

THE DEVELOPMENT OF PROPHYLACTIC AND THERAPEUTIC VACCINE USING CELL DERIVED EXTRACELLULAR VESICLES

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By
Muzaffer Yıldırım
August 2022

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

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ABSTRACT

The Development of Prophylactic and Therapeutic Vaccine using Cell Derived Extracellular Vesicles

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Ph.D. in Molecular Biology and Genetics

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The primary aim of this thesis is to extend the breadth of *in vivo* application of externally loaded cell-line derived and tumor derived exosomes as a prophylactic and therapeutic carrier against cancer treatment. Exosomes with a size between 30 to 150 nm are small extracellular vesicles secreted by all types of mammalian cells. They mediate a novel mode of intercellular communication through their bioactive cargos such as lipids, nucleic acids, metabolites, and proteins, which can be delivered to the target cells. Exosomes have successfully served as immunotherapeutic nanocarriers in cancer treatment using their natural delivery capabilities. Furthermore, they are attractive as a delivery system because of their stability in circulation, biocompatibility, and low toxicity. In the first part of this thesis, we used exosomes as a nanocarrier system to develop cancer vaccines in a therapeutic murine melanoma cancer treatment. We show that lyophilization of exosomes together with the CpG ODN, model antigen OVA and lipidic ligand alpha-galactosylceramide (α GC) followed by controlled reconstitution is successfully accomplished. We analyzed the effect of the lyophilization on a cell line-derived exosomes and we characterized the exosomes by using bead-based technique via flow cytometry, qNano, Scanning electron microscopy, and western blotting. We showed that lyophilization does not harm exosomes' vesicular integrity and fundamental biological features. Furthermore, we tested the biodistribution and activating capacity of encapsulated exosomes in mouse PEC, mesenteric lymph node, and spleen cells. We found out that loaded exosomes are mostly taken up by antigen-presenting cells. Also, we showed that loading CpG ODN into exosomes significantly improves APC activation markers in macrophages, B cells, and DCs and induced significantly higher IFN γ production from mouse mLN and splenocytes. Finally, we tested the therapeutic utility of the CpG ODN, OVA and α GC encapsulating exosome in the B16F10-OVA melanoma tumor-bearing mice. We found out that therapeutic vaccination with triple (CpG ODN, OVA, and α GC) ligand encapsulating exosomes suppressed the progression of established melanoma tumors in mice.

Moreover, our triple ligand loaded exosomes triggered Th-1 biased anti-IgG OVA immunity and converted immune cells in tumor microenvironment to the tumor-suppressing phenotype. In the second part of this thesis, we used tumor-derived exosomes (TEXs) as an immunotherapeutic cancer vaccine. These nanovesicles are inherently possesses rich tumor antigen reservoirs. Due to their undesirable features such as poor or limited immunogenicity as well as facilitation of cancer development via mediating communication between tumor cells, TEXs could be transformed into an effective immune adjuvant delivery system that initiates a strong humoral and cell-mediated tumor-specific immune response. In this study, we evaluated to immunogenicity of 4T1/Her2 cell-derived exosomes upon loading them with two potent immuno adjuvant, a TLR9 ligand, K-type CpG ODN and a TLR3 ligand, p(I:C). We showed that engineered TEXs co-encapsulating both ligands displayed boosted immunostimulatory properties by activating antigen-specific primary and memory T cell responses. Furthermore, our exosome-based vaccine candidate elicited robust Th1-biased immunity as evidenced by elevated secretion of IgG2a and IFN γ . In a therapeutic breast cancer model, we found out that administration of 4T1 tumor derived exosomes loaded with CpG ODN and p(I:C) to animals regressed tumor growth in 4T1 tumor-bearing mice. As a result, this work implicated that an exosome based therapeutic vaccine promoted strong cellular and humoral anti-tumor immunity that is sufficient to reverse established tumors. The last part of this thesis, we studied the therapeutic potential of cell line-derived exosomes loaded with superparamagnetic iron oxide nanoparticles(SPION) and immunostimulatory ligands. We showed that loading SPION enhanced the in vitro delivery of exosomes within immune cells. Also, we found out that spleen cells incubated with exosomes encapsulating with SPION and CpG ODN induced significantly higher levels of IL-12 and IFN γ . Finally, we tested our exosomal vaccine candidate in human hepatocellular carcinoma tumor model in athymic mice. We showed that TLR3 and TLR9 ligands encapsulated with SPION loaded exosomes induced pronounced innate immune activation and regressed tumors and improve survival rate of treated mice.

Keywords: Extracellular vesicles, Exosomes, Vaccine carrier, Anti-tumor therapy, SPION, Tumor derived Exosomes, TLR ligands

ÖZET

Hücre Kökenli Hücre Dışı Kesecikler Kullanılarak Profilaktik ve Terapötik Aşı Geliştirilmesi

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Bu tezin temel amacı, kanser tedavisine karşı profilaktik ve terapötik bir taşıyıcı olarak, dıştan yüklenmiş olan hücre hattından ve tümör hattından üretilmiş eksozomların *in vivo* uygulanabilirliğinin genişletilmesidir. 30 ila 150 nm arasında bir boyuta sahip eksozomlar, her tür memeli hücresi tarafından salgılanan küçük hücre dışı keseciklerdir. Eksozomlar lipidler, nükleik asitler, metabolitler ve proteinler gibi biyoaktif kargoları aracılığıyla yeni bir hücreler arası iletişim moduna aracılık ederler. Eksozomlar, doğal dağıtım yeteneklerini kullanarak kanser tedavisinde immün terapötik nano taşıyıcılar olarak başarılı bir şekilde hizmet etmektedirler. Ayrıca, eksozomlar dolaşımdaki kararlılıkları, biyouyumlulukları ve düşük toksisiteleri nedeniyle bir dağıtım sistemi olarak ilgi çekicidirler. Bu tezin ilk bölümünde, terapötik bir murin melanom kanser aşısı geliştirmek için eksozomları bir nano taşıyıcı sistem olarak kullandık. Eksozomları CpG ODN, model antijen OVA ve lipidik ligand alfa-galaktosilseramid (aGC) ile liyofilizasyon yöntemi sayesinde başarıyla yükledik. Liyofilizasyonun hücre hattından üretilmiş eksozomlar üzerindeki etkisini akış sitometrisi, qNano, taramalı elektron mikroskobu ve western blot yöntemi ile analiz ettik. Liyofilizasyonun eksozomların veziküller bütünlüğüne ve temel biyolojik özelliklerine zarar vermediğini bulduk. Ayrıca, fare PEC, mezenterik lenf düğümü ve dalak hücrelerinde yüklenmiş eksozomların biyolojik dağılımını ve bağışıklık hücrelerini aktive etme kapasitesini test ettik. Yüklü eksozomların çoğunlukla antijen sunan hücreler (APC) tarafından alındığını gösterdik. Ayrıca, CpG ODN'nin eksozomlara yüklenmesinin makrofajlarda, B hücrelerinde ve dendritik hücreler' de (DC) APC aktivasyon belirteçlerini önemli ölçüde artırdığını ve fare mezenterik lenf düğümü ve splenositlerinden önemli ölçüde IFN γ üretimini indüklediğini gösterdik. Son olarak, B16F10-OVA melanom tümörü taşıyan farelerde, CpG ODN, OVA ve aGC yüklü eksozomların terapötik etkisini test ettik. Üçlü (CpG ODN, OVA ve aGC) ligand yüklü eksozomlarla terapötik aşılamanın, farelerde yerleşik melanom tümörlerinin ilerlemesini baskıladığını bulduk. Ayrıca, üçlü ligand yüklü eksozomlar, Th-1 destekli anti-IgG OVA

başıklığını tetikledi ve tümör mikro çevresindeki başıklık hücrelerini tümör baskılayıcı fenotipe dönüştürdü. Bu tezin ikinci bölümünde, immün terapötik bir kanser aşısı olarak tümör kaynaklı eksozomlar (TEX'ler) kullandık. Bu hücre dışı kesecikler doğal zengin tümör antijen rezervuarlarına sahiptir. Zayıf veya sınırlı başıklık oluşturma gibi istenmeyen özellikleri ve tümör hücreleri arasındaki iletişime aracılık ederek kanser gelişimini kolaylaştırması nedeniyle, TEX'ler güçlü humoral ve hücre aracılı tümöre özgü başıklık tepkisini başlatan etkili bir immün adjuvan dağıtım sistemine dönüştürülebilir. Bu çalışmada, 4T1/Her2 hücre kökenli eksozomlar iki güçlü immün adjuvan CpG ODN ve p(I:C) ile yüklendikten sonra bu eksozomların başıklık oluşturma kapasitelerini değerlendirdik. Her iki ligand ile yüklenmiş TEX'ler antijene özgü birincil ve bellek T hücresi yanıtlarını aktive etti ve güçlendirilmiş immün sistemi uyarıcı özellikler gösterdi. Ayrıca, eksozom bazlı aşı adayımız, yüksek IgG2a ve IFN γ salgılayarak güçlü Th1-destekli başıklık ortaya çıkardı. CpG ODN ve p(I:C) yüklü 4T1 tümör kökenli eksozomların meme kanseri modelinde terapötik olarak farelere uygulanmasının, 4T1 tümör taşıyan farelerde tümör büyümesini gerilediğini bulduk. Sonuç olarak, bu çalışma, eksozom bazlı bir terapötik aşının, yerleşik tümörleri tersine çevirmek için yeterli olan güçlü hücre ve humoral anti-tümör başıklığını desteklediğini ortaya koydu. Bu tezin son bölümünde, süper paramanyetik demir oksit nanopartiküller (SPION) ve immün sistemi uyarıcı ligandlarla yüklü hücre hattından üretilmiş eksozomların terapötik potansiyelini inceledik. Öncelikle, SPION yüklemesinin eksozomların başıklık hücreleri tarafından alınımını arttırdığını gösterdik. Ayrıca, SPION ve CpG ODN yüklü eksozomlarla inkübe edilen dalak hücrelerinin, önemli ölçüde yüksek IL-12 ve IFN γ indüklediğini bulduk. Son olarak, atimik farelerde oluşturulan insan hepatoselüler karsinom tümör modelinde eksozomal aşı adayımızı test ettik. SPION, TLR3 ve TLR9 ligandları ile yüklenmiş eksozomlar doğuştan gelen başıklık aktivasyonunu indükledi. Ayrıca, hepatoselüler karsinom tümör oluşturan atimik farelerde tümörlerin gerilediği ve tedavi edilen farelerin hayatta kalma oranını artırdığını gösterdik.

Anahtar Kelimer: Hücre Dışı Kesecikler, Eksozomlar, Aşı Taşıyıcı Sistemler, Anti-tümör Tedavisi, Süper Paramanyetik demir Oksit Nanopartiküller, Tümör kaynaklı Eksozomlar, TLR Ligandları



To my wife and daughter...

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Abbreviations

α GC	α -Galactosylceramide
AMA 1	Apical Membrane Antigen 1
AFM	Atomic Force Microscopy
ALIX	ALG-2 Interacting Protein X
APC	Antigen Presenting Cells
BM	Bone Marrow
CD	Cluster of Differentiation
CSFE	Carboxyfluorescein Succinimidyl Ester
CX3CL 1	CX3C Chemokine Ligand
CCL 5	CC-Chemokine Ligand 5
DNA	Deoxyribonucleic Acid
DC	Dendritic Cell
ELISA	Enzyme-linked Immunosorbent Assay
EV	Extracellular Vesicle
ESCRT	Endosomal Sorting Complex required for Transport
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
FasL	Fas Ligand
HPV	Human Papilloma Virus
HSP	Heat Shock Protein
IRF 3	Interferon Regulatory Factor 3

Ig	Immunoglobulin
ISEV	International Society of Extracellular Vesicles
ICAM 3	Intercellular adhesion molecule 3
ILV	Intraluminal Vesicle
IL	Interleukin
IFN	Interferon
MDA-5	Melanoma-Differentiation Associate Gene 5
MRI	Magnetic Resonance Imaging
miRNA	Micro Ribonucleic acid
mLN	Mesenteric Lymph Node
MSC	Mesenchymal Stem Cell
MV	Microvesicles
MISEV 2018	The Minimal Information for Studies of EVs 2018
MVB	Multivesicular Body
MHC	Major Histocompatibility Complex
MDSC	Myeloid derived Suppressor Cell
NSCLC	Non-Small Cell Lung Cancer
NKT	Natural Killer T Cell
nSmase 2	Neutral Sphingomyelinase 2
NK	Natural Killer Cell
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
Poly I:C	Polyinosinic:polycytidylic Acid

PEG	Phospholipid-polyethylene Glycol
PEC	Peritoneal Exudate Cell
PAMP	Pathogen Associated Molecular Patterns
PD-L1	Programmed Death Ligand 1
PTEN	Phosphatase and Tensin Homolog
PDPK 1	3-Phosphoinositide-dependent Protein Kinase 1
RIP 1	Receptor Interacting Protein 1
RIG I	Retinoic acid-inducible Protein I
rRNA	Ribosomal Ribonucleic acid
RNA	Ribonucleic acid
SPION	Super paramagnetic Iron oxide Nanoparticle
SEM	Scanning Electron Microscopy
TLR	Toll-like Receptor
TRAF 6	TNF Receptor Associated Factor
TRPS	Tunable-Resistive Pulse Sensing
TEM	Transmission Electron Microscopy
TSG 101	Tumor Susceptibility Gene 101
TCR	T Cell Receptor
Th1	T Helper 1 Cell
TEX	Tumor-derived Exosome
TGF- β	Transforming Growth Factor
VTE	Venous Thromboembolism
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

1.1 Extracellular Vesicles

Extracellular vesicles (EVs) were discovered in the 1940s while preliminary studies were working on the biological significance of the thromboplastin protein of blood [1]. Different speeds of centrifugation affected the clotting times of plasma. Prolonged high-speed centrifugation (150 min at 31,000g) significantly extended the clotting time of the supernatant [1]. Also, the clotting times shortened when the pellet containing clotting factor was added to the plasma. This indicated that cell-free plasma contains a subcellular fraction that promotes blood clotting [1]. After more than 20 years later, this subcellular fraction was identified by electron microscopy. It originated from platelets that consisted of small vesicles.[2]. These vesicles were reported to have a diameter between 20 and 50 nm and are called “platelet dust” [2]. Ten years later, fetal calf serum was also shown to contain numerous microvesicles ranging from 30 to 60 nm [3].

Further studies have shown that EVs are membrane-derived vesicles released by cells into the extracellular space. They have essential roles in cell-cell communication and regulate various biological processes. [4,5] All types of cells, prokaryotic and eukaryotic cells, secrete EVs in their environment. EVs produced by eukaryotic cells have been investigated in far more depth. All mammalian cell types studied so far, including endothelial cells [6], epithelial cells [7], neuronal cells [8], and mesenchymal stem cells (MSCs) [9], have been found to secrete EVs. Also, EVs can be found in biological fluids such as saliva [10], synovial fluid [11], blood [12], and urine [13].

1.1.1 Classes and biogenesis of EVs

Recent studies in the EVs area suggest that EVs can be classified into three central populations: microvesicles, apoptotic bodies, and exosomes. EV populations are based on their biogenesis mechanisms and biophysical properties (Fig 1.1). A lipid bilayer encloses all EVs. They range from 50 nm to 2,000 nm in diameter [15].

The International Society of Extracellular Vesicles (ISEV) has published guidelines to help researchers characterize EVs. The Minimal Information for Studies of EVs 2018 (MISEV2018) has advised EV researchers to consider the use of terminology that indicates the physical characteristics of EVs. For example, small EVs (exosomes) might be designated as <100 nm or <200 nm and should be considered the presence of specific biochemical compositions or the condition or the cell of origin [15].

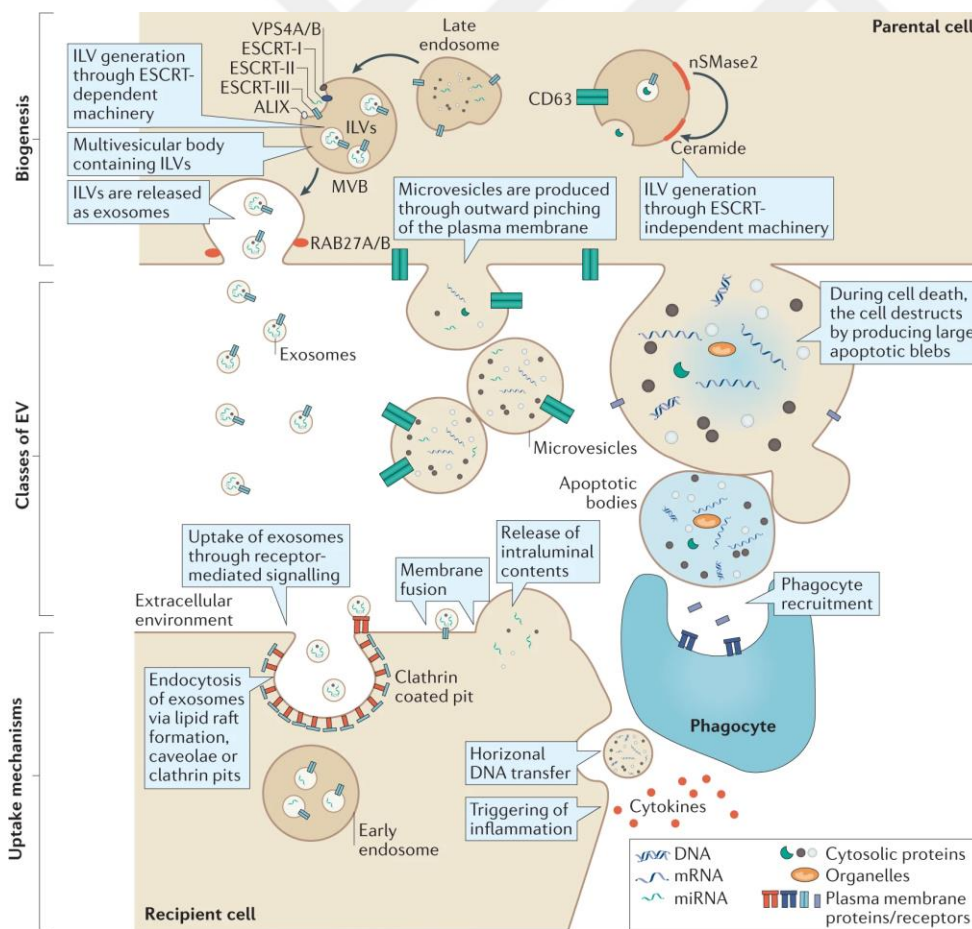


Figure 1.1 Classes of EVs, their biogenesis and features, and methods of isolating them. Adopted from [14]

1.1.1.1 Microvesicles

Microvesicles (MVs) range from 150 nm to larger than 1,000 nm. Their average size is 250–400 nm [16]. Microvesicles also can be referred to as large EVs by MISEV2018 [15]. Microvesicles are produced through the outward budding of the cell membrane (Fig 1.1). Their cargo is distributed randomly. Microvesicles are characterized by plasma membrane receptors, lipid composition, and molecules reflecting their cellular origin. MVs derived by cell membrane express, selectins, CD40 molecule, cytoskeletal protein, and integrins. Their membranes are also enriched in phosphatidylserine, cholesterol, and diacylglycerol [17,18]. MVs cargo consists of biomolecules trafficked towards the plasma membrane and contain mRNA and fragmented ribosomal RNA (rRNA) [19,20].

Microvesicles can be isolated from conditioned media and biological fluids. Firstly, Apoptotic bodies are removed, and the supernatant is centrifugated at 10,000g-50,000g. There are different strategies available to characterize MVs. MVs are characterized by nanoparticle tracking analysis to calculate size distributions and numbers of MVs in suspension, western blot to detect specific cytoskeletal proteins, electron microscopy-based approaches to show morphology, and flow cytometry combined with antibodies against surface markers. MVs are also studied on their cargo, such as bioactive lipid content, protein, and mRNA species, by employing “omics” technologies.

1.1.1.2 Apoptotic Bodies

When cells undergo apoptosis, they release phosphatidylserine exposing vesicles, often called apoptotic bodies. The significant difference between apoptotic bodies and other extracellular vesicles is their size. Apoptotic bodies represent the largest class of EVs. Studies show that the diameter of apoptotic bodies is reported to range between 1 and 5 μm [21,22].

They are produced through the characteristic blebbing of the apoptotic cell membrane during cell death (Fig. 1.1). Apoptotic vesicles originating from the plasma membrane contain DNA and histones. Apoptotic vesicles originating from the endoplasmic reticulum do not contain DNA and histones but expose immature glycoepitopes [23]. Apoptotic bodies are removed through phagocytosis by macrophage recognition of plasma membrane markers [24]. One of the reasons for developing a systemic autoimmune disease is the inappropriate clearance of apoptotic bodies.

After removing cell debris, apoptotic bodies can be isolated at low centrifuge speeds of 2,000g–4,500g. They are favorable for almost all cellular markers. Therefore, size is essential in distinguishing apoptotic bodies from other EVs.

Studies indicated that Apoptotic bodies express CX3C chemokine ligand 1 (CX3CL1) and intercellular adhesion molecule 3 (ICAM3). Thus, phagocytic cells bind these receptors for engulfment [25,26]. Unlike the microvesicles and exosomes, apoptotic bodies contain an abundant 18S and 28S rRNA. Chromatin can be observed within large round apoptotic bodies under transmission electron microscopy [27].

Recently, Studies speculated that apoptotic cells interact with other cells through apoptotic bodies to propagate tumorigenicity and horizontal DNA transfer [28] and promote inflammation [29].

1.1.1.3 Exosomes

Exosomes with a size between 30 to 150 nm are small extracellular vesicles secreted (any small EVs irrespective of their biogenesis) by all types of mammalian cells [30]. Exosomes have been visualized as cup-shaped under the TEM. They are surrounded by a phospholipid membrane containing high sphingomyelin levels, cholesterol, detergent-resistant lipid rafts, and ceramide [31]. Exosomes have the same membrane protein as the cell.

Exosomes are characterized by the presence of membrane transport and fusion proteins such as flotillin, annexins, GTPases, and Rab. Exosomes also contain components of the endosomal sorting complex required for transport (ESCRT) complex, such as tumor susceptibility gene 101 (TSG101), Alix, heat shock proteins (HSPs), tetraspanins including CD63, CD81, CD9, and integrins [32-35].

