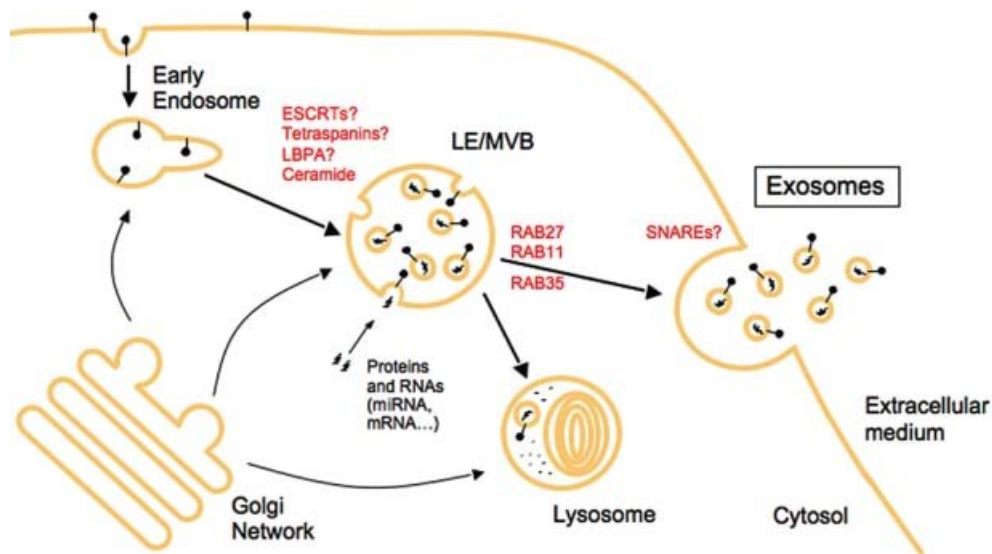
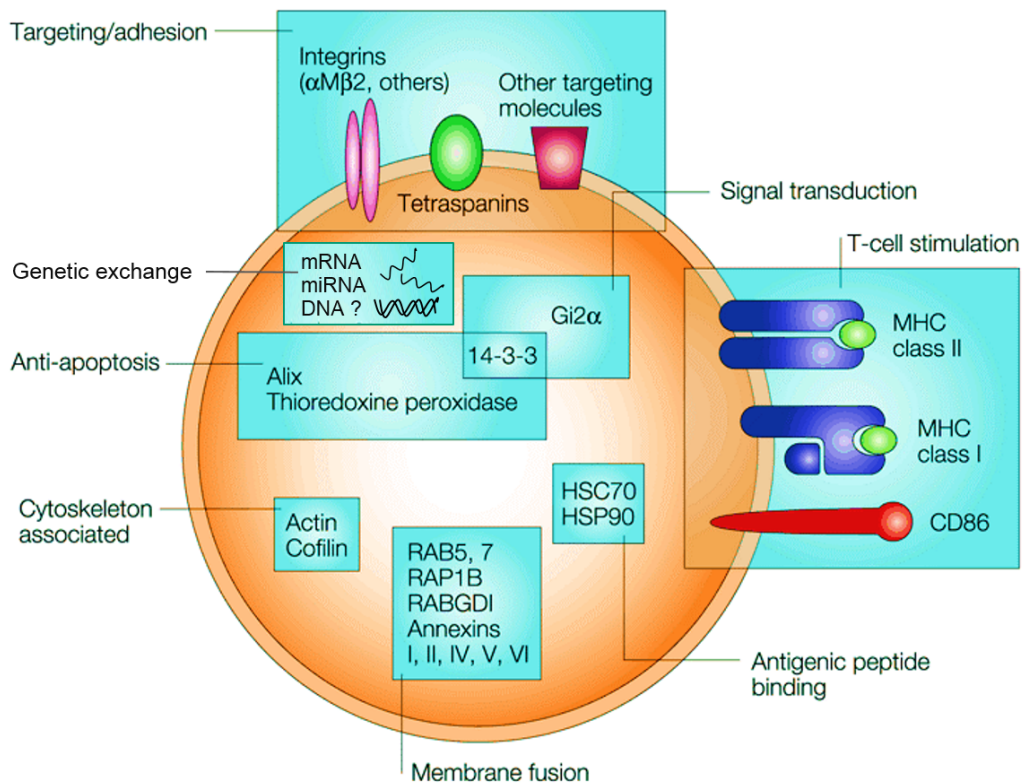


Figure 1.10: Exosomes originate from invaginating MVBs under control of complex events and factors. ESCRTs, tetraspanins and the lipid ceramide are the essential molecules for exosome formation. Also, a complex of SNARE proteins are most probably involved in Membrane fusion and subsequent secretion of exosomes. Image adapted from [193].

most abundant protein family found on exosomes [201]. Tetraspanin is a highly conserved membrane protein between species, which spans the membrane four times and act as scaffolding proteins, anchoring multiple proteins to a location on the plasma membrane [202]. The tetraspanins CD9, CD63, CD81, CD82 and CD151 are highly enriched on exosomes in a similar way as lipid rafts [203]. Heat shock proteins have been identified in exosomes, which are believed to have a role in proper protein folding. Integrins, cytoskeletal proteins, subunits of trimeric G proteins are also found on exosomes. Transferrin Moreover, immune cells derived exosomes contain specific proteins which have roles on immune regulation. B lymphocytes, DCs, APCs, MCs secrete exosomes enriched MHC class molecules, whereas platelet and cytotoxic T cell derived exosomes contain von Willebrand factors [204] and granzymes [205]. Killer proteins such as FasL and perforin have been identified on NK cell derived exosomes [206]. Co-stimulatory molecules such as CD40, CD80 and CD86 are found on the surface of the exosomes of APCs. Functional mRNAs and small RNAs including microRNAs are also found in the lumen of the exosomes [207]. Specific miRNAs are enriched or reduced in the exosomes derived from different cell lines of same origin. Therefore, those specific miRNAs and the mechanism behind selective sorting of miRNA into exosomes is an important research area at the moment. A proposed exosome composition is depicted in



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Figure 1.11: A proposed structure of exosome with functional transmembrane and cytosolic molecules. The indicated composition is not exhaustive and may differ depending upon parent cell and stimulus. Image modified from [200].

Figure 1.11. Recent studies also reported that neural cells [208] and myoblasts [209] secrete exosomes carrying mitochondrial DNA (mtDNA). Although the role of exosomal mtDNA has not been identified yet, it has been suggested that it can reach target cell's cytosol and be imported into the mitochondria [210]. As another point, exosomes are specialized nanovesicles with protective mechanism against complement lysis. For example, B cell exosomes have been shown to contain glycosylphosphatidylinositol (GPI)-anchored regulators such as CD55 and CD59 which provide protection against complement system [211]. Composition of exosomes have gained importance as biomarkers or diagnostic tools for various diseases such as renal diseases [212], Parkinson's disease [213] and ovarian cancer [214] lately.

1.10.3 Exosome function

Exosome secretion had been regarded as a random way of shedding waste substances until their various functions were discovered in cell communication. Exosomes have important properties such as small size, unique protein content and protection against complement lysis, which makes them great signal or molecule carriers. Exosomes can transfer information between cells in the form of proteins like the MHC:antigen complex or nucleic acids such as mRNA and small RNAs. Although every aspect of exosomes have not been discovered yet, they are considered as important mediators for various physiological processes. Like microparticles exosomes interact with target cell through different mechanisms in order to transfer signal and mediate cell-to-cell communication. They can activate intracellular signaling by interacting target cell membrane receptors, release their content into cytoplasm by fusing cell membrane, internalized through phagocytosis, macropinocytosis or receptor-mediated endocytosis. Additionally, exosomal membrane proteins can be cleaved by extracellular proteases and exposed ligands can bind to target cell receptors [127,215](see Figure 1.7). Although exosomes have important roles in diverse physiological mechanisms, their function in immune responses and their applications for therapeutic approaches are the most focused research areas at the moment.

It has been shown that exosomes play an important role in regulation of immune responses in both innate and adaptive immunity. Exosomes are involved in spreading of self and non-self antigens to many sites of the body due to their small size. Exosomes which are secreted from APCs such as B cells and DCs can activate T-cells by direct binding of exosomal MHC-peptide complexes to the T-cell receptor [102,216–218]. Also, antigen bearing exosomes can be internalized and processed by APCs, in order to activate T-cells [219]. Additionally, the origin of the exosomes determine the impact, as mature DC exosomes induce T-cell activation more effectively than immature DC exosomes [220, 221]. Moreover, exosomes derived from mycoplasma infected DCs could induce both T-cell and B-cell proliferation. Additionally, exosomes derived from mycoplasma infected DCs could induce both T-cell and B-cell proliferation.

Exosomes are associated with various antigens according to the disease or infection type. Pathogen-infected cell exosomes carry pathogen specific antigens, which can be processed and associated by DCs to activate T-cells [222,223]. Furthermore, it has been demonstrated that tumor-derived exosomes can also be processed by DCs and provide antitumoral immune

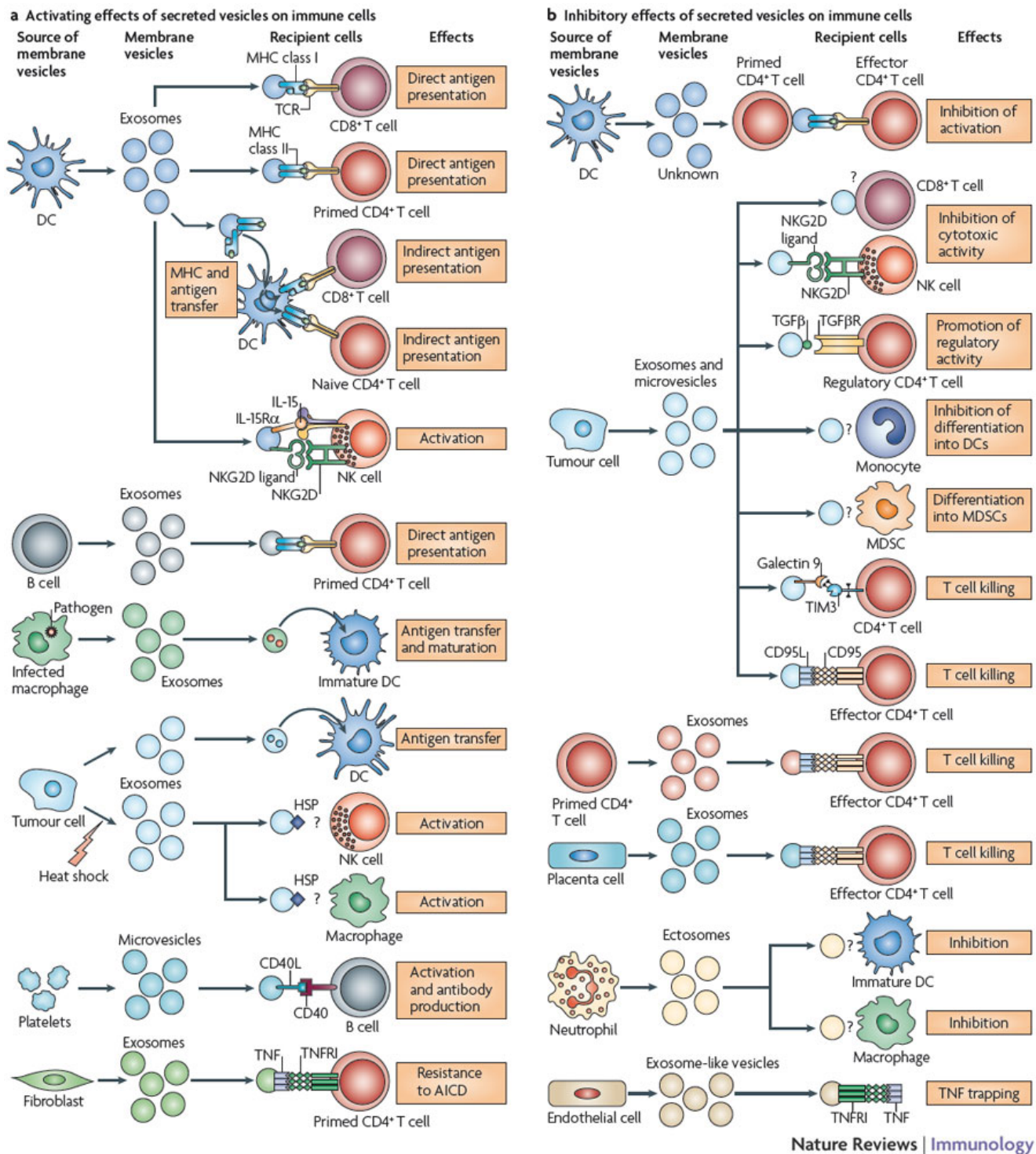


Figure 1.12: The observed effects of extracellular vesicles on immune cell *in vitro*. a) Immunostimulatory actions of vesicles secreted from various sources. b) Inhibitory and suppressive functions of vesicles derived from several cellular sources. Image adapted from [224].

responses by activating T-cells through presentation of tumor specific antigens [225–227]. On the other hand, current studies demonstrate opposite effect of tumor-derived exosomes, as mediating immunosuppressive responses in favor of tumor growth such as decreasing proliferation of T-lymphocytes [137,228–230] and NK cells [231,232] or promoting differentiation of repressive immune cells like Treg [233] and myeloid [234] cells. Current exosome isolation methods are not able to differentiate exosomes from MPs completely. Therefore, small MP subpopulations mixed with exosomes may be the reason behind such controversial results.

Microorganisms tend to exploit utilities of their host in order to reproduce and escape immune system. In 2003, Gould et al hypothesized that exosome secretion pathways are included in cellular trafficking of certain retroviruses [235]. For instance, HIV uses exosome pathway for production of virions which have been shown to be assembled in MVBs and secreted through exosomes. Exosomes derived from HIV-infected macrophages share same phenotypes with healthy macrophage exosomes [236,237]. In the light of those results, it can be suggested that HIV uses same pathway with exosomes. In another study, it was reported that immature DC derived exosome associated HIV can infect CD4+ T-cells and interestingly they are 10 fold more infectious than free virus particles [238].

In contrast the general view of exosomes as immune stimulators, their role in immune tolerance has been observed in growing number of studies. For instance, the prostate gland epithelial cells secrete exosome-like vesicles known as prostasomes into seminal fluid [239]. It has been reported that prostasomes in semen protects sperms from the female immune defense system by inhibiting NK cell function [240,241]. Also, placental explants secrete submicron sized vesicles which inhibit NK lymphocytes, possibly preventing immune response against fetus [242]. In milk and colostrum, immunosuppressive exosomes with T-cell inhibiting actions were identified [183]. Moreover, exosome-like vesicles present in the bronchoalveolar fluid of the lung, bearing tolerizing molecules against allergens or causing proinflammatory actions on airway epithelial cells [243]. Role of exosomes in the development of immunological tolerance to orally fed antigens also reported. CD4+ T cells have known to have crucial role in the induction of peripheral tolerance to non-self antigens originated from the microbial and dietary antigens in the intestinal lumen [244–246]. However, the events behind the presentation of antigens to T-cells was unclear. It has been reported that small intestinal epithelial cells process non-host antigens and release them into the circulation as MHC class II-antigen complexes on surface of exosome-like vesicles, termed tolerosomes [193,247].

1.10.4 Exosome immunotherapy

Potential of exosomes as immunotherapeutic agents were first described in 1998 when tumor growth in mice were reversed by injection of tumor peptide loaded DC exosomes [248]. Although producing humoral responses against tumor cells by using tumor-derived exosomes is a promising study, there are concerns about tumor exosomes because of their potential tumor growth favoring actions [229]. Therefore instead of direct administration of tumor exosomes, they are pulsed onto ex vivo matured DCs [249].

Moreover, it has been reported that exosomes derived from heat stressed tumor cell can chemoattract and stimulate DCs and T-cells more effectively than conventional tumor exosomes [250]. Currently exosome field is focused on immunotherapeutic application of DC exosomes (also called dexosomes) in various diseases such as cancer and autoimmune conditions. A direct comparison between tumor exosomes and dexosomes revealed that dexosomes were better stimulators of cytotoxic T-cells compared to tumor exosomes [251]. Loading of harvested dexosomes with tumor specific peptides provide promising anti-tumor response [252]. Peptide loaded DC exosomes demonstrated promising outcomes in two clinical trials conducted with patients with non-small cell lung cancer and metastatic melanoma [253–255].

Additionally, exosomes have provided protection against microbial infections as prophylactic vaccines. Antigen pulsed DCs have a great immunotherapeutic potential as source of exosomes in the field of vaccination. For instance, *Toxoplasma gondii* pulsed DC derived exosomes induced efficient and antigen specific Th1 response which provides protection against toxoplasmosis [256]. Exosomes have great potential as inducers of humoral responses. In various studies exosomes have been shown to induce protective immunoglobulin responses against infections as vaccines [224, 257, 258]. Administration of exosomes from diphtheria toxoid (DT) pulsed DC derived exosomes induced DT-specific Ig responses [257].

Although, exosomes have been generally studied as inducers of protective immune responses, they have also great potential against anti-inflammatory conditions. Exosomes derived from adenovirus-transduced DC expressing IL-4 suppressed the inflammatory delayed type hypersensitivity reaction(DTP) and reduced the severity in the murine collagen induced arthritis model by modulating the activity of APC and T-cells through a MHC class II and Fas ligand/Fas dependent mechanism [259]. In same murine model, IL-10 treated or fas ligand transduced DC derived exosomes also demonstrated similar inhibitory effect on

severity of the disease [260, 261]. Moreover, exosomes are potential anti-inflammatory drugs carriers with in effective and targeted treatment inflammatory diseases. Exosomes carrying curcumin, an anti-inflammatory agent were shown to deliver curcumin to specifically to activated myeloid cells *in vivo* [262]. In another study, curcumin loaded exosomes have been in treatment of brain inflammatory diseases in mice [263].

Together with, loading of various compounds and peptides to exosomes or treating cells which are source of exosomes, the manipulation of exosome phenotypes is another promising application of therapeutics. Exosome display technology is one of the most novel and advanced method for manipulation of exosome protein content. This technology enables the tailoring of exosomes in production stage with new desirable properties through transfection of cells [264] under favor of Lactadherin C1C2 domains localized on exosomes.

Chapter 2

Aim of the Study

Extracellular vesicles (EVs) are membrane surrounded structures secreted by almost all cell types, which are mediators of intercellular communication and involved in diverse biological functions. Depending on their secretion mechanism and size, EVs are divided into two main classes: exosomes and microparticles. Each EV has specific functions according to their originated cell type and environmental stimuli. Despite their diverse roles in a large variety physiological functions, immunomodulatory effect of exosomes and microvesicles in infections and inflammatory conditions is in the spotlight of the EV studies. Determining the functions of diverse EVs with distinct sources is essential to reveal their potential use in therapeutic applications and diagnostics in diseases.

The aim of this study was to reveal potential role of microparticles in transmission of pathogenic molecules in extracellular milieu during infection by testing immunostimulatory properties of different TLR ligand associated microparticles on mouse splenocytes and human blood peripheral mononuclear cells. Uptake and internalization of MPs by immune cells is the phenomenon behind immunomodulatory actions of MPs, thus we aimed to reveal absorption kinetics of distinct MPs by different immune cell. We also proposed to demonstrate protective and conveying impact of MPs on DNA delivery by loading A151, a synthetic inhibitory ODN to enhance its immunosuppressive effect in TLR triggered immune activation.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Immunomodulatory Agents

Table 3.1: Length and sequence of Synthetic ODNs.

ODN	Brand	Length	Sequence
K-23	Alpha DNA (Canada)	12 mer	5'-TCGAGCGTTCTC-3'
A151	NIH (U.S.)	24 mer	5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'

Table 3.2: List of TLR ligands.

Ligand	Source	Brand
LTA	<i>S.aureus</i>	Invivogen, USA
Zymosan	<i>S.cerevisiae</i>	Invivogen, USA
PGN	<i>B.subtilis</i>	Fluka, Switzerland
Poly(I:C)(HMW)	Synthetic	GE Healthcare
LPS	<i>E. coli</i>	Sigma
R848	synthetic	Invivogen, USA
Bacterial DNA	<i>P. pentosaceus</i>	-

3.1.2 ELISA Reagents

Table 3.3: Concentrations of ELISA coating Ab and Recombinant antibodies for Mouse.

Antibody	Brand	Coating	Recombinant (starting)
TNF- α	Biolegend	4 $\mu\text{g/ml}$	500 ng/ml
IL-6	Biolegend	2 $\mu\text{g/ml}$	1000 ng/ml
IL-12	Biolegend	4 $\mu\text{g/ml}$	500 ng/ml

Table 3.4: Concentrations of ELISA coating Ab and Recombinant antibodies for Human.

