

**IMMUNOMODULATORY POTENTIAL OF HUMAN UMBILICAL CORD
TISSUE-DERIVED MESENCHYMAL STROMAL CELL (UCX[®]) EXOSOMES
IN COMBINATION WITH IMMUNOSUPPRESSIVE “A151”
OLIGODEOXYNUCLEOTIDE**

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IN
MOLECULAR BIOLOGY AND GENETICS

By
Özlem Bulut
July 2019

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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

IMMUNOMODULATORY POTENTIAL OF HUMAN UMBILICAL CORD TISSUE-DERIVED MESENCHYMAL STROMAL CELL (UCX[®]) EXOSOMES IN COMBINATION WITH IMMUNOSUPPRESSIVE “A151” OLIGODEOXYNUCLEOTIDE

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Mesenchymal stromal or stem cells (MSCs) modulate immune responses apart from their regenerative capacities. Accumulating evidence suggests that MSCs exert their paracrine effects through extracellular vesicles known as exosomes. In this study, we utilized a particular human umbilical cord tissue-derived MSC type termed as UCX[®]. UCX[®] is superior to the gold-standard bone marrow-derived MSCs in terms of immunosuppressive properties. We aimed to characterize and employ UCX[®] exosomes as cell-free immunosuppressive therapeutic agents. Another aim was to compare the functionalities of exosomes either isolated from 2-dimensional (2D) cultures or isolated from 3-dimensional (3D) spheroid cultures. 3D culture provides better cell-to-cell and cell-to-matrix interactions thereby mimics the *in vivo* environment better. A synthetic oligodeoxynucleotide called A151 ODN, which consists of 4 repeats of the mammalian telomeric TTAGGG motif, has broad immunosuppressive effects. Delivery of A151 ODN within liposomes or exosomes protects it from degradation by nucleases and improve the desired outcome. We also aimed to combine the immunomodulatory potentials of UCX[®] exosomes and A151 ODN through direct loading of A151 ODN into exosomes with ~95% efficiency via a dehydration-rehydration-based lyophilization method. First, we determined the binding and internalization kinetics of exosomes with different immune cells. 3D-exosomes interacted with the target cells much faster and more efficiently. Next, we investigated how UCX[®] exosomes influence Toll-like receptor (TLR) signaling in mouse splenocytes and bone marrow-derived macrophages (BMDMs). 3D-exosomes compared to 2D-exosomes were more potent to suppress IFN γ , IL6, IL12, and to a lesser extent TNF α production mediated by TLR1/2, TLR4, TLR7/8 and TLR9 but not by TLR3 triggering. A151 ODN-loading to either 2D- or 3D-exosomes improved the inhibition of all the above mentioned pro-inflammatory cytokines. Especially 3D-exosomes downregulated co-stimulatory molecules CD80 and CD86 along with MHC-II on BMDMs following TLR stimulation. Macrophage polarization experiments revealed that UCX[®] exosomes reprogram BMDMs to produce high amounts of nitric oxide and arginase-1 which are the key immunomodulatory factors induced by myeloid-derived suppressor cells (MDSCs) to inhibit T- and NK-cell activity. Besides shifting macrophages to an MDSC-like suppressive phenotype, exosomes also supported expansion of MDSC populations *in vivo* upon intraperitoneal injection. Next, we tested the therapeutic efficiency of UCX[®] exosomes with or without A151 ODN in zymosan-induced peritonitis and dextran sodium sulfate (DSS)-induced colitis models in

mice. Exosomes could not alleviate zymosan-induced peritonitis which is an acute and severe inflammation. However, 3D-exosomes and A151 ODN-loaded versions of both 2D- and 3D-exosomes remarkably prevented DSS-induced colitis progression. A151 ODN itself was also therapeutic, albeit to a lesser degree. Standalone 3D-exosomes and A151 ODN-loaded exosomes prevented weight loss and colon shortening. All treatments except for 2D-exosomes could restore DSS-induced loss of T-cell numbers and cytokine-producing capacities in mesenteric lymph nodes and spleen. All treatments except for A151 ODN prevented DSS-induced macrophage accumulation in the lymph nodes. 3D-exosomes and A151 ODN-loaded versions of both exosomes normalized serum IL6 levels while only A151 ODN-loaded 3D-exosomes could impact the cytokine production capacities of macrophages. Finally, we tested the effects of UCX[®] exosomes with or without A151 ODN on wound healing. *In vitro*, 2D- and 3D-exosomes differentially upregulated the productions of wound healing-related cytokines and growth factors such as IL1 α , TGF β and VEGF α from fibroblast and keratinocyte cell lines. *In vivo*, in an excisional wound healing model, free or A151 ODN-loaded exosomes did not accelerate wound closure. However, they caused systemic immunosuppression at the late stages of wound healing. Systemic outcomes include reduced inflammatory capacity of macrophages and higher granulocytic MDSC numbers in spleen. A151 ODN-loaded 3D-exosomes also reduced T-cell numbers in spleen and pro-inflammatory cytokine levels in circulation. Taken together, this study revealed that 2D- but more importantly 3D-culturing of umbilical cord MSCs result in functionally different exosomes, 3D culture-derived exosomes display higher immunosuppressive potential, A151 ODN-loading into these exosomes improves immunosuppressive capacity and A151 ODN-loaded UCX[®] exosomes could be a valuable therapeutic agent for inflammatory and autoimmune disorders.

Keywords: Mesenchymal stromal cells, mesenchymal stem cells, 3D culture, exosomes, A151 ODN, TLR, MDSCs, inflammation, immunosuppressive, immunomodulatory

ÖZET

İNSAN GÖBEK KORDONU DOKUSUNDAN ELDE EDİLEN MEZENKİMAL KÖK HÜCRE (UCX[®]) EKSOZOMLARININ İMMÜNBASKILAYICI OLİGODEOKSİNÜKLEOTİD A151 İLE BİRLİKTE İMMÜN DÜZENLEYİCİ ETKİLERİ

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Mezenkimal stromal hücreler veya mezenkimal kök hücreler (MKHler) doku yenileyici özelliklerinin yanında immün yanıtı düzenleyici özelliklere de sahiptir. Araştırmalar MKHlerin etkilerinin çoğunun parakrin bir şekilde ve eksozom adı verilen hücre dışı kesecikler ile sağlandığını göstermektedir. Bu çalışmada, patentli ve özel bir MKH türü olan, göbek kordonu dokusundan elde edilen ve UCX[®] adı verilen MKHler kullanılmıştır. UCX[®] hücreleri immün baskılayıcılık açısından altın standart olan kemik iliği MKHlerinden üstündür. Çalışmanın amacı UCX[®] eksozomlarını karakterize etmek ve immün düzenleyici terapi yöntemi olarak test etmektir. Bir başka amaç da 2 boyutlu (2D) hücre kültürü ile 3 boyutlu (3D) hücre kültüründen elde edilen eksozomların fonksiyonel olarak karşılaştırılmasıdır. 3D kültür hücreler arasında ve hücreler ile hücre dışı matris arasında daha fazla etkileşim sağladığı için canlı organizmadaki duruma daha yakındır. A151 ODN adı verilen ve memeli telomerlerindeki TTAGGG motifinin 4 tekrarından oluşan sentetik oligodeoksinükleotid geniş kapsamlı immün baskılayıcı etkilere sahiptir. A151 ODN'in lipozom veya eksozomlara yüklenmesi nükleazlar tarafından parçalanmasını önleyerek etkinliğini artırmaktadır. Bu çalışmada, A151 ODN'i UCX[®] eksozomlarına yükleyerek iki ajanın immün düzenleyici etkilerini birleştirmek ve sinerjik etki elde etmek amaçlanmıştır. Grubumuzca geliştirilen yükleme tekniğiyle A151 ODN eksozomlara yaklaşık %95 verimle yüklenebilmiştir. Öncelikle, farklı immün hücrelerin UCX[®] eksozomlarına bağlanma ve eksozomları içine alma kapasiteleri incelenmiş ve 3D eksozomların hedef hücrelerle daha çabuk ve daha fazla etkileşime girdiği görülmüştür. Eksozomların Toll-benzeri reseptör (TLR) yolaklarına etkisi fare dalak hücrelerinde ve kemik iliğinden farklılaştırılmış makrofajlarda (BMDMler) incelendiğinde, eksozomların TLR1/2, TLR4, TLR7/8 ve TLR9 yolaklarının aktivasyonu sonucu gerçekleşen IFN γ , IL6, IL12, ve daha az ölçüde TNF α üretimini bastırabildiği ve 3D eksozomların genel olarak 2D eksozomlardan daha etkin olduğu görülmüştür. Eksozomlara A151 ODN yüklenmesi enflamatuvar sitokinlerin baskılanmasını artırmıştır. Ayrıca eksozomlar, özellikle 3D eksozomlar, BMDMlerde TLR yolaklarının aktive edilmesi üzerine artan yardımcı uyarıcı CD80 ve CD86 molekülleri ile antijen sunucu MHC-II molekülünün miktarlarını azaltmıştır. Makrofaj polarizasyonu deneyleri ise eksozomların BMDMleri yüksek miktarda nitrik oksit ve arjinaz üretecek şekilde programladığını göstermiştir. Bu iki faktör, myeloid kökenli baskılayıcı hücrelerin (MDSCler) T ve NK hücrelerini baskılamakta kullandıkları ana faktörlerdir. Makrofajları MDSC benzeri baskılayıcı bir fenotipe

çekmelerinin yanı sıra, eksozomlar farelerde karın içi enjeksiyon yoluyla uygulandıklarında MDSC popülasyonlarını artırmışlardır. UCX® eksozomlarının A151 ODN varlığında ve yokluğunda immün düzenleyici etkileri farelerde zymosan ile uyarılmış peritonit modeli ve dekstran sodyum sülfat ile uyarılmış kolit modelinde test edilmiştir. Akut ve şiddetli bir enflamasyon olan peritonit modelinde eksozomlar enflamasyonu baskılayamasa da, kolit modelinde özellikle tek başına 3D-eksozomlar ve iki eksozom tipinin de A151 ODN yüklü halleri hastalığın ilerlemesini durdurabilmişlerdir. Boş veya A151 ODN yüklü 3D-eksozomlar kilo kaybını ve kolon boyutlarındaki kısılmayı engellemiş, lenf nodları ve dalaktaki DSS kaynaklı T-hücre kaybını ve T-hücrelerinden sitokin salgımadaki disregülasyonu onarabilmiştir. A151 ODN yüklenmiş veya yüklenmemiş bütün eksozomlar ise lenf nodlarına DSS kaynaklı makrofaj birikmesini önlemiştir. 3D eksozomlar ve iki eksozom tipinin de A151 ODN yüklü halleri serumda DSS ile artan IL6 miktarını normalize edebilmiş ama sadece A151 ODN yüklü 3D-eksozomlar makrofajların tekrar uyarım sonucu sitokin üretim kapasitesinde etkili olmuştur. Son olarak, eksozomların A151 ODN varlığında ve yokluğunda yara iyileşmesine etkileri araştırılmıştır. 2D ve 3D eksozomlar fibroblast ve keratinosit hücre hatlarında yara iyileşmesi ile alakalı IL1 α , TGF β ve VEGF α gibi faktörlerin üretimini farklı derecelerde artırmıştır. Farede yara iyileşmesi modelinde A151 ODN varlığında ya da yokluğunda eksozomlar yara iyileşmesini hızlandırmamış olsa da iyileşmenin son safhalarında sistemik bir immün baskılanmaya sebep olmuşlardır. Bu sistemik etkilerin içerisinde makrofajların enflamatuar kapasitlerinde azalış ve granülositik MDSC popülasyonunda artış vardır. A151 ODN yüklü 3D eksozomlar ayrıca T hücre sayılarını ve dolaşımdaki enflamatuar sitokin miktarlarını da düşürmüştür. Topluca ele alındığında bu çalışma 2D ve 3D hücre kültürlerinden elde edilen eksozomların fonksiyonel açıdan farklı olduğunu, 3D kültürden izole edilen eksozomların daha immün baskılayıcı olduğunu, A151 ODN yüklenmesinin eksozomların immün baskılayıcı kapasitlerini artırdığını ve A151 ODN yüklü UCX® eksozomlarının enflamatuar ve otoimmün hastalıklarda değerli bir tedavi yaklaşımı olabileceğini göstermektedir.

Anahtar Sözcükler: Mezenkimal stromal hücre, mezenkimal kök hücre, 3 boyutlu hücre kültürü, eksozom, A151 ODN, TLR, MDSC, enflamasyon, immün baskılayıcı, immün düzenleyici



To my beloved family

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Abbreviations

α GC	α -galactosylceramide
2D	Two dimensional
3D	Three dimensional
ACK	Ammonium-chloride-potassium
AICDA	Activation induced cytidine deaminase
AP-1	Activator protein 1
ARG1	Arginase 1
ATMP	Advanced therapy medicinal product
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
BP	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
cGAS	Cyclic GAMP synthase
CHIL3	Chitinase-like 3
CIA	Collagen-induced arthritis
CM	Conditioned media
COX2	Prostaglandin-endoperoxide synthase 2 / Cyclooxygenase 2
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DSS	Dextran sodium sulfate
DTH	Delayed-type hypersensitivity
ECM	Extracellular matrix
EGF	Epidermal growth factor

ESC	Embryonic stem cell
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
FasL	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSC	Forward scatter
G-MDSC	Granulocytic myeloid-derived suppressor cell
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPx	Glutathione peroxidase
GvHD	Graft-versus-host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HMGB1	High mobility group protein B1
HSC	Hematopoietic stem cell
HSP	Heat-shock protein
I/R	Ischemia/reperfusion
ICAM	Intercellular adhesion molecule
IFI16	IFN γ -inducible protein 16
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ILV	Intraluminal vesicle
iNKT	Invariant natural killer T
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IRF	Interferon regulatory factor
IV	Intravenous
KGF	Keratinocyte growth factor
LBPA	Lysobisphosphatidic acid
LIF	Leukemia inhibitory factor

LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
M-CSF	Macrophage colony stimulating factor
M-MDSC	Monocytic myeloid-derived suppressor cell
MBV	Multivesicular body
MCP1	Monocyte chemoattractant protein 1
MDSC	Myeloid-derived suppressor cell
MEM	Minimum essential medium
MHC	Major histocompatibility complex
miRNA	microRNA
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal/stem cell
MyD88	Myeloid differentiation factor 88
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated T-cells
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAM3CSK4	Pam3CysSerLys4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-L1	Programmed death-ligand 1
PDGF	Platelet-derived growth factor
PEC	Peritoneal exudate cell
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C

PMA	Phorbol 12-myristate 13-acetate
PNPP	p-nitrophenyl phosphate
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
R848	Resiquimod
RA	Rheumatoid arthritis
RCF	Relative centrifugal force
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SA-ALP	Streptavidin - alkaline phosphatase
SCF	Stem cell factor
SD	Standard deviation
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
SOD	Superoxide dismutase
SP-DiOC	3,3'-dioctadecyl-5,5'-di(4-sulfophenyl)oxacarbocyanine sodium salt
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
Sup ODN	Immunosuppressive oligodeoxynucleotide
TBS	Tris buffered saline
TBS-T	Tris buffered saline - Tween 20
TCR	T-cell receptor
TGF β	Transforming growth factor beta
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor

TNBS	2,4,6-trinitro benzene sulfonic acid
TNF α	Tumor necrosis factor alpha
Treg	Regulatory T-cell
TRIF	TIR domain-containing adaptor protein
TSG	Tumor susceptibility gene
UC	Umbilical cord
UCX [®]	Human umbilical cord tissue-derived mesenchymal stromal cells
VEGF	Vascular endothelial growth factor



Chapter 1

Introduction

1.1 The Immune System

The immune system is made up of myriads of molecules and effector cells that work to detect and eliminate infectious agents or toxins and thus protect the body from the damage they cause. Immune cells, also termed leukocytes, originate from pluripotent hematopoietic stem cells (HSCs) in the bone marrow and most of them develop and mature there. Mature immune cells can circulate in blood and the lymphatic system or they can be tissue-resident cells. HSCs give rise to two main leukocyte lineages: myeloid and lymphoid. B- and T-lymphocytes, natural killer cells and innate lymphoid cells (ILCs) belong to the lymphoid lineage whereas platelets, erythrocytes, neutrophils, eosinophils, basophils, mast cells, monocytes and macrophages originate from the myeloid lineage [1]. Dendritic cells (DCs) are generally considered to be myeloid cells but they may also originate from lymphoid progenitors [2].

In terms of antigen specificity, rapidity and the length of response, immune system is divided into innate and adaptive arms. Innate immunity recognizes common microbial patterns via various pattern recognition receptors (PRRs) and mounts a response that is not antigen-specific while cells of adaptive immunity have specific antigen receptors and they are capable of developing immunological memory for the antigens that they recognize. Innate responses occur more acutely upon immediate recognition of a pathogen or danger-associated molecular pattern but adaptive responses require days for proliferation and activation of antigen-specific cells upon antigen recognition and for memory cell formation [3]. Although the adaptive response occurs later, the protection it confers can be lifelong through memory B- and T-cells.

1.1.1 Innate Immunity

Many invading pathogens are detected and eliminated by the innate immune system within hours or even minutes before they could cause any damage. Earlier innate immune defense includes physical mucosal and epithelial barriers to prevent the entry of pathogens. Epithelial cells and phagocytes at these first lines of defense secrete antimicrobial peptides and proteins causing the disruption of cell walls or membranes of bacteria and fungi [4]. Complement system comprising of many plasma proteins is another component of the innate immunity. The complement cascade marks pathogens for lysis or phagocytosis [5]. Since many pathogens have evolved to evade these defense mechanisms, physical and chemical barriers may fail. In that case, the pathogen-associated molecular patterns (PAMPs) present on pathogens or danger-associated molecular patterns (DAMPs) resulting from pathogen- or injury-caused tissue damage are recognized by germline-encoded pattern recognition receptors (PRRs) found

on innate immune cells [6]. Once activated through PAMP or DAMP recognition, phagocytes may destroy pathogens directly. They also produce numerous chemokines, cytokines and upregulate co-stimulatory molecules in order to help initiate an adaptive response if innate immunity fails to clear the infection.

1.1.1.1 Pattern Recognition Receptors (PRRs)

PRRs that recognize PAMPs and DAMPs are mainly expressed on phagocytes which are dendritic cells, macrophages and neutrophils. In terms of downstream function, PRRs can be classified in two groups as phagocytic PRRs and signaling PRRs. Phagocytic receptors include scavenger receptors that can bind to numerous bacterial ligands, complement receptors recognizing complement-coated pathogens, C-type lectins such as Dectin-1 which recognizes β -glucans of fungal cell walls and mannose receptor which recognizes mannosylated ligands found on bacteria, fungi and viruses [7, 8].

The most heavily studied signaling PRRs are Toll-like receptors (TLRs). They are described in detail in Section 1.1.1.1.1. NOD-like receptors (NLRs) are another class of PRRs. Cytoplasmic NOD1 and NOD2 can recognize bacterial cell wall components and lead to production of pro-inflammatory cytokines through NF κ B pathway [9]. Some NLR proteins such as NLRP3, NLRP1, NLRP6 and NLRC4 react to various cellular damage-associated signals and induce pro-inflammatory cytokine production and cell death through a multiprotein complex called the inflammasome [10]. RIG-I-like receptors (RLRs), another PRR family, are able to sense viral RNA in cytoplasm and induce production of type I interferons [11]. Type I interferons are also induced when cyclic GAMP synthase (cGAS) recognizes cytosolic bacterial or viral DNA and activates the stimulator of interferon genes (STING) [12].

1.1.1.1.1 Toll-like Receptors (TLRs)

Toll receptor was initially discovered in *Drosophila melanogaster* as an inducer of host defense mechanisms [13]. Then, its homologs Toll-like receptors (TLRs) were identified in mammals. Humans express 10 TLRs while mice express 12. Each TLR recognizes distinct PAMPs. TLRs are found either on the cell surface or on the endosomal membrane. Extracellular regions of TLRs are composed of leucine-rich repeats (LRR) and the cytoplasmic tails contain a TIR domain which can interact with other TIR domain-containing signaling molecules such as MyD88 and TRIF. TLRs might act as homodimers or heterodimers such as TLR1:TLR2 and TLR2:TLR6. Signaling events downstream of TLR activation result in the activation of interferon regulatory factor (IRF), nuclear factor κ -light-chain-enhancer of activated T-cells (NF- κ B) and activator protein 1 (AP-1) transcription factors leading to expression of type I interferons or pro-inflammatory cytokines such as tumor necrosis factor α (TNF α), IL-6 and IL-12 [14]. Signaling pathways of TLRs are displayed in detail in Figure 1.1.

Cell surface mammalian TLRs are TLR1, TLR2, TLR4, TLR5 and TLR6. Heterodimers of TLR1 or TLR6 with TLR2 recognize various PAMPs including lipoteichoic acid of Gram-positive bacteria,

diacyl or triacyl lipopeptides of Gram-negative bacteria, lipomannans of mycobacteria and β -glucans of fungal or bacterial cell walls [15]. They are found in macrophages, DCs, mast cells and granulocytes. TLR4, also found on macrophages, DCs, mast cells and eosinophils, binds to lipopolysaccharide (LPS) of Gram-negative bacteria but it also needs an accessory protein called MD-2 [16]. TLR5 which is the receptor for bacterial flagellin is present on macrophages, DCs and epithelial cells [17]. The endosomal TLRs TLR3, TLR7, TLR8 and TLR9 are all nucleic acid sensors. TLR3 which is mainly expressed on macrophages, DCs and epithelial cells is activated by viral double-stranded RNA [18]. TLR7 and TLR9 are expressed in T-cells, plasmacytoid DCs and eosinophils while TLR8 is mainly found in macrophages and neutrophils. TLR7 and TLR8 sense single-stranded RNA of viral origin and in cases such as tissue damage they also respond to self-derived single stranded RNA [19]. TLR9's ligand is the unmethylated CpG motifs present in bacterial DNA [20]. Synthetic ligands are available for studying TLRs such as PAM3CSK4 for TLR1:TLR2, poly(I:C) for TLR3, Resiquimod (R848) for TLR7/8 and CpG oligodeoxynucleotides (ODNs) for TLR9 all of which are used in this study. Apart from the hematopoietic cells mentioned above, certain types of TLRs, primarily TLR3 and TLR4, can be found on other cell types including mesenchymal stem cells, fibroblasts and keratinocytes all of which are addressed in this study [21, 22]. TLR functions on these cells are critical for immune modulation, tissue remodeling and wound healing which is explained in more detail in Sections 1.2.3 and 1.5.3.

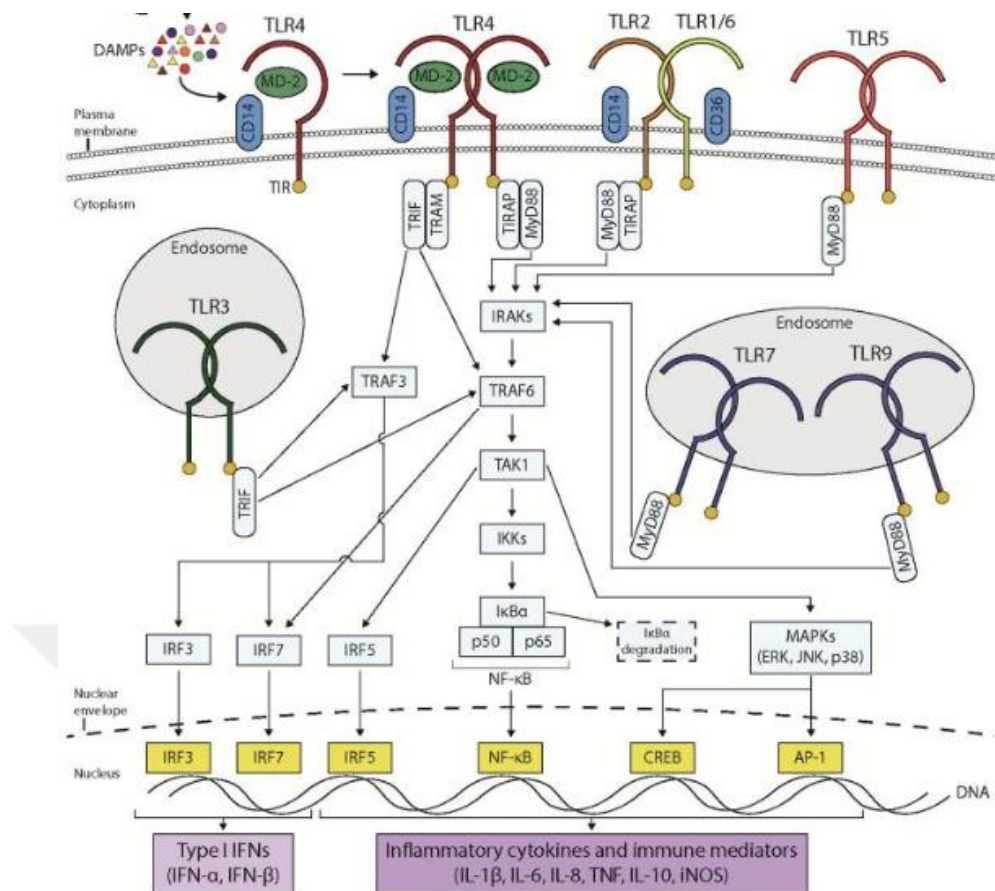


Figure 1.1. Signaling pathways of TLRs 1-9 [23].

1.1.1.2 Anti-Inflammatory Myeloid Cells

1.1.1.2.1 Alternatively Activated Macrophages (M2)

Cells of monocyte/macrophage lineage have great plasticity and therefore heterogeneity in the populations. Differential states of macrophage activation have been broadly divided into two and termed as classically activated macrophages (M1) and alternatively activated macrophages (M2) [24].

M1 activation is induced by type 1 T helper (Th1) cells producing the pro-inflammatory cytokine IFN γ in response to intracellular infection. Microbial patterns such as LPS and cytokines like TNF α also contribute to M1 induction. M1 cells produce microbicidal nitric oxide (NO) and pro-inflammatory cytokines such as IL-6 and TNF α thereby contributing to type 1 inflammation and tumor resistance [25].

Alternative M2 activation is promoted by Th2 cytokines IL-4 and IL-13. M2-macrophages promote type 2 immunity, parasitic clearance, tissue repair and remodeling, and tumor progression in the form of tumor-associated macrophages [26]. They produce anti-inflammatory cytokines such as IL-10, transforming growth factor β (TGF β), IL-1 receptor antagonist (IL-1Ra), and upregulate scavenger receptors and mannose receptor CD206 which aid endocytosis and clearance [27, 28]. M2-macrophages are critically different from M1-macrophages in terms of arginine metabolism. Arginase-1 (Arg1) which metabolizes l-arginine is upregulated in M2 macrophages. Arginine metabolites ornithine and proline

help tissue remodeling and repair by increasing tissue contractility and promoting collagen synthesis [29]. Moreover, arginase-1 inhibits T-cell responses by depleting l-arginine required for T-cell receptor (TCR) expression and function [30].

Even after macrophages are polarized into M1 or M2 state, it is not a terminal differentiation. Due to this high degree of plasticity depending on environmental signals, ways of manipulating the M1/M2 balance provide attractive therapeutic opportunities.

1.1.1.2.2 Myeloid Derived Suppressor Cells (MDSCs)

MDSCs are an intriguing heterogeneous population of immature myeloid cells with immunosuppressive functions that are expanded in cases of infection, inflammation and cancer in order to negatively regulate the immune response [31]. MDSCs emerge when usual differentiation of immature myeloid cells to dendritic cells, macrophages or granulocytes is blocked due to a pathological condition. However, under appropriate cytokine milieu, MDSCs can differentiate into mature myeloid cell populations. In mice, two MDSC subpopulations are defined: granulocytic MDSCs (G-MDSCs) that are polymorphonuclear and $CD11b^+ Ly6G^+ Ly6C^{low}$, and monocytic MDSCs (M-MDSCs) $CD11b^+ Ly6G^- Ly6C^{high}$ with monocyte-like morphology [32]. Signals that induce MDSC expansion include IL-6, macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2) [33]. All these factors activate JAK/STAT signaling, particularly signal transducer and activator of transcription 3 (STAT3) which is crucial for MDSC expansion and function [34].

Main players of MDSC-mediated immunosuppression are arginase-1 and inducible nitric oxide synthase both of which metabolize l-arginine. Arginase-1 converts l-arginine to l-ornithine and urea while iNOS generates citrulline and NO [35]. MDSCs inhibit T-cell function through l-arginine depletion causing CD3 ζ -chain downregulation and cell cycle arrest while also inhibiting T-cell proliferation and IFN γ production [36]. NO produced by iNOS blocks T-cell proliferation via disrupting JAK3/STAT5 signaling [37]. Another mechanism of T-cell inhibition by MDSCs depend on the engagement of programmed death-ligand 1 (PD-L1) upregulated on the MDSC surface with its receptor PD-1 on T-lymphocytes [38]. MDSCs also induce regulatory T-cell (Treg) development by producing TGF β and IL-10 [39]. The remarkable T-cell suppression capacity of MDSCs involving both contact-dependent and independent mechanisms make these cells appealing therapy targets.

1.2 Mesenchymal Stromal Cells (MSCs)

Mesenchymal stromal cells, also called mesenchymal stem cells (MSCs), are multipotent plastic-adherent cells capable of self-renewal and differentiation to mesenchymal lineages, mainly adipogenic, chondrogenic and osteogenic [40]. In addition to this tri-lineage differentiation potential, MSCs can also give rise to other cell types including hepatocytes [41] and neurons [42]. Different MSC populations with distinct phenotypes and differentiation capacities exist in the body in various niches (detailed in

Section 1.2.1). However, these cells share common features that are used to characterize a cell as an MSC. According to International Society for Cellular Therapy's minimal criteria, in addition to having self-renewal and tri-lineage differentiation capacity, MSCs are characterized as expressing CD73, CD90 and CD105 surface markers while lacking the pan-leukocyte marker CD45, primitive hematopoietic and endothelial marker CD34, monocytic marker CD14, T-cell marker CD19 and class II major histocompatibility complex (MHC) molecule HLA-DR [43]. Due to their remarkable differentiation capacity, MSCs have long been an interest for regenerative medicine purposes. In the past two decades, their role in immune system has also attracted a great amount of attention. Regenerative and immunomodulatory capacities of MSCs are discussed in more detail in the following sections.

1.2.1 MSC Types and Niches in Human Body

MSCs were first discovered in the bone marrow in 1970 as colony forming units that are able to form fibroblastoid colonies [44]. Since then, they have been isolated from umbilical cord blood [45], umbilical cord tissue Wharton's jelly [46], adipose tissue [47], dental pulp [48], synovial fluid [49], amniotic fluid [50], endometrium [51], menstrual blood [52] and placenta [53]. MSCs of varied sources appear to have differences in gene expression profiles, secretomes and differentiation capacities [54]. For instance, umbilical cord MSCs (UC-MSCs) has higher proliferation capacity and could be cultured for longer periods *in vitro* in contrast to bone marrow MSCs (BM-MSCs) [55, 56]. Moreover, UC-MSCs from Wharton's jelly and adipose tissue MSCs are more potent immuno-suppressors compared to BM-MSCs [57, 58]. Comparative studies imply that the source of MSCs should be carefully considered for the clinical applications being pursued.

A stem cell niche is a defined microenvironment supporting and regulating stem cell self-renewal and differentiation upon relevant stimuli. Other cell types present in the niche also help maintain stem cell behavior. The most studied stem cell niche in the body is the bone marrow hosting hematopoietic stem cells (HSCs). MSCs also contribute to the bone marrow niche but the particular MSC niche is proposed to be perivascular. MSCs are found to reside around the microvasculature of their various tissues of origin such as adipose tissue, dental pulp and placenta [59], however, they are also reported in articular cartilage which is an avascular tissue [60]. Therefore, the existence of distinct tissue-specific niches instead of a generic niche is likely and sensible considering that MSCs are not a homogenous population like HSCs but rather have distinct phenotypes, functions and migratory abilities depending on the tissue of origin.

1.2.2 Human Umbilical Cord Tissue-Derived MSCs (UCX[®])

UCX[®] cells that are used in this study are derived from the human umbilical cord tissue Wharton's jelly and isolated with an optimized and patented method [61]. The procedure results in a high yield of cells after a single adhesion and expansion phase in a much shorter period compared to the techniques that have been in use so far. MSC cultures are problematic in the sense that they lose their stemness and

therapeutic abilities in late passages. UCX[®] are advantageous in that way, since they can be cultured up to 50 generations and still preserve their genomic stability, self-renewal and differentiation capacities, and immunomodulatory effects [62]. Therefore, it is a reproducible and robust method for stem cell banking and use in clinical studies. The procedure was further refined for the production of UCX[®] as an advanced therapy medicinal product (ATMP) safe for autologous and allogeneic therapy [63].

Several preclinical studies demonstrated the therapeutic potentials of UCX[®]. UCX[®] transplantation had cardioprotective effects in a murine myocardial infarction model [64]. This was due to pro-angiogenic and anti-apoptotic paracrine mechanisms of UCX[®]. UCX[®] was also shown to have motogenic and proliferative effects on BM-MSCs, keratinocytes and fibroblasts, therefore displaying potency for tissue regeneration *in vivo* [65]. In addition to producing molecules such as hepatocyte growth factor (HGF), TGF β , VEGF and IL-6 that play important roles during different stages of wound healing, UCX[®], in contrast to BM-MSCs, secrete high amounts of keratinocyte growth factor (KGF), epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) which act in earlier phases of the wound healing process. UCX[®] were also proved to be less immunogenic than BM-MSCs and more potent immunosuppressors. In an λ -carrageenan-induced acute inflammation model, UCX[®] showed greater immunosuppressive action compared to BM-MSCs [62]. The same study revealed enhanced expression of several immunomodulatory proteins such as CD274 (PD-L1), CD273 (PD-L2), leukemia inhibitory factor and TGF β 2 by UCX[®]. The reports so far imply that UCX[®] might be a more potent alternative for regenerative and immunomodulatory clinical applications compared to the current gold standard BM-MSCs.

1.2.2.1 3D Culturing of UCX[®] Cells

A three-dimensional (3D) culture system for UCX[®] where the cells self-assemble into spheroids inside spinner flasks was developed and characterized in an attempt to allow more cell-to-cell interactions, mimicking the complex *in vivo* environment better than the traditional two-dimensional (2D) adherent culture system. Some previous studies reported that 3D culturing of MSCs results in critical changes in gene expression profiles and enhances anti-inflammatory properties and therapeutic potential [66, 67] while retaining the multi-lineage differentiation capacity [68].

3D-cultured UCX[®] cells are found to secrete higher amounts of VEGF α , matrix metalloproteinases (MMPs) 2 and 9, HGF, TGF β 1, FGF2, and granulocyte colony stimulating factor (G-CSF) which are critical for tissue remodeling, angiogenesis and immune regulation [69]. Furthermore, conditioned media of UCX[®] 3D culture (CM3D) was able to induce keratinocyte and fibroblast migration better than CM of UCX[®] 2D culture (CM2D). The same study demonstrates more complete tissue regeneration by CM3D compared to CM2D in a rat excisional wound model. Another recent study revealed that CM3D contains more anti-inflammatory proteins such as LIF, IL-10 and alleviates a murine model of rheumatoid arthritis (RA), while CM2D contains higher levels of IL-6, IL-21 and monocyte

chemoattractant protein 1 (MCP1) which have pro-inflammatory effects in RA [70]. In summary, 3D-culturing strategy could be utilized to further potentiate the regenerative and immunomodulatory actions of UCX[®] for clinical applications.

1.2.3 Immunomodulatory Actions of MSCs

MSCs are seen as promising therapeutic agents for inflammatory and autoimmune disorders, although they may exert pro-inflammatory effects in some cases depending on the factors in the microenvironment. During inflammation and tissue injury, PAMPs and DAMPs trigger the recruitment and response of immune cells via TLR signaling. In a similar way, MSCs are thought to be regulated by the TLRs they express, particularly TLR3 and TLR4. A classification similar to the M1/M2 paradigm of macrophages has been proposed for MSCs where the MSCs with pro-inflammatory responses are termed MSC1 and the anti-inflammatory MSCs are termed MSC2 [71]. TLR-4 primed MSCs (MSC1) secrete higher amounts of pro-inflammatory cytokines and chemokines such as IL-6, CXCL8, CXCL10 and RANTES, and support immune cell migration and activation. In contrast, TLR-3 primed MSCs (MSC2) potentially produce immunosuppressive molecules including indoleamine 2,3-dioxygenase (IDO) and PGE2. IDO is involved in tryptophan catabolism and generates suppressive tryptophan catabolites that are cytotoxic for T-cells [72]. PGE2 inhibits IL-2 production and responsiveness in T-cells while also suppressing NK, macrophage, DC and granulocyte functions [73]. Interactions of MSCs with innate and adaptive immunity are detailed in sections 1.2.3.1 and 1.2.3.2.

1.2.3.1 Interactions of MSCs with Innate Immunity

In homeostatic conditions and during inflammation or injury, MSCs regulate numerous aspects of innate immunity. For instance, MSCs can partially inhibit complement cascade and protect themselves from complement-mediated lysis via cell surface expression of complement inhibitors CD46, CD55 and CD59 and secretion of the soluble mediator Factor H [74, 75]. MSCs recruit neutrophils to wound sites by secreting CXCL8, causing quick neutrophil accumulation and microbial clearance at the wound site [76]. On the other hand, they can also restrain neutrophil-mediated excess tissue damage by producing superoxide dismutase 3 (SOD3) which reduces superoxide anion presence, inhibits neutrophil extracellular trap formation and release of tissue-damaging proteases [77]. MSCs can promote the migration of monocytes and macrophages to wound sites by secreting chemokines such as CCL2 (MCP-1), CCL3 and CCL12 to enhance wound repair [78]. Additionally, MSCs inhibit cytokine production, IgE secretion and degranulation of mast cells, which are the key players of allergic inflammation, in a prostaglandin-endoperoxide synthase 2 (COX2), PGE2 and TGF β 1-dependent manner [79, 80]. IL-2 induced T-cell proliferation, their cytokine production and cytotoxicity are all inhibited by MSCs which cause downregulation of activating NK receptors such as NKG2D in an IDO and PGE2-dependent manner [81]. Upon contact with MSCs, T-cells gain CD73 expression which leads to anti-inflammatory adenosine production [82, 83]. Moreover, TLR4 expression and activation in MSCs were found to be correlated with T-cell suppression [84].

MSCs downregulate the expressions of CD80, CD86, HLA-DR and CD40 in DCs, prevent their differentiation from monocytes and their maturation, decrease their IL-12 producing and T-cell activating capacities [85]. MSCs can skew mature DCs into a regulatory phenotype in a Jagged-2 or IL-10 dependent way [86, 87]. IL-6 was also suggested to drive a tolerogenic DC phenotype through SOCS1 activation leading to impaired TLR4 signaling and blocked DC maturation [88], although another study identified PGE2 but not IL-6 as the key mediator of DC maturation inhibition [89]. Another regulatory cell type induced by MSCs is alternatively activated M2 macrophages. When co-cultured with MSCs, macrophages upregulate CD206 and Arg1 expression, IL-6 and IL-10 secretion, while decreasing MCP-1, TNF α , IL-1 β and iNOS production [90, 91]. Lactate-mediated metabolic reprogramming of macrophages, PGE2 interaction with EP2 and EP4 receptors, and TSG-6 mediated NF- κ B have all been shown to contribute to the induction of anti-inflammatory M2 macrophage phenotype [92-94]. IL-1Ra secreted by MSCs was also found to be important for M1 to M2 polarization [95]. Interactions of MSCs with cell types of innate immunity are summarized in Figure 1.2.

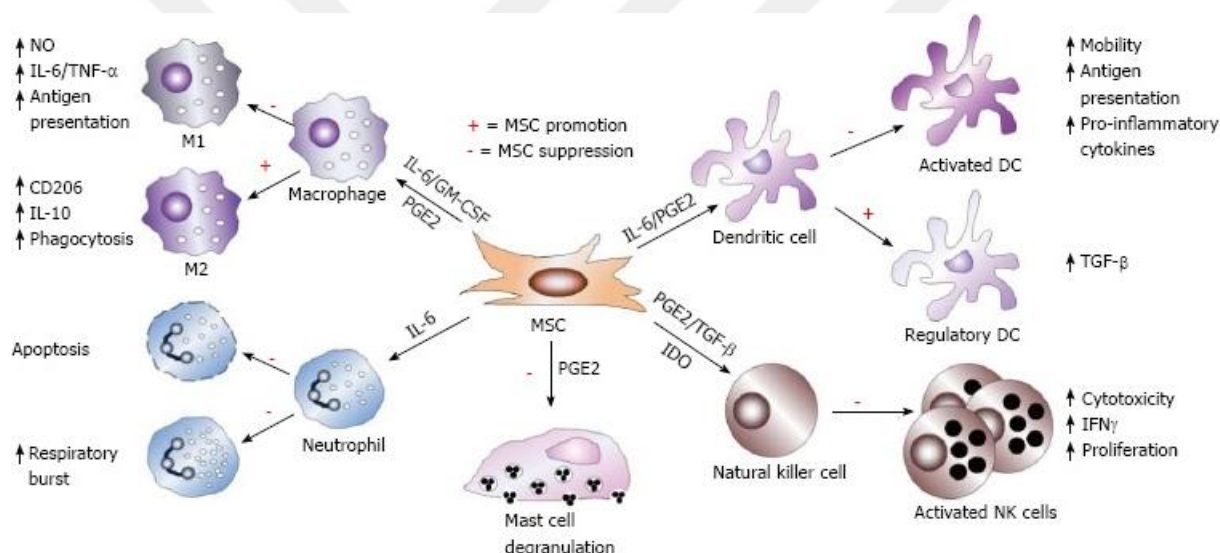


Figure 1.2. Regulation of innate immune cells by MSCs [96].

1.2.3.2 Interactions of MSCs with Adaptive Immunity

MSCs do not only regulate innate immunity but also have suppressive actions on the adaptive arm of immunity. MSCs inhibit CD4⁺ and CD8⁺ T-cell proliferation causing cell cycle arrest [97, 98], trigger the apoptosis of activated T-cells via IDO-mediated tryptophan catabolism and FasL-Fas engagement [99, 100], inhibit Th1 and Th17 differentiation of CD4⁺ T-cells while promoting Treg generation and IL-10 production [101]. Of note, suppressive action of MSCs on T-cells require the presence of inflammatory cytokines such as TNF α , IL-1 α or IL-1 β in the environment. T-cell attracting chemokines and iNOS are induced by these cytokines so that NO produced by iNOS suppresses the functions of T-cells migrating to the vicinity of MSCs [102]. MSCs exposed to IFN γ , TNF α and IL-6 were also found

to have higher IDO production [103]. Another note of interest is that the key mediator of MSC-mediated suppression is IDO in human and primate MSCs, while it is iNOS in mouse and rat MSCs [104].

T-cell proliferation and function is also inhibited by MSCs. MSCs inhibit T-cell proliferation through cell cycle arrest, prevent antibody production, and decrease the chemotactic abilities of T-cells by downregulating chemokine receptors CXCR4, CXCR5, and CCR7 [105]. Phosphorylation of elements in ERK1/2 and p38 pathways involved in T-cell viability is also decreased by MSCs [106]. Inhibition of antibody production from plasma cells by MSCs is attributed to CCL2-dependent inhibition of STAT3 and activation of the transcription factor PAX5 [107]. IL-1Ra, which contributes to MSC-mediated M2 polarization, is also important for inhibition of T-cell differentiation into plasma cells [95]. Another mechanism of T-cell suppression is PD-L1/PD-1 engagement which requires direct contact with MSCs and is enhanced in the presence of IFN γ [108]. Lastly, generation of IL-10 producing regulatory T-cells (Bregs) are also promoted by MSCs [109]. Interactions of MSCs with cell types of adaptive immunity are summarized in Figure 1.3.

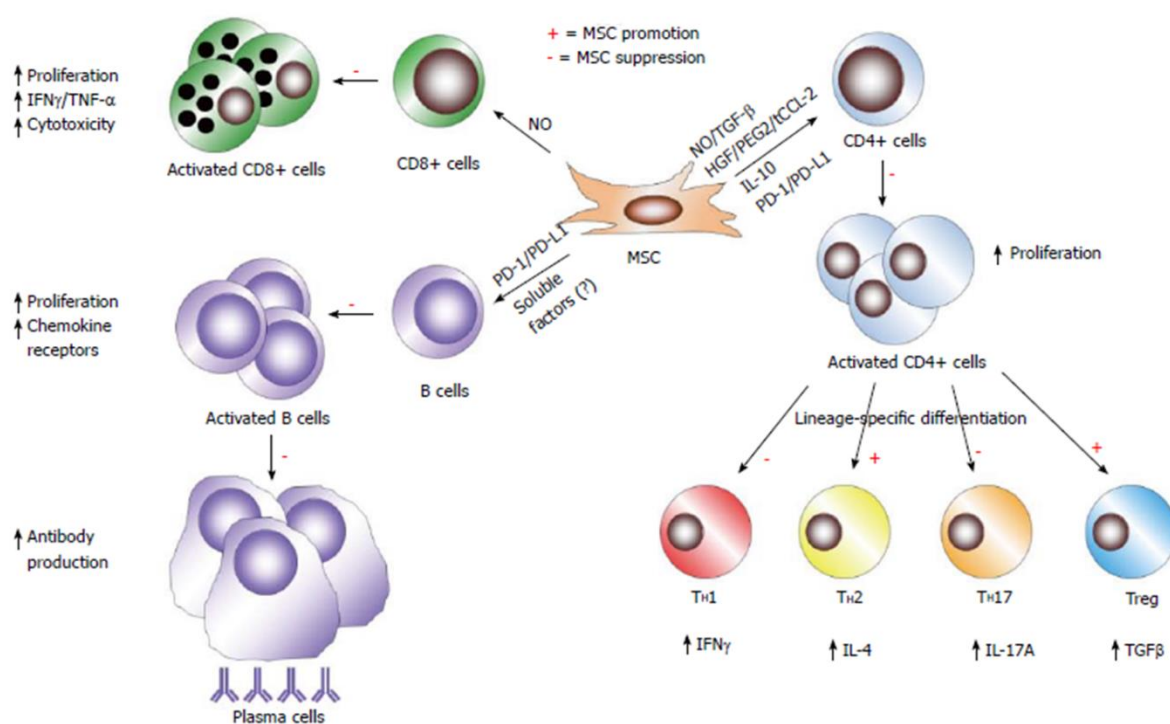


Figure 1.3. Regulation of adaptive immune cells by MSCs [96].

1.2.4 Clinical Uses of MSCs

MSCs have long been proven effective with animal models for many conditions. As a result, they are being heavily investigated in clinical trials. A search at ClinicalTrials.gov with the term “mesenchymal stem cell” results in over 900 clinical trials completed, ongoing or planned. Scope of these trials involve neurological, rheumatological, cardiovascular, hematological, inflammatory and autoimmune conditions as well as transplant rejection.

MSCs have been an interest for cartilage, bone, liver and heart regeneration due to their ability to differentiate to the cell types of these tissues. MSCs were found to engraft to the bone and promote bone growth in patients with osteogenesis imperfecta, also known as brittle bone disease, who received allogeneic bone marrow transplants [110]. Also, prenatal MSC transplantation to the fetus and postnatal booster transplantation could ameliorate skeletal damage [111]. Moreover, autologous MSC treatment with intraarticular injection was favorable to the existing treatments in terms of cartilage quality increase and pain relief for osteoarthritis patients suffering from articular cartilage degeneration [112]. UC-MSC transplantation was shown to improve liver regeneration and function in liver cirrhosis and acute-on-chronic liver failure patients [113, 114]. Furthermore, intracoronary injection of autologous BM-MSCs with or without endothelial progenitor cells improved cardiac contractility in myocardial infarction cases [115, 116]. Also for ischemic cardiomyopathy, both allogeneic and autologous BM-MSC transplantations were safe and improved the functional capacity of the heart with the autologous treatment being more effective [117].

Graft-versus-host disease (GvHD), a form of severe and lethal transplant rejection occurring after HSC transplantations, could be regressed with BM-MSC treatment irrespective of the MHC compatibility of the MSC donor [118]. MSC treatment causes immunosuppression, particularly Treg expansion, but do not increase the risk of viral infections or tumor relapse [119]. MSCs are also effective in preventing rejection in organ transplants. Autologous BM-MSCs resulted in less incidence of acute rejection, faster recovery of renal function and reduced risk of opportunistic infections in patients that underwent kidney transplants [120]. Interestingly, MSC treatment resulted in higher B-cell levels in a similar study [121]. Additionally, MSCs have shown promise for pancreatic islet cell transplantation done for the treatment of type 1 diabetes by increasing the graft vascularization aside from their immunosuppressive action [122]. Due to their remarkable immunosuppressive capacities, MSCs have also been clinically tested for inflammatory and autoimmune diseases. For instance, autologous BM-MSC treatment was able to reduce disease activity and improve mucosal healing in inflammatory Crohn's disease patients. This therapeutic efficiency was attributable to a systemic and mucosal increase in Tregs [123]. Moreover, allogeneic BM-MSC treatment to severe and drug-resistant systemic lupus erythematosus patients reduced the rates of organ dysfunction and relapse while inducing clinical remission [124]. Immunomodulatory effects of BM-MSCs were also observed in multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) patients. Upon treatment, lymphocyte proliferation and expressions of activation markers on DCs were reduced, while circulating Treg frequency was increased by MSCs [125].

1.3 Exosomes

Extracellular vesicles (EV), including microvesicles and exosomes, are small vesicles originating from endosomes or from direct budding of the plasma membrane. They enclose a part of the cytosol of the cell of origin with a lipid bilayer. Therefore, EVs can contain lipids, transmembrane and cytosolic

proteins, genomic and mitochondrial DNAs, mRNAs, long non-coding RNAs and miRNAs (Figure 1.4). EVs are a lately appreciated, very efficient and evolutionarily conserved form of intercellular communication employed by all kinds of prokaryotic and eukaryotic cells. In mammals, they can be isolated from every bodily fluid including urine, saliva, synovial fluid, cerebrospinal fluid, bronchoalveolar fluid, nasal fluid, uterine fluid, amniotic fluid, breast milk, bile, seminal plasma and blood where they carry out various physiological roles [126].

Although for a long time there has been ambiguity in the field in terms of EV classification and nomenclature, currently EVs are broadly classified into two groups: microvesicles and exosomes. Microvesicles, called oncosomes when released from cancer cells, can range between 50 and 1000 nm in diameter but the size can reach up to 10 μ m in the case of oncosomes [127]. Exosomes are much smaller and range between 50 and 150 nm in diameter. Microvesicles and exosomes also differ in their biogenesis processes which are discussed in detail in Section 1.3.1.

EVs are critical in the maintenance of homeostasis and their secretion dynamics and cargo are altered in pathological conditions. They are known to play important roles in pathogenesis of diseases and also in immune defense against insults. Roles of EVs in health and disease are summarized in Section 1.3.2. Because of their unique ability to carry critical biological molecules and genetic information over long distances while protecting their cargo from degradation that would normally occur in the extracellular spaces, EVs are attractive targets for diagnostic and therapeutic purposes which will be outlined in Sections 1.3.3 and 1.3.4. For the sake of this exosome-based study, the following sections will focus more on exosomes as the EV type of interest.

1.3.1 Exosome Biogenesis

In contrast to microvesicles arising from direct budding out of the cell membrane sequestering a part of the cytosol, exosomes are generated inside maturing endosomes, called multivesicular bodies (MBVs), and termed as intraluminal vesicles (ILVs) at that stage. ILVs arise inside MVBs through invagination of the MVB membrane and results in the encapsulation of a small portion of the cytosol. MVBs can potentially fuse with lysosomes and result in the degradation of the content. However, they can also fuse with the cell membrane resulting in the release of ILVs which are called exosomes when secreted (Figure 1.4) [128]. Studies imply different MVB subpopulations bearing distinct markers coexisting in cells. While some are driven into lysosomal degradation, some are targeted for exocytosis although the mechanisms directing the fate are not fully clear yet. One study suggests that cholesterol-enriched MVBs follow the exocytosis route [129] and another study identified lysobisphosphatidic acid (LBPA) as a marker for MVBs destined for lysosomal degradation [130]. Interestingly, further studies revealed that LBPA controls the cholesterol-bearing capacity of endosomes [131].

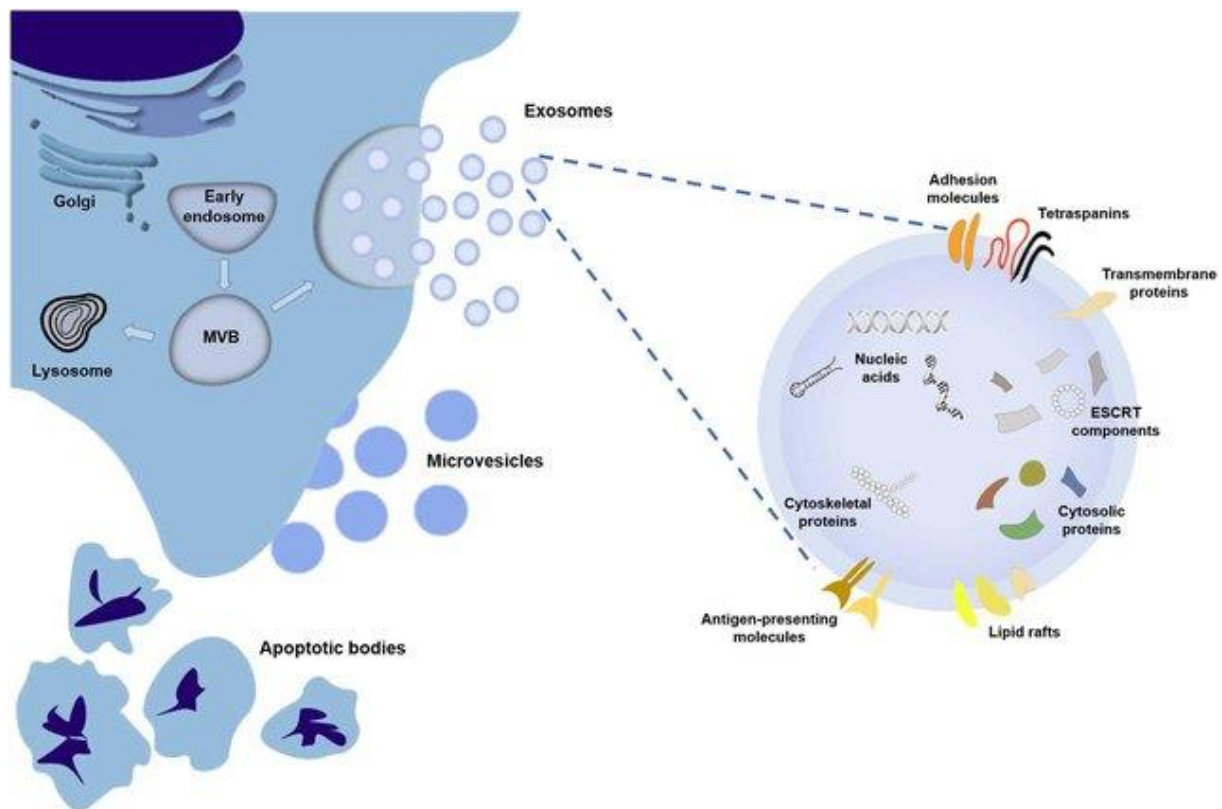


Figure 1.4. Composition and biogenesis of exosomes [132].