Exosomes are heterogeneous populations produced by two different mechanisms (Fig 1.1). They are named ‘classical’ or ‘non-classical’ exosomes. Classical exosomes are formed through the endosomal pathway. They are produced through inward budding of the late endosomal membrane to create the multivesicular body (MVB) (Fig. 1.1). The endosomal sorting complex required for transport (ESCRT) proteins, including ESCRT-0, ESCRT-I (tumor susceptibility gene 101 (TSG101)), ESCRT-II, and ESCRT-III. They are involved in recruiting cargo and forming the inward budding of the late endosomal membrane [36]. ESCRT-0, ESCRT-I, and ESCRT-II are the proteins responsible for recognizing and loading ubiquitinated cargo into the lumen of endosomes.

ESCRT-II proteins activate the assembly of ESCRT-III proteins. ESCRT-III proteins then recruit accessory proteins such as ALG-2-interacting protein X (ALIX) and VPS4 to arrange the formation of intraluminal vesicles (ILVs) in MVBs.

ESCRT-independent pathways also produce exosomes. Neutral sphingomyelinase 2 (nSmase2) proteins form sphingomyelinase hydrolysis and ceramide to mediate the inward budding of MVB in ESCRT-independent pathways [37]. Then, ILVs are created through the spontaneous negative curvature to the membrane of the MVB by ceramide. Next, The MVB fuses with the cellular membrane and releases the ILVs into the extracellular space [38].

Exosome secretion at the plasma membrane is required RAS-related proteins RAB27A and RAB27B. ILVs can package cytosolic proteins, specific proteins, endosomal proteins, and genomic cargo during their formation in the MVB. ILVs are the miniature of the parental cells [39]. Since tetraspanins are involved in recycling routes between the plasma membrane and several cellular organelles, they have an essential role in determining EV cargo [40,41].

After removing apoptotic bodies and microvesicles, exosomes are generally isolated by ultracentrifugation at 100,000g-200,000g. They can be further purified by an Optiprep gradient [42]. Tetraspanins such as CD63, CD9, and CD81, proteins involved in MVB formation like ALIX, TSG101, and membrane trafficking proteins, are highly enriched into exosomes [43].

Table 1.1. Differential characteristics of EVs [44]

	Exosome	Microvesicles	Apoptotic bodies
Size	30-100 nm	100-1000 nm	1-5 μ m
Origin	MVB fusion with the PM	Budding from PM	Budding from PM
Markers	Tetraspanins (CD9, CD63, CD81), Alix, TSG101, HSP70	Annexin V, Integrin, Selectin, CD40 ligand	Annexin V, DNA, caspase 3, Histones
Isolation method	Ultracentrifugation (100,000g)	Ultracentrifugation (10,000g-50,000g)	Ultracentrifugation (6,000g-10,000g)

1.1.2 Physiological Functions of EV

When researchers studied the loss of transferrin receptors 30 years ago, they identified the EVs, but they thought it was just cell debris [45]. However, EV research attracts attention after B cell-derived exosomes can present antigens to T cells [46]. Eventually, EVs released from healthy and disease conditions have essential roles in ensuring cell-to-cell communication [47]. They also regulate biological processes from immune response to cellular maintenance [48].

1.1.2.1 Cell-cell Communication

Extracellular vesicles released by cells have an essential mediator of cell-to-cell communication [49]. They transfer EV cargos containing lipids, proteins, and regulatory RNA between cells [50]. This presents a new scenario for our understanding of signal and molecule transfers between local and long-term cells.

All cell types secrete EVs into their environment, and studies show that these have a vital role in physiological and pathophysiological processes.

EVs use different mechanisms to mediate cell-cell communication (Fig 1.2). They can directly fuse with the plasma membrane of recipient cells to allow the exchange of transmembrane proteins and lipids [52]. Endocytosis is another way to EV-uptake mechanisms [39]. Ligand/receptor interactions may also contribute to the EV-uptake mechanism [31].

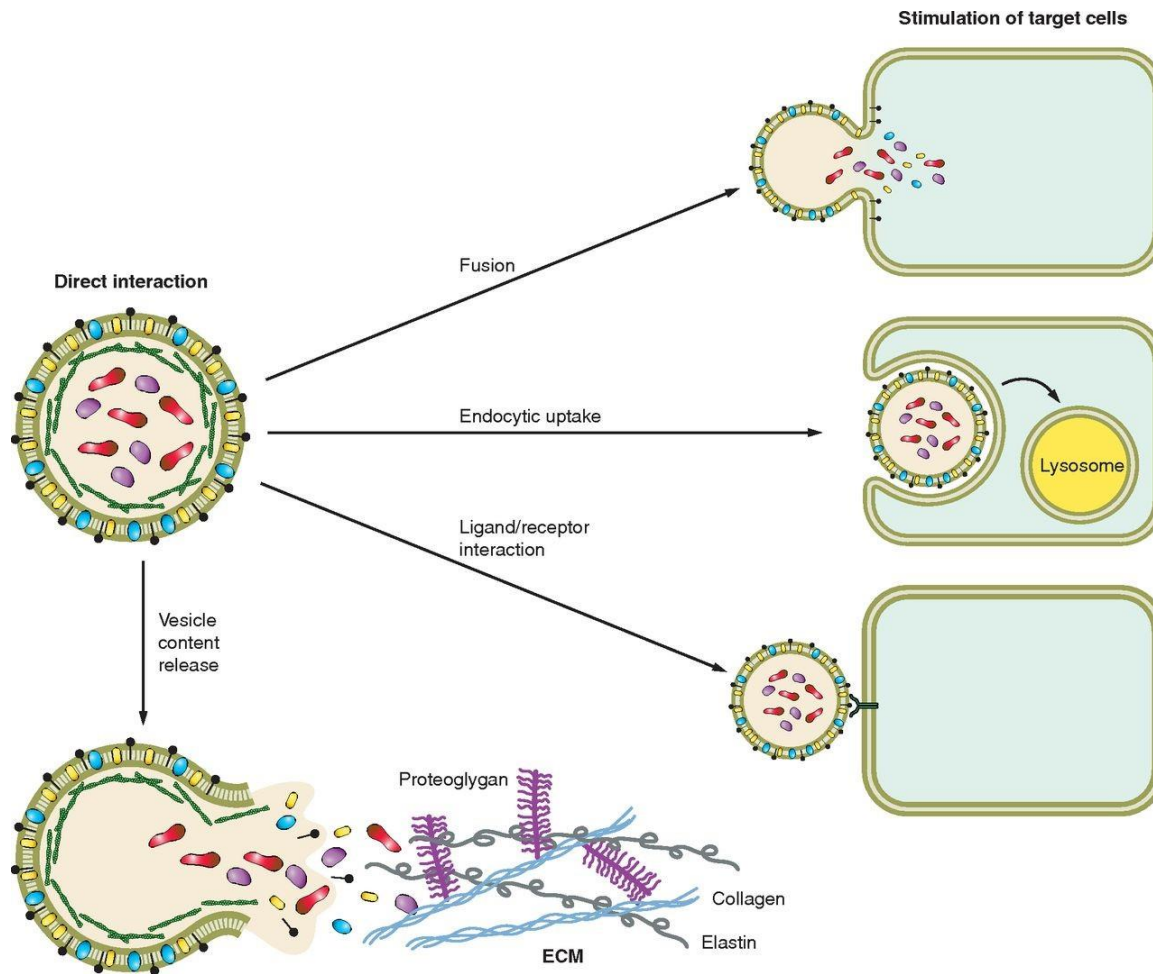


Figure 1.2 Mechanisms whereby EVs achieve their biological effects. (Adopted from [51])

1.1.2.2 Tissue development and maintenance

Packaging cell-surface receptors such as heparan sulfate proteoglycans onto the surface of EVs are facilitated by delivering growth factors and adhesion proteins to the suitable tissues, which can contribute to exosome–cell interactions [53,54]. Transfer of the functional EV cargo can regulate the expression of the extracellular matrix [50]. This can control cellular migration, proliferation, and organ morphogenesis [48,55,56]. For example, EVs derived from bone marrow, or stromal stem cells contain specific miRNA cargoes that control osteoblastic differentiation to promote osteoblast activity and bone regeneration [57]. EVs derived from astrocytes in the brain have specific proteins that promote synaptic transmission and neuronal survival [58]. Also, there is increasing evidence that endothelial lineage cells direct organ development by relying on a cargo of EVs containing miRNA and protein [59].

The role of EVs in cell-to-cell communication has been intensively studied in the past decade. Further studies suggest that EVs can act as cellular waste bags for cellular systems. Maintaining cellular homeostasis is a vital biological process by removing harmful cellular contents. This is especially true for pathophysiological environments like accumulating misfolded proteins within neurons during neurodegenerative diseases.

EVs contribute to removing harmful cytoplasmic DNA in normal cells as a self-defense mechanism for physiological maintenance [60]. DNASE2A is required for autophagy-mediated delivery of DNA to lysosomes to remove the EV containing damaged self-DNA [61,62].

Researchers should better understand EVs' role in physiological waste removal since balancing the role of EV secretion and uptake within the disease system has recently been well studied. This role of EVs can be researched by observing the removal or uptake of non-physiological doses of exogenous EV load designed for therapeutics.

1.1.2.2 Immune Response

The immune system is the defense mechanism against viral or bacterial infections. Non-immune and immune-associated cells are involved in this response. Generally, antigen-presenting cells directly interact with T cells and natural killer (NK) cells through cell surface proteins to regulate an immune response [63]. Numerous types of immune cells release EVs in their environment. The physiological role of EVs within the immune system is complex. EVs containing HLA molecules are critical for initiating antigen presentation and immune response. They can mediate the exchange of membrane and cytosolic compartments over long-distance without cells [64,65]. Researchers showed that dendritic cell-derived extracellular vesicles elicited anti-tumor effects and suppressed tumor growth in a cell-dependent manner in vivo. This is most likely through heat shock proteins and integrins [66,67].

The adaptive immune response is initiated by professional antigen-presenting cells. Dendritic cells are the most powerful APCs. They efficiently trigger T cells' activation, differentiation, and proliferation [68,69]. The immune synapse is a communication mechanism that relies on contact-dependent antigen presentation between APCs and T cells [70,71]. EVs carrying ligands and receptors can simultaneously interact with cell-surface molecules on recipient cells. This is a typical example of contact-dependent cellular activation [72]. DC-derived EVs were the first

described and most extensively studied EVs in the context of the immune response [66,67,73]. The researchers indicated that T cell stimulation efficiency by DCs is enhanced by DC-EVs exchange. This showed that EVs have a critical role in coordinating immune function [74,76].

Antigen-dependent interaction with cognate major histocompatibility complexes (MHC) on APCs is mediated by the accumulation of the T cell receptors (TCRs) on the surface of EVs [77]. Secretion of MHC and CD9 by APCs as EVs result in the transfer of these molecules to interacting T cells [41].

DC-derived EVs can make stable interaction with TCR complexes on T cells even in the absence of intact APCs, thus can activate T cells [78,79]. APC-derived EVs preserves the original topology of APCs. This provides the exposing the extracellular domain of MHC molecules on the vesicle surface. The researcher proposed that EVs secreted from peripheral DCs sensitize immature lymph node-resident DCs with specific interaction between peptide-MHC complexes before they arrive at lymph nodes [79]. The spreading of antigen-specific MHC complexes between DCs by EVs magnified T cell activation and induced a potent immune response.

EVs derived from macrophages also contain MHC class II heterodimer bound to antigenic peptides, which perform a similar function to DC-derived EVs. This contributes the activation of primed CD8⁺ and CD4⁺ T cells through specific TCR-peptide interaction [80,82]. Additionally, specific peptide-MHC complexes can be transferred to follicular DCs, and stromal cells in lymph nodes through EVs mediated antigen presentation. This primes CD4⁺ T cells and activates B cells [83,84].

Moreover, several studies showed that DC-derived EVs enhanced T cell activation and humoral responses through MHC class I and II molecules [65,85]. EVs derived from mature DCs activate T cells more efficiently than immature DCs. This indicated that EVs mediated T cell activation depends on DC developmental stage [86,87]. These studies highlight that APC-derived EVs play a significant role in antigen presentation and immune activation, even if their effects are direct or indirect.

EVs derived from T cells contribute to the crosstalk between various innate immune cells and can activate these cells. Human mast cells are stimulated through T cell-derived EVs containing LFA-1 molecules. This causes mast cell degranulation and releases IL8 and oncostatin M in a MAPK

signaling-dependent manner. Therefore, this study suggests that EVs can carry mast cell-activating factors like their original cells [88]. On the contrary, the function of DCs can be modulated by uptake of T cell-derived EVs, which leads to down-regulation of peptide-MHC I complex expression and induces DCs apoptosis through the Fas/Fas ligand (FasL) pathway [89].

Despite several studies focusing on this issue, the molecular mechanism of T cell-derived EVs on the immune response is little known. EVs derived from human resting T cells can contain cytoskeletal-related proteins. Additionally, EVs derived from activated T cells can contain RAS signaling pathway-related proteins such as ZAP70, RASGRP1, and AKT involved in immune receptor signaling and metabolisms. Thus, these EVs can activate MAPK/ERK pathway in recipient mast cells and non-activated T cells [90]. Therefore, researchers should study how T and cell-derived EVs affect the immune response. This case can be used for a therapeutic benefit such as activating or suppressing immune responses in various diseases.

Few studies are focusing on B cell-derived EVs. Activated B cell-derived EVs efficiently induced primed antigen-specific T cells. This role of EVs suggests that EVs derived from B cells are the modulator of an existing immune response [46,92]. Recently, one study showed that B cell-derived EVs mediate cytotoxic T cell immunity. In this study, EVs derived from B cells containing MHC class I molecules collaborate with host MHC class I expressed on splenic CD8 α ⁺ DCs. This result in antigen presentation to CD8⁺ T cells [93]. A recent study showed a correlation between EVs derived from B cells in plasma of patients with solid tumors and B cell responses and responsiveness to immunotherapies [94]. EVs isolated from the cancer patients' plasma contain mostly immunoglobulins, and specific immunoglobulins can be discriminated between health and cancer patients [95]. These studies show that adaptive immune cell-derived EVs can transport immunomodulatory components that enhance immune response among various cell types (Fig 1.3).

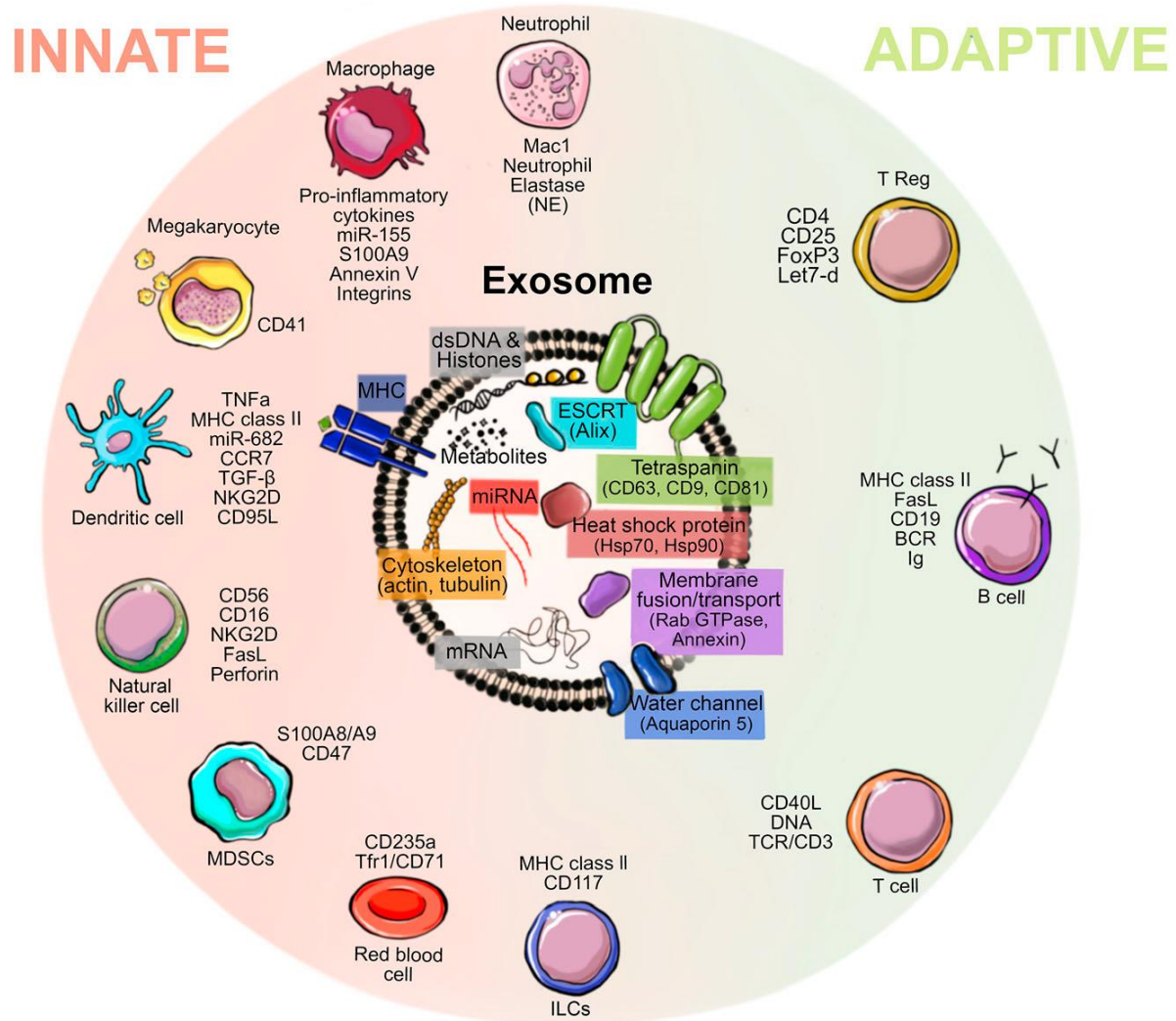


Figure 1.3. Immune cell-derived EVs cargo. EVs carry membrane, cytosolic, and even nuclear molecules such as DNA, RNA, lipids, and proteins, present inside and outside the vesicle. (Adopted from [91])

Immune cell-derived EVs have diverse cargo such as cytokines, proteins, lipids, and nucleic acids. Their pleiotropic function relies on their cargo content. EVs can protect specific cytokines from degradation, which provides increased half-lives of cytokines in circulation and delivers cytokines to particular cells. Studies showed that EVs derived from DCs and macrophages could contain pro-form and mature pro-inflammatory IL-1 β and IL-18 cytokines through distinct mechanisms [96,97]. IL-1 β and IL-18 cytokines are required for inflammasome activation. Therefore, EVs packaged IL-1 β , and IL-18 precursors prepare the way for inflammasome activation [98].

miRNAs are another most studied EVs cargo because of their well-described regulatory functions. The first described source of mRNA and miRNA packaged in EVs among immune cells are from mast cells. In these studies, secreted EVs containing mRNA and miRNA resulted in activation and degranulation of recipient cells [4,99]. It is now clear that immune cell types have a unique profile of miRNA in their EVs cargo, which is a specific target and function. For example, macrophages secrete miR-155 packaged in EVs in response to endotoxin stimulation. These EVs promote inflammatory response to LPS in vivo [100,101]. In short, EVs provide miRNA transfer between cells, regulating gene expression and generating proper inflammatory responses.

Lipids are an essential part of the EV membrane. Studies also showed that Lipids could be enriched within EVs [102,103]. One study indicated that EVs derived from asthmatic patients during chronic airway inflammation had altered lipid composition than healthy control. This suggests that EVs' lipid composition may affect their function [104].

Immune cell-derived EVs also have immunosuppressive effects in response to various stimuli. EVs derived from immune cells can behave differently against pathogens or the presence of tumors to prevent excessive immune response (Fig 1.4). T regulatory cells (Treg) are specialized immune cells that regulate self-tolerance and suppress exaggerated immune responses. Because of this reason, EVs derived from Treg cells can have the capacity to suppress T helper 1 (Th1) cell proliferation and IFN- γ cytokine secretion [105]. Treg-derived EVs can also induce tolerogenic DCs [106].

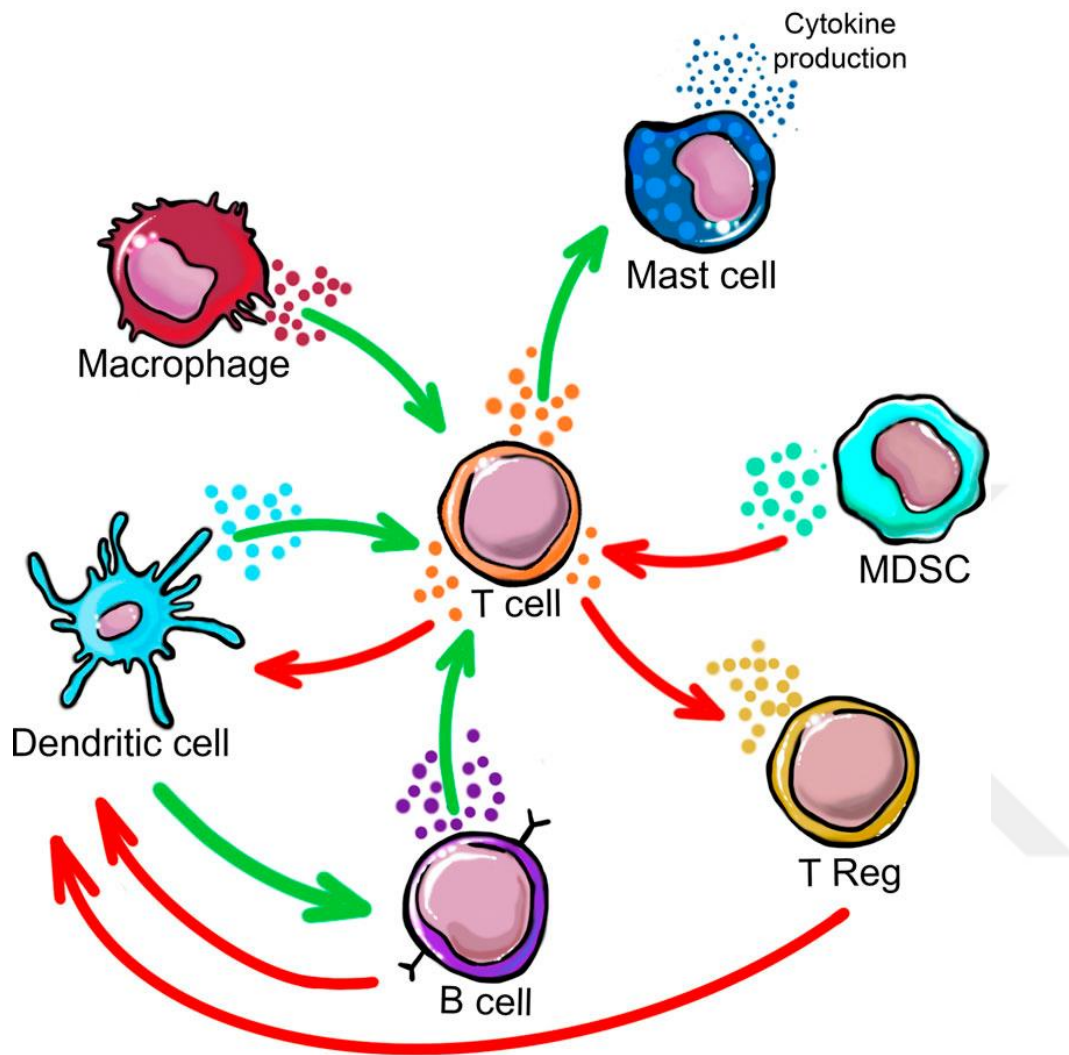


Figure 1.4. Immune cell-derived EVs orchestrate immune responses. Immune cell-derived EVs balance immune activation (green arrows) and immune suppression (red arrows). (Adopted from [91])

Other immune cell types regulate immune suppression called myeloid-derived suppressor cells (MDSCs). MDSCs prevent T cell activation. They also polarize macrophages toward tumor-promoting phenotypes [107]. Therefore, MDSCs-derived EVs also have a suppressive effect on immune cells [108]. For example, CD47 is a molecule that protects cells from phagocytosis. CD47 expressed on MDSC-derived EVs is a chemoattractant for MDSCs to maintain immunosuppressive capacity [109].