Antibody	Brand	Coating	Recombinant (starting)
TNF- α	Endogen	3 $\mu\text{g/ml}$	1000 ng/ml
IL-6	Endogen	4 $\mu\text{g/ml}$	1000 ng/ml
IFN- α	MapTech	5 $\mu\text{g/ml}$	250 ng/ml
IFN- γ	BD	10 $\mu\text{g/ml}$	500 ng/ml

3.2 Methods

3.2.1 Cytokine ELISA

For stimulation assays, cells (Splenocytes, PBMCs, RAWs) were counted and cultured into 96 cell culture plate wells equally (Generally 2×10^5 RAW cells and 4×10^5 splenocytes and PBMCs per well in 100 μl RPMI1640 medium with 5% FBS). Then, stimulants were prepared and added onto cells. Plates were incubated about 24 hours. During incubation of cells the first two steps of cytokine ELISA were completed. High binding polysorp (Nunc, Denmark) or Immulon 2HB plates (Thermo Scientific, USA) were coated with monoclonal antibodies (50 $\mu\text{l/well}$) for O/N at $+4^\circ\text{C}$. Monoclonal antibodies were prepared from stocks with working concentrations by diluting with PBS. Then plates were incubated at RT for about 15 minutes. By rapidly turning upside down the plates, the unattached monoclonal antibodies were discarded into sink. 200 μl of blocking buffer was added to each well in order to block the naked surfaces. After 2-3 hours incubation blocking buffer was discarded and plates were washed 5 times with washing buffer for 5 minutes incubation in each time. Then plates were rinsed with ddH₂O three times. By gently tapping to tissue papers, plates were dried. 50 μl of serial dilutions of recombinant cytokines (in PBS) and cell culture supernatant were added and incubated 4 h at RT or o/n at $+4^\circ\text{C}$. Plates were washed and dried. Biotin conjugated antibodies were diluted in T-cell buffer with 1:1000 dilution factor (for 1 λ stock).

Streptavidin-AKP solutions were also prepared in T-cell buffer with 1:3000 dilution factor and kept at $+4C^{\circ}$. After 2 hours of incubation at RT, plates were washed and dried. 50 μ l of previously prepared Streptavidin-AKP solution was added to each well. Plates were incubated for 1 h at RT then washed and dried. PNPP (Thermo) substrate solution was prepared according to the manual and 50 μ l of it was transferred into wells. After color formation was observed plates were read at ELISA reader (BioTek) at 405 nm with time intervals. Used concentration of ELISA reagents are listed in Table 3.3 and 3.4.

3.2.2 NO detection by Griess Method

After *in vitro* stimulation assays conducted with RAW cell lines, plates were centrifuged at 400 g. 25 μ l of cell culture supernatant was transferred to 96 well microtiter plates. 25 μ l of Griess Reagent (Sigma-Aldrich) was added onto each sample. After 15 min incubation plates were read by Elisa Reader at 405 nm (BioTek).

3.2.3 Flow Cytometry

3.2.3.1 Fixation of Cells

After cell treatments of cells ended, cell were centrifuged and aspirated. Pellets were agitated and 100 μ l of 4% paraformaldehyde (Invitrogen, USA) was added while vortexing. Cells were incubated 15 min at RT and then 2 ml PBS-BSA Azide (FACS Buffer; Appendix A) was added. After cells were centrifuged and aspirated, the pellets were dissolved in 1 ml FACS Buffer and stored at $+4C^{\circ}$ (7 days at max) before further stainings.

3.2.3.2 Surface Marker Staining Cells

Fixed of live cells were centrifuged and supernatants were aspirated. Depending on the staining combinations, cells were separated into tubes (about 2×10^5 cells per staining). Live cells should be worked on ice in order to maintain their viability. Pellets were suspended in freshly prepared 100 μ l FACS Buffer containing 1 μ l/ml of fluorochrome conjugated antibodies for specific cell markers and incubated for 30 min at RT in dark. 1 ml FACS Buffer

was added and cells were centrifuged and aspirated. Pellets were suspended in 200 μ l FACS Buffer and transferred to 96-well U Bottom Plates, then analyzed on BD Accuri C6 Flow Cytometer.

3.2.3.3 Apoptosis and Necrosis Detection: Annexin-V, PI Staining

Cells were washed twice with cold PBS then resuspended in 1x Annexin-V binding buffer at a concentration of 2×10^6 cells per ml. 200 μ l of the cell suspension was transferred to 1.5 ml tubes.

3.2.3.4 Surface Marker Staining of Microvesicles

Microparticles, apoptotic blebs and exosomes were transferred to 1.5 ml tubes and required amount of fluorochrome conjugated antibodies (CD81, Annexin-V) and Annexin-V Binding Buffer(1X final conc.) added at 1 μ l/ml concentration in 100 μ l. Microvesicles were incubated for 1 hour at RT in dark. 1 ml 1X Annexin-V Binding Buffer added and tubes were centrifuged at 16.000g(Microvesicles and A.Blebs) or 150.000g(Exosomes) for 1 hour. Supernatant was discarded and pellets were dissolved in Annexin-V Binding Buffer and analyzed by flow cytometry.

3.2.4 Preparation of Single Cell Suspensions of Mouse Spleen Cells

The spleens were removed from male C57BL/6 mice after sacrificing by cervical dislocation and transferred to cold RPMI medium containing 2% FBS in 35x15mm cell culture petri dishes. In order to obtain single cell suspensions, spleens were disrupted with sterile syringe plungers by circular moves under lumen-air hood. Then all of the cells except the big scraps collapsed to petri dishes bottom were transferred to 15 ml falcon tubes. In order to wash cells, total volumes were completed to 10 ml with RPMI containing 2% FBS. The cells were centrifuged at 1500 rpm for 5 min. The supernatants were discarded and the pellet gently disrupted and homogenized with media. After one more wash step, cells were dissolved in 1 ml warm RPMI medium supplemented with 5% FBS. Cells were counted to be used in

further experiments. (Hyclone, USA)

3.2.5 PBMC Isolation

Blood samples were transferred into 50 ml falcons. Empty falcons were filled with 15 ml of histopaque. 22.5 ml of blood was slowly poured on top of each histopaque layer. The histopaque and blood should not be mixed, therefore the falcons have to be tilted slightly and immobilized. Samples were centrifuged at 1800 RPM for 30 min (The brake setting should be off.). The supernatant was aspirated into 50 ml. Four different layers formed consisting of plasma, PBMC and basophile containing cloudy buffy coat, histopaque and red blood cells from top to bottom respectively. The buffy coat was carefully transferred to new falcons avoiding contamination from other layers. 2% FBS RPMI medium was added till reaching total volume of 50 ml and mixture was centrifuged at 1800 RPM for 10 min. Then, supernatant was discarded without disturbing the pellet. Pellet was washed two times by resuspending with 10 ml and centrifugation.

3.2.6 Maintenance of Cell Lines

3.2.6.1 RAW 264.7

RAW 264.7 (ATCC®[®], TIB-71™) is an Abelson murine leukemia virus transformed mouse macrophage cell line. Cells were cultured in RPMI1640 media with 10% (thawing) or 5% (sustaining) FBS. Subcultures were prepared by scraping after discarding culture medium. Cells were washed with 5% FBS media and appropriate aliquots of the cell suspension was added to new flasks.

3.2.6.2 EG-7

EG.7 (ATCC®[®], CRL-2113™) is a specific cell line derived from EL4, a mouse lymphoma cell line. EG.7 was stably transfected with pAc-neo-OVA which carries a complete copy of chicken ovalbumin (OVA) mRNA and neomycin (G-418) resistance gene. Cells were cultured in RPMI1640 media with 10% (thawing) or 5% (sustaining) FBS and supplemented with

with 1mg/ml G-418 (Hyclone). Culture maintenance was performed by refreshing the media in every 2-3 days. In order to avoid clumping of cells media was suspended everyday by pipetting up and down.

3.2.6.3 NIH-3T3

NIH/3T3 (ATCC®CRL-1658™) is a mouse embryonic fibroblast cell line which is very useful in DNA transfection studies. Cells were cultured in DMEM-HG media with 10% FBS. Cells should not exceed 80% confluency. For subculturing, cell culture medium was removed and cell layer was briefly rinsed with warm sterile PBS(HyClone, USA) in order to remove all traces of serum which contains trypsin inhibitors. 1 ml of 0.25% Trypsin-EDTA solution is added and incubated at 37C for 5-10 min. In order to avoid clumping cells should not be agitated by hitting the flask during this step. 9 ml complete medium was added and cells were centrifuged. Supernatant was discarded and pellets dissolved in fresh medium by gently pipetting. Appropriate aliquots of the cell suspension was transferred to new flasks.

3.2.6.4 Jurkat

Jurkat, Clone E6-1 (ATCC®TIB-152™) is a human T lymphocyte cell line that are used to study acute T cell leukemia, T cell signaling. Cells were cultured in complete RPMI1640 medium with 10% FBS as suspensions. For subculturing, fresh media addition or medium replacement is required. Cell cultures should be maintained at a cell concentration between 1×10^5 and 1×10^6 viable cells/ml. Culture medium should be renewed every 2 or 3 days.

3.2.6.5 CAL-1

CAL-1 is a plasmacytoid dendritic cell line established from a blastic natural killer cell lymphoma patient. Cells were cultured in RPMI1640 media with 10% (thawing) or 5% (sustaining) FBS. For subculturing, fresh medium addition and replacement is required. In order to avoid clumping, cell medium should be suspended every day. A subcultivation ratio of 1:5 to 1:10 is recommended in every 3-4 days.

3.2.6.6 HT-29

HT-29 (ATCC®HTB-38™) is a human colon adeno carcinoma cell line. Cells were cultured in DMEM-HG media with 10% FBS. For subculturing, cell culture medium was removed and cell layer was briefly rinsed with warm sterile PBS(HyClone, USA). 1 ml of 0.25% Trypsin-EDTA solution is added and incubated at 37 °C for 5-10 min. 9 ml complete medium was added and cells were centrifuged. Supernatant was discarded and pellets dissolved in fresh medium by gently pipetting. Appropriate aliquots of the cell suspension was transferred to new flasks.

3.2.7 Microvesicle Isolation

In order to get rid of cell debris the cell culture supernatants were collected into 15ml or 50ml falcon tubes and centrifuged at 500g for 10 mins at 4 °C. Supernatants were transferred to new falcon tubes without disrupting the pellets and centrifuged at 1500g for 10 mins at +4 °C. Supernatants were transferred to new falcon tubes. Falcon tubes were put into liquid nitrogen and then transferred to −80 °C freezers. If the microvesicle isolation is carried on same day, cell debris free supernatants can be kept at +4 °C for a few hours.

3.2.7.1 Microparticles and Apoptotic Blebs

Cell debris free cell culture supernatant was thawed in warm water. Extensive vortexing was applied in order to separate clumped microvesicles. Supernatant transfers were carried under laminar-air hood in order to avoid contamination. Supernatant was transferred to sterile ultracentrifugation tubes and centrifuged at 16.000 g for 1h. After centrifugation, supernatant was transferred to new falcon tubes for exosome isolation and the pellet were dissolved in high volume cold PBS and again centrifuged at 16.000 g for 1 hour. Supernatant was discarded and ultracentrifuge tubes were left upside down for 5-10 minutes for air dry. Pellets dissolved in sterile cold PBS(HyClone). After counting microparticles were preserved at −80 °C as aliquots.

3.2.7.2 Exosomes

To isolate exosomes microparticle free supernatants were transferred to ultracentrifuge tubes and centrifuged at 100,000g for 90 mins. Supernatants were discarded and pellets were dissolved in large volumes of cold PBS and centrifuged at 100,000g for 90 mins. Supernatants were discarded and tubes were left upside down for 5-10 minutes for air dry. Pellets dissolved in sterile cold PBS. After counting exosomes were stored into -80C freezers as aliquots.

3.2.7.3 CFSE Staining and Quantification of Microvesicles

50 μ l of isolated microvesicles were mixed with previously prepared 50 μ M CFSE (Biolegend,US) dye with 1:1 ratio and incubated 30 min at RT. Then for quenching, samples were diluted 15 times with ice cold RPMI1640 medium containing 10% FBS. Tubes were centrifuged and pellets were gently dissolved in cold 0.5% BSA-PBS gently. 20 μ l aliquots were taken and 1:40, 1:80, 1:160, 1:320 dilutions were prepared with PBS in 96-well U bottom plates as duplicates. Microvesicles were counted on BD Accuri C6 through FL1 channel(FSC threshold: 5000). It is very difficult to analyze too dense and too diluted samples. Therefore, the two serial dilutions that have 2:1 ratio were taken as references in order to calculate total vesicles. In Figure 3.1 there is an example analyze of CFSE stained RAW 264.7 derived vesicles. The best fitting dilutions (40-80) were taken as references (Figure 3.2)

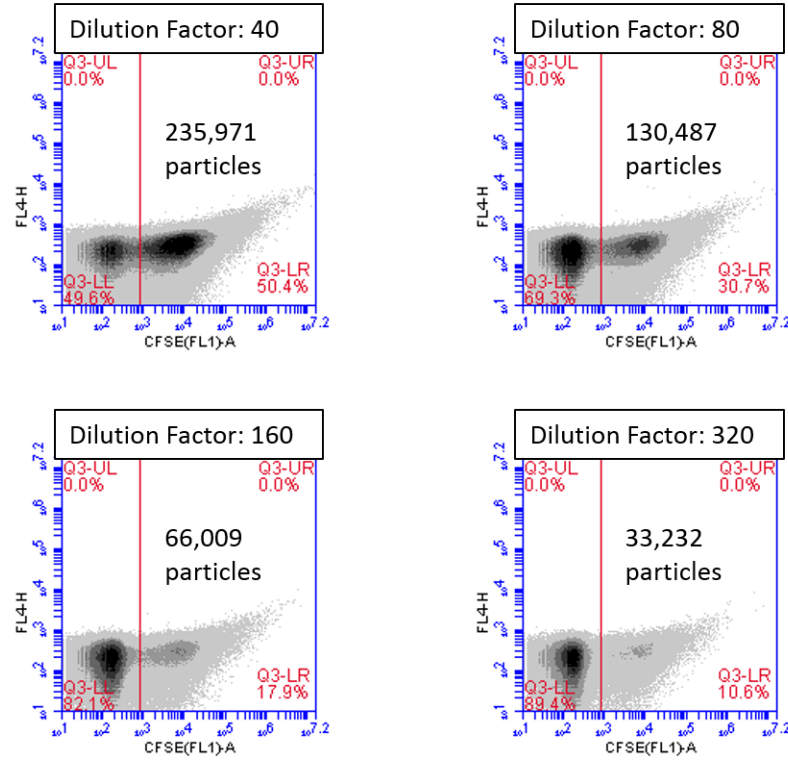


Figure 3.1: Quantification of CFSE stained microparticles with different dilutions.

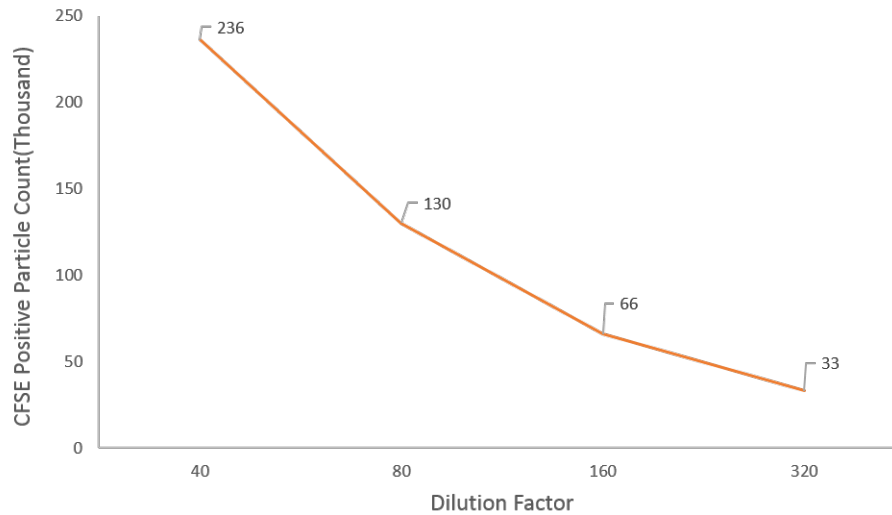


Figure 3.2: CFSE positive microparticle countings. Reference counting were determined by best fitting ratios between serial dilutions.

3.2.8 SP-DiOC Staining of Extracellular Vesicles

Microvesicles or exosomes were completed to 1 ml and 1 μ l of 1 μ g/ml aliquots of SP-DiOC was added. After 30 minutes incubation at RT and dark the samples were completed to 30 ml and centrifuged at relevant speeds. The supernatant was discarded. Pellet was agitated and suspended in PBS.

3.2.9 Atomic Force Microscopy (AFM)

After cells were washed and resuspended in cell culture medium, 20 μ l of cells were transferred into 980 μ l isotonic cell counting in flow cytometer tubes. If cells are derived from mice spleen 1 drop of zapoclopin was added to each tube in order to puncture and eliminate red blood cells. Tubes were vortexed and a volume of 50 μ l was read by the flow cytometer. The chart was set to FSS-C to SSC-C hight and cell population was gated. The number of dots in the gated area was multiplied with dilution factor in order to find total cell number in 1 ml cell suspension.

3.2.10 Binding & Uptake and Internalization Assays

Binding & Uptake and internalization studies were done by using either SP-DiOC and CFSE stained microvesicles. SP-DiOC is a green fluorescent lyophilic dye which can stain vesicular membranes. Stained MPs can either bind to the surface of the cells or be internalized. Trypan Blue is a dye which can penetrate into dead cells and determine cell viability. It is also used for quenching of extracellular fluorescence signaling. To determine the proportion of internalized vesicles, extracellular vesicle fuorescence was quenched with trypan blue (0.01% final concentration). Quenching with trypan blue was only done with live cells. Since dead cells also acquire trypan blue, internalization fluorescence signal would be lost. To determine binding & uptake and internalization fluorescence was measured before and after the addition of trypan blue. CFSE is membrane permeable dye which covalently bind to proteins inside and outside of the vesicles. CFSE stained MPs were incubated with spleen cells or injected intraperitoneally. Depending on the incubation time, cells were fixed (isolated first for in vivo) and stained with surface markers. Cells were analyzed for fluorescence with flow cytometry.

3.2.11 Association of TLR ligands with Microparticles

TLR ligands and microvesicles were mixed in desired concentrations in eppendorf tubes in a total volume of 200 μ l. They were incubated for 30 min at 37 °C. Then, 1.3 ml PBS was added into each mixture tubes were centrifuged at 16,000 g. Supernatants were discarded and pellets were agitated. Required amount of PBS added for final concentration and tubes were vortexed. During the association of TLR ligands with microparticles it was presumed that 100% of ligands were linked to microparticles although about 1% percent association was expected at the furthest. Used TLR ligands are listed in Table 3.2.

3.2.12 Cell Counting

3.2.12.1 Hemocytometer

After primary cells and cell lines were washed and resuspended in fresh medium(1 ml- 10 ml), small aliquots were transferred to 1.5 ml tubes and diluted (10 to 50 folds) with fresh medium. Then 10 μ l from diluted cells were dropped onto hemocytometer surface. In order to count viable cells, before dropping the diluted cell suspension onto hemacytometer, 10 μ l of it was mixed with 10 μ l Trypan Blue(HyClone) and then 10 μ l from the mixture was used for counting. Blue colored cells are apoptotic or necrotic cells.

3.2.12.2 Flow Cytometry

After cells were washed and resuspended in cell culture medium, 20 μ l of cell solution was suspended in 980 μ l Isoton II Diluent Buffer in flow cytometer tubes. If cells are derived from mice spleen 1 drop of ZAP-OGLOBIN II Lytic Reagent (red blood cell lytic agent) was added to each tube in order to puncture and eliminate red blood cells. Tubes were vortexed and a volume of 50ul was read by the flow cytometer, BD Accuri C6. The chart was set to FSS-C to SSC-C height and cell population was gated. The total number of dots in the gated area was multiplied with 1000 in order to find cell number/ml in cell suspension.

3.2.13 Cargo Loading to Extracellular Vesicles

The microvesicles and A151 were mixed with the desired amount in 1.2 ml tubes. For example, generally per 10ug of A151 , 2 million microvesicles were added. If there are more than one sample, all mixtures volumes were equalized by adding sterile PBS. The caps of the tubes were replaced with caps with holes. Mixture was frozen in liquid nitrogen and loaded into freeze-drier and lyophilized o/n. Then 30 μ l sterile ddH₂O was added and vortexed for 30 secs. Then samples were vortexed for 10 secs in every 5 minutes(repeated 5 times). Then sterile PBS added to finalize volume. If cargo is nucleotide based, the samples were centrifuged and supernatant was analyzed with NanoDrop-1000(Thermo) in order to determine loading efficiency. The general methodology about A151 loading and further analysis is summarized in Figure 3.3.

3.2.14 Micro BCA Protein Assay

Standards were prepared in tubes as shown in Table 3.5. Serial dilutions (with 1:2 or 1:3 ratio for each dilution) of microvesicles were staged in tubes. 10 μ l from each standard and sample dilutions were transferred to 96 well qPCR plates as duplicates or triplicates. Then working reagent(WR) was prepared according to the number of total used wells(well count X 10 μ l). To prepare WR, 25 part MA, 24 part MB and 1 part MC solution were mixed. 10 μ l of WR was added to each well. Plate was sealed and incubated 2h at 37 °C. Then samples were analyzed with NanoDrop-1000(Thermo) by using Micro BCA Program in the software.