Creation of ILVs require a family of proteins called endosomal sorting complexes required for transport (ESCRT) recruited to the endosomal membrane. Other proteins regulating the endocytic pathway and targeting of exosome cargo to the endosomes include syntenin, syndecan and ALIX [133]. Packaging of EV cargo with the help of ESCRT system also involves ubiquitination as a key mechanism. Ubiquitination is thought to identify and recruit proteins to the newly forming MVBs. Other post-translational modifications such as palmitoylation and farnesylation are additional mechanisms believed to be contributing to protein sorting during exosome biogenesis [134]. Mechanisms of miRNA sorting into exosomes are also being revealed. For instance, proteins hnRNPA2B1 and YBX1 were identified as modulators of miRNA sorting into exosomes by recognizing specific motifs on miRNAs [135, 136]. Moreover, expression of some cargo proteins such as MHC-II aids in the recruitment of the sorting machinery and promotes MVB and ILV formation and exosome secretion [137].

1.3.2 Role of Exosomes in Health and Disease

Since exosomes are present in bodily fluids and carried in circulation, they are able to help the host cells to exert paracrine and endocrine functions. When exosomes reach the target cells, they may either interact directly by binding or may be internalized by fusion with the target plasma membrane or by endocytosis and consequently release their content into the target cell. Exosomes regulate various healthy physiological processes including antigen presentation [138], blood coagulation [139], gamete maturation [140] and immune tolerance towards fetus during pregnancy [141, 142]. Regular exosome

secretion from cells is also critical for the removal of harmful cytoplasmic DNA which induces immune response through DNA sensors when accumulated and results in reactive oxygen species (ROS) build-up [143].

In cases of cancer, exosomes regulate critical tumor-related processes such as angiogenesis, immune suppression in the microenvironment and metastasis. Hypoxic tumor cells secrete exosomes with angiogenic and metastatic properties and thus manipulate the microenvironment to promote tumor growth and invasion [144]. Melanoma exosomes were shown to alter the phenotype of bone marrow progenitor cells to be pro-vasculogenic and pro-metastatic [145]. A striking study revealed that the integrin profile of tumor-derived exosomes determine which organ the tumor will metastasize and that the arriving exosomes help establish the pre-metastatic niche when they are taken up by the organ-specific cells [146]. Moreover, tumor-derived exosomes contribute to immune evasion by inducing CD8⁺ cytotoxic T-cell apoptosis through Fas ligand (FasL) [147] and T-cell inhibition through PD-L1 expressed on the exosomes [148]. Also, tumor-derived exosomes trigger the activation and potentiate the immunosuppressive function of MDSCs in the tumor microenvironment through PGE2 and TGFβ [149] or by triggering STAT3 activation through Hsp72 [150].

Exosomes also modulate inflammation and autoimmunity. EVs originating from damaged or stressed tissues help the induction and maintenance of inflammation by carrying DAMPs such as high mobility group protein B1 (HMGB1) and heat-shock proteins (HSPs) [151]. In case of autoimmune diseases, exosomes are known to carry autoantigens and facilitate their distribution. For example, exosomes from synovial fluids of patients with rheumatoid diseases contain citrullinated proteins which serve as autoantigens in rheumatoid arthritis (RA) [152]. Pathogens such as bacteria, fungi and protozoa are able to secrete EVs that contain PAMPs and antigens inducing immune responses. Also, virally or bacterially infected cells secrete exosomes carrying virulence factors and distributing the infection to the uninfected neighboring cells [153]. HIV-1 infected cells secrete exosomes containing the accessory protein negative factor (Nef) to trigger apoptosis in uninfected CD4⁺ T-cells [154]. They were also found to contribute to the activation of latent HIV-1 reservoirs [155]. Exosomes can also facilitate the transmission of Hepatitis B and C by carrying viral nucleic acids from infected cells [156, 157]. Apart from cancer, autoimmunity and infection, exosomes have roles in numerous other conditions such as allergic, cardiovascular and neurodegenerative diseases. Treatment strategies utilizing exosomes for these diseases are discussed in Section 1.3.3.

1.3.3 Therapeutic Applications of Exosomes

Intrinsically immunosuppressive exosomes derived from tolerogenic cells are attractive treatment opportunities for inflammatory disorders. For instance, systemic or local administration of exosomes derived from genetically modified FasL-expressing DCs or IDO-expressing tolerogenic DCs were able to inhibit collagen-induced arthritis and delayed-type hypersensitivity in mice [158, 159]. EVs from

commensal bacteria *Bacteroides fragilis* induce T-cell tolerance and were effective in an experimental colitis model [160]. MSC-derived exosomes are one of the most heavily investigated exosome types due to their intrinsic regenerative and immunosuppressive capacities. Studies with MSC exosomes will be detailed in Section 1.3.5.

Immunostimulatory exosomes are used for vaccination against infectious diseases and cancer therapy. Exosomes from *Mycobacterium tuberculosis*-infected macrophages or macrophages pulsed with *M. tuberculosis* proteins protect mice against *M. tuberculosis* infection [161]. Exosomes from DCs pulsed with *Toxoplasma gondii* antigens proved to be an effective vaccination for congenital toxoplasmosis in mice [162]. EVs of a mutant *Neisseria meningitidis* strain are investigated in clinic as a promising meningitis vaccine [163]. DC exosomes loaded with iNKT-cell ligand α -galactosylceramide (α GC) and model antigen ovalbumin (OVA) induced both adaptive antitumor immunity and innate immune response dependent on NK, iNKT and $\gamma\delta$ T-cells in a mouse model [164]. Exosomes of DCs activated by tumor-derived chaperone-rich cell lysates were able to elicit a potent adaptive antitumor response in a mice intracranial glioma model [165]. Besides triggering adaptive immunity, DC exosomes are also able to enter tumor cells and increase their immunogenicity [166].

Apart from numerous promising pre-clinical studies and many clinical trials investigating exosomes as biomarkers for diagnostics and prognostics, there are several exosome-based clinical studies in different phases. Details of these trials are given in Table 1.1.

Table 1.1. Completed, ongoing and planned clinical trials utilizing exosomes or microvesicles as therapeutic agents (data collected from ClinicalTrials.gov).

Condition	Treatment	Administration	Phase	Identifier	Status
non small cell lung cancer	antigen-loaded exosomes	DC intradermal	phase 2	NCT01159288	completed
pancreas cancer with KrasG12D mutation	MSC exosomes with KrasG12D siRNA	intravenous (IV)	phase 1	NCT03608631	not yet recruiting
malignant pleural effusion and malignant ascites	chemotherapeutic drugs packaged in tumor cell microvesicles	perfusion to pleural or peritoneal cavity	phase 2	NCT01854866	unknown
malignant pleural effusion	cisplatin packaged in tumor cell microvesicles	not specified	phase 2	NCT02657460	recruiting
colon cancer	curcumin conjugated with plant exosomes	oral	phase 1	NCT01294072	active, not recruiting
acute ischemic stroke	miR-124-enriched MSC exosomes	IV	n/a	NCT03384433	not yet recruiting
diabetes mellitus type 1	MSC exosomes	IV	phase 2&3	NCT02138331	unknown
macular hole	MSC exosomes	dripping into vitreous cavity	early phase 1	NCT03437759	recruiting
bronchopulmonary dysplasia	bone marrow-derived MSC EVs	IV	phase 1	NCT03857841	not yet recruiting
cutaneous ulcer	exosome-rich plasma	topical	early phase 1	NCT02565264	recruiting
polycystic ovary syndrome	ginger and/or aloe exosomes	not specified	n/a	NCT03493984	not yet recruiting
oral mucositis associated with chemoradiation	grape exosomes	oral	phase 1	NCT01668849	active, not recruiting

1.3.4 Use of Exosomes as Nanocarriers

Exosomes are stable in circulation, they protect the contents from degradation, they have cell targeting molecules and can easily pass natural barriers such as the blood-brain barrier. All these features make exosomes excellent agents for drug delivery. A study showed that encapsulation into exosomes through direct mixing increase the bioavailability and anti-inflammatory activity of curcumin in a mouse septic shock model [167]. In another study, exosomes were used to encapsulate curcumin and a STAT3 inhibitor for efficient intranasal delivery to microglia in three neuroinflammation models [168]. Furthermore, host cells can be genetically engineered and loaded with the desired molecules. Brain-specific gene silencing was achieved in mouse brains when exosomes from DCs engineered to express a neuron-specific targeting peptide were loaded with a therapeutic siRNA by electroporation and given intravenously [169]. Exosomes purified from immature DCs engineered to express a targeting peptide fused with the exosomal membrane protein Lamp2b were loaded with doxorubicin via electroporation. Although the loading efficiency was only 20%, exosomes achieved efficient targeting and doxorubicin delivery, reducing tumor growth with low toxicity [170].

Currently used methods for drug loading into exosomes include feeding the host cell with the desired molecule for later release in exosomes, driving the sorting of desired proteins or RNAs by incorporating certain motifs to trigger the sorting machinery, expression of a fusion protein consisting of the protein of interest and a well-known exosomal protein, direct mixing of exosomes with hydrophobic molecules, and electroporation [171, 172]. In this study, we employ a novel method based on lyophilization and controlled reconstitution for oligodeoxynucleotide (ODN) loading into exosomes with more than 90% efficiency (see Section 2.2.6 and Figure 3.10).

1.3.5 Therapeutic Potential of MSC-derived Exosomes

MSCs have long been an interest for regenerative medicine applications. Although their therapeutic capacity was initially attributed to their differentiation potential into many cell types depending on the environmental signals, it is now appreciated that MSCs exert their effects mainly by paracrine action which involves exosomes along with soluble secreted proteins [173]. Therefore, MSC-derived exosomes are now in focus as a safer cell-free alternative to regular MSC-based treatments. First study reporting the therapeutic efficiency of MSC exosomes was done in 2010 in a mouse myocardial ischemia/reperfusion (I/R) injury model where the exosomes were able to reduce the infarct size [174]. Since then, MSC exosomes proved to be effective in various disease models and progressed to clinical trials for type 1 diabetes mellitus, refractory macular holes, bronchopulmonary dysplasia and acute ischemic stroke (Table 1.1).

In a mouse I/R injury model, human embryonic stem cell (ESC)-derived-MSC exosomes decreased oxidative stress, induced anti-apoptotic pathways, increased myocardial viability through PI3K/Akt pathway, and enhanced cardiac function [175]. Furthermore, two rat myocardial infarction models revealed that BM-MSCs promote angiogenesis and enhance blood flow [176] while restraining inflammatory T-cell response [177]. Exosomes of BM-MSCs engineered to overexpress GATA-4 were rich in anti-apoptotic miRNAs, particularly miR-19a, efficiently delivered them to cardiomyocytes, increased cardiomyocyte survival and restored cardiac function [178]. Human endometrium MSC exosomes exerted similar effects through miR-21-driven PTEN inhibition [179]. In an interesting study, cardiac stem cells were pre-treated with BM-MSC exosomes and then transplanted into rats with myocardial infarction. Pre-treatment of transplanted cardiac stem cells with MSC exosomes significantly reduced cardiac fibrosis and enhanced long-term cardiac function [180]. BM-MSC exosomes were also protective in hypoxic pulmonary hypertension by suppressing STAT3 signaling induced by hypoxia [181].

MSC exosomes are also promising treatments for neurological diseases. In rat and mouse stroke models, systemic BM-MSC administration promoted angiogenesis, neurogenesis and led to functional recovery [182, 183]. In utero administration of human BM-MSC EVs protected the fetal brain in an ovine model of preterm hypoxic-ischemic brain injury [184]. BM-MSC exosomes improved neurovascular plasticity, sensorimotor functions and spatial learning after traumatic brain injury in rats [185]. Human adipose-derived MSC exosomes were shown to carry neprilysin, a β -amyloid-degrading enzyme, which implicates therapeutic potential for Alzheimer's disease [186].

Furthermore, human UC-MSCs also suppressed hepatic inflammation, decreased collagen deposition and TGF β levels in a mouse liver fibrosis model [187]. In another liver injury model, ESC-derived-MSC exosomes inhibited hepatocyte apoptosis, increased their proliferation and promoted liver regeneration [188]. Human UC-MSC exosomes also rescued mice from oxidant hepatic injury in a glutathione peroxidase1 (GPX1)-dependent manner [189]. In a cisplatin-induced nephrotoxicity model, human UC-MSC exosomes decreased oxidative stress and apoptosis while promoting cell proliferation by activating ERK1/2 [190]. In another study with UC-MSC exosomes and cisplatin-induced cytotoxicity, exosomes increased autophagy and decreased the levels of pro-inflammatory IL-1 β and TNF α levels [191].

More recently, potential of MSC exosomes on rheumatic models has started to attract attention. Intraarticular injection of ESC-derived-MSC exosomes promoted extracellular matrix (ECM) deposition and complete cartilage regeneration in rats with osteochondral defects [192]. BM-MSC exosomes inhibit antibody production, T-cell proliferation and enhance Treg populations *in vitro* and alleviate inflammation in a CIA model *in vivo* [193]. In a mouse osteoarthritis model, BM-MSC exosomes protected mice from bone and cartilage degradation. *In vitro* they were able to increase type II collagen and aggrecan while decreasing detrimental MMP-13 and iNOS [194]. Although MSC exosomes have

already proved to be excellent therapeutic agents in many disease conditions, functional mechanisms of the exerted effects are not fully clear but must be determined in order to harness the full potential of these vesicles.

1.4 Immunosuppressive Oligodeoxynucleotides (Sup ODNs)

Alleviating excessive immune activation holds great importance for the treatment of autoimmune and inflammatory disorders. For this reason, there are several synthetic immunosuppressive oligodeoxynucleotides (Sup ODNs) developed to inhibit immune responses broadly or in a target-specific manner. The archetypal broad-acting Sup ODN A151 ODN will be discussed in detail in Section 1.4.1. H154 is a TLR9-specific Sup ODN effectively suppressing bacterial DNA-induced TLR9 signaling and inflammation [195]. Some other Sup ODN types compete for ligand binding to TLR9, therefore block the activation of downstream transcription factors such as AP-1 and inhibit the production of pro-inflammatory cytokines [196, 197]. Another class of Sup ODNs contain GpC or GpG motifs instead of the immunostimulatory CpG motifs found in bacterial DNA. They support Th2 immunity while strongly inhibiting Th1 [198, 199]. One GpC ODN promotes tolerogenic phenotype in DCs through modulating TLR7/TRIF signaling and promotes Treg generation via DC-derived IDO [200]. Nucleotide backbone, size and sequence of ODNs are determinants of the action mechanisms and therapeutic efficacy [201]. For instance, poly-G sequences and microsatellite-mimicking sequences are known to confer suppressive abilities to ODNs [202, 203].

1.4.1 A151 ODN

1.4.1.1 Mechanisms of Action

Suppressive activity of A151 ODN containing 4 repeats of mammalian telomeric TTAGGG sequence whose suppressive ability was dependent on forming a G-tetrad was first reported in 2003 by Gursel et al. [204]. Initially, A151 ODN-mediated immunosuppression was attributed solely to blocking the interaction between CpG ODN and TLR9. However, it was later revealed that A151 ODN has much broader effects in the cell rather than just inhibiting TLR9 signaling. A151 ODN was shown to bind to STATs 1, 3 and 4, inhibiting their phosphorylation induced by IFN γ and IL-12, therefore subsequently blocking Th1 cytokine production [205, 206]. Blocking of STAT1 activation by A151 ODN also supports Treg generation [207]. A151 ODN promotes Th17 generation by inhibiting the negative STAT3 regulator suppressor of cytokine signaling 3 (SOCS3) [208]. Furthermore, A151 ODN blocks B-lymphocyte proliferation, plasma cell differentiation, antibody production and class switching [209]. A151 ODN-mediated inhibition of class switching was attributed to the downregulation of activation induced cytidine deaminase (AICDA). Other identified targets of A151 ODN are AIM2 and IFN γ -inducible protein-16 (IFI16) both of which are intracellular DNA sensors important for bacterial and viral DNA recognition [210, 211]. A151 ODN was shown to bind AIM2 and prevent the formation of AIM2 inflammasome complex. Therefore, IL-1 β and IL-18 maturation due to inflammasome activation,

type I interferon and $\text{TNF}\alpha$ production due to cytosolic dsDNA recognition were all abrogated in DCs and macrophages [212]. Recently, another study revealed that A151 ODN also competes with cytosolic DNA for the activation of cGAS and consequently inhibit type I interferon production in monocytes [213]. Broad cellular effects of A151 ODN are visually summarized in Figure 1.5.

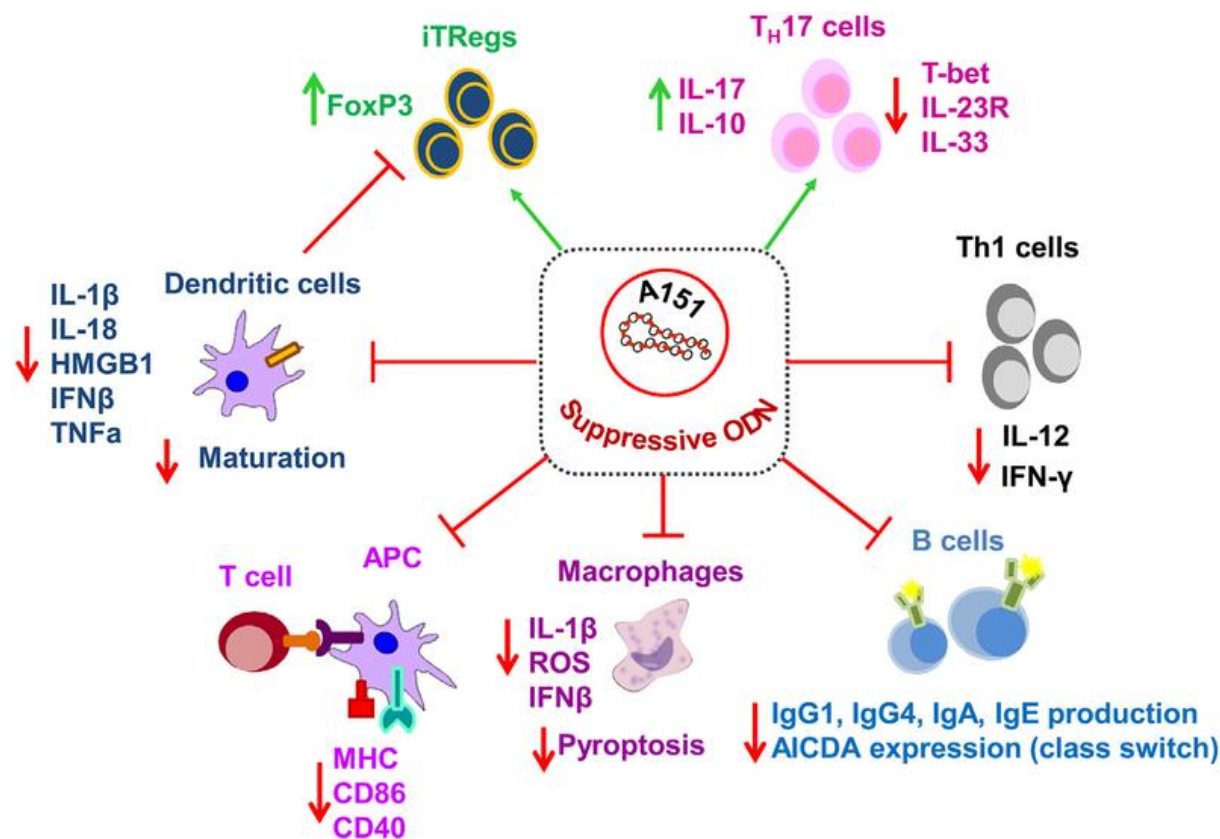


Figure 1.5. Mechanisms of A151 ODN-mediated immunosuppression [201].

Additionally, a recent study by our group revealed new insights into A151 ODN's cellular actions. Many elements of PI3K/Akt/mTOR signaling, including mTOR, are downregulated at transcription level upon A151 ODN exposure. A151 ODN also inhibits mTOR phosphorylation in macrophages and T-cells. Moreover, A151 ODN decreases both glycolytic capacity and oxidative phosphorylation in the cell, driving an energetically inert state. These new findings imply that A151 ODN metabolically reprograms the cells into an inert state and this most likely contributes to immunological suppression (data under review for publication).

1.4.1.2 Therapeutic Applications

A151 ODN was tested in many disease models including autoimmune disorders, infections, toxic shock, atherosclerosis and stroke. In lupus-prone NZB/NZW mice, A151 ODN treatment decreased autoantibody production, reduced IFN γ and IL-12 secretion and delayed glomerulonephritis progression [214]. Intraarticular injection of A151 ODN reduced the severity of CpG-induced arthritis and CIA decreasing immune cell infiltration, autoantibody production, and secretion of IFN γ and TNF α [215, 216]. In two different uveitis models, A151 ODN inhibited pro-inflammatory cytokine production and

relieved ocular inflammation [217, 218]. In an interesting study, A151 ODN was encapsulated into acid-stable nano capsules for oral delivery and long-term administration was able to inhibit the development of atopic dermatitis. A151 ODN-encapsulated nano capsules prevented STAT6 phosphorylation, therefore blocking IL-4 signaling and attenuating pro-allergic Th2 immunity [219]. Furthermore, A151 ODN was able to reduce infectious burden in *Candida albicans*-infected animals by promoting anti-fungal Th17 immunity [208]. In *Toxoplasma gondii*-induced gut inflammation and dextran sodium sulfate (DSS)-induced colitis, A151 ODN was able to maintain the gut epithelium integrity and decrease pro-inflammatory cytokine production including IL-6, IL-22, TNF α and IFN γ [220]. A151 ODN-treated mice were also protected from LPS-induced lethal endotoxic shock [206]. In ApoE-knockout atherosclerotic mice, A151 ODN was able to minimize atherosclerotic lesions, reduce MCP-1 and VCAM levels which are markers of atherosclerotic inflammation, and inhibit Th1 cytokine secretion [221]. A151 ODN pre-administration limited the development of ischemic brain injury in stroke-prone rats with an observed inhibition of NLRP3 inflammasome [222]. Moreover, studies with inflammation-induced skin carcinogenesis and silicosis-induced lung cancer models suggested A151 ODN as an effective drug for preventing inflammation-induced cancers [223, 224]. Lastly, A151 ODN was found to enhance the anti-proliferative and pro-apoptotic effects of several conventional anti-cancer drugs in a human non-small cell lung cancer cell (NSCLC) line [225]. Thus, A151 ODN might also be of use as an additional treatment strategy for some cancer types.

1.5 Disease Conditions Modeled in the Study

1.5.1 Ulcerative Colitis

Ulcerative colitis (UC) is a chronic inflammatory disorder affecting the colon. In contrast to the other broadly seen inflammatory bowel disease type Crohn's disease, which may involve inflammation in any part of the gastrointestinal tract, inflammation in UC is limited to the rectum and the colonic mucosa [226]. Incidence of UC is around 0.3% in USA while it goes up to 0.5% in Europe, and it has been increasing in newly industrializing countries in South America, Asia and Africa [227]. Rectal bleeding, abdominal pain and diarrhea are the most common clinical manifestations of UC, while histological features include mucosal inflammation, distortion of crypt architecture and abscesses in the crypts [226]. Although the etiology of the disease is not very clear, risk factors include cigarette smoking, dietary factors such as sugar intake, perinatal and childhood infections [228]. 5-aminosalicylic acid, oral corticosteroids, TNF α blocker Infliximab, purine synthesis inhibitor Azathioprine, probiotics *Escherichia coli* Nissle 1917 and *Lactobacillus* GG are effective treatments used in clinic for the remission of UC [229].

The three most common mouse models to study colitis are induced by 2,4,6-trinitro benzene sulfonic acid (TNBS), oxazolone and dextran sodium sulfate (DSS). TNBS-induced colitis can be triggered by intrarectal injection of TNBS in ethanol. As ethanol breaks the mucosal barrier, TNBS haptinize colonic

proteins, making them immunogenic. Response to TNBS is CD4⁺ T-cell-dependent and characterized by Th1 dominance [230]. Oxazolone is also rectally administered in ethanol. It is characterized by high death rates, diarrhea and weight loss. This is a Th2-dominant model where IL-4, IL-5 and IL-13 productions are elevated [231]. DSS is administered orally in drinking water for several days and causes ulcerations, weight loss, rectal bleeding, and diarrhea [232]. DSS is toxic to epithelial cells and directly disrupts the crypt integrity. In acute DSS colitis, levels of TNF- α , IL-6, IL-17 and the chemokine KC (CXCL1) are elevated [233]. T or B-lymphocyte-deficient animals are able to develop DSS-induced colitis which means the disease progression is not entirely dependent on adaptive response in contrast to the other two models [234]. This is the reason that DSS-induced colitis is a fitting model to study the contributions of innate immune cell types which are the main foci of this study.

1.5.2 Peritonitis

Peritonitis is inflammation of the inner lining of abdominal cavity called peritoneum. It can develop due to bacterial or fungal infection during various conditions such as surgery, peritoneal dialysis, cirrhosis, ascites or ruptured appendix [235-237]. Peritonitis models in animals are fast and easy ways to study processes related to acute inflammation. Most commonly used peritonitis model is a sterile, self-resolving inflammation triggered by zymosan which is a glucan preparation from the cell wall of *Saccharomyces cerevisiae*. Intraperitoneal injection of zymosan results in acute inflammatory response peaking at 4-6 hours and completely resolving within 48-72 hours. Zymosan-induced peritonitis is characterized by severe neutrophil and monocyte infiltration, peritoneal increase in chemokines such as KC and RANTES and cytokines such as TNF α , IL-1 β and IL-12 [238].

1.5.3 Wound Healing

Wound healing is a complex process which can be divided into three phases: inflammatory phase, proliferative phase and remodeling phase. In the inflammatory phase, there are platelet accumulation, coagulation and infiltration of leukocytes which are mainly neutrophils, macrophages and T-lymphocytes [239]. Proliferative phase is when the new tissue formation starts with the help of epithelial cells, fibroblasts and macrophages. In this phase, wound contraction, angiogenesis, re-epithelization, ECM production and deposition occur [240]. Finally, ECM and vascular remodeling slowly restore the tissue back to its pre-injury architecture and strength.

1.5.3.1 Inflammation in Wound Healing

Inflammation is necessary for the normal healing process although its excess can cause pathological healing. Upon injury, tissue-resident macrophages are stimulated by the DAMPs released because of injury and recognized with TLRs and other PRRs [241]. Chemokines are produced to attract leukocytes to the wound. An important purpose of inflammation is clearance of apoptotic cells and debris in the wound site by phagocytic cells [242]. Neutrophils are the first cells to quickly infiltrate to the wound followed by monocytes that differentiate into macrophages in the tissue [243]. Macrophages have a

double-sided role in the wound healing process. When exposed to inflammatory signal such as DAMPs, pro-inflammatory M1-macrophages produce IL-1 β , IL-6, IL-12, TNF α and NO to promote inflammation [244]. After the early stages, alternatively activated M2-macrophages and other populations displaying some of both M1 and M2 characteristics are also present in the wound [245]. They promote the resolution of inflammation and tissue remodeling by producing VEGF, TGF β and IL-10 [246]. Mast cells also arrive to the wound and degranulate. They support not only the acute inflammation but also angiogenesis and re-epithelization due to factors they release such as TNF α , VEGF, platelet-derived growth factor (PDGF), TGF β and FGF-2 [247]. Later, neutrophil dominance diminishes and Tregs accumulate in the wound where they start to suppress inflammatory macrophage activity, promote re-epithelization and therefore mediate the ending of the inflammatory phase [248].

Resident non-immune cells fibroblasts and keratinocytes also contribute to the inflammatory phase through TLR signaling. Keratinocytes express every TLR except for TLRs 7 and 8, and they produce TNF α , CXCL8, CXCL9, CXCL10 and CCL2 upon TLR activation [249, 250]. During injury, TLR4 is further upregulated in keratinocytes [251]. Moreover, fibroblasts express the full range of TLRs and can produce numerous cytokines and chemokines including IFN γ , IL-6, CCL2, CXCL8 and CXCL10 [249, 252, 253].

Even though inflammatory phase is critical for clearance and prevention of infections in the wound site, activities of inflammatory cells can also lead to fibrosis, excessive scar formation or chronic wounds [254]. Therefore, the delicate balance of inflammation and the suppression of it must be carefully taken into consideration while developing therapeutics for scarless and fast wound healing.

1.5.3.2 MSCs in Wound Healing

MSCs have various active roles in the wound healing process and they have been therapeutically used to enhance it. Many studies showed that MSCs accelerate wound closure and ameliorate pathogenic wounds such as diabetic wounds [255-257]. One way they enhance wound healing is the promotion of angiogenesis via pro-angiogenic VEGF, HGF and FGF2 production [256, 258]. MSCs also contribute to the resolution of inflammation. Mechanisms of MSC-mediated immune suppression were explained in Section 1.2.3. MSC-treated wounds have lower levels of pro-inflammatory TNF α , IL-1 β and the intercellular adhesion molecule 1 (ICAM1) which mediates leukocyte binding to fibroblasts [256, 259]. They also induce fibroblasts to produce ECM proteins fibronectin, elastin and collagen as well as glutathione peroxidase (GPx) and SOD which relieve oxidative stress and prevents ulcerative wound pathogenesis [260]. MSCs do not only contribute to wound healing through paracrine effects but also can help skin regeneration by differentiating into keratinocytes, epithelial and endothelial cells [258, 261]. Mechanisms by which MSCs enhance wound healing are summarized in Figure 1.6. Wound healing-promoting potential of UCX[®] cells which are the MSC cell type used in this study was previously discussed in detail in Section 1.2.2.

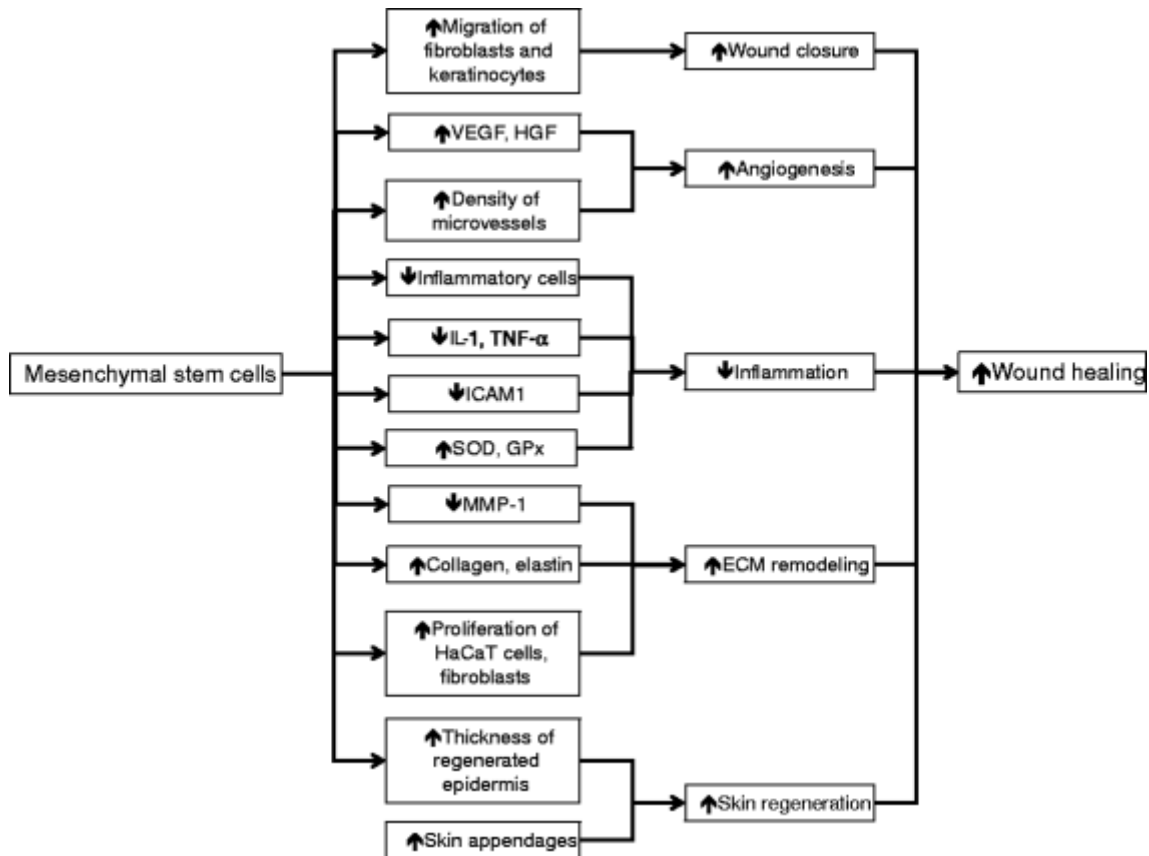


Figure 1.6. Mechanisms of wound healing promotion by MSCs [262].

1.6 Aims and Outline of the Study

MSCs have a wide-range of anti-inflammatory actions on innate and adaptive immune cells. In recent years, most of the well-known regenerative functions of MSCs were revealed to be exerted by paracrine action through exosomes. Studies with MSC exosomes prove their efficiency as a cell-free alternative to MSC-based therapies. The first aim of the study was characterizing the immunomodulatory effects of exosomes derived from human umbilical cord tissue-derived MSCs termed UCX[®] with a particular focus on their effects on macrophages. The second aim was to reveal the functional differences between UCX[®] exosomes derived from regular 2D culture and *in vivo*-mimicking 3D spheroid culture. The third aim was to investigate whether the immunomodulatory actions of UCX[®] exosomes can be enhanced through the loading of immunosuppressive ODN A151 ODN into the exosomes. Since exosome encapsulation increases the stability of bioactive compounds *in vivo*, this would also enhance the potency of A151 ODN itself. Lastly, we aimed to test the *in vivo* therapeutic potential of A151 ODN-loaded or naïve UCX[®] exosomes in three different animal models of inflammation.

For the purposes outlined above, firstly, 2D- and 3D-culture-derived UCX[®] exosomes were characterized in terms of their size, surface protein expressions and uptake kinetics by immune cells. Dynamic light scattering (DLS), atomic force microscopy (AFM) and flow cytometry were utilized for characterization purposes. A151 ODN was loaded into exosomes with a novel and easy method

developed by our group and the loading efficiency was determined with fluorescent labeling and flow cytometric analysis. Immunomodulatory effects of MSCs on TLR signaling were analyzed by stimulating mouse splenocytes or bone marrow-derived macrophages (BMDMs) with TLR2, TLR3, TLR4, TLR7 or TLR9 ligands and incubating with free exosomes, exosomes mixed with A151 ODN, or exosomes loaded with A151 ODN. Cytokine release was determined with ELISA and expressions of certain surface markers were determined with flow cytometry. Moreover, fibroblast and keratinocyte cell lines were incubated with exosomes with or without A151 ODN to observe the changes in cytokine production and levels of wound healing-related gene expressions which MSCs are known to promote. How UCX[®] exosomes in the presence or absence of A151 ODN influence macrophage polarization was investigated by incubating BMDMs in M1 or M2-inducing conditions with or without exosome/A151 ODN formulations. M1/M2 phenotype markers were investigated with qRT-PCR, ELISA and Griess assay.

In order to determine how 2D- and 3D-exosomes with or without A151 ODN affect immune cell populations *in vivo*, they were injected intraperitoneally (IP) into mice and the changes in peritoneal cell populations were analyzed with flow cytometric analyses of collected peritoneal exudate cells. In light of promising *in vitro* results, anti-inflammatory potentials of IP-injected exosome/A151 ODN formulations were assessed in DSS-induced colitis and zymosan-induced peritonitis models in mice. Lastly, immunomodulatory and regenerative capacities of subcutaneously-injected exosome/A151 ODN formulations were assessed in an excisional wound healing model in mice. In all *in vivo* studies, serum cytokine levels were measured with ELISA and changes in local and systemic immune cell populations were determined with flow cytometry.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cell Culture Media and Solutions

RPMI 1640 with L-glutamine and phenol red (#21875-034), high glucose DMEM with L-glutamine, pyruvate and phenol red (#41966-029), FBS (#10270-106, Lot 42G6172K), Sodium Pyruvate (100mM, #11360-070), MEM Non-Essential Amino Acids (100X, #11140-050), Penicillin-Streptomycin (10,000 U/mL, #15140-122), 10X Trypsin-EDTA without phenol red (0.5%, #15400-054), DPBS (#14190-250) were obtained from Gibco, Thermo Fisher Scientific (Waltham, MA, USA). HEPES buffer (1M, #03-025-1B) was purchased from Biological Industries (Beit-Haemek, Israel). Pyrogen Free Cell Culture Water (#LM-T1707/500) was from Biosera (Nuaille, France). Complete RPMI or DMEM media were prepared by supplementing the base media with varying amounts of FBS and with Sodium Pyruvate, Non-Essential Amino Acids, Penicillin-Streptomycin and HEPES all of which 1/100 diluted. FBS was heat-inactivated at 56°C for 1 hour prior to use. FBS content of the media and trypsin concentration used for passaging is specified for each cell type in the Section 2.2.1. Lymphocyte Separation Medium (density 1.077 g/ml, #LSM-A) for PBMC isolation was purchased from Capricorn (Ebsdorfergrund, Germany).

2.1.2 TLR Ligands

Types of TLR ligands used in cell culture experiments excluding CpG ODNs, their target TLRs, *in vitro* working concentrations and vendors are listed in Table 2.1. CpG ODN sequence targeting TLR9 and immunosuppressive A151 ODN are given in Table 2.2.

Table 2.1. List of TLR ligands used throughout the study

Ligand	Target TLR	Working Concentration	Company	Catalog #
Pam3CSK4	TLR1/2	1 µg/ml	InvivoGen (San Diego, CA, USA)	tlrl-pms
Polyinosinic:polycytidylic acid (Poly I:C)	TLR3	30 µg/ml	Amersham (Amersham, UK)	27-4732-01
Lipopolysaccharides from Escherichia coli O55:B5 (LPS)	TLR4	1 µg/ml	Sigma-Aldrich (St. Louis, MO, USA)	L-2880
R848	TLR7/8	1 µg/ml	Sigma-Aldrich	SML0196

Table 2.2. List of ODNs used throughout the study

Oligodeoxynucleotide (ODNs)	Sequence	Working Concentration	Company
K23	TCGAGCGTTCTC	1 μ M	Alpha DNA (Montreal, Canada)
A151 ODN	TTAGGGTTAGGGTTAGGGTTAGGG	1.5 μ M	Alpha DNA

2.1.3 Flow Cytometry Antibodies

All antibodies used for flow cytometry analyses are listed in Table 2.3.

Table 2.3. Human and mouse-specific flow cytometry antibodies used throughout the study

Target Protein	Fluorescence Conjugate	Catalog #	Clone	Company
Human CD4	Brilliant Violet 570	300534	RPA-T4	Biolegend (San Diego, CA, USA)
Human CD8	Pacific Blue	301023	RPA-T8	Biolegend
Human CD9	PE	312106	HI9a	Biolegend
Human CD11c	PE	301606	3.9	Biolegend
Human CD14	APC/Cy7	301820	M5E2	Biolegend
Human CD19	PE/Cy7	302216	HIB19	Biolegend
Human CD55	PE	311308	JS11	Biolegend
Human CD81	PE	349506	5A6	Biolegend
Human CD274 (PD-L1)	PE	329706	29E.2A3	Biolegend
Mouse CD3	Pacific Blue	100214	17A2	Biolegend
Mouse CD4	APC	100516	RM4-5	Biolegend
Mouse CD4	Brilliant Violet 785	100453	GK1.5	Biolegend
Mouse CD8a	PE/Cy5	100710	53-6.7	Biolegend
Mouse CD8a	APC/Cy7	100714	53-6.7	Biolegend
Mouse CD11b	FITC	35-0112-U500	M1/70	Tonbo Biosciences (San Diego, CA, USA)
Mouse CD11b	PE	101208	M1/70	Biolegend
Mouse CD11b	Brilliant Violet 605	101257	M1/70	Biolegend
Mouse CD11c	FITC	117306	N418	Biolegend
Mouse CD11c	PE	50-0114-U100	N418	Tonbo Biosciences
Mouse CD11c	Pacific Blue	117322	N418	Biolegend
Mouse CD25	PE/Cy5	102010	PC61	Biolegend

Mouse CD25	Brilliant Violet 605	102036	PC61	Biolegend
Mouse CD45	Brilliant Violet 605	103155	30-F11	Biolegend
Mouse CD45R (B220)	PE/Cy5	103210	RA3-6B2	Biolegend
Mouse CD49b	PE	108908	DX5	Biolegend
Mouse CD80	FITC	104706	16-10A1	Biolegend
Mouse CD86	PE/Cy5	105016	GL-1	Biolegend
Mouse CD127	FITC	135008	A7R34	Biolegend
Mouse CD206	APC	141708	C068C2	Biolegend
Mouse CD274 (PD-L1)	Brilliant Violet 421	124315	10F.9G2	Biolegend
Mouse CD274 (PD-L1)	Brilliant Violet 605	124321	10F.9G2	Biolegend
Mouse F4/80	PE/Cy7	123114	BM8	Biolegend
Mouse F4/80	APC/Cy7	123118	BM8	Biolegend
Mouse I-A/I-E (MHC-II)	PE	107608	M5/114.15.2	Biolegend
Mouse I-A/I-E (MHC-II)	Brilliant Violet 650	107641	M5/114.15.2	Biolegend
Mouse Ly6C	Brilliant Violet 785	128041	HK1.4	Biolegend
Mouse Ly6G	PE	127608	1A8	Biolegend
Mouse Ly6G	PE/Cy7	127618	1A8	Biolegend
Mouse NK-1.1	APC	108710	PK136	Biolegend
Mouse TCR β	PE	109208	H57-597	Biolegend
Mouse TCR β	Pacific Blue	109226	H57-597	Biolegend
Mouse IgG1, κ	PE	400112	MOPC-21	Biolegend
Mouse IgG2b, κ	PE	401208	MG2b-57	Biolegend

2.1.4 ELISA Antibodies and Reagents

Antibodies, recombinant standards and reagents used for cytokine ELISA experiments and their working conditions are listed in Table 2.4.

Table 2.4. Human and mouse-specific ELISA antibodies and reagents used throughout the study

Product	Catalog #	Clone	Company	Working Concentration
Anti-human TNF α	3510-1A-20	TNF3/4	Mabtech (Nacka, Sweden)	2 μ g/ml
Anti-human TNF α -biotin	3510-1A-20	TNF5	Mabtech	1:1000
Human recombinant TNF α	3510-1A-20	-	Mabtech	12.5 ng/ml
Anti-human IL-10	3430-1A-20	9D7	Mabtech	2 μ g/ml

Anti-human IL-10 -biotin	3430-1A-20	12G8	Mabtech	1:1000
Human recombinant IL-10	3430-1A-20	-	Mabtech	8 ng/ml
Anti-human TGF β	3550-1A-20	MT593	Mabtech	2 μ g/ml
Anti-human TGF β -biotin	3550-1A-20	MT517	Mabtech	1:1000
Human recombinant TGF β	3550-1A-20	-	Mabtech	1.25 nM
Anti-mouse IFN γ	3321-1A-20	AN18	Mabtech	1 μ g/ml
Anti-mouse IFN γ -biotin	3321-1A-20	R4-6A2	Mabtech	1:1000
Mouse recombinant IFN γ	3321-1A-20	-	Mabtech	20 ng/ml
Anti-mouse TNF α	3511-1A-20	MT1C8/23C9	Mabtech	2 μ g/ml
Anti-mouse TNF α -biotin	3511-1A-20	MT11B10	Mabtech	1:1000
Mouse recombinant TNF α	3511-1A-20	-	Mabtech	25-100 ng/ml
Anti-mouse IL-6	504502	MP5-20F3	Biolegend	2 μ g/ml
Anti-mouse IL-6-biotin	504602	MP5-32C11	Biolegend	1:1000
Mouse recombinant IL-6	575702	-	Biolegend	100 ng/ml
Anti-mouse IL-10	504902	JES5-2A5	Biolegend	4 μ g/ml
Anti-mouse IL-10-biotin	505004	JES5-16E3	Biolegend	1:1000
Mouse recombinant IL-10	575804	-	Biolegend	625 ng/ml
Anti-mouse IL-12	3451-1A-20	C15.6	Mabtech	2 μ g/ml
Anti-mouse IL-12-biotin	3451-1A-20	C17.8	Mabtech	1:1000
Mouse recombinant IL-12	573102	-	Biolegend	10 ng/ml
Streptavidin-ALP	3310-8	-	Mabtech	1:1000
PNPP	34047	-	Thermo Scientific	1 tablet/5 ml

2.1.5 PCR Primers

Primers used for RT-qPCR and their optimal conditions are given in Table 2.5. All primers were synthesized by Oligomer Biotechnology (Ankara, Turkey).

Table 2.5. Human and mouse-specific PCR primers and conditions used throughout the study

Gene	Primer	Sequence 5'→3'	Product Length	Annealing Temperature
Human β -actin	Forward	GATGAGATTGGCATGGCTTT	100 bp	60 °C
	Reverse	CACCTTCACCGTTCCAGTTT		
Human IL1a	Forward	CTTCTGGGAAACTCACGGCA	92 bp	62 °C
	Reverse	AGCACACCCAGTAGTCTTGC		
Human IL10	Forward	AAGCCTGACCACGCTTTCTA	122 bp	60 °C

	Reverse	GCTCCCTGGTTTCTCTTCCT		
Human Ccl2	Forward	TCATAGCAGCCACCTTCATTC	190 bp	62 °C
	Reverse	CAGATCTCCTTGGCCACAAT		
Mouse β -actin	Forward	TTCGTTGCCGGTCCACACCC	90 bp	55 °C
	Reverse	GCTTTGCACATGCCGGAGCC		
Mouse Arg1	Forward	TTTCTCAAAAGGACAGCCTCG	177 bp	60 °C
	Reverse	ACAGACCGTGGGTCTTCAC		
Mouse Ccl2	Forward	TGACCCCAAGAAGGAATGGG	105 bp	60 °C
	Reverse	GACCTTAGGGCAGATGCAGTT		
Mouse Chil3	Forward	CAGAAGCTCTCCAGAAGCAATC	139 bp	60 °C
	Reverse	GCCTGTCCTTAGCCCAACT		
Mouse Cd274 (Pdl1)	Forward	CACCTTGCTACGGGCGTTTAC	133 bp	60 °C
	Reverse	CCCAGTACACCACTAACGCA		
Mouse Il1a	Forward	AGTCAACTCATTGGCGCTTG	127 bp	58 °C
	Reverse	GAGAGAGATGGTCAATGGCAGA		
Mouse IL10	Forward	CAGTACAGCCGGGAAGACAA	179 bp	60 °C
	Reverse	TGGCAACCCAAGTAACCCTTA		
Mouse Nos2	Forward	GGTGAAGGGACTGAGCTGTT	103 bp	62 °C
	Reverse	ACGTTCTCCGTTCTCTTGAC		
Mouse Tgfb1	Forward	CCTGAGTGGCTGTCTTTTGA	70 bp	55 °C
	Reverse	CCTGTATTCCGTCTCCTTGG		

2.2 Methods

2.2.1 Cell Culture

See Section 2.1.1 for all reagents used in cell culture and the compositions of complete RPMI and DMEM media. All cell cultures were kept in a humidified incubator with 5% CO₂ at 37°C.

2.2.1.1 2D UCX[®] Cell Culture

For expansion, UCX[®] cells were cultured in α -MEM medium with 15% FBS. Media were changed twice weekly and cells were sub cultured when they reached 80% confluency. 1X Trypsin/EDTA was used for 5 minutes at 37°C for the detachment of cells during sub culturing

Before they were used for conditioning media for exosome collection, UCX[®] cells were cultured in α -MEM medium with 15% FBS for 24 hours. After that, FBS concentration was adjusted to 5% and the culture was left for another 3 days. Then, medium was replaced with basal α -MEM with no FBS. 48 hours later, conditioned medium was collected.

2.2.1.2 3D UCX[®] Cell Culture

For 3D spheroid culture, cells obtained from 2D-culture were inoculated at a concentration of 1×10^6 cells/ml to spinner flasks with ball impeller. Culture medium was the same as 2D-culture medium which was α -MEM with 15% FBS. Spinner flasks were stirred at 80 RPM to promote spheroid formation for 24 hours. After 24 hours, half of the culture medium was changed with fresh medium and the spinning speed was kept at 110 RPM in order to maintain spheroids smaller than 350 μ m in diameter. Similar to the 2D-culture, FBS content of the medium was gradually decreased to zero before CM production as

described in Section 2.2.1.1. 3D-cultures were only kept until CM collection and were not sub cultured again. For repeated CM production, new inoculations to 3D-culture were performed.

2.2.1.3 NIH3T3 Fibroblast Cell Line Culture

NIH3T3 cells were cultured in complete DMEM medium with 4 g/L glucose and 10% FBS. Cells were split 1/6 for sub culturing and were passaged every 2-3 days when they reach 80-90% confluency. 2X Trypsin/EDTA was used for 3 minutes at 37°C for the detachment of cells during sub culturing. Cell counting was done with a hemocytometer as described in Section 2.2.1.11.

2.2.1.4 HaCaT Keratinocyte Cell Line Culture

HaCaT cells were cultured in complete DMEM medium with 4 g/L glucose and 10% FBS. Cells were split 1/6 for sub culturing and were passaged every 3-4 days when they reach 80-90% confluency. 1X Trypsin/EDTA was used for 8-10 minutes at 37°C for the detachment of cells during sub culturing. Cell counting was done with a hemocytometer as described in Section 2.2.1.11.

2.2.1.5 Bone Marrow Derived Macrophage (BMDM) Generation

6-8 weeks old C57BL/6 mice were euthanized by cervical dislocation and femur and tibia bones were removed. The ends of the bones were cut and bone marrows were obtained by flushing out cell culture media through the bones with a 25G syringe. Cells were washed with cell culture media, centrifuged at 300 RCF for 5 minutes and resuspended with ACK Lysis Buffer (Lonza, Basel, Switzerland). After 1 minute of incubation, cells were washed and centrifuged again at 300 RCF for 5 minutes. Cells were seeded on ultra-low attachment 24 well plates (#3473, Corning, NY, USA) as 5×10^5 cells/well in 1 ml/well 30% L929 CM diluted in RPMI medium with 10% FBS. After 3 days, a medium addition of 1/3 of the original medium amount was done. At the end of 7 days in total, attached cells were collected upon 30 minutes of incubation with ice-cold 10 mM EDTA, centrifuged at 300 RCF for 5 minutes, and macrophage marker expressions were analyzed with flow cytometry upon staining with anti-CD11b and anti-F4/80 antibodies (see Table 2.3).

For the production of L929 CM, L929 cells were seeded in T175 flasks at a density of 5×10^5 cells/flask in 50 ml/flask RPMI medium with 5% FBS. No medium change was performed until the cells are confluent in 6-7 days. Once they were confluent, the CM was collected and centrifuged at 300 RCF for 5 minutes. The supernatant was filtered with 0.2 μ m filters and kept at -20°C until their use in BMDM generation.

2.2.1.6 Single Cell Isolation from Mouse Spleen

8-10 weeks old BALB/c or C57BL/6 mice were euthanized by cervical dislocation and spleens were surgically removed. Single cell suspensions were prepared by mashing the spleen with the back of a sterile syringe plunger inside RPMI medium with 2% FBS. After transferring the homogenate to a 15 ml falcon tube, cells were washed with medium and centrifuged at 300 RCF for 5 minutes. After

repeating the washing step one more time, cells were resuspended in 1 ml of culture medium and counted with flow cytometer (see Section 2.2.1.11).

2.2.1.7 Isolation of Mouse Peritoneal Exudate Cells (PECs)

10 ml of DPBS was injected into the peritoneal cavities of 6-8 weeks old C57BL/6 mice and the fluid was re-collected with the syringe after slight agitation. Cells were precipitated at 300 RCF for 5 minutes. If presence of red blood cells were visible in the pellet, cells were treated with ACK Lysis Buffer for 30 seconds, washed with culture media and centrifuged again at 300 RCF for 5 minutes. Cells were counted with flow cytometer as described in Section 2.2.1.11.

2.2.1.8 Isolation of Mouse Mesenteric Lymph Nodes

8-10 weeks old C57BL/6 mice were sacrificed and mesenteric lymph nodes were surgically removed. Single cell suspensions were prepared by mashing the lymph nodes between two glass microscope slides and subsequently washing and collecting the homogenate with RPMI medium with 2% FBS. After transferring the homogenate to a 15 ml falcon tube, cells were washed with medium and centrifuged at 300 RCF for 5 minutes. After repeating the washing step one more time, cells were resuspended in 1 ml of culture medium, filtered with 0.7 μ m filters, and counted with flow cytometer (see Section 2.2.1.11).