Studies showed that EVs containing miR-146a exchange between bone marrow (BM) derived DCs inhibit the immune activation in response to pathogen-associated molecular patterns (PAMPs) such as bacterial endotoxin [100]. EVs containing immunosuppressive molecules such as death-ligand FasL, NKG2D, and TGF- β derived from immature DCs can inhibit macrophages, natural killer (NK) cells, and neutrophils in response to tumors. [110,111].

Programmed death ligand 1 (PD-L1) is a crucial immune evasion molecule. Up-regulation of PD-L1 interacts with the corresponding PD-1 receptor on T cells to suppress T cell activation. Therefore, tumor cells have upregulated expression of PD-L1 molecules to hijack T cell activation. Anti-PD-1 antibodies are used as promising immunotherapy for cancer treatment. [112]. For this reason, resistance to anti-PD-1/PD-L1 antibody therapy in cancer treatment can be overcome by understanding the mechanism of EVs containing PD-L1 molecules for the success of immunotherapies.

As a result, EVs can either activate or suppress immune responses with respect to originating cell type (Fig 1.4).

1.1.3 Role of EVs in disease

EVs are essential mediators of cell-to-cell communication. They also contribute to the disease's progress. EVs derived from dysfunctional cell spreads their pathological cargos into the environment. After finding that increased cancer invasiveness, metastasis, and disease progression correlate with the secretion of EVs, EVs' role in cancer studies gained attention.

1.1.3.1 EVs in cancer

Tumor growth can be accelerated by tumor-derived EVs (TEXs), and this can promote tumor metastasis by manipulating neighboring tissues to create a favorable tumor microenvironment. An increasing number of studies indicate TEXs contain functional oncogenic RNAs and oncoproteins. These studies demonstrated that TEXs promote the generation of premetastatic niches that mimic the traits of the primary tumor [5,113,114,115]. Since TEXs travel long distances within the lymphatic system or bloodstream, secondary metastatic sites can be predicted [116]. Also, analyzing the integrin expression profile of TEXs can give a clue about their target cells in specific distant organs [117].

Tumor-derived EVs have a crucial role in the colonization of metastatic tumors. Even if studying the pathogenic role of TEXs is challenging, there is increasing evidence that the subpopulation of EVs may have different pathophysiological effects [118,119]. Additional tumorigenic content packaged in several subpopulations of EVs can work in harmony to escape from the primary site. Thus, they can promote the pre-metastatic niche to invade neighboring tissues. For instance, Study found out that Wnt10b from Wnt family proteins is significantly increased in EVs. These EVs can induce an epithelial-mesenchymal transition to promote tumor invasion [120]. Another study showed that microvesicles containing phosphorylated epidermal growth factor receptor (EGFR) contribute to the tumor microenvironment to suppress annexin A, which occurs in head and neck cancer tissues [121]. Furthermore, tumor-derived microvesicles containing vascular endothelial growth factor (VEGF) promote angiogenesis and tumor growth in neighboring cells [122]. In addition, different types of cells can take up different populations of EVs [117,123]. For example, EVs derived from pancreatic ductal adenocarcinoma are mainly taken up by macrophages located in the liver. These EVs can contribute to the pre-metastatic setting. Bone-marrow-derived cells can be recruited to the liver in a pre-metastatic environment. Thus, secretion of TGF β can be upregulated, leading to activation of fibrosis and further tumor burden [123].

Many studies focus on how EVs select cancer-promoting cargo and how their cargo content changes in cancer development. Some studies proposed that cancer cells can hijack the ESCRT pathway to increase EV secretion—consequently, this increased upregulation of EVs containing cancer-related molecules [124]. EVs released from primary tumor cells can arrange cell to cell transfer of oncogenic factors through the ESCRT-mediated pathway to healthy neighboring cells. C-src, a proto-oncogene that regulates cell migration, growth, and adhesion, can be encapsulated into EVs by interacting with ALIX. Thus, it can increase EV secretion to promote tumor growth [124]. Deleting the ALIX causes a decrease in EVs expressed PD-L1. This result decreases tumor-associated immunosuppression and suppresses tumor growth in vivo models of breast cancer [125]. Also, another study showed that silencing CHMP4B and VPS4A, which are ESCRT-III assembly components, decreased the β -catenin release into EVs. Reducing the release of β -catenin into EVs inhibited cell migration and metastasis of hepatocellular carcinoma [126].

RNA and small RNA, particularly miRNAs, can be packaged within EVs. These RNAs regulating several biological pathways are functional in recipient cells [4,127]. For instance, EVs secreted from KRAS mutated colorectal cancer cells affect healthy cells through miRNAs enriched in EVs. This causes the induction of proteins involved in cell growth and migration [115]. In different studies, EVs derived from prostate cancer stem cells are highly expressed in miR-100-5p and miR-21-5p. When these EVs are cultured with normal fibroblasts cells, they form pre-metastatic niches and promote cell migration [128]. Furthermore, miR-143, miR-21, and miR-378e packaged in EVs stimulate epithelial-mesenchymal transition phenotype in breast cancer cells [129]. These results showed that miRNAs packaged in EVs could induce epithelial-mesenchymal transition, contribute to pre-metastatic niches, and increase cell invasion and migration.

There are lots of oncogenic factors explored in tumor-derived EVs supporting tumor growth [130,131]. Researchers showed that human brain microvascular endothelial cells take up to TEXs derived from primary glioblastoma cells. In this study, EVs cargo contains angiogenic proteins such as interleukin-6 (IL6), angiogenin, and IL8. This induces angiogenesis in human brain microvascular endothelial cells [5]. EVs enriched oncogenic factors also proliferate stromal cells. The proliferation of stromal cells provides the vessels and connective tissue with nutritional support. In another study, researchers found out that adipose-tissue-derived MSCs induce tumor-supportive cytokines such as VEGF, transforming growth factor- β (TGF β) and CC-chemokine

ligand 5 (CCL5) when cells incubated with EVs isolated from breast adenocarcinoma cell lines [114].

Lung, breast, and melanoma cancers are highly metastatic cancer that spread to the brain even if the brain is protected by the blood-brain barrier [132]. Few studies are focusing on how EVs bypass the blood-brain barrier. Astrocytes play an essential role in regulating blood-brain barrier permeability [133,134]. Researchers explored that EVs derived from deregulated astrocyte cells promote metastatic brain cancer. These EVs contain upregulated phosphatase and tensin homolog (PTEN)-targeting miRNA(miR-19a). Therefore, the expression of PTEN, a tumor suppressor, is lost in metastatic brain tumors by transferring these EVs [135]. In different studies, Tumor-derived EVs bypassed blood-brain barriers by moving miRNAs that target the proteins involved in actin remodeling and tight junctions [136,137]. In these studies, EVs derived from highly metastatic human mammary tumor cells have elevated miR-181c. miR-181c causes the downregulation of its target 3-phosphoinositide-dependent protein kinase 1 (PDPK1). Thus, actin dynamics can be reorganized to allow for extravasation [136]. In addition, EVs containing miR-105 derived from cancer cells downregulates the tight junction protein zonula occludens protein 1 (ZO1) to break the barrier function of endothelial monolayers [137].

1.1.4 Clinical application of EVs

Extracellular vesicles have therapeutic potential in cancer and immune disorders because of their bioactive cargos. Since EVs have intercellular communication capacity, they are also being investigated for the delivery of exogenous therapeutic loads such as siRNA [138], anti-inflammatory drugs [139], and tumor associated antigens for inducing immune response [140]. EVs can also be explored as a source of biomarkers and prognostic potential in disease [141,142].

1.1.4.1 Therapeutic application of EVs

Potential therapeutic applications of EVs are currently a very active area of research due to the role of EVs in biological functions. EVs can be used for vaccine development and potential exogenous cargo loading. However, there are some challenges in each of these approaches. Furthermore, EVs' pharmacokinetics and therapeutic efficacy should be extensively studied to become clinically approved therapeutic agents since the field of EVs is just in its infancy. Nevertheless, Since EVs have played a significant role as a mediator in cell-cell communication and modulate immune responses, they are ideal candidates for cancer immunotherapies.

1.1.4.1.1 Cancer

EVs derived from DCs express functional MHC class I and II molecules and T cell costimulatory molecules. Therefore, Since DC-derived EVs contain MHC class I and II molecules together with costimulatory molecules, researchers mainly explored these EVs to induce potent antigen-specific T cell response and tumor suppression in vivo for the immunotherapeutic application [66,67,143]. The efficacy of EVs derived from immature DC loaded with tumor-associated antigen peptides was explored for melanoma and non-small cell lung cancer (NSCLC) patients in two separate phase 1 clinical trial [140,144]. These studies found that DC-derived EVs have little therapeutic benefit because of poor immunostimulation, even if EVs are nontoxic to patients. In another study, patients with NSCLC were treated with EVs derived from IFN- γ -matured DC in phase 2 clinical trials. Similar to the previous phase 1 studies, IFN- γ -matured DC-derived EVs had a limited activation effect on T cells and NK cells [145]. For these reasons, researchers focused on how the efficiency of DC-derived EVs can be enhanced. For example, the Maturation of DCs with TLR ligands such as CpG and poly(I: C) secretes EVs containing a higher concentration of MHC molecules. This potentiates CD8⁺ T cell response and generates potent anti-tumor immunity in the murine

melanoma model [146]. In addition, α -galactosylceramide, a potent stimulator for invariant NKT cells, was used to engineer DC-derived EVs to induce a robust antigen-specific antitumor response in murine melanoma [147]. A recent study showed that tumor growth of mouse hepatocellular carcinoma could be suppressed via α -fetoprotein-expressing DCs derived EVs [148]. Furthermore, EVs derived from murine DCs loaded with HPV early antigen 7 peptide produced an anti-tumor cytotoxic T lymphocyte (CTL) response in human papillomavirus (HPV)-driven cervical cancer and generated a protective and therapeutic immune response against cervical cancer in vivo [149]. In short, these approaches can overcome the limited efficacy of DC-derived EVs for clinical use.

EVs derived from NK cells also have great potential for anti-tumor therapy. Researchers showed that perforin and FasL packaged into NK cell-derived EVs generate anti-tumor effects in glioblastoma and melanoma xenograft models [150]. Since NK cell-derived EVs were taken up by tumor cells but not immune cells, they could be carriers for drug delivery and targeted therapy [151].

EVs are ideal candidates for drug delivery and immune therapy due to their selective cell targeting capacity and immune stimulation. However, the potential tumorigenic effects of EVs should be explored carefully. Also, large-scale production of good manufacturing practice-grade EVs is quite challenging [152]. For these reasons, researchers focused on alternative sources of EVs. Since platelet cells are anucleated, EVs derived from platelets should be considered an alternative delivery system and reduce safety concerns. Moreover, autologous platelet-derived EVs are already in circulation, which can minimize immunogenicity concerns [153].

Recently, researchers started to test immune cell-derived EVs to treat early-stage pneumonia caused by severe acute respiratory syndrome coronavirus 2 in a phase 1/2 clinical trial (NCT04389385). This clinical trial activates and expands donor T cells by stimulating viral peptide fragments and cytokines. In this way, EVs potentially containing potent immune mediators such as IFN- γ from T cells are isolated and investigated to control disease progression if they are administrated early in the disease.

EVs can be used for preventive vaccine studies. Researchers tested that vaccination with EVs isolated from plasmacytoma cells for its efficacy in preventing tumor growth rather than eradicating already established tumors. When mice are challenged with wild-type tumors, %80 of mice vaccinated with EVs derived from plasmacytoma cells were protected. This protection is

provided by the generation of specific cytotoxic T cells. This protective effect was not seen in severe combined immunodeficiency mice [154].

Tumor-derived EVs can also be used to generate an anti-tumor immune response. EVs isolated from ascites of patients with colorectal cancer were tested in phase I clinical trial. This study treated cancer patients with only tumor-derived EVs and EVs plus granulocyte-macrophage colony-stimulating factor. Researchers found that patients treated with combination therapy raised tumor-specific anti-tumor CD8⁺ T cell response compared to patients treated with only tumor-derived EVs [155].

EVs have numerous advantages in immunotherapies compared to cell-based therapies [156,157]. Since EVs do not replicate themselves after injection, the risk of tumor formation is reduced, unlike cells. Furthermore, EVs have lipid bilayer membrane that increases their stability and protects their cargo from degradation in the extracellular space. These are the essential features for an efficient delivery system. EVs can be easily manipulated to load exogenous RNAs, proteins, and chemotherapies [158]. Researchers showed that EVs derived from DC encapsulated with doxorubicin were used as a tumor target via expression of a Lamp2b- α v integrin-specific iRGD peptide fusion protein in a breast cancer xenograft model [159]. In another study, paclitaxel encapsulated in a macrophage cell line-derived EVs were taken up by cancer cells and reduced tumor growth in the lung cancer model [160].

1.1.4.1.2 Inflammation and immune response

The therapeutic effect of EVs in several immune-related diseases is another well-studied area for clinical applications. Several studies showed that in mouse models of experimental arthritis, genetically modified EVs expressing FasL, interleukin-4, or tryptophan-degrading enzyme indoleamine 2,3-dioxygenase isolated from mouse DCs have both anti-inflammatory and immunosuppressive effects when they are injected systemically in mouse [111,161,162]. In another study, mouse DCs were treated with recombinant murine interleukin-10 or transduced with an adenovirus expressing interleukin-10. The intra-articular injection of EVs isolated from these DCs repressed the murine collagen-induced arthritis and suppressed delayed-type hypersensitivity in the mouse. These results indicated that EVs from modified DCs could efficiently repress autoimmune and inflammatory responses in vivo [163].

In addition, researchers demonstrated that immature DCs release lactadherin packaged EVs. Lactadherin is a molecule that is required to opsonize apoptotic cells for phagocytosis. Low levels of Lactadherin in the blood and spleen of mice were associated with impaired apoptotic clearance. In an experimental sepsis model, EVs derived from immature DCs were administered into mice. Injection of EVs enhanced phagocytosis of apoptotic cells and decreased mortality compared to lactadherin deficient mice. This finding showed that EVs from immature DCs could reduce acute systemic inflammatory response by raising apoptotic cell clearance [164].

1.1.4.2 Prognosis and biomarkers

Cancer patients have a higher risk of developing venous thromboembolism (VTE). Increased tissue factor-initiated coagulation is one of the reasons for establishing VTE in many cancer patients [165]. Several studies found that tissue factor packaged tumor-derived EVs were discovered in peripheral blood of cancer patients, and tumor-derived EVs containing tissue factors are associated with venous thromboembolic events [166,167].

EVs can be used for the identification of cancer aggressiveness in prognosis. For instance, a higher level of CD24 in ovarian cancer patients causes reduced survival rates, which indicates the worst prognosis [168]. A study found that EVs isolated from ascites of ovarian cancer patients containing CD24 and EpCAM were correlated with aggressiveness of ovarian cancer [169]. In another study, EVs derived from mouse melanoma cells promote metastatic behavior of tumor cells through receptor tyrosine kinase MET. When the expression of Met in exosomes was decreased, the pro-metastatic behavior of the bone marrow cell was also reduced [170].

EVs as a diagnostic biomarker is a rapidly growing area in EV research. Researchers have put considerable effort into potential EV biomarkers in diseases. For instance, tau phosphorylated at Thr181 is a well-known biomarker of EVs isolated from Alzheimer's patients [171]. Moreover, EVs derived from the urine of prostate cancer patients contain prostate-specific antigens [172]. Ovarian cancer patients have increased levels of EVs containing claudin in their peripheral blood [173]. EVs derived from ascites of colorectal cancer patients give clues about tumor development [174]; however, how these biomarkers are clinically suitable needs further investigation.

1.1.5 Immunostimulatory Ligands

Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition receptors. They are identified in various species, such as mammals. Pathogen-associated molecular patterns (PAMPs) are ligands that are conserved structural motifs of microorganisms for TLRs. Antigen-presenting cells like DC, macrophages, and B cells express several TLRs. TLRs can mediate cellular activation and cytokine production in response to pathogens. TLR ligands are vital in boosting potent adaptive immune response by maturing professional antigen-presenting cells. Therefore, TLR ligands are mainly studied as vaccine adjuvants because of enhancing immune responses against tumor cells and pathogens. TLR 3 ligand polyinosinic: polycytidylic acid (poly I: C) and TLR9 ligand CpG ODNs have been shown strong adjuvant effects to boost standard vaccination protocols in preclinical and clinical settings [175,176].

1.1.5.1 Polyinosinic: polycytidylic acid (poly I: C)

Double-stranded RNA (dsRNA) is a pathogen-associated molecular pattern associated with a viral infection. Polyinosinic-polycytidylic acid (poly(I: C)) is a synthetic analog of double-stranded RNA. Poly(I: C) is structurally similar to dsRNA.

Poly(I: C) is recognized by endosomal Toll-like receptor 3 (TLR3). Poly(I: C) activates numerous signaling pathways upon recognition by its receptor TLR3 [178,179]. When Poly(I: C) binds to its receptor TLR3, TLR3 activates the transcription factor interferon regulatory factor 3 (IRF3) by the adapter protein Toll-IL-1 receptor (TIR) domain-containing adapter (TRIF, also known as TICAM-1) [180]. Thus, type I IFNs are generated by activation of IRF3 (Fig 1.5). Moreover, upon recognition of Poly(I: C) by TLR3, TNF receptor-associated factor 6 (TRAF6) or receptor-interacting protein 1 (RIP1) are recruited to activate transcription factors NF- κ B and AP-1 [181]. Thus, activating these pathways can produce inflammatory cytokines and chemokines such as TNF- α , IL-6, and CXCL10. Additionally, cytosolic RNA helicases retinoic acid-inducible protein I (RIG-I) and melanoma differentiation-associate gene 5 (MDA-5) pathways can be induced by poly(I: C) to produce Type I IFNs [182]. (Fig 1.5)

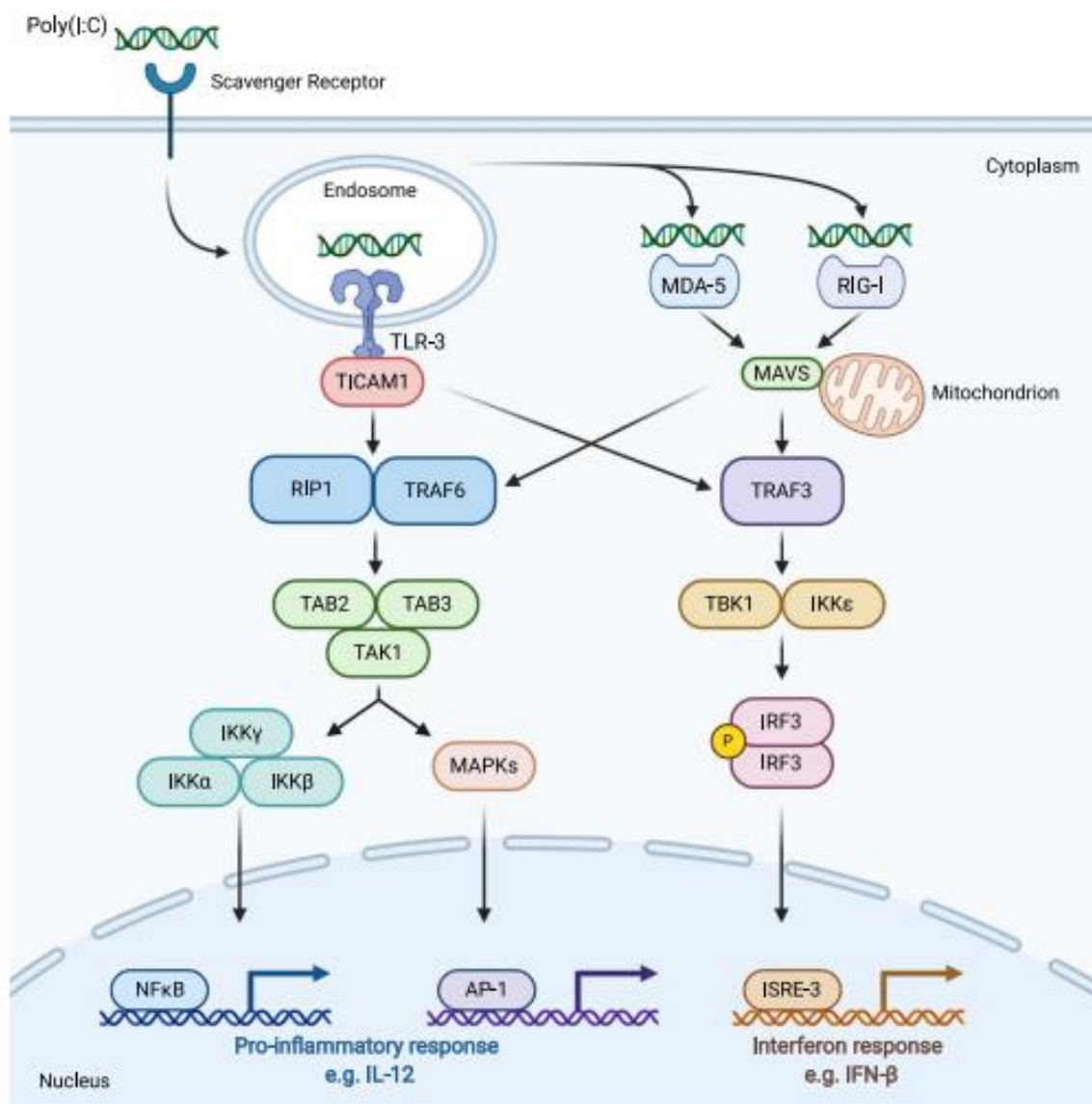


Figure 1.5 Poly(I: C) signaling through TLR-3, MDA-5, and RIG-I generates a pro-inflammatory and interferon response. (Adopted from [177])

Poly(I: C) is a highly potent vaccine adjuvant because of its ability to induce multiple inflammatory pathways. Poly(I: C) is used to treat and protect against tumors due to its vaccine immunogenicity effects. The development of adaptive immunity is essential to enhance vaccine-specific responses against tumors and pathogens by generating potent innate immune responses [183]. Activating TLR3 pathways by stimulating poly (I: C) induces strong type I and III interferon and Th1 cytokine responses. Since poly (I: C) is a Th1-inducing adjuvant and leads to producing a type I interferon,

it has a critical role in antigen processing, APC maturation, and the generating antigen-specific B and T cells to treat tumors and pathogens [184,185].

