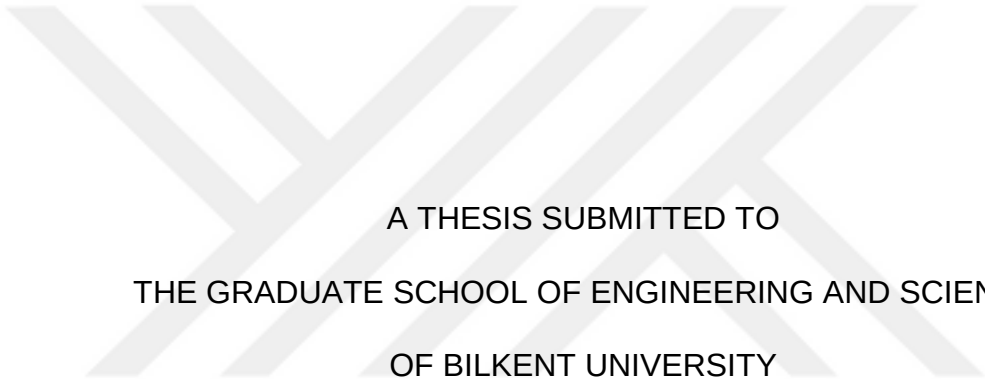


**THERAPEUTIC AND DIAGNOSTIC
APPLICATIONS OF
EXTRACELLULAR VESICLES**



A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MOLECULAR BIOLOGY AND GENETICS

By
Tamer Kahraman
March, 2016

**THERAPEUTIC AND DIAGNOSTIC APPLICATIONS OF EXTRACELLULAR
VESICLES**

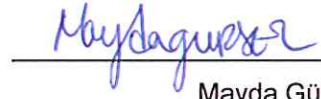
By Tamer Kahraman

March, 2016

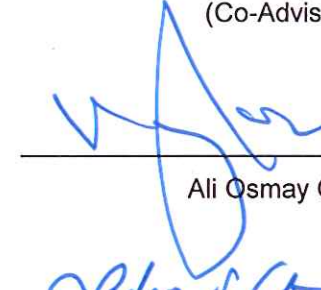
We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.



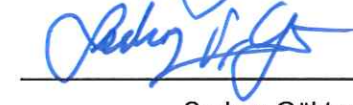
İhsan Gürsel
(Advisor)



Mayda Gürsel
(Co-Advisor)



Ali Osmay Güre



Serkan Göktuna



Gülay Özcengiz



Kamil Can Akçalı

Approved for the Graduate School of Engineering and Science

Prof. Dr. Levent Onural

Director of the Graduate School of Engineering and Science

ABSTRACT

THERAPEUTIC AND DIAGNOSTIC APPLICATIONS OF EXTRACELLULAR VESICLES

Tamer Kahraman

Ph.D. in Molecular Biology and Genetics

Supervisor: İhsan Gürsel

Co-supervisor: Mayda Gürsel

March, 2016

Extracellular vesicles (EV), consisting of exosomes and microvesicles, are secreted biological nanovesicles and assumed plethora of physiological functions ranging from transport of cargo, regulating distant cell communication, and altering immune response. Accumulating evidence suggests that extracellular vesicles may participate in disease pathogenesis of inflammatory diseases. Moreover, accumulating evidence suggests that EVs are promising nanocarrier capable of modulating immune response. This thesis aims to harness EVs in immunotherapeutic and diagnostic applications. Behçet's Disease (BD) activity is manifested with sustained, over exuberant immune activation, yet the underlying mechanisms leading to active BD state is poorly defined. Herein, we show that the human cathelicidin derived antimicrobial peptide LL37 and EVs are elevated in BD plasma. Our data suggested that majority of LL37 is associated with EVs. This association drives plasma EVs to immune cells, enhancing pathologic and sustained immune response, thereby leading to aggravating BD pathology. Stimulation of healthy PBMC with active BD patient EVs induced heightened IL1 β , IFN α , IL6 and IP10 secretion compared to healthy and inactive BD EVs. Remarkably, when mixed with LL37, healthy plasma-EVs triggered a robust immune activation replicating the pathology inducing properties of BD EVs. Findings of this study could be of clinical interest in the management of BD, implicating that LL37/EV association as one of the major contributors of BD pathogenesis and might be used as a diagnostic read-out to stratify the severity of BD patients.

EVs, more specifically exosomes, suggested as new tools for biomedical applications such as drug/vaccine carrier vesicles. However, efforts to engineer cells to express desired cargo in/on these secreted exosomes or induce physical complexation with candidate bioactive agents or even use of membrane-breaching techniques such as electroporation to load exosomes with desirable cargo showed limited in vivo performance. Here we developed a mild and simple technique enabling external loading of any type of desired bioactive molecule within exosomes at high yield. Using this approach, we exploited therapeutic potential of exosomes encapsulating CpG ODN together with a protein antigen as a vaccine cancer for preventive tumor therapy. CpG ODN loaded within exosomes displayed pronounced in-vitro activity as evidenced by up to 6-fold higher IL6 and IL12 secretion from splenocytes as well as increased IFN α secretion from pDCs. Exosomes protected CpG ODN from digestion by DNase-I up to 90%. In order to demonstrate improved in-vivo activity, exosomes co-encapsulating CpG ODN and ovalbumin were tested

as a potential vaccine vector against EG-7 thymoma. Animals that received Exo(CpG ODN+OVA) vaccine led to a magnified and persistent Th1-biased anti-OVA IgG responses that was sufficient to fully protect mice from EG-7 derived tumor challenge even after 24 weeks post-booster injection as opposed to free vaccine combination. Our results suggest that EVs could be of clinical interest in both prognosis and management of BD, implicating LL37/EV association as one of the major contributors of BD pathogenesis. In addition, our studies related with exosomes present a platform that opens a new avenue to personalized cell-free therapeutic intervention and could be developed to harbor other therapeutically important molecules ranging from plasmid to mRNA or si/miRNA for more effective therapeutic modality development in the clinic against debilitating diseases ranging from cancer to infectious diseases.

Keywords: Extracellular vesicles, Microvesicles, Exosomes, Behçet's Disease, LL-37, Vaccine Carrier, Anti-tumor therapy

ÖZET

HÜCRE DIŐI KESECİKLERİN TERAPÖTİK VE DİAGNOSTİK UYGULAMALARI

Tamer Kahraman
Moleküler Biyoloji ve Genetik Bölümü, Doktora
Tez Danışmanı: İhsan Gürsel
Eş Tez Danışmanı: Mayda Gürsel
Mart, 2016

Hücre dışı kesecikler (EV), eksozom ve mikrokkeseciklerden oluşan, hücreler tarafından salgılanan doğal nano kesecikler olup hücreler arası iletişim, kargo taşıma ve immün yanıtı düzenleme gibi birçok biyolojik olayda görev adıkları bilinmektedir. Yapılan çalışmalar EV'lerin, otoimmün ve/veya otoenflammatuvar hastalıkların patogeneğinde rol aldıklarını göstermektedir. Bunun yanı sıra EV'lerin immün yanıtları düzenleyebilecek karakterde doğal nano taşıyıcılar olarak kullanılabileceğini öneren çalışmalar bulunmaktadır. Bu tezin amacı EV'lerin potansiyel diagnostik ve terapötik uygulamalarını araştırmaktır. BD sürekli ve yüksek immün aktivasyon ile karakterize olan bir hastalık olmasına rağmen hastalığa neden olan mekanizmalar tam anlamıyla aydınlatılamamıştır. Bu çalışmada, bir anti-mikrobial peptid olan LL37 ve EV'lerin hastalık seyri ile paralel bir şekilde arttığını gösterdik. Plazmadaki LL37'nin EV'ler ile assosiyе olduğu belirlenmiştir. LL37 bağlanmış plazma EV'lerinin immün hücreler tarafından daha yüksek oranda ve verimle internalize olmasını sağlayarak patolojik ve sürekli etkin bir immün yanıtın oluşmasına sebep olarak BD patogeneğinde hastalığın şiddetini yönlendirmektedir. Aktif BD hastalarından izole edilen EV'ler sağlıklı periferik kan hücrelerinden (PBMC) IL1 β , IFN α , IL6 ve IP10 salgılanmasına neden olmaktadır. Sağlıklı EV'lerle LL37 karıştırıldığında bu EVlerin BD plazmasından elde edilen EV'ler gibi davranarak patolojik etkiye sebep olduğu bulunmuştur. EV ve LL37'nin BD'deki rolü, klinikte BD tanısı ve hastalığın durumunu tespit etmek için kullanılabilecek bir diagnostik bulgudur.

Yakın zamanda yapılan çalışmalar, eksozomların ilaç veya aşı taşıyıcı vektörler olarak kullanılabilecek yeni nesil nanotaşıyıcılar olabileceklerini iddia etmektedir. Ancak literatürdeki teknikler eksozomlara istenilen kargonun etkin olarak yüklenmesini sağlamamaktadır. Biz bu çalışma ile eksozomlara istenilen kargonun yüksek verimle yüklenmesini sağlayacak basit ve moleküler yapılara zarar vermeyen bir teknik geliştirerek eksozomları immün tedavide kullanmayı amaçladık. Bu teknik kullanılarak eksozomlara CpG ODN yanında antijende yüklenerek, koruyucu aşı olarak, terapötik potansiyelleri araştırılmıştır. Eksozomlara enkapsüle edilmiş CpG ODN'lerin fare dalak hücrelerinden salınan IL6 ve IL12 cevaplarını yükseltirken, pDC'lerden de IFN α salgımını 6 kat artırmıştır. Eksozomlar, CpG ODN'leri DNase-I atağından %90'dan fazla koruduğu elde edilmiştir. CpG ODN'ler ovalbumin (OVA) ile birlikte eksozomlara yüklenerek potansiyel aşı vektörü olarak kullanılan çalışmada Exo(CpG ODN+OVA) enjekte edilen hayvanlar daha güçlü ve uzun süreli Th1 karakterli anti-OVA immün yanıtı oluşturmaktadır. Bu immün yanıt ise,

hayvanları OVA ifade eden EG-7 hücre hattının oluşturduğu timoma'ya karşı aşılardan 24 hafta sonra bile koruyabilmektedir. Bu tez kapsamında yapılan çalışmalar, EV'lerin hem diagnostik/prognostik, hem de terapötik amaçlı kullanım potansiyellerinin yüksek olduğunu göstermektedir. BD hastalarında LL37/EV ilişkisi, hastalık patogenezi ile ilgili önemli bir bilgi verirken, hem BD tanısında, hem de hastalık seyrinin takibinde kullanılabileceklerini önermektedir. Bunun yanı sıra tez'in ikinci kısmında yürütülen çalışmamızda eksozomların hücre kullanılmayan kişiselleştirilmiş yeni nesil terapilerde kullanılabileceklerini göstermektedir. Ayrıca eksozomlara istenilen her çeşit kargonun (siRNA, plasmid ve protein gibi) yüklenmesini sağlayan yeni bir teknik ortaya çıkararak, bu keseciklerin terapötik kullanım potansiyellerini genişletmektedir.

Anahtar Kelimeler: Hücre dışı kesecikler, Mikrokesecikler, Eksozom, Behçet Sendromu, LL-37, Aşı taşıyıcı, Anti-tümör terapi



To my precious family...

Acknowledgements

I wish to thank, first and foremost, my advisor Prof. Dr. İhsan Gürsel for giving me the opportunity to work in his lab and for his guidance, support, encouragement and patience throughout my PhD work. It would be impossible for me to finish this study without his academic and life experience. I consider it an honor and privilege to work with him as well as know him.

I would like to thank Prof. Dr. Mayda Gürsel for her scientific assistance during my thesis project as well as being my co-advisor. She has been very enthusiastic to share her knowledge and support. I would be in debt for my life of both my advisor and co-advisor because of their teachings in past 8 years.

I would like to thank my thesis committee members; Prof. Dr. Kamil Can Akçalı and Prof. Dr. Gülay Özçengiz for sharing their experience, academic knowledge and guidance throughout the progress of my thesis project.

I would also like to express my gratitude to my jury members Assoc. Prof. Ali Güre, Assist. Prof. Serkan Göktuna, Assoc. Prof. Işık Yuluğ and Prof. Dr. Dicle Güç for their critical reading as well as valuable evaluation of my thesis.

Moreover, I would like to thank to past and present THORLAB group members Fuat, Gizem, Hande, Erdem, Kutay, Fırat, Gözde, Banu, Arda, İhsan, Mehmet, Begüm Han, Defne, Kübra, Hakan, Elif, Begüm, Muzaffer, Fehime and MG Group members, Bilgi, Esin, and Soner for their support and friendship.

I am truly thankful to all of my dearest friends, especially Ece, Emre Onat, Işıl, Şerif, Elif, İrem, Nilüfer, Gurbet, Gökhan, Sinem, Bala, Tolga, Atıl, Derya, Sinan, Onur and Muammer for their full support and friendship during this long journey. Thanks to all MBG family members for the great working atmosphere and technical support.

I would like to thank veterinarians Burcu, Emre and Gamze for their help and guidance in animal experiments, our MBG lab manager Bilge, and technicians Abdullah Amca, Turan Amca for their support in technical issues.

I would like to express my deepest gratitude to my family for their invaluable and endless love, support and faith in me throughout my life. Without them none of these achievements would be possible to accomplish.

Last but not least, I would like to express my deepest love to my wife, Deniz, and thank for her patience and efforts to cheer me up as well as helping in my Ph.D. work. Her love and support means a lot to me and is one of the driving forces for my achievements and accomplishments.

This work was supported by TÜBİTAK (SBAG 111S206)



Contents

Chapter 1. Introduction.....	1
1.1. Extracellular Vesicles Secreted from Cells.....	1
1.1.1. Different types of extracellular vesicles	2
1.1.2. Characteristics of exosomes and microvesicles.....	3
1.1.3. Interaction of extracellular vesicles with cells.....	6
1.2. Physiological Roles of Microvesicles and Exosomes in Health and Disease.....	8
1.2.1. Role of microvesicles in disease pathology.....	10
1.2.2. Exosomes and immune system	15
1.3. Extracellular Vesicles in Clinical Applications	22
1.3.1. Therapeutic applications of unmodified EVs	22
1.3.2. Modified exosomes as therapeutic agents.....	24
1.3.3. Diagnostic and prognostic potential of EVs.....	27
1.4. Immunostimulatory Ligands	29
1.4.1. CpG ODN and immune system	29
1.4.2. CpG ODNs as vaccine adjuvants	30
1.4.3. Cyclic-di-GMP	31
1.5. Behçet's Disease and Its Molecular Background	32
1.6. Asthma, Atopic Dermatitis and Their Molecular Pathogenesis	33
1.7. Subject and Outline of This Thesis	33
Chapter 2. Materials and Methods	35
2.1. Patients and Controls.....	35
2.1.1. BD patients and controls	35
2.1.2. Asthma, AD patients and controls	37

2.2. Animals and Cell Culture.....	38
2.2.1. Animals	38
2.2.2. Cell culture	38
2.3. Isolation of EVs and Exosomes.....	41
2.3.1. Isolation of EVs and exosomes from plasma and cell culture	41
2.3.2. Determining protein concentration of exosomes.....	42
2.4. Characterization of Exosomes	42
2.4.1. Analysis of size distribution of exosomes by DLS and AFM.....	42
2.4.2. Analysis of exosomal markers by flow cytometer	42
2.5. TLR9 and STING Ligand Loading within Exosomes.....	44
2.5.1. Ligands	44
2.5.2. Dehydration rehydration technology for ligand loading within exosomes	45
2.6. Flow Cytometry Studies	47
2.6.1. Analysis of EVs and exosomes by flow cytometer	47
2.6.2. Flow cytometry studies of PBMCs, RAW264.7 cells and splenocytes ..	49
2.7. Confocal Microscopy Studies	51
2.8. ELISA Studies.....	51
2.8.1. Cytokine ELISA.....	51
2.8.2. anti-OVA IgG ELISA.....	54
2.9. Immunization Studies.....	54
2.10. EG-7 Challenge Experiments.....	55
2.11. Statistical Analysis	55
Chapter 3. Results	56
3.1. Role of Extracellular Vesicles in Behçet's Disease	56
3.1.1. BD patients have elevated circulating EV numbers	56

3.1.2. Platelet derived EVs constitute the majority of circulating EVs in BD plasma	58
3.1.3. EVs are internalized by PBMCs triggering IL6 secretion.....	62
3.1.4. Active BD patient plasma LL37 levels are elevated and majority of LL37 is associated with EVs.....	64
3.1.5. Elevated concentration of EVs, increased IL6 secretion and LL37 concentrations correlates with disease severity.....	65
3.1.6. LL37 interacts with plasma EV enhancing their cellular uptake to increase IL6 secretion from PBMCs	68
3.1.7. LL37/healthy EV complex mimics active BD EVs and triggers IFN α from pDC as well as IL1 β and IP10 from monocytes	71
3.2. Role of Extracellular Vesicles in Allergic Diseases	73
3.2.1. Decreased levels of EVs in allergic diseases.....	73
3.2.2. Cellular origin of EVs varies in allergic patients	75
3.3. EXOSOLIGOS: A novel Exosome-based Delivery Platform for Effective Immune Modulation	76
3.3.1. Exosomes can be isolated from supernatant of RAW264.7 cell line with high yield	76
3.3.2. A TLR9 ligand CpG ODN D35 can be encapsulated within exosomes	79
3.3.3. Mouse spleen cells efficiently internalizes CpG ODN loaded exosomes	81
3.3.4. D-Type CpG ODN encapsulation within exosomes improves TLR9 mediated immunostimulatory activity.....	83
3.3.5. Blocking scavenger receptor attenuates D35 dependent immune activation.....	87
3.3.6. Exosomes protects D35 form DNase-I digestion	90
3.3.7. Co-encapsulating antigen and CpG ODN adjuvant within exosome elicits better and extended antigen-specific immunity.....	91
3.3.8. Exosomal vaccine protects mice from EG-7 induced tumors	96
3.4. Enhancing in vitro Internalization of STING ligand c-di-GMP either within Exosomes or complexing with peptide Arg(9)	101

3.4.1. Improved cellular delivery of c-di-GMP by Arg(9) and exosomes.....	101
3.4.2. Encapsulation increases stimulatory effect of c-di-GMP and synergizes with CpG ODNs	103
Chapter 4. Discussion	107
4.1. Involvement of EV in Behçet`s Diseases.....	107
4.2. Involvement of EV in Allergic Diseases	110
4.3. Development of EVs as drug carriers.....	111
References	117
Appendices.....	138
Appendix A	139
Appendix B	142
Appendix C	144
Curriculum Vitae and Publications	145

List of Figures

Figure 1.1. Different types of extracellular vesicles are secreted by live cells.....	3
Figure 1.2. Different interaction mechanisms between cells and EVs.....	7
Figure 1.3. Physiological role of EVs in health and disease	9
Figure 1.4. Participation of MVs in arthritic joint	13
Figure 1.5. MVs participate in tumor pathogenesis.....	15
Figure 1.6. Immune response inducing effects of exosomes.....	19
Figure 1.7. Suppressive effects of exosomes on immune cells	21
Figure 1.8. Therapeutic applications of unmodified EVs.....	24
Figure 1.9. Modification of exosomes for therapeutic purposes.....	26
Figure 1.10. Localization and induced pathways of D- and K-type ODNs.....	30
Figure 2.1. Representative picture of grids in hemocytometer.....	40
Figure 2.2. Representative scheme showing bead based analysis of exosomes using flow cytometer	44
Figure 2.3. Dehydration rehydration loading method is given schematically.....	46
Figure 2.4. Gating strategy used during analysis of EVs	48
Figure 2.5. Immunization and bleeding schedule	55
Figure 3.1. Plasma concentration of EVs are elevated in BD patients.....	57
Figure 3.2. The analyses of cellular origin of EVs at different disease stages..	59
Figure 3.3. The cellular origin of plasma EVs alters with disease severity	60
Figure 3.4. Absolute concentration (EV#/ml plasma) of specific cell derived EVs vary with disease state.....	61
Figure 3.5. EVs are efficiently internalized by PBMCs.....	63
Figure 3.6. BD EVs trigger IL6 secretion from PBMCs	64
Figure 3.7. BD plasma contains elevated levels of antimicrobial peptide LL37 which is mostly associated with EVs.	65

Figure 3.8. The LL37 levels, IL6 induction capacity of EVs and concentration of EV in plasma correlates with disease activity.....	67
Figure 3.9. LL37 promotes uptake of EVs by immune cells.....	68
Figure 3.10. Different immune cells internalize EV/LL37 complex at different levels	69
Figure 3.11. Complexation of healthy EVs with LL37 elicits IL6 secretion from PBMCs	70
Figure 3.12. LL37/EV triggers IFN α from pDC, IL1 β and IP10 from monocytes.....	72
Figure 3.13. Allergic patients have reduced level of plasma EVs	74
Figure 3.14. Cellular origin of EVs vary in allergic patients.....	76
Figure 3.15. Exosomes can be isolated from RAW264.7 cells	78
Figure 3.16. D-Type CpG ODN could be loaded at high efficiency without disrupting exosomal integrity	80
Figure 3.17. Both CpG ODN D35 and exosomes co-localizes within same compartment upon internalization by cells	82
Figure 3.18. Encapsulation into exosomes improved immune stimulatory capacity of D35 CpG ODN.....	87
Figure 3.19. DS attenuates D35 mediated immune activation.....	89
Figure 3.20. D35 is protected from DNase-I digestion when delivered within exosomes	91
Figure 3.21. Exo(D35+OVA) vaccine induced stronger Th1-biased immune response 2 weeks after post booster immunization.....	93
Figure 3.22. Exo(D35+OVA) vaccine yielded higher anti-OVA IgGs at 10 weeks post-booster injection.....	94
Figure 3.23. Persistent anti-OVA IgG responses induced by Exo(D35+OVA) vaccine	95
Figure 3.24. Exo(35+OVA) vaccine confers protection from EG-7 tumor engraftment	100
Figure 3.25. Exosomes deliver c-di-GMP within cells.....	102
Figure 3.26. Exosome encapsulation enhances stimulatory effect of c-di-GMP and further synergizes with CpG ODN mixing	106
Figure 4.1. Potential biomedical application of EVs elaborated in this thesis.....	116

Figure A1. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 2 weeks post-booster bleeding	139
Figure A2. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 10 weeks post-booster bleeding	140
Figure A3. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 24 weeks post-booster bleeding	141
Figure B. Reciprocal titers of (A) total IgG, (B) IgG1 and (C) IgG2c of each mouse at 2-, 10- and 24-weeks post-booster sera	142
Figure C. Immune modulation mediated by Arg(9) mixing.....	144

List of Tables

Table 1.1. Characteristics of exosomes and microvesicles.....	5
Table 1.2. Tumor antigens found in EVs of cancer patients.....	28
Table 2.1. Clinical evaluations of the BD Patients.....	37
Table 2.2. Exosome-CpG ODN mixing conditions.	45
Table 2.3. ELISA reagents and their working conditions.....	53



Abbreviations

aBD	active BD
aChR	Acetylcholine receptor
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
AFM	Atomic Force Microscopy
AICD	Activation induced cell death
AIM-2	Absent in melanoma 2
allo	Allogeneic
ALP	Alkaline phosphatase
AnV	Annexin-V
APC	Antigen presenting cell
aPL	Antiphospholipid antibody
APS	Antiphospholipid syndrome
ATP	Adenosine three phosphate
BAL	Bronchoalveolar fluid
BAT3	HLA-B associated transcript 3
BB	Binding Buffer
BD	Behçet's Disease
BSA	Bovine serum albumin
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
c-di-GMP	cyclic-di-GMP
CeA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
cGAS	Cyclic GMP-AMP synthase
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DIC	Differential interference contrast
DLS	Dynamic Light Scattering
DNA	Deoxynucleic acid
EAMG	Experimental autoimmune myasthenia gravis
EDTA	Ethylenediamine tetraacetic acid

EGFR2	Epidermal growth factor receptor 2
EGFRvIII	Epidermal growth factor receptor variant III
EMV	Endothelial derived MV
ERK	Extracellular signal-regulated kinase
EV	Extracellular vesicles
FACS	Flourescence assosicated cell sorting
FasL	Fas ligand
FB	FACS Buffer
gp100	Glycoprotein 100
GvHD	Graft versus host disease
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICAM1	Intercellular adhesion molecule 1
IDH1	Isocitrate dehydrogenase 1
IFN	Interferon
Ig	immunoglobulin
IL	Interleukin
IL15R α	IL-15 receptor α -chain
IP10	Interferon gamma-induced protein 10
IRF7	Interferon regulatory factor 7
iBD	inactive BD
L1CAM	L1 cell adhesion molecule
LEAF	Low Endotoxin, Azide-Free
LFA1	Lymphocyte function-associated antigen 1
LMV	Leukocyte derived MV
LPS	Lipopolysaccharide
LSM	Lyphocyte separation medium
MAGE3	Melanoma-associated antigen 3
MAGE6	Melanoma-associated antigen 6
MART1	Melanoma antigen recognized by T cells 1
MCP1	Monocyte chemoattractant protein-1
MDA5	Melanoma differentiation-associated protein 5

MD-DC	Monocyte derived DC
MDSC	Myeloid-derived suppressor cell
MFGE8	Milk fat globule epidermal growth factor 8
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
miRNA	MicroRNA
MMP	Metalloproteinase
MMV	Monocyte derived MV
mRNA	messenger RNA
MSC	Mesenchymal stem cell
MSC-EV	Mesenchymal stem cell derived EV
MV	Microvesicle
MVB	Multivesicular bodies
MyD88	Myeloid differentiation primary response 88
NK	Natural killer
NKG2D	Natural killer group 2D
NS	Not significant
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PBMC	Peripheral Blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PDPN	Podoplanin
PI3	Phosphoinositide 3
PMV	Platelet derived MV
PNPP	p-nitrophenyl phosphate
PS	Phosphatidylserine
PSGL1	P-selectin glycoprotein ligand-1
RA	Rheumatic arthritis

RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RT	Room Temperature
siRNA	small interfering RNA
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
TF	Tissue factor
TGFβ	transforming growth factor β
Th	T-helper
TIM1	T cell immunoglobulin domain and mucin domain protein 1
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TMV	Tumor derived MV
TNFR1	Tumor necrosis factor receptor type I
TNFα	Tumor necrosis factor α
Treg	Regulatory T cell
TSG101	Tumor susceptibility gene 101
TSLP	Thymic stromal lymphopoietin
TYRP1	Tyrosinase-related protein 1
ULBP1	UL16 binding protein 1
VAMP3	Vesicle associated membrane protein 3
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1. Extracellular Vesicles Secreted from Cells

The studies performed on bone marrow chimeras, which showed that thymocytes could acquire host-derived MHC molecules, established for the first time, almost 30 years ago, that membrane proteins are exchanged between cells [1]. Since then several were published showing how cellular membranes exchanged between cells by either direct cell to cell contact or secreted extracellular vesicles (EV). The functional relevance of transferring materials between cells are induction, amplification or modulation of cellular responses, and acquisition of new properties by the recipient cells, such as migratory or metastatic abilities. Migration and metastatic capabilities are two examples that can be acquired by internalizing EVs. In addition, it was shown that EVs are merely just small particles carrying membrane proteins but also contains mRNAs and microRNAs [2]. This finding raised exciting possibility of genetic material carrying EVs could alter behaviors of the recipient cell. Altogether these studies showed that EVs are not just garbage disposal system but a common communication channel of cells [3], which was recently identified.

Further studies showed that EVs can be secreted constitutively from most cell types such as platelets [4], B-cells [3], tumors [5], neutrophils [6], dendritic cells (DCs) [7], macrophages [8] and T-cells [9]. In addition, upon external stimuli EVs secretion from cells can be induced, enhanced [10] or dampened [11]. Because of the stability of these vesicles, EVs can be isolated from most of the biological fluids such as serum [12], plasma [13], urine [14], saliva [15], amniotic fluid[16], synovial fluid [17], breast milk [18], nasal [19] and bronchoalveolar lavage (BAL) [20] fluids.

1.1.1. Different types of extracellular vesicles

Recent updates in the literature suggest that EVs are very heterogeneous population consisting of exosomes [21], microvesicles [4], ectosomes [22], membrane particles [23], exosome-like vesicles [24] and apoptotic bodies [25] (Figure 1.1). EVs are different from carrier vesicles, which bud out from internal compartments and transfer materials intracellularly, as these vesicles are secreted by live cells except apoptotic bodies as seen in Figure 1.1. Although this heterogeneity of the population and some similar features cause confusion during classification of vesicles in the literature, these vesicles has diverse properties differentiating them from others. All of these vesicles have spherical shape and are covered with lipid bilayer. Regardless of type and donor cell of EVs, they originate from either plasma membrane or lumens of intracellular organelles of the cell. Therefore, these vesicles contain the exact components as the originating lumen. In addition, the membrane orientation of the vesicles is as the same as originating lumen. Unrelated to the originating compartment of the cell, all of these vesicles contain cytosolic and luminal content of the cell. Therefore, these vesicles can be considered as miniature of the originating cell.

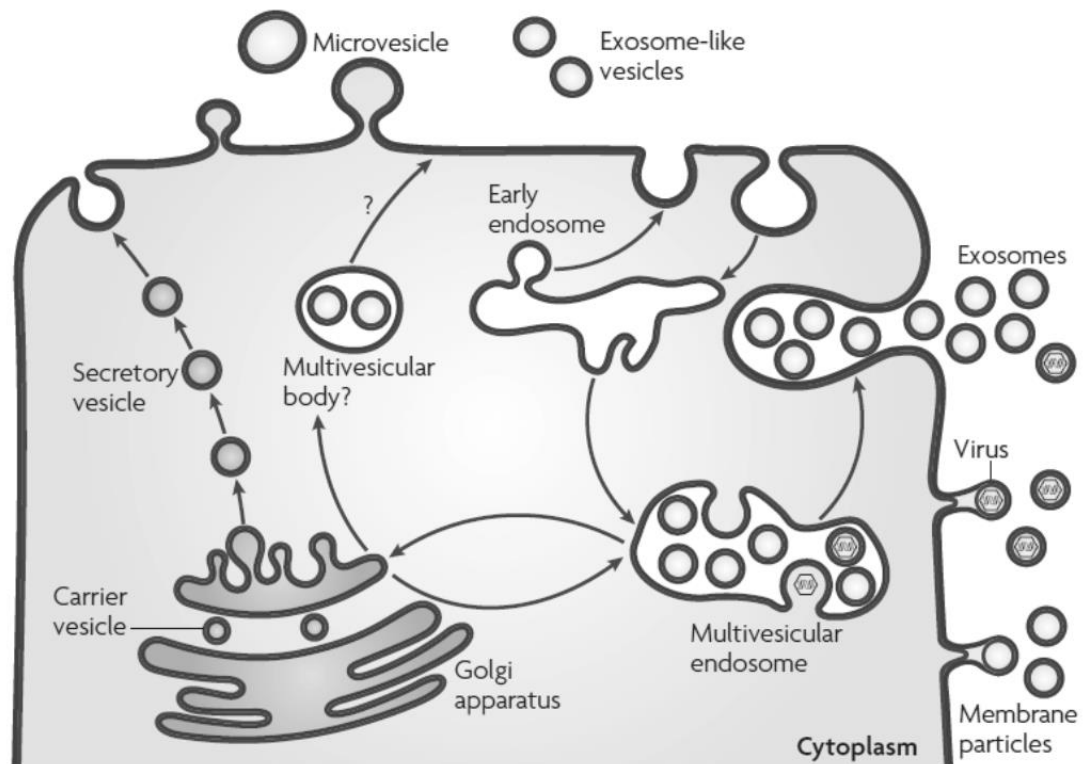


Figure 1.1. Different types of extracellular vesicles are secreted by live cells. Extracellular vesicles are originating from either plasma membrane or intracellular lumens. Plasma membrane give rise to microvesicles, membrane particles and phages. On the other hand, intracellular lumens forms multivesicular bodies which produces exosomes by inward budding. Carrier vesicles and secretory vesicles are not classified as extracellular vesicles since they do not traffic cellular material between cells. (Adopted from [26])

1.1.2. Characteristics of exosomes and microvesicles

Although all of these different types of EVs have crucial physiological roles, exosomes and microvesicles (MV) are of important in the context of this thesis. Both of these vesicles have been proposed to have essential roles in maintaining physiological homeostasis [27]. However, they have diverse physicochemical and molecular properties (Table 1.1). Exosomes are the smallest population of EVs ranging from 30nm to 100nm in diameter and originating from intracellular compartments called multivesicular bodies (MVB) [28]. On the other hand, microvesicles are produced by outwards budding of plasma membrane hence they are larger than exosomes (from 100nm to 1000nm) [29]. Because of their size difference sedimentation rates of these vesicles are also different from each other. MVs can be isolated from fluids at lesser centrifugation speeds than exosomes. Exosomes has specific density of 1.13-1.19 g/ml [21] hence can be purified by

sucrose gradient. Although both vesicles express cell specific markers of originating cell, exosome can be characterized by presence of endosomal proteins such as tetraspanins [30], Alix [31], TSG101 [32]. The other abundant proteins expressed in exosomes are heat shock proteins (HSPs) [33] and major histocompatibility complex (MHC) molecules [34]. On the other hand, MVs have similar surface composition as originating cell other than exposed phosphatidylserine (PS) lipids on the surface. In live cells, PS was faced inside of the cell in the presence of ATP by flippase although it tends to face outside because of its negative charge. Therefore, in both exosomes and MVs, PS faces outside of the leaflet in the absence of ATP [35]. As a result, both can be isolated and identified by Annexin-V (AnV) staining from body fluid and cell culture supernatants. Apart from PS, unlike MVs, exosomes have also enhanced lipid rafts, cholesterol, sphingomyelin and ceramide in its membrane [36]. These biophysical and chemical differentiates exosomes and MVs.

Table 1.1. Characteristics of exosomes and microvesicles.

	Exosomes	Microvesicles
Size	30-100 nm	100-1,000 nm
Density in sucrose	1.13-1.19 g/ml	ND
Appearance*	Cup shape	Irregular shape
Sedimentation	100,000 x g	30,000 x g
Lipid composition	• Contains lipid rafts • Enriched in cholesterol, sphingomyelin and ceramide • Flipped out PS	Flipped out PS
Main protein markers	• Tetraspanins; CD9, CD63, CD81, CD82 • Alix • TSG101 • 14-3-3 proteins • MHC molecules • HSPs	• β1 integrins • Selectins • CD40 ligand • VAMP3 • Actin and tubulin
Intracellular origin	Internal compartments (MVB)	Plasma membrane

**Indicated appearances were taken by electron microscope. ND; not determined, PS; phosphatidylserine, MHC; major histocompatibility complex, TSG101; tumor susceptibility gene 101, HSPs; heat shock proteins, MVB; multivesicular bodies*

1.1.3. Interaction of extracellular vesicles with cells

Different types of interaction of EVs, especially exosomes and MVs, have been proposed explaining how the vesicles internalized by cells. Direct internalization, receptor mediated endocytosis and fusion are possible interaction and internalization pathways of EVs into cells (Figure 1.2).

Evidence suggest that EVs mostly internalized by endocytosis [37]. Clathrin-mediated and caveolin-dependent endocytosis of the vesicles are the most common mechanisms of EV uptake. Internalization by endocytosis occurs early and very fast that EV internalization even after 15min incubation period was seen [38]. Internalization via endocytosis is shown to be energy dependent [39] and active process [40] since incubating cells with EVs at cooler temperatures and fixation of cells attenuates EV uptake by cells. In addition, using several different inhibitory molecules such as heparin [41], cytochalasin D [42], wortmanin [38], AnV [12] and EDTA [42, 43] blocks EVs entry into cells.

Studies showed that interaction between EV molecules and cell surface receptors are important during internalization. Intercellular adhesion molecule 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1) interaction plays crucial role in interaction between cells and EVs. It was shown that ICAM1 expressing exosomes can be captured by T-cells via LFA1 [44]. Alternatively, cells expressing ICAM1 such as B-cells was also shown to interact with EV integrins ($\beta 1$ and $\beta 2$) and internalizing EVs via this interaction [45].

PS molecules on both exosomes and MVs are another target for both cellular and extracellular receptors hence play important role in EV-cell interactions. Exposed PS molecules are targets for both cellular and soluble receptors. T cell immunoglobulin domain and mucin domain protein 1 (TIM1) on activated lymphocytes as well as CD36 and TIM4 on phagocytes are PS binding receptors that can facilitate binding and internalization of exosomes and MVs [46-48]. Other than these cell surface receptors, soluble protein milk fat globule epidermal growth factor 8 (MFGE8) can bind to PS molecules through its carboxy-terminal factorv/vIII-like region [49]. MFGE8 can also bind to the integrins ($\alpha \beta 3$ and $\alpha \beta 5$) on the macrophages in turn mediating internalization of EVs by macrophages.

After internalization by cells through indicated mechanisms, exosomes and MVs can go under different fates. Mostly occurred one is degradation in endosomes. This degradation releases the cargo of EVs into endosomes and make vesicular proteins available for antigen processing. Upon antigen processing, vesicular protein epitopes can be presented by recipient cells via MHC class II molecules. Another fate is to fuse with cells and releasing their cargo directly to cytosol of the recipient cell. This can result in recognition by cytosolic receptors by cargo of EVs hence activating or suppressing recipient cell. As a result, interaction and internalization of an EV with cell modifies the behavior of the recipient cell; therefore, making EVs, especially microvesicles and exosomes, crucial in maintaining homeostasis as well as disease pathology.

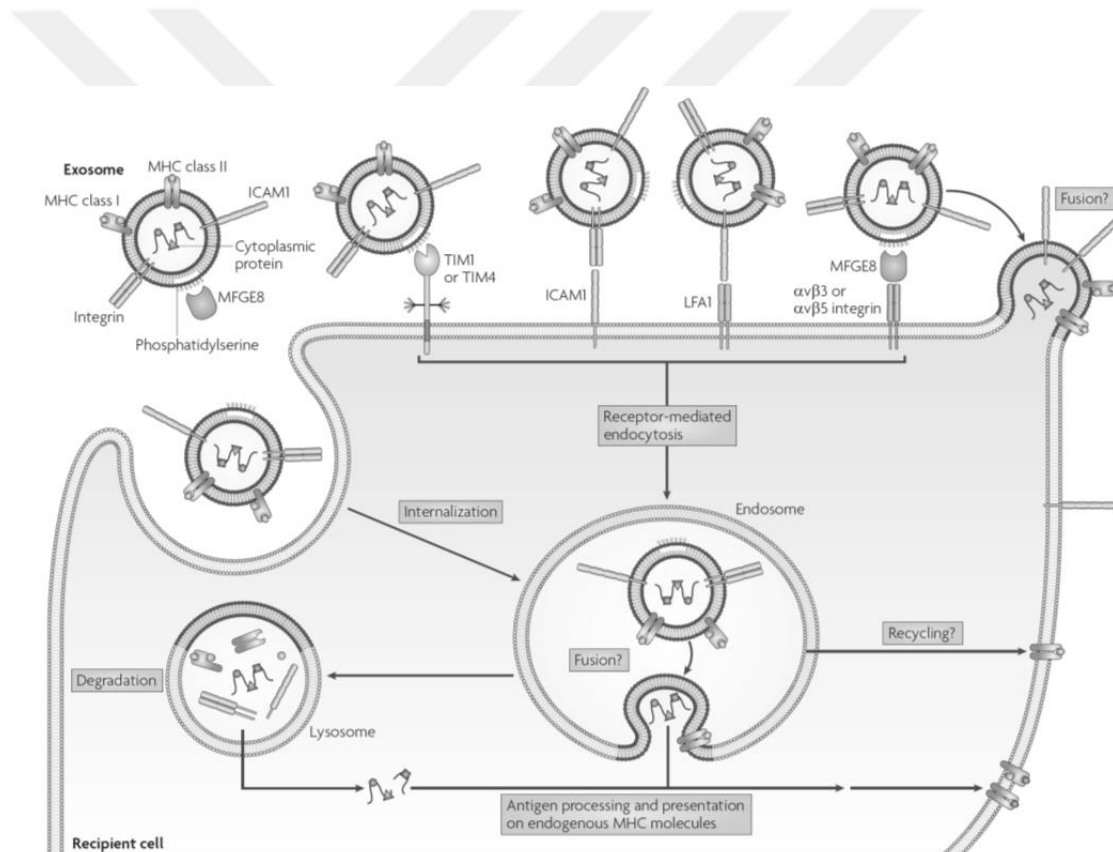


Figure 1.2. Different interaction mechanisms between cells and EVs. EVs are internalized by cells by direct internalization, receptor mediated endocytosis or fusion mechanisms. EVs can be directly pinocytosed by phagocytes to endosomes and then degraded in liposomes. Another internalization mechanism leading to same outcome is receptor mediated endocytosis of EVs into endosomes of recipient cells. Indicated receptors on cells and target molecules on EVs plays crucial roles in these interaction. After binding onto the cell EVs can be fused with plasma membrane, hence releasing its cargo directly into cytosol of the cell. In all of these scenarios, EVs modifies behavior of the recipient cell's behaviors. (Adopted from [26])

1.2. Physiological Roles of Microvesicles and Exosomes in Health and Disease

EVs were initially regarded as nothing more than cellular debris with no biological importance when first identified in 30 years ago as reticulocytes disposing transferrin receptor via small vesicle secretion [50, 51]. After showing that B-cell derived exosomes can present antigens to T-cells [3], EVs research gain interest. Eventually these vesicles were acknowledged to have important physiological roles in cell to cell communication by transfer of lipids, proteins and genetic materials between cells [52-54]. In-vitro and in-vivo studies performed on EVs showed that they have important roles not only in normal physiological process but also in stem cell maintenance [55], tissue repair [56], immune surveillance [3] and blood coagulation [57], as well as in the pathology underlying several diseases [58] (Figure 1.3).

Roles of exosomes and MVs in disease pathology have been intensively studied. Circulating MVs are mostly related with the pathogenesis of autoimmune diseases and vascular diseases as well as identification of disease related biomarkers [58, 59]. On the other hand, exosomes are mostly regarded as fine tuners of homeostasis of immune system as some types of exosomes activate adaptive immune system while others are suppressing [26]. Having crucial role in homeostasis both in healthy and disease, exosomes have been target for wide variety of biopharmaceutical applications such as isolation of disease markers [60], drug and vaccine delivery [61, 62] and cancer therapy.

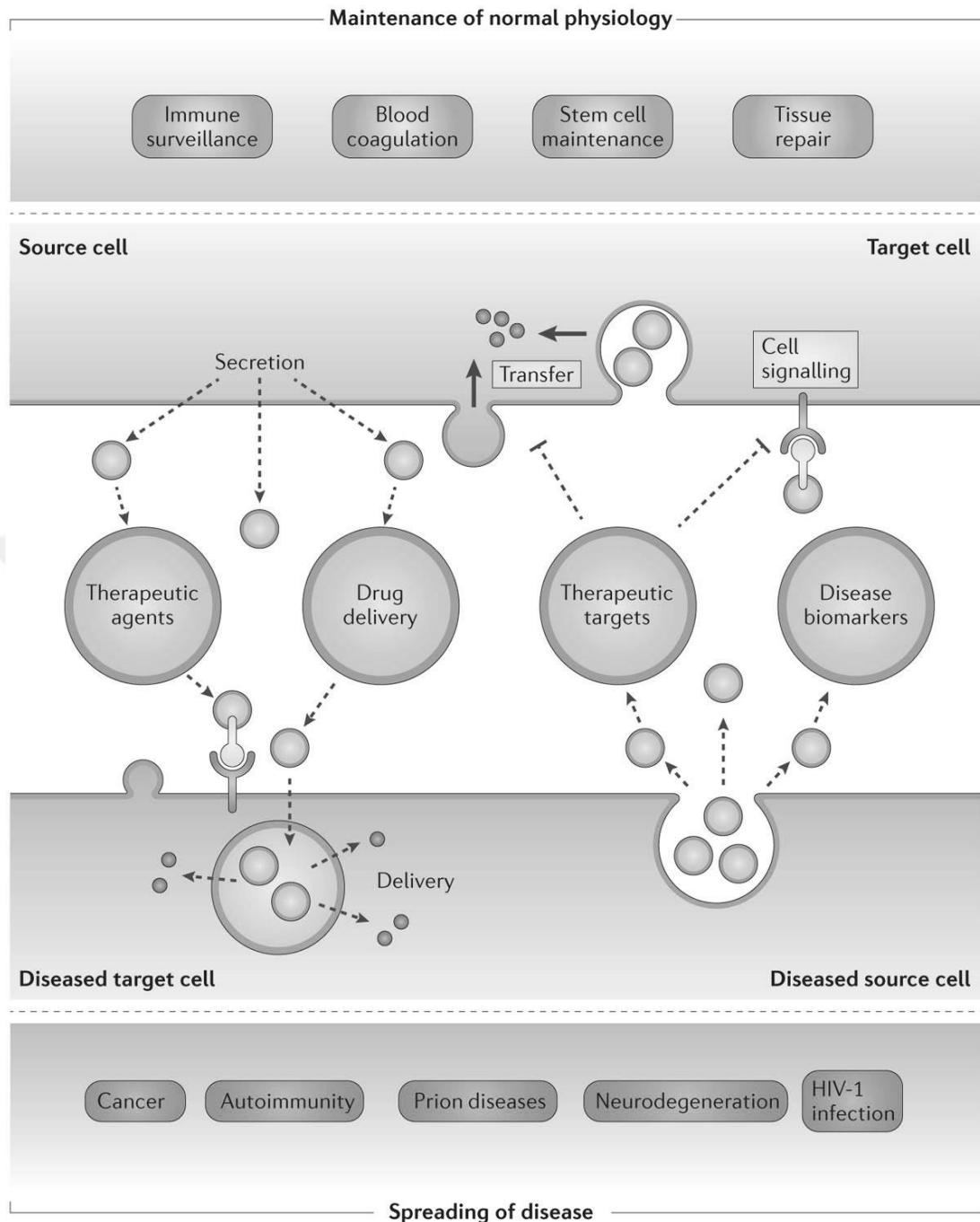


Figure 1.3. Physiological role of EVs in health and disease. EVs play an important role in maintaining homeostasis as signal transducing vector. In normal physiology, these vesicles monitoring and fine tuning immune system, participating in tissue repair as well as blood coagulation. They have been shown to have important function even in stem cell maintenance. On the other hand, these vesicles play also important role in spreading and progression of diseases such as infections, autoimmune and neurodegenerative diseases as well as cancer. The function and effect of EVs on recipient cell depends on the origin of the EV. (Adopted from [63])

1.2.1. Role of microvesicles in disease pathology

Because of their crucial roles in homeostasis, circulating MVs were extensively studied in pathogenesis of vascular and rheumatic diseases as well as in cancer.

1.2.1.1. Microvesicles and vascular involvement

The importance of MVs in homeostasis was first shown in patients with Scott syndrome, which is bleeding disorder [4]. In these patients, decreased levels of MVs was report due to atypical function of scramblase enzyme which is responsible for PS expression and partly generation of MVs [22]. On the other hand, platelet derived MVs (PMV) were increased in the plasma of patients with hemophilia and idiopathic thrombocytic purpura during acute bleeding occurrences [23]. These data suggest that MVs play crucial role in coagulation and thrombosis. Further findings have been supported this hypothesis. PMVs was shown to participate in catalysis of prothrombin to thrombin via high expression of PS molecules, which has binding sites for coagulation factors II, Va, and Xa [24]. Furthermore, not only PMVs but also endothelial (EMV), leukocyte (LMV) and monocyte (MMV) derived MVs contribute coagulation process. Recent studies showed that MVs carry also tissue factor (TF) and P-selectin glycoprotein ligand 1 (PSGL1) apart from PS molecules [64]. TF and PSGL1 molecules plays crucial role in modulating clot formation. TF and PSGL1 carrying MVs was found to accumulate in plasma upon cell injury [65]. These MVs shown to participate in clot formation by binding P-selectins on platelets [66] Because of this procoagulant activity of MVs, they participate in thrombic tendencies of rheumatic diseases.

Because of procoagulant activities of MVs, their participation in pathogenesis of antiphospholipid syndrome (APS) was studied. These studies showed that EMVs and PMVs was increased in the plasma of patients with APS [67, 68]. LMVs expressing CD14 was also reported to be increased in the plasma of patients with APS [69] Furthermore, PMV elevation was found to be linked with history of thrombosis in the patients with antiphospholipid antibodies (aPL) whereas EMV increase was independent of aPL [68]. These findings highlight a possibility of using PMVs as a biomarker for thrombosis in individuals with APS.

1.2.1.2. Role of microvesicles in rheumatic diseases

It was shown that MVs can act as signaling vesicles in the pathogenesis of several rheumatic diseases. Upon releasing from cells via membrane blebbing, MVs mediate various functions and cellular communication. Significantly, MVs can modulate inflammation, vascular activity, thrombosis as well as angiogenesis during the pathology of rheumatic diseases. Considering these functions of MVs, studies found that patients showed increased levels of MVs in plasma in rheumatic diseases. More importantly, it was found that this increased levels of MVs correlates with disease severity [21].

Cardiovascular complications are hallmark of rheumatic diseases like rheumatic arthritis (RA) and systemic lupus erythematosus (SLE). Cardiovascular involvement during pathogenesis reflect defective coagulation and thrombosis. During atherosclerosis, activated platelets secrete increased levels of MVs as a result of high shear stress. PMVs was shown to stimulate endothelial cells and monocyte to increase expression of adhesion molecules [70]. Increased adhesion molecules on both endothelial and immune cells increase attachment of immune cells to artery wall, penetrate arteriosclerotic plaque and exuberate inflammation. In addition, PMVs might promote increased thrombosis and clot formation since levels of these vesicles was reported to increase dramatically in patients with rheumatic diseases especially RA [71] and SLE [72].

Role of MVs in pathogenesis of rheumatic disease is not restricted to cardiovascular aspect of these diseases but also plays crucial modulatory activity in inflamed joints. There are numerous evidences suggesting that inflammation in joints might be augmented because of pro-inflammatory nature of MVs released from platelets, granulocytes, monocytes and other immune cells in patients [73]. Involvement of MVs in inflamed joints was shown to be multi parametric as these vesicles stimulate synoviocytes to secrete cytokines and chemokines to attract more leukocytes, activate compliment system in the joint and induce/enhance expression of matrix metalloproteinases (MMPs) (Figure 1.4).

In-vitro studies showed that upon stimulation with arthritic joint MVs, synoviocytes secrete chemokine and cytokines including IL6, IL8, CCL2, CCL5, CCL8, ICAM1 and VEGF [74]. These cytokines and chemokines recruits more leukocytes to the inflamed joint. It was also shown that particular MVs are responsible for stimulation

of synoviocytes and release of inflammatory molecules that attracts and activates leukocytes as THP1 (human macrophage/monocyte cell line) but not CEM (human lymphocyte cell line) derived MVs modulate secretion of B-cell activating factor from synoviocytes [75].

Increased levels of C1q, C4 and C3-binding MVs was found in arthritic synovial fluid of patients with RA [76]. Correlation between increased C1q-binding MVs and IgG/IgM bound MVs in synovial fluid suggests that MVs activate classical pathway of complement system. Therefore, more leukocytes are recruited to and activated in arthritic joint further augmenting inflammation.

Another pathological role of MVs in arthritis might be induction of MMP expression and secretion from synoviocytes. In-vitro studies confirmed that MVs originated from monocytes and T-cells of arthritic joint stimulate fibroblast like synoviocytes to express and secrete MMP3, MMP9 and MMP13 [77]. Release of these molecules could destruct joints of patients with rheumatic disease. This hypothesis was confirmed by the inverse correlation between modified Rodnan skin thickness of systemic sclerosis (SSc) patients and levels of MVs in synovial fluid [78].

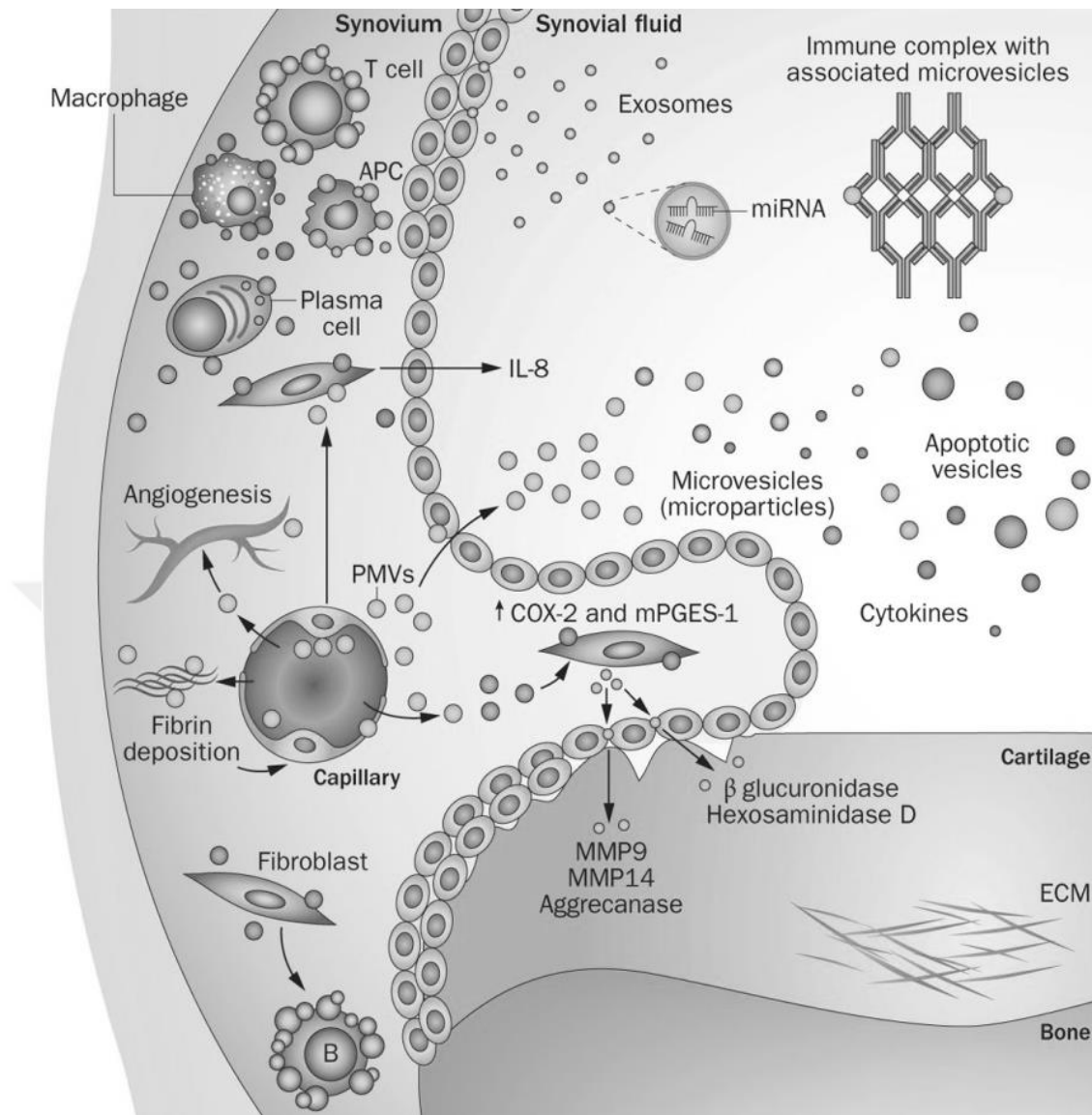


Figure 1.4. Participation of MVs in arthritic joint. Synovial fluid MVs stimulate synoviocytes to secrete chemokines and cytokines as well as carrying cytokines such as IL-1 β and IL-8 themselves. Furthermore, they form immune complexes with immunoglobulins hence activates classical complement system. MMP expression by synoviocytes was also found to be enhanced upon stimulation with circulating and synovial MVs of patients with RA. (Adopted from [79])

1.2.1.3. Microvesicles and cancer

Tumor derived (TMVs) and host derived MVs participates in pathogenesis of cancer. Healthy cell derived MVs induce tumor invasion, angiogenesis and thrombogenicity while tumor cell derived MVs modulate cell activation, endothelial cell dysfunction, apoptosis, thrombogenicity and immune suppression (Figure 1.5).

Spontaneous clot formation both in veins and arteries of cancer patients are widely seen complications. One of the main reason for clot formation is increased expression of TF in tumor and healthy cells of cancer patients [80]. Therefore, TF and PSGL1 expressing MVs might participate in abnormal coagulation events seen in cancer patients. This was proven by infusing fluorescently labelled tumor MVs to mice and inducing thrombus formation [81]. The study showed that tumor derived TF and PSGL1 expressing MVs shorten bleeding time as well as participation thrombus formation evident by fluorescent thrombi occurrence. More strikingly, it was found that TF bearing MVs are the sole source of TF activity seen in cancer patients [82]. These studies shows that both tumor- and healthy cell-derived MVs participates in abnormal coagulation events seen in cancer patients.

TMVs mediate cellular dysfunction and apoptosis of several cells especially immune cells [83]. Cancer cells mediate apoptosis of Fas expressing immune cells by secreting Fas ligand (FasL) bearing MVs [84]. Several reports showed that FasL induced apoptosis via TMVs is common mechanism among cancer types. TMVs isolated from serum of patients with oral cancers kills T-cells by Fas-FasL interaction [85]. Similar situations were seen with colorectal cancer cell- and melanoma cell-derived TMVs inducing apoptosis of activated T-cells and lymphoid cells, respectively [86, 87]. In addition, TMVs suppress immune cells. It is shown that TMVs attenuate monocyte differentiation into DCs [88]. Upon incubation with tumor derived MVs CD14⁺ monocytes differentiated into CD14⁺ MHC low suppressive cells. Similarly, human melanoma and colorectal carcinoma cell derived TMVs mediate generation of M2 macrophages suppressing activated immune cells [89].

It was shown that in cancer patients PMVs, EMVs and TMVs carry growth factors, TF and MMPs that can modulate angiogenesis. TF can stimulate angiogenesis via its cytoplasmic domain independent of its pro-coagulant effect [90]. Tumor growth was attenuated upon using TF-inhibiting factors showing importance of increase TF expression in cancer [80]. MVs in cancer patients may induce angiogenesis via TF cascade. Other than TF, PMVs induce angiogenesis via PI3 kinase, Src kinase, and ERK both *in-vitro* and *in-vivo* [91]. Inhibiting VEGF and PDGF activity attenuated PMV induced angiogenesis. PMVs was also shown to enhance invasiveness and metastasis of breast and lung cancers [92, 93]. TMVs carry CD147, which induce MMP expression, and truncated form of oncogenic epidermal growth factor receptor

variant III (EGFRvIII). As a result, these vesicles induce expression and secretion of MMPs by endothelial cells as well as transfer oncogenic activity [5, 94].

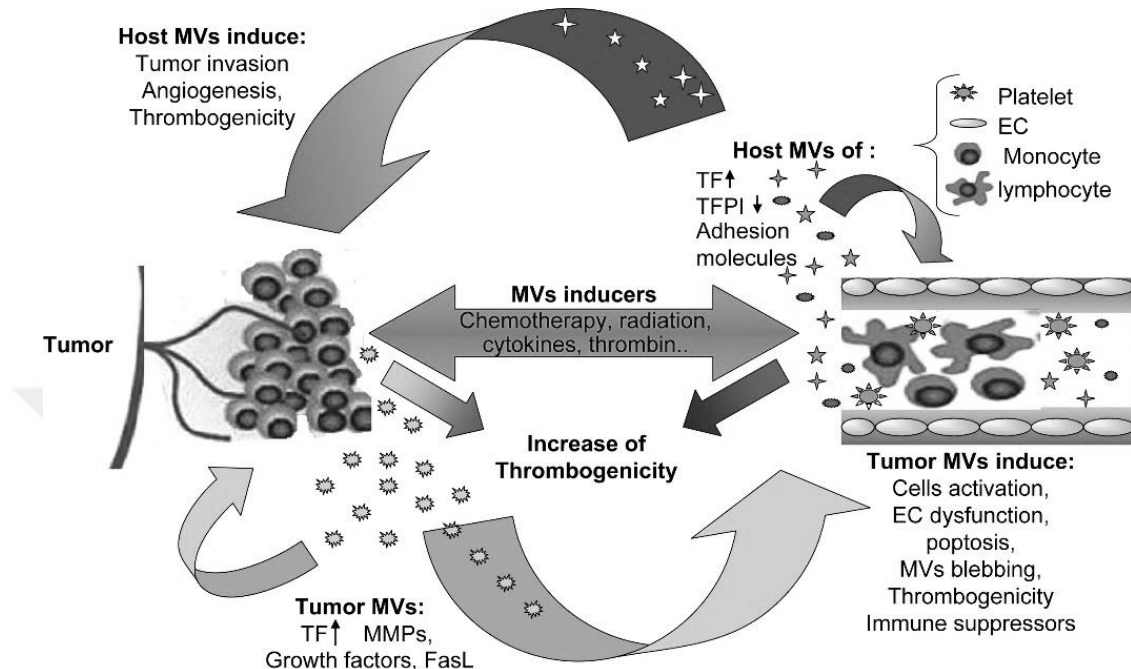


Figure 1.5. MVs participate in tumor pathogenesis. MV release are stimulated by external stimuli such as chemotherapy, radiation, cytokines, thrombin and so. Tumor cells secrete MVs carrying TF, growth factor, FasL and MMPs while healthy cells release MVs with increased TF and adhesion molecules. Both tumor- and healthy cell-derived MVs contribute pathogenesis of cancer. (Adopted from [95])

1.2.2. Exosomes and immune system

Exosomes are proposed to participate in several stages of immune response as suggested by both *in-vitro*, performed by exosomes isolated from body fluids and cell culture supernatants, and *in-vivo* studies. These studies showed that with respect to originating cell's type and state exosomes can either activate (Figure 1.6) or suppress (Figure 1.7) immune responses.

1.2.2.1. Activating effects of exosomes on immune cells

It was shown that exosomes exert their effect on immune cells in several ways; some vesicles carry peptide-MHC complexes to modulate antigen presentation

while others bear proteins and other ligands to activate immune cells antigen independent manner (Figure 1.6).

Exosomes derived from tumor cells was shown to be source for tumor antigens [96]. Release of exosomes from tumor cells enable tumor specific antigens accessible to APCs otherwise could not encounter. These exosomes are tumor derived antigen source for antigen presenting cells (APCs). Exosomes released from tumor cells can carry surface antigens like epidermal growth factor receptor 2 (EGFR2) and carcinoembryonic antigen (CeA) or antigens from endosomes such as melanoma antigen recognized by T cells 1 (MART1), glycoprotein 100 (gp100) and tyrosinase-related protein 1 (TYRP1). Upon internalization by APCs, exosomal cargo containing tumor antigen released and mounted onto MHC complexes then activates antigen specific T-cells mediating CTL response against tumor cells [97]. Moreover, exosomes released from infected cells also transfer antigens of pathogens to APCs and then activates CD4⁺ T-cells indirectly via DCs. Exosomes released from *M. tuberculosis* infected macrophages contain pathogen derived antigens and contribute to activation of CD4⁺ and CD8⁺ T cells both *in-vitro* and *in-vivo*.

It was also shown that any cell type bearing MHC molecule can secrete exosomes carrying peptide-MHC complexes on their surface. Therefore, these exosomes can present antigens directly to T-cells. DCs originating exosomes was shown to activate CD8⁺ T-cell clones alone or together with allogeneic MHC expressing DCs showing that peptide-MHC complexes on exosomes are functional [98, 99]. Not only DCs derived exosomes but also exosomes derived from other APCs can activate CD4⁺ T cells by presenting peptide-MHC II complexes on their surface [3, 100]. APC derived exosomes can directly trigger immune by antigen presenting to cognate T-cell clones/lines and pre-activated CD4⁺ T cells; however, they need recipient DCs to activate naïve CD4⁺ T cells [101, 102]. In addition, it was also found that exosomes can transfer peptide-MHC class II molecules to MHC lacking DCs in order to gain ability to activate CD4⁺ T cells [103]. Interestingly, exosomes derived from mature DCs are more potent inducers of both CD4⁺ and CD8⁺ T-cell mediated immune responses with respect to exosomes originating from immature DCs due to expression of co-stimulatory molecules [100, 104].

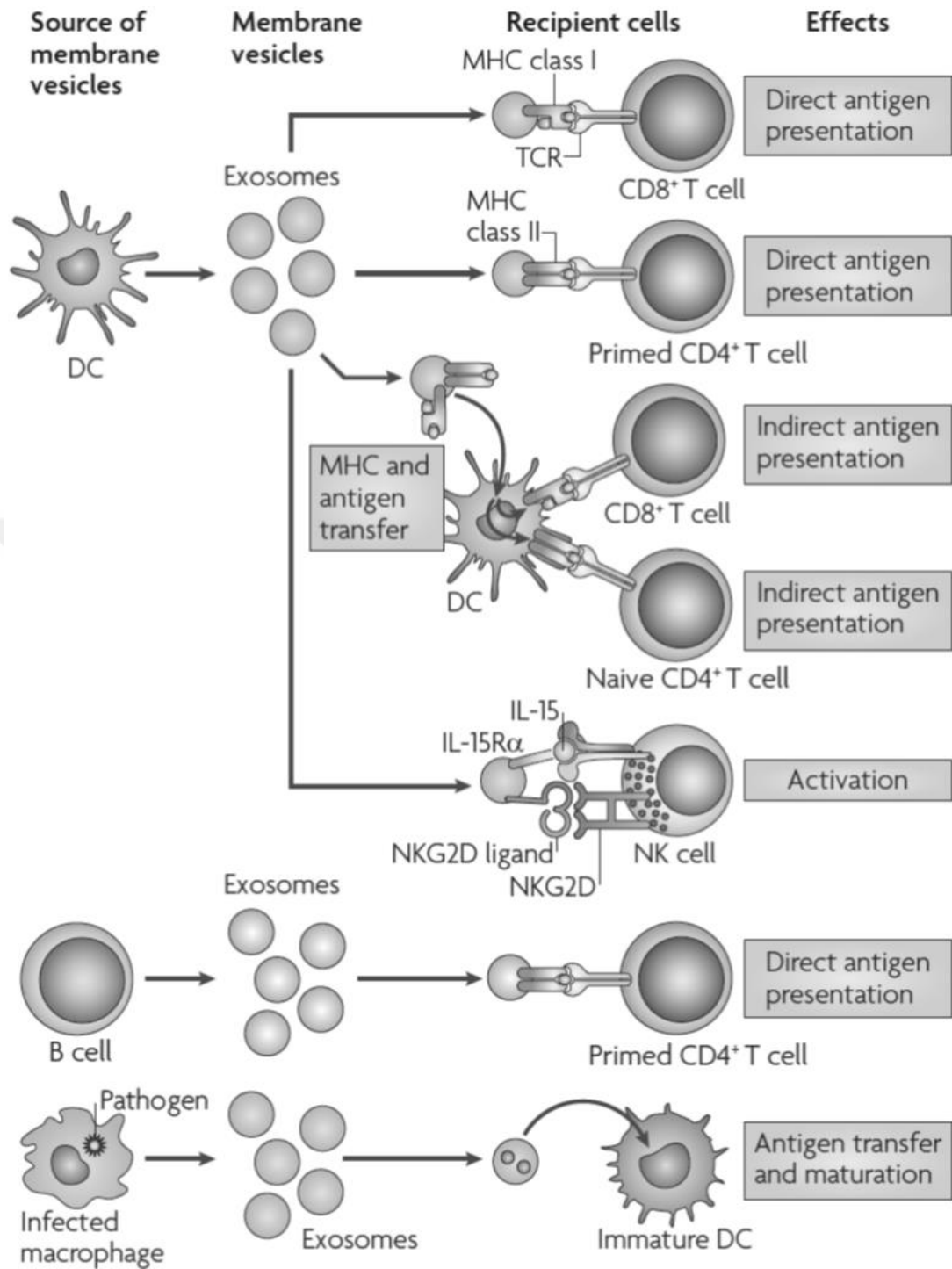
Exosomes has been shown to induce immune activation independent antigens. Pathogen infected macrophages known to secrete exosomes containing molecules

inducing secretion of pro-inflammatory cytokines by receiving macrophages [105]. This observation becomes important as *Mycoplasma arginine* or *Mycoplasma orale* infected cultured cells secrete exosomes capable of inducing clonal expansion of B and T-cell clones as well as maturing DCs [106].

Exosomes derived from immature DCs express functional NK cell activating receptors. In several studies, DCs derived exosomes secreted upon heat shock activate NK cells via HLA-B associated transcript 3 (BAT3) or UL16 binding protein 1 (ULBP1), MHC class I polypeptide-related sequence A (MICA) and MICB [107, 108]. However, these molecules are not enough to activate NK cells, it is of important to exosomes express IL-15 receptor α -chain (IL-15R α) simultaneously to bind exogenous IL-15 thus activate and proliferate NK cells [108].

Under stress induced conditions, tumor cells reported to secrete exosomes with immune activating roles. Heat shocked tumor cells secreted exosomes bearing heat shock protein (HSP70). These exosomes shown to induce NK cell activity and tumor necrosis factor α (TNF α) release from macrophages [109, 110]. Lymphoma and carcinoma cells was also reported to secrete immunogenic exosomes under stress induced conditions that can effectively induce CeA specific CTL response [111].

Other than APC and tumor derived exosomes, exosomes originating from fibroblast was also reported with immunogenic functions. Fibroblast isolated from synovial fluids of RA patients release exosomes expressing membrane bound TNF. These exosomes bind to primed CD4⁺ T-cells through TNF-TNFR1 (Tumor Necrosis Factor Receptor Type I) interaction and make T cells resistant against activation induced cell death (AICD) [112].



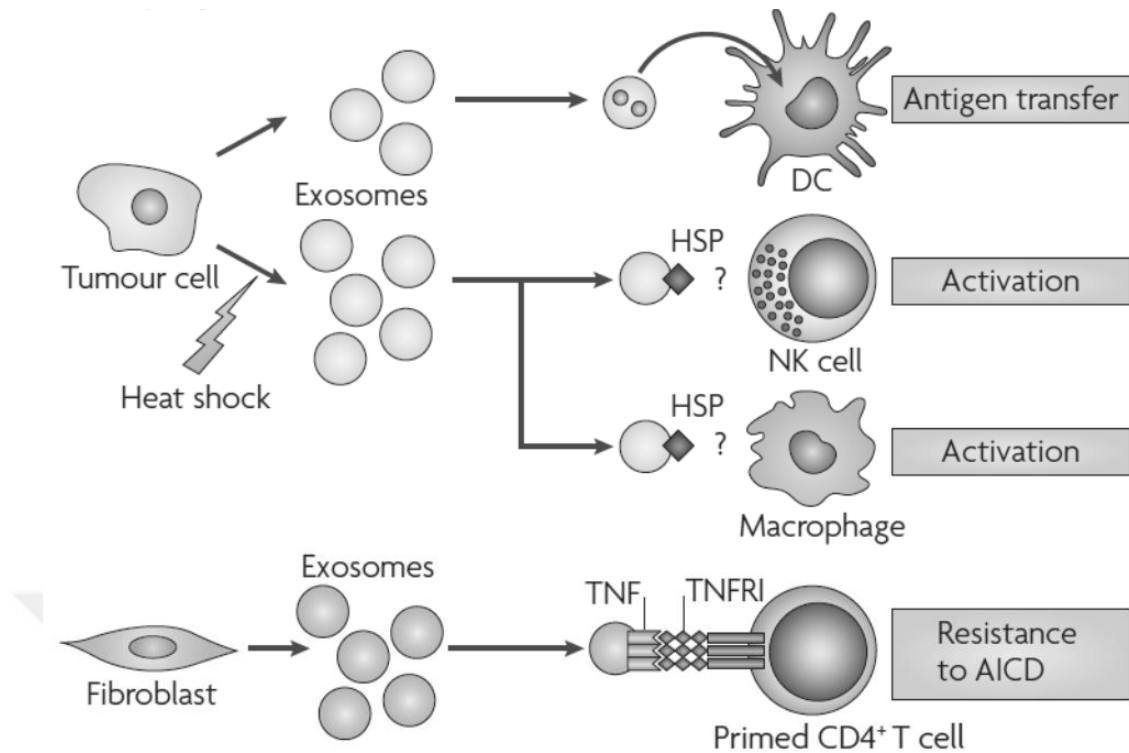


Figure 1.6. Immune response inducing effects of exosomes. Exosomes originating from different types of cells display various activating effects on immune cells, including direct antigen or peptide-MHC complex transfer to DCs leading to indirect antigen presentation, direct peptide-MHC complex presentation to both CD4⁺ and CD8⁺ T cells, activation of NK cells, macrophages and B cells, maturation of DCs, and TNF-dependent protection of CD4⁺ T cells against AICD. (Adopted from [26])

1.2.2.2. Suppressive effects of exosomes on immune cells

As well as promoting immune responses, exosomes can inhibit immune activation. Early studies on exosomes revealed that tumor derived exosomes are capable of immunosuppressive properties [113]. Exosomes isolated from tumor cell lines or tumor-bearing patients were reported to mediate T-cell apoptosis via FasL [114] and galectin-9 [115], in-vitro. In addition, exosomes can attenuate IL-2-induced T cell proliferation [116], promote T-cell to differentiate into regulatory T-cells [117] hence inhibiting immune responses. As shown in tumor derived exosomes mediate apoptosis of activated tumor specific T cells via Treg activation [118]. In addition, tumor exosomes can attenuate cytotoxic activities of CD8⁺ T cells and NK cells [119]. Expression of NK2GD ligands and membrane bound form of transforming growth factor β (TGF β) on exosome are of important for inhibition of cytotoxicity. Tumor exosomes downregulates NK2GD receptor on NK-cells via its ligands

expressed on the surface hence impairing NK-cell cytotoxicity [120]. Furthermore, membrane bound form of TGF β on tumor exosomes is responsible for suppression of several immune cells. Exosomes impair myeloid precursor differentiation into DCs and mediate myeloid-derived suppressor cell (MDSC) differentiation as well as Treg differentiation via TGF β on their surface [88, 121-123]. Heat shock protein 70 (Hsp70) is another protein carried by exosomes inducing immune suppression. Hsp70 carrying tumor derived exosomes induce activation of MDSC via producing p-Stat3 in a TLR2-MyD88 dependent manner [124].

