PLATELET-DERIVED MICROPARTICLES DIFFERENTIALLY REGULATE MACROPHAGE POLARIZATION

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By

Elif Senem Köksal

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We certify that we have read this thesis and that in our 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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September, 2016

Platelet-derived microparticles (PMPs) shed from platelets upon activation and constitute almost 90% of the circulating microparticles. Due to their versatile cargo, PMPs were associated with the generation of immunosuppressive microenvironment and and thought to promote tumor growth. They are also potential candidates for prevention and treatment of autoimmune diseases. Macrophages are one of the enigmatic cells of the immune system. They are either categorized as ‘M1-type’, mediating an inflammatory environment or ‘M2-type’, mediating an immune suppressive environment. Cardinal signals resulting M1-tropic or M2-tropic macrophage differentiation is not fully understood. However, it is crucial to understand the inducers of macrophage polarization for therapeutic approaches. We aimed to understand the interaction between PMPs with macrophages and wished to understand mechanistic alterations upon macrophages engage with PMPs.

In this thesis, we showed that activated human platelets released microparticles and they were internalized by macrophages differentiated from THP1 monocytic cell line. Internalized PMPs co-localized with late endosomes. The phagocytic capacity of M2-polarized THP1 macrophages were greater than M1-polarized macrophages. Strikingly, when THP1 derived macrophages were treated with standalone PMPs our results revealed that these macrophage were unable to mount any detectable cytokine secretion related to M1 or M2 type identity. This prompted us to encapsulate TLR agonists within PMPs and harness them as a carrier system. Different TLR ligands including TLR7 (sensing ssRNA) and TLR9 (sensing ss/ds DNA expressing CpG motifs) ligands were incorporated within PMPs via dehydration-rehydration method that was developed in our laboratory. Upon screening of several TLR agonist candidates on healthy donor PBMCs as well as on purified monocytes, we found that M1-like macrophage differentiation was TLR9 agonist D-type CpG oligodeoxynucleotide loaded PMP dependent whereas M2-like macrophage differentiation was dependent on TLR7 agonist R848 loaded PMPs.

In conclusion, this work implicated that PMP treatment of macrophages loaded with suitable ligand combinations might regulate M1/M2 type macrophage differentiation and could be used efficiently either to control tumor development (M1) or to alleviate symptoms of auto-immune/auto-inflammatory diseases (M2).

Keywords: Microparticles, platelets, macrophages, M1-M2 macrophage polarization

Özet

TROMBOSİT MİKROKESECİKLERİNİN M1- VE M2-BENZERİ MAKROFAJ KUTUPLAŞMASINA ETKİLERİ

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Trombosit microkesecikleri (PMP), trombositlerin aktifleşmesiyle salgılanırlar ve kan dolaşımındaki mikrokeseciklerin neredeyse %90'ını oluştururlar. PMPler kargolarından dolayı bağışıklık sistemini bastıran mikro ortamların üretimi ve tümör büyümesi ile ilişkilendirilmiştir. Ancak PMPler oto-bağışıklık hastalıklarının önlenmesi ve tedavisi için potansiyel adaylar arasında bulunmaktadır. Makrofajlar bağışıklık sisteminin anlaşılmaz hücrelerinden biridir. Makrofajlar M1 pro-inflamatuar ve M2 immunsupresif olarak kategorize edilmiştir. M1-M2 makrofaj kutuplaşmasına neden olan sinyaller tamamen anlaşılmış değildir. Ancak tedavi amaçlı makrofaj kutuplaşmasını indükleyen uyarıları anlamak önemlidir. Biz bu çalışmada makrofajlar ile PMPlerin arasındaki etkileşimi anlamayı amaçladık. Ayrıca PMPlerin temas ettikleri makrofajlardaki değişimleri anlamak istedik.

Bu tez ile aktif insan trombositlerinden salınan mikrokeseciklerin THP1 monositik hücre hattından üretilmiş makrofajlar ile etkileşime girdiklerini gösterdik.

Makrofajlar tarafından hücre içine alınan PMPler, geç endozoma yerleştiler. M2 makrofajına kutuplaştırılmış olan THP1 hücreleri daha gelişmiş fagositik aktivite göstererek daha fazla PMPyi hücre içine aldı. Ayrıca, yaptığımız fonksiyonel çalışmalar PMPlerin pro-inflamatuar M1 ve immunsupresif M2 makrofajlarına kutuplaşmasına veya herhangi bir immün yanıt oluşturmasına neden olmadığını ortaya çıkarmıştır. Bu nedenle, biz PMPleri taşıyıcı sistemler olarak kullanmaya karar verdik ve onları TLR (Toll-like receptor) agonistleri ile birlikte kullandık. TLR agonistlerinden ssRNA'ya bağlanan TLR7 ve CpG'ye sahip ss/dsRNA'ya bağlanan TLR9 agonistini PMP moleküllerinin içine yüklemek için laboratuvarımızda geliştirilen dehidrasyon-rehidrasyon yöntemini kullandık. Farklı sağlıklı donörlerden aldığımız periferik kan hücreleri ve monositleri üzerinde farklı TLR agonistlerini taradığımızda TLR9 agonisti D-tipi CpG oligonükleotit yüklü PMPlerin M1-benzeri ve TLR7-8 agonisti R848 yüklü PMPlerin M2-benzeri immün tepkisi gösterdikleri gözlendi.

Sonuç olarak bulgularımız, makrofajların uygun ligand kombinasyonları ile yüklü PMPler ile muamele edilmesi durumunda M1-M2 makrofaj kutuplaşmasının düzenlenebileceğini göstermiştir. Böylece M1 makrofajları ile tümör gelişimini kontrol edebilir ya da M2 makrofajları ile oto-immün/oto-inflamatuar hastalıkların belirtilerini hafifletebiliriz.

Anahtar sözcükler: Mikrokesecikler. , trombositler, makrofajlar, M1-M2 makrofaj kutuplaşması

To my precious family…

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Abbreviations

Ab	Antibody
ACD	Acid-Citrate-Dextrose buffer
ALR	AIM2-like receptors
AP1	Activator protein 1
APC	Antigen presenting cells
BCA	Bicinchoninic acid assay
bp	Base pair
BSA	C-type lectin receptors
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptors
CMAF	Transcription factor Maf
CpG ODN	CpG Oligodeoxynucleotide
CREB	CAMP responsive element binding protein
CSF1	Colony stimulating factor 1

CXCL	C-X-C motif chemokine ligand
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMS	Demarcation membrane system
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
EV	Extracellular vesicle
FBS	Fetal bovine serum
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIF-1	Hypoxia-inducible factor 1
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobin
IKK	IκB kinase

IL	Interleukin
IP10	Interferon gamma-induced protein 10
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
M1	M1-type macrophage
M2	M2-type macrophage
MACS	Magnetic-activated cell sorting
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
MEG-01	Human bone marrow megakaryoblast cell line
MHC	Major histocompatibility complex
MKK	Mitogen activator kinase
MP	Microparticle
MyD88	Myeloid differentiation primary response 88
NEAA	Non-essential amino acid
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor Kappa B
NK	Natural killer cell
NKT	Natural killer T cell

NLR	Nucleotide-binding oligomerization domain (Nod)-like receptors
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-Strep	Penicillin and streptomycin
PKC	Protein kinase C
PMA	Phorbol 12-myristate
PMP	Platelet-microparticles
PNPP	p-Nitrophenyl Phosphate
Poly (I:C)	Polyinosinic-polycytidylic acid
PPAR	Peroxisome proliferator-activated receptors
PRP	Platelet rich plasma
PRR	Pattern recognition receptors
PS	Phosphotidylserine
qPCR	Quantitative PCR
RIP1	Receptor interacting Serine/Threonine kinase 1
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SA-ALP	Streptavidin−Alkaline Phosphatase

SSC	Side scatter
STAT	Signal transducer and activator of transcription
TAE	Tris-Acetate-EDTA
TBK	TANK binding kinase 1
TCR	T-cell receptors
Th	T helper
THP1	Human peripheral blood monocyte cell line
TIR	Toll-interleukin-1 receptor
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptors
TNFa	Tumor necrosis factor
TPO	Thrombopoietin
TRAM	Translocation associated membrane protein
TRAP-6	Thrombin receptor activator for peptide 6
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
UV	Ultraviolet

Chapter 1

Introduction

1.1 The immune system

The immune system defends us against infections throughout our life with an interactive network of cells and molecules. These network recognizes, repels and eradicates pathogens (bacteria, viruses, fungi, and parasites) and wide variety of foreign molecules. The immune system is classified into two subsystems as adaptive and innate immunity. They differ from each other in terms of the speed and specificity of the reaction [1-2].

The innate immune system is the first line of host defense if pathogen passes the physical barriers of the body. It gives immediate but unspecific response with cellular, biochemical and physical defense mechanisms. The diverse cellular component of innate immune system includes granulocytes (basophils, eosinophils and neutrophils), mast cells, monocytes/macrophages, dendritic cells (DCs), γδ T cells and natural killer cells (NK cells) [3-5]. Innate immune response is triggered when pathogen-associated molecular patterns (PAMPs) are identified via pattern recognition receptors (PRR). Upon detection of PAMPs, inflammatory response is initiated, pathogen is killed with phagocytosis. Pro-inflammatory mediators are released and innate and adaptive immune cells are activated. Then the danger is resolved and homeostasis is restored [6].

The adaptive immune system is the second line of host defense. It gives antigen specific responses through B and T lymphocytes and cytokines and antibodies secreted from them. While T cells are providing cell-mediated immunity and recognize antigen specifically with T-cell receptors (TCRs) on them, B cells provide humoral immunity with secreted antibodies (Igs) and again recognize antigen specifically. Unlike innate immunity, adaptive immune response takes several days or weeks. Also, it has memory and this leads to rapid response in second exposure [6-7]. The adaptive immune response is awakened by innate immune system upon detection of pathogens. Antigen presenting cells (APCs) mostly DCs act as the link between them. It phagocytes, processes them by cutting them into small peptides and presents antigen to the lymphocytes on major histocompatibility complex (MHC) protein and provide required co-stimulatory signals [6, 8-9].

1.2 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) used by the innate immune system are germ-line encoded receptors that are specialized to recognize PAMPs or DAMPs and discriminate between self and non-self. They are expressed on the cell surface, in intracellular compartments like endosome, lysosome and cytoplasm or secreted into the circulation or tissue fluids. They opsonize, activate complement and coagulation cascade, phagocyte, activate pro-inflammatory signaling pathways and induce apoptosis [4]. Overall, they clear pathogens and restore homeostasis. Also, they can activate both innate and adaptive immunity [10]. PRRs are divided into several families; Toll-like receptors (TLR), AIM2-like receptors (ALR), RIG-I like receptors, nucleotide-binding oligomerization domain (Nod)-like receptors (NLR), C-type lectin receptors (CLR) and cytosolic DNA sensors. TLRs are the first and best characterized PRR family [11-13].

1.2.1 Toll-like Receptors

TLRs are named because they are similar to *Toll* and discovered in the fruit fly *Drosophila melanogaster*. They are type 1 transmembrane receptors with PAMP interacting leucine-rich ectodomain folded into β-sheets, transmembrane domain and cytoplasmic Toll-interleukin-1 receptor (TIR) domain which signals through adaptive molecules like MyD88 (Myeloid differentiation primary response 88) , TIRAP (Toll-

Interleukin 1 receptor (TIR) domain containing adaptor protein), TRIF (TIR-domain-containing adapter-inducing interferon-β) and TRAM (Translocation associated membrane protein). They all induce different immune response. They recognize PAMPs including lipids, lipoproteins, proteins and nucleic acids derived from bacteria, virus, fungi or parasites.13 mammalian TLRs have been identified. While humans have TLR1-10, mice have TLR1-13 except TLR10 [10-12, 14-16]

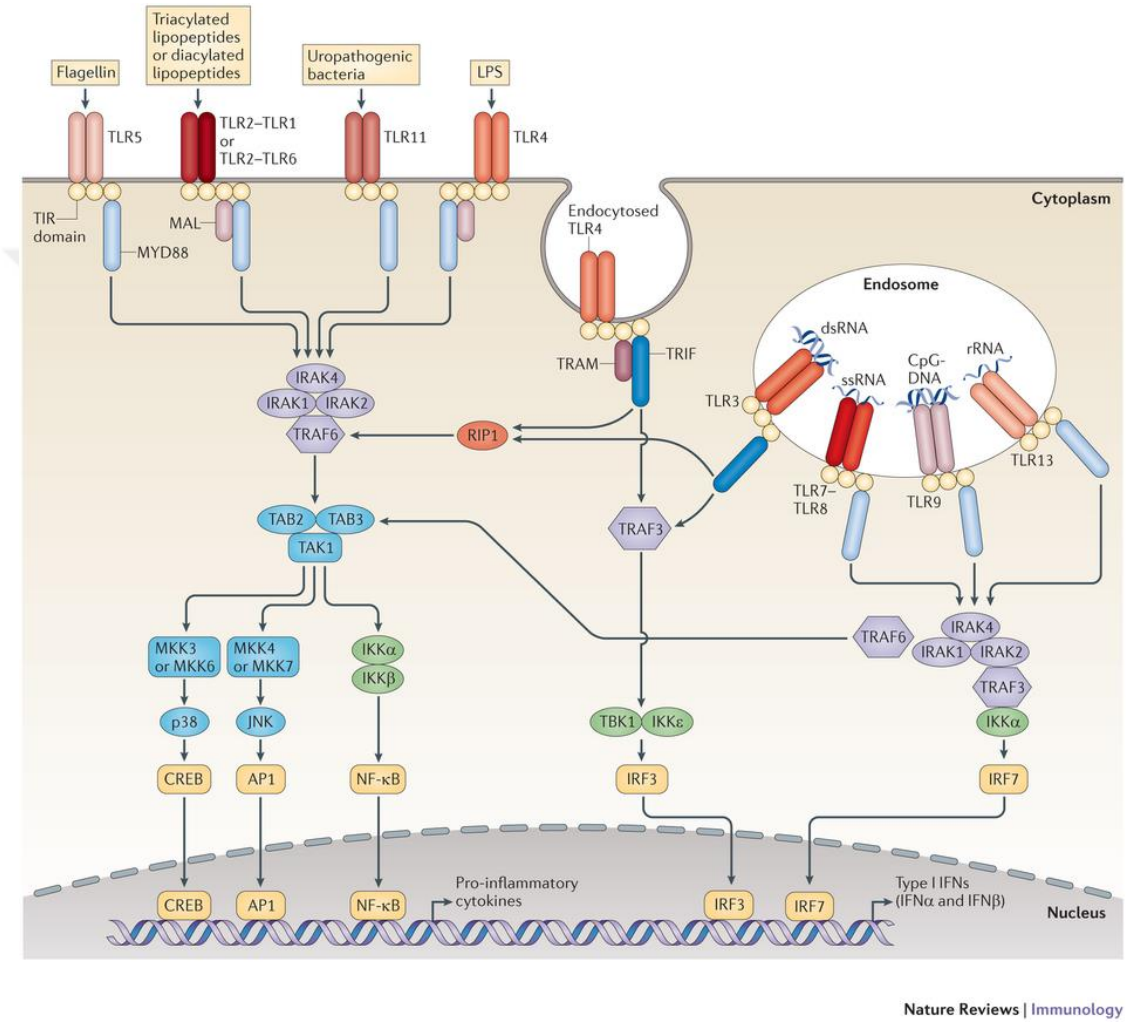

Figure 1.1: Cellular localization, PAMPs, adaptor molecules and signaling pathways of TLRs [20].

TLR1-2 and TLR2-6 form heterodimers and found in cell surface. Also, TLR5, TLR11 and TLR4 are found in the cell surface and recognize their respective ligands there. TLR7-8 heterodimer, TLR3, TLR9 and TLR13 are found and sense PAMPs in endosome. TLR4 which recognizes LPS is found both on cell surface and in

endosome. Following ligand recognition by leucine rich domain, TIR domain signals to adaptor proteins. MyD88 is used by all TLRs except TLR3 and leads to activation of NF-κB (Nuclear factor kappa B) and MAP (Mitogen activated protein) kinase and induces secretion of inflammatory cytokines. TLR3 and 4 use TRIF to activate another pathway which activates NF-κB and IRF3 (Interferon regulatory factor) and induces type I IFN (Interferon) and inflammatory cytokine productions. TLR4 recruits all four adaptors and activate both MyD88 and TRIF dependent pathways. [18-20]

1.3 Immunmodulatory synthetic DNA motif

DNA has complex effects on the immune system. TLR9 recognizes both immune stimulatory or suppressive synthetic DNA motifs which are promising agents for vaccine development and treatment of inflammatory conditions.

1.3.1 Immune stimulatory synthetic DNA motifs

Bacterial DNA contains immune stimulatory unmethylated CpG motifs which are rare in eukaryotic DNA. During infection, these CpG motifs are recognized by TLR9 and immune cascade is initiated. Triggered immune cells proliferate, mature and secrete wide variety of cytokines, chemokines and antibodies. This immune stimulatory activity of CpG motifs in bacteria is mimicked by synthetic CpG oligodeoxynucleotides (ODNs) [21-23]. These ODNs have unmethylated CpG dinucleotide flanked by two 5’ purines and two 3’ pyrimidines. They induce IL12 and type II interferon which promote Th1 dependent cytotoxic T cell response and IL6 which promotes B cell activation and antibody secretion. CpG motifs can boost antigen specific immune responses when co-administered with vaccines. Also they show promising results as anti-allergens, anticancer and immune-protective agents [24]. There are two mostly studied classes of CpG ODNs [25-28].

K-type ODNs encode multiple CpG motifs on a phosphorothioate backbone. They have longer in vivo half-life due to the backbone. They trigger plasmacytoid DCs to differentiate and produce TNFα, and B cells to proliferate and secrete IgM. Also, they induce monocytes to proliferate and secrete IL6 [25-28].

D-type ODNs encodes a mixed phosphodiester/phosphorothioate backbone containing a single CpG motif flanked by palindromic sequences and have poly G tails at the 3' and 5' ends. They trigger plasmacytoid DCs to mature and secrete IFN-α. They don't have any effect on B cells unlike K-type ODNs. They induce monocytes to differentiate into mature DCs. While K-type ODNs are fatly transported to the late endosome from early endosome, B-type ODNs stay longer in early endosome [25-28].

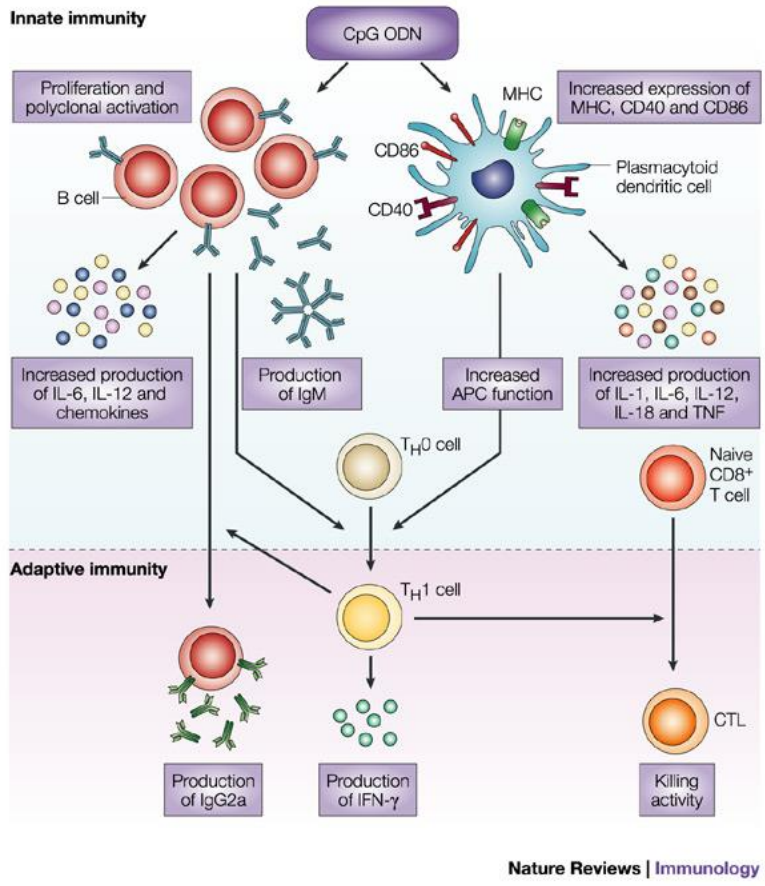

Figure 1.2: Mechanism of CpG ODNs to facilitate immune responses [28].

1.3.2 Immune suppressive synthetic DNA motifs

CpG induced immune activation can be damaging to tissues, enable to the development of autoimmune disease and increase sensitivity to immune shock if not appropriately regulated. Telomeres at the ends of the mammalian chromosomes have TTAGGG motifs which was shown to act as immune suppressant. Synthetic ODN

containing TTAGGG multimers inhibit activation and differentiation of macrophages, DCs, B cells and T cells and production of pro-inflammatory cytokines [29-31].
A151 is the best studied suppressive ODN. It has four TTAGGG motifs. It has been used for the treatment of autoimmune and infectious diseases, toxic shock, organ specific inflammation and others [32].