Researchers showed that treating cancer patients with high dose poly (I: C) in phase I-II clinical trials causes severe side effects such as renal failure and coagulopathies [186]. For this reason, poly-IC12U, which is a safer derivative of poly (I: C), was produced to treat cancer patients. Poly-IC12U induces mature and functional DCs in cancer patients [187], and it was well tolerated by cancer patients involved in phase I-II clinical trials [188].

1.1.5.2 CpG ODN

Innate immunity is the first-line defense mechanism against infectious pathogens. The innate immune response initiates the development of adaptive immunity. The adaptative immune response is characterized by antigen-specific B and T cells. Thus, high-affinity antibodies and specific cytotoxic T cell responses against pathogens are produced, and eventually, long-lasting memory is occurred to protect against following infectious [189].

Bacterial DNA containing unmethylated CG dinucleotides (CpG motifs) is a pathogen-associated molecular pattern that activates the innate immune system [190,191]. Unmethylated CpG motifs are recognized by Toll-like receptor 9 (TLR9), which triggers a protective immune response to eliminate the pathogens [192,193,194]. Synthetic oligodeoxynucleotides (ODNs) expressing unmethylated CpG motifs is a short single-stranded DNA that mimics the immunostimulatory activity of bacterial DNA [195,196,197]. CpG ODNs reach the endosomal compartment of the cell to activate TLR9 signaling cascades [198].

TLR9 is primarily expressed in plasmacytoid dendritic cells and B cells in humans [199,200], whereas TLR9 is defined by multiple myeloid lineage cells such as DCs, macrophages, and monocytes in mice [201,202]. CpG ODNs contribute to the induction of innate and adaptive immune responses by directly activating B cells and plasmacytoid dendritic cells. Furthermore, CpG ODNs indirectly contribute to NK cells, T cells, and monocyte/macrophage maturation, proliferation, and differentiation (Fig 1.6)

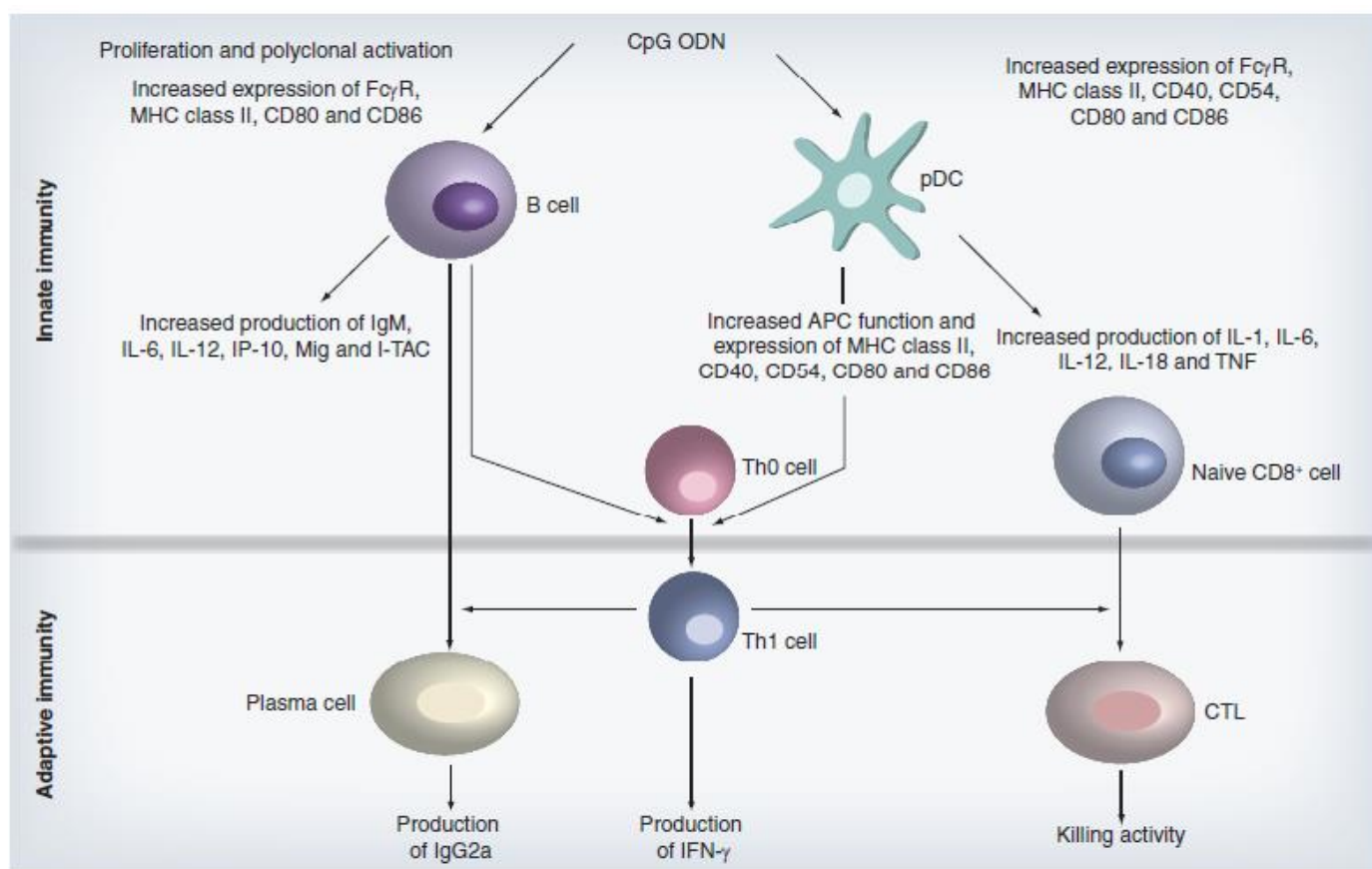


Figure 1.6 Mechanism by which CpG oligodeoxynucleotides facilitate innate and adaptive immune responses. (Adopted from [203])

There are four synthetic CpG ODNs classes with distinct structural and biological properties [204,205,206]. Most studied synthetic ODNs are K type and D type CpG ODNs. K-type ODNs, also called B type, have a phosphorothioate backbone containing CpG motifs. Using a phosphorothioate backbone provides nuclease digestion resistance than a native phosphodiester backbone. Thus, the half-life of CpG ODNs in vivo is enhanced compared to the phosphodiester backbone [207]. Stimulation of plasmacytoid dendritic cells with K-type ODNs produces $\text{TNF}\alpha$ and promotes differentiation of plasmacytoid dendritic cells. Stimulation of B cells with K-type ODNs secrete IgM and proliferate B cells [204,208].

D type ODN, also called A-type, has a mixed phosphodiester/phosphorothioate backbone containing a single CpG motif flanked by palindromic sequences and has poly G tails at the 3' and 5' ends [204]. D type ODNs do not affect B cells, whereas stimulation of plasmacytoid dendritic

cells with D type ODNs secrete IFN α and promotes maturation of plasmacytoid dendritic cells [209,210].

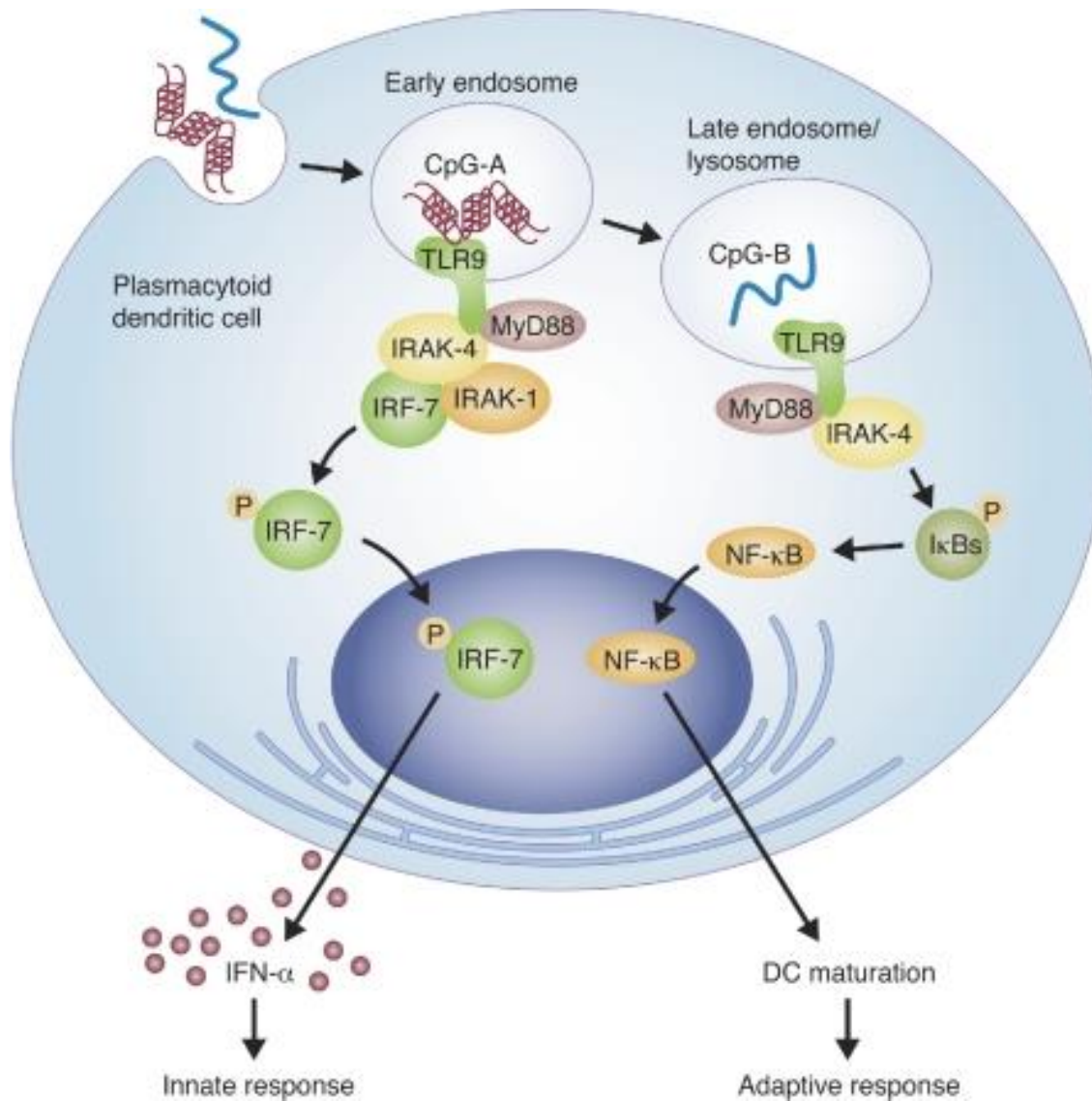


Figure 1.7 CpG oligonucleotides in early or late endosomes induce innate or adaptive immune responses in plasmacytoid dendritic cells. (Adopted from [211])

K-type ODNs and D-type ODNs behave differently in plasmacytoid dendritic cells due to the retention times of CpG ODN/ TLR-9 complexes in the endosomes [204,212]. D-type ODNs stay for a more extended period in early endosomes, whereas K-type ODNs are rapidly transported into late endosomes [213].

C-type ODNs and P-type ODNs are other synthetic ODNs described [206,208]. C-type ODNs have properties in common with both K- and D-type ODNs. C-type ODNs have a phosphorothioate backbone and contain palindromic CpG motifs. C-type ODNs stimulate plasmacytoid dendritic cells to produce IFN α and B cells to secrete IL6 [208,214]. C-type ODNs are stayed in both early and late endosomes [212]. P-type ODNs have two palindromic sequences. This provides a higher-ordered structure for P-type ODNs. P-type ODNs activate plasmacytoid dendritic cells to produce higher IFN α production than C-type ODNs [206].

The therapeutic potential of CpG ODNs has been extensively studied in clinical trials [215]. K-type ODNs are mainly studied in human clinical trials as a vaccine adjuvant for infectious diseases and cancer [216]. In phase I/II study of Engerix B, adults immunized with the CpG adjuvanted vaccine developed higher primary and secondary serum antibody responses than those vaccinated alone [217,218]. In another phase I study, the codelivery of CpG ODN with apical membrane antigen (AMA) 1 induced a significantly stronger antibody when compared with AMA1 alone since AMA1 is a malaria vaccine candidate and is poorly immunogenic when injected in adults [219]. CpG ODNs were also studied as vaccine adjuvants in immunocompromised HIV-infected adults. Immunocompromised HIV-infected adults immunized with CpG ODNs adjuvanted Engerix B significantly enhanced the antibody and T cell responses compared to adults who do not get a response to Engerix B alone [220]. Furthermore, When CpG ODNs were administered with a 23-valent pneumococcal polysaccharide vaccine (PPV-23) and pneumococcal conjugate vaccine, they enhanced the antibody-specific IgG response of HIV-infected adults [221].

Several clinical studies focus on using CpG ODNs in combination with cancer vaccines. Since tumor-reactive CD8⁺ T cells play a crucial role in eradicating tumors, researchers investigated that melanoma antigen A adjuvanted with CpG ODN in incomplete Freund's adjuvant produced a more robust and more rapid antigen-specific CD8⁺ T cell response in patients with melanoma compared to patients treated with vaccine alone [222]. Moreover, New York-esophageal cancer (NY-ESO)-1 protein adjuvanted with CpG ODNs and montanide significantly improved tumor-specific antibody response in the patients. Also, NY-ESO-1-specific CD8⁺ T cell responses were detected in a subset of patients [223]. As a result, clinical trials demonstrated that CpG ODNs could improve the immunogenicity of tumor vaccines and infectious vaccines.

1.1.5.3 α -Galactosylceramide

Natural killer T cells (NKT) are characterized by the expression of both a T cell receptor and NK1.1 receptor. Invariant TCRs are encoded by the majority of murine and human NKT cells. Murine NKT cells encode V α 14 and J α 18 gene segments in their TCR, whereas human NKT cells encode V α 24 and J α 18 segments in their TCR, and they are called invariant NKT (iNKT) cells [224,225]. CD1d is an MHC class I-like molecule that is only characterized ligand of iNKT cells [226]. Many immune cells, such as DCs, macrophages, and B and T cells, constitutively express the CD1d molecule [227,228]. CD1d molecules are recognized by all iNKT cells, and iNKT cells are restricted by CD1d molecules, whereas there are non-CD1d-restricted NKT cells that do not depend on the recognition of CD1d molecules [225].

α -galactosylceramide (α -GalCer) is a synthetic glycolipid identified in an Okinawan marine sponge, and it was shown to bind CD1d molecules [225,229]. α -GalCer represented by CD1d molecule activates both murine and human iNKT cells in vitro and in vivo [230,231,232]. Activating iNKT cells in vivo by α -GalCer produce a large number of IFN γ and IL4 cytokine [229,231].

α -GalCer-activated iNKT cells have a protective role against tumors, autoimmune diseases, and infectious diseases [233,234,235]. Activation of iNKT cells by α -GalCer induces full maturation of DCs. α -GalCer increases the expression of co-stimulatory molecules, including CD40, CD80, and CD86, and MHC class II molecules on DCs [236]. Therefore, α -GalCer can act as an adjuvant.

Researchers demonstrated that α -GalCer-activated iNKT cells induce malaria-specific T cell responses and provide protective anti-malarial immunity. The adjuvant effects of α -GalCer in this study rely on CD1d molecules, IFN γ , and iNKT cells [237]. Moreover, in other studies, the adjuvant effects of α -GalCer on T cell responses can rely on possible CD40–CD40L interaction, IFN γ , and type I interferon secretion [236,238]. Thus, these interactions can induce the full maturation of DC and can be responsible for enhancing the adjuvant activity of glycolipids.

α -GalCer is explored in several clinical trials against cancer and infectious diseases due to its adjuvant effect. Direct intravenous administration of α -GalCer into cancer and chronic hepatitis patients did not have beneficial clinical outcomes [239,240]. Furthermore, the transfer of DCs pulsed with α -GalCer into cancer patients did not display any beneficial anti-tumor effects in

clinical trials [241]. The low number of iNKT cells in patients could result from these failures. Therefore, in other clinical trials, APCs were activated with α -GalCer in the presence of autologous iNKT cells. This activated iNKT cells and α -GalCer-pulsed APC induced significant antitumor immunity when given to patients as a combined therapy [242].

As a result, the potential of iNKT cells against tumors should be studied extensively, and the adjuvant properties of α -GalCer can be explored carefully for the potential role of tumor regression.

1.1.6 Superparamagnetic iron oxide nanoparticles (SPIONs) and their therapeutic potential

In its elemental form, iron can exist in two states, Fe⁺² and Fe⁺³. Fe₃O₄ is a magnetite form of iron, and they are primarily used for biomedical applications because of its magnetic properties. Some iron oxide nanoparticles (IONPs) have a superparamagnetic feature that undergoes magnetization only under a magnetic field. These are called superparamagnetic iron oxide nanoparticles (SPIONs), and they are used as a contrast agent in magnetic resonance imaging (MRI) for diagnostic applications [243,244]. Labeled SPIONs can be used to image organs and tissues due to the magnetic property of iron oxide [245]. United States Food and Drug Administration (FDA) approved several SPION-based formulations for imaging contrast agents [246]. Intravenous administration of SPIONs can be helpful for MRI application due to their small size, and researchers focused on SPIONs for cell-specific tracking via MRI. For instance, dextran-coated SPIONs can accumulate in immune and stem cells without significant toxicity. These properties of SPIONs provide a platform that tracks cancer patients in MRI [247].

SPIONs also can create suitable platforms for therapeutic applications apart from diagnostic use. Targeted drug delivery for various cancer treatments makes SPIONs an area of extensive research due to their magnetic properties [248,249]. The surface of SPIONs can be engineered to obtain longer circulation time and hence better accumulation in tumors and engineered with tumor-targeting moieties to enhance their accumulation in the tumor area [250].

The external magnetic field can be applied to SPIONs to guide SPIONs accumulation in tumors. This can generate hyperthermia due to magnetic field alteration. Elimination of cancer cells by heat provides an additional benefit to cancer therapy. Also, hyperthermia can promote a tumor-specific

immune response that regresses secondary tumors [251]. To improve an antitumor response, hyperthermia can also be combined with SPIONs containing cytotoxic drugs and surface coating. The study indicated that SPIONs coating with a phospholipid–polyethylene glycol (PEG) and doxorubicin maintain drug release and promote cancer cell apoptosis. This is further improved by hyperthermia [252]. Furthermore, in another study, cytotoxic T lymphocytes loaded with SPIONs can be used as a tool to eliminate HIV-infected latent reservoirs through thermal ablation [253].

Gene and biomarker-specific targeting of SPIONs is another method for therapeutic applications of SPIONs. For example, SPIONs loaded with mitoxantrone can be manipulated to target apoptosis and necrosis in tumor cells without affecting healthy peripheral blood leukocytes [254]. Engineered SPIONs can also be explored as a possible vaccine adjuvant because of switching macrophages to the M1 phenotype [255]. Recently, Researchers focused on the SPIONs formulation for anticancer treatment. For example, Ferumoxytol is a superparamagnetic iron oxide nanoparticle with a polyglucose sorbitol carboxymethylether coating. It is used to treat iron deficiency anemia in chronic kidney disease and regress tumor growth in mammary and lung cancer by promoting pro-inflammatory M1 macrophage polarization in tumor tissues [256]. In another study, Rats immunized with DCs pulsed with heat shock protein 70 coated SPIONs and glioma tumor lysate slowed down tumor progression in glioma-bearing rats and improved the survival rate of rats [257]. Consequently, SPIONs can offer an approach for developing a therapy that could boost their therapeutic impact.

1.1.7 Aims of this thesis

Exosomes with a size between 30 to 150 nm are small extracellular vesicles secreted (any small EVs irrespective of their biogenesis) by all types of mammalian cells. They mediate a novel mode of intercellular communication through their bioactive cargos such as lipids, nucleic acids, metabolites, and proteins, which can be delivered to the target cells. Exosomes have successfully served as immunotherapeutic nanocarriers in cancer treatment using their natural delivery capabilities. Furthermore, they are attractive as a delivery system because of their stability in circulation, biocompatibility, and low toxicity.