Exosomes derived from other than tumor cells have also suppressive properties. Expression FasL on the exosomes released from activated T-cell induce apoptosis of neighbor T-cells hence participating in AICD [125]. Other than activated T-cells, MDSCs secrete immunosuppressive exosomes. Exosomes released from immature DCs were also shown to suppress immune responses as well as mediating immune tolerance. In experimental autoimmune myasthenia gravis (EAMG), immature DC derived exosomes attenuates activity of acetylcholine receptor (aChR) reactive lymphocytes [126]. These exosomes in combination with rapamycin can delay cardiac allograft rejection and induce tolerance [127].

Recently, CD4⁺CD25⁺Foxp3⁺ Tregs shown to release exosomes like other immune cells [128]. TCR activation of Tregs resulted in release of exosomes characterized by CD73 expression contributing their immune suppression. These exosomes have the capacity to attenuate DC induced cytotoxicity of CD8⁺ T-cells. Releasing exosomes is of important in suppressive capacity of Tregs since blocking exosomes secretion was found to attenuate their suppressive function [129]. CD25 and CTLA4 was also found on Treg derived exosomes suggesting that they might also inhibit activated CD4⁺ and CD8⁺ T-cells CTLA4 dependent manner as their progenitor cells do [128]. APCs were shown to endow new novel functional properties via cross-dressing these high CD25 expression Treg derived exosomes [130]. Upon cross-dressing APCs binds free IL2 in the environment and leading to apoptosis of effector T-cells.

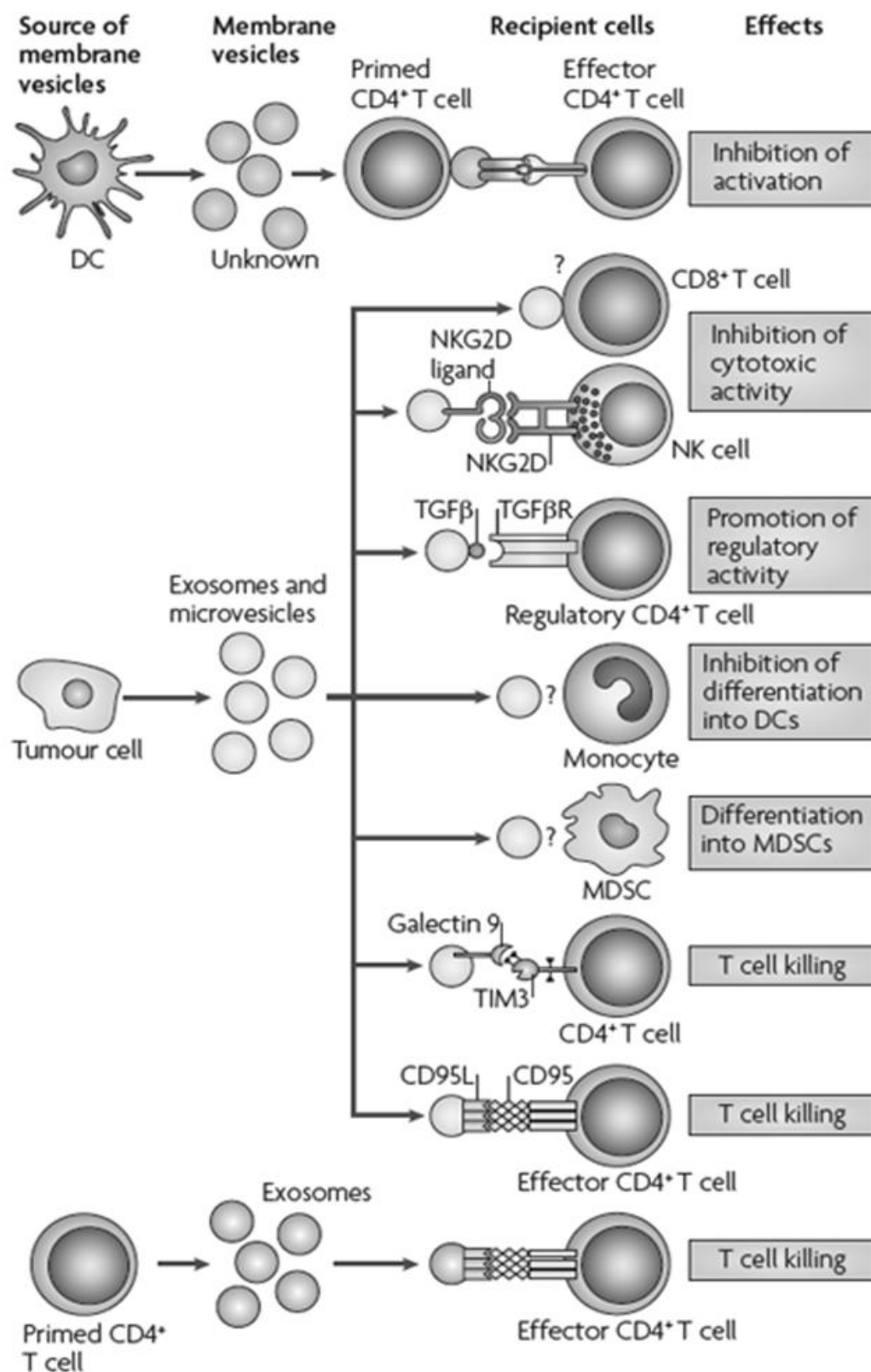


Figure 1.7. Suppressive effects of exosomes on immune cells. Exosomes can attenuate immune responses in various ways, inhibiting cytotoxicity of CD8⁺ T-cell and NK cells, promoting Treg proliferation, impairing DC generation, favoring MDSC differentiation and proliferation as well as inducing apoptosis of activated T-cells. (Adopted from [26])

1.3. Extracellular Vesicles in Clinical Applications

1.3.1. Therapeutic applications of unmodified EVs

The roles in intercellular communication, immunomodulatory effects and the cargo of EVs as well as participation in homeostasis in both health and disease suggest that they can be used in several therapeutic applications. As being natural vectors for genetic materials (mRNAs and miRNAs), proteins and lipids, exosomes are stable vesicles as evidenced by existing in every body fluid which strengthens the idea of using them as therapeutic agents. Therefore, these vesicles can be used for several applications including immune-modulatory and regenerative, immune tolerance and anti-tumor therapies.

Increasing evidence suggests that EVs derived from particular cell type that is in appropriate state can be used by itself for immune-modulation in autoimmune and inflammatory diseases as well as regenerative purposes (Figure 1.8). EVs derived from MSCs, endothelial progenitors, Tregs, DCs as well as of many other cell types, are promising therapeutic agents in regenerative medicine and immune therapy. In many studies, human EVs show promise in animal models and the effect seen was conserved between species.

Mesenchymal stem cell (MSC) derived EVs (MSC-EVs) were the most extensively studied population in this aspect. Today several clinical trials were under investigation that uses MSC-EVs as therapeutic agents [131]. First studies investigating therapeutic potential of MSC-EVs showed that these vesicles reduce infarct size in myocardial ischemia/reperfusion model and protect against tubular injury in experimental model of acute kidney injury [132, 133]. It was demonstrated that these vesicles exert anti-apoptotic activities comparable to their originating cells hence induce protection against tissue destruction. Furthermore, this protective effect increased in MSC-EV rich fragment rather than depleted one confirming that this effect is MSC-EV dependent [132]. It was also found that MSC-EVs indirectly activate Tregs via M2 macrophage polarization [134]. Additionally, MSC-EVs was found to inhibit activity of NK cells and other immune cells in graft versus host disease (GvHD) patients leading to be tested on steroid resistant GvHD patients [135].

Currently, Treg exosomes are proposed to be used in transplantation tolerance. Tregs, themselves, are trying to be used to prevent rejection of grafts; however, upon administration they tend to lose their suppressive ability due to the inflammatory environment [136]. Since Treg exosomes exhibit enormous suppressive ability and do not effected by the inflammatory environment, these vesicles can be used as alternative or complementary therapy against rejection of transplant. Although, using these exosomes in man is a long way away, several studies showed promising results. Adoptive transfer of autologous Treg derived exosomes was shown to increase survival and function of kidney transplants [137].

As mentioned above EVs derived from mature DCs exhibit immune inducer functions due to expression of MHC complexes as well as co-stimulatory molecules. When these vesicles were harvested from tumor antigen pulsed DC, they induced rejection of tumor via activation of tumor-specific T-cells upon administration to tumor engrafted mice [138]. This finding encouraged 2 phase I clinical trial to be investigated. EVs of antigenic tumor peptides patient's monocyte-derived DCs (MD-DC) was used for anti-tumor therapy against melanoma and non-small cell lung carcinoma [139-141]. Recently, IFN γ treated MD-DC derived EVs were expressing higher amount of ICAM1, CD40 and B7 molecules hence have higher stimulatory capacity. Therefore, EVs isolated from antigenic tumor antigen pulsed IFN γ -matured MD-DCs were used for phase II clinical trials to treat non-small lung carcinoma [142]. In addition to mature DC derived EVs, human NK cell originated EVs have been reported to promote immune stimulation leading to lysing of the human tumor cells *in-vitro* [143]. Because of these findings and promising clinical trials, EVs, isolated from appropriate cell type and state, are potential tools for next-generation anti-tumor therapies.

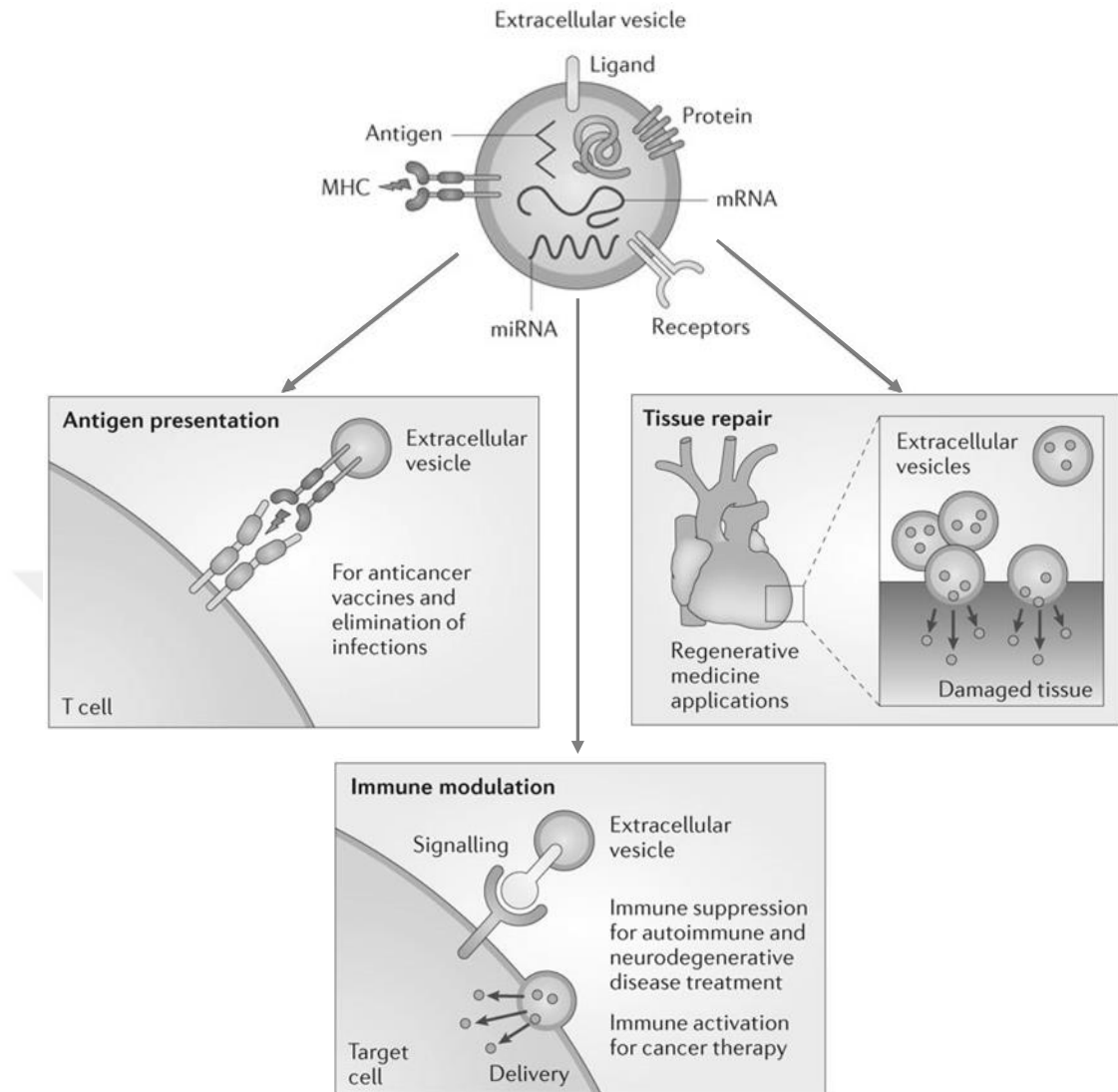


Figure 1.8. Therapeutic applications of unmodified EVs. EVs derived from tumor antigen pulsed APCs can be used to activate T-cells against tumor cells. Using MSC-EVs was seen to complementary in regenerative medicine as well as immune modulation. EVs can be used as immune stimulatory or suppressive depending on the originating cell and its state. (Adopted from [63])

1.3.2. Modified exosomes as therapeutic agents

There are several studies that support the notion that exosomes can deliver immunomodulatory drugs to cells, thus can be used as therapeutic vehicles (Figure 1.9). Naturally occurring exosomes becomes very suited candidates for drug delivery purposes. One of several reasons is the exchange of genetic material mediated by exosomes [144]. In addition, the ability of exosome to carry its cargo into recipient cells and mediate different cellular responses demonstrated in various

studies [2, 62, 98, 145]. Melanoma derived exosomes target sentinel lymph nodes in order to promote tumor metastasis [146] which indicates targeted delivery of exosomal cargo into the recipient cells.

There are two strategies introduced to load exosomes with drugs; in vivo loading and in vitro loading. In in vivo loading, the interested drug's gene introduced into exosome secreting cells which limits the variety of drugs to be loaded to RNA and proteins [147, 148]. On the other hand, in in-vitro loading strategies, drug of interest loaded into exosomes by physical entrapment [61, 149] or membrane breaching techniques [150-152]. Curcumin was loaded into EL-4 derived exosomes by mixing at room temperature. Encapsulating curcumin into exosomes was shown to enhance its anti-inflammatory effect [61]. This complex was also shown to penetrate blood-brain barrier into the brain and mediate reduced LPS-induced brain inflammation [149]. Likewise, it has been demonstrated that siRNA [153] and miRNA [154] loaded exosomes mediate attenuation of tumor growth.

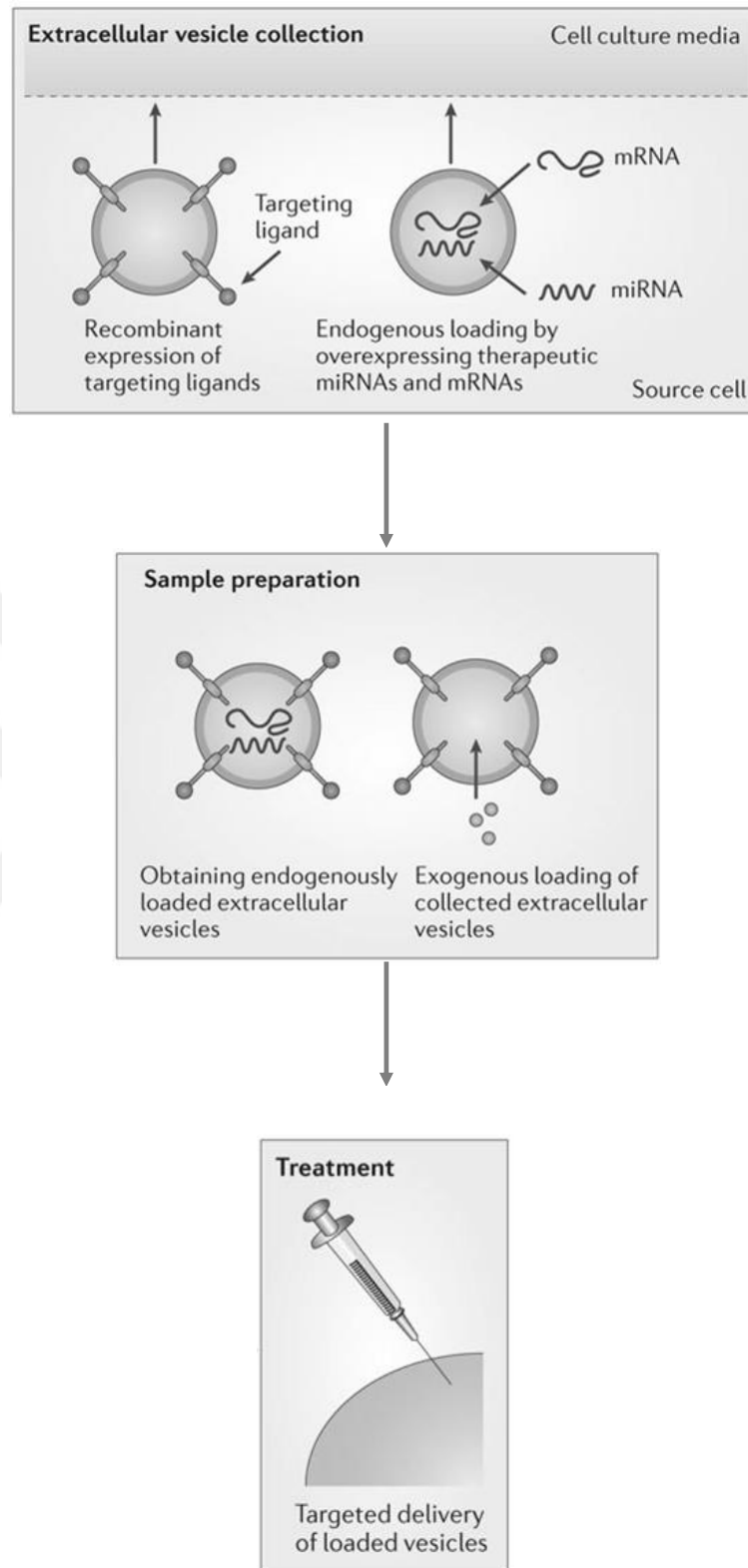


Figure 1.9. Modification of exosomes for therapeutic purposes. *Modification of originating cell, endogenous loading and exogenous loading methods are the common way to engineer exosomes to use as therapeutic vesicles.(Adopted from [63])*

1.3.3. Diagnostic and prognostic potential of EVs

As mentioned above, EVs can be detected from all biological fluids including plasma, serum, saliva, urine. EVs are released to body fluids constitutively into body fluids and changing with respect to extracellular environment both in health and disease. These vesicles have been reported to be participating pathogenesis of many diseases including autoimmune and auto-inflammatory diseases, cancer and neurodegenerative diseases. This raises the question of whether EVs can be used as diagnostic tools for detecting and screening of certain diseases.

It is known that miRNA profile of a cancer cell is different from healthy counterpart and differentially expressed miRNA profiles have been identified from biopsy samples. However, using identified miRNA molecules as diagnostic or screening tools is impossible for two major; i) obtaining biopsy for most cancer types is extremely invasive and ii) miRNAs have very short half-life in body fluids [155]. However, since EVs mimics their originating cell and carry/transfer miRNA molecules, they can be used as source for these cancer specific miRNA molecules. Several studies confirmed that EVs carrying identified cancer-specific miRNAs can be isolated from serum/plasma of patient. In non-small cell lung carcinoma patients miR-1, 25, 30d, 223, 486 and 499 was found to be differentially expressed compared to healthy samples in the EVs [156, 157]. Exosomes from serum of prostate cancer patients carry miR21, 107, 141, 181a-2, 301a, 326, 331-3p, 625 and 2110 molecules that can be used to identify and prognose cancer in patient [158]. In addition to non-small cell lung carcinoma and prostate cancer, glioblastoma[59], lung squamous carcinoma [159] and hepatocellular carcinoma [160] patients were identified with EVs transferring cancer specific miRNA in their bloodstream. These findings indicate that EVs has a huge potential to be used in diagnosis as well as prognosis of cancers in near future.

In addition to miRNA molecules, EVs was found to carry cancer-specific proteins in the patients that can be harvested from plasma/serum [161]. Several studies were conducted to investigate EVs for identification of tumor antigens. In Table 1.2, promising tumor antigens found differentially expressed in EVs of cancers patients with respect to healthy samples is outlined.

Table 1.2. Tumor antigens found in EVs of cancer patients.

Disease	Markers	Vesicle Type	Ref.
Ovarian cancer	L1CAM, CD24, ADAM10	Exosome	[162]
	TGF β 1, MAGE3/6	Exosome	[163]
	Claudin-4	MVs	[164]
Glioblastoma	EGFRvIII	Exosome	[59]
	EGFR, EGFRvIII, PDPN, IDH1	MVs	[165]
Melanoma	CD63 and Caveolin 1	Exosome	[166]
Oral cancer	FasL	MVs	[85]
Gastric cancer	HER-2/neu, CCR6	MVs	[167]
Cancer-associated thrombosis	Tissue factor (CD142)	MVs	[168]

L1CAM; L1 cell adhesion molecule, ADAM10; A disintegrin and metalloproteinase domain-containing protein 10, TGF β 1; Transforming growth factor β 1, MAGE3/6; Heterodimer of Melanoma-associated antigen 3 and 6, EGFRvIII; Epidermal growth factor receptor variant III, PDPN; Podoplanin, FasL; Fas Ligand,

1.4. Immunostimulatory Ligands

There are several ligands activating immune cells; however, in the context of this thesis CpG oligodeoxynucleotide (ODN) and cyclic-di-GMP (c-di-GMP) is of important.

1.4.1. CpG ODN and immune system

Nucleic acid fragments from bacteria have shown to exhibit anti-tumor effects [169]. Initially, it was thought that the palindromic regions of bacterial DNA is responsible for activating immune cells but it turned out that sequences of unmethylated CpG flanked by two purines and two pyrimidines on 5' and 3' side, respectively, triggers immune responses [170]. In mammalian DNA, these CpG sequences are 20 fold less frequent as well as methylated attenuating any immune stimulatory effects. This difference in frequency and methylation status have been led to evolutionary conserved recognition of bacterial DNA vs mammalian DNA by Toll-like receptor 9 (TLR9) [171]. Bacterial DNA or synthetic ODNs mimicking bacterial DNA (CpG ODN) bearing CpG motifs induce immune cells to secrete pro-inflammatory and T-helper (Th) 1 biased cytokines [172].

All types of CpG ODNs contain at least one unmethylated CpG motifs but differentiate according to their flanking regions. With that respect there are two major types of CpG ODN; D-type (also known as A-class) and K-type (also known as B-class). In addition to these new type of (C-class) CpG ODN was described recently. D-type ODNs possess poly G tail enabling them to generate nanoparticle like structures whereas K-type ODNs may contain more than one CpG motif [173]. D type ODNs upon internalization retains in early endosome, due to their distinct structure, where interacts MyD88/IRF7 complexes leading secretion of $\text{INF}\alpha$ from pDCs, which was hallmark of D type ODN induced immune responses [174]. On the other hand, immune responses induced by K-type ODNs characterized by secretion of high amounts of $\text{TNF}\alpha$ from pDCs due to relocation to the late endosomes [175]. The pathway D- and K-type ODNs undertake after internalization and immune stimulatory pathways they induced outlined in Figure 1.10. Recently described C-class ODNs, on the other hand, combines advantages of D- and K-type ODN but to a lesser extent [176].

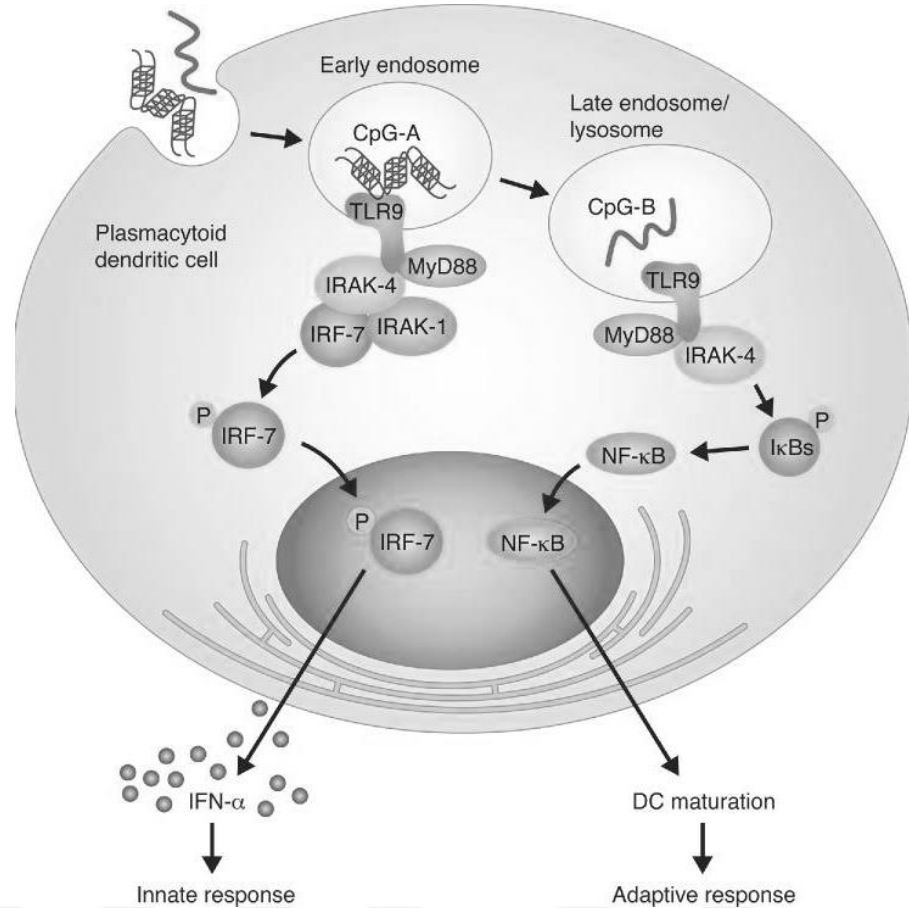


Figure 1.10. Localization and induced pathways of D- and K-type ODNs. *D-type ODN* localize early endosomes and induce secretion of type I interferons via MyD88/IRF-7 pathway. On the other hand, *K-type ODNs* interact with TLR9 in late endosome triggering NF-κB to secrete TNFα and IL6 to induce DC maturation.

1.4.2. CpG ODNs as vaccine adjuvants

Acting as ligand for TLR9, CpG ODNs directly activate pDCs and B-cell in humans and indirectly induce maturation and proliferation of NK cells, T-cells and macrophages [177, 178]. It was shown that CpG ODN stimulation upregulates expression of co-stimulatory molecules including MHC class II, CD40, CD80 and CD86 in APCs [170, 179, 180]. CpG ODNs activates B-cells by upregulating secretion of IgM, IL6 and IL10 as well as enhancing expression of Fc receptor [181]. CpG ODNs also induce secretion of IL1β, IL6, IL18 and TNFα from pDCs. More remarkably, they induce production and secretion of Th1 cytokines of IL12 and IFNγ [182]. These effects suggest that CpG ODNs can increase immunogenicity of conventional antigens used in vaccines.

There are more than 600 preclinical studies demonstrating increased immunogenicity of CpG ODN vaccines. Induction of CpG ODN into vaccines enhanced the humoral immune response against pathogens including influenza virus, anthrax, leishmania, measles virus, orthopox virus, tetanus toxoid and lymphocytic choriomeningitis virus [182-185].

Although, CpG ODNs performed well in preclinical trials. In clinical settings, they have several drawbacks. One of them is their short half-time in body fluids. Upon administration 90% of them are degraded within 30 min by nucleases which limits their potential as vaccine adjuvants. The other major drawback is their absorption by serum proteins hampering bio-distribution of CpG ODNs *in-vivo*.

1.4.3. Cyclic-di-GMP

c-di-GMP is a bacterial product working as secondary messenger. It was first identified in *Gluconacetobacter xylinus* [186]. Studies showed that c-di-GMP has immunomodulatory effects inducing innate immune responses [187]. In the presence of c-di-GMP, immature DCs showed upregulation of MHC class II, CD80 and CD86 as well as maturation marker of CD83. In addition, c-di-GMP stimulates DCs to secrete cytokines and chemokines including IL8, IL12, IFN γ , MCP1, IP10 and RANTES. c-di-GMP matured DCs was observed to exhibit enhanced capacity to activate T-cells. It was shown that these immunomodulatory functions of c-di-GMP is related with activation of p38 mitogen activated kinase protein in DCs [188]. Other than DCs, c-di-GMP activates also monocytes/macrophages to secrete IL6 and IFN β [189]. These functions of c-di-GMP makes them potential candidates as vaccine adjuvants. Studies showed that vaccine preparations containing c-di-GMP performs comparable to potent vaccine adjuvants of LPS and CpG ODN. Immune responses induced by c-di-GMP containing vaccines were more biased towards Th1 than other potent vaccine adjuvants

Although, c-di-GMP is a very potent immune cell inducer, it has an important drawback in usage as vaccine adjuvant. One of them is its negatively charged nature attenuates its passage through cell membrane. As a result, in order to obtain appreciable amount of immune stimulation it has to be used at high concentrations or need to be transfected [189, 190].

1.5. Behçet's Disease and Its Molecular Background

Behçet's disease (BD) is a chronic systemic inflammatory disorder classified among the vasculitides. Its worldwide distribution is restricted to the Far East Asia, Mediterranean basin and the Middle East [191]. The disease is widely characterized by occurrence of, oral aphthae, genital ulcers, skin lesions, and uveitis. Although it is possible to diagnose BD according to its clinical manifestations, the molecular mechanism behind its pathology is still not very well understood [192]. A complex genetic background leading to a pro-inflammatory, innate immune-system-derived activation perpetuated by adaptive immune responses against environmental and auto-antigens is accepted to be the hallmark of BD [193]. An augmented cytokine storm in BD with an elevated levels of pro-inflammatory cytokines, such as IL1 β , IL6, IL8, IL10, IL12, IL15, IL18, and TNF α is an indirect evidence of activation of different immune cell types [194], suggesting that BD clinical manifestations are a result of a complex interaction of a variety of immune cells. Although it may vary according to the type of the lesion and time of sampling, predominant cell type in the inflammatory lesions of BD patients is the CD4⁺ T lymphocytes. As in line with this observation, it was shown that injury to the skin of BD patients elicits a Th1 cell response owing to IL12 mediated production of IFN γ by CD4⁺ T cells [195]. It has also been demonstrated that an intensity of Th1-type polarization of immune response in BD is being correlated with disease activity [194, 196].

As similar to other forms of vasculitis, endothelial cell injury and resultant endothelial dysfunction due to vasculitis is regarded as one of the underlying features of BD [197-199]. The main mechanism of endothelial dysfunction has been shown to be associated with immune-mediated vascular injury, increased expression of pro-inflammatory and Th1 cytokines, adhesion molecules, and reactive oxygen species [199]. Thrombophlebitis, either of the superficial or deep veins, develops approximately in one-third of patients with BD. The pathogenesis of thrombophlebitis has been the subject of controversy since some claim that it is the result of pro-coagulant factors whether other thinks that it is caused by endothelial inflammation [192, 199]. However, no single coagulation abnormality has been described up to now apart from a decrease in thrombolysis [200].

1.6. Asthma, Atopic Dermatitis and Their Molecular Pathogenesis

Asthma is a chronic inflammatory disorder that affects the airways of atopic individuals upon exposure to allergens. Physiological manifestations of asthma are episodic and reversible airway obstruction and bronchial hyper-responsiveness [201]. At the childhood, the disease can be manifested with other allergic diseases such as atopic dermatitis and seasonal rhinitis [202]. The best characterized form of asthma is allergic asthma. There is a complex immune pathogenesis behind allergic inflammation. In the most recent model of this pathogenesis, allergic inflammation is initiated by the epithelial cells lined through the airway by the release of thymic stromal lymphopoietin (TSLP), IL33, and IL25 in response to allergens [203]. Major role of TSLP was proposed is to mediate migration of dendritic cells (DCs) to the area in turn promoting Th2 polarization of helper T cells. Th2 cells stimulate B cells to secrete antigen specific immunoglobulin E (IgE) molecules. Antigen specific-IgE, in turn, binds to mast cells and basophils in the airway, augmenting local allergic responses. On the other hand, the IL33 and IL25 produced by epithelial cells promote IL13 and IL5 release from IL25 receptor (IL25R) expressing natural helper cells in the airway [204], which then promote Th2 cell polarization and further secretion of IL4, IL5, and IL13 locally. In the process, memory T and B cells are generated that can facilitate more rapid responses to repeated stimulation and disease chronicity [205].

1.7. Subject and Outline of This Thesis

In the first part of this thesis, levels, origin and function of EVs in Th1 and Th2 biased disorders was analyzed. As mentioned above, studies revealed that levels of circulating EVs were elevated in several rheumatologic diseases, such as rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren's syndrome, vasculitis and anti-phospholipid syndrome [206-217]. However, the implications of elevated EV levels were not fully understood. In addition, all these diseases are coupled with vascular abnormalities that might EVs are accounted for. Since inflammation, endothelial dysfunction, and thrombotic tendency are collectively regarded as cardinal features of BD, we speculated that the levels and source of EVs along with their cargo might influence the course of BD in terms of disease activity and severity.

Since BD and other diseases are characterized as Th1 biased immune responses, we analyzed levels and differential origin in Th2 biased disorders. In this pursuit, plasma was collected from patients with asthma and atopic dermatitis as well as healthy controls. Their EVs composition was analyzed by flow cytometer as well.

In the second part of this thesis, we pursued the potential of exosomes to be used as vaccine carrier vesicles. To date, modified exosomes secreted from engineered cells were employed as the sole source for delivery purposes. Although this may offer some advantages, secreted exosomes of this sort, does not ensure the presence of cargo of interest in all particles, thus external loading that permits adjustment of the amount of internalized cargo per vesicle is superior approach for in vivo applications. In addition, current protocols propose exosomes as delivery vehicles for RNA molecules but not DNA molecules.

Present study describes a very efficient and mild approach to load/encapsulate CpG ODN within exosomes. Here, we demonstrate that ODN loaded vesicles can efficiently deliver their cargo to relevant immune cells and can also improve ODN internalization into target cells. Moreover, we demonstrated that the stability of these labile oligonucleotides were significantly enhanced along with their immunostimulatory action upon delivery. As a proof of concept, we utilized these vesicles as natural vaccine carrier vesicles that co-encapsulate CpG ODN along with ovalbumin. This platform offers a mild and easy approach that allows external loading of valuable oligonucleotides within naturally occurring exosomes and provide several advantages over currently existing synthetic delivery systems.

Finally, the potential of exosomes as drug carrier systems was tested with c-di-GMP as well. Since these molecules cannot penetrate into cells because of their negatively charged nature, exosomes were used as carrier systems for these molecules. Using exosomes increased both internalization and activity of these molecules.

Chapter 2

Materials and Methods

2.1. Patients and Controls

2.1.1. BD patients and controls

Consecutive patients, who were regularly examined at the Department of Rheumatology in Gulhane Military Medical Academy and had an established diagnosis of BD (according to the criteria of the International Study Group for Behçet's Disease [218]) were referred for the study after they had their last clinical evaluation to determine clinical disease status (See Table 2.1). For this study, peripheral blood of 72 BD patients (Active; n=35, Inactive; n=37) and 22 healthy control individuals were included. Patients having active disease was defined by the presence of two and more clinical characteristics such as; oral ulcers, genital ulcers, papulopustular or pseudo-follicular cutaneous lesions, active vascular disease and active ocular involvement. The patients who had no symptoms related with BD at the time of sampling and receiving steady medication for at least 1 month were classified as having an inactive disease. All patients were further categorized according to their organ of involvement as i) mucocutaneous, ii) ocular, and iii) vascular. With regard to the classification of those patients with inactive disease, patients' detailed clinical history was investigated with respect to either deep vein

thrombosis or arterial aneurysm and evidence for uveitis was sought. Subjects with hypertension, diabetes mellitus, renal disease or a previous history of coronary artery disease or myocardial infarction were excluded. The healthy control group included 22 individuals, matched for age and sex. Baseline characteristics of patients with BD and healthy subjects at inclusion are summarized in Table 2.1. The study was approved by the Institutional Review Board, and all participants provided their informed consents.



Table 2.1. Clinical evaluations of the BD Patients.

	Healthy (n=22)	Inactive BD (n=37)	Active BD (n=35)
Sex (M)	22	37	35
Age (year, mean±SD)	22±5	22±5	19±6
Disease Duration (year, mean±SD)	N/A	11±5	8±2
Disease Progression			
Mucosa	N/A	9	16
Vein	N/A	12	7
Eye	N/A	16	12
Treatment			
Colchicine	N/A	21	24
Azathioprine	N/A	14	11
Cyclophosphamide	N/A	2	0

2.1.2. Asthma, AD patients and controls

Patients and healthy peripheral blood were obtained from Department of Pediatric Allergy in Ankara Dışkapı Hospital (Child Health and Medicine Hematology Oncology Training and Research Hospital). For this study, peripheral blood of asthma (n=8) and AD (n=9) patients at ages between 8 and 14, as well as age matched healthy controls (n=12) were collected. From each donor, either patient or healthy, 10-20ml peripheral blood collected into vacutainers containing sodium citrate. The study was approved by the Institutional Review Board, and all participants provided their informed consents.

2.2. Animals and Cell Culture

2.2.1. Animals

Adult C57BL/6 (male, 6–8 weeks old) were kept in the Bilkent University animal housing facility of the Department of Molecular Biology and Genetics. Mice were housed under controlled conditions at 22°C with 12 h light and 12 h dark cycles and provided with unlimited access of food and water. All experimental procedures were approved by the animal ethical committee of Bilkent University (Bil-AEC/Protocol# 2011/061).

2.2.2. Cell culture

2.2.2.1. Isolation of PBMCs and plasma from peripheral blood

20-50ml peripheral blood was collected from each donor either in K3EDTA or in heparin or in sodium citrate containing vacutainers. In order to isolate PBMCs from peripheral blood, lymphocyte separation medium (LSM, Lonza, Cat#17-829E) was used. 3 volumes of peripheral blood were slowly layered on top of 2 volumes of LSM and centrifuged for 30min at 600 x g at room temperature (RT) at the break off setting. Plasma, the top phase, was collected into new falcon tube and used for exosome and extracellular vesicles (EV) isolation as described below sections. The cloudy interface between plasma and LSM, that contained mononuclear cells were slowly transferred into a new 50 ml falcon tube by using a sterile Pasteur pipette (LP Italiana, Cat#135030). Tubes were then filled with 2% FBS supplemented RPMI-1640 and centrifuged at RT for 10min at 600Xg. Supernatants were removed and washed twice (at RT for 10min at 600Xg). After washing steps, pellets containing PBMCs were re-suspended in 10% FBS containing RPMI-1640 and cells were counted either by hemocytometer (with the addition of trypan blue) or by flow cytometer.

2.2.2.2. Culture of RAW264.7 murine macrophage cells for supernatant collection

RAW 264.7 murine macrophage cells were obtained from ATCC (ATCC® TIB-71™). Cells were cultured with RPMI-1640 supplemented with 10% FBS, 50 mg/ml

penicillin/streptomycin, 2mM L-glutamine, 10mM HEPES buffer, 0,11 mg/ml sodium pyruvate. All of the media components were centrifuged at 100,000Xg for 4h in order to remove unrelated particles that could interfere or contaminate the isolation procedure throughout the exosome isolation protocol. Cells were seeded into 10 and 15mm petri dishes (5×10^6 and 10×10^6 RAW cells, respectively) and were first incubated for 2h in 7ml and 13ml cell culture media (10 and 15mm petri dishes, respectively) in order to attach to the surface of the cell culture plates. At the end of 2h, culture media was aspirated and fresh 30 and 60ml culture media was added onto the plates (10 and 15mm petri dishes, respectively). After 3 days, cultured media was collected for exosome isolation and cells were passaged for another round of exosome isolation as described above.

2.2.2.3. Preparation of single cell suspension from spleens

Spleens were surgically removed from C57BL/6 male mice. Single cell suspensions were prepared by smashing spleen by back of a syringe plunger in warm 2% FBS supplemented RPMI-1640 media. Cells were transferred to a new 15ml falcon tube and filled with 12ml media. Samples were sit still for 30 seconds to precipitate the remaining tissue debris. After removing tissue debris, splenocytes were centrifuged at 600Xg for 10min at RT. Supernatant was removed and cells were washed. Cell pellet was gently disrupted with 1ml fresh media and counted either with hemocytometer (with the addition of trypan blue) or by flow cytometer.

2.2.2.4. Cell counting

After dislodging RAW264.7 cells from petri dishes or preparing single cell suspension of splenocytes, they were suspended in 1 or 10ml of cell culture media depending on how they will be counted.

Cells, dissolved in 10ml culture media, were diluted 5-fold and mixed with trypan blue (Lonza, Cat#17-942E) at 1:1 ratio. Trypan blue was used to exclude dead cells during counting on hemocytometer. 10 μ l of 10-fold diluted cell suspension was mounted on hemocytometer. The number of cells, inside the bold red squares (0.1mm³/each) represented in Figure 2.1, was counted under the light microscope. The total cell number was calculated from counted cell number according to following formula;

$$\text{Total Cell \#} = \left(\frac{\text{Counted Cell \#}}{4} \right) \times 10^4 \times 10(\text{Dilution Factor}) \times 10(\text{Volume})$$

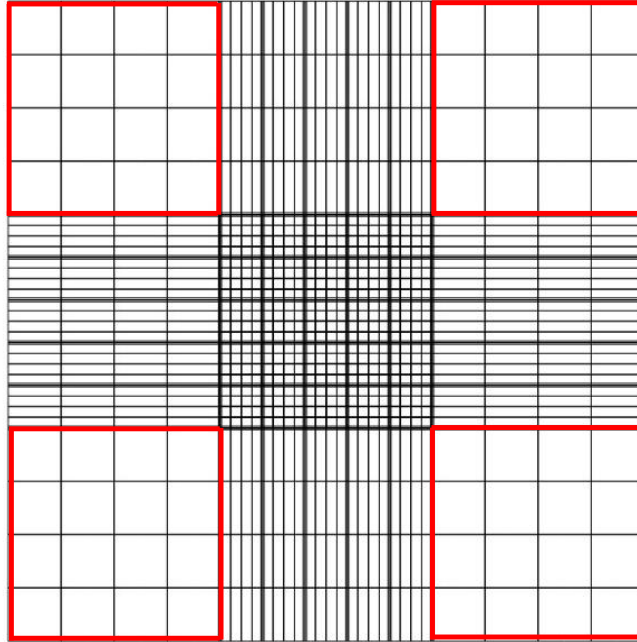


Figure 2.1. Representative picture of grids in hemocytometer. Cells residing within the red squares were included in the counting.

In some experiments, cells were counted by flow cytometer (BD Accuri™ C6, San Jose, USA). Isolated or extracted cells were dissolved in 1ml culture media. 20µl of cell suspension was added into 10ml Isoton II Diluent Buffer (Beckmann Coulter, Cat# 8546719) containing 3 drops of Zap-Oglobin II Lytic Reagent (Beckmann Coulter, Cat# 7546138). 100µl of this 500X diluted cell solution was used during counting. Live cells were gated, excluding apoptotic cells and cell debris clusters. Total number of cells were calculated by multiplying counted cell number by 5000 (the formula is given below).

$$\text{Total Cell \#} = \left(\frac{\text{Counted Cell \#}}{\text{Used Volume (\mu l)}} \right) \times 500 (\text{Dilution Factor}) \times 1000 (\text{Volume (\mu l)})$$

2.3. Isolation of EVs and Exosomes

2.3.1. Isolation of EVs and exosomes from plasma and cell culture

Isolation of EVs and exosomes from plasma and cell culture supernatants were performed as described below. After isolation of plasma from peripheral blood or collection of cell culture supernatant from culture media, they were centrifuged at 1500Xg for 10min at RT to remove remaining cells or cell debris. Supernatants were collected to new tubes, leaving 1 ml aliquot behind the tubes. At this stage, EV and exosome containing solutions could be snap-frozen following suspending them in liquid nitrogen and could be kept at -80°C for further use. However, at time of EV and exosome isolation frozen tubes must be thawed very slowly overnight at +4°C in order to prevent EV and/or exosome rupture before isolation.

Before the start of the isolation step, all plasma and cell culture supernatants (either fresh or thawed) must be vortexed vigorously. This step is necessary, and it helps to dissolve vesicle clumps and furthermore help to separate proteins that was remained in the culture from EVs and exosome nanovesicles. At the end of vortexing, samples were transferred to ultracentrifuge tubes (Beckmann Coulter, Cat# 326823). and were filled with 1X Ca²⁺/Mg²⁺ free 1X, PBS (35ml, Lonza, Cat#17-516F) or PBS-citrate buffer (10mM tri-sodium citrate PBS @ pH=7.4) and centrifuged at 10,000Xg for 10min at 4°C. At the end of this step, the cell debris and/or other contaminating large vesicles such as apoptotic blebs were removed. 1X PBS was used as diluent solution for isolation of vesicles from cell culture supernatant while 1X PBS-citrate was used during isolation from plasma. Moreover, all liquids used throughout the purification steps were pre-filtered using 0.2µm filters and then centrifuged at 100,000Xg for two hours.

After centrifugation 10,000Xg, 32ml of the supernatant was transferred to a new ultracentrifuge tube leaving approximately 3ml liquid behind. The solution was re-centrifuged at 100,000Xg for 90min at 4°C. At the end of this second ultra-centrifuge step, EVs were sedimented. In order to further purify exosomes instead of EVs, an extra 0.2µm filtration and centrifugation at 30,000Xg for 30min was conducted before the second centrifugation. The EV and exosome pellets were resuspended and washed in order to get rid of from the contaminating proteins. Obtained EVs or

exosomes were, then, dissolved in desired volumes of 1X PBS or if they were from plasma in 1X PBS-citrate and stored @ 4°C for further use.

2.3.2. Determining protein concentration of exosomes

Since exosomes are too small to be quantified by flow cytometer, quantity of exosomes isolated from cell culture supernatants or from plasma was determined according to exosomal protein content. The protein concentration of isolated exosomes was determined with Micro BCA™ Protein Assay Kit (Pierce, Cat#23235). In brief, the exosomes were diluted by 4X with nuclease free ddH₂O (Lonza, Cat#51200) and mixed with working reagent at 1:1 volumetric ratio. Samples were incubated along with BSA standards (prepared as instructed by the manufacturer) for 2h at 37°C. After cooling to RT, the absorbance of samples was measured at 562nm by spectrophotometer (Synergy HT, Biotek, Virginia, USA). The concentration of samples was determined by BSA standard curve.

2.4. Characterization of Exosomes

2.4.1. Analysis of size distribution of exosomes by DLS and AFM

In order to analyze size of exosomes, dynamic light scattering (DLS) and atomic force microscopy (AFM) was used. For DLS studies, exosome solutions were diluted 100X by 1X PBS and transferred into folded capillary zeta cells (Malvern Ins., Cat#DTS1070). Then, hydrated size of exosomes was analyzed using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

The dehydrated size of exosomes was determined by AFM using XE-100E model AFM (PSIA with XEI 1.6 software incorporated) in non-contact mode with a 0.73–0.79 Hz scanning rate. Exosome samples for AFM studies were prepared by dropping 10µl of solution containing 1µg exosomes onto mica slides and either 5x5 µM or 1x1 µM and in some experiments 0.5x0.5 µM areas were scanned.

2.4.2. Analysis of exosomal markers by flow cytometer

Since exosomes are too small to be analyzed by flow cytometer, a bead-based strategy was performed to analyze exosomal markers. Carboxyl latex beads,

purchased from Life Technologies (Cat#C37282), was used for coupling with antibodies against exosomal markers such as CD9. After washing latex beads twice, 40µg (1µl) carboxyl latex beads were incubated with 1µg LEAF purified anti-CD9 (Biolegend, Cat#124802) antibody in 1X PBS overnight at RT. Of note, it is of great importance that incubation should be done with gentle mixing to prevent beads to precipitate but at the same time, the mixing rate must be adjuvated in such a way that it should not harm the beads. Then latex beads/anti-CD9 complex were blocked with 5% BSA (Roche, Cat#10735094001) containing 1X PBS by incubating with gentle mixing for overnight at RT in order to cover unbound surface. Coated and blocked beads were washed twice with 1X PBS by centrifuging at 3000Xg for 10min at RT. The coated beads were dissolved in Storage Buffer (1X PBS containing 0.1% glycine and 0.1% NaN₃, pH; 7.2) at final concentration of 3x10⁵ beads/µl.

10µg exosomal protein containing intact exosomes were incubated with 1µl of bead solution in 100µl FACS Buffer (FB; 1X PBS containing 5% BSA and 0.05% Tween 20) with gentle mixing at RT for 30min. Exosome bound beads were centrifuge at 3000Xg for 10min and supernatant was removed. Beads were washed with FB, twice. After that exosome/bead complex were incubated with anti-CD81-PE antibody or AnV-PE in FB or in 1X Binding Buffer (BB), respectively, for 30min with gentle mixing at RT. The schematic representation of final complex is shown in Figure 2.2. After incubation period, complexes were centrifuged, washed twice and analyzed by BD Accuri C6 flow cytometer.

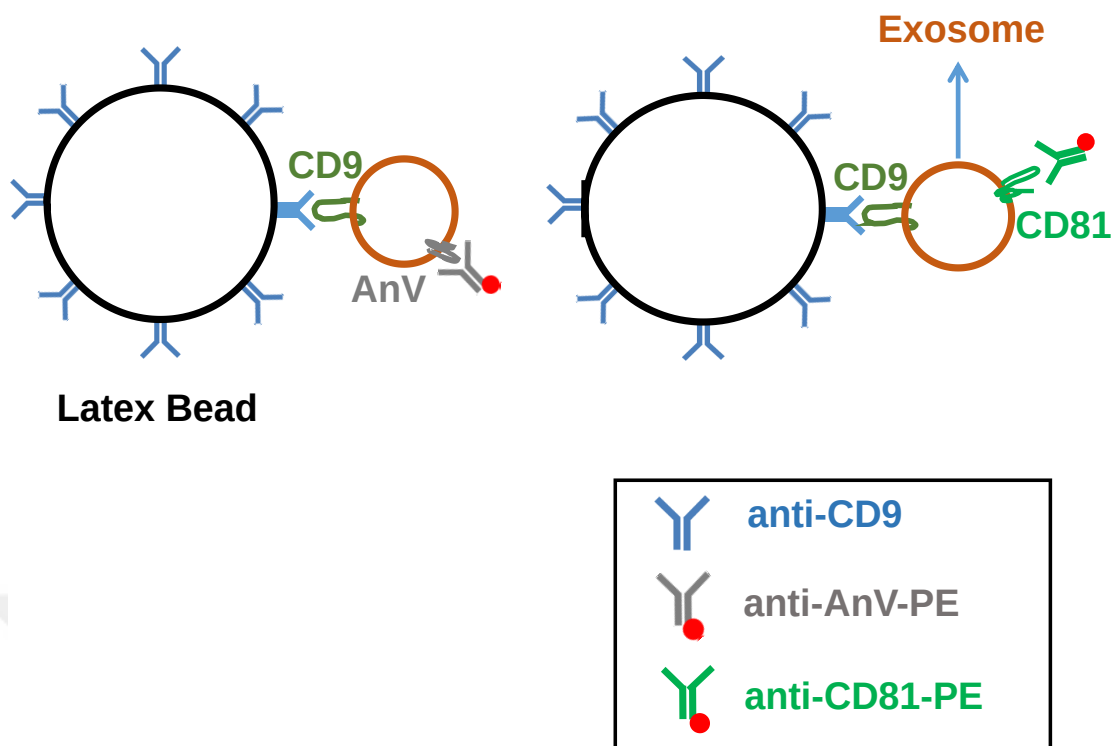


Figure 2.2. Representative scheme showing bead based analysis of exosomes using flow cytometer. In order to discriminate exosomes from noise and debris during flow acquisition exosomes bound with 3 μ m latex beads were analyzed for exosomal-specific markers with flow cytometer.

2.5. TLR9 and STING Ligand Loading within Exosomes

2.5.1. Ligands

In order to improve internalization, stability and in vitro or in vivo immunostimulatory activity of TLR9 and STING ligands, they were loaded within exosomes. The K-Type CpG ODNs; i) K3 (5'-ATCGACTCTCGAGCGTTCTC-3'), ii) K23 (5'-TCGAGCGTTCTC-3'), and D-Type CpG ODNs; i) D35 (5'-GGTCGATCGATCGAGGGGGG-3'), as well as STING ligand, c-di-GMP, were used during the exosome encapsulation experiments. The unlabeled or Cy-5 or FAM labeled CpG ODNs were either synthesized in house on a Mermade6 oligonucleotide synthesizer (XXX, XXX, USA) or purchased from Alpha DNA, (Canada). The unlabeled or FAM-labeled cyclic-di-GMP was purchased from BioLog (Cat#C057, Cat#F009, respectively, Germany). While labeled reagents were

used in confocal and flow cytometry studies unlabeled reagents were used in functional assays.

2.5.2. Dehydration rehydration technology for ligand loading within exosomes

Table 2.2 summarizes our initial CpG ODN loading conditions within exosomes. Figure 2.3 schematically represent the critical steps leading to ligand loaded exosomes.

Table 2.2. Exosome-CpG ODN mixing conditions.

RATIO	Exosome Mass	CpG ODN Mass
2:1	20µg	10µg
1:1	20µg	20µg
1:2	20µg	40µg

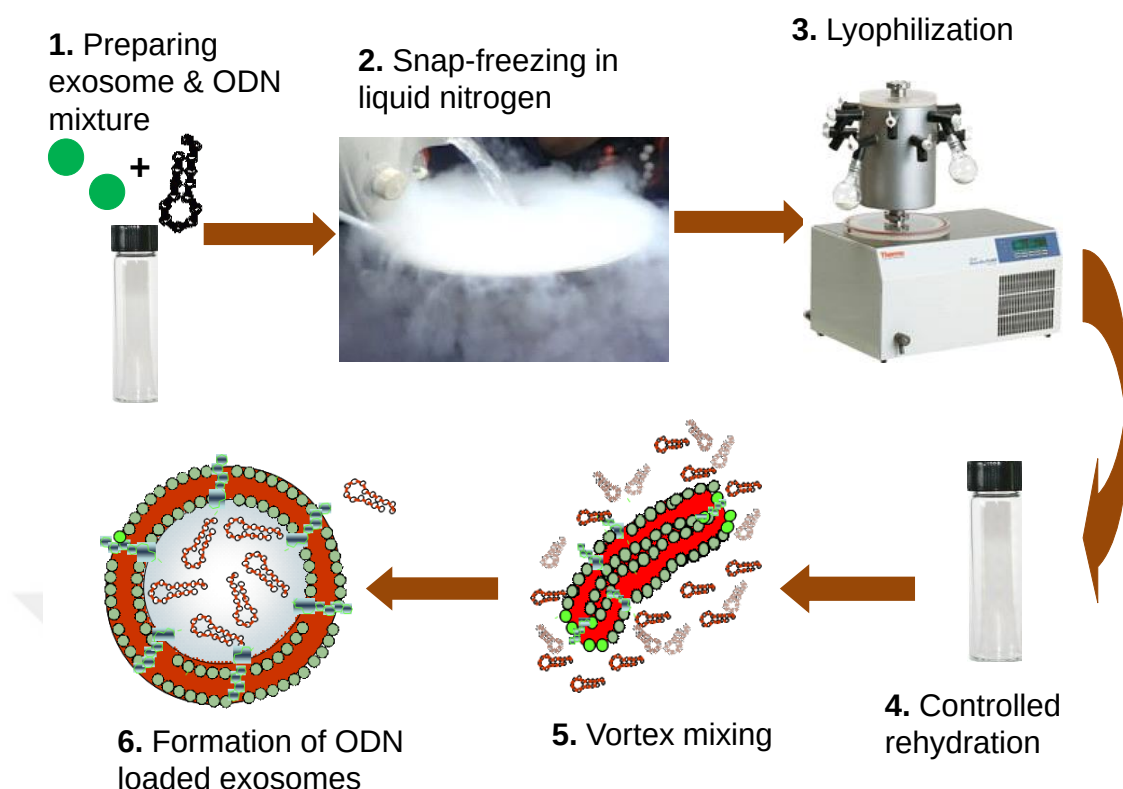


Figure 2.3. Dehydration rehydration loading method is given schematically. *Essential steps of the method used in loading of exosomes with CpG ODNs is outlined.*

In brief, exosomes and ligands were mixed at 1:1 ratio (w/w), which was found to be the optimum condition. After mixing exosomes and ligands, the solution was snap-frozen in liquid nitrogen and then lyophilized overnight using VirTis BenchTop K Freeze Dryer (SP Industries, New York, USA). At this step, exosome/ligand powder is obtained, and there is no ligand loading within exosomes. Ligand encapsulation within the exosomes was achieved during controlled-rehydration step. Nuclease free ddH₂O (1:10 ratio of original solution volume) was added onto dehydrated exosome/ligand powder and vigorously vortexed for 15 s every 3min for a duration of 20min at RT. At the end of 20min, PBS (1:10 original exosome/ODN solution volume) was added and gently vortexed 15s every 3min for 10min in order to regain the buoyancy of the solution. Loaded exosomes were diluted to desired ligand concentration by adding required volume of 1X PBS. Exosome formulations were stored at 4°C until use.

2.6. Flow Cytometry Studies

2.6.1. Analysis of EVs and exosomes by flow cytometer

2.6.1.1. EV counting from patient plasma

Our initial optimization studies were performed with standard calibration beads of known concentration (1×10^7 beads/ml, Sigma LB-11, 1.1 μm in diameter). We acquired the # of events on a FACS Calibur (BD Bioscience, San Jose, USA) or BD Accuri C6 flow cytometer using a 60 sec acquisition time at a “fast-flow” setting and determined the volume utilized for a run at regular setting of fast acquisition rate. We calculated mean $\pm$ SD of volume utilized by performing 30 independent runs each week. After verification of volume utilized, we quantified AnV positive EVs from each individual and multiplied with the dilution factor to determine the # of EVs/ml plasma.

2.6.1.2. Identification of cellular origin of EV from patient and healthy plasma

For the identification of cellular origin of EVs, samples were double-stained with AnV (FITC or PE conjugated, BD Bioscience, Cat#556419 or 556421, respectively) and antibodies against one of the cell surface markers. i) CD42a-PE (platelet, BD Pharmingen, Cat#558819), ii) CD31-FITC (endothelial cells, Biolegend, Cat#303104), iii) CD69-PE (early activation marker, Biolegend, Cat# 310906), iv) CD105-FITC (epithelial cells, Biolegend, Cat#323203), v) CD3-FITC (T-cells, BD Pharmingen, Cat#555339), vi) CD14-FITC (monocytes, BD Pharmingen, Cat#555397) and vii) CD9-PE (eosinophils, Biolegend, Cat#312106) were mixed with AnV antibody. During double staining compatible fluorochrome conjugated antibody pairs were selected. Isolated EVs dissolved in proper amount of 1X PBS and then mixed with 2X AnV Binding Buffer (BB) at 1:1 (v/v ratio). AnV Binding Buffer was prepared as 10X stock solution (0.1M HEPES, 1.4M NaCl, 25mM CaCl_2 in distilled H_2O , pH 7.4) and stored in fridge for a month.

25 μl EV aliquots were incubated with 75 μl of staining cocktail (containing of 2 μl AnV and 2 μl of one of the above mentioned cell surface markers in 1X BB) for 30 min at RT in dark. Following staining, 500 μl of 1X BB was added onto stained EVs and were analyzed by FACS Calibur using a 60 sec acquisition time at a “fast-flow”

setting and data evaluation was performed using the CellQuest Pro Software (BD Bioscience, San Jose, USA). Of note, in order to gate-out air bubbles, electrical noise and contaminating particles, a very stringent FSC-SSC gating strategy was used as indicated by the "R2 EV-associated particle gate" (Figure 2.4). In addition, to gate out particles > 1 μ m, latex beads (Sigma LB-11) with 1.1 μ m diameter were used to define our EV associated gate. The optimized acquisition settings were controlled weekly. In some experiments, data acquisition was performed on an Accuri C6 Flow cytometer with similar results to those obtained by FACS Calibur.

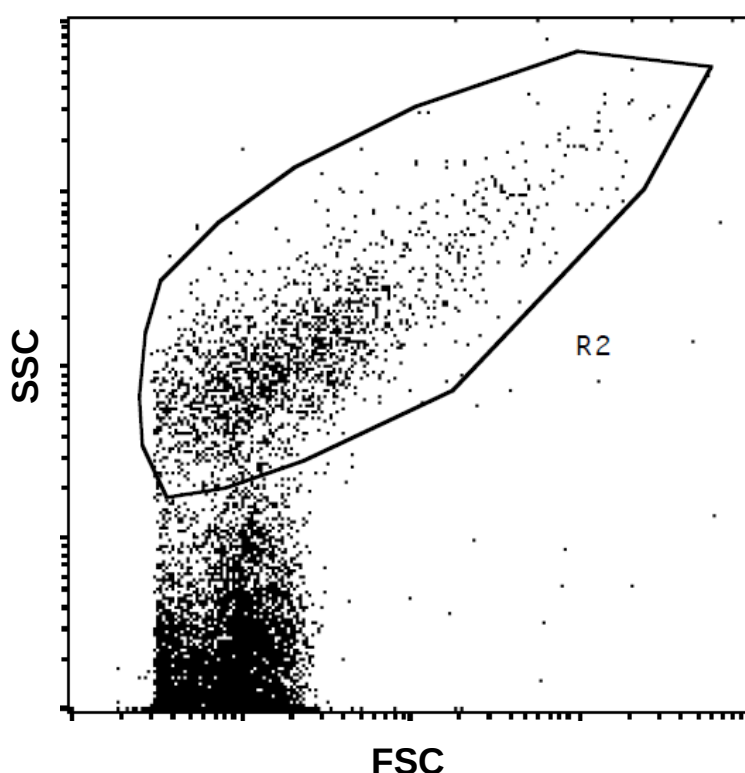


Figure 2.4. Gating strategy used during analysis of EVs. *Representative dot plot showing selective gate used to discriminate EV population from contaminating particles and electrical noise of FACS Calibur.*

2.6.1.3. Staining EVs and exosomes with lipophilic dyes

EVs were stained with CM-Dil (Thermo, Cat# C-7001) and SP-DiOC (Thermo, Cat#D7778) while exosomes were labeled only with SP-DiOC in order to assess cellular internalization of the vesicles. Staining protocol for both lipophilic dyes were same. Vesicles dissolved in 1X PBS were incubated with dyes at 1 μ M concentration

for 30min at 37°C. After incubation, vesicles were washed with 1X PBS once in order to remove the unbound dye.

2.6.1.4. Loading exosomes with CFSE

Exosomes were loaded with CFSE to evaluate cell specific uptake of vesicles. Exosome pellet was dissolved in 1ml 1% BSA containing 1X PBS and vigorously vortex to dissolve vesicle clumps. Exosome solution (1ml) was mixed with equal volume of 20 μ M CFSE solution. 2ml of 10 μ M CFSE containing exosome solution was incubated for 30min at 37°C. After incubation, solution was diluted 16X with exosome free 10% FBS containing RPMI-1640 to quench free CFSE and transferred to an ultracentrifuge tube. Exosomes as previously mentioned were washed once by ultra-centrifugation (@ 100,000Xg for 90min). The pellet was dissolved in desired amount of 1% BSA containing 1X PBS.

2.6.2. Flow cytometry studies of PBMCs, RAW264.7 cells and splenocytes

2.6.2.1. EV and exosome internalization by cells

2x10⁶/ml PBMCs incubated with CM-Dil (In) 2x10⁶/ml PBMCs were incubated with CM-Dil stained EVs for 2 to 8h at 1:1.5 to 1:100 (cell:EV) ratio at 37°C. After incubation period, cells were centrifuged at 600Xg for 10min to remove unbound/uninternalized EVs and internalization kinetics were analyzed by BD Accuri C6.

Similarly, internalization kinetics of free and LL37 complexed EVs into PBMCs was performed using lipophilic dye, SP-DiOC. SP-DiOC labeled exosomes, free or complexed with LL37, incubated with 2x10⁶/ml PBMCs for 1-8h at 1:2 (Cell:EV) concentration (at 37°C). Assessment of exosome internalization by BD Accuri C6 flow cytometer was performed in the presence of Trypan Blue (Lonza, Cat#17-942E) in order to exclude bound but not internalized vesicles.

Internalization kinetics of different variant of RAW264.7 derived exosomes into splenocytes was performed in order to show D35 loading procedure do not effect exosome's cellular uptake. 2x10⁶/ml splenocytes were incubated with 20 μ g/ml D35-

Cy5, or Exo_{SP-DiOC} or Exo(D35-Cy5)_{SP-DiOC} for 1-24h at 37°C. Internalization kinetics were examined by BD Accuri C6 in the presence of Trypan Blue.

Effect of exosome encapsulation on c-di-GMP internalization by splenocytes were also analyzed by incubating cells (2×10^6 cells/ml) with 15, 5 and 1.66 μ M of FAM-c-di-GMP or Exo(FAM-c-di-GMP) for 2h at 37°C. The cells were then stained with 2 μ g/ml anti-MHC-II-PE (Biolegend, Cat#107608). MHC-II⁺ cells were gated and their FAM expressions were assessed using BD Accuri C6 Cytometer.

2.6.2.2. Analysis of cell specific uptake of EV and EV/LL37 complexes

Cell specific uptake of EV or EV/LL37 complex was also determined by incubating PBMCs with CFSE loaded EV or EV/LL37 for 2h and then staining them with antibodies against cell specific markers. PBMCs were stained with 2 μ g/ml i) anti-CD19-PE/Cy5 (Biolegend, Cat# 302210) (B-cell), ii) anti-CD11c-PE (Biolegend, Cat#301606) (Dendritic cells), iii) anti-CD14-PE (Biolegend, Cat# 325606) (monocytes) and iv) anti-CD3-APC (Immunostep, Cat# 3A1-20T) (T-cells) and analyzed for CFSE positivity in specific cell types by BD Accuri C6.

2.6.2.3 Intracellular cytokine staining of splenocytes

EVs isolated from healthy donors (with or without LL-37 loading) were incubated with healthy PBMCs. To assess IL-1 β and IP10 production following stimulation, cells were treated with Brefeldin-A either 6h (for IL-1 β) or 10h (for IP10) (5 μ g/ml, Biolegend, Cat#420601). Of note, Brefeldin-A was added at t=0 and t= 6h of incubation for IL1 β (total of 6h stimulation) and for IP10 (total of 16h stimulation), respectively. The cells were then fixed with Fix & Perm Medium A (Invitrogen, Cat# GAS001S100) and were first stained for monocytes (CD14-PE, Biolegend, Cat#325606) and then to detect the level of intracellular IL-1 β stained with IL-1 β -FITC (e-Biosciences, Cat# 12-7018-81) in the presence of saponin (0.1%). To determine intracellular IP10, cells were first stained with IP10-Biotin (BD, Cat# 555048) and then with SA-FITC (Biolegend, Cat# 405202) in the presence of saponin (0.3%).

IFN α secreting cells were analyzed with Miltenyi Biotech's IFN α Secretion Assay and Detection Kit (PE-labeled, Miltenyi, Cat# 130-094-161) from PBMCs in accordance with kit instructions. Additionally, in order to identify IFN α secreting

pDCs, cells were stained with BDCA-2-FITC (Biolegend, Cat# 354208) and then analyzed by BD Accuri C6 flow cytometer.

2.6.2.4 Analysis of MHC-II and CD86 expressions on splenocytes

Splenocytes (2×10^6 cells/ml) were stimulated with three doses (15, 5 and 1.66 μ M) of free c-di-GMP, Exo(c-di-GMP) with or without 1 μ M K3-CpG ODN for 24h at 37°C. Stand-alone K3 CpG ODN was used as control treatment. After 24h of incubation, cells were stained with 2 μ g/ml of anti-MHC-II-FITC (Biolegend, Cat# 107606) and anti-CD86-PE/Cy5 (Biolegend, Cat# 105016) antibodies and analyzed with BD Accuri C6.

2.7. Confocal Microscopy Studies

Confocal Microscopy studies were performed to visualize the internalization of both EVs and exosomes within healthy PBMCs or within splenocytes, respectively. In these studies, i) CM-Dil labeled EVs loaded or unloaded with Cy5-labeled D35 CpG ODN were used on PBMCs or ii) SP-DiOC labeled exosomes loaded or unloaded with Cy5-labeled D35 CpG ODN were incubated either with healthy PBMCs or with splenocytes, respectively, for 8h at 37°C. After incubation cells were centrifuged to eliminate free EVs and exosomes. Following treatments, cells were fixed with Fix & Perm Medium A (Thermo, Cat# GAS001S100), washed, pelleted again and finally suspended in 20 μ l 1X PBS. A fraction of cell solution (5-10 μ l) was spread onto slides and let it air dry in a dark place. 5 μ l of ProLong® Gold Anti-fade Mountant (Thermo, Cat# P36934) reagent was added onto dehydrated samples and covered with coverslip without introducing any air bubble. After stabilizing coverslip with colorless nail polish, sample were ready for visualization with confocal microscope. All of the images were taken by LSM 500 confocal microscope (Zeiss, Oberkochen, Germany).

2.8. ELISA Studies

2.8.1. Cytokine ELISA

After the stimulation of cells with various stimulants for indicated time periods, cells were centrifuged at 600Xg for 5min, if otherwise indicated. Supernatants, leaving

10-15µl behind, were transferred into new plates and stored at –20°C until further use. Properties, brands, clones and concentrations of antibodies and chemicals used in different human/mouse cytokine ELISA were indicated in Table 2.3. The ELISA protocol used for all cytokines were adapted from [219]. Briefly, high binding PolySorp (Thermo, Cat#475094) were coated with capture antibodies (anti-cytokine monoclonal antibodies) by adding antibodies diluted in 1X PBS at 50 µl/well and incubating for 4h at RT or overnight at +4°C. Then wells were blocked with 200 µl blocking buffer (5% BSA and 0.05% Tween 20 in 1X PBS) for 2h at RT or at +4°C for overnight. Before adding supernatants and serially diluted standards (recombinant proteins, 11 duplicates), plates were washed with Wash Buffer (0,05% Tween in 1X PBS) 5 times for 3min intervals and rinsed with ddH₂O. Cell supernatants and standards were added 50 µl/well and incubated for 2h at RT or overnight at +4°C. Plates were washed as previously mentioned. Afterwards, tracker antibodies (biotinylated anti-cytokine monoclonal antibodies), which were diluted in indicated concentration by Blocking Buffer, were added to wells at 50 µl/well and incubated for 2h at RT or overnight at +4°C. Again followed by washing as described earlier. 1:1000 diluted Streptavidin Alkaline-Phosphatase (SA-ALP) or 1:200 diluted Streptavidin Horseradish Peroxidase (SA-HRP) in Blocking Buffer should be kept at least 1h prior to use at RT, can also be kept at +4°C for overnight. Then they were added into plates as 50 µl/well and incubated for 1-2h at RT (or overnight in +4°C). After washing the plates, PNPP (for SA-ALP detection) or TMB (for HRP detection) substrates were prepared according to manufacturer's protocol and 50 µl/well aliquots were added into each wells. Color development was followed by multiple optical density readings at 405nm for PNPP (or at 450nm for TMB) until recombinant cytokine standards reach a saturation. The concentration of cytokines for each sample were calculated based on standard curves. In case of using TMB substrate, stop solution (2N sulfuric acid) was added to block color development and absorbance reading was taken at OD 450 nm. Data were collected by µQuant (BioTek, Virginia, USA) or SpectraMax (Molecular Devices, California, USA) spectrophotometers.