2.2.1.9 Single Cell Isolation from Mouse Skin

8-10 weeks old BALB/c mice with skin wounds were sacrificed and the wound areas were removed with surgical scissors. Tissues were thinly sliced with a surgical blade and incubated with Liberase™ DL enzymatic solution (#5466202001, Roche Life Science, Indianapolis, IN, USA) diluted with RPMI medium with 2% FBS so that the enzyme activity was 2.6 Wünsch units. Each sample was incubated in a 200 μ l enzyme solution in a 1.5 ml Eppendorf tube at 37 °C for 2.5 hours with intermittent heavy vortexing every 20 minutes. After 2.5 hours, 200 μ l 0.5% trypsin/EDTA was added to the samples and incubated at 37°C for another 15 minutes. After vigorous vortexing at the end of the incubation, homogenates were passed through 40 μ m cell strainers (#352340, Corning), enzymes were diluted by addition of 3-4 ml of culture medium or DPBS, centrifuged at 500 RCF for 5 minutes, resuspended in 1 ml of culture medium and counted with flow cytometer (see Section 2.2.1.11).

2.2.1.10 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Blood

Blood was collected from healthy donors with informed consent and slowly layered onto the lymphocyte separation medium with 1:1.5 (blood:medium) volume ratio and centrifuged at 600 RCF for 30 minutes. The cloudy interface containing PBMCs between the upper plasma layer and the lower separation medium layer was transferred to a separate falcon tube with a sterile Pasteur pipette. RPMI medium with 2% FBS was added and the cells were centrifuged at 300 RCF for 5 minutes. This washing step was repeated one more time to discard any remaining separation medium. Cells were resuspended in 1 ml of culture medium and counted with flow cytometer (see Section 2.2.1.11).

2.2.1.11 Cell Counting

For cell lines, hemocytometer was used for counting. After resuspending cell pellets in 1 ml of culture medium, 10 μ l of the cell suspension was taken and diluted 1:10 by adding 90 μ l culture medium. Another 1:2 dilution was made by adding 100 μ l trypan blue to be able to distinguish live cells from the dead cells under microscope. 10 μ l of the final suspension was placed on a hemocytometer chamber and observed under a light microscope. The areas counted are represented in Figure 2.1. Total cell number was calculated with the formula below.

$$\text{Total cell \#} = \frac{\text{Counted cell \#}}{4} \times 10^4 \times 20 \text{ (dilution factor)} \times 1 \text{ (ml, resuspension volume)}$$

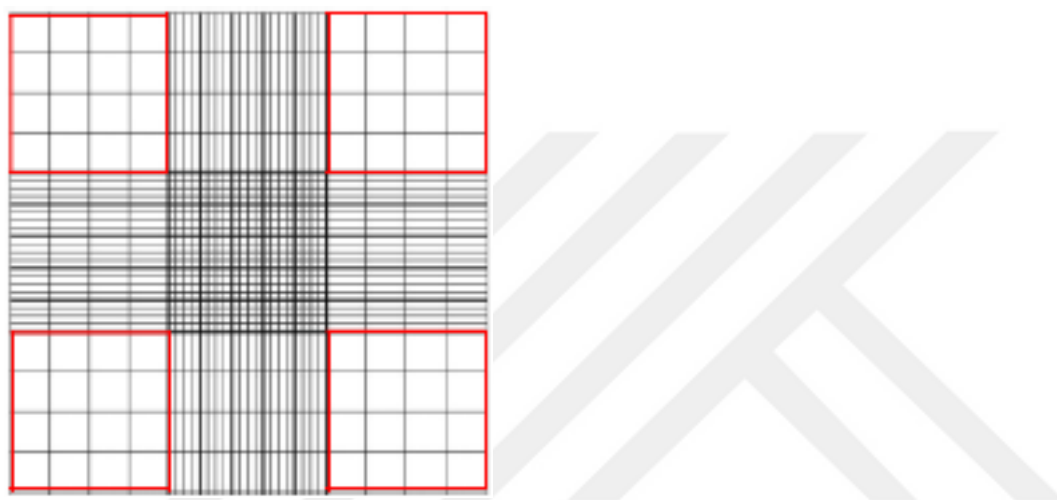


Figure 2.1. Representative figure of the counting chambers of hemocytometer. Areas designated with red squares were used for counting cells.

For bone marrow cells, BMDMs, PECs, splenocytes, lymph node cells, skin isolate cells and PBMCs, NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA) was used for counting. After cells were resuspended in 1 ml culture medium, different dilutions were done according to each cell type. 100 μ l of the diluted cell suspension was counted with the flow cytometer. Gating was done only to include live cells and doublet discrimination was performed to make sure only single cells are counted as particles by the cytometer. A representative gating strategy for cell counting is shown in Figure 2.2. Total cell number was calculated by multiplying the count in the live singlet gate and the dilution factor.

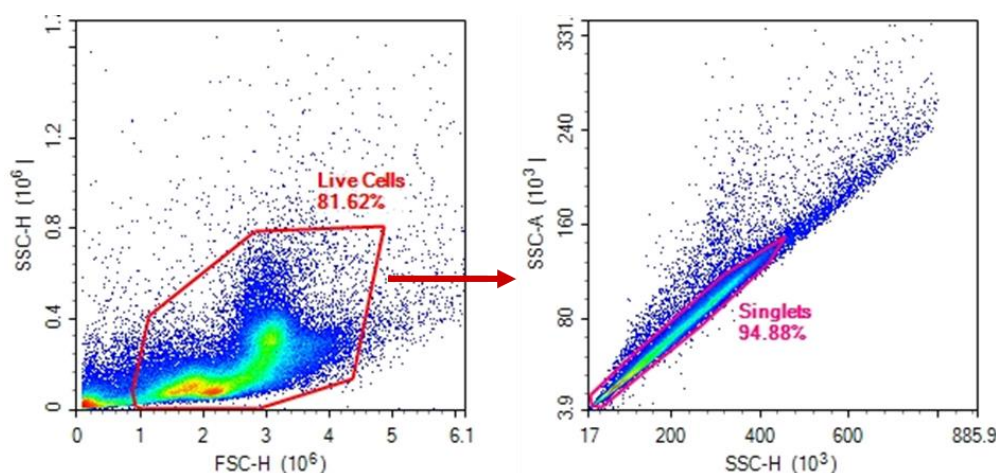


Figure 2.2. Representative gating strategy for cell counting. Number of cells in the ‘singlets’ gate taken from ‘live cells’ gate is considered for counting. This representative figure depicts murine bone marrow cells. FSC: forward scatter, SSC: side scatter, H: height.

2.2.2 Isolation of Exosomes from UCX[®] Conditioned Media

Conditioned media from 2D- or 3D-UCX[®] cultures were concentrated with Amicon centrifugal filters with 3KDa cutoff (#UFC900324, Millipore, Burlington, MA, USA) by centrifugation at 3000 RCF until the volume is equal to or less than 1 ml. For isolation of exosomes from concentrated CM, Exo-spin Midi-Columns (#EX04, Cell Guidance Systems, Cambridge, UK) were used according to the manufacturer’s instructions. Briefly, columns were first washed with DPBS twice and allowed to drain under gravity. When no fluid remained above, CM was applied onto the column and allowed to drain under gravity. 2 ml DPBS was applied and column was drained with gravity. At this point, exosomes have not passed through the column yet and the eluate contained only DPBS. The waste eluate was discarded and the column was placed into a new 15 ml falcon tube. 5 ml DPBS was added on top of the column and drained with gravity to collect the exosome fraction.

2.2.3 Determination of Exosomal Protein Content

Exosomal protein content was determined with Pierce BCA Protein Assay Kit (#23225, Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, BSA standard dilutions ranging between 2000 and 2 µg/ml were placed in 96 well plates as 25 µl/well and samples were placed as 5 µl/well. Both standards and samples were mixed with 200 µl of the working reagent. The plate was incubated in dark at 37°C for 30 minutes and then cooled down at room temperature for 15 minutes. Absorbance at 562 nm was measured with Synergy HT microplate reader (BioTek, Winooski, VT, USA). The concentrations of the samples were calculated according to the standard curve and then multiplied by 5.

2.2.4 Characterization of Exosomes

2.2.4.1 Size Distribution Analysis by Dynamic Light Scattering

Exosomes were diluted with DPBS to a concentration of 0.005 µg/µl. Size distribution measurements were done with Zetasizer Nano ZS (Malvern, Worcestershire, UK) in triplicates for each sample.

2.2.4.2 Atomic Force Microscopy (AFM)

Exosomes with various dilutions in DPBS were adsorbed onto mica sheets and dried at room temperature. Images were obtained in non-contact dynamic mode with ambient AFM (NanoMagnetics Instruments, Oxford, UK). Obtained images were further analyzed using NMI ImageAnalyzer (NanoMagnetics Instruments).

2.2.4.3 Analysis of Surface Markers by Flow Cytometry

A bead-based detection method was used for better detection of exosomes with flow cytometry. Carboxyl latex beads (#C37282, Thermo Fisher Scientific) were coated with purified anti-human CD63 antibody to be able to capture exosomes. Latex beads were mixed with anti-CD63 (#353014, Biolegend) at a 1µl:1µg (bead:antibody) ratio. The volume was completed to 50 µl with PBS and the mixture was incubated for 30 minutes at RT. After that, the volume was increased to 500 µl with PBS and the mixture was placed on a rotator at a low speed to incubate overnight. After overnight incubation, the bead-antibody mixture was precipitated at 10000 RCF for 10 minutes and resuspended with PBS containing 5% BSA for blocking. Upon 4 hours of incubation at RT, coated beads were precipitated again at 10000 RCF for 10 minutes, resuspended in PBS containing 1% BSA and stored at 4°C. The final resuspension volume was 10 times of the initial volume of beads that was used.

For each staining, 1 µg exosome was mixed with 1 µl of the final coated bead solution. Volume was increased with PBS up to 50 µl and the mixture was incubated at RT for 30 minutes. Then the volume was increased to 500 µl and the mixture was slowly rotated overnight to let the exosomes and the beads conjugate. After overnight conjugation, exosome-bead conjugates were incubated with fluorochrome antibodies for human CD9, CD63, CD81, and their appropriate isotype controls (see Table 2.3 for antibodies) at a concentration of 1 µg/ml in 100 µl volume for 1 hour at RT in the dark. After 1 hour of incubation, samples were washed with PBS and centrifuged at 10000 RCF for 5 minutes, then resuspended in 100 µl of PBS and analyzed with NovoCyt flow cytometer.

2.2.5 Determination of Exosome Binding and Uptake with Mouse Splenocytes, BMDMs and Human PBMCs

In order to stain the membranes of exosomes to make them visible for flow cytometric analyses, the lipophilic dye SP-DiOC₁₈ (#D7778, Invitrogen, Carlsbad, CA, USA) was mixed with exosomes at a ratio of 1µg:10µg (SP-DiOC:exosome). The mixture was incubated for 60 minutes at 37°C in dark. The

solution was diluted with PBS and ultracentrifuged for 90 minutes at 10^5 RCF to remove unbound dye. Exosome pellet was dissolved in PBS and the volume is adjusted according to further applications.

Human PBMCs, mouse splenocytes and mouse BMDMs were seeded to 96 well plates as 2.5×10^5 cells/well. Cells were stimulated with 5 or 20 $\mu\text{g/ml}$ SP-DiOC-stained UCX[®] exosomes in a volume of 250 μl /well for 2, 4, 8, 16 or 24 hours. After incubations were done, splenocytes were mixed with fluorochrome antibodies for B220, TCR β , CD4, CD8, CD11c and F4/80, while PBMCs were mixed with fluorochrome antibodies for CD4, CD8, CD11c, CD14 and CD19 in a volume of 100 μl in which all antibodies were at a concentration of 1 $\mu\text{g/ml}$. Details of the antibodies are given in Table 2.3. Cell-antibody mixtures were incubated at dark for 30 minutes at 4°C, washed with PBS and centrifuged at 300 RCF for 5 minutes, resuspended in 100 μl PBS and analyzed with NovoCyte flow cytometer. Then, the SP-DiOC positivity was analyzed in the gates for each immune cell type. Also, fluorescence exosome signals in the cells were visualized with EVOS FL Auto cell imaging system (Thermo Fisher Scientific).

2.2.6 Loading of A151 ODN into Exosomes

Cargo loading into exosomes was done with a controlled dehydration-rehydration method developed in our laboratory. A schematic representation is given in Figure 2.3. Briefly, exosomes and A151 ODN was mixed at 1 μg :1 μg or 2 μg :1 μg (exosome:A151 ODN) ratio and snap-frozen in liquid nitrogen. The mixture was lyophilized overnight with VirTis BenchTop K freeze dryer (SP Scientific, Warminster, PA, USA). For the controlled reconstitution, nuclease-free ddH₂O at the 1:10 volume of the original lyophilized solution was added to the powder and vigorously vortexed for 15 seconds. The solution was left at RT for 30 minutes with intermittent vortexing for 15 seconds every 5 minutes. After 30 minutes, DPBS at the same volume as the ddH₂O was added and gently vortexed for 15 seconds. After resting the solution for 15 minutes at RT, volume was adjusted as desired with DPBS and the samples were stored at 4°C up to 2 weeks.

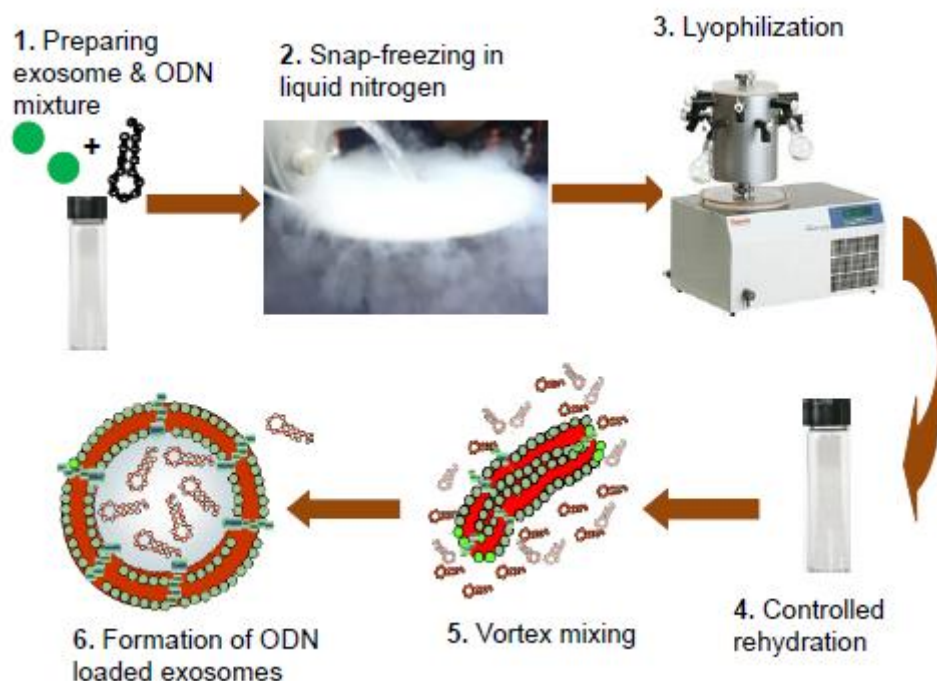


Figure 2.3. Exosome loading with controlled rehydration-dehydration method [263].

2.2.7 Determination of A151 ODN Loading Efficiency into Exosomes

Cy3-labelled A151 ODN was loaded into UCX[®] exosomes at 2 μ g:1 μ g or 1 μ g:1 μ g (exosome:A151 ODN) ratio as described in Section 2.2.6. After reconstitution, exosomes were stained with SP-DiOC as described in Section 2.2.5. Cy3 positivity in SP-DiOC positive particles were analyzed with NovoCyt flow cytometer.

2.2.8 Determination of Immunomodulatory Effects of UCX[®] Exosomes

2.2.8.1 Determination of the Presence of Immunomodulatory Surface Proteins on Exosomes

Exosomes were captured with carboxyl latex beads coated with anti-CD63 antibodies as described in Section 2.2.4.2. Exosome-bead conjugates were then stained with anti-human PD-L1 and anti-human CD55 antibodies and their appropriate isotype controls (see Table 2.3 for antibodies) in the same way it is described in Section 2.2.4.2 and analyzed with NovoCyt flow cytometer.

2.2.8.2 Determination of UCX[®] Exosomes' Effect on BMDM Polarization

BMDMs at M₀-state were generated as described in Section 2.2.1.5. Treatments were done in 24 well plates with 5x10⁵ cells/well. They were treated with A151 ODN alone, 2D- or 3D-exosomes alone, 2D- or 3D-exosomes mixed with A151 ODN, and 2D- or 3D-exosomes loaded with A151 ODN in the presence or absence of LPS or IL4/IL13 for 24 hours. Exosomes were used at 20 μ g/ml concentration and A151 ODN was used as 1.5 μ M. LPS was used as 100 ng/ml to drive M1-differentiation, IL4 and IL13 were used as 10 ng/ml to drive M2-differentiation. In order to determine M1- or M2-like signatures

of macrophages after 24 hours, cytokine ELISA (see Section 2.2.8.5) for IL6, TNF α and IL10 production, RT-qPCR (see Section 2.2.8.6) for Nos2, Arg1, Chil3, Pdl1 genes and Griess Assay (see Section 2.2.8.8) were performed.

2.2.8.3 Determination of UCX[®] Exosomes' Effect on Cytokine Production from Fibroblasts and Keratinocytes

In 12 well plates, NIH3T3 fibroblasts or HaCaT keratinocytes were placed as 5x10⁵ cells/well, stimulated with Pam3CSK4, poly(I:C) and LPS at concentrations given in Table 2.1 and treated with A151 ODN, 2D-exosomes, 3D-exosomes or A151 ODN-loaded forms of the exosomes for 24 hours. Exosome concentrations were 20 μ g/ml. After 24 hours, cytokine ELISA (see Section 2.2.8.5) for TNF α and IL10 production, RT-qPCR (see Section 2.2.8.6) for Tgfb, IL1a and Ccl2 genes, and Griess Assay (see Section 2.2.8.8) were performed.

2.2.8.4 In Vitro Stimulations with TLR Ligands and UCX[®] Exosomes

In 96 well plates, splenocytes and were placed as 2,5x10⁵ cells/well and BMDMs as 2x10⁵ cells/well. The cells were stimulated with the TLR ligands and ODNs given in Table 2.1 and Table 2.2 at the indicated working concentrations and treated with A151 ODN alone, 2D- or 3D-exosomes alone, 2D- or 3D-exosomes mixed with A151 ODN, and 2D- or 3D-exosomes loaded with A151 ODN for 24 hours. Exosomes were used at 20 μ g/ml concentration. After the incubation, cytokine ELISA (see Section 2.2.8.5) for IFN γ , IL6, TNF α and IL10 production and flow cytometric analyses (see Section 2.2.8.7) for MHC-II, CD80, CD86, PD-L1 were performed (see Table 2.3 for antibodies).

2.2.8.5 Determination of Cytokine Levels by ELISA

All antibodies, recombinant standards and reagents with their optimized working concentrations are given in Table 2.4. PolySorp Nunc-Immuno 96 well plates (#475094, Thermo Fisher Scientific) were coated with purified antibodies for the desired protein overnight at 4°C. Antibodies were diluted with DPBS in order to achieve the working concentration. Plates were coated with the diluted antibodies as 50 μ l/well. After overnight incubation, fluids in the wells were discarded and plates were blocked with 200 μ l/well blocker solution (5% BSA and 0.05% Tween 20 in 1X PBS) for 2 hours at RT. After blocking, plates were washed 3 times for 3-5 minutes each with washing buffer (0.05% Tween 20 in 1X PBS) and rinsed with ddH₂O 3 times. Cell culture supernatants or blood sera and serially 1:2 diluted recombinant protein standards were added in wells as 50 μ l/well. Serial dilutions of the standards were made with the blocker solution which was also used as the blank sample. After overnight incubation at 4°C, plates were washed as described previously, biotinylated antibodies diluted in the blocking solution were added as 50 μ l/well and incubated for 2 hours at RT. After another washing step, 50 μ l/well SA-ALP diluted in the blocker solution was added to the wells and incubated for 1 hour at RT. After washing, PNPP substrate was added as 50 μ l/well and color development was followed by frequently

taking measurements at 405 nm with the ThermoMax microplate reader (Molecular Devices, San Jose, CA, USA) until the recombinant standards reached saturation.

2.2.8.6 Determination of Gene Expression

2.2.8.6.1 RNA Isolation and cDNA Synthesis

Total RNA isolation was performed with TRIzol Reagent (#15596-018, Thermo Fisher Scientific). Briefly, cell pellets were lysed in 1 ml TRIzol Reagent by pipetting until the sample is homogenous, 200 µl chloroform was added and the tubes were shaken vigorously for 15 seconds, the mixture was rested at RT for 3 minutes and then centrifuged at 13000 RPM for 15 minutes at 4 °C for phase separation. All centrifugations hereafter were also done at 4°C. Clear upper layer was transferred into a new Eppendorf tube without disturbing the middle layer of DNA, mixed with 500 µl isopropanol, rested for 10 minutes at RT and centrifuged at 13000 RPM for 10 minutes. Isopropanol was sucked, the pellet was washed with 1 ml 75% ethanol and centrifuged for 5 minutes at 8000 RPM. After this washing step was repeated with 96% ethanol, all fluid was sucked and the pellet was left to dry at RT. Finally, the RNA pellet was resuspended with nuclease-free ddH₂O and RNA concentration was measured with NanoDrop One (Thermo Fisher Scientific). RNAs were then stored at -80°C.

cDNA synthesis was performed with RevertAid RT Reverse Transcription Kit (#K1691, Thermo Fisher Scientific) by exactly following the manufacturer's instructions. For each reaction, 1 µg RNA was used. cDNAs were then stored at -20°C up to 1 year and for longer periods of storage at -80°C.

2.2.8.6.2 RT-qPCR

All primers, sequences and their optimum conditions are listed in Table 2.5. qPCR reactions were made with LightCycler 480 SYBR Green I Master Mix (#04887352001, Roche Life Science). Components of each qPCR reaction are listed in Table 2.6 and the steps of the reaction are given in Table 2.7. LightCycler 480 (Roche Life Science) instrument was used for qPCR reactions.

Table 2.6. Components and differential volumes of the qPCR reaction

Reagent	Volume
LightCycler 480 SYBR Green I Master Mix	5 µl
Nuclease-free ddH ₂ O	3 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
cDNA	1 µl
Total Volume	10 µl

Table 2.7. Experimental conditions of the qPCR reaction

Step	Temperature	Duration	Cycle #
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	45
Annealing	varies (see Table 2.6)	20 seconds	
Elongation	72°C	20 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	-

2.2.8.7 Analysis of Cell Surface Markers by Flow Cytometry

If the flow cytometric analyses were not done immediately after an experiment, the cells were fixed for storage up to 10 days. Fixation was performed with FIX & PERM Cell Fixation & Permeabilization Kit Medium A (#GAS001S100, Thermo Fisher Scientific) or IntraCell fixation reagent (#INTRA-500T, Immunostep) according to manufacturers' instructions. Briefly, cells were mixed with varying amounts of fixation reagent depending on the cell count, vortexed and incubated for 15-30 minutes at RT depending on the fixation agent used. After the incubation, cells were washed by adding 1-2 ml of FACS buffer (1% BSA and 0.05% sodium azide in 1X PBS) and centrifuged for 5 minutes at 300 RCF. The pellet was resuspended in 1 ml FACS buffer and stored at 4°C up to 10 days.

All flow cytometry antibodies that were used in the studies are given in Table 2.3. Antibody concentrations were always adjusted to 1 µg/ml. Desired fluorochrome antibodies were mixed with cells in a volume of 100 µl for each sample. In order to prevent non-specific signals by blocking Fc receptors on the cells, TruStain FcX antibodies (#101320 for mouse, #422301 for human, Biolegend) were included in the mix as 0.5 µl per sample. The mixtures were incubated for 30 minutes in the dark at RT if the cells were fixed, at 4°C if the cells were alive. After incubation, live cells were washed with 1 ml DPBS and fixed cells with FACS buffer and centrifuged for 10 minutes at 300 RCF. Once the supernatant was aspirated, live cells were resuspended in 100 µl DPBS and kept on ice, fixed cells were resuspended in 100 µl FACS buffer. Stainings were analyzed with NovoCyt flow cytometer.

2.2.8.8 Measurement of Nitric Oxide Levels by Griess Assay

10 mM 100X stock standard solution was prepared with 34.5 mg NaNO₂ and 50 ml ddH₂O. To use in the assay, stock standard was diluted 1:10 with ddH₂O so the highest standard (1X) was 100 µM. Then, the 1X standard was 1:2 serial diluted 6 times. Standards and culture supernatant samples were added to a 96 well plate as 50 µl/well. ddH₂O was used as the blank sample. Then, 50 µl Griess Reagent

(#03553, Fluka, St. Gallen, Switzerland) was added to each well. After incubation at RT for 15 minutes, reading at 540 nm was acquired with Synergy HT microplate reader.

2.2.9 In Vivo Experiments

BALB/c and C57BL/6 mice were housed in the Bilkent University Animal Facility in the Department of Molecular Biology and Genetics. Mice were exposed to 12-hour light/dark cycles at 22°C. They were provided with food and water ad libitum unless specified otherwise for certain experiments. All experimental procedures were approved by the Bilkent University Animal Ethics Committee (Bil-AEC Protocol no. 246).

2.2.9.1 Determination of Cell Phenotypes Following Intraperitoneal Injection of UCX[®] Exosomes

8-10 weeks old male C57BL/6 mice were intraperitoneally (IP) injected with A151 ODN, 2D/3D-exosomes, 2D/3D-exosomes mixed with A151 ODN, or 2D/3D-exosomes loaded with A151 ODN. 10 µg/mouse exosome and 10 µg/mouse A151 ODN were used for injections. 24 hours later, animals were sacrificed by cervical dislocation and PECs were collected as described in Section 2.2.1.7 and stained with anti CD45, TCRβ, B220, CD11b, F4/80, Ly6G and Ly6C antibodies (see Table 2.3) as described in Section 2.2.8.7 to observe the T-cell, T-cell, macrophage and MDSC phenotypes.

2.2.9.2 Dextran Sulfate Sodium (DSS)-Induced Colitis Model

3% (gram/liter) colitis-grade dextran sulfate sodium salt (#160110, MP Biomedicals, Santa Ana, CA, USA) was dissolved in autoclaved drinking water of 8-10 weeks old male C57BL/6 mice. DSS-containing water was given to the mice for 4 days in order to induce colitis and then replaced with fresh regular drinking water for 3 days before the sacrifice of animals. The day that DSS administration started was counted as day 0. A151 ODN, 2D/3D-exosomes, or 2D/3D-exosomes loaded with A151 ODN were IP injected as 30 µg/mouse in a volume of 200 µl on day 0 and day 2. At day 7, mice were sacrificed. Cardiac puncture blood was used for cytokine ELISA, single cell suspensions obtained from spleen (Section 2.2.1.6) and mesenteric lymph nodes (Section 2.2.1.8) were fixed and stained with relevant antibodies (Table 2.3) in order to analyze T- and B-lymphocyte, neutrophil, macrophage, DC and MDSC populations and their activating and inhibiting surface markers. Also, splenocytes were seeded on 96 well plates as 2.5×10^5 cells/well and stimulated with 20 ng/ml PMA along with 1 µg/ml ionomycin, 1 µg/ml LPS, 1 µg/ml R848 or 1 µM K23 CpG ODN for 24 hours. IFNγ, IL6, TNFα and IL10 levels in the culture supernatants were determined with ELISA. Lastly, colons were removed for length measurements and preparation of Swiss-roll samples for histological analyses as detailedly explained by Whitem et al. [264].

2.2.9.3 Zymosan-Induced Peritonitis Model

8-10 weeks old male C57BL/6 mice were pre-treated with IP injections of 2D/3D-exosomes as 30 µg/mouse in a volume of 200 µl on day -1. On day 0, 100 µg/mouse zymosan (#tlrl-zyn, InvivoGen)

was IP injected to induce acute inflammation in the peritoneum. 6 hours later, mice were sacrificed and PECs were collected as described in Section 2.2.1.7. Neutrophil, macrophage and MDSC phenotypes in the peritoneal cavity were determined by staining PEC cells as in Section 2.2.9.1.

2.2.9.4 Excisional Wound Healing Model

8-10 weeks old female BALB/c mice were shaved and anesthetized with ketamine and xylazine by the veterinary specialist. Circular punch biopsies were performed with 6 mm punches (#P625, Acuderm, Fort Lauderdale, FL, USA) and silicone wound splints (#476684, Grace Bio-Labs, Bend, OR, USA) were surgically stitched around the wound in order to prevent contraction. The day that punch biopsies were performed was considered as day 1. 2D/3D-exosome-depleted CM, A151 ODN, 2D/3D-exosomes, or 2D/3D-exosomes loaded with A151 ODN were injected subcutaneously to the wound edges as 30 µg/mouse on day 1 and day 3. Each wound was photographed every day and the areas were digitally measured using public ImageJ software. 3 animals from each group were sacrificed on day 7 while the remaining 7 were sacrificed on day 14. Each wound area was surgically removed and half of it was placed in 10% buffered formalin for histological analyses. The rest of the wound was used for single cell isolation (see Section 2.2.1.9). Cardiac puncture blood was used for cytokine ELISA, single cell suspensions obtained from spleen (Section 2.2.1.6) and skin tissue were stained with relevant antibodies (Table 2.3) in order to analyze T and B-lymphocyte, neutrophil, macrophage and MDSC populations and their activating and inhibiting surface markers. Skin cells were stained live whereas spleen cells were fixed for staining within 10 days.

2.2.10 Statistical Analysis

In order to compare multiple treatment groups to a control group, One-way ANOVA with Dunnett's multiple comparison test was used. For the comparison between two treatment groups, Student's unpaired t test was employed. p values less than 0.05 were considered significant. All statistical analyses and plotting were performed with GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA).

Chapter 3

Results

3.1 Characterization of UCX[®] Exosomes

3.1.1 Size Distribution

Size distribution of UCX[®] exosomes before and after A151 ODN loading (method described in Section 2.2.6) was investigated via dynamic light scattering (DLS) in aqueous state and via atomic force microscopy (AFM) in dry state.

DLS data revealed that 2D-exosomes were smaller than 3D-exosomes with diameters of 104.6 ± 11.9 and 188.1 ± 31.09 nm, respectively (Figure 3.1). The peaks indicate only one population per sample which means that the exosome solutions were fairly homogenous. After A151 ODN loading, average size of 2D-exosomes increased to 225.5 ± 53.86 nm while it was 248.2 ± 61.31 nm for loaded 3D-exosomes. A151 ODN loading appears to have increased the sizes of 2D-exosomes more than 100% but the increase in 3D-exosomes' size was around 30%. Of note, loading of A151 ODN either within 2D or within 3D-exosomes significantly increased size variation as evidenced by the increase in the standard deviation values (Figure 3.1 left up vs left down or right up vs right down values). Another readout that could be obtained from DLS instruments was the polydispersity index (PDI) which was 0.52 for 2D-exosomes, 0.12 for 3D-exosomes, 0.42 for A151 ODN loaded 2D-exosomes and 0.35 for A151 ODN loaded 3D-exosomes. PDI is a measure of heterogeneity, 0 representing a completely uniform sample and 1 representing a highly polydisperse sample [265]. A151 ODN loading apparently increased the PDI 3-fold for 3D-exosomes although PDI values did not differ critically for 2D-exosomes.

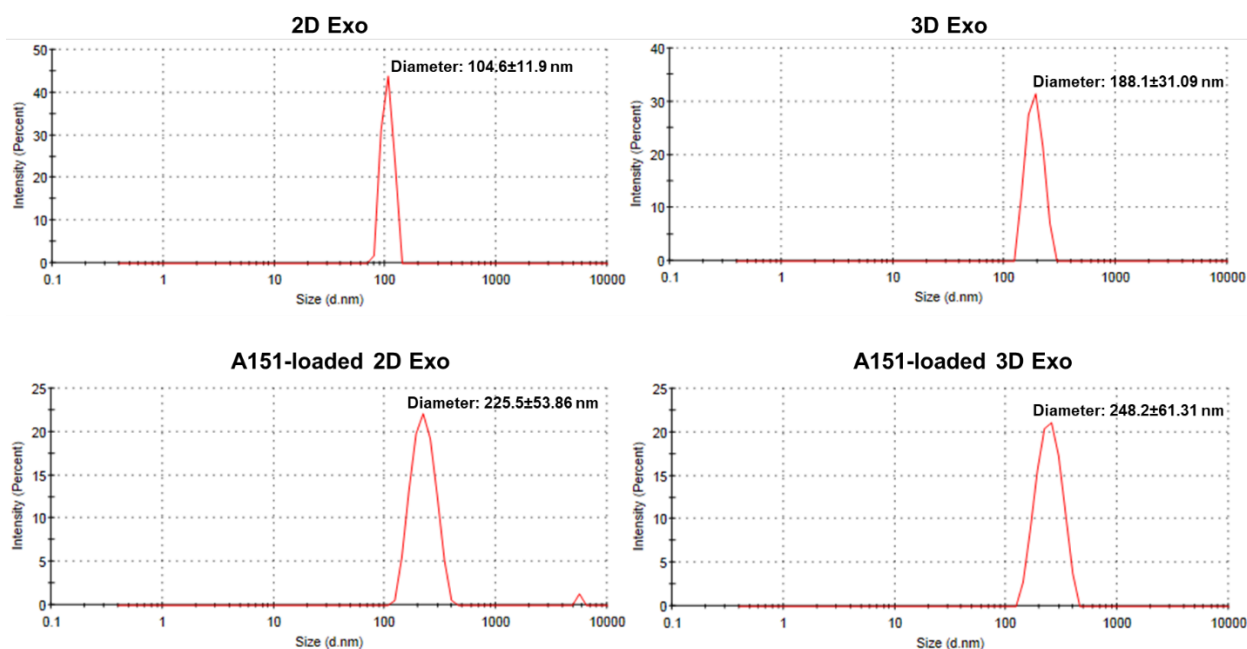


Figure 3.1. Size distribution of UCX exosomes before and after A151 ODN loading. Size distribution of free and A151 ODN loaded exosomes diluted in DPBS in a final concentration of 0.005 $\mu\text{g}/\mu\text{l}$ was determined via dynamic light scattering (DLS). Distributions are represented as percent intensity. Measurements were done in triplicates and representative measurements are shown. Exo: exosome.

AFM images, although not available for 2D-exosomes, support the DLS data (Figure 3.2). Imaging revealed that A151 ODN loading into exosomes increased the particle sizes, both in diameter and height, and resulted in a more heterogenous population as the PDI values from DLS measurements also indicated (Figure 3.2). After loading, particles got bigger and lost their well-rounded shapes. The differences in size among the loaded particles might be due to different amounts of A151 ODN encapsulated by the particles. Visible diameters of particles in the AFM images goes up to 400 nm which is much higher than the diameter values obtained in aqueous state with DLS. As the particles are attached on a surface and are spread over it during AFM imaging, it is feasible that their shapes and sizes are more distorted compared to their aqueous state.

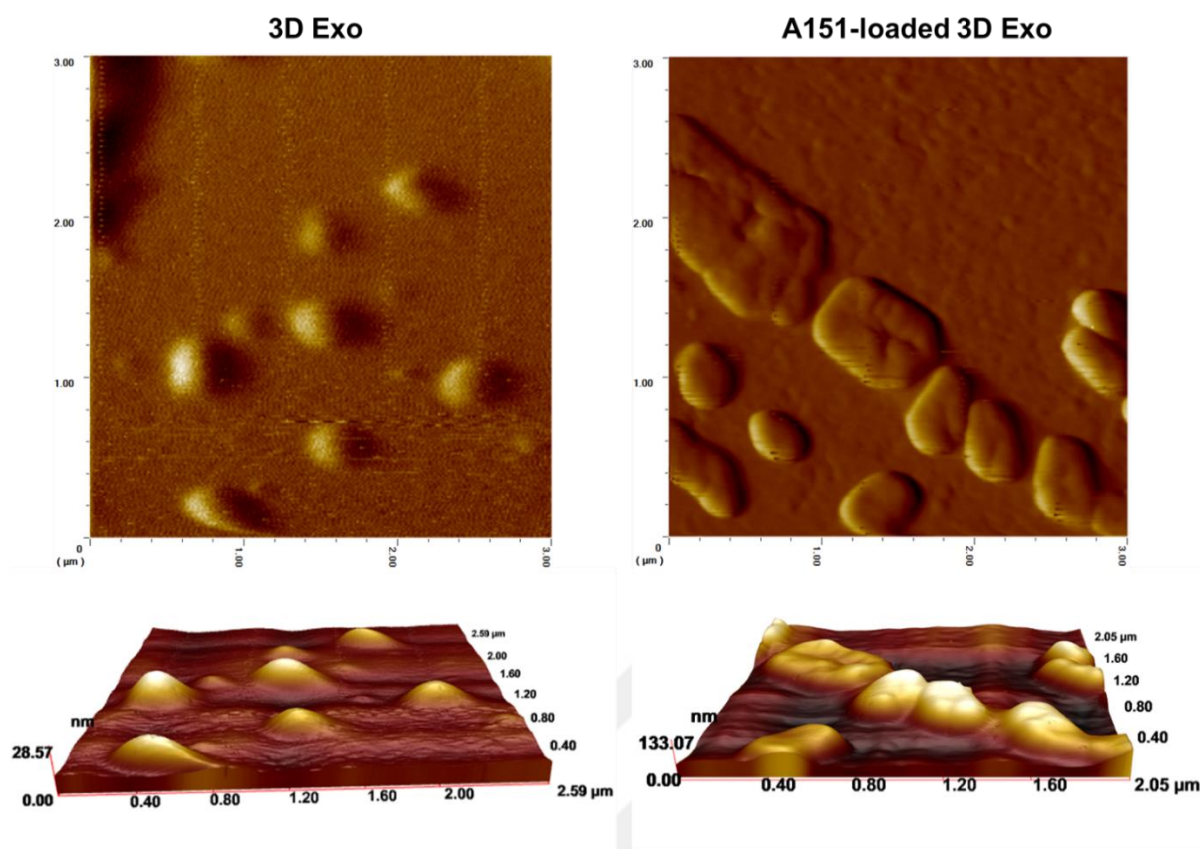


Figure 3.2. AFM images of 3D UCX exosomes before and after A151 ODN loading. Images of exosome samples dried on mica surface were obtained in non-contact dynamic mode. Both two dimensional (upper row) and three dimensional (lower row) images are shown for each sample. Exo: exosome.

3.1.2 Surface Protein Characterization

Exosomes are known to be enriched in tetraspanin proteins such as CD9, CD63 and CD81 which have roles in membrane organization and are commonly used as exosome surface markers [266]. Thus, we wanted to confirm that UCX[®] exosome samples indeed carry these tetraspanins and that the A151 ODN loading procedure does not impair the integrity of the membrane and the bound proteins. Since visualizing exosomes with flow cytometry is hard due to their small sizes, we utilized a bead-based system to observe the presence of our markers of interest. Exosomes were first captured by anti-CD63-coated carboxyl latex beads and then stained with fluorochrome anti-CD9 and anti-CD81 antibodies as described in Section 2.2.4.3.

Both CD9 and CD81 were found to be present on the 2D- and 3D-exosome samples (Figure 3.3). Mean fluorescence intensity values (MFI values) belonging to the fluorochrome-labeled tetraspanin-specific antibodies were at least 2-fold higher than the signal obtained from the antibody isotype control non-specifically bound to the beads. After A151 ODN loading, although fold changes slightly decreased for the 3D sample, both 2D- and 3D-exosome samples were still positive for CD9 and CD81. In short, both

2D and 3D culture-derived UCX[®] exosomes display cardinal exosome-specific surface markers and A151 ODN loading procedure does not significantly affect the expression frequencies of these proteins.

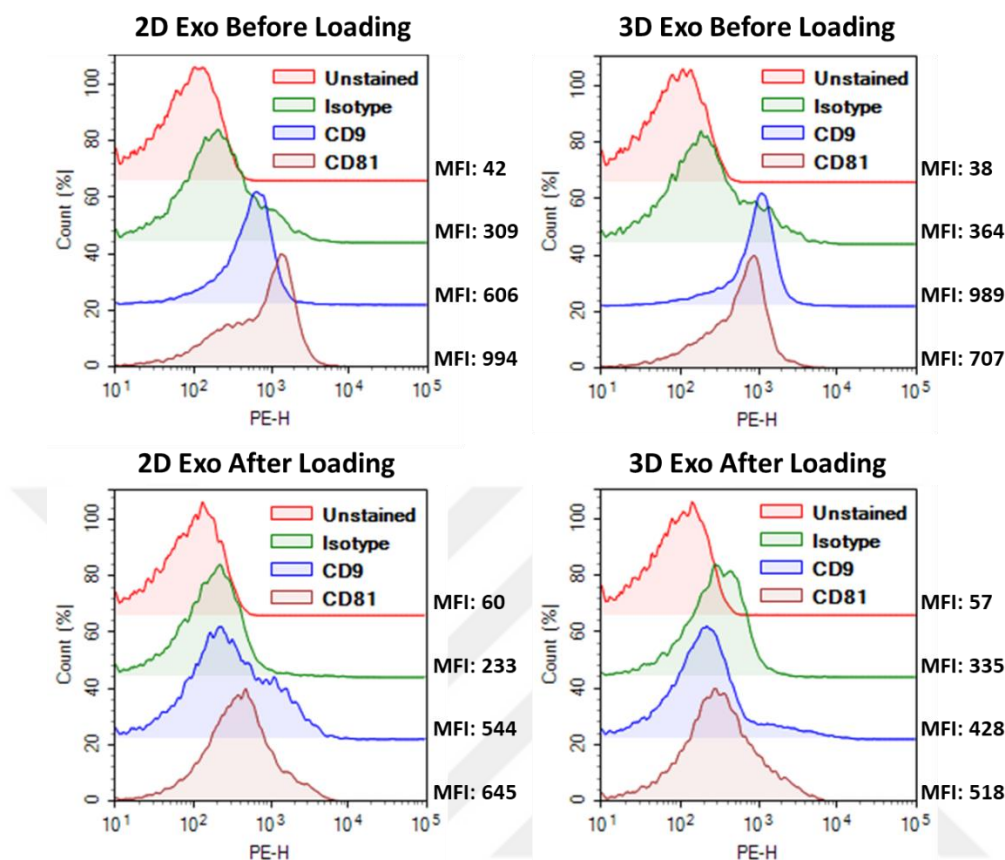


Figure 3.3. Presence of conventional exosome-specific surface markers on UCX exosomes before or after the lyophilization based loading procedure. Histogram plots showing flow cytometric analyses of 2D- and 3D-exosomes, before (upper panels) or after (lower panels) A151 ODN loading, captured with anti-CD63 coated latex beads and stained with PE-labeled anti-CD9, anti-CD81 and their appropriate isotype controls. Data are representative of three independent staining experiments. Exo: exosome, MFI: mean fluorescence intensity.

With the same bead-based staining technique, we wanted to confirm the presence of two immunomodulatory proteins which were shown to be present on UCX[®] exosomes with mass spectrometric analyses performed by our collaborators (data not shown). PD-L1, an inhibitory ligand acting by binding to the PD-1 receptor on T-cells, is known to induce immune tolerance by inhibiting T-cell activation and proliferation, and it is overexpressed in immune-privileged tissues such as placenta and in tumors in order to establish an immunosuppressive environment [267]. CD55, also called the decay-accelerating factor, blocks the complement cascade and prevents the formation of the membrane attack complex. It was also shown to suppress T-cell immunity [268]. Moreover, CD55 is present on MSCs and helps protect them from complement-dependent lysis [74]. Confirming the proteomics data, both PD-L1 and CD55 were found to be present on 2D and 3D UCX[®] exosomes and A151 ODN loading did not alter the surface expression levels of both proteins (Figure 3.4). Although direct comparison of expression levels between samples were not possible due to the limitations of bead-based analyses rather

than individual exosome monitoring, both types of exosomes surely contain the immunosuppressive PD-L1 and CD55 proteins before and after A151 ODN loading. Direct engagement of PD-L1 on exosome surface with PD-1 on T-lymphocytes might confer these exosomes a T-cell suppression ability while CD55 would protect the circulating exosomes from complement-dependent destruction, thereby improves its circulation time.

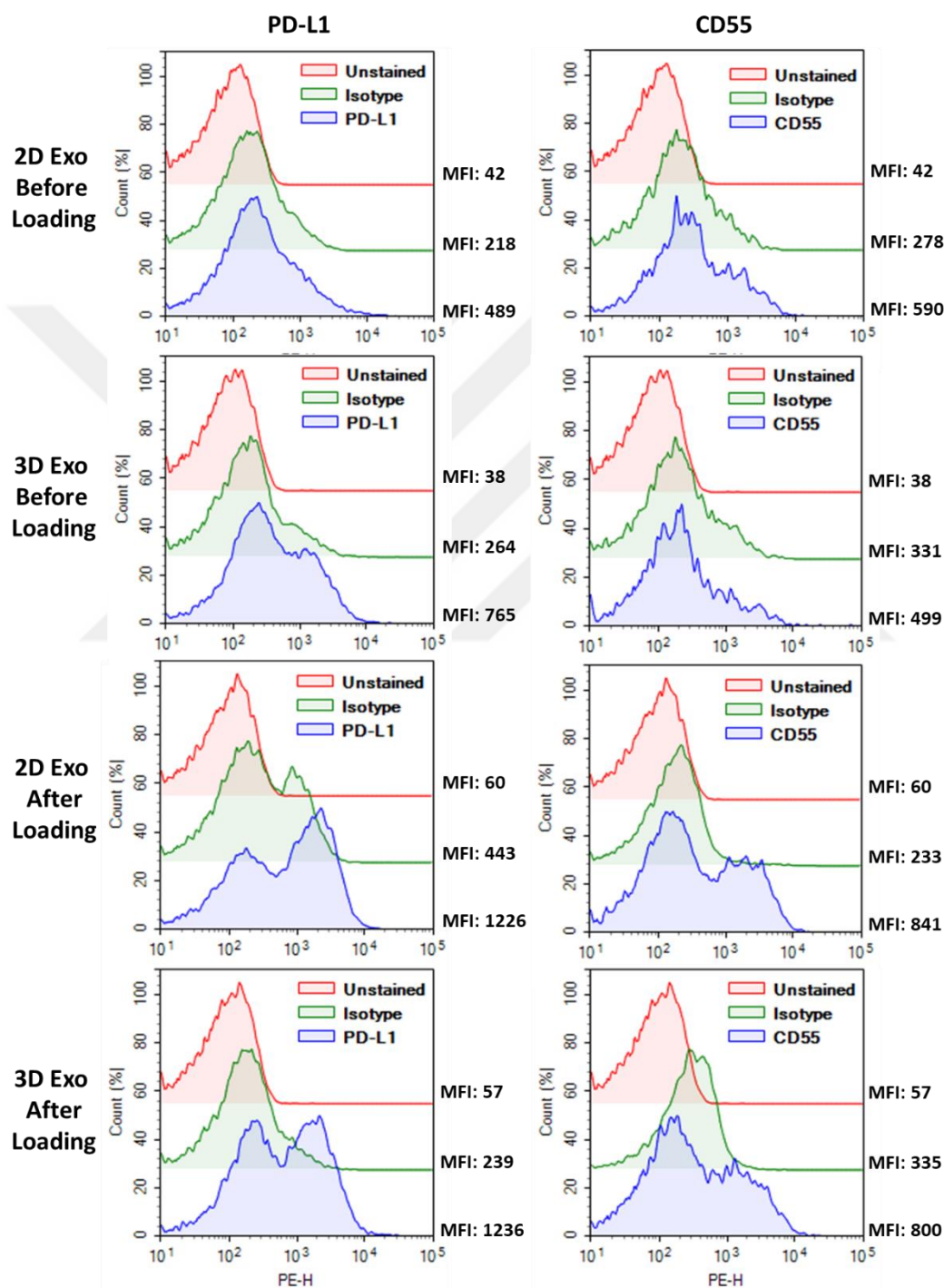


Figure 3.4. Presence of immunomodulatory proteins PD-L1 and CD55 on 2D- or 3D-exosomes before or after the lyophilization based loading procedure. Histogram plots showing flow cytometric analyses of 2D- and 3D-exosomes, before or after A151 ODN loading, captured with anti-CD63 coated

latex beads and stained with PE-labeled anti-PD-L1, anti-CD55 and their appropriate isotype controls. Data is representative of three independent staining experiments. Exo: exosome, MFI: mean fluorescence intensity.

3.2 Exosome Binding and Uptake Kinetics

In order to determine the time- and dose-dependent binding and uptake kinetics of UCX[®] exosomes by different types of immune cells, exosomes labeled with the lipophilic fluorescent dye SP-DiOC were incubated with two heterogeneous immune cell populations which were human PBMCs and mouse splenocytes (see Section 2.2.5 for the method). Moreover, since mouse bone marrow-derived macrophages (BMDMs) were frequently used throughout this study, their binding and uptake potential for the exosomes were also investigated. The results presented in the following figures represent collective values of both bound and internalized exosome signals.

3.2.1 Exosome Binding and Uptake by Immune Cell Populations in Human PBMCs

Extracellular vesicles can directly bind to a surface molecule on the target cell or they can be internalized through various mechanisms including lipid raft-mediated internalization, clathrin-dependent endocytosis, macropinocytosis and phagocytosis [269]. Therefore, phagocytic immune cells such as dendritic cells, monocytes and macrophages are expected to display high exosome internalization rates. In line with this, nearly 70% of DCs and 100% of monocytes in circulating human PBMCs were exosome positive after 16-24 hours of incubation (Figure 3.5). B-cells, which are also suggested to have phagocytic capacities [270], could reach to 60% exosome positivity after 24 hours of high dose (20 µg/ml) 3D-exosome incubation. Interestingly, exosome positivity in cytotoxic CD8⁺ T-cells could also reach nearly 60%. This might represent direct binding rather than internalization since UCX[®] exosomes carry proteins such as PD-L1 able to bind T-cell surfaces (Figure 3.4). The lowest uptake and/or binding rate in PBMCs were of CD4⁺ helper T-cells with a maximum exosome positivity of 10-15% (Figure 3.5). Overall, after 24 hours of high-dose exosome incubation, around 40% of the whole PBMCs were exosome positive.

For all cell types analyzed, binding and/or uptake of exosomes were dose-dependent. The maximum binding/uptake level reached with the low dose 5 µg/ml in 24 hours could be reached in 4-8 hours with the high exosome dose 20 µg/ml (Figure 3.5). With the high exosome dose, exosome positivity in cells did not increase dramatically after 16 hours since it already reached the maximum but the time-dependency of the uptake/binding could clearly be observed with the low dose. Differences between 2D- and 3D-exosomes were also more distinct with the low exosome dose. For all cell types except for B-cells, binding and/or uptake of 3D-exosomes were faster compared to 2D-exosomes. The maximum exosome positivity achieved after 24 hours was higher with 3D-exosomes for T- and B-lymphocytes while it was similar to 2D-exosomes for DCs and monocytes. Representative flow cytometry plots

displaying exosome-negative and exosome-positive cell populations were given in Figure 3.6. In summary, our results revealed that, UCX[®] exosomes interact and/or taken up by phagocytic and non-phagocytic immune cell types in human blood with varying efficiencies and the binding and/or uptake of 3D-exosomes were faster and higher compared to 2D-exosome counterpart.

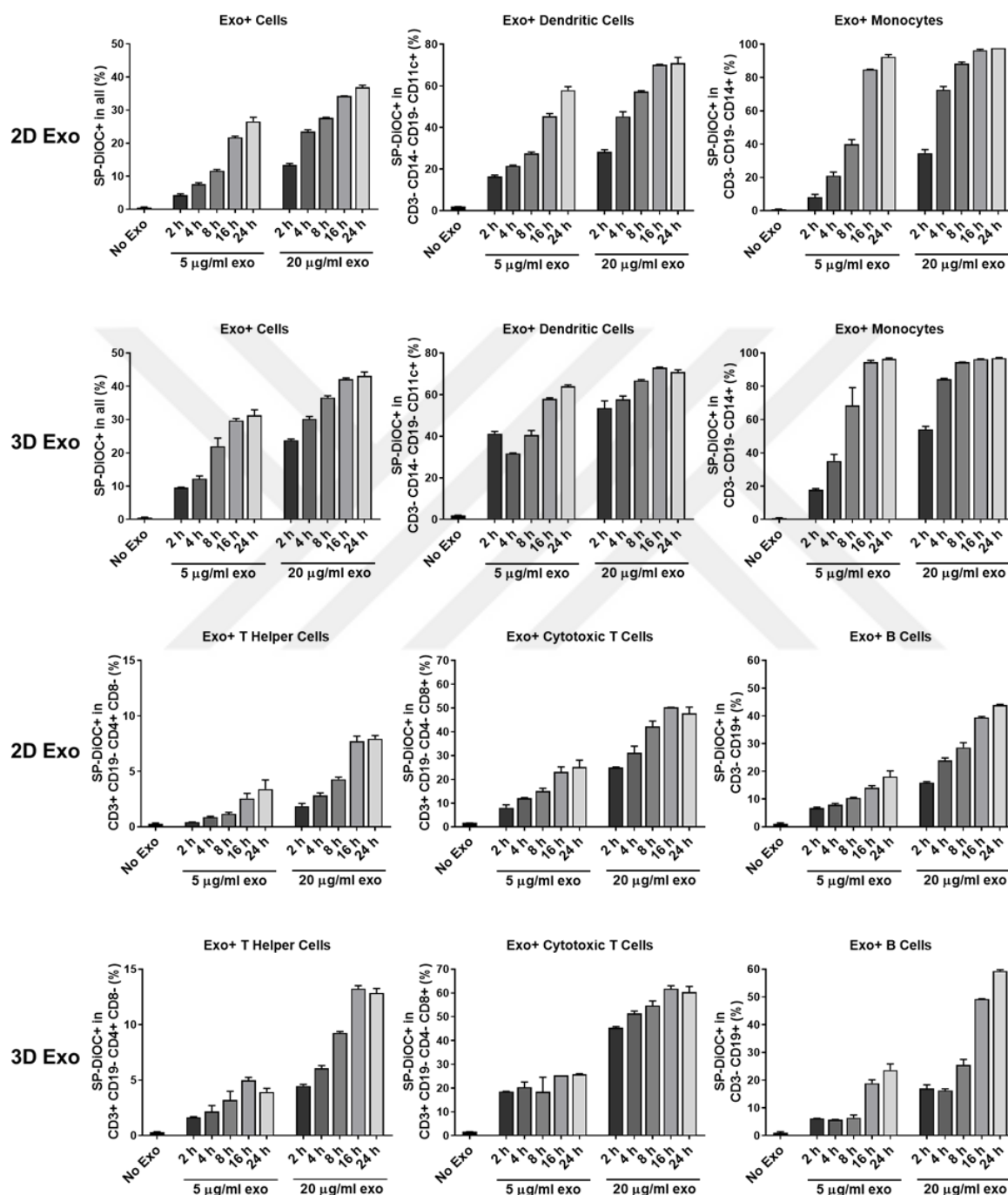


Figure 3.5. Time- and dose-dependent binding and uptake of 2D- and 3D-exosomes by different cell populations in human PBMCs. SP-DiOC-labeled exosomes were incubated with 2.5×10^5 cells/well PBMCs in a concentration of 5 or 20 µg/ml for the indicated time points. Exosome positivity

in different cell populations was determined via flow cytometry upon surface marker staining. Experiment was conducted in triplicates. Exo: exosome, h: hour.