For this purpose, in the first part of this thesis, exosomes are used as a nanocarrier system to develop cancer vaccines in a therapeutic murine melanoma cancer treatment. The effect of the lyophilization on a cell line-derived exosomes and physical characterization of exosomes were analyzed using bead-based technique via flow cytometry, qNano, SEM, and western blotting. Encapsulation of exosomes with CpG ODN, model protein antigen OVA, and lipidic ligand α -GalCer was accomplished. Biodistribution and activating capacity of encapsulated exosomes were characterized in mouse PEC, mesenteric lymph node, and spleen cells. The therapeutic contribution of a cell line-derived exosomes loaded with CpG ODN, OVA, and α -GalCer in the murine B16-OVA melanoma tumor model was evaluated. The role of triple ligand-loaded exosomes on cellular and humoral immunity of melanoma-bearing mice after treatment was analyzed by antigen-specific ELISA assays. Furthermore, the phenotype changes of immune cells in the tumor microenvironment after treating B16-Ova bearing mice with CpG ODN, OVA, and α -GalCer loaded exosomes were explored by tumor-infiltrating leukocytes assay via using flow cytometry.

In the second part of this thesis, tumor-derived exosomes were used as an appropriate tumor-specific antigen source to induce immunity against tumor cells since they can even present endogenous tumor-specific neoantigens. The simultaneous delivery of tumor cell-derived exosomes and adjuvants to the same immune cells could effectively induce a potent anti-tumor immune responses immune response. Therefore, 4T1/Her2 cell-derived exosomes were co-encapsulated with CpG ODN and p(I: C) to assess their prophylactic and therapeutic effect on the 4T1-bearing mice. The role of the 4T1/Her2 derived exosomes loaded with CpG ODN and p(I: C) on mice's cellular and humoral immunity was explored through antigen-specific IgG ELISA and antigen-specific recall assay. Moreover, since 4T1/Her2 specific memory T-cell is one of the

significant contributing cell populations to eliminating Her2 expressing tumor cells, 4T1/Her2 antigen-specific CD4⁺ memory T cell percentages were analyzed in the immunized mice splenocytes through flow cytometry. Additionally, the contribution of CpG ODN and poly(I: C) loaded 4T1/Her2 derived exosomes on the regressing of 4T1 breast cancer in mice was studied.

The last part of this thesis explored the therapeutic potential of cell line-derived exosomes loaded with SPION and immunostimulatory ligands. The internalization and immunostimulatory activities of unloaded or CpG ODN, poly(I: C), and SPIONs packed exosomes were analyzed on RAW264.7 murine macrophage-like cell line or splenocytes at various time and dose intervals. Furthermore, the therapeutic efficacy of exosomes loaded with SPIONs and TLR ligands (i.e., Exo(pI:C+CpG+SPION) were tested on HUH7-bearing athymic mice.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents

Cytokine antibody pairs and recombinant proteins for ELISA studies (Table 2.1) were purchased from Mabtech (Sweden).

Table 2.1 Monoclonal antibody pairs and recombinant proteins used throughout the thesis studies.

Product	Catalog number	Clone	Company/Country
Anti-mouse IL-6	3361-1A-20	MT26B2	Mabtech/Sweden
Anti-mouse IL-12/-23(p40)	3451-1A-20	C15.6	Mabtech/Sweden
Anti-mouse IFN γ	3321-1A-20	AN18	Mabtech/Sweden
Biotin anti-mouse IL-6	3361-1A-20	32C11	Mabtech/Sweden
Biotin anti-mouse IL-12/-23(p40)	3451-1A-20	C17.8	Mabtech/Sweden
Biotin anti-mouse IFN γ	3321-1A-20	R4-6A2	Mabtech/Sweden
Recombinant mouse IL-6	3361-1A-20		Mabtech/Sweden
Recombinant mouse IL-12	3451-1A-20		Mabtech/Sweden
Recombinant mouse IFN γ	3321-1A-20		Mabtech/Sweden

Monoclonal antibodies used in IgG ELISA (Table 2.2) studies were purchased from Southern Biotech (USA).

Table 2.2 Monoclonal antibodies used throughout the thesis studies for IgG ELISA

Product	Catalog number	Company/Country
Anti-mouse IgG AP	1030-04	Southern Biotech/USA
Anti-mouse IgG1 AP	1070-04	Southern Biotech/USA
Anti-mouse IgG2a AP	1080-04	Southern Biotech/USA
Anti-mouse IgG2c AP	1079-04	Southern Biotech/USA

Streptavidin-alkaline phosphatases (SA-ALPs) were obtained from Mabtech (Sweden). Substrate for alkaline phosphatases, p-nitrophenyl phosphate disodium hexahydrate (PNPP), was purchased from Thermo Fisher Scientific (USA).

Fluorochrome-labeled antibodies (Table 2.3) were from Biolegend (USA) and BD (USA).

Table 2.3 Fluorochrome-conjugated antibodies for cell surface staining and intracellular staining were used throughout the thesis studies.

Product	Catalog number	Company/Country
Anti-mouse B220	103208	Biolegend/USA
Anti-mouse CD1d	123509	Biolegend/USA
Anti-mouse CD3	100214	Biolegend/USA
Anti-mouse CD4	100406	Biolegend/USA
Anti-mouse CD8a	553033	BD/USA
Anti-mouse CD9	124806	Biolegend/USA
Anti-mouse CD11b	101208	Biolegend/USA
Anti-mouse CD11c	553802	BD/USA
Anti-mouse CD19	115508	Biolegend/USA
Anti-mouse CD28	553297	BD/USA
Anti-mouse CD44	103027	Biolegend/USA

Anti-mouse CD45	103114	Biolegend/USA
Anti-mouse CD54	116108	Biolegend/USA
Anti-mouse CD62L	104438	Biolegend/USA
Anti-mouse CD63	143904	Biolegend/USA
Anti-mouse CD69	104543	Biolegend/USA
Anti-mouse CD80	104714	Biolegend/USA
Anti-mouse CD81	104906	Biolegend/USA
Anti-mouse CD86	105030	Biolegend/USA
Anti-mouse CD115	135524	Biolegend/USA
Anti-mouse I-A/I-E(MHC-II)	107606	Biolegend/USA
Anti-mouse H-2Kd (MHC-I)	116607	Biolegend/USA
Anti-mouse IFN γ	554413	BD/USA
Anti-mouse TCR β	109226	Biolegend/USA
Anti-mouse NK1.1	108724	Biolegend/USA
Anti-mouse F4/80	123108	Biolegend/USA
Anti-mouse Ly6C	128041	Biolegend/USA
Anti-mouse LY6G	127608	Biolegend/USA
PE Rat IgG2b, κ Isotype Ctrl Antibody	400607	Biolegend/USA
PE Rat IgG2a, κ Isotype Ctrl Antibody	400508	Biolegend/USA
PE Armenian Hamster IgG Isotype Ctrl Antibody	400907	Biolegend/USA
FITC Rat IgG2b, κ Isotype Ctrl Antibody	400606	Biolegend/USA
PE Mouse IgG2a, κ Isotype Ctrl Antibody	400211	Biolegend/USA

2.1.2 Cell culture media and general solution components

RPMI-1640, DPBS (Dulbecco's Phosphate-Buffered Saline,1x), DNase/RNase free water, High glucose DMEM (Dulbecco's Modified Eagle Medium), HEPES, L-glutamine, Na-pyruvate, penicillin/streptomycin, the non-essential amino acid solution was from Gibco (USA). Fetal Bovine Serum (FBS) was obtained from Biowest (USA) and Gibco (USA).

2.1.3 Ligands

In vitro and *in vivo* experiments, TLR ligand, iNKT ligand, and SPION were used for stimulation and anti-cancer vaccine studies. D-type CpG ODN as TLR9 ligand (D35-5'-ggTGCATCGATGCAGGGGgg-3', Alpha DNA, lower letters represent phosphodiester linkages, capital letters phosphorothioate linkages), K-type CpG ODN as TLR9 ligand (Alpha DNA, K3-5'-ATCGACTCTCGAGCGTTCTC-3') and iNKT cell ligand α -Galactosylceramide (KRN7000 α GC, Avanti, Catalog # 260377) were used TLR ligands and iNKT ligand loaded exosome experiments. Polyinosinic: polycytidylic acid (poly(I: C); TLR3 ligand) were obtained from Amersham (UK). All ligands were endotoxin-free.

Chicken ovalbumin was obtained from Sigma (USA), and Imject OVA[™] was purchased from Thermo Scientific (USA). Iron oxide (II, III), magnetic nanoparticle solution (SPION), was from Sigma (Catalog # 725331-5ML). OVA (257 264) (SIINFEKL peptide) was obtained from Biopeptide (USA, Catalog # 01141510583). Her2-E75 peptides were synthesized from Immunostep (Spain).

2.1.4 Western Blot Antibodies and Solutions

Prestained protein ladder and PVDF transfer membranes were purchased from Thermo Scientific (USA). ECL Prime Western Blotting Detection Reagents were purchased from Cytiva Amersham (UK). The visualization of the membranes was performed by Amersham Imager 600. Western Blot antibodies were purchased from Cell Signaling (USA) and Abcam (UK) and listed in Table 2.4.

Product	Catalog number	Company/Country	Working Condition
HER2/ErbB2(29D8) Rabbit mAb	2165S	CST/USA	1:1000
Alix (3A9) Mouse mAb	2171S	CST/USA	1:1000
RecombinantAnti-TSG101 antibody	ab125011	Abcam/UK	1:1000
Anti-mouse IgG, HRP-linked Antibody	7076S	CST/USA	1:5000
Anti-rabbit IgG, HRP-linked Antibody	7074S	CST/USA	1:5000

2.2 METHODS

2.2.1 Mouse strains

Male or female BALB/c, C57BL/6 mice (6-8 weeks, Wild type), and female athymic nude mice (6-12 weeks old) were housed in the animal holding facility of the Bilkent University, Department of Molecular Biology and Genetics. Housing conditions for all mice were at 22°C with 12 h light and 12 h dark cycles, and they were provided with unlimited access to food and water. All experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC). They were used for the in vivo experiments and isolated primary spleen cells, PECs, and mesenteric Lymph Nodes for in vitro studies throughout this thesis.

2.2.2 Exosome isolation from RAW 264.7, 4T1/Her2, and 4T1-Luc cell line

Exosomes were isolated from RAW264.7, 4T1/Her2, and 4T1-Luc cell lines via the differential ultracentrifugation method. Following supernatant collection from cultured cells, they were centrifuged at 1500 x g for 10 min at RT to discard the cell debris. Supernatants were transferred to ultracentrifugation tubes (Beckmann Coulter) and centrifuged at 10000 x g for 10 min at +4°C to remove any large particles, followed by second centrifugation at 30000 x g for 30 min. This generated a pellet consisting of medium EVs and an upper aqueous phase containing the exosomes. The supernatant was collected and passed through a 0.2 µm filter to ensure the removal of larger particles. To sediment exosome, filtered supernatant was centrifuged at 100000 x g for 90 min at +4°C. The pellet was washed once with PBS at 100000 x g for another 90 min at +4°C. Following washing, supernatants were aspirated, and the exosome pellet was resuspended in an appropriate volume of Ca²⁺/Mg²⁺ free 1x PBS and used in further studies.

2.2.3 Exosome characterization

2.2.3.1 Size measurement by DLS and qNano

To analyze the size of exosomes by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern, UK), exosomes were diluted in PBS, and triplicate readings of samples were recorded.

Tunable-resistive pulse sensing (TRPS) measurements were executed with the qNano Gold (IZON S/N 601A) system and analyzed with the IZON Control Suite 3.4.2.48 software (IZON Science

LTD). The calibration particles, IZON coating solution, wetting solution, and nanopores were used according to the manufacturer's protocols. 35 μ L of 1:1000 diluted calibration particles (CPC100, IZON Reagent Kit, RK3-167) or exosome liquid was loaded, and each sample was recorded.

2.2.3.2 Atomic Force Microscopy

Purified exosomes were diluted at 1:100 in PBS and adsorbed onto mica sheets. According to the manufacturer's instructions, the adsorbed samples were air-dried, and micrometer-scale AFM imaging was conducted in non-contact dynamic mode (NanoMagnetics Instruments). Scans were analyzed using the NMI Image Analyzer software.

2.2.3.3 Scanning Electron Microscopy

Purified exosomes were verified by scanning electron microscopy (SEM). Ten μ L of the exosome solution were deposited on silica surfaces. After evaporation, samples were sputter-coated with 8 nm of Au/Pd alloy using a precision coating system (Gatan Inc, USA).

2.2.3.4 Transmission Electron Microscopy

Exosomes were fixed in 2% glutaraldehyde for 30 min at RT, fixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol (30–100%), and embedded in Epon 812 resin. Sections were stained with uranyl acetate and lead citrate. Imaging was performed at 80 kV using a JEOL-JEM 1400 transmission electron microscope. Digital images of the specimens were acquired using a CCD camera (Gatan Inc.).

2.2.3.5 Bead-based flow cytometry technique to determine the specific exosomal markers

Forty μ L of carboxyl modified latex beads (Life Technologies) were incubated with 40 μ g of LEAF purified anti-mouse CD81 antibody (Biolegend) at RT for 30 min. Then, the volume was adjusted to 500 μ L with 1x PBS, and the bead-antibody mix was incubated overnight at RT by continuous rotation. Following incubation, beads were blocked with 5% BSA containing 1x PBS for 4 h at RT by rotation. To analyze the specific molecules on exosomes, two μ g of exosomes were mixed with anti-mouse CD81 conjugated beads and incubated at RT overnight. Following washing by centrifugation at 13200 x g, bead-associated exosomes were incubated separately with two μ g/mL of anti-mouse CD1d, anti-mouse CD9, anti-mouse CD11b, anti-mouse CD11c, anti-mouse CD54, anti-mouse CD63, anti-mouse CD81, anti-mouse I-A/I-E, anti-mouse H-2Kd antibodies in sodium

azide containing FACS buffer or with their corresponding isotype controls for 30 min at RT. Then, the exosome-bead complexes were washed with 1x PBS and analyzed with Novocyte D3000 (Agilent, California, USA).

2.2.3.6 Western blot for determining the presence of exosomal markers

Total cellular and exosomal proteins were extracted using a RIPA lysis buffer, and samples were vortexed and sonicated. Protein concentration was determined by BCA Protein Assay Reagent Kit (Thermo Scientific). Twenty-five µg of cell lysate, medium EVs lysate, or exosome lysate were separated on 10% SDS-polyacrylamide gel for 90 min at 120V following 10 min run on 5% SDS-polyacrylamide resolving gel and then were transferred to PVDF membranes (Thermo Scientific) by applying 100V for 90min. Membranes were blocked with 5% BSA containing 1x PBS-T for 2 h at RT. Then, blocked membranes were incubated with primary antibodies against TSG101 (Abcam), Alix (Cell Signaling), and Her2 (Cell Signaling) prepared in 5% BSA containing 1x PBS-T at a 1:1000 dilution at +4°C overnight. Following primary antibody incubation, membranes were washed and incubated with 1:5000 diluted secondary antibodies, HRP conjugated anti-mouse IgG (Cell Signaling), and HRP-conjugated anti-rabbit IgG in 5% BSA supplemented 1x PBS-T at RT for 1 h. Membranes were developed using the chemiluminescent detection kit, ECL+ from Amersham, and visualized by Amersham Chemiluminescence Imager 600 (GE, Amersham).

2.2.4 Cell culture

2.2.4.1 Splenocyte isolation

Spleens were surgically removed from C57BL/6 and Balb/c mice. Single-cell suspensions were prepared by smashing the spleen by the 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 media. Samples were waited for 30 seconds to precipitate the remaining tissue debris. After removing tissue debris, splenocytes were centrifuged at 600Xg for 10min at RT. The supernatant was removed, and cells were washed. The cell pellet was gently disrupted with 1ml new media and counted with the flow cytometer.

2.2.4.2 Cell lines

2.2.4.2.1 B16-OVA

B16-OVA cells are derived from B16-F10 (ATCC-CRL6475) melanoma cell line. B16-OVA were stably transfected with plasmids that carry a copy of chicken OVA. Cells were cultured in 10% FBS containing RPMI-1640 media. The media was refreshed every 2 days. When cells reached around 80% confluency, cells were passaged a 1:10 ratio following trypsinization.

2.2.4.2.2 RAW 264.7

RAW 264.7 (ATCC-TIB 71) cultured with 10% FBS containing RPMI-1640 medium. The media was refreshed every 2-3 days. When cells reached around 80% confluency, cells were passaged 1:5 ratio by gently scraping.

2.2.4.2.3 HUH-7

The human hepatocellular carcinoma cell line was cultured in %10 FBS containing High Glucose DMEM. The media was refreshed every 2-3 days. When cells were ed around 80% confluency, cells were passaged 1:5 ratio following trypsinization.

2.2.4.2.4 4T1-Luc and 4T1/Her2

4T1-Luc and 4T1/Her2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). GFP-tagged HER2 expressing mouse breast cancer cell line (4T1-HER2) was kindly provided by Prof. Michael Kershaw (Cancer Immunology Program, Peter MacCallum Cancer Center, Victoria, Australia) [ref1, ref2]. 4T1-luc cell line is originally developed by G Gary Sahagian [ref3].

2.2.4.3 Mesenteric Lymph Node isolation

6-8 weeks old C57BL/6 mice were sacrificed, and mesenteric lymph nodes were surgically removed. The single-cell suspension was prepared by mashing the lymph nodes between two glass micro slides and washing and collecting the cells with 2% FBS containing RPMI-1640 media. After transferring the cells to a new falcon, cells were washed with fresh media and centrifuged at

300x g for 5 min. After repeating the washing step one more time, cells were resuspended in 1 ml of culture media and analyzed with Novocyte 3000.

2.2.4.4 Preparation of PECs

To evaluate the biodistribution and activating capacity of CpG-loaded exosomes on peritoneal immune cells, CFSE loaded or 30 µg K3 CpG ODN loaded exosomes (1:1 ratio loading) were injected intraperitoneally into mice. Then, After the 24 hours of injection, PECs were collected by injecting and recollecting 10 ml of ice-cold 2% FBS supplemented RPMI-1640 media into euthanized mice. Recovered media was centrifugated 2 times with warm 2% FBS containing complete media at 300x g for 5 min. The cell pellet was resuspended with 5% FBS containing media, and they were stained with macrophage, DC, B cell, and neutrophil-specific mouse antibodies.

2.2.4.5 Cell counting

The pellet is resuspended with a fresh medium after the cell line or primary cells are taken and centrifuged. 20-50 µL cell pellet is put into cell cuvette containing 5 ml Isoton II Diluent buffer. If the pellet contains red blood cells, 2 drops of ZAPOGLOBIN II Lytic Reagent are used to burst red blood cells. Novocyte 3000 Flow Cytometry analyzes 50 µL from the mixture. After arranging the threshold to get rid of dead cells, the numbers of gated cells are multiplied with the appropriate dilution rate to obtain the actual number of cells.

2.2.5 TLR9, OVA, and iNKT ligand loading within Exosome

Thirty µg of exosome were mixed with 5 µg OVA, 10 µg CpG ODN, and 0.3 µg αGC. The mixture was snap-frozen in liquid nitrogen and lyophilized overnight in VirTis Bench Top K Freeze Dryer (SP Industries, PA, USA). The exosome-ligand powder mixture was rehydrated in a controlled fashion by adding nuclease-free ddH₂O at a 1:10 ratio of the pre-lyophilized solution volume. Then, this rehydrated mixture was vortexed for 15 seconds every 2 min for 20 min. Next, 1x Ca²⁺/Mg²⁺ free PBS, identical to the amount of ddH₂O used at the beginning of the rehydration process, was added, and the mixture was incubated at RT for another 10 min. Then, the mixture was vortexed for 15 seconds, and the controlled rehydration process was completed with the addition of 1x Ca²⁺/Mg²⁺ free PBS to adjust the desired concentration of ligand encapsulated

exosome solution. As such prepared exosomal formulations were maintained at +4°C until further use.

2.2.6 TLR9, TLR3, and SPION loading within Exosome

Thirty µg of exosome were mixed with 30 µg K3 CpG ODN, 30 µg poly(I: C), and 5 µg SPION. The mixture was snap-frozen in liquid nitrogen and lyophilized overnight in VirTis Bench Top K Freeze Dryer (SP Industries, PA, USA). The exosome-ligand powder mixture was rehydrated in a controlled fashion by adding nuclease-free ddH₂O. Then, this rehydrated mixture was vortexed for 15 seconds every 2 min for 20 min. Next, 1x Ca²⁺/Mg²⁺ free PBS was added, and the mixture was incubated at RT for another 10 min. Then, the mixture was vortexed for 15 seconds, and the controlled rehydration process was completed with the addition of 1x Ca²⁺/Mg²⁺ free PBS to adjust the desired concentration of ligand encapsulated exosome solution. As such prepared exosome formulations were maintained at +4°C until further use.

2.2.7 Loading Exosome with CFSE

Exosomes were loaded with CFSE to determine cell-specific uptake of vesicles. Exosome pellet was dissolved in 1ml 1% BSA containing 1X PBS. Exosome solution (1ml) was mixed with an equal volume of 20µM CFSE solution. 2ml of 10µM CFSE containing exosome solution was incubated for 30min at 37°C. After incubation, the solution was diluted 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 at 100,000Xg for 90min. The pellet was dissolved in the desired amount of 1% BSA containing 1X PBS.

2.2.8 Cell stimulation studies

2.2.8.1 Stimulation of mouse splenocyte with SIINFEKL

In stimulation assays, splenocytes (2x10⁶ cell/mL in 200 µL) from vaccinated animals were incubated with 5 µg/mL SIINFEKL for 72 hours in a CO₂ incubator. After 72 hours, the supernatant is taken to perform IFNγ cytokine ELISA to evaluate the cytotoxic T cell response.

2.2.8.2 Stimulation of mouse splenocytes with CpG and SPION loaded exosome

Splenocytes isolated from wild type C57BL/6 mice were seeded at 2×10^6 cell/ml on 96-well cell culture plates and stimulated with free D35CpG ODN, exosome-encapsulated CpG ODN, or exosome-encapsulated CpG ODN together with SPION (1 µg/ml per ligand; 4 µg exosome/1 µg ligand) for 12 hours at 37 °C in a 5% CO₂ incubator. After stimulation, supernatants were collected for ELISA analysis of IL-12 and IFN-γ secretion.

2.2.8.3 Stimulation of mouse splenocytes with 4T1/her2 isolated exosome

Splenocytes (2×10^6 cell/mL in 250 µL) were stimulated with 10 µg/mL 4T1/Her2 cell-derived exosome for 72 h at 37°C in a 5% CO₂ incubator. After stimulation, the supernatants were collected and used for IFNγ cytokine ELISA.