Table 2.3. ELISA reagents and their working conditions.

Product Name	Brand (Cat#)	Clone	Working Conc.
Anti-human IL6	MabTech (3450-1a-20)	13A5	4 µg/ml
Anti-human IL6 Biotin	MabTech (3450-1a-20)	39C5	1 µg/ml
Human recombinant IL6	MabTech (3450-1a-20)	N/A	1000 ng/ml
LL37 ELISA kit	HyCult (HK321-02)	N/A	Coating Ab; Pre-coated Tracker Ab; not-known SA-HRP; 1:1000 dilution Standard; 100 ng/ml
Anti-mouse IL6	Biolegend (504502)	MP5-20F3	4 µg/ml
Anti-mouse IL6 Biotin	Biolegend (504602)	MP5- 32C11	1 µg/ml
Mouse recombinant IL6	Biolegend (563401)	N/A	1:1000 dilution
Anti-mouse IL12	Biolegend (505202)	C15.6	4 µg/ml
Anti-mouse IL12 Biotin	Biolegend (505302)	C17.8	1 µg/ml
Mouse recombinant IL12	Biolegend (563401)	N/A	400 ng/ml
Anti-mouse IgG-ALP	Southern BioTech (1030-04)	N/A	1 µg/ml
Anti-mouse IgG1-ALP	Southern BioTech (1070-04)	N/A	1 µg/ml
Anti-mouse IgG2c-ALP	Southern BioTech (1079-04)	N/A	1 µg/ml
SA-ALP	MabTech (3310-8)	N/A	1:1000 dilution
PNPP	Thermo (34047)	N/A	1 tablet/5ml

2.8.2. anti-OVA IgG ELISA

In anti-OVA total IgG, IgG1 and IgG2c ELISA, MaxiSorp plates (Thermo, Cat# 469949) were used. Each well was coated with 50 μ l of OVA (10 μ g/ml) diluted in 1X PBS and incubated for 4h at RT or overnight at +4°C. Blocking and washing steps were same as cytokine ELISA as mentioned in Section 2.8.1. Instead of culture supernatants, 7X diluted mouse sera from each animal was pipetted on the first row and then 4-fold serial dilution was performed on vertical axis without exceeding 50 μ l sample volume. After adding and preparing dilution of mouse sera, samples were incubated for 4h at RT or overnight at +4°C. After washing, ALP conjugated anti-mouse IgG, IgG1 or IgG2c antibodies diluted in T-cell Buffer (5% FBS and 0.05% Tween 20 in 1X PBS) were added as indicated in Table 2.3. Samples were incubated for 2h at RT or overnight at +4°C. Of note these ALP conjugated antibodies should also be prepared ahead of time as mentioned in Section 2.8.1. After the addition of PNPP substrate, yellow color formation was read at 405nm with SpectraMax spectrophotometer.

2.9. Immunization Studies

Adult male C57BL/6 mice (10 mice/group) were injected i.p. either with saline, D35 + OVA or encapsulated from Exo(D35 + OVA). For each mouse, 20 μ g exosomes were loaded with 10 μ g D35 and 5 μ g OVA. Mice, in the D35 + OVA, were also injected with same amount of D35 and OVA as in Exo(D35 + OVA). The immunization schedule is given in Figure 2.5. One day before booster injection (@d = 13) animals were bled from tail vein and sera was saved for further use. The next day booster injection was performed. Similarly, 2nd, 10th and 24th week post-booster bleedings were also performed. Sera from each bleeding was obtained by incubating venous blood at 37°C for 1-2h and then centrifuging at 2,000 x g for 5min. anti-OVA total IgG, IgG1, and IgG2c ELISA was performed to assess magnitude of immunization strength and duration of anti-OVA immunity.

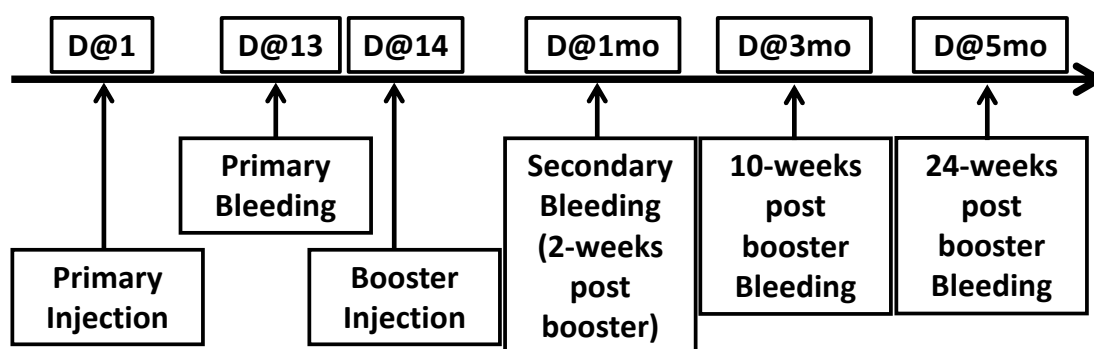


Figure 2.5. Immunization and bleeding schedule. Mice were injected i.p. with 10 μ g D35 and 5 μ g OVA in free or exosome encapsulated form. Bleedings were performed 1 day before, 2-week post, 10-week post and 24-week post booster injection.

2.10. EG-7 Challenge Experiments

After immunizing mice against model antigen OVA, at 10- and 24-weeks post booster mice (5 mice/group) were challenged with s.c. injection of 5x10⁶ EG-7 cells in 100 μ l PBS. EG-7 cells were inoculated to the back of the animals since they are expressing and secreting OVA. C57BL/6 mice immunized with E.G7-OVA cells give rise to H-2 Kb restricted cytotoxic lymphocytes specific for the OVA 258-276 peptide (SIINFEKL). After EG-7 cell inoculation, mice were investigated twice a day until tumor formation was observed and once a day after all mice in the saline group developed tumors. The tumor development was followed by measuring the length, width and height of tumors. First experiment (10-week post booster) was terminated when any mouse tumor reached 2000 mm³ whereas the challenge experiment at 24-week post booster mice was used for survival analysis.

2.11. Statistical Analysis

All data analyses were carried out using Sigma Stat 3.5 (Systat Software Inc.) program. In order to assess significance between active BD, inactive BD and healthy groups, one-way ANOVA test was used, while student's t-test was applied to analyze significance between sub-groups of active BD vs. inactive BD. p values ≤ 0.05 was set to be statistically significant.

For mice and cell-line experiments student's t-test was applied between treatment and control groups. p values ≤ 0.05 was set to be statistically significant.

Chapter 3

Results

3.1. Role of Extracellular Vesicles in Behçet's Disease

3.1.1. BD patients have elevated circulating EV numbers

To assess whether plasma EV concentrations correlated with BD severity, EV numbers in plasma of 72 BD patients (Active; n=35, Inactive; n=37) and 22 healthy controls were determined by flow cytometry. Results are presented in Figure 3.1.

As it is shown in Figure 3.1A, EV concentrations (number of EV/ml) in plasma were significantly higher in active BD ($3.9 \pm 0.77 \times 10^5$, n=35) and inactive BD ($2.05 \pm 0.46 \times 10^5$, n=37) patients compared to healthy individuals ($0.78 \pm 0.25 \times 10^5$, n=22, $p < 0.001$, active BD vs. healthy, and inactive BD vs. healthy). Plasma EV concentrations in active BD were consistently higher than the inactive BD group ($p < 0.01$) and this increase (1.75-2.2 fold) was valid in all patient sub-groups suggesting that elevated EV levels was a common feature of active disease and was not dependent on organ involvement. The distribution of EV concentration of each individual in each group is given as dot plot in Figure 3.1B.

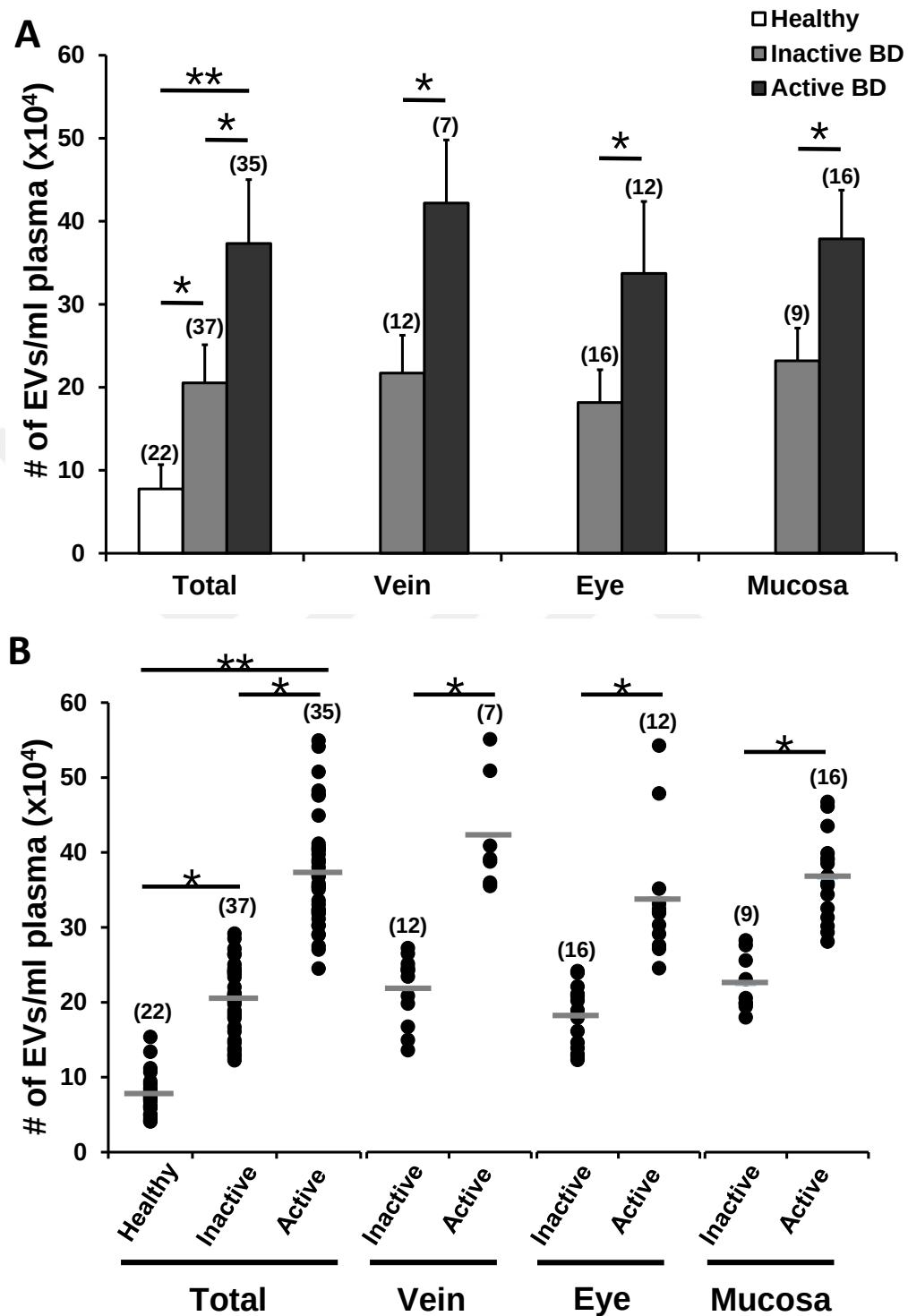


Figure 3.1. Plasma concentration of EVs are elevated in BD patients. Plasma EVs were purified and stained with AnV-FITC as described in Materials and Methods. Number of EVs per ml plasma was calculated following flow cytometry analyses of AnV positive EV signals. (A) Bar graphs of the mean and standard deviation of each group. (B) Concentration of EV for each individual is shown (each dot represents a donor). The number of healthy

individuals or BD patients investigated was shown in parentheses. * $p < 0.01$ and ** $p < 0.001$ for comparison groups.

3.1.2. Platelet derived EVs constitute the majority of circulating EVs in BD plasma

EVs are known to be secreted from multiple cell types; including platelets, endothelial cells, monocytes, and T cells. However, composition of plasma EV in health and disease is not fully revealed. We sought to explore the relative contribution of each cell types to the cumulative EV plasma concentration, and hoped to unravel the difference depending on disease BD activity. To this end, isolated EVs from healthy individuals, inactive and active BD patients were stained with different cell specific markers. Their relative ratios were analyzed by flow cytometry. The representative dot-plots of selected EV samples from donors were given in Figure 3.2.

Flow cytometric analysis of stained EV populations revealed that platelets were the most common source of plasma EVs in all groups (CD42a⁺, Figure 3.3). Platelet-derived EV percentages correlated with disease state and were found to be significantly elevated in BD patients ($52.6 \pm 4.5\%$ and $65.4 \pm 3.8\%$, inactive and active BD, respectively) when compared to healthy controls ($41.5 \pm 4.3\%$, $p < 0.001$ for all comparisons). Similarly, significant increases were found for EVs derived from T cells (Figure 3.3, 1.49 ± 0.36 , 4.14 ± 0.77 and $5.25 \pm 1.24\%$ for healthy, inactive and active BD, respectively; $p < 0.001$) and monocytes (Figure 3.3, 1.39 ± 0.32 , 3.27 ± 0.42 and $5.7 \pm 0.56\%$ for healthy, inactive and active BD, respectively; $p < 0.001$). In contrast, endothelial and/or epithelial derived EVs in BD plasma, (i.e. CD31⁺ and CD105⁺) were lower than those observed in healthy controls ($p < 0.001$ for all comparisons).

Next, relative numbers of cell-type specific EVs in 1ml plasma of inactive and active BD patients as opposed to healthy controls were also determined (Figure 3.4). The exact number of EVs were found as described in materials methods chapter for each different cell type and for each individual. Number of EVs originating specific type of cell for each individual is given in Figure 3.4A. This analysis showed us that number of platelet-derived EVs were 3.5 and 7-fold higher in BD plasma (inactive and active, respectively), while monocyte and T-cell derived EVs were 7-11 and 21-22 fold (inactive and active, respectively) more when compared to healthy EVs

($p < 0.001$, Figure 3.4B). Collectively, these findings suggest that the significant increases found for EVs derived from platelets, T cells and monocytes in BD correlate with the activation state of these cells and may contribute to pathogenesis.

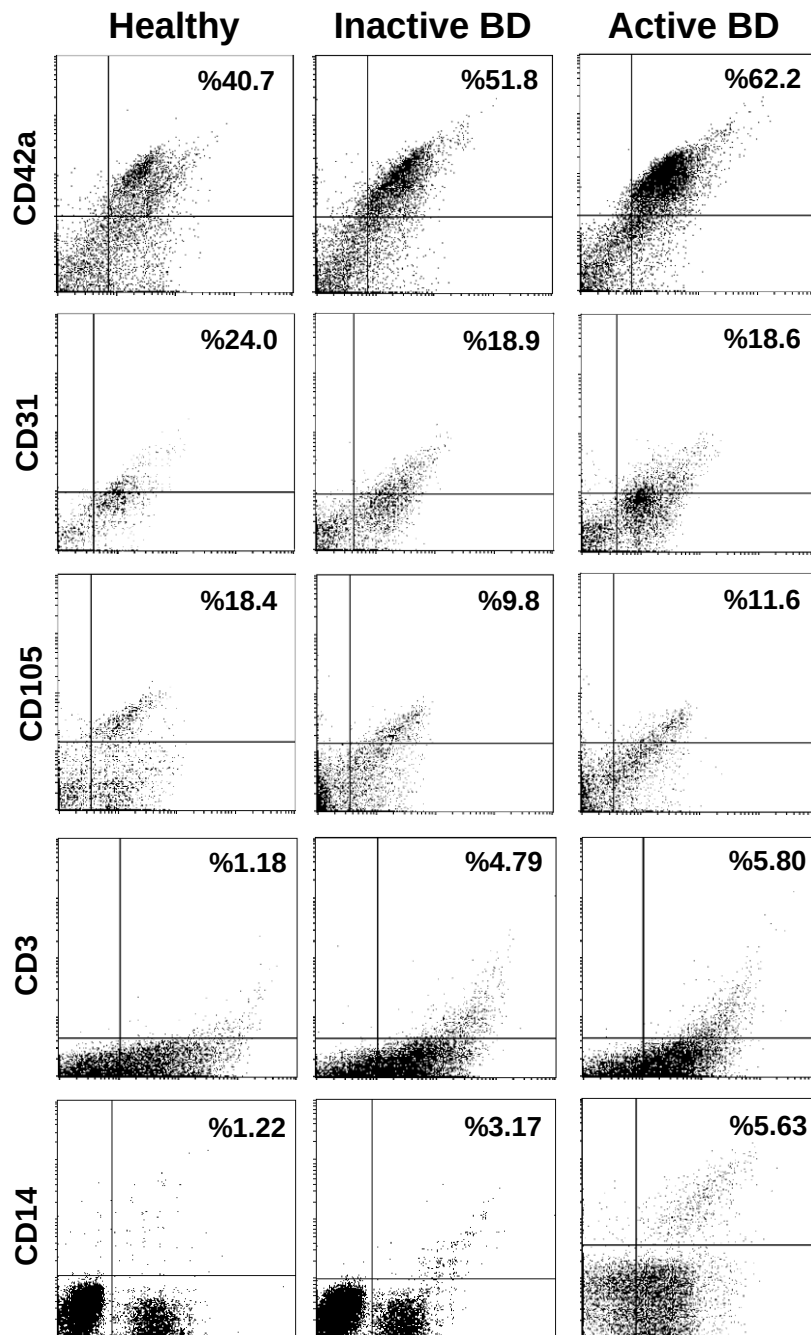


Figure 3.2. The analyses of cellular origin of EVs at different disease stages. *In order to evaluate cellular origin of EVs and relating them to disease states they were stained with AnV/Cell-specific marker antibodies. EVs isolated from the plasma of healthy (left panel), inactive BD (middle panel), and active BD (right panel) donors were first stained with AnV*

(FITC or PE) and then stained against one of the following cell specific marker antibodies against CD42a⁺(platelet), CD31⁺(endothelial), CD105⁺(epithelial), CD3⁺(T-cell) and CD14⁺(monocyte) (PE or FITC labeled) and analyzed by flow cytometry. Representative dot plots of each cell specific EVs are presented.

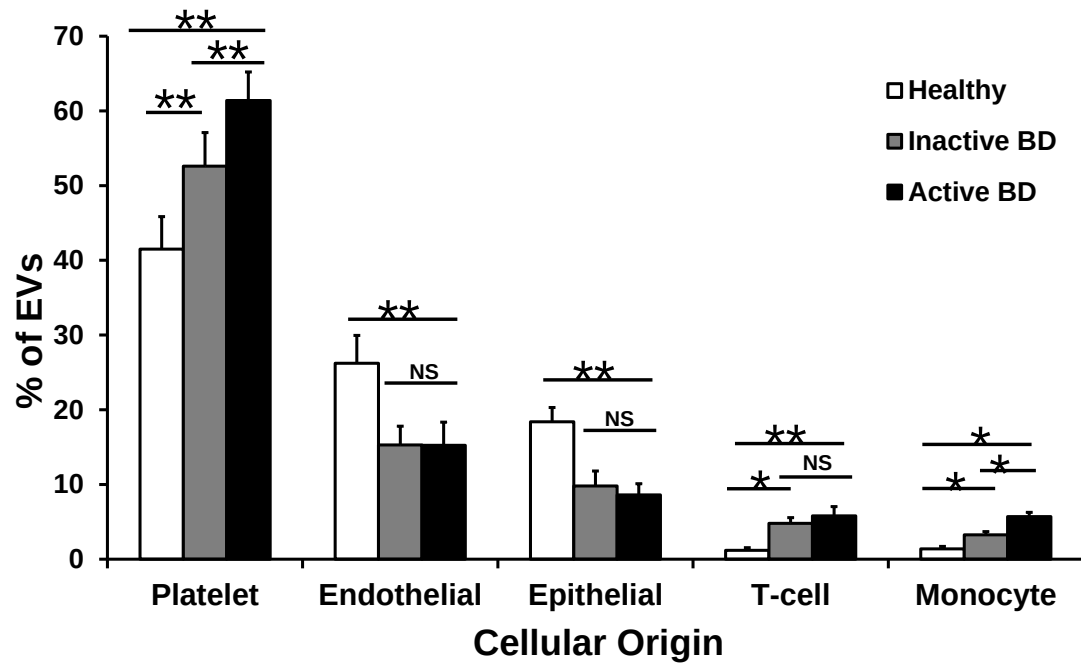


Figure 3.3. The cellular origin of plasma EVs alters with disease severity. EVs were stained with corresponding cell type specific surface markers and AnV. Data is represented as the mean percentage of AnV positive EVs derived from each cell type $\pm$ SD for all samples. Individual dot-plots were used to calculate CD3 (T-cells), CD14 (monocytes), CD42a (platelets), CD31 (endothelial cells) and CD105 (epithelial cells) derived EV ratios over total EVs from healthy (n=22), inactive BD (n=37) and active BD (n=35) donors. * $p < 0.01$, ** $p < 0.001$ based on student's t-test analyses of healthy vs. inactive BD, healthy vs. active BD and inactive BD vs. active BD. NS indicates not-significant.

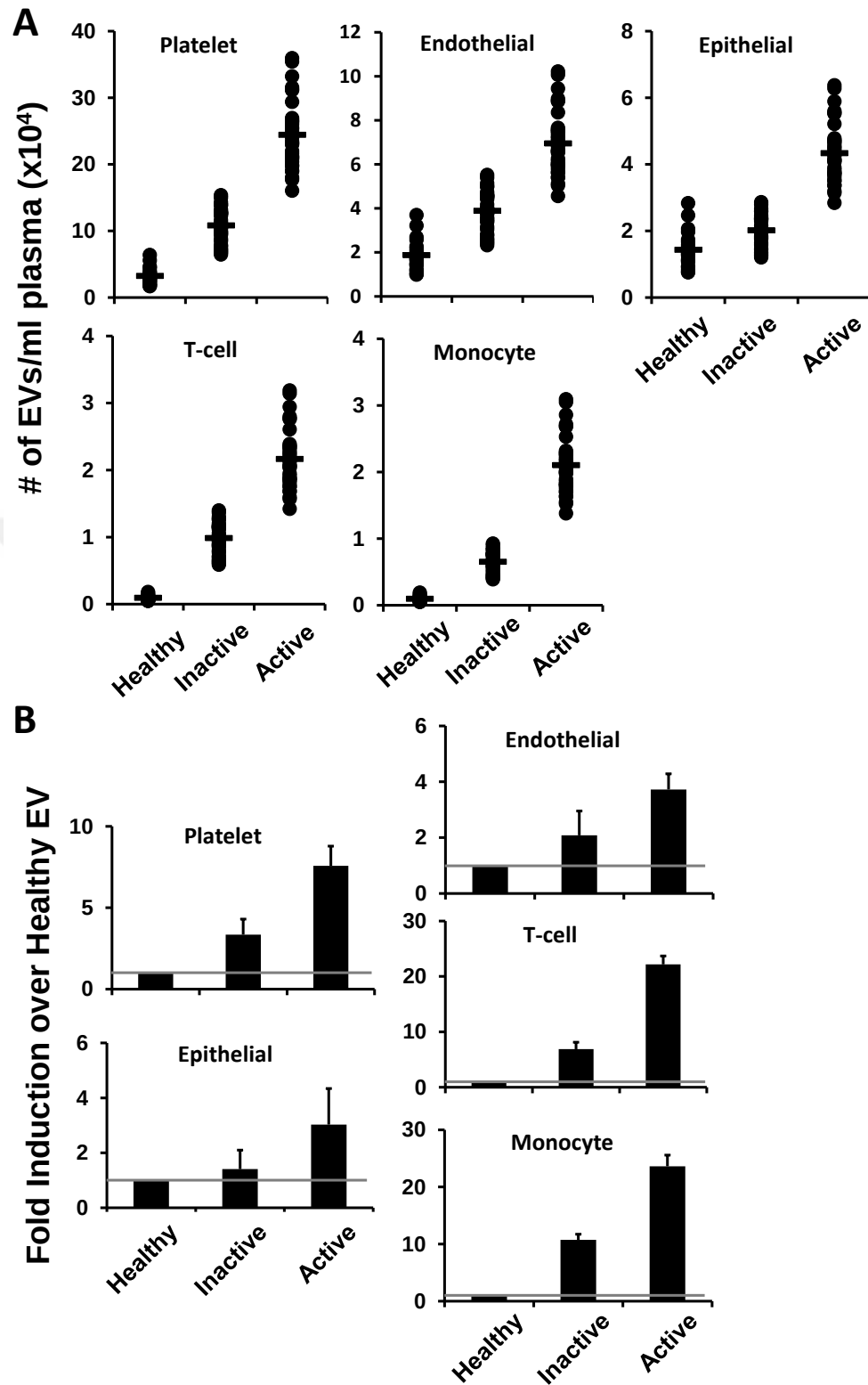


Figure 3.4. Absolute concentration (EV#/ml plasma) of specific cell derived EVs vary with disease state. (A) the distribution of concentrations of cell type-specific EVs for each individual based on their absolute numbers/ml plasma. (B) Cell-specific fold change of EV concentration in BD patients over healthy control levels.

3.1.3. EVs are internalized by PBMCs triggering IL6 secretion

The underlying reasons for the heightened and sustained immune activation in BD patients remain elusive. We established that circulating EV concentrations are elevated especially in active BD. We next attempted to explore the role of heightened EV concentration as a culprit in sustained immune activation in BD patients. To test this possibility, initial experiments focused on internalization studies using fluorescently labeled extracellular vesicles that were isolated from active BD patient plasmas. For this, lipophilic dye, CM-Dil, labeled EVs were incubated with healthy PBMCs at various cell to EV ratios and their internalization patterns were analyzed by confocal microscopy and by flow cytometry (Figure 3.5A-B). Photomicrographs revealed that active BD EVs were effectively internalized by the immune cells (Figure 3.5A). By using different cell to EV ratios, the kinetic of this internalization was also established. Additionally, flow cytometry analysis supported confocal images and demonstrated that PBMCs internalized EVs up to ~40% within 8h when 1:100 cell to EV ratio was used (Figure 3.5B). In addition, this internalization process was found to be independent of incubation time since there is not any significant difference between 2, 4 and 8 h in terms of EV uptake (Figure 3.5B).

To further explore whether active BD EVs were capable of inducing an immune response, we incubated EVs, isolated from healthy, inactive or active BD individuals, with healthy PBMCs for 12 h and determined EV-induced IL6 secretion from culture supernatants by ELISA. Results revealed that autologous and allogeneic healthy plasma-derived EVs triggered lower but detectable levels of IL6 production from healthy PBMC (Figure 3.6, 49.9 ± 19.0 and 116.1 ± 35.4 , ng/ml, respectively). In contrast, inactive and active BD patients triggered 2- and 3.3-fold more IL6 (Figure 3.6, 235.1 ± 38.0 and 384.9 ± 51.3 , ng/ml, respectively) when compared to allogeneic healthy EVs ($p < 0.001$ for all comparisons). This result showed that EVs are internalized by human PBMCs and change the behavior of the cell. In this case, EVs isolated from BD patient internalized by healthy PBMCs and induced more pronounced IL6 secretion with respect to allogeneic healthy EVs. The underlying cause for this elevation is elusive.

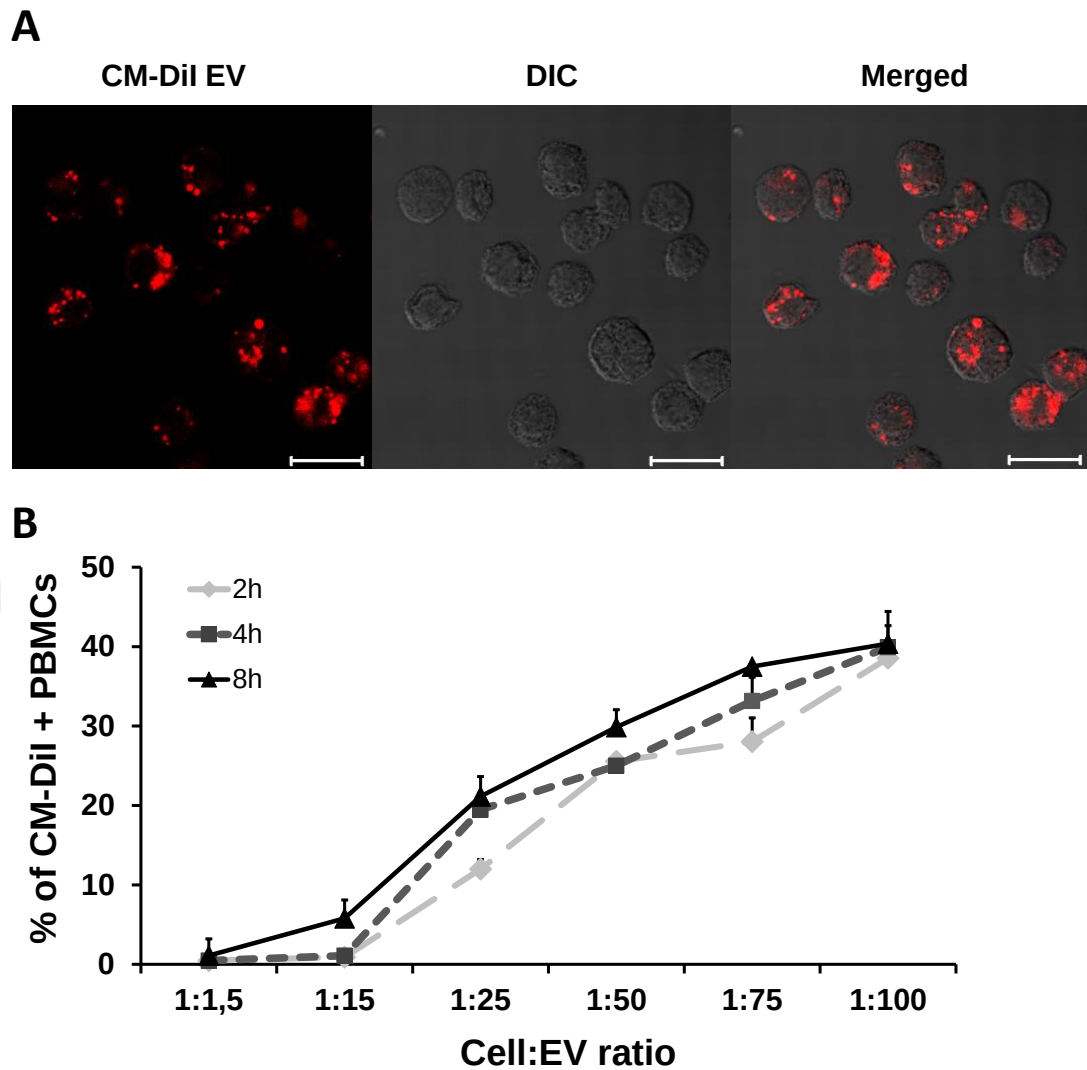


Figure 3.5. EVs are efficiently internalized by PBMCs. (A) Confocal image showing EV internalization by PBMC. Left panel, CM-Dil labeled EV; middle panel, DIC image of the cells; right panel, merged image (Scale bar=20 μ m). (B) Kinetics of EV internalization by PBMCs at different cell:EV ratios. PBMCs were incubated with CM-Dil labeled EVs at various cell:EV ratios up to 8h in culture and percentages of CM-Dil+ cells were assessed by flow cytometry.

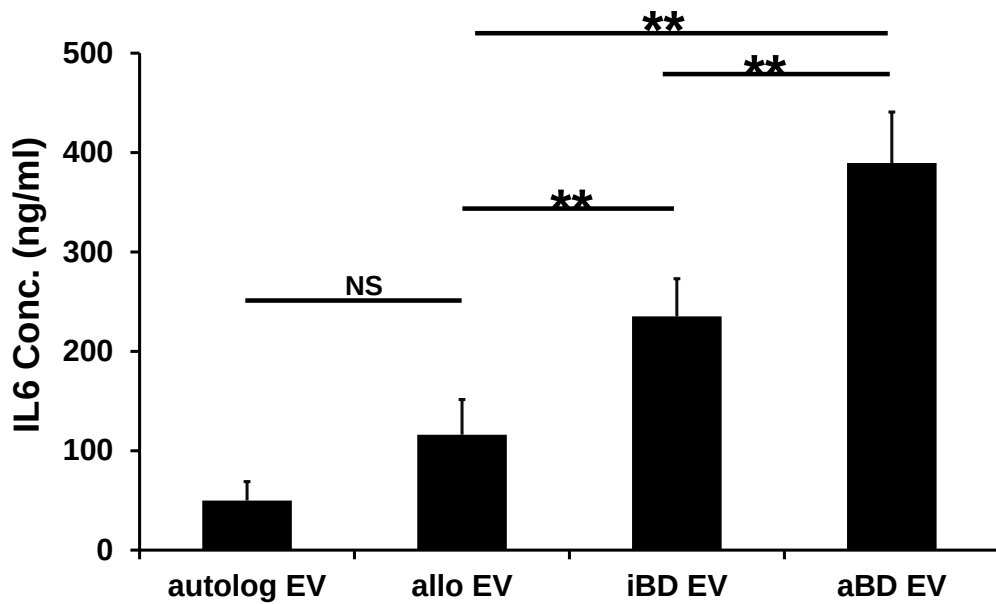


Figure 3.6. BD EVs trigger IL6 secretion from PBMCs. Healthy PBMCs were incubated with autologous or allogeneic EVs at a cell:EV ratio of 1:2 for 12 h. IL6 production was assessed from the culture supernatants by ELISA. (autolog EV; autologous EVs from healthy donor; alloEV; allogeneic EVs from healthy donor; inactive BD EVs (iBD EV); active BD EVs (aBD EV). ** $p < 0.001$ for all comparisons NS: not significant.

3.1.4. Active BD patient plasma LL37 levels are elevated and majority of LL37 is associated with EVs

After establishing that circulating EVs are elevated in active BD plasma and are mediating exaggerated cytokine secretion from PBMCs, we embarked on identifying the factor(s) that might contribute to BD EV-associated immune activation. For this, we analyzed the plasma levels of LL37, an anti-microbial peptide associated with the pathogenesis of several autoimmune/inflammatory diseases, including psoriasis, SLE, RA and atherosclerosis [220]. Plasma LL37 concentrations were found to be 39 ± 8 , 80 ± 11 and 207 ± 16 ng/ml in healthy, inactive and active BD plasma samples, respectively (Figure 3.7A). This data suggests that plasma levels of LL37 in BD patients increased 2- (inactive) and 5- (active) fold with respect to healthy individuals.

Considering that LL37 is a cationic peptide and EVs are enriched in negatively charged phospholipids [221], we next analyzed the possibility of EV/LL37 interaction and determined the levels of LL37 in EV-depleted plasma and in the EV fraction.

Strikingly, ~2/3 of LL37 in the plasma was found to be associated with EVs in active BD (Figure 3.7B).

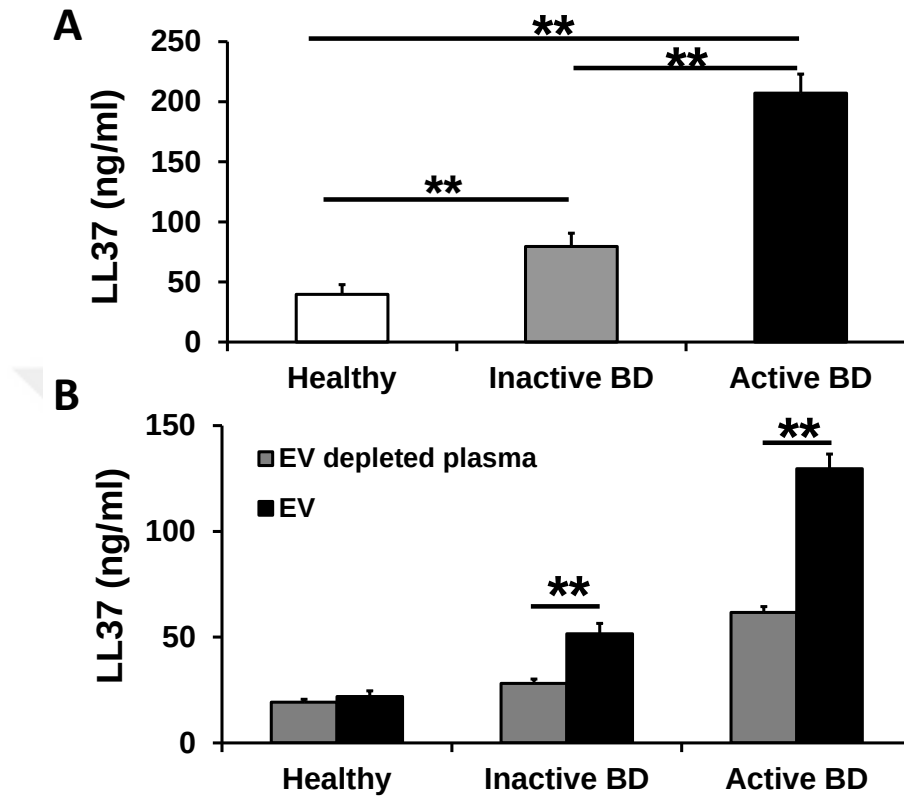


Figure 3.7. BD plasma contains elevated levels of antimicrobial peptide LL37 which is mostly associated with EVs. (A) LL37 concentrations (ng/ml) in plasma of BD patients (active BD $n=12$), (inactive BD $n=12$) and control healthy subjects ($n=12$) were assessed by LL37 ELISA kit. (B) Relative LL37 concentrations (ng/ml) in EV depleted plasma vs. EV

3.1.5. Elevated concentration of EVs, increased IL6 secretion and LL37 concentrations correlates with disease severity

Until now, it is demonstrated that the concentration of EVs in plasma increases with disease progression (Section 3.1.1). Increased IL6 production was observed when healthy PBMCs were incubated with EVs isolated from BD patients (Section 3.1.3). As the culprit for this enhanced IL6 secretory capacity, LL37 levels was found to be increased and anchored to EVs in the plasma of BD patients (Section 3.1.4).

By taking these findings into account, the relationship between LL37 levels, EV concentration in plasma, and pathological cytokine induction capacity of EVs was analyzed using 36 individuals (n= 12 for healthy, inactive BD and active BD groups). It was found that all three parameters correlated with each other as well as disease severity significantly (Figure 3.8). The increase in LL37 levels results in more pronounced secretion of IL6 from PBMCs correlating with disease severity (Figure 3.8A, R^2 : 0.9275 obtained from best fitted line of the plot). LL37 concentration in plasma was also found to be correlated with EV levels in plasma of individuals in addition to the severity of disease progression (Figure 3.8B, $R^2 = 0,9463$ obtained from best fitted line of the plot). Similarly, as seen in Figure 3.8 A and 3.8 B, increased EV levels and IL6 induction capacities showed strong correlation with disease severity (Figure 3.8C, $R^2 = 0,9437$ obtained from best fitted line of the plot).

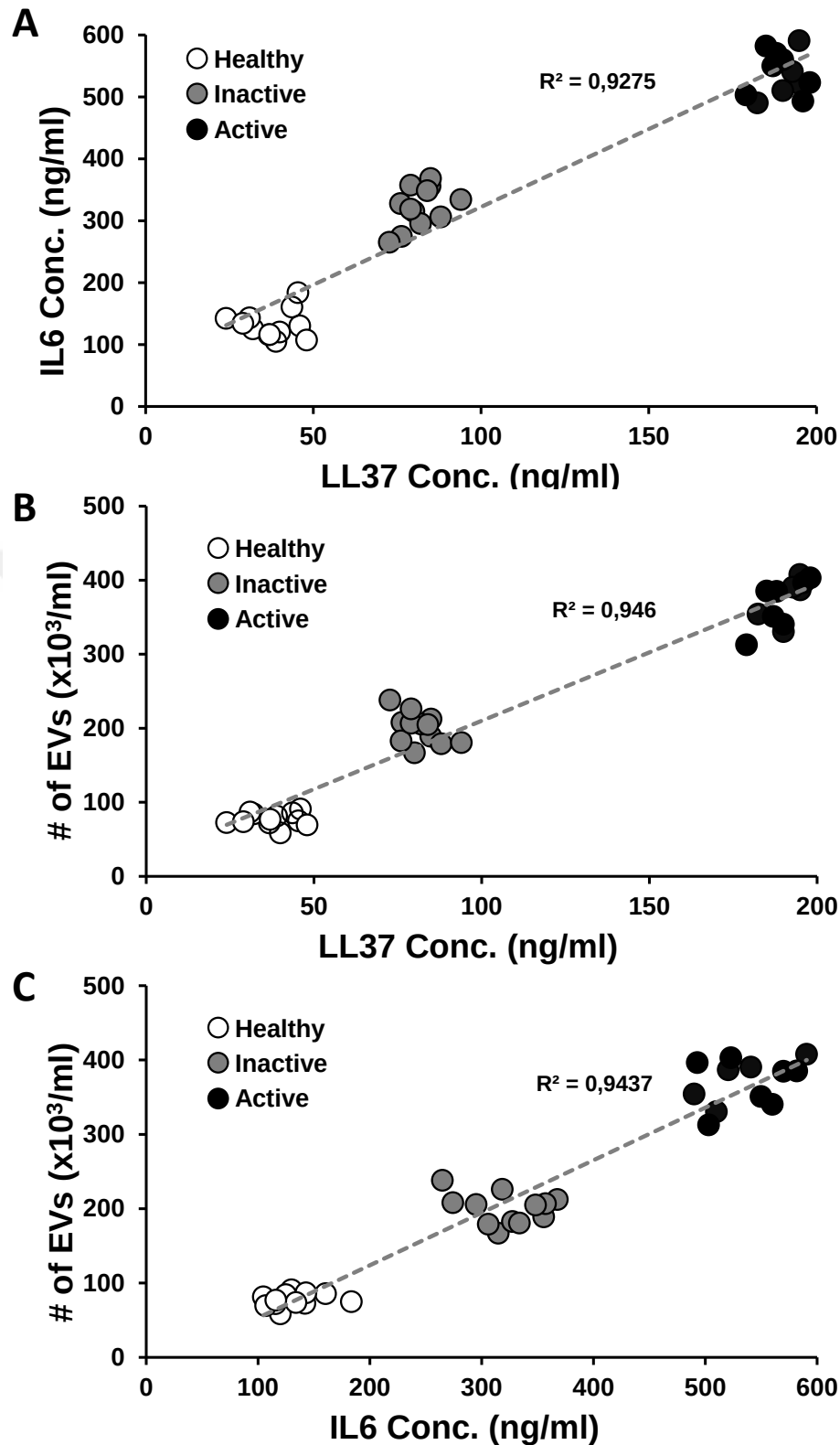


Figure 3.8. The LL37 levels, IL6 induction capacity of EVs and concentration of EV in plasma correlates with disease activity. (A) Correlation between disease severity and plasma LL37 levels in relation to IL6 induction ($n=12$ for all groups, $R^2 = 0,9275$). (B) Correlation between # of EVs and LL37 levels as opposed to disease state ($n=12$ for all group, $R^2 = 0,9463$) (C) Correlation between # of EVs and IL6 concentration as opposed to disease state ($n=12$ for all group, $R^2 = 0,9437$).

3.1.6. LL37 interacts with plasma EV enhancing their cellular uptake to increase IL6 secretion from PBMCs

LL37 was previously shown to interact with and enhance delivery of nucleic acids to immune cells [222]. To assess whether a similar mechanism could operate for EVs, SP-DiOC labeled healthy EVs were either used as such or mixed with LL37 peptide (endotoxin free) prior to incubation with healthy donor PBMCs for 1-8 h. Internalization kinetics of LL37-free and LL37 associated-EVs into PBMCs were assessed by flow cytometer. It was found that in the presence of LL37, the uptake of EVs were enhanced more than 4 folds within 8h incubation period as shown in Figure 3.9A ($p<0.001$). The representative dot plots of EV internalized PBMCs at 2, 4 and 8 h were shown in Figure 3.9B.

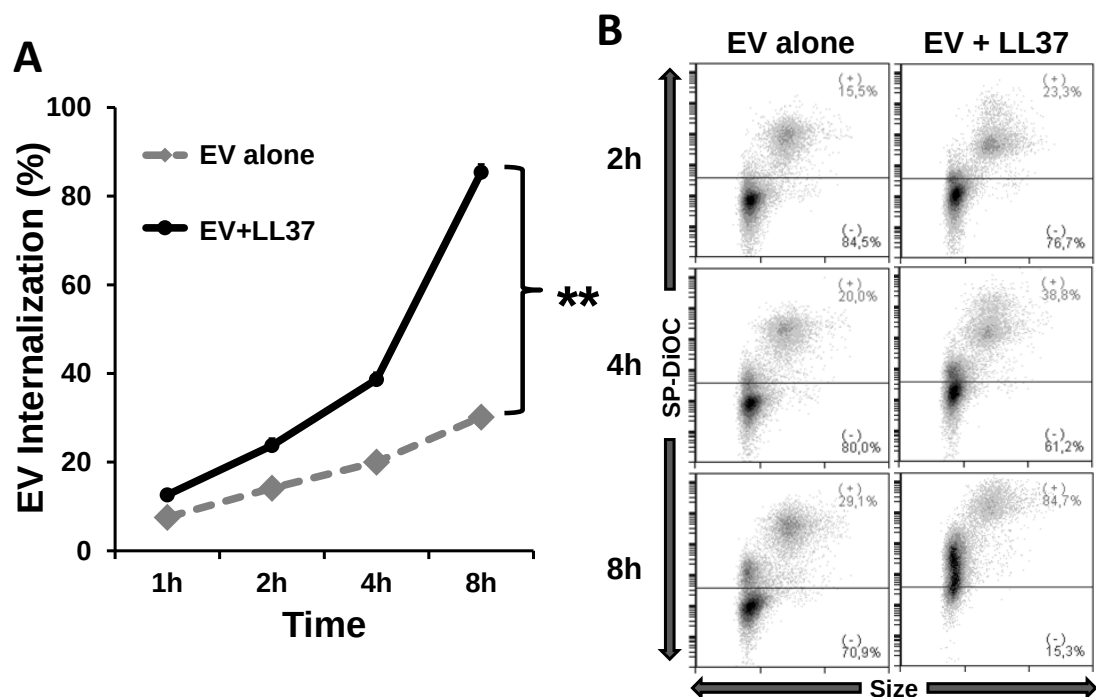


Figure 3.9. LL37 promotes uptake of EVs by immune cells. (A) Time-dependent EV uptake by PBMCs with or without LL37 was analyzed by flow cytometry. PBMCs (1:2; Cell:EV ratio) were incubated as described in the Method Section, and SP-DiOC signal positive cells were analyzed at indicated time points. The data was plotted as Average $\pm$ SD of 6 independent experiments. ** $p<0.001$, (Student's T-test between EV vs EV+LL37 treatments) (B) Representative dot plots at 2, 4 and 8 h incubations.

After establishing that LL37 helps to bring more EVs within PBMCs, next we wished to detect the relative internalization of associated-EVs by different peripheral immune cells. This time EVs were loaded with CFSE and then incubated with LL37. EV or LL37/EV were incubated with PBMCs for 2 h, then cells were fixed and stained with different antibodies against B-cell, dendritic cell (DC), monocyte and T-cell markers. CFSE positivity of each population was assessed by flow cytometer. Results showed that EV delivery to B-cells, monocytes, DCs and to a lesser extent to T-cells were significantly enhanced in the presence of LL37 (Figure 3.10A). The representative dot plots showing CFSE positivity vs cell type was presented in Figure 3.10B.

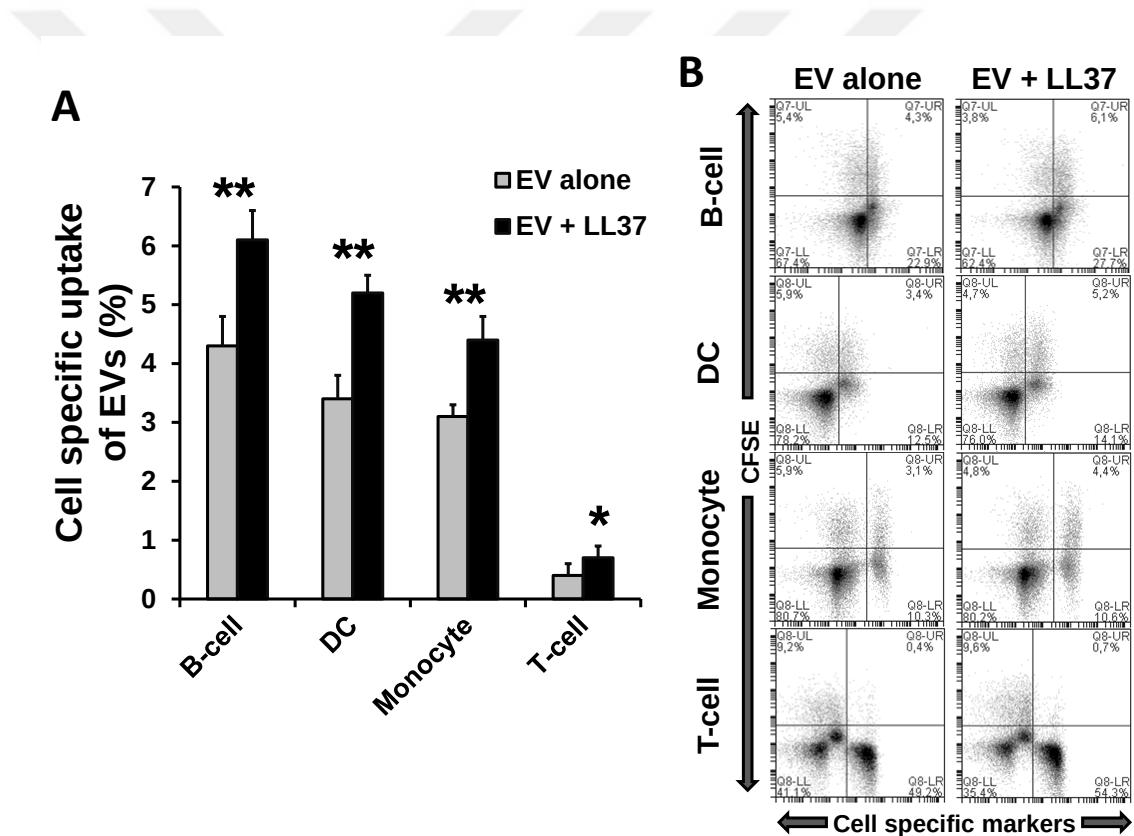


Figure 3.10. Different immune cells internalize EV/LL37 complex at different levels. (A) Uncomplexed or LL37 complexed and CFSE-labeled EVs were incubated with healthy PBMCs at a 1:2 ratio (Cell:EV) for 2h. Cells were stained with anti-CD45R (B-cells), anti-CD11c (DC), anti-CD14 (monocytes), and anti-CD3 (T-cells) antibodies. CFSE positive cells were gated to determine EV internalization within specific cell types by flow cytometry. * $p < 0.01$, and ** $p < 0.001$ (for EV vs EV/LL37 groups comparisons). (B) Representative dot plots of cell-specific internalization.

Next, to establish whether this enhanced LL37-mediated cellular internalization had an impact on subsequent cytokine induction, healthy EVs were mixed with increasing concentrations of LL37 or its scrambled-control prior to co-culture with healthy PBMCs. As seen in Figure 3.11, LL37/EV combination induced a significantly higher IL6 secretion from PBMCs in a dose-dependent manner ($p < 0.001$). This induction was specific to LL37 since the scrambled peptide (SCR)/EV treatment did not yield appreciable amounts of IL6 (Figure 3.11). Although free LL37 also triggered IL6 release at these doses, EV/LL37 combination triggered 2.3-2.2 fold more IL6 than the free peptide at both doses (150 and 450 ng, respectively, Figure 3.11).

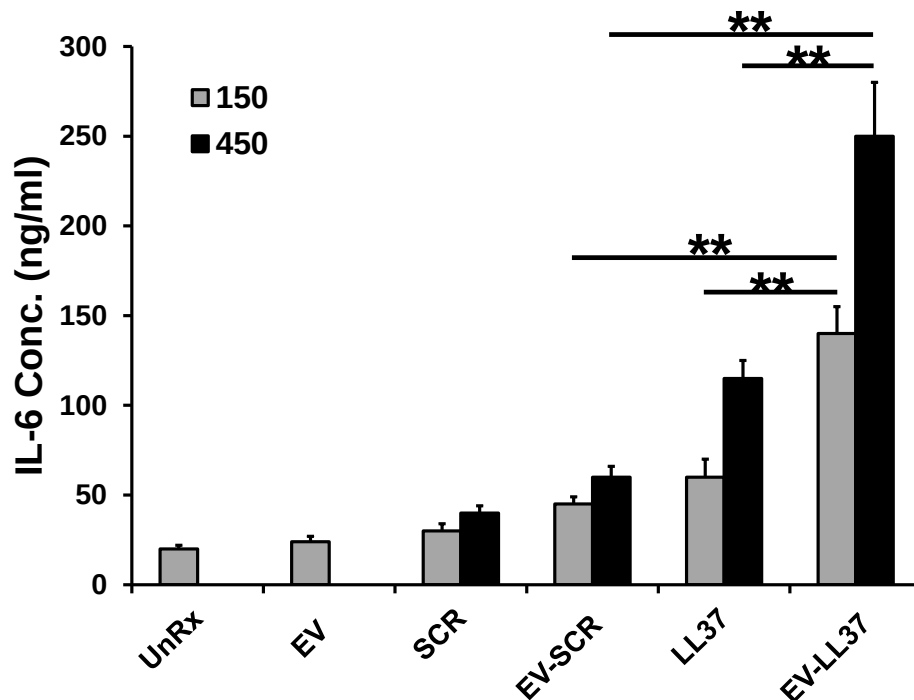


Figure 3.11. Complexation of healthy EVs with LL37 elicits IL6 secretion from PBMCs. EVs isolated from healthy plasma were complexed overnight at 4°C either with 150 ng (gray bars) or with 450 ng (black bars) LL37 (or scrambled control peptide-SCR), prior to incubation with healthy PBMCs for 12h. IL6 levels were assessed from culture supernatants by ELISA. $**p < 0.001$ for all comparisons

3.1.7. LL37/healthy EV complex mimics active BD EVs and triggers IFN α from pDC as well as IL1 β and IP10 from monocytes

Our findings implicated that LL37 associated EVs are capable of inducing inflammatory immune response from healthy cells. To identify the cell types activated by LL37/EV complexes, healthy donor EVs were complexed with LL37, (mimicking active BD EVs) and subsequently incubated with autologous healthy PBMCs. As expected, healthy EVs devoid of LL37 failed to induce any detectable IFN α production from pDCs or IL1 β from monocytes, respectively (Figure 3.12A-B). However, LL37/EV complexes induced >2.5 fold more IL1 β , and >20 fold more IFN α -secreting cells (Figure 3.12A-B). Similarly, IP10 producing monocytes were also significantly elevated in response to LL37/EV (Figure 3.12C).

Collectively, our findings established that active BD patient plasma contains elevated numbers of circulating EVs, and majority of these EVs are associated with LL37. LL37 facilitate more efficient internalization to immune cells, thereby eliciting more robust and sustained immune activation leading to the exacerbation of Behçet`s disease severity.

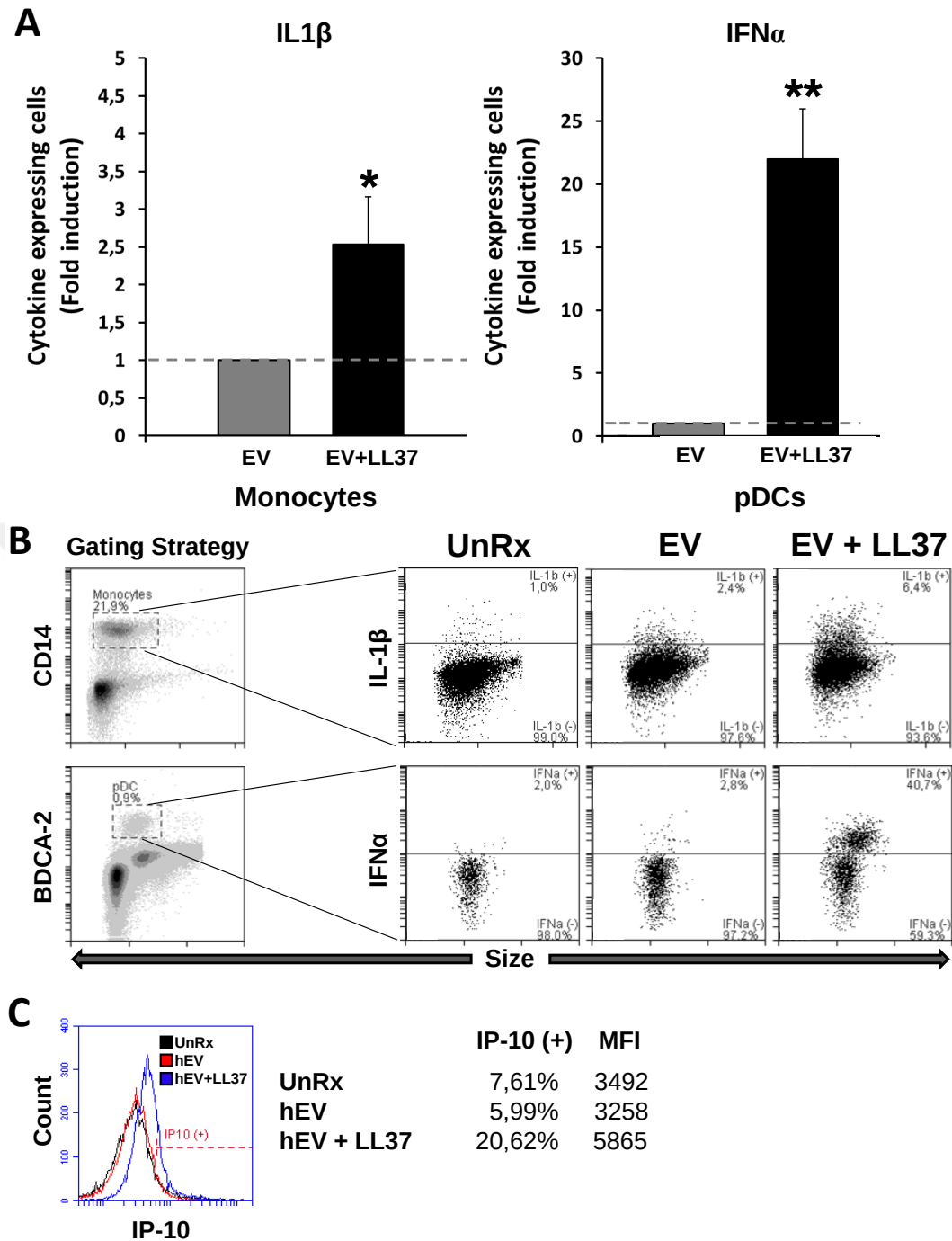


Figure 3.12. LL37/EV triggers IFN α from pDC, IL1 β and IP10 from monocytes. (A) Bar graphs showing average fold induction of IL-1 β and IFN α secreting cells. The data is represented as AVE $\pm$ SD of 6 independent experiments. * p <0.01, ** p <0.001 (comparing EV vs LL37/EV treatments) (B) Gating strategy used to analyze IL-1 β secreting monocytes (CD14) and IFN α secreting pDCs (BDCA-2), (C) Histogram showing the increased IP-10 production in response to LL37/EV from monocytes.

3.2. Role of Extracellular Vesicles in Allergic Diseases

3.2.1. Decreased levels of EVs in allergic diseases

Since it was elucidated that elevated levels of EVs in BD as well as in other autoimmune/inflammatory diseases contributes to heightened Th-1 biased immunity, we attempted to unravel the roles of EVs in diseases showing uncontrolled Th-2 biased immunity such as allergic diseases. To examine possible roles of EVs in allergic diseases such as atopic dermatitis (AD) and asthma, peripheral blood samples were collected from 29 volunteers consisting of 12 healthy individuals, 8 AD and 9 asthma patients. The levels and origin of EVs were examined from these peripheral blood samples.

When the level of EVs in plasma was analyzed from healthy vs allergic patient, it was found that level of EVs show inverse correlation with disease type. Concentration of EVs (# of EV/ml) in plasma were significantly higher in healthy individuals ($7.20 \pm 1.06 \times 10^4$) compared to AD ($2.37 \pm 0.61 \times 10^4$) and asthma ($1.94 \pm 0.50 \times 10^4$, $p < 0.001$, healthy vs., AD and healthy vs. asthma) as shown in Figure 3.13.

Plasma EV concentrations in healthy individuals were 3 and 3.7 fold higher than AD and asthma patients, respectively (Figure 3.13A). AD and asthma patient's EV levels were similar to each other (Figure 3.13A). The EV # of each individual were presented in Figure 3.13B.

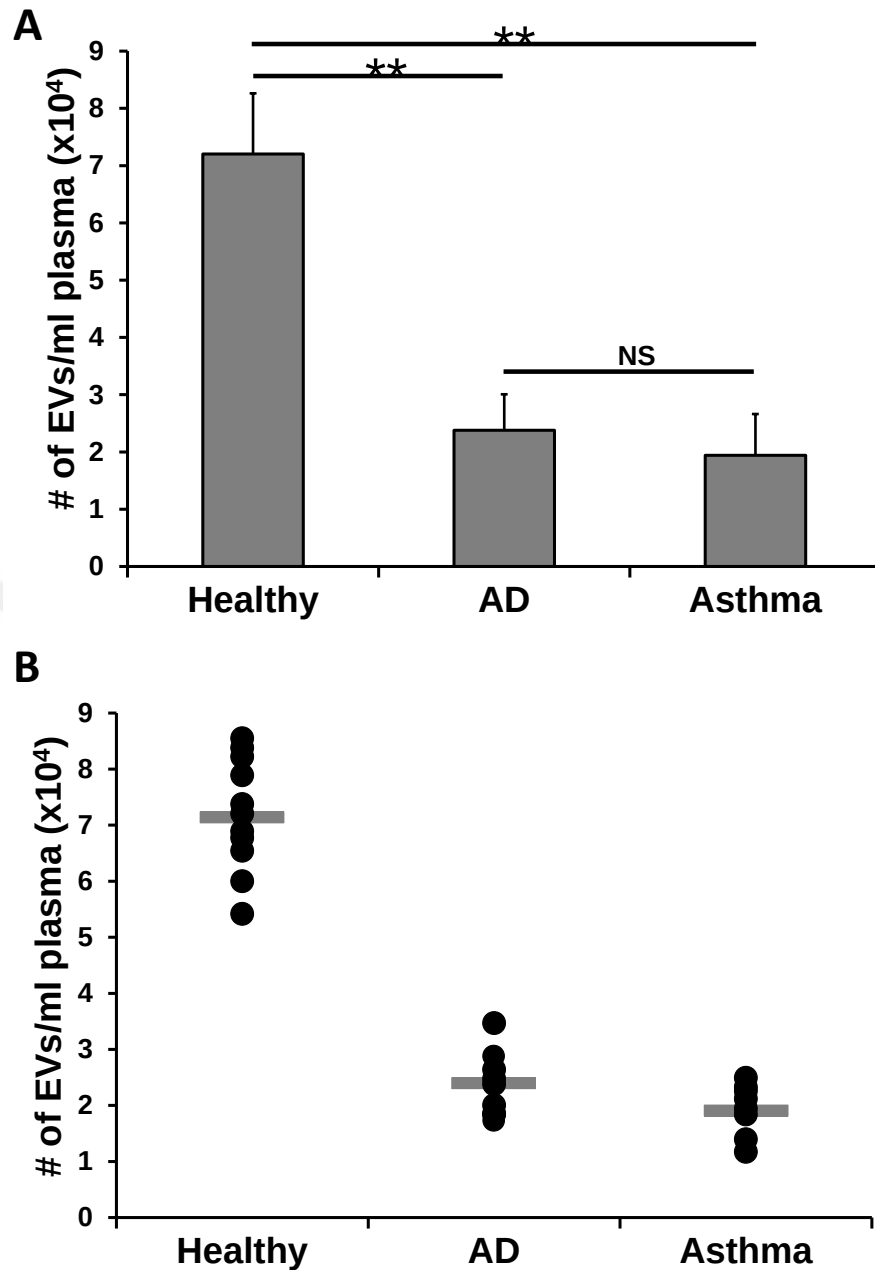


Figure 3.13. Allergic patients have reduced level of plasma EVs. (A) The total number of EVs was given for healthy ($n=12$), AD ($n=8$) and asthma ($n=9$) samples as bar graph. (B) Distribution of EV concentration for each individual as represented by a dot was shown ** $p<0.001$ for comparison groups.

3.2.2. Cellular origin of EVs varies in allergic patients

Next, we checked whether the cellular origin of plasma EVs liberated in allergic patients in contrast to healthy individuals differ. The isolated EVs were stained with different cell specific markers; CD42a for platelets, CD9 for eosinophils, CD14 for monocytes, CD69 for activated T-cells and CD31 for endothelial cells. EVs first stained both with AnV and cell specific markers and then were analyzed by flow cytometer.

As shown in Figure 3.14, flow analysis revealed that platelets are the dominant cell type contributing to plasma EVs both in healthy individuals and allergic patients. However, contribution of platelet derived EVs to total EV population in healthy (67.34 ± 7.96 %) decreases in allergic patients (AD; 52.29 ± 8.27 %, asthma; 51.36 ± 8.14 %, $p < 0.01$ for healthy vs. AD and healthy vs asthma comparisons). When we checked the second dominant EV source, we found that it was eosinophils. In allergic patients (AD; 43.62 ± 3.84 %, asthma; 45.70 ± 3.22 %, $p < 0.01$ for healthy vs. AD and healthy vs asthma comparisons) in contrast to healthy individuals (32.33 ± 4.31 %, $p < 0.01$ for healthy vs. AD and healthy vs asthma comparisons) there was a significantly higher eosinophil-specific circulating EVs.

Although, other cell types albeit to a lesser extent contributed to the total plasma EV amount, their percentages changed dramatically with respect to disease type (Figure 3.14). Percentage of monocyte derived EVs were increased 5- and 11-fold in AD (0.58 ± 0.27 %) and asthma (1.76 ± 0.43 %) patients, respectively, in contrast to healthy individuals (0.11 ± 0.05 %, $p < 0.01$ for AD vs. healthy, $p < 0.001$ for asthma vs. AD and asthma vs. healthy). Similarly, endothelial derived EVs were also found to be heightened in allergic patients. There was 3- and 18- fold increase in AD (0.46 ± 0.18 %) and asthma (2.90 ± 0.53 %) patient's levels compared to healthy subjects (0.16 ± 0.09 %, $p < 0.01$ for AD vs. healthy, $p < 0.001$ for asthma vs. AD and asthma vs. healthy). The percent of EVs originated from activated T-cell were also found to be elevated in plasma of AD and asthma patients by 4- and 6-fold compared to healthy individuals ($p < 0.01$ AD vs. healthy and asthma vs. healthy).

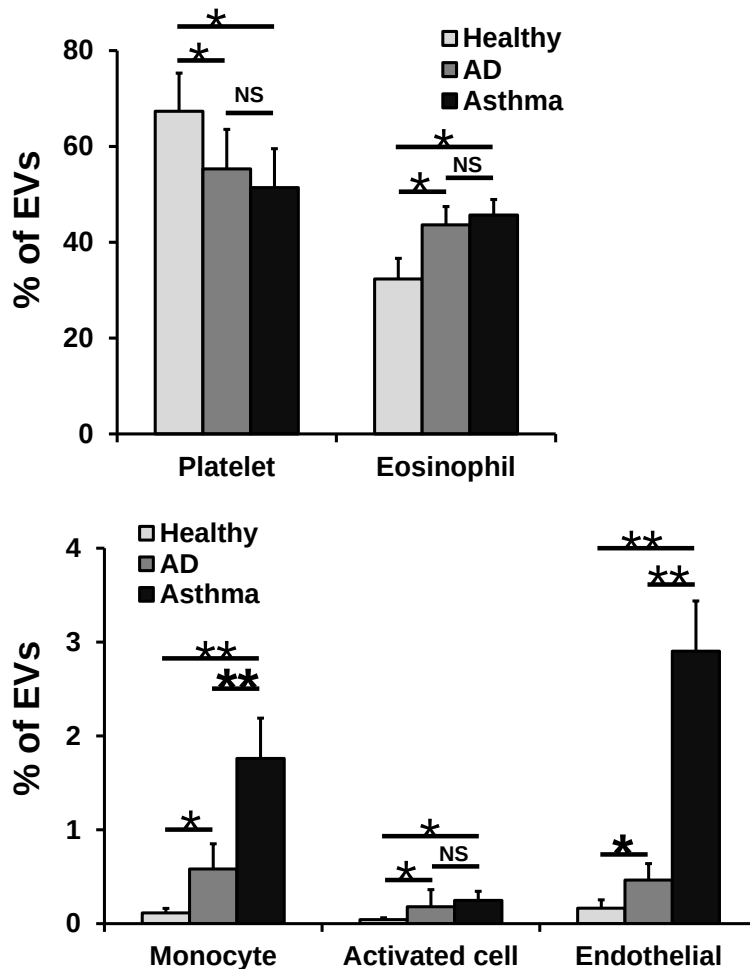


Figure 3.14. Cellular origin of EVs vary in allergic patients. The bar graph shows the relationship between disease states to that of EVs secreted from different cell types. The data depicts the percentage of EVs derived from each specific cell. The following cell-surface markers were used to stain eosinophil (CD9), monocyte (CD14), platelet (CD42a), T-cell activation marker (CD69), and endothelial cell (CD31) specific EVs isolated from healthy individuals (n=12), AD (n=8) and asthma (n=9) patients. * $p < 0.01$ and ** $p < 0.001$ for indicated comparisons.

3.3. EXOSOLIGOS: A novel Exosome-based Delivery Platform for Effective Immune Modulation

3.3.1. Exosomes can be isolated from supernatant of RAW264.7 cell line with high yield

Since exosomes are proposed as new mediators of cell to cell communications, transferring cargo between cells, we reasoned that these natural nanovesicles could be engineered and used as drug or vaccine delivery vehicles. To test this

hypothesis, exosomes were isolated from cell culture supernatants of mouse macrophage like cell line, RAW264.7. The purified pellet was analyzed to verify that indeed we are obtaining exosomes from the culture supernatants. DLS and AFM analyses as well as surface marker analysis by flow cytometer were performed in order to confirm that pure exosome pellet was obtained after differential centrifugation.

The hydrated and dry state sizes of exosome was confirmed. DLS analysis revealed that the hydrated size of obtained pellet (89.72 ± 27.85 nm) was within the acceptable range for exosomes (Figure 3.15A). Furthermore, AFM images confirmed the desiccated size of vesicles were between 30-90 nm, which was also in accordance with the literature [26], (Figure 3.15B).

Another gold standard procedure to further identify the identity of these cell-line derived nanovesicles was to check the surface expression of tetraspanins and phosphatidylserine. The RAW264.7 and isolated exosomes were stained with anti-CD81-PE (binds to tetraspanins) and AnV-PE (binds to PS) and analyzed by flow cytometer. Since exosomes are smaller than flow cytometer's detection limit, exosome staining was performed by anti-CD9 coated beads (3 μ m) that was hybridized prior to staining with anti-CD81-PE or with AnV-PE. By including this step, isolated vesicles were tested for expression of CD9 as well. Flow analyses, as expected, demonstrated high AnV and CD81 co-expression on exosomes (MFIs; 1850 and 33851, respectively) but not on RAW264.7 cells (MFIs; 181 and 274, respectively) (Figure 3.15).