Table 3.5: Standard Preparation for Micro BCA Protein Assay

Standard Name	Standard Conc. (mg/ml)	Diluent (PBS)	Solute (BSA)
A	0.2	90 μ l	10 μ l (2 mg/ml)
B	0.04	160 μ l	40 μ l from A
C	0.02	80 μ l	80 μ l from B
D	0.01	80 μ l	80 μ l from C
E	0.005	80 μ l	80 μ l from D
F	0.0025	80 μ l	80 μ l from E
G	0.0012	96 μ l	64 μ l from F
Blank	-	50 μ l	-

MV isolation and A151 loading Protocol

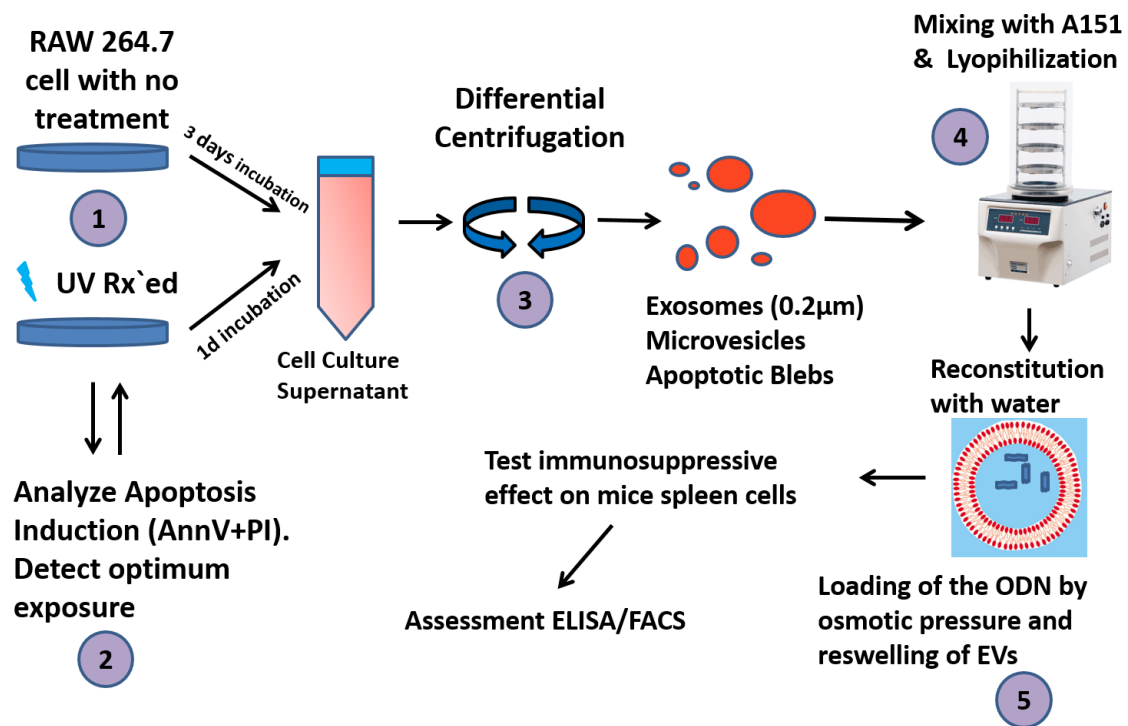


Figure 3.3: EV isolation and A151 loading protocol.

Chapter 4

Results & Discussion

The present thesis aims to characterize and investigate the immunological properties of extracellular vesicles (EVs) secreted from various cell types. To reach this goal firstly, i) microparticles, ii) apoptotic blebs and iii) exosomes were isolated and purified from supernatants of various mouse and human cell lines. Next, in order to delineate the transmission of microbial/pathogenic by-products associated onto EVs and also to investigate immunomodulatory character of secreted EVs on naive immune cells *in vitro* stimulation assays were conducted. Furthermore, upon injection, *in vivo* fates of EVs were analyzed. Finally, their potential application as a drug delivery vehicle was explored.

4.1 Physicochemical analyses of the extracellular vesicles

The size and shape analyses of the isolated vesicles were investigated under atomic force microscopy (AFM). In Figure 4.1, 3D topography of EVs were demonstrated. The height of exosomes and MPs were approximately 40 and 80 nm, respectively. In phase 2D topography, as expected (Figure 4.2), it was clearly observed that MPs were much bigger than secreted exosomes (Figure 4.1 and 4.2).

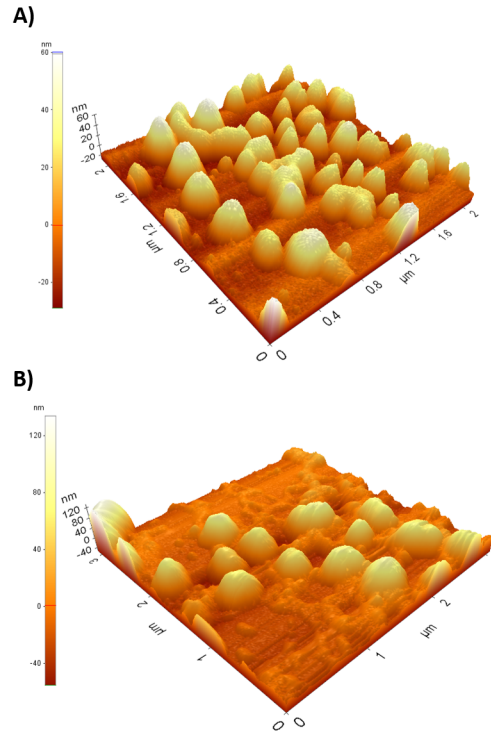


Figure 4.1: 3D Topography of RAW derived extracellular vesicles. A) Exosome, B) MP

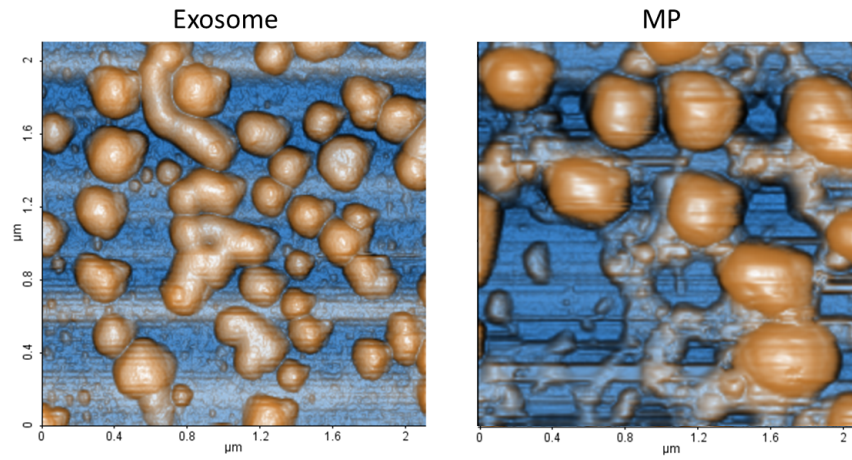


Figure 4.2: AFM photomicrographs of phase 2D topography of RAW derived exosomes(left) and MPs(right).

While diameter of exosomes varied between 50-200 nm, MPs size distribution was found to be in the range of 200-500 nm. Since our AFM analyses procedure required sample preparation to be in dry state, these investigations demonstrated that the topographic height was lower and diameter was bigger than expected sizes with respect to exosomes' hydrated forms.

In addition to AFM analyses, physico-chemical characterization of EVs was also investigated by dynamic light scattering (DSL) method. Of note, this analysis method allows us to gather information of EV particles suspended in aqueous state, in addition to their surface charge distributions. In order to determine zeta potential and size, exosomes were suspended in PBS, and their hydrated sizes were determined to be ≈ 256 nm (Figure 4.3A). As expected, the average zeta potential value of exosomes was found to peak around -29mV (Figure 4.3B) strongly correlated with the biological membranes' zeta-potential, confirming that our isolated EVs stem from plasma membrane based vesicles [265].

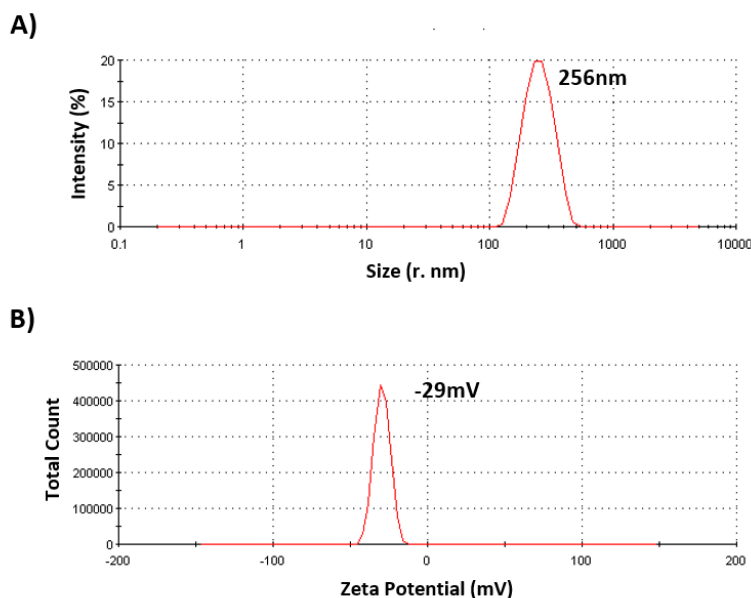


Figure 4.3: Zeta potential and size of the exosomes derived from RAW 264.7 cells. (A)Size distribution by intensity. (B)Zeta potential distribution. Measurements were made by Malvern Nano ZS.

4.2 Uptake and Internalization studies with EVs

Previous studies demonstrated that EVs were taken up by target cells at a different rate and level [266]. It is important to determine internalization kinetics of vesicles in order to reveal possible mechanisms, different functions and understand the potency as a drug delivery vehicles. If EVs from various cells are differentially taken up by distinct immune cells it could be monitored by double labeling of these cells upon incubating them with labeled EVs. For that internalization and uptake experiments were performed with SP-DiOC- a green fluorescent membrane dye -labeled vesicles. Incubations of EVs and mouse spleen cells were varied in these experiments (i.e. different EV:Cell ratios were used).

As seen in Figure 4.4, after 8h of incubation of EVs with RAW cells, apoptotic exosomes demonstrated the maximum rate of uptake by spleen cells. Exosomes derived from apoptotic cells demonstrated the highest uptake whereas normal exosomes indicated the lowest value. Also, uptake of the apoptotic blebs were greater than MPs. The uptake of apoptotic EVs was an unexpected observation. Both MFI and SP-DiOC signals at cell:EV ratio of 1:64 the top highest binding was observed with apoptotic EVs.

While binding is a critical step for vesicle interaction with the target cells, internalization of that bound vesicle is also important. Figure 4.5 summarizes the findings of the studies on internalization. In order to detect internalization of vesicles, cells were stained with Trypan Blue (TB) after incubation with SP-DiOC labeled EVs (see Section 3.2.10 for details). Consistent with the results in Figure 4.4, apoptotic exosomes entered into cells (at 1:64 cell:EV ratio) significantly higher than other vesicles . Again, apoptotic blebs internalized slightly better than MPs. While 80% of the spleen cells were positive for apoptotic exosome signal by 8 h, only 10% was bound on the surface and 70% were internalized. Strikingly, for apoptotic blebs, and other EVs the degree of internalization was not as impressive as apoptotic exosomes. For apoptotic blebs 50% of spleen cells were positive for SP-DiOC signal (Fig 4.4) but only 15% of spleen cells internalized these blebs after 8h of *in vitro* incubation at 1:64 ratio (please compare Fig 4.4A vs Fig 4.5A yellow line).

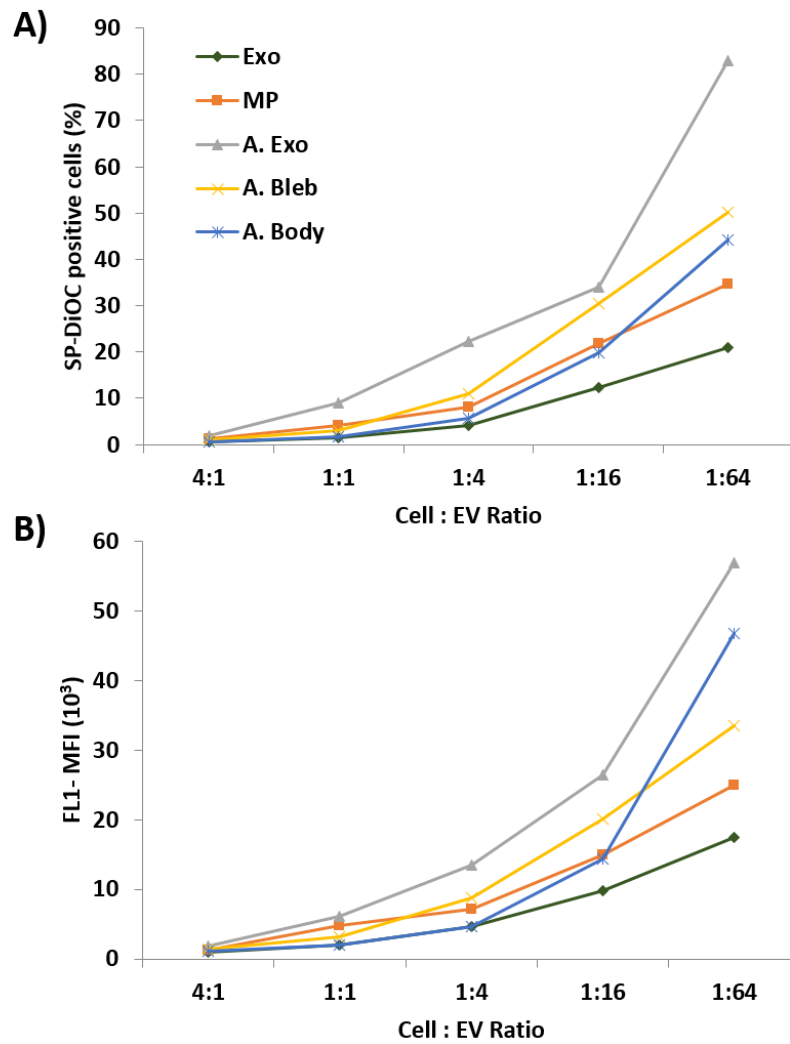


Figure 4.4: Uptake of RAW derived different EVs by mouse spleen cells. SP-DiOC labeled EVs incubated with splenocytes at different EV:Cell ratios for 8 hours and cells were analyzed with flow cytometry for SP-DiOC signal. A) Percentage of SP-DiOC positive cells, B) Mean fluorescence intensity (MFI) values. A: Apoptotic forms of EVs

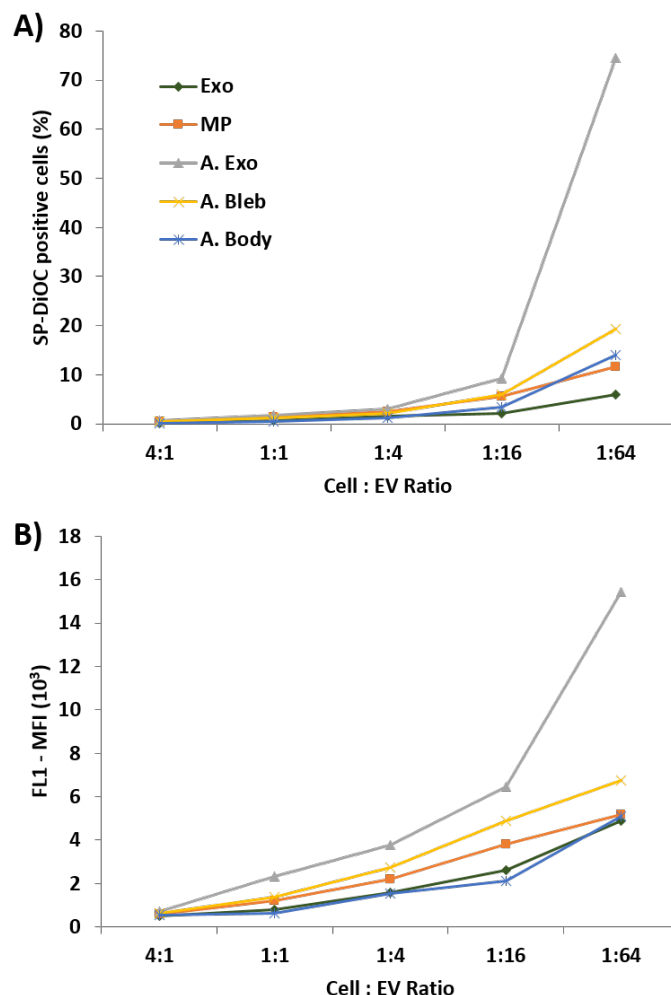


Figure 4.5: Internalization of RAW derived different EVs by mouse spleen cell. SP-DiOC labeled EVs were incubated with splenocytes with different EV:Cell ratios for 8 hours. Before analyzing cells were stained with Trypan Blue. A) Percentage of SP-DiOC positive cells, B) Mean fluorescence intensity (MFI) values. A: Apoptotic forms of EVs

A similar experiment with more time points were planned to distinguish the level of binding and internalization kinetics of EVs at two different cell:EV ratio. Data revealed that RAW derived MPs internalized more rapidly than exosomes and apoptotic blebs (Figure 4.6) within spleen cells. The binding to and internalization by cells increased as the EV ratio increased from 1:4 to 1:16 at early time points (2 or 4h), but subsided at late time point (8h incubation). Interestingly, binding of MPs reached 20% positivity (@ 1:4 ratio) by 2h of incubation, but when MP ratio was used at 1:16, the cells bound > 45% of vesicles. At the end of 8h incubation, MPs were increasingly taken up by cells at low cell:MP ratio, whereas,

at 1:16 cell:MP ratio it was saturated (Figure 4.6A). Internalization kinetics of EVs by spleen cells showed slightly different profile compared to uptake data. As seen in Figure 4.6A, all EV types progressively bound to spleen cells as the incubation time prolonged irrespective of cell:EV ratio, however, internalization of EVs by immune cells increased and peaked at 4h post-incubation and by 8h the levels either dropped or remained unchanged for all EV types at both Cell:EV ratios (Figure 4.6B).

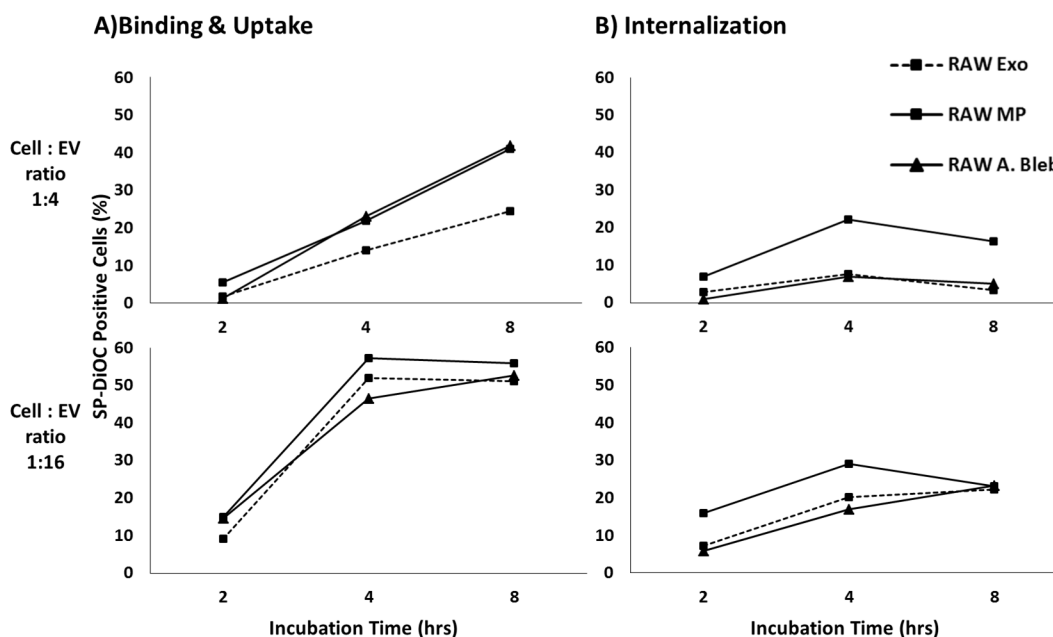


Figure 4.6: Uptake and Internalization kinetics of RAW derived normal and apoptotic vesicles. SP-DiOC labeled exosomes, MPs and A.blebs were incubated with mice spleen cells for 2, 4 and 8 hours with two different concentrations. A) Uptake of vesicles. B) Internalization. A.bleb: Apoptotic Bleb

4.2.1 Influence of cell source of EVs on binding and internalization by spleen cells

It is a known fact that different cells secrete EVs at different amount and rate depending on that specific cell's physiological state. These EVs do regulate different biological processes upon internalization by target cells. So far in this study we demonstrated that macrophage derived extracellular vesicles do enter into different cells at a different rate and level, but we have never compared different cell secreted EV entry on target cells.