2.2.9 Internalization assay of RAW 264.7 and splenocytes with SPION loaded exosome

SP-DiOC labeling was performed by adding 1 µM reagent into the exosome solution and incubating at 37°C for 15mins. Exosome preparations were washed with 1x PBS to remove the unloaded SPION, CpG ODN, and SP-DiOC. For binding and uptake experiments, 4×10^5 RAW 264.7 cells were incubated with 0.2 µg, 1 µg, and 5 µg SPION encapsulated into exosomes for 30 minutes, 4 hours, and 8 hours. 4×10^5 splenocytes were incubated with 1 µg of SPION encapsulated into exosomes for 30 minutes, 4 hours, and 8 hours. CpG ODN and SP-DiOC positive cells were recorded using BD Accuri C6 flow cytometer.

2.2.10 Flow cytometry studies

2.2.10.1 Intracellular cytokine staining of mouse splenocytes

10^6 cells/mL of splenocytes in a medium containing Brefeldin A (5 µg/mL, was included in the last 6 hours) was stimulated with 4T1/Her2 derived exosome (10 µg/mL) for 24 h at 37°C. After stimulation, cells were washed two times with stain buffer (2% FCS in PBS). Cells were stained with 1 µg/mL anti-mouse CD45-PE/Cy7, anti-mouse CD3-Pacific Blue, anti-mouse CD4-FITC, anti-mouse CD8-PE, anti-mouse CD44-APC/Cy7, anti-mouse CD62L-BV605 for 30 min at 4°C. The cells were washed with stain buffer and fixed with Cytofix/Cytoperm solution (BD, USA). Fixed cells were washed two times with Perm/Wash buffer (BD, USA) and then stained with 1 µg/mL anti-mouse IFNγ-FITC antibody for 30 min at 4 °C. The cells were washed with Perm/Wash

buffer and suspended in 300 μ L stain buffer for flow cytometric analysis with Novocyte D3000 (Agilent, California, USA).

2.2.10.2 Analysis of tumor-infiltrating leukocytes by flow cytometry

Stock liberase DL solution (Roche) was diluted at 1:26 with RPMI-1640 so that the working concentration was 0.5 Wunsch (1ml solution/tumor). Tumors were resected, dissected into small pieces, and placed in 6 well plates. 1ml liberase working solution was added to each well, and tumors were processed. Homogenates were transferred to tubes and incubated for 30 min at 37°C. Digests were passed through 35 μ m cell strainer capped tubes by washing two times with sodium azide containing FACS buffer. Then, single-cell suspensions were fixed with Fix & Perm Medium A (Invitrogen), and cells were stained with antibodies presented in Table 2.3. Data were acquired on a Novocyte 3000 (ACEA Biosciences) flow cytometer.

2.2.11 ELISA studies

2.2.11.1 Cytokine ELISA

After stimulating cells with various stimulants for indicated periods, cells were centrifuged at 600Xg for 5min, and supernatants were transferred into new plates and stored at –20°C until further use. High binding PolySorp plates (Thermo, USA) were coated with anti-cytokine monoclonal antibodies by adding antibodies diluted in 1X PBS at 50 μ L/well and incubating overnight at +4°C. Then wells were blocked with 200 μ L blocking buffer (5% BSA and 0.05% Tween 20 in 1X PBS) for 2h at RT. Before adding supernatants and serially diluted standards, plates were washed with Wash Buffer (0.05% Tween in 1X PBS) 4 times for 3 min intervals and rinsed 2 times with ddH₂O. Cell supernatants and standards were added to 50 μ L/well and incubated overnight at +4°C. Plates were washed as previously mentioned. Biotinylated anti-cytokine monoclonal antibodies diluted in Blocking Buffer were added to wells at 50 μ L/well and incubated for 2h at RT. Again, followed by washing as described earlier. 1:1000 diluted Streptavidin Alkaline-Phosphatase (SA-ALP) in Blocking Buffer were added into plates as 50 μ L/well and incubated for 1h at RT. After washing the plates, PNPP substrates were prepared according to the manufacturer's protocol, and 50 μ L/well was added to each well. Color development was followed by multiple optical density readings at

405nm until recombinant cytokine standards reached saturation. The concentration of cytokines for each sample was calculated based on standard curves. Data were collected by SpectraMax (Molecular Devices, California, USA) spectrophotometers.

2.2.11.2 Anti-OVA IgG ELISA

Immulon 2HB microtiter plates were coated with 10 µg/ml OVA in PBS (200 µl/well). The plates were blocked with PBS-5% BSA. Supernatants from cultured cells or serially diluted sera from immunized mice were distributed into wells (50 µl/well). Following incubation with ALP labeled goat anti-mouse IgG, IgG1, and IgG2c (Southern Biotech) for anti-OVA IgG ELISA, plates were developed using the PNPP (Thermo Scientific) substrate. Optical density values for samples detected on an ELISA reader (SpectraMax, Molecular Devices) at 405nm.

2.2.11.3 Anti-75 and Her2 IgG ELISA

Immulon 2 HB microtiter plates (Thermo Scientific, San Jose, CA, USA) were coated with Her2 E75 peptide (40µg/mL), 4T1/Her2 cell lysate (10 µg/mL), and 4T1/Her2 exosome lysate (10 µg/mL) respectively, and then blocked with PBS-BSA. Serially diluted standards and culture supernatants or serially diluted mouse sera were added to plates overnight at 4 °C. Bound IgG subclasses were detected using alkaline phosphatase streptavidin-conjugated anti-mouse IgG (1:1000), anti-mouse IgG1 (1:1000), and anti-mouse IgG2a (1:1000) antibodies and developed by the addition of PNPP substrate.

2.2.12 In Vivo studies

2.2.12.1 Prophylactic immunization study

6–8-week-old female Balb/c mice (4 per group) were vaccinated 2 times in 2-week intervals intraperitoneally (I.P) with PBS, only 30 µg TEX, free form of 30 µg TEX+30 µg CpG ODN and 30 µg poly(I: C) or TEX loaded with CpG ODN + poly(I: C). Two weeks after the last booster dose, all mice were sacrificed, and their splenocytes and blood serum were collected aseptically to detect cellular immune responses.

2.2.12.2 Established melanoma tumor model

Female C57Bl/6 mice (12 mice/group) were subcutaneously injected with 1×10^6 OVA expressing B16-F10 cells on day zero. Palpable tumors developed on days 6-7. Tumor-bearing mice were then intraperitoneally injected with a mixture of 5 μ g OVA, 10 μ g D35 CpG ODN, and 0.3 μ g α GC or their encapsulated counterparts in 30 μ g of exosome. Before repeating this treatment on day 15, mice were bled from tail veins, and sera were kept at -20°C for anti-OVA IgG ELISA. Tumor development was monitored at 2-3 days intervals until the tumor sizes reached 2000 mm^3 , after which the mice were sacrificed. Tumor volumes were calculated as $\text{length} \times \text{width} \times \text{height}$.

2.2.12.3 HCC xenograft in Athymic mice

Male Athymic Nude mice (6-8 weeks old) were subcutaneously injected with 4×10^6 Huh7 cells at the back of the mice. Tumor development was observed. When tumors became palpable and measurable, mice were injected 4 times intraperitoneally either with saline, the mixture of 30 μ g p(I: C), 30 μ g CpG ODN and 5 μ g SPION or their encapsulated counterparts into 30 μ g of exosome. Tumor size was measured with digital calipers until the tumor sizes reached 2000 mm^3 , and mice were sacrificed. Tumor volumes were calculated as $\text{length} \times \text{width} \times \text{height}$.

2.2.12.4 Established breast cancer tumor model

6–8-week-old female Balb/c mice were injected with 2×10^5 4T1-Luc tumor cells into right mammary fat pads orthotopically with 5–6 mice per group. Tumor-bearing mice were then intraperitoneally injected with a PBS and 30 μ g TEX loaded with 30 μ g CpG ODN and 30 μ g poly(I: C) on day 0, day 3, day 6, day 10, day 17, day 24, and day 31 after tumor inoculation. Tumor growth was monitored by measuring the tumor volume with a caliper after tumors become palpable. Tumor volumes were calculated as $\text{length} \times \text{width}^2/2$.

2.2.12.5 In Vivo imaging of breast cancer tumor model

For the IVIS spectrum, Caliper Lifesciences Lumina III series was used to visualize tumor growth, and each mouse received 150 mg Luciferin/kg according to the datasheet suggestion.

2.2.13 Statistical analysis

One-way ANOVA with Kruskal Wallis and Mann–Whitney tests was employed to assess the significance of differences among the groups. Survival analysis was performed using a log-rank test (GraphPad Prism version 9, San Diego, California). $P < 0.05$ was statistically significant.



CHAPTER 3

RESULTS

PART-I: Application of Exosome as a Therapeutic Anti-Cancer Vaccine in a murine melanoma tumor model

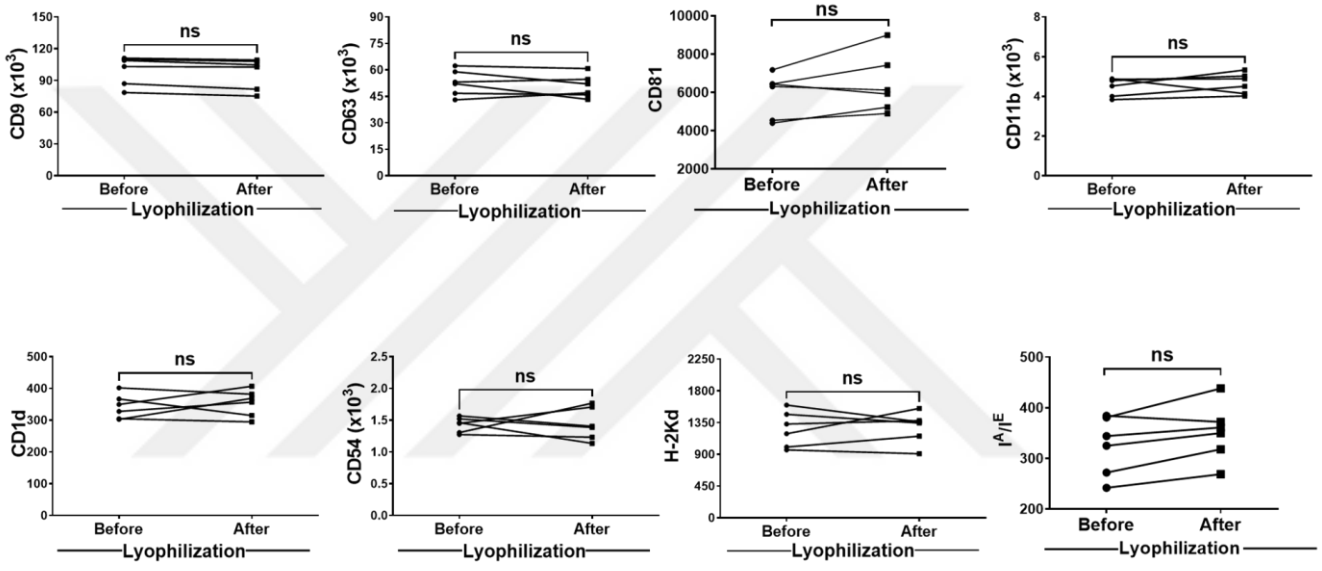
3.1.1. Characterization of Exosome isolated from the RAW264.7 cell line

Lyophilization is a well-established fluid removal process that enables the long-term preservation of biological samples. To establish a platform for long-term protection of exosomes, we aimed to investigate the possible harmful effects of the lyophilization process on the exosomal membrane and the vesicular integrity. To address these, comparative biochemical and biophysical analyses were performed on non-lyophilized versus lyophilized exosome batches. Exosomes were collected from the murine macrophage-like cell line RAW264.7 supernatants via differential centrifugation and were subject to analysis of i) surface marker expression by flow cytometry, ii) size distribution and concentration amount by TRPS technology, iii) morphological analyses by Scanning electron microscopy (SEM) and iv) exosome-specific protein expression by immunoblotting (Fig 3.1).

Anti-CD81 conjugated latex bead-captured exosomes were stained for CD9, CD63, CD81, CD11b, CD1d, CD54, MHCI, and MHCII surface expression marker proteins. (Fig 3.1A) and analyzed using flow cytometry. Lyophilized and non-lyophilized exosome batches had similar surface marker expression levels (n=6 for each test group) (Fig 3.1A). While non-lyophilized exosomes had an average size of around 100-110nm, an upward shift in size was measured after ligand loading. (Fig 3.1B). TRPS analysis also showed that lyophilized and non-lyophilized exosome

batches had similar particles in the exosome solution. (Fig 3.1B). SEM studies revealed that exosomes retained their vesicular structure after lyophilization (Fig 3.1C). Similarly, Alix protein expression (Fig 3.1D) was identical between non-lyophilized and lyophilized exosomes. These results collectively indicate that lyophilization is a gentle technique that does not harm exosomes' vesicular integrity and fundamental biological features.

A.



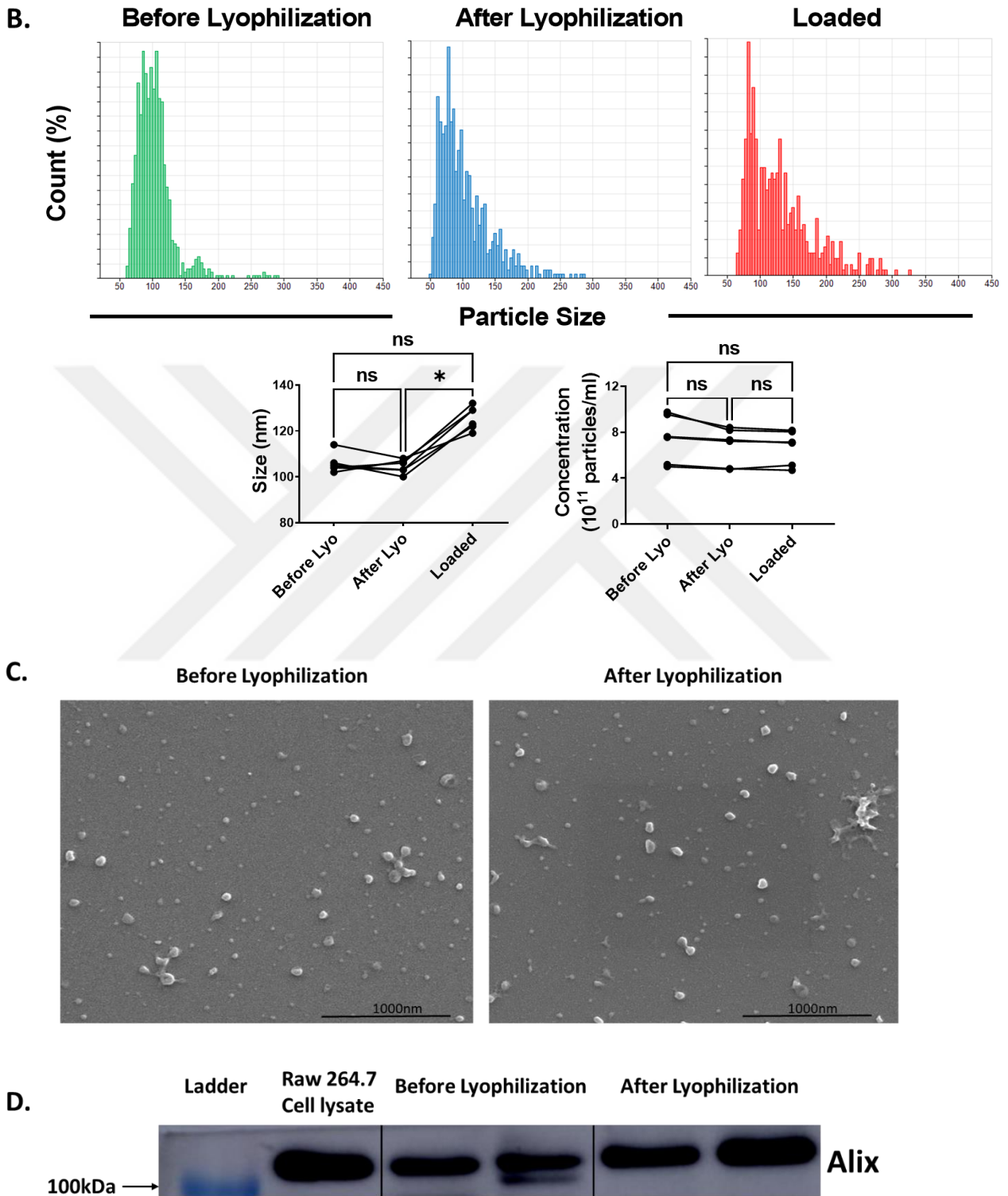


Figure 3.1. Characterization of exosomes isolated from the RAW264.7 cell line. A) Non-lyophilized and lyophilized exosomes coupled to anti-CD81 conjugated beads were phenotyped using cell surface-specific fluorescent antibodies and flow cytometry. Individual symbols represent

the MFI ratio of the antibody to its specific isotype control staining of separate exosome batches. CD9, CD63, CD81, CD11b, CD1d, CD54, MHCI, and MHCII molecule expression ratios with respect to their specific isotype controls before and after Lyophilization. Statistical significance was tested using Wilcoxon matched-pairs signed-rank test. **B)** The size and concentration of fresh, lyophilized, and loaded RAW264.7 derived exosomes were analyzed by TRPS technology showing the effect of lyophilization and loading on concentration and hydrated size distribution. Statistical significance was tested using Holm-Šídák's multiple comparisons test. Ns; non-significant, *, $p < 0.05$ **C)** A representative scanning electron microscopy (SEM) image of non-lyophilized and lyophilized exosomes. The scale bar represents 1000 nm. **D)** 20 μ g of proteins isolated from non-lyophilized and lyophilized exosomes were analyzed by western blot to test the presence of Alix.

3.1.2. Biodistribution of exosomes encapsulated with CpG ODN and OVA

Next, we explored which cell types can take up CpG ODN and OVA-loaded exosomes. For this purpose, exosomes were loaded with Cy5-conjugated CpG ODN, and DQ labeled Ovalbumin. Labeled CpG ODN and OVA-loaded exosomes were administered into the animals via *i.p.* injection. After 24 hours of injection, PECs were collected, and both the CpG ODN signal and OVA signal taken up by cells were analyzed by flow cytometry. Figure 3.2 shows that CpG ODN and OVA encapsulated exosomes were taken up by primarily macrophages, B cells, and Dendritic cells. Also, neutrophils and natural killer cells take up a small number of loaded exosomes. Consequently, loaded exosomes are mostly taken up by antigen-presenting cells (APCs).

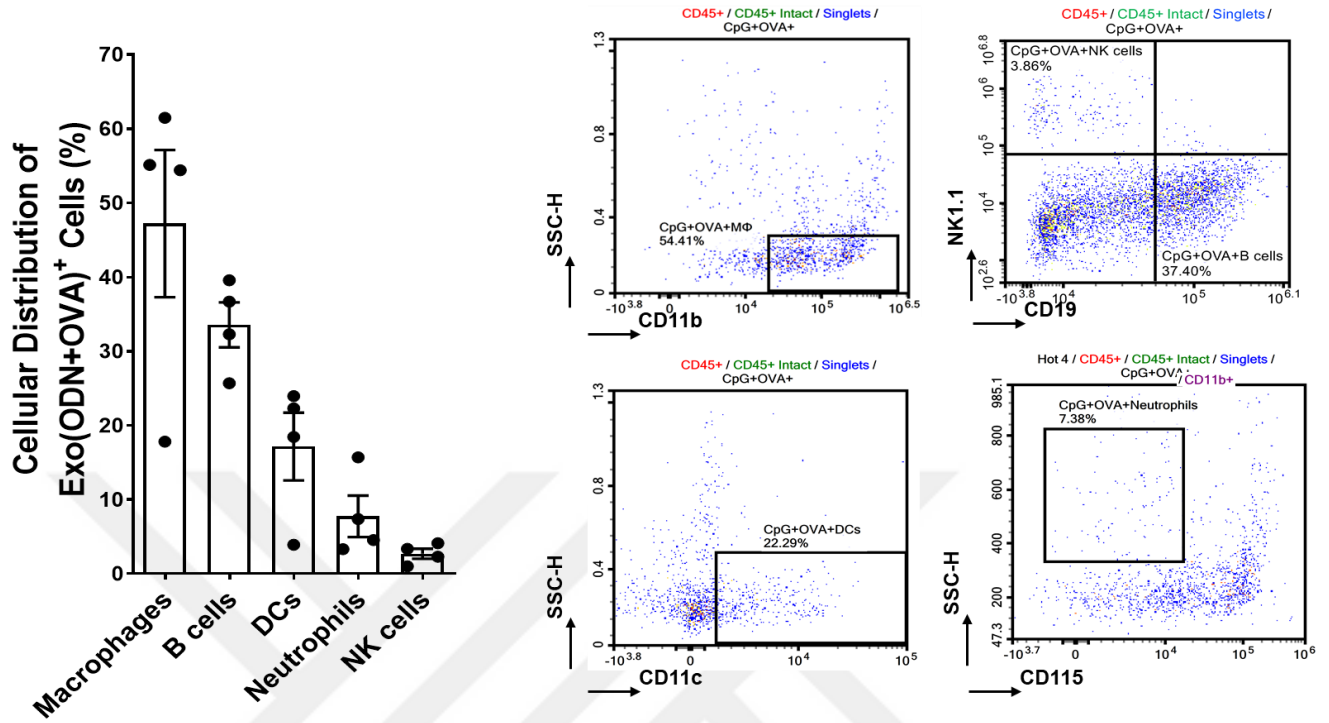


Figure 3.2. Biodistribution of exosomes encapsulated with CpG ODN and OVA. Labeled ODN and OVA-loaded exosomes were administered to the animals via *i.p.* injection. After 24h of injection, PECs were collected and stained against cell-specific antigens. Cell-specific uptake was analyzed and reported as a bar graph with each dot representing an individual mouse on the left while representative density plots are on the right side.

3.1.3. Activating capacity of exosomes encapsulated with CpG ODN

After we observed that CpG ODN and OVA-loaded exosomes were efficiently taken up by immune cells, we then investigated whether CpG ODN-loaded exosomes improved cellular immune activation. Therefore, CpG ODN-loaded exosomes were administered *i.p.* into the animals. After 24 hours of injection, PEC, spleen, and mesenteric lymph nodes were collected to investigate cellular activation. Flow cytometry analyzed APC activation markers CD80 and CD86 and MHC-II expression in macrophages, B cells, and DCs in PECs and splenocytes. (Fig 3.3A and Fig 3.3B). IFN γ expression levels upon CpG ODN loaded exosome stimulation was assessed in CD4⁺ and CD8⁺ T cells isolated from mLN and spleen. (Fig 3.3C). All data shows that loading CpG ODN

into exosomes significantly improves APC activation markers in macrophages, B cells, and DCs. Also, flow cytometry analysis indicated that exosome-loaded CpG ODN elicited significantly higher IFN γ production from mouse mLN and splenocytes.