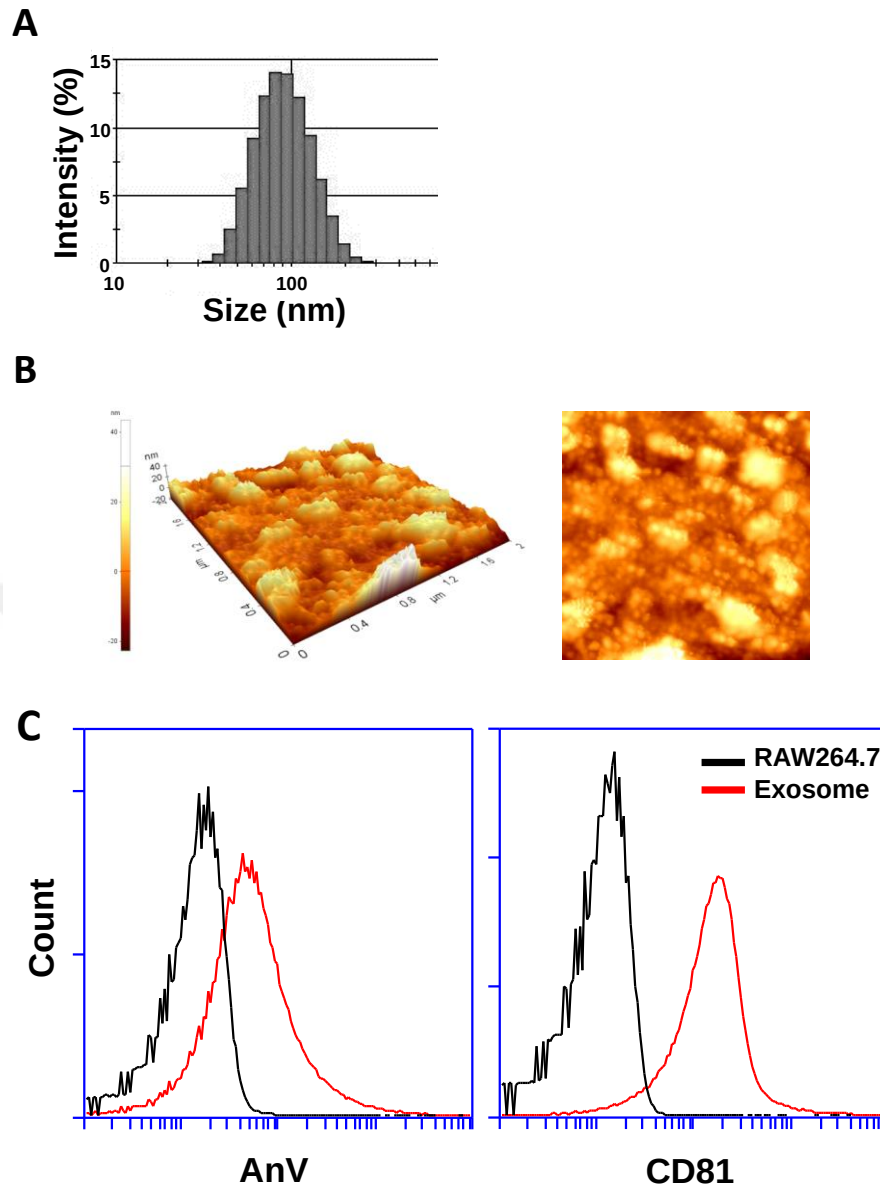


Figure 3.15. Exosomes can be isolated from RAW264.7 cells. (A) DLS analysis showing hydrated size distribution of RAW264.7 derived exosomes. (B) AFM topography image of dehydrated exosomes (Area: $2 \times 2 \mu\text{m}$). (C) FACS analysis showing the exosomal surface expression of AnV and CD81 from RAW264.7 cells

3.3.2. A TLR9 ligand CpG ODN D35 can be encapsulated within exosomes

After showing that exosomes can be isolated from mouse macrophage cell-line cell supernatants, the feasibility of CpG ODN encapsulation by dehydration and rehydration technology that was initially developed for liposome loading [223], was sought.

Initially, K-Type and D-Type CpG ODNs (K3, K23 and D35, respectively) were tested to determine the % encapsulation within exosomes by mixing ODN:Exo ratios (1:2, 1:1 and 2:1, w/w). Upon dehydration-rehydration vesicle technology (DRV method), CpG ODN exosomes (Exo(CpG)) were separated from the encapsulation environment by ultracentrifugation and remaining free CpG ODN concentration in the supernatant was assessed by NanoDrop™ in order to obtain encapsulation efficiency. Among the tested ODNs, D35 was found to be the only CpG ODN type that could be encapsulated within exosomes with high ODN yield (~70%). The other ODNs, K3 and K23, were loaded at substantially much lower levels. On the other hand, mixing exosomes and D35 CpG ODN at 1:1 ratio was found to be the optimum condition (Figure 3.16A). More than 30% more loading was achieved when D35 was used at 1:1, rather than at 1:2 mixing ratio ($71.34 \pm 2.31\%$ vs. $54.48 \pm 4.57\%$, $p < 0.001$). However, there was not significant difference in encapsulation efficiency when the ODN amount was increased to 2:1 ratio ($70.23 \pm 2.85\%$).

After optimizing the D35 CpG ODN encapsulating within exosomes via DRV technology, we checked whether the encapsulation method hampered exosome integrity and physical features. The effect of dehydration-rehydration method on size distribution of exosomes was determined by DLS and the size of dehydrated Exo(D35) was found to be around 80 nm (77.19 ± 23.42 nm) (Figure 3.16B). When compared with the size of freshly isolated exosomes (Exo; 89.72 ± 27.85 nm), there was no significant size change after DRV procedure (Figure 3.16C).

Furthermore, retaining of cardinal surface staining markers of reconstituted exosomes were also analyzed by interacting Exo(D35) with anti-CD9 coated latex beads and then stained either with anti-CD81-PE or with AnV-PE. Flow analyses confirmed that reconstituted exosome vesicles expressed both CD9 and CD81 as well as surface exposed phosphatidylserine lipids (Figure 3.16D)

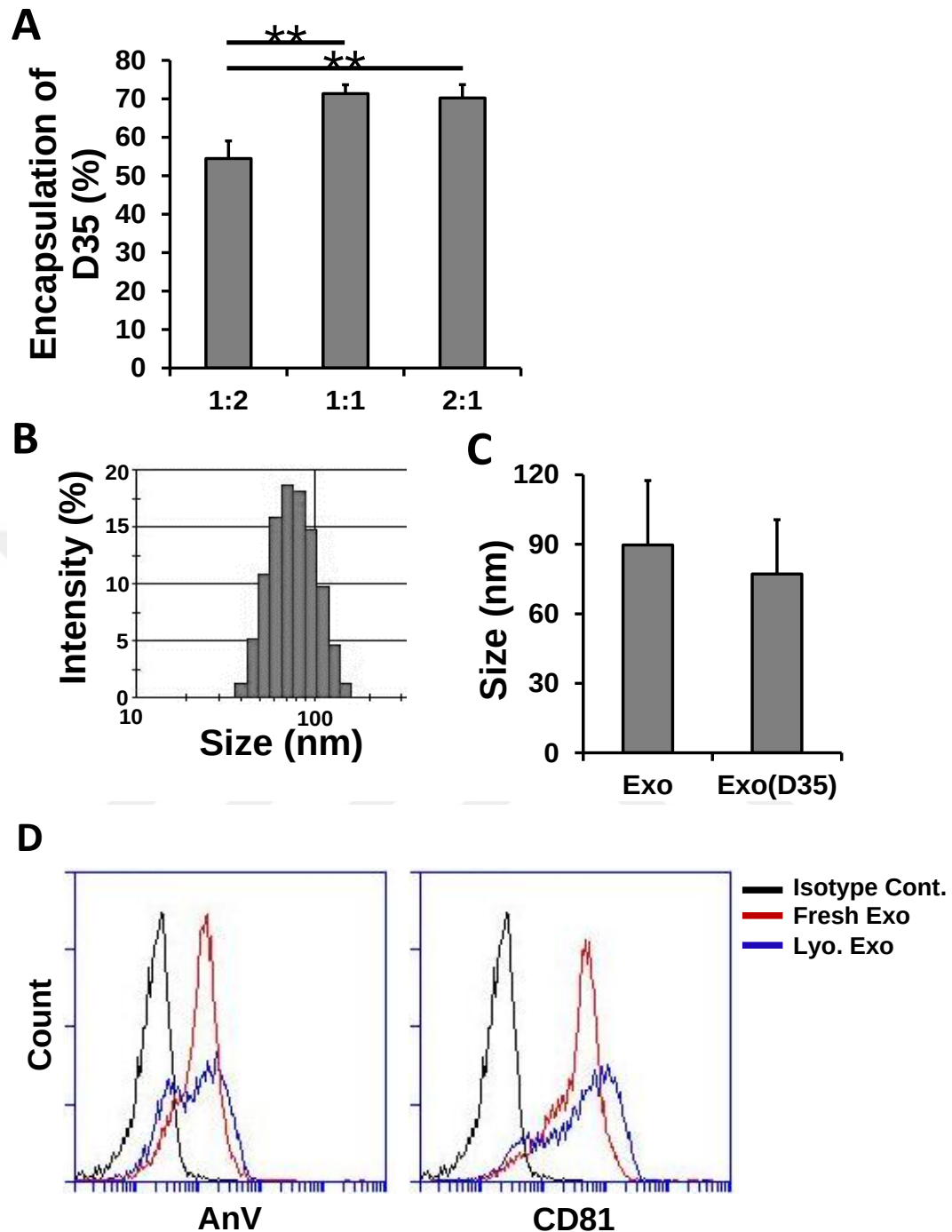
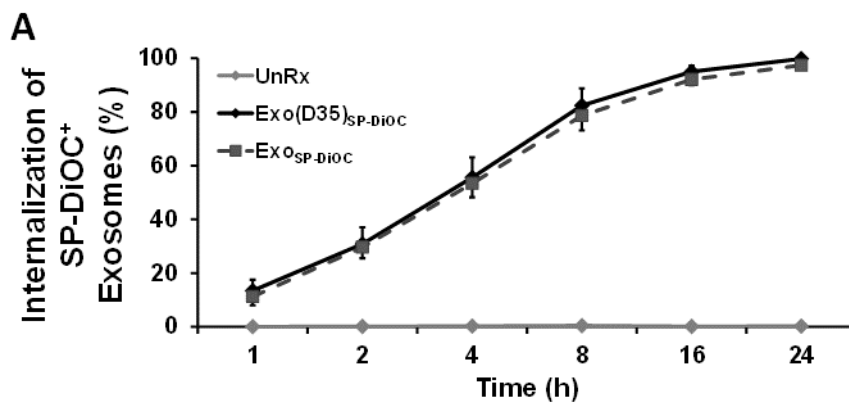


Figure 3.16. D-Type CpG ODN could be loaded at high efficiency without disrupting exosomal integrity. (A) Bar graph showing loading efficiency of D35 within exosomes at various ODN:Exo ratios (** $p < 0.001$ for indicated comparisons) (B) Size distribution of rehydrated D35 containing exosomes by DLS. (C) Bar graph comparing size distribution of freshly isolated RAW exosomes to that of lyophilized and then reconstituted D35 containing exosomes. (D) Histograms comparing surface expression of AnV and CD81 of freshly un lyophilized exosomes vs. reconstituted exosomes.

3.3.3. Mouse spleen cells efficiently internalizes CpG ODN loaded exosomes

Next, we examined whether splenocytes can take up D-Type CpG ODN loaded exosomes (Exo(D35) hereafter). For this purpose, exosomes were initially loaded with Cy5-conjugated ODNs and then stained with lipophilic dye, SP-DiOC (Exo(D35-Cy5)_{SP-DiOC}). Splenocytes were then incubated up to 24h in culture with Cy5 conjugated free ODNs, Exo_{SP-DiOC} (unloaded exosomes) or Exo(D35-Cy5)_{SP-DiOC} and cells were analyzed either by flow cytometry or by confocal microscopy for the internalization of exosomes. Exo(D35-Cy5)_{SP-DiOC} were internalized by cells starting from 1h of incubation and reached a plateau by 16h (99.26 ± 0.34% of immune cells were double positive for Cy5/SP-DiOC signals, Figure 3.17A). In Figure 3.17B, representative dot plots at the end of 24 h of incubation are presented. Consistent with flow analysis, both exosome signal and CpG ODN signal (SP-DiOC and Cy5, respectively) was co-localized within target cells as revealed by confocal microscopy (Figure 3.17C). Here, yellow spots indicated co-localized signals coming from SP-DiOC and Cy5.



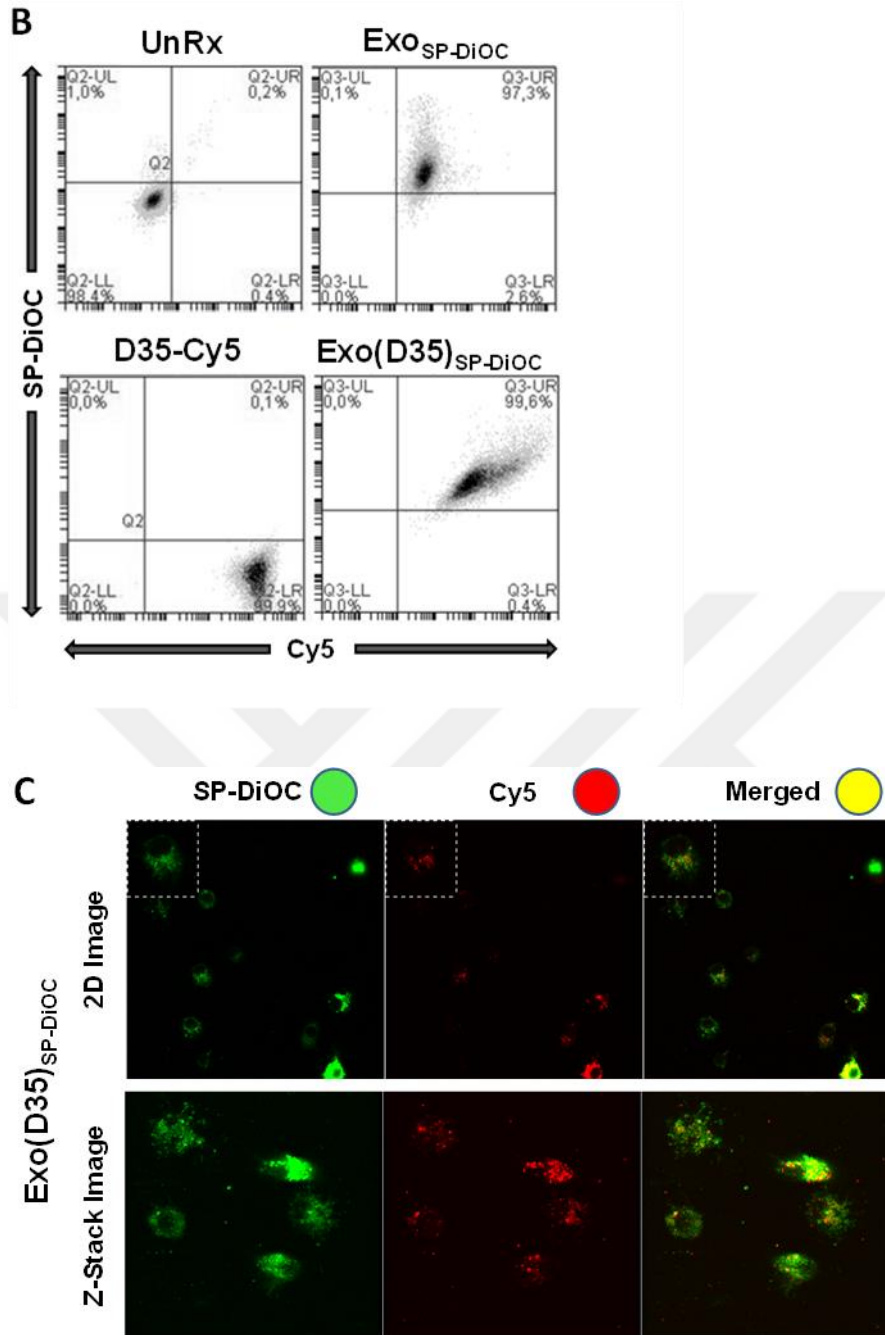


Figure 3.17. Both CpG ODN D35 and exosomes co-localizes within same compartment upon internalization by cells. Binding uptake kinetics of ODN loaded exosomes by immune cells were assessed by flow cytometry and internalization ability was analyzed by confocal microscopy. (A) Internalization kinetics of D35 loaded exosomes over time. Exosomes were stained with SP-DiOC. (B) Representative dot plots of untreated (upper left), exosomes (upper right), free D35 (lower left), and D35 loaded exosomes (lower right) treated cells. Double positivity for Cy5 and SP-DiOC signals indicated that they are co-localized by cells at the end of 24 h of incubation in culture. (C) The confocal analyses of 2D (upper panel) and 3D (lower panel) images of cells that was incubated with D35 loaded exosomes for 8h. NOTE: merged (yellow spots) image indicated the co-localized sub-cellular compartments within a cell.

3.3.4. D-Type CpG ODN encapsulation within exosomes improves TLR9 mediated immunostimulatory activity

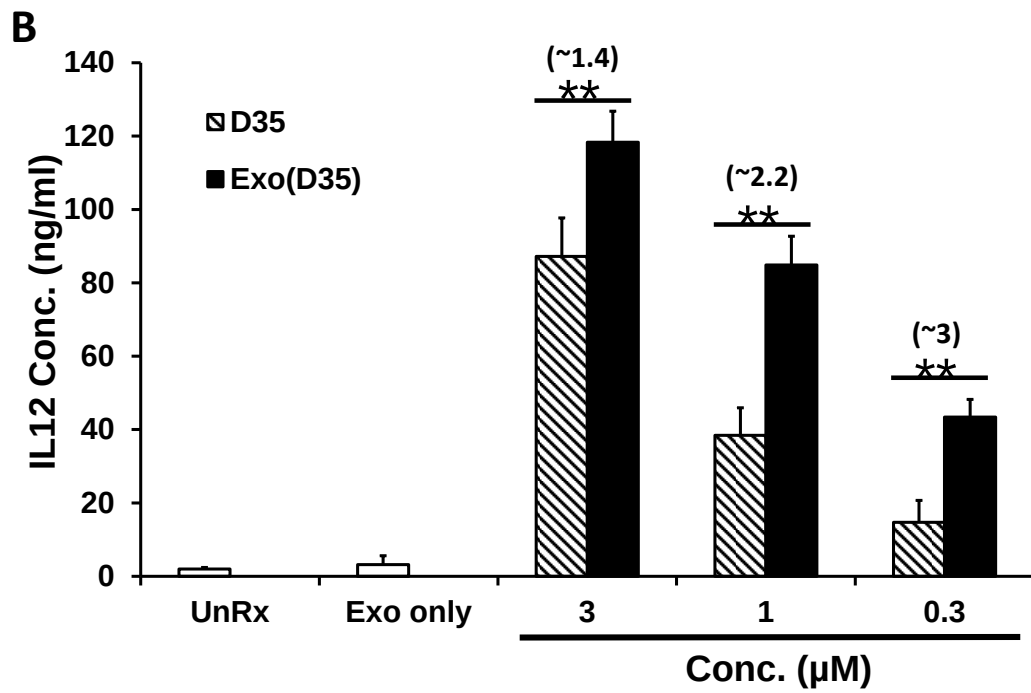
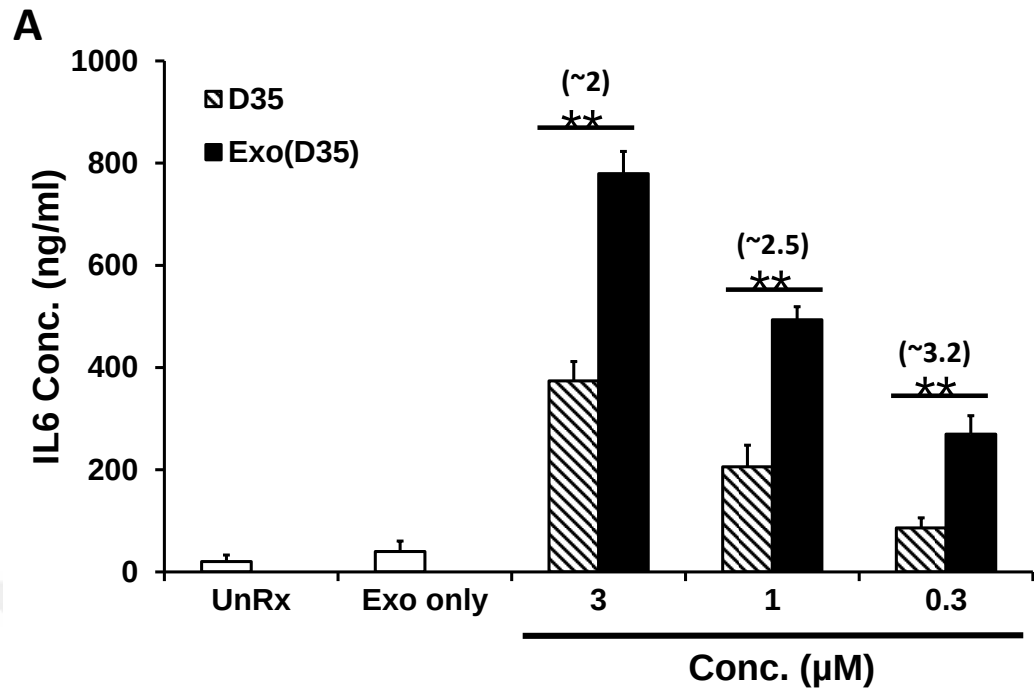
After we observed that exosomes could efficiently enter and deliver its cargo to the target cells, we then investigated whether ODN loading improves immune stimulatory properties of D-Type CpG ODN on mouse spleen cells. Stimulation assays either with free or exosomal D35 at various doses revealed that there was a dose-dependent amplification of CpG-mediated IL6 or IL12 production (Figure 3-18). Even at 3 μ M dose, an optimum dose for free D35, cells stimulated with Exo(D35) secreted 2-fold higher IL6 (780 $\pm$ 43 ng/ml vs. 360 $\pm$ 38 ng/ml, p <0.001). This difference was much pronounced when the CpG ODN was titrated by 10 fold. A 3.3-fold increase in IL6 secretion was observed at 0.3 μ M concentration of D35 (270 $\pm$ 36 ng/ml vs. 83 $\pm$ 38 ng/ml, p <0.001, Figure 3.18A). Similar observation was also seen in IL12 secretion. At 3 μ M dose, Exo(D35) stimulated cells secreted 1.4-fold higher IL12 compared to free form (118 $\pm$ 9 ng/ml vs. 87 $\pm$ 10 ng/ml, p <0.001) and when 0.3 μ M CpG ODN stimulation was used the exosomal D35 induction capacity go up 3.25-fold compared to free D35 induction capacity (270 $\pm$ 36 ng/ml vs. 83 $\pm$ 38 ng/ml, p <0.001, Figure 3.18B).

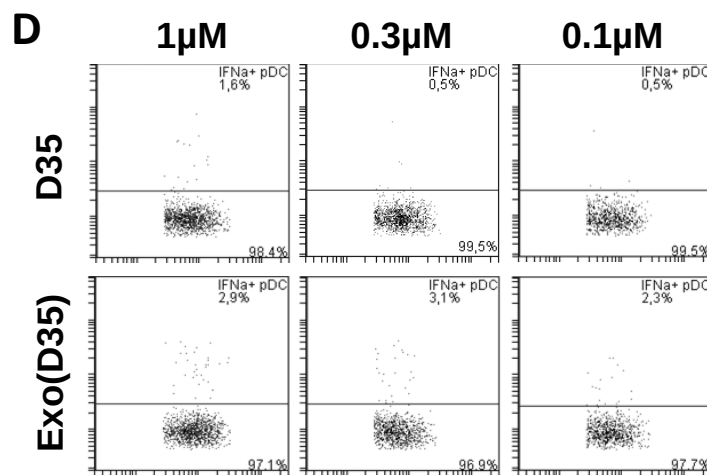
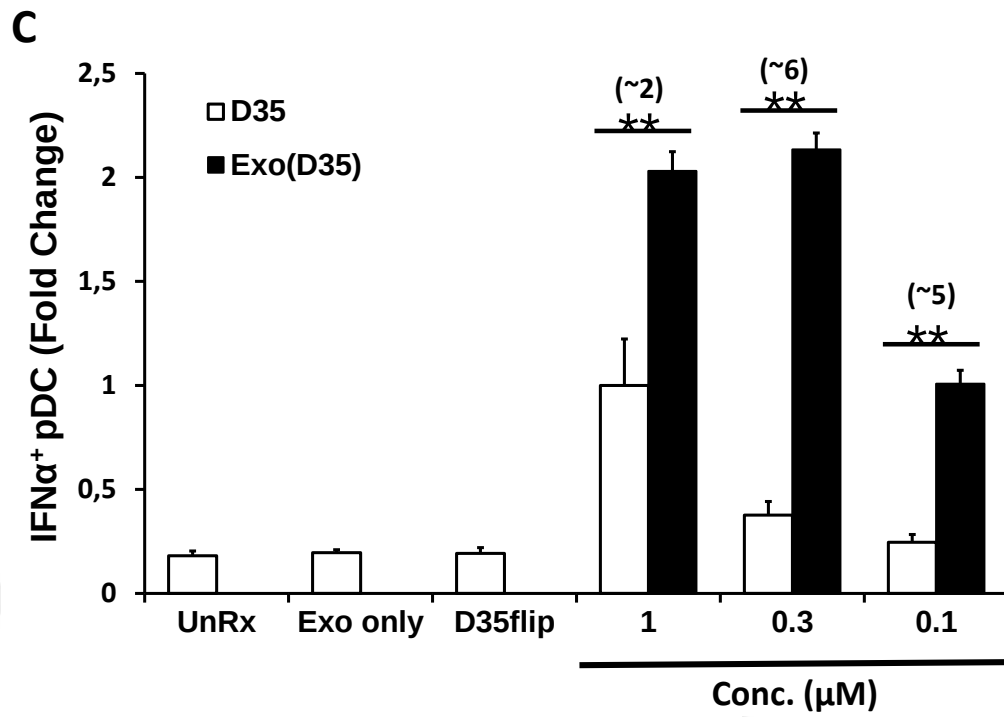
Since IFN α secretion from pDCs is one of the hallmarks implicating stimulatory activity mediated by D-Type CpG ODN [224], we next checked the IFN α production levels upon free or exosomal D35 treatment by intracellular cytokine staining of mouse pDC cells. Splenocytes were stimulated with D35 or Exo(D35) in the presence of Brefeldin-A for 6 h. Then cells were fixed and stained with anti-B220-PE/Cy5 and anti-CD123-PE along with anti-IFN α -FITC. Flow cytometric analyses suggested that higher numbers of B220⁺/CD123⁺ pDCs were capable of producing intracellular IFN α upon incubating with Exo(D35) than free D35 counterpart. For all doses (1.0-0.1 μ M ODN), 2X, 6X, and 5X more IFN α ⁺-pDCs were detected from Exo(D35) treated samples, respectively. Similar to cytokine ELISA responses, intracellular IFN α difference was more pronounced at low doses of Exo(D35) treatment (p <0.001, Figure 3.18C, & Fig. 3.18D).

Since Exosomes could act as a depot system for D35 CpG ODNs upon loading, we hoped that the magnitude and the speed of CpG-triggered immune activation could be enhanced when the cells were treated with Exo(D35). Indeed, exosomal D35, as opposed to its free counterpart mediated more rapid and sustained cytokine

production and as evidenced by IL12 secretion kinetics ($p < 0.001$, Figure 3.18E). While it required 8-16h to detect IL12 in the supernatant of cells that was treated with low dose free D35 (0.3 μ M), the exosomal counterpart led to detectable IL12 secretion by 4h post-treatment. Strikingly, by 16h incubation a 10-fold more IL12 was present in the Exo(D35) supernatants, to that of D35 alone supernatants (~100ng/ml vs ~10ng/ml; Figure 3.18E). This stimulatory capacity was even more superior at 8h. While free D35 failed to induce splenocytes to secrete detectable IL12, Exo(D35) triggered ~32 ng/ml ($p < 0.001$, Figure 3.18E).

Of note all these effects were dependent on D35 presence since unloaded exosomes or exosomes loaded with control ODNs did not yield any appreciable amounts of cytokine production in these assays (data not shown).





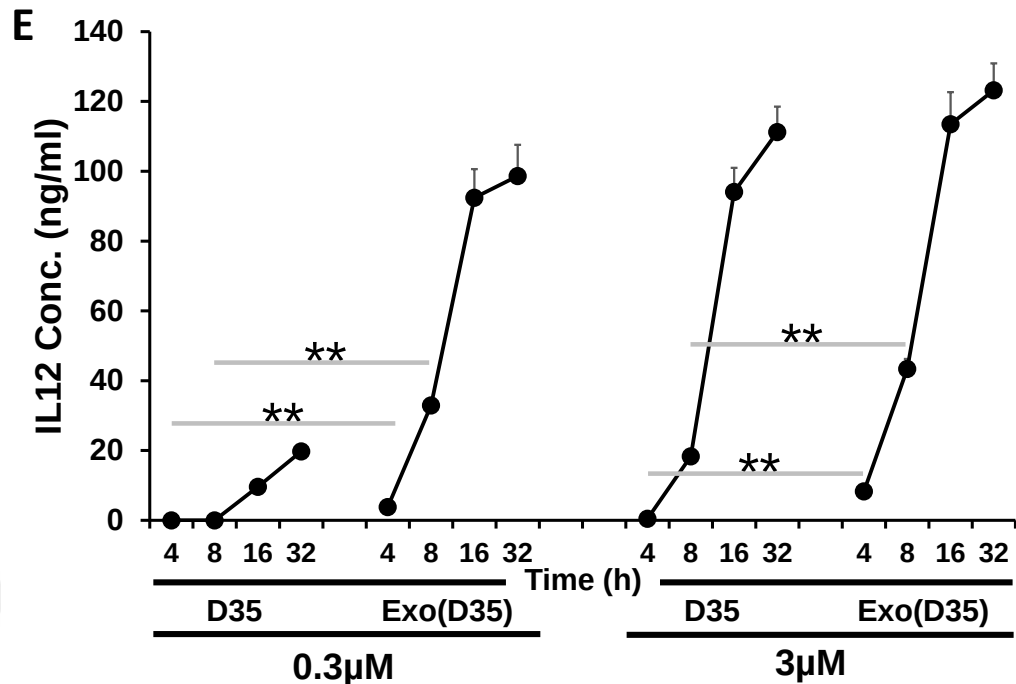


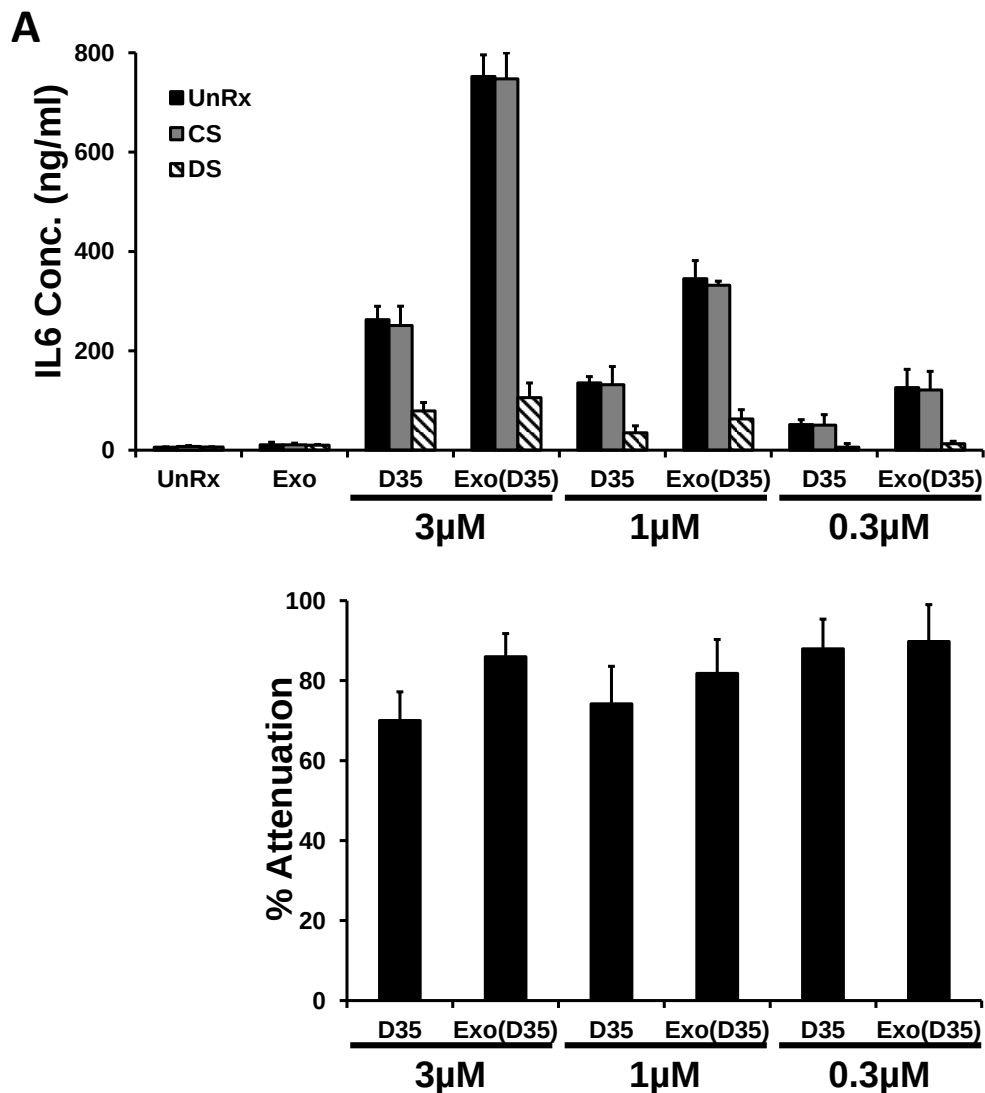
Figure 3.18. Encapsulation into exosomes improved immune stimulatory capacity of D35 CpG ODN. Immune stimulatory properties of free D35 vs. Exo(D35) was compared. (A) IL6 and (B) IL12 secretion from splenocytes upon stimulating with free and encapsulated forms of ODN D35 at indicated concentrations for 36h. (C) Bar graph showing fold change of IFN α secreting pDCs upon intracellular cytokine staining. (D) Representative dot plots of IFN α ICS analyses. (E) Time course IL12 secretion after incubation of splenocytes for indicated time periods. ** $p < 0.001$ for all indicated comparisons. ($p < 0.001$ for all comparisons)

3.3.5. Blocking scavenger receptor attenuates D35 dependent immune activation

Until now, we established that RAW-exosomes can be engineered (post-purification from cell supernatants) to harbor CpG ODN as their cargo. The exosomes led to improved cellular binding and subsequent ODN uptake which was then translated to superior in vitro immune stimulation mediated by CpG ODN. We next wished to delineate the mechanism of internalization leading to effective stimulation.

Since exosomes are negatively charged due to flipped out PS, we reasoned that studying the contribution of scavenger receptors (SRs) on nanovesicle internalization would be the most reasonable approach. Therefore, we tested whether blockage of this pathway mitigates immune stimulation mediated by Exo(D35).

Dextran sulfate (DS) is one of the classical ligands of scavenger receptors. In this experiment, next to DC, as a sulfated macromolecule, chondroitin sulfate (CS) was used as control group. Here, splenocytes were first incubated either with DS or CS (80 $\mu\text{g/ml}$) for 30 min at 37°C and then the cells were washed and stimulated (0.3-3.0 μM) either with i) D35 or ii) Exo(D35) for 24h in culture. As depicted in Figure 3.19A-B, pre-treatment of spleen cells with a scavenger receptor ligand (i.e. DS) and subsequently stimulating them either with D35 or with Exo(D35) attenuated both IL6 and IL12 production. This is SR mediated since CS, as expected did not mitigate D35-mediated immune activation. DS treatment attenuated 70-90 % of IL6 (Figure 3.19A) and IL12 (Figure 3.19A) secretion from spleen cells irrespective of ODN dose ($p < 0.001$).



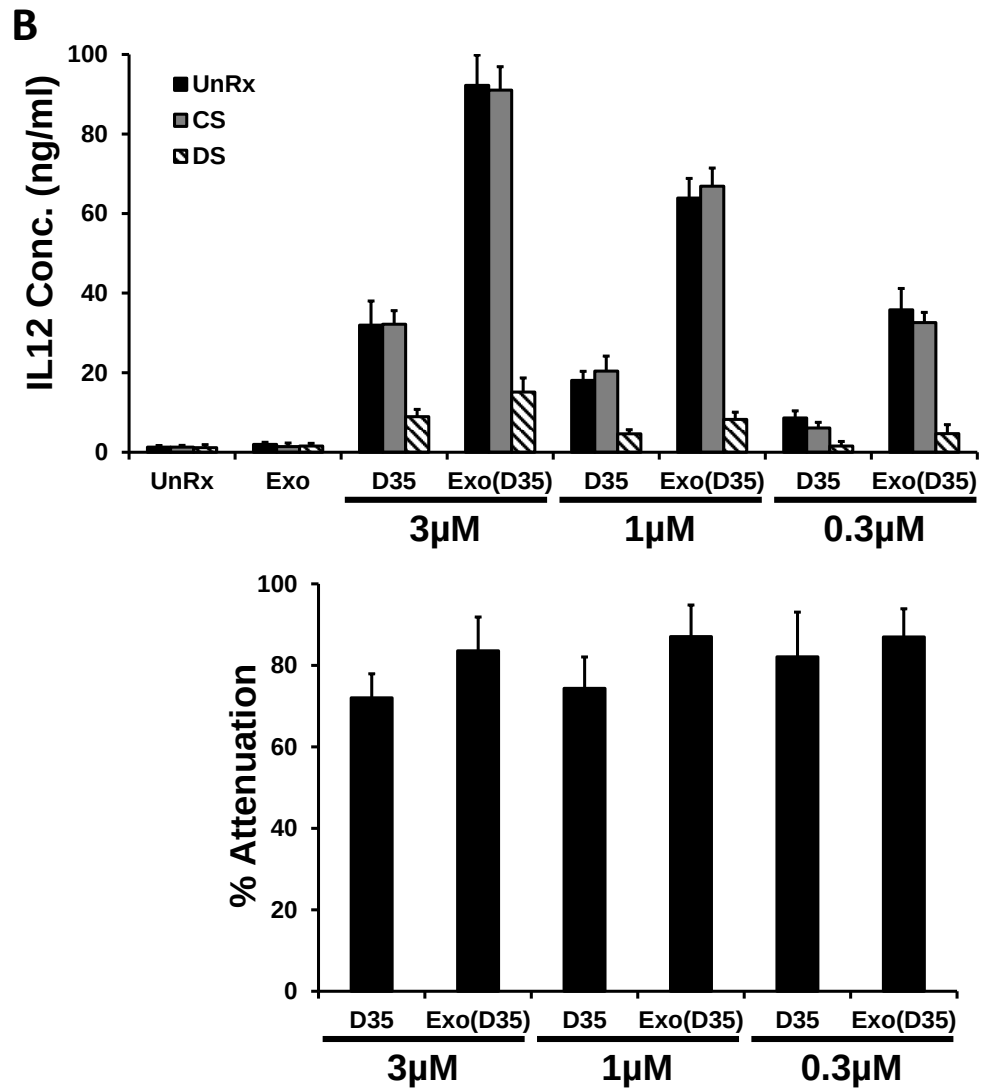
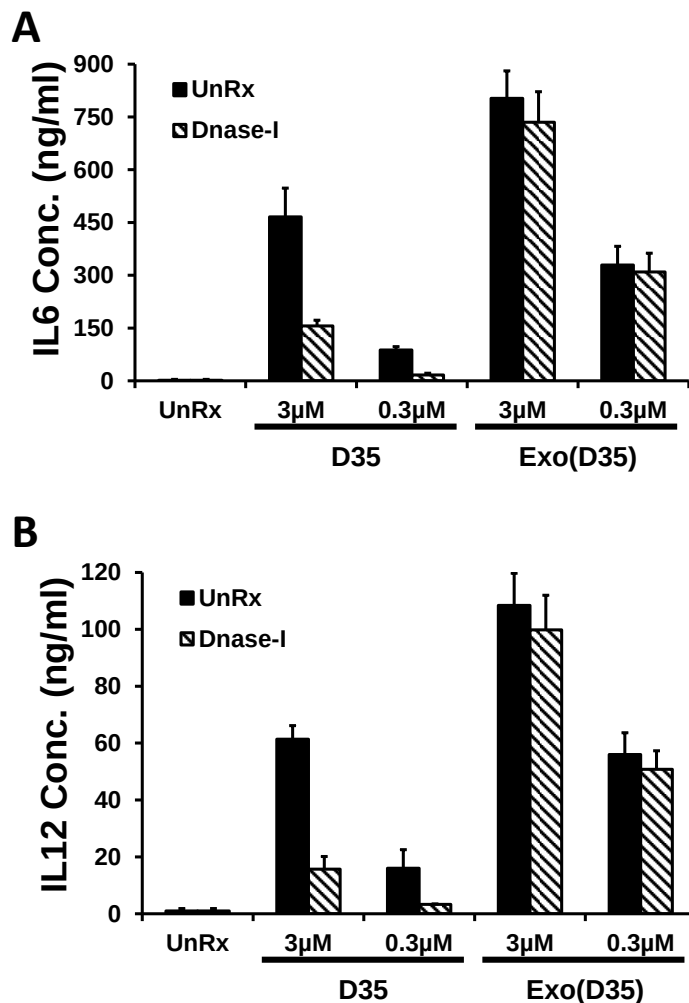


Figure 3.19. DS attenuates D35 mediated immune activation. Cytokine production of splenocytes pre-treated with DS or CS upon stimulation with D35 or Exo(D35) is given as bar graphs. (A) IL6, and (B) IL12 secretion from cells and % attenuation of immune activation by DS. CS is a sulfated macromolecule used as a control of DS. $p < 0.001$ for comparison of DS vs. CS and UnRx in all groups and doses.

3.3.6. Exosomes protects D35 from DNase-I digestion

Nuclease digestion is one of the prime reasons that dampen in-vivo activity of injected CpG ODNs [225]. Since one of the major reasons for loading CpG ODNs within exosomes was to achieve stability along with protection from digestion, we tested the in vitro protective capability of ODN upon exosome loading.

In these experiments, D35 and Exo(D35) were first treated with DNase-I for 30min and then used on splenocyte stimulation assay. As determined by IL6 and IL12 ELISA, ODN loading within exosomes protected D35 from DNase-I digestion (Figure 3.20A-B and C) and retained over >90% of its original inductive capacity for both cytokines (Figure 3.20C, $p < 0.001$ for comparisons of D35 vs. Exo(D35) in both cytokines and concentrations). On the contrary, as expected DNase-I digestion reduced the original activity of free D35 by ~60-80%.



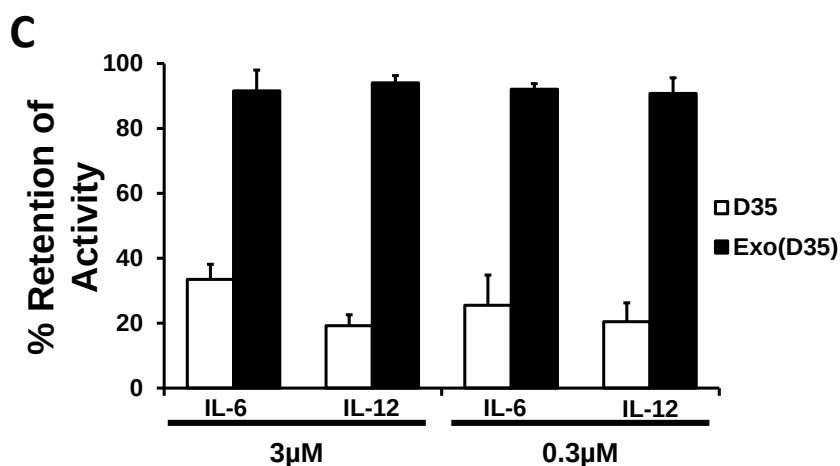


Figure 3.20. D35 is protected from DNase-I digestion when delivered within exosomes. Cytokine production of splenocytes upon stimulation with DNase-I treated either D35 or Exo(D35). (A) IL6 and (B) IL12 secretion from cells is shown as bar graphs. (C) % Retention of immune activity mediated by encapsulation of D35 into exosomes is shown. $p < 0.001$ for comparisons of D35 vs. Exo(D35) in both cytokines and for all concentrations.

3.3.7. Co-encapsulating antigen and CpG ODN adjuvant within exosome elicits better and extended antigen-specific immunity

After observing these improved activities of exosomal CpG ODN, we wished to test its in vivo performance. One of the cardinal features of using carrier systems in vaccine formulations is to ensure that the adjuvant and antigen is internalized simultaneously by that relevant APC. This delivery boosts APC function along with co-stimulatory molecule upregulation and initiates antigen dependent effective B- and T-cell priming, leading to more pronounced effector function and subsequently long lasting antigen-specific memory formation. Therefore, we thought to use exosomes encapsulate both adjuvant +/- an antigen and test whether it will initiate superior antigen-dependent immune response on mice. Ovalbumin (OVA) was loaded as the model antigen along with D35 CpG ODN within exosomes. C57BL/6 mice were immunized either with i) PBS only, ii) free D35+OVA or iii) Exo(D35+OVA). Two weeks after primary injection, animals were boosted with the same formulations. Anti-OVA specific total IgG, and IgG subtypes (IgG1 and IgG2c) were analyzed from sera of animals by ELISA at 2, 10, and 24 weeks post- booster injection.

As seen in Figure 3.21, anti-OVA specific total IgG, IgG1 and IgG2c subtypes were significantly boosted by the Exo(D35+OVA) group even after 2-week post-booster injection when compared to free D35+OVA in solution group (Figure 3.20A). The average immune response at indicated dilutions of sera for each group and IgG subtype is given in Figure 3.21A. The individual responses of mice for each IgG subtypes at 2, 10 and 24-week post booster is given in Appendix A (Figure A1, A2 and A3, respectively). Analysis of reciprocal titers for OVA specific total IgG, IgG1 and IgG2 at 2-week post booster, showed that Exo(D35+OVA) mediated 12-fold increase in anti-OVA IgG levels with respect to free form (287.522 ± 42.966 vs. 24.649 ± 21.351 , Figure 3.21B, $p < 0.001$). Of important note, ~5 and 16-fold increase in IgG1 (4.974 ± 2.109 vs. 1.033 ± 1.397 , $p < 0.001$) and IgG2c (125.537 ± 43273 vs. 7.826 ± 7.907 , $p < 0.001$) levels were observed when immunized with encapsulated vaccine formulation rather than free form (Figure 3.21B). The reciprocal titer values for each mouse were shown in Appendix B.

This anti-OVA immunity not only magnified by exosome formulation but also biased towards Th1. Higher anti-OVA IgG2c to IgG1 ratio ($\text{IgG2c/IgG1} > 1$) implicates a stronger Th1 immune response. When mice immunized with D35 encapsulated exosomal vaccines instead of free D35 vaccine, the IgG2c/IgG1 ratio increased almost 5-fold (4.36 ± 1.14 vs. 0.92 ± 0.48 , Figure 3.31C, $p < 0.001$).

In addition to amplifying the antigen-specific Ab titers along with Th1-biased immunity, the anti-OVA specific immune response persisted over time when the animals were injected with exosomal (D35+OVA) vaccine. When the sera collected and analyzed at 10 weeks (Figure 3.22) or 24-weeks (Figure 3.23) post booster bleedings similar results were obtained to what was observed at 2-weeks post-booster injection.

When these results were taken together, our exosome vaccine ensured simultaneous internalization of CpG adjuvant plus OVA antigen APCs, thereby led to stronger and persistent Th-1 biased anti-OVA immunity.

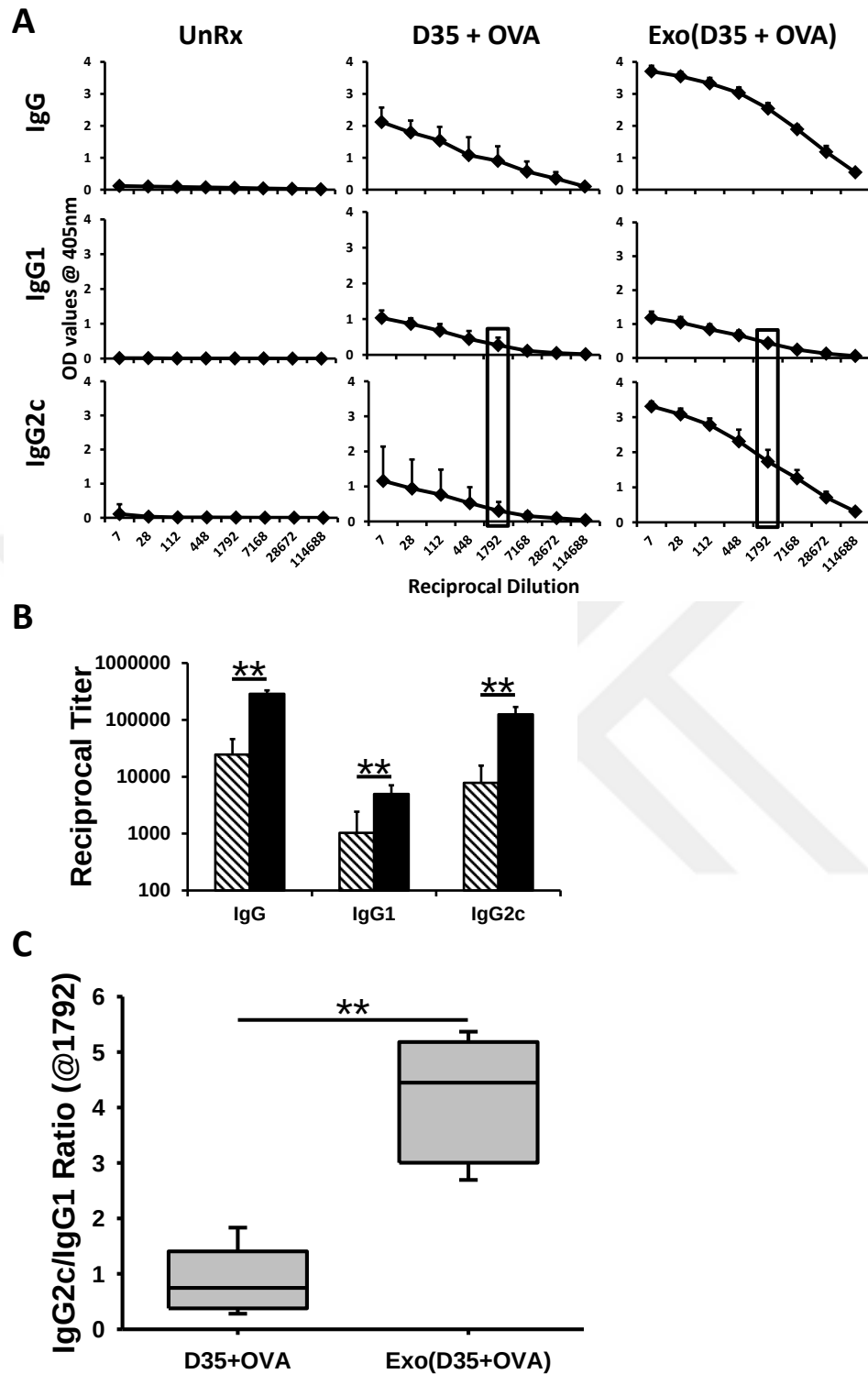


Figure 3.21. Exo(D35+OVA) vaccine induced stronger Th-1 biased immune response 2 weeks after post booster immunization. Sera was collected 2 weeks after booster injection. (A) Average OD readings of total IgG, IgG1 and IgG2c titers at indicated dilutions. (B) The mean $\pm$ SD reciprocal sera titers for IgG, IgG1 and IgG2c. (C) Box-plots of IgG2c/IgG1 ratio at 1792 reciprocal dilution of mice sera. $**p < 0.001$ for indicated comparisons; (N=10 mice/group).

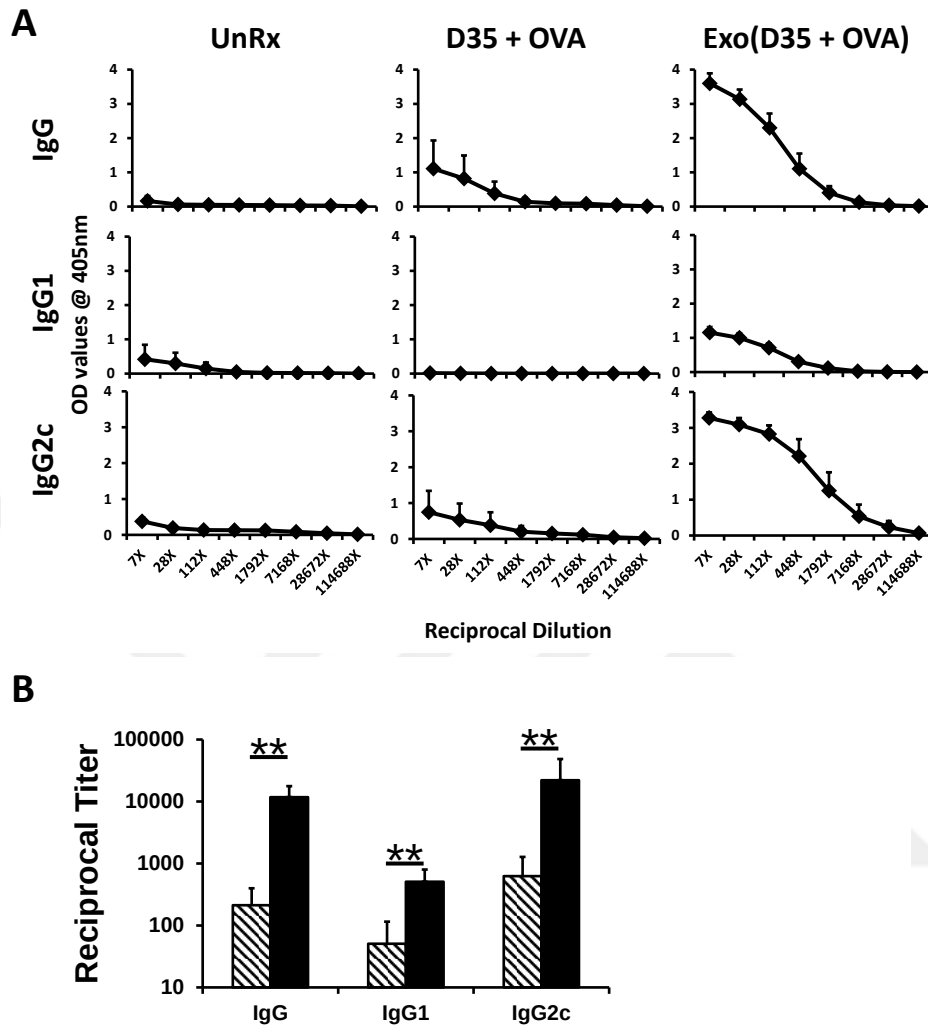


Figure 3.22. Exo(D35+OVA) vaccine yielded higher anti-OVA IgGs at 10 weeks post-booster injection. Sera was collected 10 weeks after booster injection. (A) Average OD readings of total IgG, IgG1 and IgG2c titers at indicated dilutions. (B) The mean $\pm$ SD reciprocal sera titers for IgG, IgG1 and IgG2c. (C) Box-plots of IgG2c/IgG1 ratio at 1792 reciprocal dilution of mice sera. ** $p < 0.001$ for indicated comparisons; (N=10mice/group).

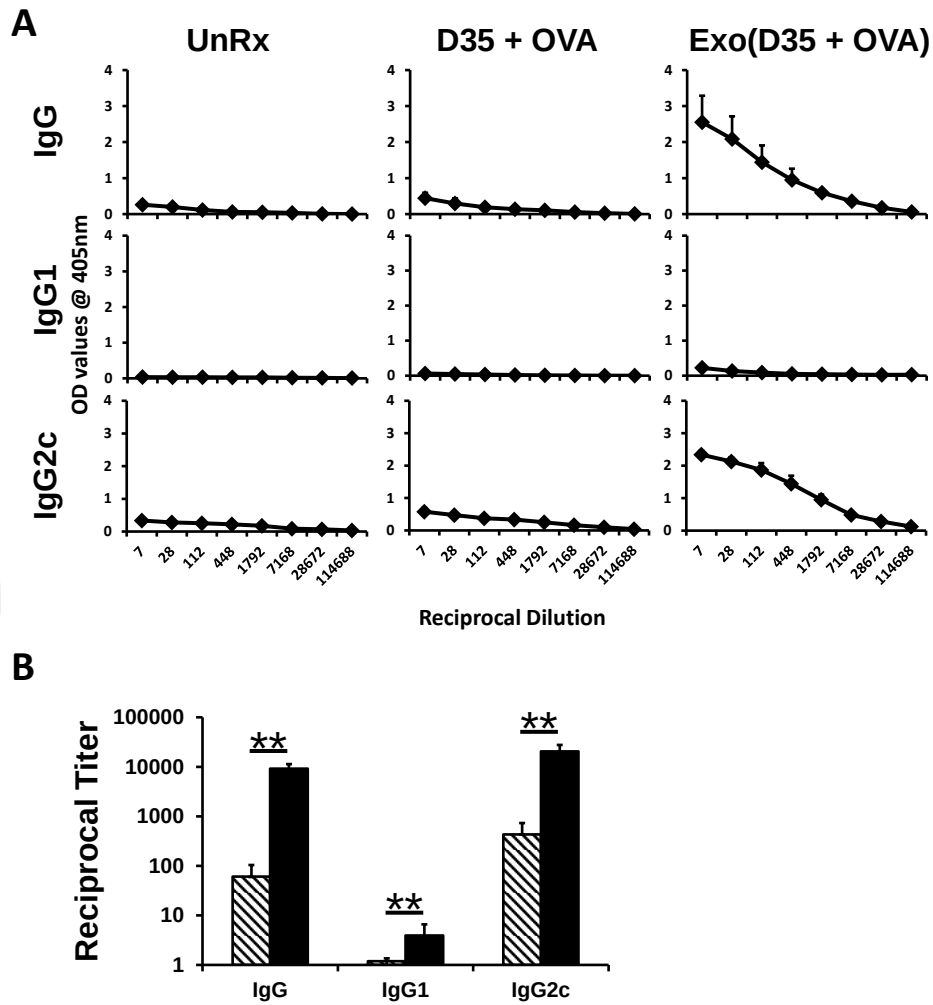


Figure 3.23. Persistent anti-OVA IgG responses induced by Exo(D35+OVA) vaccine. Sera was collected 24 weeks post- booster injection. (A) Average OD readings of total IgG, IgG1 and IgG2c titers at indicated dilutions. (B) The mean $\pm$ SD reciprocal sera titers for IgG, IgG1 and IgG2c. ** p <0.001 for indicated comparisons; (N=5mice/group).

3.3.8. Exosomal vaccine protects mice from EG-7 induced tumors

Our utmost aim was to demonstrate that encapsulating adjuvant and antigen within exosomes would more potent antigen specific immune response thereby could initiate long-term protection of immunized animals from tumor development. In our immunization studies we intentionally selected OVA as the model antigen. We postulated that if sufficient and sustained anti-OVA immunity is established, we could harness these mice to tumor challenge experiment and ask the question whether developed anti-OVA immunity against a single epitope is sufficient to control tumor development. To test this hypothesis, EG-7 cells, (an OVA expressing EL-4 thymoma cells), were subcutaneously inoculated onto the back of OVA immunized mice at 5×10^6 EG-7 cells/mice and tumor development was followed. Two independent challenge experiment was performed, OVA immunized mice at i) 10 (N=5 mice/group) and ii) at 24 weeks (N=5 mice/group) after booster injection was used during these experiments. While most of the animals rejected tumor growth that had exosomal vaccine, all PBS (untreated) and D35+OVA mixture treated animals developed EG-7 tumors. The representative pictures of tumor-bearing mice from each group at 10th day after palpable tumor formation is shown in Figure 3.24A. While tumor developments at day=5 was observed in PBS and D35+OVA immunized groups following post-engraftment no tumor was visible for exosomal vaccine immunized animals. Moreover, tumor volumes progressively grew for the duration of experiment for those groups indicating that OVA specific priming is unable to control tumor development. Strikingly all exosomal vaccine immunized animals challenged at either 10 weeks (Figure 3.24B) or 24 weeks (Figure 3.24C) after booster immunization remained free or almost free of tumor development for the entire duration of the experiment. The protection was also supported by tumor volume measurements at day=15 for both challenge experiments (Figure 3.24D). The survival rate of mice was followed for 3 months after EG-7 inoculation. While untreated and D35+OVA mixture immunized groups succumb to tumor burden by two months after tumor inoculation, 60% of animals that had Exo(D35+OVA) survived until the cessation of the experiment (Figure 3.24E). these findings suggested that of 60% of the Exo(D35+OVA) treated animals had sufficient effector CD8⁺ T cells to clear tumor engraftment.

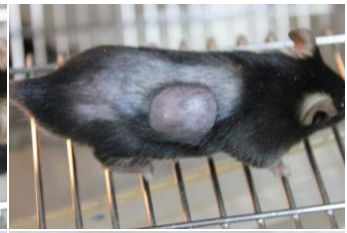
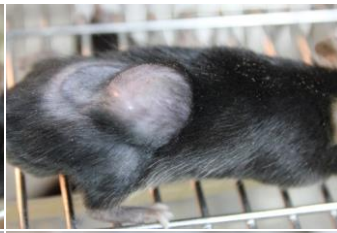
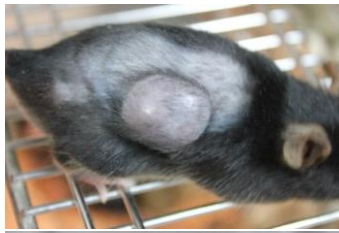
A

m1

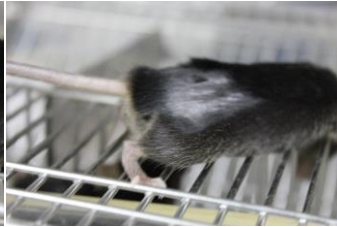
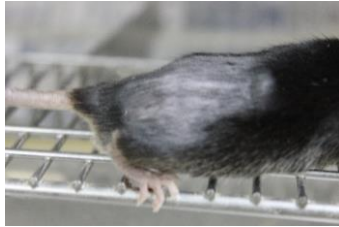
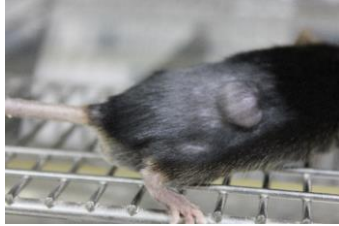
m2

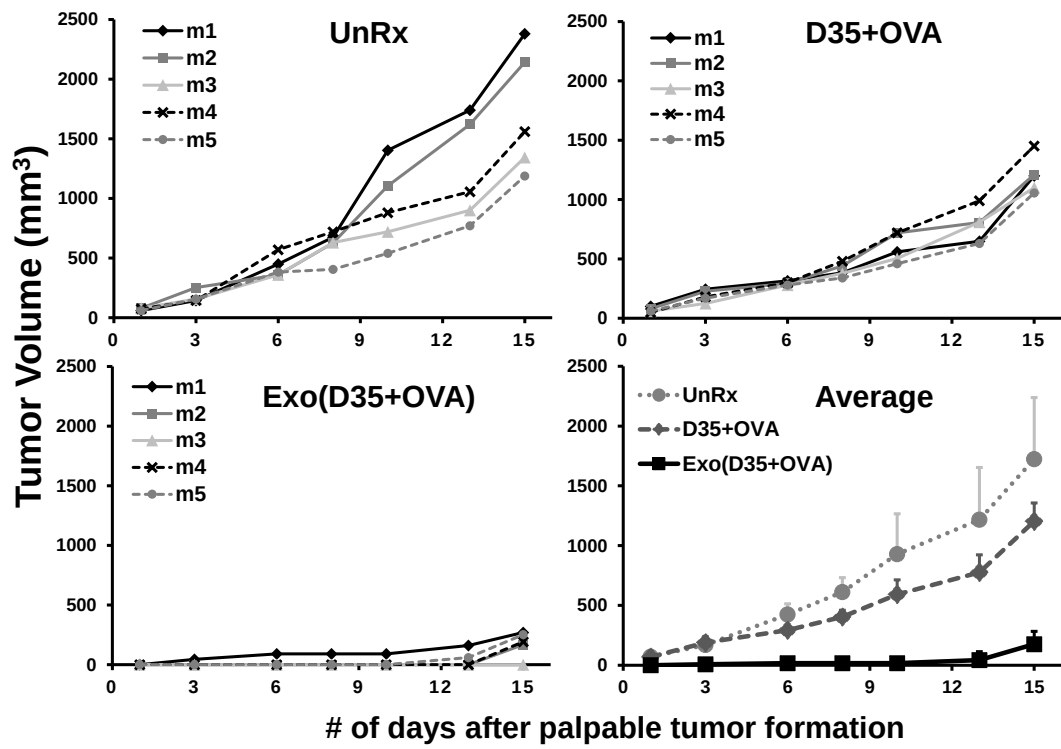
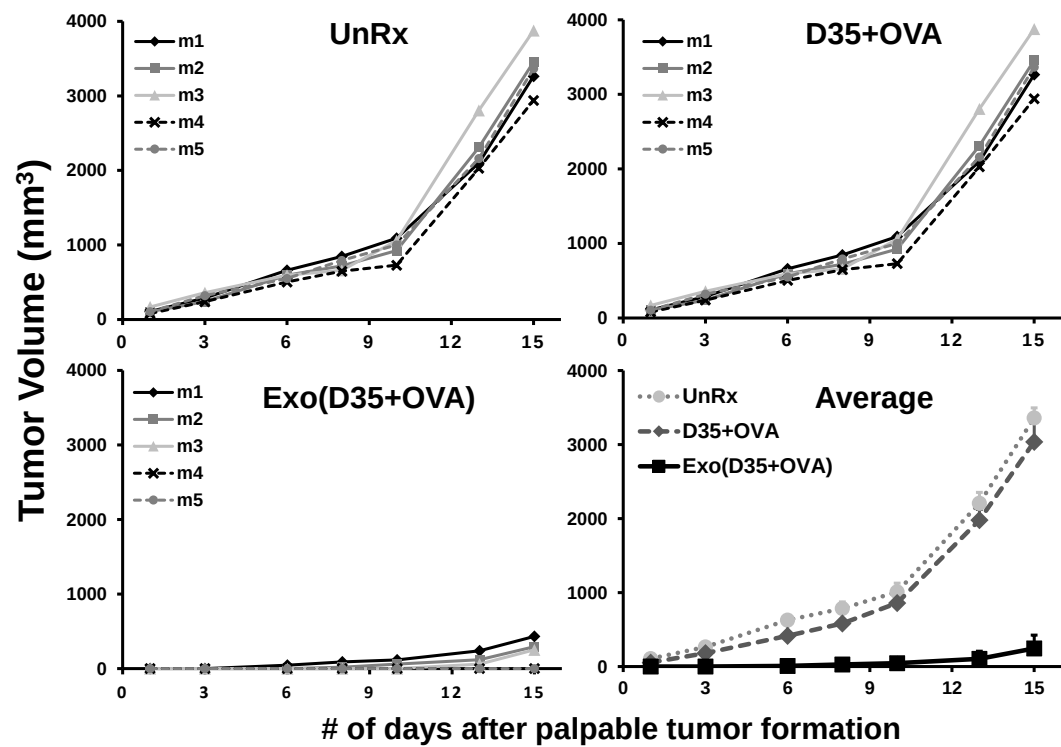
m3

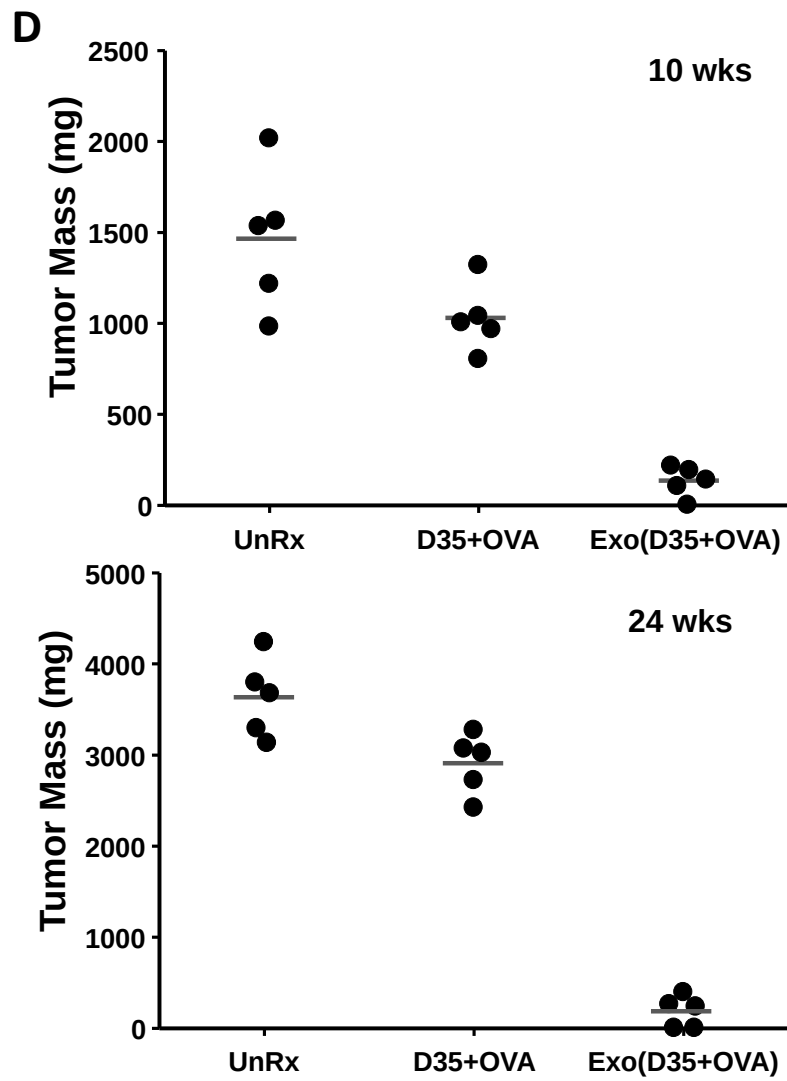
UnRx



Exo(D35+OVA) D35+OVA



B**C**



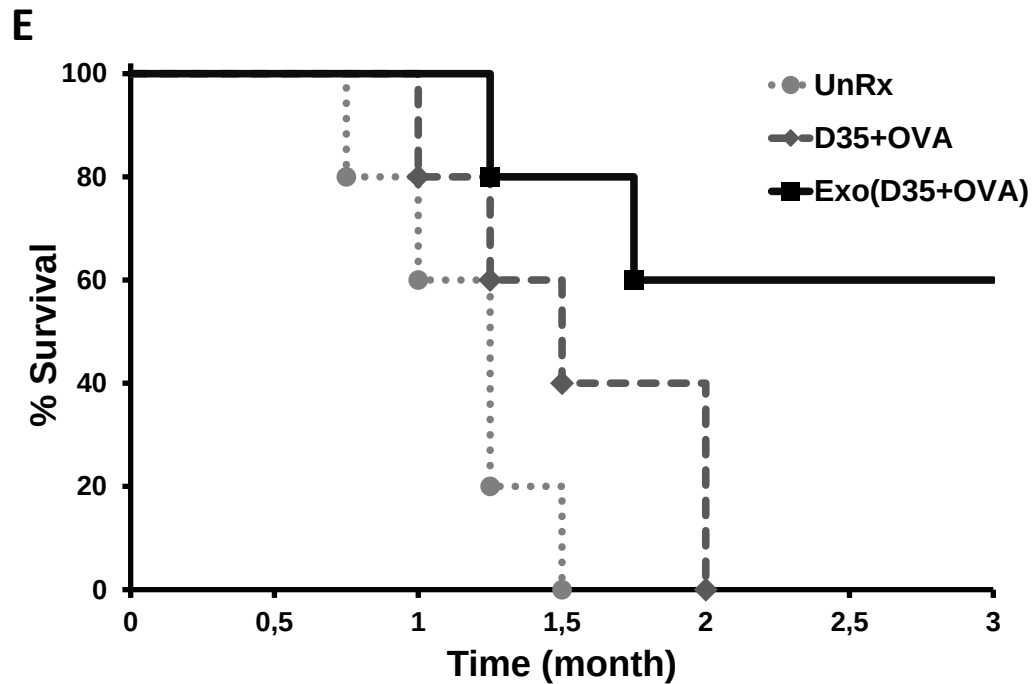


Figure 3.24. Exo(35+OVA) vaccine confers protection from EG-7 tumor engraftment. OVA immunized mice were s.c. injected with EG-7 (5×10^6 cells/mice) at 10 weeks ($n = 5$ mice/group) and 24 weeks ($n = 5$ mice/group) post booster injection and their tumor progressions were followed. A) Representative pictures of tumor bearing mice at day=10 after palpable tumor formation (3 mice/group) challenged at 10 weeks after booster injection. B) Average tumor volumes of each animal (10wks post booster challenge). C) Average tumor volumes of each animal (24wks post booster challenge). D) Tumor mass at day=15 for 10 (upper graph) and 24 (lower graph) week post-booster challenged animals E) Long-term survival curve of EG-7 bearing mice at 24 weeks post- booster challenge.