1.4 Monocytes & macrophages

1.4.1 Monocytes

Monocytes are originated from myeloid precursor in the bone marrow and fetal liver. They are released into the blood circulation to help tissue homeostasis and immunity. They are key players during inflammation and pathogen challenge. Their development completely depends on colony stimulating factor 1 (CSF1) which are secreted from stromal cells within the blood and tissues. Their half-life in the circulation is very short so they continuously migrate into tissues and form macrophages and DCs. [3, 33-34]

There are different subpopulations of circulatory monocytes. Human monocytes can be divided into classical ($CD14^{++}$ $CD16^{+}$), intermediate ($CD14^{++}$ $CD16^{+}$) and nonclassical ($CD14^{+}$ $CD16^{++}$) subsets [3, 35-36].

1.4.2 Macrophages

Macrophages which have roles in every process in organism's biology including development, homeostasis, tissue repair and immune response, are derived from monocytes. They take different names depending on their tissue localization like osteoclasts in bone, alveolar macrophages in lung, Kupffer cells in liver, Langerhans cells in skin, microglia in brain. They are the most plastic cells of the hematopoietic system and can adapt to the environmental signals very fast. Their differentiation from monocytes depends on growth factors GM-CSF (Granulocyte-macrophage colony-stimulating factor) or M-CSF (Macrophage colony-stimulating factor) initially. Then they are polarized to different macrophage subsets by priming with IFNγ or IL4 and IL13. Macrophages are classified according to their functions as M1 classically activated macrophages which are primed with IFNγ and M2 alternatively activated macrophages which are primed with IL4 and IL13 [37]. Although it is a

limited definition for complexity of macrophages, it is parallel to the T helper cell polarization as Th1 and Th2. Also, recently it has been shown that these M1-M2 macrophages are highly dynamic and can switch to each other upon received signal. This new phenotype is called as M3 switch phenotype [38].

THP1 human monocytic cell line can be used for macrophage differentiation studies. It differentiates into macrophages with PMA treatment. During this process, monocytes in suspension, adhere to the culture plates, stop proliferating and enhance their phagocytic potential. Also, they start producing pro-inflammatory signals [39-41].

1.4.2.1 M1 immune stimulatory macrophages

Macrophages polarize into classically activated M1 macrophages when they encounter a pathogen and sense it through PRRs. They generate inflammatory response. GM-CSF or IFNγ and LPS co-stimulation induces polarization of M1 macrophages. Upon activation of the TLR or other receptors' signaling pathway, STAT1 and NF-kB transcription factors are activated and pro-inflammatory mediators are produced. They secrete IL12 and IL23 highly but show low IL10 secretion. They efficiently produce reactive oxygen and nitrogen intermediates. It induces Th1 response by releasing pro-inflammatory molecules like IL1β, IL6, IL12, IFNγ and TNFα and co-stimulatory molecules CD86 and MHCII in order to present antigen to T cells [42-49].

In the presence of an infection or injury, monocytes are recruited to the site and differentiate into M1 macrophages in order to clear invading organisms. If these M1 macrophages cannot be stopped after elimination, they can cause worsening of the inflammatory condition or induce auto inflammatory disease such as inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis and type I diabetes mellitus. Also, continuous autoimmune disease can induce spontaneous cancers due to highly produced reactive oxygen intermediates [42-46].

1.4.2.2 M2 immune suppressive macrophages

Alternatively activated M2 macrophages are immune-modulatory and mediate Th2 type immune response. IL4 and IL13 produced from various innate immune cells

including mast cells, basophils, esosinophils, NKT cells and macrophages are the inducers of M2 polarization. Activation of STAT3 and STAT6 predominates M2 polarization. M2 macrophages secrete low IL12 and IL23 and high IL10 [42-46]. Since macrophage polarization studies are still under investigation and due to high plasticity, cell surface markers are controversial. Macrophage mannose receptor CD206 are the mostly used cell surface markers for M2 macrophages [50-51]. CD163 is a M2 marker when it is found in combination with CMAF transcription factor [47]. Comparing to M1 macrophages, M2 macrophages have higher phagocytic activity and this has been shown with nanoparticles [52].

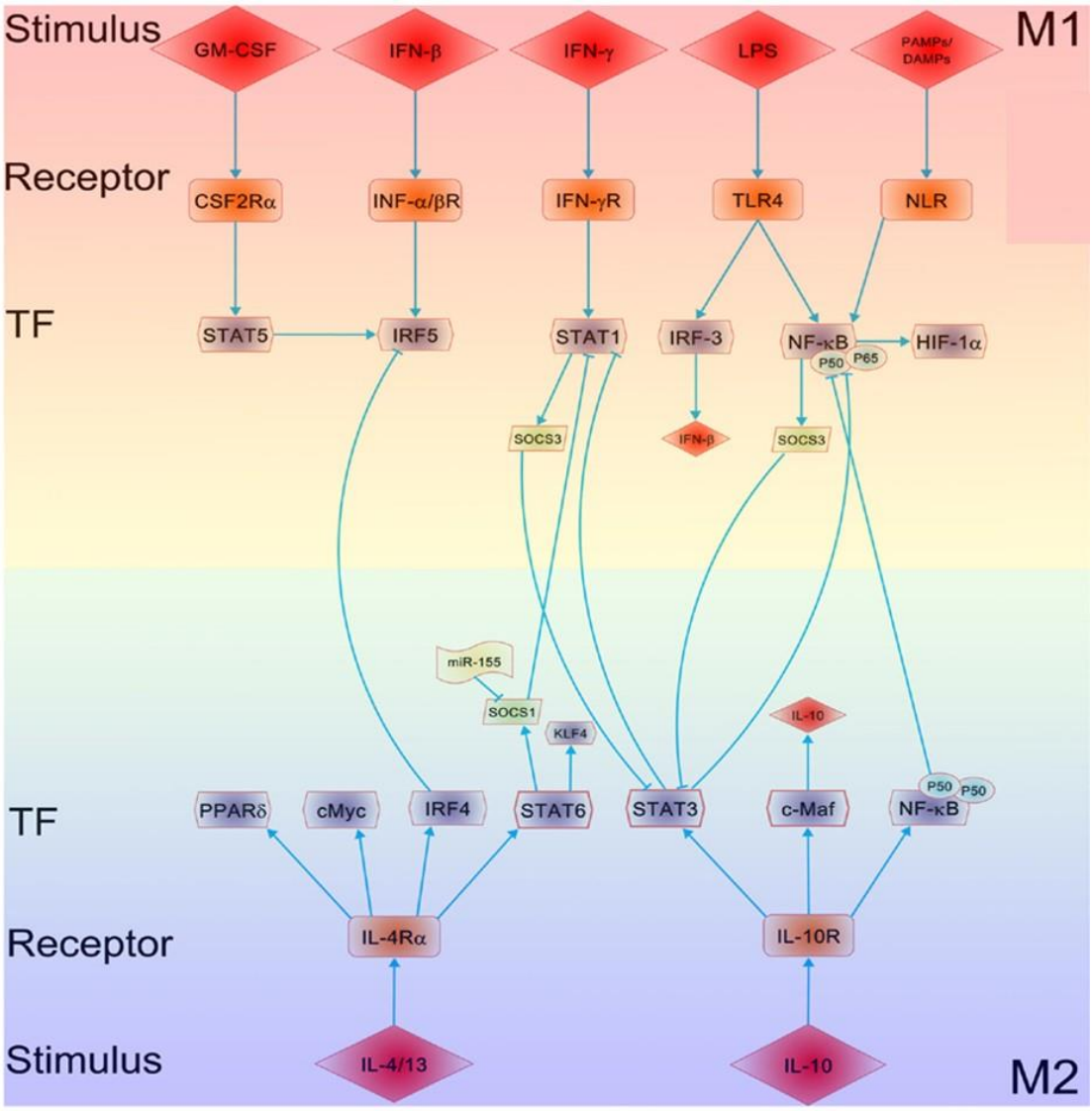

Figure 1.3: Mechanisms of macrophage polarization [49].

Peroxisome proliferator-activator receptor γ (PPARγ) and PPARδ is a ligand activated nuclear receptor and it is highly expressed in macrophages and platelets. Upon activation, it mediates macrophage polarization towards M2 [53-59]. Also, M2 inducers IL4 and IL13 activates PPARγ by producing its ligands like 13-(S)-hydroxyoctadecadienoic acid and 15-hydroxyeicosatetraenoic acid [54]. Moreover, PPARγ inhibits the production of inflammatory cytokines [55].

In case of cancer, macrophages contribute to the tumor initiation, progression and metastasis. However, after tumor is established, macrophages turn into tumor associated macrophages which are one of the subtypes of M2 immune suppressive macrophages. They contribute to the angiogenesis, invasion of local environment and metastasis to other parts of the body [41-45].

1.5 Megakaryocytes & platelets

1.5.1 Megakaryocytes

Megakaryocytes develop from hematopoietic stem cells that reside in the bone marrow. Commitment to megakaryocyte lineage is indicated by the expression of CD61 (integrin β3, GPIIIa) and CD41 (integrin αIIb, GPIIb). During megakaryopoiesis, cytokines like GM-CSF, IL3, IL6, IL11, IL12, thrombopoietin (TPO), transcription factors like GATA-1, RUNX1 and others play crucial roles. Megakaryocytes are very large cells with 50-100 um diameter. Also, they make endomitosis and amplify their DNA up to 128N because they have to express platelet proteins before platelet secretion. For all these functions, they increase their volume. [60-61].

1.5.2 Platelets

Platelets are the small, anucleated, 2-3 μm, discoid cells found in the blood circulation. They are secreted from megakaryocytes and found around 150,000-400,000 per μl of blood in a healthy adult. TPO is the primary cytokine which regulates the platelet release from megakaryocytes. Since their life span is short and about a week, they are constitutively produced by megakaryocytes in different sites including bone marrow, blood and lung. Although platelets are lack of nucleus, they are secreted with mRNAs and translational machinery which enable them to produce

their own protein. There are around 20 million proteins per platelet. Upon activation, smooth surface of platelets become spiny due to calcium influx. Storage granules including alpha-granules, dense granules and lysosomes secrete different molecules like cytokines, chemokines, coagulation factors which will mediate platelet function [62].

Thousands of platelets are released from megakaryocytes and there are several proposed mechanisms about their production. The first model suggests that platelets released by budding from the megakaryocyte membrane. Second model suggests that demarcation membrane system (DMS) leads to cytoplasmic fragmentation of megakaryocytes. These subdivided cytoplasm is enveloped by its own membrane and forms platelets. The third model suggests that platelets are released from formed proplatelets which are long and thin cytoplasmic processes emanating from megakaryocytes [63-65].

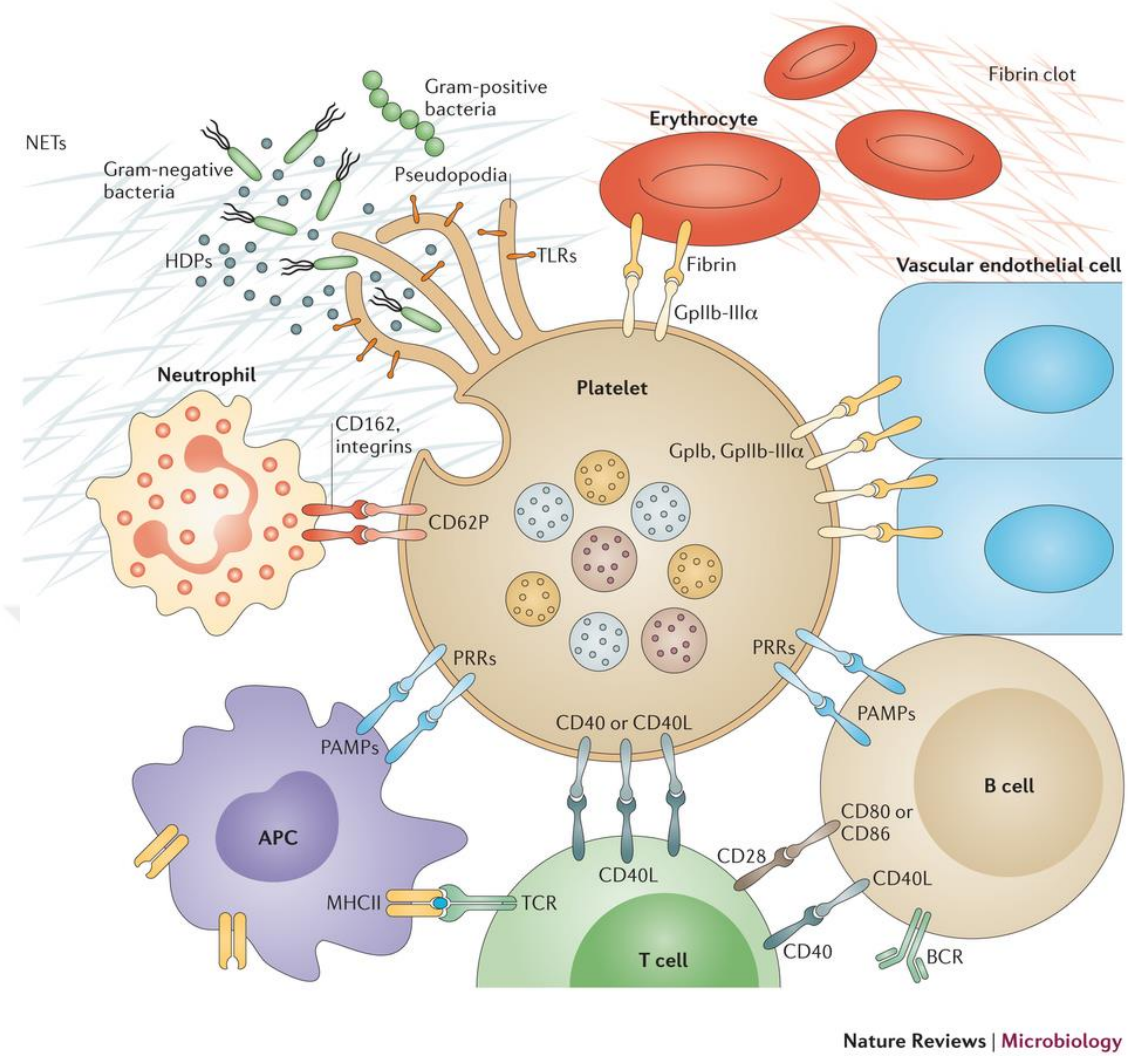

Figure 1.4: Functions of platelets [66].

Platelets have a role in various processes including hemostasis, wound repair, blood clotting and immune surveillance. They interact with different cells to function. For blood clotting in case of an injury, they sense and activated platelets accumulate at the site. With the glycoproteins on their surface they mediate fibrin clot formation [62]. In case of an infection, they again accumulate to the site and engage with pathogens with their PRRs and orchestrate immune cells. Platelets express TLR2, TLR3, TLR4, TLR7 and TLR9 [67-69]. They release host-defense peptides to call for immune cells including neutrophils, APCs, T and B cells to reestablish homeostasis. Platelets induce neutrophils for NET production. Also, it helps antigen processing by

APCs and with the help of CD40 or its ligand, it coordinates the T cell polarization and then the B cell activation for antibody production [68-69].

Human megakaryoblastic leukemia cell line MEG-01 when induced with PMA activating PKC (protein kinase C) or other agents can make megakaryopoiesis and show polyploidy, proplatelet formation and elevated expression of CD41, CD61. Also, they show reduced cell proliferation and enhanced cell adherence. Resulting megakaryocytes can secrete platelet-like particles [70-73].

1.6 Extracellular vesicles

Almost all cells release extracellular vesicles (EVs) upon activation. EVs are found in all human body fluids such as saliva, blood, urine and breast milk. Depending on their cellular origin, EVs express cell specific markers like CD14 in monocyte and CD41, CD42, CD61 in platelet EVs. They mediate intercellular communications [74-75].

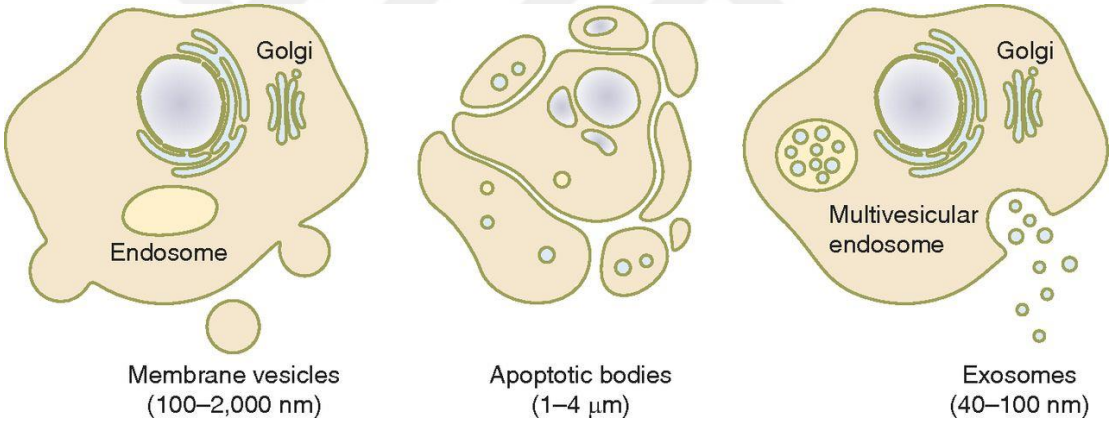

Figure 1.5: Different type of extracellular vesicles [76].

EVs are divided into three groups as exosomes, microparticles and apoptotic blebs. Microparticles are generated from cell membrane by budding and they are around 100-2000 nm in diameter. The release of MPs is triggered by the increased calcium ions in cells. Scramblase induces loss of cell membrane asymmetry and as a result phosphotidylserine (PS) on membrane is exposed with budding of microparticles. Exosomes are released by the fusion of multivesicular body containing exosomes with the cell membrane. Exosomes are the smallest EVs. While MPs can fully reflect the membrane composition of cellular origin, exosomes cannot. They both carry

nucleic acids, lipids and proteins as a cargo. Apoptotic bodies are formed from apoptotic cells by outward blebbing of membrane. They are bigger than MPs and they carry nuclear fractions and cell organelles [77-78].

EVs function in various processes mainly intercellular communication. Also, they play role in angiogenesis, coagulation, waste management, survival, inflammation and immune responses. Tumor-derived exosomes contribute to the survival of cancer cells by releasing the toxic drugs or by controlling the immune response. They inhibit DC production by monocytes, NK and T cell activities. Also, they induce Treg and MDSC development. But they could also transfer tumor antigen to DCs and activate T cells. EVs secreted from pathogen infected macrophages stimulate other immune cells to the site of infection by carrying pro-inflammatory message, while EVs secreted from tumor activated macrophages carry immune suppressive message and prevent immune activation [80-82].

1.6.1 Platelet-derived microparticles

Platelet microparticles (PMPs) are released from activated platelets and like their progenitor platelets, they function in homeostasis and immunity. They constitute almost 90-95% of circulating microparticles. They express surface glycoproteins CD41, CD42 and CD61 which are also megakaryocyte lineage markers [83-84].

During their circulation in the blood interacts with many blood cells including leukocytes, lymphocytes and endothelial cells. Fusion of PMP to the target cell results in transfer of PMPs internal composition. PMPs are shown to have PPARγ (Peroxisome proliferator-activated receptors) and deliver it to the monocytes. Monocytes that received PPARγ differentiated into macrophages and showed less inflammatory cytokine production. However, for such transcription factors to fully function, signal or ligand may be required. Also, the delivered cargo of PMPs could be short lived and if the effect is not permanent, it may not be recognized [85-86]. Platelet-membrane functionalized particles which are like PMPs are shown to be very effective in drug delivery to the circulating tumor cells. Since these are usually metastatic tumor cells, they are very important in prevention of metastasis [87]. Also, PMPs deliver growth factors which would further induce tumor growth or enable

tissue regeneration. Platelet MPs promote angiogenesis meaning new blood vessel formation and help tumor growth [88-91].