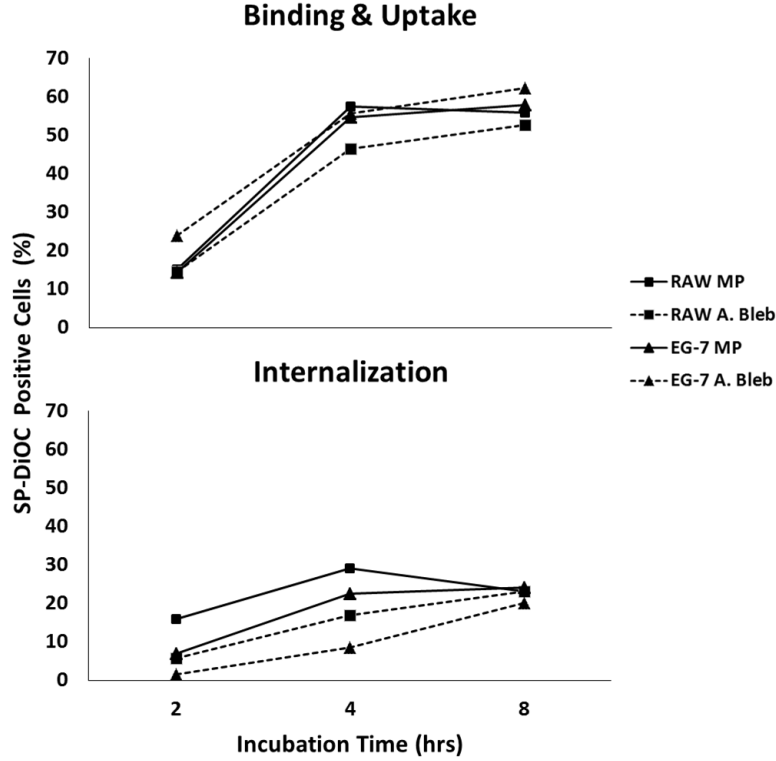


Figure 4.7: Uptake and Internalization kinetics of RAW and EG-7 derived normal and apoptotic vesicles. SP-DiOC labeled MPs and A.blebs were incubated with mice spleen cells for 2, 4 and 8 hours. Cell:EV ratio is 1:16. A.bleb: Apoptotic Bleb

To understand whether different cells that secrete their EVs differentially internalized by a target cells, EVs from EG-7 and RAW cells were purified and their binding and internalization kinetics were tested on spleen cell culture. Similar to previous section Cell:EV ratio was kept at 1:16, and incubation time was continued up to 8 h. As seen in Figure 4.12, while uptake kinetics for both EV sets showed similar trend irrespective of EV type, internalization kinetics was slightly different. RAW derived EVs (MP and Apoptotic blebs) at earlier time points internalized by spleen cells much faster than EG-7 EVs. There were 2 fold more RAW EV positive cells at 2h, compared to EG-7 positive spleen cells. This difference was lost by 8h and both EG-7 and RAW EVs internalized at similar levels (30% cells were positive for EVs irrespective of cell source, Fig. 4.7).

4.2.2 Studies on differential internalization of EVs by immune cells

After establishing that immune cells do take up secreted EVs and internalize them differentially, we wished to identify the cell types internalizing these vesicles. Of note, in our previous experiments EVs were labeled with the lipophilic dye SP-DiOC, but this dye could not be compensated during cell specific staining procedure, so for these experiments we had to shift to another fluorescent dye known as carboxyfluorescein succinimidyl ester (CFSE). CFSE can be retained within vesicles for extremely long periods due to its covalent bond between succinimidyl groups and lysine residues of vesicular molecules. Also, once it was incorporated within vesicles, it cannot be transferred to adjacent vesicles unless they fuse. CFSE labeled MPs from mouse i) macrophage (RAW), ii) T-cell (EG-7) and iii) fibroblast (NIH-3T3) cell lines, were incubated with spleen cells (@ 1:8 ratio for up to 16 h). Internalization kinetics by each immune cell types: i) B cells (B220+), ii) T-cells (CD+), iii) Macrophages (CD11b+), and iv) Dendritic Cells (CD11c+) were studied by FACS against each cell-specific surface marker by fluorochrome conjugated antibody. By 16 h of incubation of different EVs isolated from different cells it was found that over 30% of spleen cells took up NIH-3T3 MPs this was followed by EG-7 and RAW MPs (Figure 4.8). Interestingly, EG-7 MPs were taken up by more cells according to the RAW MPs which was exactly

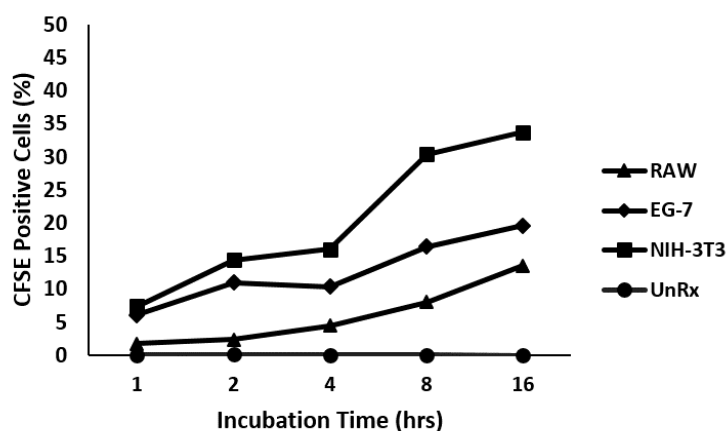


Figure 4.8: Internalization of CFSE labeled RAW, EG-7 and NIH-3T3 MPs to mouse spleen cells. CFSE-MPs were incubated with mice splenocytes *in vitro* for 1, 2, 4, 8, 16 hours. Cell:MP ratio was 1:8. Then cells were analyzed for CFSE positivity by flow cytometry.

opposite in previous SP-DiOC labeled internalization experiments. This is another example to demonstrate that the dye used to label MPs may impact the outcome of the experiment.

4.2.2.1 MP Internalization by B cells

Total B cell ratio among spleen cells did not affected by vesicle treatment (Figure 4.9). When CFSE positive cells were gated, to B cells, 90 % was positive for NIH-3T3-MP, 85 % for EG-7 and 70 % for RAW MPs within 16 hour. Entry kinetics of CFSE+ MPs to B220+ cells was as follows: NIH-3T3 > EG-7 > RAW.

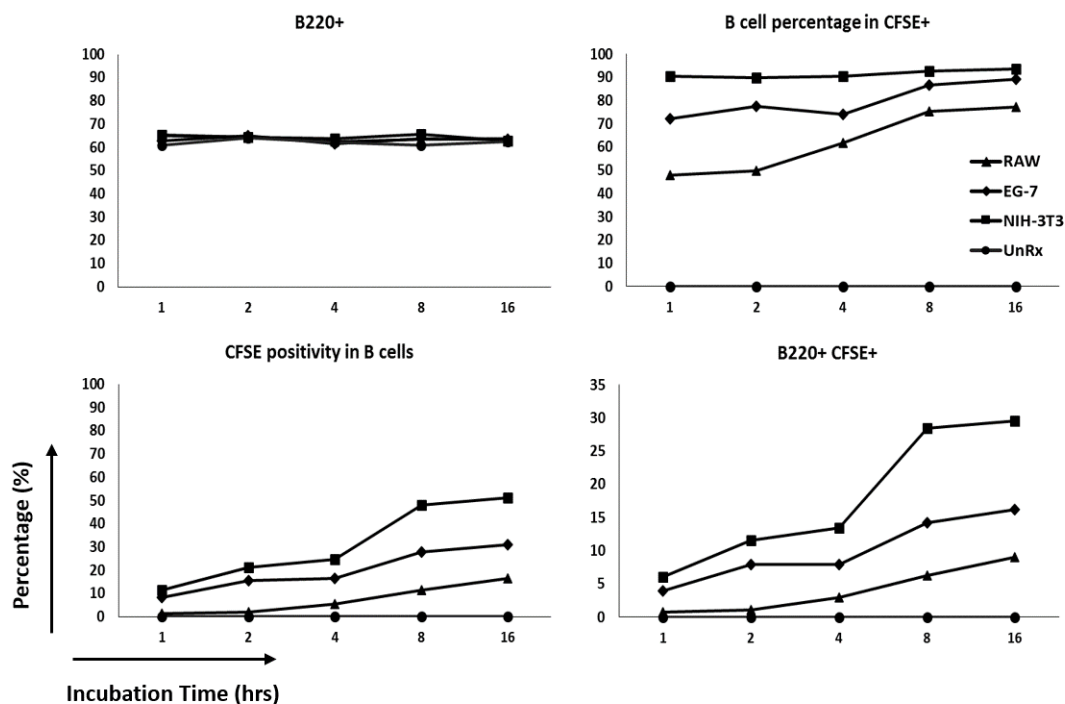


Figure 4.9: Analysis of internalization of MPs to B cells specifically. Cells incubated with CFSE-MPs were fixed and stained with B220-APC Ab. For more details see also Fig 4.8.

4.2.2.2 MP Internalization by Splenic T-cells

Contrary to B-cells, T-cells does not readily took up MPS. The level of MP accumulation was in the range of 3-5% of all T-cells for the entire incubation period (Figure 4.10). T-cells mostly

took RAW MPs (Figure 4.10, top right panel) for the entire incubation period (1-16h). For RAW MPs uptake, T-cell ratio among CFSE+ cells decreased by time indicating that, the rate of early entry of MPs by T-cells at the onset of incubation was much faster than late stages of incubation. Opposite to what was seen for B-cells, NIH-3T3 cells were least internalized by T-cells at early incubation times, it did recover by 16 h of incubation. CFSE positivity among T cells demonstrated that EG-7 MPs reach the highest accumulation at 2nd hour of incubation and then began to subside.

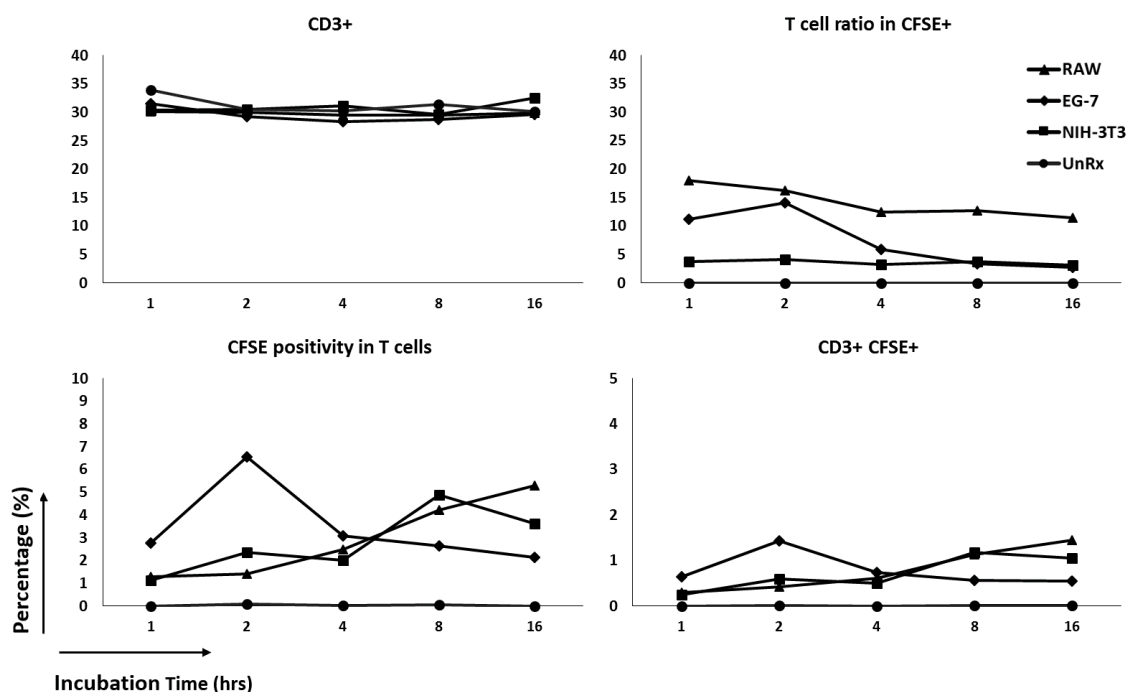


Figure 4.10: Analysis of internalization of MPs to T cells specifically. Cells incubated with CFSE-MPs were fixed and stained with CD3-PE Ab. For more details see also Fig 4.8.

4.2.2.3 MP Internalization by Splenic DC-cells

Total DC ratio in mouse spleen ranges between 1 to 2% of all spleen cells. Upon MP administration very small accumulation was observed between groups. When CFSE+ gated DC cells were examined, MP + ratio was around 22% for RAW MPs and 5% for EG-7 and NIH-3T3 MPs at 1st hour (Figure 4.11). Interestingly, at the end of 16th hours, DC ratio in CFSE+ cells was around 10% for RAW, 4% for NIH-3T3 and 2% for EG-7 MPs. This

significant decrease, points out the existence of differential internalization kinetics of various microparticles. By 16h, CFSE positivity among DCs was around 70% for RAW, and NIH-3T3 and 20% for EG-7 MPs which suggests that macrophage and fibroblast derived MPs internalized by DCs significantly higher than T-cell MPs (Figure 4.11 lower left panel).

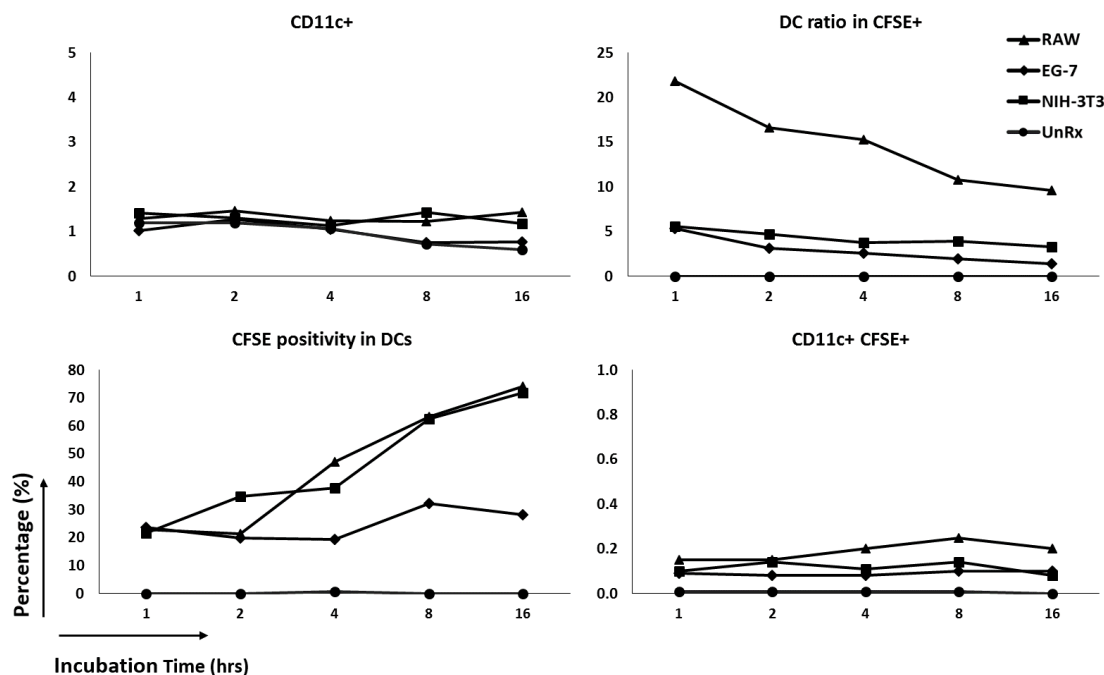


Figure 4.11: Analysis of internalization of MPs to DCs specifically. Cells incubated with CFSE-MPs were fixed and stained with CD11c-PE Ab. For more details see also Fig 4.8.

4.2.2.4 MP Internalization by Splenic Macrophages

Internalization of RAW MPs by splenic macrophages was above 35% for the entire experiment duration (Figure 4.12, upper right panel). Interestingly, NIH3T3 and EG7 cells internalized within macrophages far less than RAW MPs (less than 10% of macrophages took up these cells). CFSE positivity in macrophages rose from $\approx 10\%$ (at 1 h) to $> 70\%$ (at 16 h) for RAW MPs, this value was only 40% for NIH3T3 MPs. Interestingly EG7 MPs never exceeded 10% in splenic macrophages (Figure 4.12 bottom left panel).

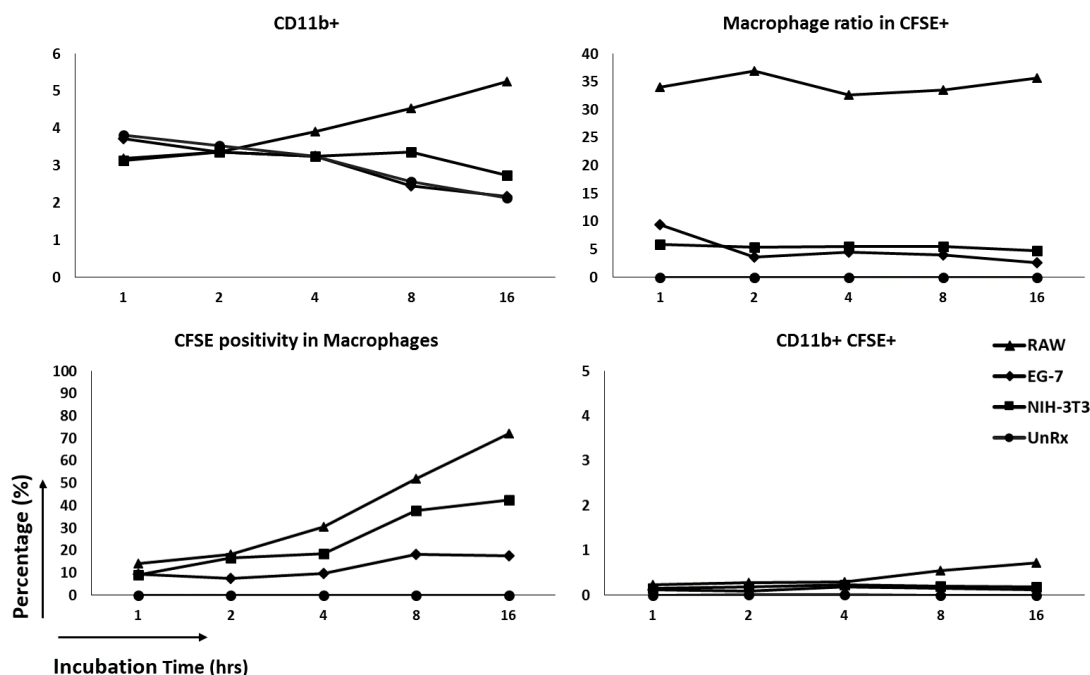


Figure 4.12: Analysis of internalization of MPs to macrophages specifically. Cells incubated with CFSE-MPs were fixed and stained with CD11b-PE Ab. For more details see also Fig 4.8.

4.2.2.5 Internalization of different MPs by ICAM-1 expressing splenic cells.

ICAM-1 (Intercellular Adhesion Molecule 1 or CD54) is a glycoprotein which is typically expressed on endothelial cells and immune cells. After CFSE labeled MP administration of spleen cells, CD54 positive cells were gated and analyzed for CFSE signal as a control (Figure 4.13), since most of the spleen cells are CD54 positive. As expected, internalization kinetics of different MPs by ICAM-1+ cells were parallel to total values obtained for the whole spleen analyses (please compare Figure 4.8 with lower left panel of Figure 4.13).