3.4. Enhancing in vitro Internalization of STING Ligand c-di-GMP either within Exosomes or Complexing with Peptide Arg(9)

3.4.1. Improved cellular delivery of c-di-GMP by Arg(9) and exosomes

Cyclic di-guanosine monophosphate (c-di-GMP) is a well-known member of the cyclic dinucleotides (CDNs hereafter) triggering Type-I IFN through the cytosolic nucleic acid sensor STING via IRF3 axis. There is a growing interest to harness CDNs in immune therapy or develop them as vaccine adjuvants. However, one of the major hurdles for its utilization is their limited penetration rate into the cell cytosol, hampering its wide use. In order to obtain an immune response at low doses, c-di-GMP has to be transfected into cell cytosol, which is not a feasible approach since transfection reagents are detrimental to cells. Moreover, in vivo application is not possible since FDA prevents its in vivo use for humans. Therefore, we embarked on a project to resolve this problem. We planned to increase internalization capacity of c-di-GMP either by complexing with Arg(9) or loading within exosomes, thereby induce its effective delivery within immune cells and hopefully improve its immunostimulatory potential.

Arg(9) is a cationic peptide that can complex with c-di-GMP at ease hence overcoming membrane impermeability. c-di-GMP was mixed with Arg(9) at 1:2 molar ratio in order to facilitate complexing. Splenocytes were incubated with free (in the presence of lipofectamine) or complexed form containing 15 μ M FAM-c-di-GMP for 2h. Flow analysis after incubation showed that Arg(9) mediated 4-fold higher internalization of c-di-GMP by MHC-II⁺ cells with respect to transfected free c-di-GMP (Figure 3.25A).

To check whether exosomes improved cellular uptake of the, c-di-GMP, RAW264.7 derived exosomes were mixed at 1:1 ratio (w:w) with FAM conjugated c-di-GMP. Reconstituted exosomes (Exo(FAM-c-di-GMP)) and free FAM-c-di-GMP incubated with splenocytes for 2h at 15, 5 and 1.66 μ M concentration. Free c-di-GMP incubated with splenocytes in the presence of lipofectamine to mediate transfection. After 2h, cells were stained with anti-MHC-II-PE and then analyzed with flow cytometer. The results revealed that at doses of 15 and 5 μ M, all of the MHC-II⁺ cells internalized STING ligand form, whereas less than 3% of cells were positive for c-di-

GMP when incubated at the highest dose. At 1.66 μ M concentration, the effect of using exosomes became more apparent. The c-di-GMP entry within target cells rose to over 100-fold higher for the exosomal ligand as compared to transfected ligand (22.1 ± 0.7 % vs. 0.2 ± 0.1 %, $p<0.001$, Figure 3.25B). This data shows that at very low doses, at which transfection fails to induce any positive read-out, exosomes mediate internalization of c-di-GMP with high efficacy.

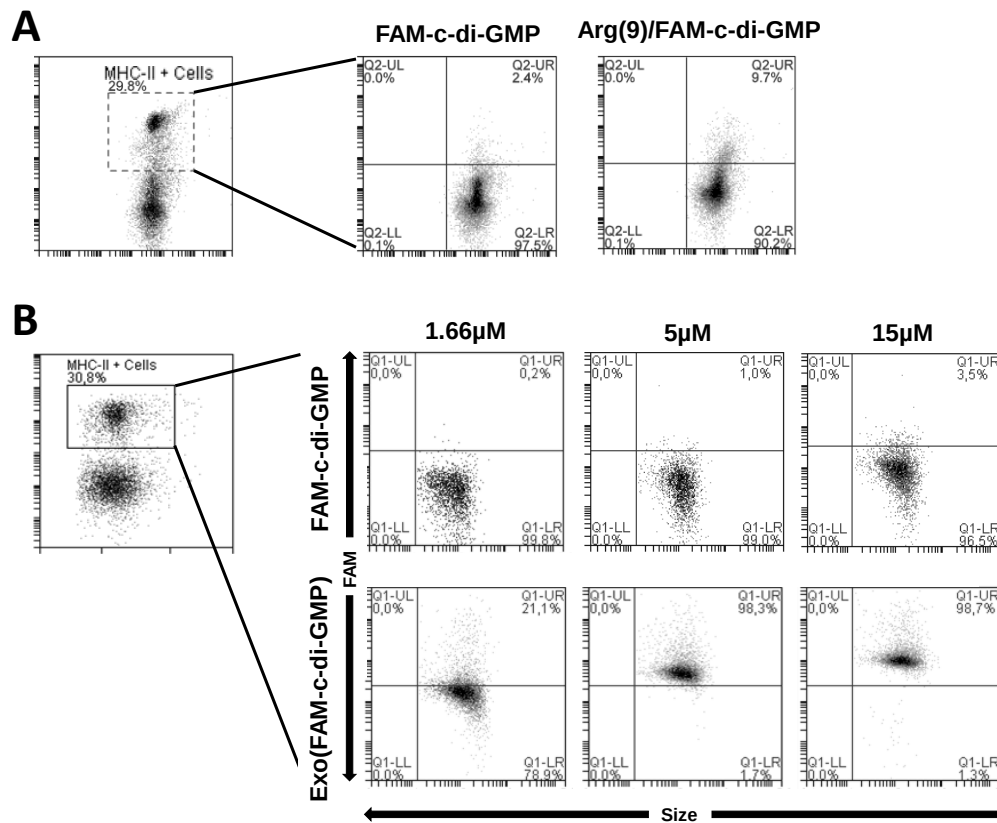


Figure 3.25. Arg(9) and exosomes improve c-di-GMP delivery to target cells. (A) Internalization of FAM-c-di-GMP by MHC-II⁺ cell mediated by lipofectamine (free form) and Arg(9). MHC-II⁺ cells were gated as indicated. (B) Exo(FAM-c-di-GMP) (lower panel) and FAM-c-di-GMP (in the presence of lipofectamine, upper panel) incubated with splenocytes (2×10^6 cells/ml) at indicated concentrations for 2h. MHC-II⁺ cells were gated and investigated for c-di-GMP internalization. Data are representative of 2 independent experiments

3.4.2. Encapsulation increases stimulatory effect of c-di-GMP and synergizes with CpG ODNs

Since mixing either with Arg(9) or encapsulating within exosomes mediate transfer of c-di-GMP into cells even at very low concentrations, we next examined whether this enhances in vitro immunostimulatory action of c-di-GMP. To achieve this, splenocytes were incubated with mixed, encapsulated or free form of c-di-GMP at 15, 5 and 1.66 μ M concentrations for 24h. Furthermore, we also explored the possibility of synergistic effect of CpG ODN on both Exo(c-di-GMP) and c-di-GMP but not Arg(9)/c-di-GMP complex. Therefore, 1 μ M CpG ODN containing groups were also included in the experiment. After 24h incubation with i) c-di-GMP, ii) Exo(c-di-GMP), iii) CpG ODN, iv) CpG ODN+c-di-GMP or v) CpG + Exo(c-di-GMP), upregulation of co-stimulatory molecules and pro-inflammatory cytokine production was determined from the cells and supernatants, respectively.

In order to assess APC function of the cells, upon stimulation, splenocytes were stained with anti-MHC-II and anti-CD86 antibodies and analyzed by cytometry. Flow analysis revealed that while complexing with Arg(9) does not induce upregulation of co-stimulatory molecules as shown in Appendix C (Figure C-A), encapsulation within exosomes enhanced immunostimulatory effect of c-di-GMP (Figure 3.26A) which was further amplified by CpG ODN addition (Figure 3.26A-B). When stimulated with Exo(c-di-GMP), MHC-II⁺/CD86⁺ cells increased at all tested doses (15 μ M; 8.1 $\pm$ 0.5 vs. 5.6 $\pm$ 0.8, 5 μ M; 5.8 $\pm$ 0.7 vs. 4.1 $\pm$ 0.6, 1.66 μ M; 3.5 $\pm$ 0.3 vs. 2.1 $\pm$ 0.4, p <0.01 for all comparisons, (Figure 3.26B).

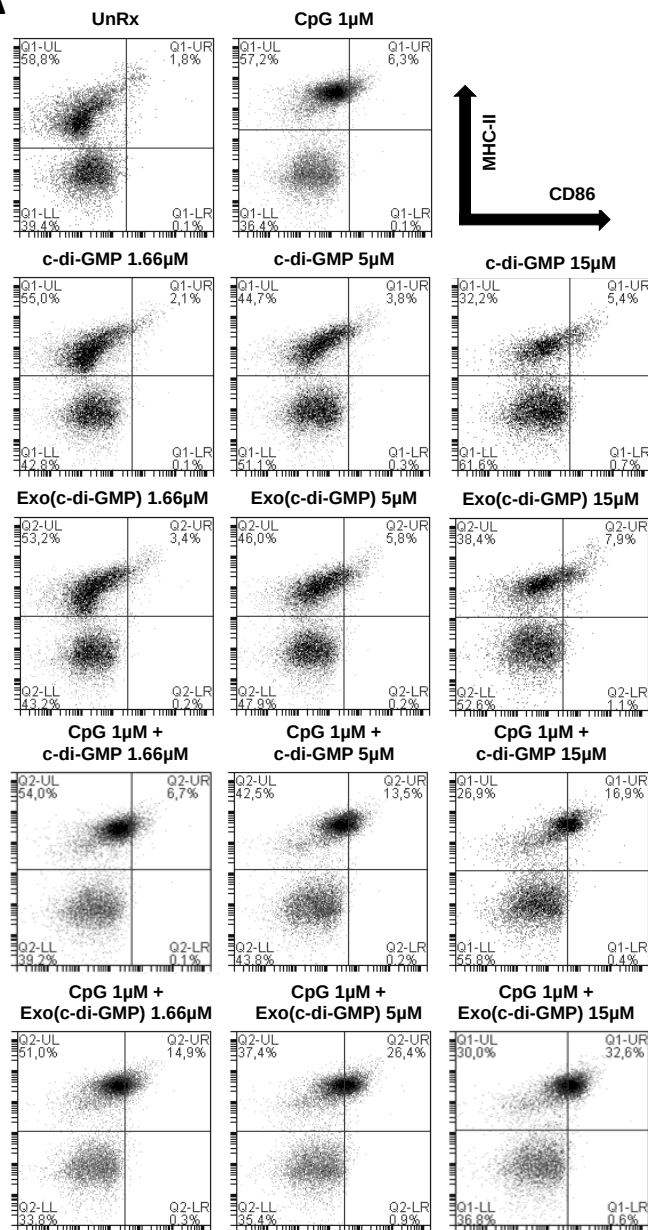
Both encapsulated and free form of c-di-GMP synergized with CpG ODN during MHC-II⁺/CD86⁺ upregulation. However, level of double positive cells when stimulated with encapsulated form mixed with CpG ODN (i.e. CpG + Exo(c-di-GMP)) was increased 2-fold more compared to ODN + c-di-GMP group. The fold induction rate of co-stimulation was more at lower doses (Figure 3.26B).

TNF α , IL12 and IL6 secretion from cell supernatants were analyzed by ELISA. Consistent with level of double positivity, while encapsulation within exosomes increased c-di-GMP-dependent TNF α secretion from splenocytes by more than 3-fold even at low doses, Arg(9) mixing did not enhance immunostimulatory capacity at low doses (Figure C-B). This effect was not apparent for either IL6 and IL12

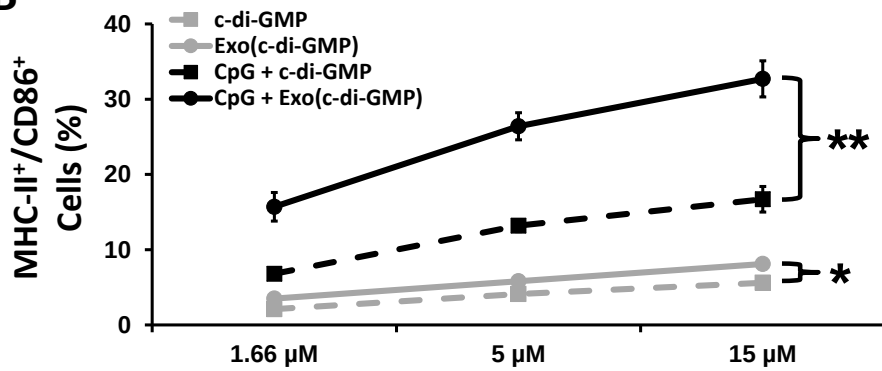
productions, since free or exosomal c-di-GMP treatments led to similar cytokine levels. As expected exosome loaded with c-di-GMP synergized better with CpG ODN addition and induced higher levels of pro-inflammatory cytokines from spleen cells (Figure 3.26C).



A



B



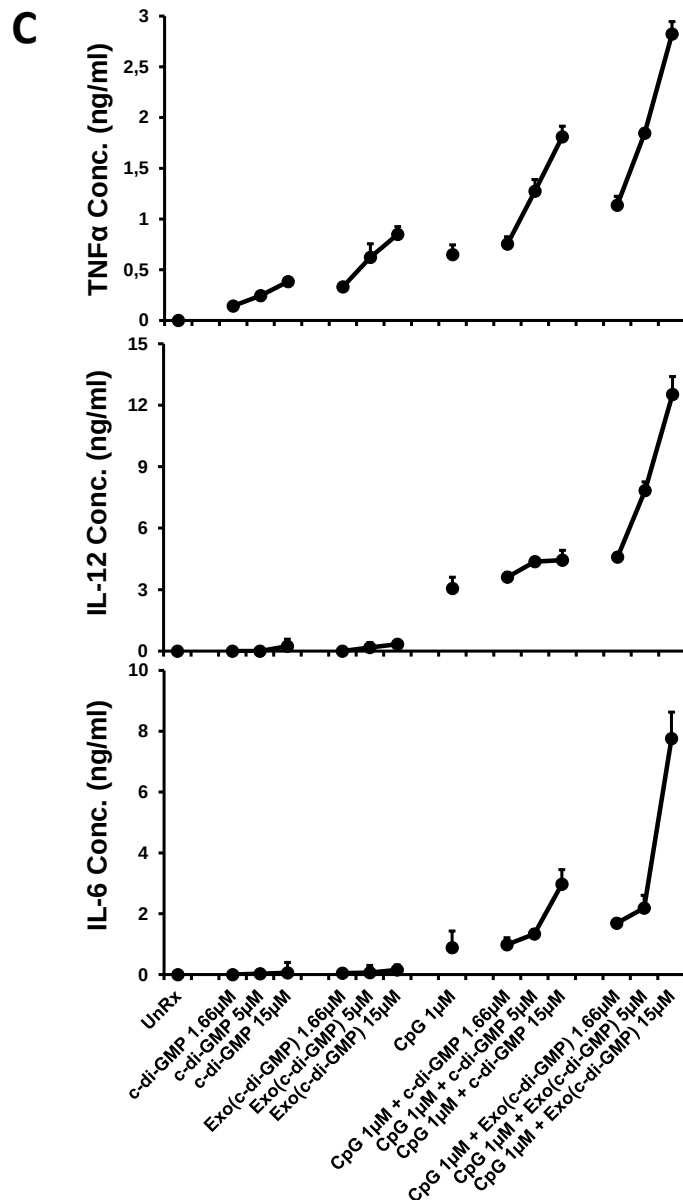


Figure 3.26. Exosome encapsulation enhances stimulatory effect of c-di-GMP and further synergizes with CpG ODN mixing. Indicated doses of Exo(c-di-GMP) and c-di-GMP incubated with splenocytes combined either with CpG ODN or as such forms for 24h. Cells were stained with anti-MHC-II and anti-CD86 antibodies for co-stimulatory function analyses. The culture supernatants were used for cytokine ELISA. (A) Representative dot plots of independent four experiments that gave similar results. (B) The effect of c-di-GMP concentration with or without CpG ODN inclusion on MHC-II/CD86 expression. (C) TNF α , IL12 and IL6 secretion capacity in response to free or exosomal c-di-GMP +/- CpG ODN. 24h culture supernatants were studied by ELISA. Data represented as mean $\pm$ SD of four independent experiment. * p <0.01 and ** p <0.001 for indicated comparisons.

Chapter 4

Discussion

4.1. Involvement of EV in Behçet's Diseases

EVs have recently been implicated as novel extracellular mediators of distant intercellular communication [226]. Since EVs circulate and remain intact in peripheral blood, they are capable of transferring labile cargo to distant targets, thereby altering the physiology of the recipient cells [26]. Because of their modulatory effects on recipient cell, these vesicles play important role in regulating homeostasis both in health and disease. Recent studies revealed that numbers of circulating EVs were elevated in several rheumatologic diseases, such as RA, SLE, primary Sjögren's syndrome, vasculitis and APS [206-217]. However, the implications of elevated EV levels were not fully understood. In this thesis, within the context of BD patients, we asked the question whether this increase in EV levels is influencing the severity and pathogenicity of the disease. To our knowledge, this study is the first to demonstrate that EVs are not just elevated in plasma of BD patients but also associated with LL-37 and thus participate and exacerbate disease pathology.

Several studies demonstrated that EVs not only possess bioactive materials on their surface but also harbor biologically active cargo within their lumen. These include nucleic acids (both RNA and DNA) or proteins such as, cytokines, growth factors

and cell or tissue specific antigens. Recently, miRNAs were also reported to be delivered via these nanovesicular structures [227-229]. EVs are considered to have important functions in establishment of homeostasis in health and could play potentially detrimental roles during disease states [230, 231]. Data presented herein are in support of these observations and extend our current knowledge of EV-disease associations. They support the notion that EVs or EV associated molecules such as proteins and miRNAs can be used in clinic for diagnostic and therapeutic purposes.

Our findings could be of clinical interest for several reasons. First, plasma EV numbers were found to be elevated in BD patients (Figure 3.1), which correlated with disease severity (higher EV numbers in active BD patients). Vascular involvement in almost 25% of BD patient, which is poorly understood [232], might be caused by increased levels of pro-coagulant EVs as implicated in the pathology of RA and APS. Findings presented in Figure 3.2 in which activated platelet- and monocyte-derived EV levels are shown to correlate with disease activity support this hypothesis. Increased EV levels from activated monocytes was previously reported *in-vitro* [10], our finding in BD patients shows that upon activation both monocytes and platelets secrete higher amount of EVs to the extracellular environment *in-vivo*. Since diseased cells are the main thrombotic factor expressing cells, increased EVs from these cells might cause or exacerbate thrombotic events seen in BD patients [233]. Taken together, it is conceivable that a plasma EV number- and origin-based stratification of BD could more precisely identify inactive and active disease states as well as probability of thrombosis and could aid in pharmacological management of this disease.

By itself, increased EV levels could not mean functional relevance to disease pathogenesis. Several reports suggested that although in most cases EVs are not underlying condition, they participate and regulate the pathogenesis of the disease via mediating cellular interactions and modifying behavior of the recipient cell [234-237]. Our findings support this view as BD patient derived EVs stimulated cells to secrete pro-inflammatory cytokines from healthy PBMCs. Moreover, the stimulatory capacity of EVs enhanced with increased disease severity (Figure 3.6). This result shows that EVs are not just bystanders in the disease but regulate the severity of BD by their immune activating properties as in other inflammatory diseases.

Differential cargo between healthy derived EVs and pathological BD derived EVs have to cause this functional divergence.

Although LL37 is a cationic anti-microbial peptide generally known to be secreted during pathogenic insult [238-240], the data presented here demonstrate that plasma LL37 levels are elevated in BD (Figure 3.7). This finding is somehow expected since there are reports showing exacerbated BD symptoms as well as disease initiation in genetically susceptible individuals after severe bacterial infections [241, 242]. As in the case of EVs, LL37 is found to be interacted with EVs in the plasma of BD patients. More importantly, it was found that pathological activity of EVs correlate directly with its interaction with LL37 as well as disease severity (Figure 3.8). This information could be of value to follow patient responses to treatment. Of interest, the only published data that investigated a role of LL37 in BD revealed elevated LL37 levels in saliva of BD patients which correlated with the number of monthly oral ulcers [243]. Consistent with this observation, our results also revealed a correlation between LL37 levels and disease severity. Of note, LL37 levels were reported to be higher during sterile inflammation [244] suggesting a potential role for exacerbating BD pathogenesis. LL37 was also shown to be activator of P2X7 receptor and transient ATP release from cells which were needed to IL-1 β maturation and release from monocytes [245]. In addition, this cationic peptide was demonstrated to contribute to IL-1 β , IL6, MCP-1 and MCP-3 production [246]. In this context, our results also established a link between elevated LL37 levels and enhanced cytokine production as well as a therapeutic target for BD.

To our knowledge, this study is also the first to show that LL37 can interact with EVs, enhancing their cellular delivery and immune stimulatory activity (Figure 3.9-11). As stated above, EVs are known carriers of self-nucleic acids. It is conceivable that LL37-mediated enhanced EV delivery to immune cells could activate several endosomal and/or cytosolic RNA and DNA-dependent nucleic acid sensors, including TLR7, TLR9, RIG-I, MDA-5, cGAS or AIM-2, leading to production of BD-associated cytokines (i.e. IL6, IL-1 β and IFN α), contributing to disease severity. Supporting this view, it was shown that high levels of LL37 present in psoriatic skin formed complexes with genomic DNA liberated from dying cells, and enhanced DNA delivery to pDCs, triggering a TLR9-mediated interferon alpha response in psoriatic patients [247].

The anionic charge of EVs provides a surface convenient for LL37 complexation. Our findings implicate that LL37-mediated enhanced delivery of circulating EVs to immune cells contribute to sustain and exacerbate immune activation in active BD. EV-LL37 complexes not only increasing pro-inflammatory cytokines in the environment but also increase IL-1 β and IP-10 secreting monocytes as well as IFN α secreting pDCs sharply. As well as contributing local pro-inflammatory environment by modulating secretion of cytokines, EV-LL37 complexes increase number of inflammatory immune cells. Furthermore, these complexes might induce recruitment of Th-1 and cytotoxic T-cells [248, 249]. Therefore, targeting both EVs and peptide could be of therapeutic value in the management of this complex disease as well as prognostic tools for the management of the disease.

4.2. Involvement of EV in Allergic Diseases

In Th-2 biased diseases, the level of circulating EVs was found to be decreased (Figure 3.13). According to our findings, EVs were effected differently according to nature of the disease. Contradicting our observation, several studies proposed that EVs participate in exacerbation of allergic diseases as bronchoalveolar lavage fluid (BAL) derived EVs contribute to IL-4, IL-13 and leukotrine production in asthma patients [250, 251]. However, in these two studies and others, the role and function of BAL derived EVs was investigated unlike plasma EVs. In addition, we found that in plasma of allergic patient's platelet derived EVs are also decreased unlike BD patients. This dichotomy between BD and Allergic diseases is probably due to the differences in the pathogenesis of these diseases, since allergy is not related with peripheral circulation. Deep vein thrombosis events are also seen in asthma patients but these occurrences are mostly restricted to epithelial surface of airways. Therefore, decrease rather than an increase in circulating EV levels could be understandable in terms of asthma and AD. However, using peripheral blood EVs for diagnostic and therapeutic applications against these diseases is feasible since obtaining bronchoalveolar lavage fluid is harder and invasive procedure. Our results showed percentage-wise increase in eosinophil derived EVs in plasma of allergic patients. Therapeutic applications such as administering drugs via peripheral blood EVs or identifying disease specific markers (miRNA or protein) using eosinophil derived circulating EVs might be a promising research field.

4.3. Development of EVs as drug carriers

In the second part of this study, exosomes were engineered and then utilized as vaccine carrier vehicles. Exosomes were already shown to carry labile molecules and mediating intercellular communication. Therefore, these vesicles can be considered as nature's nanocarriers [231]. Because of these properties and functions, modulating these vesicles as drug or vaccine carrier is an enthusiastic and promising field which is intensively studied [231, 252]. However, there are two main obstacles in using them in therapeutic applications; i) selecting proper cell type as source for exosomes and ii) absence of effective encapsulation methods for entrapment of exogenous molecules within exosomes. In this study, we introduced a new and a very mild encapsulation technique and exosomes purified from RAW264.7 cells were developed as nanocarriers for CpG ODNs. In addition, for the first time we convincingly demonstrated that exosomes carry and deliver D35 type CpG ODN to immune cells and enhance its immune stimulatory activity both *in-vitro* and *in-vivo*.

Selecting proper cell type as exosome source is crucial in therapeutic approaches since exosomes display immunomodulatory effects depending on originating cell type and physiological state [26]. According to therapeutic application, cell type should be changed since contradictory results because of nature of exosomes might be obtained. For example, immature or tolerogenic DC and tumor cell derived exosomes can be used to control autoimmune and inflammatory diseases like RA because of their innate suppressive properties [253]. However, using these vesicles in cancer therapy might exacerbate the disease by further promoting suppressive environment [88, 117, 120, 254, 255]. In cancer therapies using mature DC derived exosomes should be used because of their T-cell activating/priming properties [256]. Clinical trials performed on both non-small cell lung carcinoma and melanoma patients proved this notion, phase I clinical trials using tumor antigen pulsed immature DC derived exosomes showed limited anti-tumor activity due to inadequate co-stimulatory molecules [139, 140]. However, phase II clinical trials using tumor antigen pulsed IFN γ -matured DC exosomes performed better T-cell activation and increased post therapy survival as well as tumor clearance in some patients [142, 257]. Therefore, in order to use exosomes as delivery vehicles, we choose murine macrophage cell line, RAW264.7, as source of our exosomes because of their immunological inertness under normal (unstimulated) conditions.

Isolated particles from these cells were also controlled according to exosomal criteria in order to claim that exosomes can be isolated from these cells by conventional methods [258]. Our results proved that we can isolate exosomes from RAW264.7 cells (Figure 3.15). In addition, splenocyte stimulation assays further confirmed that these vesicles by themselves do not induce any immune response (Figure 3.18).

We used dehydration-rehydration method to encapsulate CpG ODNs as well as OVA antigen within exosomes which offers advantages over other loading methods used in the literature. One of the accepted methods is modifying originating cell to express molecule of interest so that secreted exosomes from these cells will also express the molecule [259, 260]. However, this limits the variety of molecules used for loading since cells do not have the ability to synthesize all molecules but just proteins and RNAs. In addition, this approach does not guarantee efficient loading within every secreted exosomes since there is a selection mechanism in molecule sorting into exosomes [261]. Furthermore, with this approach the physiological state of the cell from batch to batch may vary, thus the yield or the loading efficiency vary accordingly. External loading of drugs via mixing or electroporation is the second approach that enables loading of wider range of molecules [149, 262, 263]. Nevertheless, mixing is not suitable for efficient vaccine development since it is impossible to target antigens within exosomes unless they are lipid soluble or cationic. Although, transferring molecules across plasma membrane of cells via electroporation is a logical approach, it may cause aggregation of exosomes as well as raise stability or integrity issues [264]. On the other hand, dehydration-rehydration method used to introduce external molecules within exosomes overcomes these obstacles. Any desired molecules can be loaded into exosomes via this method. In this study we have shown that synthetic DNA molecules (D35) and proteins (OVA) can be encapsulated within exosomes at high efficiencies (Figure 3.16). In addition, there are studies showing encapsulation of plasmids and siRNAs within liposomes via this method [265, 266]. This approach is very mild in mediating loading of molecules into exosomes unlike electroporation. Our DLS analysis and flow cytometry results confirmed that dehydration and rehydration approach of exosomes did not alter either their size or their molecular composition (Figure 3.16). Therefore, it is possible to obtain engineered exosomes post purification sharing very similar physicochemical properties to that of original exosomes, for therapeutic applications.

As evidenced by our immunization experiments, antigens and adjuvants could be simultaneously encapsulated within exosomes externally (Figure 3.21). Therefore, this method facilitates loading of any desired molecule within exosomes in a single step process.

Our findings showed that encapsulating CpG ODNs within exosomes enhanced their *in-vitro* stimulatory capacity as seen by the increased pro-inflammatory cytokine release as well as number of IFN α producing pDCs (Figure 3.18). The most fascinating read-out from these experiments was that Exosome loaded with D35 CpG ODN (Exo(D35)). induced splenocytes to secrete comparable IL6 and IL12 levels to 10-fold higher concentration of free D35 counterpart. Moreover, earlier/faster immune response was obtained when cells were stimulated with Exo(D35). These findings were quite promising since the major drawback of *in-vivo* applications was rapid elimination of CpG ODN upon administration. Increase in IFN α producing pDCs was of special importance for effective vaccination since Type-I IFNs participate in maturation of DCs, induction of effector and memory T-cell as well as promoting humoral immunity [267]. Moreover, the enhanced immune activity might be also due protection of D35 ODN via exosome encapsulation from DNase-I degradation as shown by the *in vitro* digestion studies (Figure 3.20). Taken together, enhanced Exo(D35) *in-vitro* performance implicated that the exosomal depot system may act as promising vaccine adjuvants.

The encapsulated D35 was then used with OVA as a candidate tumor vaccine. Thanks to mild and non-detrimental procedure of the dehydration-rehydration technique, we loaded both adjuvant and antigen within same exosome with high encapsulation efficiency. Then, Exo(D35+OVA) and free vaccine was administered to mice via *i.p.* injection. As expected, encapsulated vaccine formulation induced better anti-OVA response compared to free vaccine group, as evidenced by higher anti-OVA IgG titers (Figure 3.21). In addition to mediating enhanced immune response against OVA, mice immunized with encapsulated vaccine formulation showed persistent anti-OVA response even at 24 weeks after booster injection (Figure 3.24). There are several reasons why encapsulated vaccine formulation performed better *in-vivo* setting. Encapsulation of OVA vaccine provided enhanced protection against DNase-I degradation, as proved in *in-vitro* studies, preventing pre-mature elimination of vaccine from the system. Moreover, encapsulating adjuvant and antigen within the same exosome and delivering to a relevant cell,

provided simultaneous presentation of both antigen and co-stimulatory signals to relevant APCs. Exosomes, because of their surface composition, offers passive targeting to APCs. Exosomes bear molecules like PS, LFA1, and integrins providing targeting to macrophages, DCs and B-cells as well as T-cells [268]. This natural ability of exosomes provided over 90% of encapsulated vaccine to be captured and internalized by CD11b⁺ F4/80⁺ peritoneal macrophages rather than immunologically irrelevant cells upon administration. As a result, this targeted simultaneous delivery of vaccine components mediates strong and lengthened immunization against OVA.

Another aspect of immunization provided by encapsulated exosome vaccine was the nature of anti-OVA immune response. Analysis of IgG2c/IgG1 ratios of mice post-booster immunization and thereafter revealed that only exosome encapsulation generated Th1 biased immune response which was also persisted more than 24 weeks after booster injection (Figure 3.21C). Ability of encapsulated D35 to stimulate immune cells, to secrete faster and higher levels of IL12, which is crucial for driving Th1 response. both IFN α secreting pDCs and long-term Th1 dominance helped mediate elimination of transfected cells.

We performed challenge experiments using OVA expressing thymoma cell line called EG-7 to the OVA immunized mice. Of note, these experiments were performed with 5×10^6 EG-7 inoculation, which was rather a high number of such an aggressive tumor model. Challenge experiments performed at 10 and 24 weeks after booster injections showed that in fact just one antigen is enough, if one can mediate strong immunity against the cancer cell, inducing protection against tumor formation. Exosomal vaccine immunized mice performed better in both experiments with respect to free vaccine (Figure 3.24). The anti-tumor activity induced by free vaccine waned off by the time we inoculated EG-7 cells since their tumor sizes were close to PBS immunized animals. On the other hand, encapsulated vaccine immunized mice gave reproducible results in both experiments. More importantly, in addition to smaller tumor sizes, 3 out of 5 mice were tumor free at the termination time of experiments. Fascinatingly, in the latter experiment, which was continued for 3 months, 2 out of 5 mice were tumor free with the survival ratio of 60%. The challenge experiments further demonstrated that using exosomes as delivery vessels for these labile molecules is sufficient to boost their in-vivo capacity enabling them to induce strong and lengthen immune response capable of clearing aggressive cancer cells. This enhanced ability to clear tumor cells might be due to

increased amount of OVA specific effector/memory CD8⁺ cytotoxic T cells as well as activated NK cells.

Dehydration-rehydration method was also tested in loading c-di-GMP, which is also another therapeutically important molecule, within exosomes. The c-di-GMP was shown to be a very potent inducer of Type-I IFNs [189, 269]. This induction capacity makes c-di-GMP a potent candidate as vaccine adjuvant. However, due to its negatively charged nature, c-di-GMP cannot penetrate through cell membrane and trigger its cytosolic sensor, STING and subsequent IFN α secretion [190]. Both mixing with Arg(9) and encapsulating within exosomes bypassed entry problem as evidenced by higher internalization of Arg(9)/c-di-GMP and Exo(c-di-GMP) within APCs compared to lipofectamine assisted free c-di-GMP (Figure 3.25). However, exosomal formulation mediated uptake of c-di-GMP by almost all APCs, only ~10% of APCs were positive for c-di-GMP. This higher internalization translated into increased activation of MHC-II⁺/CD86⁺ on APCs as well as higher pro-inflammatory cytokine secretion by Exo(c-di-GMP) but not Arg(9)/c-di-GMP (Figure 3.26 and Figure A5). This immune activation could be further improved by synergistic effect of CpG ODNs (Figure 3.26). This extra piece of evidence with c-di-GMP further proved the global applicability of loading method within exosomes as described in this thesis.

To summarize, dehydration-rehydration method provided a means for loading exosomes externally with ligands of interest with high efficiency without altering their chemical or physical properties. With the choice of proper cell source, modified exosomes can be produced to be used for a wide variety of therapeutic applications such as cancer immunotherapy, vaccination against pathogens, management of autoimmune diseases, regenerative medicine as well as cosmetics.

In this thesis, our studies proved that EVs are excellent tools for both diagnostic and therapeutic applications in bio-medicine as outlined in Figure 4.1. For example, patient derived EVs could be used to identify disease specific marker(s) to generate next-generation diagnostic tools as well as prognostic kits. In the therapeutic aspect, patient derived EVs could be engineered as effective carrier vectors for drugs or vaccines. EVs could open new avenues in personalized medicine as they can be obtained from any biological fluid. Furthermore, when combined with the loading techniques described in this thesis, EVs become very promising and versatile tools for therapeutic approaches.

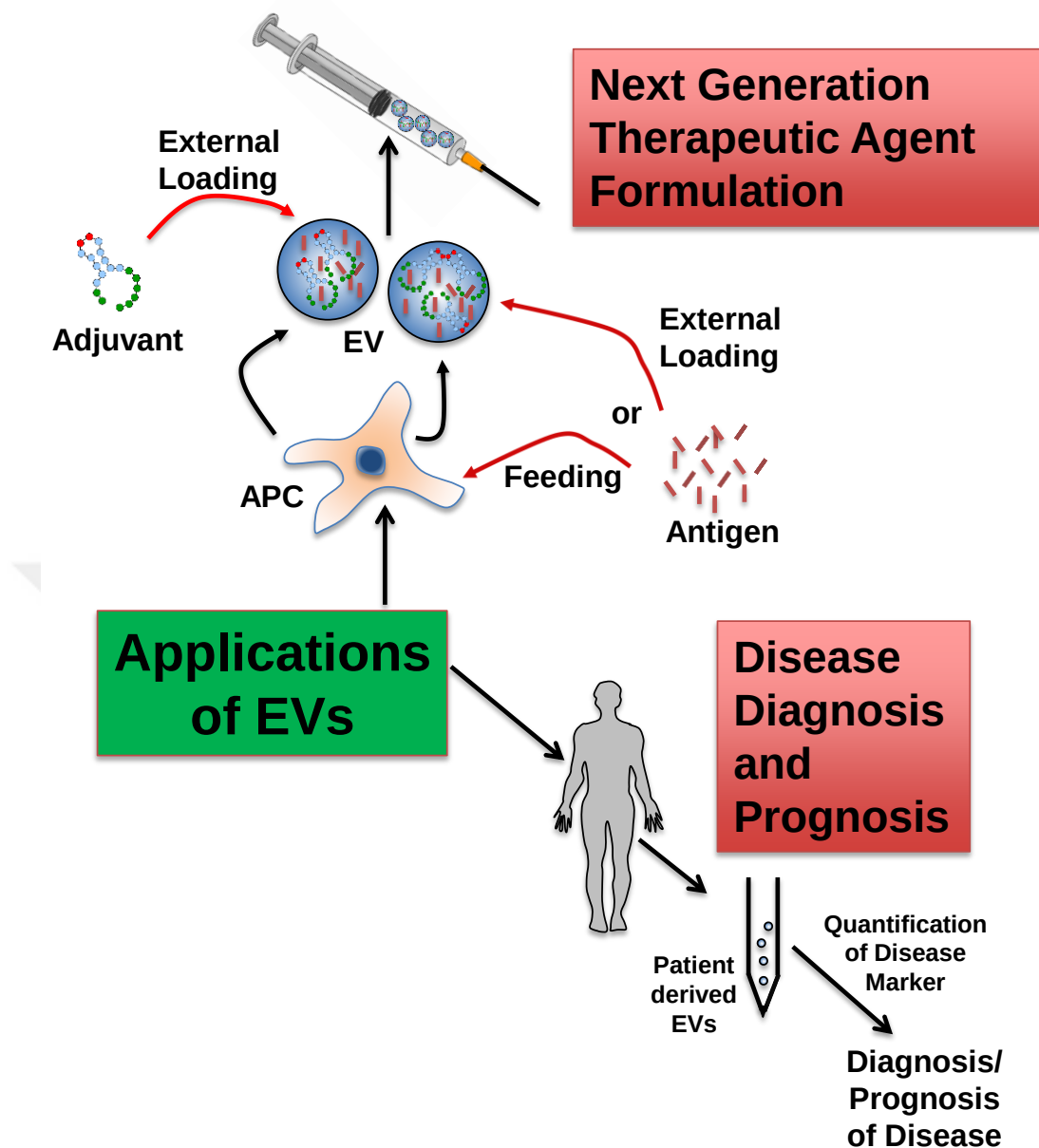


Figure 4.1. Potential biomedical application of EVs elaborated in this thesis. *Patient derived EVs can be used as diagnostic markers. In addition, both patient or cell line derived EVs can be modified to be used various therapeutic applications.*

References

- [1] Sharrow SO, Mathieson BJ, Singer A. Cell surface appearance of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras. *J Immunol.* 1981 Apr;126(4):1327-35.
- [2] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007 Jun;9(6):654-9.
- [3] Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med.* 1996 Mar 1;183(3):1161-72.
- [4] Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood.* 1999 Dec 1;94(11):3791-9.
- [5] Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol.* 2008 May;10(5):619-24.
- [6] Hess C, Sadallah S, Hefti A, Landmann R, Schifferli JA. Ectosomes released by human neutrophils are specialized functional units. *J Immunol.* 1999 Oct 15;163(8):4564-73.
- [7] Obregon C, Rothen-Rutishauser B, Gitahi SK, Gehr P, Nicod LP. Exovesicles from human activated dendritic cells fuse with resting dendritic cells, allowing them to present alloantigens. *Am J Pathol.* 2006 Dec;169(6):2127-36.

- [8] Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood*. 2007 Nov 1;110(9):3234-44.
- [9] Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol*. 2002 Apr 1;168(7):3235-41.
- [10] Gauley J, Pisetsky DS. The release of microparticles by RAW 264.7 macrophage cells stimulated with TLR ligands. *J Leukoc Biol*. 2010 Jun;87(6):1115-23.
- [11] Tramontano AF, O'Leary J, Black AD, Muniyappa R, Cutaia MV, El-Sherif N. Statin decreases endothelial microparticle release from human coronary artery endothelial cells: implication for the Rho-kinase pathway. *Biochem Biophys Res Commun*. 2004 Jul 16;320(1):34-8.
- [12] Keller S, Ridinger J, Rupp AK, Janssen JW, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. *J Transl Med*. 2011;9:86.
- [13] Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005 Jul;17(7):879-87.
- [14] Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 2004 Sep 7;101(36):13368-73.
- [15] Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med*. 2011;9:9.
- [16] Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, et al. CD24 is a marker of exosomes secreted into urine and amniotic fluid. *Kidney Int*. 2007 Nov;72(9):1095-102.
- [17] Skriner K, Adolph K, Jungblut PR, Burmester GR. Association of citrullinated proteins with synovial exosomes. *Arthritis Rheum*. 2006 Dec;54(12):3809-14.
- [18] Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, et al. Immune-related microRNAs are abundant in breast milk exosomes. *Int J Biol Sci*. 2012;8(1):118-23.
- [19] Lasser C, O'Neil SE, Ekerljung L, Ekstrom K, Sjostrand M, Lotvall J. RNA-containing exosomes in human nasal secretions. *Am J Rhinol Allergy*. 2011 Mar-Apr;25(2):89-93.
- [20] Qazi KR, Torregrosa Paredes P, Dahlberg B, Grunewald J, Eklund A, Gabrielsson S. Proinflammatory exosomes in bronchoalveolar lavage fluid of patients with sarcoidosis. *Thorax*. 2010 Nov;65(11):1016-24.

- [21] Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006 Apr;Chapter 3:Unit 3 22.
- [22] Gasser O, Hess C, Miot S, Deon C, Sanchez JC, Schifferli JA. Characterisation and properties of ectosomes released by human polymorphonuclear neutrophils. *Exp Cell Res.* 2003 May 1;285(2):243-57.
- [23] Marzesco AM, Janich P, Wilsch-Brauninger M, Dubreuil V, Langenfeld K, Corbeil D, et al. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. *J Cell Sci.* 2005 Jul 1;118(Pt 13):2849-58.
- [24] Hawari FI, Rouhani FN, Cui X, Yu ZX, Buckley C, Kaler M, et al. Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors. *Proc Natl Acad Sci U S A.* 2004 Feb 3;101(5):1297-302.
- [25] Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol.* 2001 Jun 15;166(12):7309-18.
- [26] Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009 Aug;9(8):581-93.
- [27] Shifrin DA, Jr., Demory Beckler M, Coffey RJ, Tyska MJ. Extracellular vesicles: communication, coercion, and conditioning. *Mol Biol Cell.* 2013 May;24(9):1253-9.
- [28] Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J Proteome Res.* 2008 Dec;7(12):5157-66.
- [29] Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2009 Feb;19(2):43-51.
- [30] Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem.* 1998 Aug 7;273(32):20121-7.
- [31] Geminard C, De Gassart A, Blanc L, Vidal M. Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. *Traffic.* 2004 Mar;5(3):181-93.
- [32] Lasser C, Eldh M, Lotvall J. Isolation and characterization of RNA-containing exosomes. *J Vis Exp.* 2012(59):e3037.
- [33] Merendino AM, Bucchieri F, Campanella C, Marciano V, Ribbene A, David S, et al. Hsp60 is actively secreted by human tumor cells. *PloS one.* 2010;5(2):e9247.

- [34] Segura E, Guerin C, Hogg N, Amigorena S, Thery C. CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. *J Immunol.* 2007 Aug 1;179(3):1489-96.
- [35] Devaux PF, Herrmann A, Ohlwein N, Kozlov MM. How lipid flippases can modulate membrane structure. *Biochim Biophys Acta.* 2008 Jul-Aug;1778(7-8):1591-600.
- [36] Record M, Carayon K, Poirot M, Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiological processes. *Biochim Biophys Acta.* 2014 Jan;1841(1):108-20.
- [37] Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB, Papworth GD, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood.* 2004 Nov 15;104(10):3257-66.
- [38] Feng D, Zhao WL, Ye YY, Bai XC, Liu RQ, Chang LF, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic.* 2010 May;11(5):675-87.
- [39] Tian T, Zhu YL, Hu FH, Wang YY, Huang NP, Xiao ZD. Dynamics of exosome internalization and trafficking. *J Cell Physiol.* 2013 Jul;228(7):1487-95.
- [40] Pan Q, Ramakrishnaiah V, Henry S, Fouraschen S, de Ruiter PE, Kwekkeboom J, et al. Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi). *Gut.* 2012 Sep;61(9):1330-9.
- [41] Christianson HC, Svensson KJ, van Kuppevelt TH, Li JP, Belting M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc Natl Acad Sci U S A.* 2013 Oct 22;110(43):17380-5.
- [42] Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood.* 2012 Jan 19;119(3):756-66.
- [43] Barres C, Blanc L, Bette-Bobillo P, Andre S, Mamoun R, Gabius HJ, et al. Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. *Blood.* 2010 Jan 21;115(3):696-705.
- [44] Nolte-'t Hoen EN, Buschow SI, Anderton SM, Stoorvogel W, Wauben MH. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood.* 2009 Feb 26;113(9):1977-81.
- [45] Clayton A, Turkes A, Dewitt S, Steadman R, Mason MD, Hallett MB. Adhesion and signaling by B cell-derived exosomes: the role of integrins. *FASEB J.* 2004 Jun;18(9):977-9.
- [46] Tait JF, Smith C. Phosphatidylserine receptors: role of CD36 in binding of anionic phospholipid vesicles to monocytic cells. *J Biol Chem.* 1999 Jan 29;274(5):3048-54.

- [47] Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. *Nature*. 2007 Nov 15;450(7168):435-9.
- [48] Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, et al. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity*. 2007 Dec;27(6):927-40.
- [49] Veron P, Segura E, Sugano G, Amigorena S, Thery C. Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells. *Blood Cells Mol Dis*. 2005 Sep-Oct;35(2):81-8.
- [50] Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983 Jul;33(3):967-78.
- [51] Harding C, Heuser J, Stahl P. Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur J Cell Biol*. 1984 Nov;35(2):256-63.
- [52] Bobrie A, Colombo M, Raposo G, Thery C. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic*. 2011 Dec;12(12):1659-68.
- [53] Lee Y, El Andaloussi S, Wood MJ. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet*. 2012 Oct 15;21(R1):R125-34.
- [54] Ratajczak MZ, Kucia M, Jadczyk T, Greco NJ, Wojakowski W, Tendera M, et al. Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies? *Leukemia*. 2012 Jun;26(6):1166-73.
- [55] Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*. 2006 May;20(5):847-56.
- [56] Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C, et al. Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant*. 2011 May;26(5):1474-83.
- [57] Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood*. 2005 Sep 1;106(5):1604-11.
- [58] Puddu P, Puddu GM, Cravero E, Muscari S, Muscari A. The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. *Can J Cardiol*. 2010 Apr;26(4):140-5.
- [59] Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote

tumour growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2008 Dec;10(12):1470-6.

- [60] An T, Qin S, Xu Y, Tang Y, Huang Y, Situ B, et al. Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis. *J Extracell Vesicles.* 2015;4:27522.
- [61] Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther.* 2010 Sep;18(9):1606-14.
- [62] Andre F, Chaput N, Scharzt NE, Flament C, Aubert N, Bernard J, et al. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol.* 2004 Feb 15;172(4):2126-36.
- [63] S ELA, Mager I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov.* 2013 May;12(5):347-57.
- [64] Kovar M, Boyman O, Shen X, Hwang I, Kohler R, Sprent J. Direct stimulation of T cells by membrane vesicles from antigen-presenting cells. *Proc Natl Acad Sci U S A.* 2006 Aug 1;103(31):11671-6.
- [65] Sprent J. Direct stimulation of naive T cells by antigen-presenting cell vesicles. *Blood Cells Mol Dis.* 2005 Jul-Aug;35(1):17-20.
- [66] Hwang I, Shen X, Sprent J. Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. *Proc Natl Acad Sci U S A.* 2003 May 27;100(11):6670-5.
- [67] Dignat-George F, Camoin-Jau L, Sabatier F, Arnoux D, Anfosso F, Bardin N, et al. Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb Haemost.* 2004 Apr;91(4):667-73.
- [68] Jy W, Tiede M, Bidot CJ, Horstman LL, Jimenez JJ, Chirinos J, et al. Platelet activation rather than endothelial injury identifies risk of thrombosis in subjects positive for antiphospholipid antibodies. *Thromb Res.* 2007;121(3):319-25.
- [69] Nagahama M, Nomura S, Kanazawa S, Ozaki Y, Kagawa H, Fukuhara S. Significance of anti-oxidized LDL antibody and monocyte-derived microparticles in anti-phospholipid antibody syndrome. *Autoimmunity.* 2003 May;36(3):125-31.
- [70] Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis.* 2001 Oct;158(2):277-87.
- [71] Knijff-Dutmer EA, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, van de Laar MA. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. *Arthritis Rheum.* 2002 Jun;46(6):1498-503.

- [72] Nagahama M, Nomura S, Ozaki Y, Yoshimura C, Kagawa H, Fukuhara S. Platelet activation markers and soluble adhesion molecules in patients with systemic lupus erythematosus. *Autoimmunity*. 2001;33(2):85-94.
- [73] Boilard E, Blanco P, Nigrovic PA. Platelets: active players in the pathogenesis of arthritis and SLE. *Nat Rev Rheumatol*. 2012 Sep;8(9):534-42.
- [74] Berckmans RJ, Nieuwland R, Kraan MC, Schaap MC, Pots D, Smeets TJ, et al. Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes. *Arthritis Res Ther*. 2005;7(3):R536-44.
- [75] Messer L, Alsaleh G, Freyssinet JM, Zobairi F, Leray I, Gottenberg JE, et al. Microparticle-induced release of B-lymphocyte regulators by rheumatoid synoviocytes. *Arthritis Res Ther*. 2009;11(2):R40.
- [76] Biro E, Nieuwland R, Tak PP, Pronk LM, Schaap MC, Sturk A, et al. Activated complement components and complement activator molecules on the surface of cell-derived microparticles in patients with rheumatoid arthritis and healthy individuals. *Ann Rheum Dis*. 2007 Aug;66(8):1085-92.
- [77] Distler JH, Jungel A, Huber LC, Seemayer CA, Reich CF, 3rd, Gay RE, et al. The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. *Proc Natl Acad Sci U S A*. 2005 Feb 22;102(8):2892-7.
- [78] Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis Rheum*. 2008 Sep;58(9):2845-53.
- [79] Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol*. 2014 Jun;10(6):356-64.
- [80] Belting M, Ahamed J, Ruf W. Signaling of the tissue factor coagulation pathway in angiogenesis and cancer. *Arterioscler Thromb Vasc Biol*. 2005 Aug;25(8):1545-50.
- [81] Thomas GM, Panicot-Dubois L, Lacroix R, Dignat-George F, Lombardo D, Dubois C. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. *J Exp Med*. 2009 Aug 31;206(9):1913-27.
- [82] Yu JL, Rak JW. Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human cancer cells. *J Thromb Haemost*. 2004 Nov;2(11):2065-7.
- [83] Huber LC, Jungel A, Distler JH, Moritz F, Gay RE, Michel BA, et al. The role of membrane lipids in the induction of macrophage apoptosis by microparticles. *Apoptosis*. 2007 Feb;12(2):363-74.
- [84] Abrahams VM, Straszewski-Chavez SL, Guller S, Mor G. First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis. *Mol Hum Reprod*. 2004 Jan;10(1):55-63.

- [85] Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin Cancer Res*. 2005 Feb 1;11(3):1010-20.
- [86] Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology*. 2005 Jun;128(7):1796-804.
- [87] Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med*. 2002 May 20;195(10):1303-16.
- [88] Valenti R, Huber V, Filipazzi P, Pilla L, Sovenia G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res*. 2006 Sep 15;66(18):9290-8.
- [89] Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res*. 2007 Apr 1;67(7):2912-5.
- [90] Milsom C, Yu J, May L, Magnus N, Rak J. Diverse roles of tissue factor-expressing cell subsets in tumor progression. *Semin Thromb Hemost*. 2008 Mar;34(2):170-81.
- [91] Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. *Cardiovasc Res*. 2005 Jul 1;67(1):30-8.
- [92] Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M, Ratajczak MZ. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. *Transfusion*. 2006 Jul;46(7):1199-209.
- [93] Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int J Cancer*. 2005 Feb 20;113(5):752-60.
- [94] Millimaggi D, Mari M, D'Ascenzo S, Carosa E, Jannini EA, Zucker S, et al. Tumor vesicle-associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia*. 2007 Apr;9(4):349-57.
- [95] Aharon A, Brenner B. Microparticles, thrombosis and cancer. *Best Pract Res Clin Haematol*. 2009 Mar;22(1):61-9.
- [96] Andre F, Scharz NE, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet*. 2002 Jul 27;360(9329):295-305.
- [97] Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med*. 2001 Mar;7(3):297-303.

- [98] Chaput N, Scharzt NE, Andre F, Taieb J, Novault S, Bonnaventure P, et al. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. *J Immunol.* 2004 Feb 15;172(4):2137-46.
- [99] Utsugi-Kobukai S, Fujimaki H, Hotta C, Nakazawa M, Minami M. MHC class I-mediated exogenous antigen presentation by exosomes secreted from immature and mature bone marrow derived dendritic cells. *Immunol Lett.* 2003 Oct 31;89(2-3):125-31.
- [100] Admyre C, Johansson SM, Paulie S, Gabrielsson S. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol.* 2006 Jul;36(7):1772-81.
- [101] Muntasell A, Berger AC, Roche PA. T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. *EMBO J.* 2007 Oct 3;26(19):4263-72.
- [102] Segura E, Nicco C, Lombard B, Veron P, Raposo G, Batteux F, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood.* 2005 Jul 1;106(1):216-23.
- [103] Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol.* 2002 Dec;3(12):1156-62.
- [104] Montecalvo A, Shufesky WJ, Stolz DB, Sullivan MG, Wang Z, Divito SJ, et al. Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition. *J Immunol.* 2008 Mar 1;180(5):3081-90.
- [105] Bhatnagar S, Schorey JS. Exosomes released from infected macrophages contain *Mycobacterium avium* glycopeptidolipids and are proinflammatory. *J Biol Chem.* 2007 Aug 31;282(35):25779-89.
- [106] Quah BJ, O'Neill HC. Mycoplasma contaminants present in exosome preparations induce polyclonal B cell responses. *J Leukoc Biol.* 2007 Nov;82(5):1070-82.
- [107] Simhadri VR, Reiners KS, Hansen HP, Topolar D, Simhadri VL, Nohroudi K, et al. Dendritic cells release HLA-B-associated transcript-3 positive exosomes to regulate natural killer function. *PloS one.* 2008;3(10):e3377.
- [108] Viaud S, Terme M, Flament C, Taieb J, Andre F, Novault S, et al. Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Ralpha. *PloS one.* 2009;4(3):e4942.
- [109] Gastpar R, Gehrman M, Bausero MA, Asea A, Gross C, Schroeder JA, et al. Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. *Cancer Res.* 2005 Jun 15;65(12):5238-47.
- [110] Vega VL, Rodriguez-Silva M, Frey T, Gehrman M, Diaz JC, Steinem C, et al. Hsp70 translocates into the plasma membrane after stress and is

released into the extracellular environment in a membrane-associated form that activates macrophages. *J Immunol.* 2008 Mar 15;180(6):4299-307.

- [111] Dai S, Wan T, Wang B, Zhou X, Xiu F, Chen T, et al. More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. *Clin Cancer Res.* 2005 Oct 15;11(20):7554-63.
- [112] Zhang HG, Liu C, Su K, Yu S, Zhang L, Zhang S, et al. A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death. *J Immunol.* 2006 Jun 15;176(12):7385-93.
- [113] Poutsika DD, Schroder EW, Taylor DD, Levy EM, Black PH. Membrane vesicles shed by murine melanoma cells selectively inhibit the expression of Ia antigen by macrophages. *J Immunol.* 1985 Jan;134(1):138-44.
- [114] Czystowska M, Han J, Szczepanski MJ, Szajnik M, Quadrini K, Brandwein H, et al. IRX-2, a novel immunotherapeutic, protects human T cells from tumor-induced cell death. *Cell Death Differ.* 2009 May;16(5):708-18.
- [115] Klibi J, Niki T, Riedel A, Pioche-Durieu C, Souquere S, Rubinstein E, et al. Blood diffusion and Th1-suppressive effects of galectin-9-containing exosomes released by Epstein-Barr virus-infected nasopharyngeal carcinoma cells. *Blood.* 2009 Feb 26;113(9):1957-66.
- [116] Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. *Cancer Res.* 2007 Aug 1;67(15):7458-66.
- [117] Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL. Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg). *PloS one.* 2010;5(7):e11469.
- [118] Wieckowski EU, Visus C, Szajnik M, Szczepanski MJ, Storkus WJ, Whiteside TL. Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes. *J Immunol.* 2009 Sep 15;183(6):3720-30.
- [119] Ashiru O, Boutet P, Fernandez-Messina L, Aguera-Gonzalez S, Skepper JN, Vales-Gomez M, et al. Natural killer cell cytotoxicity is suppressed by exposure to the human NKG2D ligand MICA*008 that is shed by tumor cells in exosomes. *Cancer Res.* 2010 Jan 15;70(2):481-9.
- [120] Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z. Human tumor-derived exosomes down-modulate NKG2D expression. *J Immunol.* 2008 Jun 1;180(11):7249-58.
- [121] Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, et al. Tumor exosomes inhibit differentiation of bone marrow dendritic cells. *J Immunol.* 2007 Jun 1;178(11):6867-75.
- [122] Xiang X, Liu Y, Zhuang X, Zhang S, Michalek S, Taylor DD, et al. TLR2-mediated expansion of MDSCs is dependent on the source of tumor exosomes. *Am J Pathol.* 2010 Oct;177(4):1606-10.

- [123] Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, et al. Induction of myeloid-derived suppressor cells by tumor exosomes. *Int J Cancer*. 2009 Jun 1;124(11):2621-33.
- [124] Diao J, Yang X, Song X, Chen S, He Y, Wang Q, et al. Exosomal Hsp70 mediates immunosuppressive activity of the myeloid-derived suppressor cells via phosphorylation of Stat3. *Med Oncol*. 2015 Feb;32(2):453.
- [125] Monleon I, Martinez-Lorenzo MJ, Monteagudo L, Lasierra P, Taules M, Iturralde M, et al. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J Immunol*. 2001 Dec 15;167(12):6736-44.
- [126] Bu N, Wu HQ, Zhang GL, Zhan SQ, Zhang R, Fan QY, et al. Immature dendritic cell exosomes suppress experimental autoimmune myasthenia gravis. *J Neuroimmunol*. 2015 Aug 15;285:71-5.
- [127] Li X, Li JJ, Yang JY, Wang DS, Zhao W, Song WJ, et al. Tolerance induction by exosomes from immature dendritic cells and rapamycin in a mouse cardiac allograft model. *PloS one*. 2012;7(8):e44045.
- [128] Smyth LA, Ratnasothy K, Tsang JY, Boardman D, Warley A, Lechler R, et al. CD73 expression on extracellular vesicles derived from CD4⁺ CD25⁺ Foxp3⁺ T cells contributes to their regulatory function. *Eur J Immunol*. 2013 Sep;43(9):2430-40.
- [129] Okoye IS, Coomes SM, Pelly VS, Czieso S, Papayannopoulos V, Tolmachova T, et al. MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. *Immunity*. 2014 Jul 17;41(1):89-103.
- [130] Nolte-'t Hoen EN, Wagenaar-Hilbers JP, Peters PJ, Gadella BM, van Eden W, Wauben MH. Uptake of membrane molecules from T cells endows antigen-presenting cells with novel functional properties. *Eur J Immunol*. 2004 Nov;34(11):3115-25.
- [131] Heldring N, Mager I, Wood MJ, Le Blanc K, Andaloussi SE. Therapeutic Potential of Multipotent Mesenchymal Stromal Cells and Their Extracellular Vesicles. *Hum Gene Ther*. 2015 Aug;26(8):506-17.
- [132] Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res*. 2010 May;4(3):214-22.
- [133] Bruno S, Grange C, Deregibus MC, Calogero RA, Saviozzi S, Collino F, et al. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol*. 2009 May;20(5):1053-67.
- [134] Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev*. 2014 Jun 1;23(11):1233-44.
- [135] Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia*. 2014 Apr;28(4):970-3.

- [136] Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol.* 2009 Sep;10(9):1000-7.
- [137] Yu X, Huang C, Song B, Xiao Y, Fang M, Feng J, et al. CD4+CD25+ regulatory T cells-derived exosomes prolonged kidney allograft survival in a rat model. *Cell Immunol.* 2013 Sep-Oct;285(1-2):62-8.
- [138] Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med.* 1998 May;4(5):594-600.
- [139] Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J Transl Med.* 2005 Mar 2;3(1):10.
- [140] Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med.* 2005 Feb 21;3(1):9.
- [141] Lamparski HG, Metha-Damani A, Yao JY, Patel S, Hsu DH, Ruegg C, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods.* 2002 Dec 15;270(2):211-26.
- [142] Viaud S, Ploix S, Lapierre V, Thery C, Commere PH, Tramalloni D, et al. Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-gamma. *J Immunother.* 2011 Jan;34(1):65-75.
- [143] Lugini L, Cecchetti S, Huber V, Luciani F, Macchia G, Spadaro F, et al. Immune surveillance properties of human NK cell-derived exosomes. *J Immunol.* 2012 Sep 15;189(6):2833-42.
- [144] Simons M, Raposo G. Exosomes--vesicular carriers for intercellular communication. *Curr Opin Cell Biol.* 2009 Aug;21(4):575-81.
- [145] Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol.* 2002 Aug;2(8):569-79.
- [146] Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. *Cancer Res.* 2011 Jun 1;71(11):3792-801.
- [147] Fang Y, Wu N, Gan X, Yan W, Morrell JC, Gould SJ. Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. *PLoS Biol.* 2007 Jun;5(6):e158.
- [148] Shen B, Wu N, Yang JM, Gould SJ. Protein targeting to exosomes/microvesicles by plasma membrane anchors. *J Biol Chem.* 2011 Apr 22;286(16):14383-95.
- [149] Zhuang X, Xiang X, Grizzle W, Sun D, Zhang S, Axtell RC, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-

inflammatory drugs from the nasal region to the brain. *Mol Ther.* 2011 Oct;19(10):1769-79.

- [150] Wahlgren J, Karlson TD, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen P, et al. Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Res.* 2012 May 22.
- [151] El Andaloussi S, Lakhal S, Mager I, Wood MJ. Exosomes for targeted siRNA delivery across biological barriers. *Adv Drug Deliv Rev.* 2012 Aug 18.
- [152] Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol.* 2011 Apr;29(4):341-5.
- [153] Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y, et al. Microvesicle-mediated RNA molecule delivery system using monocytes/macrophages. *Mol Ther.* 2011 Feb;19(2):395-9.
- [154] Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem.* 2010 Jun 4;285(23):17442-52.
- [155] Kondkar AA, Abu-Amero KK. Utility of circulating microRNAs as clinical biomarkers for cardiovascular diseases. *Biomed Res Int.* 2015;2015:821823.
- [156] Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol.* 2010 Apr 1;28(10):1721-6.
- [157] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 2008 Oct;18(10):997-1006.
- [158] Hessvik NP, Sandvig K, Llorente A. Exosomal miRNAs as Biomarkers for Prostate Cancer. *Front Genet.* 2013;4:36.
- [159] Aushev VN, Zborovskaya IB, Laktionov KK, Girard N, Cros MP, Herceg Z, et al. Comparisons of microRNA patterns in plasma before and after tumor removal reveal new biomarkers of lung squamous cell carcinoma. *PLoS One.* 2013;8(10):e78649.
- [160] Wang H, Hou L, Li A, Duan Y, Gao H, Song X. Expression of Serum Exosomal MicroRNA-21 in Human Hepatocellular Carcinoma. *Biomed Res Int.* 2014;2014:864894.
- [161] Revenfeld AL, Baek R, Nielsen MH, Stensballe A, Varming K, Jorgensen M. Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. *Clin Ther.* 2014 Jun 1;36(6):830-46.
- [162] Keller S, Konig AK, Marme F, Runz S, Wolterink S, Koensgen D, et al. Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett.* 2009 Jun 8;278(1):73-81.

- [163] Szajnik M, Derbis M, Lach M, Patalas P, Michalak M, Drzewiecka H, et al. Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy. *Gynecol Obstet (Sunnyvale)*. 2013 Apr 29;Suppl 4:3.
- [164] Li J, Sherman-Baust CA, Tsai-Turton M, Bristow RE, Roden RB, Morin PJ. Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. *BMC Cancer*. 2009;9:244.
- [165] Shao H, Chung J, Balaj L, Charest A, Bigner DD, Carter BS, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med*. 2012 Dec;18(12):1835-40.
- [166] Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PloS one*. 2009;4(4):e5219.
- [167] Baran J, Baj-Krzyworzeka M, Weglarczyk K, Szatanek R, Zembala M, Barbasz J, et al. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. *Cancer Immunol Immunother*. 2010 Jun;59(6):841-50.
- [168] Tesselaar ME, Romijn FP, Van Der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost*. 2007 Mar;5(3):520-7.
- [169] Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity. *J Immunol*. 1992 Jun 15;148(12):4072-6.
- [170] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*. 1995 Apr 6;374(6522):546-9.
- [171] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature*. 2000 Dec 7;408(6813):740-5.
- [172] Klinman DM, Currie D, Gursel I, Verthelyi D. Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunol Rev*. 2004 Jun;199:201-16.
- [173] Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J Immunol*. 2001 Feb 15;166(4):2372-7.
- [174] Krug A, Rothenfusser S, Hornung V, Jahrsdorfer B, Blackwell S, Ballas ZK, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol*. 2001 Jul;31(7):2154-63.
- [175] Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol*. 2000 Jan 15;164(2):944-53.

- [176] Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, et al. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- α induction in plasmacytoid dendritic cells. *Eur J Immunol*. 2003 Jun;33(6):1633-41.
- [177] Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M, et al. Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J Immunol*. 2001 Oct 1;167(7):3555-8.
- [178] Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A*. 1996 Apr 2;93(7):2879-83.
- [179] Kobayashi H, Horner AA, Takabayashi K, Nguyen MD, Huang E, Cinman N, et al. Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell Immunol*. 1999 Nov 25;198(1):69-75.
- [180] Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J Immunol*. 1998 Jan 15;160(2):870-6.
- [181] Klinman DM. Adjuvant activity of CpG oligodeoxynucleotides. *Int Rev Immunol*. 2006 May-Aug;25(3-4):135-54.
- [182] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med*. 1997 Nov 17;186(10):1623-31.
- [183] von Hunolstein C, Mariotti S, Teloni R, Alfarone G, Romagnoli G, Orefici G, et al. The adjuvant effect of synthetic oligodeoxynucleotide containing CpG motif converts the anti-Haemophilus influenzae type b glycoconjugates into efficient anti-polysaccharide and anti-carrier polyvalent vaccines. *Vaccine*. 2001 Apr 30;19(23-24):3058-66.
- [184] Eastcott JW, Holmberg CJ, Dewhirst FE, Esch TR, Smith DJ, Taubman MA. Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. *Vaccine*. 2001 Feb 8;19(13-14):1636-42.
- [185] Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB, et al. CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infect Immun*. 2005 Feb;73(2):828-33.
- [186] Amikam D, Benziman M. Cyclic diguanylic acid and cellulose synthesis in *Agrobacterium tumefaciens*. *J Bacteriol*. 1989 Dec;171(12):6649-55.
- [187] Karaolis DK, Newstead MW, Zeng X, Hyodo M, Hayakawa Y, Bhan U, et al. Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infect Immun*. 2007 Oct;75(10):4942-50.

- [188] Karaolis DK, Means TK, Yang D, Takahashi M, Yoshimura T, Muraille E, et al. Bacterial c-di-GMP is an immunostimulatory molecule. *J Immunol*. 2007 Feb 15;178(4):2171-81.
- [189] McWhirter SM, Barbalat R, Monroe KM, Fontana MF, Hyodo M, Joncker NT, et al. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J Exp Med*. 2009 Aug 31;206(9):1899-911.
- [190] Kumagai Y, Matsuo J, Hayakawa Y, Rikihisa Y. Cyclic di-GMP signaling regulates invasion by *Ehrlichia chaffeensis* of human monocytes. *J Bacteriol*. 2010 Aug;192(16):4122-33.
- [191] Kaklamani VG, Vaiopoulos G, Kaklamanis PG. Behcet's Disease. *Seminars in arthritis and rheumatism*. 1998 Feb;27(4):197-217.
- [192] Yazici Y, Yurdakul S, Yazici H. Behcet's syndrome. *Current rheumatology reports*. Dec;12(6):429-35.
- [193] Direskeneli H. Autoimmunity vs autoinflammation in Behcet's disease: do we oversimplify a complex disorder? *Rheumatology (Oxford, England)*. 2006 Dec;45(12):1461-5.
- [194] Hamzaoui K, Hamzaoui A, Guemira F, Bessioud M, Hamza M, Ayed K. Cytokine profile in Behcet's disease patients. Relationship with disease activity. *Scandinavian journal of rheumatology*. 2002;31(4):205-10.
- [195] Melikoglu M, Uysal S, Krueger JG, Kaplan G, Gogus F, Yazici H, et al. Characterization of the divergent wound-healing responses occurring in the pathergy reaction and normal healthy volunteers. *J Immunol*. 2006 Nov 1;177(9):6415-21.
- [196] Raziuddin S, al-Dalaan A, Bahabri S, Siraj AK, al-Sedairy S. Divergent cytokine production profile in Behcet's disease. Altered Th1/Th2 cell cytokine pattern. *The Journal of rheumatology*. 1998 Feb;25(2):329-33.
- [197] Schmitz-Huebner U, Knop J. Evidence for an endothelial cell dysfunction in association with Behcet's disease. *Thrombosis research*. 1984 May 15;34(4):277-85.
- [198] Haznedaroglu IC, Ozcebe OI, Ozdemir O, Celik I, Dundar SV, Kirazli S. Impaired haemostatic kinetics and endothelial function in Behcet's disease. *Journal of internal medicine*. 1996 Oct;240(4):181-7.
- [199] Kiraz S, Ertenli I, Ozturk MA, Haznedaroglu IC, Celik I, Calguneri M. Pathological haemostasis and "prothrombotic state" in Behcet's disease. *Thrombosis research*. 2002 Jan 15;105(2):125-33.
- [200] Yurdakul S, Hekim N, Soysal T, Fresko I, Bavunoglu I, Ozbakir F, et al. Fibrinolytic activity and d-dimer levels in Behcet's syndrome. *Clinical and experimental rheumatology*. 2005 Jul-Aug;23(4 Suppl 38):S53-8.
- [201] Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*. 2001 Dec;15(6):985-95.

- [202] Locksley RM. Asthma and allergic inflammation. *Cell*. 2010 Mar 19;140(6):777-83.
- [203] Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature*. 2010 Jan 28;463(7280):540-4.
- [204] Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisle CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences of the United States of America*. 2010 Jun 22;107(25):11489-94.
- [205] Bhakta NR, Woodruff PG. Human asthma phenotypes: from the clinic, to cytokines, and back again. *Immunol Rev*. 2011 Jul;242(1):220-32.
- [206] Berckmans RJ, Nieuwland R, Kraan MC, Schaap MC, Pots D, Smeets TJ, et al. Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes. *Arthritis research & therapy*. 2005;7(3):R536-44.
- [207] Berckmans RJ, Nieuwland R, Tak PP, Boing AN, Romijn FP, Kraan MC, et al. Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism. *Arthritis and rheumatism*. 2002 Nov;46(11):2857-66.
- [208] Brogan PA, Dillon MJ. Endothelial microparticles and the diagnosis of the vasculitides. *Internal medicine (Tokyo, Japan)*. 2004 Dec;43(12):1115-9.
- [209] Brogan PA, Shah V, Brachet C, Harnden A, Mant D, Klein N, et al. Endothelial and platelet microparticles in vasculitis of the young. *Arthritis and rheumatism*. 2004 Mar;50(3):927-36.
- [210] Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *The Journal of clinical investigation*. 1999 Jul;104(1):93-102.
- [211] Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis and rheumatism*. 2008 Sep;58(9):2845-53.
- [212] Knijff-Dutmer EA, Koerts J, Nieuwland R, Kalsbeek-Batenburg EM, van de Laar MA. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. *Arthritis and rheumatism*. 2002 Jun;46(6):1498-503.
- [213] Larkin M. Raised endothelial microparticles an early marker for multiple sclerosis? *Lancet*. 2001 May 26;357(9269):1679.
- [214] Minagar A, Jy W, Jimenez JJ, Sheremata WA, Mauro LM, Mao WW, et al. Elevated plasma endothelial microparticles in multiple sclerosis. *Neurology*. 2001 May 22;56(10):1319-24.