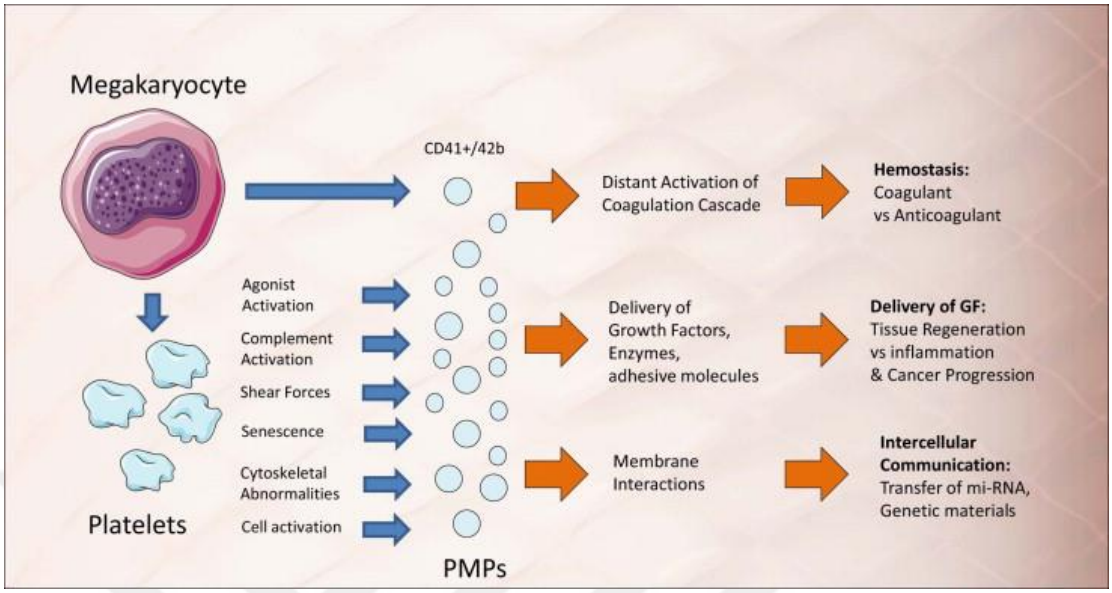

Figure 1.6: Mechanism of PMP generation from platelets [88].

1.7 Aim of the study

Macrophages are the key modulator and effector cells and their activation influences multiple subset of immune cells shaping their response. Changes in their polarization status are associated with many pathophysiological conditions. Therefore, understanding how to regulate M1/M2 type macrophage polarization would be beneficial either to control tumor development (M1) or to alleviate symptoms of auto-immune/auto-inflammatory diseases (M2).

In this thesis, we tried to exploit highly phagocytic nature of macrophages through the highest microparticle secreting cells, platelets. Since almost 95% of microparticles in circulation are derived from platelets and most of these microparticles are phagocytosed by macrophages, we postulated that PMP ingestion could alter the fate of the macrophages and facilitate their polarization tendencies either towards M1 or M20like phenotype. Therefore, we aimed to regulate macrophage polarization by using different TLR agonists in platelet-derived microparticles. Initially, we showed the interaction kinetics and intracellular localization of PMPs in macrophages. Then

with functional studies, we aimed to show immune suppressive activity of PMPs on macrophage polarization. Our results demonstrated that standalone PMPs are insufficient to induce M2 polarization by them. However, when TLR ligands were included within PMPs we found that macrophage polarization could be modulated With the screening of several TLR agonists on PBMCs and monocytes from different healthy donors, this work established that M1-like phenotype was generated following TLR9 ligand loaded PMP treatment and M2-like phenotype was obtained following TLR7 agonist loaded PMP treatment.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Laboratory & Cell Culture Reagents and Materials

Ultracentrifuge rotor tubes highly used for EV isolation, Zap-OGLOBIN® II Lytic reagent and Z-PAK Isoton II diluent used for cell counting were purchased from Beckman Coulter, USA. 10 mL BD Vacutainer® EDTA blood collection tubes used for blood collection were purchased from BD, USA. Human CD14 microbeads and other MACS equipment were purchased from Milteny Biotech, Germany. Media used in cell culture DMEM and RPMI1640, together with FBS, PBS, 2-mercaptoethanol and L-glutamine were purchased from Gibco, USA. Other supplemental reagents Hepes, Na Pyruvate, NEAA, Pen-Strep and Trypsin were purchased from Lonza, Switzerland. Trypan blue used for cell counting and Hypure Molecular Biology Grade Water were purchased from Hyclone. All plastic materials used in cell culture like flasks, microplates, scrapers, serological pipets, filters were purchased either from Costar Corporation, UK or Corning Life Sciences Inc., USA. Mr. Frosty freezing container was purchased from Thermo Scientific, USA and 2 ml screw cap cryo vials and tissue culture dishes were purchased from Greiner bio-one, Austria.

2.1.2 Recombinants and Other Agents

Recombinant human IL-4, IL-13 and IFN-γ were purchased from Biolegend, USA. Recombinant human GM-CSF and M-CSF for primary monocyte culture were purchased from Endogen, USA and Tonbo, USA. PMA and Ionomycin were supplied from Sigma, USA. Platelet activator peptide TRAP-6 were purchased from Anaspec, USA.

2.1.3 CpG ODNs

CpG ODNs were supplied from several companies. Their sequence and length information were provided in Table 2.1.

ODN	**Brand**	**Length**	**Sequence**
K23-PS	Alpha-DNA, Canada	12mer	TCGAGCGTTCTC
D35-3CG	Alpha-DNA, Canada	20mer	GGTCGATCGATCGAGGGGGG
A151	NIH, USA	24mer	TTAGGGTTAGGGTTAGGGTTAGGG

Table 2.1: List of CpG ODNs.

2.1.4 PRR Ligands

Ligands used for cell stimulations were listed in Table 2.2.

Ligand	**Source**	**Brand**
Poly (I:C) (HMW)	*B. Subtilis*	Fluka, USA
LPS	*E. coli*	Sigma, USA
R848	Synthetic	Invivogen, USA

Pam3Csk4	Synthetic	Invivogen, USA

Table 2.2: List of PRR ligands.

2.1.5 Inhibitors

Inhibitors used for platelet internalization experiments were listed in Table 2.3 together with working concentrations and corresponding incubation time. They were prepared in nuclease free water.

Inhibitor	**Brand**	**Working concentration**	**Incubation time**
Fucoidan	Sigma, USA	40 µg/ml	30 min
Dextran Sulfate	Sigma, USA	40 µg/ml	30 min
Chondroitin Sulfate	Sigma, USA	40 µg/ml	30 min
Sodium Azide	Merck, USA	0.1 M	90 min
Sucrose	Sigma, USA	0.45 M	30 min

Table 2.3: List of inhibitors used for internalization experiments.

2.1.6 BCA

BCA and Micro BCA Protein Assay kits were purchased from Pierce, USA and assays were performed on flat bottom 96 well plates purchased from RatioLab, Germany.

2.1.7 Flow Cytometry

Staining antibodies used for flow cytometry analysis were purchased from various companies. They were listed in Table 2.4 together with their brand and catalog number. Brefeldin A used for intracellular cytokine staining were supplied from

Sigma, USA. Fix and Perm Medium A and B were purchased from Invitrogen, USA. 3.5 μM 4%w/v Carboxyl latex beads for flow cytometry analysis of EVs were purchased from Life Technologies, USA.

Dye	**Species**	**Brand**	**Cat #**
Anti-Annexin-V-PE		Biolegend, USA	640908
Anti-Annexin-V-FITC		Biolegend, USA	640906
Anti-CD9-PE	Human	Biolegend, USA	312106
Anti-CD11b-FITC	Human	Tonbo, USA	35-0112-U500
Anti-CD14-PE	Human	Biolegend, USA	325606
Anti-CD63 Purified	Human	Biolegend, USA	312002
Anti-CD63-PE	Human	Biolegend, USA	353004
Anti-CD86-PE	Human	Biolegend, USA	310906
Anti-CD80-FITC	Human	Biolegend, USA	305206
Anti-CD81 Purified	Human	Biolegend, USA	349502
Anti-CD86-Alexa488	Human	Biolegend, USA	305414
Anti-HLA-DQ-FITC	Human	Biolegend, USA	318104
Anti-HLA-DQ-PE	Human	Biolegend, USA	318106
Anti-IL-12/IL-23 p40-FITC	Human	Biolegend, USA	501804
Anti-CD206-PerCP/Cy5.5	Human	Biolegend, USA	321122
Anti-IL-10-PE	Human	Biolegend, USA	506804
Anti-Alix Purified	Human	Biolegend, USA	634502

CFSE		Biolegend, USA	79898
Propidium Iodide		Sigma, USA	P4170

Table 2.4: List of flow cytometry antibodies.

2.1.8 Confocal Microscopy

Transferrin TexasRed and Lysotracker gree DND26 used to stain early and late endosomes were purchased from Molecular Probes, USA and SP-DiOC were purchased from Invitrogen, USA.

2.1.9 Determination of Gene Expression

RNA isolation was performed with Trizol reagent purchased from Life Technologies, USA. ProtoScript M-MulV cDNA Synthesis Kit and OneTaq Quick-Load 2x Master mix with Standard Buffer were purchased from NEB, USA. For qPCR analysis, 2x DyNAmo HS SYBR Green qPCR mix were purchased from Thermo Scientific, USA. 6x gel loading dye and DNA ladders were purchased from Fermentas, USA.

2.1.9.1 Primers

Primers were designed with Primer3 Input v.0.4.0 program (http://frodo.wi.mit.edu/primer3/input.htm) using the cDNA sequences of human genes available at the Ensembl™ database. Each primer pair was blasted (http://www.ncbi.nlm.nih.gov/BLAST/ or http://genome.ucsc.edu/) against the human genome. Primers used for human TLR profiling and gene expression analysis were listed in Table 2.5.

Gene Name	**Direction**	**Sequence**	**Product size (bp)**
TLR1	F	CATAACTCTGCTGATCGTCACC	491
TLR1	R	TGCTAGGAATGGAGTACTGCG	

TLR2	F	GATGACTCTACCAGATGCCTCC	745
TLR2	R	CAGAAGAATGAGAATGGCAGC	
TLR3	F	GCTGTCCACCACCAGCAATA	691
TLR3	R	CCTTCGGAGCATCAGTCGTT	
TLR4	F	TTACCTGTGTGACTCTCCATCC	529
TLR4	R	CAGAAGAATGAGAATGGCAGC	
TLR5	F	CCTTGACTATTGACAAGGAGGC	718
TLR5	R	TTGTAGGCAAGGTTCAGAACC	
TLR6	F	TCTCATGACGAAGGATATGCC	578
TLR6	R	CGATCAGCAGAGTTATGTTGC	
TLR7	F	ACGAACACCACGAACCTCAC	725
TLR7	R	GGCACATGCTGAAGAGAGTTAC	
TLR8	F	GGGAACATCAGCAAGACCCA	94
TLR8	R	GGCTGCAGGAGCTATTTTGC	
TLR9	F	CAACAACCTCACTGTGGTGC	514
TLR9	R	GAGTGAGCGGAAGAAGATGC	
TLR10	F	GAACTGATGACCAACTGCTCC	557
TLR10	R	GAAGTCTTGATTCCATCACGC	
CXCL11	F	AAGCAGTGAAAGTGGCAGAT	141
CXCL11	R	TAAGCCTTGCTTGCTTCGAT	
CCL18	F	GCTGCCTCGTCTATACCTCC	113

CCL18	R	CCGGCCTCTCTTGGTTAGGA	
β-actin	F	CGACAACGGCTCCGGCATGT	105
β-actin	R	ACCATCACGCCCTGGTGCCT	

Table 2.5: List of human primers used for PCR and qPCR.

2.1.10 Reagents for ELISA

2HB ELISA plates used for ELISA were purchased from SPL Life Sciences, Korea. SA-ALP was purchased from MabTech, Sweden and PNPP Tablets and 5X Diethanolamine Buffer were purchased from Thermo Scientific, USA. Monoclonal antibodies, biotinylated antibodies and recombinant proteins were purchased from variety of companies. Information showing the company name, catalog number and working conditions were listed in Table 2.6.

Antibody Name	**Brand**	**Cat #**	**Working concentration**
Anti-Pan IFN-α Ab	MabTech, Sweden	3425-1a-20	5 µg/ml in PBS
Pan IFN-α Biotinylated Ab	MabTech, Sweden	3425-1a-20	1:1000 diluted in T cell buffer
Recombinant Pan IFN-α	MabTech, Sweden	3425-1A-20	20 ng/ml in Blocker
Anti- IL-10 Ab	Endogen, USA	M011	4 µg/ml in PBS
IL-10 Biotinylated hAb	Endogen, USA	M011B	1:1000 diluted in T cell buffer
Recombinant IL-10	Biolegend, USA	571004	250 ng/ml in Blocker

Anti- IP-10 Ab	BD, USA	555046	2 μg/ml in PBS
IP-10 Biotinylated Ab	BD, USA	555048	1:1000 diluted in T cell buffer
Recombinant IP-10	BD, USA	551130	100 ng/ml in Blocker
Anti- IL-6 Ab	Biolegend, USA	501102	2 μg/ml in PBS
IL-6 Biotinylated Ab	Biolegend, USA	501202	1:1000 diluted in T cell buffer
Recombinant IL-6	Biolegend, USA	570802	60 ng/ml in Blocker
Anti- IL-12 Ab	Biolegend, USA	511002	5 μg/ml in PBS
IL-12 Biotinylated Ab	Biolegend, USA	508802	1:1000 diluted in T cell buffer
Recombinant IL-12	Biolegend, USA	573002	250 ng/ml in Blocker
Anti-IFN-γ Ab	Biolegend, USA	507502	4 μg/ml in PBS
IFN-γ Biotinylated Ab	Biolegend, USA	502504	1:1000 diluted in T cell buffer
Recombinant IFN-γ	Biolegend, USA	570202	500 ng/ml in Blocker

Table 2.6: List of human ELISA reagents.

2.2 Solutions, Buffers and Culture Media

2.2.1 Cell Culture Media

High Glucose DMEM and RPMI-1640 (Lonza)

- 2,.5 or 10% FBS inactivated at 55 °C
- 50 g/ml Penicillin/Streptomycin
- 10 mM HEPES
- 0,.11 mg/ml Na Pyruvate
- 1% Non-Essential Amino Acids Solution
- 2 mM L-Glutamine

All ingredient were added into 500 ml medium and stored at +4 °C.

2.2.2 Platelet Isolation Buffers

ACD Buffer (Acid-Citrate-Dextrose)

- 39 mM Citric acid
- 75 mM Sodium citrate
- 135 mM Dextrose

The pH of the solution was adjusted to 7.4 and stored at +4 °C. Before using, solution was warmed up to RT.

Platelet Wash Buffer

- 10 mM Sodium citrate
- 150 mM NaCl
- 1 mM EDTA
- 1% (w/v) Dextrose

The pH of the solution was adjusted to 7.4 and stored at +4 °C. Before using, solution was warmed up to RT.

Tyrode's Buffer

- 134 mM NaCl
- 12 mM $NaHCO_3$
- 2.9 mM KCl
- 0.34 mM Na_2HPO_4
- 1 mM $MgCl_2$
- 10 mM HEPES

The pH of the solution was adjusted to 7.4 and stored at +4 °C. Before using, solution was warmed up to RT.

2X Platelet Lysis Buffer

- 2% NP40
- 30 mM HEPES
- 150 mM NaCl
- 2 mM EDTA

The pH of the solution was adjusted to 7.4 and stored at +4 °C.

2.2.3 Flow Cytometry Buffers

PBS-BSA-Na azide Buffer

- 500 ml 1x PBS
- 5g BSA (1%)
- 125 mg Sodium Azide (0.25%)

Solution was stirred until fully homogenized and stored at +4 °C.

10X Annexin-V Binding Buffer

- 0.1 M HEPES
- 1.4 M NaCl
- 25 mM CaCl

Ingredients were dissolved in ddH_2O, pH was adjusted to 7.4 and stored at +4 °C. Before use, solution was diluted to 1x with ddH_2O.

2.2.4 Agarose Gel Electrophoresis

50X TAE (Tris-Acetate-EDTA)

- 242 g Tris ($C_4H_{11}NO_3$)
- 37.2 g Tritiplex 3 (EDTA= $C_{10}H_{14}N_2Na_2O_2 \cdot 2H_2O$)
- 57.1 ml Glacial acetic acid

Ingredients were dissolved in 1 lt ddH_2O, autoclaved and stored at RT. Before use, solution was diluted to 1x with ddH_2O.

2.2.5 ELISA Buffers

Blocking Buffer

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

Mixture was stirred until fully homogenized and stored in -20 °C.

T-cell Buffer

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

Mixture was stirred until fully homogenized and stored in -20 °C.

Wash Buffer

- 500 ml 10x PBS
- 2.5 ml Tween20
- 4.5 lt ddH_2O

Buffer was prepared freshly and used.

10X PBS (Phosphate Buffered Saline)

- 80 g NaCl
- 2 g KCl
- 8,01 g Na_2HPO_4 . $2H_2O$
- 2 g KH_2PO_4
- 1 lt ddH_2O

pH of the solution was adjusted to 6.8 and autoclaved. It was stored at RT.

2.3 Methods

2.3.1 Maintenance of Cell Lines

2.3.1.1 MEG-01

MEG-01 (ATCC®, CRL-2021) is a human bone marrow megakaryoblast cell line. Cells were sustained in a RPMI1640 media with 10% regular FBS. Cells cultured for EV isolation were maintained in media with ultra-centrifuged FBS. These half adherent half suspension cells were subcultured when they reach 90% confluence with fresh media following scraping. Cells collected at 300 g with 5 min centrifugation were seeded on appropriate concentration back to new flasks.

2.3.1.2 THP-1

THP-1 (ATCC®, TIB-202) is a human peripheral blood monocyte cell line. Subcultures of this suspension cells were maintained with addition of RMI1640 media with 10% regular FBS or with replacement of media after centrifugation at 150 g for 5 min. Subculturing was done every 2-3 days and cell concentration was kept between 3×10^5- 9×10^5 viable cells/ml.

2.3.2 Cryopreservation and Thawing of Cells

Cryotubes taken from liquid nitrogen were immediately put in water bath, which was pre-heated to 37 °C. After the ice in the vials melted, cells were transferred into a 15 ml falcon tube having 9 ml of the preferred media. Then, the falcon was centrifuged at 300 g for 5 min. Supernatant containing DMSO was aspirated and the cell pellet

was resuspended with fresh complete media. Cells were seeded in preferred culture plate and placed into the incubator, which was set for 37 °C with 5% CO_2 maintenance.

Cells reached around 60-80% confluency and 80-90% viability could be frozen. Cells were collected with a suitable method and pelleted with centrifugation. Then the cell pellet was resuspended in 10% DMSO in FBS and transferred into cryovials labeled beforehand. Cryovials were placed in Ms. Frosty Freezing Container and placed to -80 °C fridges. 24 h later cells were transferred into liquid nitrogen in order to store them for longer periods of time.

2.3.3 Cell Counting

2.3.3.1 Hemocytometer

After the cell lines and primary cells were washed and pelleted and resuspended in 1-10 ml of fresh media. 10 µl from cells were mixed with 10 µl Trypan Blue to distinguish live and dead cells. 10 µl from this suspension were loaded on hemocytometer and the number of cells was determined by counting under light microscope. Then the total cell number was calculated with the help of counted cell number and dilution factor.

2.3.3.2 Flow Cytometer

After the suspension cell lines and primary cells were washed and pelleted and resuspended in 1-10 ml of fresh media. Aliquots taken from cell line suspensions were directly counted on flow cytometer. With the help of total count in the live cell gated area and dilution factor, total cell number was determined. For primary cells, 20 µl of cell suspension was mixed with10 ml Isoton II Diluent buffer and 2-3 drops of ZAP-OGLOBIN II Lytic Reagent was added to lyse red blood cells. Following gentle tilting of the vial up and down for 5-6 times, cells were then counted on the flow cytometer. Live cells were gated, apoptotic cells or cell clusters were ignored. Again with the help of total count in the gated area and dilution factor, total cell number was determined.

2.3.4 PBMC Isolation

Blood samples were collected into BD vacutainers with EDTA and isolations were done within 30 min. 9 ml whole blood was slowly transferred into a 15 ml falcon having 6 ml histopaque. Samples were centrifuged at 300 g for 30 min with no brake. Plasma layer at the top was removed and the buffy coat at the middle containing PBMCs were transferred to new 50 ml falcon avoiding contamination from other layers. 2% FBS RPMI medium was added till reaching total volume of 50 ml and mixture was centrifuged at 300 g for 10 min. Then, supernatant was discarded without disturbing the pellet. Pellet was washed again, counted in flow cytometer in isotonic solution and adjusted to certain concentration depending on the intended experiments.

2.3.5 Platelet Isolation and Storage

Platelet isolation were done from whole blood samples of healthy donors. Bloods were collected into BD vacutainers with EDTA and isolations were done within 30 min. Whole blood was transferred into a 15 ml falcon having 1/10 volume of ACD buffer. PRP was isolated by centrifugation at 900 g for 5 min with no brake. PRP was transferred into a new 15 ml falcon carefully. Platelets were collected with 15 min centrifugation at 1700 g. Platelet poor plasma were discarded and platelets were washed with 1 ml platelet wash buffer gently without disrupting the pellet. They were counted in flow cytometer and frozen in 5% DMSO in FBS until further use.

Platelet isolation were also done from MEG-01 cell line. Cells grown for 3 days were collected by scraping. Cells were discarded after centrifugation at 300 g for 5 min. Remaining cell debris was discarded at 500 g for 5 min and then platelets were collected at 1900 g for 15 min.

2.3.6 Platelet activation and PMP Isolation

Thawed platelets were collected by centrifugation at 1700 g for 15 min. Platelets were incubated with 50 μM TRAP6 in 500 μl RPMI1640 filtered through 0.2 μm for 30 min at 37 °C. 5 ml RPMI1640 filtered through 0.2 μm filter were added and inactive platelets were collected with centrifugation at 1700 g for 15 min. Supernatant containing PMPs were transferred into ultracentrifuge tubes and volume was adjusted

with sterile PBS. PMPs were collected from the bottom after 30.000 g for 45 min and resuspended in 200-400 µl of sterile PBS.