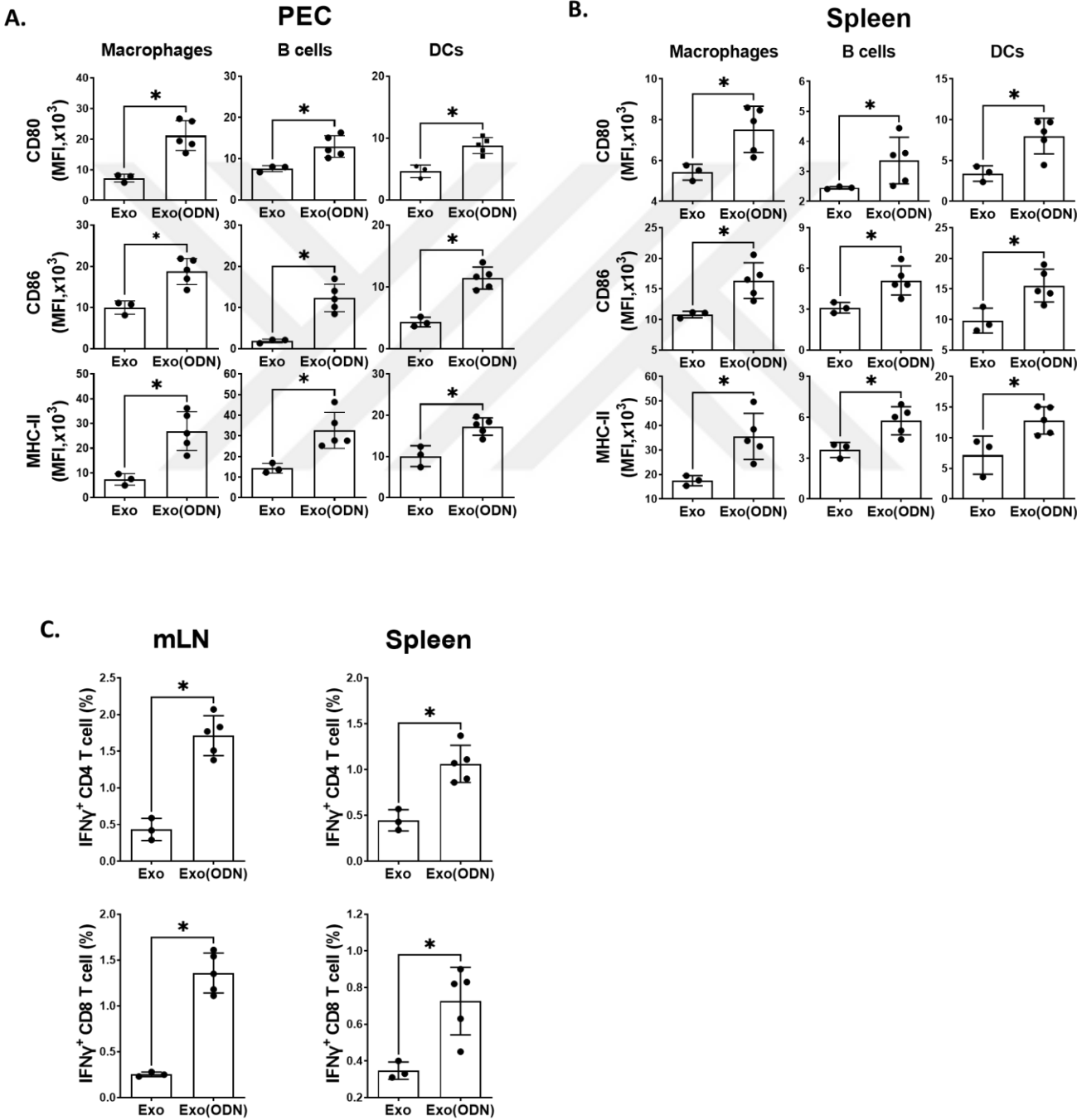


Figure 3.3. Activating capacity of exosomes encapsulated with CpG ODN. CpG ODN-loaded exosomes were administered *i.p.* into the animals. After 24h of injection, PEC, Spleen, and mLN were collected to investigate cellular activation. APC activation markers and MHC-II expression were analyzed in macrophages, B cells, and DCs in PECs **(A)** and splenocytes **(B)**. IFN γ expression levels upon CpG ODN loaded exosome stimulation was assessed in CD4⁺ and CD8⁺ T cells isolated from mLN and spleen **(C)**. Statistical analysis was conducted using the Mann-Whitney test. *; $p < 0.05$

3.1.4. Therapeutic vaccination with triple (CpG ODN, OVA, and α GC) ligand encapsulating exosomes suppresses the progression of established melanoma tumors in mice.

We next explored whether the raised immunity via exosome vaccine is sufficient to control in-vivo tumor development. Although it represents a clinically relevant scenario, regressing or eliminating established tumors is challenging. Therefore, we included a third antigenic component -a glycolipid antigen- α -galactosylceramide (α GC) that can activate iNKT cells against tumors within the encapsulation mixture. We postulated that our therapeutic vaccine formulation would generate more potent anti-tumor immunity.

The therapeutic utility of the triple ligand encapsulating exosome vaccine construct was tested in the B16F10-OVA melanoma tumor-bearing mice. Exosome-encapsulated CpG ODN in a therapeutic cancer vaccine model, purified exosomes were first mixed with a model cancer antigen OVA and an adjuvant CpG ODN and α GC. Following palpable tumor formation, mice were treated twice (on days 7 and 13) either with a mixture of 5 μ g OVA/10 μ g CpG ODN/0.3 μ g α GC or their encapsulated counterparts within 30 μ g of exosomes. As seen in Figure 4, compared to PBS or OVA+ODN+ α GC mixture treated group, immunization with exosomal construct showed a significantly longer delay in tumor development (Fig 3.4A-C). In another experiment aiming to evaluate the benefit of the exosome vaccine in the survival of mice, animals (n=7/group) were followed until all mice died because of the tumor. At the end of day 53, mice treated with OVA+ODN+ α GC loaded exosomes had a significantly higher survival rate than the OVA+ODN+ α GC and control group. (Fig 3.4D).

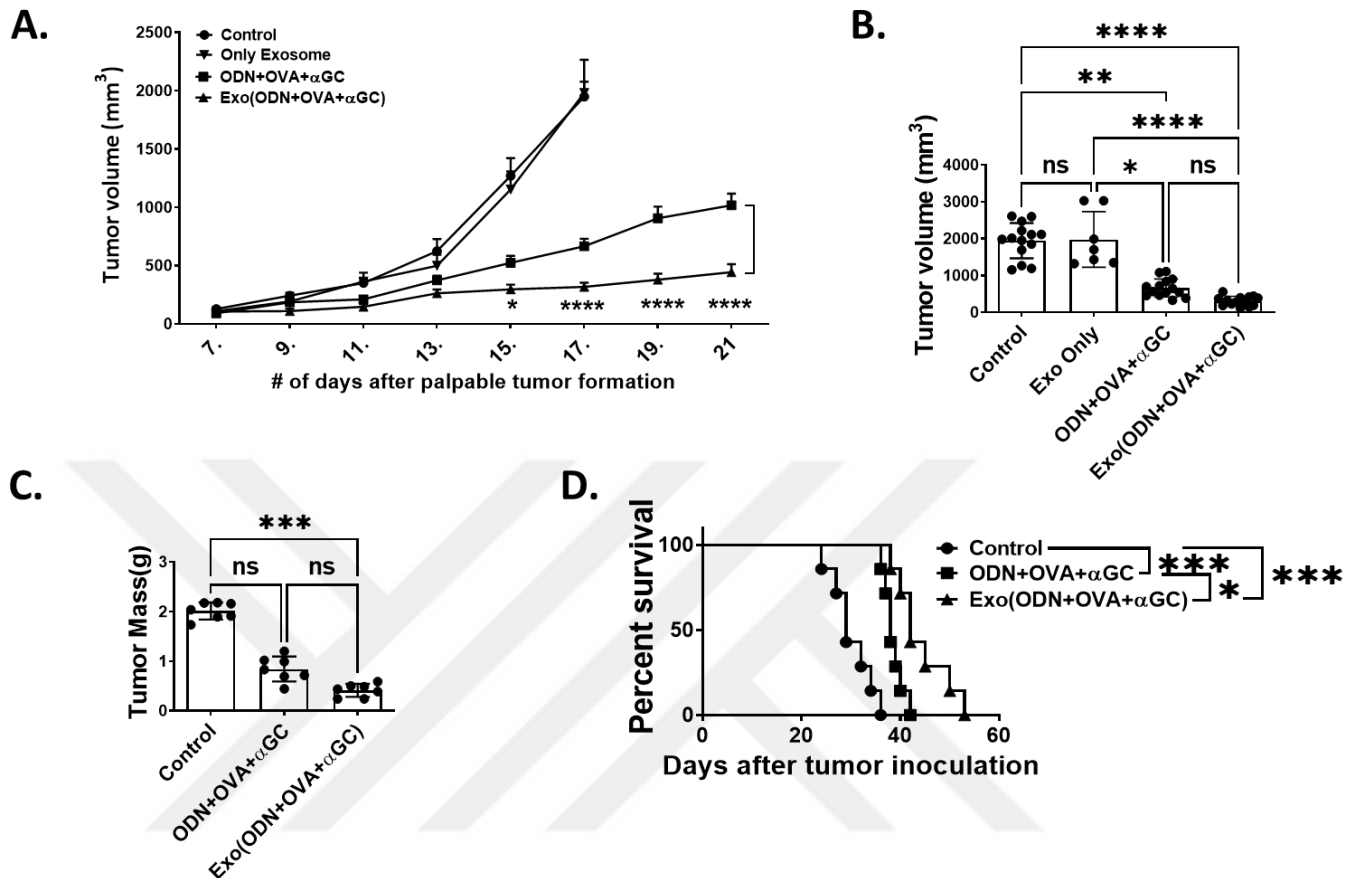


Figure 3.4. Therapeutic vaccination with triple (ODN, OVA, and α GC) ligand encapsulating exosomes suppresses the progression of established melanoma tumors in mice. **A)** Line graph showing average tumor volumes of mice following subcutaneous B16F10-OVA tumor cell injection (n=14). **B)** Tumor volumes at day=17 post palpable tumor formation. **C)** Tumor mass of individual mice at the time of sacrifice. **D)** Long-term Kaplan-Meier survival curve of mice (n=7) immunized either with free or exosome-loaded ODN+OVA α GC. Two-way ANOVA with Bonferroni correction was used to compare tumor volume changes over time. Statistical analysis was conducted using a one-way ANOVA non-parametric Kruskal Wallis comparison test. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001.

3.1.5. Triple ligand loaded exosomes triggers Th-1 biased anti-IgG OVA immunity

One week after primary injection into B16F10-OVA bearing mice, the animal was bled from their tail, and sera were isolated for anti-OVA IgG ELISA. OVA-specific antibody titers verified the

development of a Th1-biased after a single injection of exosomal vaccine ($\text{IgG2c/IgG1} > 1.15 \pm 0.3$, Fig 3.5A-D). Furthermore, the splenic CD8^+ T-cells in exosomal vaccine immunized mice produced higher levels of $\text{IFN}\gamma$ in response to OVA MHC-I peptide stimulation (Fig 3.5E). This result showed that our exosome vaccine led to stronger Th-1 biased anti-OVA immunity.

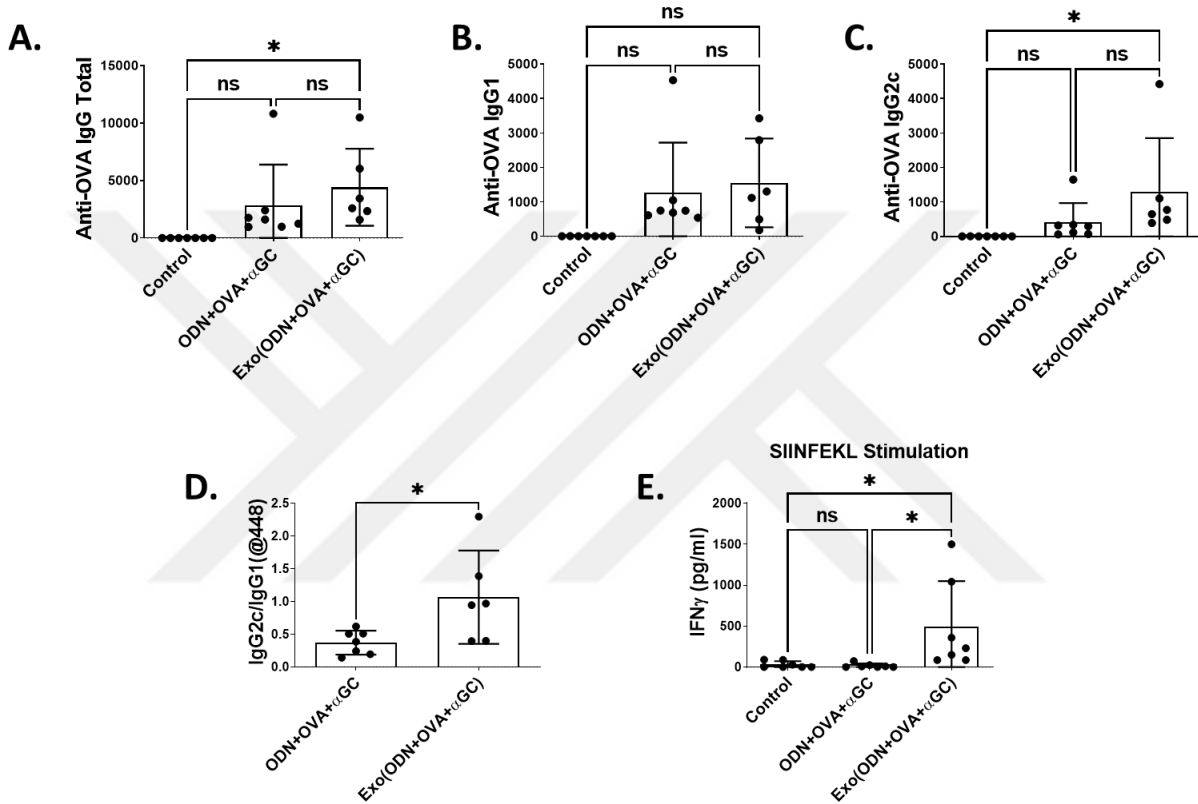


Figure 3.5. Triple ligand-loaded exosomes trigger Th-1 biased anti-IgG OVA immunity.

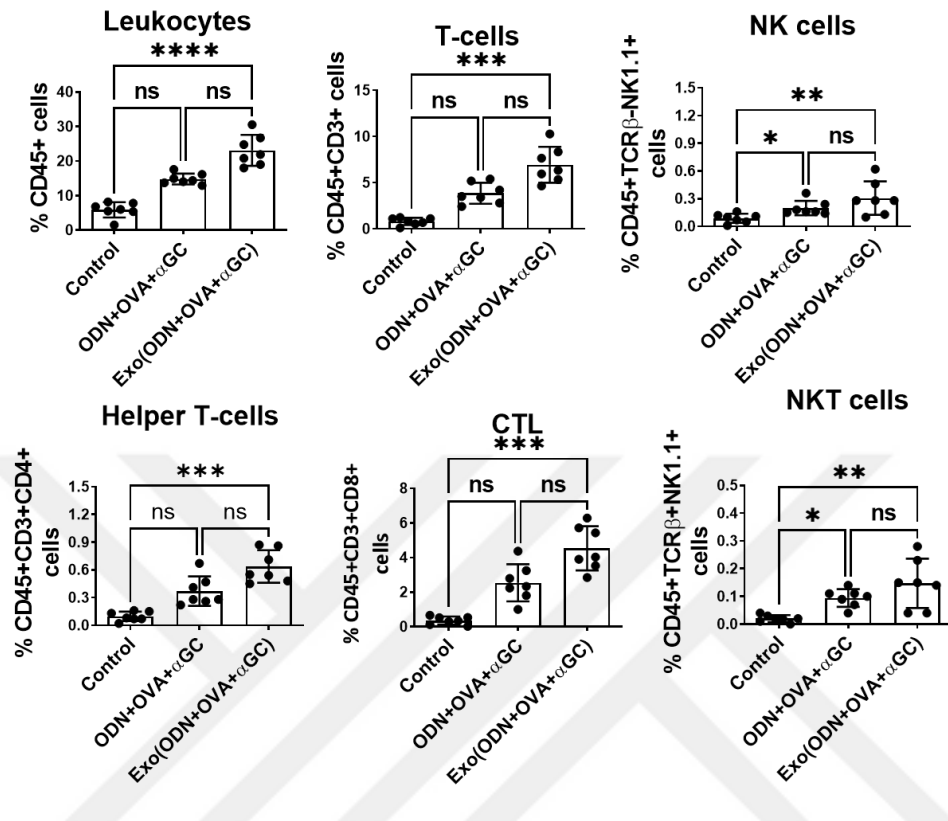
Serum anti-OVA IgG responses following a single immunization (day 15 after palpable tumor formation) with PBS control, a non-exosomal free mixture of ODN+OVA+αGC, and exosomal form of ODN+OVA+αGC. Reciprocal titers of **A)** Total IgG, **B)** IgG1, **C)** IgG2c. **D)** IgG2c/IgG1 ratio between groups. **E)** $\text{IFN}\gamma$ secretion profile of MHC-I specific peptide treated splenic CD8^+ cells. One representative experiment out of two independent studies is presented ($N=7$ in each study group). One-way ANOVA and non-parametric Kruskal Wallis comparison were performed to assess the significance between the treatment groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.1.6. Tumor infiltrated leukocytes analysis in tumor micro-niche of mice treated either with free or co-encapsulated CpG ODN, OVA, and α GC into exosomes

We next investigated how the exosomal vaccine affects the immune cells of the tumor microenvironment. When the control group's tumor volume reached 2000 mm³, the animal was sacrificed, and tumors were resected and dissected into small pieces. Tumor cells were isolated by liberase solution and analyzed by flow cytometry. The percent of tumor-infiltrating cells, including leukocytes, pan T-cells, NK cells, Helper and cytotoxic T cells, and NKT cell levels, were determined using flow cytometric analysis and cell subset-specific markers (Fig 3.6A). Tumor-associated CD4⁺, CD8⁺ T-cells, and NK- and NKT-cells (Fig 3.6A) were substantially higher in exosome treated group tumors when compared to untreated or OVA+ODN+ α GC mixture treated mice. Further investigation of the tumor-cell suspensions demonstrated that pan- and tumor-associated macrophages (TAMs), myeloid-derived inflammatory monocytic cells, and M1-macrophages were significantly higher in exosome construct vaccinated animals compared to those in control or free mixture treated groups (Fig 3.6B). MDSC and M2-like macrophages were not significantly different in exosome immunized animals compared to those in OVA+ODN+ α GC mixture or control received animals. (Fig 3.6B)

Our data indicate that exosomes could shelter multiple ligands by dehydration controlled-rehydration technology. As such, prepared exosomes provide sufficient immune-activating signals to elicit antigen-specific humoral and cellular immunity necessary to suppress the progression of established tumors.

A.



B.

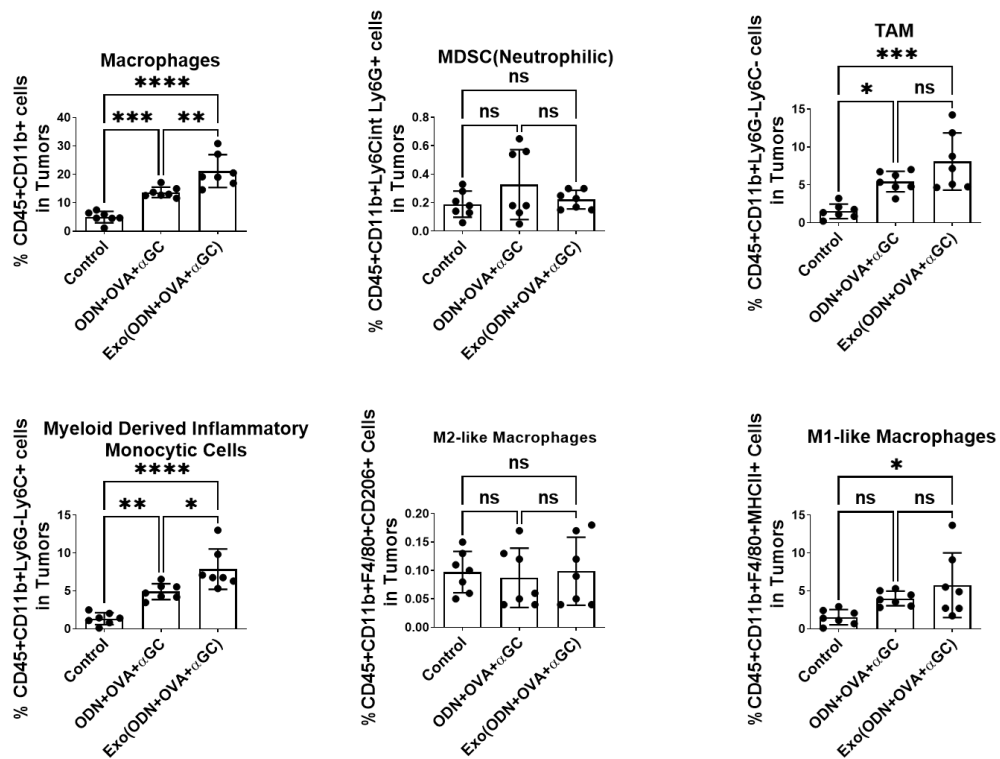


Figure 3.6. The tumor infiltrated leukocytes analysis in tumor micro-niche of mice. A) Leukocytes, T-cells, NK cells, CD4+T-cells, CD8+T-cells, and iNKT cells on day 21 in the tumor microenvironment were analyzed by flow cytometry. **B)** Pan macrophages, MDSC neutrophilic subset, Tumor-Associated Macrophages, Myeloid-derived inflammatory monocytic cells, M2-like macrophages, and M1-like macrophages were compared in mice tumor microenvironment on day21. One representative experiment out of two independent studies is presented (n=7 in each study group). One-way ANOVA and non-parametric Kruskal Wallis comparison were performed to assess the significance between the treatment groups. NS: Non-significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

PART-II: Application of breast cancer-derived exosomes (TEXs) as a Prophylactic and Therapeutic Anti-Cancer Vaccine

3.2.1. Characterization of tumor-derived exosomes isolated from 4T1/Her2 cell line

Exosomes were isolated from the 4T1/Her2 cell culture supernatants using the differential ultracentrifugation method described in Material and Methods. Isolated exosomes were subjected to analysis of i) surface marker expression by flow cytometry, ii) exosome-specific protein and Her2 expression by immunoblotting, iii) size distribution by DLS, and iv) morphological analysis by SEM. Exosomes were verified by detecting the expression of positive protein markers CD81, CD9 (Fig 3.7A), and TSG101 (Fig 3.7B). Tumor cell-derived exosomes are an excellent source of cellular components that stimulate the immune response, such as tumor-associated antigens. To check whether the isolated exosome pools carry antigen, we confirmed that purified exosomes of 4T1/Her2 cells also express Her2, whereas isolated exosomes of RAW264.7 as a negative control do not (Fig 3.7B). The size of exosomes abundantly ranged from 40 to 190 nm (Ave. Mean Size: 70 ± 20 nm, Fig 3.7C). The purified exosomes exhibited typical round-shaped structures under scanning electron microscopy (SEM) (Fig 3.7D). These findings collectively support that extracellular nanovesicles derived from the 4T1/Her2 cell line display convincing exosome features.