- [215] Sellam J, Proulle V, Jungel A, Ittah M, Miceli Richard C, Gottenberg JE, et al. Increased levels of circulating microparticles in primary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis research & therapy*. 2009;11(5):R156.
- [216] van Eijk IC, Tushuizen ME, Sturk A, Dijkmans BA, Boers M, Voskuyl AE, et al. Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis. *Annals of the rheumatic diseases*. 2009 Nov 16.
- [217] Dye JR, Ullal AJ, Pisetsky DS. The Role of Microparticles in the Pathogenesis of Rheumatoid Arthritis and SLE. *Scandinavian Journal of Immunology*. 2013 May 14.
- [218] Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. *Lancet*. 1990 May 5;335(8697):1078-80.
- [219] Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol*. 2003 Aug 1;171(3):1393-400.
- [220] Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol*. 2013 Nov 15;191(10):4895-901.
- [221] Lynch SF, Ludlam CA. Plasma microparticles and vascular disorders. *Br J Haematol*. 2007 Apr;137(1):36-48.
- [222] Hurtado P, Peh CA. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. *J Immunol*. 2010 Feb 1;184(3):1425-35.
- [223] Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J Immunol*. 2001 Sep 15;167(6):3324-8.
- [224] Gursel M, Gursel I, Mostowski HS, Klinman DM. CXCL16 influences the nature and specificity of CpG-induced immune activation. *J Immunol*. 2006 Aug 1;177(3):1575-80.
- [225] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther*. 1999 Oct 10;10(15):2461-80.
- [226] Distler JH, Huber LC, Gay S, Distler O, Pisetsky DS. Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity*. 2006 Dec;39(8):683-90.
- [227] Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends in cell biology*. 2009 Feb;19(2):43-51.
- [228] Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. *PloS one*. 2008;3(11):e3694.

- [229] Reich CF, 3rd, Pisetsky DS. The content of DNA and RNA in microparticles released by Jurkat and HL-60 cells undergoing in vitro apoptosis. *Experimental cell research*. 2009 Mar 10;315(5):760-8.
- [230] Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. *Physiology (Bethesda, Md)*. 2005 Feb;20:22-7.
- [231] Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borrás FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015;4:27066.
- [232] Espinosa G, Font J, Tassies D, Vidaller A, Deulofeu R, Lopez-Soto A, et al. Vascular involvement in Behcet's disease: relation with thrombophilic factors, coagulation activation, and thrombomodulin. *Am J Med*. 2002 Jan;112(1):37-43.
- [233] Wu X, Li G, Huang X, Wang L, Liu W, Zhao Y, et al. Behcet's disease complicated with thrombosis: a report of 93 Chinese cases. *Medicine (Baltimore)*. 2014 Dec;93(28):e263.
- [234] Danielson KM, Das S. Extracellular Vesicles in Heart Disease: Excitement for the Future ? *Exosomes Microvesicles*. 2014;2(1).
- [235] Gaceb A, Martinez MC, Andriantsitohaina R. Extracellular vesicles: new players in cardiovascular diseases. *Int J Biochem Cell Biol*. 2014 May;50:24-8.
- [236] Geddings JE, Mackman N. New players in haemostasis and thrombosis. *Thromb Haemost*. 2014 Apr 1;111(4):570-4.
- [237] Beyer C, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatic diseases. *Nat Rev Rheumatol*. 2010 Jan;6(1):21-9.
- [238] Dean SN, Bishop BM, van Hoek ML. Susceptibility of *Pseudomonas aeruginosa* Biofilm to Alpha-Helical Peptides: D-enantiomer of LL-37. *Front Microbiol*. 2011;2:128.
- [239] Wong JH, Ng TB, Legowska A, Rolka K, Hui M, Cho CH. Antifungal action of human cathelicidin fragment (LL13-37) on *Candida albicans*. *Peptides*. 2011 Oct;32(10):1996-2002.
- [240] Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, et al. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PloS one*. 2011;6(10):e25333.
- [241] Singer O. Cogan and Behcet syndromes. *Rheum Dis Clin North Am*. 2015;41(1):75-91, viii.
- [242] Direskeneli H. Behcet's disease: infectious aetiology, new autoantigens, and HLA-B51. *Ann Rheum Dis*. 2001 Nov;60(11):996-1002.
- [243] Mumcu G, Cimilli H, Karacayli U, Inanc N, Ture-Ozdemir F, Eksioglu-Demiralp E, et al. Salivary levels of antimicrobial peptides Hnp 1-3, LI-37 and S100 in Behcet's disease. *Arch Oral Biol*. 2012 Jun;57(6):642-6.

- [244] Vandamme D, Landuyt B, Luyten W, Schoofs L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cellular Immunology*. 2012 Nov;280(1):22-35.
- [245] Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol*. 2004 Apr 15;172(8):4987-94.
- [246] Yu J, Mookherjee N, Wee K, Bowdish DM, Pistolic J, Li Y, et al. Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. *Journal of Immunology*. 2007 Dec 1;179(11):7684-91.
- [247] Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. 2007 Oct 4;449(7162):564-9.
- [248] Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol*. 2015 Aug;15(8):471-85.
- [249] Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol*. 2002 Apr 1;168(7):3195-204.
- [250] Torregrosa Paredes P, Esser J, Admyre C, Nord M, Rahman QK, Lukic A, et al. Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. *Allergy*. 2012 Jul;67(7):911-9.
- [251] Kulshreshtha A, Ahmad T, Agrawal A, Ghosh B. Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol*. 2013 Apr;131(4):1194-203, 203 e1-14.
- [252] Delcayre A, Le Pecq JB. Exosomes as novel therapeutic nanodevices. *Curr Opin Mol Ther*. 2006 Feb;8(1):31-8.
- [253] Yang C, Robbins PD. Immunosuppressive exosomes: a new approach for treating arthritis. *Int J Rheumatol*. 2012;2012:573528.
- [254] Taylor DD, Gercel-Taylor C, Lyons KS, Stanson J, Whiteside TL. T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors. *Clin Cancer Res*. 2003 Nov 1;9(14):5113-9.
- [255] Liu C, Yu S, Zinn K, Wang J, Zhang L, Jia Y, et al. Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol*. 2006 Feb 1;176(3):1375-85.
- [256] Romagnoli GG, Zelante BB, Toniolo PA, Migliori IK, Barbuto JA. Dendritic Cell-Derived Exosomes may be a Tool for Cancer Immunotherapy by Converting Tumor Cells into Immunogenic Targets. *Front Immunol*. 2014;5:692.

- [257] Pitt JM, Charrier M, Viaud S, Andre F, Besse B, Chaput N, et al. Dendritic cell-derived exosomes as immunotherapies in the fight against cancer. *J Immunol*. 2014 Aug 1;193(3):1006-11.
- [258] Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 2013;2.
- [259] Cho JA, Yeo DJ, Son HY, Kim HW, Jung DS, Ko JK, et al. Exosomes: a new delivery system for tumor antigens in cancer immunotherapy. *Int J Cancer*. 2005 Apr 20;114(4):613-22.
- [260] Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, et al. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther*. 2006 Feb;13(2):289-300.
- [261] Villarroya-Beltri C, Baixauli F, Gutierrez-Vazquez C, Sanchez-Madrid F, Mittelbrunn M. Sorting it out: regulation of exosome loading. *Semin Cancer Biol*. 2014 Oct;28:3-13.
- [262] El-Andaloussi S, Lee Y, Lakhali-Littleton S, Li J, Seow Y, Gardiner C, et al. Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat Protoc*. 2012 Dec;7(12):2112-26.
- [263] Momen-Heravi F, Bala S, Bukong T, Szabo G. Exosome-mediated delivery of functionally active miRNA-155 inhibitor to macrophages. *Nanomedicine*. 2014 Oct;10(7):1517-27.
- [264] Johnsen KB, Gudbergsson JM, Skov MN, Christiansen G, Gurevich L, Moos T, et al. Evaluation of electroporation-induced adverse effects on adipose-derived stem cell exosomes. *Cytotechnology*. 2016 Feb 8.
- [265] Gregoriadis G, McCormack B, Obrenovich M, Perrie Y. Entrapment of plasmid DNA vaccines into liposomes by dehydration/rehydration. *Methods Mol Med*. 2000;29:305-11.
- [266] Landen CN, Jr., Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, et al. Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery. *Cancer Res*. 2005 Aug 1;65(15):6910-8.
- [267] Gungor B, Yagci FC, Tincer G, Bayyurt B, Alpdundar E, Yildiz S, et al. CpG ODN nanorings induce IFN α from plasmacytoid dendritic cells and demonstrate potent vaccine adjuvant activity. *Sci Transl Med*. 2014 May 7;6(235):235ra61.
- [268] Chaput N, Thery C. Exosomes: immune properties and potential clinical implementations. *Semin Immunopathol*. 2011 Sep;33(5):419-40.
- [269] Yildiz S, Alpdundar E, Gungor B, Kahraman T, Bayyurt B, Gursel I, et al. Enhanced immunostimulatory activity of cyclic dinucleotides on mouse cells when complexed with a cell-penetrating peptide or combined with CpG. *Eur J Immunol*. 2015 Apr;45(4):1170-9.

Appendices

Appendix A

Figure A1. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 2 weeks post-booster bleeding

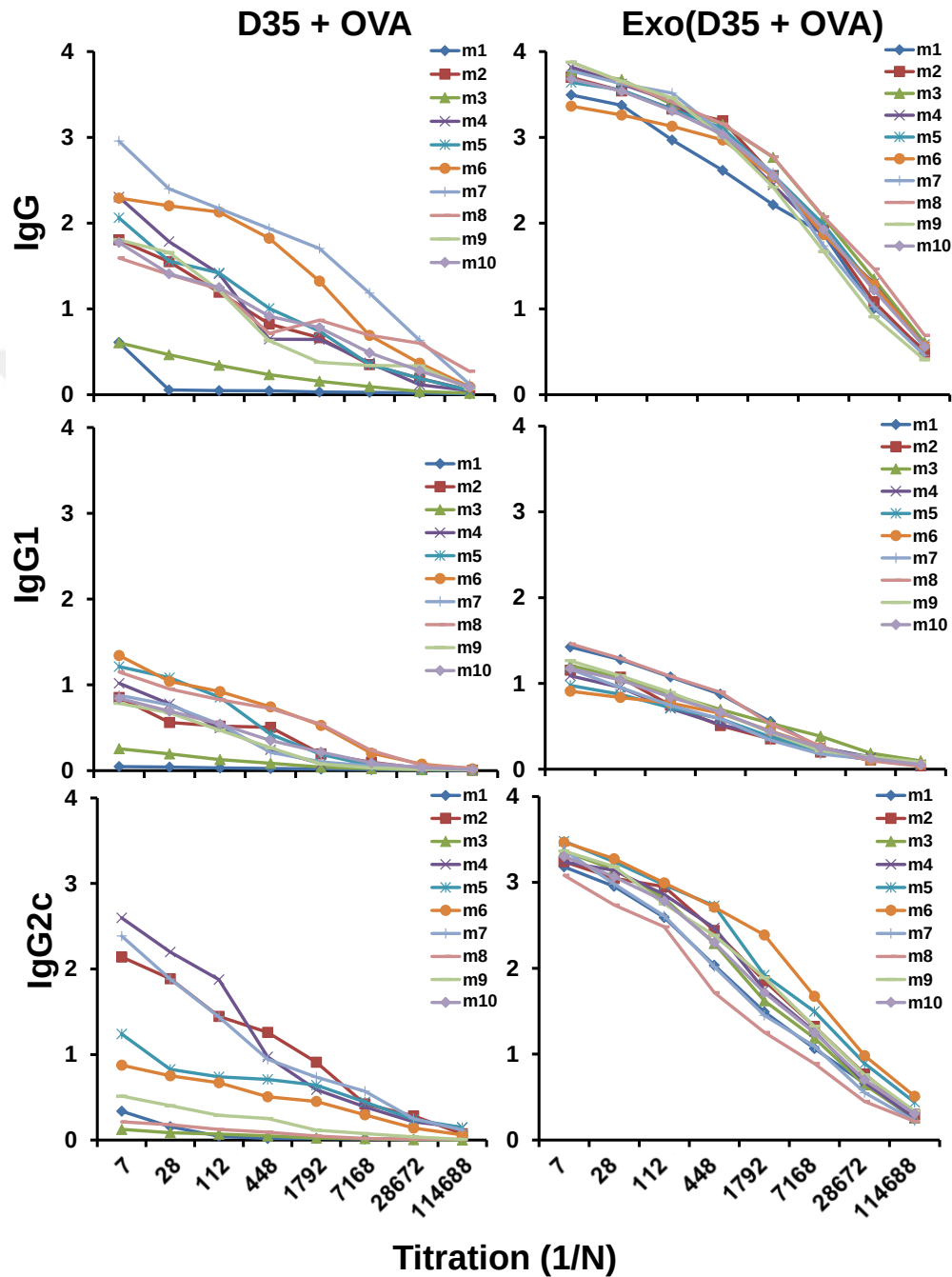


Figure A2. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 10 weeks post-booster bleeding

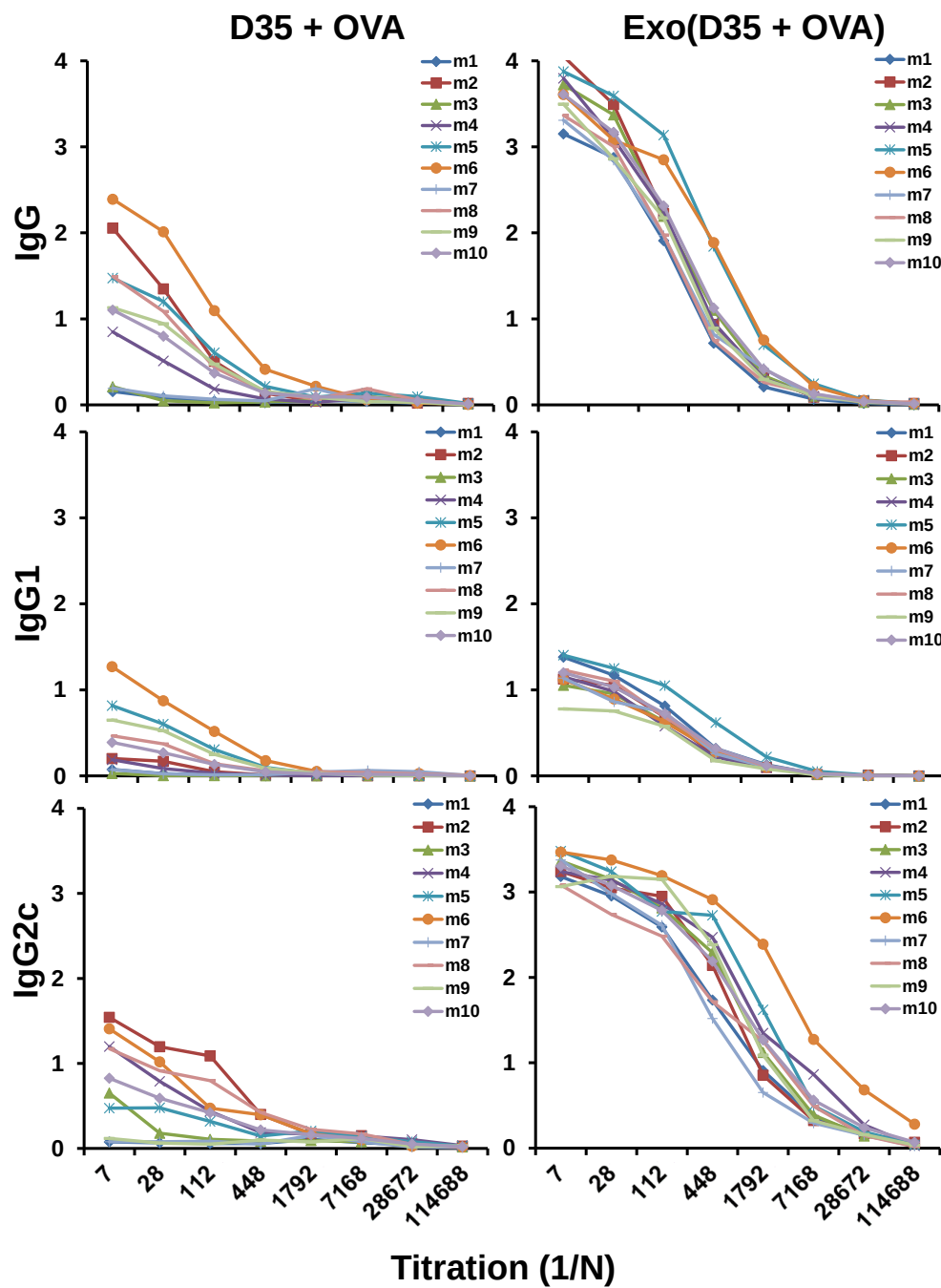
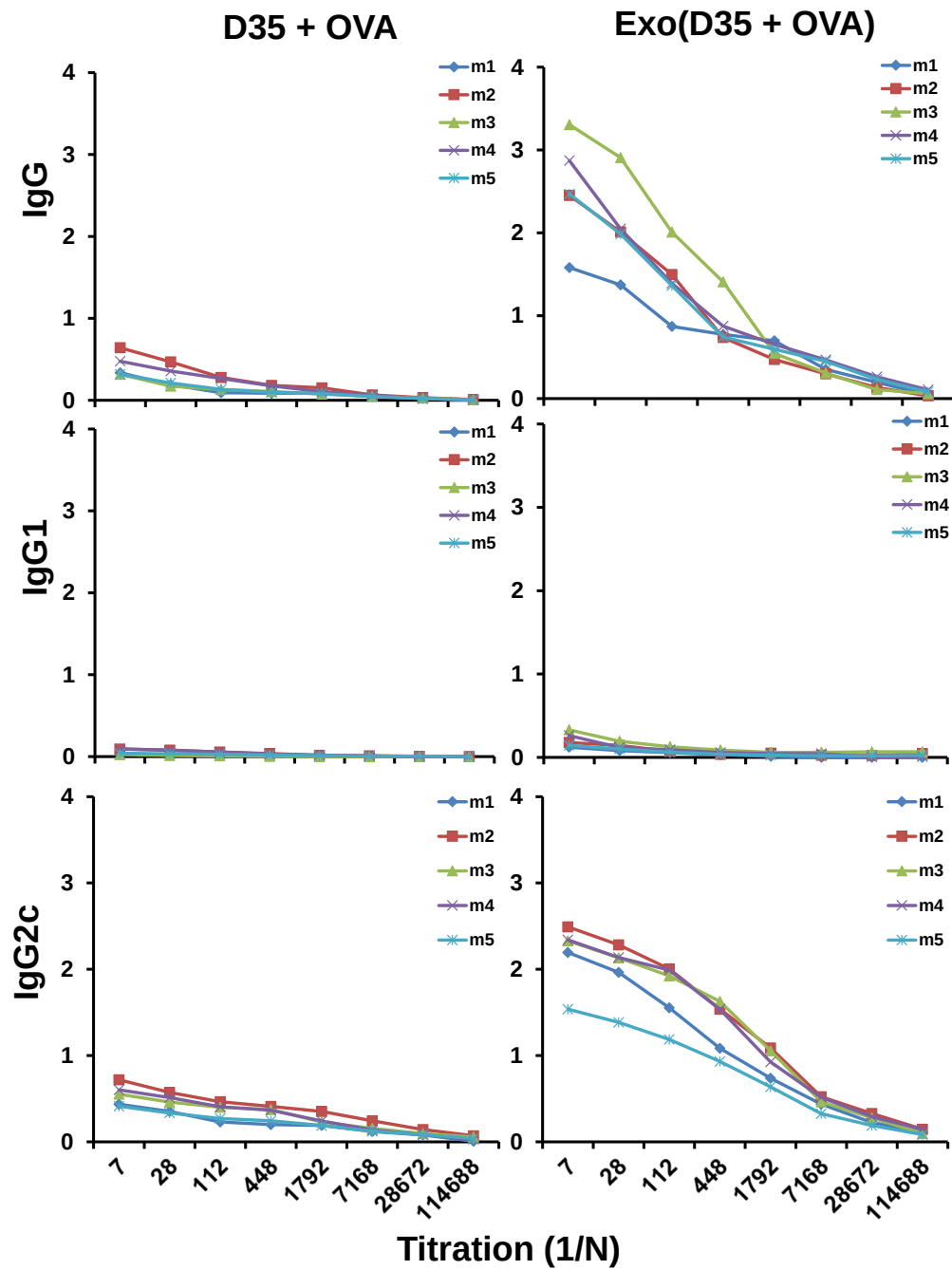


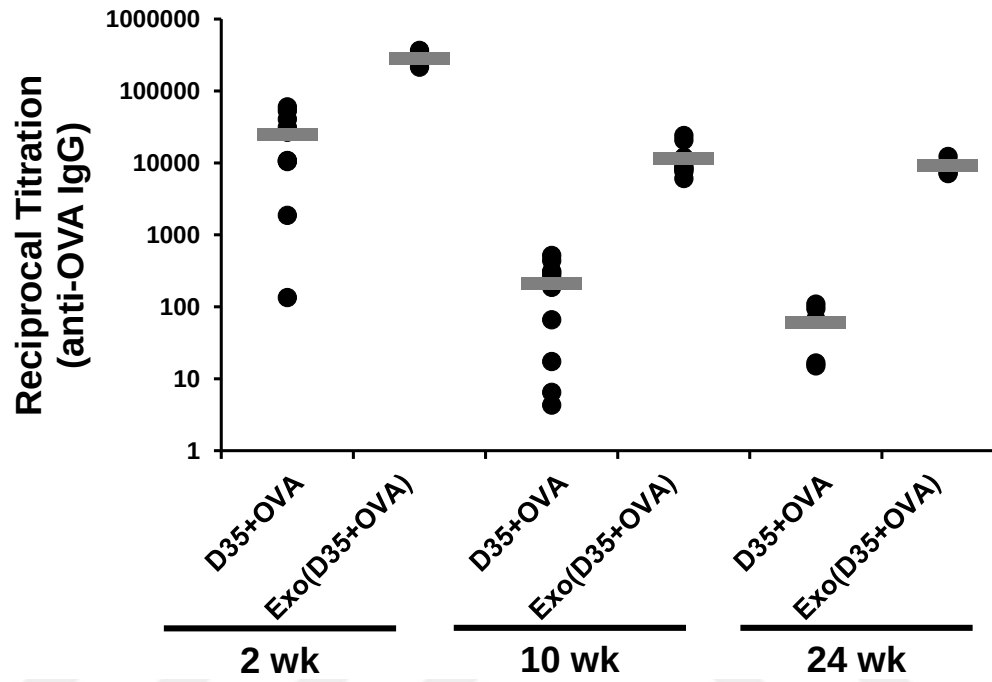
Figure A3. Anti-OVA total IgG, IgG1 and IgG2c responses of each mouse upon immunization with D35 + OVA or Exo(D35 + OVA) at 24 weeks post-booster bleeding.



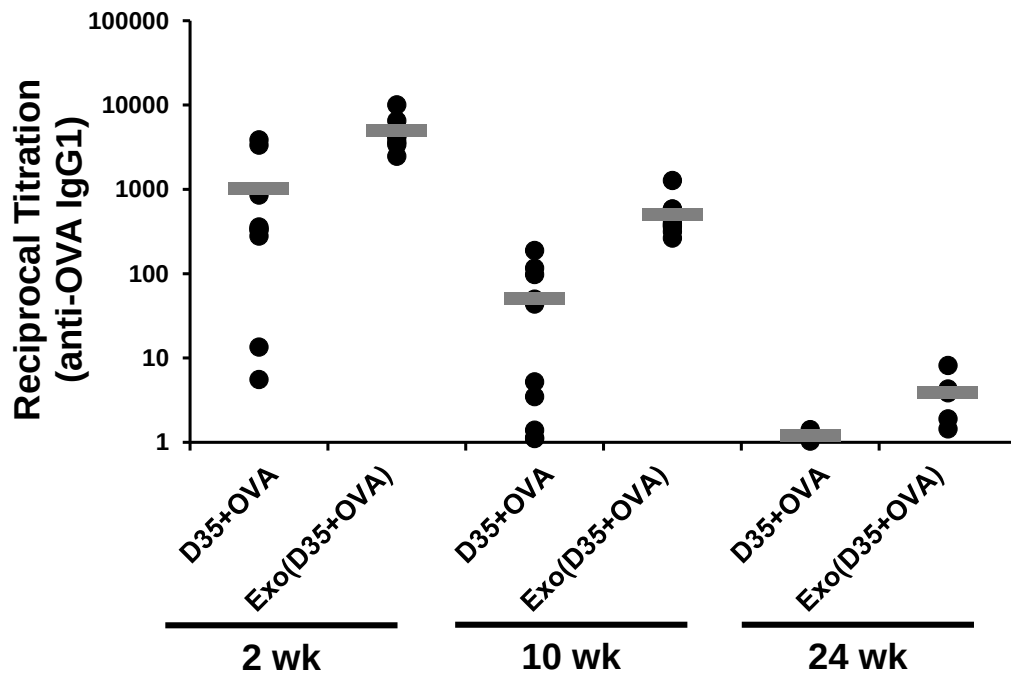
Appendix B

Figure B. Reciprocal titers of (A) total IgG, (B) IgG1 and (C) IgG2c of each mouse at 2-, 10- and 24-weeks post-booster sera

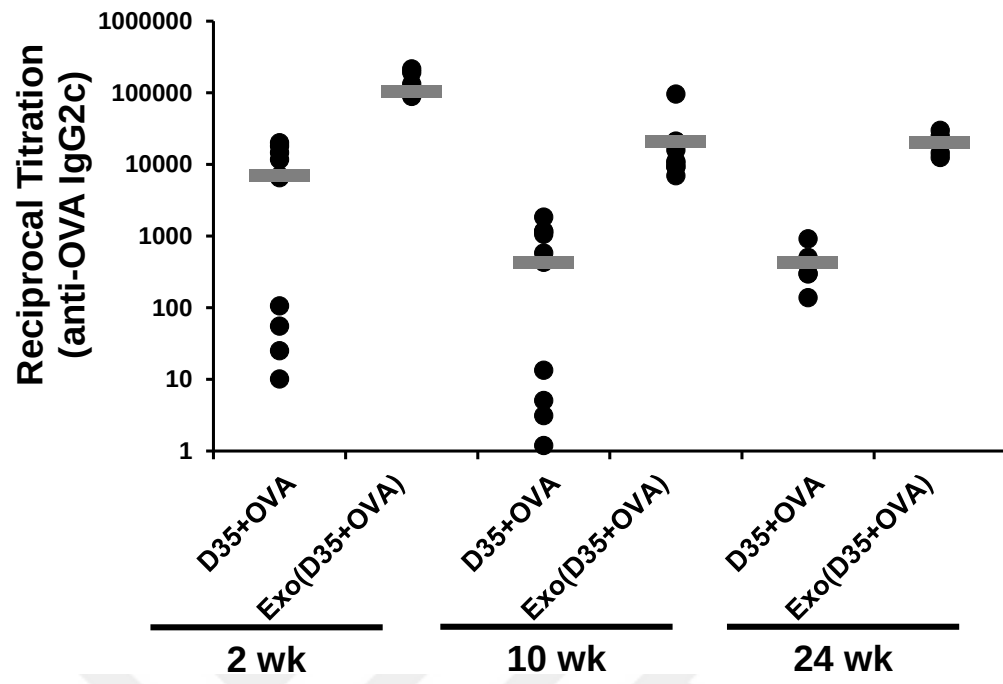
A



B

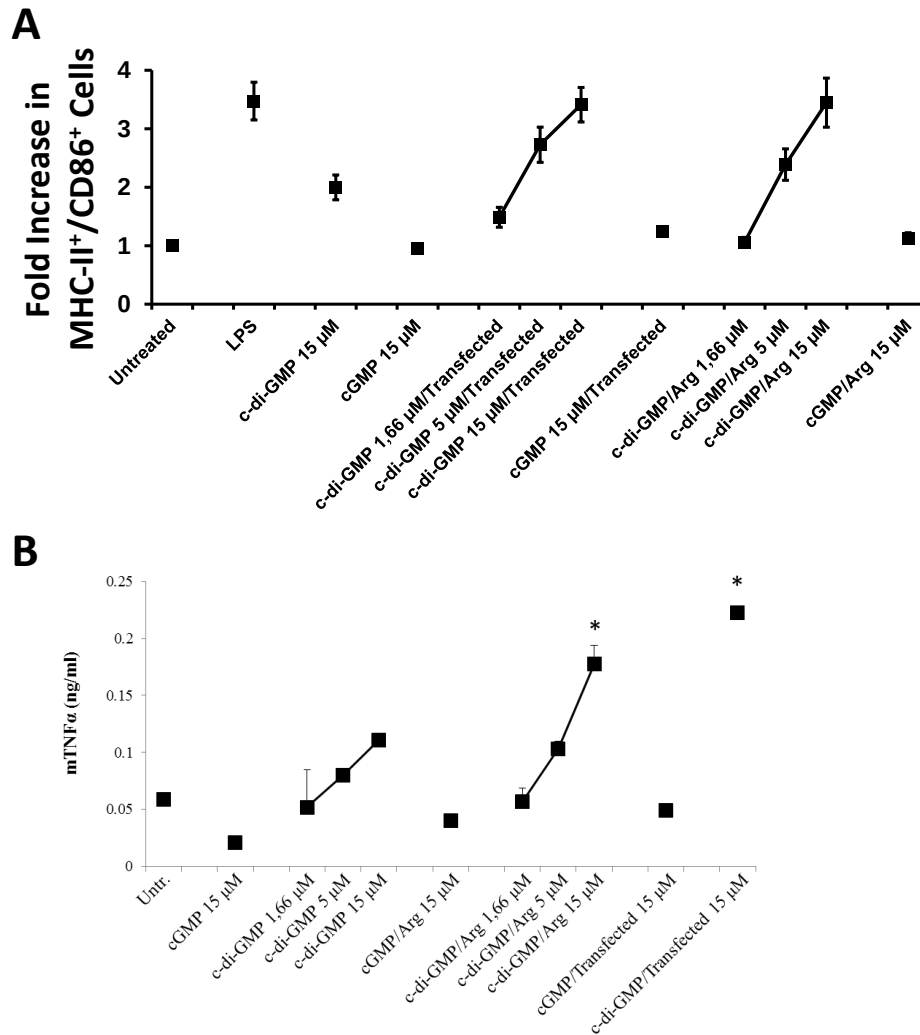


C



Appendix C

Figure C. Immune modulation mediated by Arg(9) mixing. Arg(9)/c-di-GMP is incubated with splenocytes for 24h at indicated concentrations. (A) MHC-II/CD86 positivity of APCs was assessed by flow cytometry. (B) TNF α secretion upon stimulation with mixed and free c-di-GMP was assessed by ELISA.



Curriculum Vitae and Publications

PERSONAL INFORMATION

Name Surname; Tamer KAHRAMAN

Date of Birth; 18.01.1984

Nationality; Turkish and Bulgarian

Languages spoken; Turkish (Mother tongue)
English (Fluent)

Contact info

Address; Bilkent University, Faculty of Science
Department of Molecular Biology and Genetics
06800 Bilkent Ankara
Turkey

Mobile Phone; +90 555 346 2897

Work Phone; +90 312 290 2396

E-mail; tamerk@bilkent.edu.tr
tamer@thorvacs.com

AFFILIATIONS

2014 -... ExoDiag Biotech. Ltd. Şti., CEO

2013 -... Thorvacs Biotech. Ltd. Şti., Head of R&D Department

2007 -... Bilkent University, Department of Molecular Biology and Genetics,
PhD Student

EDUCATION & TRAINING

2015 **METU Teknokent San Francisco Office,** Entrepreneurship & U.S. Business Culture Boot Camp, Jan 26th-Feb 6th 2015, San Francisco, USA

2014 **Tübitak MRE,** Lean Entrepreneurship Training, 12-14 Aralık 2014, Gebze, Turkey

2007-... **Bilkent University,** Department of Molecular Biology and Genetics, Ankara, Turkey (PhD Student)

2002-2007 **Bilkent University,** Department of Molecular Biology and Genetics, Ankara, Turkey (Undergraduate) **Degree obtained; B.S.**

1999-2002 **Çanakkale Fen Lisesi,** Çanakkale, Turkey (High School)

WORK EXPERIENCES

2014 -... **Project Manager,** Tübitak 1512 - 2140157; “Development of next generation prostate cancer diagnostic kit”. Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

2014 -2015 **Research Assistant,** Tübitak SBAG 111S978; “Investigation of Effects of Allergen Specific Immunotherapy on Cytokine Profile, Exosomes and T Lymphocytes”. Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

2011 - 2013 **Research Assistant,** Tübitak SBAG 111S236; “Vaccination and anti-cancer applications of DNA encapsulated exosomes”. Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

- 2008 - 2010** **Research Assistant**, Tübitak SBAG 108S316; “Biomedical applications of DNA nanoparticles”. Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
- 2007 -2106** **PhD Student**; Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
Advisor: Ass. Prof. İhsan Gürsel, “Therapeutic and Diagnostic use of Extracelleular Vesicles”
- 2006 - 2007** **Senior Student**; Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey
Advisor: Assoc. Prof Uygur H. Tazebay, “Effect of iodine on breast and stomach defects”
- 2006** **Summer Trainee**; Department of Medical Biology, Cerrahpaşa Medical School, Istanbul, Turkey
Advisor: Prof. Melek Öztürk, “Direct relationship between MMP1 Gene polymorphism and metastasis ability of small cell lung carcinoma”
- 2005** **Summer Trainee**; Department of Medical Biology, Cerrahpaşa Medical School, Istanbul, Turkey
Advisor: Prof. Melek Öztürk, “Effects of Vitamin D receptor polymorphism on Alzheimer Disease”

PUBLICATIONS

Article Publications

- **T. Kahraman, I. Simsek, F. C. Yagci, C. Ozen, A. Dinc, M. Gursel, S. Gay, I. Gursel.** “Circulating LL37 targets plasma extracellular vesicles to immune cells and intensifies behçet’s disease severity” (Submitted to Journal of Extracellular Vesicles)
- **S. Yildiz, E. Alpdundar, B. Gungor, T. Kahraman, B. Bayyurt, I. Gursel, M. Gursel.** “Enhanced immunostimulatory activity of cyclic dinucleotides on mouse cells when complexed with a cell-penetrating peptide or combined with CpG”. *Eur J Immunol.* 2015 Apr;45(4):1170-9
- **T. Kahraman, G. Gucluler, I. Gursel.** [Exosomes: Natural Nanovesicle Candidates Used in the Diagnosis and Treatment] (Turkish). *Turkish J Immunol.* 2014 Aug; 2(2):34-40.
- **G. Tincer, S. Yerlikaya, F.C. Yagci, T. Kahraman, O.M. Atanur, O. Erbatur, I. Gursel.** “Immunostimulatory activity of polysaccharide-poly(I:C) nanoparticles”. *Biomaterials.* 2011 Jun;32(18):4275-82.
- **I. Gursel, F.C. Yagci, G. Tincer, T. Kahraman, M. Gursel.** [Novel applications of DNA nanoparticles in nanomedicine and nanobiotechnology] (Turkish). *Science and Technology Journal (by TÜBİTAK, Turkey)*, 520:44-48, March 2011.

Selected Oral Presentations

- **T. Kahraman, D. Bayık, F. C. Yagci, M. Gursel, I. Simsek, A. Dinc, I. Gursel.** [Role of LL-37 containing extracellular vesicles on Behcet's Disease Pathogenesis] (Turkish), *23th Turkish Congress of Immunology*, 27-30 April 2015, Antalya, Turkey
- **T. Kahraman, G. Gucluler, B. Bayyurt, I. Gursel.** "Magnified and Persistent Th1-biased Immunity Mediated by Exosome Vaccine" *MIMIC-II*, 27-30 April 2014, Antalya, Turkey
- **G. Gucluler, M. Sahin, T. Kahraman, A. Gursel, I. Gursel.** "Enhanced Immunostimulatory Properties of Extracellular Vesicles Harboring Different TLR Ligands" *MIMIC-II*, 27-30 April 2014, Antalya, Turkey
- **G. Gucluler, T. Kahraman, D. Bayık, B. H. Horuluoglu, A. Gursel, I. Gursel.** "CpG ODN loaded extracellular nanovesicles: enhanced immunotherapeutic activity" *6th ILS International Conference*, 14-17 December 2013, London, UK
- **T. Kahraman, G. Gucluler, M. Gursel, C. Ozen, I. Gursel.** [Transfer of CpG oligos to cells via exosomes] (Turkish) *21th Turkish Congress of Immunology*, April 2011, Turk J Immunol 1:15 Supplement 2011 p80
- **T. Kahraman, I. Simsek, M. Gursel, H. Erdem, A. Dinc, S. Gay, I. Gursel.** "Characterization of Source and Level of Plasma Microparticles from Allergic and Autoimmune patients: relation to disease severity" *Workshop on Frontiers in Nanomedicine and Nanobiotechnology*, Ankara, 13-16 January 2010
- **T. Kahraman, I. Simsek, M. Gursel, A. Dinc, S. Gay, I. Gursel.** "Characterization of source and level of plasma nanoparticles from Behçet's Patients; Relation to Disease Severity" *5th National Nano-science and Nanotechnology Congress*, p122, Eskisehir, Turkey, 8-12 June 2009.

Selected Poster Publications

1. **T. Kahraman, G. Gucluler, I. Gursel.** "Magnified and persistent Th1-biased anti-OVA immunity mediated by co-encapsulated adjuvant and antigen in exosomes" *4th European Congress of Immunology*. 06-09 September 2015, Vienna, Austria
2. **G. Gucluler, T. Kahraman, D. Bayık, M. Gursel, I. Gursel.** "Improved immunostimulatory activity of D-type CpG oligonucleotides encapsulated into exosomes in cancer treatment" *4th European Congress of Immunology*. 06-09 September 2015, Vienna, Austria
3. **I. Gursel, T. Kahraman, I. Simsek, M. Gursel, A. Dinc.** "LL-37 associated extracellular vesicles regulate Behçet's Disease severity" *ISEV 2015 Annual Meeting*. 23-26 April 2015, Bethesda, MD, U.S.A.
4. **D. Bayık, T. Kahraman, G. Gucluler, D.M. Klinman, I. Gursel.** "Targeting and sub-cellular localization of cell-derived microparticles to macrophages." *ISEV 2015 Annual Meeting*. 23-26 April 2015, Bethesda, MD, U.S.A.
5. **F.C. Yagci, M.M. Aydin, T. Kahraman, K.C. Akcali, I. Gursel.** "Inhibitory Effects of Immunosuppressive ODN A151 in CCl₄ Induced Liver Fibrosis" *MIMIC-II*, 27-30 April 2014, Antalya, Turkey
6. **T. Kahraman, G. Gucluler, D. Bayık, A. Gursel, M. Gursel, I. Gursel.** "EXOLIGOS: a CpG oligonucleotide loaded exosome nanovesicles delivery

- platform suitable for effective immune modulation” (Poster Presentation) *ICI2013*, 22-27 August 2013, Milano, Italy
7. **T. Kahraman, Y. C. Yagci, I. Gursel.** “Plasma microparticles and LL-37 levels regulate Behcet’s Disease severity” (Poster Presentation) *ICI2013*, 22-27 August 2013, Milano, Italy
 8. **G. Gucluler, T. Kahraman, D. Bayik, A. Gursel, M. Gursel, I. Gursel.** “D type CpG oligonucleotide loaded EG-7 exosomes act as strong vaccine adjuvant and anti-cancer agent” (Poster Presentation) *ICI2013*, 22-27 August 2013, Milano, Italy
 9. **T. Kahraman, G. Gucluler, B. Bayyurt, M. Gursel, I. Gursel.** [Adjuvant effect of CpG DNA encapsulated exosomes] (Turkish) (Poster Presentation) 22. *National Congress of Immunology*, 27-30 April 2013, Izmir, Turkey
 10. **G. Gucluler, T. Kahraman, D. Bayik, A. Gursel, M. Gursel, I. Gursel.** [Vaccine adjuvant and anti-cancer applications of CpG loaded EG-7 exosomes] (Turkish) (Poster Presentation) 22. *National Congress of Immunology*, 27-30 April 2013, Izmir, Turkey
 11. **G. Gucluler, T. Kahraman, I. Gursel.** “ODN-loaded exosome nanovesicles: A novel anticancer and vaccine delivery platform” (Poster Presentation) *Innate Immunity 2012: from Evolution to Revolution*, 31 October - 4 November 2012, Sorrento, Italy
 12. **T. Kahraman, G. Gucluler, I. Gursel.** “A novel ODN delivery platform: CpG-ODN-loaded exosome nanovesicles” (Poster Presentation) *CIMT2012*, May 2012, Mainz, Germany
 13. **G. Gucluler, T. Kahraman, I. Gursel.** “Immunostimulatory activity of CpG-ODNs is enhanced via encapsulation into nanovesicles” (Poster Presentation) *EMBO-EMBL Symposium: New Perspectives on the Immune Response to Infection*, May 2012, Heidelberg, Germany
 14. **T. Kahraman, M. Erkocoglu, D. Azkur, C. N. Kocabas, I. Gursel** “Contribution of Plasma Microparticles to the Clinical Manifestation of Allergic Diseases” (Poster Presentation) *MIMIC2012*, April 2012, Antalya
 15. **T. Kahraman, B. Bayyurt, V. Ibrahimova, D. Tuncel, I. Gursel.** “CpG Loaded Fluorescent Polymeric Nanoparticles: a theranostic drug delivery system suitable for TLR based therapies” (Poster Presentation) *MIMIC2012*, April 2012, Antalya
 16. **G. Gucluler, T. Kahraman, I. Gursel.** “CpG ODN Loaded Exosome Nanovesicles: Enhanced Immunostimulatory Activity” (Poster Presentation) *MIMIC2012*, April 2012, Antalya. **Best Poster Award**
 17. **T. Kahraman, G. Gucluler, Esin Alpdundar, Mayda Gursel, I. Gursel.** “Encapsulating CpG-ODNs into nanovesicles enhances their immunostimulatory action” (Poster Presentation) *BIOMED 2011*, November 2011, Ankara
 18. **T. Kahraman, D. Azkur, C. Ozcan, C. N. Kocabas, M. Gursel, I. Gursel.** “Relationship between Level of Plasma Microparticles and the Clinical Manifestation of Allergic Diseases” (Poster Presentation) *EAACI Congress 2011*, June 2011, Istanbul.
 19. **T. Kahraman, I. Simsek, M. Gursel, H. Erdem, S. Pay, A. Dinc, S. Gay, C. Özcan, C. N. Kocabas, I. Gursel.** “Microparticles; immune regulatory roles in autoimmune and allergic diseases” (Poster Presentation). *14th International Congress of Immunology*, Abstract book 4 p106, Kobe, Japan, 22-27 August 2010.

20. **T. Kahraman, I. Simsek, M. Gursel, H. Erdem, S. Pay, A. Dinc, S. Gay, I. Gursel.** “Contribution of source and level of plasma microparticles to the severity of Behçet’s and other inflammatory rheumatoid diseases” (Poster presentation). *World Immune Regulation Meeting-III*, p42, Davos, Switzerland, March 2009.
21. **I. Simsek, T. Kahraman, M. Gursel, I. Gursel, H. Erdem, S. Pay, A. Dinc, S. Gay.** “*The relation between clinical findings and severity of Behçet Syndrome and the origin and level of plasma microparticles*” (Oral presentation). 9th National Congress of Rheumatology, p125, September 2008. Best Research Prize

SCHOLARSHIPS and AWARDS

- Turkish Immunology Society, Bursary (300 Euro) to attend European Congress of Immunology 2015, Vienna, Austria
- Turkish Immunology Society, Oral Presentation Bursary (600 Euro) to attend Turkish Congress of Immunology 2015, Antalya Turkey
- YFYİ 2014 (New Ideas New Jobs 2014), Teknokent İstanbul USA Boot Camp Special Award, 8th November 2014, Ankara, Turkey
- TUBITAK BIGG 1512 Entrepreneurship Multi-Phase Program Grant (99180 TL), Project #; 2140157
- EFIS Bursary to attend 2nd International Molecular Immunology & Immunogenetics Congress, 27-30 April 2014, Antalya, Turkey
- The Best Poster Award, MIMIC-II, 27-30 April 2014, Antalya, Turkey
- The Best Podium Presentation Award, 6th ILS International Conference, 14-17 December 2013, London, UK
- Scholarship for attending ICI2013, Turkish Society of Immunology (500 Euro)
- EFIS ICI2013 Milano Travel Award (600 Euro)
- The Best Poster Award, MIMIC2012, April 2012 Antalya
- Turkish Immunology Society, Full Scholarship to attend MIMIC2012, Antalya Turkey
- Turkish Immunology Society, Full Scholarship to attend 21th Turkish Congress of Immunology 2011
- EFIS ICI2010 Kobe Travel Award (1000 Euro)
- Best Research Award, 9th National Congress of Rheumatology, Sept, 2008, Antalya, Turkey
- Bilkent University, Department of Molecular Biology and Genetics, PhD Program Full Scholarship
- Bilkent University, Department of Molecular Biology and Genetics, Undergraduate Program Full Scholarship (entered by National Entrance Exam; 69th out of 1.5M people)

SOCIETY MEMBERSHIPS

- Turkish Society of Immunology
- The American Association of Immunologists (2010-2012)

CIRCULATING LL37 TARGETS PLASMA EXTRACELLULAR VESICLES TO IMMUNE CELLS AND INTENSIFIES BEHÇET'S DISEASE SEVERITY

Tamer Kahraman¹, Ismail Simsek², Fuat Cem Yagci¹, Can Ozen³, Ayhan Dinc², Mayda Gursel⁴, Stephen Gay⁵, Ihsan Gursel¹

¹ Bilkent University, Science Faculty, Department of Molecular Biology and Genetics, Ankara, Turkey

² Gulhane School of Medicine, Division of Rheumatology, Ankara, Turkey

³ Department of Biotechnology, Middle East Technical University, Ankara, Turkey

⁴ Department of Biological Sciences, Middle East Technical University, Ankara, Turkey

⁵ Department of Rheumatology, University Hospital Zurich,

Corresponding Author:

Prof. Dr. Ihsan Gursel

Therapeutic Oligonucleotide Research Lab, Department of Molecular Biology and Genetics, Science Faculty, Bilkent University, 06800 Ankara, Turkey.

Web address; www.thorlab.bilkent.edu.tr

Tel; +90-312-290 24 08

Fax; +90-312-266 50 97

E-mail; ihsangursel@bilkent.edu.tr

ABSTRACT

Behçet's Disease (BD) activity is manifested with sustained, over exuberant immune activation, yet the underlying mechanisms leading to active BD state is poorly defined. Herein, we show that the human cathelicidin derived antimicrobial peptide LL-37 associates with and directs plasma extracellular vesicles (EV) to immune cells, thereby leading to enhanced immune activation aggravating BD pathology. Notably, disease activity correlated with elevated levels of circulating LL-37 and EV plasma concentration. Stimulation of healthy PBMC with active BD patient EVs induced heightened IL1 β , IFN α , IL6 and IP10 secretion compared to healthy and inactive BD EVs. Remarkably, when mixed with LL-37, healthy plasma-EVs triggered a robust immune activation replicating the pathology inducing properties of BD EVs. Findings of this study could be of clinical interest in the management of BD, implicating LL-37/EV association as one of the major contributors of BD pathogenesis.

Keywords:

Extracellular Vesicles, Behçet's Disease, Autoimmune, Immune activation, LL-37, Cytokine

Abbreviations:

BD, Behçet's Disease; EV, Extracellular Vesicle; BB, Binding Buffer; AnV, Annexin V; autologEV, autologous extracellular vesicles; alloEV, allogeneic extracellular vesicles

INTRODUCTION

Behçet's Disease (BD) is a complex multi-organ chronic inflammatory condition of unknown etiology wherein the genetic background and environmental factors are thought to be important contributors for disease pathogenesis (1-5). Recent findings suggest that a predominance of Th1/Th17-type immune polarizations and elevated levels of associated cytokines correlate with disease activity (6-9). In this context, plasmapheresis has been shown to induce rapid short-term remission suggesting that an unidentified plasma-associated factor could be a trigger of flare-ups (10). Herein, we hypothesized that one such plasma-related factor contributing to disease activity and severity could be the circulating extracellular vesicles (EV).

EVs are small vesicles (diameter of 30nm to 1µm) constitutively released from various cell types and are considered as key players mediating cell-to-cell communication (11-13). Accumulating evidence suggests that plasma EV levels are altered during disease states (12). For instance, the number of EVs released from cultured immune cells increases dramatically upon stimulation with TNF α or IL1 β . Moreover, several apoptosis inducers contribute EV secretion (13-17). In addition, stimulation of RAW 264.7 cells by TLR ligands intensify EV release (18). EVs have several roles in immune modulation along with stimulation of coagulation, and modulation of vascular functions (19-25). In general, their cargo is loaded with an array of bioactive molecules capable of regulating target cell behavior (23-27). Recent studies revealed that numbers of circulating EVs were elevated in several rheumatologic diseases, such as rheumatoid arthritis, systemic lupus erythematosus, primary Sjögren's syndrome, vasculitis and anti-phospholipid syndrome (20-31). The implications of elevated EV levels were not fully understood.

Since inflammation, endothelial dysfunction, and thrombotic tendency are collectively regarded as cardinal features of BD, we speculated that the levels and source of EVs along with their cargo might influence the course of BD in terms of disease activity and severity.

In this study, we report that circulating EV numbers correlate with BD severity. Furthermore, we show that the human cathelicidin group of anti-microbial peptide LL-37 associates with EVs, enhancing their internalization by immune cells, initiating a robust pro-inflammatory cytokine milieu.

MATERIALS AND METHODS

Patients and controls.

Consecutive patients, who were regularly examined at the Department of Rheumatology had an established diagnosis of BD (according to the criteria of the International Study Group for Behçet's Disease (32)) were referred for the study after they had their last clinical evaluation to determine clinical disease status (See Table I). For this study, plasma of 72 BD patients (Active; N=35, Inactive; N=37) and 22 healthy control individuals were included. Patients having active disease was defined by the presence of two and more clinical characteristics such as; oral ulcers, genital ulcers, papulopustular or pseudo-follicular cutaneous lesions, active vascular disease and active ocular involvement. The patients who had no symptoms related with BD at the time of sampling and receiving steady medication for at least 1 month were classified as having an inactive disease. All patients were further categorized according to their organ of involvement as i) mucocutaneous, ii) ocular, and iii) vascular. With regard to the classification of those patients with inactive disease, patients' detailed clinical history was investigated with respect to either deep vein thrombosis or arterial aneurysm and evidence for uveitis was sought. Subjects with hypertension, diabetes mellitus, renal disease or a previous history of coronary artery disease or myocardial infarction were excluded. The healthy control group included 22 individuals, matched for age and sex. Baseline characteristics of patients with BD and healthy subjects at inclusion are summarized in Table I. The study was approved by the Institutional Review Board, and all participants provided their informed consents.

Collection of platelet poor plasma from PBMCs.

Fresh blood samples were centrifuged at 400g for 10 min; plasma was separated and further centrifuged at 1500g for 10 min to obtain platelet poor plasma (PPP). The PPP was collected and divided into 1.5 ml aliquots, snap-frozen in liquid nitrogen and were kept at -80°C.

Isolation of EVs by differential centrifugation.

EV isolation from PPP was based on differential centrifugation method (33). All reagents used throughout the purification were filtered using 0.2 µm filters and then centrifuged at 100,000g for two hours. Briefly, PPPs were thawed centrifuged at 10,000g for 10 min to get rid of cell debris. 1ml of the supernatant were 35x diluted by PBS-citrate buffer (10mM tri-sodium citrate in Ca²⁺/Mg²⁺ free PBS, pH=7.4) and were centrifuged at 100,000g for 1h (XL90 Beckman ultracentrifuge with a SW28 rotor with 6 x 38.5 ml polyallomer tubes, Beckman Instruments, Fullerton, CA). The supernatant was discarded, and the pellet was washed once with PBS-citrate buffer. The pellet (containing EV fraction) was then dissolved in 150 µl of 1X Binding Buffer (BB) (10X BB was composed of 0.1M HEPES, 1.4M NaCl and 25mM CaCl₂, pH=7.4) and analyzed by flow cytometer.

FACS analyses of labeled EVs.

For the identification and quantification of EVs, samples were double-stained with Annexin V (AnV-PE or FITC conjugated, Biolegend) and an antibody against one of the following cell surface markers: PE-CD42a (platelet, BD Pharmingen), FITC-CD31 (endothelial cells, Biolegend), PE-CD69 (early activated cells, Biolegend) PE-CD105 (epithelial/endothelial cells, Biolegend), PE-CD3 (T-cells, BD Pharmingen) and PE-CD14 (monocytes, BD Pharmingen). 25 µl EVs aliquots were incubated with staining cocktail containing of 5 µl AnV (as instructed in the manufacturers protocol) and 1 µg/ml of one of the indicated cell

surface markers) for 30 min at RT in dark. Following staining, EVs were resuspended in 500 µl of 1X Binding Buffer, particles were counted on a FACS Calibur flow cytometer using a 60 sec acquisition time at a “medium-flow” setting and data evaluation was performed using the CellQuest Pro Software. Of note, in order to gate-out air bubbles, electrical noise and contaminating particles, a very stringent FSC-SSC gating strategy was used as indicated by the "R2 EV-associated particle gate" (Supplementary Fig. 1). In addition, to gate out particles > 1µm, latex beads (Sigma LB-11) with 1.1 µm diameter were used to define our EV associated gate. The optimized acquisition settings were controlled weekly. In some experiments, data acquisition was performed on an Accuri C6 Flow cytometer with similar results to those obtained using the FACS Calibur.

Calculation of EV number in one ml plasma

Our initial optimization studies were performed with standard calibration beads of known concentration (1×10^7 beads/ml, Sigma LB-11, 1.1 µm in diameter). We acquired the # of events (@ 1 min) and determined the volume utilized for that run. After verification, we acquired AnV positive EVs and multiplied with the dilution factor to determine the # of EVs/ml plasma.

EV internalization by PBMCs

Purified EVs were stained with the lipophilic cell tracer CM-Dil dye (1 µM) (Invitrogen), washed 2 times in 35 ml PBS and centrifuged at 100.000g for 1 h. Following re-suspension in 200 µl of endotoxin-free PBS, as such prepared labelled particles were incubated with 2×10^6 /ml PBMCs at the indicated ratios for various time points. Internalization was deduced from the CM-Dil⁺ cell numbers by flow cytometer. For confocal microscopy studies, cells were incubated on cover slips with labeled EVs for 8h, washed, fixed and studied using a Zeiss LSM5 confocal microscope.

Human ELISA studies

EVs from active BD, inactive BD and healthy subjects were incubated with healthy PBMCs and autologous PBMCs (2×10^6 /ml) for 12h at a ratio of 1:2 (Cell:EV) in 96-well flat bottom culture plates. LPS (5 µg/ml) and PGN (5 µg/ml) were used as positive controls throughout these assays. Culture supernatants were collected and IL6 production was determined by ELISA (34). The concentration of LL-37 from plasma and EV samples was measured by a human LL-37 ELISA kit (HyCult Biotech), according to manufacturer's protocol.

Intracellular cytokine staining

Healthy EVs with or without LL-37 were used to stimulate healthy PBMCs for determining IP10, IL1β and IFNα positive cells. Healthy PBMCs were stimulated with EV or EV/LL-37 complex for 6h and 16h in the presence of Brefeldin-A (5 µg/ml, Biolegend, Cat#420601) for IL1β and IP10 analysis, respectively. Of note, Brefeldin-A was added at t=0 and t= 6th hour of incubation for IL1β and for IP10, respectively. The cells were then fixed with Fix & Perm Medium A (Invitrogen, Cat# GAS001S100). Cells were first stained with CD14-PE (Biolegend, Cat#325606) and then to detect the level of intracellular IL1β or IP10 were stained with IL1β-FITC (e-Biosciences, Cat# 12-7018-81) or IP10-Biotin (BD, Cat# 555048) then SA-FITC (Biolegend, Cat# 405202) in the presence of saponin (0.1%).

IFN α secreting cells were analyzed with Miltenyi Biotech's IFN α Secretion Assay and Detection Kit (PE-labelled, Miltenyi, Cat# 130-094-161) from PBMCs in accordance with kit instructions. Additionally, in order to identify IFN α secreting pDCs, cells were stained with BDCA-2-FITC (Biolegend, Cat# 354208) and then analyzed by BD Accuri C6 flow cytometer.

Statistical analysis

All statistical data analyses were carried out using the Sigma Stat 3.5 (Systat Software Inc.). In order to assess significance between active BD, inactive BD and healthy groups, one way ANOVA test was used, while student's t-test was applied to analyze significance between sub-groups of active BD vs. inactive BD. p values ≤ 0.05 was set to be statistically significant.



RESULTS

BD patients have elevated circulating EV numbers

To assess whether plasma EV concentrations correlated with BD severity, EV numbers in plasma of 72 BD patients (Active; N=35, Inactive; N=37) and 22 healthy controls were determined by flow cytometry.

Results presented in Figure 1 and Supplementary Table I show that total plasma EV concentrations (number of EV/ml) were significantly higher in active BD ($3.9 \pm 0.77 \times 10^5$) and inactive BD ($2.05 \pm 0.46 \times 10^5$) compared to healthy plasma EVs ($0.78 \pm 0.25 \times 10^5$, $p < 0.001$, active vs. healthy, and inactive vs. healthy). Total EV numbers in active BD were consistently higher than the inactive BD group ($P < 0.01$) and this increase (1.75-2.2 fold) was valid in all patient sub-groups suggesting that elevated EV levels was a common feature of active disease and was not dependent on organ involvement (Figure 1 and Supplementary Table I, $P < 0.01$ for all tested sub-groups).

Platelet derived EVs constitute the majority of circulating EVs in BD plasma

EVs are believed to be secreted from multiple cell types, including platelets, endothelial cells, monocytes, and T cells. However, contribution of each population to the circulating EV pool can vary depending on disease activity. To elucidate whether source of EVs varied with respect to BD severity, isolated particles from healthy (N=22), inactive (N=35) and active BD patients (N=37) were stained with different cell specific markers and their relative ratios were confirmed by flow cytometry. The gating strategy for Flow cytometric analysis and representative dot-plots of individual plasma EV stainings are detailed in Supplementary Fig. 1 and Supplementary Fig. 2. Flow cytometric analysis stained EV populations revealed that platelets were the most common source of EV population in all groups (CD42a⁺, Figure 2). Platelet-derived EV percentages correlated with disease state and were found to be significantly elevated in BD patients ($52.6 \pm 4.5\%$ and $65.4 \pm 3.8\%$, inactive and active BD, respectively) when compared to healthy controls ($41.5 \pm 4.3\%$, $P < 0.001$ for all comparisons). Similarly, significant increases were found for EVs derived from T cells (Figure 2A, 1.49 ± 0.36 , 4.14 ± 0.77 and $5.25 \pm 1.24\%$ for healthy, inactive and active BD, respectively; $p < 0.001$) and monocytes (Figure 2A, 1.39 ± 0.32 , 3.27 ± 0.42 and $5.7 \pm 0.56\%$ for healthy, inactive and active BD, respectively; $p < 0.001$). In contrast, endothelial and/or epithelial derived EVs in BD plasma, (i.e. CD31⁺ and CD105⁺) were lower than those observed in healthy controls ($p < 0.001$ for all comparisons).

Next, relative numbers of cell-type specific EVs in 1ml plasma of inactive and active BD patients as opposed to healthy controls were also determined (Figure 2B, Supplementary Fig. 3). Number of platelet-derived EVs were 3.5 and 7-fold higher in BD plasma (inactive and active, respectively), while monocyte and T-cell derived EVs were 11-7 and 21-22 (inactive and active, respectively) fold more when compared to healthy EVs ($p < 0.001$). Collectively, these findings suggest that the significant increases found for extracellular vesicles derived from platelets, T cells and monocytes in BD correlate with the activation state of these cells and may contribute to pathogenesis.

EVs are internalized by PBMCs triggering IL-6 secretion

The underlying reasons for the heightened and sustained immune activation in BD patients remain elusive. We established that circulating EV concentrations are elevated especially in

active BD. We next attempted to explore the role of heightened EV concentration as a culprit in sustained immune activation in BD patients. To test this possibility, initial experiments focused on BD EV internalization studies using fluorescently labelled extracellular vesicles. For this, lipophilic CM-DiI dye labeled EVs were incubated with healthy PBMCs at various cell:EV ratios and their internalization patterns were analyzed using confocal microscopy and flow cytometry (Figure 3A-B). Photomicrographs revealed that BD EVs were effectively internalized by the immune cells (Figure 3A) and up to ~40% of all PBMCs took up EVs within 8h (Figure 3B).

To further explore whether active BD EVs were capable of inducing an immune response, we incubated EVs isolated from healthy, inactive or active BD with healthy PBMCs for 12 h and determined EV-induced cytokine secretion from culture supernatants. Results revealed that autologous and allogeneic healthy plasma-derived EVs triggered lower but detectable levels of IL-6 production from healthy PBMC (Figure 4, 49.9 ± 19.0 and 116.1 ± 35.4 , ng/ml, respectively). In contrast, inactive and active BD patients triggered 2- and 3.3-fold more IL-6 (Figure 3, 235.1 ± 38.0 and 384.9 ± 51.3 , ng/ml, respectively) when compared to allogeneic healthy EVs ($p < 0.001$ for all comparisons). These findings demonstrate that EVs can interact with and are internalized by human PBMCs and this interaction leads to a more pronounced IL-6 production when EVs were of BD origin.

Active BD patient plasma LL-37 levels are elevated and majority of LL-37 is associated with EVs

After establishing that circulating EVs are elevated in active BD plasma and are mediating exaggerated cytokine secretion from PBMCs, we embarked on identifying the factor(s) that might contribute to BD EV-associated immune activation. For this, we analyzed the plasma levels of LL-37, an anti-microbial peptide associated with the pathogenesis of several autoimmune/inflammatory diseases, including psoriasis, systemic lupus erythematosus, rheumatoid arthritis and atherosclerosis (35). LL-37 concentrations were found to be 39 ± 8 , 80 ± 11 and 207 ± 16 ng/ml in healthy, inactive and active BD plasma samples, respectively (Figure 5A), representing a 2- (inactive) and 5- (active) fold increase.

Considering that LL-37 is a cationic peptide and EVs are enriched in negatively charged phospholipids (36), we next analyzed the possibility of EV/LL-37 interaction and determined the levels of LL-37 in EV-depleted plasma and in the EV fraction. Strikingly, ~2/3 of LL-37 was associated with EVs in active BD (Figure 5B).

LL-37 was previously shown to interact with and enhance delivery of nucleic acids to immune cells (37). To assess whether a similar mechanism could operate for EVs, SP-DiOC labelled healthy EVs were either used as such or mixed with LL-37 peptide (endotoxin free) prior to incubation with healthy donor PBMCs. LL-37-free and LL-37 associated-EV internalization patterns in peripheral blood dendritic cell (DC), macrophage and T-cell populations were assessed with flow cytometer 1 h after co-incubation. Results showed that EV delivery to macrophages, DCs and to a lesser extent to T-cells were significantly enhanced in the presence of LL-37 (Figure 6A). Furthermore, internalization enhancing effect of LL-37 was more prominent in PBMCs when incubation period was extended to 8 h (Fig 6B, and Supplementary Fig. 4).

Next, to establish whether this enhanced LL-37-mediated cellular internalization had an impact on subsequent cytokine induction, healthy EVs were mixed with increasing concentrations of LL-37 or its scrambled-control prior to co-culture with healthy PBMCs. As

seen in Figure 7, LL-37/EV combination induced a significantly higher IL6 secretion from PBMCs in a dose dependent manner ($p < 0.01$). This induction was specific to LL-37 since the scrambled peptide (SCR)/EV combination did not yield appreciable amounts of IL-6 (Fig. 7A). Although free LL-37 also triggered IL6 release at these doses, EV/LL-37 combination triggered 2.3-2.2 fold more IL6 than the free peptide at both doses (150 and 450 ng, respectively, Fig. 7A).

Taken together, these findings demonstrate that, i) LL-37 levels, ii) EVs concentration, iii) pathological cytokine production are well correlated with disease severity (Fig. 7B, R^2 : 0.9275, and Supplementary Figs. 5A & B).

Active BD EVs triggers IFN α from pDC, IL1 β and IP10 from monocytes/ macrophages

Our findings implicated that LL-37 associated EVs are capable of inducing elevated immune activation. To identify the cell types activated by LL-37/EV complexes, healthy donor EVs were complexed with LL-37, (mimicking active BD EVs) and subsequently incubated with autologous healthy PBMCs. As expected, healthy EVs devoid of LL-37 failed to induce any detectable IFN α or IL1 β (Figure 8). However, LL-37/EV complexes induced >2 fold more IL1 β , and >20 fold more IFN α production from monocytes and plasmacytoid dendritic cells, respectively. Similarly, IP10 production from monocytes were elevated only in response to LL-37-EV (Supplementary Fig. 6, > 2 fold more IP10 was secreted).

Collectively, our findings established that active BD patient plasmas contain elevated numbers of circulating EVs, and majority of these EVs are associated with LL-37. LL-37 drives EVs specifically and efficiently to immune cells, inducing a more robust and sustained immune activation thereby exacerbating Behçet's disease severity.

DISCUSSION

EVs have recently been implicated as novel extracellular mediators of distant intercellular communication (11). Since EVs circulate and remain intact in peripheral blood, they are capable of transferring labile cargo to distant targets, thereby altering the physiology of the recipient cells. This study is the first to demonstrate that EVs are elevated in the peripheral blood of BD patients, suggesting their potential involvement in disease pathogenesis.

Several studies demonstrated that EVs not only possess bioactive materials on their surface but also harbour biologically active cargo within their lumen. These include nucleic acids (both RNA and DNA) or proteins such as, cytokines, growth factors and cell or tissue specific antigens. Recently, miRNAs were also reported to be delivered via these structures (38-40). EVs are considered to have important functions in establishment of homeostasis in health and could play potentially detrimental roles during disease states (41, 42). Data presented herein are in support of these observations and extend our current knowledge of EV-disease associations.

Our findings could be of clinical interest for several reasons. First, plasma EV numbers were found to be elevated in BD patients, which correlated with disease severity (higher EV numbers in active BD patients). It is conceivable that a plasma EV number-based stratification of BD could more precisely identify inactive and active disease states and could aid in pharmacological management of this disease.

Second, although LL-37 is a cationic anti-microbial peptide generally known to be secreted during pathogenic insult, (43-45) the data presented here demonstrate that plasma LL-37 levels are elevated in BD. As in the case of EVs, this information could be of value to follow patient responses to treatment. Of interest, the only published data that investigated a role of LL-37 in BD revealed elevated LL-37 levels in saliva of BD patients which correlated with the number of monthly oral ulcers (46). Consistent with this observation, our results also revealed a correlation between LL-37 levels and disease severity. Of note, LL-37 levels were reported to be higher during sterile inflammation (47). In addition, this cationic peptide was demonstrated to contribute to IL-1 β , IL-6, MCP-1 and MCP-3 production (48). In this context, our results also established a link between elevated LL-37 levels and enhanced cytokine production.

Third, to our knowledge this paper is the first to show that LL-37 can interact with EVs, enhancing their cellular delivery and immune stimulatory activity. As stated before, EVs are known carriers of self-nucleic acids. It is conceivable that LL-37-mediated enhanced EV delivery to immune cells could activate several endosomal and/or cytosolic RNA and DNA-dependent nucleic acid sensors, including TLR7, TLR9, RIG-I, MDA-5, cGAS or AIM-2, leading to production of BD-associated cytokines (i.e. IL-6, IL-1 β and IFN α), contributing to disease severity. Supporting this view, it was shown that high levels of LL-37 present in psoriatic skin formed complexes with genomic DNA liberated from dying cells, and enhanced DNA delivery to pDCs, triggering a TLR9-mediated interferon alpha response in psoriatic patients (49).

The anionic charge of EVs provides a surface convenient for LL-37 complexation. Our findings implicate that LL-37-mediated enhanced delivery of circulating EVs to immune cells contribute to sustain and exacerbate immune activation in active BD. Therefore, targeting this peptide could be of therapeutic value in the management of this complex disease.

ACKNOWLEDGEMENTS

Gizem Tincer and Deniz C. Yildirim are sincerely acknowledged for their critical reading of the manuscript and feedbacks during the study.

DISCLOSURE STATEMENT

There is no competing interest.



REFERENCES

1. Comarmond, C., B. Wechsler, B. Bodaghi, P. Cacoub, and D. Saadoun. Biotherapies in Behçet's disease. *Autoimmun Rev.* 2014; 13: 762-769.
2. Hatemi, G., P. A. Merkel, V. Hamuryudan, M. Boers, H. Direskeneli, S. Z. Aydin, et al. Outcome measures used in clinical trials for Behçet syndrome: a systematic review. *J Rheumatol.* 2014; 41: 599-612.
3. Direskeneli, H. Autoimmunity vs autoinflammation in Behçet's disease: do we oversimplify a complex disorder? *Rheumatology.* 2006; 45: 1461-1465.
4. Emmi, G., E. Silvestri, D. Squatrito, M. M. D'Elia, L. Ciucciarelli, D. Prisco, et al. Behçet's syndrome pathophysiology and potential therapeutic targets. *Intern Emerg Med.* 2014; 9: 257-265.
5. Yazici, Y., S. Yurdakul, and H. Yazici. Behçet's syndrome. *Curr Rheumatol Rep.* 2010; 12: 429-435.
6. Hamzaoui, K., A. Hamzaoui, F. Guemira, M. Bessiod, M. Hamza, and K. Ayed. Cytokine profile in Behçet's disease patients. Relationship with disease activity. *Scand J Rheumatol.* 2002; 31: 205-210.
7. Hatemi, G., Y. Yazici, and H. Yazici. Behçet's syndrome. *Rheum Dis Clin North Am.* 2013; 39: 245-261.
8. Melikoglu, M., S. Uysal, J. G. Krueger, G. Kaplan, F. Gogus, H. Yazici, et al. Characterization of the divergent wound-healing responses occurring in the pathergy reaction and normal healthy volunteers. *J Immunol.* 2006; 177: 6415-6421.
9. Raziuddin, S., A. al-Dalaan, S. Bahabri, A. K. Siraj, and S. al-Sedairy. Divergent cytokine production profile in Behçet's disease. Altered Th1/Th2 cell cytokine pattern. *J Rheumatol.* 1998; 25: 329-333.
10. Raizman, M. B., and C. S. Foster. Plasma exchange in the therapy of Behçet's disease. *Graefes Arch Clin Exp Ophthalmol.* 1989; 27: 360-363.
11. Distler, J. H., L. C. Huber, S. Gay, O. Distler, and D. S. Pisetsky. Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity.* 2006; 39: 683-690.
12. Thery, C., M. Ostrowski, and E. Segura. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009; 9: 581-593.
13. Distler, J. H., A. Jungel, L. C. Huber, C. A. Seemayer, C. F. Reich, 3rd, R. E. Gay, et al. The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. *Proc Natl Acad Sci U S A.* 2005; 102: 2892-2897.
14. Barry, O. P., D. Pratico, R. C. Savani, and G. A. FitzGerald. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest.* 1998; 102: 136-144.
15. Duval, A., D. Helley, L. Capron, P. Youinou, Y. Renaudineau, S. Dubucquoi, et al. Endothelial dysfunction in systemic lupus patients with low disease activity: evaluation by quantification and characterization of circulating endothelial microparticles, role of anti-endothelial cell antibodies. *Rheumatology (Oxford).* 2010; 49: 1049-1055.
16. Ullal, A. J., and D. S. Pisetsky. The release of microparticles by Jurkat leukemia T cells treated with staurosporine and related kinase inhibitors to induce apoptosis. *Apoptosis.* 2010; 15: 586-596.
17. VanWijk, M. J., E. VanBavel, A. Sturk, and R. Nieuwland. Microparticles in cardiovascular diseases. *Cardiovasc Res.* 2003; 59: 277-287.
18. Gauley, J., and D. S. Pisetsky. The release of microparticles by RAW 264.7 macrophage cells stimulated with TLR ligands. *J Leukoc Biol.* 2010; 87: 1115-1123.
19. Beyer, C., and D. S. Pisetsky. The role of microparticles in the pathogenesis of rheumatic diseases. *Nat Rev Rheumatol.* 2010; 6: 21-29.
20. Berckmans, R. J., R. Nieuwland, M. C. Kraan, M. C. Schaap, D. Pots, T. J. Smeets, et al. Synovial microparticles from arthritic patients modulate chemokine and cytokine release by synoviocytes. *Arthritis Res Ther.* 2005; 7: R536-544.