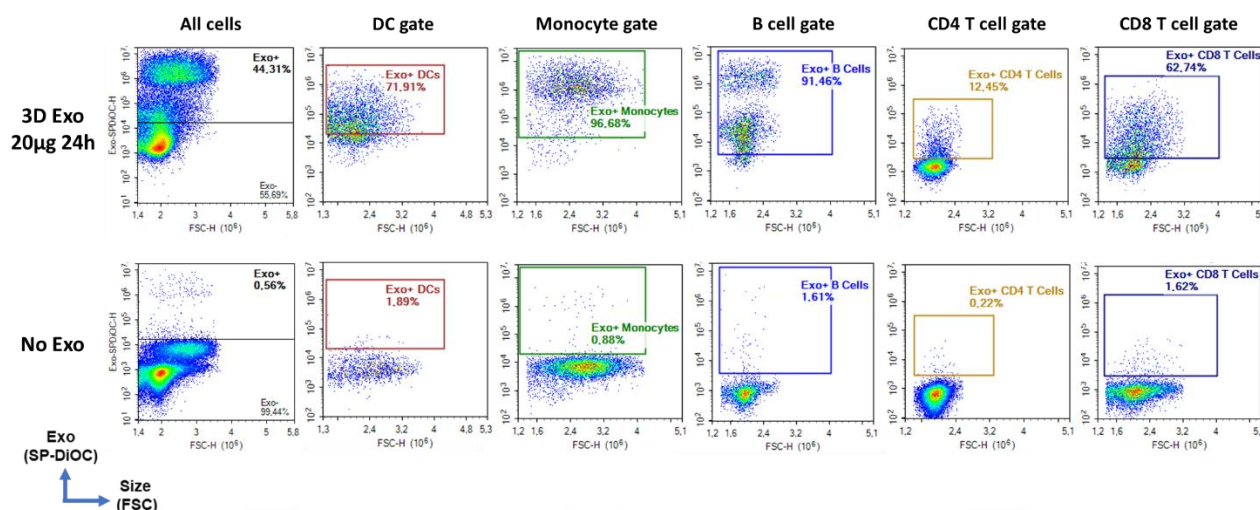


Figure 3.6. Representative flow cytometry plots related to Figure 3.5 showing exosome positivity in different cell types. PBMCs incubated with 20 µg/ml 3D-exosomes for 24 hours are shown as the representative positive sample. PBMCs incubated without any exosome for the same time period are shown as the negative example. Exo: exosome, h: hour, DC: dendritic cell, FSC: forward scatter.

3.2.2 Exosome Binding and Uptake by Immune Cell Populations in Mouse Splenocytes

Similar to the case with PBMCs, phagocytic cells in spleen which include DCs and macrophages were expected to be the cell types with the highest exosome uptake among splenocytes. Indeed, exosome positive DCs could reach 90% of the DC population after 24 hours while exosome positivity in macrophages went up to 70% (Figure 3.7). Follicular T-cells of spleens are also able to perform phagocytic action [271] and our results revealed that exosome positivity in splenic T-cells could reach up to 70% (Figure 3.7). Maximal exosome binding/uptake by CD4⁺ helper T-cells was 25-30% whereas it was 15-20% by CD8⁺ cytotoxic T-cells. Overall, maximal exosome positivity in the whole splenocyte population was around 25-30%.

For all cell types, dose-dependency of binding and/or uptake of exosomes were apparent. The maximum binding/uptake level reached with the low dose 5 µg/ml in 16-24 hours could be reached in just 2 hours when using the high exosome dose which was 20 µg/ml (Figure 3.7). With the high exosome dose, exosome positivity in cells did not increase dramatically throughout the rest of the incubation time since it reached the maximum in 2-4 hours. Only in macrophages, longer incubation resulted in improved exosome uptake. Differences between 2D and 3D-exosomes were more apparent with the low exosome dose. For all cell types, 3D-exosomes bound to or entered the cells faster compared to 2D-exosomes and the maximum exosome positivity achieved after 24 hours was always higher with 3D-exosomes. Representative flow cytometry plots displaying exosome-negative and exosome-positive cell populations are given in Figure 3.8.

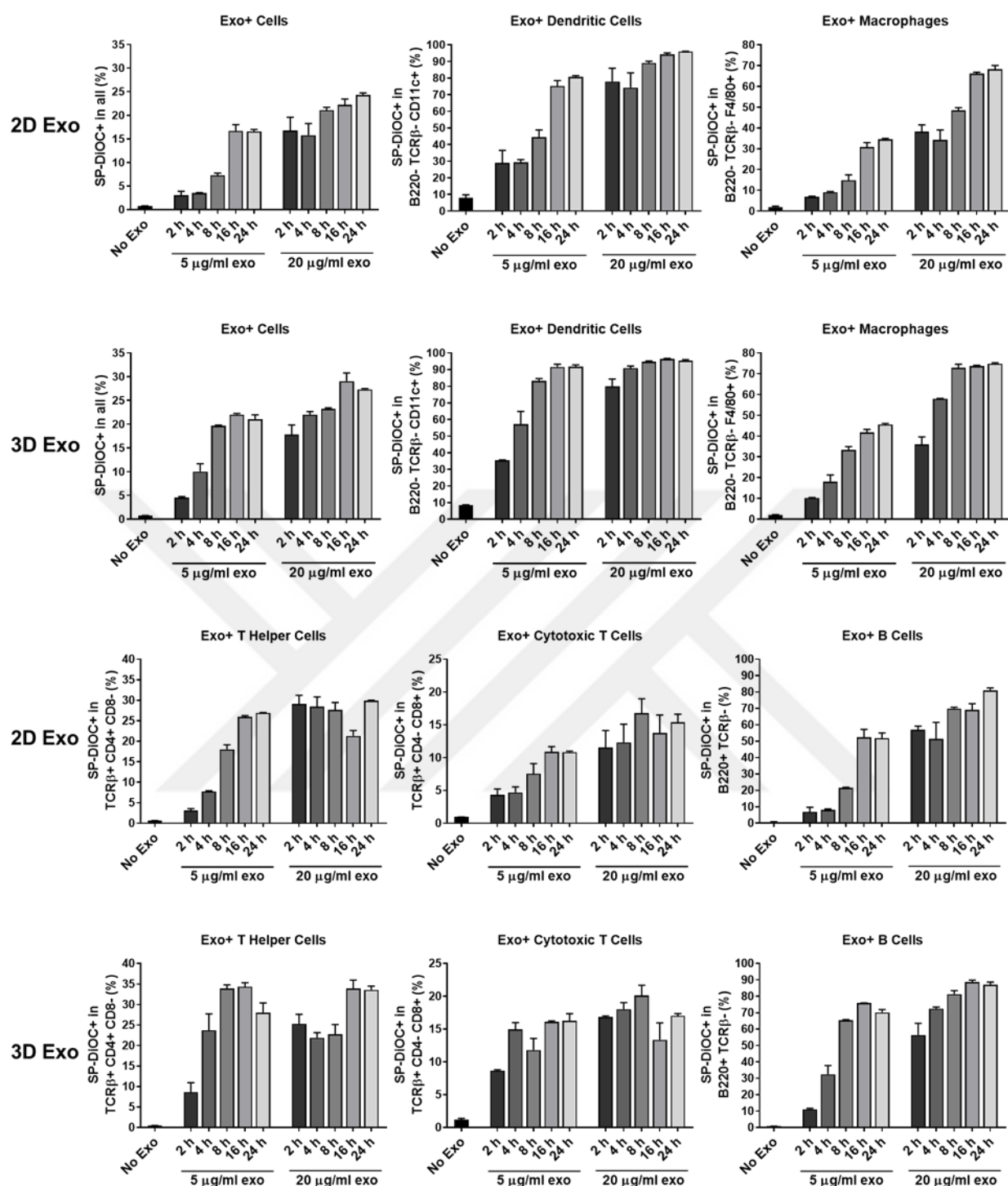


Figure 3.7. Time- and dose-dependent binding and uptake of 2D- and 3D-exosomes by different immune cell populations in mouse splenocytes. SP-DiOC-labeled exosomes were incubated with 2.5×10^5 cells/well splenocytes in a concentration of 5 or 20 $\mu\text{g/ml}$ for the indicated time points. Exosome positivity in different populations was determined via flow cytometry upon surface marker staining. Experiment was conducted in triplicates. Exo: exosome, h: hour.

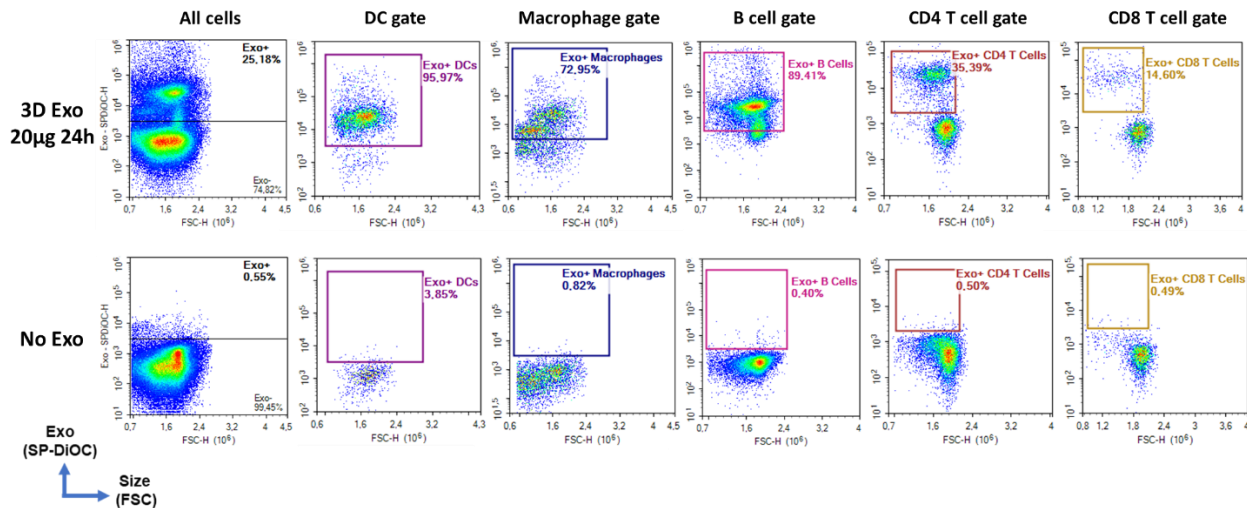


Figure 3.8. Representative flow cytometry plots related to Figure 3.7 showing exosome positivity in different cell types. Splenocytes incubated with 20 µg/ml 3D-exosomes for 24 hours are shown as the representative positive sample. Splenocytes incubated without any exosome for the same time period are shown as the negative example. Exo: exosome, h: hour, DC: dendritic cell, FSC: forward scatter.

Compared to the human PBMC data, exosome binding/uptake rate of the DC, B-cell and T-helper cell populations of the mouse spleen were higher. However, the overall exosome positivity in the whole population could reach up to 40% in PBMCs while it was only 25-30% in splenocytes. Although the UCX[®] exosomes are human-derived and their uptake by human immune cells are slightly higher, we decided to use mouse-derived immune cells for further experiments since mouse samples are more easily accessible in higher amounts. For all further *in vitro* studies, 20 µg/ml exosome dose was used and the minimal time of incubation was 24 hours so that maximum binding and internalization would be achieved and the possible effects would be more pronounced.

3.2.3 Exosome Binding and Uptake by Mouse BMDMs

Since we had planned to use mouse BMDMs for most of the *in vitro* experiments, their binding and uptake potential of UCX[®] exosomes were also evaluated. Their capacity of exosome uptake and binding turned out to be even higher than primary mouse macrophages obtained from the spleen. Nearly 100% of the cells were positive for 2D-exosomes in 4 hours while it only took 2 hours for 3D-exosomes (Figure 3.9). Furthermore, a distinct exosome-high population was observed in the flow cytometric analyses. Representative plots showing this population are displayed in Figure 3.9. When the exosome-high population was investigated, 90% of the BMDMs were in this gate after 8 hours of incubation with 3D-exosomes or 16 hours of incubation with 2D-exosomes. At the end of 24 hours of incubation with either type of exosome, 90-100% of the cells were exosome-high. Thanks to their fast and efficient uptake of exosomes, we confidently used BMDMs to study the immunomodulatory potentials of UCX[®] exosomes in further experiments.

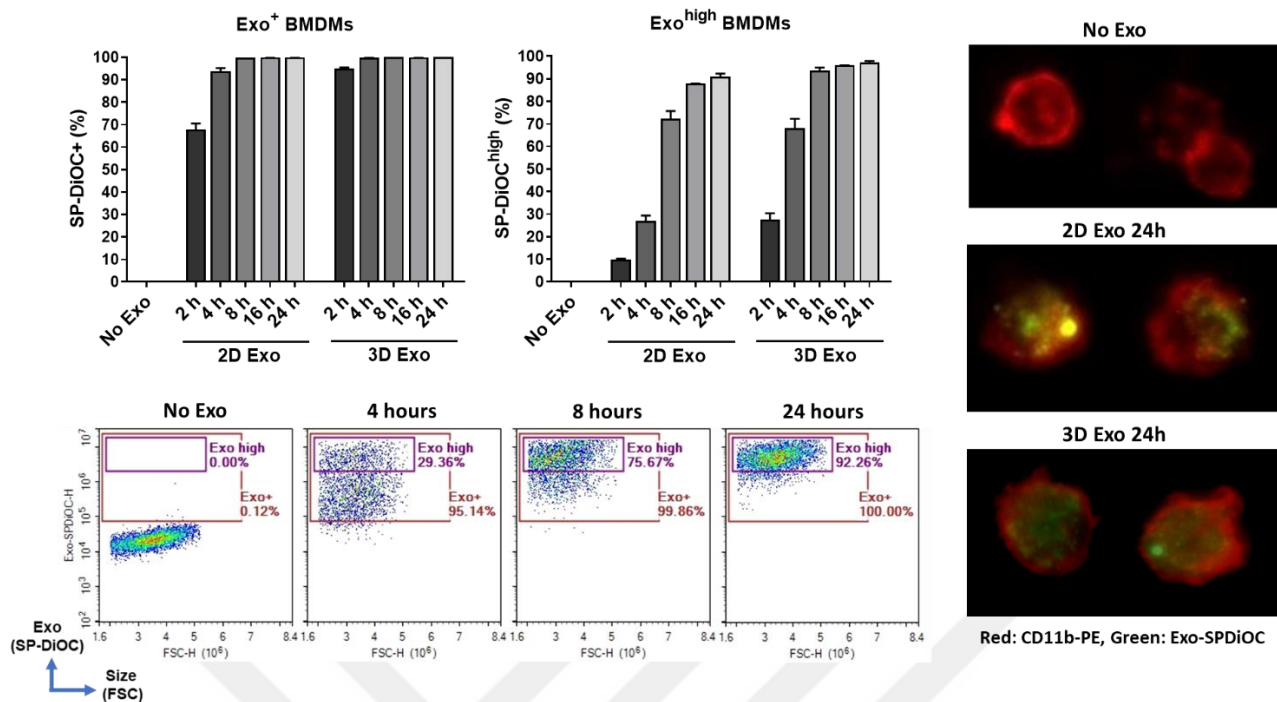


Figure 3.9. Time-dependent binding and uptake of 2D- and 3D-exosomes by mouse BMDMs. SP-DiOC-labeled exosomes were incubated with 2.5×10^5 cells/well BMDMs in a concentration of $20 \mu\text{g/ml}$ for the indicated time points. Exosome positivity in the $\text{CD11b}^+ \text{F4/80}^+$ BMDM population was determined via flow cytometry upon surface marker staining. Representative flow cytometry plots belonging to BMDMs incubated with no exosomes or 2D-exosomes showing exosome positive and exosome-high populations are provided (lower left). Representative fluorescence microscopy images displaying exosome negative and positive cells are shown on the right panels. Experiment was conducted in triplicates. BMDM: Bone marrow-derived macrophage, Exo: exosome, h: hour.

3.3 Determination of A151 ODN Loading Efficiency into Exosomes

Since exosomal encapsulation of bioactive molecules including nucleic acids improve their stability and provide efficient delivery while remaining biocompatible and safe [272], we aimed to use UCX[®] exosomes and the immunosuppressive A151 ODN in conjunction for the further studies. In order to first determine if A151 ODN could efficiently be loaded within the exosomes via lyophilization-based method developed in our laboratory (Section 2.2.6), SP-DiOC labeled fluorescent exosomes were loaded with Cy3-labeled A151 ODN and the loading efficiency was determined by analyzing the overlapping Cy3 and SP-DiOC signals using Novocyte flow cytometer (Section 2.2.7).

Using either 1:1 or 2:1 exosome:A151 ODN ratio (wt/wt) did not differ much for 3D-exosomes in terms of loading efficiency but more A151 ODN resulted in slightly more encapsulation into 2D-exosomes (Figure 3.10). With lower A151 ODN concentration (2:1 ratio), loading efficiency was higher with 3D-exosomes compared to 2D-exosomes. However, in all the conditions irrespective of the exosome type and loading ratio, A151 ODN was present in over 90% of the exosomes which is a nearly perfect loading efficiency. This data proves that A151 ODN can be loaded into UCX exosomes with very high efficiency

and provides a reliable basis for further studies. For the following *in vitro* experiments, exosome:A151 ODN ratio was kept at 2:1 in order to test if a substantial synergistic effect would be observed with lower A151 ODN use.

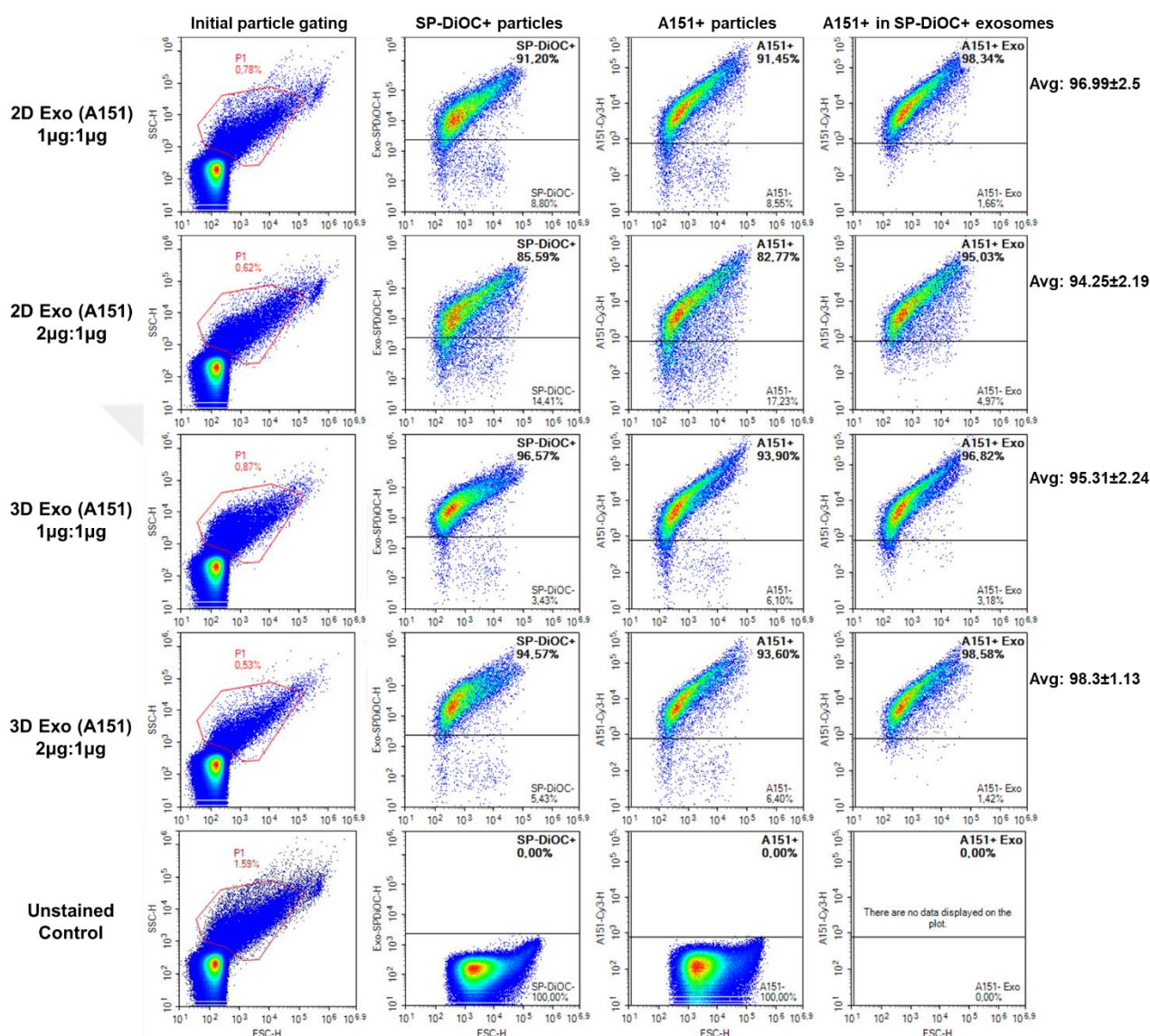


Figure 3.10. Representative flow cytometry plots displaying the loading efficiency of A151 ODN into 2D- and 3D-exosomes. SP-DiOC-labeled fluorescent 2D and 3D-exosomes were loaded with Cy3-labeled fluorescent A151 ODN at different ratios, remaining free A151 ODN was removed with ultracentrifugation, and A151 ODN positivity inside exosomes was analyzed via flow cytometry. Indicated ratios on the left side are exosome:A151 ODN ratios in terms of µg. The experiment was conducted in triplicates and the average loading efficiencies are given on the right side. Exo: exosome, Avg: average, FSC: forward scatter, SSC: side scatter.

3.4 Immunomodulatory Effects of UCX[®] Exosomes on TLR Signaling

3.4.1 Dose-Dependency Studies

Preliminary experiments with UCX[®] exosomes indicated a suppressive ability on TLR7 in terms of IL6 and IL12 secretion (Figure A1, Appendix A) and A151 ODN was already known to be a TLR9 inhibitor

[201]. We wanted to further validate the suppressive effects of 3D-exosomes and A151 ODN while also testing if they act synergistically to suppress TLR7 and TLR9 mediated immune activation either in free mixture combination form or in exosome loaded form. To this end, mouse splenocytes were stimulated with 1 µg/ml TLR7/8 ligand R848 or 1 µM TLR9 ligand K23 CpG ODN and then incubated with varying doses of A151 ODN, 3D-exosomes, or their combinations for 24 hours. Highest doses for A151 ODN were set as 1.5 µM (~10 µg/ml) and for 3D-exosomes 20 µg/ml. Three serial dilutions were adjusted for each suppressive agent and were labelled as i) high (1.5 µM for A151, 20 µg/ml for exosome), ii) medium (0.5 µM for A151, 6.667 µg/ml for exosome) and iii) low (0.167 µM for A151, 2.22 µg/ml for exosome) doses. Similarly three different doses of free A151 ODN or unloaded exosome was used as standalone control groups (Figures 3.11 and 3.12).

Both A151 ODN and the exosomes were able to suppress IL6 secretion (Figure 3.11, upper left panel) triggered by R848 in a dose-dependent manner with the highest doses being more suppressive. For the exosome-A151 mixture ~50% and for the encapsulated counterpart ~75% reduction was detected for IL6 secretion (Figure 3.11, upper left panel violet and purple bars). A151 ODN-loaded exosome was the more potent among the groups for IL6 inhibition. Unfortunately, none of the treatment groups and doses were effective in suppressing R848-induced IL12 production and a clear dose-dependent pattern was not observed.

Upon K23 stimulation, all groups except unencapsulated exosome treatment groups were very potent inhibitors of IL6 production either at medium or at high doses (Figure 3.11, upper right panel). This effect seems to be a consequence of A151 ODN presence. Of note, even the lowest dose of the A151 ODN loaded exosome treatment significantly shut down IL6 secretion almost by half while the lowest doses of A151 ODN alone or the free exosome-A151 mixture treatments could not induce this suppressive effect, implying that the synergistic activity is required to turn off cytokine secretion from R848-stimulated spleen cells. Unexpectedly, only the highest doses of all treatments were effective on IL12 inhibition, even for A151 (Figure 3.11, lower right panel). In summary, suppressive actions of A151 ODN, 3D-exosomes and their combinations are dose-dependent and A151 ODN-loading into exosomes combines and potentiates the immunosuppressive abilities of A151 ODN and the exosomes.

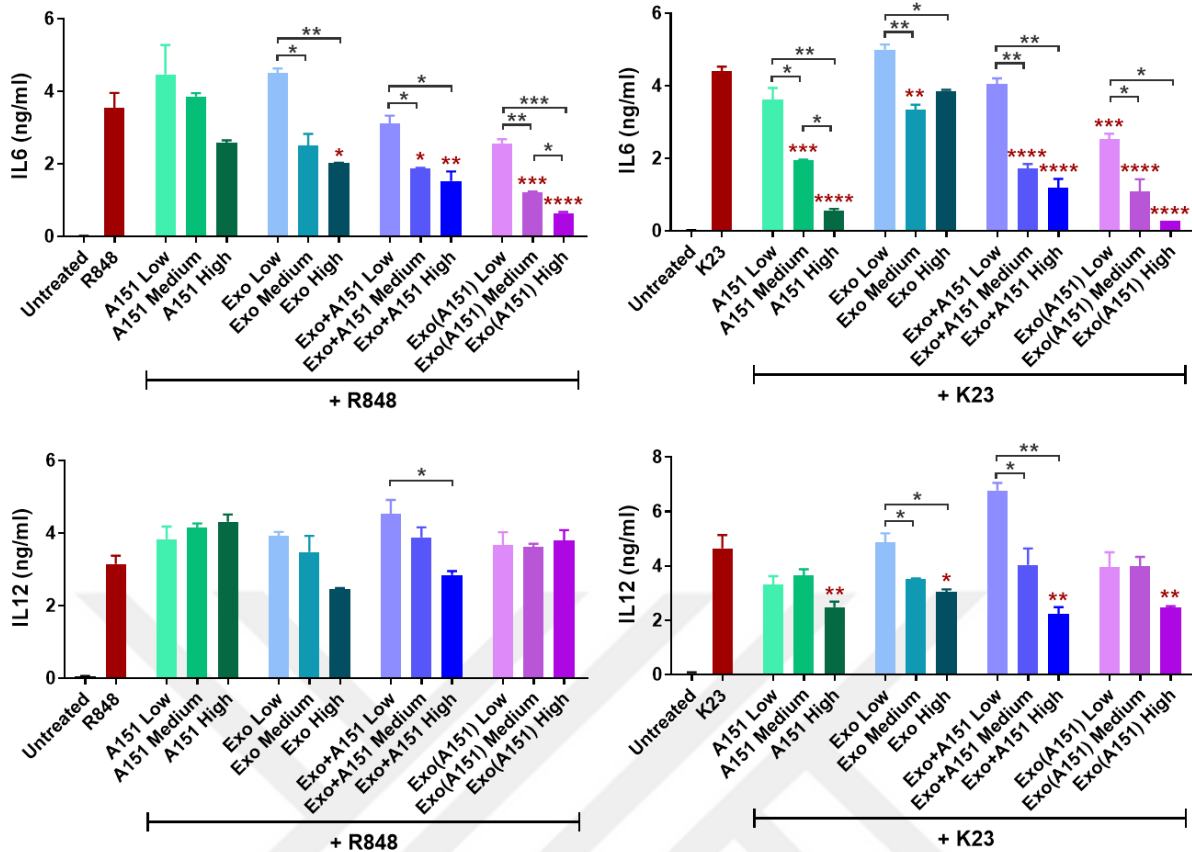


Figure 3.11. Dose-dependent effects of A151 ODN, 3D-exosome and their combinations on IL6 and IL12 secretions from mouse splenocytes upon stimulation with TLR7/8 ligand R848 and TLR9 ligand K23. Splenocytes were stimulated with 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 3 doses of A151 ODN, 3D-exosome or their free or encapsulated combinations for 24 hours. High doses were 1.5 μ M (~10 μ g/ml) for A151 ODN and 20 μ g/ml for 3D-exosome. Medium and low doses were 1/3 of the previous dose. Cytokine levels from culture supernatants were determined with ELISA. Experiment was conducted in triplicates. Red stars indicate the differences compared to the R848-only or K23-only group. Exo: 3D-exosome, Exo+A151: free mixtures of 3D-exosome and A151 ODN, Exo(A151): A151 ODN-loaded within 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

The same set of experiments were also performed with BMDMs. Since splenocyte population is a heterogeneous population of immune cells apart from macrophages, BMDMs were generated to particularly investigate the effects of A151 ODN and exosomes on macrophages. After both R848 and K23 stimulations, A151 ODN was able to inhibit IL6 secretion from BMDMs only with medium and high doses with the highest dose being more effective (Figure 3.12, upper panels). Exosomes suppressed IL6 even with the lowest dose and no clear dose-dependent further potentiation was observed. The lowest dose of free mixture of exosomes with A151 ODN caused mediocre suppression while medium and high doses almost completely abolished R848- and K23- induced IL6 secretion. A151-ODN loaded exosome formulation was able to abolish IL6 secretion even when the lowest dose was used.

A151 ODN was ineffective in inhibiting R848-induced IL12 secretion regardless of the dose (Figure 3.12, lower left panel). However, alone and A151 ODN-loaded exosomes were inhibitory in a dose-

dependent manner while the free mixture moderately suppressed IL12 with all doses. After K23 stimulation, exosomes were moderately effective in decreasing IL12 levels with medium and high doses but a dose-dependent and more potent suppression was observed with all the other groups (Figure 3.12, lower right panel). High dose of A151 ODN alone or in conjunction with exosomes in free mixture or loaded form was extremely powerful as an inhibitor of K23-induced IL12 production. In light of these data, we decided to use the highest doses for the following experiments and to further investigate the effects of A151 ODN-exosome combinations, in particular the loaded formulation, as more promising immunosuppressive agents.

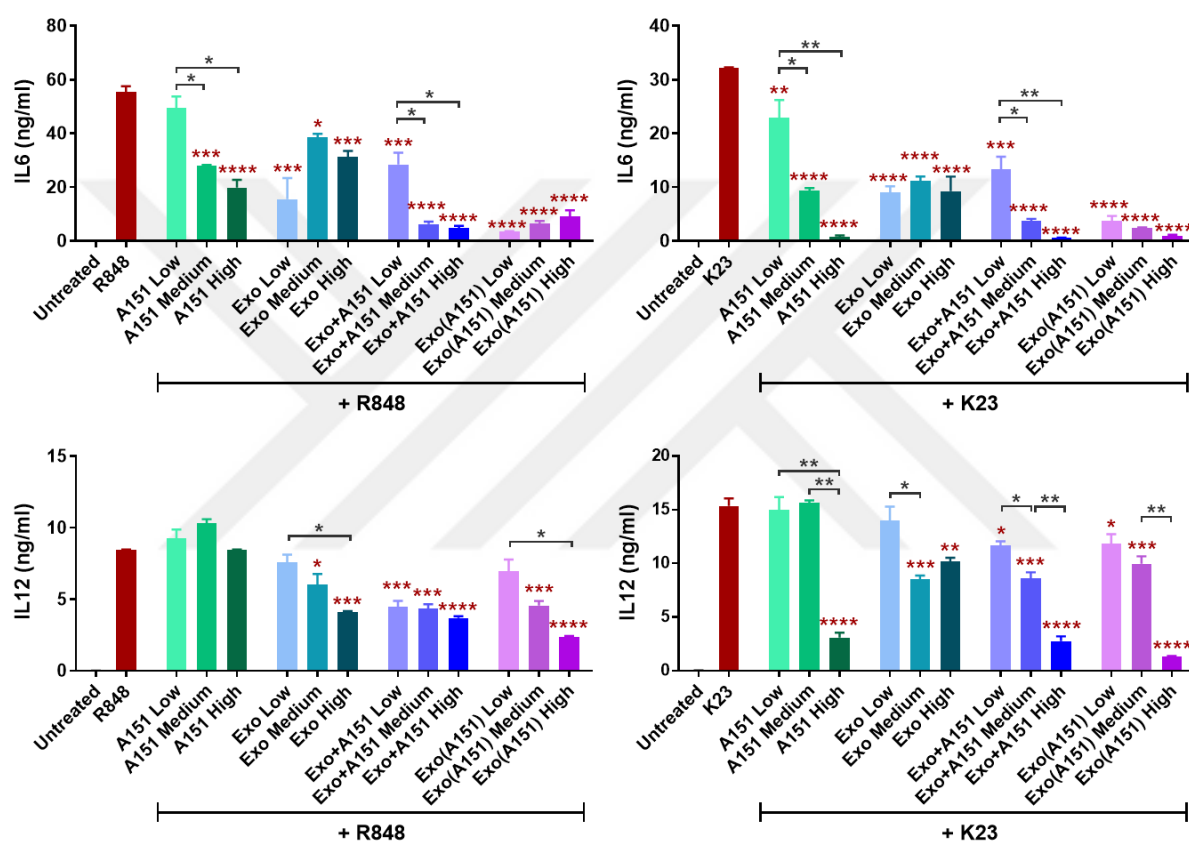


Figure 3.12. Dose-dependent effects of A151 ODN, 3D-exosome and their combinations on IL6 and IL12 secretions from mouse BMDMs upon stimulation with TLR7/8 ligand R848 and TLR9 ligand K23. BMDMs were stimulated with 1 µg/ml R848 or 1 µM K23 CpG ODN and treated with 3 doses of A151 ODN, 3D-exosome or their free or encapsulated combinations for 24 hours. High doses were 1.5 µM (~10 µg/ml) for A151 ODN and 20 µg/ml for 3D-exosome. Medium and low doses were 1/3 of the previous dose. Cytokine levels from culture supernatants were determined with ELISA. Experiment was conducted in triplicates. Red stars indicate the differences compared to the R848-only or K23-only group. Exo: 3D-exosome, Exo+A151: free mixtures of 3D-exosome and A151 ODN, Exo(A151): A151 ODN-loaded within 3D-exosome. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

3.4.2 Effects of Free or A151 ODN-Loaded UCX[®] Exosomes on Mouse Splenocytes

We wanted to investigate further the antagonistic effects of 2D- and 3D-cell culture-derived UCX[®] exosomes alone or in combination with A151 ODN against TLR ligands such as i) TLR1/2 ligand: Pam3CSK4, ii) TLR3 ligand: poly(I:C), iii) TLR4 ligand: LPS, iv) TLR7/8 ligand R848: and v) TLR9 ligand: K23 CpG ODN mediated immune activation on spleen cells.

Initially, immunomodulatory actions of UCX[®] exosomes appeared to be dependent on the severity of inflammation. When no TLR pathway was stimulated, both 2D- and 3D- exosomes caused an increase in IFN γ levels of the spleen cells in culture (Figure 3.13, upper left panel). In contrast, they suppressed IFN γ very potently when any TLR pathway other than TLR3 was stimulated (Figure 3.13). Of note, IFN γ -inducing capacity of the exosomes in a non-inflammatory environment could be dampened by combining them with A151 ODN (Figure 3.13, upper left panel). A151 ODN, 2D- and 3D-exosomes were all capable of significantly inhibiting IFN γ secretion induced by Pam3CSK4 and R848 up to 80% and 60-70% respectively (Figure 3.13, middle panels). However, loading of A151 ODN into the exosomes led to improved suppression compared to either the single treatments or to the free mixtures of exosomes with A151 ODN. A151 ODN loading within exosomes also potentiated the suppression on LPS-induced IFN γ secretion (Figure 3.13, lower left panel). Although 2D-exosome was ineffective as a standalone treatment, its mixture with A151 ODN and especially its A151 ODN-loaded form decreased LPS-induced IFN γ levels ~60% and ~70% respectively. Exosomes alone or in mixture with A151 ODN could not decrease K23-mediated IFN γ while either A151 ODN alone or its exosome-loaded counterpart were capable of significantly suppressing immune response (Figure 3.13, lower right panel). Interestingly, 3D-exosome actually increased the IFN γ levels further but either mixing it with A151 ODN or A151 ODN loading converted it to a potent suppressor. Another interesting observation is that 2D-exosomes strongly potentiated the stimulatory action of poly(I:C) almost 4-fold (Figure 3.13, upper right panel). This phenomenon was attenuated slightly when the exosomes were mixed with A151 ODN and it was completely abolished by A151 ODN-loading. Since UCX[®] cells express high amounts of TLR3 (Figure A2, Appendix A) which is an endosomal TLR, it might be possible that TLR3 is also incorporated to the exosome membranes and the exosomes help deliver poly(I:C) in the culture environment or probably exosome-associated TLR3 molecules to the target cells thereby potentiating the IFN γ stimulation. It is essential to investigate further how A151 ODN encapsulation within exosomes but not the mixing shuts of TLR3 mediated IFN γ production by immune cells.

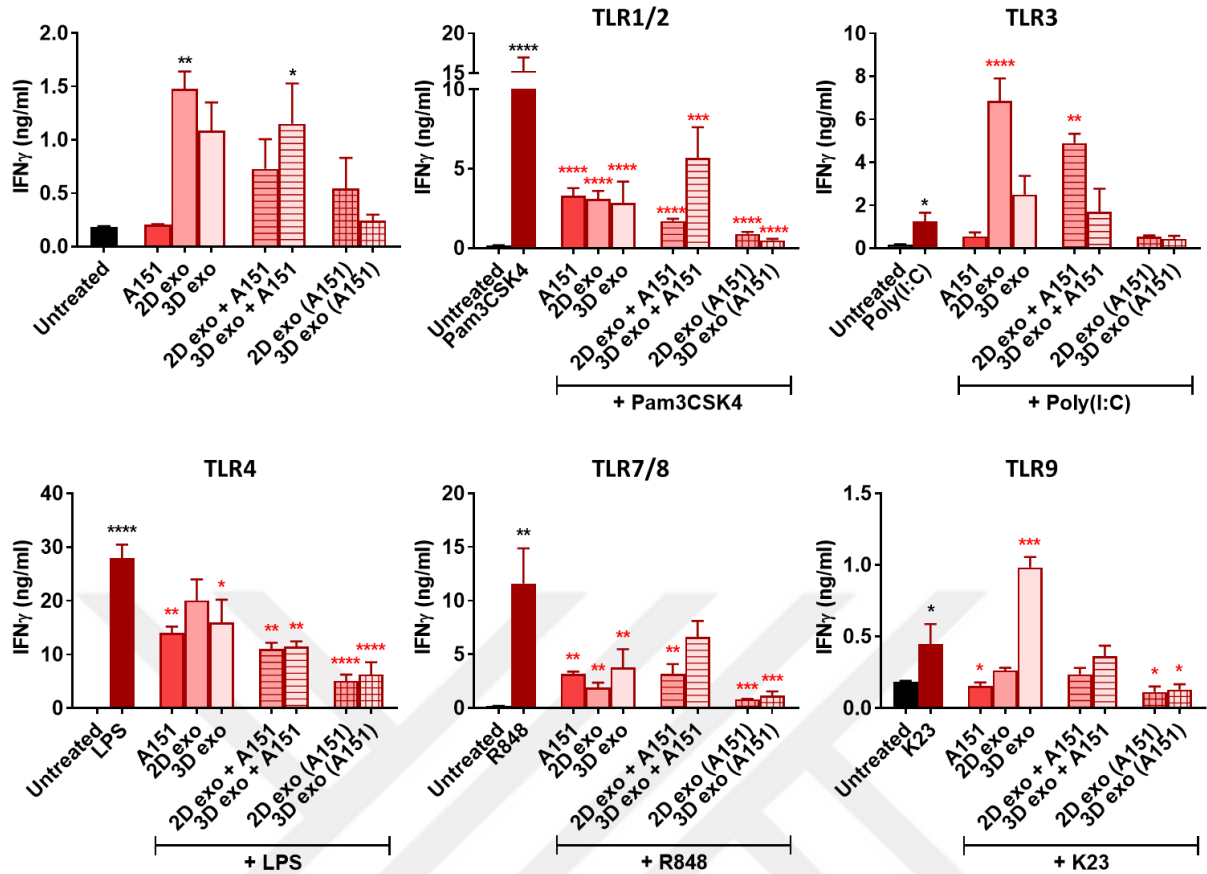


Figure 3.13. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on IFN γ secretion from splenocytes upon stimulation with various TLR ligands. Splenocytes (10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to the untreated control. Red stars represent the difference compared to the standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Similar to IFN γ , 2D- and 3D-exosomes also induced IL6 secretion from splenocytes when there was no inflammatory signal induced by TLR triggering and this could be downregulated either by adding A151 ODN through mixing or by loading (Figure 3.14). While 2D-exosomes potentiated the IFN γ secretion upon TLR3 stimulation (Figure 3.13, upper right panel), only 3D-exosomes increased IL6 secretion (~2-fold) synergistically with poly(I:C) (Figure 3.14, upper right panel). This too could be alleviated with the addition of A151 ODN in mixed or loaded form. On Pam3CSK4-induced IL6 secretion, 3D-exosomes were moderately suppressive while the A151 ODN-loaded versions of both 2D- and 3D-exosomes almost completely shut off IL6 production (Figure 3.14, upper middle panel). Only 3D-exosomes could decrease (~50%) LPS-induced IL6 production alone, however, 2D-exosomes were more effective when combined with A151 ODN especially in loaded form which achieved ~85%

suppression (Figure 3.14, lower left panel). Surprisingly, A151 ODN did not improve the suppressive action of 3D-exosomes in this case. Only 2D-exosomes were able to inhibit R848-induced IL6 secretion alone and their potency was critically improved after A151 ODN loading (Figure 3.14, lower middle panel). Although 3D-exosomes alone or mixed with A151 ODN were unable to inhibit IL6 secretion upon TLR7/8 stimulation, the A151 ODN-loaded form of 3D-exosomes were as effective as A151 ODN-loaded 2D-exosomes achieving ~85% suppression. As a well-known TLR9 antagonist, A151 ODN strongly repressed K23-induced IL6 production (Figure 3.14, lower right panel). Even though none of the exosomes were suppressive in this condition, A151 ODN-encapsulating exosomes were better inhibitors than A151 ODN alone. This is probably due to protection of A151 ODN from degradation and improved delivery by exosomes.

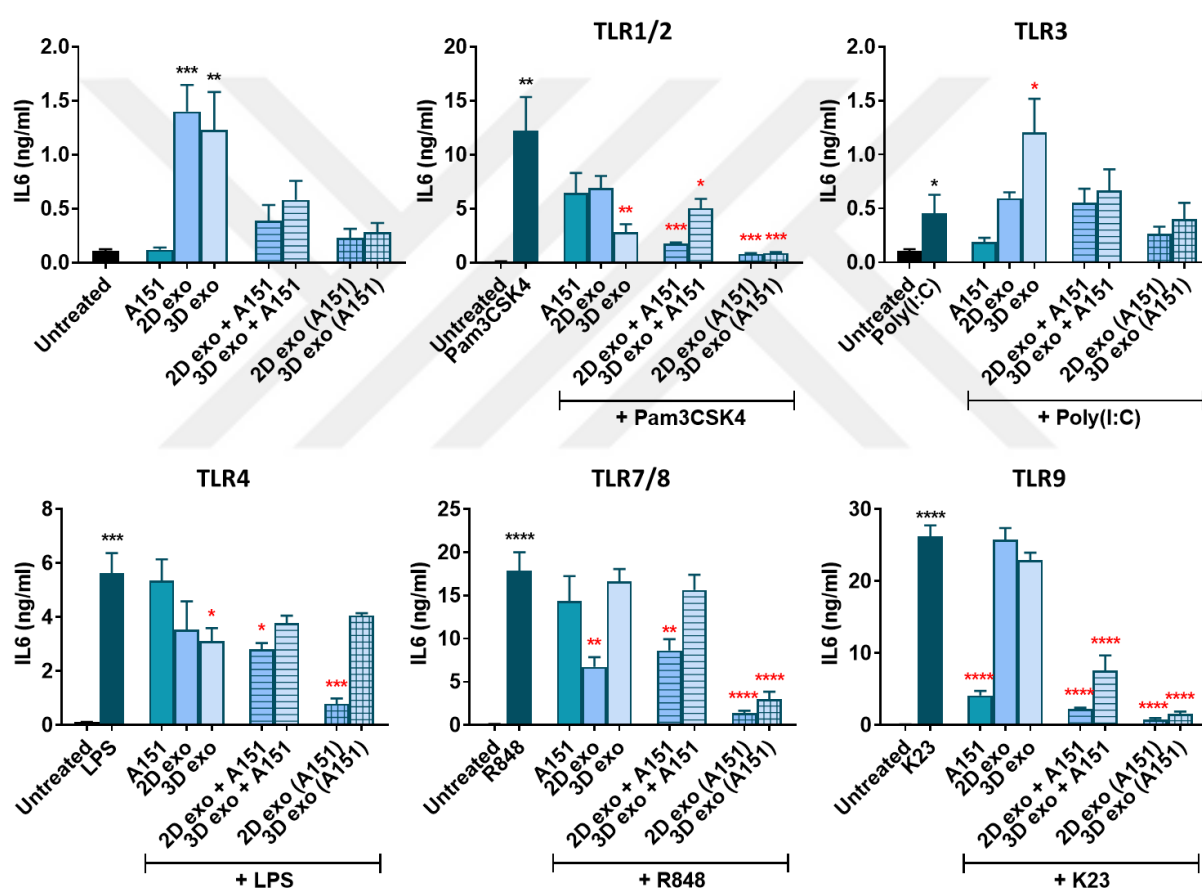


Figure 3.14. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on IL6 secretion from splenocytes upon stimulation with various TLR ligands. Splenocytes (10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to the untreated control. Red stars represent the difference compared to the standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo:

exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Except for TLR9, IL12 production induced by all other TLR ligands could not be suppressed either by A151 ODN, by exosomes or by their free or loaded combinations (Figure 3.15). In fact, 2D-exosomes consistently augmented IL12 levels both in the absence and presence of TLR ligands. Although A151 ODN-loading could limit this activity, it could not always abolish it. Even though 2D-exosomes were IL12-stimulatory upon TLR9 activation as well, A151 ODN and 3D-exosomes alone or combined were able to reduce IL12 levels around 30-35% (Figure 3.15, lower right panel). A151 ODN-loaded form of 2D-exosomes were also suppressive although less effective than A151 ODN-loaded 3D-exosomes.

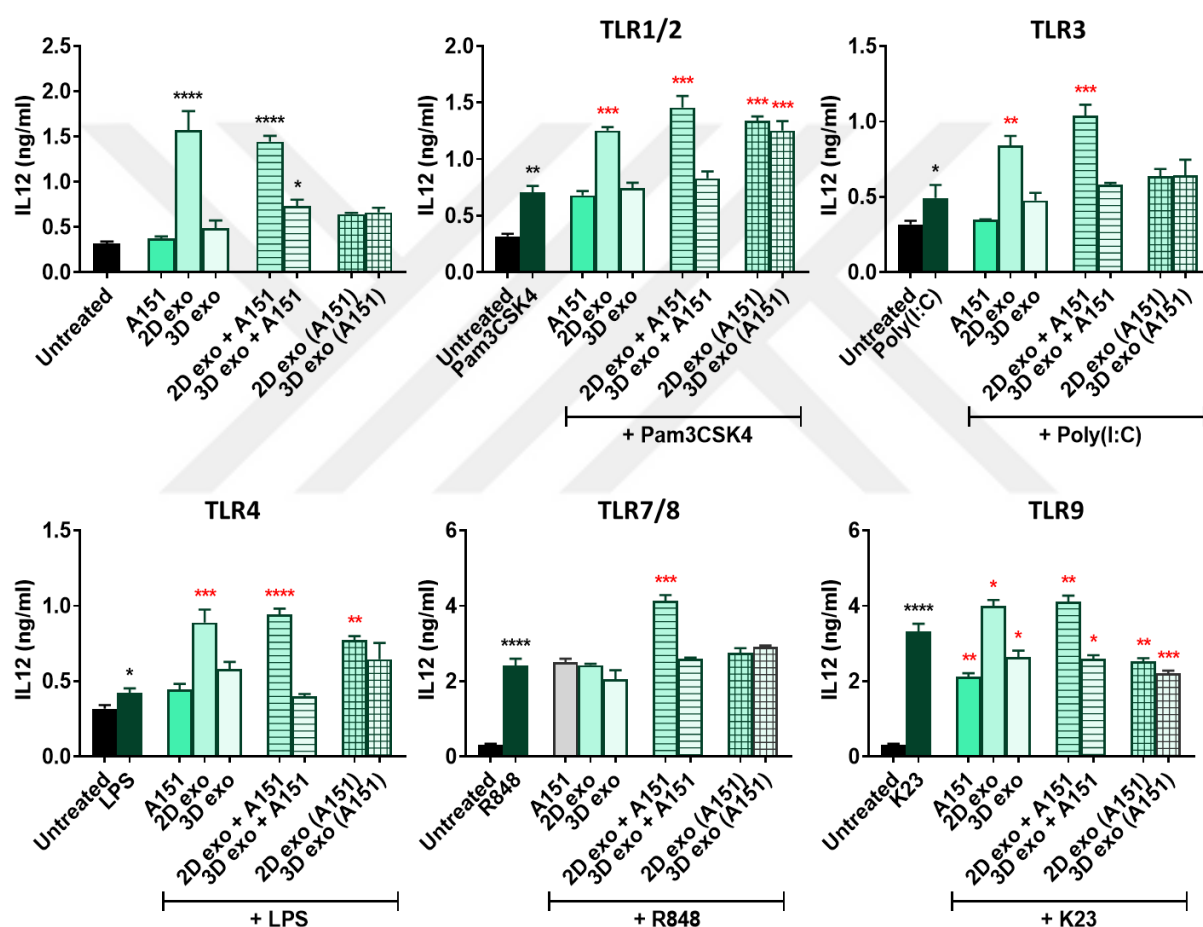


Figure 3.15. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on IL12 secretion from splenocytes upon stimulation with various TLR ligands. Splenocytes (10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to the untreated control. Red stars represent the difference compared to the standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo:

exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Similar to IL12, exosomes were not able to suppress TNF α secretion from splenocytes upon TLR signaling (Figure 3.16). As seen for other cytokines, 2D-exosomes acted in synergy with poly(I:C) unless they were loaded with A151 ODN (Figure 3.16, upper right panel). Without the presence of a stimulant, both exosomes significantly induced TNF α production unless they were loaded with A151 ODN (Figure 3.16, upper left panel). The only observed suppressive activities were of A151 ODN-loaded forms of both 2D- and 3D-exosomes on TLR4- and TLR9-induced TNF α secretion (Figure 3.16, lower left and lower right panels). They were inhibitory on TLR4 around 50% and TLR9 around 60%. As expected, A151 ODN alone was also significantly suppressive on TLR9 (Figure 3.16, lower right panel).