2.3.7 Exosome and MP Isolation

Cells cultured for MP isolation were kept in media with FBS that is ultra-centrifuged overnight at 28,000 g. Supernatant of cells were collected when cells reach ~80% confluency. Cells were pelleted first at 300 g for 5 min, cell-free supernatant was taken. Then to get rid of any cell debris supernatant was centrifuged at 1500 g for 10 min and stored at -80 °C after snap-freezing in liquid nitrogen until the day before isolation. One day before EV isolation, supernatants were put on +4 °C overnight for slow thawing. Isolations were done using either 15 or 30 ml sterile tubes. Supernatants were loaded into tubes and placed inside the ultracentrifuge tubes cleaned with 70% EtOH. The lids were closed and tubes were placed carefully into rotor and then rotor to the ultracentrifuge. In order to discard any remaining contaminants, samples were centrifuged at 10,000 g for 10min. Supernatants were taken into new tubes without touching to the bottom and samples were centrifuged at 30,000 g for 30 min to get MPs. After this step the 2-3 ml at the bottom of the tube was taken for MP and top portion was taken for exosome isolation if desired. MP portion was mixed well and sterile PBS was added for washing the MP pellet. To collect MPs, samples were centrifuged at 30,000 g for 45 min and supernatant was aspirated without touching to the MP pellet at the bottom. MPs were resuspended in 200-500 µl of sterile PBS and snap-frozen in liquid nitrogen and stored at -80 °C for further use.

2.3.8 BCA and Micro BCA Protein Assay

BCA and Micro BCA protein assays were used to assess protein concentration of EVs and other samples. Both assays were done according to the manufacturer's protocol. Basically, BSA standards were prepared at different concentrations. Working reagents were prepared according to the kit's manual. Then suggested amount of working reagent plus suggested amount of standards were added on each well of micro-plates. Samples were also added on working reagent at the same of volume of standards. Micro-plates were incubated at 37 °C for 30 min for BCA and 2 h for micro BCA assay. After plates were cooled to RT, the absorbance of samples were

measured at 562 nm by spectrophotometer (Synergy HT). The concentration of samples was determined by BSA standard curve.

2.3.9 Cargo Loading to Extracellular Vesicles

MPs and exosomes were mixed with desired amount of TLR ligands in 1.2 ml tubes. If there are more than one sample, all samples' volumes were equalized by addition of sterile PBS. Immediately after mixing ligands with EVs, the tubes were covered with parafilms and holes were opened on top. Mixture was frozen in liquid nitrogen and placed in freeze-drier for overnight lyophilization. For reconstitution of the powdered samples, sterile water in 1/10 of initial mixture volume was added. Samples were vortexed for ~15 seconds every 2 min for 20 min Then the same of volume of sterile PBS was added and samples were incubated for 10 min at RT. After the incubation, samples were vortexed for ~15 seconds and they were ready for further use in treatments.

2.3.10 Flow Cytometry

2.3.10.1 Fixation of Cells

Cells were collected by 5min centrifugation at 300 g. Supernatant was aspirated and cells were further washed with PBS. 50 µl Fixation Medium A per $1x10^6$ cells were added on pelleted cells while vortexing and cells were incubated at RT for 15 min. 20 volumes of PBS-BSA-Na-Azide buffer was added on cells to wash and fixed cells were centrifuged at 500 g for 5 min. Cells were resuspended in 100 µl PBS-BSA-Na-Azide buffer, directly stained or stored at +4 °C maximum for a week.

2.3.10.2 Surface Marker Staining of Cells

0.1 µg of staining antibodies were added on $1x10^6$ fixed cells in 100 µl PBS-BSA-Na-Azide buffer. Cells were incubated 30 min at RT and dark. 2 ml PBS-BSA-Na-Azide buffer was added to wash and cells were collected at 500 g for 5 min. Then cells were resuspended in PBS and analyzed in flow cytometer.

2.3.10.3 Intracellular Cytokine Staining

For detection of intracellular cytokines, cells were treated with Brefeldin-A 6 h before the end of the incubation. After Brefeldin-A treatment, cells were collected with centrifugation at 300 g for 5 min and fixed with Fixation Medium A for 15 min at RT. Fixed cells were washed with PBS-BSA-Na-Azide buffer containing 0.3% saponin and centrifuged at 500 g for 5 min. Cells were resuspended in Permeabilization Medium B for permeabilization and at the same time, 0.1 μg of staining antibodies were added per on $1x10^6$ cells in 100 μl. Staining was done at RT and dark for 30 min. 2ml PBS-BSA-Na-Azide buffer was added to wash and cells were collected in 5 min at 500 g. Then cells were resuspended in PBS and analyzed in flow cytometer.

2.3.10.4 Surface Marker Staining of EVs with Latex Beads

For catching EVs, latex beads were coated with monoclonal antibodies. 5 μl of latex beads were taken from stock, washed with PBS and collected at 12,000 g for 10 min. 5 μg antibody of interest were added on beads and volume was completed to 500 μl with PBS in an eppendorf tube. Samples were incubated overnight on rotator at RT. Then antibody conjugated beads were centrifuged at 12,000 g for 10 min. They were blocked with 5% BSA in PBS again on rotator and RT for 5 h. Beads were collected with centrifugation and resuspended in 50 μl 1% BSA in PBS. They were stored at +4 °C until further use. Before analysis, 1 μl of antibody conjugated beads were mixed with 1 μg of EVs and volume was completed to 500 μl with PBS in an eppendorf tube. Then samples were incubated overnight on rotator at RT and EV captured latex beads were obtained with centrifugation. They were stained with staining antibodies and analyzed in flow cytometer.

2.3.11 SP-DiOC Staining of PMPs

PMPs were stained with SP-DiOC to analyze cellular internalization of the vesicles. PMPs dissolved in sterile PBS were incubated with dye at 1 μM concentration for 30 min at 37 °C. After incubation, vesicles were washed with PBS once in order to remove the unbound dye and collected by ultracentrifugation.

2.3.12 Binding & Uptake and Internalization Assays

This assays were done by using SP-DiOC stained microparticles. Stained MPs can either bind to the surface of the cells or be internalized. PMA activated THP-1 macrophages were cells were co-incubated with stained MPs. Cells were collected at different time points and directly counted on flow cytometer to measure binding & uptake. However, to determine the proportion of internalized vesicles, extracellular fluorescence signal was quenched with addition of trypan blue (0.01% final concentration) and then cells were counted again on flow cytometer.

2.3.13 Analysis of PMP Localization with Confocal Microscopy

THP1 cells were seeded on coverslips placed inside 6-well cell culture plates. SP-DiOC stained PMPs were co-incubated with cells for 4 h. After co-incubation, cells were washed twice with PBS and fixed by addition of 150 µl Fixation Medium A. Fixation was done at RT for 15 min. Then cells were washed twice with PBS and for late endosome visualization Transferrin-red dye was added in 200 µl. It was incubated for 30 min at 37 °C. Cells were washed again and visualized under confocal microscope. For visualization of early endosome, lysotracker-red was directly added on washed cells without fixation. It was incubated for 30 min at +4 °C, washed and visualized under confocal microscope. Images taken from confocal microscope were analyzed in ImageJ.

2.3.14 Determination of Gene Expression

2.3.14.1 Total RNA Isolation

Cells were collected and centrifuged at 300 g for 5 min at +4 °C. Supernatants were gently discarded without disrupting the pellet and cells were extensively mixed and lysed with 1 ml TRIzol. Cell lysates were transferred into eppendorf tubes. At this step, samples could be stored at -80 °C. Directly or after thawing, samples were handled at +4 °C all the time. 200 µl chloroform was added into each 1 ml TRIzol containing tubes and shaken vigorously for 15 sec and incubated at RT for 3 min. Samples were centrifuged at 13,200 rpm for 17 min. Subsequently, the clear upper aqueous phase was transferred into a new eppendorf tube and total RNA was precipitated by adding 500 µl of isopropanol, gently inverted up-down for proper

mixing, incubated at RT for 10 min and centrifuged for 12 min at 13.200 rpm. Supernatant was removed and the pellet was washed with 1 ml of 75% EtOH, vortexed for 10 sec, again centrifuged at 8000 rpm for 7 min. After centrifugation ethanol was removed and pellet was washed again with 1ml >99,8% EtOH followed by centrifugation at 8000 rpm for 7 min. After the last centrifugation, the pellet was left under laminar flow hood to air-dry. Purified total RNA then dissolved with 20-30 µl RNase/DNase free water. The OD measurements of the samples were carried out at 260 nm and 280 nm with the NanoDrop® ND-1000 spectrophotometer. Total RNA samples were expected to give 1.8-2.0 ratio at A260/280 which was the indicator of minimal DNA, protein or chemical organic solvent contaminations. RNA samples were stored at -80 °C for further use.

2.3.14.2 cDNA Synthesis

ProtoScript First Strand cDNA synthesis kit were used to synthesize cDNA from total RNA samples according to the manufacturers' protocol. 2 µl oligo dT was mixed with up to 1 µg of total RNA and volume was adjusted to 8 µl. Pre-denaturation step was done at 70 °C for 5 min. Then 10 µl M-MulV reaction mix and 2 µl M-MulV enzyme mix were added. For cDNA synthesis, samples were incubated at 42 °C for 60 min, 80 °C for 5 min and 4°C for 10 min. cDNA samples were preserved at -20 °C.

2.3.14.3 PCR

For each gene, a specific primer pairs (Table 2.5) and optimal PCR conditions were set and following mixing the reagents, PCR was performed on MJ Mini thermocycler (Bio-Rad, USA). The protocol for PCR sample preparation is shown in Table2.7. PCR conditions in Table 2.8 were used. PCR products kept at +4 °C until they were analyzed with agarose gel electrophoresis.

Reagent	**Volume**
Quick Load Taq 2x Master Mix	12,5 µl
Nuclease-free H2O	9.5 µl

10pmol Forward Primer	1 µl
10pmol Reverse Primer	1 µl
cDNA	1 µl
Total Volume	25 µl

Table 2.7: Sample PCR reaction.

Step	**Temperature**	**Duration**	**Cycle**
Initial Denaturation	94 °C	2 min	1x
Denaturation	94 °C	30 sec	30x
Annealing	β-actin 60 °C TLR6 61.2 °C TLR1,2,4,5 61.9 °C TLR3,8 62 °C TLR9,10 62.4 °C	30 sec	
Elongation	68 °C	1 min	
Final Extension	68 °C	5 min	1x
Hold	4 °C	∞	

Table 2.8: PCR reaction conditions.

2.3.15.4 qPCR

For each gene, a specific primer pairs (Table 2.5) and optimal qPCR conditions were set and following mixing the reagents, PCR was performed. The protocol for sample preparation is shown in Table 2.9 and conditions in Table 2.10.

Reagent	**Volume**
DyNAmo HS SYBR Green qPCR Master Mix	12,5 µl
Nuclease-free H2O	9.5 µl
10pmol Forward Primer	1 µl
10pmol Reverse Primer	1 µl
cDNA	1 µl
Total Volume	25 µl

Table 2.9: Sample qPCR reaction.

Step	**Temperature**	**Duration**	**Cycle**
Initial Denaturation	95 °C	15 min	1x
Denaturation	95 °C	10 sec	30x
Annealing	65 °C	30 sec	
Elongation	72 °C	30 sec	
Final Extension	72 °C	5 min	1x
Hold	4 °C	∞	

Table 2.10: qPCR reaction conditions.

2.3.14.5 Agarose Gel Electrophoresis

1-3% agarose gel was prepared with 1x TAE buffer depending on the expected product size and 1 mg/ml ethidium bromide solution. Gel lanes were loaded with 10-30 µl of PCR product mixed with loading dye. 10 µl of the 10, 100 and 1000 bp DNA ladders were used as markers and the gels were ran for 20-60 min at 100-120 V power. Visualization of the gels were performed under UV transilluminator (Vilber Lourmat, France) with BIO-PROFILE Bio-1D V 11.9 software.

2.3.15 Cytokine ELISA

After stimulation assay done in 96-well cell culture plates with RPMI1640 media supplied with 5% regular FBS were finished, supernatants of the cells were stored at -20 °C until further use for cytokine ELISA. SPL ELISA plates were coated with 50 µl/well monoclonal antibodies diluted with PBS from stock and kept overnight at +4 °C. After coating, the solutions were discarded to the sink and plates were blocked with 200 µl/well blocking buffer 2 h at RT. Blocking buffer was discarded and plates were washed 5 times with 3 min incubation in each with washing buffer. Plates were rinsed with ddH2O for 5 times and dried by tapping gently on tissue papers. Thawed cell supernatants were added at this step as 50 µl/well and recombinant cytokines were prepared at desired concentration with serial dilution in blocking buffer. Also, 50 µl PBS was added to one well on each plate as blank. Plates were incubated overnight at +4 °C. Plates were washed, dried and biotin conjugated antibodies diluted 1:1000 with T cell buffer from stock were added as 50 µl/well and incubated 2 h at RT. Plates were washed, dried again and streptavidin-AKP solutions also prepared in T-cell buffer with 1:1000 dilution factor were added 50 µl/well. It was kept for 1 h at RT and then plates were washed and dried last time. PNPP substrate solution was prepared according to the manual and 50 µl of it was transferred into wells. After color formation was observed plates were read at ELISA reader (BioTek) at 405 nm with time intervals.

2.3.16 Macrophage Differentiation and Polarization from THP-1

2.3.16.1 Macrophage Differentiation

THP-1 cells were seeded as 50,000 cells per well in 96-well plate in 250 µl 5% regular RPMI1640 media. Cells were treated with 50 ng/ml PMA for 24 h at 37 °C in a CO_2 incubator. Next day, medium of cells were replaced with fresh 5% regular RPMI1640 media. Cells were rested for 24 h for further differentiation and then they were ready for polarization.

2.3.16.2 M1-M2 Polarization

Differentiated THP-1 cells were treated with M1 (20 ng/ml IFNγ and 100 ng/ml LPS) and M2 (20 ng/ml IL4) supplements at the third day and incubated for further 24 h for polarization.

2.3.17 Monocyte Isolation from PBMC

CD14 monocyte isolation was done with MACS human CD14 microbeads according to the manufacturer's protocol. Basically, PBMCs were resuspended MACS buffer and then mixed with CD14 microbeads. 80 µl buffer and 20 µl microbeads were used for every $10x10^6$ PBMCs and up to $100x10^6$ cells were used per isolation. Cells were incubated for 15 min at +4 °C. After incubation, 2 ml MACS buffer was added on cells and they were centrifuged at 300 g for 10 min. Bead conjugated cells were resuspended in 500 µl of MACS buffer and loaded onto MS column which was prepared before by rinsing with 500 µl buffer. Unlabeled cells that pass through were collected at the bottom. 500 µl buffer were added three times when reservoir was empty to wash. Column was then removed from the separator and placed into a collection tube. 2 ml buffer was pipetted on the column and labeled cells were flushed out by pushing the plunger. Cells were collected by centrifugation at 300 g for 5 min and used for desired experiment after their purity was checked with CD14 staining.

2.3.18 Macrophage Differentiation and Polarization from Human Monocytes

2.3.18.1 Macrophage Differentiation

CD14+ monocytes isolated from PBMCs were seeded as 65,000 cells per well in 96-well plate in 250 µl 5% regular RPMI1640 media. Cells were treated either with 25 ng/ml GM-CSF for 3 days or 5 days at 37 °C in a CO_2 incubator for differentiation.

2.3.18.2 M1-M2 Polarization

Differentiated human monocytes were treated with M1 (20 ng/ml IFNγ and 100 ng/ml LPS) and M2 (20 ng/ml IL4) supplements or other reagents to test at the third or fifth day and incubated for further 48 h for polarization.

Chapter 3

Results

3.1 TLR expression analysis of THP-1, MEG-01 cell lines and healthy PBMCs

THP-1 monocyte and MEG-01 megakaryoblast cell lines were used throughout this thesis and we wanted to screen their TLR expressions, initially. (Figure 3.1) Healthy human PBMCs were used as positive control because they express all TLRs. THP-1 macrophages also expressed all TLRs however, expressions of some were very low. However, they can increase after stimulation [92]. MEG-01 cell line showed expression of only TLR2 in our analysis. But it was also shown to have TLR1 and 6 in very low amount and they could only be detected in flow cytometer [93]. With the help of this TLR expression panel, we could use TLR agonists in PBMC and monocyte treatments. Since MEG-01 cell line didn't express most of the TLRs, we didn't see basal cytokine response coming from platelets or PMPs.

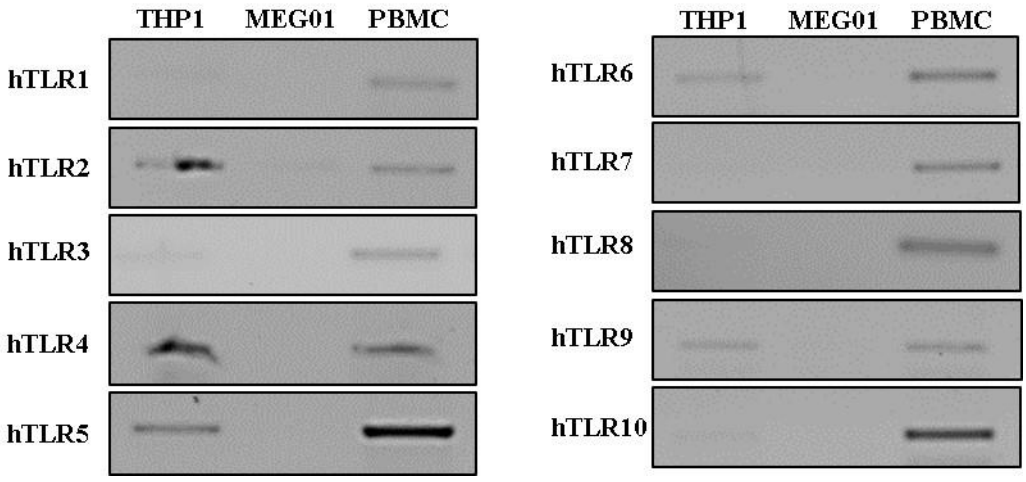

Figure 3.1: TLR expression panel of THP-1, MEG-01 cell lines and healthy PBMC. *RNA isolation from these cells were done with Trizol and equal amount of RNA was used for cDNA synthesis. PCR amplified genes were detected via agarose gel electrophoresis.*

3.2 Efforts to collect platelet and PMP

To assess the effect of platelet-derived MPs on macrophages, platelets and PMPs were collected. Whole blood collected from healthy donors were used to isolate PRP. With the help of PRP, the gates for platelets and PMPs were drawn and these gates shown in Figure 3.2 were used throughout this thesis to count platelets in flow cytometer. Count of PMPs were not healthy because there is also junk material appeared at that size in flow cytometer. Since whole blood was difficult to obtain regularly, performing experiment with MEG-01 derived platelets and PMPs were more logical. However, when cultured MEG-01 cells were collected and platelets were pelleted at appropriate force like whole blood platelets, expected populations didn't appear. MEG-01 PMPs were again so small and not appropriate to analyze and count in flow cytometer. Also, directly isolated MEG-01 MP from culture supernatant had events in both platelet and PMP size.

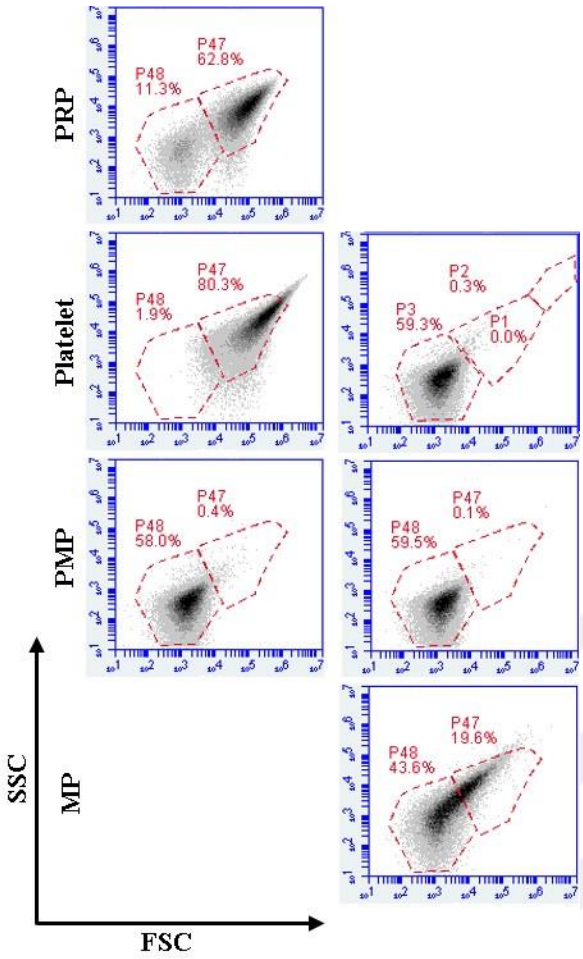

Figure 3.2: Flow cytometry analysis of platelet and PMP isolation from whole blood of healthy individuals and MEG-01 cell culture supernatant. *PRP, platelets, isolated PMPs and MPs at different centrifugation steps were analyzed according to their size in flow cytometer immediately after isolation.*

In order to increase the amount of platelets secreted from MEG-01 human megakaryoblast cell line, it was differentiated into megakaryocyte. Because megakaryocytes can form pro-platelets and secrete platelets (Figure 3.3.A). Differentiation was done with PMA and Pam3Csk4 [94-96]. MEG-01 cells were treated with suggested amounts of either PMA or Pam3Csk4 for 3 h and then cultured for 3 days. Results didn't show presence of more platelets secreted into the culture media, there was a slight increase. Interestingly, Pam3Csk4 treated cells had pro-platelet formations but platelets were not detected (Figure 3.3.B).