21. Berckmans, R. J., R. Nieuwland, P. P. Tak, A. N. Boing, F. P. Romijn, M. C. Kraan, et al. Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism. *Arthritis Rheum.* 2002; 46: 2857-2866.
22. Brogan, P. A., V. Shah, C. Brachet, A. Harnden, D. Mant, N. Klein, et al. Endothelial and platelet microparticles in vasculitis of the young. *Arthritis Rheum.* 2004; 50: 927-936.
23. Brogan, P. A., and M. J. Dillon. Endothelial microparticles and the diagnosis of the vasculitides. *Intern Med.* 2004; 43: 1115-1119.
24. Combes, V., A. C. Simon, G. E. Grau, D. Arnoux, L. Camoin, F. Sabatier, et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest.* 1999; 104: 93-102.
25. Guiducci, S., J. H. Distler, A. Jungel, D. Huscher, L. C. Huber, B. A. Michel, et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. *Arthritis Rheum.* 2008; 58: 2845-2853.
26. Knijff-Dutmer, E. A., J. Koerts, R. Nieuwland, E. M. Kalsbeek-Batenburg, and M. A. van de Laar. Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis. *Arthritis Rheum.* 2002; 46: 1498-1503.
27. Larkin, M. Raised endothelial microparticles an early marker for multiple sclerosis? *Lancet.* 2001; 357: 1679.
28. Minagar, A., W. Jy, J. J. Jimenez, W. A. Sheremata, L. M. Mauro, W. W. Mao, et al. Elevated plasma endothelial microparticles in multiple sclerosis. *Neurology.* 2001; 56: 1319-1324.
29. Sellam, J., V. Proulle, A. Jungel, M. Ittah, C. Miceli Richard, J. E. Gottenberg, et al. Increased levels of circulating microparticles in primary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity. *Arthritis Res Ther.* 2009; 11: R156.
30. van Eijk, I. C., M. E. Tushuizen, A. Sturk, B. A. Dijkmans, M. Boers, A. E. Voskuyl, et al. Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis. *Ann Rheum Dis.* 2010; 69: 1378-1382.
31. Dye, J. R., A. J. Ullal, and D. S. Pisetsky. The role of microparticles in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus. *Scand J Immunol.* 2013; 78: 140-148.
32. Criteria for diagnosis of Behçet's disease. International Study Group for Behçet's Disease. *Lancet.* 1990; 335: 1078-1080.
33. Thery, C., S. Amigorena, G. Raposo, and A. Clayton. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006; Chapter 3: Unit 3 22.
34. Gursel, I., M. Gursel, H. Yamada, K. J. Ishii, F. Takeshita, and D. M. Klinman. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol.* 2003; 171: 1393-1400.
35. Kahlenberg, J. M., and M. J. Kaplan. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol.* 2013; 191: 4895-4901.
36. Lynch, S. F., and C. A. Ludlam. Plasma microparticles and vascular disorders. *Br J Haematol.* 2007; 137: 36-48.
37. Hurtado, P., and C. A. Peh. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. *J Immunol.* 2010; 184: 1425-1435.
38. Cocucci, E., G. Racchetti, and J. Meldolesi. Shedding microvesicles: artefacts no more. *Trends in Cell Biology.* 2009; 19: 43-51.
39. Hunter, M. P., N. Ismail, X. Zhang, B. D. Aguda, E. J. Lee, L. Yu, et al. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS ONE.* 2008; 3: e3694.
40. Reich, C. F., 3rd, and D. S. Pisetsky. The content of DNA and RNA in microparticles released by Jurkat and HL-60 cells undergoing in vitro apoptosis. *Exp Cell Res.* 2009; 315: 760-768.

41. Hugel, B., M. C. Martinez, C. Kunzelmann, and J. M. Freyssinet. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)*. 2005; 20: 22-27.
42. Yanez-Mo, M., P. R. Siljander, Z. Andreu, A. B. Zavec, F. E. Borrás, E. I. Buzas, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles*. 2015; 4: 27066.
43. Dean, S. N., B. M. Bishop, and M. L. van Hoek. Susceptibility of *Pseudomonas aeruginosa* Biofilm to Alpha-Helical Peptides: D-enantiomer of LL-37. *Front Microbiol*. 2011; 2: 128.
44. Wong, J. H., T. B. Ng, A. Legowska, K. Rolka, M. Hui, and C. H. Cho. Antifungal action of human cathelicidin fragment (LL13-37) on *Candida albicans*. *Peptides*. 2011; 32: 1996-2002.
45. Barlow, P. G., P. Svoboda, A. Mackellar, A. A. Nash, I. A. York, J. Pohl, et al. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE*. 2011; 6: e25333.
46. Mumcu, G., H. Cimilli, U. Karacayli, N. Inanc, F. Ture-Ozdemir, E. Eksioglu-Demiralp, et al. Salivary levels of antimicrobial peptides Hnp 1-3, LI-37 and S100 in Behçet's disease. *Arch Oral Biol*. 2012; 57: 642-646.
47. Vandamme, D., B. Landuyt, W. Luyten, and L. Schoofs. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol*. 2012; 280: 22-35.
48. Yu, J., N. Mookherjee, K. Wee, D. M. Bowdish, J. Pistolic, Y. Li, et al. Host defense peptide LL-37, in synergy with inflammatory mediator IL-1 β , augments immune responses by multiple pathways. *J Immunol*. 2007; 179: 7684-7691.
49. Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y. H. Wang, B. Homey, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. 2007; 449: 564-569.

FIGURE LEGENDS

Figure 1. EVs numbers are elevated in BD plasma. Plasma EVs were purified and stained with Annexin V-FITC as described in Materials and Methods. Number of EVs per ml plasma was calculated following FACS analyses of AnV positive EV signals. Numbers in parentheses represent number of subjects studied. * $p < 0.01$ and ** $p < 0.001$ for comparison groups.

Figure 2. Extent of EV secretion from cells of various origin deviate according to disease activity. (A) EVs were stained with corresponding cell type specific surface markers and AnV. Data is represented as the mean percentage of AnV positive EVs derived from each cell type $\pm$ SD for all samples. Individual dot-plots were used to calculate CD3 (T-cells), CD14 (monocytes), CD42a (platelets), CD31 (endothelial cells) and CD105 (epithelial cells) derived EV ratios over total EVs from healthy ($n=22$), inactive BD ($n=37$) and active BD ($n=35$) donors. * $p < 0.01$, ** $p < 0.001$ based on student's t-test analyses of healthy vs. inactive BD, healthy vs. active BD and inactive BD vs. active BD. NS indicates not-significant. (B) Concentrations of cell type-specific EVs were plotted based on their absolute numbers per ml plasma. $p < 0.001$ based on One Way ANOVA.

Figure 3. EVs are efficiently internalized by PBMCs. (A) Confocal image showing EV internalization in PBMC. Left panel, CM-DiI labelled EV; middle panel, DIC image of the cells; right panel, merged image (Scale bar = 20 μ m). (B) Kinetics of EV internalization in PBMCs at different EV:cell ratios. PBMCs were incubated with CM-DiI labelled EVs at various cell:EV ratios. Percentages of CM-DiI+ cells were assessed after indicated incubation periods by flow cytometry.

Figure 4. BD EVs trigger IL6 secretion from PBMCs. Healthy PBMCs were incubated with autologous or allogeneic EVs at a cell:EV ratio of 1:2 for 12 h. IL6 production was assessed from the culture supernatants by ELISA. (autolog EV; autologous EVs from healthy donor; alloEV; allogeneic EVs from healthy donor; inactive BD EVs (iBD EV); active BD EVs (aBD EV)). ** $p < 0.001$ for all comparisons.

Figure 5. BD plasma contains elevated levels of antimicrobial peptide LL-37 which is mostly associated with EVs. (A) LL-37 concentrations (ng/ml) in plasma of BD patients (active BD $n=12$), (inactive BD $n=12$) and control healthy subjects ($n=12$) were assessed by LL-37 ELISA kit. (B) Relative LL-37 concentrations (ng/ml) in EV depleted plasma vs. EV fractions. ** $p < 0.001$ for all comparisons.

Figure 6. LL-37 promotes uptake of EVs into immune cells. (A) SpDiOC labeled LL-37 free EVs or EVs complexed with LL-37 were incubated with healthy PBMCs at a 1:1 ratio (Cell:EV) for 1h. Cells were stained with PE-labeled anti-CD11c (DC), anti-CD14 (monocytes), and anti-CD3 (T-cells) antibodies and SpDiOC positive cells were gated to determine EV internalization within specific cell types by flow cytometry. * $p < 0.01$, and ** $p < 0.001$ (for EV vs EV+LL37 groups comparisons). (B) EV uptake in PBMCs is facilitated by LL-37 over time. PBMCs (1:1; Cell:EV ratio) were incubated as described above, and SpDiOC signal positive cells were analyzed by flow cytometry at indicated time points. Data is represented as Average $\pm$ SEM of 6 independent experiments. ** $p < 0.001$, (Student's T-test between EV vs EV+LL37 treatments)

Figure 7. Complexation of healthy EVs with LL-37 converts them to an immunostimulatory phenotype, triggering IL6 production from PBMCs. (A) EVs isolated from healthy plasma were complexed overnight at 4°C either with 150 ng (gray bars) or with 450 ng (black bars) LL-37, prior to incubation with healthy PBMCs for 12h. IL6 levels were assessed from culture supernatants by ELISA. (SCR: scrambled peptide). ** $p < 0.001$ for all comparisons (B) Correlation between disease severity and plasma LL-37 levels in relation to IL6 induction ($n = 12$ for all groups). $R^2 = 0.9275$ was obtained from the best-fitted line of the plot.

Figure 8. LL-37/EVs induce IFN α from pDCs and IL1 β from monocytes. PBMCs were incubated either with Healthy EVs or with Healthy EV/LL37 complexes. Brefeldin A was added to the culture medium at the time of incubation in order to detect intracellular IL1 β production. Cells were stained for CD14 and then permeabilized and stained against anti-IL1 β after fixing. CD14 $^{+}$ cells were gated and analyzed for IL1 β levels. To detect intracellular IFN α producing pDCs, fixed cells were first stained with BDCA-2 Ab, and then IFN α detection kit protocol (from Miltenyi Biotec) was performed. To assess intracellular IFN α from pDC, BDCA-2 $^{+}$ cells were gated and analyzed with flow cytometry. The data is represented as AVE+SEM of 6 independent experiments. *p<0.01, **p<0.001(comparing EV vs EV+LL37 treatments)



Table I. Clinical evaluations of the BD Patients

	Healthy (n=22)	Inactive BD (n=37)	Active BD (n=35)
Sex (M)	22	37	35
Age (year, mean±SD)	22±5	22±5	19±6
Disease Duration (year, mean±SD)	N/A	11±5	8±2
Disease Progression			
Mucosa	N/A	9	16
Vein	N/A	12	7
Eye	N/A	16	12
Treatment			
Colchicine	N/A	21	24
Azathioprine	N/A	14	11
Cyclophosphamide	N/A	2	0

Figure 1.

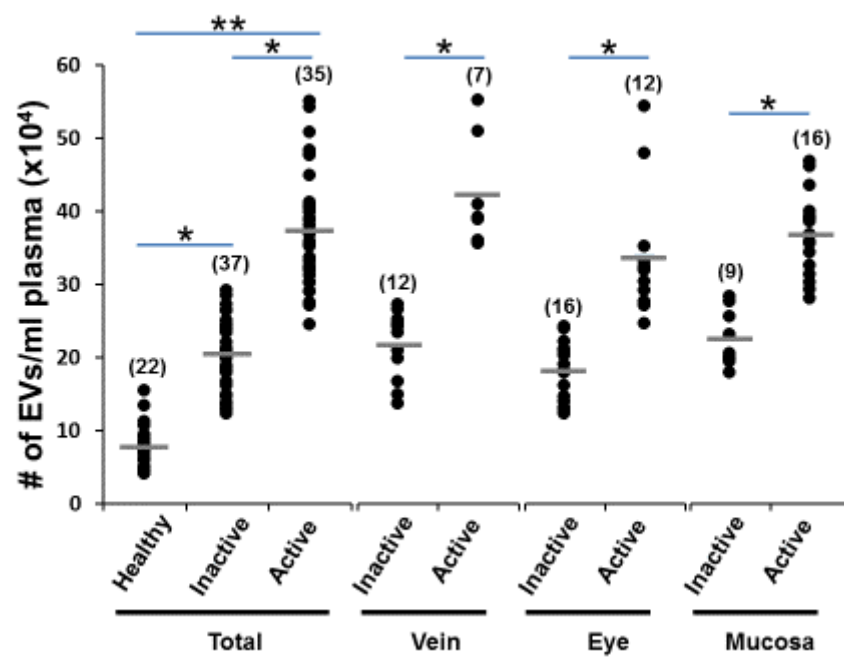
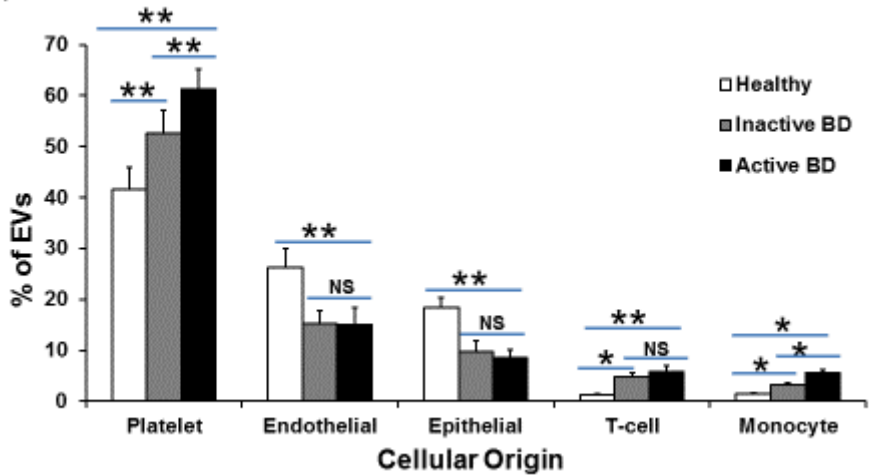


Figure 2.

A)



B)

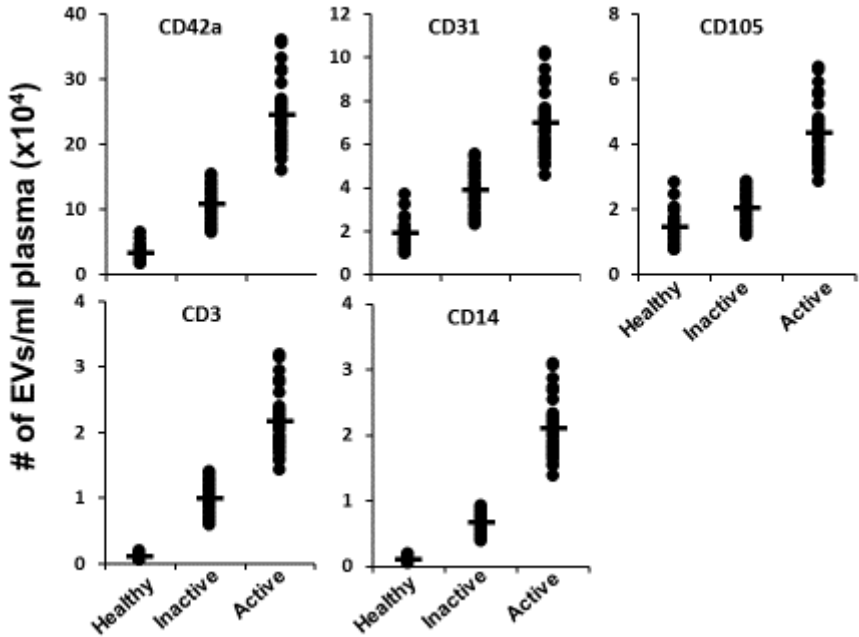


Figure 3.

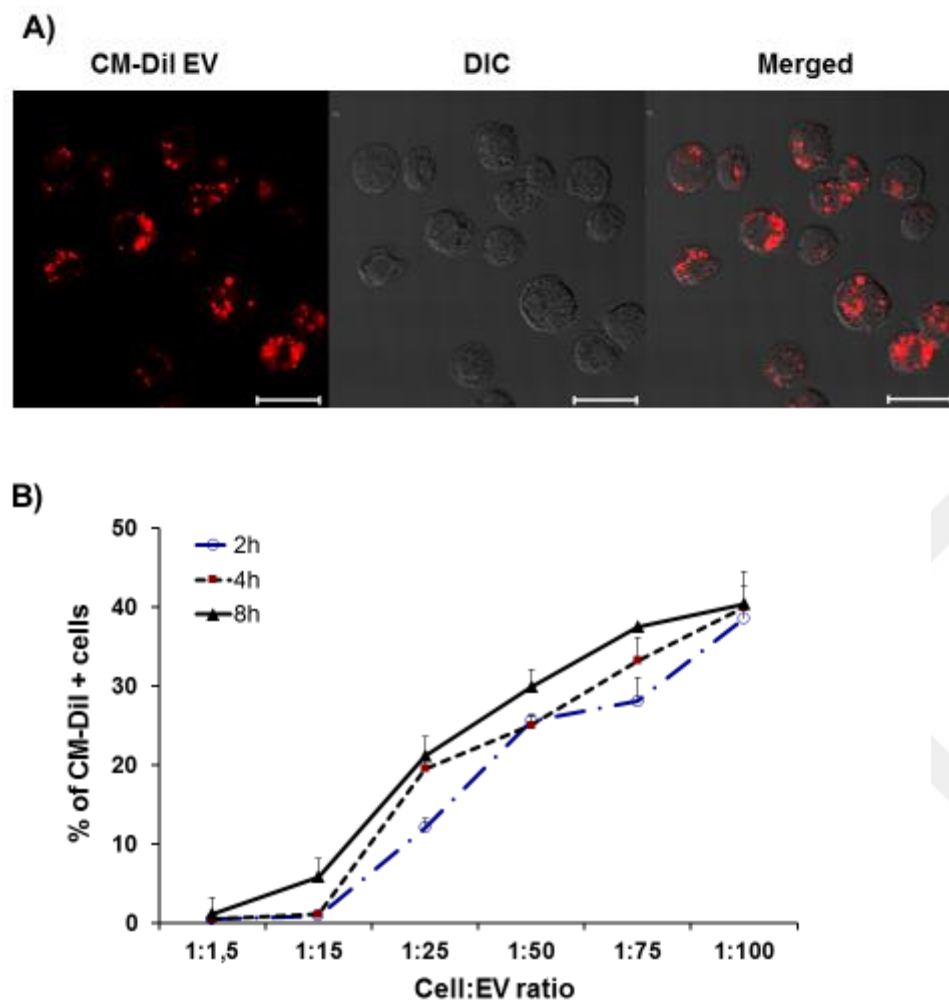


Figure 4.

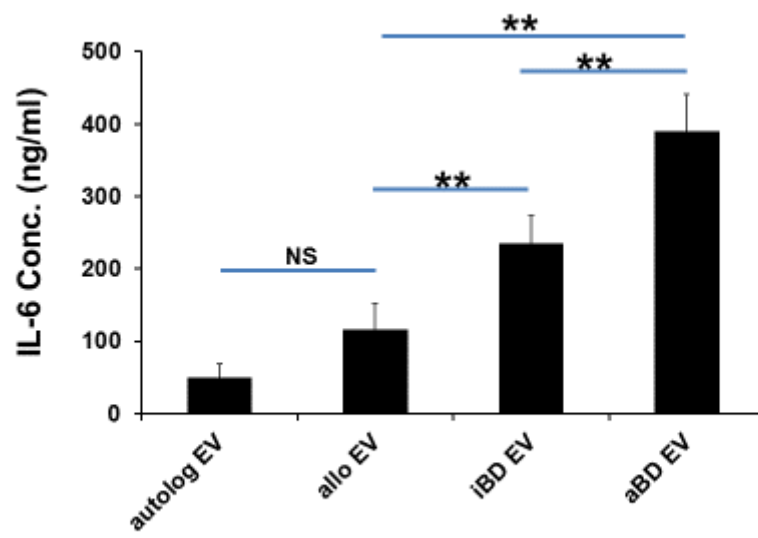


Figure 5.

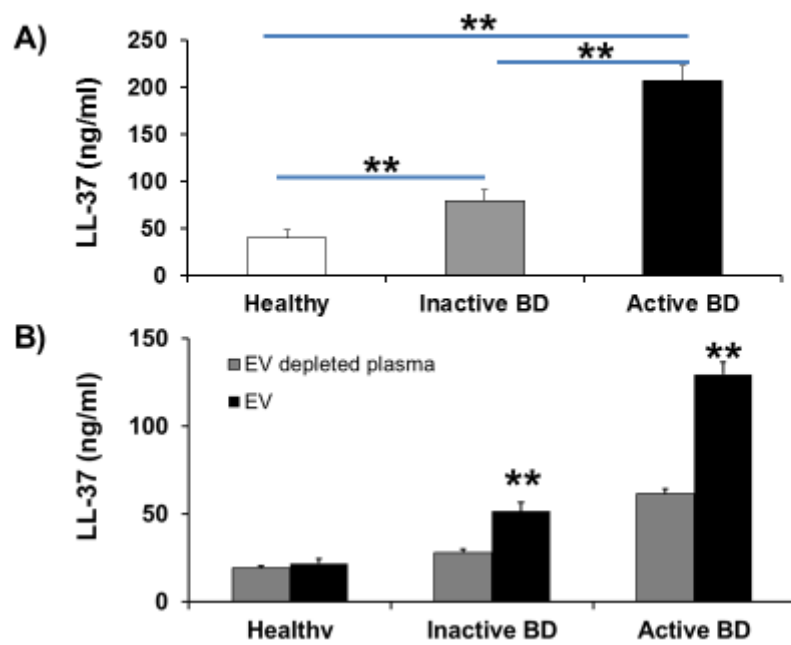


Figure 6.

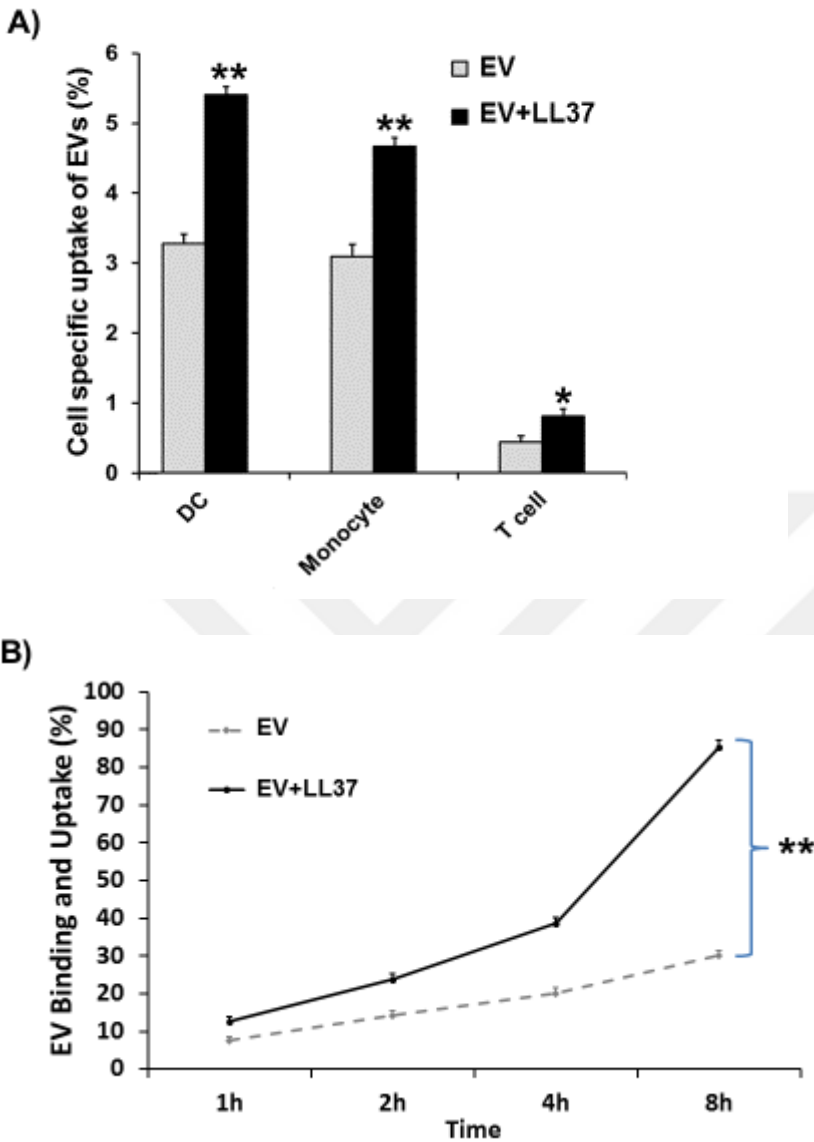


Figure 7.

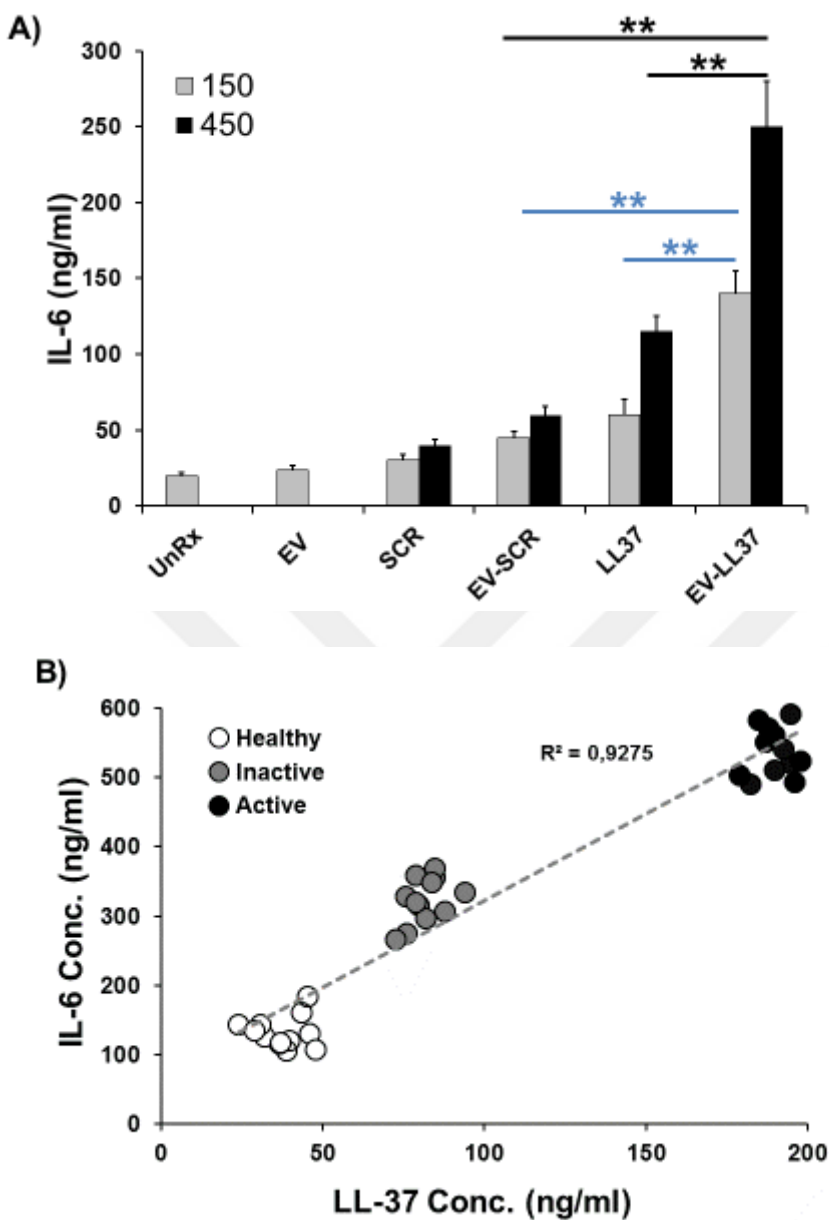
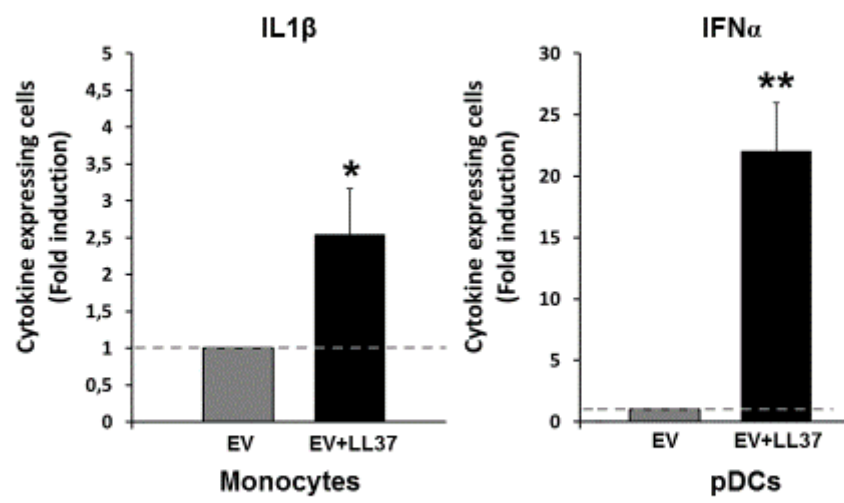


Figure 8.

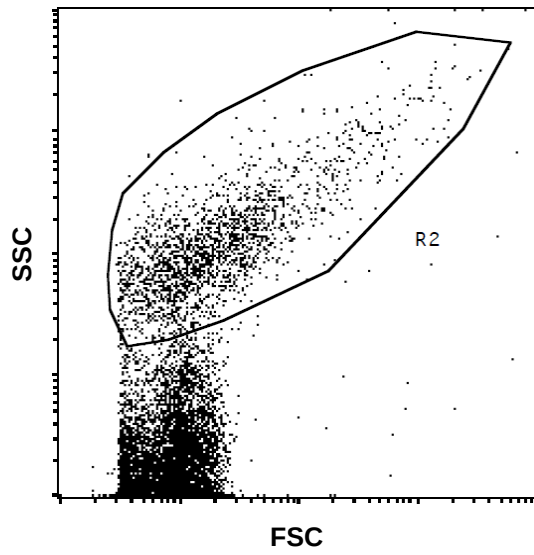


Supplementary Materials

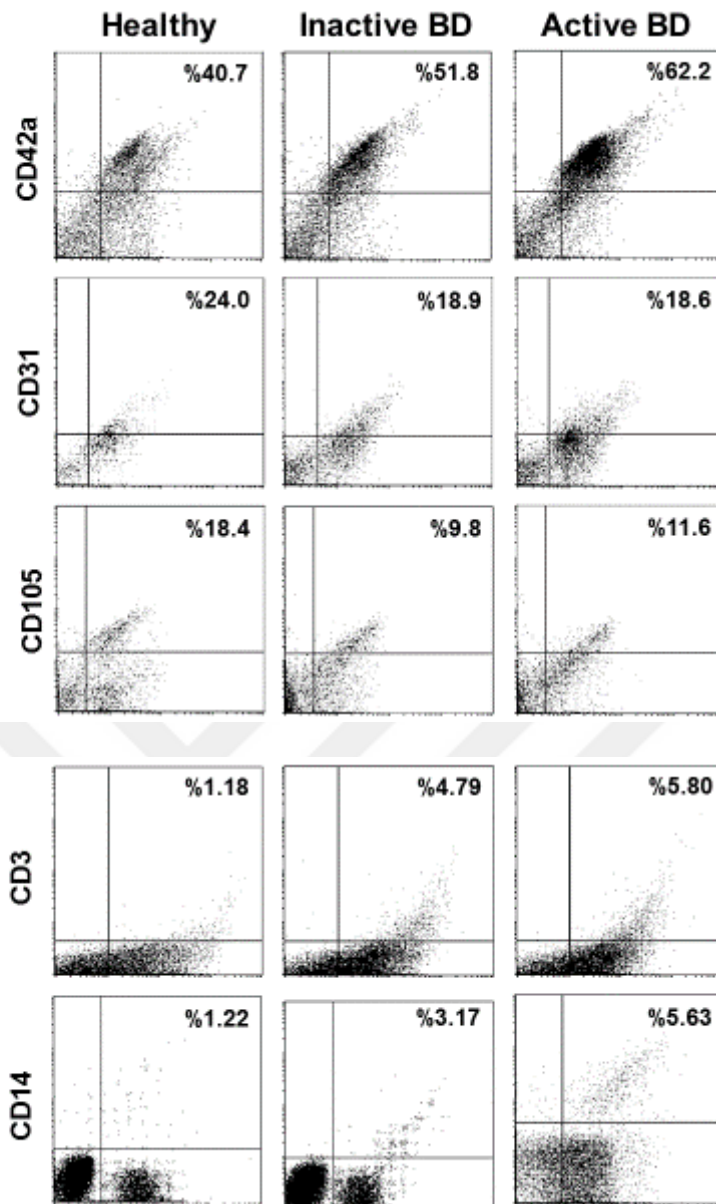
Supplementary Table I: Number of EVs per ml plasma of study groups

Disease State	Total EV	Vein	Eye	Mucosa
Healthy	77675 ± 25419	-	-	-
Inactive	205327 ± 45906	217101 ± 45560	181547 ± 39627	231906 ± 39387
Active	373161 ± 77037	422005 ± 75968	337185 ± 86689	378774 ± 58738

The data is shown as mean ± SD for each group.

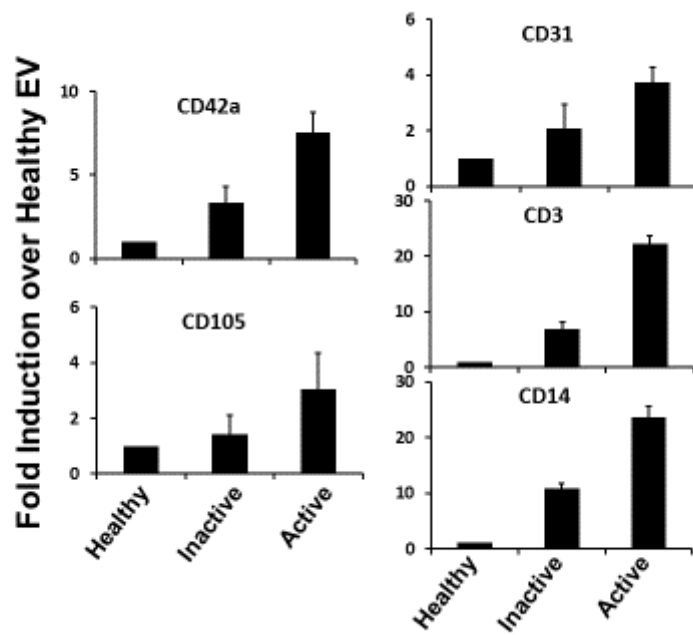


Supplementary Figure 1. Representative dot plots showing; A) Selective gate used to discriminate EV population from contaminating particles and electrical noise of FACS Calibur.

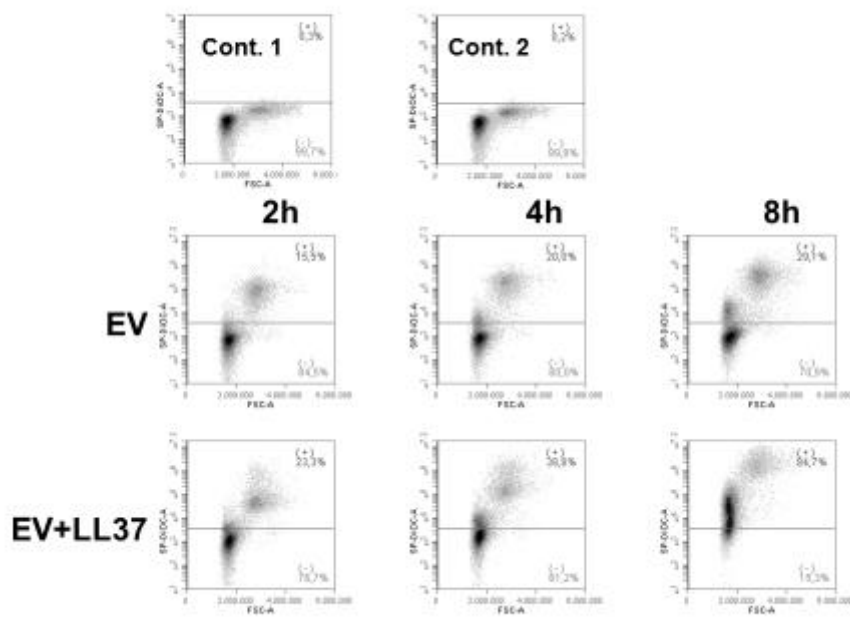


Supplementary Figure 2. The cellular origin of EVs at different disease states.

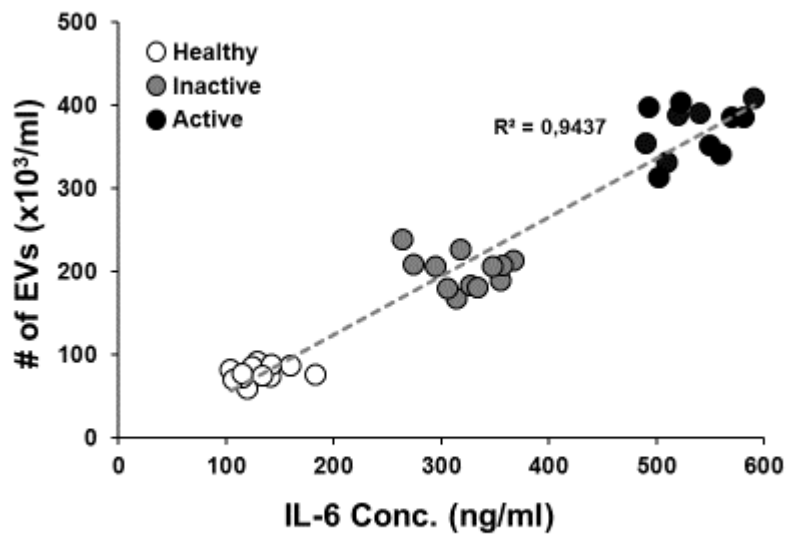
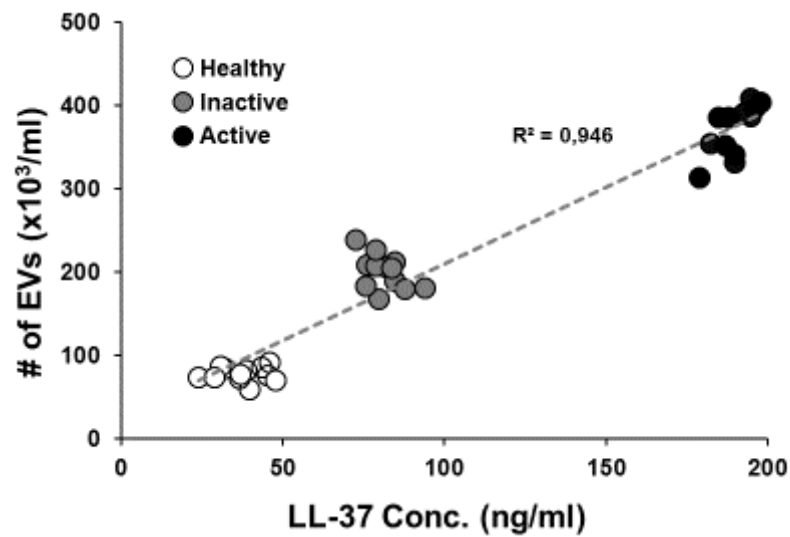
CD42a⁺, CD31⁺, CD105⁺, CD3⁺ and CD14⁺ EVs isolated from the plasma of healthy (left panel), inactive BD (middle panel), active BD (right panel) were first stained with AnV-FITC (or PE in some experiments) and then stained against above mentioned cell specific marker antibodies (PE-labelled or in some experiments FITC labelled) and analyzed by Flow Cytometry. Representative dot plots of each cell specific EVs are presented.



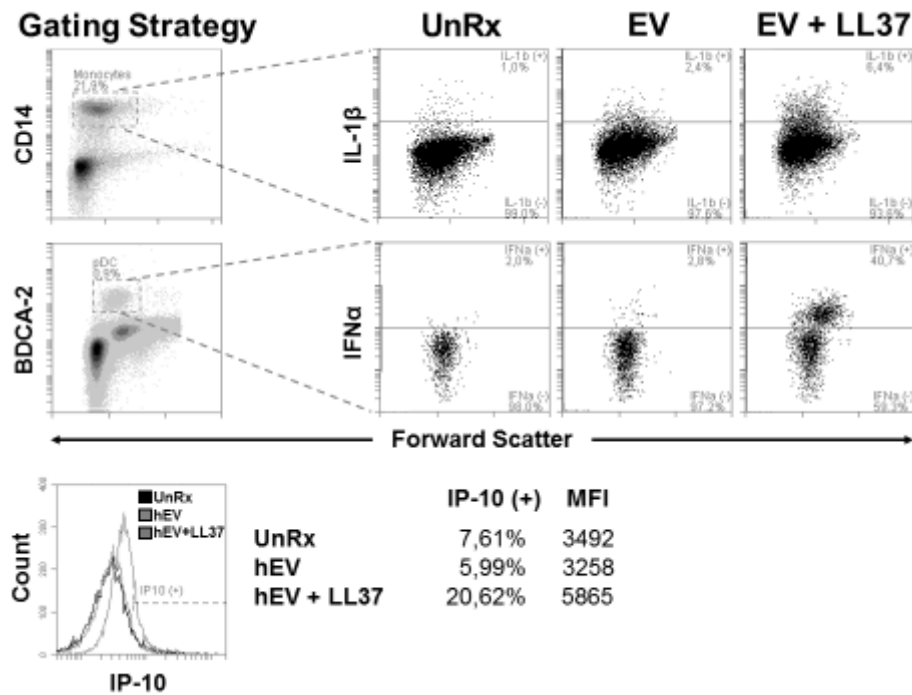
Supplementary Figure 3. Fold induction over Healthy EVs were plotted by taking the EV concentration of each individual (from inactive and active BD patients) in relation to healthy donor EV numbers based on per ml plasma.



Supplementary Figure 4. Flow cytometric dot-plots of time-dependent internalization of SpDiOC-labeled EV or EV+LL37 within PBMCs. The data is representative of 6 independent experiments with similar results.



Supplementary Figure 5. (A) Correlation between EV # vs LL-37 concentration as opposed to disease state. **(B)** Correlation between EV # vs IL-6 concentration as opposed to disease state.



Supplementary Figure 6. Gating strategy and representative dot-plots and histograms of intracellular cytokine staining for IL1 β , IFN α and IP10 from monocytes (IL1 β and IP10) and pDCs (IFN α).

Enhanced immunostimulatory activity of cyclic dinucleotides on mouse cells when complexed with a cell-penetrating peptide or combined with CpG

Soner Yildiz¹, Esin Alpdundar¹, Bilgi Gungor¹, Tamer Kahraman²,
Banu Bayyurt², Ihsan Gursel² and Mayda Gursel¹

¹ Department of Biological Sciences, Middle East Technical University, Ankara, Turkey

² Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

Recognition of pathogen-derived nucleic acids by immune cells is critical for the activation of protective innate immune responses. Bacterial cyclic dinucleotides (CDNs) are small nucleic acids that are directly recognized by the cytosolic DNA sensor STING (stimulator of IFN genes), initiating a response characterized by proinflammatory cytokine and type I IFN production. Strategies to improve the immune stimulatory activities of CDNs can further their potential for clinical development. Here, we demonstrate that a simple complex of cyclic-di-GMP with a cell-penetrating peptide enhances both cellular delivery and biological activity of the cyclic-di-GMP in murine splenocytes. Furthermore, our findings establish that activation of the TLR-dependent and TLR-independent DNA recognition pathways through combined use of CpG oligonucleotide (ODN) and CDN results in synergistic activity, augmenting cytokine production (IFN- α/β , IL-6, TNF- α , IP-10), costimulatory molecule upregulation (MHC class II, CD86), and antigen-specific humoral and cellular immunity. Results presented herein indicate that 3'3'-cGAMP, a recently identified bacterial CDN, is a superior stimulator of IFN genes ligand than cyclic-di-GMP in human PBMCs. Collectively, these findings suggest that the immune-stimulatory properties of CDNs can be augmented through peptide complexation or synergistic use with CpG oligonucleotide and may be of interest for the development of CDN-based immunotherapeutic agents.

Keywords: Arginine peptide (nona-arginine) · cGAMP · CpG ODN · Cyclic-di-GMP · Immunostimulation



See accompanying article by Temizoz et al.



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Detection of pathogen-derived nucleic acids by innate immune cells is critical for the initiation of protective responses against bacterial, viral, and fungal pathogens. Pathogen-derived nucleic acids are sensed based on their sequence, structure, nucleotide

modifications, and their intracellular localization. So far, several nucleic acid sensors have been identified, including the endosomal TLRs TLR3, TLR7/TLR8, and TLR9 that recognize dsRNA, ssRNA, and CpG-containing DNA; cytosolic dsRNA sensors RIG-I, MDA5, LGP2; and a plethora of cytosolic receptors dedicated for dsDNA recognition (DAI, AIM2, RNA polymerase III, IFI16, DEAD-box helicase DDX41) [1]. Signaling in response to cytosolic DNA depends on the expression of the adaptor protein STING (stimulator of IFN genes, also known as TMEM173, MPYS, MITA and ERIS) and proceeds through the TBK1–IRF3 axis,

Correspondence: Dr. Mayda Gursel
e-mail: mgursel@metu.edu.tr

culminating in type I IFN production [2, 3]. STING itself is not a DNA-recognition molecule. However, it can directly recognize cyclic dinucleotides (CDNs) such as the bacteria-derived molecule cyclic-di-GMP (c-di-GMP) [4]. Although CDNs were thought to function as universal secondary signaling molecules only in bacteria, recent discoveries suggest that a new type of CDN called cyclic AMP-GMP (cGAMP) plays an important role in mammalian cells [5]. Specifically, cytosolic DNA has been shown to trigger the synthesis cGAMP from ATP and GTP by an enzyme called cGAMP synthetase (cGAS), leading to STING–TBK1–IRF3 dependent induction of IFN response [6, 7].

C-di-GMP has interesting immune-stimulatory properties [8] and numerous studies explored its in vivo immunotherapeutic potential [9, 10]. In one such study, pretreatment of mice with c-di-GMP resulted in a striking protective effect against subsequent systemic or mucosal bacterial challenge with *Staphylococcus aureus*, confirming the stand-alone immune-protective potential of this molecule [11].

Myeloid dendritic cells (DCs) respond to c-di-GMP stimulation by upregulating costimulatory molecule expression and secreting proinflammatory cytokines TNF- α , IL-12, IFN- γ , IP-10, and IL-8 [8]. c-di-GMP was also shown to trigger IL-6 and IFN- β secretion in various cell types including mouse peritoneal exudate cells, RAW 264.7 (Mouse leukemic monocyte macrophage cell line), and myeloid DCs [8, 12]. Similar cytokine secretion profiles were also observed in hPBMCs and mouse splenocytes [12]. Vaccine adjuvant studies conducted with c-di-GMP demonstrated favorable antigen-specific IgG responses comparable to those seen with potent vaccine adjuvants (such as LPS and CpG oligonucleotide (ODN)). The elicited response was reportedly skewed toward IgG2a, indicating a TH1-dominated response [8, 13]. C-di-GMP was also tested as a mucosal vaccine adjuvant for the inactivated influenza H5N1 virosomal vaccine and was shown to induce local and systemic H5N1-specific humoral and cellular immune responses in mice [14].

Despite the favorable immune-stimulatory properties of c-di-GMP, the molecule contains two negatively charged phosphate groups that restrict its passage through the plasma membrane. Therefore, to benefit from its favorable immunostimulatory properties the CDN has to be used at relatively high concentrations or must be transfected to the cytosol [12, 15]. Here, we explored the possibility of using two different strategies that would improve the intracellular delivery and/or boost the immunostimulatory activity of c-di-GMP. Specifically, we show that complexation with the cationic peptide nona-arginine improves cellular uptake and enhances the vaccine adjuvant activity of the STING ligand. Moreover, we demonstrate that c-di-GMP shows synergistic immunostimulatory activity when used in combination with the TLR9 ligand, CpG oligodeoxynucleotide (CpG ODN). Our results further demonstrate that c-di-GMP is less effective in human cells than in mouse but the response can be greatly improved by replacing this CDN with another bacteria-derived second messenger, 3'3'-cGAMP. These findings may be of interest for the development of CDN-based vaccine adjuvants.

Results

Complexing c-di-GMP to arginine(9) peptide improves delivery to murine cells and immunostimulation

The physicochemical properties of native c-di-GMP prevent its free passage across cellular membranes [12]. Since the target receptor STING resides on the ER membrane, the ligand (c-di-GMP) has to be transfected into the cytosol in order to trigger an observable response at low doses [15]. To bypass the need of transfection, we evaluated the feasibility of a simple complexation strategy that would enhance the cellular entry of the molecule. To this end, we chose various cationic peptides/polymers, Arg(9), Lys(9), and polyEthyleneImine (pEI) and Tat_(47–57), as complexation agents since these molecules are well-known membrane transduction agents. For example, Arg(9) (or nona-arginine) is a positively charged small peptide, with known cell-penetrating properties [16, 17]. Lys(9) and pEI are also positively charged and can act as transfection reagents for their associated cargo [18, 19]. To evaluate whether these molecules could form complexes with the anionic c-di-GMP, fluorescently labeled form of the cyclic di-nucleotide (2'-Fluo-AHC-c-di-GMP; abbreviated as FAM-c-di-GMP) was mixed with different molar ratios (1:1, 1:2) of Arg(9), Lys(9), or pEI and complexation was followed on an agarose gel. Assessment of complex formation was done based on band shifts of the samples relative to the reference sample (FAM-c-di-GMP alone). Results showed that of the three cationic molecules tested, only Arg(9) caused a significant band shift, indicative of complex formation (Fig. 1A, upper gel image).

C-di-GMP is capable of forming bimolecular, tetramolecular, and octamolecular structures in the presence of monovalent cations, such as Na⁺ and K⁺ [20]. To assess whether c-di-GMP/Arg(9) complexation may be further enhanced in the presence of such ions, c-di-GMP was mixed with arginine at molar ratios of 1:1, 1:2, and 1:4 either in the presence or absence of KCl. Furthermore, to investigate the contribution of the arginine residues during complexation, another arginine-rich cationic cell-penetrating peptide Tat_(47–57) was tested. Tat peptide is an HIV Tat protein-derived 11-mer peptide with a net charge of +8 and has the following sequence: YGRKKRRQRRR [21]. Being rich in arginine residues, it was included in the study to see whether it would reproduce the same effect observed with nona-arginine. The gel image of one representative study is shown in Figure 1A (lower gel image). Compared to Arg(9), Tat_(47–57) formed inefficient complexes even when used at the highest ratio of 1:4. On the other hand, K⁺ ions had no effect on the complexation since comparable amounts of band shifts took place with or without K⁺ (Fig. 1A).

Next, cellular internalization properties of the Arg(9) complexes and the free ligand were tested in mouse splenocytes using the FAM-labeled CDN. Antigen-presenting cells (APCs) were stained and gated based on their MHC class II expression, and percentage of APCs internalizing the CDN was determined prior to and following Trypan blue quenching in order to enable discrimination between the surface bound and internalized and

complexes (highest dose) significantly increased the percentage of MHC Class II/CD86 double-positive cells relative to the same concentration of free c-di-GMP ($p < 0.05$). Of note, a 3× lower dose (5 μ M) of c-di-GMP/Arg(9) showed equivalent stimulatory activity observed with 15 μ M dose of the free drug (Fig. 1C and Supporting Information Fig. 2A). The immunostimulatory activity of c-di-GMP/Arg(9) was comparable to Lipofectamine 2000 transfected groups, suggesting that the active drug was efficiently transferred to the cytosol (Fig. 1C and Supporting Information Fig. 2B). Of note, the majority of cells that upregulated MHC Class II and CD86 were B220⁺ (Supporting Information Fig. 2C).

The negative controls (cGMP, lipofectamine 2000 transfected cGMP, and cGMP/Arg(9)) did not change the expression levels of the cell-surface markers, indicating that the response was specific to the CDN and not to the complexation agents. Similarly, Arg(9)-associated c-di-GMP stimulated significantly higher levels of IL-12 and TNF- α production from mouse splenocytes when compared to the free drug ($p < 0.05$, Supporting Information Fig. 3). Neither the free ligand nor its Arg(9) complexes induced detectable IL-6 secretion (Supporting Information Fig. 3). Of note, complexes prepared using a 1:4 molar ratio did not differ from those prepared at 1:2 ratio in terms of immune activation (data not shown) and were not cytotoxic (1% cell death) whereas transfection of c-di-GMP with Lipofectamine 2000 resulted in 10% cell death (Supporting Information Fig. 4). Collectively, these findings show that complexation with Arg(9) peptide increases the cytosolic availability and the immunostimulatory activity of c-di-GMP and may replace the need to transfect the drug through the use of cytotoxic lipid-based transfection reagents.

C-di-GMP synergizes with CpG ODN to upregulate costimulatory markers and proinflammatory cytokines

Emerging evidence suggests that to induce an effective immune response, use of multiple PRR ligands can result in synergistic effects that modulate the innate and the adaptive immunity [22]. Based on this information, we explored the possibility of synergism between c-di-GMP and CpG ODN mediated immune activation. To evaluate this, mouse splenocytes were treated with increasing concentrations of free c-di-GMP plus a fixed concentration of CpG ODN (optimum dose of 1 μ M) and 24 h later, percentage of MHC class II/CD86-expressing cells were compared to those stimulated with single ligands. Flow cytometric analyses showed that CpG ODN alone induced 5.5% of MHC class II/CD86 double-positives as opposed to 2.1% observed with the unstimulated control (Fig. 2A, upper panels). Cells stimulated with three different doses of c-di-GMP alone showed a dose-dependent increase in the number of double-positive cells (Fig. 2A; middle panels; 3.1, 5.4, and 7.3%). C-di-GMP synergized with CpG-ODN to induce significantly elevated levels of double-positive cells (Fig. 2A; lower panels; 5.3, 9.3, 19.6%). The combination of 15 μ M c-di-GMP and 1 μ M CpG ODN improved marker upregulation approximately ninefold and ~3.5-fold when compared to unstimulated

or CpG ODN stimulated samples, respectively. Consistently, IL-6 and TNF- α secretion was drastically increased with combined use of c-di-GMP and CpG ODN (Fig. 2B, $p < 0.05$), whereas IL-12 levels remained unchanged. Synergism for TNF- α production was evident in the MHC class II/CD11c double-positive DC population, whereas CpG ODN stimulated IL-12-producing cell percentage was not affected by the addition of c-di-GMP (Supporting Information Fig. 5). These results suggest that combined use of STING and TLR9 agonists may augment the magnitude of innate immune responses except for IL-12 production. CpG-ODN induced IL-12 production was previously shown to be independent of MAPK, PI3K, and IRF signaling pathways [23]. It is conceivable that STING-dependent TBK1–IRF3 activation would therefore be ineffective in augmenting the magnitude of CpG ODN induced IL-12 production.

In vivo vaccine adjuvant activities of Arg(9)/c-di-GMP, and combined c-di-GMP+CpG

To test the in vivo immunostimulatory activities of Arg(9)/c-di-GMP and the CDN/CpG combination, C57BL/6 mice were immunized intraperitoneally with ovalbumin (OVA), or OVA plus the adjuvants on days 0 and 14, and OVA-specific antibody responses were determined 2 weeks after the booster injection. To enable assessment of a possible in vivo synergistic activity in the CDN/CpG adjuvanted group, a low dose of CpG ODN was administered to mice (i.e. 10 μ g/mouse). For the antibody subclasses to be measured, IgG1 and IgG2c were chosen since class switching to these subtypes depends on the type of T-cell help provided to B lymphocytes (Th2 vs. Th1, respectively). Sera of individual mice were serially diluted and OD versus reciprocal serial dilution curves were generated (Supporting Information Fig. 6). Response of individual mice from each group was compared using a serum dilution of 1/32 768 (Fig. 3A). Both c-di-GMP and its complexes increased OVA-specific IgG1 levels ($p < 0.05$). IgG1 response of c-di-GMP/Arg group was significantly higher than the free c-di-GMP group ($p < 0.05$, Fig. 3A). CpG ODN alone or its combination with c-di-GMP had no significant effect on this subclass. Since c-di-GMP augments the OVA-specific IgG1 production only when used alone but not when combined with CpG ODN, the response in the synergy group may have been directed toward IgG2c class switching. Consistent with this idea, combined use of CpG ODN and c-di-GMP induced significantly elevated IgG2c levels when compared to single-ligand adjuvants ($p < 0.05$ for both groups, Fig. 3A). C-di-GMP/Arg(9) did not improve OVA-specific IgG2c response when compared to the free cyclic nucleotide, suggesting that complexation only augmented Th2-dependent IgG1 responses but had no effect on Th1-dependent IgG2c production.

To assess the extent of OVA-specific cell-mediated immunity generated by vaccination, immunized mice were challenged subcutaneously on day 51 with the OVA expressing mouse thymoma cell line, EG.7. Tumor volumes were then measured every second day for 14 days. As seen in Figure 3B, all c-di-GMP adjuvanted groups demonstrated significant reduction in tumor growth

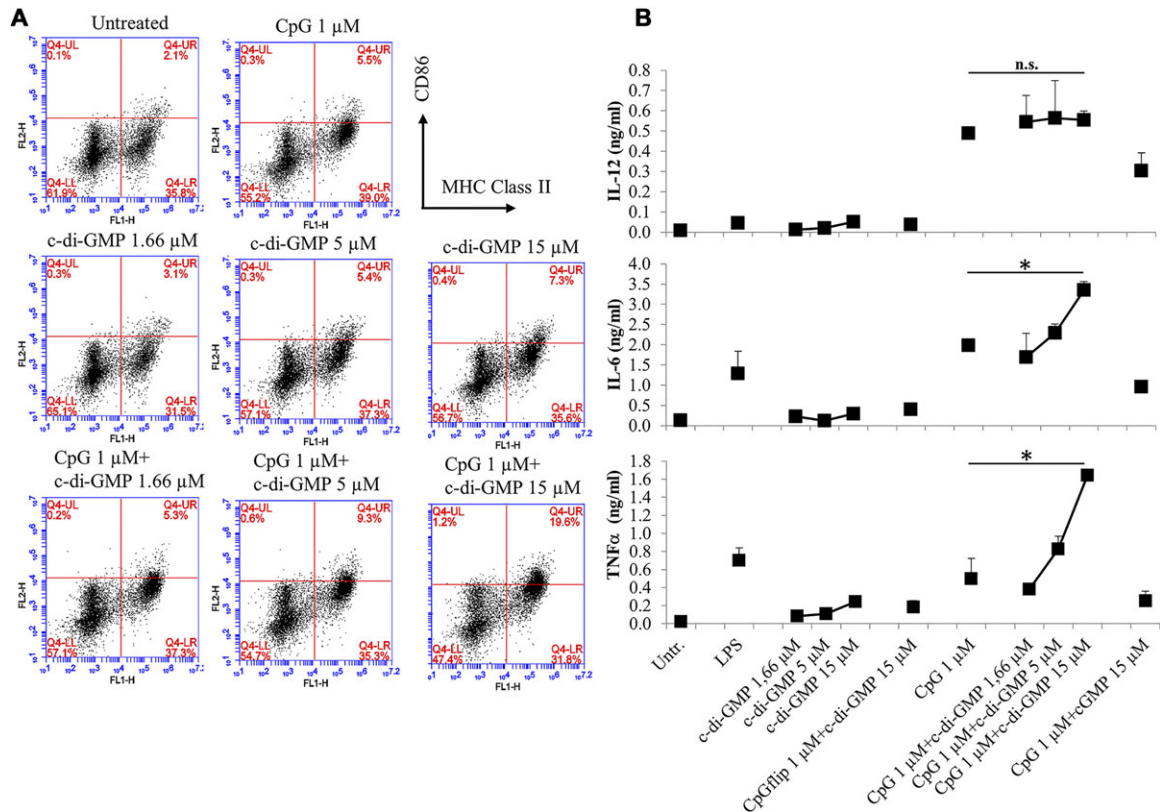


Figure 2. Combined use of CpG ODN and c-di-GMP promote synergistic activation of immune cells. (A) Indicated doses of CDN and CpG ODN were cultured with mouse splenocytes (2×10^6 cells/mL) for 24 h. Cells were stained for MHC class II and CD86 expression and analyzed by flow cytometry. Data are representative of four independent experiments, each giving similar results. (B) Culture supernatants from stimulated cells (as in A) were quantified for cytokine production by ELISA. Data are shown as mean $\pm$ SD of four independent experiments, each performed with two replicates. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. * $p < 0.05$.

($p < 0.05$). Based on individual tumor volume measurements (Fig. 3C, day 12), the most potent formulations that triggered an antitumor response were the Arg(9)/c-di-GMP and the CDN/CpG adjuvanted groups. The sizes of tumors in these groups were negligible when compared to single-ligand adjuvants (Fig. 3D). Consistent with the ability of c-di-GMP, Arg(9)/c-di-GMP, and the c-di-GMP/CpG to induce antitumor immunity, ex vivo restimulation of spleen cells from immunized mice with the SIINFEKL peptide resulted in significantly higher levels of peptide-specific IFN- γ production when compared to OVA alone, suggesting increased tumor-specific CD8 T-cell responses (Supporting Information Fig. 7, $p < 0.05$).

3'3'-cGAMP is a more effective STING ligand than c-di-GMP in both human and mouse cells

To determine whether human cells also responded similarly to c-di-GMP stimulation, preliminary experiments were conducted on human PBMCs using identical doses of the CDN that showed activity in mouse cells. However, under these conditions, we observed a very modest response in terms of IL-6 and TNF- α production or for MHC Class II/CD86 upregulation (data not shown).

To determine if this low response was directed only to c-di-GMP, we stimulated hPBMCs with the classical (c-di-GMP) and the alternate CDN 3'3'-cGAMP, with or without transfection and assessed the levels of cytokines produced (IL-8, IFN- α , IL-6, IL-10, IL-1 β , and TNF- α) using cytometric bead array. Similar to our preliminary findings, c-di-GMP failed to trigger substantial levels of cytokine production even when transfected (Fig. 4A). In contrast, 3'3'-cGAMP was very effective in stimulating the secretion of all tested cytokines even without transfection (Fig. 4A).

Synergism between 3'3'-cGAMP and CpG ODN was also tested comparative to response generated using c-di-GMP. Human CD14⁺ peripheral blood monocytes did not produce any IP-10 in response to c-di-GMP (Fig. 4B). In contrast, CpG ODN and 3'3'-cGAMP triggered similar amounts (Fig. 4B, ~9 and 11% of cells, respectively; Supporting Information Fig. 8 MFIs of 2284 ± 1250 and 5058 ± 2260 , respectively) of IP-10 production from monocytes. Interestingly, c-di-GMP suppressed CpG-ODN mediated chemokine production (Fig. 4B, from 8.8 to 4.4% of cells; Supporting Information Figure 8 MFI decreased from 2284 ± 1250 to 1314 ± 1028). The amount of IP-10 produced by cells increased by 2.5-fold (Supporting Information Fig. 8 MFI of $13\,015 \pm 3297$) for the combined group (cGAMP+CpG-ODN) comparative to cGAMP alone. Further examination of IL-6 and IFN- α

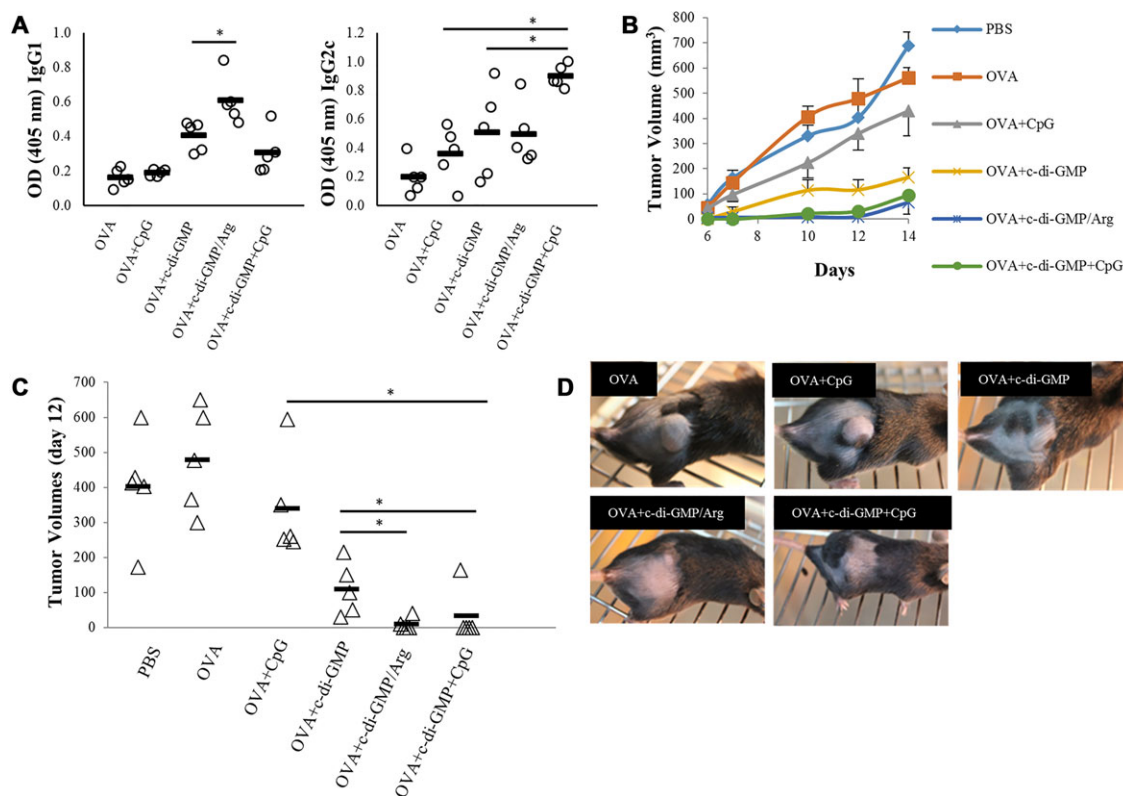


Figure 3. OVA-specific humoral and cellular immune responses generated in immunized mice. C57BL/6 mice were immunized intraperitoneally on days 0 and 14 with OVA (7.5 μ g/mouse) or OVA mixed with free or Arg(9)/c-di-GMP (15 μ g/mouse) or CpG ODN (10 μ g/mouse). (A) OVA-specific IgG1 and IgG2c responses (OD values at 1/32768 serum dilution) were determined by ELISA from sera collected on day 42. Each symbol represents an individual mouse and bars represent means. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. * $p < 0.05$. (B–D) On day 51, immunized mice were challenged with subcutaneous E.G7-OVA (4×10^6 /mouse) thymoma cells. (B) Tumor volumes were measured using a caliper every second day and recorded as length $\times$ width $\times$ height. Data are shown as mean $\pm$ SEM of five mice from a single experiment. (C) Tumor volumes of individual mice on day 12 are shown, with bar representing mean. Significance among groups was based on the nonparametric Mann–Whitney U test and * $p < 0.05$. (D) Representative images of tumors present in indicated groups. (B–D) Data shown are from a single experiment.

production from human cells showed no measurable response to c-di-GMP or its combination with CpG-ODN (Fig. 4C). However, 3′/3′-cGAMP stimulated human peripheral blood cells to secrete IL-6 and IFN- α in a dose-dependent manner and this response was modestly augmented in the presence of CpG ODN although this increase was not statistically significant ($p < 0.05$, Fig. 4C). Mouse spleen cells upregulated MHC class II and CD86 in response to free c-di-GMP but did not secrete measurable amounts of IFN- α/β (Fig. 4C). Consistent with our previous results, co-administration with CpG ODN showed synergistic activity and amplified the aforementioned responses. When used alone, 3′/3′-cGAMP was more effective than c-di-GMP in inducing type I IFN production (Fig. 4C) and synergized with CpG ODN to generate higher levels of cytokine production and costimulator molecule upregulation.

Although the vaccine adjuvant activity of 3′/3′-cGAMP was not tested in vivo, in vitro the mouse cells responded well to this CDN. Collectively, data presented here suggest that both mouse and human cells respond to 3′/3′-cGAMP stimulation and 3′/3′-cGAMP could be a more effective immunostimulatory agent than c-di-GMP for human use.

Discussion

CDNs produced by bacteria act as PAMPs and elicit an innate immune response that is critical for effective host defense against infection. Seminal work by Karaolis et al. demonstrated that c-di-GMP-induced proinflammatory cytokine/type I IFN production from immune cells provided protection against extracellular bacterial pathogens and elicited a Th1-biased antibody response to co-injected antigen [8, 9]. Further work provided evidence for c-di-GMP's role in promoting cellular and humoral immunity in vaccinated mice [14, 24–26]. These studies are of great interest for the development of CDN-based vaccine adjuvants for human use. Although CDNs have great potential as novel immunostimulatory agents, strategies to improve their resistance to digestion by phosphodiesterases and/or enhance their delivery to the cytosol are still required. For example, attempts to increase the intracellular half-life through synthesis of mono- and bisphosphorothioate analogs resulted in attenuation of the elicited inflammatory response [27], warranting adoption of alternate approaches. To overcome this challenge, we evaluated the feasibility of a simple cell-penetrating peptide and CDN complexation strategy that

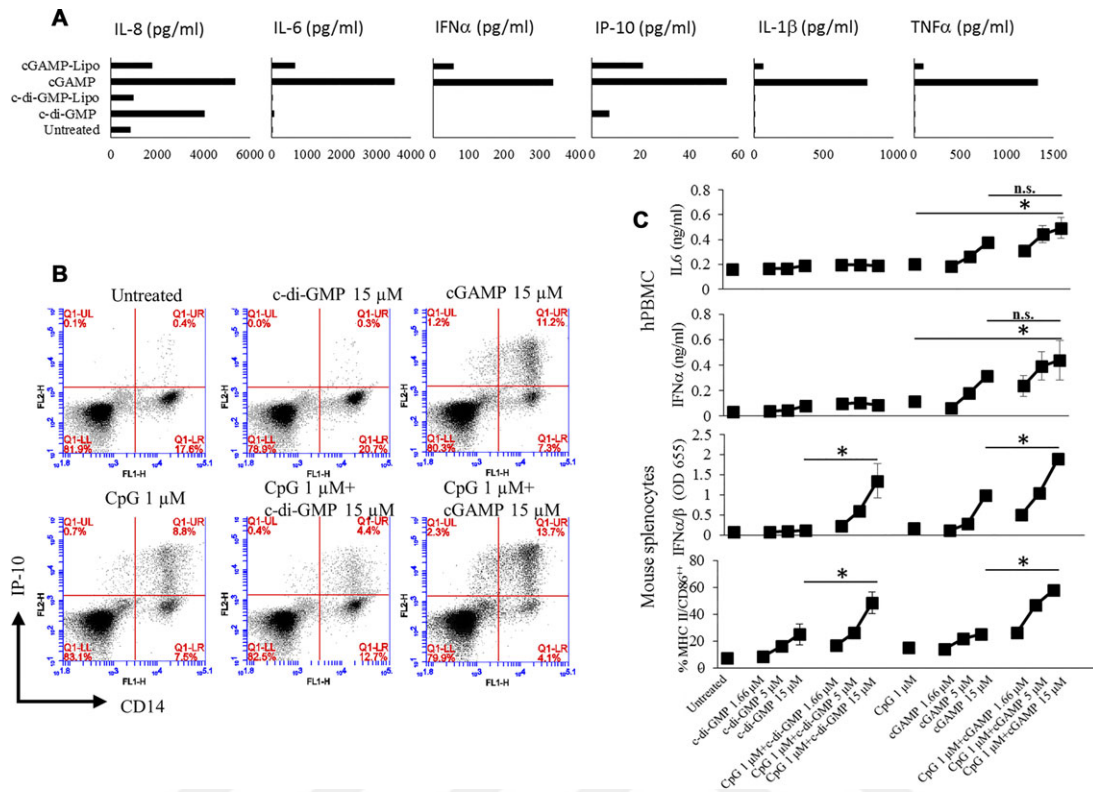


Figure 4. Immunostimulatory activities of c-di-GMP, 3'3'-cGAMP, and their combination with CpG ODN in human PBMCs and mouse splenocytes. Indicated doses of CDN and CpG ODN were cultured with human PBMCs or mouse splenocytes ($1-2 \times 10^6$ cells/mL) for 24 h. (A) Cytokine production from human PBMCs was evaluated by cytometric bead array. Representative plots (of three different individuals) show internal fluorescence intensity of capture beads (IL-8, IFN- α , IL-6, IP-10, IL-1 β , and TNF- α) versus cytokine levels (analyte intensity). (B) Indicated doses of CDN and CpG ODN were cultured with PBMCs (2×10^6 cells/mL) for 7 h and brefeldin A (10 μ g/mL) was added for the last 2 h of stimulation. IP-10-producing CD14 $^{+}$ cells were evaluated by flow cytometry. Data are representative of three different individuals. (C) Cytokine secretion patterns of human PBMCs and mouse splenocytes were evaluated by ELISA. Bioactive mouse IFN- α/β was assessed using the B16-Blue IFN- α/β reporter cells (Invivogen). Data are shown as mean $\pm$ SD of three independent experiments, each performed with two replicates. Significance among groups was tested using one-way ANOVA and post hoc Bonferroni method. * $p < 0.05$.

would enhance the cellular delivery of the molecule. C-di-GMP is known to form supramolecular aggregates stabilized by G-quadruplexes especially in the presence of intercalators [28, 29]. Of the cationic peptide or polymer intercalators tested herein, only the 9-mer arginine peptide aggregated c-di-GMP into different polymorphs. Our results suggest that c-di-GMP/Arg(9) complexation is not a simple electrochemical attraction process and might involve specific interaction of the CDN with the positively charged guanidinium group of Arginine [30]. This protonated form of guanidine is an excellent anion binder and can form two sets of double-hydrogen bonds with phosphate anion residues in what is known as the "arginine fork" binding mode [31]. C-di-GMP possesses two phosphate groups and thus would be considered as an ideal molecule for "arginine fork" binding. Of interest, Arg is the only amino acid with this functional group, and the absence of the guanidino group in Lys(9) may explain lack of complex formation even if the net charges of both peptides are identical (+9).