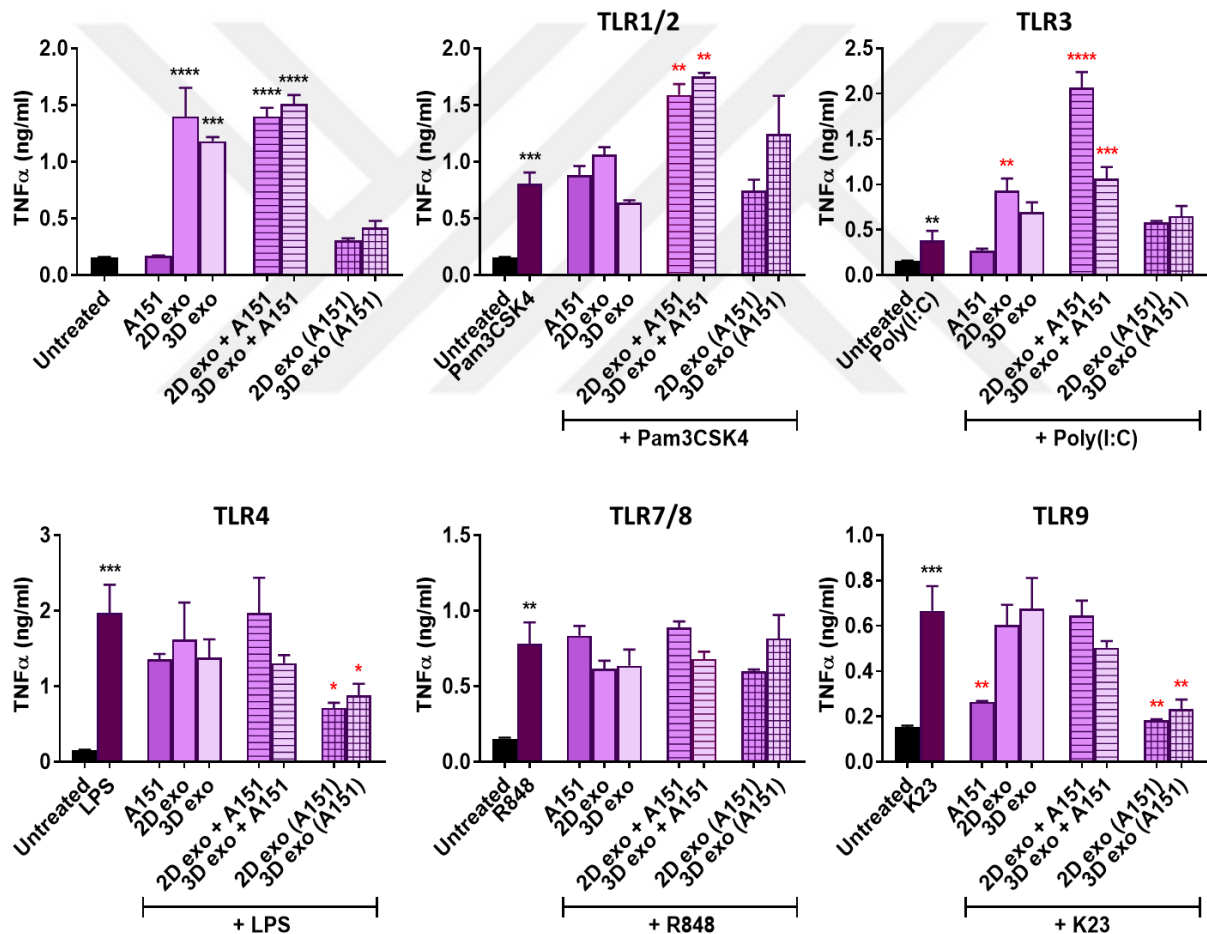


Figure 3.16. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on TNF α secretion from splenocytes upon stimulation with various TLR ligands. Splenocytes (10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to the untreated control. Red stars represent the difference compared to the standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo:

exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

In summary, UCX[®] exosomes as such in non-inflammatory culture conditions induced inflammatory cytokine production but they were potent suppressors of IFN γ and IL6 in cases of TLR-mediated inflammation. Their immunosuppressive actions could slightly be improved with free A151 ODN addition but loading of A151 ODN into exosomes consistently improved the suppressive capacity critically. Collectively these results suggest that synergistic suppressive actions of A151 ODN and the exosomes became apparent only when A151 ODN was encapsulated within exosomes. Furthermore, immunogenic actions of exosomes in non-inflammatory conditions could also be reversed by A151 ODN loading. Since the literature suggests that MSCs are suppressive only when there is sufficient inflammatory signal such as pro-inflammatory cytokines TNF α and IFN γ in the environment, and that MSCs constitutively express high amounts of IL6 in homeostatic conditions [21], our results regarding the immunomodulatory behavior of UCX[®] exosomes are rational considering the activation state of the host cells. Lastly, these results revealed that 2D- and 3D-culture-derived exosomes based on the type of stimulatory signal existing in the culture environment may behave differently in terms of their immunomodulatory actions.

3.4.3 Effects of Free or A151 ODN-Loaded UCX[®] Exosomes on Mouse BMDMs

The same set of experiments performed with splenocytes were later performed with BMDMs to observe the immunomodulatory actions of UCX[®] exosomes on macrophages. Similar to the results with splenocytes, 2D-exosomes caused IL6 production from BMDMs in the absence of any inflammatory signal and this was down-modulated by A151 ODN loading within these exosomes (Figure 3.17, upper left panel). Synergism of exosomes with TLR3 was more pronounced in BMDMs. Both 2D and 3D-exosomes severely upregulated IL6 production ~ 4 -fold upon poly(I:C) stimulation (Figure 3.17, upper right panel). Strikingly, contrary to the findings with splenocytes, A151 ODN mixing or loading could not prevent this synergism. In terms of inhibiting IL6 secretion, A151 ODN and 2D-exosomes for TLR2, 3D-exosomes for TLR4, A151 ODN and both types of exosomes for TLR7/8 and TLR9 specific stimulations were significantly effective (Figure 3.17, upper middle panel and lower panels). However, A151 ODN loading within either of these exosomes did not significantly improve these suppressive effects. Although not as critical as the effects seen with splenocytes, combining or loading of A151 ODN conferred suppressive ability to 3D-exosomes on TLR2, and to 2D-exosomes on TLR4 activation (Figure 3.17, upper middle and lower left panels). Overall, the experiments with BMDMs revealed that free A151 ODN addition was as effective as A151 ODN loading, another contrasting finding to the splenocyte study.

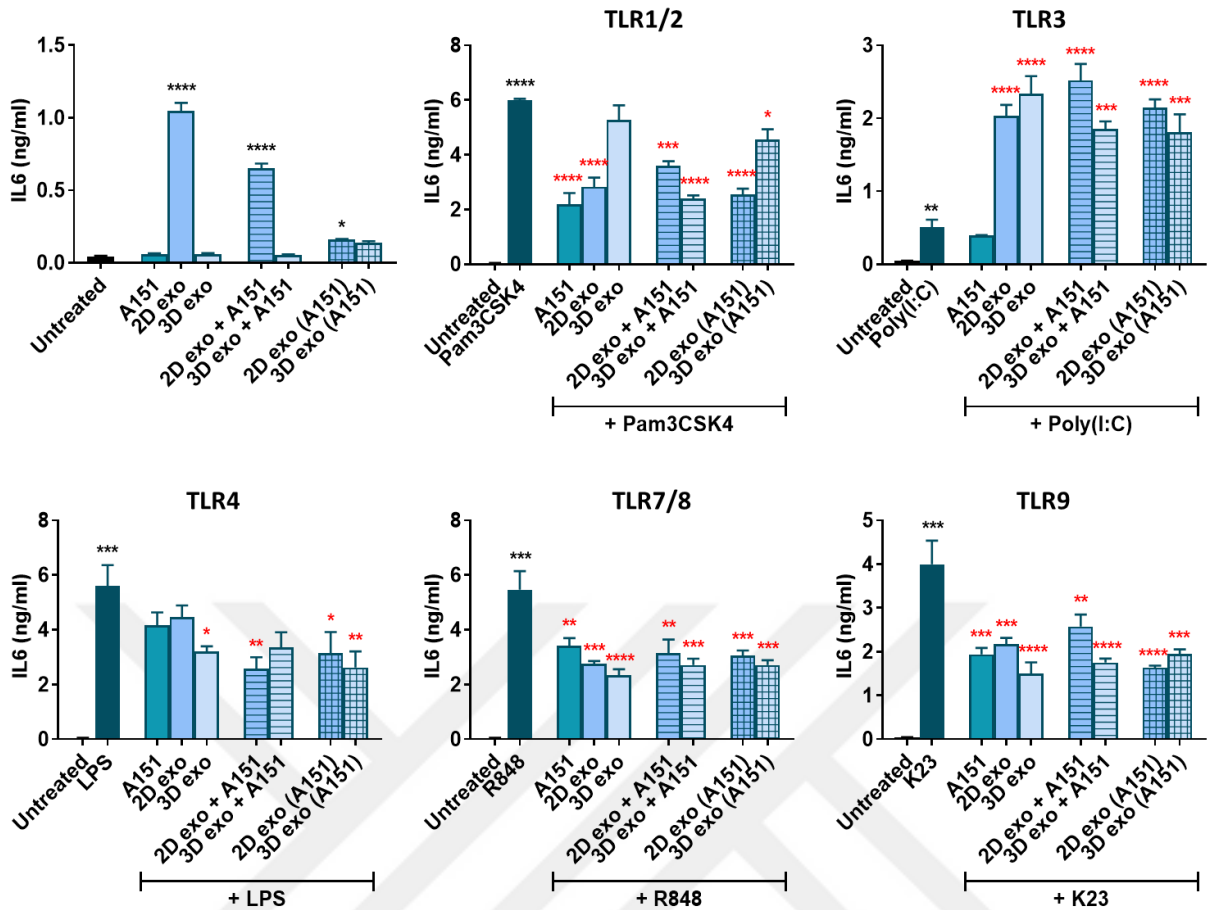


Figure 3.17. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on IL6 secretion from BMDMs upon stimulation with various TLR ligands. BMDMs (0.8×10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to untreated control. Red stars represent the difference compared to standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Consistent with previous observations, 2D-exosomes augmented IL12 secretion in the absence of TLR-triggering (Figure 3.18, upper left panel). Furthermore, it increased TLR3-dependent IL12 production from BMDMs (Figure 3.18, upper right panel). These were partially prevented following A151 ODN loading. Although 2D- or 3D-exosomes were not able to decrease IL12 levels secreted by splenocytes (Figure 3.15), IL12 production from macrophages upon Pam3CSK4 (TLR1/2L), R848 (TLR7/8L) and K23(TLR9L) stimulations was significantly suppressed by both types of exosomes (Figure 3.18, upper middle, lower middle and lower right panels, ~50%, 50% and 65% suppression, respectively). In general, A151 ODN loading did not dramatically improve the suppression mediated by exosomes but it did double the efficiency of 2D-exosomes on TLR1/2 suppression (Figure 3.18, upper middle panel). While only 3D-exosomes could inhibit LPS-mediated IL12 production alone up to 50%, A151 ODN

loading conferred a comparable suppressive ability to 2D-exosomes as well (Figure 3.18, lower left panel). Overall, 3D-exosomes were more potent when administered alone but A151 ODN-loaded forms of both 2D and 3D-exosome types were equally potent in suppressing macrophage-derived IL12 secretion upon Pam3CSK4 (TLR1/2L), LPS (TLR4L), R848 (TLR7/8L) and K23 (TLR9L) stimulations.

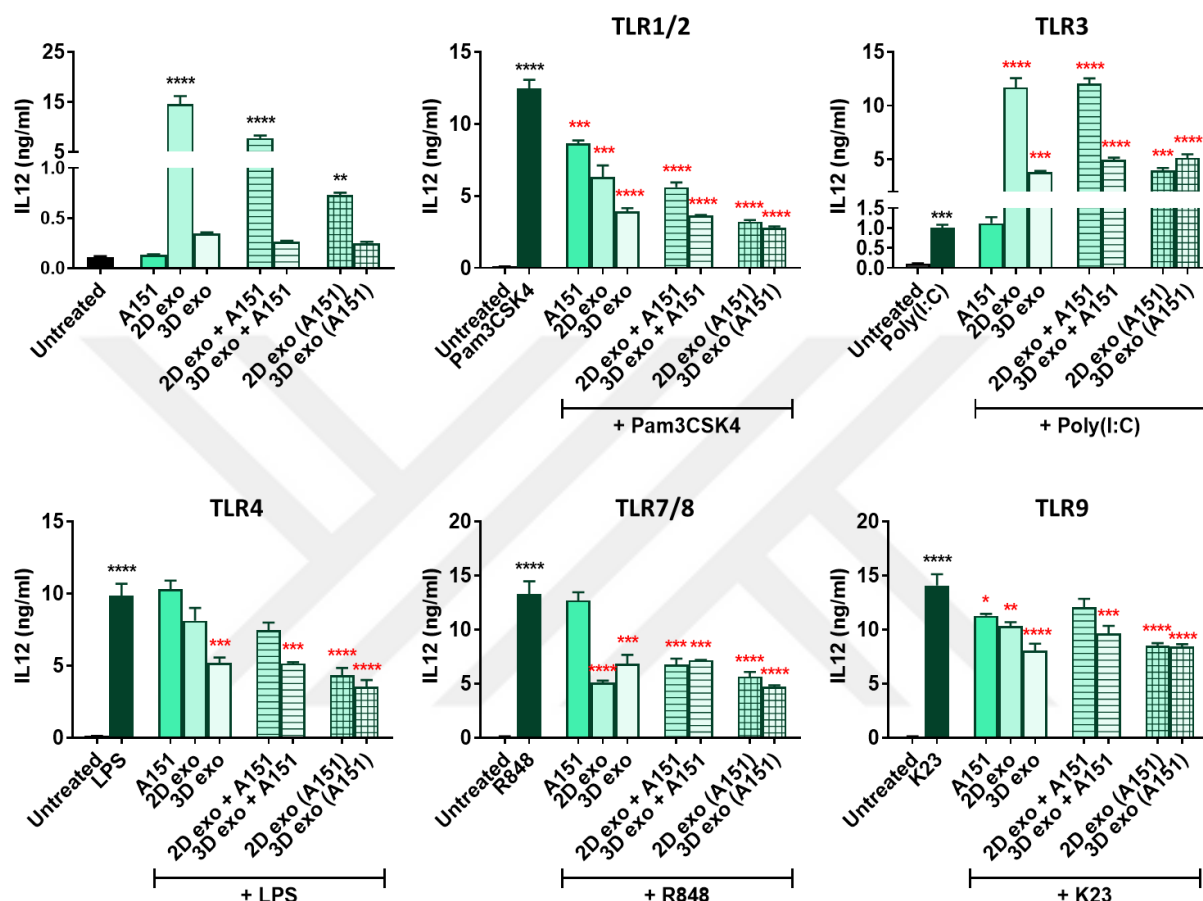


Figure 3.18. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on IL12 secretion from BMDMs upon stimulation with various TLR ligands. BMDMs (0.8×10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to untreated control. Red stars represent the difference compared to standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Similar to the results obtained with splenocyte assays (Figure 3.16), the cytokine exosomes were least effective in suppressing was TNF α from BMDMs (Figure 3.19). When incubated as such, 2D-exosomes triggered TNF α production which could be limited by A151 ODN loading (Figure 3.19, upper left panel). Interestingly, in the presence of poly(I:C), A151 ODN addition or loading further promoted the synergism of exosomes and TLR3 activation instead of repressing it (Figure 3.19, upper right panel).

None of the treatments were effective in inhibiting TLR1/2-dependent TNF α production (Figure 3.19, upper middle panel). Both types of exosomes alone or combined with A151 ODN moderately (~30%) decreased TNF α levels upon LPS stimulation (Figure 3.19, lower left panel). While only 2D-exosomes were able to inhibit R848-induced TNF α secretion (~40% inhibition), A151 ODN-loaded forms of both exosome types were equally effective (Figure 3.19, lower middle panel). Conversely, only 3D-exosomes could inhibit K23-induced TNF α secretion alone but this ability was not observed with the A151 ODN-loaded form (Figure 3.19, lower right panel). However, A151 ODN loading to 2D-exosomes led to reduction of K23-induced TNF α levels.

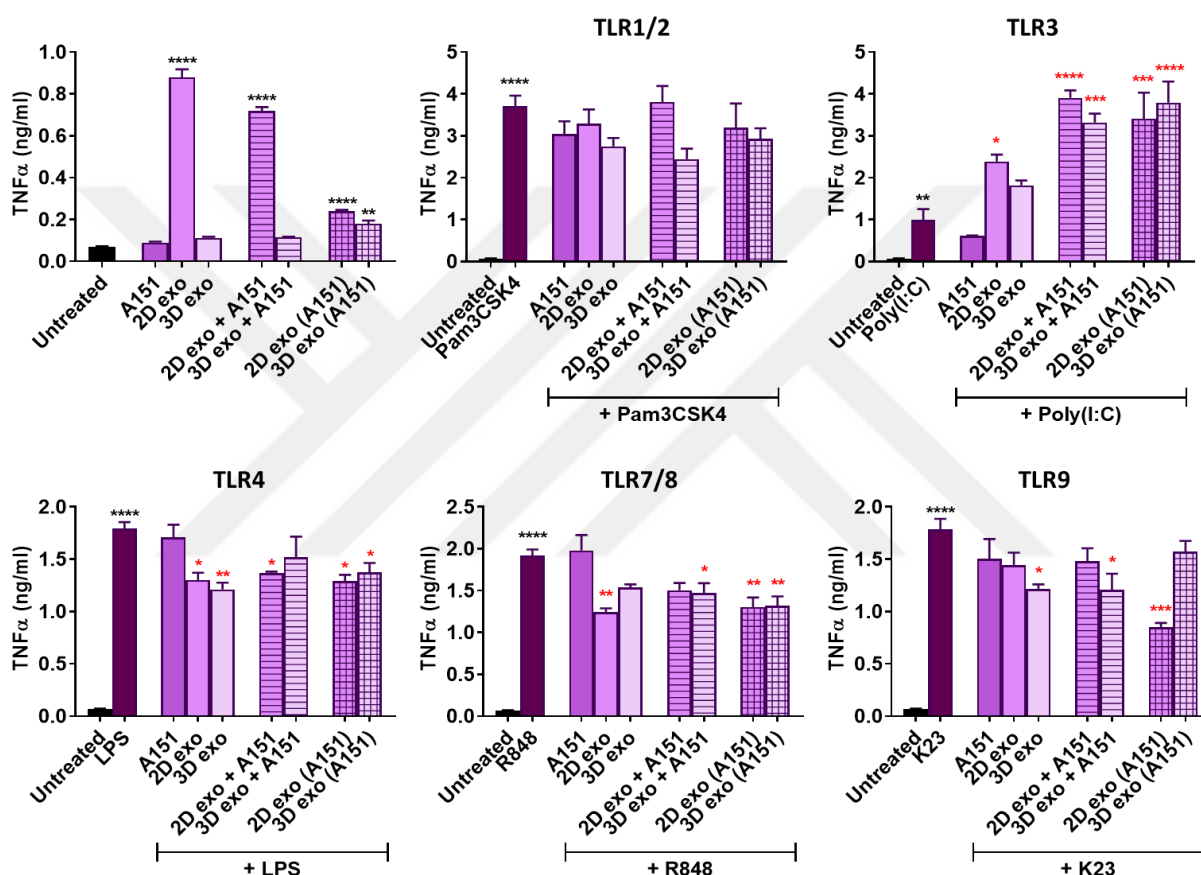


Figure 3.19. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on TNF α secretion from BMDMs upon stimulation with various TLR ligands. BMDMs (0.8×10^6 cells/ml) were stimulated with 1 μ g/ml Pam3CSK4, 30 μ g/ml poly(I:C), 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their free or loaded combinations for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Black stars represent statistical difference compared to untreated control. Red stars represent the difference compared to standalone TLR ligand. Experiment was performed in triplicates. TLR: Toll-like receptor, Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

When taken together, our findings implied that the most pronounced effect of both exosome types was the suppression of IL6 and IL12 secretions especially upon TLR1/2, TLR7/8 and TLR9 activation in

BMDMs. In the absence of a TLR ligand, 2D-exosomes had a stimulating effect on macrophages which could be partially downregulated by A151 ODN incorporation. Although A151 ODN loading did not dramatically contribute to the existing suppressive effects of exosomes on macrophages, it is important to note that loading prevents macrophage activation by exosomes in the absence of any TLR mediated stimulus. On BMDMs, 2D- and 3D-exosomes displayed differential activities. The exosomes from 3D-spheroid culture supernatant, showed more suppressive ability under active inflammation and were less immunogenic under steady-state culture conditions.

Apart from measuring the cytokine secretion levels, we checked the variations of the cell surface marker expression levels of macrophages following stimulation with TLR ligands and subsequent treatment with A151 ODN, UCX[®] exosomes or their free or loaded combinations. While MHC molecules provide the first signal by interacting with the T-cell receptor, CD80 and CD86 molecules which are also called B7-1 and B7-2 respectively bind to CD28 on T-cells and provide co-stimulatory signal for T-cell activation. Expression of co-stimulatory and MHC molecules increase in antigen-presenting cells such as dendritic cells, monocytes and macrophages upon activation by TLR-triggering [273]. Surface expressions of CD80, CD86 and MHC-II on BMDMs were measured by staining the cells with fluorochrome-labelled specific antibodies and are represented as histogram plots obtained via flow cytometry in the following figures (Figures 3.20 - 3.25).

Without stimulation with any TLR ligand, 2D and 3D-exosomes both upregulated CD80 and MHC-II (Figure 3.20, left and right panels). The increases caused by 2D-exosomes were higher but could be limited by A151 ODN addition or loading. These results are in line with the ELISA results where 2D-exosomes promoted pro-inflammatory cytokine production from BMDMs which could be attenuated by A151 ODN addition and especially loading (Figures 3.17-3.19). A151 ODN decreased CD80 expression even below the basal level while it did not affect MHC-II expression and slightly increased CD86 expression (Figure 3.20). 2D-exosomes also slightly increased CD86 levels unless they were mixed or loaded with A151 ODN (Figure 3.20, middle panel). Interestingly and counterintuitively to the cytokine ELISA results, A151 ODN-loaded form of 3D-exosomes upregulated all three activation markers (Figure 3.20) although it did not induce pro-inflammatory cytokine production as a result of macrophage activation (Figures 3.17-3.19).

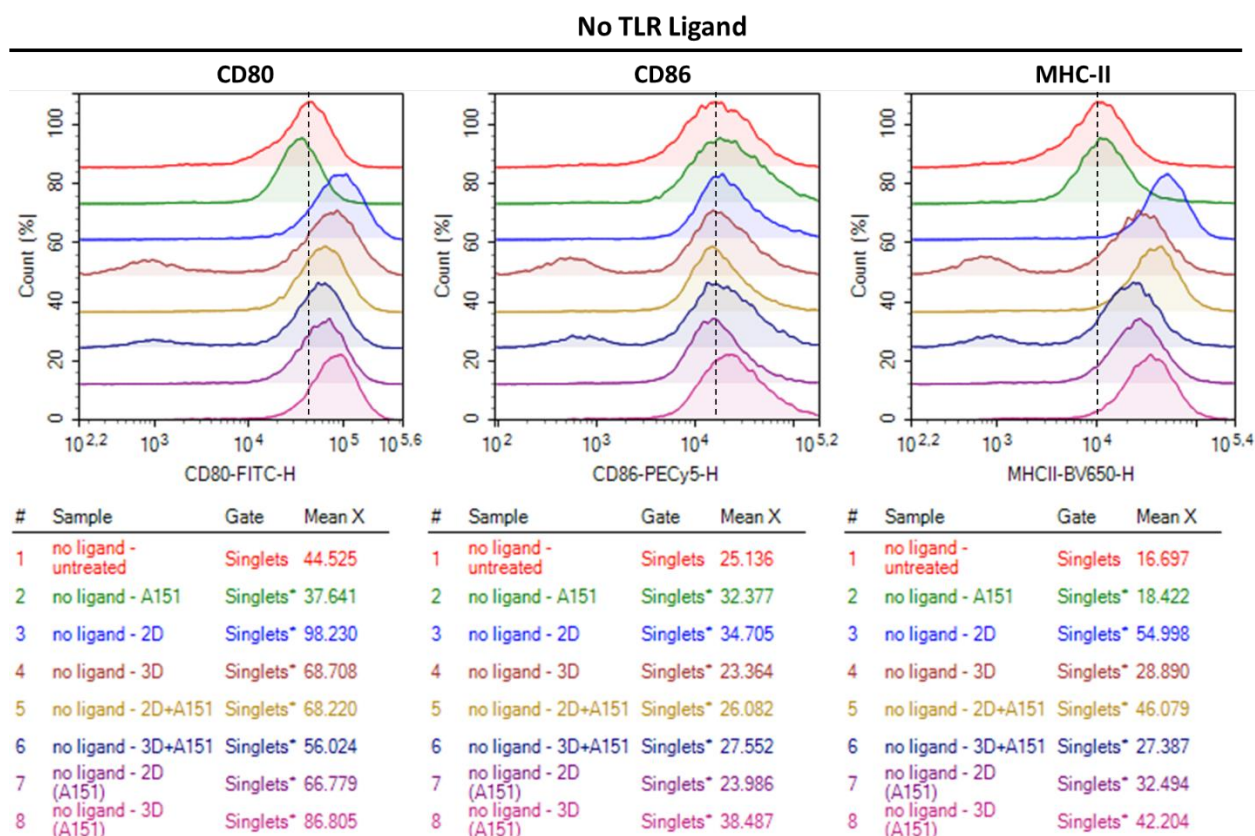


Figure 3.20. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs. Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) treated with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the untreated group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D (A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

Upon TLR1/2 activation with Pam3CSK4, all three activation markers were upregulated (Figure 3.21). All the treatment groups were able to decrease CD80 and CD86 levels (Figure 3.21, left and middle panels). Both types of exosomes were more effective compared to A151 ODN alone. A151 ODN addition or loading did not significantly improved the suppressive capacities of exosomes. Although all groups could also downregulate MHC-II expression, the extent was much lower (Figure 3.21, right panel). However, similarly to CD80 and CD86, MHC-II was downregulated better with both types of exosomes compared to A151 ODN, while A151 ODN addition did not improve the effect of exosomes but rather slightly worsened it.

Stimulated with Pam3CSK4

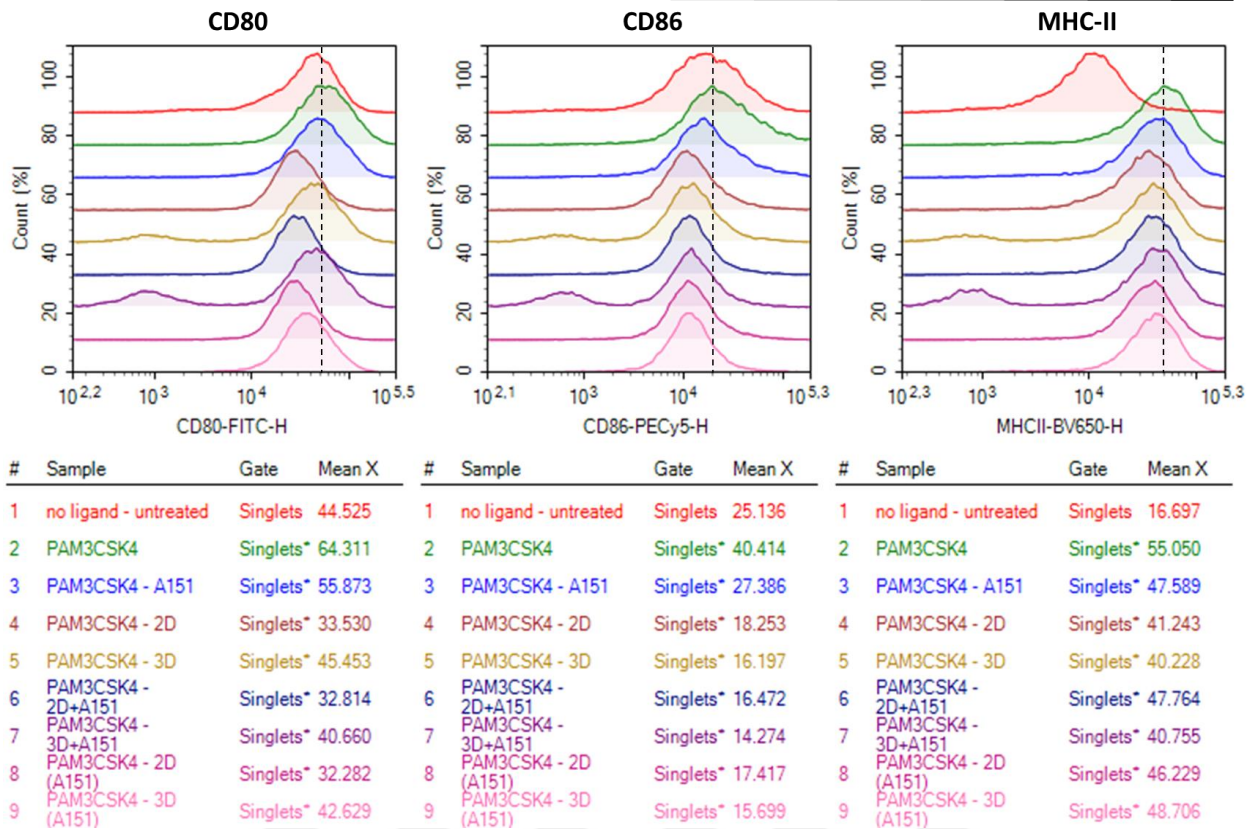


Figure 3.21. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs upon stimulation with Pam3CSK4. Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) stimulated with 1 μ g/ml Pam3CSK4 and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the ligand-only group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D (A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

TLR3 activation by poly(I:C) mildly increased CD80 levels but upregulated CD86 and MHC-II 23.5- and 8.5-fold respectively (Figure 3.22). A151 ODN, 3D-exosomes and their combinations could downregulate CD80 while 2D-exosomes further upregulated it unless they were mixed or loaded with A151 ODN (Figure 3.22, left panel). Both types of exosomes but not A151 ODN were able to reduce CD86 and MHC-II levels (Figure 3.22, middle and right panels). While 3D-exosomes were more effective in reducing MHC-II expression, 2D-exosomes were more efficient for CD86 inhibition and could lower the protein level more than 50%. Interestingly, A151 ODN addition or loading limited the suppressive capacities of exosomes on CD86 and MHC-II. Another notable observation was that treatment with 3D-exosomes resulted in populations completely negative for CD80, CD86 and MHC-II seen as separate peaks at the left side of histogram plots. While A151 ODN addition reduced these populations, A151 ODN-loading completely prevented their existence. Exosomes being suppressive

against TLR3 while A151 ODN limits this feature do not fit the ELISA results implying that different signaling cascades are involved in the suppressive abilities of these proteins. Apparently, exosomes synergized with poly(I:C) for pro-inflammatory cytokine production (Figures 3.17-3.19) but they decreased BMDMs' capacity of antigen-presentation and T-cell activation (Figure 3.22).

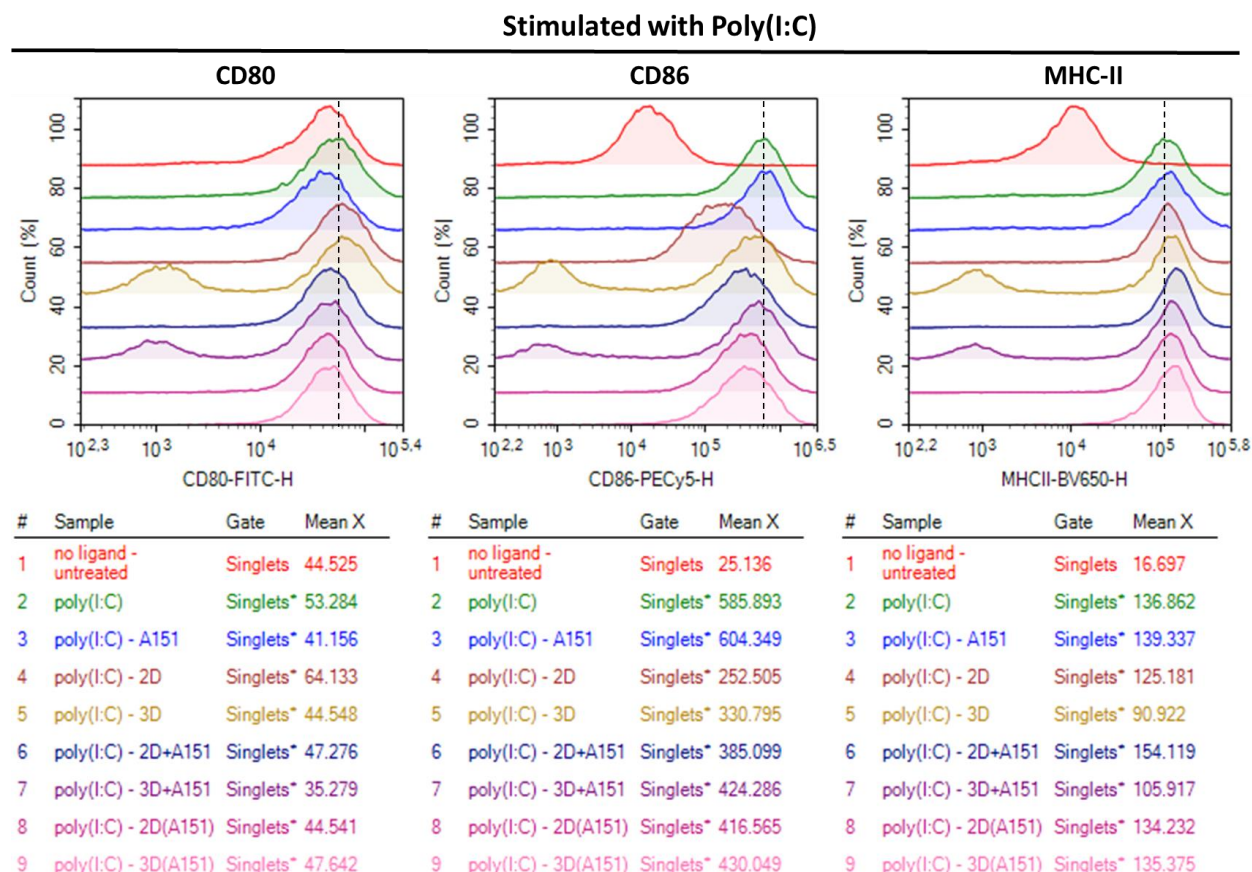


Figure 3.22. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs upon stimulation with poly(I:C). Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) stimulated with $30 \mu\text{g/ml}$ poly(I:C) and treated with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the ligand-only group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D(A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

TLR4 activation by LPS increased the levels of all three activation markers on BMDM surface (Figure 3.23). All treatment groups could decrease CD80 levels but 3D-exosomes alone and 2D-exosomes mixed with A151 ODN were the most effective, achieving $\sim 40\%$ downregulation (Figure 3.23, left panel). This was also the case for CD86 expression although the differences in CD86 levels were more subtle ($\sim 25\%$) compared to CD80 (Figure 3.23, middle panel). 3D-exosomes being more effective in decreasing the activation markers compared to 2D-exosomes alone were in line with the ELISA results revealing that 3D- but not 2D-exosomes alone could significantly decrease IL6 and IL12 production

from BMDMs (Figures 3.17 and 3.18). MHC-II levels could be reduced by A151 ODN, 3D-exosomes and their free mixture (Figure 3.23, right panel). For all three markers, A151 ODN loading did not improve downregulation and even hindered the capacity of exosomes. Again, a negative population for all three markers were observed in cells treated with 3D-exosomes alone and to a lesser degree in cells treated with the mixture of 3D-exosomes and A151 ODN.

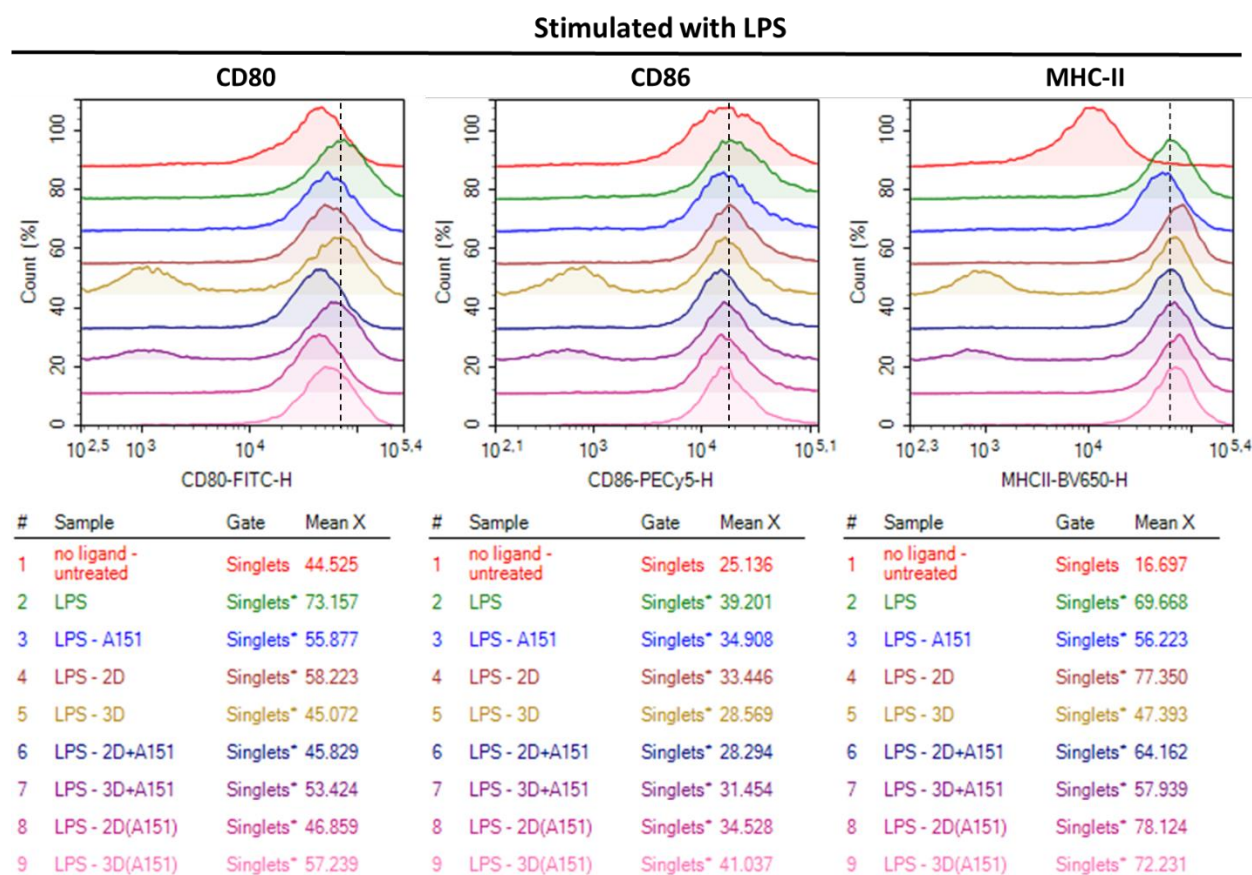


Figure 3.23. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs upon stimulation with LPS. Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) stimulated with 1 μ g/ml LPS and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the ligand-only group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D (A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

R848 stimulation induced higher CD80 and MHC-II expression but not CD86 (Figure 3.24). All treatment groups could reduce CD86 levels except for A151 ODN alone (Figure 3.24, middle panel). Mixing exosomes with A151 ODN improved suppression whereas loading was less efficient. Treatments apart from free mixture of 3D-exosomes and A151 ODN did not cause a critical decrease in MHC-II levels (Figure 3.24, right panel). Even though CD86 levels were not increased upon stimulation, all treatments, especially 3D-exosomes alone and in mixture with A151 ODN brought the expression to

even lower levels than the basal condition (Figure 3.24, middle panel). Although both types of exosomes were very potent in suppressing IL6 and IL12 production upon R848 stimulation (Figures 3.17 and 3.18), they were not as effective in downregulating the co-stimulatory markers (Figure 3.24).

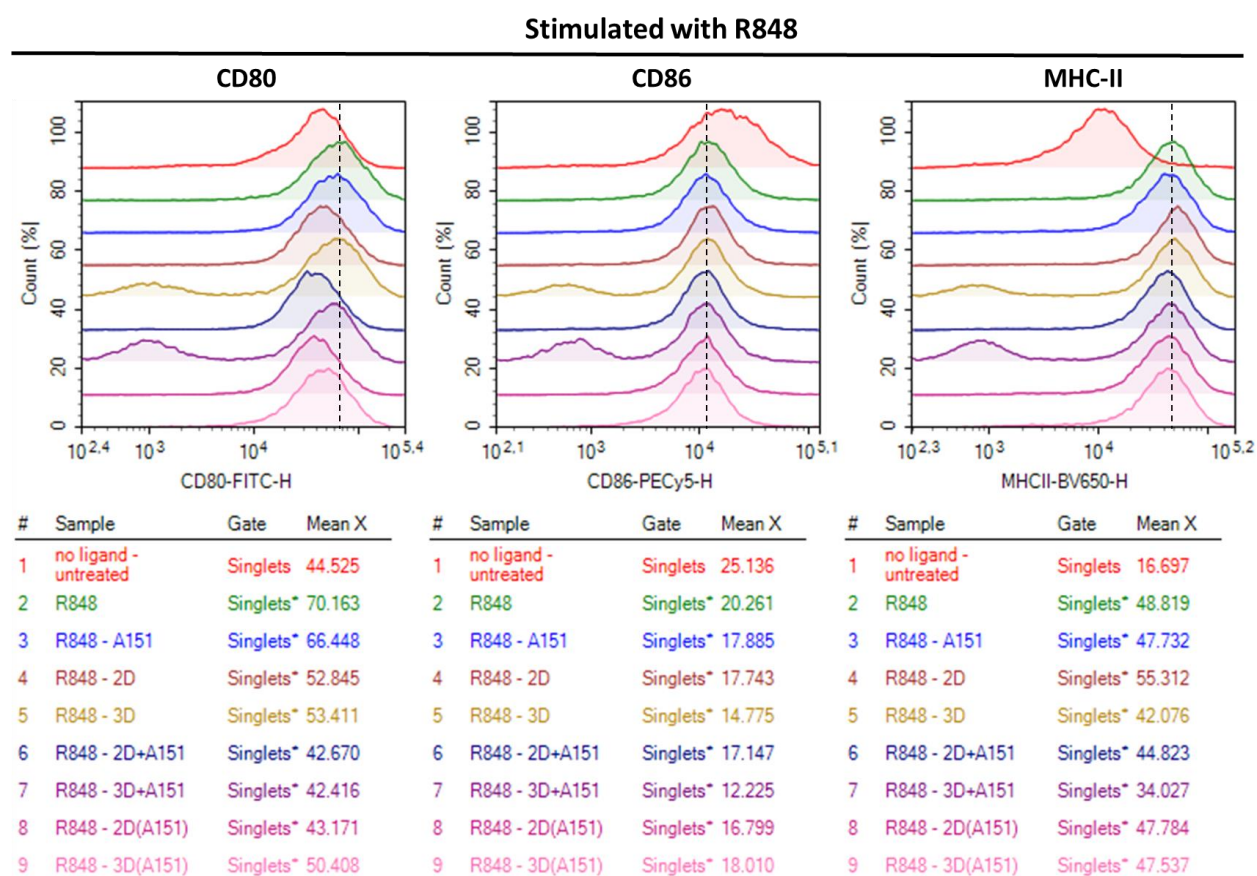


Figure 3.24. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs upon stimulation with R848. Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) stimulated with 1 μ g/ml R848 and treated with 1.5 μ M A151 ODN, 20 μ g/ml exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the ligand-only group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D(A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

Similar to R848, K23 only upregulated CD80 and MHC-II (Figure 3.25). Both exosome types could downregulate CD80 around 20% and this capacity was increased to 30-35% by the addition or loading of potent TLR9 antagonist A151 ODN (Figure 3.25, left panel). Free addition of A151 ODN was more effective than loading. Similar to the R848 stimulation results, treatments could lower the CD86 expressions further than the basal level (Figure 3.25, middle panel). Exosome and A151 ODN mixtures were the most efficient treatments in that regard. MHC-II expression could be downregulated most efficiently (~30%) by 3D-exosomes alone or in free mixture with A151 ODN, although A151 ODN addition and loading to 2D-exosomes led to a subtler downregulation (~15%) (Figure 3.25, right panel).

As in the other TLR stimulation conditions, 3D-exosomes alone and with A151 ODN resulted in a negative population peak for all three markers.

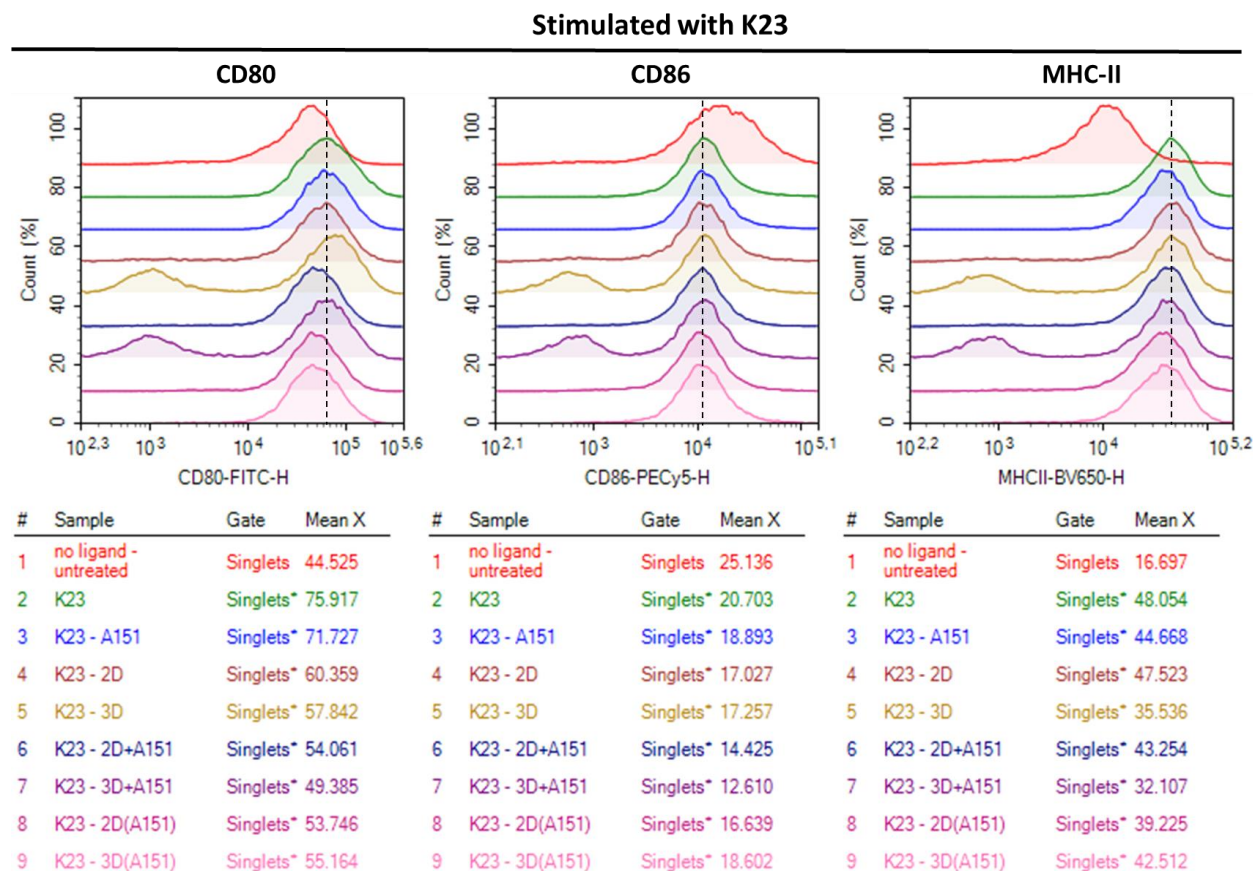


Figure 3.25. Effects of A151 ODN, 2D- or 3D-exosomes and their free or loaded combinations on CD80, CD86 and MHC-II cell surface expressions in BMDMs upon stimulation with K23 CpG ODN. Representative histogram plots showing fluorescence intensities of CD80, CD86 and MHC-II cell surface expressions in BMDMs (0.8×10^6 cells/ml) stimulated with $1 \mu\text{M}$ K23 CpG ODN and treated with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosome or their combinations for 24 hours. Cells were stained with fluorochrome antibodies and surface protein expressions were determined via flow cytometry. Dotted lines represent the peak fluorescence signal of the ligand-only group. 2D or 3D: 2D- or 3D-exosome, 2D or 3D + A151 ODN: free mixture of 2D- or 3D-exosome and A151 ODN, 2D(A151) or 3D (A151): A151 ODN-loaded 2D- or 3D-exosome, Mean X: Mean fluorescence intensity of the parameter on the X axis.

To summarize, 2D- and 3D-exosomes differentially regulated the expression of CD80, CD86 and MHC-II levels in macrophages upon activation via TLR signaling. 3D-exosomes were more potent inhibitors than 2D-exosomes in most conditions. Exosomes' effects on cytokine secretion profiles and activation markers did not always correlated. A151 ODN loading did not improve the downregulatory action of exosomes on the activation markers while it could potentiate pro-inflammatory cytokine suppression. Overall, in various conditions of TLR-mediated inflammation, UCX[®] exosomes displayed distinct inhibitory potentials in decreasing the antigen presentation and T-cell activating capacities of

macrophages as well as potent suppression of pro-inflammatory cytokine production such as IL6 and IL12.

3.5 Effects of Free or A151 ODN-Loaded UCX[®] Exosomes on Fibroblasts and Keratinocytes

Fibroblasts and keratinocytes are two skin cell types that play major roles in wound healing. UCX[®] cells, especially in 3D-culture, were previously shown to abundantly secrete proteins such as VEGF α , TGF β 1, FGF2, KGF, EGF and HGF which are important for angiogenesis and tissue remodeling, and to promote migration of fibroblasts and keratinocytes [65, 69]. In light of that, we wanted to test the therapeutic ability of UCX[®] exosomes on wound healing. Before the *in vivo* experiments, we wanted to investigate if the exosomes affect the production of wound healing-related growth factors, cytokines and chemokines from fibroblasts and keratinocytes. As model cell lines, NIH3T3 fibroblast cell line and HaCaT keratinocyte cell line were utilized.

Nitric oxide (NO) is suggested to be important for healthy wound healing especially in the early stage. Inhibition of nitric oxide synthase enzymes impair wound healing and studies have found lower NO levels in chronic wounds caused by diabetes or malnutrition [274]. The factors we investigated also include IL1 α , TNF α , CCL2, IL10, TGF β and VEGF. Mainly keratinocytes but also fibroblasts can produce the inflammatory cytokines such as TNF α and IL1 α that are elevated in the wound during the inflammatory phase and also help re-epithelization apart from inflammation [275, 276]. The monocyte/macrophage chemoattractant CCL2 promotes macrophage infiltration to the wound and is deficient in chronic wounds [277]. The anti-inflammatory cytokine IL10 promotes scarless healing [278]. TGF β produced by both keratinocytes and fibroblasts promotes numerous processes during wound healing including granulation tissue formation, keratinocyte proliferation and migration, collagen production and deposition [244]. Lastly, VEGF produced by both cell types is the key mediator of angiogenesis while also promoting immune cell recruitment to the wound [276].

After NIH3T3 or HaCaT cells were incubated with A151 ODN, 2D- or 3D-culture-derived UCX[®] exosomes, or A151 ODN-loaded exosomes for 24 hours, NO levels in the culture were indirectly determined by measuring its metabolite nitrite via Griess Assay since NO itself has a very short half-life, TNF α , IL10 and TGF β concentrations were determined with ELISA, and the mRNA levels of TGF β , IL1 α , CCL2 and VEGF α were measured by RT-qPCR due to unavailability of ELISA reagents for these proteins.

In NIH3T3 fibroblasts, 3D-exosomes decreased NO production by half while A151 ODN itself and A151 ODN-loaded versions of both exosome types significantly elevated NO production (Figure 3.26, left panel). Both exosomes dramatically (almost 8-fold) increased TNF α secretion from fibroblasts while A151 ODN loading slightly hindered it (Figure 3.26, middle panel). A151 ODN and 3D-exosomes could

elevate IL10 secretion 35-40% but a similar effect was not achieved with their combinations (Figure 3.26, right panel).

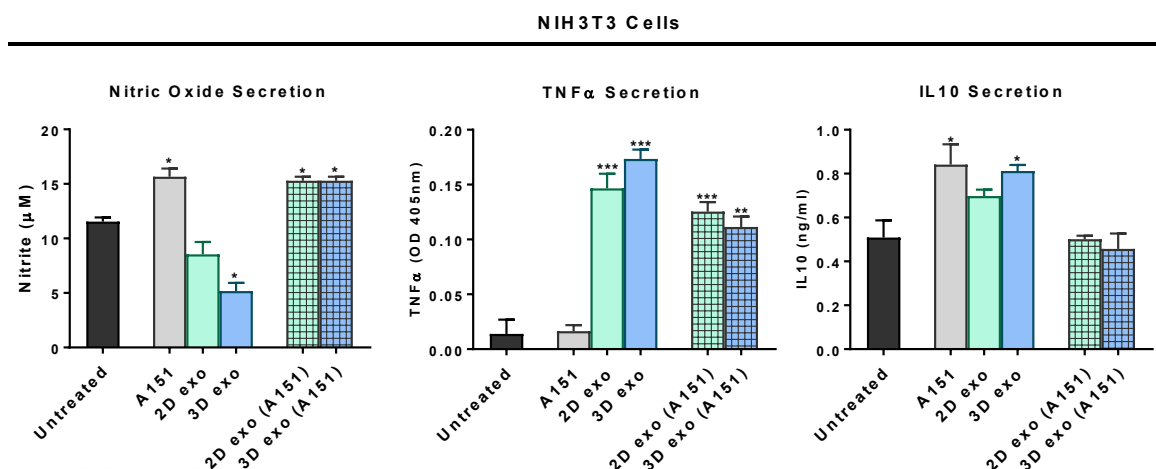


Figure 3.26. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on nitric oxide and cytokine production from NIH3T3 fibroblast cell line. NIH3T3 cells were incubated (0.5×10^6 cells/ml) with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosomes or A151 ODN-loaded exosomes for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Nitric oxide secretion was indirectly determined with Griess assay measuring the nitric oxide metabolite nitrite. Stars represent statistical difference compared to untreated control. Experiment was conducted in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

In terms of gene expression, only 2D-exosomes with or without A151 ODN were able to significantly increase TGF β levels in fibroblasts (Figure 3.27, upper left panel). In contrast, only 3D-exosomes could elevate (~3-fold) IL1 α mRNA levels alone but not with A151 ODN (Figure 3.27, upper right panel). Interestingly, A151 ODN or the exosomes by themselves did not affect CCL2 production, however, A151 ODN-loaded form of both exosomes significantly increased it (Figure 3.27, lower left panel). Both types of exosomes dramatically induced VEGF α expression at least 3-fold but A151 ODN loading completely abolished this induction (Figure 3.27, lower right panel).