Figure 4.14 and Figure 4.15 summarizes dot-plots of cell population specific associations of different MPs after 8 and 16 h incubation, respectively. Figure 4.16 represents pie chart distribution of MP treated CFSE positive cell ratios at 8 h. As clearly demonstrated in these figures there are distinct affinities of various MPs for different immune cells. Strikingly, all MPs types irrespective of their sources greatly internalized by B cells in comparison to other immune cells. Very less proportion of EG-7 and NIH-3T3 MPs were taken by macrophages,

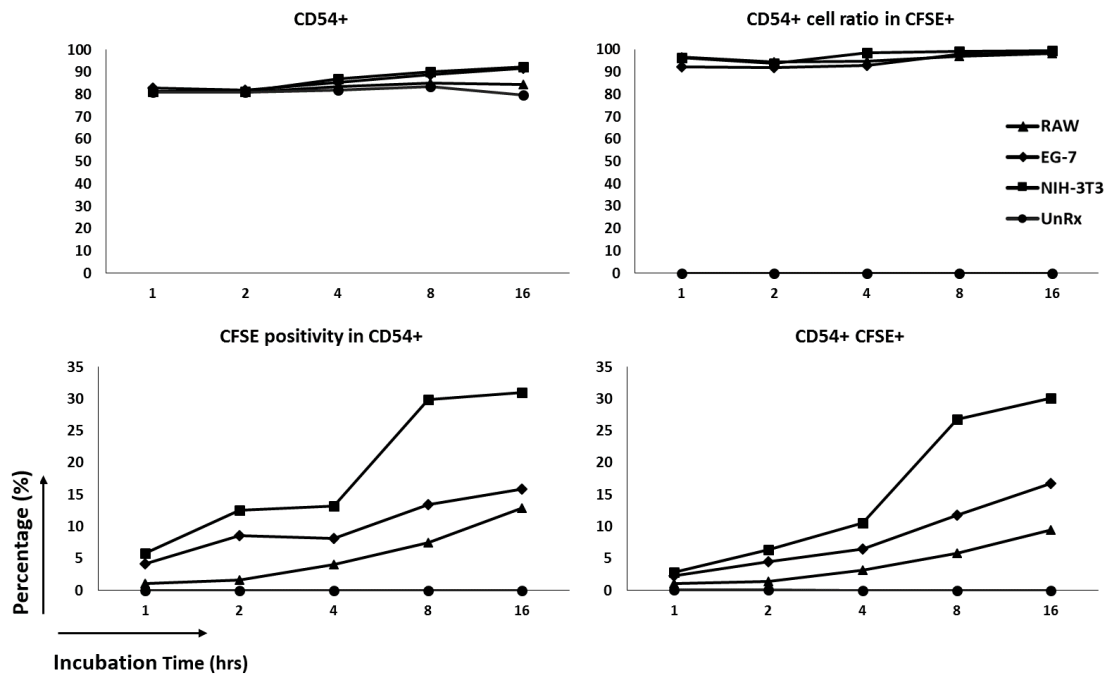


Figure 4.13: Internalization of CFSE-MPs to CD54 surface protein presenting spleen cells. Cells incubated with CFSE-MPs were fixed and stained with CD54-PE Ab. For more details see also Fig 4.8.

DCs and T-cells. However, notable percentage of RAW specific MPs penetrated especially to macrophages.

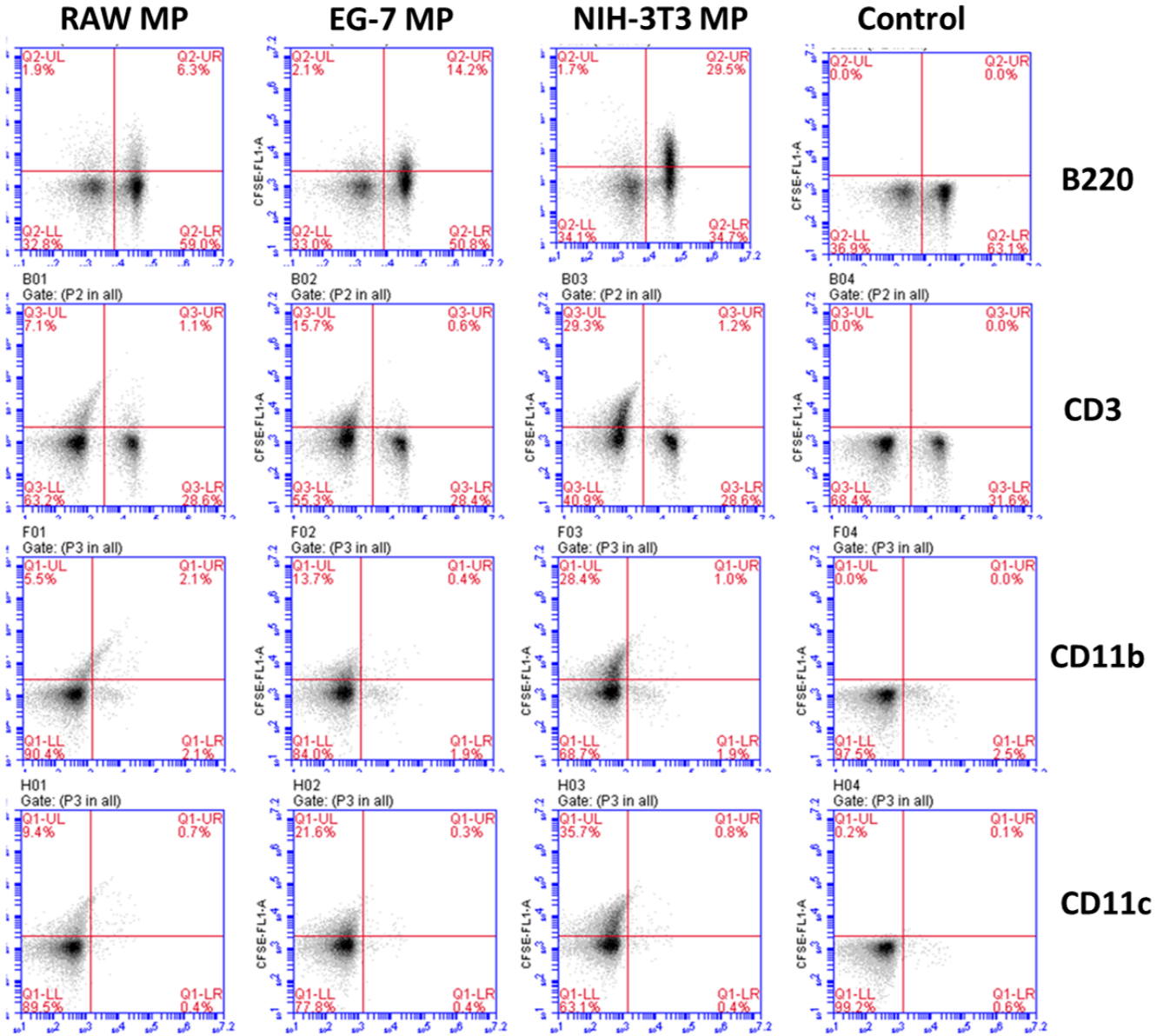


Figure 4.14: Dot plots of different splenic cells positive for CFSE-labeled MPs at 8h. For more details see also Fig 4.8.

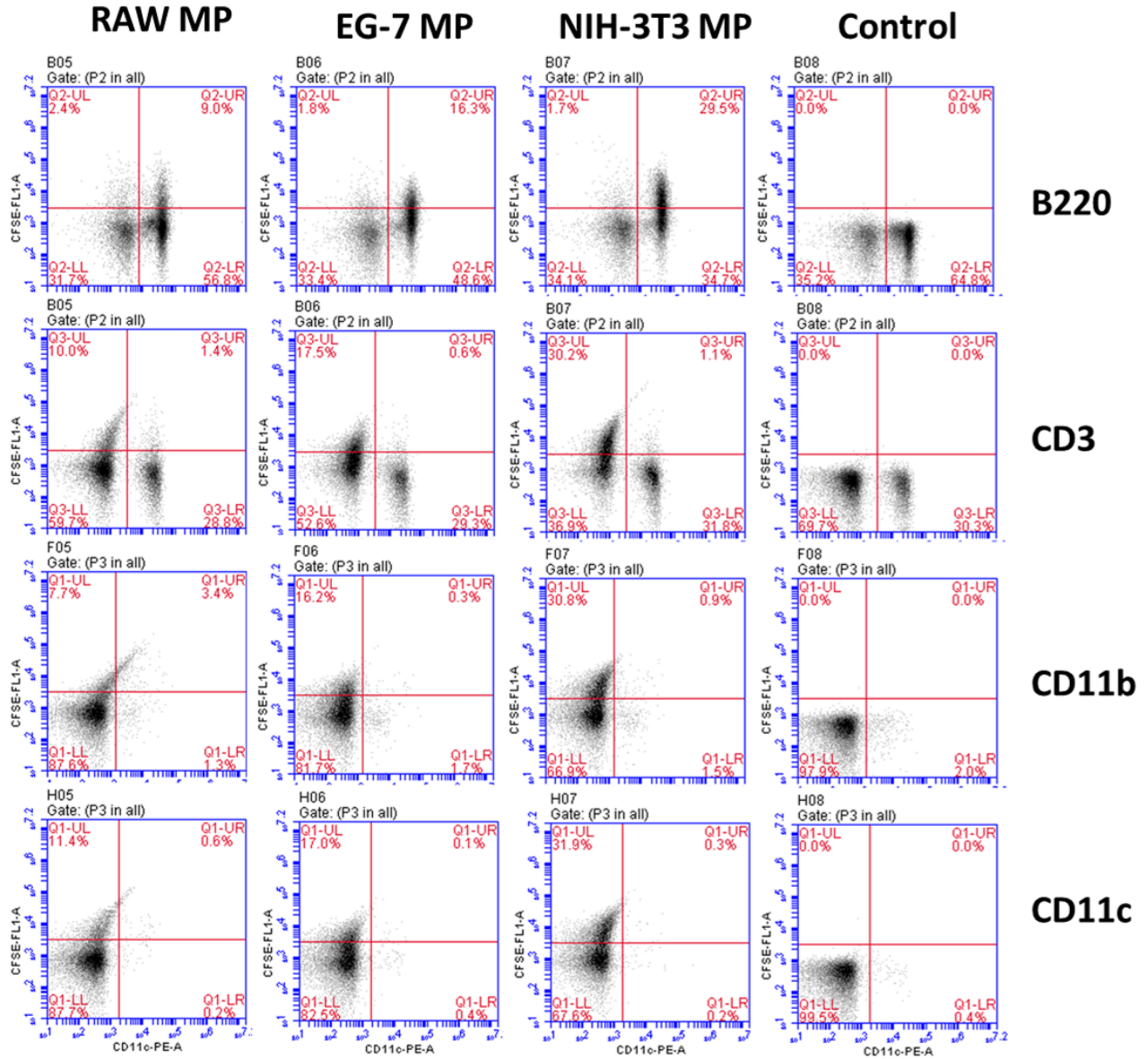


Figure 4.15: Dot plots of different splenic cells positive for CFSE-labeled MPs at 16h. For details see Figure 4.17.

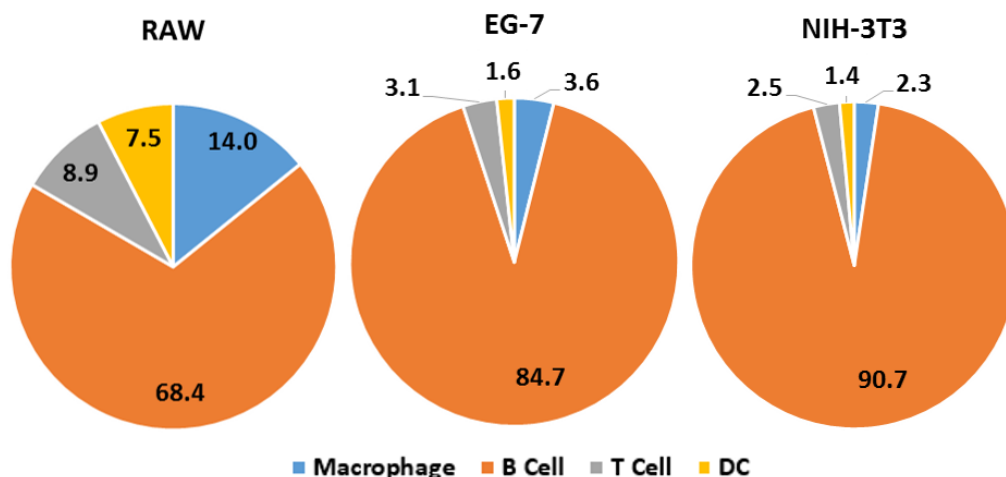


Figure 4.16: Immune cell ratios among MP treated CFSE positive cells. RAW, EG-7 and NIH-3T3 derived MPs were given to spleen cells *in vitro*. After 8 hours of incubation cells were fixed and stained with specific cell markers. Cell:MP ratio is 1:8.

4.2.3 *In vivo* distribution of MPs upon intraperitoneal administration to mice

In vitro experiments with MPs and spleen cells demonstrated that vesicles enter into spleen cells differentially. The fate of MPs upon *in vivo* injection was not explored in great depth. the internalization kinetics was essential in order to harness MPs for therapeutic applications. We used CFSE labeled MPs from 3 different cell lines and injected intraperitoneally to C57BL/6 mice (100 million particles /animal). After 24 hours, mice were dissected to examine spleen, lymph nodes (LN) and peritoneal exudate cells (PEC). Flow cytometry analysis on collected PEC demonstrated that RAW derived MPs internalize to 9.5% of cells whereas 2.5% of PECs were CFSE positive after injected with either EG-7 or NIH-3T3 MPs (Figure 4.17). As expected the CFSE signals were very small among spleen and LN cells (data not shown). Consistent with *in vitro* data there was a differential internalization pattern for MPs isolated from different cell source. Fluorescence intensity analyses of harvested cells indicated that, of three MPs types, RAW MPs were the highest among others in terms of internalization. (Figure 4.18).

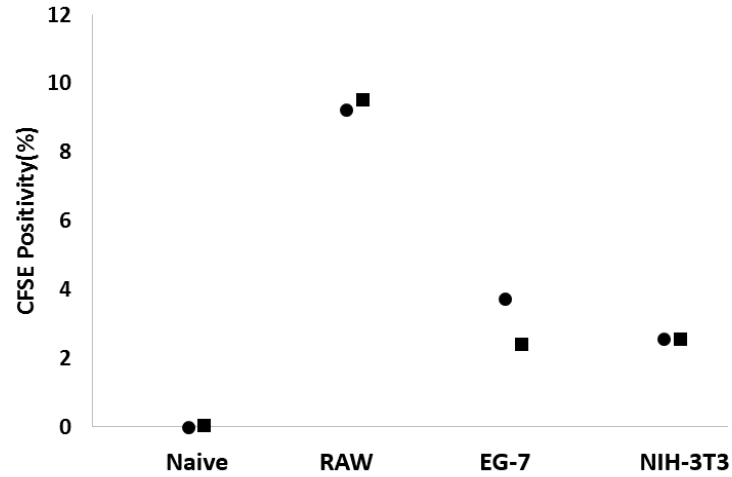


Figure 4.17: CFSE positivity of PECs. CFSE labeled RAW, EG-7, NIH-3T3 MPs (10×10^7 /mouse) were administered to C57BL/6 mice intraperitoneally. 24 h later, cells were recovered, and monitored by flow cytometry.

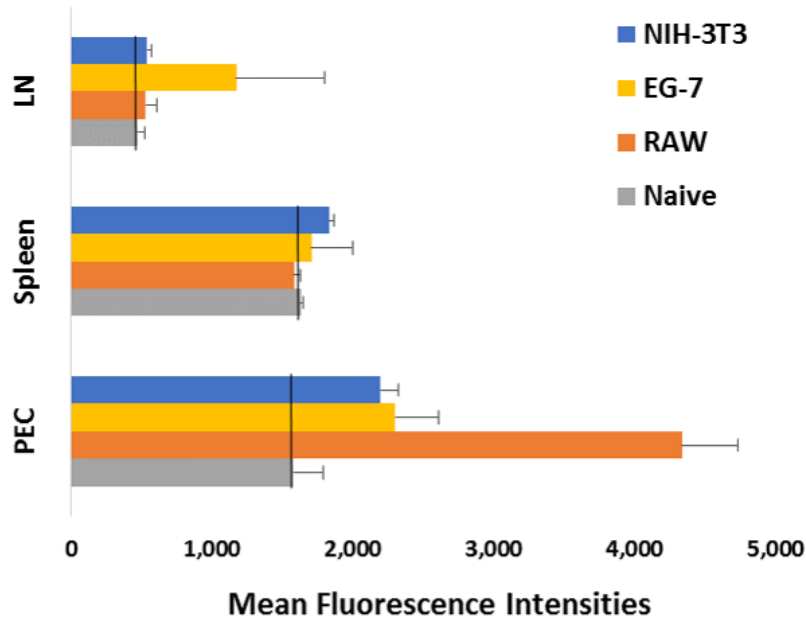


Figure 4.18: Mean fluorescence intensities (MFI) of PEC, Spleen and LN cells dissected from MP treated mice. CFSE labeled RAW, EG-7, NIH-3T3 MPs were injected to C57BL/6 mice intraperitoneally. After 24 hours uptake was monitored by flow cytometry analysis of cell fluorescence intensity.

Cell specific uptake was analyzed by staining spleen cells with specific cell surface marker antibodies. As seen in Table 4.1, PEC macrophages took up nearly all MP post-24h injection. Notably, PEC resident macrophages took up over 95% of CFSE signal independent of the source of the secreted MP.

Taken together our findings implicated that MPs are potential nanocarriers and can be internalized *in vivo* for further immunotherapeutic interventions.

Table 4.1: CD11b, CD11c, CD3, B220 cell population percentages among CFSE positive cells. For more details see also Fig 4.17

	RAW MP	EG-7 MP	NIH-3T3 MP
B-cell	0.5 ± 0.1	1.8 ± 0.5	0.3 ± 0.2
T-cell	1 ± 0.2	1.7 ± 0.2	0.2 ± 0.1
Macrophage	98.8 ± 0.5	96 ± 1.5	95 ± 1
DC	1.6 ± 0.0	3.4 ± 0.5	3.6 ± 3.4

4.3 EVs transmit TLR ligands to distant cells and induce immune activation

Recent studies demonstrated that EVs are capable of mediating cell-to-cell communication [267] during immune responses. We postulated that cells infected by microbes or upon ingesting bacterial by products freely circulating in biological milieu subsequently secrete EVs harboring different TLR ligands. The critical step is to distinguish whether these secreted EVs reaches distant sites and contribute to the pronunciation of the ongoing immune activation upon internalization by other naive (or uninfected) immune cells. To test this hypothesis a preliminary experiment was designed. RAW cells were incubated overnight with $3 \mu\text{g}/\text{ml}$ LPS. The next day secreted MPs were isolated, purified from the supernatant and counted. These MPs were then incubated with freshly cultured RAW cells at a ratio of 1:1 (cells:MP number ratio). 24 hours after stimulation with MPs cells were harvested and IL-6 secretion along with nitric oxide production was determined from the supernatant by ELISA and Griess method, respectively. As seen in Figure 4.19, MPs induced appreciable amounts of IL-6 and NO from RAW cells. This induction is dependent on the cargo since empty MPs did not initiate any activation. This data along with the data presented in

Figure 4.19 suggested that cell-derived extracellular vesicles might be non-immunogenic or immunogenic depending on the state of that cell. It also implied that if a cell is exposed to bacterial by-products (such as LPS a TLR4 ligand) it has the capacity to transmit this ligand via its secreted extracellular vesicles to other neighboring cells and it is sufficient to trigger an immune cascade depending on the associated ligand.

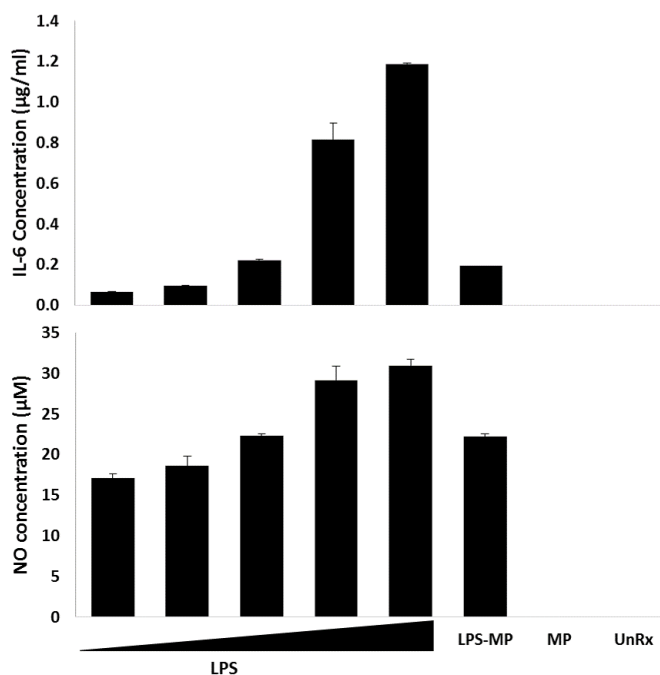


Figure 4.19: MPs secreted from LPS exposed RAW cells (LPS-MP) can transmit its ligand to other cells and demonstrate immunostimulatory activity. RAW cells treated with $3\mu\text{g/ml}$ LPS for o/n, were harvested and MPs. LPS-harboring MPs were transferred to a freshly cultured cells at a 1:1 MP:cell ratio. The supernatant of these MP treated cells were collected and assayed for IL-6 and NO production. Free LPS ($0.001\text{-}5\mu\text{g/ml}$, 5x dil.) was used as a standard control to compare stimulatory activity of LPS associated MPs.

4.3.1 Effects of EVs on murine immune system cells

Current studies supported that MPs may play a very significant role during infections. Activated T-cell MPs can activate macrophages or MPs from stimulated macrophage can trigger naive T-cells [224]. Moreover, extracellular vesicles were reported to carry nucleic acids such as mRNAs or miRNAs, and contribute immune modulation [268]. However, it is not clear whether MPs have role in transportation or presentation of bacterial products. After this

encouraging preliminary finding we planed to examine such function of microvesicles after incubating RAW 264.7, CAL-1 and EG-7; a macrophage, a pDC and a T-cell representative member of the cell lines, respectively with various TLR ligands. The following ligands were used throughout this set of experiments: i) LPS, (TLR4L), ii) poly(I:C), (TLR3L), iii) PGN, (TLR2/6), iv) Zymosan, (TLR2L), v) R848, (TLR7/8L), and vi) bacterial DNA, (TLR9L)

MPs purified from above mentioned cell lines were incubated with the ligands for 30 mins at 37 °C. They were washed to remove excess unassociated ligands and then used for stimulation assays. These ligand associated MPs were then incubated with freshly cultured RAW cells (Figure 4.20), or spleen cells (Figure 4.21) for 24h. Supernatants were saved for NO, IL-6 and IL-12 detection by Griess method, or ELISA, respectively.