The finding that Arg(9) uniquely forms complexes with c-di-GMP is an interesting one and is in support of published results showing the importance of Arginine 231/232 in STING-mediated response to the cyclic nucleotide [32]. Absence of covalent

bonding between c-di-GMP and Arg(9) is of benefit since the complexes would be readily dissociable in cellular compartments and the ligand may then interact with its receptor. Furthermore, Arg(9) would also be well suited for c-di-GMP delivery since it can bypass endosomal barriers, and transfer its cargo into the cytosol [33]. Data presented herein demonstrate that c-di-GMP/Arg(9) complexes are internalized approximately fourfold more efficiently than the free molecule and the overall potency of the CDN is increased.

Combined activation of multiple PRRs can result in synergistic effects, triggering robust adaptive immune responses [34–36]. Following this concept, we chose to activate the TLR-dependent and TLR-independent DNA recognition pathways through combined use of CpG ODN and CDNs. In contrast to A-class CpG ODN that induce type I IFN in pDC via TLR9-MyD88-IRF7 pathway, recognition of conventional CpG-ODN (Class B) by endosomal TLR9 initiates a signaling cascade where MyD88-dependent recruitment of IRAK-4/IRAK-1/TRAF6 leads to the activation of TAK1 (TGF- β -activated kinase). TAK-1-dependent activation of NF- κ B (through IKK $\alpha/\beta/\gamma$) and MAP kinases results in the production of proinflammatory cytokines [37, 38]. Conversely, all pathways of cytosolic

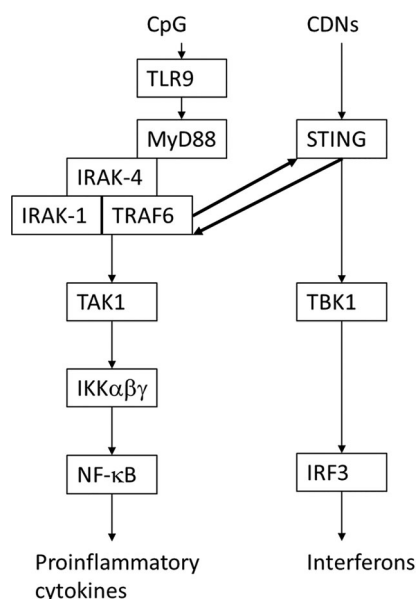


Figure 5. Proposed mechanism of synergism between CpG ODN and CDN dependent signaling pathways. CpG-ODN initiates a TLR9-mediated signaling cascade where MyD88-dependent recruitment of IRAK-4/IRAK-1/TRAF6 leads to the activation of TAK1 (left). TAK1 phosphorylates I κ B kinase, which in turn activates NF κ B leading to proinflammatory cytokine production. STING recognizes CDNs, recruits and activates TBK1 (right). TBK1 in turn phosphorylates and activates IRF3, leading to IFN gene transcription. TRAF6 may also be recruited to the STING–TBK1 complex, facilitating the activation of NF- κ B and IRF-3. Since TRAF6 has a role in activation of both CpG ODN and CDN induced signaling, when cells are stimulated with both ligands, the triggered pathways may synergize through a TRAF6-dependent process.

DNA recognition and CDN sensing converge on STING. Activated STING recruits TBK1 and IRF-3, which in turn results in transcription of IFN- β and proinflammatory cytokines [7, 12]. Recent evidence suggests that TRAF6 may be recruited to the STING–TBK1 complex, facilitating the activation of NF- κ B and IRF-3 (through TBK1) [39]. TRAF6 is an important molecule that converges signals from various PRRs, affecting DC maturation and induction of cytokine production [40]. Our results established that combined use of CpG ODN and CDNs promotes synergistic activation of immune cells. TRAF6 appears to be essential for the activation of CpG ODN and CDN-induced downstream signaling cascades. It is therefore conceivable that the endosomal and the cytosolic DNA detection pathways synergize through a TRAF6-dependent process (Fig. 5).

Our data revealed 3′/cGAMP to be a more effective STING ligand than c-di-GMP. Whether this is due to a difference in membrane permeabilities of the ligands or their affinity to STING remains to be determined.

Vaccine adjuvants are essential for enhancing and directing the adaptative immune response to antigens. Yet, most vaccines are still supplemented with the Th2-skewing adjuvant alum. In this context, CDNs constitute an important class of molecules with a potential to improve vaccine efficacy. Here, we showed that cellular delivery and immune-stimulatory properties of CDNs can be augmented by either Arg(9) peptide complexation, or synergistic

use with CpG ODN, suggesting that such strategies may be of value for further clinical development.

Materials and Methods

Reagents and cell lines

Cyclic diguanosine monophosphate (c-di-GMP), 2′-O-(6-[Fluoresceinyl] amino hexyl carbamoyl)-cyclic diguanosine monophosphate (FAM-c-di-GMP), and guanosine-2′, 3′-cyclic monophosphate (cGMP) were purchased from Biolog. 3′/cGAMP (cyclic [G(3′,5′)pA(3′,5′)p]) was from Invivogen. CpG ODN K3 ATCGACTCTCGAGCGTTCTC and its flipped control were from Alpha DNA. LPS (Sigma) and polyinosidic acid:cytidilic acid (polyI:C, Amersham) were used as positive controls. Lipofectamine 2000 was purchased from Invitrogen, and used according to the manufacturer's protocol. Nonamer arginine peptide (Arg(9)), polylysine peptide (Lys(9)), Tat_(47–57), and OVA were obtained from Anaspec. B16-Blue IFN- α/β cells that allow the detection of bioactive murine type I IFNs were obtained from Invivogen and used according to the manufacturer's protocol.

Visualization of complexes by agarose gel electrophoresis (AGE)

Complexes were prepared by mixing 0.67 μ g of 2′-O-(6-[Fluoresceinyl] amino hexyl carbamoyl)-cyclic diguanosine monophosphate (FAM-c-di-GMP) with cationic peptides Arg(9), Lys(9), Tat_(47–57), or PEI at 1:1, 1:2, and 1:4 molar ratios. Samples were incubated for 30 min at room temperature, in order to allow for the complexation to proceed and then were loaded onto a 2% agarose gel. Gels were resolved at 70 V for 30 min.

Cells and culture conditions

All hPBMC experiments were conducted following approval from the Bilkent University Human Studies Ethical Committee and with the informed consent of all participants. Human PBMCs were isolated from healthy volunteers using density gradient centrifugation. E.G7-OVA cells (derived from C57BL/6 (H-2b) mouse lymphoma), PBMCs, and single-cell spleen suspensions were cultured in RPMI 1640 medium containing 5% FCS, 50 U/mL penicillin, 50 μ g/mL streptomycin, 0.3 μ g/mL L-glutamine, 1 μ M nonessential amino acids, 1 μ M sodium pyruvate, 10 mM HEPES, and 10^{-5} M 2-ME.

Analysis of cellular internalization of c-di-GMP and its complexes

Complexes of c-di-GMP/Arg (molar ratio of 1:2) were prepared using either free c-di-GMP or FAM-c-di-GMP. Splenocytes

(1×10^6 /mL) were incubated for 2 h at 37°C with 15 μ M of the stimulants. Following incubation, cells were washed and analyzed using an Accuri C6 Flow Cytometer. To detect internalized CDN, surface-bound ODN-FAM signal was quenched using 0.2% Trypan blue (mixed 1:1 with the sample).

Determination of in vitro stimulatory activity of c-di-GMP and cGAMP

Splenocytes or hPBMCs (4×10^5 cells/well) were incubated with various concentration of c-di-GMP, CpG, or cGAMP with appropriate controls in a 96-well plate for 24 h. Culture supernatants were collected and analyzed by ELISA using capture and detection antibodies for mIL6, mIL12p40, mTNF- α , hIL6 (Biolegend), or hPAN α (Mabtech). In some experiments, cytokines were detected using the BD Biosciences Cytometric Bead Array Human Flex Set (IL-8, IFN- α , IP-10, IL-6, IL-1 β , and TNF- α). For intracellular cytokine staining, cells were stimulated in the presence of brefeldin A (10 μ g/mL). For flow cytometric analysis, cells were fixed (and permeabilized where necessary) and stained with conjugated antibodies (1 μ g/mL final concentration) PE-mI-A/I-E, Alexa Fluor 647-mCD86, PE-hIP10, and/or FITC-hCD14 (Biolegend).

Immunization studies

All animal studies were conducted with prior approval of the animal ethics committee of Bilkent University. Six- to eight-week-old C57BL/6 mice were immunized intraperitoneally on days 0 and 14 with 100 μ L OVA (7.5 μ g/mouse) or OVA mixed with free or Arg(9)/c-di-GMP (15 μ g/mouse) or CpG ODN (10 μ g/mouse). To follow OVA-specific antibody responses, sera were collected on day 42 and antibody levels were determined by ELISA. Briefly, Immulon 1B plates were coated overnight with 50 μ L of OVA (10 μ g/mL) at 4°C and blocked with PBS-5% BSA. Eight-time-diluted mouse sera were serially diluted fourfold and specific antibodies were detected using goat anti-mouse IgG1 or IgG2a alkaline phosphatase conjugate (1:3000 dilution) followed by PNPP substrate addition. Color development was followed at OD 405 nm using a microplate reader. Thirty-four days after the booster injection (day 51), mice were challenged with subcutaneous E.G7-OVA (4×10^6 /mouse) thymoma cells. After the tumors reached a palpable size (~ 50 mm³, ~ 1 week after tumor initiation), tumor volumes were measured using a caliper every other day and recorded as length $\times$ width $\times$ height.

Statistical Analysis

Data were statistically analyzed with the IBM SPSS v22.0 software using two-tailed unpaired Student's *t*-test, one-way ANOVA with Bonferroni posttest or Mann–Whitney *U* test. For all comparisons, 95% confidence intervals were used and *p*-values <0.05 were

considered significant. Statistical tests used to determine significance are provided in individual figure legends.

Acknowledgments: We thank Ihsan Cihan Ayanoglu for technical assistance with statistical analyses. M.G. and I.G. received partial funding from TUBITAK projects 113S207, 111S151, and 113S305.

Conflict of interest: M.G. and I.G. are among the co-inventors of patents concerning the activity of CpG ODN, including their use as vaccine adjuvants. The rights to all such patents have been transferred to the US government. All other authors declare no financial or commercial conflict of interests.

References

- Barbalat, R., Ewald, S. E., Mouchess, M. L. and Barton, G. M., Nucleic acid recognition by the innate immune system. *Annu. Rev. Immunol.* 2011. **29**: 185–214.
- Ishikawa, H., Ma, Z. and Barber, G. N., STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 2009. **461**: 788–792.
- Bhat, N. and Fitzgerald, K. A., Recognition of cytosolic DNA by cGAS and other STING-dependent sensors. *Eur. J. Immunol.* 2014. **3**: 634–640.
- Burdette, D. L., Monroe, K. M., Sotelo-Troha, K., Iwig, J. S., Eckert, B., Hyodo, M., Hayakawa, Y. et al., STING is a direct innate immune sensor of cyclic di-GMP. *Nature* 2011. **478**: 515–518.
- Wu, J., Sun, L., Chen, X., Du, F., Shim H., Chen, C. and Chen, Z. J., Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 2013. **339**: 826–830.
- Diner, E. J., Burdette, D. L., Wilson, S. C., Monroe, K. M., Kellenberger, C. A., Hyodo, M., Hayakawa, Y. et al., The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. *Cell Rep.* 2013. **3**: 1355–1361.
- Sun, L., Wu, J., Du, F., Chen, X. and Chen, Z. J., Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 2013. **339**: 786–791.
- Karaolis, D. K., Means, T. K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., Philpott, D. et al., Bacterial c-di-GMP is an immunostimulatory molecule. *J. Immunol.* 2007. **178**: 2171–2181.
- Karaolis, D. K., Newstead, M. W., Zeng, X., Hyodo, M., Hayakawa, Y., Bhan, U., Liang, H. et al., Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infect. Immun.* 2007. **75**: 4942–4950.
- Ogunniyi, A. D., Paton, J. C., Kirby, A. C., McCullers, J. A., Cook, J., Hyodo, M., Hayakawa, Y. et al., c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. *Vaccine* 2008. **26**: 4676–4685.
- Hu, D. L., Narita, K., Hyodo, M., Hayakawa, Y., Nakane, A. and Karaolis, D. K., c-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infection. *Vaccine* 2009. **27**: 4867–4873.

- 12 McWhirter, S. M., Barbalat, R., Monroe, K. M., Fontana, M. F., Hyodo, M., Joncker, N. T., Ishii, K. J. et al., A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J. Exp. Med.* 2009. **206**: 1899–1911.
- 13 Gray, P. M., Forrest, G., Wisniewski, T., Porter, G., Freed, D. C., DeMartino, J. A., Zaller, D. M. et al., Evidence for cyclic diguanylate as a vaccine adjuvant with novel immunostimulatory activities. *Cell Immunol.* 2012. **278**(1–2):113–119.
- 14 Pedersen, G. K., Ebensen, T., Gjeraker, I. H., Svindland, S., Bredholt, G., Guzmán, C. A. and Cox, R. J., Evaluation of the sublingual route for administration of influenza H5N1 virosomes in combination with the bacterial second messenger c-di-GMP. *PLoS One* 2011. **6**: e26973.
- 15 Kumagai, Y., Matsuo, J., Hayakawa, Y. and Rikihisa, Y., Cyclic di-GMP signaling regulates invasion by *Ehrlichia chaffeensis* of human monocytes. *J. Bacteriol.* 2010. **192**: 4122–4133.
- 16 Zorko, M. and Langel, U., Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv. Drug Deliv. Rev.* 2005. **57**: 529–545.
- 17 Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V. and Lebleu, B., Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* 2003. **278**: 585–590.
- 18 Zauner, W., Wagner, E. and Ogris, M., Polylysine-based transfection systems utilizing receptor-mediated delivery. *Adv. Drug Deliv. Rev.* 1998. **30**: 97–113.
- 19 Akinc, A., Thomas, M., Klibanov, A. M. and Langer, R., Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* 2005. **7**: 657–663.
- 20 Zhang, Z., Kim, S., Gaffney, B. L. and Jones, R. A., Polymorphism of the signaling molecule c-di-GMP. *J. Am. Chem. Soc.* 2006. **128**: 7015–7024.
- 21 Brooks, H., Lebleu, B. and Vivès, E., Tat peptide-mediated cellular delivery: back to basics. *Adv. Drug Deliv. Rev.* 2005. **57**: 559–577.
- 22 Trinchieri, G. and Sher, A., Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 2007. **7**: 179–190.
- 23 Bode, K. A., Schmitz, F., Vargas, L., Heeg, K. and Dalpke, A. H., Kinetic of RelA activation controls magnitude of TLR-mediated IL-12p40 induction. *J. Immunol.* 2009. **182**: 2176–2184.
- 24 Ebensen, T., Schulze, K., Riese, P., Morr, M. and Guzmán, C. A., The bacterial second messenger c-di-GMP exhibits promising activity as a mucosal adjuvant. *Clin. Vaccine Immunol.* 2007. **14**: 952–958.
- 25 Libanova, R., Ebensen, T., Schulze, K., Bruhn, D., Nörder, M., Yevsa, T., Morr, M. and Guzmán, C. A., The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. *Vaccine* 2010. **28**: 2249–2258.
- 26 Madhun, A. S., Haaheim, L. R., Nøstbakken, J. K., Ebensen, T., Chichester, J., Yusibov, V., Guzman, C. A. et al., Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 CD4⁺ cells and strong mucosal and systemic antibody responses in mice. *Vaccine* 2011. **29**: 4973–4982.
- 27 Yan, H., Wang, X., KuoLee, R. and Chen, W., Synthesis and immunostimulatory properties of the phosphorothioate analogues of c-di-GMP. *Bioorg. Med. Chem. Lett.* 2008. **18**: 5631–5634.
- 28 Yan, H. and Chen, W., 3', 5'-Cyclic diguanylic acid: a small nucleotide that makes big impacts. *Chem. Soc. Rev.* 2010. **39**: 2914–2924.
- 29 Nakayama, S., Kelsey, I., Wang, J. and Sintim, H. O., c-di-GMP can form remarkably stable G-quadruplexes at physiological conditions in the presence of some planar intercalators. *Chem. Commun. (Camb.)* 2011. **47**: 4766–4768.
- 30 Benach, J., Swaminathan, S. S., Tamayo, R., Handelman, S. K., Foltz-Stogniew, E., Ramos, J. E., Forouhar, F. et al., The structural basis of cyclic diguanylate signal transduction by PilZ domains. *Eur. Mol. Biol. Organ. J.* 2007. **26**: 5153–5166.
- 31 Steed, J. W. and Atwood, J. L. (Eds.), *Supramolecular chemistry* (2nd edn.), John Wiley, Chichester: 2009, p 229.
- 32 Ouyang, S., Song, X., Wang, Y., Ru, H., Shaw, N., Jiang, Y., Niu, F. et al., Structural analysis of the STING adaptor protein reveals a hydrophobic dimer interface and mode of cyclic di-GMP binding. *Immunity* 2012. **36**: 1073–1086.
- 33 El-Sayed, A., Futaki, S. and Harashima, H., Delivery of macromolecules using Arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment. *AAPS J.* 2009. **11**: 13–22.
- 34 Arsenault, R. J., Kogut, M. H. and He, H., Combined CpG and poly I:C stimulation of monocytes results in unique signaling activation not observed with the individual ligands. *Cell Signal.* 2013. **25**: 2246–2254.
- 35 Nourizadeh, M., Masoumi, F., Memarian, A., Alimoghaddam, K., Moazzeni, S. M. and Hadjati, J., Synergistic effect of Toll-like receptor 4 and 7/8 agonists is necessary to generate potent blast-derived dendritic cells in Acute Myeloid Leukemia. *Leuk. Res.* 2012. **36**: 1193–1199.
- 36 Kasturi, S. P., Skountzou, I., Albrecht, R. A., Koutsonanos, D., Hua, T., Nakaya, H. I., Ravindran, R. et al., Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* 2011. **470**: 543–547.
- 37 Koyama, S., Ishii, K. J., Coban, C., and Akira, S., Innate immune response to viral infection. *Cytokine* 2008. **43**: 336–341.
- 38 Desmet, C. J. and Ishii, K. J., Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. *Nat. Rev. Immunol.* 2012. **12**: 479–491.
- 39 Abe, T. and Barber, G. N., Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF- κ B activation through TBK1. *J. Virol.* 2014. **88**: 5328–5341.
- 40 Kobayashi, T., Walsh, M. C. and Choi, Y., The role of TRAF6 in signal transduction and the immune response. *Microbes Infect.* 2004. **6**: 1333–1338.

Abbreviations: AIM2: Absent in Melanoma 2 · c-di-GMP: cyclic-di-GMP · CDN: cyclic dinucleotide · cGAMP: cyclic GMP-AMP · DAI: DNA-dependent activator of IFN-regulatory factors · DEAD: D-E-A-D (asp-glu-ala-asp) · ERIS: Endoplasmic Reticulum Interferon Stimulator · FAM-c-di-GMP: 2'-Fluo-AHC-c-di-GMP · IFI16: Interferon-Inducible myeloid differentiation transcriptional activator · IRF: Interferon regulatory factor 3 · LGP2: Laboratory of Genetics and Physiology 2 · MDA5: Melanoma Differentiation-Associated protein 5 · MITA: Mediator of IRF3 Activation · MPYS: M-P-Y-S (met-pro-tyr-ser) · ODN: oligonucleotide · pEI: polyethylenimine · RIG-I: Retinoic Acid Inducible Gene-I · STING: stimulator of IFN genes · TMEM173: Transmembrane Protein 173

Full correspondence: Dr. Mayda Gursel, Department of Biological Sciences, Middle East Technical University, Ankara 06800, Turkey
Fax: +90-312-210-7976
e-mail: mgursel@metu.edu.tr

See accompanying article:
<http://dx.doi.org/10.1002/eji.201445132>

Received: 15/8/2014
Revised: 12/12/2014
Accepted: 7/1/2015
Accepted article online: 9/1/2015



Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles

Gizem Tincer^a, Seda Yerlikaya^{a,1}, Fuat C. Yagci^a, Tamer Kahraman^a, Osman M. Atanur^b, Oktay Erbatur^b, Ihsan Gursel^{a,*}

^a Biotherapeutic ODN Research Laboratory, Molecular Biology and Genetics Department, Faculty of Sciences, Bilkent University, Bilkent, Ankara 06800, Turkey

^b Chemistry Department, Science Faculty, Çukurova University, Balcalı, Adana 01330, Turkey

ARTICLE INFO

Article history:

Received 27 November 2010

Accepted 12 January 2011

Available online 2 April 2011

Keywords:

Polysaccharides

Poly(I:C)

Nanoparticles

Targeted delivery system

TLR

Immunogenicity

ABSTRACT

Immunostimulatory properties of mushroom derived polysaccharides (PS) as stand-alone agents were tested. Next, PS were nanocomplexed with poly(I:C) (pIC) to yield stable nanoparticles around 200 nm in size evidenced by atomic force microscopy and dynamic light scattering analyses. PSs were selectively engaged by cells expressing TLR2 and initiated NFκB dependent signaling cascade leading to a Th1-biased cytokine/chemokine secretion in addition to bactericidal nitric oxide (NO) production from macrophages. Moreover, cells treated with nanoparticles led to synergistic IL6, production and up-regulation of TNFα, MIP3α, IFNγ and IP10 transcript expression. In mice, PS-Ovalbumin-pIC formulation surpassed anti-OVA IgG responses when compared to either PS-OVA or pIC-OVA mediated immunity. Our results revealed that signal transduction initiated both by TLR2 and TLR3 via co-delivery of pIC by PS in nanoparticle depot delivery system is an effective immunization strategy. The present work implicate that the PS and nucleic acid based nanoparticle approach along with protein antigens can be harnessed to prevent infectious diseases.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Toll-like receptors (TLRs) are the most extensively studied pathogen recognition receptors (PRRs) that recognizes specific microbial associated molecular patterns (MAMPs) including lipopolysaccharides, peptidoglycans, lipoproteins, flagellin, unmethylated CpG motifs or viral RNA/DNA that triggers innate immune response [1–3]. TLRs are subcategorized as endosomal or intracellular receptors depending on their site of expression. TLR3, 7/8 and 9 are specialized to sense pathogens via their nucleic acids moieties [4]. TLR3, a member of endosome-associated TLR is activated directly in response to dsRNA, or synthetic poly(riboinosinic:polyribocytidylic acid) (pIC) [5] and are harnessed as vaccine adjuvant, anti-cancer or anti-allergic therapeutic agents. When these ligands are given *in vivo*, they are rapidly cleared by nucleases, and could be adsorbed by serum proteins hampering their *in vivo* therapeutic

applications [6]. In order to improve their immune stimulatory potential repeated high doses are necessary, however, in many instances this may cause undesirable side effects including septic shock [7]. Nucleic acid backbone modifications such as phosphorothioate linkages are another widely accepted approach but this not only induces undesirable side effects such as granuloma formation, temporary splenomegaly, and lymphadenopathy but also increases the cost of the final product [8–11].

Several strategies were proposed as carriers for labile nucleic acids attempting to increase their *in vivo* performances [12–14]. Among many, liposome encapsulation, biodegradable nano/micro carriers and soluble macromolecules with cationic moieties were widely studied [12–14]. We and others demonstrated that, uptake, duration and *in vivo* immunostimulatory activity of oligodeoxynucleotides enhanced when encapsulated in sterically stabilized cationic liposomes (SSCL) [15–17]. Although co-administration of pIC with liposomes induced anti-viral immunity and activated effective CD8⁺ T cells *in vivo* [18], still several obstacles prevent these depot delivery systems enter into clinic. Of note, batch to batch variation, limited shelf-life, difficulty in reproducible reconstitution and sterility are the major concerns from FDA stand point. Furthermore, organic solvents used during the production of certain formulations are of major concern [19–21].

In the present study, natural amphiphilic polysaccharides purified from mushrooms were studied to assess their immunostimulatory

Abbreviations: AFM, Atomic Force Microscopy; BMDC, Bone marrow derived dendritic cell; HEK, Human embryonic kidney; NOD, Nucleotide-binding oligomerization domain; ODN, Oligodeoxynucleotide; PEC, Peritoneal exudate cells; PGN, Peptidoglycan; pIC, Poly(riboinosinic:ribocytidylic acid); PS, Polysaccharide.

* Corresponding author. Tel.: +90 312 290 24 08; fax: +90 312 266 50 97.

E-mail address: ihsangursel@bilkent.edu.tr (I. Gursel).

¹ Present address: Department of Molecular Biology, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland.

potential. Later, a nanoparticle delivery system was formulated to efficiently harbour pIC. The TLR2 mediated PS targeting and subsequent initiation of signal transduction cascade and ability of the nanoparticles to induce synergistic immune activation was evaluated. Furthermore, in an immunization study in mice co-delivering a model protein antigen ovalbumin (OVA), immunogenicity of PS-OVA-pIC nanoparticles was compared to either PS-OVA or pIC-OVA mediated immune response.

2. Materials and methods

2.1. Materials

All cell culture media components were from Hyclone (USA). Cytokine, IgG ELISA were from Endogen and Southern Biotech, respectively (USA). TLR ligands were obtained from several vendors: peptidoglycan (PGN) (Fluka, Switzerland), pIC (Amersham, UK). β -Glucan based polysaccharides were provided by Prof. Oktay Erbatır (Cukurova University, Chem Dept., Adana, Turkey). A high pressure/high temperature stainless steel reactor (Parr 4575 HT/HP) with 500 ml volume was used for extraction of polysaccharide at subcritical water conditions from different mushrooms. The sugar content of the extracts was determined by HPLC (Varian Prostar210, equipped with a RI detector) against monosaccharide standards and protein determination by Lowry assay. Monosaccharide analyses of the PS yielded mainly glucose, in addition to mannose and galactose. The purity was found to be >93%. Following protease digestion to eliminate protein contamination purity reached over 97%. Molecular weight determination was carried out by high performance size exclusion chromatography (on a HPSEC-MALLS system). In this study, polysaccharides were abbreviated as PS1 to PS4. Their physicochemical characteristics were as follow: i) *Ganoderma lucidum* (Alata strain)-PS1 (Ave. MWt: 2.9×10^6 Da and pKa: 6.64), ii) *G. lucidum* (Balcalı strain)-PS2 (Ave. MWt: 3.8×10^6 Da and pKa: 6.99), iii) *Shiitake*-PS3 (Ave. MWt: 1.6×10^6 Da and pKa: 6.67), and iv) *G. lucidum* (Alata strain)-PS4 (Ave. MWt: 5.2×10^6 Da and pKa: 6.69). Endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml). PS nanocomplexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4 °C. Unbound pIC was filtered (Microspin G-25 column) free nucleic acid concentration was measured by Nanodrop™ from the eluent. pIC incorporation was over 90% in all preliminary trials.

2.2. Mice

Adult C57BL/6 and BALB/C mice (female, 6–8 weeks old) were housed in Department of Molecular Biology and Genetics, facility and were provided with unlimited access of food and water. All experimental procedures were approved by the animal ethical committee of Bilkent University (Bil-AEC/Protocol#: 2006/027). RAW 264.7 cells (ATCC) or splenocytes were cultured with RPMI 1640 supplemented with 5% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.11 mg/ml sodium pyruvate. HEK 293 or stably *hTLR2* transfected cells (Invivogen, USA), were kept in high-glucose DMEM media with 10% FBS.

2.3. Immunization

Adult female C57/BL6 mice (5/group) were injected ip with of PS, pIC (15 μ g each) or PS-pIC nanocomplex combined with 7.5 μ g of OVA. One day before booster injection ($d = 13$) animals were bled and next day injected with the same formulations. On day 28 mice were bled. Sera from the primary and secondary bleedings were studied for total IgG, IgG1, and IgG2a by ELISA.

2.4. ELISA and NO assays

Immulon 2 HB microtitre plates (Thermo Scientific, USA) were coated with anti-cytokine or anti-IgG antibodies and then blocked with PBS-BSA 1% [17,35]. Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine Ab (TNF α : XT22 and IL6: 20F3 clones) followed by phosphatase-streptavidin (Perbio Pierce, USA) whereas bound IgG subclasses were detected using phosphatase-conjugated anti-IgG, IgG1, and IgG2a antibodies as described elsewhere [17]. Nitric oxide detection by Griess method was conducted on RAW 264.7 cells (10^5 /ml) after 6–48 h of incubation as described by the suppliers (Promega WI, USA).

2.5. Luciferase assay

hTLR2 expressing HEK cells were transfected using FuGENE6 with p5xLucNFkB luciferase as described in manufacturer's protocol. Following overnight stimulations, cells were further incubated for 24–36 h and were harvested and assayed for luciferase activity (Roche, Germany). Plots were generated from relative light units.

2.6. Cytokine and chemokine RT-PCR

Total RNA was extracted from the cells that were treated either with PS or with pIC nanocomplexes for 2–6 h. They were reverse-transcribed and amplified to obtain cDNA in a standard PCR reaction for 30 cycles using primers for murine specific target genes (Supplementary Table S2) as previously described [35,36]. PCR amplified material was separated on 2% agarose gels and visualized under UV light after ethidium bromide staining.

2.7. Atomic force microscopy (AFM) and size measurement studies of the nanocomplexes

pIC, PS4 and their nanocomplexes were diluted in DNase/RNase free H₂O and were deposited on silicon wafer. Following complete drying images were taken by using non-contact mode XE-100E model AFM (PSIA with XEI 1.6 software incorporated) with a 0.73–0.79 Hz scanning rate. The scanning area sizes were in $1 \times 1 \mu\text{m}$. Particle size analyses of the generated nanocomplexes were measured using dynamic light scattering method on a zetasizer (Model: Nano ZS, Malvern, UK).

2.8. Statistical analysis

Statistical significant differences between groups were determined using Student's t- test analysis via SigmaSTAT software. *P* values < 0.05 were considered significant.

3. Results

3.1. TNF α and nitric oxide production by polysaccharides

Initial experiments were designed to understand the immunostimulatory potential of the four PS candidates in dose-titration (20–0.02 $\mu\text{g/ml}$) assays. A well established positive control peptidoglycan (PGN) was run in parallel to compare the response raised by β -Glucan polymers. Fig. 1 shows that when RAW cells were treated with different PSs for 24 h, a dose dependent TNF α (Fig. 1A) and NO (Fig. 1B) production were induced especially by PS2 and PS4. PS4 was the most active among other tested PSs at doses

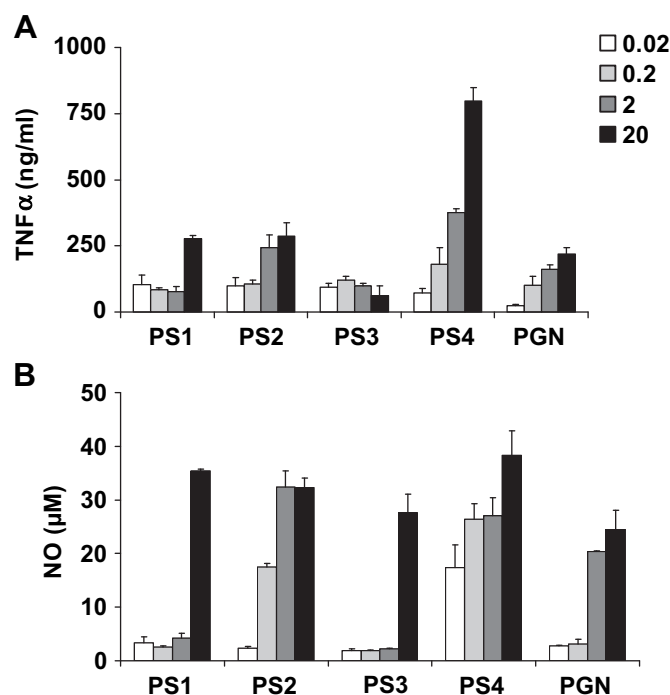


Fig. 1. Dose dependent TNF α and NO induction from RAW cells following 24 h post-stimulation with different β -Glucan polymers. A) TNF α and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean $\pm$ SEM) of triplicate samples treated with different stimulants (0.02–20 $\mu\text{g/ml}$). PS4 vs PS2 comparison gave a *p* < 0.001 for TNF α (at all doses) and *p* < 0.004 for NO (at 0.02 $\mu\text{g/ml}$).

greater or equal to 0.2 $\mu\text{g/ml}$ (Fig. 1A). In the case of NO induction PS4 was superior (at all doses) than the rest of the stimulants including positive control PGN (Fig. 1B).

3.2. PS mediated upregulation of various cytokine/chemokine transcripts

To examine the kinetics of PS driven immune activation RAW cells were incubated with different β -Glucan stimulants. The culture fluids collected at different time intervals (6, 12, 24, 36, and 48 h after stimulation) after 2 $\mu\text{g/ml}$ PS treatments were assayed for TNF α (Fig. 2A) and bactericidal mediator NO (Fig. 2B). As seen in Fig. 2A, as early as 6 h post-stimulation all PS types secreted substantial amount of TNF α . Among the tested groups, only PS4 type continued to increase TNF α level by 48 h. Similarly, nitric oxide secretion profiles of different PS types gave a time-dependent response. Again, consistent with Fig. 1, only PS2 and PS4 (Fig. 2B) displayed time-dependent NO production from macrophages. Also these β -Glucans' effects on mRNA transcript upregulation of TLRs as well as various cytokines were assessed after 4 h incubation. When TLR transcript levels of an untreated mouse splenocytes were compared to that of PS treated cells' transcript levels PS2 and PS4 treatment highly upregulated expression of *tlr1*, 2, 5, 7, and 9 genes (Supplementary Figure S1A). Moreover, compared to untreated vs PS treated cells, mRNA band intensities for *il-15*, *il-18*, and TNF α significantly increased only upon PS4 treatment (Supplementary Figure S1B). These results suggested that PS4 and then PS2 polysaccharides were the most potent stimulants.

3.3. Engagement of PS by TLR2

Several β -Glucan polymers were reported to trigger signaling cascade either using cell surface expressed TLR2 (alone or engaging

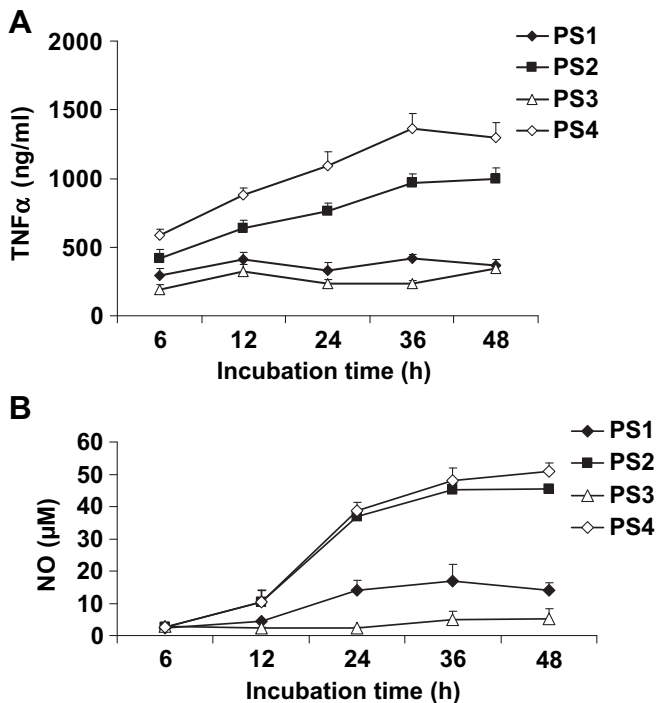


Fig. 2. Time dependent TNF α and NO release by RAW cells following different PS treatments at 2 $\mu\text{g/ml}$. A) TNF α and B) NO levels were assessed by ELISA and Griess assay respectively. Result represents combination of at least three independent experiments (mean $\pm$ SEM) of triplicate samples treated at different time points (6 h, 12 h, 24 h, 36 h & 48 h). PS4 vs PS1, PS2 (except 12 h) or PS3 comparison gave a $p < 0.01$ for TNF α (at all time points) and for NO, PS4 vs PS2 is NS, and PS4 vs PS1 or PS3 is $p < 0.001$ (at all time points except 6 h).

TLR1 or TLR6), TLR4 or even cytosolic sensors such as NOD1 and NOD2 [22,31]. To differentiate between these alternatives HEK cells stably expressing hTLR2, hTLR4 or TLR2/6 genes were transfected with p5xLucNF κ B and in subsequent experiments co-transfected with pcDNA3NOD1 or pcDNA3NOD2 or mock plasmids to check whether these PS polymers either engages only through TLRs or additionally interact with the cytosolic NOD sensors. In Fig. 3, NF κ B-mediated luciferase activity studies demonstrated that PS triggers via TLR2 receptor (Fig. 3A) and furthermore regulates IL8 production (Fig. 3B). Expression of TLR6 did not improve TLR2 mediated promoter activation (*data not shown*). Consistent with previous results, PS4 is the most potent inducer. Of note, other TLRs or NODs were not contributing to this immune cascade since subsequent experiments demonstrated that there was no further significant increase (either luciferase activity or IL8 production) mediated by PS treatment of cells expressing TLR4, or NOD1 or NOD2 (*data not shown*).

3.4. PS4 activity upon in vivo administration to mice

These four PSs were given to mice (2 $\mu\text{g/ml}$) and 6 h later PEC and spleen cells were recovered and incubated in culture without further stimulation to evaluate cytokine production or chemokine message transcript upregulation by ELISA and RT-PCR, respectively. Consistent with previous observations, PS4 was the most active stimulant among the tested polysaccharides. It yielded the highest IL6 production from PECs after 48 h *ex-vivo* incubation (Fig. 4A). The spleen cells TNF α and IP10 transcript levels were significantly upregulated when PS1, 2, and 3 vs PS4 band intensities were

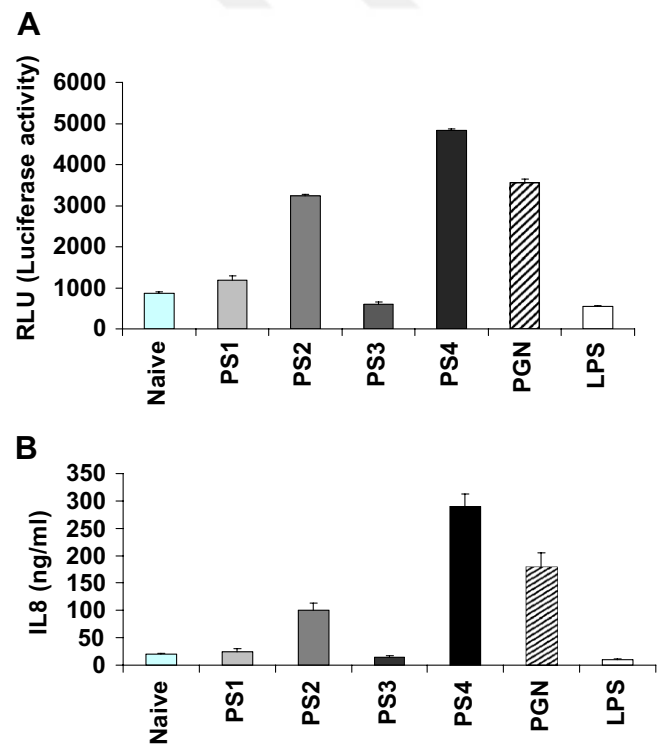


Fig. 3. PS mediated promoter assay and IL8 production by hTLR2-HEK cells. A) Relative luciferase activity after PS treatment of stable hTLR2-HEK cells. Cells were transfected with p5xLucNF κ B plasmid for 24 h and stimulated with 5 $\mu\text{g/ml}$ of each PS for 12 h. B) IL8 production following PS treatment. Stable hTLR2-HEK cells were stimulated with 5 $\mu\text{g/ml}$ of each PS for 24 h. IL8 ELISA was studied from the culture supernatants. Data represents combination of at least two independent experiments (mean $\pm$ SEM) of triplicate samples treated with different stimulants (PS1–4, PGN and LPS). PS4 vs PS1, 2, 3 and LPS gave a $p < 0.001$.

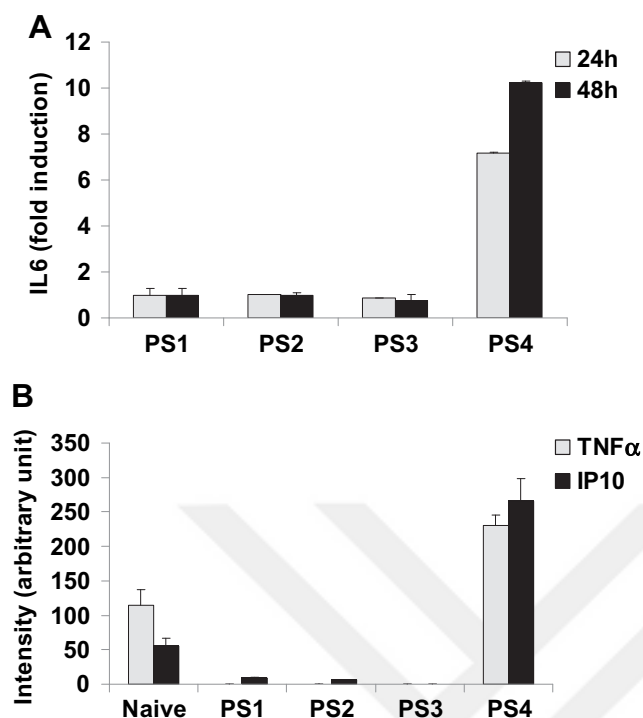


Fig. 4. *In vivo* immunostimulatory activity of polysaccharides. A) Six hours after i.p. PS injection (2 µg/ml), PECs were isolated (3 mice/group). Cells (4×10^6 /ml) were incubated *ex-vivo* for further 24 h or 48 h, IL6 levels from supernatants were measured by cytokine ELISA. Fold induction of IL6 production over naive cells was plotted (naive cells induced 56 ± 13 @ 24 h and 164 ± 23 @ 48 h pg/ml IL6). B) mRNA levels of TNFα and IP10 were assessed by RT-PCR from spleen cells. Densitometric measurements (obtained from 3 independent mice) for the gel band intensities were plotted. PS4 vs PS1, 2 and 3 gave a $p < 0.001$.

compared, (Fig. 4B). Although previously PS2 activity in culture was similar to PS4, surprisingly it failed to reproduce this trend when administered *in vivo*. When *in vitro* and *in vivo* data are collectively considered, among four tested PS candidates, PS4 consistently displayed the highest immunostimulatory performance, and was selected to develop PS4-pIC nanoparticles.

3.5. Physical characterization of PS4-pIC nanocomplexes

When nucleic acids are injected *in vivo* they are either subjected to premature digestion by nucleases or adsorbed by serum proteins, therefore, hampering their bioavailability. Accumulating evidence strongly suggested that their *in vivo* applications are limited due to their labile nature. Strategies to improve their stability as well as retaining activity and facilitate their cellular internalization without premature clearance are of great importance to pursue these ligands into clinical use [15,17]. To establish whether PS4-pIC complexes form stable nanoparticles their physical characteristics by i) AFM and by ii) dynamic light scattering techniques (Zetasizer Nano, ZS™) were studied. AFM photomicrographs revealed that PS4 appeared as individual nanoparticles around 50–70 nm in size (Fig. 5). This was further confirmed by Zetasizer and was found to be ca. 40 ± 20 nm (Ave $\pm$ SEM) (Supplementary Figure S2). PS4 nanocomplex with pIC led to an increase in the nanoparticle size from 40 nm to 165 nm (Supplementary Figure S2). The stability of these nanocomplexes was also studied by dynamic light scattering method using Zetasizer. There was no significant change in the size of the generated nanocomplexes over a period of two weeks indicating that there was no spontaneous fusion (or disintegration) of the individual particles when suspended either in PBS or in FBS

supplemented media (Supplementary Table S1, and data not shown).

3.6. Synergistic immune activation by PS4-pIC nanoparticles

Next we analyzed the immunostimulatory effect of PS4 nanocomplexes harbouring one of the labile nucleic acid ligands, pIC. Whether it improves pIC dependent immune activation to that of its free form is of great interest. As presented in Fig. 6, PS4-pIC nanoparticles induced synergistic activation on both RAW 264.7 or mouse spleen cells upon *in vitro* or *in vivo* treatments. PS4-pIC nanocomplexes led to a significantly high IL6 production (Fig. 6 and data not shown). It is important to note that pIC alone, when given at this concentration could not produce any detectable IL6, however, the nanoparticles yielded 1250 ± 57 ng/ml of IL6 secretion. This amount is >6x fold more cytokine production to that of PS4 alone stimulation (Fig. 6A left panel). Furthermore, macrophages stimulated by these nanocomplexes induced very strong bactericidal mediator, NO compared to either of the PS4 alone or pIC alone treatments (Supplementary Figure S3). When spleen cells were incubated with PS4-pIC nanocomplexes, the gene message levels of *tnfa*, *il15*, *il18*, *mip3α*, and *ip10* were significantly upregulated (compared to either PS4 alone or pIC alone treatments, Fig. 6B). The activity of these nanocomplexes were further analyzed following ip injection of mice (3/group) and checking IFNγ transcript level from recovered spleen cells (Fig. 6C). Consistently, PS4-pIC nanocomplex treated animals displayed the highest *ifnγ* message. These results strongly support the synergistic activation mediated by the nanocomplex.

3.7. Immunization with PS4-pIC nanoparticles

To examine whether the PS4-pIC nanoparticles could act as an effective immune adjuvant/delivery system, ovalbumin (OVA) was loaded as the model antigen and C57BL/6 mice were immunized. Primary and secondary bleeding sera were analyzed to detect the levels of total IgG, IgG1 and IgG2a. As seen in Fig. 7, total IgG and anti-OVA specific IgG subtypes were significantly boosted by the PS4-OVA-pIC nanoparticles when compared to dual combination groups (i.e. pIC + OVA or PS + OVA). This was evident even after primary immunization, since there was 2–3-fold more anti-OVA Ab induction at 1/2000 antibody titre, (Fig. 7A). As expected, significantly higher magnitude of total IgG, IgG1 as well as IgG2a titers were obtained after booster injection. In Fig. 7B, at titre 1/8000, all IgG types were improved at least 2-fold and up when mice were immunized with the nanocomplex. These data implied that generated stable nanoparticles ensured simultaneous internalization of the adjuvant (signal 1) and antigen (signal 2) by antigen presenting cells (APCs), thereby led to stronger anti-OVA immunity.

4. Discussion

Natural polysaccharides extracted from mushrooms was shown to activate host immune system leading to production of various Th1-biased cytokines [23]. One approach to induce an improved immune response is to ensure that these formulations achieve simultaneous presentation of antigen and adjuvant to relevant APCs. Developing such systems that act in synergy with the loaded adjuvant may further improve the success of the therapy and help host defence [14,24–26].

In this study, PS/Nucleic acid ligand nanocomplexes were designed to solve several key problems associated with poor *in vivo* performance. Polysaccharides used in this study are; i) naturally occurring, readily available, easily extracted in bulk ii) cheap iii) does not require sophisticated formulation technology as it is water soluble iv) readily undergoes stable interaction with charged

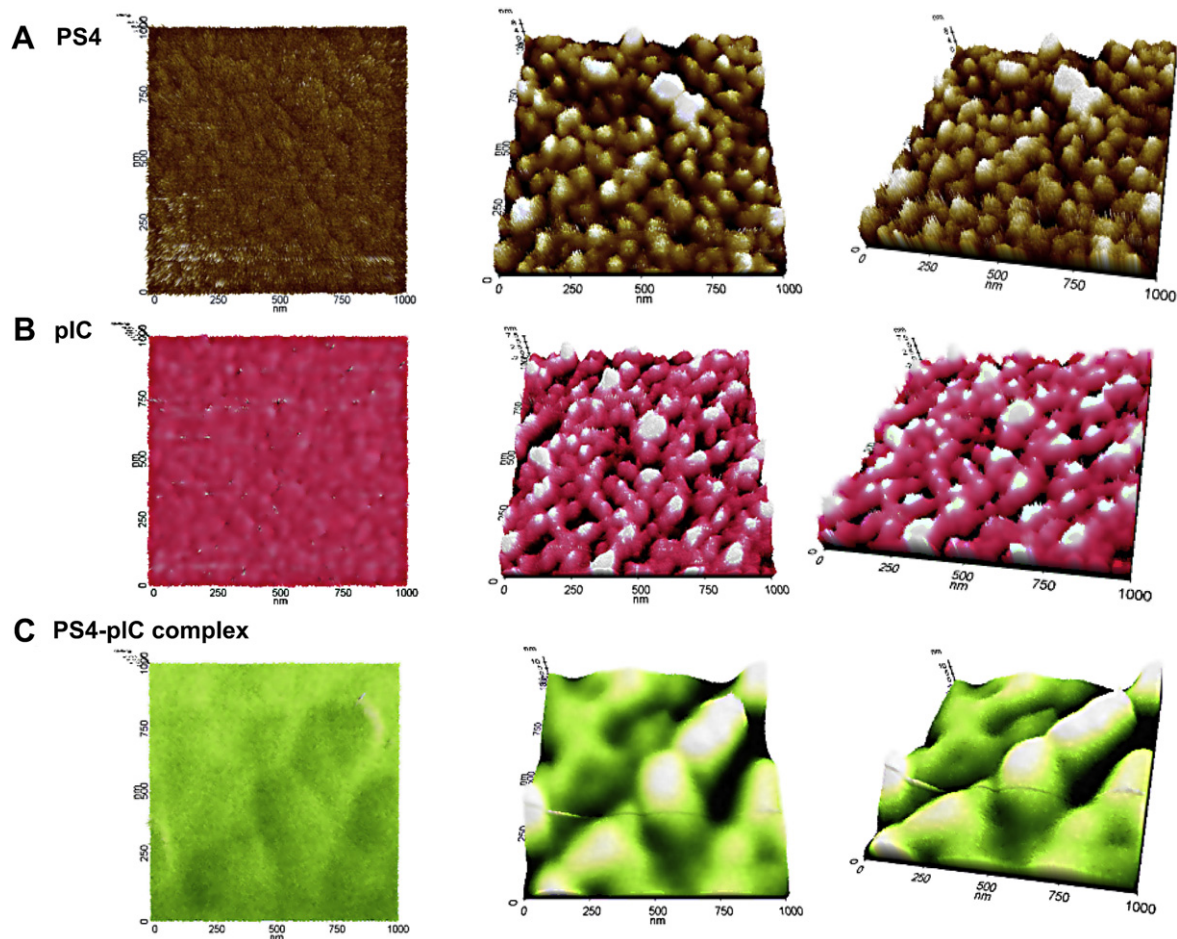


Fig. 5. AFM photomicrographs of phase, 2D phase (left) and 3D topography (middle and right) images. A) PS4, B) pIC and C) PS4-pIC nanoparticles at 1000 nm² area. Images were recorded under contact mode.

macromolecules due to their amphiphilic nature v) effectively engage by cells expressing TLR2 on their surface (i.e. natural targeting), vi) the labile cargo is protected from premature degradation/elimination and finally vii) act as a co-adjuvant in vaccine formulations along with the adjuvant to augment Ag-dependent immune activation. Our findings strongly suggest that all these key features are fulfilled by the developed formulation.

Recent studies showed that a natural β -glucan polysaccharide isolated from *Schizophyllum* modified with peptides and cholesterol formed a complex with TLR9 ligand, CpG ODNs and significantly elevated secretion of cytokines (IL12, IL6 and TNF α) from BMDCs, and murine macrophages [27,28]. These compounds were proposed to trigger several immune cells through various receptors including complement receptor (CR3), scavenger receptors, as well as TLR4 or TLR2/6 [29–32]. In other study, *G. lucidum* polysaccharides enhanced CD14 mediated endocytosis of LPS and promoted TLR2/4 signal transduction of cytokine expression [31].

Contrary to previous reports, our findings suggested that mushroom derived PS polymers recognized only via TLR2 but not via TLR4 or TLR2/6. Furthermore, to date, there is no document reporting the collaborative activation of TLR3 ligand with PS obtained from edible mushrooms. Moreover, in the present study plain PS compounds were used with no further modification (i.e. cholesterol or peptide). The cytokine secretion panels (dose-dependent and time-dependent findings) presented in Figs. 1 and 2 clearly suggested that PS4 is the most potent stimulant. Moreover, in HEK cell system we established

that these PS types only interact with TLR2 (Fig. 3) but not with other cell surface or cytosolic receptors (NOD1 or 2). In the literature, receptor usage of PS is still elusive. This is probably due to the problem associated with the extraction of PS from different sources. Similar compounds were reported to trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells through various receptors including Dectin-1 and scavenger receptors [31,32]. We have attempted to delineate the contribution of PS mediated activation via the scavenger receptor CXCL16. It was found that it does not involve in PS mediated cytokine production, since there was no up or down regulation of the *cxcl16* gene transcript (Supplementary Figure S2). However, Dectin-1 has not been studied here, and must be checked to resolve its involvement.

We further assessed the nitric oxide production mediated by different PS compounds. Since NO is an important regulator and mediator of macrophage directed cytotoxicity against microbes and tumour cells, detecting the most potent NO inducer as well as establishing the breadth of activation pattern of PS4 was very critical [33,34]. The results of *in vitro* findings were reproduced *in vivo* (Figs. 1, 2 and 4). Among all tested PSs, consistently PS4 triggered the highest IL6 production in addition to NO secretion as well as enhanced several key cytokine/chemokine gene expression in PECs and splenocytes. This is the first example to demonstrate that a polysaccharide extracted from mushrooms induced strong NO production from immune cells.

PS4-pIC nanoparticles supported the added effect of formulating them as a carrier depot system. In addition to IL6 production by

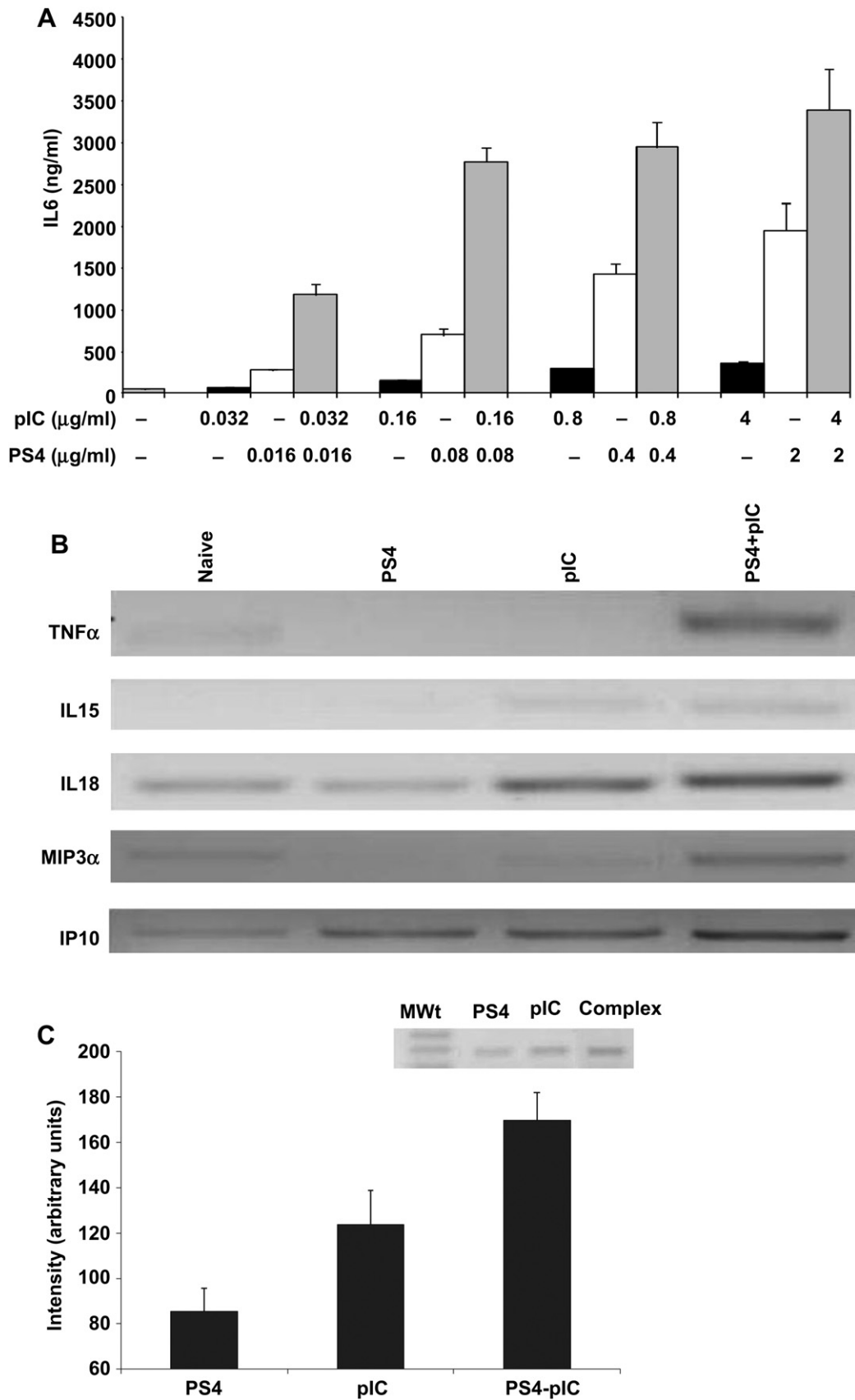


Fig. 6. PS-pIC nanoparticles induced higher IL6 production and strong gene upregulation of various cytokine and chemokines after *in vitro* and *in vivo* stimulations. A) RAW cell supernatants from 36 h culture were analyzed by ELISA. Result represents combination of at least two independent experiments (mean $\pm$ SEM) of triplicate samples. $p < 0.002$ nanocomplexes vs nucleic acid alone treated groups. B) After stimulation with PS4 (0.016 μ g/ml) or PS4-pI:C (0.16 μ g/ml each) for 4 h in culture, total RNA from spleen cells were subjected to RT-PCR and cDNA were amplified against gene-specific primers. C) PS4-pIC nanoparticles (2 μ g each) was injected to mice and spleen cells were recovered 18 h post-treatment for IFN γ transcript analyses (inlet; representative band intensities from a single animal). Band intensities were reported as average of 3 mice/group.

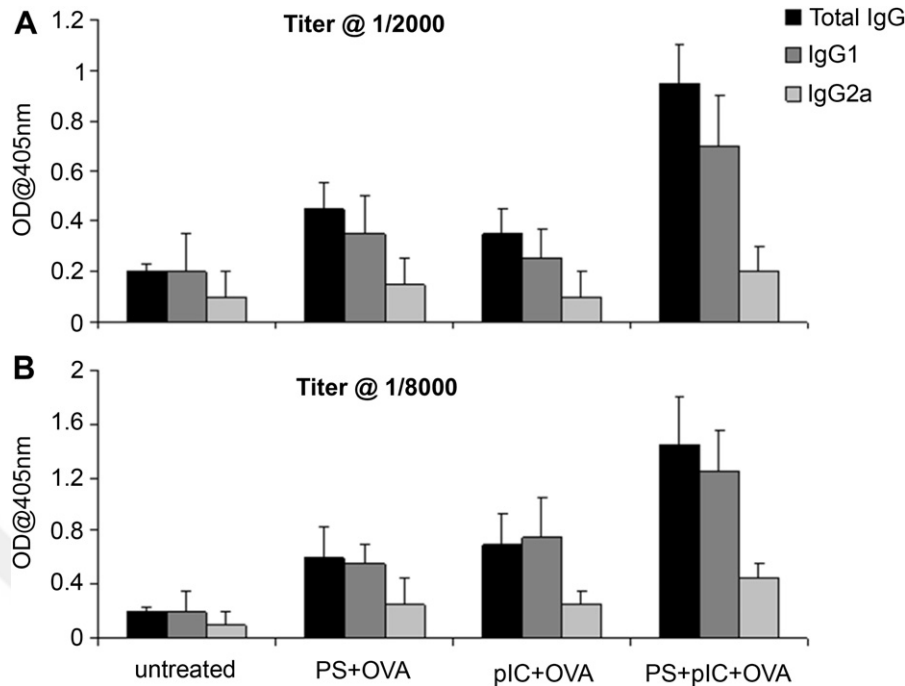


Fig. 7. IgG anti-OVA response of immunized mice. Animals were injected i.p with 15 μ g of PS4 + pIC combination or PS4-pIC nanoparticles incorporating 10 μ g OVA. Serum anti-OVA titers at day 13 (A) and day 28 (B) were determined by Total IgG, IgG1 and IgG2a ELISA. Data represents the average $\pm$ SD serum IgG anti-OVA response (five mice per group).

immune cells compared to their alone treatments (Fig. 6A), data also demonstrated that several cardinal chemokine and cytokine mRNA messages were upregulated (Fig. 6B). The *in vivo* benefit of PS4-pIC nanocomplexes were documented in *ex vivo* study along with model antigen immunization study. Data suggested that these formulations could improve primary and booster immunity against OVA antigen (Fig. 7). This is primarily due to the fact that, the present formulation helped avoiding premature clearance and digestion of its cargo as well as increased stability and efficient delivery of OVA + adjuvant simultaneously to APCs, thereby resulting more pronounced immune response.

5. Conclusions

The present work established that combination of mushroom derived polysaccharide and pIC forms stable nanocomplexes and triggered upregulation of inflammatory cytokines, and bactericidal mediators as evidenced by dose and time-dependent kinetic profiles of, IL6, IL18, TNF α and NO productions. Immune enhancement of PS is dependent on TLR2 signaling, compounded by the pIC mediated TLR3 signaling resulted stronger pro-inflammatory effect of the formulation. When used *in vivo* as a protein vaccine carrier, nanocomplex led to elevated IgG titers against ovalbumin antigen in mice, compared to either PS + OVA or pIC + OVA immunizations. In conclusion, present system can be formulated with other clinically valuable bioactive agents such as siRNA, plasmids, and different nucleic acid based TLR ligands, along with peptides or proteins as vaccine carriers to control infectious pathogenic insults.

Acknowledgements

This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant No: 108S316). IG received grant support from EU/FP6/MC IRG (Grant # 036615) and EU 7th Framework Project UNAM-Regpot (Grant # 203953). GT and FCY received scholarship grants from SANTEZ (0448-STZ-2009-2)

and TÜBİTAK. We thank to Dr. Aykutlu Dana and his group members for their invaluable support during AFM studies. Drs. Can K. Akcali and Mayda Gursel are sincerely acknowledged for their critical reading of the manuscript.

Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biomaterials.2011.01.028.

References

- [1] Janeway Jr CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216.
- [2] Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001;1:135–45.
- [3] Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;17:1–14.
- [4] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* 2008;3:352–63.
- [5] Schmidt KN, Leung B, Kwong M, Zarembka KA, Satyal S, Navas TA, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J Immunol* 2004;172:138–43.
- [6] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther* 1999;10:2461–80.
- [7] Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, et al. Bacterial DNA causes septic shock. *Nature* 1997;386:336–7.
- [8] Krieg AM, Efler SM, Wittpoth M, Adhami AI, M.J., Davis HL. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J Immunother* 2004;27:460–71.
- [9] Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF, et al. Immune stimulation—a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des* 1997;12:421–32.
- [10] Sparwasser T, Hultner L, Koch ES, Luz A, Lipford GB, Wagner H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol* 1999;162:2368–74.
- [11] Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J Immunol* 2000;165:1228–35.

- [12] Borges O, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. *Int Immunopharmacol* 2008;8:1773–80.
- [13] Fischer S, Schlosser E, Mueller M, Csaba N, Merkle HP, Groettrup M, et al. Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. *J Drug Target* 2009;17:652–61.
- [14] Schlosser E, Mueller M, Fischer S, Basta S, Busch DH, Gander B, et al. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 2008;26:1626–37.
- [15] Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J Immunol* 2001;167:3324–8.
- [16] Suzuki Y, Wakita D, Chamoto K, Narita Y, Tsuji T, Takeshima T, et al. Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. *Cancer Res* 2004;64:8754–60.
- [17] Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB, et al. CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infect Immun* 2005;73:828–33.
- [18] Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J Immunol* 2006;176:7335–45.
- [19] Mozafari MR. Liposomes: an overview of manufacturing techniques. *Cell Mol Biol Lett* 2005;10:711–9.
- [20] Crommelin DJ, Storm G. Liposomes: from the bench to the bed. *J Liposome Res* 2003;13:33–6.
- [21] Chirila TV, Rakoczy PE, Garrett KL, Lou X, Constable IJ. The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides. *Biomaterials* 2002;23:321–42.
- [22] Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Nunez G. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* 2008;28:246–57.
- [23] Lin ZB, Zhang HN. Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. *Acta Pharmacol Sin* 2004;25:1387–95.
- [24] Bourquin C, Anz D, Zwiorek K, Lanz AL, Fuchs S, Weigel S, et al. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. *J Immunol* 2008;181:2990–8.
- [25] Wilson KD, de Jong SD, Tam YK. Lipid-based delivery of CpG oligonucleotides enhances immunotherapeutic efficacy. *Adv Drug Deliv Rev* 2009;61:233–42.
- [26] Mizu M, Koumoto K, Anada T, Matsumoto T, Numata M, Shinkai S, et al. A polysaccharide carrier for immunostimulatory CpG DNAs to enhance cytokine secretion. *J Am Chem Soc* 2004;126:8372–3.
- [27] Shimada N, Coban C, Takeda Y, Mizu M, Minari J, Anada T, et al. A polysaccharide carrier to effectively deliver native phosphodiester CpG DNA to antigen-presenting cells. *Bioconjug Chem* 2007;18:1280–6.
- [28] Mizu M, Koumoto K, Kimura T, Sakurai K, Shinkai S. Protection of polynucleotides against nuclease-mediated hydrolysis by complexation with schizophyllan. *Biomaterials* 2004;25:3109–16.
- [29] Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. *J Hematol Oncol* 2009;2:25.
- [30] Zhu XL, Lin ZB. Effects of *Ganoderma lucidum* polysaccharides on proliferation and cytotoxicity of cytokine-induced killer cells. *Acta Pharmacol Sin* 2005;26:1130–7.
- [31] Hua KF, Hsu HY, Chao LK, Chen ST, Yang WB, Hsu J, et al. *Ganoderma lucidum* polysaccharides enhance CD14 endocytosis of LPS and promote TLR4 signal transduction of cytokine expression. *J Cell Physiol* 2007;212:537–50.
- [32] Lin YL, Liang YC, Lee SS, Chiang BL. Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. *J Leukoc Biol* 2005;78:533–43.
- [33] Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992;6:3051–64.
- [34] Chin, M. P., Schauer, D. B. and Deen, W. M., Nitric oxide, oxygen, and superoxide formation and consumption in macrophages and colonic epithelial cells. *Chem Res Toxicol*.
- [35] Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol* 2003;171:1393–400.
- [36] Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;25:386–401.

Permissions to the Copyrighted Materials



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Membrane vesicles as conveyors of immune responses

Author: Clotilde Thery, Matias Ostrowski and Elodie Segura

Publication: Nature Reviews Immunology

Publisher: Nature Publishing Group

Date: Jun 5, 2009

Copyright © 2009, Rights Managed by Nature Publishing Group

Logged in as:
Tamer Kahraman
Bilkent University
Account #:
3001009329

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Bilkent University -- Tamer Kahraman ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3830801418432
License date	Mar 16, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Membrane vesicles as conveyors of immune responses
Licensed content author	Clotilde Thery, Matias Ostrowski and Elodie Segura
Licensed content date	Jun 5, 2009
Type of Use	reuse in a dissertation / thesis
Volume number	9
Issue number	8
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
High-res required	no
Figures	Figure 1, 3 and 4
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Therapeutic and Diagnostic Applications of Extracellular Vesicles
Expected completion date	Mar 2016
Estimated size (number of pages)	200
Total	0.00 USD

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2016 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#). [Terms and Conditions](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Extracellular vesicles: biology and emerging therapeutic opportunities

Author: Samir EL Andaloussi, Imre Mäger, Xandra O. Breakefield, Matthew J. A. Wood

Publication: Nature Reviews Drug Discovery

Publisher: Nature Publishing Group

Date: Apr 15, 2013

Copyright © 2013, Rights Managed by Nature Publishing Group

Logged in as:
Tamer Kahraman
Bilkent University
Account #:
3001009329

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Bilkent University -- Tamer Kahraman ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3830810708100
License date	Mar 16, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Drug Discovery
Licensed content title	Extracellular vesicles: biology and emerging therapeutic opportunities
Licensed content author	Samir EL Andaloussi, Imre Mäger, Xandra O. Breakefield, Matthew J. A. Wood
Licensed content date	Apr 15, 2013
Type of Use	reuse in a dissertation / thesis
Volume number	12
Issue number	5
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Therapeutic and Diagnostic Applications of Extracellular Vesicles
Expected completion date	Mar 2016
Estimated size (number of pages)	200
Total	0.00 USD

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2016 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#). [Terms and Conditions](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Emerging role of extracellular vesicles in inflammatory diseases

Author: Edit I. Buzas, Bence György, György Nagy, András Falus, Steffen Gay

Logged in as:
Tamer Kahraman
Bilkent University
Account #: 3001009329

[LOGOUT](#)

Publication: Nature Reviews Rheumatology

Publisher: Nature Publishing Group

Date: Feb 18, 2014

Copyright © 2014, Rights Managed by Nature Publishing Group

Order Completed

Thank you very much for your order.

This is a License Agreement between Bilkent University -- Tamer Kahraman ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3830810904285
License date	Mar 16, 2016
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Rheumatology
Licensed content title	Emerging role of extracellular vesicles in inflammatory diseases
Licensed content author	Edit I. Buzas, Bence György, György Nagy, András Falus, Steffen Gay
Licensed content date	Feb 18, 2014
Type of Use	reuse in a dissertation / thesis
Volume number	10
Issue number	6
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 3
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Therapeutic and Diagnostic Applications of Extracellular Vesicles
Expected completion date	Mar 2016
Estimated size (number of pages)	200
Total	0.00 USD

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2016 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#). [Terms and Conditions](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com



RightsLink®

[Home](#)
[Account Info](#)
[Help](#)


Title: Microparticles, thrombosis and cancer
Author: Anat Aharon, Benjamin Brenner
Publication: Best Practice & Research Clinical Haematology
Publisher: Elsevier
Date: March 2009
 Copyright © 2008 Elsevier Ltd. All rights reserved.

Logged in as:
 Tamer Kahraman
 Bilkent University
 Account #: 3001009329

[LOGOUT](#)

Order Completed

Thank you very much for your order.

This is a License Agreement between Bilkent University -- Tamer Kahraman ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3830811134399
License date	Mar 16, 2016
Licensed content publisher	Elsevier
Licensed content publication	Best Practice & Research Clinical Haematology
Licensed content title	Microparticles, thrombosis and cancer
Licensed content author	Anat Aharon, Benjamin Brenner
Licensed content date	March 2009
Licensed content volume number	22
Licensed content issue number	1
Number of pages	9
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	Figure 2
Title of your thesis/dissertation	Therapeutic and Diagnostic Applications of Extracellular Vesicles
Expected completion date	Mar 2016
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

[ORDER MORE...](#)
[CLOSE WINDOW](#)

Copyright © 2016 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#). [Terms and Conditions](#).
 Comments? We would like to hear from you. E-mail us at customercare@copyright.com