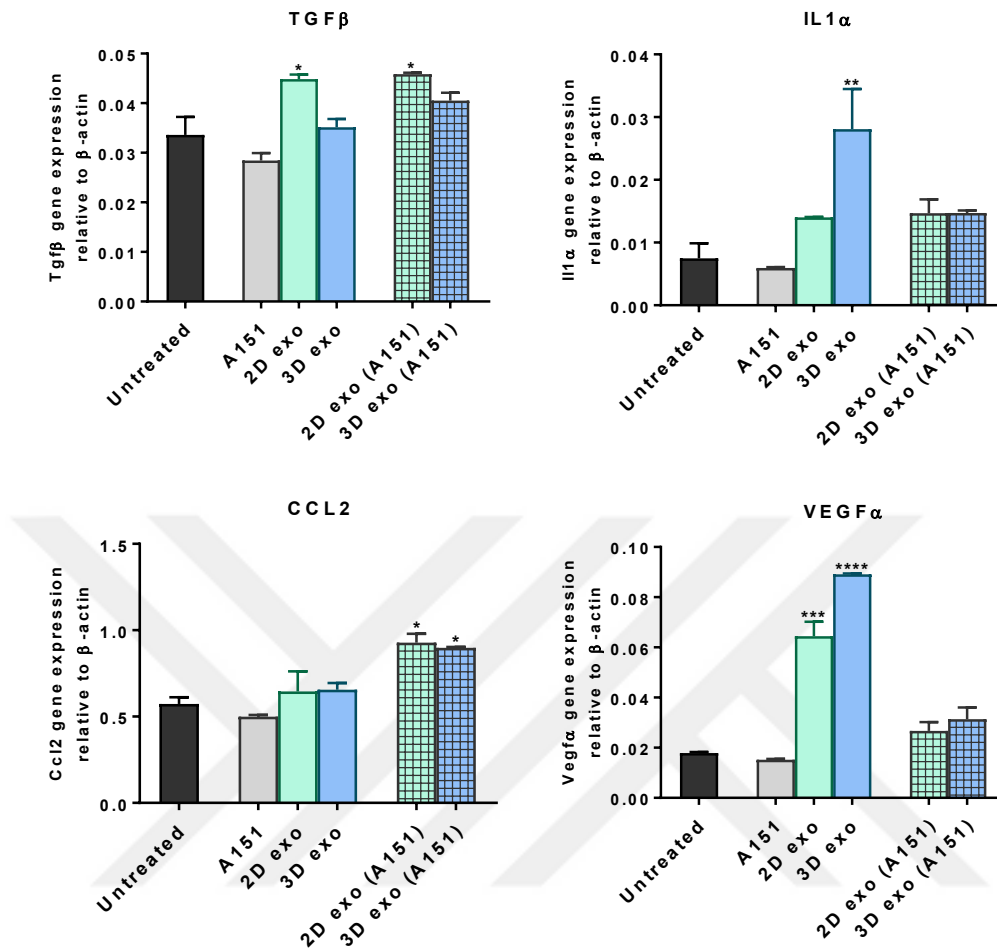


Figure 3.27. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on wound healing-related gene expressions in NIH3T3 fibroblast cell line. NIH3T3 cells were incubated (0.5×10^6 cells/ml) with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosome or A151 ODN-loaded exosomes for 24 hours. Gene expressions relative to β -actin were determined with RT-qPCR using efficiency-corrected comparative CT method. Stars represent statistical difference compared to untreated control. Experiment was conducted in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Similar to the fibroblast data, only A151 ODN but not the exosomes could induce NO secretion from HaCaT keratinocytes (Figure 3.28, upper left panel). Contrary to the case with fibroblasts, the NO-inducing capability was lost when A151 ODN was used in conjunction with the exosomes. None of the treatments altered IL10 levels (Figure 3.28, lower left panel), while only 3D-exosomes could mildly increase TNF α secretion (Figure 3.28, upper right panel). Both types of exosomes, 3D-exosomes much more potently (~ 2 -fold), elevated TGF β production while A151 ODN loading limited this ability (Figure 3.28, lower right panel).

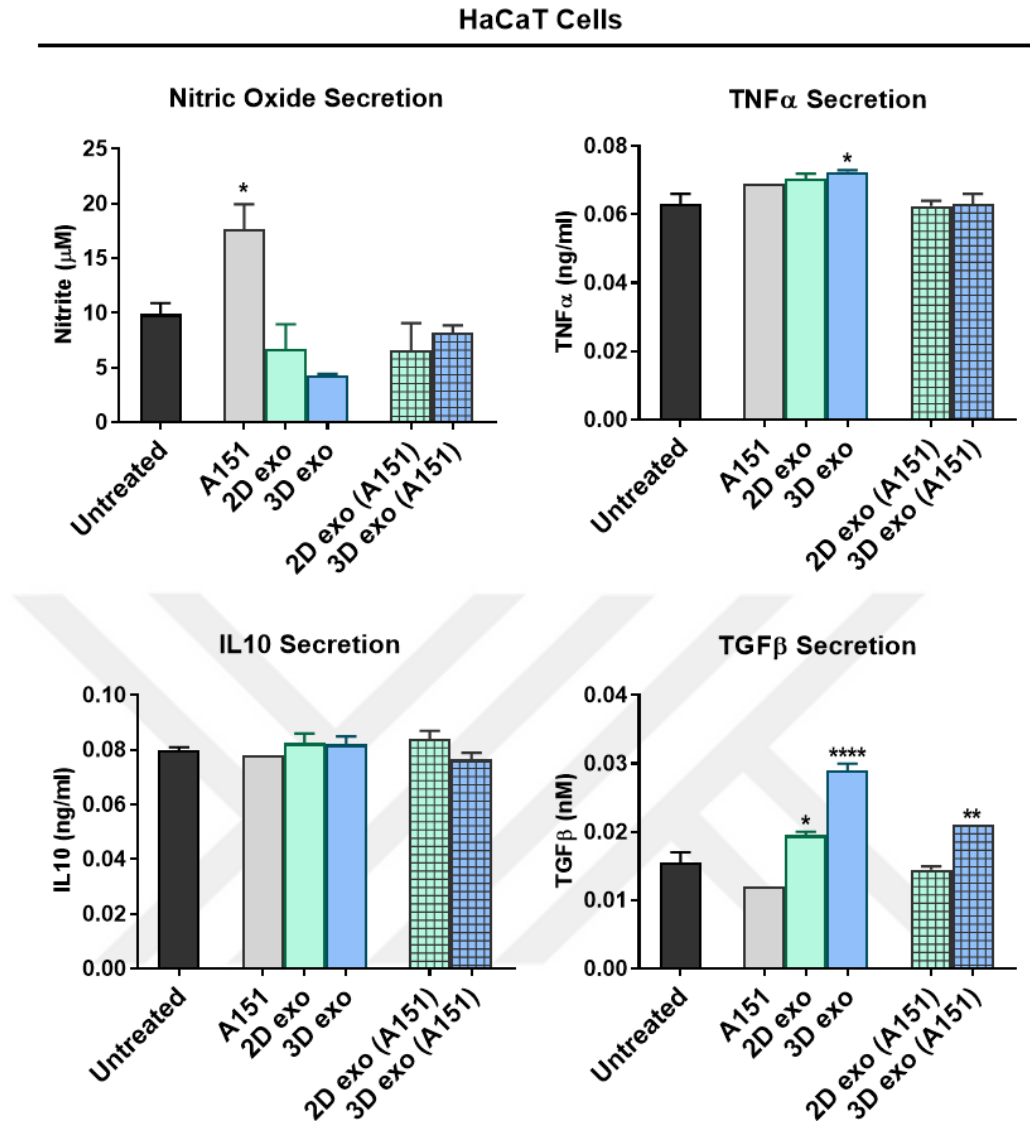


Figure 3.28. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on nitric oxide and cytokine production from HaCaT keratinocyte cell line. HaCaT cells were incubated (0.5×10^6 cells/ml) with 1.5μ M A151 ODN, 20μ g/ml exosome or A151 ODN-loaded exosomes for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Nitric oxide secretion was indirectly determined with Griess assay measuring the nitric oxide metabolite nitrite. Stars represent statistical difference compared to untreated control. Experiment was conducted in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

No significant change in VEGF α expression was observed in keratinocytes (Figure 3.29, right panel) although exosomes were very potent inducers of VEGF α in fibroblasts (Figure 3.27, lower right panel). Moreover, only A151 ODN-loaded 3D-exosomes could elevate CCL2 expression (Figure 3.29, middle panel). Similar to the case in fibroblasts, 2D- and especially 3D-exosomes caused nearly 2-fold and 3-fold increases in IL1 α expression levels respectively (Figure 3.29, left panel).

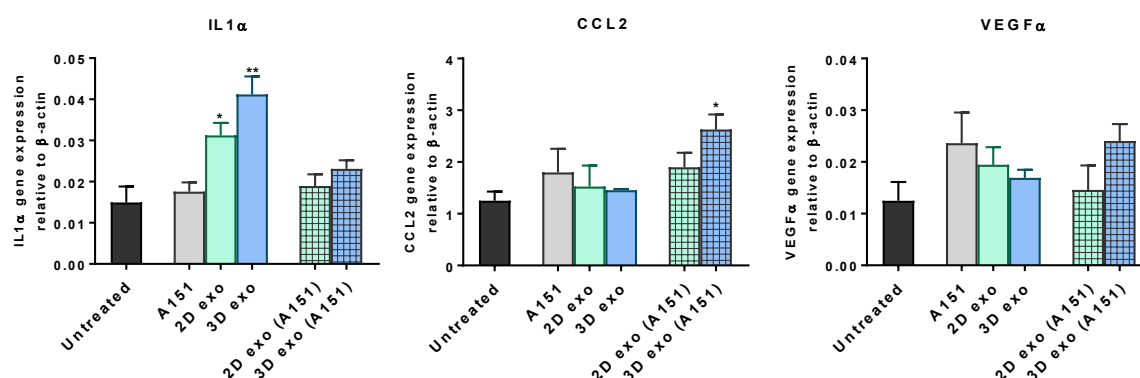


Figure 3.29. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on wound healing-related gene expressions in HaCaT keratinocyte cell line. HaCaT cells were incubated (0.5×10^6 cells/ml) with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosomes or A151 ODN-loaded exosomes for 24 hours. Gene expressions relative to β -actin were determined with RT-qPCR using efficiency-corrected comparative CT method. Stars represent statistical difference compared to untreated control. Experiment was conducted in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. * $p \leq 0.05$, ** $p \leq 0.01$.

Taken together, 2D- and 3D-exosomes influenced fibroblasts and keratinocytes differently and they had more prominent effects on fibroblasts. 3D-exosomes appeared more potent than 2D-exosomes in most cases. Exosomes could promote various steps of wound healing by their ability to induce VEGF α , IL1 α , TNF α , TGF β and IL10 production from the cells such as fibroblasts and keratinocytes in the wound site. Although A151 ODN exerted positive effects on NO and IL10, A151 ODN loading into the exosomes abolished or limited the beneficial effects of the exosomes. Previously explained results had suggested that A151 ODN loading improves the immunomodulatory actions of exosomes in many ways. However, fibroblast and keratinocyte studies implied that it might be counterintuitive to use the loaded versions of exosomes for wound healing purposes.

3.6 Effects of UCX[®] Exosomes on Macrophage Polarization

MSCs are known to promote the anti-inflammatory, pro-angiogenic and regenerative activation state of macrophages which is termed as M2 (see Section 1.1.1.2.1). When co-cultured with macrophages, MSCs increase M2 markers such as Arg1 and IL10 while downregulating M1 markers such as iNOS, IL6 and TNF α [90]. We hypothesized that UCX[®] exosomes could likewise induce M2-differentiation. While complete differentiation may take 3-4 days in cell culture conditions, changes in M1/M2 gene signatures and cytokine production capacities are easily detectable after 24 hours. To test our hypothesis, we incubated naive BMDMs with A151 ODN, 2D/3D-exosomes or their A151 ODN-loaded forms in the presence or absence of M1- or M2-inducing factors. Off note, to trigger M1-differentiation, 100 ng/ml LPS was used whereas for M2-differentiation, 10 ng/ml IL4 and 10 ng/ml IL13 mixture were used. Free mixture of A151 ODN and exosomes were not included as separate treatments in this

experiment and in subsequent experiments since previous *in vitro* results indicated that loading is generally more efficient in inducing synergistic immunomodulatory effects of A151 ODN and exosomes compared to simple mixing.

Griess assay revealed that, in the absence of a polarizing agent, 3D-exosomes increased NO production ca. 3-fold (Figure 3.30, upper left panel, gray bars). Although it was not comparable to LPS-induced NO levels which were nearly 10 times higher, the increase caused by 3D-exosomes was significant. Interestingly, when used with IL4 and IL13, 2D- and 3D-exosomes both elevated ~6-fold the NO amount secreted by BMDMs (Figure 3.30, upper left panel, blue bars). A151 ODN prevented that effect when loaded within exosomes. 2D-exosomes by themselves led to a massive IL6 secretion which was even higher than the LPS-induced IL6 levels (Figure 3.30, upper right panel). This was not the case in the TLR experiments performed with BMDMs (Figure 3.17, lower left panel) but it is of importance to note that the LPS dose used for polarization was 1/10 of the dose used in the previous TLR experiments. Presence of IL4 and IL13 partially limited but could not block the IL6-inducing ability of 2D-exosomes (Figure 3.30, upper right panel, blue bars). An unexpected result was that A151 ODN-loaded 3D-exosomes coupled with IL4 and IL13 also induced IL6 secretion. As in Figure 3.19, 2D- but not 3D-exosomes dramatically induced TNF α secretion from BMDMs, at a level even higher than the M1-specific positive control LPS-specific induction, while as expected A151 ODN loading blocked this response (Figure 3.30, lower left panel). Interestingly but consistently concerning NO secretion data, addition of IL4 and IL13 triggered much higher TNF α production by 3D-exosomes (Figure 3.30, lower left panel, blue bars). IL10 is an anti-inflammatory cytokine produced alongside the pro-inflammatory cytokines during infection and inflammation in order to limit the overexuberant and persistent immune response. Apart from as such LPS stimulation group, 2D-exosomes were the only treatment to elevate IL10 secretion in the presence or absence of polarizing agents (Figure 3.30, lower right panel). Since 2D-exosomes also induced high amounts of IL6 and TNF α , observing IL10 secretion at the same time was not very unexpected. When taken together, these results indicated that 2D-exosomes might be skewing macrophages to a more inflammatory phenotype while 3D-exosomes were able to increase NO which is considered as a cardinal M1-signature.

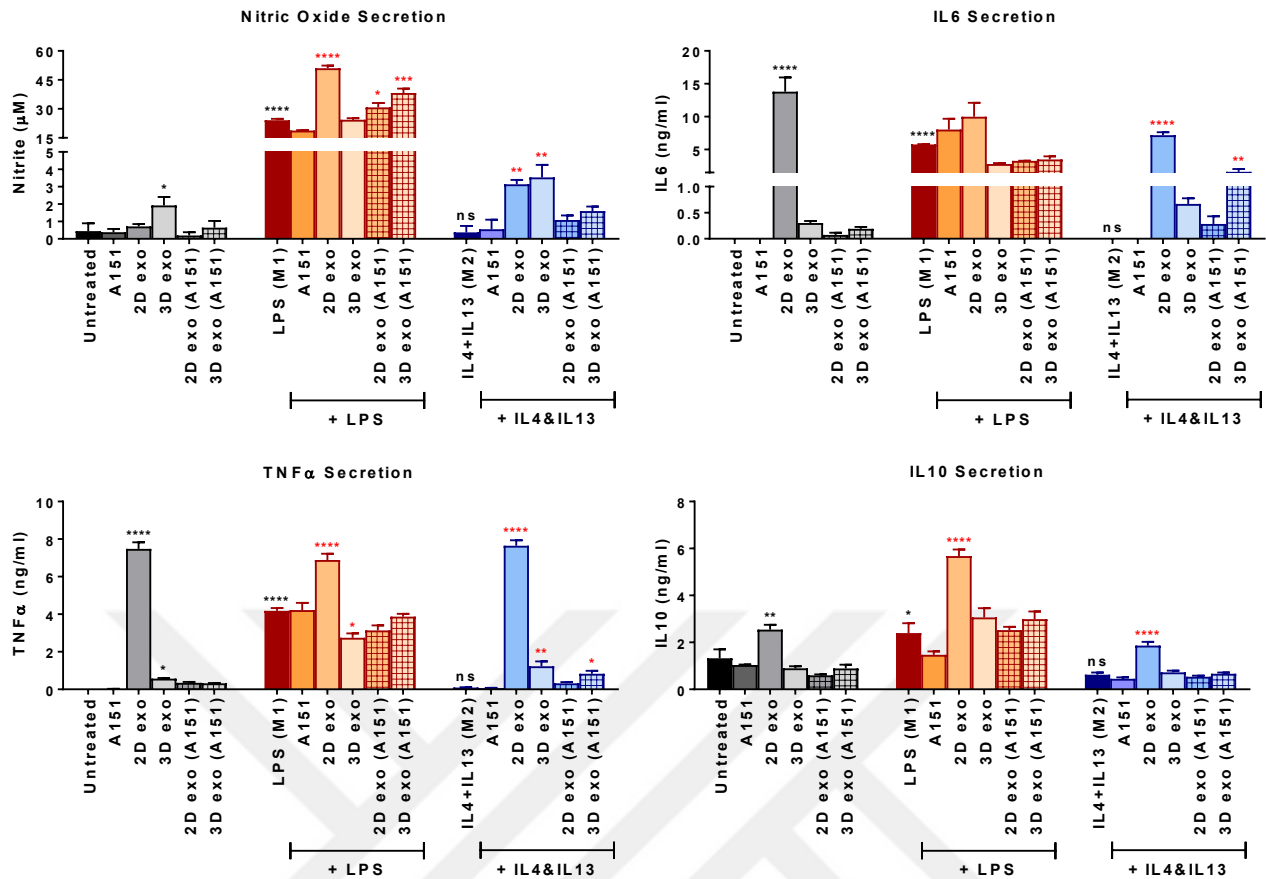


Figure 3.30. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on M1/M2 polarization in terms of nitric oxide and cytokine secretions. BMDMs (1×10^6 cells/ml) were incubated with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosomes or A151 ODN-loaded exosomes for 24 hours in the absence or presence of 100 ng/ml LPS for M1-induction or 10 ng/ml IL4 and 10 ng/ml IL13 for M2-induction. Nitric oxide secretion was indirectly determined with Griess assay. Black stars indicate differences with the untreated group whereas red stars indicate differences compared to M1- or M2-control groups. Experiment was performed in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

The most widely used gene marker for M1-polarization is *Nos2* which codes for the NOS2 enzyme producing NO. In contrast, M2-macrophages do not express *Nos2* but express high amounts of Arginase-1 (*Arg1*) which is absent in M1-macrophages [279]. Chitinase-like 3 protein (*Chil3*) is another marker for M2-macrophages and is induced by Th2-biased cytokines such as IL4 and IL13. [280]. Furthermore, studies also suggested that PD-L1 might be important for the suppressive characteristics of M2-macrophages [281]. In the absence or presence of A151 ODN mediates immuno suppression. Both types of exosomes significantly increased *Nos2* expression alone or in both polarizing conditions (Figure 3.31, upper left panel). This was prevented when A151 ODN was loaded within exosomes. The *Nos2* expression patterns were in line with NO activity measured by Griess Assay (Figure 3.30, upper left panel). To our surprise, both exosome types also induced *Arg1* expression alone, and more than 10-fold more potently in the presence of LPS (Figure 3.31, upper right panel, gray and orange bars). Once again, when A151 ODN was loaded within but not mixed with exosomes inhibited the action of LPS. Amount

of Arg1 expression triggered by the exosomes in the presence of LPS was equal to the Arg1 level induced by M2-polarization factors. When added onto IL4 and IL13, 3D-exosomes and interestingly A151 ODN-loaded 2D-exosomes upregulated Arg1 even further compared to only IL4+IL13 treatment (Figure 3.31, upper right panel, blue bars). The M2 marker Chil3 was strongly induced in the presence of IL4 and IL13 (Figure 3.31, lower left panel). Exosomes with or without A151 ODN did not cause any change alone or with LPS, but they limited the Chil3 upregulation caused by IL4 and IL13 (Figure 3.31, lower left panel, blue bars). Both types of exosomes triggered Pdl1 expression in the absence of a polarizing factor (Figure 3.31, lower right panel, gray bars). A151 ODN loading prevented 2D-exosomes from doing so while it could only limit the 3D-exosomes partially. The pattern was similar in the presence of M2-polarizing factors (Figure 3.31, lower right panel, blue bars). However, Pdl1 overexpression caused by the exosomes was a bit higher in that case and the A151 ODN-loaded 2D-exosomes could still significantly increase the expression levels. Stimuli such as LPS and IFN γ are known to increase PD-L1 on macrophages [282] and accordingly a substantial overexpression caused by LPS was observed, as expected (Figure 3.31, lower right panel, red bar). Strikingly, when used as such in the presence of LPS, 2D-exosomes further upregulated Pdl1 expression. Again consistently, this was in accordance with the observation that 2D-exosomes further potentiated LPS to induce more NO and to secrete more TNF α (Figure 3.30, left panels) in M₀-culture.

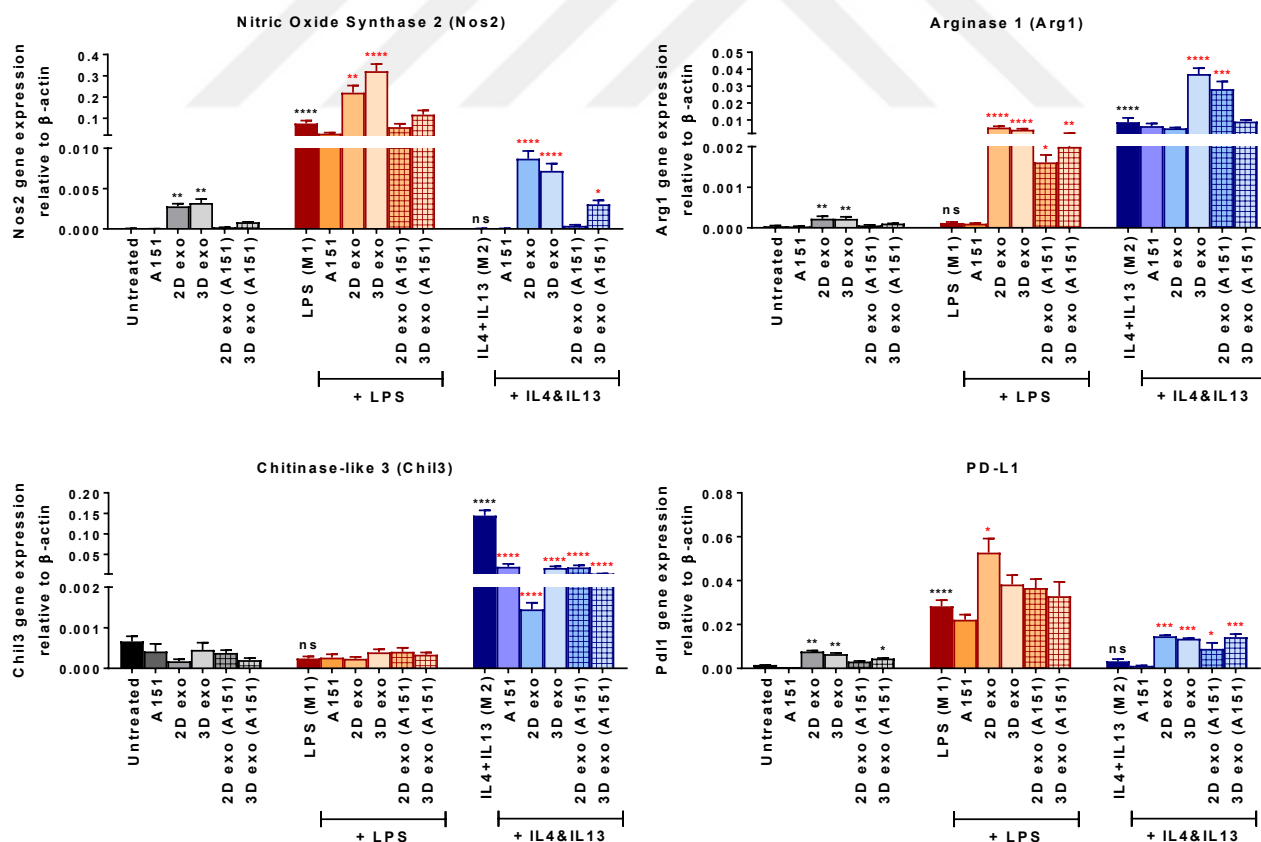


Figure 3.31. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on M1/M2 polarization in terms of mRNA signatures. BMDMs (1×10^6 cells/ml) were incubated with $1.5 \mu\text{M}$ A151 ODN, $20 \mu\text{g/ml}$ exosomes or A151 ODN-loaded exosomes for 24 hours in the absence or presence

of 100 ng/ml LPS for M1-induction or 10 ng/ml IL4 and 10 ng/ml IL13 for M2-induction. M1/M2 marker gene expressions relative to β -actin were determined with RT-qPCR using efficiency-corrected comparative CT method. Black stars indicate differences with the untreated group whereas red stars indicate differences compared to M1 or M2 control groups. Experiment was performed in triplicates. Exo: exosome, Exo(A151): A151 ODN-loaded exosome. ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

What was intriguing about these results was that exosomes induce M1 markers such as NO, Nos2 and IL6 while at the same time triggers an M2-marker, Arg1 but down-regulate another M2-marker Chil3. These results indicated that, contrary to our expectations, exosomes did not significantly skew the M1/M2 balance towards either to the M1- or to M2-phenotype. Instead, exosomes appeared to be inducing an MDSC-like phenotype in macrophages. MDSCs are a heterogeneous group of immature myeloid cells with immunosuppressive functions, particularly on T-cells, which are mainly mediated by NO and Arginase 1 (see Section 1.1.1.2.2). The macrophages producing high amounts of both Arginase 1 and NO but no Chil3 as a result of exosome treatment implied that UCX[®] exosomes might trigger an MDSC-like behavior rather than M2-polarization on macrophages.

3.7 Changes in Cell Phenotypes of Mouse PECs Following

Intraperitoneal Injection of Free or A151 ODN-Loaded UCX[®] Exosomes

Next, we wanted to investigate how UCX[®] exosomes in the presence or absence of A151 ODN affect the immune cell populations *in vivo*. To this end, 2D- or 3D-culture-derived exosomes, A151 ODN, free A151 ODN-exosome mixtures or A151 ODN-loaded versions of exosomes were intraperitoneally injected to C57/BL6 mice as 30 μ g/mouse. After 24 hours, peritoneal exudate cells (PECs) were collected as described in Section 2.2.1.7. B1-type B-cells and macrophages constitute majority of the cells in the PEC population [283]. Granulocytic or myeloid derived MDSCs can also mobilize to the peritoneal cavity when they are expanded in cases such as cancer [284]. Therefore, macrophage, T-cell and MDSC populations along with macrophage activation markers were analyzed with flow cytometry.

B-cells were slightly higher in all groups except for PBS-injected control group (Figure 3.32, upper left panel). Except for the group injected with 2D-exosomes, these increases were not statistically significant due to in-group variation. 2D-exosomes caused a 3-fold increase in T-cell numbers. Granulocyte-like type of MDSCs termed G-MDSCs, characterized as CD11b⁺ Ly6G⁺ Ly6C^{low} in mice, were expanded more than 10-fold by 2D-exosome injection (Figure 3.32 upper middle panel and Figure 3.33). Although 3D-exosomes did not induce G-MDSCs alone, A151 ODN addition and loading led to significantly higher G-MDSC within PECs. In contrast, A151 ODN addition and especially loading to 2D-exosomes prevented G-MDSC expansion. Monocytic type of MDSCs called M-MDSCs which are characterized as CD11b⁺ Ly6G⁻ Ly6C^{high} in mice were significantly elevated by both types of exosomes (Figure 3.32 upper right panel and Figure 3.33). Expansion of the M-MDSC population was partially limited by A151 ODN addition or loading. Macrophage population characterized as CD11b⁺ F4/80⁺ also increased around

5-fold upon 2D-exosome injection (Figure 3.32, lower left panel) similarly to T-cells (Figure 3.32, upper left panel). However, the surface expressions of inflammatory marker MHC-II and suppressive marker PD-L1 on macrophages were not altered by any of the injections (Figure 3.32, lower middle and right panels).

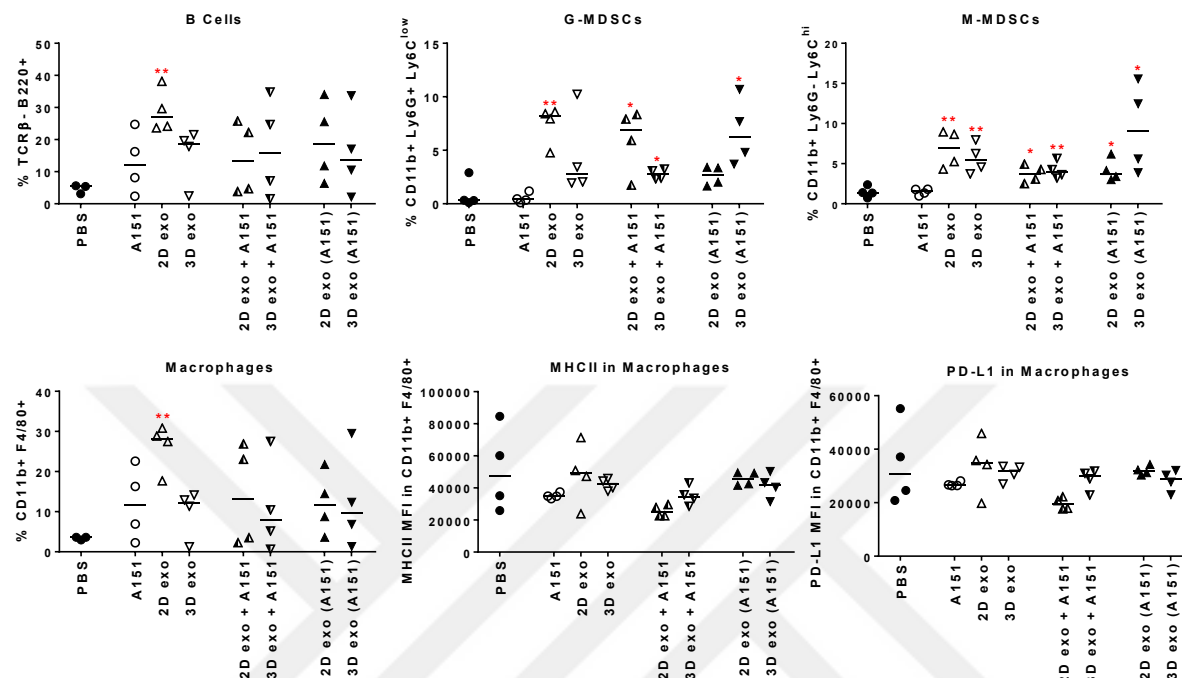


Figure 3.32. Effects of A151 ODN, 2D- or 3D-exosomes and their combinations on mouse peritoneal cell populations. 30 μ g/mouse A151 ODN, 30 μ g/mouse exosome or their combinations were administered via intraperitoneal injection to C57BL/6 mice. Peritoneal exudate cells (PECs) were collected 24 hours later. Cell populations and activation markers were analyzed via flow cytometry upon surface marker staining. Stars indicate differences with the PBS-treated control group. Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome, MFI: mean fluorescence intensity, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell. * $p \leq 0.05$, ** $p \leq 0.01$.

These results fitted well with the *in vitro* experiments indicating an MDSC-inducing ability of UCX[®] exosomes (Figures 3.30 and 3.31). Also in line with the *in vitro* experiments, 2D-exosomes appeared to be more inflammatory, increasing B1-cells and macrophage levels, while 3D-exosomes only induced MDSC expansion without any other effect (Figure 3.32). A151 ODN consistently impaired the actions of 2D- and 3D-exosomes *in vitro* and *in vivo*. Representative flow cytometry plots displaying the MDSC populations and their change due to injections are presented in Figure 3.33.

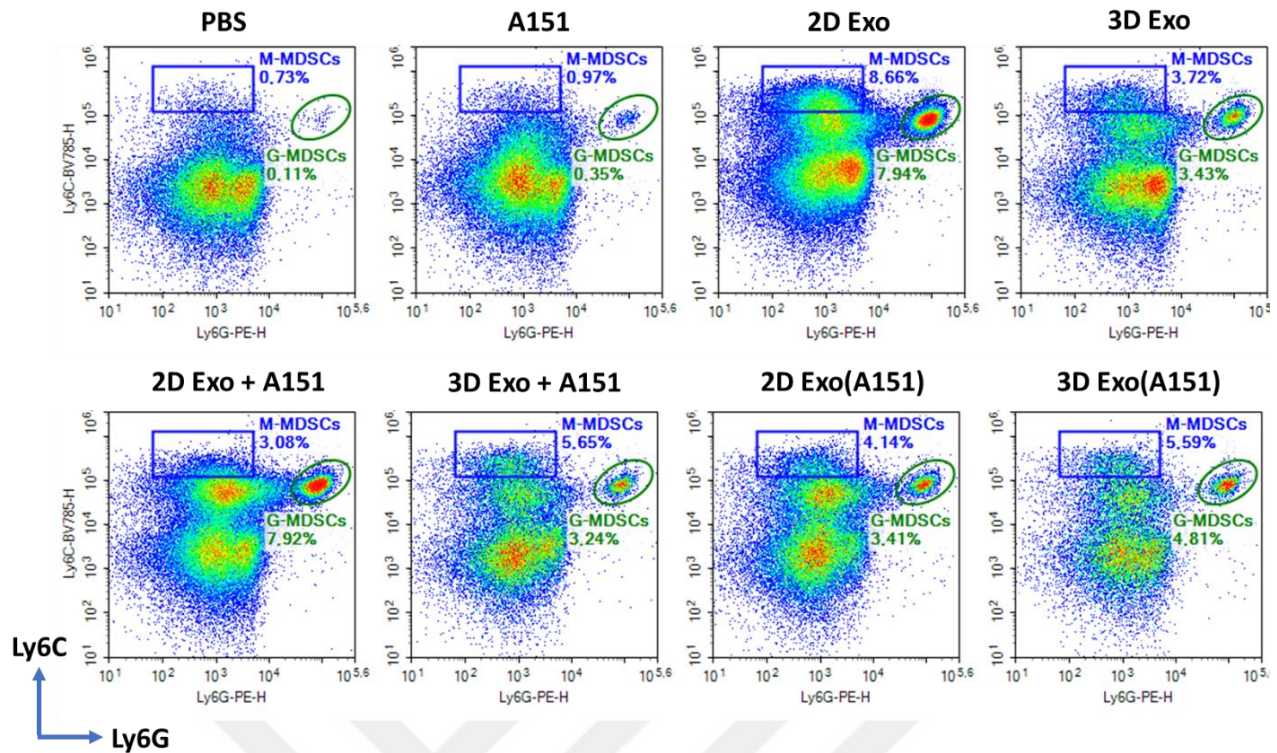


Figure 3.33. Representative flow cytometry plots displaying the effects of A151 ODN, 2D- or 3D-exosomes and their combinations on MDSC populations in PECs. 30 $\mu\text{g}/\text{mouse}$ A151 ODN, 30 $\mu\text{g}/\text{mouse}$ exosome or their combinations were administered via intraperitoneal injection to C57BL/6 mice. Peritoneal exudate cells (PECs) were collected 24 hours later and MDSC populations were analyzed via flow cytometry upon surface marker staining. Displayed Ly6G/Ly6C plots were gated from the CD11b^+ population. Exo: exosome, Exo+A151: free mixture of exosome and A151 ODN, Exo(A151): A151 ODN-loaded exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell.

3.8 *In Vivo* Immunomodulatory Potential of Free or A151 ODN-Loaded UCX[®] Exosomes in DSS-Induced Colitis Model

In light of the *in vitro* results, we wanted to test the immunomodulatory capacities of UCX[®] exosomes with or without A151 ODN in two auto-inflammatory models in mice. First, zymosan-induced peritonitis model was tested (see Sections 1.5.2 and 2.2.9.3). Unfortunately, exosomes did not prevent the neutrophil infiltration and macrophage death caused by zymosan in that model. Results are presented in Figure A3, Appendix A. Since zymosan-induced peritoneal inflammation is very acute and severe, we decided to go on with a more progressive disease model. Dextran sodium sulfate (DSS)-induced colitis (see Section 1.5.1) is an inflammatory bowel disease model reproducibly induced in mice by oral administration of DSS in drinking water. As detailed in Section 2.2.9.2, C57BL/6 mice were provided with drinking water containing 3% DSS for 4 days followed by clean drinking water for 3 days. Considering the beginning of DSS administration as day 0, treatments of exosome, A151 ODN or their

combinations were applied at day 0 and day 2. A151 ODN, 2D- or 3D-exosomes, or A151 ODN-loaded 2D- or 3D-exosomes were intraperitoneally injected as 30 µg/mouse in a 200 µl of saline.

Shortening of the colons and weight loss are two of the main indications of disease progression in DSS-induced colitis [264]. DSS caused critical shortening of the colons (~30%) while also causing necrotic lesions (Figure 3.34, upper and lower left panels). These animals also experienced bloody diarrhea for the last 3 days of the experiment. A151 ODN and 2D-exosome treatments were able to partially prevent excessive shortening of the colons but there were still visible necrotic areas (Figure 3.34, upper and lower left panels). Of note, one animal in the 2D-exosome-treated group had severe bloody diarrhea and died before the termination of the experiment. Colons of the animals treated with 3D-exosomes or A151 ODN loaded forms of both 2D- or 3D-exosomes appeared almost identical to the group that has not received DSS in terms of length and color (Figure 3.34, upper panel, compare upper left-most panel to upper right-most panel). Although macroscopic differences were apparent, only the A151 ODN-loaded 2D-exosomes prevented colon shortening enough to be statistically significant (Figure 3.34, lower left panel). Control group that was not exposed to DSS experienced no weight loss during the course of the experiment while the DSS-only group lost 20% of their weights (Figure 3.34, lower right panel). 2D-exosome-treated group also lost a similar portion of their weights but A151 ODN treatment could lower the weight loss by half. 3D-exosomes and A151 ODN-loaded versions of both exosomes significantly improved the well-being of the animals, preventing weight loss almost completely.

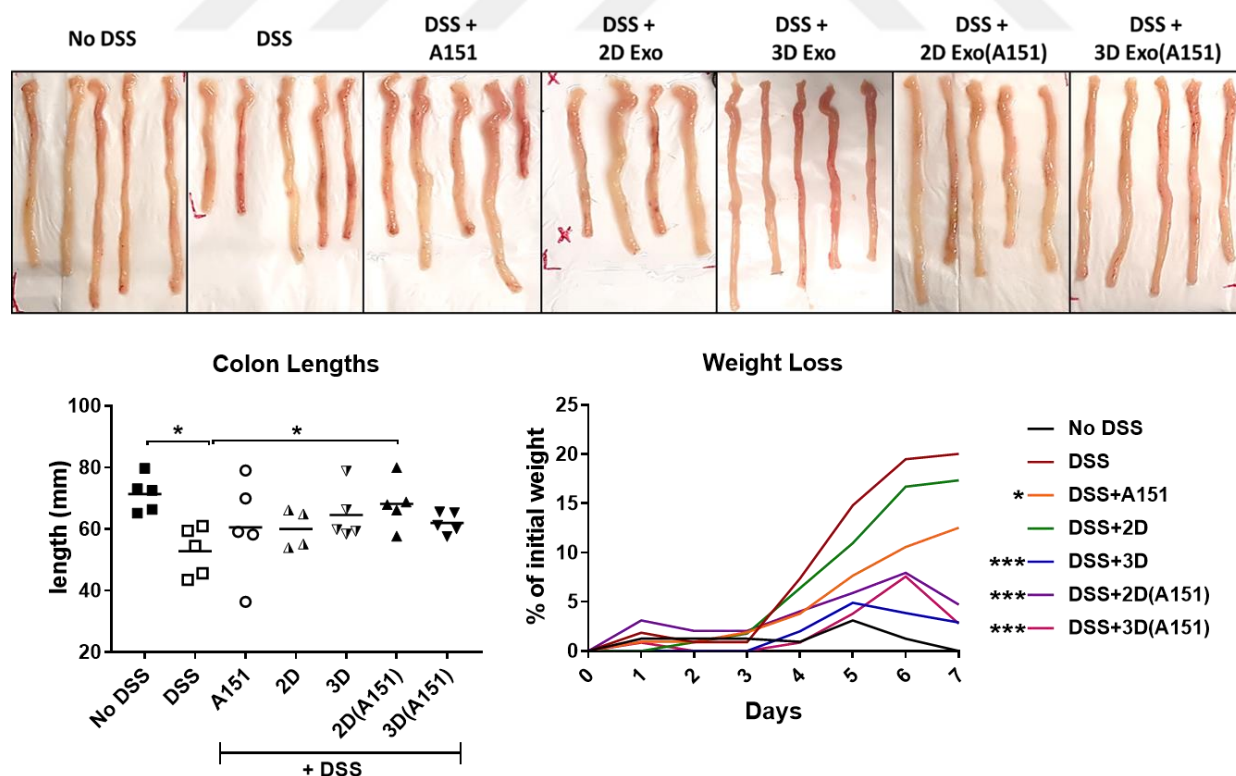


Figure 3.34. Therapeutic effects of A151 ODN, 2D- or 3D-exosomes and their A151 ODN-loaded forms on mice with DSS-induced colitis. 3% DSS was administered to C57BL/6 mice in drinking

water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 µg/mouse A151 ODN, free 2D- or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. Animals were weighed daily. At day 7, colons were taken out, flushed with PBS, flattened out, measured with a digital caliper and photographed. Weight loss is shown as the mean of each group and differences of treatment groups compared to the DSS group at day 7 are indicated next to the color legend. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, *** $p \leq 0.001$.

Histological changes that occur in the colon tissue upon DSS exposure include the infiltration of inflammatory cells, disruption of crypt architecture, epithelial erosion and ulceration [285]. After colons were removed, they were prepared according to the Swiss roll protocol [264] and stained with hematoxylin and eosin. As expected, DSS led to inflammatory cell infiltration into lamina propria and submucosa, crypt damage, and epithelial erosion (Figure 3.35). Histological scoring based on Laroui et al. [286] revealed that 3D-exosomes and A151 ODN-loaded 2D-exosomes reduced inflammation, all treatments prevented crypt damage and free or A151 ODN-loaded 3D-exosomes decreased the incidence of epithelial erosion (Figure 3.35). Total histological scoring proved 3D-exosomes and A151 ODN-loaded 2D- and 3D-exosomes as the most effective treatments, albeit only 3D-exosome treatment reduced the score in a statistically significant degree. Representative histological images provided in Figure 3.35 help to display the epithelial erosion and loss of crypt architecture due to DSS treatment and their recovery with free or A151 ODN-loaded exosome treatment.

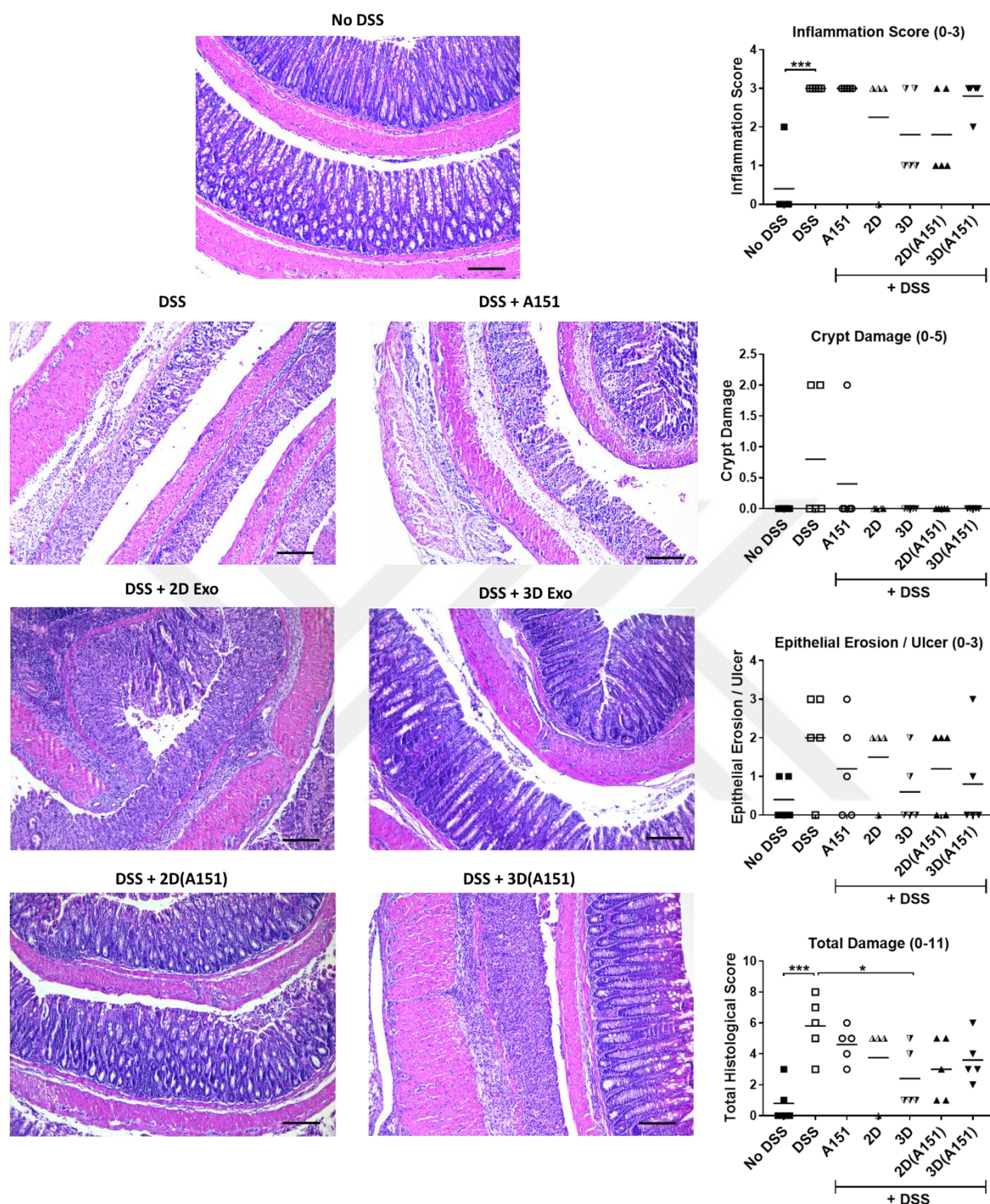


Figure 3.35. Histological scoring and representative histological images displaying the therapeutic effects of A151 ODN, 2D- or 3D-exosomes and their A151 ODN-loaded forms on mice with DSS-induced colitis. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 μ g/mouse A151 ODN, free 2D- or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, colons were taken out, flushed with PBS, cut longitudinally, rolled as Swiss rolls and placed in 10% buffered formalin for subsequent hematoxylin & eosin staining. Scale bars represent 20 μ m. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-

exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, *** $p \leq 0.001$.

We wanted to determine the exact actions of UCX[®] exosomes and A151 ODN on the immune system through which they prevented disease progression. To this end, single cells from spleens and mesenteric lymph nodes of the animals were isolated at the end of the experiment as described in Sections 2.2.1.6 and 2.2.1.8. Spleen and lymph nodes are secondary lymphoid organs harboring antigen presenting cells and lymphocytes. In the mesenteric lymph nodes of DSS-treated animals, an average 35% reduction in T-cell numbers was observed (Figure 3.36, upper left panel). When sub-populations CD4⁺ and CD8⁺ T-cells were quantified, there was an apparent decrease in both populations upon DSS exposure (Figure 3.36, upper right and lower left panels). All the treatments except for 2D-exosomes were able to prevent T-cell loss. In terms of CD4⁺ T-cells, 3D-exosomes and the A151 ODN-loaded versions of both exosomes were more efficient than A151 ODN in restoring the T-cell numbers. Although A151 ODN, 3D-exosomes and A151 ODN-loaded 2D-exosomes could effectively prevent CD8⁺ T-cell loss, A151 ODN-loaded 3D-exosomes were the more potent in that regard. We also wanted to see if the regulatory T-cells were expanded by the treatments. However, only 2D-exosomes seem to have slightly increased Treg numbers although it was not statistically significant (Figure 3.36, lower right panel).

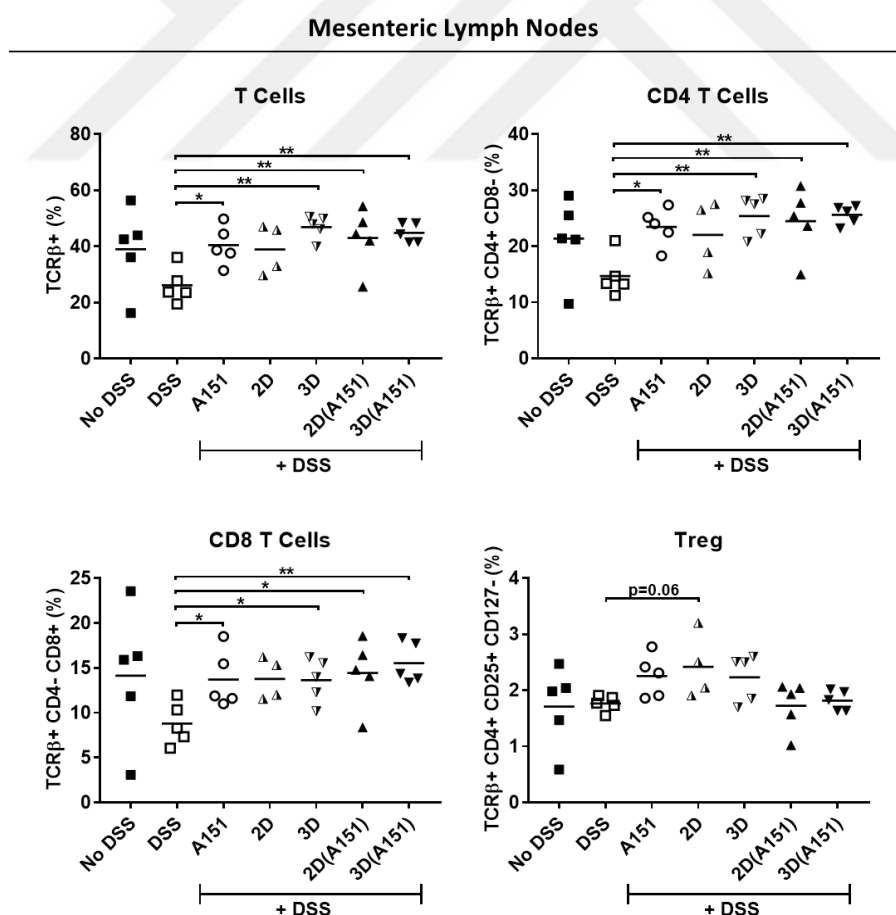


Figure 3.36. T-lymphocyte populations in the mesenteric lymph nodes of mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was

administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 $\mu\text{g}/\text{mouse}$ A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, lymph nodes were taken out and cell populations were analyzed via flow cytometry. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, Treg: regulatory T-cell. * $p \leq 0.05$, ** $p \leq 0.01$.

Myeloid cell populations in the mesenteric lymph nodes which are DCs and macrophages were also investigated. There was no significant change in DC population or their MHC-II expression which indicates antigen-presentation capacity (Figure 3.37, upper panels). DSS caused a significant elevation of macrophages in the lymph nodes (Figure 3.37, lower left panel). Both types of exosomes and their A151 ODN-loaded forms appeared to have brought the macrophage numbers close to basal levels but it was not found statistically significant compared to the DSS-only group probably due to low sample size. There was no alteration caused by DSS or the treatments on macrophage MHC-II levels (Figure 3.37, lower right panel), similar to DCs.

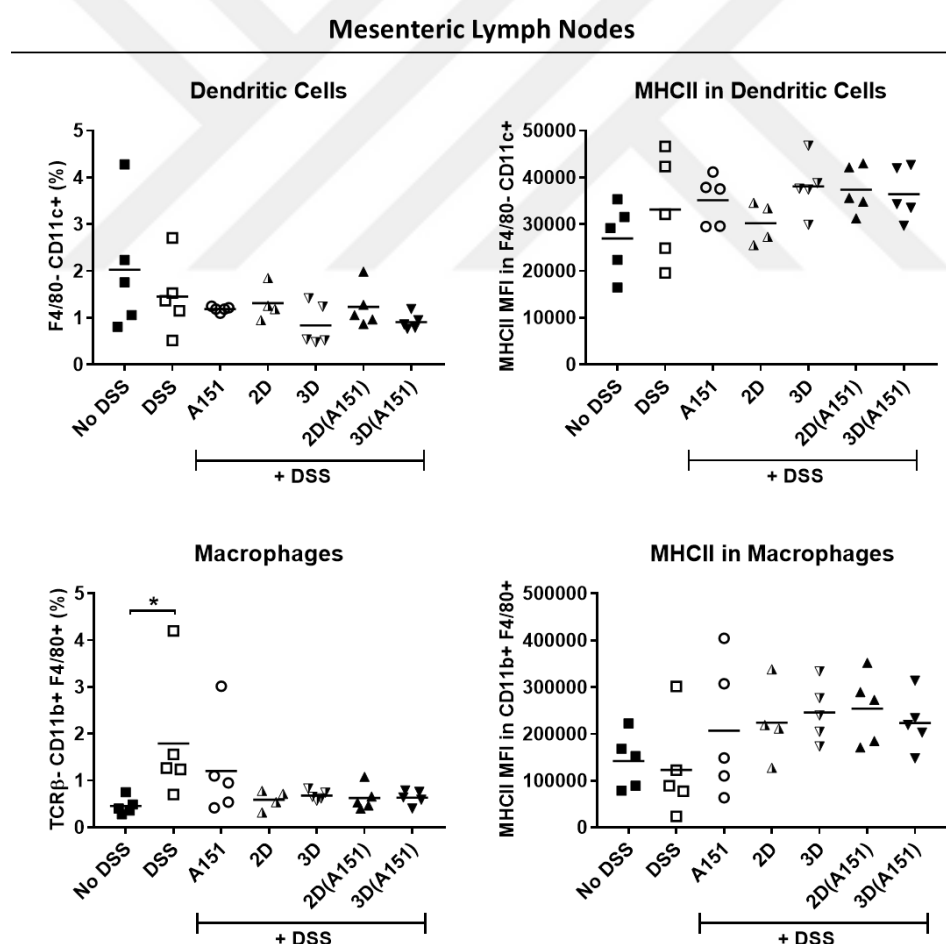


Figure 3.37. Dendritic cell and macrophage populations in the mesenteric lymph nodes of mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as

day 0, and 30 $\mu\text{g}/\text{mouse}$ A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, lymph nodes were taken out and cell populations were analyzed via flow cytometry. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. $*p \leq 0.05$.