In this experiment, it is important to note that the associations of the ligands on to MPs were considered to be 100%, and the concentrations of each ligand in induction experiments were based on this assumption. Another important point to note that the pDC cell line CAL-1 is a human cell line, the MPs from this cell was intentionally included in these experiments to check the interspecies effect of secreted vesicles on immune activation.

As seen in Figure 4.20A, LPS treated MPs derived from all cells led to nitric oxide (NO) secretion similar to free LPS. PGN treated MPs also induced slight NO production but no IL-6 and IL-12 (Figure 4.20B,C). Interestingly, Poly(I:C) treated EG-7 MPs provided NO, IL-6 and IL-12 secretion which is similar to the free Poly(I:C). Contrarily, Poly(I:C) treated RAW and CAL-1 MPs did not cause any immune activation on RAW cells. Of note, untreated EG-7 MPs were capable of NO production but it did not induce either IL-6 or IL-12 secretion. Our results suggests that LPS can bind to the macrophage, pDC and T-cell derived microparticles even after 30 mins of incubation and LPS bearing MPs provide an immune activation on cells that did not have exposed to LPS. Another important observation is that T-cell derived MPs have the ability to specifically present dsRNA to macrophages and trigger their synergistic activation, since in our hands only poly(I:C) treated EG-7 MPs stimulated NO, IL-6 and IL-12 production from RAW cells (Figure 4.20A-C).

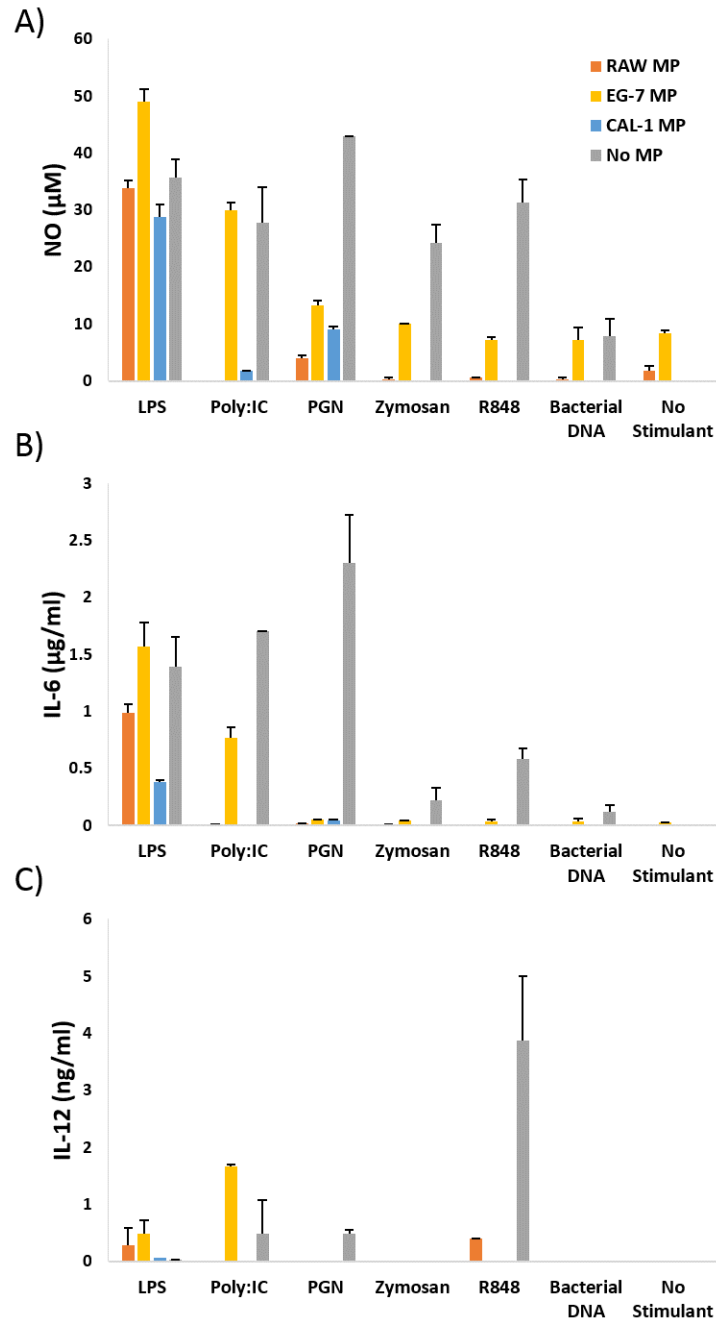


Figure 4.20: Immunostimulatory properties of MPs incubated with TLR ligands. RAW, EG-7 and CAL-1 derived MPs were incubated with TLR ligands for 30 min at 37°C . Different ligand associated 10^6 MPs were administered onto fresh RAW cells (2×10^5 cells in μl media). After 24 hours of incubation supernatant were collected for A) Griess Assay, B) IL-6 and C) IL-12 ELISA. MPs were incubated in $5 \mu\text{g/ml}$ LPS, PGN, Zymosan, R848, Bacterial DNA and $100 \mu\text{g/ml}$ Poly(I:C). Stimulation concentrations: LPS($0.2 \mu\text{g/ml}$), Poly(I:C)($20 \mu\text{g/ml}$), PGN($1 \mu\text{g/ml}$), Zymosan($1 \mu\text{g/ml}$), R848 ($1 \mu\text{g/ml}$), DNA ($1 \mu\text{g/ml}$).

Ligand treated MPs showed much different responses when incubated with spleen cells *in vitro*. Only R848 treated MPs stimulated both IL-6 and IL-12 production where zymosan treated MPs activated IL-12 secretion (Figure 4.21). Although bacterial DNA itself induced IL-6 and IL-12 production, MPs treated with DNA did not cause any activation. Spleen cells are consists of various types of immune cells and PGN, Poly(I:C), LPS did not stimulate the expression of inflammatory cytokines at detectable levels.

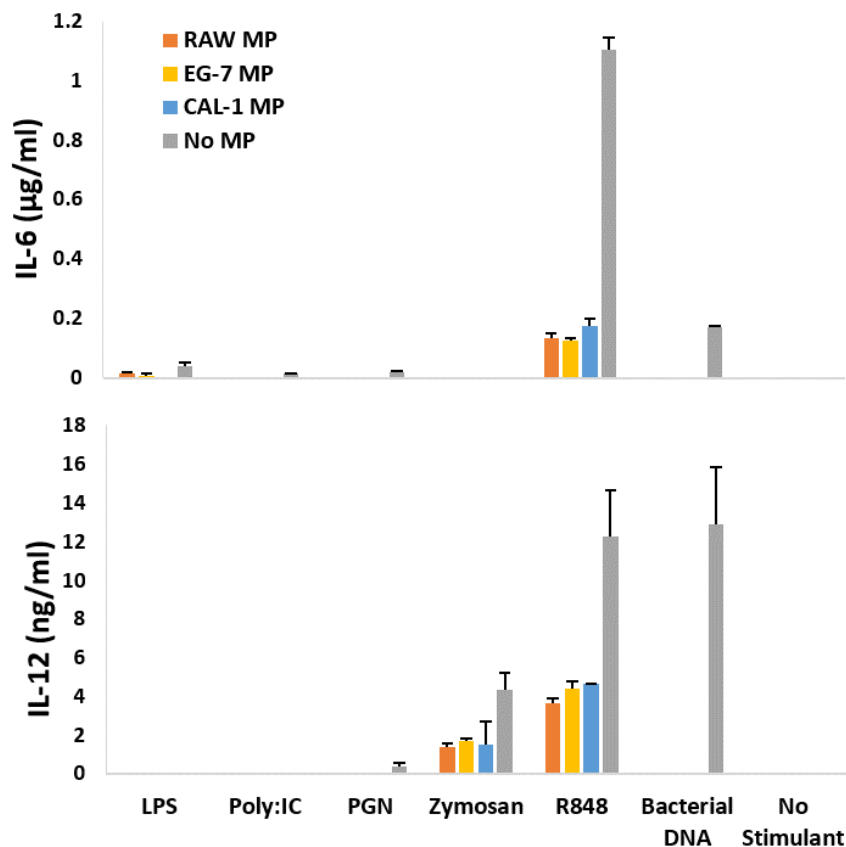


Figure 4.21: Immunostimulatory properties of MPs incubated with TLR ligands. RAW, EG-7 and CAL-1 derived MPs were incubated with TLR ligands for 30 min at 37°C. Different ligand associated 10⁶ million MP were administered onto fresh spleen cells (2x10⁵ spleen cells in 200 µl). After 24 hours of incubation supernatant were collected for A) IL-6 and B) IL-12 ELISA. MPs were incubated in 5 µg/ml LPS, PGN, Zymosan, R848, Bacterial DNA and 100 µg/ml Poly(I:C). Stimulation concentrations: LPS(1 µg/ml), Poly(I:C)(20 µg/ml), PGN(1 µg/ml), Zymosan(1 µg/ml), R848 (1 µg/ml), DNA (1 µg/ml).

When taken together (Figure 4.20 and Figure 4.21) our results suggest that circulating MPs secreted from different cells, if they are exposed to freely circulating microbial products even for a brief period are capable of adhering these ligands on their surface and could aid transmitting these ligands to other distant cells. These MPs can interact and can be internalized by different immune cells albeit at different level (or kinetics) leading to differential and synergistic immune activation. Our findings point out to a phenomenon that MPs could contribute to the amplification or dissemination of the infection and could contribute to the severity of ongoing infection *in vivo*. Of note, the possibility of differential MP internalization by naive immune cells must be separately investigated in depth in the future.

4.3.2 Effects of EVs on human immune system cells

Lastly, in this thesis the attribution of MP mediated pronounced immune activation was tested on human PBMCs. MPs derived from MEG-01 (megakaryoblast), Jurkat (T-cell) and CAL-1 (pDC) cell lines were incubated with TLR ligands and washed to get rid of unattached ligands. Notably, all TLR ligand treated MPS from different cell courses led to IL-6 and TNF- α secretion from PBMCs (Figure 4.22). A similar trend was seen for IFN- γ secretion via ligand associated MPs, except for R848-associated MPs. A much lower interferon secretion was yielded by MPs compared to free R848. None of the MPs led to any IFN α secretion. This is probably due to inability of the pDCs to internalize MPs. Consistent with mouse data this experiment also support the finding that MPs differentially regulate immune activation when they are associated with ligands of microbial world.

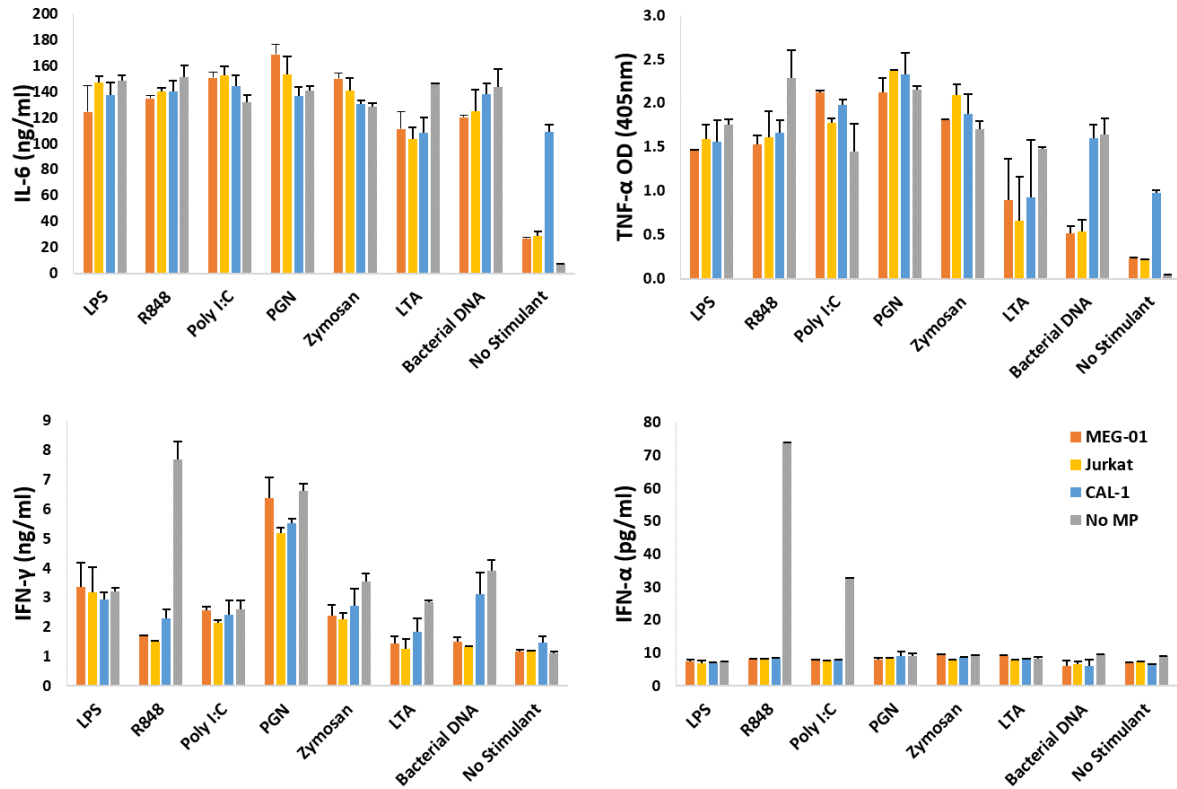


Figure 4.22: MEG-01, Jurkat, CAL-1 MPs stimulate immune activation on PBMCs after incubation with TLR ligands. 10^6 MPs were first incubated with TLR ligands for 30 min at 37°C . Then MPs were washed and administered to human PBMC cells (2×10^5 cells in $200 \mu\text{l}$ media). Supernatants from 24h culture were collected for IL-6, TNF- α , IFN- γ and IFN- α cytokine ELISA. MPs were incubated in $10 \mu\text{g/ml}$ LPS, PGN, Zymosan, R848, LTA, Bacterial DNA and $100 \mu\text{g/ml}$ Poly(I:C). Stimulation concentrations: LPS($1 \mu\text{g/ml}$), Poly(I:C)($20 \mu\text{g/ml}$), PGN($1 \mu\text{g/ml}$), Zymosan($1 \mu\text{g/ml}$), R848 ($1 \mu\text{g/ml}$), LTA($1 \mu\text{g/ml}$), DNA ($1 \mu\text{g/ml}$).

4.4 Studies with A151 ODN loaded EVs

As mentioned in the Introduction Section, A151 is a single stranded short ODN derived from mammalian telomeric repeats with a broad range of immuno- suppressive activities [86]. Although A151 is a very promising drug against auto-inflammatory or auto-immune diseases, upon *in vivo* administration it is prone to digestion and/or serum protein adsorption, hampering its *in vivo* performance due to limited bioavailability [269]. We postulated that, EVs could be utilized as a suitable nano-carrier to encapsulate A151 ODN thus, improve its *in vivo* stability and immunosuppressive performance upon injection. In order to determine whether EVs possess such potency as drug carriers, A151 was loaded into vesicles by one of our Lab's patented strategy known as "the rehydration-dehydration technology" method. Then, EV internalization and uptake kinetics were determined. Also, various EVs loaded with A151 administered to mice spleen cells simultaneously with different immunostimulatory TLR ligands and immunosuppressive potency with respect to its free A151 counterpart were analyzed.

In the literature, there are various exosome and microparticle isolation and purification protocols with different advantages and disadvantages. In this thesis, ultracentrifugation coupled with filtration technique were performed (please see materials and Method, Section 3.2.7). In our method, size is the only distinguishing feature between different particles, and it permits us to sequentially separate microparticles from exosomes in a cell supernatant pool. Therefore, MPs smaller than 200nm can interfuse with exosomes. In order to validate that the isolated products are cell derived vesicles and are clean from any contaminating cell debris, flow cytometry based characterization experiments were performed. Measuring the protein content of EVs is a popular method for quantification. However, depending on the cell type and physiological state including stress, or following different treatments on the same cell may affect the protein content expressed on that same vesicles isolated at different times from the same cell source. Therefore, in our studies we aimed to determine number of the EVs as a quantification method. For this purpose, we selected flow cytometry method to detect, quantify and characterize different extracellular vesicles from cell supernatants.

4.4.1 CD81 Expression on EVs before and after reconstitution

The tetraspanin CD81 is a surface protein which is expressed in most cell types. Any cellular vesicles which directly shed from the surface of cells should express CD81 on their surfaces. However, exosomes are secreted from multivesicular bodies to extra-cellular space and they are not expected to be positive for CD81 on their surface. Phosphatidylserine (PS) is a phospholipid component which is usually found on the cytosolic side of cell membranes. An enzyme called flippase keep the PS on the inner-leaflet but when cell dies or goes to apoptosis, PS becomes exposed on the surface of the cell. Because there is no flippase action in EVs they present PS on their surface and can be detected by Annexin V staining.

EVs isolated from mouse macrophage cell line (RAW 264.7) were stained with CD81-Ab and Annexin V (Figure 4.23). A low CD81 positivity was observed in exosomes as compared to the MPs whereas Annexin V positivity was high in all EVs, as expected.

It was unclear to distinguish whether A151 was loaded into vesicles or they adhered to the EV surface. As a control, lyophilized and then reconstituted EVs without A151 loading were also used. Reconstitution did not alter the CD81 expression on EVs, however, A151 loading caused increased CD81 and Annexin V positivity in exosomes and Apop. Blebs. We repeated the same experiment by including an extra group, exosome-like particles derived from apoptotic RAW cells (A. Exo). Normally, when cells undergo apoptosis they stop producing exosomes [270] but the pellets centrifuged at 100.000 g were collected and added to the experiment. While A151 loading did not effect CD81-Ab signal in exosomes, it did slightly in apoptotic exosomes and MPs (Figure 4.24). Unexpectedly, when A. blebs were loaded with A151, it significantly increased the CD81-Ab signaling. When we compare the CD81 expression densities on the vesicles, A151 loaded Apoptotic blebs were the highest expressing ones among others (Figure 4.25).

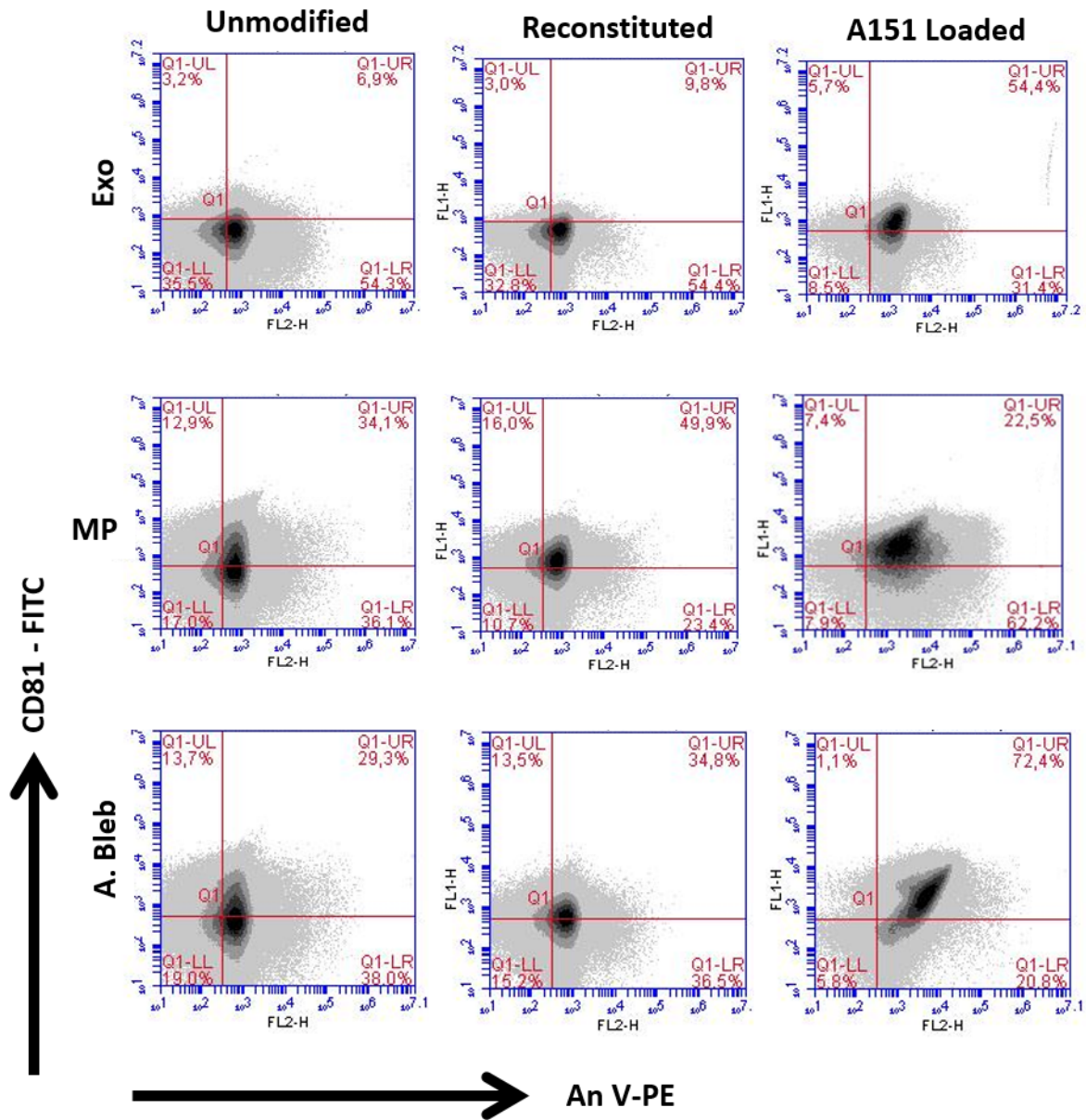


Figure 4.23: surface Characterization of Different RAW derived vesicles were labeled with CD81-PE Ab and Annexin-V-FITC. There are three samples from each vesicle type; unmodified, reconstituted and A151 loaded. Vesicles were analyzed with flow cytometer.