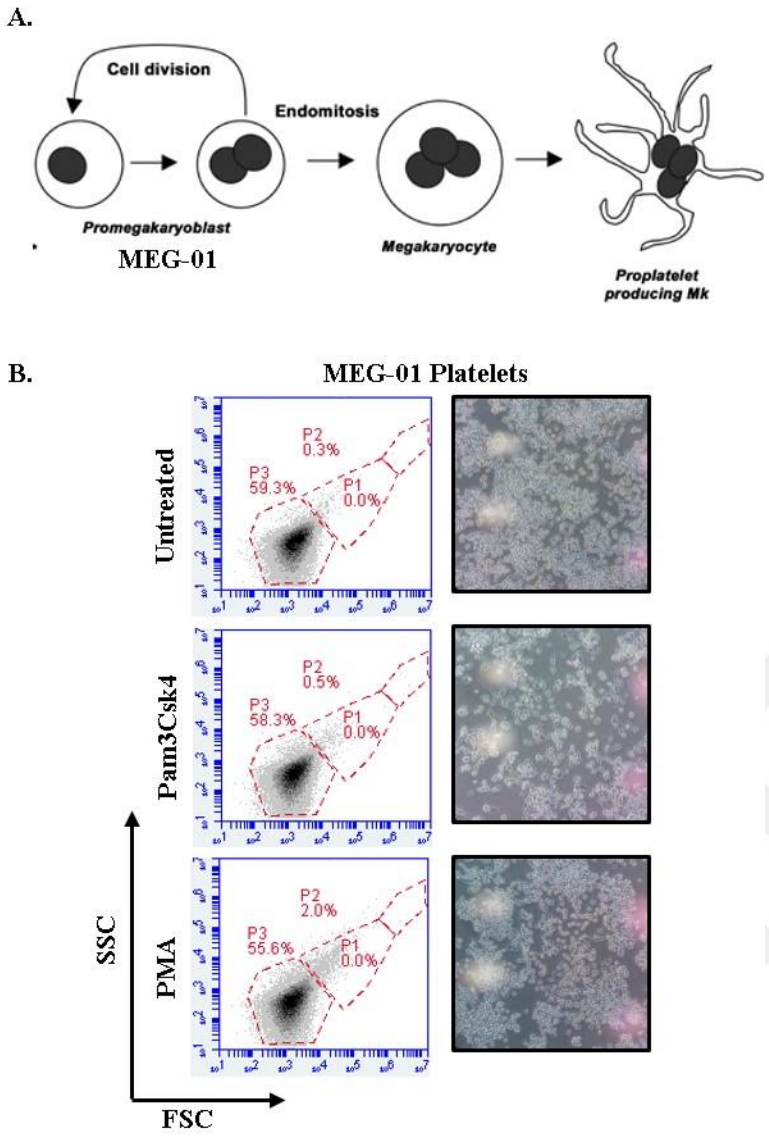

Figure 3.3: Induction of platelet secretion from MEG-01 cells. *A. Representation of megakaryocyte differentiation and pro-platelet formation. B. Megakaryocyte differentiation and pro-platelet formation was induced by 3 µg/ml Pam3Csk4 or 10 nM PMA for 3 h and cells were incubated for further 3 d. Pro-platelet formation was observed under microscopy and secreted platelets were analyzed at flow cytometry after isolation with centrifugation.*

Since platelets weren't detected, megakaryocyte markers of MEG-01 cells were analyzed. Results revealed that MEG-01 cells don't express megakaryocytic lineage markers CD42a, CD41 and CD63. PMA and Pam3Csk4 treatments also didn't increase their expression (Figure 3.4). Without any of these markers, it was

impossible to make sure that these are platelets so MEG-01 cells were abandoned and whole blood platelets were used in experiments.

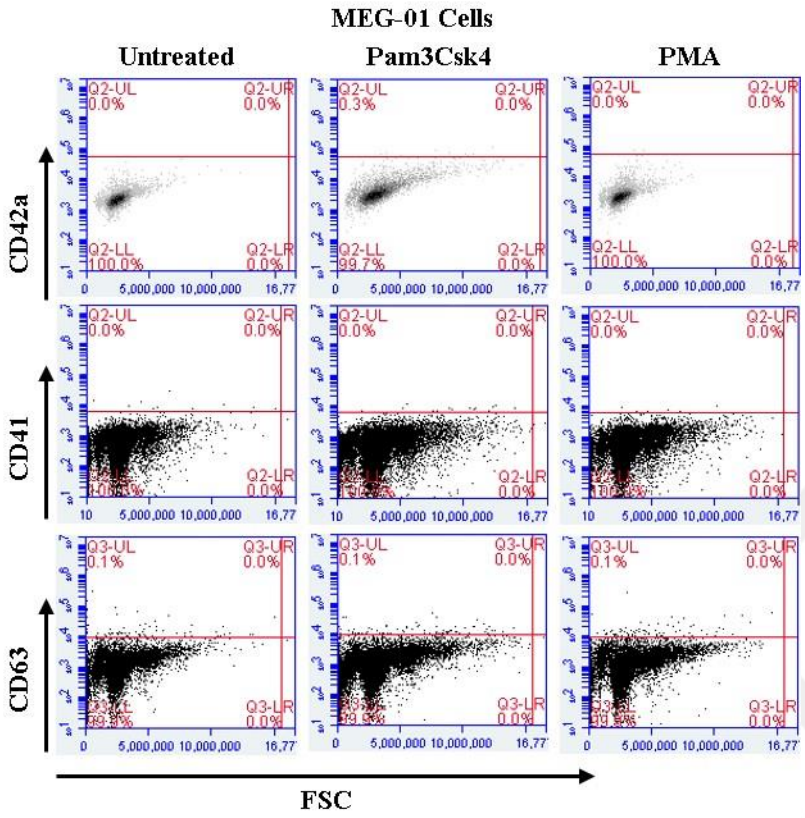

Figure 3.4: Induction of megakaryocyte differentiation from MEG-01. *Cells were induced by 3 µg/ml Pam3Csk4 or 10 nM PMA for 3 h and incubated for further 3 d. Change in megakaryocyte lineage markers CD42a, CD41 and CD63 were analyzed at flow cytometer after staining.*

After abandoning usage of MEG-01 cells, we focused on whole blood platelets (Figure 3.2). They were characterized by CD41 staining. Collected platelets were activated with TRAP6, ionomycin or PMA. However, there wasn't much difference in their activation marker CD63 expression because inhibitor wasn't used to prevent activation during the isolation procedure. Platelets were almost 100% active at the end of isolation. (Figure 3.5.A) But platelets were still activated with TRAP6 and PMPs were collected with ultra-centrifugation. For the analysis of PMPs, anti-CD41 conjugated latex beads were used. PMPs caught by beads were shown to express

megakaryocyte lineage markers like CD42a, CD41, CD63 and extracellular vesicle markers like Annexin V.

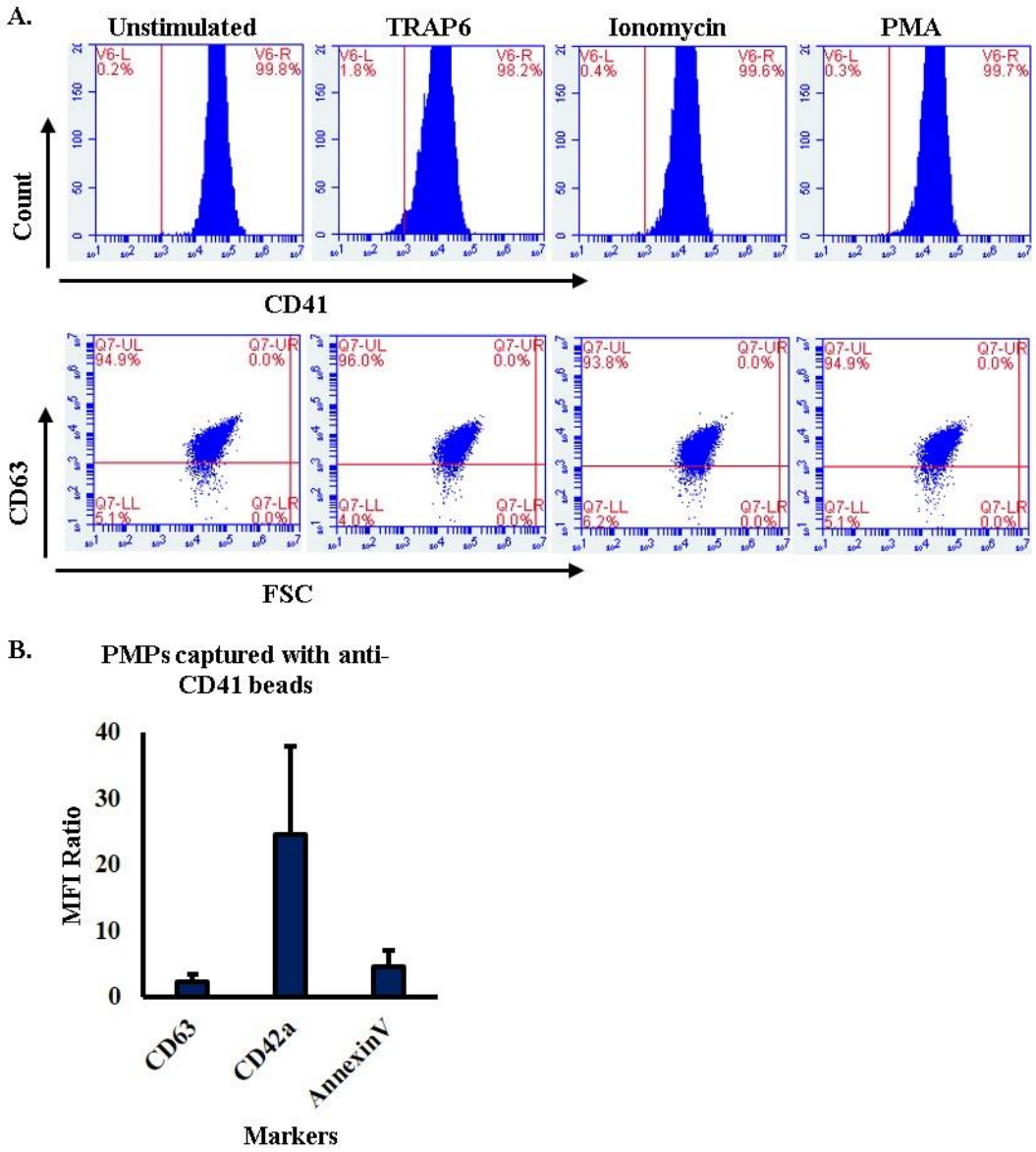

Figure 3.5: Analysis of platelets isolated from whole blood. *A. Activation of platelets freshly isolated from whole blood. Collected platelets were stimulated with 50 µM TRAP6, 10 µM ionomycin, 10 ng/ml PMA or left unstimulated. They were stained with CD41 and CD63 antibodies and analyzed in flow cytometer. B. PMPs isolated from TRAP6 activated platelets were captured with anti-CD41 conjugated latex beads and their marker expression were analyzed. Graph shows the MFI ratio of stained PMPs over isotype controls.*

3.3 Efforts to generate M0, M1 and M2 macrophages from THP-1 monocytes

THP-1 monocytes are regularly used for macrophage studies and there are many different methods published in the literature [97-98]. To find out the optimum condition for macrophage differentiation and then macrophage polarization from THP-1 monocytes, different protocols were tested in a single experiment. THP-1 monocytes were initially incubated in different amounts of PMA for 24 h then rested for further 24 h or directly treated with M1-M2 supplements. IL6 and IL10 cytokine secretions were detected with ELISA to understand their polarization status (Figure 3.6). Cells that was treated with 50 ng/ml PMA, rested for a day and then treated with LPS and IFNɣ for a day showed highest IL6 response and cells treated with 50 ng/ml PMA, rested for a day and then treated with IL4 for a day showed highest IL10 response. (Figure 3.7.C) Therefore, these protocols were used to further analyze macrophage differentiation and M1-M2 polarization from THP-1 cells. Light microscopy images showed attached macrophages and M1and M2 macrophages had different morphologies. (Figure 3.7.A) These THP-1 macrophages expressed 85% CD11b macrophage marker however, the percentage of M1 and M2 polarized macrophages checked with HLA-DQ and CD206 flow cytometer stainings were very small. (Figure 3.7.B) Like cytokine ELISA results, qPCR results also showed the expected differential expression in chemokine markers CXCL11 and CCL18. However, the number of cells that was truly differentiated was very small according to the flow cytometer data. So THP-1 monocyte cell line was not a suitable model to study M1-M2 polarization.

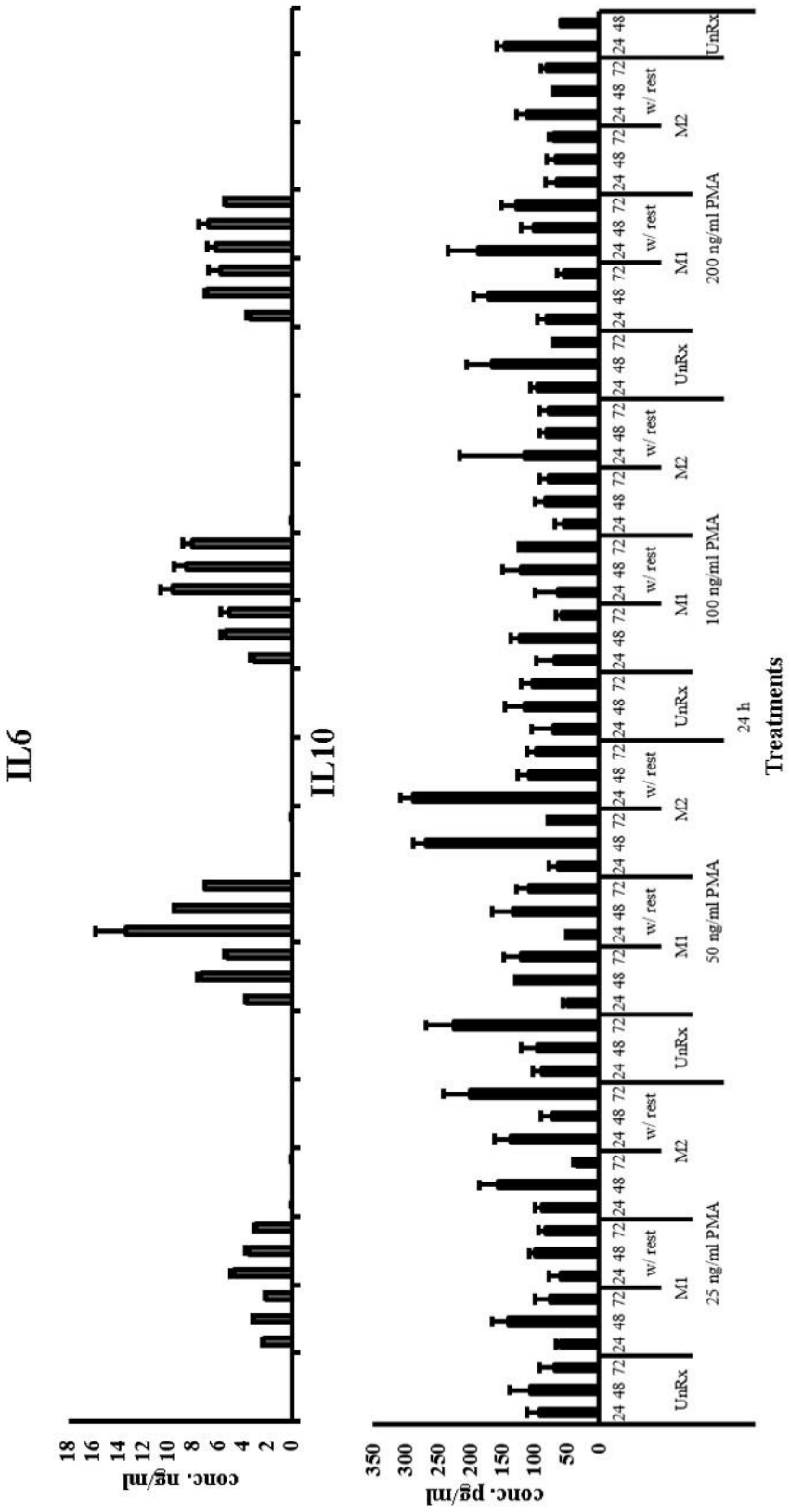

Figure 3.6: Macrophage differentiation and M1-M2 polarization of THP-1. *THP1 monocytes were activated with PMA at different concentrations. Then macrophages were rested a day or directly polarized into M1-M2 macrophages with 100 ng/ml LPS, 20 ng/ml*

IFNɣ or 20 ng/ml IL4 treatment. IL6 and IL10 cytokine secretions at different time points were determined with ELISA.

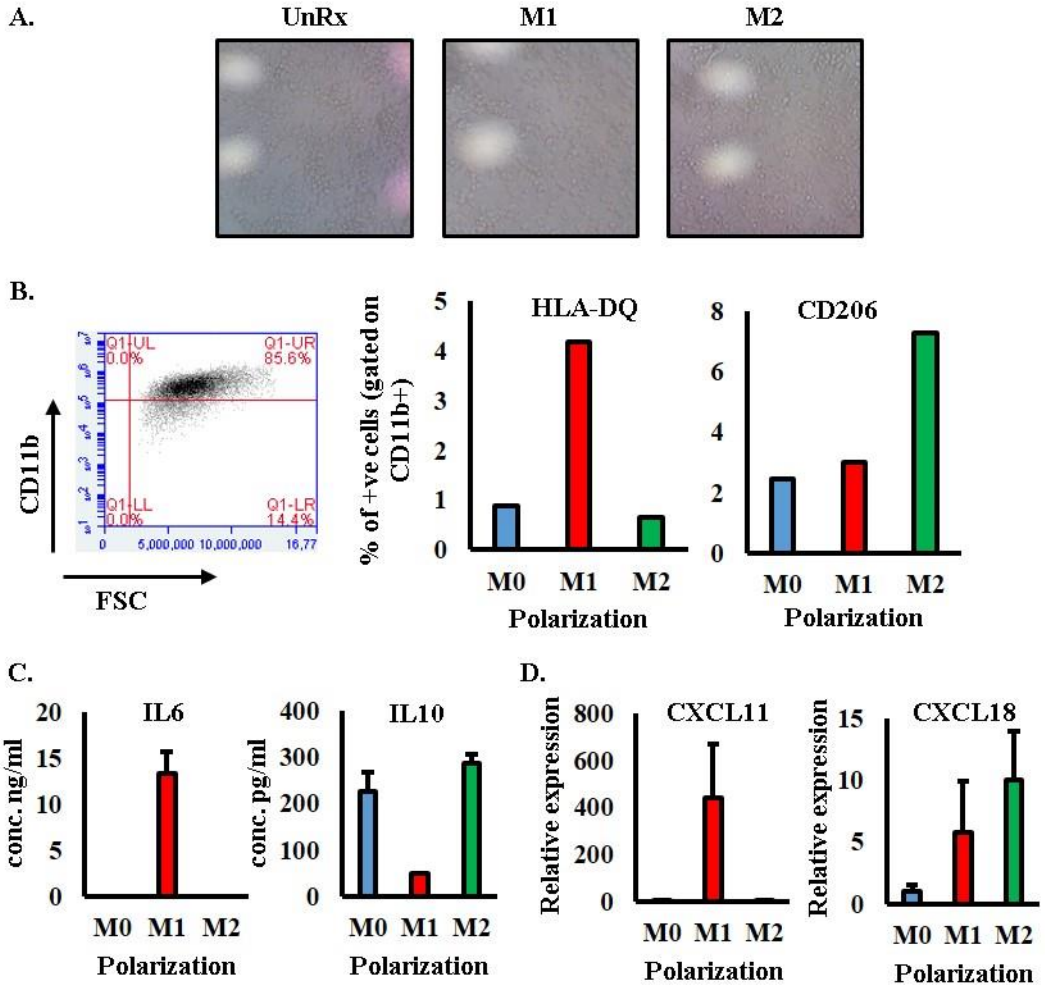

Figure 3.7: PMA-activated THP1 macrophages were polarized into M1-M2 macrophages. *A. Light microscopy images. B. CD11b, HLA-DQ and CD206 positivity were analyzed in flow cytometry. C. IL6 and IL10 cytokine secretions were determined with ELISA. D. Expression of CXCL11 and CCL18 were analyzed with RT-qPCR.*

3.4 Platelets and PMPs interact with THP-1 macrophages

When co-incubated platelets can interact with THP-1 macrophages. To confirm that increasing amounts of Sp-DiOC stained platelets were co-incubated with PMA activated THP-1 macrophages. Fluorescence microscopy and flow cytometer analysis showed that platelets interact and get internalized by macrophages. (Figure 3.8.A-B)

Macrophages that uptake platelets were seen in green under the microscope and cells were seen Sp-DiOC^{+} in flow cytometer. Also, platelet internalization didn't get affected from scavenger receptor inhibitor Fucoidan and Dextran S., active transport inhibitor NaN3 or clathrin coated pit formation inhibitor Sucrose. (Figure 3.8.C) So, the mechanism of internalization wasn't receptor or clathrin coated pit mediated or active transport.