In the spleen, 2D-exosomes increased T-cell numbers (Figure 3.38, upper left panel) which was the same as their effect on T-cell populations in PECs (Figure 3.32, upper left panel). Regarding the T-cell numbers, observations in the spleen were similar with the lymph nodes. DSS caused $\sim 40\%$ T-cell loss on average (Figure 3.38, upper right panel), more significantly than it did in the lymph nodes (Figure 3.36, upper left panel). Only A151 ODN and more potently A151 ODN-loaded 3D-exosomes could restore the loss in overall T-cell population significantly (Figure 3.38, upper right panel). Regarding CD8^+ cytotoxic T-cells, A151 ODN and A151 ODN-loaded 3D-exosomes were the most effective in preventing cell loss, although all the other treatments could also significantly protect the CD8^+ T-cells (Figure 3.38, lower left panel). The reduction in CD4^+ helper T-cell numbers induced by DSS was more striking compared to CD8^+ T-cells. DSS lowered the CD4^+ T-cell population in the spleen by half (Figure 3.38, lower middle panel). All the treatments were effective in preventing this, although A151 ODN-loaded 3D-exosomes were the most potent and kept the T-cells at healthy levels. The next most potent treatments were free 3D-exosomes and A151 ODN, respectively. As in the lymph nodes, no significant change in the Treg population in the spleens was observed (Figure 3.38, lower right panel).

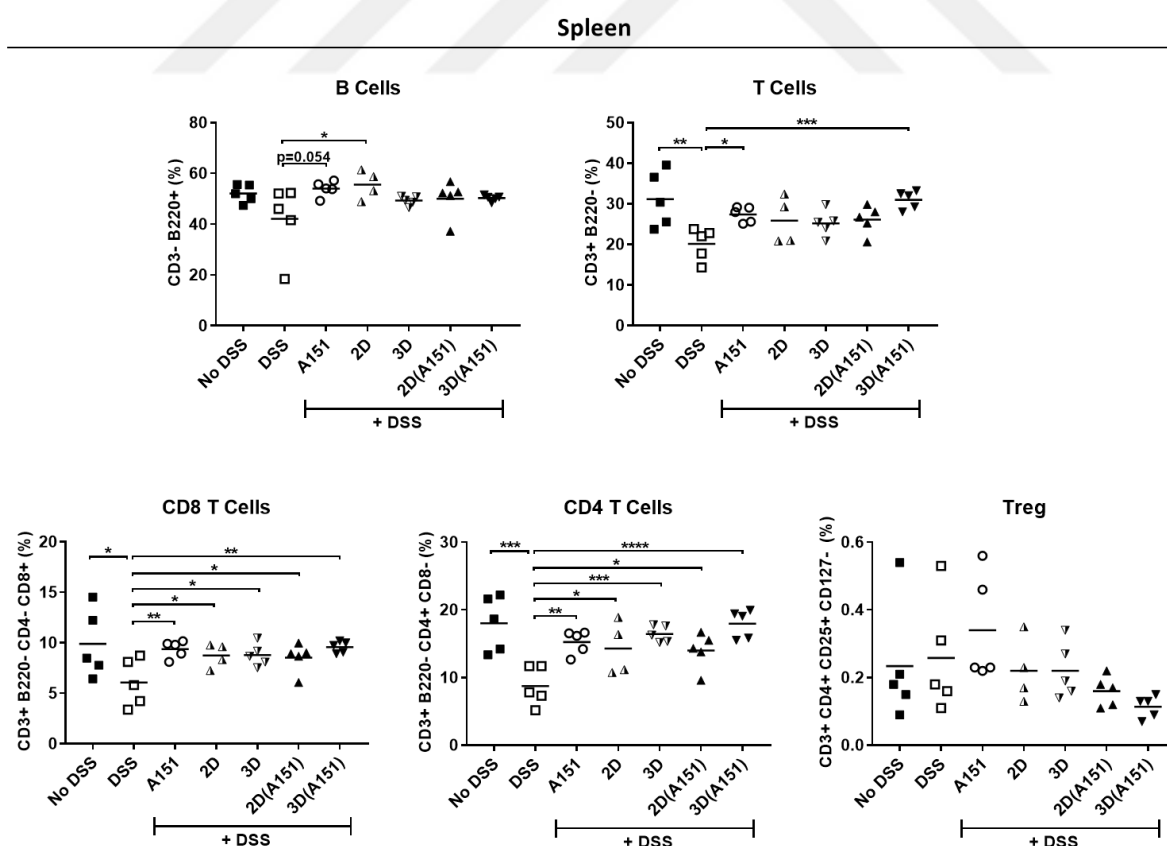


Figure 3.38. B and T-lymphocyte populations in the spleens of mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was

administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 µg/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and cell populations were analyzed via flow cytometry. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, Treg: regulatory T-cell. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

There was a slight increase in the macrophage population in spleen (Figure 3.39, upper left panel) even though it was not significant as it was in the lymph nodes (Figure 3.37, lower left panel). Interestingly, both the overall MHC-II level and the MHC^{high} sub-population of the macrophages were reduced by DSS, ~50% and ~60% on average respectively, which imply reduced inflammatory and antigen-presentation capacity of macrophages (Figure 3.39, upper middle and right panels). A151 ODN-loaded 3D-exosome treatment resulted in healthy levels of MHC^{high} macrophages. Although not statistically significant, it also restored the overall MHC expression. There was no significant alteration caused by DSS or the treatments in the surface levels of anti-inflammatory markers PD-L1 and CD206 on macrophages (Figure 3.39, middle row, left and middle panels). There was also no change in the DC population (Figure 3.39, middle row, right panel), similar to the case in the lymph nodes (Figure 3.37, upper left panel). However, DSS caused a reduction in MHC-II levels of DCs (33% on average) (Figure 3.39, lower left panel). This was almost completely prevented by the A151 ODN-loaded exosomes even though the results were not found statistically significant possibly due to low sample size. Two MDSC populations were also investigated in the spleen since exosomes showed MDSC-inducing capacities in former experiments, but neither DSS nor the treatments caused any significant change in G-MDSC or M-MDSC levels (Figure 3.39, lower middle and right panels). The ability of the treatment groups to prevent colitis progression was likely MDSC-independent.

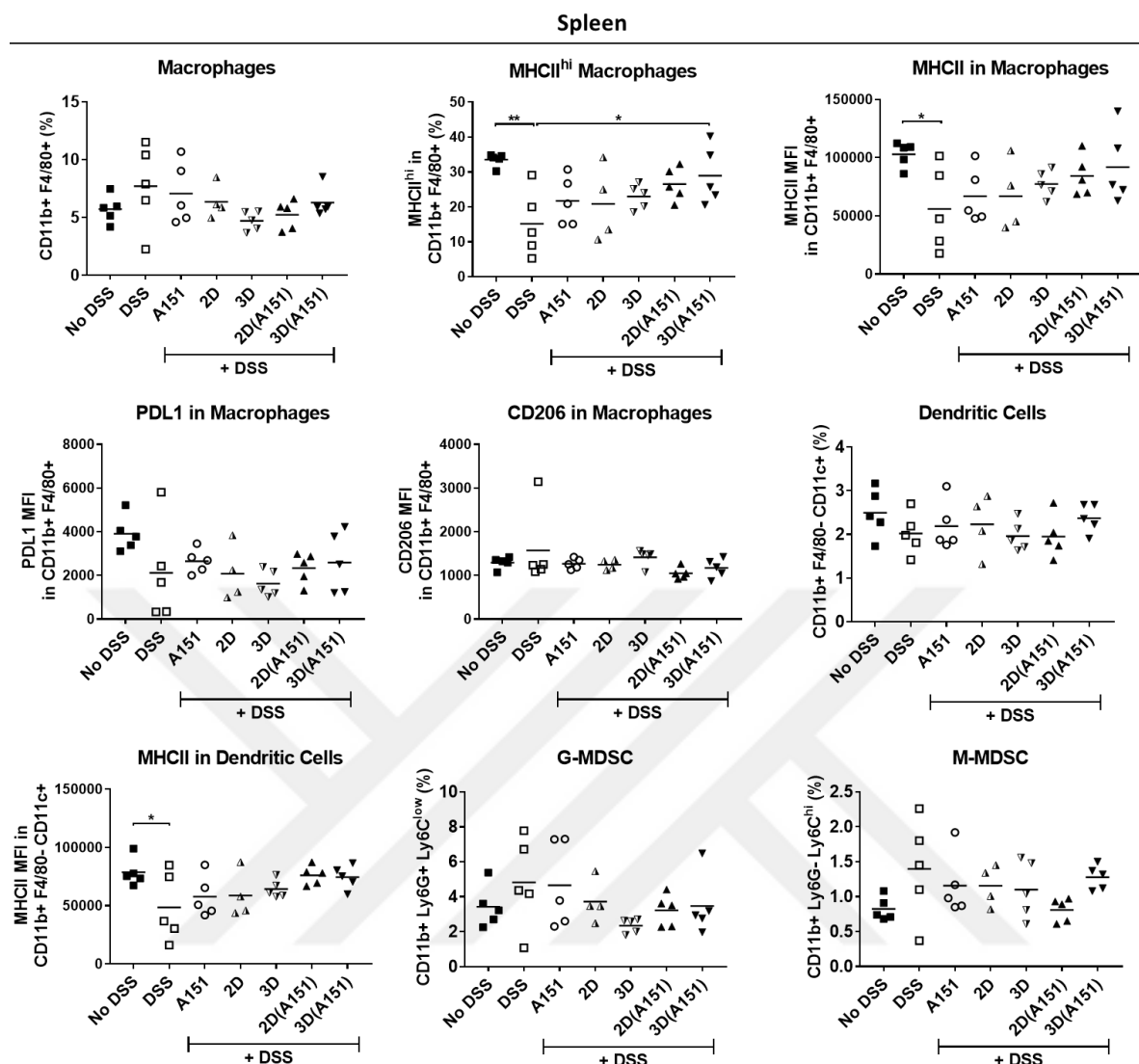


Figure 3.39. Macrophage, dendritic cell and MDSC populations in the spleens of mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 μ g/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and cell populations were analyzed via flow cytometry. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, hi: high, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell. * $p \leq 0.05$, ** $p \leq 0.01$.

Serum levels of pro-inflammatory cytokines IL6 and TNF α , which are known as key cytokines induced by DSS [287], were also measured with ELISA upon blood collection via cardiac puncture. IL6 levels were significantly elevated in DSS-exposed mice (Figure 3.40, left panel). 3D-exosomes and the A151 ODN-loaded forms of both exosomes were able to eliminate excessive IL6 secretion induced by DSS. However, contrary to the literature, no DSS-induced difference was observed in serum TNF α levels (Figure 3.40, right panel).

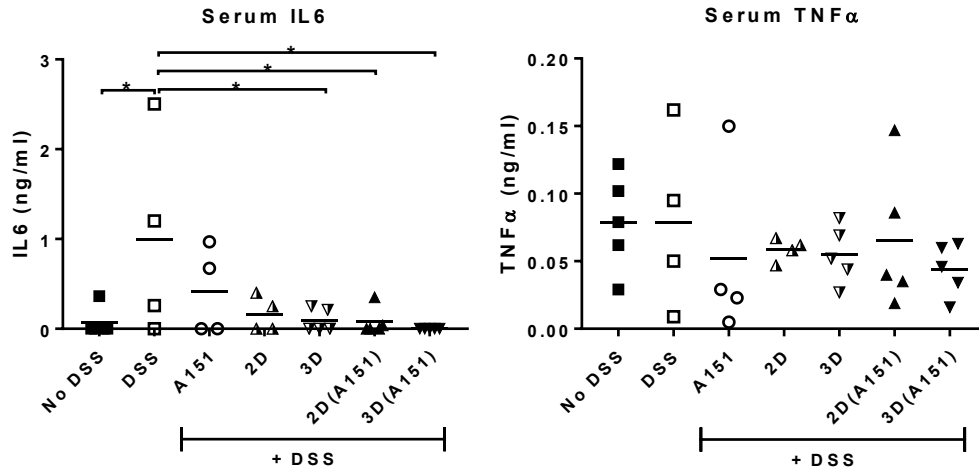


Figure 3.40. Serum IL6 and TNF α levels of mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0, and 30 μ g/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, blood was collected through cardiac puncture and serum cytokine levels were determined with ELISA. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$.

Next, we wanted to investigate the cytokine-producing capacities of the splenocytes obtained from the animals. For that purpose, splenocytes were seeded on 96 well plates as 2.5×10^5 cells/well and stimulated with 20 ng/ml PMA along with 1 μ g/ml ionomycin, 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN for 24 hours. IFN γ , IL6, TNF α and IL10 levels in the culture supernatants were determined with ELISA.

When unstimulated, there was no notable IFN γ secretion from the splenocytes of any group (Figure 3.41, upper left panel). PMA coupled with Ca^{2+} ionophore ionomycin non-specifically activates T-cells through protein kinase C (PKC) activation [288]. PMA/ionomycin potently induced T-cell dependent IFN γ production in healthy cells just as expected but the ability of cells of animals exposed to DSS to respond to PMA/ionomycin stimulation was almost 3-fold lower (Figure 3.41, upper right panel). All treatments except for 2D-exosome treatment restored the T-cell function but 3D-exosomes were the most potent followed by the A151 ODN-loaded versions of both exosomes. Interestingly, LPS and R848 stimulation did not cause high amounts of IFN γ secretion from splenocytes of healthy animals (Figure 3.41, lower left and middle panels). Still, the responses of DSS-induced animals were even lower. One significant result was that splenocytes of animals treated with A151 ODN-loaded exosomes could secrete significantly higher amounts of IFN γ upon LPS and K23 stimulation (Figure 3.41, lower left and right panels).

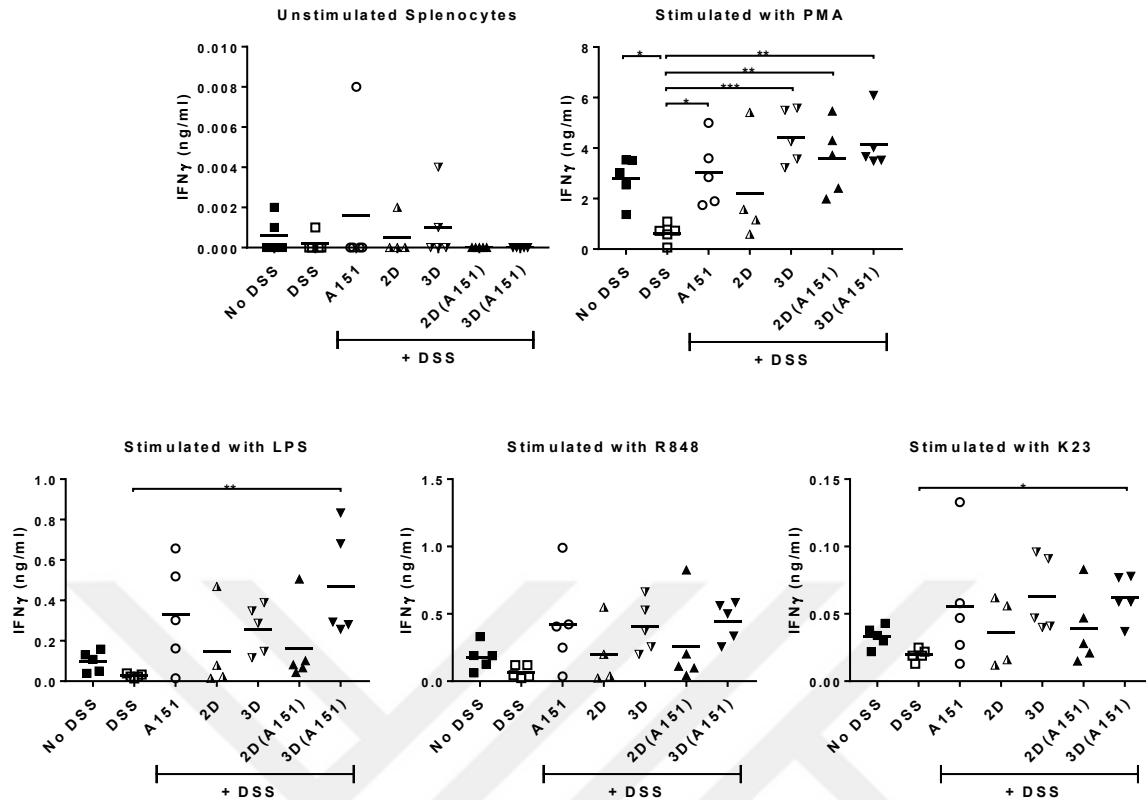


Figure 3.41. IFN γ production capacities of splenocytes isolated from mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 μ g/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and splenocytes were stimulated with 20 ng/ml PMA along with 1 μ g/ml ionomycin, 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN for 24 hours. Cytokine concentrations were determined with ELISA. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

None of the unstimulated splenocytes produced IL6 (Figure 3.42, upper left panel). Some animals from the treatment groups caused non-significant and negligible amounts of IL6 production. IL6 secretion was triggered from healthy cells by all three TLR ligands that were used (Figure 3.42, lower panels). However, IL6-producing capacities of the splenocytes of DSS-exposed animals were half of the healthy splenocytes. A151 ODN-loaded 3D-exosomes effectively increased IL6 production which was in line with the observation that A151 ODN-loaded 3D-exosomes increased the MHC^{high} macrophage population in the spleens (Figure 3.39, upper middle panel). Of note, A151 ODN treatment could also restore the IL6-producing capacities of splenocytes stimulated with R848 and K23 (Figure 3.42, lower middle and right panels).

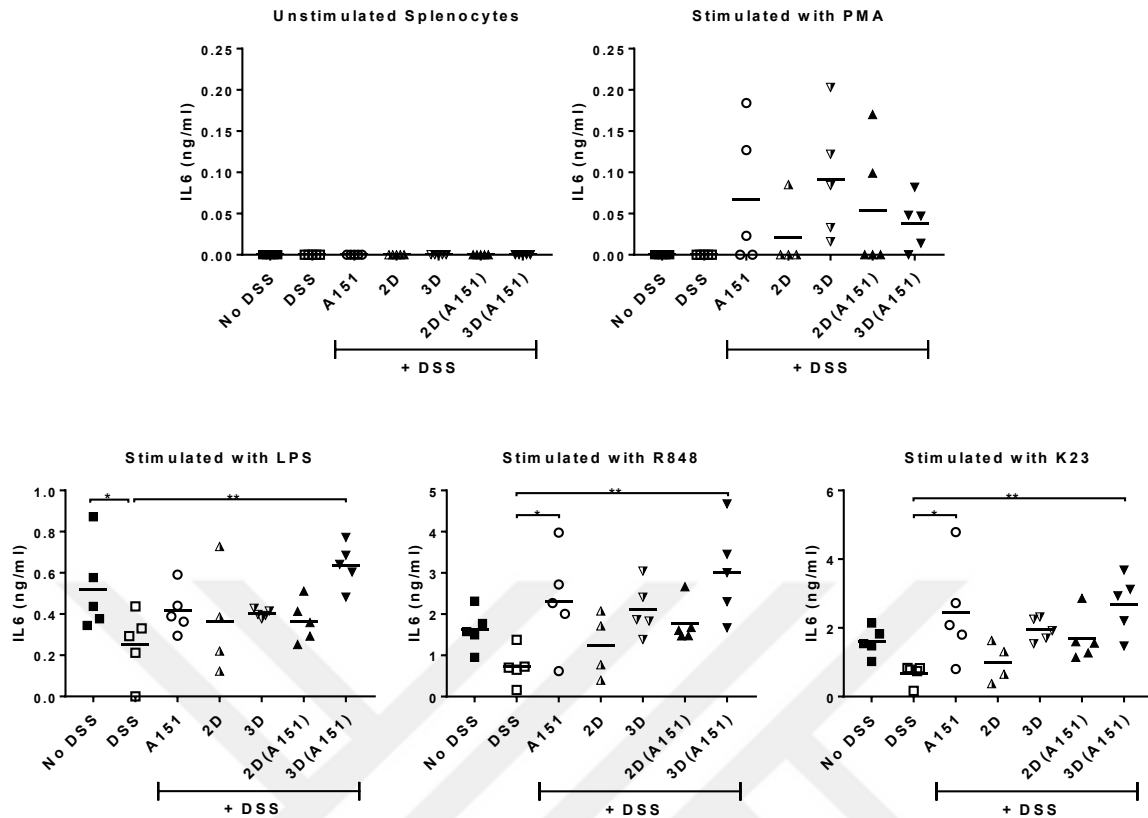


Figure 3.42. IL6 production capacities of splenocytes isolated from mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 μ g/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and splenocytes were stimulated with 20 ng/ml PMA along with 1 μ g/ml ionomycin, 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN for 24 hours. Cytokine concentrations were determined with ELISA. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$.

A151 ODN-loaded 2D-exosomes decreased basal TNF α production in the absence of a stimulant (Figure 3.43, upper left panel). PMA stimulation increased TNF α levels compared to the unstimulated splenocytes, however, all of the treatments except for 2D-exosomes elevated it further (Figure 3.43, upper right panel). Unlike IFN γ and IL6, TNF α -producing capacities of DSS-exposed animals were not critically disrupted and they responded to LPS and R848 stimulations similarly to healthy animals (Figure 3.43, lower left and middle panels). Even so, A151 ODN-loaded 3D-exosome production caused even higher TNF α secretion. On the contrary, responsiveness of the DSS-exposed group to K23 stimulation in terms of TNF α production was significantly impaired (Figure 3.43, lower right panel).

This indicated a possible TLR9 signaling impairment due to DSS. This impairment was recovered by A151 ODN-loaded 3D-exosome treatment.

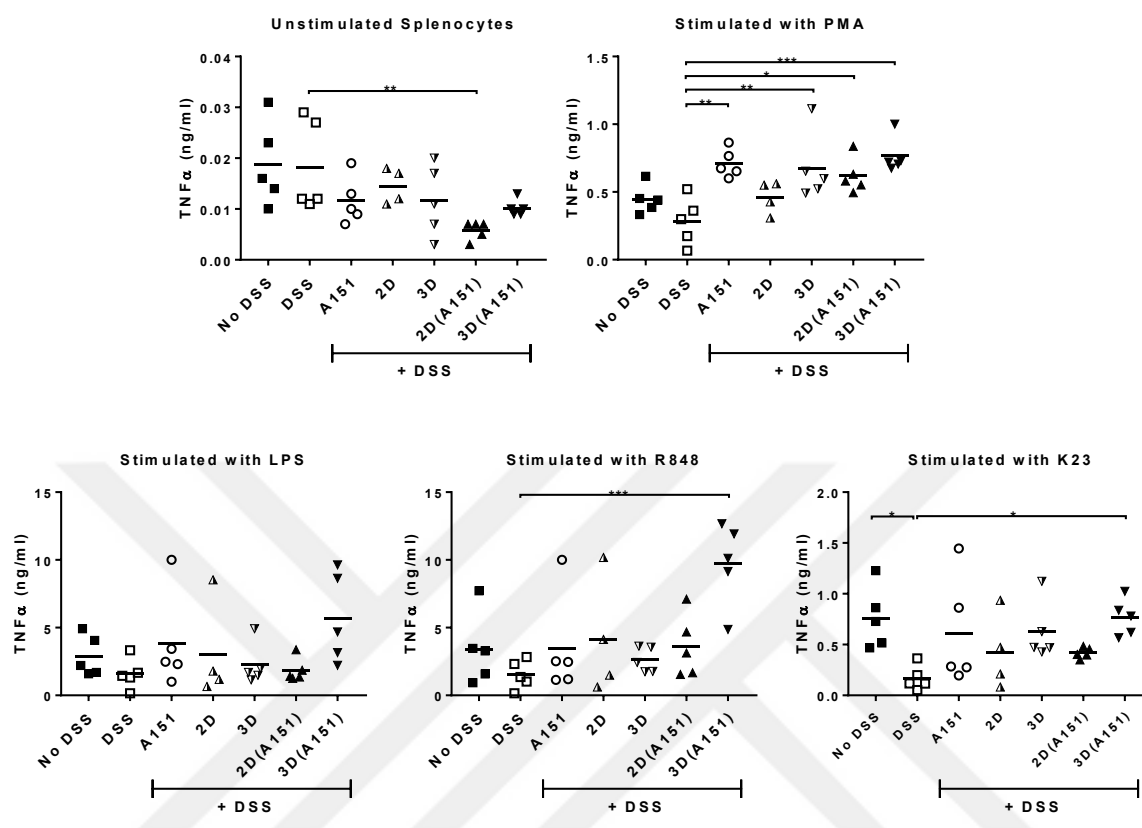


Figure 3.43. TNFα production capacities of splenocytes isolated from mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 µg/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and splenocytes were stimulated with 20 ng/ml PMA along with 1 µg/ml ionomycin, 1 µg/ml LPS, 1 µg/ml R848 or 1 µM K23 CpG ODN for 24 hours. Cytokine concentrations were determined with ELISA. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

All stimulants caused major IL10 secretion which serves to control excessive inflammation (Figure 3.44). IL10 production by the DSS-only group was slightly lower upon PMA, R848 and K23 stimulations compared to the healthy levels, even though it was not statistically significant. In each of the stimulation conditions, a dramatic IL10-inducing effect of A151 ODN was observed. Only in case of K23 stimulation, A151 ODN-loaded 3D-exosomes also promoted IL10 production (Figure 3.44, lower right panel).

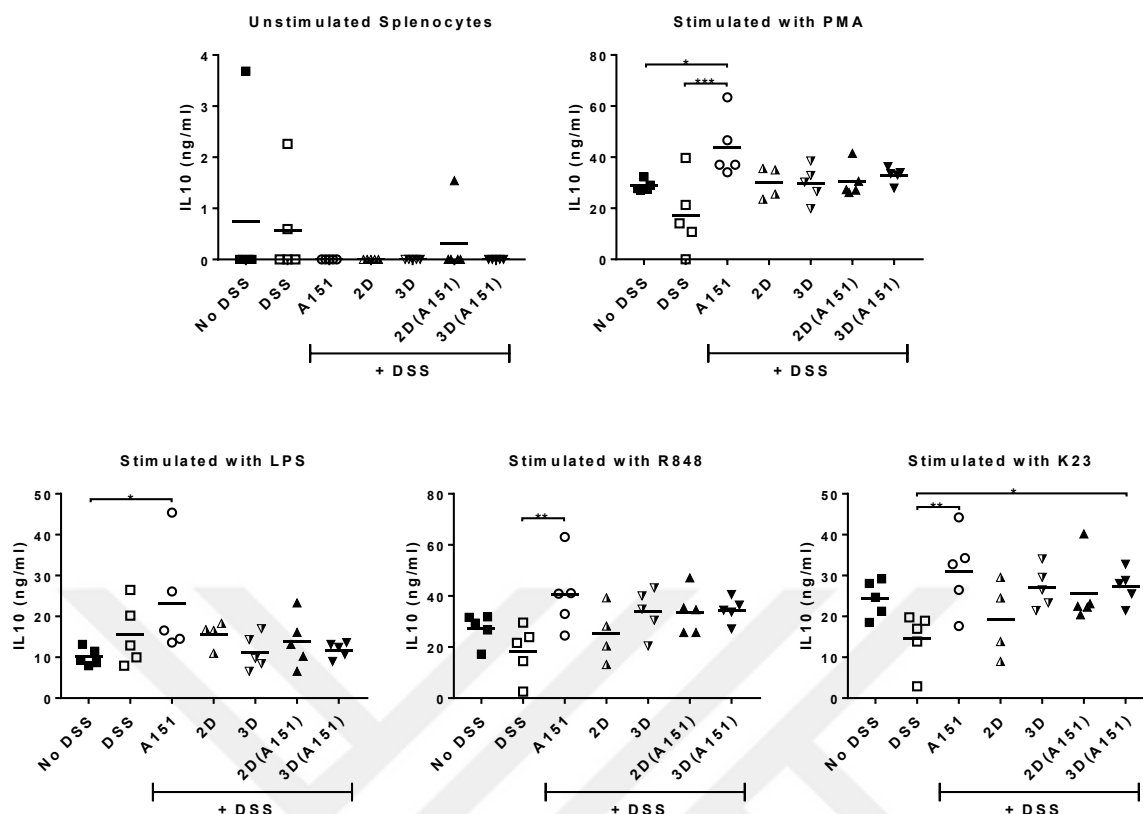


Figure 3.44. IL10 production capacities of splenocytes isolated from mice with DSS-induced colitis treated with A151 ODN, free UCX exosomes or their A151 ODN-loaded forms. 3% DSS was administered to C57BL/6 mice in drinking water for 4 days which was followed by 3 days of fresh water for epithelial recovery. The day DSS administration started was considered as day 0 and 30 μ g/mouse A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected intraperitoneally at days 0 and 2. At day 7, spleens were taken out and splenocytes were stimulated with 20 ng/ml PMA along with 1 μ g/ml ionomycin, 1 μ g/ml LPS, 1 μ g/ml R848 or 1 μ M K23 CpG ODN for 24 hours. Cytokine concentrations were determined with ELISA. DSS: dextran sodium sulfate, 2D: 2D-exosome, 3D: 3D-exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Overall, these results revealed that treatments with 3D-exosomes, A151 ODN-loaded 2D-exosomes and A151 ODN-loaded 3D-exosomes were very effective in preventing DSS-induced colitis progression and reversing the cellular alterations caused by DSS. Although A151 ODN was also able to reverse some of the pathogenic parameters, its combination with the UCX[®] exosomes, particularly with 3D-exosomes, appear to be a highly promising treatment approach for inflammatory bowel diseases such as colitis.

3.9 *In Vivo* Immunomodulatory Potential of Free or A151 ODN-Loaded UCX[®] Exosomes in Excisional Wound Healing Model

Conditioned media of UCX[®] cultures, especially 3D culture, were previously shown to promote wound regeneration [69]. Since conditioned media contain exosomes along with soluble secreted proteins, we hypothesized that exosomes could be the paracrine factors enhancing wound repair. To test this, 6 mm

circular punch biopsies were performed on female BALB/c mice. Silicone wound splints were surgically stitched around the wounds in order to prevent contraction. The day that punch biopsies were performed was considered as day 1. Exosome-depleted 2D- or 3D-conditioned media, A151 ODN, free 2D- or 3D-exosomes, or 2D- or 3D-exosomes loaded with A151 ODN were injected subcutaneously to the wound edges as 30 μ g/mouse on day 1 and day 3. Each wound was photographed every day and the areas were digitally measured using public ImageJ software. 3 animals from each group were sacrificed on day 7, while the remaining 7 animals were sacrificed on day 14.

Until day 7, all treatments except for exosome-depleted 2D CM delayed wound closure compared to PBS-injected control wounds (Figure 3.45, left panels). After day 7, differences between groups disappeared and at the end of 14 days, wound closure was not significantly different among groups (Figure 3.45, right panels). Photographs of each wound at the time of sacrifice are provided in Figures A4 and A5 in Appendix A.

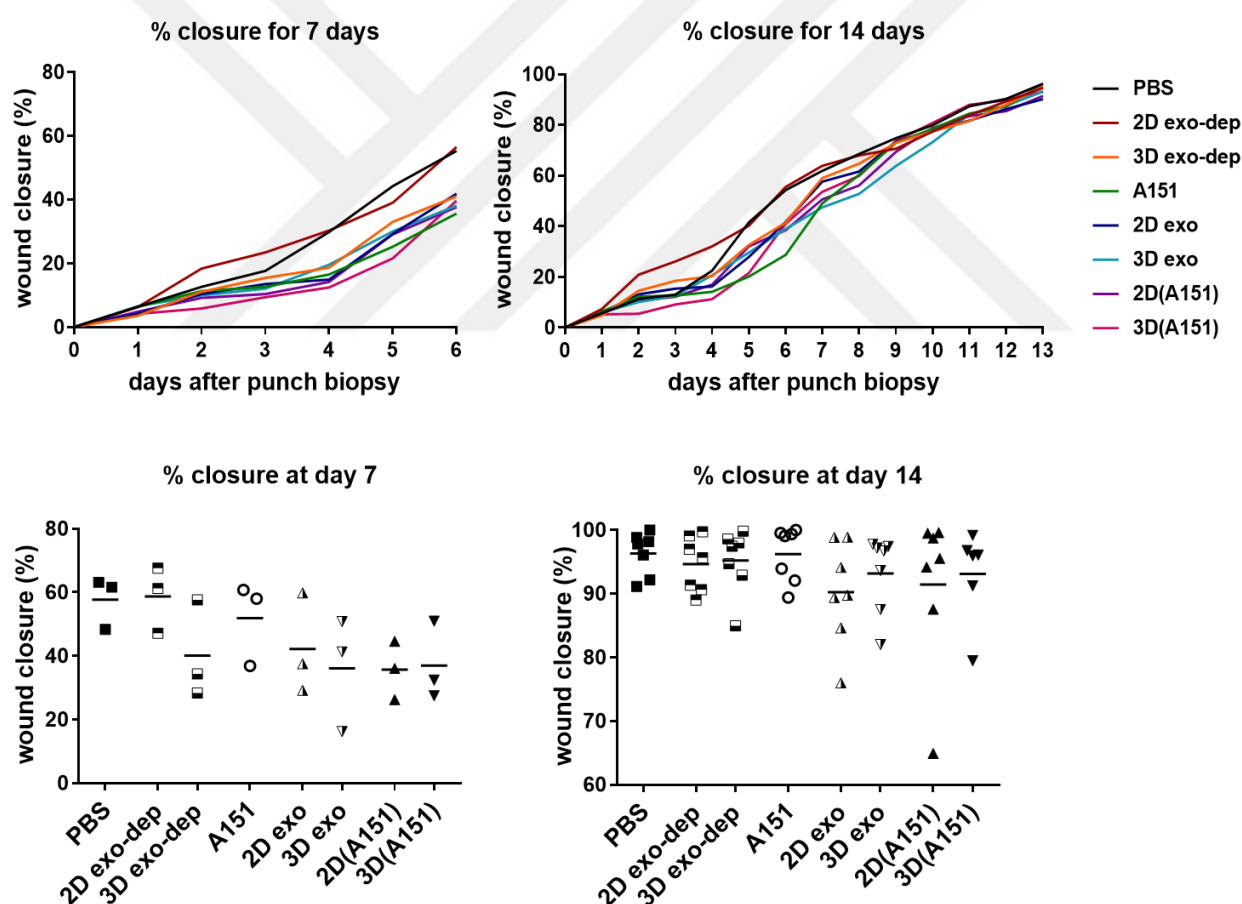


Figure 3.45. Effects of exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes and their A151 ODN-loaded forms on mice with excisional wounds. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D- or 3D-conditioned media, A151 ODN, free 2D- or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Wounds were measured daily. 3 animals per group were sacrificed at day 7, the remaining were sacrificed at day 14.

Wound closures are shown as the mean of each group. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome.

At day 7, free or A151 ODN-loaded exosome treatments appeared to have caused higher IL6 levels in circulation although the differences compared to PBS-treated group were not statistically significant except for 3D-exosome-treated group due to low sample size (Figure 3.46, upper left panel). $\text{TNF}\alpha$ levels were only significantly elevated by treatment with A151 ODN-loaded 3D-exosomes (Figure 3.46, upper middle panel). All treatments besides exosome-depleted CMs lowered IL10 levels (Figure 3.46, upper right panel). In general, the immunomodulatory treatments appeared to have delayed the resolution of the early inflammatory phase of wound healing. Interestingly, when cytokine levels at day 14 were measured, the animals treated with A151 ODN-loaded 3D-exosomes had significantly lower levels of IL6 and $\text{TNF}\alpha$ but not IL10 (Figure 3.46, lower panels).

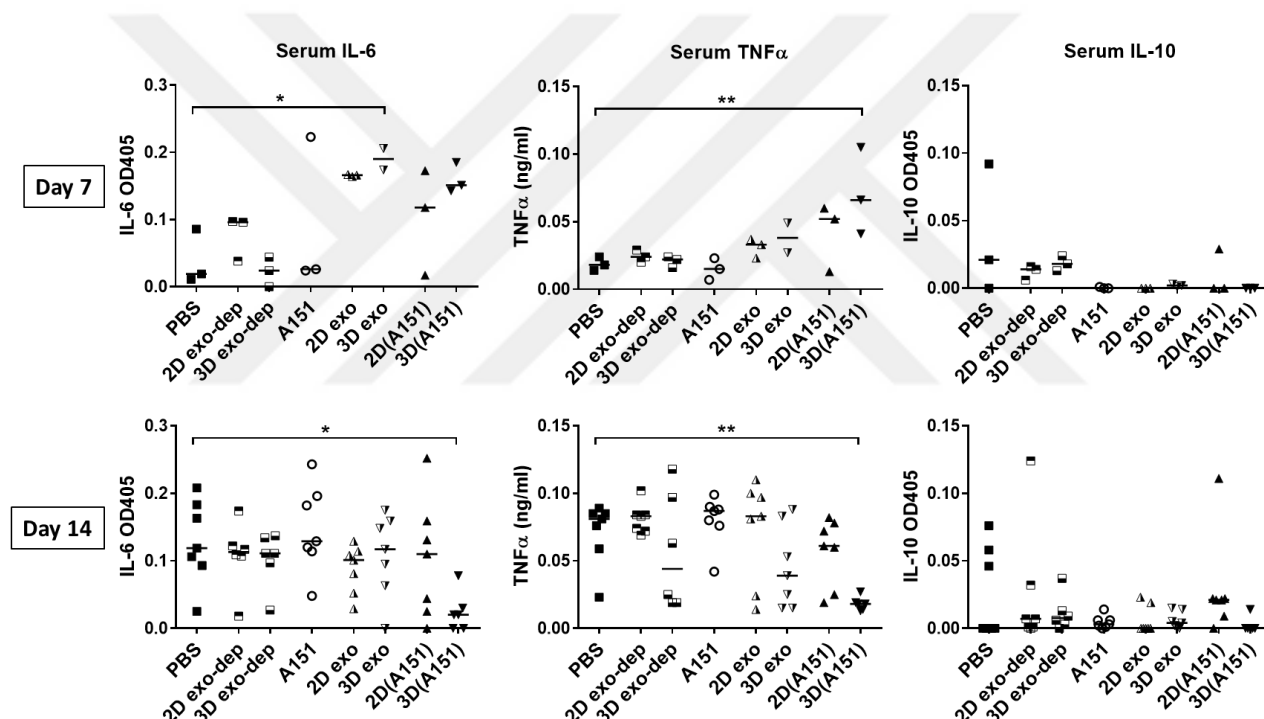


Figure 3.46. Serum IL6, $\text{TNF}\alpha$ and IL10 levels of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 $\mu\text{g}/\text{mouse}$ exosome-depleted 2D- or 3D-conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. At day 7, 3 animals per group were sacrificed while the remaining were sacrificed at day 14. Blood was collected through cardiac puncture and serum cytokine levels were determined with ELISA. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome. * $p \leq 0.05$, ** $p \leq 0.01$.

Furthermore, single cells from wound tissues were isolated after sacrifice as described in Section 2.2.1.9. Presence of neutrophils, macrophages and MDSCs along with inflammatory and anti-inflammatory

macrophage markers such as MHC-II, CD206 and PD-L1 were investigated. Unfortunately, no meaningful change was observed among groups regarding the parameters analyzed. Results of the skin cell stainings are provided in Figures A6 and A7 in Appendix A.

Splenocytes taken from the animals were also stained and investigated with flow cytometry to see any systemic effect caused by the treatments. Mostly due to low sample size of day 7 samples, no noteworthy result was obtained. Splenocyte stainings of day 7 samples are provided in Figure A8 of Appendix A.

At day 14, A151 ODN, exosome and A151 ODN-loaded exosome treatments caused slight elevation in macrophage numbers in the spleens but these were not found statistically significant (Figure 3.47, upper left panel). While M-MDSC levels always stayed the same, 3D-exosomes significantly elevated G-MDSC levels (Figure 3.47, upper middle and right panels). Even though macrophage population was not reduced, all free and A151 ODN-loaded exosomes significantly decreased MHC-II surface expression in macrophages (Figure 3.47, middle row, left panel). There was no change in the M2 markers CD206 and PD-L1 (Figure 3.47, middle row, center and right panels). Once again, this indicated that exosomes exerted their systemic effects through MDSC induction and reduction of antigen presentation capacity of macrophages rather than promoting M2 polarization. Furthermore, there was a striking decrease in the CD4⁺ T-cell populations in animals treated with A151 ODN or A151 ODN-loaded 3D-exosomes whereas CD8⁺ T-cells were unaffected (Figure 3.47, lower panels). Since Th1, Th2 and Th17 sub-populations of CD4⁺ cells were not individually investigated, it was not clear which inflammatory T-cell type or types A151 ODN and A151 ODN-loaded 3D-exosomes suppressed.

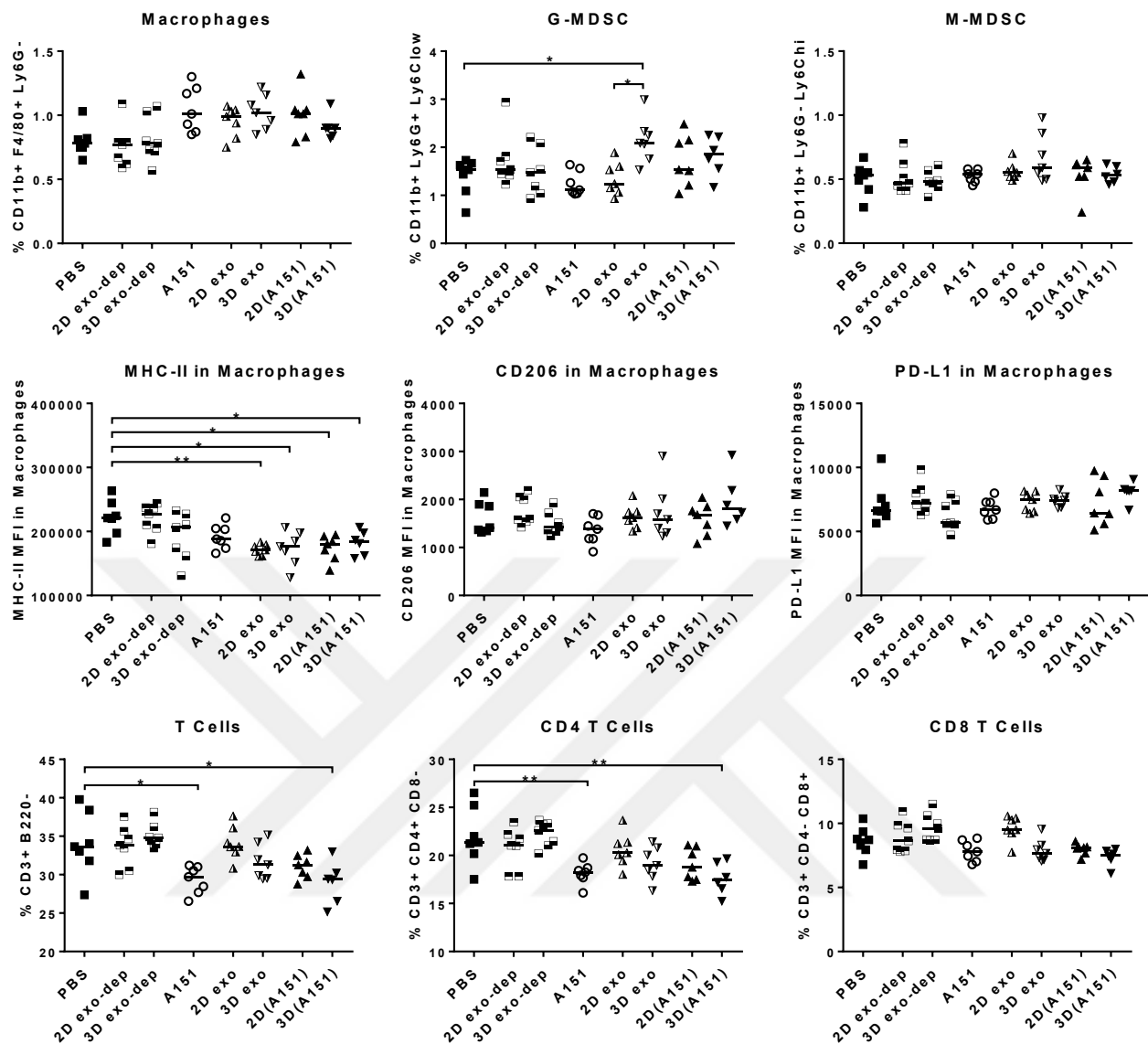


Figure 3.47. Changes in macrophage, MDSC and T-cell populations at day 14 in spleens of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Animals were sacrificed at day 14, spleens were taken out and cell populations were analyzed via flow cytometry. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell. * $p \leq 0.05$, ** $p \leq 0.01$.

To conclude, even though A151 ODN, free UCX[®] exosomes or A151 ODN-loaded exosomes did not notably accelerate wound closure, their subcutaneous injections caused significant systemic immunosuppression at late stages of wound healing. These immunosuppressive outcomes caused by free or A151 ODN-loaded exosome treatments included reduced inflammatory capacity of macrophages and higher G-MDSC numbers. However, among the treatments, only A151 ODN-loaded 3D-exosomes

were also able to reduce pro-inflammatory cytokine levels in circulation and T-lymphocyte numbers. Histological analyses of the wounds obtained from this study are still underway to observe the extent of tissue regeneration.



Chapter 4

Discussion

Being able to modulate the immune system is of critical importance in cases where exuberant immune response leads to pathological conditions such as autoimmune diseases, inflammatory diseases, chronic non-healing wounds or transplant rejection. Apart from cytokine-specific or receptor-specific antibodies in clinical use as immunosuppressants, non-specific and broad-acting inhibitors are immensely valuable for severe clinical cases. Broad-acting immunosuppressive drugs frequently used include corticosteroids which leads to inhibition of transcription factors such as NF- κ B and AP-1 thereby broadly blocking pro-inflammatory cytokine production [289], JAK inhibitors such as Tofacitinib blocking the signaling of receptors for interleukins 2, 4, 7, 9, 15, and 21 [290], and mTOR inhibitors Sirolimus and Everolimus which mediate suppression by their anti-proliferative actions and also by metabolically reprogramming innate and adaptive immune cells towards immunologically less active states [291, 292].

Another approach in wide-ranged immune modulation is immunosuppressive oligodeoxynucleotides. The archetypal example of that is A151 ODN which has been investigated by our group and many others since it was reported in 2003 by Gursel et al. [204]. A151 ODN contains a repetitive TTAGGG motif mimicking the telomeric repeats of the mammalian genome. This host-derived molecule was first reported to block bacterial CpG DNA-induced TLR9 activation and A151 ODN's regulatory function was found to be correlated with the sequence's ability to form G-tetrads. Later studies revealed a plethora of immune processes downregulated by A151 ODN (Figure 1.5). Inhibition of STATs 1,3 and 4 by A151 ODN results in downregulation of Th1-biased differentiation and cytokine production alongside promotion of Treg generation [205, 207]. A151 ODN also acts on T-cells by blocking their proliferation, differentiation to plasma cells and subsequent antibody production [209]. Moreover, A151 ODN inhibits cytosolic DNA sensor AIM2 and cGAS [212, 213]. Although some cellular actions of A151 ODN such as TLR9 and cGAS inhibition are sequence-specific, a recent study by our group revealed that one of the underlying mechanisms behind the far-reaching suppressive effects of A151 ODN, if not the only mechanism, is metabolic reprogramming mediated by inhibition of PI3K/Akt/mTOR axis (Yazar et al., under revision for publication). Many elements of the PI3K/Akt/mTOR pathway are transcriptionally downregulated upon A151 ODN exposure. Phosphorylation of mTOR is also inhibited by A151 ODN in macrophages and T-cells, although studies on T-cells are also underway. This study also revealed that A151 ODN decreases cells' capacities of both key energy production cascades, namely glycolysis and oxidative phosphorylation. Therefore, we now hypothesize that A151 ODN results in wide-range suppression of immune functions by metabolically switching immune cells to an inert or anergic state.

In preclinical studies, A151 ODN proved to be a promising therapeutic agent for many autoimmune and inflammatory disorders including glomerulonephritis [214], rheumatoid arthritis [215, 216], uveitis [217, 218], atopic dermatitis [219] and atherosclerosis [221]. A151 ODN was also found to be protective against endotoxic shock [206], fungal infections [208], infection-induced or chemically-induced gut inflammation [220], ischemic brain injury [222], and inflammation induced cancers [223, 224]. Due to these broad immunosuppressive capacities and therapeutic potentials of A151 ODN, it was one of the two agents we utilized to achieve immunomodulation in this study.

Another foundation of this study was mesenchymal stromal/stem cell (MSC)-mediated immunosuppression. Apart from their well-studied functions that promotes tissue repair and regeneration, MSCs regulates both innate and adaptive arms of the immune system in various ways (Figures 1.2 and 1.3). They are able to produce pro-inflammatory or anti-inflammatory cytokines, chemokines and enzymes depending on the inflammatory signals present in that environment [71]. On one hand, the regulatory actions of MSCs on innate immunity includes complement inhibition [75], mast cell inhibition [79, 80], blocking of NK proliferation and cytotoxicity [81], inhibiting the maturation and decreasing the antigen presentation capacities of DCs [85], and promoting the alternative M2 activation of macrophages [90, 91, 94]. On the other hand, they are able to promote inflammation during the early phases of wound healing by recruiting neutrophils, monocytes and macrophages to wound sites for microbial clearance and effective healing [76, 78]. In terms of adaptive immunity, MSCs inhibit T-cell proliferation [98], induce their apoptosis [99], inhibit Th1 and Th17 differentiation and promote Treg generation [101], prevent T-cell proliferation and antibody production [105] while supporting IL10-producing Breg generation [109].

In this study, we utilized a particular MSC variety termed UCX[®]. Unlike other MSCs, they are stable up to 50 passages without losing their self-renewal and differentiation capacities [61, 62]. Therefore, UCX[®] are more feasible for reproducible studies and eventual clinical use. UCX[®] were found to be less immunogenic and more immunosuppressive than bone marrow-derived MSCs which are considered as the gold-standard for MSC therapies. They express higher amounts of immunomodulatory proteins such as PD-L1, PD-L2, LIF and TGF β compared to BM-MSCs [62]. Culturing UCX[®] in 3-dimensional (3D) cultures alter their secretome. 3D-cultured UCX[®] secrete higher amounts of VEGF α , HGF, TGF β 1, FGF2 and G-CSF which promote angiogenesis and tissue remodeling [69]. They also secrete more of anti-inflammatory proteins LIF and IL10 but less of IL6, IL21 and chemokine CCL2 compared to 2D-cultured cells. Conditioned media of 3D UCX[®] culture (CM3D) was recently reported to be an effective anti-inflammatory treatment for rheumatoid arthritis [70]. Moreover, CM3D promoted more complete tissue regeneration in a rat excisional wound model. For these reasons, we chose to employ UCX[®] cells for our immunomodulatory applications [69].

Most of the immunomodulatory effects of MSCs are dependent on paracrine factors consisting of soluble proteins and extracellular vesicles [173]. Extracellular vesicles, including microvesicles and exosomes, are small vesicles secreted through the endosomal pathway or by direct budding of the plasma membrane. They are found in every type of bodily fluid and carry out critical physiological roles in health and disease [126]. Their unique ability to carry parts of the host cell's lipid, protein and nucleic acid content gives them functional capabilities. They are also able to carry their cargo over long distances in the body without being subject to degradation. Therefore, extracellular vesicles are intriguing candidates for cell-free therapeutics and drug delivery applications. Exosomes secreted by MSCs are being tested in pre-clinical and clinical settings due to their superior safety, low immunogenicity and tumorigenicity compared to MSC-based therapies and their ability to mimic the parent cell's functions in terms of regeneration and immunomodulation [293]. Therapeutic potentials of MSC exosomes were revealed with mouse models of myocardial ischemia/reperfusion injury [175, 177], pulmonary hypertension [181], stroke [182, 183], hypoxic-ischemic brain injury [184], liver fibrosis and injury [187, 188], nephrotoxicity [190, 191], and osteoarthritis [192, 194]. They are also being tested in clinical studies for the treatment of type 1 diabetes, acute ischemic stroke, bronchopulmonary dysplasia and macular holes (Table 1.1, ClinicalTrials.gov). In a one patient study, MSC exosomes had remarkable downgrading effects on graft-versus-host disease [294]. Exosomes of various types of MSCs including BM-MSCs, adipose-derived and ESC-derived MSCs were reported to display immunosuppressive activities by supporting the anti-inflammatory M2 phenotype of macrophages *in vitro* and *in vivo* [295-297].

Although no reports exist regarding the exosomes of UCX[®], we hypothesized that they would display potent immunomodulatory and regenerative activities mimicking the known effects of their parent cells. We also wanted to compare the exosomes isolated from 2D- and 3D-cultures in terms of protein cargo and functionality. Moreover, we hypothesized that combining UCX[®] exosomes and A151 ODN through direct loading of A151 ODN into exosomes would result in greater immunomodulatory ability. *In vitro* experiments presented in this study focused on TLR signaling and macrophage polarization, but studies with DCs and T-cells are currently underway. Both the MSC literature and A151 ODN's known abilities made us confident that UCX[®] exosomes combined with A151 ODN could be utilized as potent modulators of innate and adaptive immunity.

Prior to conducting any *in vitro* or *in vivo* experiments, we wanted to confirm that the UCX[®] exosomes fit the criteria as exosomes suitable for clinical use and that the A151 ODN loading procedure does not alter their cardinal features. DLS study revealed that 2D-exosomes are smaller than 3D-exosomes and A151 ODN loading increases the sizes of both (Figure 3.1). Of note, before and after A151 ODN loading, 3D-exosomes had lower polydispersity index (PDI) compared to 2D-exosomes. Lower PDI shows that 3D culture-derived exosomes are more uniform and they are more suitable to use as a carrier system in clinic [265]. Flow cytometric analyses proved that both exosome types carry the cardinal

exosome markers CD9 and CD81 whose presence were not disrupted by A151 ODN loading (Figure 3.3). Proteomic studies conducted by our collaborators (data not shown) indicated that UCX[®] exosomes carry several immunomodulatory proteins. Herein we validated the presence of 2 of them: T-cell inhibitory molecule PD-L1 and complement inhibitor CD55 (Figure 3.4). This data suggests that UCX[®] exosomes might have T-cell inhibitory capacities through direct binding of PD-L1 to the PD-1 receptors on T-cells, and that they could also be utilized to control complement cascade.