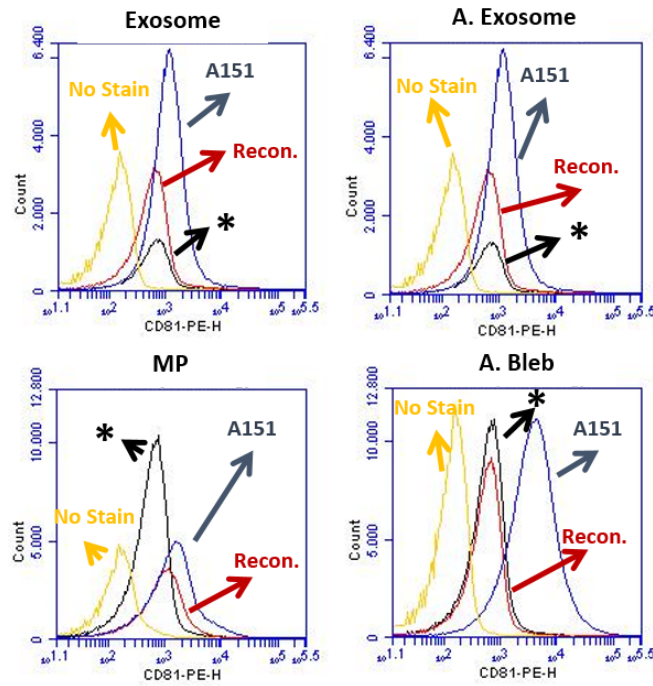


Figure 4.24: Effect of lyophilization on CD81 surface protein presentation of EVs. Different RAW derived vesicles were labeled with CD81-PE. There are three samples from each vesicle type; *: unmodified, Recons: reconstituted, A151: A151 loaded.

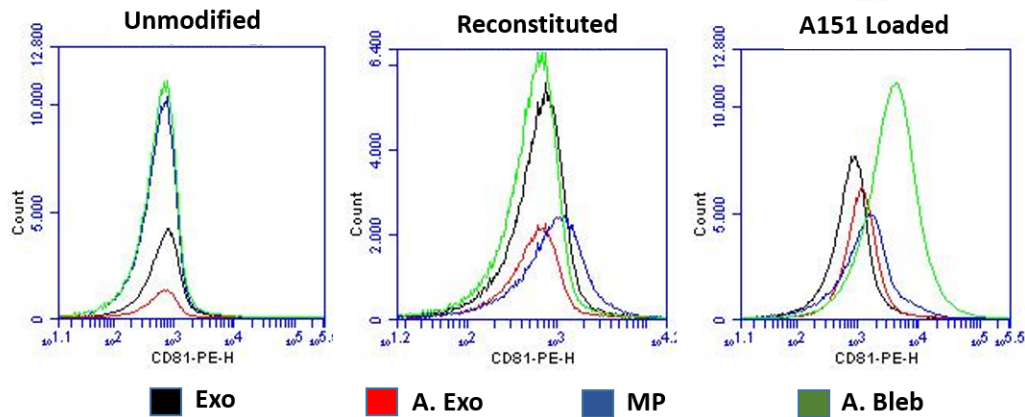


Figure 4.25: Analysis of CD81 presentation among various RAW derived vesicles. Microparticles and exosomes acquired from normal and apoptotic RAW cells were labeled with CD81-PE Ab and analyzed by flow cytometry. A: Apoptotic vesicles.

4.4.2 Nucleic Acid detection from EVs either unloaded or loaded with A151 ODN

A151 loading to the MPs and A.Blebs greatly increased the nucleic acid content (Figure 4.26). Acridine Orange (AO) is a cell membrane permeable dye which stains

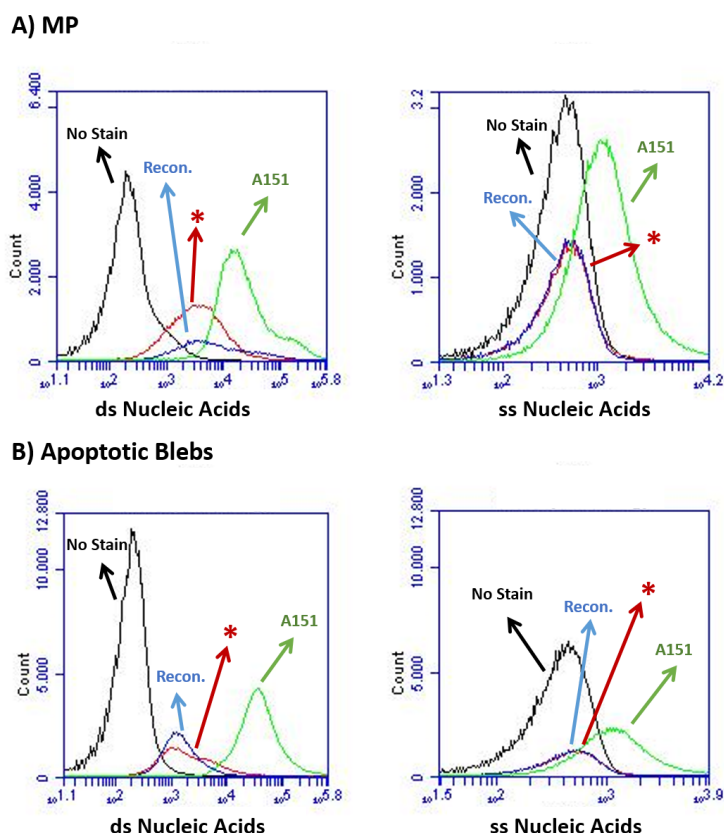
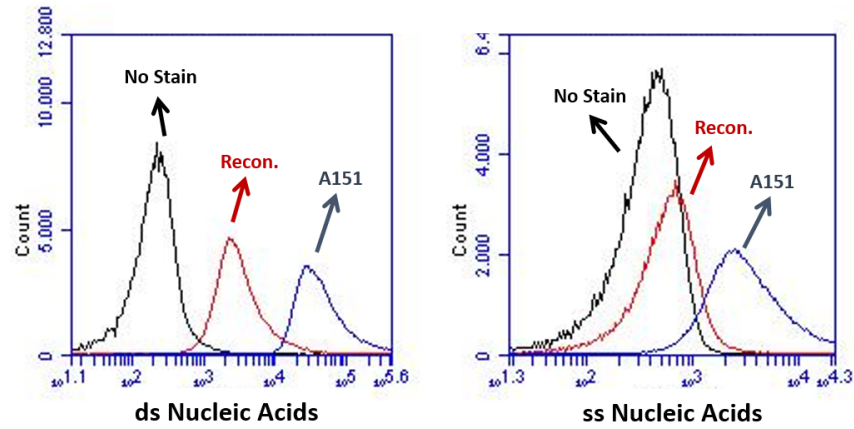


Figure 4.26: Nucleic acid content of MPs and A.Blebs derived from RAW cell lines. Unmodified, reconstituted with and without A151 vesicles were stained with AO dye. AO stains single and double stranded nucleic acids differentially. Analysis were made by Accuri C6. *: unmodified, Recons: reconstituted, A151: A151 loaded.

single and double stranded nucleic acids distinctly; while green signal indicates presence of either ds DNA or dsRNA orange signal indicates either ss DNA or ssRNA. Because A151 can accommodate complex G-tetrad structure, AO stains as if it is a double stranded structure. There is slight increase in ss nucleic acid signal probably because of the tattered A151 strands. Loading of A151 to the exosomes also demonstrated a similar increase in nucleic

acid content (Figure 4.27).

A) Exosome



B) Apoptotic Exosome

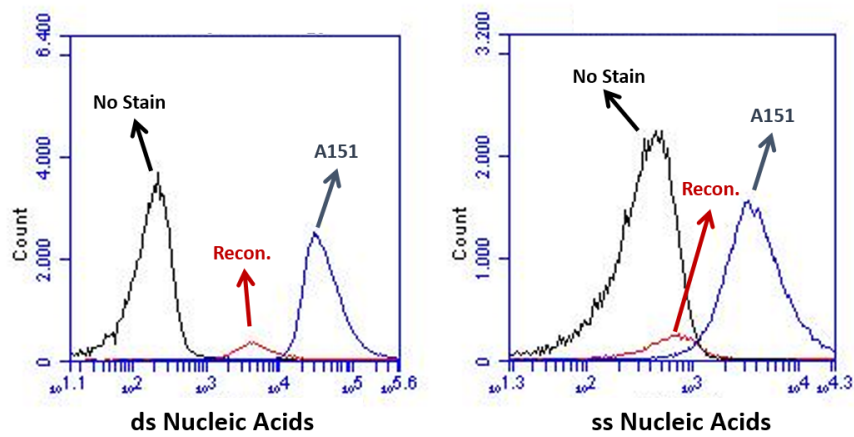


Figure 4.27: Nucleic acid content of exosomes derived from naive and apoptotic RAW cell lines. Unmodified or reconstituted with and without A151 vesicles were stained with AO dye. Recons: reconstituted, A151: A151 loaded.

4.4.3 Immunosuppressive effects of EVs associated with A151

A151 is a promising immunosuppressive agent. After observing that A151 can be loaded/associated within EVs, we planned to test the immunosuppressive effect of A151 loaded EVs on immune cells that were simultaneously stimulated with different TLR ligands. In suppression experiments 5 μ g of A151 added per one million vesicles for lyophilization and subsequent reconstitution. Data revealed that when A151 was loaded to EVs its suppressive capacity greatly augmented against either a TLR9 ligand, K23 CpG ODN (Figure 4.28) or a TLR7 ligand, R848 (Figure 4.29) mediated immune activation. As mentioned earlier, different EVs (i.e. Exosomes, MPs, Apoptotic Exosomes, Apoptotic Blebs) were loaded with A151 ODN via Rehydration-Dehydration Technology (please see Section 3.2.13 for more details) and incubated with mouse spleen cells (from C57/BL6) that were simultaneously either treated with 0.3 uM K23 CpG ODN (Figure 4.28), or 1 μ g/ml R848 ligand (Figure 4.29). Our *in vitro* suppression assay results suggested that upon K23 CpG ODN stimulation when low dose free A151 (3 μ g/ml) was added to the culture medium, it could only suppressed $\approx$ 50% of IL-6 production from splenocytes. Supporting our hypothesis, MP or apoptotic exosome-loaded with A151 counterparts led to 85% suppression of IL-6 production (please see Fig 4.28 top panel, left side). At low dose suppression of IL-12 production did not show any difference with respect to free or EV associated A151 (please see Fig 4.28 bottom panel, left side). At high doses of A151, however, all of the EV-associated A151 groups successfully blocked both IL-12 secretion, while its free counterpart could only induced 50-60% suppression (please see top and bottom right hand side panels of Fig 4.28).

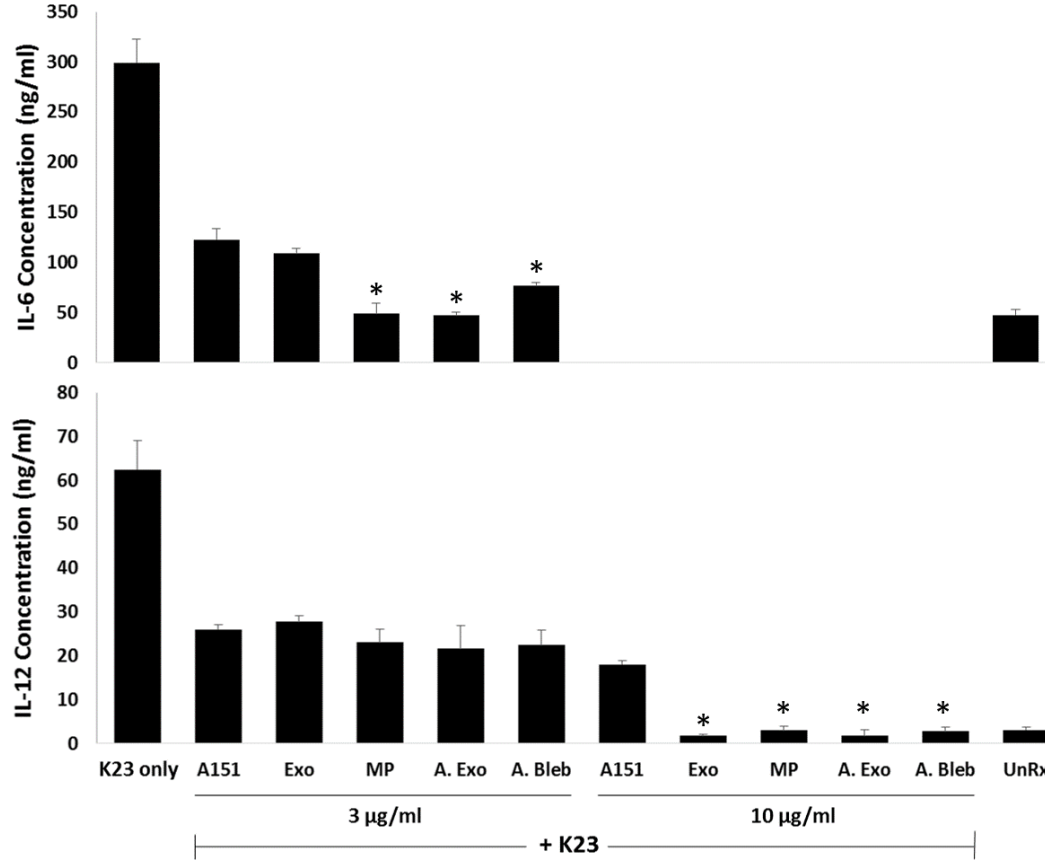


Figure 4.28: IL-6 and IL-12 secretion was suppressed by A151 loaded EVs. K23 stimulated spleen cells were co-incubated with free A151 or EV-A151 formulations for 24 h, and culture supernatants were collected and assayed by ELISA. Two concentrations of A151 were used; 3 and 10 $\mu\text{g/ml}$. K23 concentration is 0.3 $\mu\text{M/ml}$. Statistical analysis was performed using a one-way ANOVA test for multiple comparisons. $*P < 0.001$

Figure 4.29 summarizes the suppression profile upon R848 stimulation of spleen cells. EV encapsulated A151 was very effective against immune activation mediated by TLR7 ligand. While free A151 (at either doses) failed to suppress any IL-6 or IL-12 production, EV loaded A151 at 3 $\mu\text{g/ml}$ dose (left panels) was $\approx 50\%$ suppressive and at 10 $\mu\text{g/ml}$ dose (right panels), it completely shut off both IL-6 and IL-12 production (please check top and bottom panels of Fig 4.29).

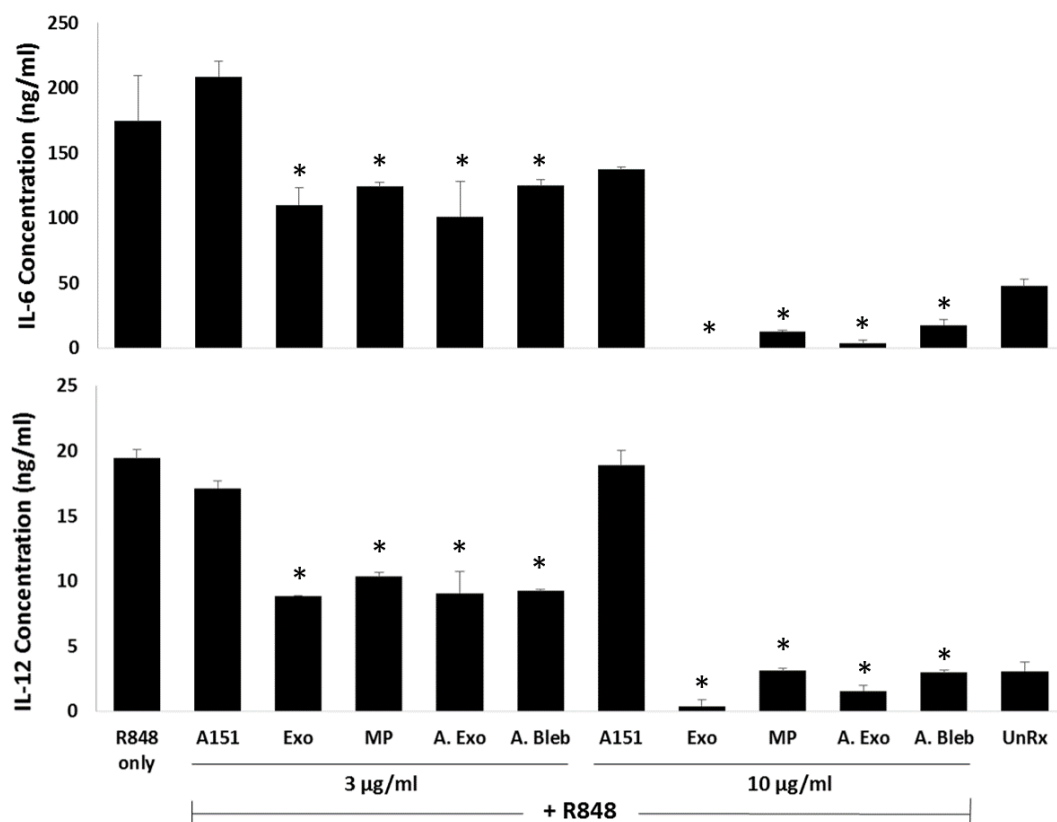


Figure 4.29: IL-6 and IL-12 secretion was suppressed by A151 loaded EVs. R848 stimulated spleen cells were co-incubated with free A151 or EV-A151 formulations for 24 h, and culture supernatants were collected and assayed by ELISA. Two concentrations of A151 were used; 3 and 10 $\mu\text{g/ml}$. R848 concentration is 1 $\mu\text{g/ml}$. Statistical analysis was performed using a one-way ANOVA test for multiple comparisons. * $P < 0.001$

To differentiate whether the immunosuppressive effect was solely regulated by associated A151 or empty EVs also contribute to the suppressive action, we designed an additional control experiment. Similar to above experiments mentioned in Figures 4.28 and 4.29, we incubated different empty EVs with spleen cells that was stimulated either with K23 (Figure 4.30) or with R848 (Figure 4.31). In these settings, EVs were devoid of any A151 ODN. As clearly seen in Figure 4.30, at low doses EVs did not show any appreciable suppression of IL-6 that was triggered by K23 CpG ODN. But significant suppression of IL-12 was mediated by these EVs (Bottom panel of Fig 4.30). As for R848 stimulated cells with empty EVs, we have not seen any EV mediated immune suppression of IL-6, but 50% IL-12 suppression was mediated by empty EVs irrespective of the dose used for suppression (Figure 4.31).

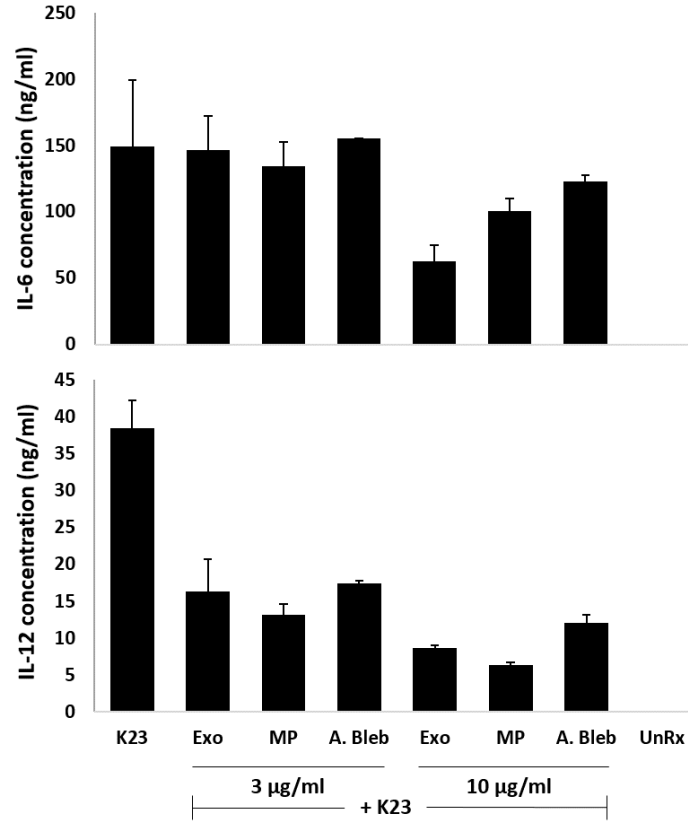


Figure 4.30: IL-6 and IL-12 secretion by mouse splenocytes treated with K23 and EVs simultaneously for 24 h. supernatants were collected and assayed ELISA. Vesicle concentrations were adjusted to 6.5×10^5 and 2×10^6 /ml, equivalent to the amounts used in Figures 4.28 and 4.29. K3 concentration was $0.3 \mu\text{M}/\text{ml}$.