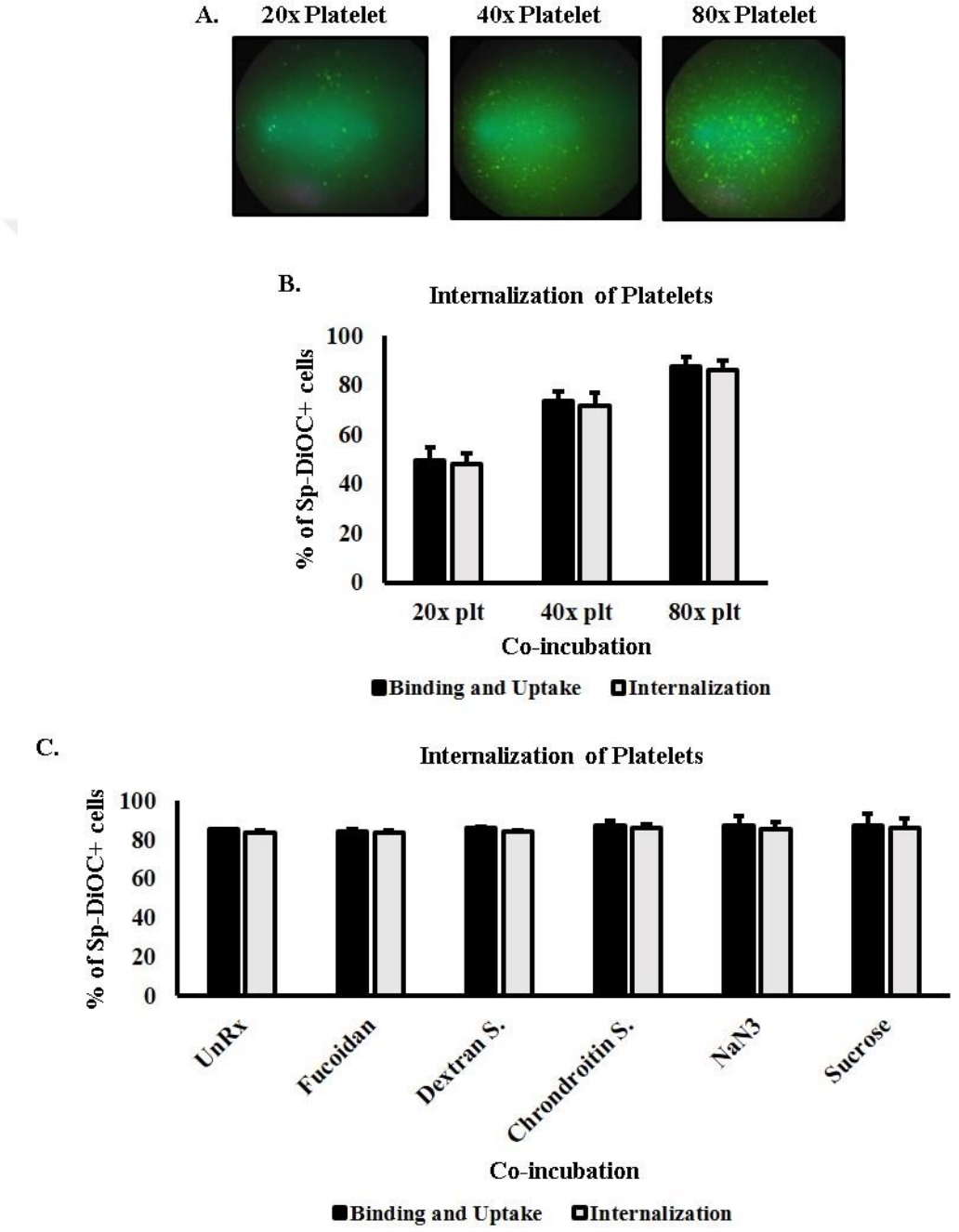

Figure 3.8: PMA-activated THP1 macrophages internalize platelets. *A. Fluorescent microscopy images of THP1 macrophages co-incubated with increasing amounts of Sp-DiOC*

stained platelets for 24 h. B. Binding, uptake and internalization analysis of THP1 macrophages co-incubated with increasing amounts of Sp-DiOC stained platelets. C. Binding, uptake and internalization analysis of THP1 macrophages co-incubated with increasing amounts of Sp-DiOC stained platelets after different inhibitor treatments.

When we analyzed the binding-uptake and internalization of PMPs, we initially tested their time-dependency. With time, THP-1 macrophages interact with and internalize more and more PMPs. After 24 h, almost all cells showed Sp-DiOC positivity in flow cytometer. But in all time points around half of the interacting PMPs were internalized by macrophages. (Figure 3.9.A) Moreover, it was shown that M2 polarization promotes nanoparticle uptake [99]. Our results confirmed that M2 polarized macrophages internalize more PMPs comparing to M1 polarized macrophages. (Figure 3.9.B)

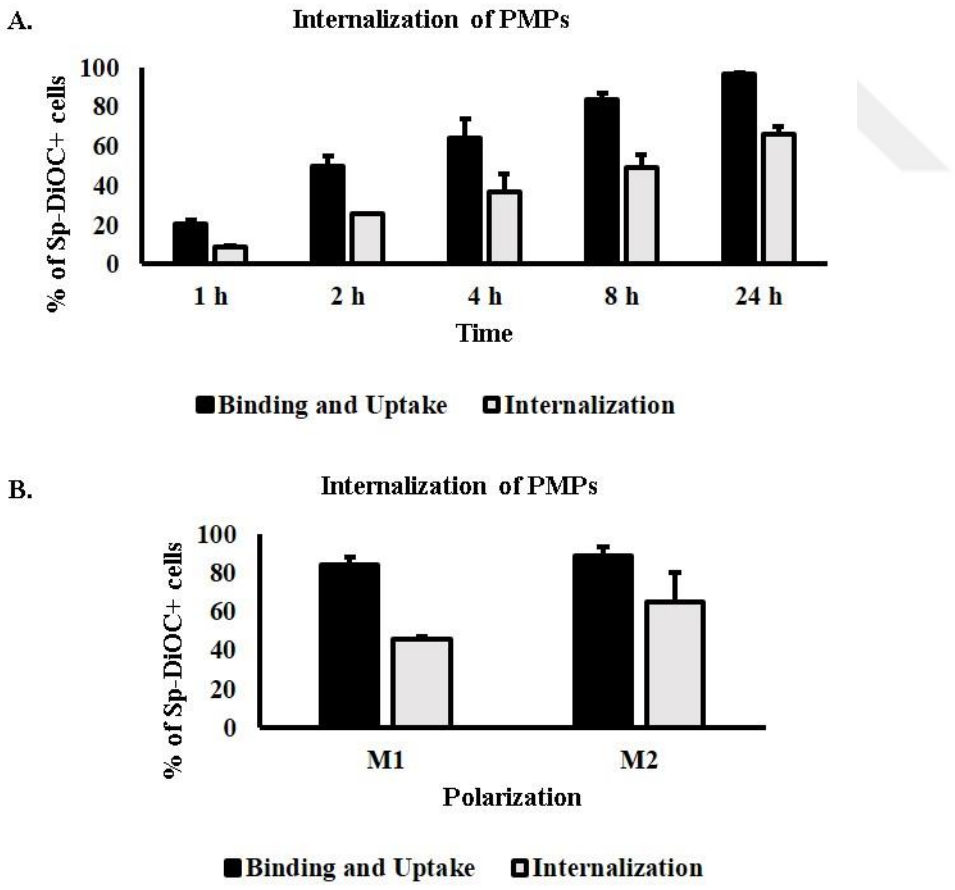

Figure 3.9: PMA-activated THP1 macrophages internalize PMPs. *A. Binding, uptake and internalization analysis of THP1 macrophages co-incubated with Sp-DiOC stained PMPs for*

different time points B. Binding, uptake and internalization analysis of polarized THP1 macrophages co-incubated with Sp-DiOC stained PMPs.

These internalized PMPs should end-up in endosomes. When activated and transferrin red or lysotracker red stained THP-1 macrophages were co-incubated with Sp-DiOC stained PMPs in different setups, we showed that internalized PMPs co-localize into late endosome. (Figure 3.10)

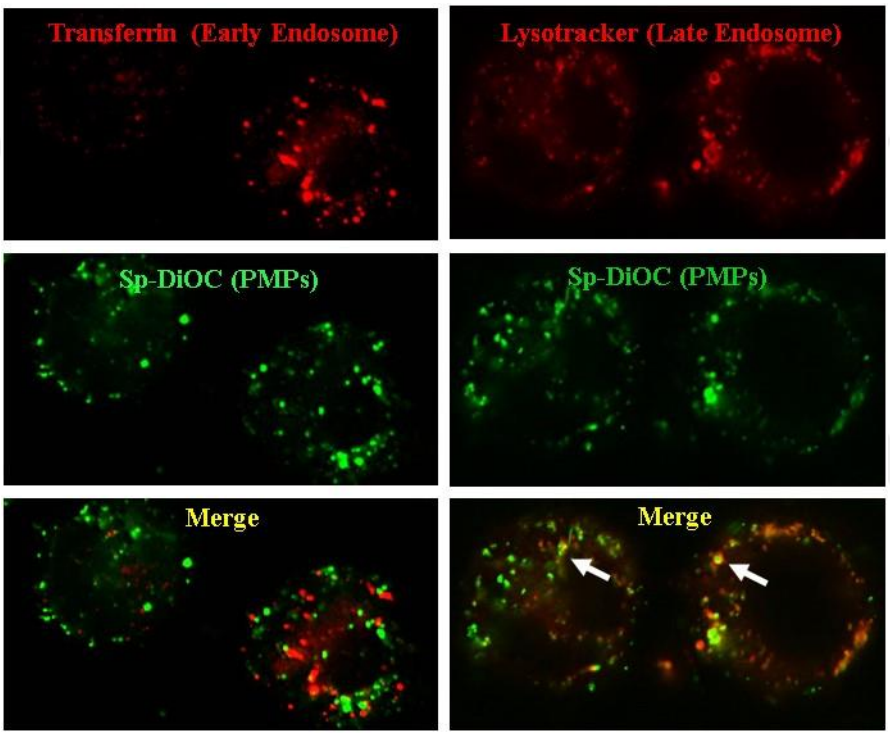

Figure 3.10: PMPs co-localize into late endosome. *PMA-activated THP1 macrophages were stained with Early (Transferrin) and Late (Lysotracker) endosome markers. Fate of the Sp-DiOC stained PMPs was determined with confocal microscope after 4 h of incubation with THP1 macrophages.*

3.5 Effects of free and ligand loaded PMPs on THP-1 macrophages

PMPs interact and get internalized by macrophages. To determine their effect on macrophages, PMPs were co-incubated in free form or as loaded with TLR2/1 agonist Pam3Csk4, TLR7/8 agonist R848 or TLR9 agonist A151 with THP-1 macrophages. PMPs didn't induce any IL6 or IL12 cytokine response from macrophages and

behaved as untreated. (Figure 3.11) Also, IL10 secretion wasn't detected from these cells. Ligand loaded or ligand mixed forms of PMPs gave different cytokine responses. Loading Pam3Csk4 into PMPs decreased its IL12 and IL6 secretion and behaved more like M2-trophic. Loading R848 into PMPs also decreased IL6 secretion. A151 could induce slight immune response from macrophages. Overall, these results initially showed that PMPs are not immune inducers alone. When loaded with ligands, they decreased the cytokine response that ligand gave in free form. However, THP-1 macrophages weren't healthy to make functional analysis. For functional studies, we determined to continue with healthy human PBMCs and monocytes.

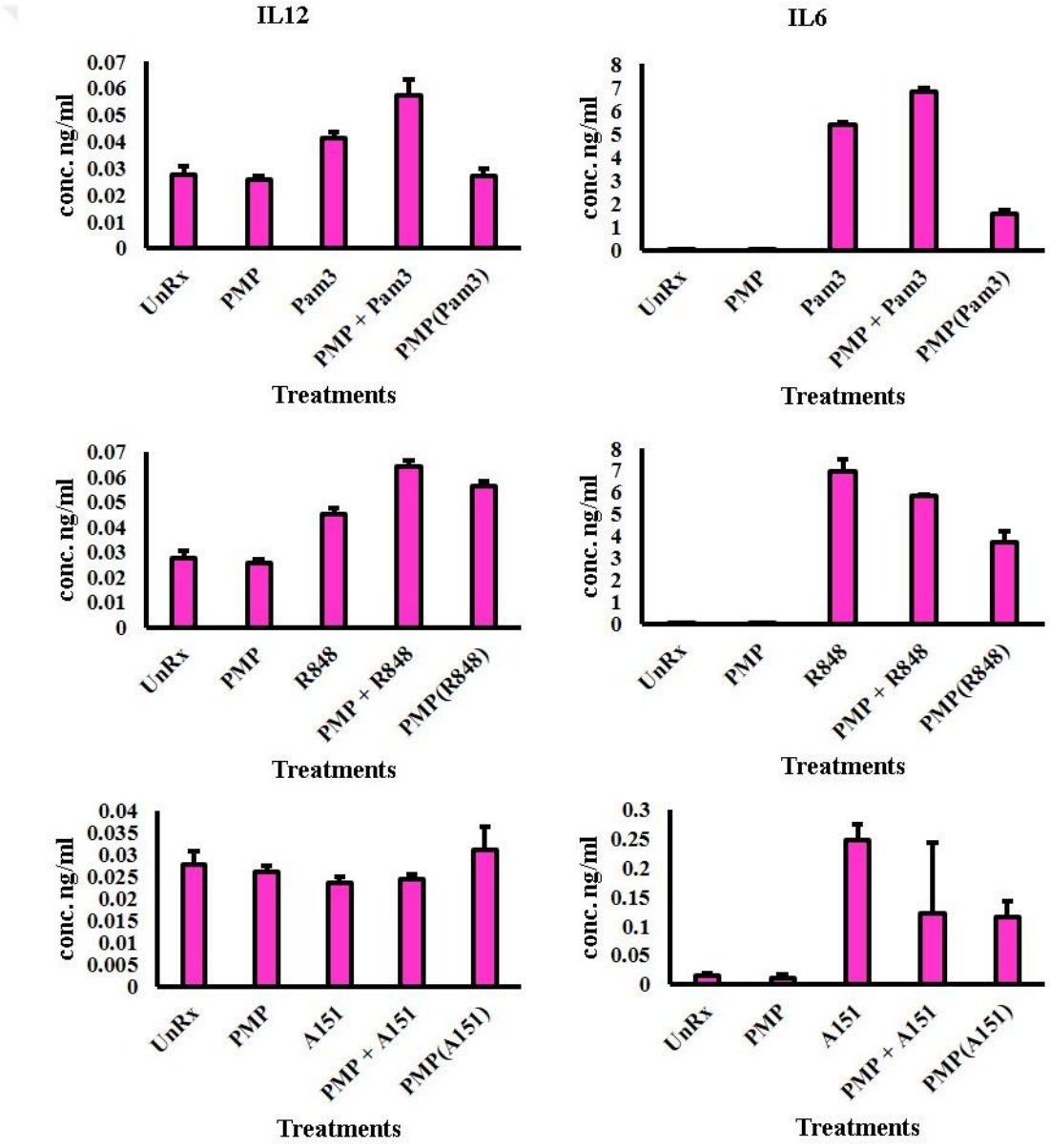

Figure 3.11: PMPs and TLR agonists induce IL6 and IL12 cytokine response. *PMA-activated THP1 macrophages were treated with A151, R848 and Pam3Csk4 in free form or in PMPs. IL6 and IL12 cytokine secretions were determined with ELISA. IL10 wasn't detected.*

3.6 Effects of free and ligand loaded PMPs on healthy PBMCs

Since THP-1 cell line was not a good choice for functional analysis, whole blood was collected from healthy individuals and PBMCs were isolated with ficol gradient. Platelets and then PMPs were isolated from TRAP6 activated platelets with centrifugation. To see the general immune response of PBMCs before going into the monocytes when co-incubated with PMPs alone or together with different PRR ligands. PMPs didn't induce any immune response alone from PBMCs like THP-1 macrophages. As TLR agonists, we tried Pam3Csk4, R848, A151, TLR3 ligand poly(I:C) and TLR9 ligand D35-3CG on three different healthy individuals. (Appendix Figure 1-3) R848 induced IL10 and D35 -3CG ODN induced type I and II interferon response. (Figure 3.12) When they were loaded in PMPs, their effect was increased significantly. PBMCs results overall showed that D type ODN induced M1 trophic and R848 induced M2 trophic immune response.

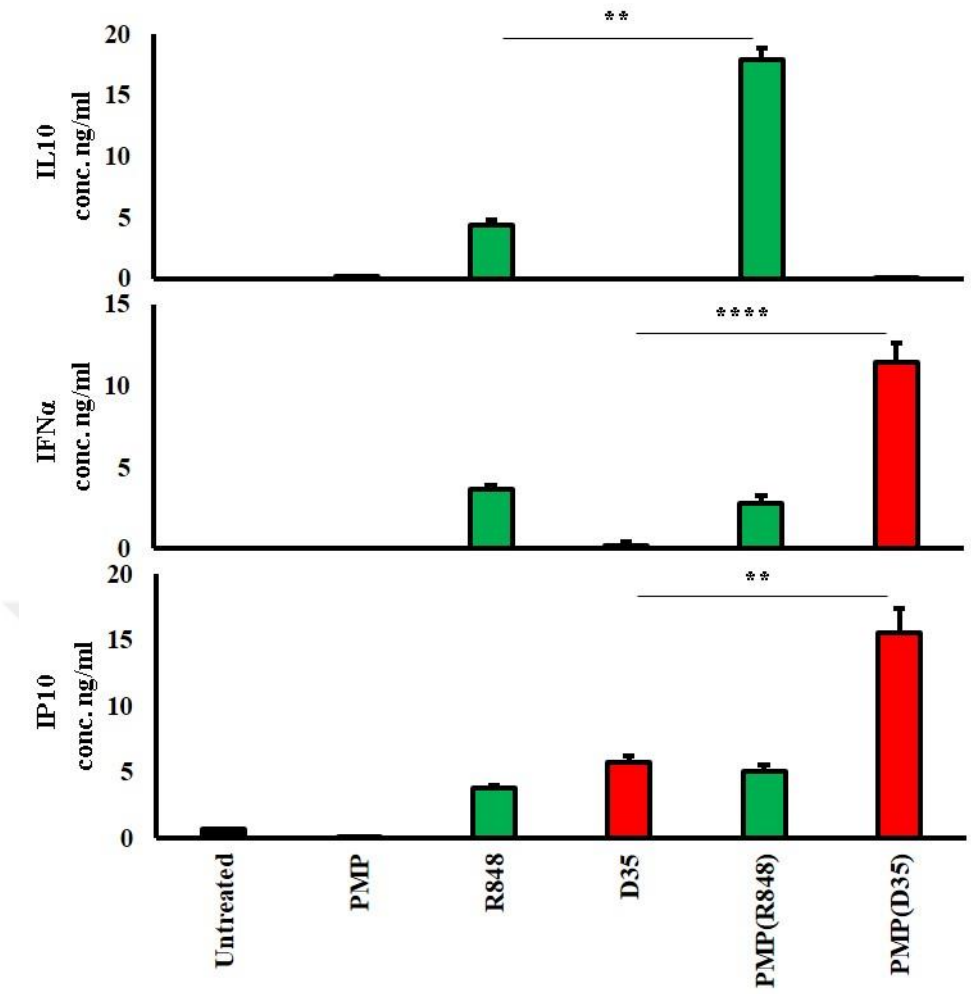

Figure 3.12: PMPs and TLR agonists induce cytokine responses from PBMCs. *PBMCs collected from healthy donors were treated with 1μM D35 and 1μg/ml R848 in free form or in PMPs for 24h. IL10, IFNα and IP10 cytokine responses were detected by ELISA. Statistical analysis by unpaired student's t-test: **P<0.01, ****P<0.0001.*