The next step in characterization was to investigate if UCX[®] exosomes can bind to or be internalized by different immune cell types. Although the exosomes are of human origin, they could efficiently be internalized and/or bound to mouse and human immune cell types including monocytes, macrophages, DCs, T-cells and T-cells (Figures 3.5 and 3.7). T-lymphocytes are not phagocytic cells that would internalize exosomes with high efficiency, however, they might be directly binding to them through receptor-ligand interactions one of which could be the interaction of PD-1 with the PD-L1 on the exosome surface. Moreover, 3D-exosomes were faster and more efficient to interact with immune cells compared to 2D-exosomes (Figures 3.5 and 3.7). This might be one of the reasons why 3D-exosomes were more potent in most of the subsequent *in vitro* and *in vivo* experiments. If the cellular uptake and/or binding of exosomes are altered after A151 ODN-loading was not investigated in the scope of this study but it would be valuable to show that the loading procedure does not adversely affect uptake and/or binding.

Currently there is no method for direct loading of bioactive agents within exosomes with near-perfect efficiency. Methods in use include feeding the host cell with the desired molecule so that they later release it in exosomes, manipulating the sorting of desired proteins or RNAs in the host cell by incorporating certain motifs to trigger the sorting machinery, expression of a fusion protein consisting of the protein of interest and a well-known exosomal protein, direct mixing with exosomes in case of hydrophobic molecules, and electroporation which is the most direct way but has low efficiency [171, 172]. A novel and direct exosome loading method developed by our group (Section 2.2.6) allows easy and efficient loading of bioactive molecules into exosomes without the need to manipulate the parent cell. With this method, we were able to load A151 ODN into UCX[®] exosomes with near-perfect efficiency (~95%) which is yet unprecedented in the exosome field (Figure 3.10).

Toll-like receptors (TLRs) are known to modulate MSC function. Expression of TLRs varies depending on the MSC source and can be upregulated in conditions such as hypoxia, infection and presence of inflammatory cytokines in the environment [298]. MSCs encounter TLR ligands at sites of injury or infection and TLR signaling affects the differentiation, proliferation, migration and immunomodulatory capacities of MSCs. For instance, TLR-3 priming of MSCs is known to increase the production of immunomodulatory molecules IDO and PGE2 [71]. Although the role of TLR signaling in MSCs have been heavily investigated, how MSCs or their exosomes regulate TLR signaling in immune cells is an

open question. We investigated the effects of UCX[®] exosomes with or without A151 ODN on TLR signaling in mouse splenocytes and BMDMs.

In BMDMs, both 2D- and 3D-exosomes efficiently suppressed TLR7/8- and TLR9-induced IL6 and IL12, and TLR1/2-induced IL12 secretions (Figures 3.17 and 3.18). On TLR4, 3D-exosomes were more suppressive in terms of IL6 and IL12 production. In most of the cases, A151 ODN loading improved the inhibition of pro-inflammatory cytokines. Direct mixing of A151 ODN with exosomes was always ineffective or less effective compared to loading in terms of generating a synergistic inhibition. Contrary to IL6 and IL12, TNF α levels were not dramatically reduced upon exosome treatment and were not improved a lot with A151 ODN loading since A151 ODN itself cannot efficiently suppress TNF α in macrophages (Figure 3.19). A very interesting observation was that the exosomes, especially 2D-exosomes, potentiated TLR3 activation and caused more cytokine production (Figures 3.17 - 3.19). Since UCX[®] cells express high amounts of TLR3 (Figure A2, Appendix A), it might be possible that TLR3 is also incorporated to the exosome membranes and the exosomes help deliver bound poly(I:C) or unbound TLR3 molecules to the target cells thereby potentiating the stimulation. Presence of TLR3 on exosomes should be validated in order to further pursue this hypothesis. When the expressions of co stimulatory molecules CD80, CD86 and the antigen-presenting molecule MHC-II were investigated in BMDMs, 2D- and 3D-exosomes showed differential suppressive capacity upon activation of BMDMs via TLR signaling (Figures 3.21 – 3.25). Overall, 3D-exosomes were more potent inhibitors of the activation markers than 2D-exosomes. Effects on cytokine secretion profiles and activation markers were not always correlated. For instance, synergism between exosomes and TLR3 was not apparent in terms of activation markers. Rather, the exosomes inhibited their expressions (Figure 3.22). Also, A151 ODN loading did not improve the downregulation of these markers while it could potentiate pro-inflammatory cytokine suppression (Figures 3.21 – 3.25).

Splenocyte population contains all types of myeloid and lymphoid immune cells therefore provides a general view of the immunomodulatory actions of what is being tested. Unlike the case in BMDMs, exosomes were not able to decrease IL12 levels except for TLR9-induced IL12 (Figure 3.15). Similar to BMDMs, TNF α was the least suppressed cytokine in splenocytes (Figure 3.16). Inhibition of TLR9 was always A151 ODN-dependent and could be achieved with A151 ODN alone or A151 ODN-loaded forms of exosomes (Figures 3.13 - 3.16). 3D-exosomes were more potent in inhibiting TLR1/2- and TLR4-induced IL6 secretion while 2D-exosomes better inhibited TLR7/8-induced IL6 production (Figure 3.14). Both exosomes could potentially decrease IFN γ produced upon TLR1/2 and TLR7/8 activation while only 3D-exosomes could do so upon TLR4 activation (Figure 3.13). Suppressive abilities of exosomes on TLR1/2, TLR4 and TLR7/8 in terms of IFN γ and IL6 production were consistently improved by A151 ODN loading (Figures 3.13 and 3.14). Direct addition of A151 ODN was generally ineffective or less effective compared to loading. Synergism of 2D-exosomes and to a

lesser extent 3D-exosome with TLR3 was also observed in splenocytes (Figures 3.13 - 3.16). However, unlike the case in BMDMs, A151 ODN loading was able to hinder this synergy.

When TLR studies are taken together, we have seen that 2D- and 3D-exosomes have differential capacities of inhibiting pro-inflammatory cytokine production and the expression of activation markers in macrophages. Exosomes and A151 ODN synergize to achieve more potent and broad-ranged suppression of TLR signaling pathways in terms of pro-inflammatory cytokine production from splenocytes and BMDMs but not the antigen-presentation capacities. These data also proved that loading of A151 ODN into exosomes is a much more efficient way to achieve synergy compared to direct mixing of A151 ODN with the exosomes. The discrepancies between BMDM and splenocyte results are attributable to cell types present in the splenocyte mixture other than macrophages. To clarify the distinct effects of UCX[®] exosomes on each immune cell type, further *in vitro* studies using isolated immune cells are required. Lastly, the inability of exosomes to inhibit TLR3 signaling while they are suppressive on TLR1/2, TLR4, TLR7/8 and TLR9 signaling might be attributable to the fact that TLR3 utilizes TRIF as the adaptor protein for the initiation of signaling events while all the other TLRs mainly utilize MyD88 adaptor protein [299]. Intracellular targets of the exosomes must be identified to have a clear picture of how the inhibitory effects are achieved.

Even though UCX[®] exosomes acted suppressively in the presence of an insult such as during active inflammation, intriguingly, they led to IFN γ , IL6, IL12 and TNF α production from splenocytes and BMDMs (Figures 3.13 – 3.19) as well as upregulation of activation markers in BMDMs (Figure 3.20) when there are no inflammatory signal. This finding is not entirely counterintuitive considering the literature suggesting that MSCs constitutively produce some pro-inflammatory cytokines including IL6 and that they acquire suppressive abilities when there is sufficient inflammatory stimulus in the environment such as pro-inflammatory cytokines TNF α and IFN γ or TLR ligands [21]. Therefore, this observation does not undermine the goal of therapeutically utilizing MSC exosomes in inflammatory and autoimmune conditions where they would exert suppressive actions. Of note, A151 ODN loading completely or partially limited the pro-inflammatory effects of exosomes in most cases. Therefore, we can suggest that A151 ODN would improve the safety profile of the exosomes in clinical use. On the other hand, inflammation-inducing potential of UCX[®] exosomes could be valuable in certain cases to modulate the sensitive balance between healthy inflammation and excess inflammation. For instance, in the wound healing process, an initial inflammatory phase is essential for effective healing. However, as the amount of inflammatory stimuli in the environment increases, suppressive actions are required to resolve the inflammation so that tissue remodeling can take place [275]. Therefore, double-edged immune regulation observed with UCX[®] exosomes could potentially be advantageous in certain physiological conditions such as the wound healing process.

Our next *in vitro* set of experiments aimed to investigate if UCX[®] exosomes drive macrophage polarization towards the anti-inflammatory, pro-angiogenic and repair-promoting M2-macrophages rather than the pro-inflammatory M1 macrophages. MSCs of different origin have been shown to promote M2 polarization via different mechanisms [90-95]. Several recent reports also suggest that MSC exosomes are capable of promoting M2 polarization [295-297]. To this end, cytokine and gene signatures related to M1 and M2 states were investigated. Exosomes increased NO production from BMDMs in neutral, M1-polarizing and M2-polarizing conditions (Figure 3.30). 2D-exosomes also severely induced IL6, TNF α and concomitant IL10 production which could all be prevented by A151 ODN loading. Cytokine profile suggested that 2D but not 3D-exosomes might actually be promoting an M1-like state. However, qRT-PCR results exhibited a different situation. Nos2 coding for nitric oxide synthase 2 which is considered an M1 marker was upregulated by both types of exosomes (Figure 3.31). However, Arg1 coding for the enzyme arginase 1 which is considered an M2 marker was also upregulated by both exosomes. These changes were seen in neutral, M1-polarizing and M2-polarizing conditions. Exosomes were also able to upregulate PD-L1. Interestingly, Chil3 which is another M2 signature gene was not overexpressed by exosome treatment. The exosomes even inhibited its expression induced by M2-polarizing factors. The simultaneous overexpression of Nos2 and Arg1 but not Chil3 is characteristic of another anti-inflammatory myeloid cell type called myeloid-derived suppressive cells (MDSCs). Of note, A151 ODN loading into the exosomes seems to have limited the induction of this MDSC-like phenotype in macrophages (Figure 3.31). MDSCs are a heterogeneous population of immature myeloid cells with immunosuppressive function whose regular differentiation into mature granulocytes, monocytes, macrophages or DCs is disrupted [31]. They are particularly critical in establishing an immune-tolerant environment during cancer. Expansion of MDSCs is triggered by STAT3 signaling [34]. A151 ODN's limiting action on the MDSC profile is plausible considering that it is a known STAT3 inhibitor [205]. Arginase 1 and NO are the key players through which MDSCs interfere with the activation and proliferation of T- and NK-cells [35-37]. T-cell inhibition mechanisms by MDSCs also involve PD-L1 interaction with PD-1 of T-cells [38]. Apart from their ability to shift macrophages to an MDSC-like profile *in vitro* (Figure 4.1), UCX[®] exosomes caused expansion of both granulocytic and monocytic MDSC subtypes when intraperitoneally (IP) injected to mice (Figures 3.32 and 3.33). A151 ODN partially limited this ability but only for 2D-exosomes. Of note, 2D-exosomes but not 3D-exosomes also caused an increase in T-cell and macrophage populations upon IP injection (Figure 3.32). Once again, 3D-exosomes or the A151 ODN-loaded versions of 2D-exosomes appear to be safer for *in vivo* applications. Of interest, a study suggested that MSCs drive DCs to an MDSC-like phenotype through the secretion of GRO γ [300]. Another study reported that MSCs contribute to immune evasion in chronic myeloid leukemia by educating MDSCs to secrete higher amounts of Arginase 1, COX2 and IL6 thereby potentiating their immunosuppressive functions [301]. To summarize, shifting macrophages and possibly DCs to an MDSC-like state (Figure

4.1) and causing MDSC expansion and activation might be some of the immunomodulatory mechanisms of UCX[®] exosomes through which they could indirectly suppress T- and NK-cell functions *in vivo*.

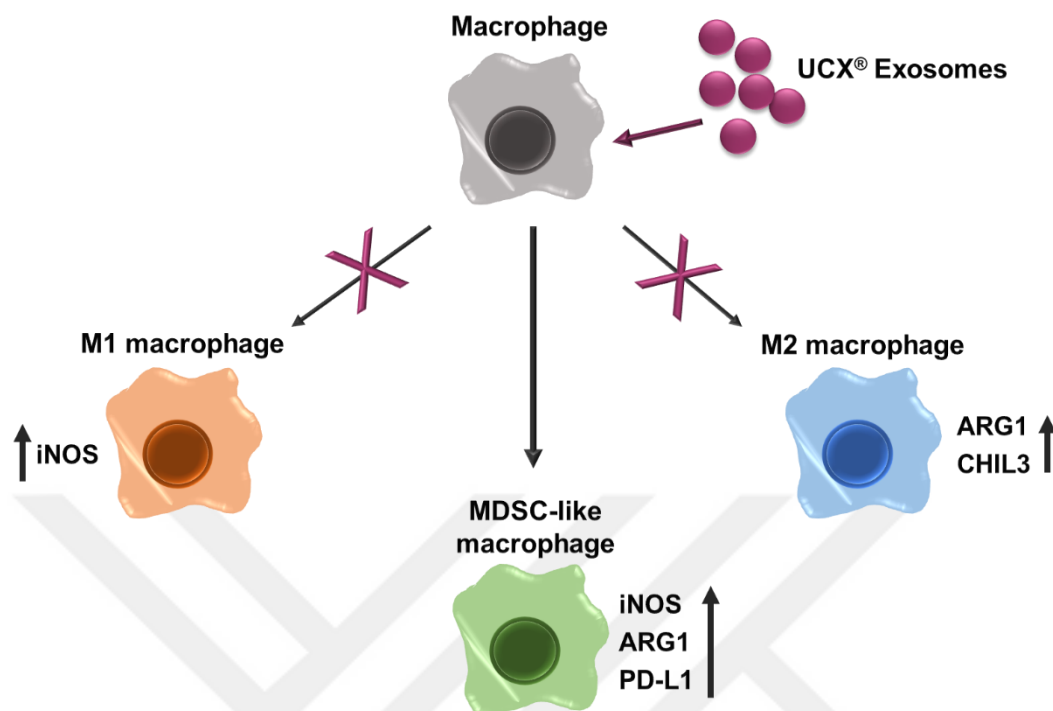


Figure 4.1. UCX exosomes promote an MDSC-like macrophage phenotype but not M1 or M2 differentiation of macrophages in vitro.

In vivo, we investigated the immunomodulatory potentials of UCX[®] exosomes with or without A151 ODN with a DSS-induced colitis model in mice. DSS is administered in the drinking water of mice for 4 days followed by 3 days of recovery period with fresh water. Increase in TNF α , IL6, IL17 and IL1 β levels, upregulation of TLRs 2 and 4, increased myeloperoxidase activity are all common inflammatory outcomes of DSS administration [302]. However, inflammation is an indirect outcome of DSS. DSS is toxic to epithelial cells and causes the disruption of epithelial barrier allowing bacteria and antigens to enter into the submucosa and mucosa causing inflammation [303]. Our setting exhibited the characteristic macroscopic outcomes of DSS-induced colitis which are weight loss, diarrhea and shortening of colons (Figure 3.34). Histological changes which included inflammatory cell infiltration, crypt damage and epithelial erosion in the colon tissue were also consistent with the literature (Figure 3.35). Unfortunately, contrary to the literature, DSS caused increased serum IL6 levels but not TNF α levels in our model (Figure 3.40). Some of the most significant outcomes of DSS observed in our model were reduced CD4⁺ and CD8⁺ T-cell numbers in mesenteric lymph nodes (Figure 3.36) and spleens (Figure 3.38) along with decreased ability of existing T-cells to produce IFN γ upon re-stimulation with PMA (Figure 3.41). DSS-induced colitis is known as a lymphocyte-independent model because T and T-cell-deficient animals are able to develop the disease [234]. Even though there is no report of a mice or rat study supporting the T-cell-related changes that we observed, T-cells of human patients with

ulcerative colitis are known to undergo activation-induced apoptosis and to have limited expansion capacity [304]. Furthermore, DSS caused reduced MHC-II expression in macrophages and DCs in spleens (Figure 3.39). This might be due to the migration of cells with higher inflammatory capacity from secondary lymphoid organs like spleen to the injury site. MHC-II decrease in splenic antigen-presenting cell types has not been reported for DSS-induced colitis but circulating monocytes of human patients with ulcerative colitis have lower MHC-II expression and it correlates with disease severity [305]. In these respects, our colitis model appropriately replicates the relative human disease condition.

IP injection of 3D-exosomes, A151 ODN-loaded 2D and 3D-exosomes, and to a lesser extent A151 ODN prevented DSS-induced weight loss and colon shortening (Figure 3.34) while also reducing the severity of inflammation, incidence of crypt damage and epithelial erosion in colon tissue even though no treatment could abolish inflammation and epithelial erosion completely (Figure 3.35). Remarkable protection of the colon tissue by free or A151 ODN-loaded exosomes might not be due to only anti-inflammatory effects on immune system, we envision that UCX exosomes probably, facilitates proliferative and regenerative program particularly at the crypt area thereby restoring DSS-induced colon injuries. Further studies are designed to investigate these options. Unloaded 3D-exosomes, A151 ODN-loaded 2D- and 3D-exosomes, and to a lesser extent A151 ODN itself also helped to recover the DSS-induced T-cell loss in mesenteric lymph nodes (Figure 3.36) and spleens (Figure 3.38). 2D-exosomes were also able to prevent T-cell loss in spleens. Moreover, reduced MHC-II expression in splenic macrophages and DCs caused by DSS could only be restored by A151 ODN-loaded 3D-exosomes (Figure 3.39). However, in lymph nodes, all treatments tended to decrease macrophage numbers increased by DSS but the differences were not enough to be statistically significant (Figure 3.37). Cellular changes related to the inhibition of colitis progression by free or A151 ODN-loaded exosomes did not include elevation of Treg or MDSC numbers (Figures 3.36, 3.38 and 3.39). When splenocytes of mice were stimulated *in vitro* with T-cell activators PMA and ionomycin, TLR4 ligand LPS, TLR7/8 ligand R848 or TLR9 ligand K23 CpG ODN, cells of DSS-exposed animals were less capable of producing IFN γ , IL6 and TNF α (Figures 3.41 – 3.43) probably due to exhaustion or anergy. Cytokine production upon TLR stimulation was only similar to the healthy levels in animals treated with A151 ODN-loaded 3D-exosomes and by A151 ODN itself to a lesser extent. Of note, A151 ODN could also induce significantly higher amounts of anti-inflammatory cytokine IL10 from stimulated splenocytes (Figure 3.44). T-cell dependent IFN γ and TNF α production triggered by PMA and ionomycin could be restored by all treatments except for 2D-exosomes (Figures 3.41 and 3.43). Overall, these results revealed that A151 ODN-loaded 2D-exosomes, A151 ODN-loaded 3D-exosomes, free 3D-exosomes but not free 2D-exosomes could be effective treatment options for ulcerative colitis. Although A151 ODN was also able to partly prevent some of the pathogenic outcomes, its combination with the UCX[®] exosomes, particularly with 3D-exosomes, appear to be a more promising treatment approach.

DSS-related pathologies that A151 ODN-loaded UCX[®] exosomes were able to prevent are summarized in Figure 4.2.

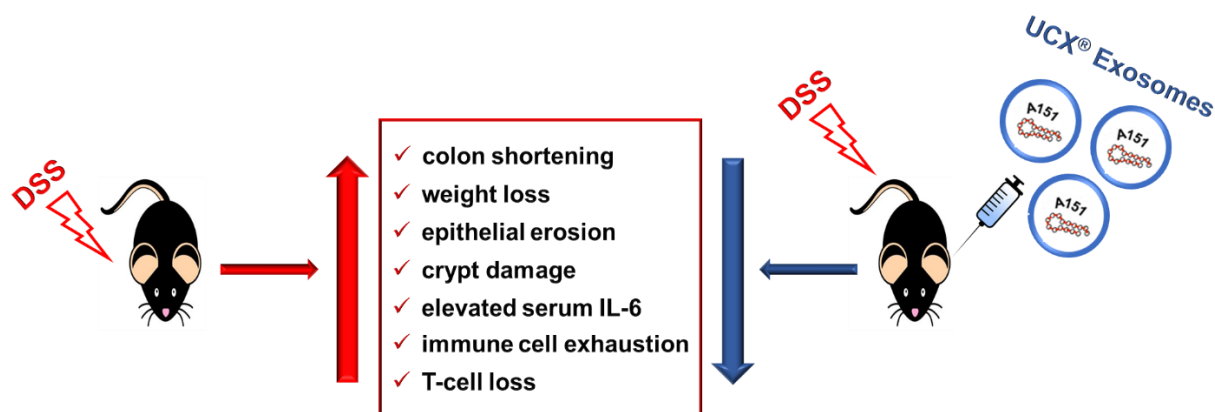


Figure 4.2. A151 ODN-loaded UCX exosomes restore DSS-induced pathologies in mice.

UCX[®] have been shown to produce molecules involved in various phases of wound healing including TGF β , IL6, VEGF α , KGF, EGF and FGF2. They also promote proliferation and migration of fibroblasts and keratinocytes which are the major skin cells contributing to wound healing [65]. Moreover, conditioned media from 3D cultured UCX[®] led to faster wound closure and more complete tissue regeneration [69]. Considering these, we hypothesized that UCX[®] exosomes might have impacts on fibroblasts and keratinocytes mimicking their parent cells. Since conditioned media involves exosomes along with soluble factors, we also wanted to test the potential of exosomes to improve wound healing. *In vitro* experiments with fibroblast cell line NIH3T3 revealed that 2D- and 3D-exosomes differentially induce IL10, TGF β and IL1 α production while they both drastically increase TNF α and VEGF α (Figures 3.26 and 3.27). These induced molecules act in different phases of healthy wound healing such as initial inflammation, later re-epithelization, angiogenesis and matrix production. A151 ODN loading limited or abolished the mentioned effects of the exosomes. The only improvement achieved by A151 loading was the increase in CCL2 levels (Figure 3.27). CCL2 drives macrophage infiltration to the wound and its deficiency is related to chronic wounds [277]. In the keratinocyte cell line HaCaT, exosomes but most potently 3D-exosomes induced more TGF β , IL1 α and TNF α production which were also hindered by A151 ODN loading (Figures 3.28 and 3.29). These results suggested that exosomes without A151 ODN could improve wound healing through their effects on fibroblasts and keratinocytes. Unfortunately, in our *in vivo* excisional wound model, wound closure was not significantly different between PBS-treated animals and animals treated with A151 ODN, exosome-depleted 2D- or 3D-conditioned media, 2D- or 3D-exosomes, or A151 ODN-loaded 2D- or 3D-exosomes (Figure 3.45). In the first 7 days, the treatments except for exosome-depleted media actually delayed wound closure but later the differences disappeared and all wounds were almost closed on day 14. The treatments appear to have delayed the resolution of the early inflammatory phase of wound healing evident by the elevated inflammatory cytokine levels at day 7 (Figure 3.46). However, when cytokine levels at day 14 were

measured, the animals treated with A151 ODN-loaded 3D-exosomes had significantly lower levels of serum IL6 and TNF α . There was also systemic suppression in cellular level. All free and A151 ODN-loaded exosomes decreased MHC-II expression, hence the inflammatory capacities of macrophages, in the spleens of animals (Figure 3.47). 3D-exosomes also elevated the granulocytic MDSC population while A151 ODN and A151 ODN-loaded 3D-exosomes decreased CD4⁺ T-cell numbers. Overall, these results imply that UCX[®] exosomes or the exosome-depleted media are not by themselves enough to significantly improve wound healing in contrast to the whole conditioned media. However subcutaneous injection of A151 ODN-loaded 3D-exosomes in particular can cause systemic immunosuppression at the late stages of wound healing which may help prevent the development of non-healing chronic wounds. Nonetheless, histological analyses of the wounds are required to clearly observe if the extent of tissue regeneration was affected by the treatments.

Undergoing and future experimental plans to further investigate the immunomodulatory actions of UCX[®] exosomes include *in vitro* experiments with DCs, T-cells and T-cells observing their proliferation, maturation, activation, cytokine and antibody production. Presence of TLRs on the exosomes should also be investigated mainly to reveal the underlying reason of their synergism with TLR3 ligand poly(I:C). Exosomes, exosome-depleted media and the whole conditioned media of 2D and 3D UCX[®] were subjected to mass spectrometry analyses by our collaborators. Initial findings indicate that all 3D culture-derived sample types contain higher protein amounts compared to their 2D-culture-derived counterparts (Figure A9, Appendix A). This confirms our rational that 3D-culture is more complex and closer to *in vivo* setting. 3D-exosomes contain 570 different proteins while the number is 415 for 2D-exosomes. Also, there are 16 proteins unique to 3D-exosomes and 4 unique to 2D-exosomes. The identities and functions of these proteins will be carefully investigated since they are likely the underlying causes of differential functions of these exosomes. Conditioned medium of 3D UCX[®] culture was recently reported to alleviate rheumatoid arthritis [70]. We plan to test the therapeutic efficiency of UCX[®] exosomes with and without A151 ODN in a mouse model of adjuvant-induced rheumatoid arthritis.

Taken together, this study revealed that 2D- and 3D-culturing of cells result in critical functional differences of the secreted exosomes, 3D culture-derived exosomes appear less immunogenic therefore safer for clinical use while also displaying higher immunosuppressive potential, A151 ODN-loading into exosomes reduce immunogenicity and improves immunosuppressive capacity. We propose A151 ODN-loaded UCX[®] exosomes, particularly 3D culture-derived exosomes, as a valuable cell-free therapeutic agent that should be investigated further and proceeded to human trials for the treatment of inflammatory and autoimmune disorders.

Appendices

Appendix A

Additional Figures

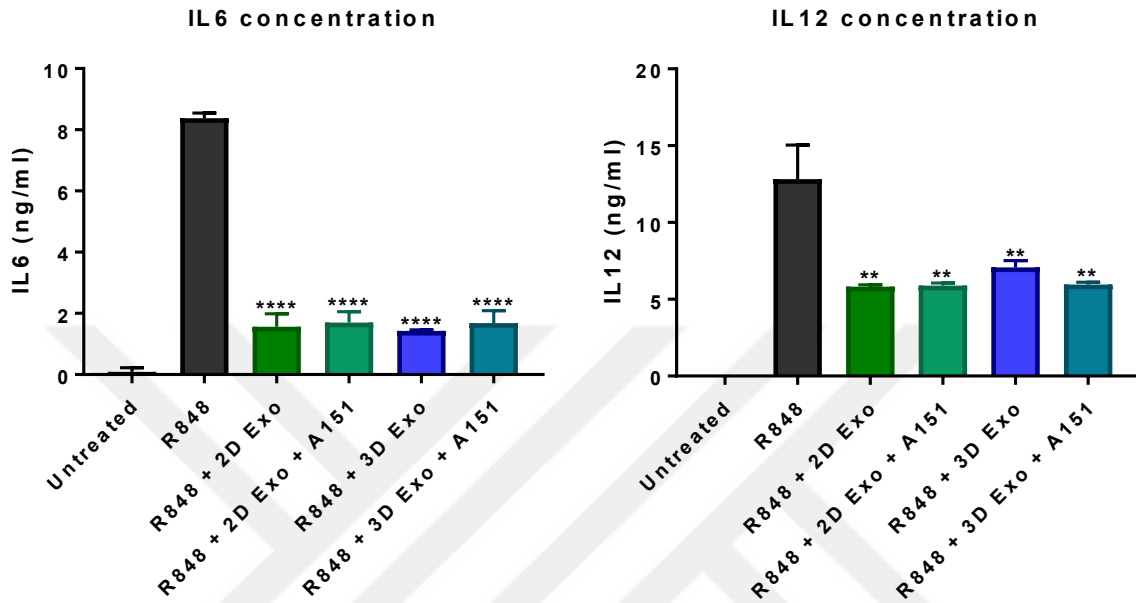


Figure A1. Effects of 2D- and 3D- UCX exosomes and their free mixtures with A151 ODN on IL6 and IL12 secretions from mouse BMDMs upon stimulation with TLR7/8 ligand R848. BMDMs were stimulated with 1 µg/ml R848 and treated with 20 µg/ml exosomes with or without 1.5 µM A151 ODN for 24 hours. Cytokine levels from culture supernatants were determined with ELISA. Stars indicate the differences compared to the R848-only group. Exo: Exosome, Exo+A151: mixtures of 3D-exosome and A151 ODN. ** $p \leq 0.01$, **** $p \leq 0.0001$.

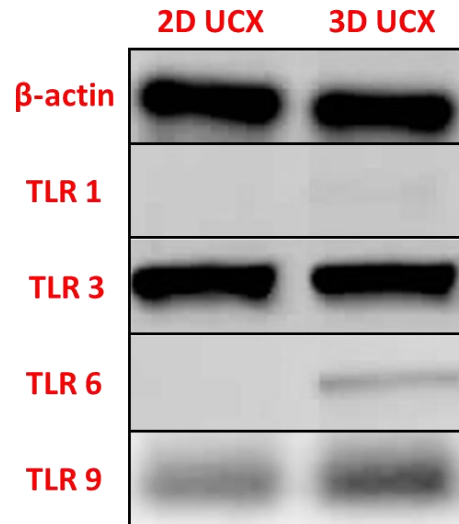


Figure A2. Agarose gel electrophoresis results showing the TLRs present on 2D- or 3D-cultured UCX cells. cDNAs generated from mRNAs isolated from 2D- or 3D-cultured UCX cells were amplified with polymerase chain reaction and separated in 1.5% agarose gel. All 10 human TLRs were tested. Only the TLRs for which amplification bands were visible are displayed. 2D: 2-dimensional, 3D: 3-dimensional, TLR: Toll-like receptor.

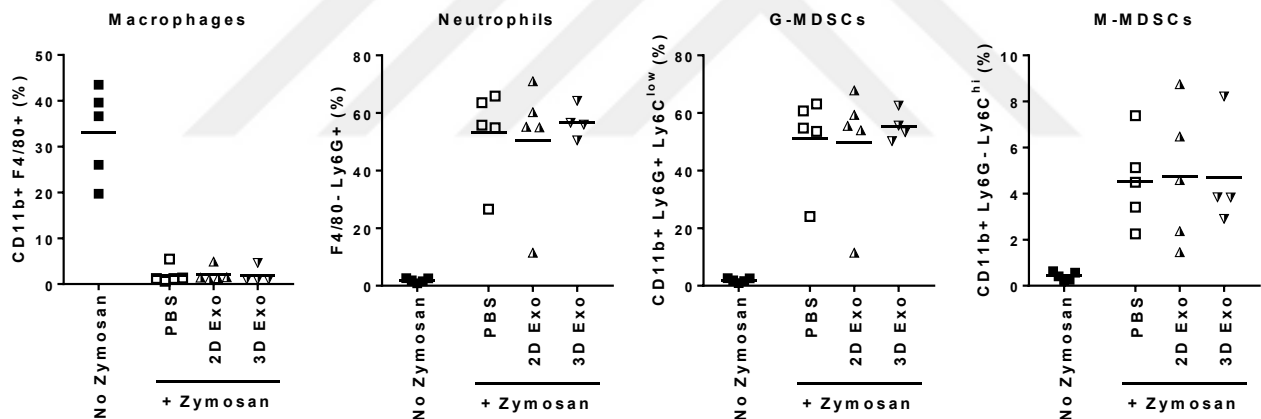


Figure A3. Effects of 2D and 3D UCX exosomes on mouse peritoneal cell populations in mice with zymosan-induced acute peritonitis. 30 µg/mouse exosomes were administered via intraperitoneal injection to C57BL/6 mice 24 hours before 100 µg/mouse zymosan injection. 6 hours after zymosan administration, peritoneal exudate cells were collected and cell populations were analyzed via flow cytometry upon surface marker staining. Exo: exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell.

Animals sacrificed at day 7

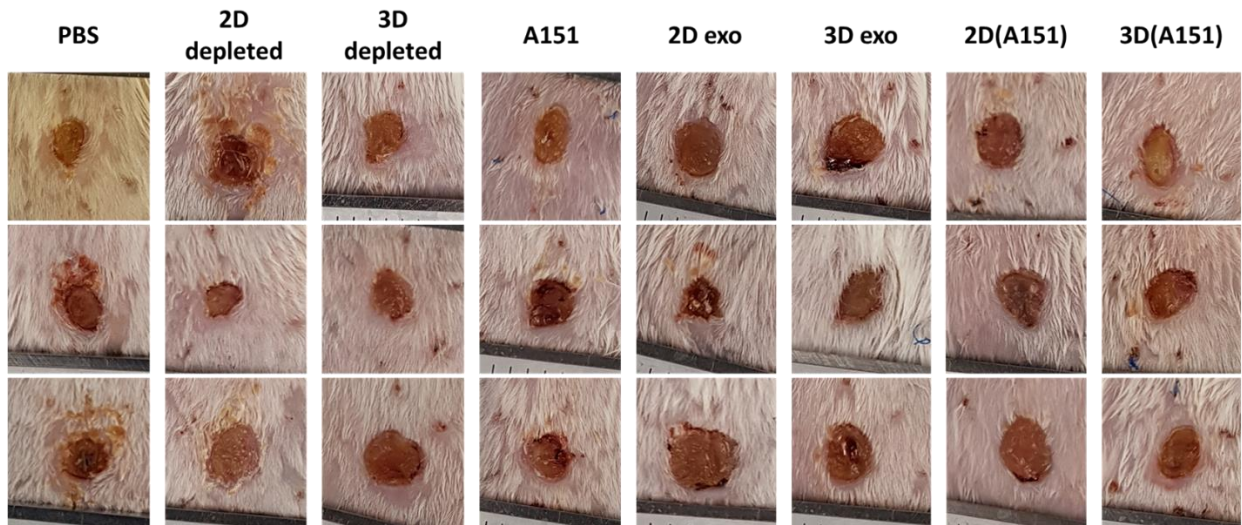


Figure A4. Wound images of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms and sacrificed at day 7. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 µg/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Wounds were photographed and measured daily. 3 animals per group were sacrificed at day 7, the remaining were sacrificed at day 14. Depleted: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome.

Animals sacrificed at day 14

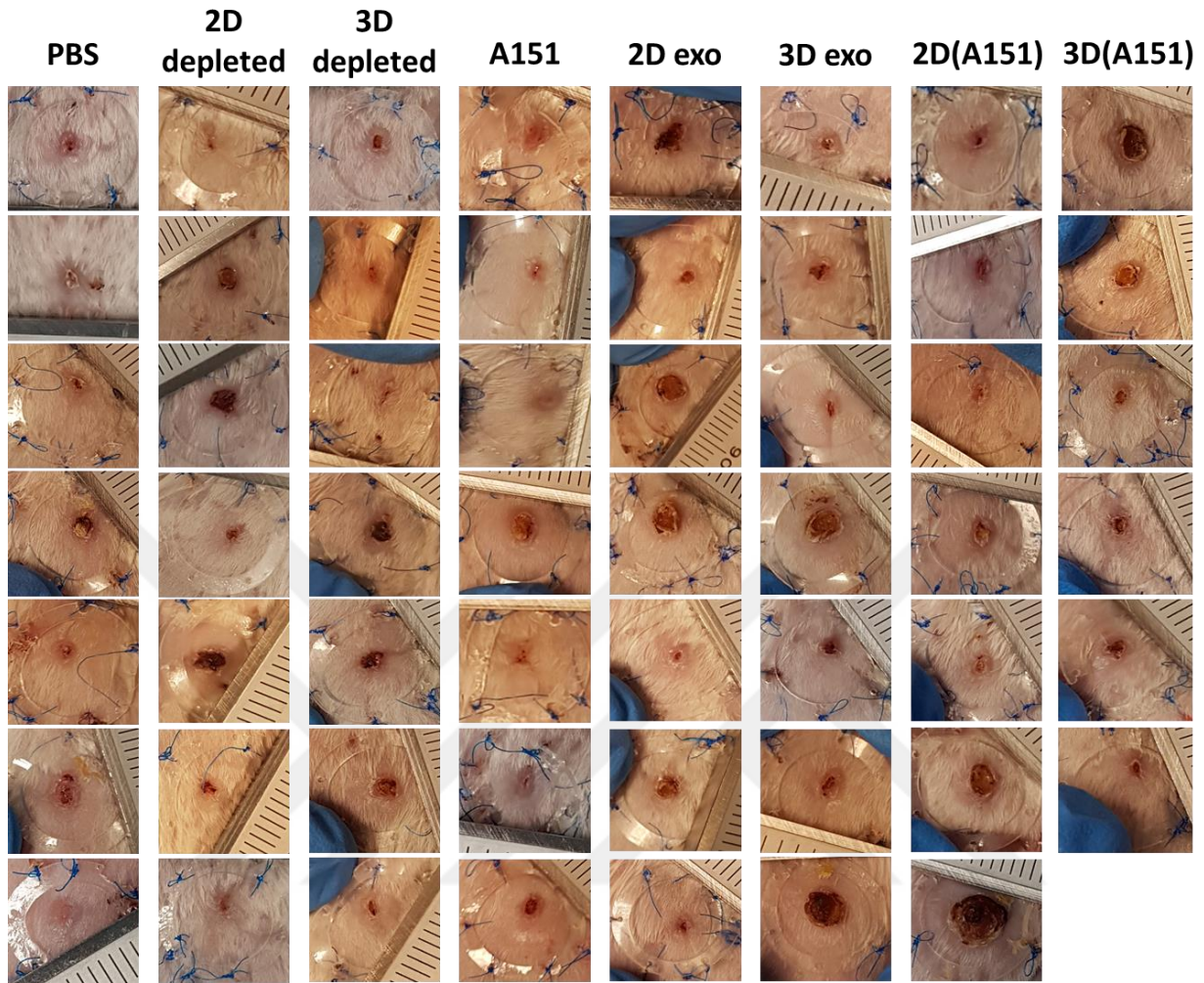


Figure A5. Wound images of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms and sacrificed at day 14. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Wounds were photographed and measured daily. 3 animals per group were sacrificed at day 7, the remaining were sacrificed at day 14. Depleted: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome.

Skin Staining – Day 7

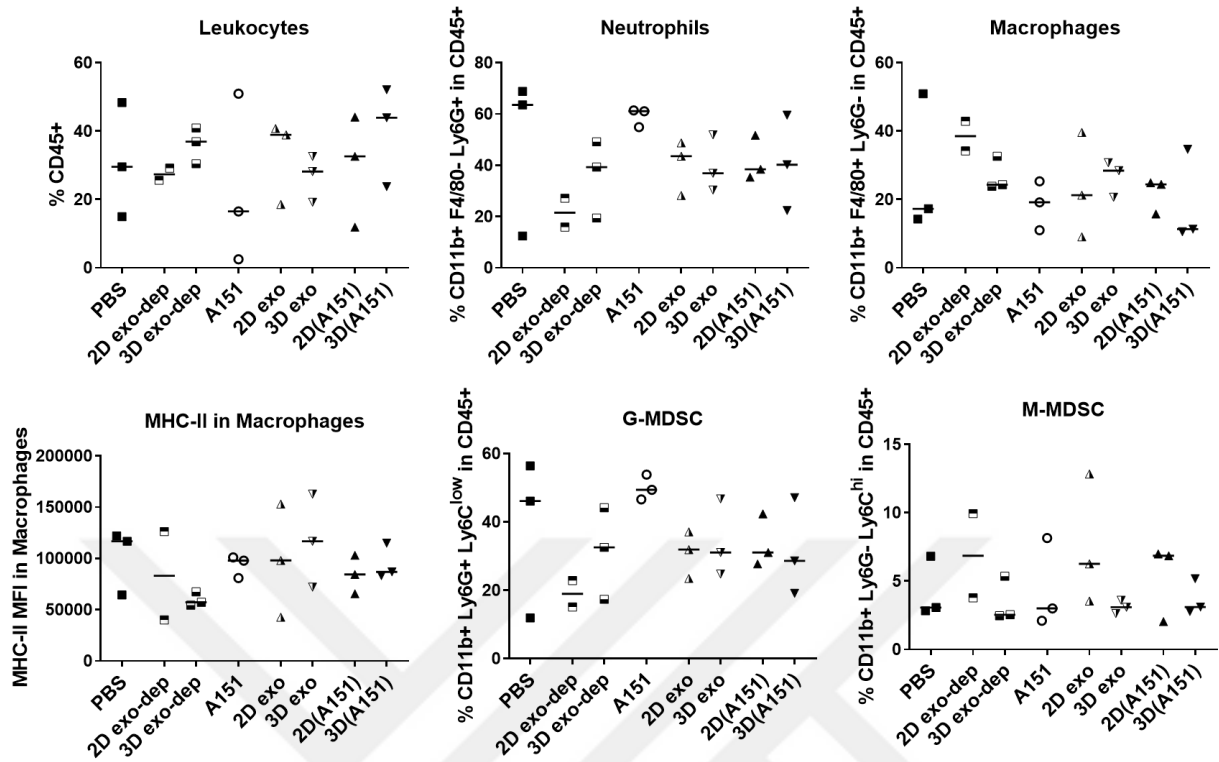


Figure A6. Leukocyte, neutrophil, macrophage and MDSC populations at day 7 in the wound tissues of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Animals were sacrificed at day 7, wound and the peripheral skin tissue were excised, single cells were isolated and cell populations were analyzed via flow cytometry. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell.

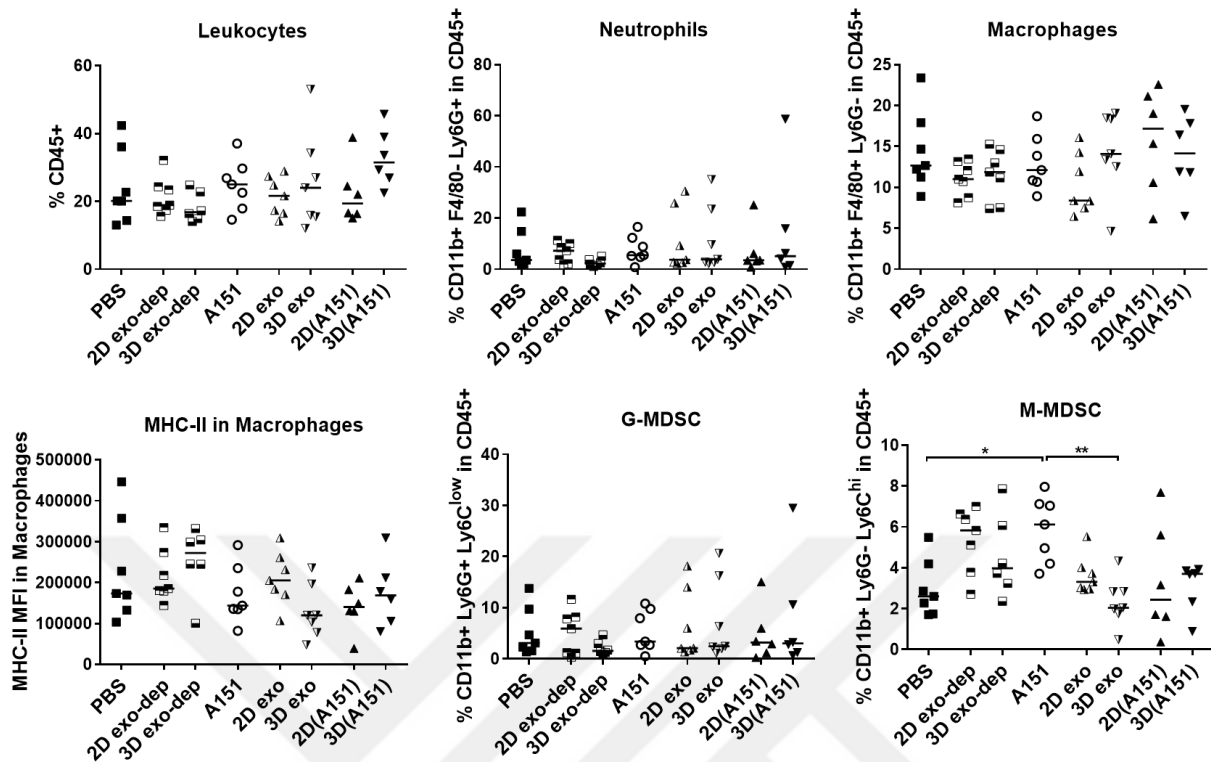


Figure A7. Leukocyte, neutrophil, macrophage and MDSC populations at day 14 in the wound tissues of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Animals were sacrificed at day 14, wound and the peripheral skin tissue were excised, single cells were isolated and cell populations were analyzed via flow cytometry. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell. * $p \leq 0.05$, ** $p \leq 0.01$.

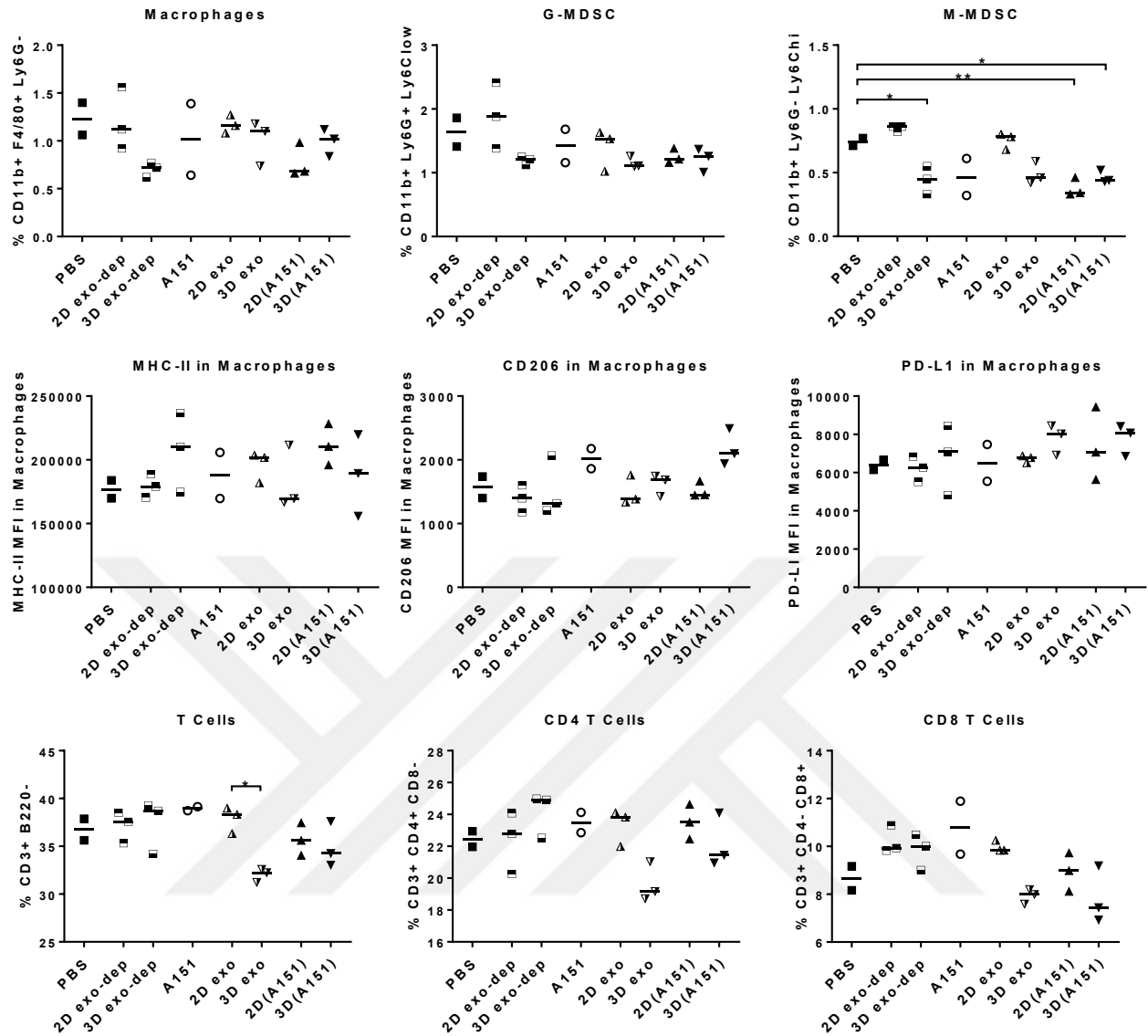


Figure A8. Macrophage, MDSC and T-cell populations at day 7 in spleens of mice with excisional wounds treated with exosome-depleted 2D- or 3D-conditioned media, A151 ODN, 2D- or 3D-exosomes or their A151 ODN-loaded forms. 6 mm punch biopsies were performed on BALB/c mice. The day of wounding was considered as day 1 and 30 μ g/mouse exosome-depleted 2D/3D conditioned media, A151 ODN, free 2D or 3D-exosomes, or their A151 ODN-loaded forms were injected subcutaneously to the wound edges at days 1 and 3. Animals were sacrificed at day 7, spleens were taken out and cell populations were analyzed via flow cytometry. Exo-dep: exosome-depleted conditioned medium, Exo: exosome, 2D(A151): A151 ODN-loaded 2D-exosome, 3D(A151): A151 ODN-loaded 3D-exosome, G-MDSC: granulocytic myeloid-derived suppressor cell, M-MDSC: monocytic myeloid-derived suppressor cell. * $p \leq 0.05$, ** $p \leq 0.01$.

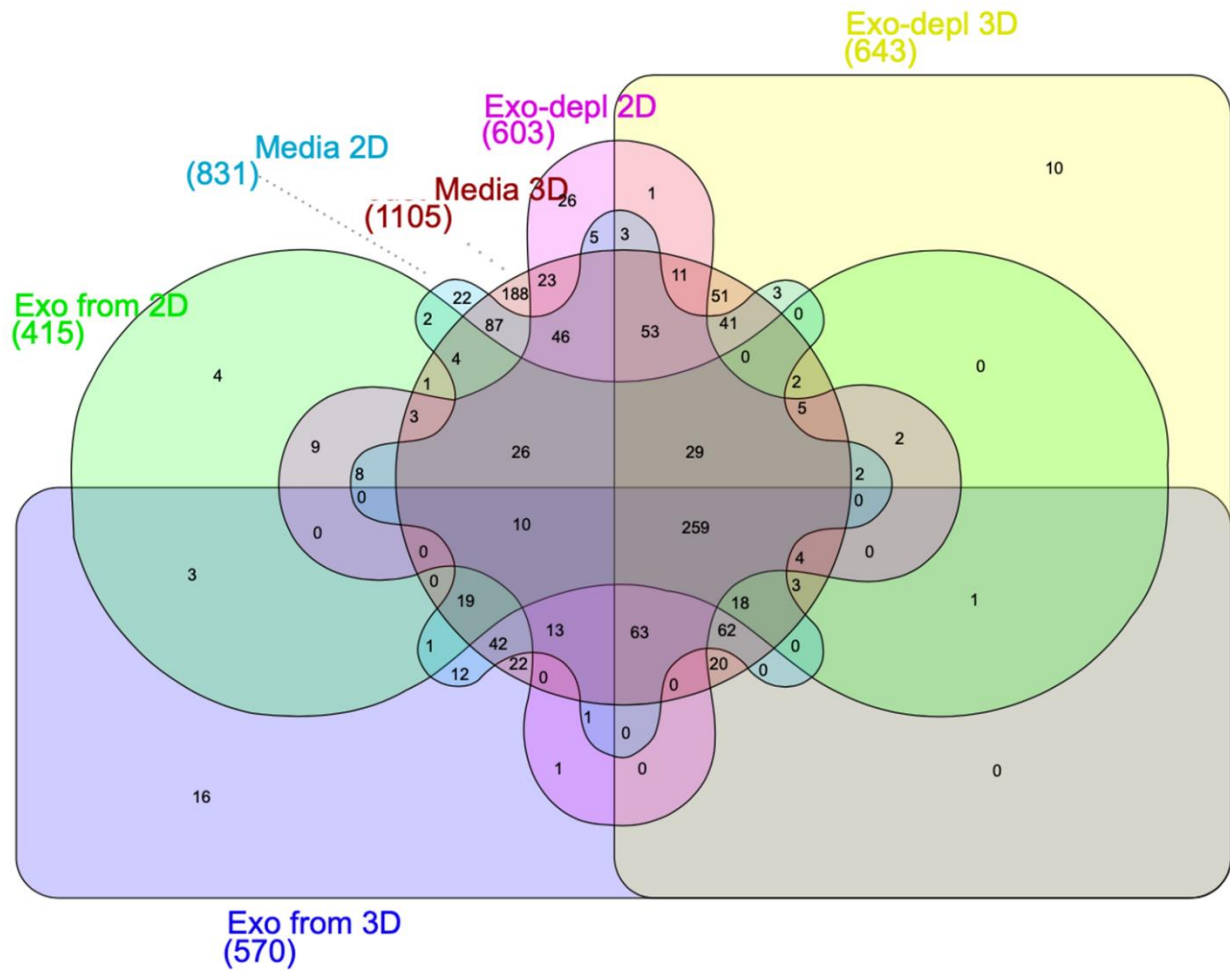


Figure A9. Venn diagram showing the unique and shared protein numbers among exosomes, exosome-depleted media and whole conditioned media obtained from 2D and 3D UCX cultures. Exo: exosome, Exo-depl: exosome-depleted conditioned media, Media: whole conditioned media.

Appendix B

Buffers for ELISA and Flow Cytometry

1. 10X Phosphate Buffered Saline (PBS):

- 80 g NaCl
- 2 g KCl
- 8.01 g Na₂HPO₄·2H₂O
- 2 g KH₂PO₄
- 1 L dH₂O

pH is set to 6.8 and the buffer is autoclaved before use.

2. 1X PBS:

- 100 ml 10X PBS
- 900 ml dH₂O

pH is set to 7.4.

3. ELISA Blocking Buffer:

- 500 ml 1X PBS
- 25 g bovine serum albumin (BSA)
- 250 µl Tween-20

4. ELISA Washing Buffer:

- 500 ml 10X PBS
- 2.5 ml Tween-20
- 4.5 L dH₂O

5. FACS Buffer for Flow Cytometry:

- 500 ml 1X PBS
- 5 g BSA
- 125 mg Na-Azide

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