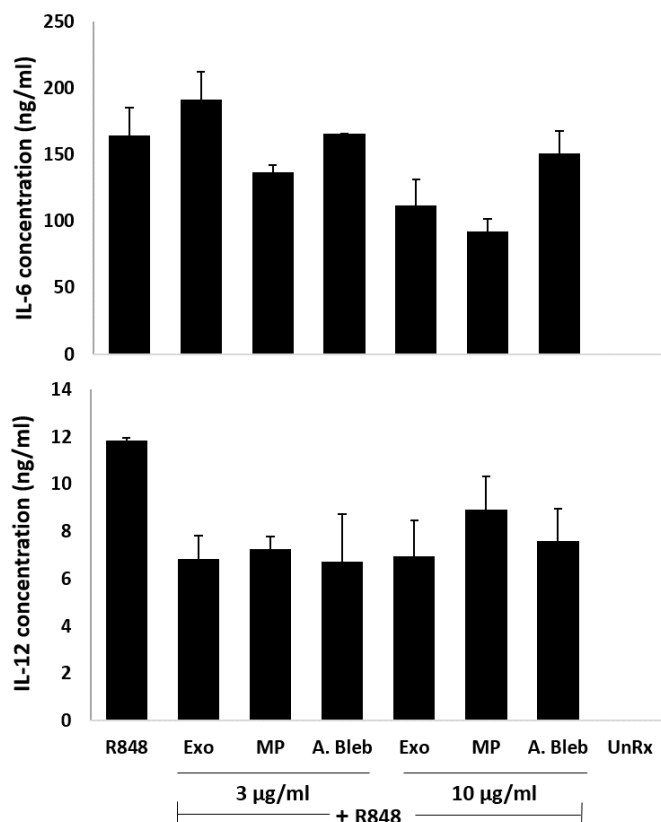


Figure 4.31: IL-6 and IL-12 secretion by mouse splenocytes treated with R848 EVs simultaneously for 24 h. 24 hours after the treatments, cell culture supernatant were removed for cytokine ELISA. Vesicle concentrations were adjusted to 6.5×10^5 and 2×10^6 /ml, equivalent to the amounts used in Figures 4.28 and 4.29. R848 concentration $1 \mu\text{g/ml}$.

Collectively, this data implicated that suppression of either TLR9 or TLR7 ligand mediated IL-6 production was regulated mainly by EV associated A151, whereas, both EVs and A151 collectively contributed suppression of IL-12 production by immune cells.

Throughout these *in vitro* assays, EV derived immune activation was not assessed. To check this possibility, spleen cells were treated with empty EVs for 24h. Secretion of IL6 and IL12 was analyzed by ELISA from culture supernatants. As expected there was no significant immune activation triggered by empty EVs (Figure 4.32).

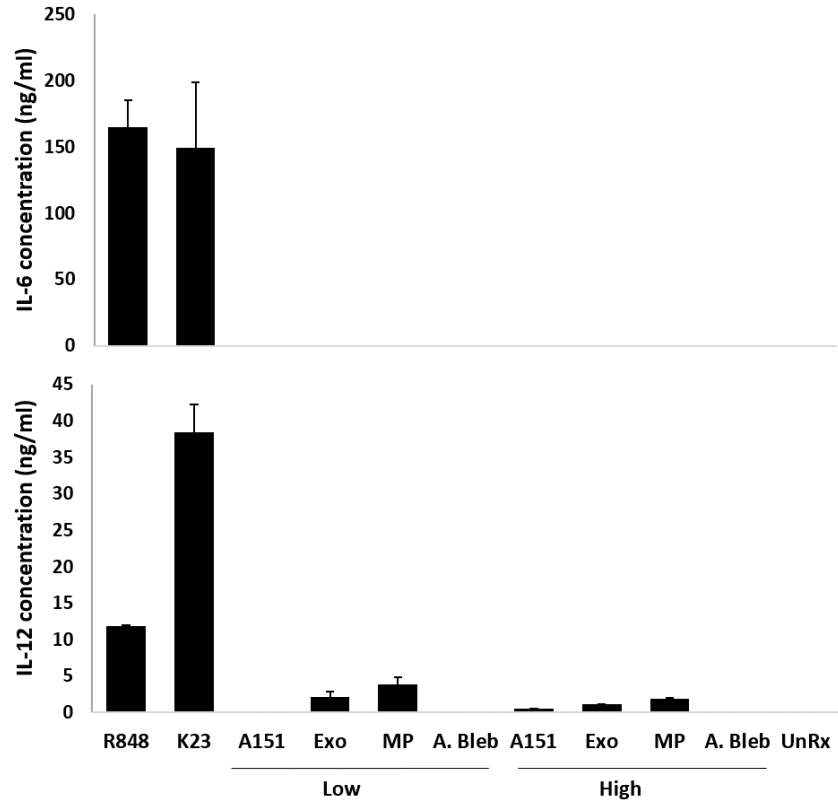


Figure 4.32: Immunostimulatory effect of RAW derived EVs on mouse spleen cells. All vesicles were administered without any ligand. After 24 hours, cell culture supernatant were removed for IL-6 and IL-12 cytokine ELISA. Two concentrations of A151 and vesicles were used; 3, 10 $\mu\text{g}/\text{ml}$ and 0.65, 2 ml/ml respectively. R848, K23 and A151 were administered as immunostimulatory and immunosuppressive controls.

Chapter 5

Conclusion

The major theme of this thesis was to characterize and understand the potential functions of extracellular vesicles (EVs) isolated from different mouse and human cell lines on immune cells. Coupled to this, we attempted to identify the immunomodulatory roles of these vesicles as suitable nanocarriers of TLR ligands that were liberated from infected cells. The *in vitro* and *in vivo* internalization kinetics and differential accumulation degrees of EVs by different immune cells were described. Based on this knowledge clues regarding to EV mediated distant transmission and propagation of ongoing infection were investigated. Lastly, in order to document that these secreted EVs could be harnessed as a suitable drug carriers, an immunosuppressive DNA motifs, namely, A151 ODN was externally loaded within EVs and their improved immunosuppressive potential against down-regulating an ongoing immune activation mediated by bacterial by-products was demonstrated in tissue culture settings.

The findings in this thesis revealed that different forms of EVs (exosomes, microparticles and their apoptotic forms) could be isolated and purified from cell culture supernatants at high yields. Moreover, their physiochemical characterization studies via DLS or AFM confirmed their vesicular nature and retention of their proper sizes even after reconstitution followed by lyophilization (i.e. size ranges were 100-150nm for exosomes, and 250-500nm for microparticles, respectively). Interaction of these EVs in culture with immune cells isolated from mouse spleens implicated that relevant target cells internalize and bind these secreted vesicles at a different rate and kinetics and both parent and target cell types are the determinative factors behind internalization and association of the cells. Kinetics of internalization and degree was dependent on Cell:MP ratio. Higher this ratio much rapid/faster internalization occurs. Our data suggested that in culture the MPs were mostly internalized by B-cells

where as T-cells did absorbed very few MPs. Also, each MPs type indicated different affinities to the target spleen cells. On one hand, NIH-3T3 derived MPs were the most absorbed one by B-cells and DCs but RAW MPs indicated the greatest affinity for macrophages, T-cells and DCs. On the other hand EG-7 MPs demonstrated the fewest affinity for all cells except B-cells. This differential internalization was critical to describe the contribution of vesicular cargo delivery to specific immune cells and subsequent alteration of the immune activation upon EV association.

Intaperitoneal(IP) injection of MPs demonstrated a completely different internalization and uptake profile. Injected MPs were mostly absorbed by peritoneal macrophages whereas peritoneal B-cells embodied very few MPs. Also, RAW MPs demonstrated significantly higher affinity for macrophages than other MPs. The distinct profiles of *in vivo* and *in vitro* internalization studies indicated distinct characteristics of same cell type on different locations, such as peritoneal and splenic B-cells. Although, the internalization studies were based on a few cell lines derived MPs and principal immune cells, we believe that our approach provided a wide perspective into the realm of EVs. Further internalization and uptake characteristics of MPs secreted from different cells may provide essential knowledge about the function of distinct MPs as well as their potential for therapeutic studies such as modified EVs and targeted drug delivery.

Stimulation experiments with MPs associated with TLR ligands indicated the affinity of cell derived MPs to TLR ligands. Also, when immunostimulatory activities of TLR ligand-associated MPs were compared with free TLR ligands we observed that MP association provides amplified immune responses. Lots of previous studies in field demonstrated that infected cells can secrete pathogenic by-products through MPs and subsequently propagate immune responses on other cell. However, our results demonstrated the potential role of MPs in amplification of immune responses through transportation and presentation of pathogenic molecules in extracellular milieu.

Loading of A151 to MPs significantly enhanced its immunosuppressive effect on TLR9, TLR7/9 triggered immune activations. Especially, when free A151 did not influence R848 based IL-6 and IL-12 secretions, MP-A151 completely inhibited proinflammatory cytokine secretion. Although, suppressive effect of MP-A151 has not been examined *in vivo* yet, our finding still indicates that MPs are potential delivery systems for therapeutic synthetic DNA motifs.

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Appendix A

Buffers, Solutions, Culture Media

Blocking Buffer (ELISA)

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

Mixture is stirred until fully homogenized. Stored in -20°C .

T-cell Buffer (ELISA)

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

Stirred until fully homogenized. Stored in -20°C .

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2.5 ml Tween20
- 4.5 lt ddH₂O

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8,01 grams Na₂HPO₄ · 2H₂O
- 2 grams KH₂PO₄
- 1 lt ddH₂O

Stirred until fully homogenized. pH is adjusted to 6.8. Filtered and autoclaved before use.

PBS-BSA-Na azide

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

Stirred until fully homogenized. Stored at +4 °C.

Annexin-V Binding Buffer [10x]

- 0.1 M HEPES
- 1.4 M NaCl

- 25 mM CaCl

Dissolved in ddH₂O, pH adjusted to 7.4 and diluted to 1x with ddH₂O before use. Stored at +4 °C.

RPMI-1640 (Lonza)

- 2 % : 10 ml FBS (FBS = inactivated at 55 °C.)
- 5 % : 25 ml FBS
- 10 % : 50 ml FBS
- 5 ml Penicillin/Streptomycin (50 g/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES (Biological Industries), (10 mM final concentration from 1M stock)
- 5 ml Na Pyruvate, (0,11 mg/ml final concentration from 100mM, 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)

Added into 500 ml medium. Stored at 4 °C.

Appendix B

Figures

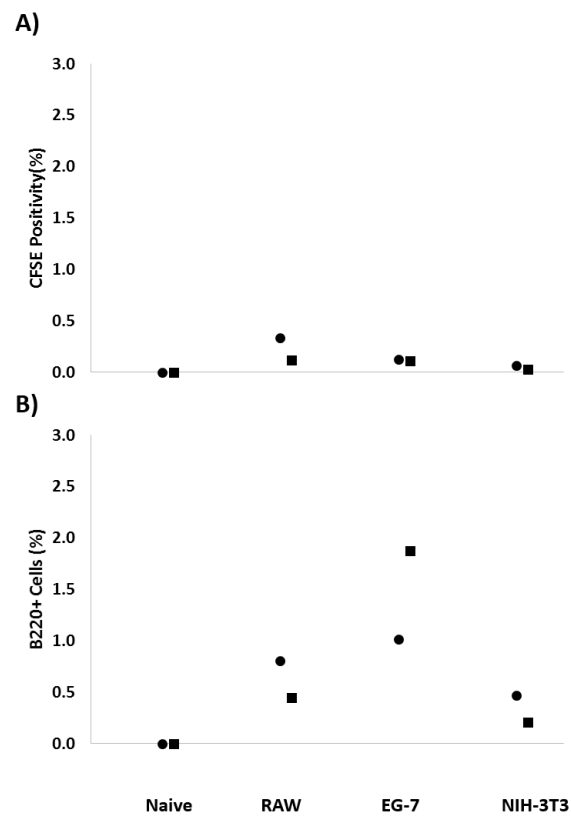


Figure B.1: MP uptake by B cells in PEC. A) CFSE positivity among gated B cells, B) B cell ratio in CFSE positive cells. For more detail see Figure 4.17

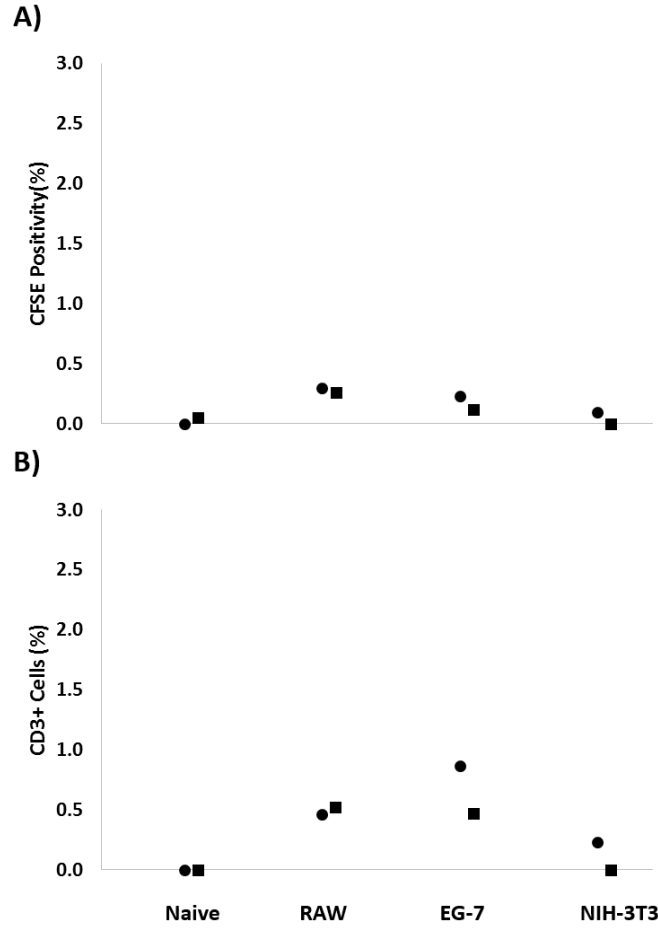


Figure B.2: MP uptake by T cells in PEC. A) CFSE positivity among gated T cells, B) T cell ratio in CFSE positive cells. For more detail see Figure 4.17

Table B.1: CD11b, CD11c, CD3, B220 cell population percentages in PEC. For more detail see Figure 4.17

	CD11b	CD11c	CD3	B220
Naive	59.9±0.9	2.0±0.6	11.1±2.2	22.5 ±0.2
RAW	56.5±5.4	1.5±0.3	16.7± 0.6	26.1 ±6.2
EG-7	57.2±0.8	1.1±0.0	11.0± 3.5	33.5 ±7.1
NIH-3T3	75.6±0.7	1.2±0.2	10.0± 0.4	16.0 ±1.0

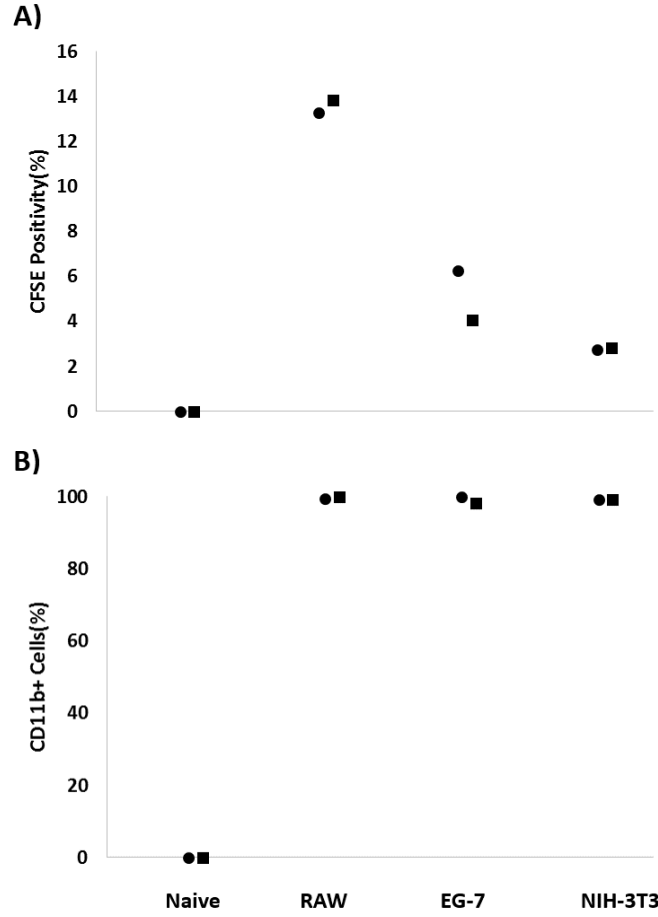


Figure B.3: MP uptake by macrophage cells in PEC. A) Percentage of macrophages which obtained CFSE-MPs, B) Macrophage ratio among CFSE positive cells. For more detail see Figure 4.17

Table B.2: CD11b, CD11c, CD3, B220 cell population percentages in spleen cells . For more detail see Figure 4.17

	CD11b	CD11c	CD3	B220
Naive	4.3 ± 0.2	3.1 ± 0.2	25.5 ± 2.1	63.1 ± 1.7
RAW	5.1 ± 0.9	2.4 ± 0.6	25.7 ± 1.9	68.4 ± 2.1
EG-7	4.9 ± 1.2	3.3 ± 0.4	23.2 ± 5.6	63.7 ± 3.7
NIH-3T3	4.5 ± 0.4	2.7 ± 0.1	24.6 ± 1.6	66 ± 1.8

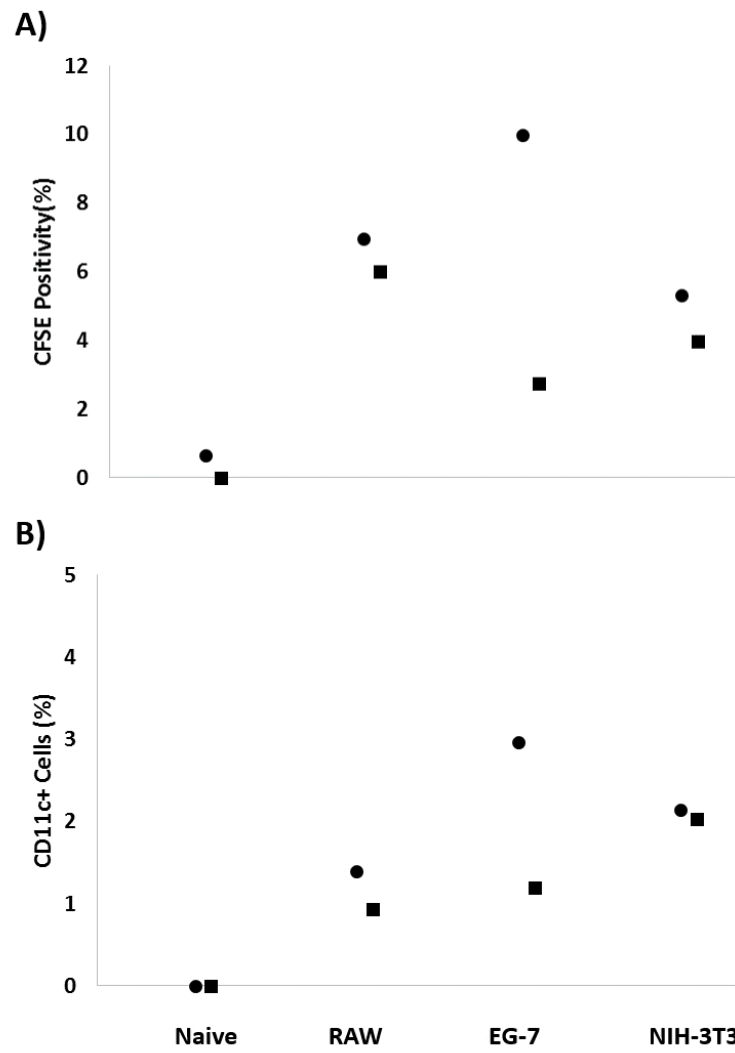


Figure B.4: Uptake of MPs by DCs in mice PEC. A) CFSE positivity in gated DCs, B) DC ratio among CFSE positive cells. For more detail see Figure 4.17

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