3.7 Effects of free and ligand loaded PMPs on healthy PBMC derived monocytes

After screening PBMC responses in different individuals and observing M1 and M2-trophic effects of D35 and R848, we continued with circulating human monocytes. CD14+ monocytes were isolated from PBMCs with magnetic sorting. Isolated monocytes were characterized with CD14, CD11b and CD16 staining in flow cytometer. (Figure 3.13.A) Then they were differentiated into macrophages in the presence of GM-CSF for 5 days and treated with D35 and R848 either in free form or as loaded in PMP for further 2 days. Flow cytometry analysis of 25F9 active

macrophage marker showed that at the end of 7 days, they were almost all active macrophages. (Figure 3.13.B)

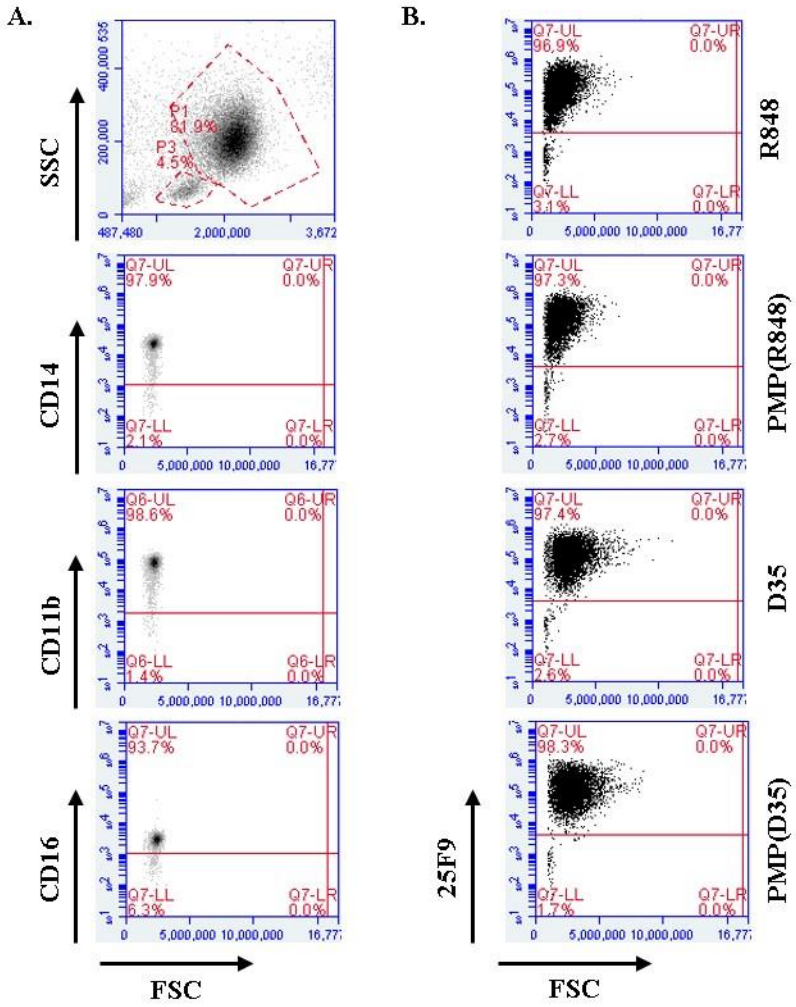

Figure 3.13: Characterization of PBMC isolated monocytes and differentiated macrophages. *A. CD14+ blood monocytes were isolated from PBMCs of healthy donors with MACS. CD14, CD11b and CD16 expression of these monocytes were checked in flow cytometry. B. Macrophage differentiation was done with 25 ng/ml GM-CSF for 5 days. Generated macrophages were treated with 1 mM D35 and 1 µg/ml R848 in free form or in PMPs for 48 h. Active macrophage marker 25F9 expression were checked in these cells.*

Monocyte responses were analyzed in 2 different healthy individuals. (Appendix Figure 4-5) ELISA results of these differentiated macrophages showed that R848 loaded in PMP induced significant IL10 secretion and M2-trophic immune response.

D35 CpG ODN induced significant type II interferon and M1-trophic immune response. Monocyte results were similar to previous PBMC results. (Figure 3.14)

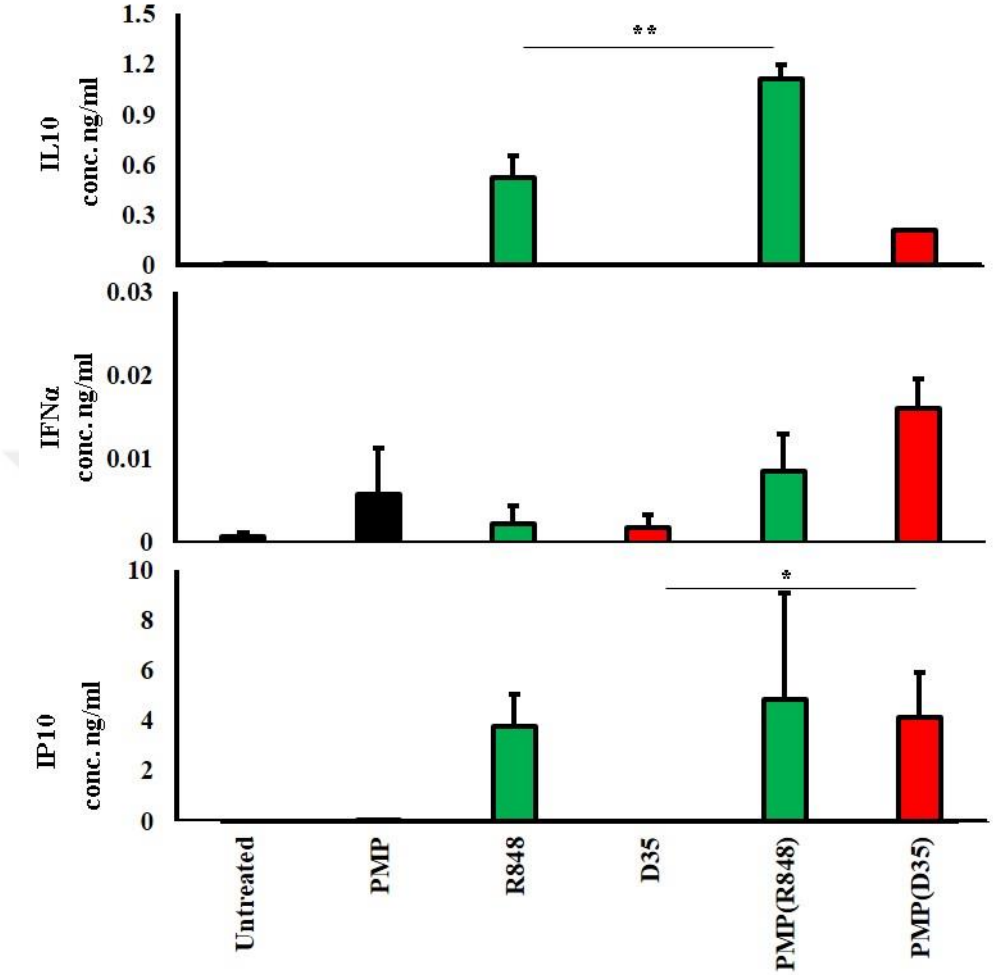

Figure 3.14: PMPs and TLR agonists induce cytokine responses from monocytes. *A. CD14+ blood monocytes were isolated from PBMCs of healthy donors with MACS. Macrophage differentiation was done with 25ng/ml GM-CSF for 5 days and percentage of active macrophages was assessed with 25F9 expression. B. Generated macrophages were treated with 1μM D35 and 1μg/ml R848 in free form or in PMPs for 48h. IL10, IFNα and IP10 cytokine responses were detected by ELISA. Statistical analysis by unpaired student's t-test: *P>0.05, **P<0.05.*

Chapter 4

Discussion

According to our previous data, macrophages are the cells which interact with and internalize microparticles the most comparing to the other cell types. In the blood circulation, they interact with PMPs highly because platelet-derived microparticles constitute almost 95% of the circulating microparticles. Therefore, this thesis aimed to investigate the effect of platelet-derived microparticles on macrophage polarization.

Firstly, we started our experiments by trying to isolate platelets from MEG-01 human megakaryoblast cell line. Since collecting whole blood from healthy donors is not always possible to freshly isolate platelets, we thought that this cell line could be useful. When we performed differential centrifugation process on the supernatant of this cell line, we couldn't collect platelets. After that we decided to stimulate these megakaryoblasts for megakaryocyte differentiated so that the platelet secretion rate increases. After Pam3Csk4 stimulation, we could observe pro-platelet formations however, again there weren't any platelets secreted to the environment. To make sure that we are working with the correct cell line, we stained megakaryocytic lineage markers that should be expressed by these cells. We observed that they don't express any of the megakaryocytic lineage markers. Also, even after stimulation for megakaryocyte differentiation, they don't increase their marker expressions and platelet secretion. Under these conditions, it was impossible to work MEG-01 cell line and characterize their differentiation status or secreted platelets. These findings showed us that MEG-01 is not a good model for platelet studies. Therefore, we used

human platelets freshly isolated from whole blood. Since we didn't use inhibitors other than EDTA containing vacutainer and citrate containing ACD buffer, collected platelets were active. We still tried to induce activation of all platelets with different approaches, however the changes were very little. Most of the platelets were already active. This wasn't important for our case because we collected microparticles secreted from active platelets. But, this study confirmed that platelets are very fragile and require professional handling. However, the microparticles collected from them were not fragile and could be easily stored in -80°C after snap-freezing in liquid nitrogen. Although the storage of these microparticles were easy, working with them is difficult. Because they are so small to count and analyze with flow cytometer. To achieve it, we used latex bead approach and coated latex beads that is big enough to be detected in flow cytometer with CD41 antibody. Then we coincubated PMPs with these coated beads and captured them with their surface CD41. We only analyzed 3 different markers on the microparticles with this approach however, much more can be done. These markers were enough to characterize PMPs.

After collecting platelets and PMPs, we started working on generation of M1-M2 polarized macrophages from THP1 human monocytic cell line. Our results confirmed that macrophage polarization is a highly dynamic process and it is crucial to catch the right time. Upon treatment with LPS, IFNɣ for M1 or IL4 for M2, macrophages start polarization however, if signal is not provided again they may change their polarization status. Our screen revealed the decrease in cytokine response after the initial 24h of treatment. Moreover, when we analyzed these cells with flow cytometer and gated on macrophages, we observed that the percentage of M1 and M2 polarized cells were very small in the population. Only 4% of the M1-primed cells were HLA-DQ+ and only 7% of the M2-primed cells were CD206+. Even though inflammatory and suppressive cytokine secretions analyzed with ELISA and chemokines analyzed with qPCR showed expected differential results, it is not ideal to work with a mix population. Additionally, THP derived macrophages are in the end cell lines. In order to make cells immortalized, you have to sacrifice certain aspects which might also affect the polarization responses and prevent us to see the real potential of monocytes. For more promising results without interfering unpolarized cells, cell sorter is needed.

Our next experiments were to see the interaction between platelets, PMPs and macrophages. Because if there is an interaction, there is also a functional effect on macrophages. Our findings showed that both platelets and platelet derived microparticles interact with macrophages. Kinetics of internalization depends on cell:platelet ratio. When this ratio was higher, faster internalization occurred. We used several inhibitors including active transport, scavenger receptor and receptor mediated endocytosis to understand the platelet uptake mechanism. However, none of these inhibitors affected platelet internalization. We previously had data on microparticle uptake mechanism, therefore we didn't use inhibitors again with PMPs. Our data which is not shown here showed that microparticles are internalized by active transport, scavenger receptor and receptor mediated endocytosis. These findings are important because the inhibitors could be used to generate desired immune response. Moreover, comparison of M1 and M2 polarized macrophages confirmed the findings in the literature and showed that M2 polarization promotes phagocytosis and particle uptake. After seeing there is an interaction between platelets and microparticles, we wanted to see where they are going in the macrophages and made confocal microscopy analysis. These results revealed PMPs localize into the late endosome of macrophages and they deliver their cargo here to induce several pathways.

Platelets and platelet-derived microparticles (PMPs) are associated with the generation of immunosuppressive micro-environment due to their growth factor and nucleic acid containing cargo. Our initial hypothesis was that macrophages contacted with PMPs should go into alternative M2 macrophage polarization. Because PMPs are shown to have nuclear receptor PPARγ and deliver it to the monocytes. Monocytes that received PPARγ differentiated into macrophages and showed less inflammatory cytokine production. Upon activation, it mediates macrophage polarization towards M2. After showing the interactions between them, we continued with functional analysis. However, our functional studies on THP1, human PBMCs and monocytes revealed that PMPs don't induce macrophage polarization towards immune stimulatory M1 and immunosuppressive M2 macrophages or any immune response alone. The reason can be the short half-life of the signal delivered by PMPs. If the effect is not permanent, it may not be recognized. For the macrophage polarization study, continuance of the signal is very crucial. Otherwise they re-

polarize. Also, for such transcription factors to fully function, signal or ligand may be required. Macrophages can produce endogenous ligand for PPARγ however, addition of ligand would be helpful to observe the immune suppressive effect generated by PMP delivered PPARγ.

Since we couldn't observe any immune response generated by PMPs alone. We decided to use PMPs as a carrier system and couple them with TLR agonists. To do that, we used the dehydration-rehydration method developed in our laboratory. Our target was macrophages, however these findings could be helpful for tumor studies as well. Because platelet-membrane functionalized particles which are like PMPs are shown to be very effective in drug delivery to the circulating tumor cells. With this loading method, drug loaded PMPs can be used for metastatic tumor cells which escape to the circulation. We screened several TLR agonists on PBMCs and monocytes from different healthy donors. M1-like response was observed from TLR9 agonist D-type CpG oligodeoxynucleotide loaded PMPs and M2-like response was observed from TLR7-8 agonist ssRNA R848 loaded PMPs. Also, loading these agonists into PMPs enhanced immune responses generated from them. Our findings indicted that PMP treatment of macrophages loaded with suitable ligand combinations might regulate M1/M2 type macrophage polarization. By regulating macrophage polarization, we could use them to control tumor development or to alleviate the symptoms of auto-immune/auto-inflammatory diseases. Also, these results showed that in case of a ssRNA virus infection, microparticles secreted from infected cells could be captured by macrophages and induce M2-trophic macrophage polarization like R848 in our experiments. This environment could help to the progression of the infection.

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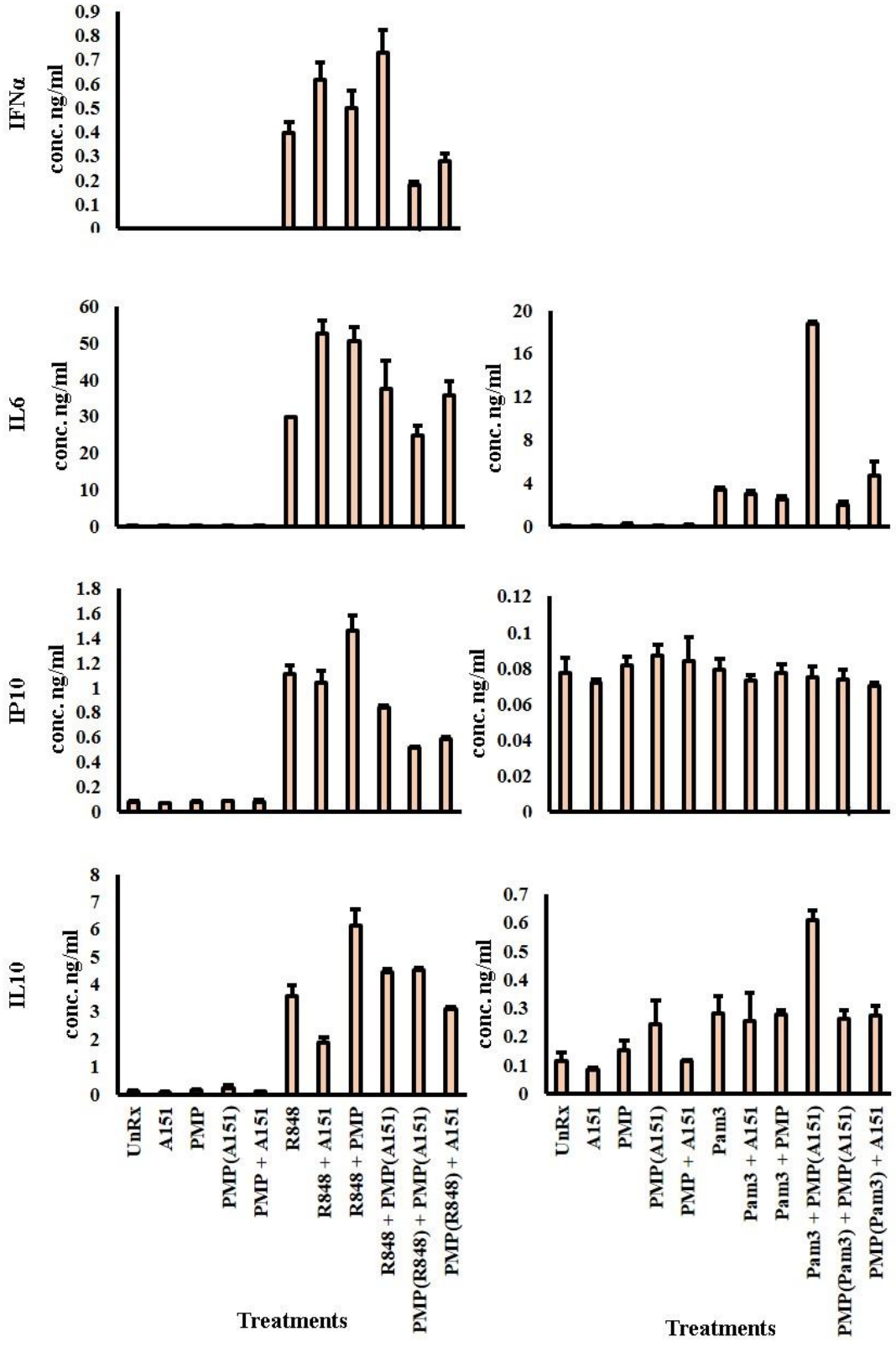
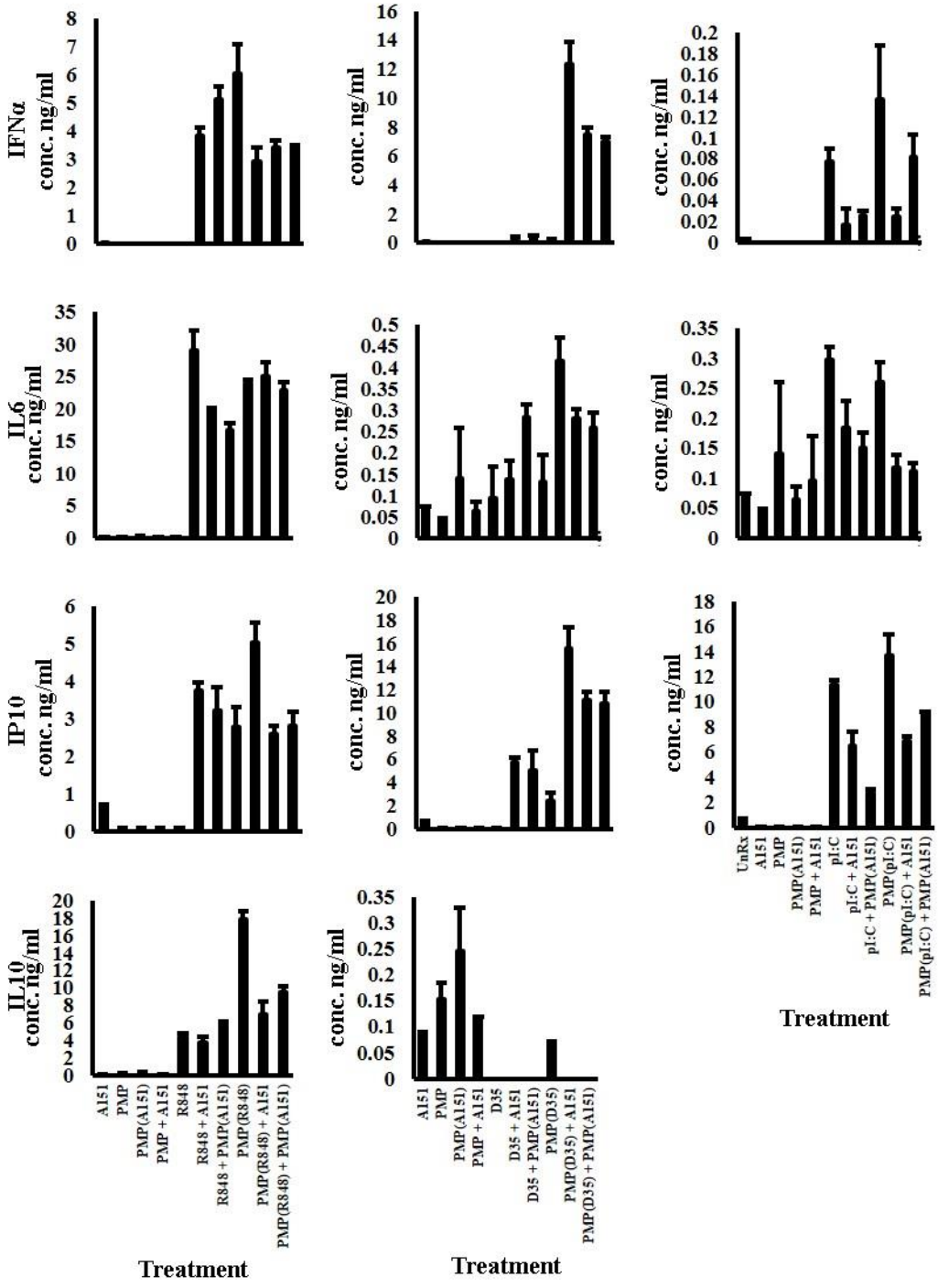
APPENDIX

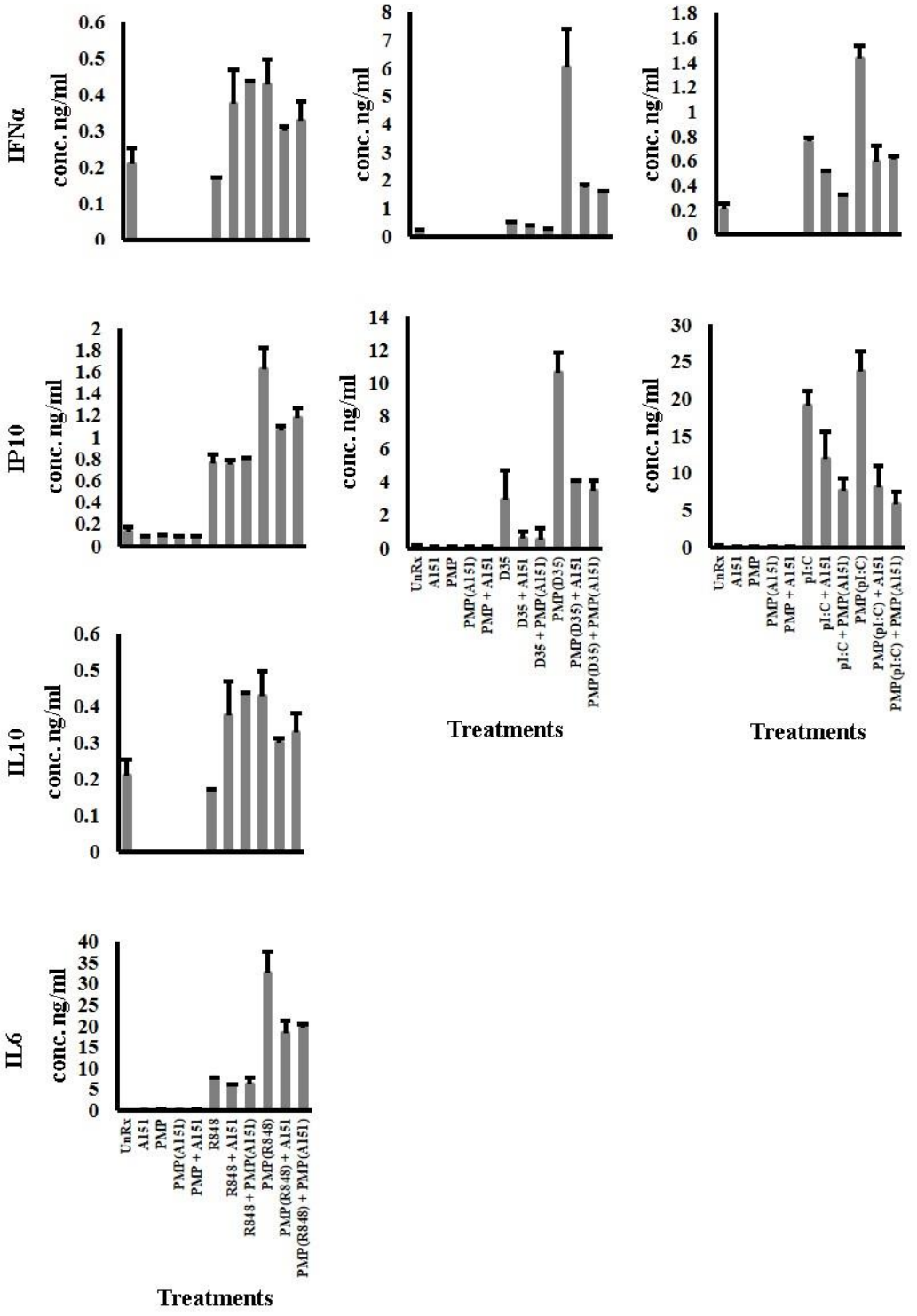


Appendix Figure 1: PMPs and TLR agonists induce cytokine responses from PBMCs. *A. PBMCs collected from healthy donor 1 were treated with 3 µg/ml Pam3CSk4 or R848 in free*

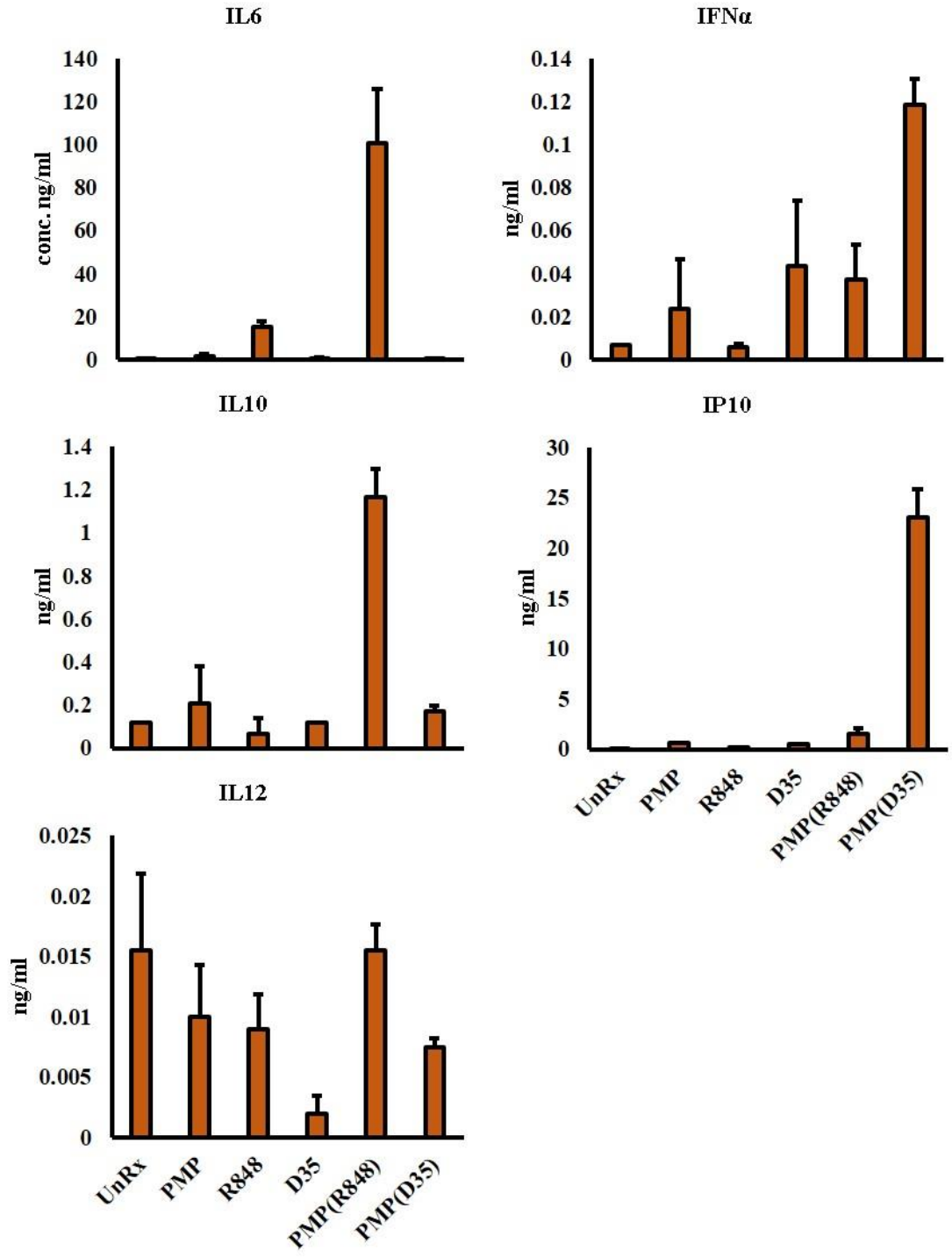
form or in PMPs and with or without 3 μM A151 for 24h. IL10, IFNα and IP10 cytokine responses were detected by ELISA. IFNα wasn't detected in Pam3Csk4 samples.



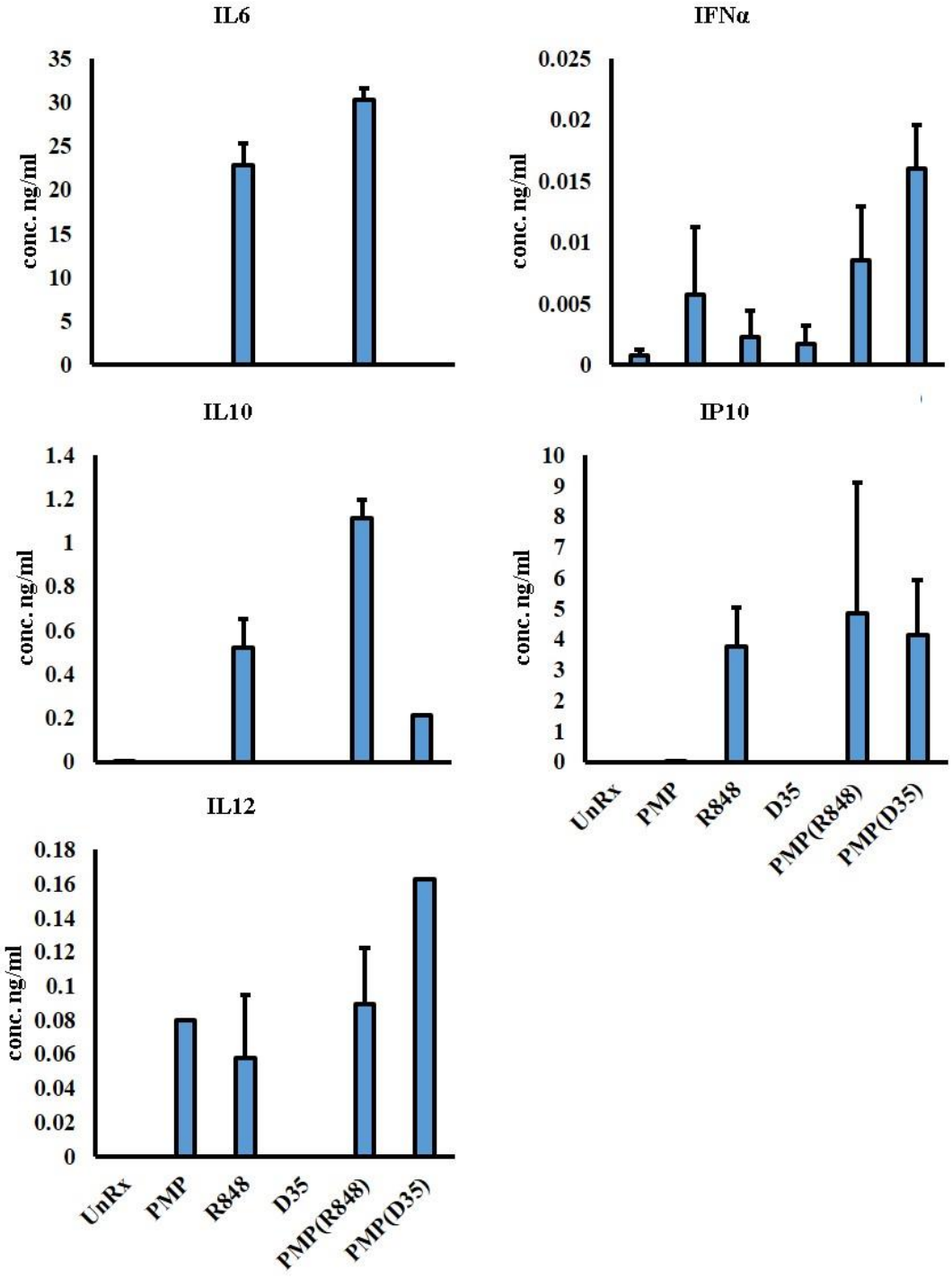
Appendix Figure 2: PMPs and TLR agonists induce cytokine responses from PBMCs. *A. PBMCs collected from healthy donor 2 were treated with 1 μg/ml R848, 1 μM D35, 30 μg/ml poly(I:C) in free form or in PMPs and with or without 3 μM A151 for 24h. IL10, IFNα and IP10 cytokine responses were detected by ELISA. IL10 wasn't detected in poly(I:C) samples.*



Appendix Figure 3: PMPs and TLR agonists induce cytokine responses from PBMCs. *A. PBMCs collected from healthy donor 3 were treated with 1 μg/ml R848, 1 μM D35, 30 μg/ml poly(I:C) in free form or in PMPs and with or without 3 μM A151 for 24h. IL10, IFNα and IP10 cytokine responses were detected by ELISA.*



Appendix Figure 4: PMPs and TLR agonists induce cytokine responses from monocytes. *CD14+ blood monocytes were isolated from PBMCs of healthy donor 1 with MACS. Macrophage differentiation was done with 25 ng/ml GM-CSF for 5 days. Generated macrophages were treated with 1 mM D35 and 1 µg/ml R848 in free form or in PMPs for 48 h .IL6, IL10, IL12, IFNα and IP10 cytokine responses were detected by ELISA.*



Appendix Figure 5: PMPs and TLR agonists induce cytokine responses from monocytes. *CD14+ blood monocytes were isolated from PBMCs of healthy donor 2 with MACS. Macrophage differentiation was done with 25 ng/ml GM-CSF for 5 days. Generated macrophages were treated with 1 mM D35 and 1 µg/ml R848 in free form or in PMPs for 48 h. IL6, IL10, IL12, IFNα and IP10 cytokine responses were detected by ELISA.*

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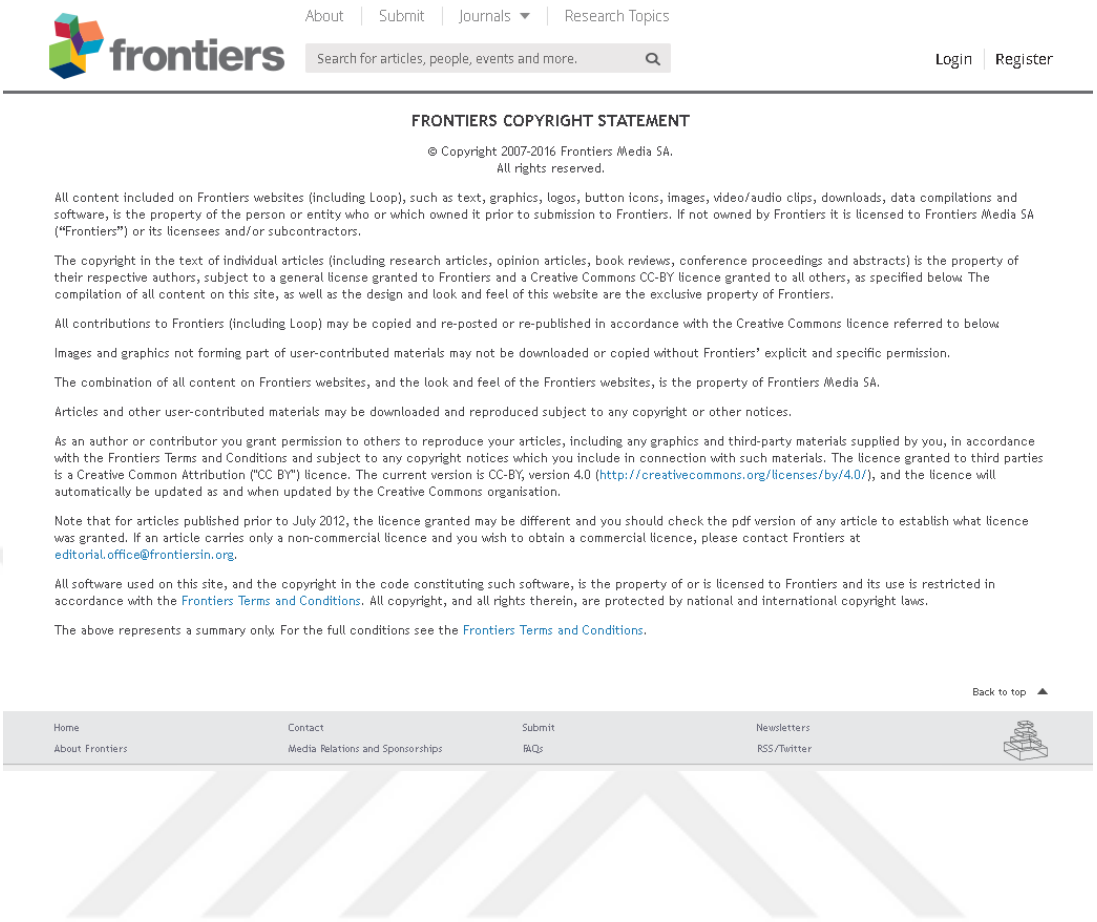

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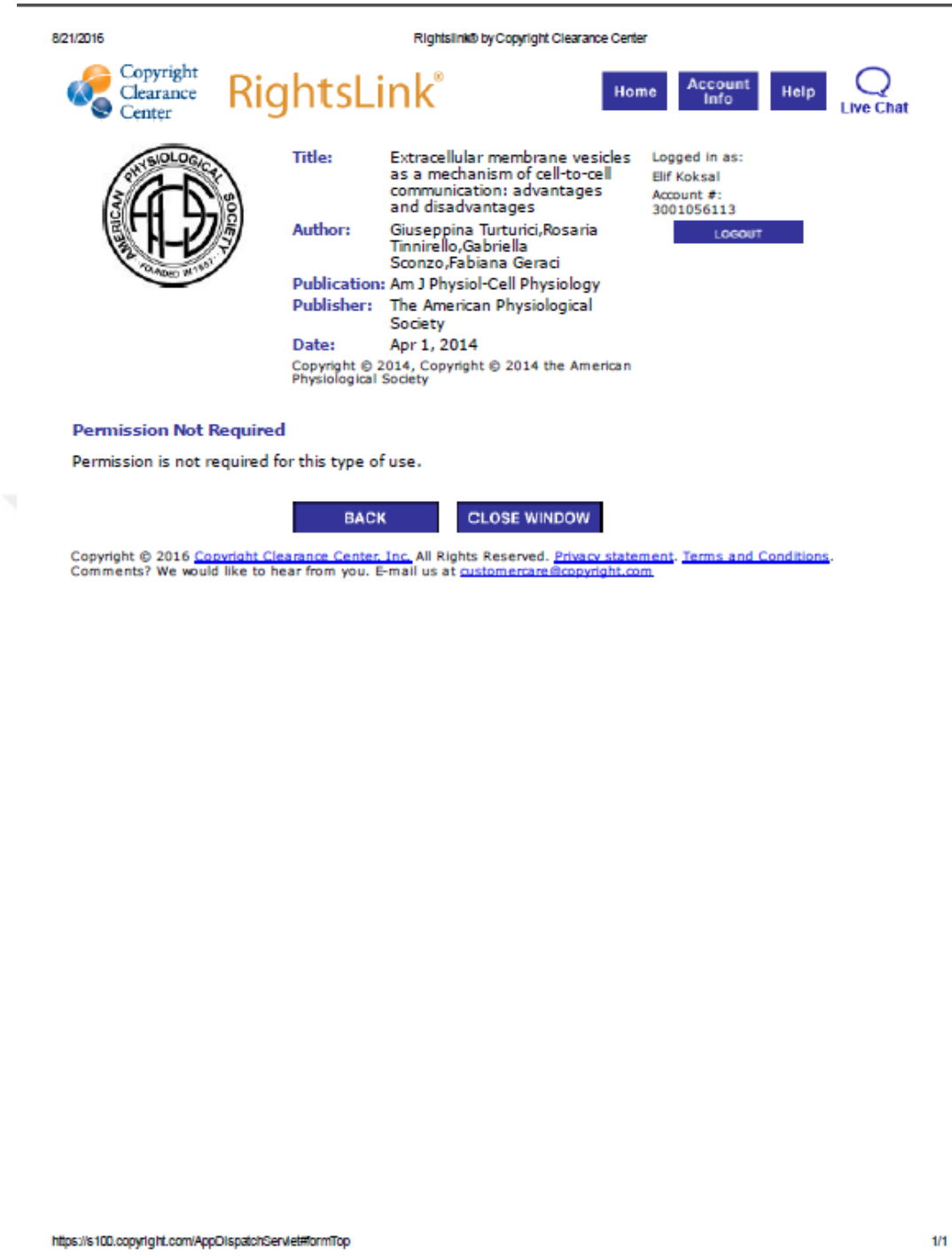

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