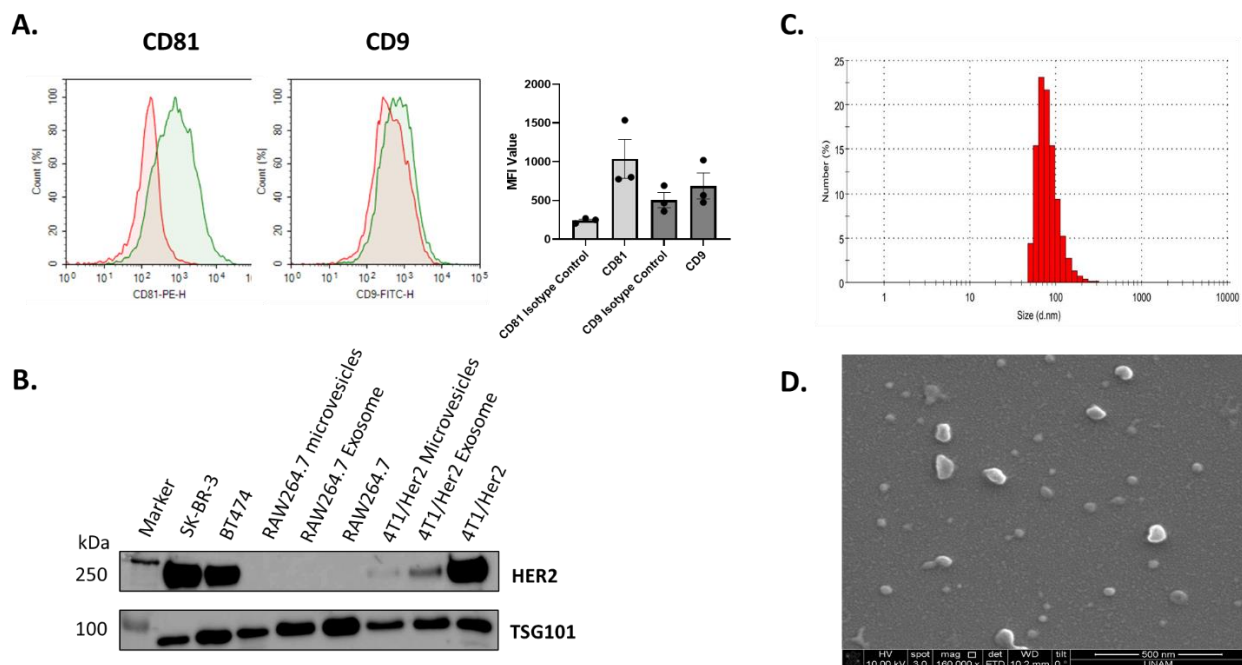


Figure 3.7. Characterization of exosomes isolated from 4T1/Her2 cell line. **A)** Exosomes coupled to anti-CD81 conjugated beads were phenotyped using flow cytometry analysis. Bar graphs represent the average MFI values of specific antibodies to isotype control staining of 3 separate exosome batches. **B)** Western blot analysis of exosomal marker TSG101 and Her2 expression in total cell extracts and cell-derived exosomes. (SK-BR-3 and BT474 cell lines were used as positive controls, and RAW264.7 cell line was used as a negative control) **C)** Dynamic Light Scattering (DLS) analysis showing the average size distribution of 4T1/Her2 cell-derived exosomes (n=3 independent batches) **D)** A representative scanning electron microscopy (SEM) image of 4T1/Her2 cell-derived exosomes. The scale bar represents 500 nm

3.2.2. Tumor cell-derived exosomes loaded with CpG ODN and poly(I: C) immunization magnified anti-IgG 4T1/Her2 and anti-IgG E75 immune responses.

We intended to develop an exosomal carrier system co-encapsulating two nucleic acid-based TLR ligands as immunoadjuvants naturally expressing tumor-associated antigen Her2 and analyzed its potency as a prophylactic and therapeutic vaccine. Previous studies have shown that tumor-derived exosomes have a dual effect on the immune system and immunosuppressive or immunostimulatory outcomes. To combine and enhance the synergistic immunostimulatory activity of TLR3 (p(I: C)) and TLR9 (CpG ODN) ligands, we co-encapsulated them within 4T1/Her2 cell-derived exosomes, thereby aiming to achieve simultaneous antigen and adjuvant delivery to the relevant antigen-presenting cell. Next, we assessed the humoral responses from sera of mice collected on day 28 following twice (day 0 and day 14) i.p. injection to Balb/c mice. 4T1/Her2-specific total IgG, IgG1, and IgG2a titers against 4T1/Her2 total cellular protein extract or 4T1/Her2 total exosome extract were analyzed by ELISA. As expected, there was no 4T1/Her2-specific antibody production in the control group, and only exosome injected animals raised strong antibody titers. Besides, the level of 4T1/Her2 specific total IgG, IgG1, and IgG2a in sera of mice immunized with the dual ligand-loaded exosomal group was significantly higher than those animals that were vaccinated with a free combination of antigen containing exosome and TLR ligands (Fig 3.8A-C and Fig 3.8E-G). We also detected a significantly elevated IgG2a/IgG1 ratio after immunization with exosomes co-encapsulating poly(I: C) and CpG ODN indicating the development of antigen-specific Th1-biased immunity ($p=0.0286$ for both antigens) (Fig 3.8D and Fig 3.8H).

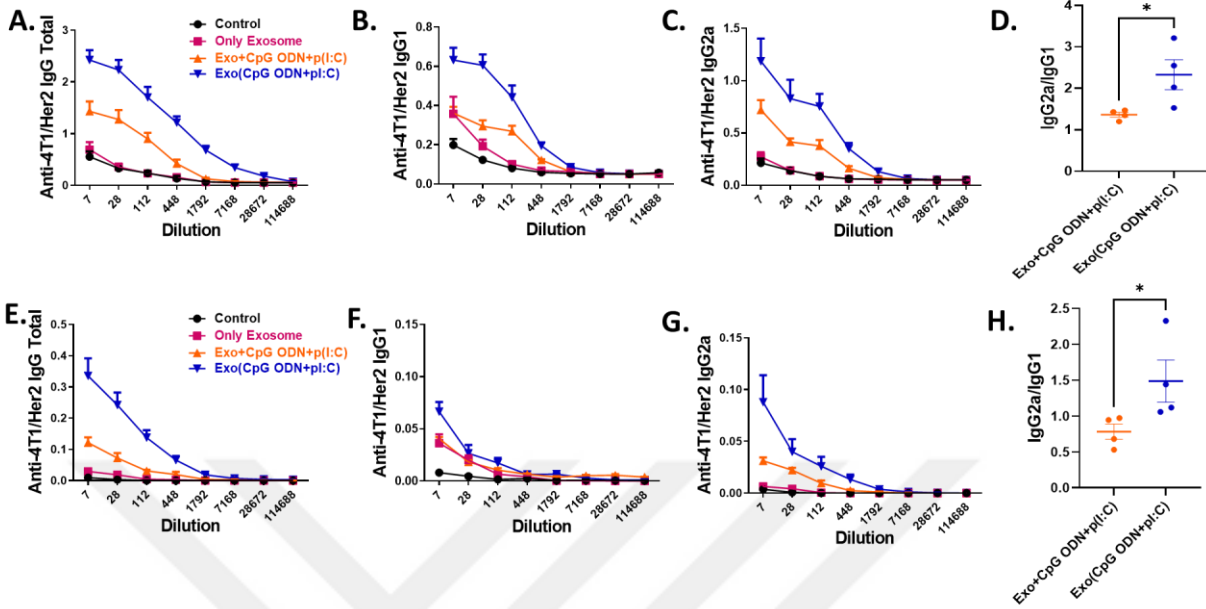


Figure 3.8. 4T1/Her2 cell-derived exosomes loaded with CpG ODN and poly(I:C) immunization generate sustained and magnified anti-4T1/Her2 immune responses. Mice (n=4 mice/group) were injected i.p. with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g pI:C or exosome loaded with CpG ODN + poly(I:C) on days 0 and day 14. Mice were sacrificed on day 28. **A-C)** Sera of immunized mice were investigated for systemic anti-4T1/Her2 antibody production against 4T1/Her2 cell lysate. End-point average titers of anti-4T1/Her2 total IgG, IgG1 and IgG2a responses of mice were shown. **D)** IgG2a/IgG1 ratio of individual mouse either immunized with free or immunized with exosomal formulation at 2 weeks after booster injection (at 1:112 dilution against 4T1/Her2 cell Lysate). Mann-Whitney test was used for statistical analysis. **E-G)** Sera of immunized mice were investigated for systemic anti-4T1/Her2 antibody production against protein extracts of 4T1/Her2 cell-derived exosome. End-point average titers of anti-4T1/Her2 total IgG, IgG1 and IgG2a responses were shown. **H)** IgG2a/IgG1 ratio of individual mouse either immunized with free or immunized with exosomal formulation at 2 weeks after booster injection (at 1:7 dilution against protein extracts of 4T1/Her2 cell-derived exosomes). Mann-Whitney test was used for statistical analysis. The data indicate the mean $\pm$ SEM (n = 4). Statistically significant differences designated as follows: ns: p > 0.05, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

Furthermore, we analyzed whether exosome loaded with CpG ODN and p(I:C) produce Her2-specific antibodies. To assess this, we used Her2-specific E75 peptide. As shown in Fig 3.9A, mice immunized with dual ligand-loaded exosomes produced significantly higher Her2/E75 specific total IgG antibody than mice immunized with only exosomes and free exosomal combination ($p=0.0286$). We detected significantly elevated IgG2a/IgG1 ratio after immunization with CpG ODN plus p(I:C) loaded exosomes ($p=0.0286$ for both antigens, Fig 3.9B). Collectively these results pointed out that CpG ODN and p(I:C) loaded exosomes elicited anti-4T1/Her2 and Her2/E75 specific Th1-biased immunity.

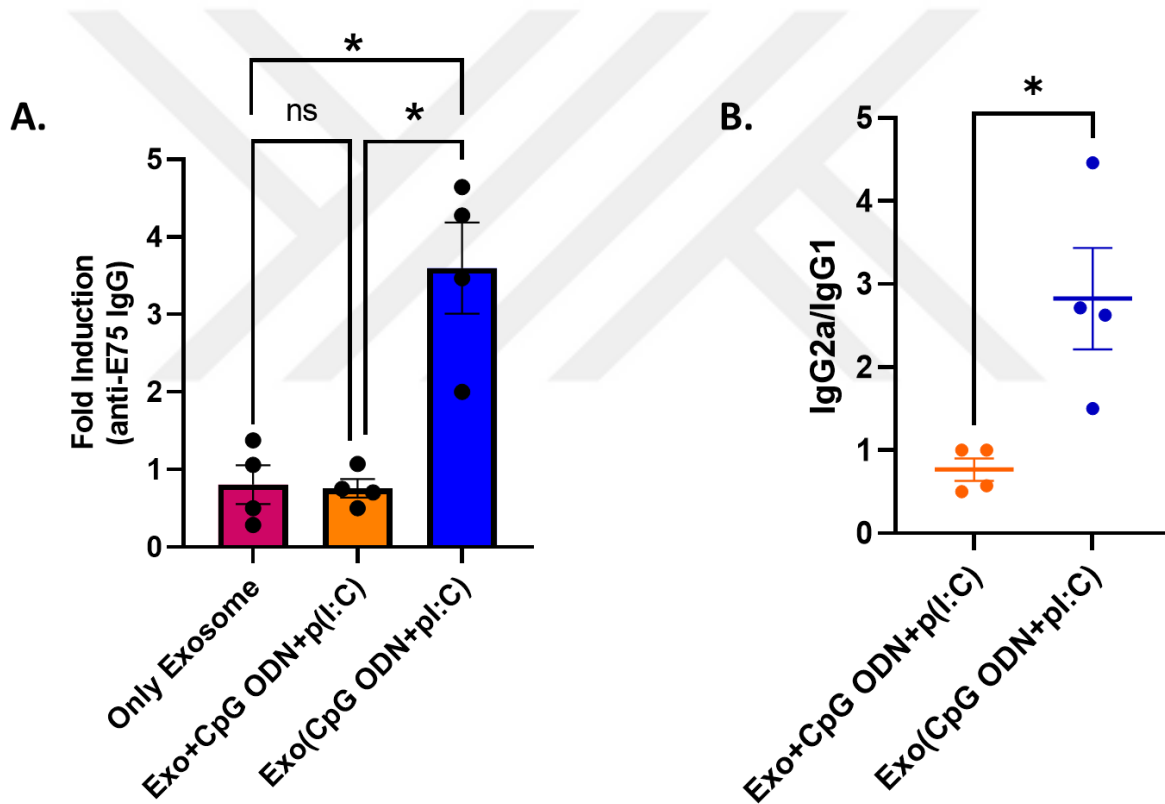


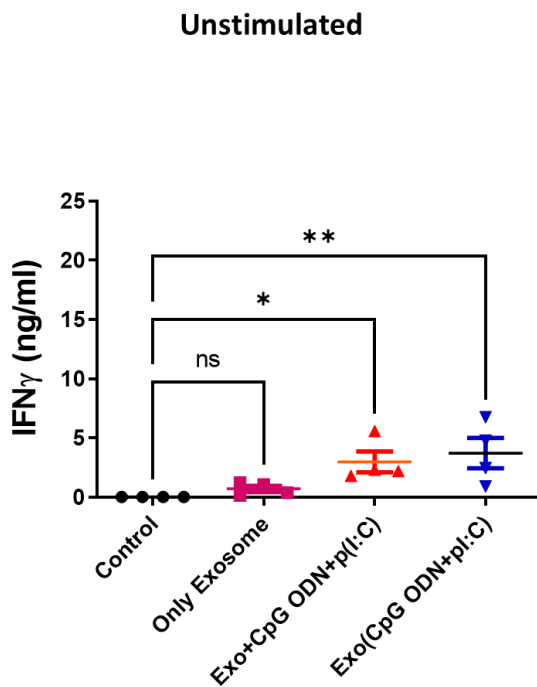
Figure 3.9. 4T1/Her2 cell-derived exosomes loaded with CpG ODN and poly(I:C) immunization magnified anti-E75 immune responses. Mice ($n=4$ mice/group) were injected i.p. with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN + poly(I:C) on days 0 and day 14. Mice were sacrificed 14 days after the booster injection **A)** Bar graphs showing fold induction over control group of sera of immunized mice for systemic anti-IgG antibody production against Her2/E75 peptide. **B)** IgG2c/IgG1 ratio of individual mouse either immunized with free or immunized with

exosomal formulation at 2 weeks after booster injection (at 1:28 dilution against Her2/E75 peptide). Mann-Whitney test was used for statistical analysis. The data indicate the mean $\pm$ SEM (n = 4). Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

3.2.3. Induction of antigen-specific IFN γ from splenocytes of animals treated with CpG ODN and poly(I:C) loaded TEXs.

To evaluate the type of immune response generated, splenocytes were isolated two weeks after the last booster injection and IFN γ production was analyzed by ELISA assay. As shown in Fig 3.10A, we detected that both non-encapsulated combination and dual ligand co-encapsulated exosome group generated more IFN γ compared to control group ($p=0.0143$, $p=0.0089$ respectively). Furthermore, Fig 3.10B shows that when the splenocytes of immunized mice were activated with 4T1/Her2 exosomes, specific IFN γ productions of non-encapsulated combination and co-encapsulated exosome group were boosted significantly compared to control groups ($p=0.0347$, $p=0.0019$, respectively).

A.



B.

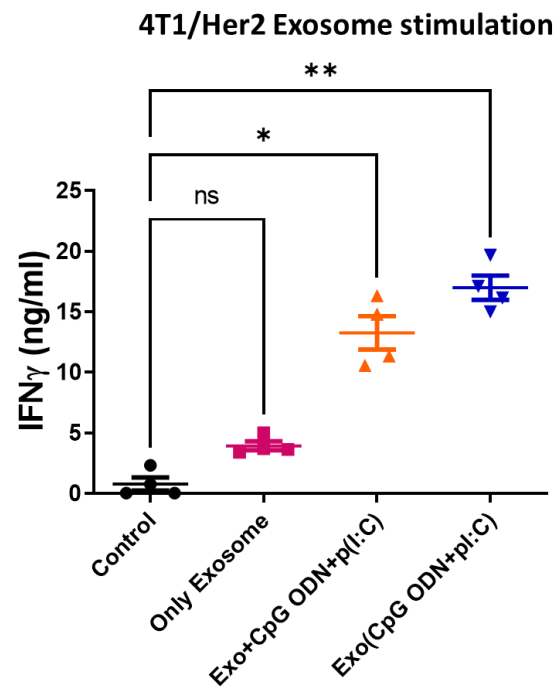


Figure 3.10. IFN γ assay on ex vivo expanded splenocytes of mice immunized two times with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN + poly(I:C) **A)** 14 days later after the booster, the spleens of mice from each group were harvested and immune responses were evaluated using an IFN γ ELISA assay. **B)** 14 days after the booster spleens of mice (n =4) were harvested and activated with 4T1/Her2 exosome and the induced immune responses were then evaluated using IFN γ ELISA assay. The data indicate the mean $\pm$ SEM (n = 4). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: p > 0.05, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

3.2.4. Antigen specific T cell responses are more pronounced by CpG ODN and poly(I:C) loaded into TEXs than their free form

To further differentiate the contribution of TLR ligand co-encapsulation within exosomes on cellular immunity that was raised against 4T1 tumors, we performed IFN γ specific intracellular cytokine staining on splenocytes. To this end, the IFN γ ⁺ T-cell subsets following staining of CD4 and CD8 were analyzed by flow cytometry. The data showed that before and after 4T1/Her2 exosome stimulation in culture the induction of IFN γ producing CD4⁺ T cells were significantly higher in mice immunized with exosomes loaded with TLR ligands compared to the control groups. (P=0.046, P=0.0032 respectively) (Fig 3.11A and Fig 3.11B).

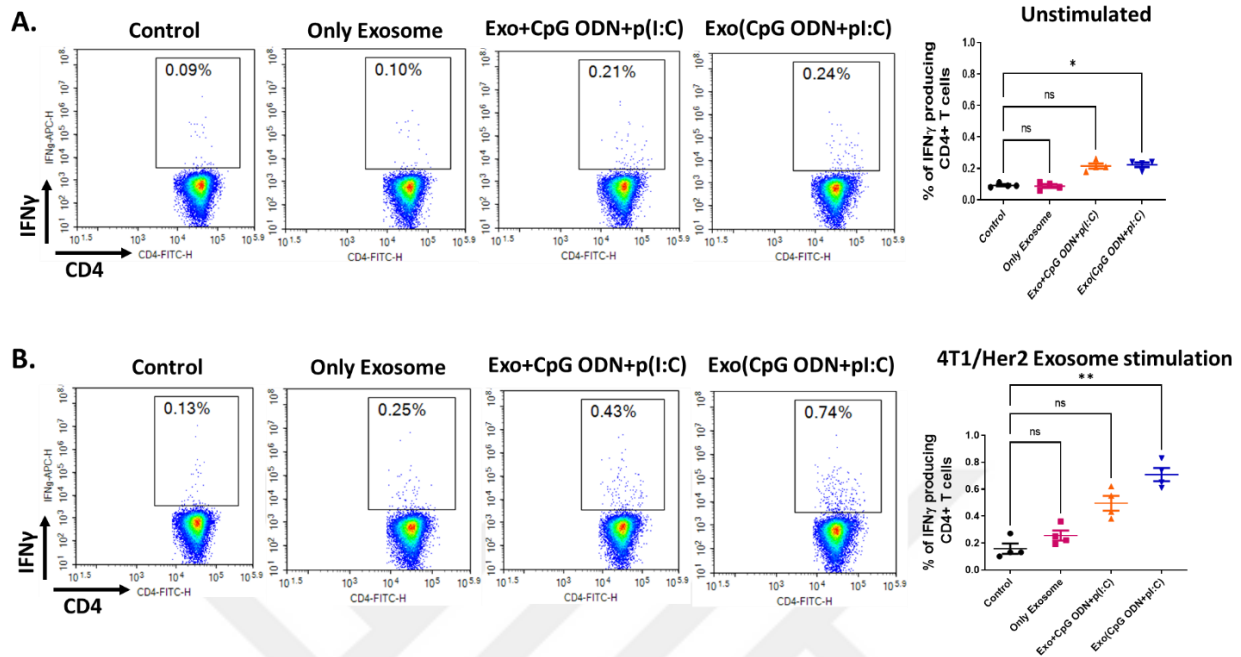


Figure 3.11. Intracellular cytokine production assay on splenocytes of mice immunized two times with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN + poly(I:C). **A)** 14 days later after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC surface markers and then with anti-IFN γ -APC antibodies. **B)** 14 days later after the booster, Isolated splenocytes of different groups of vaccinated mice were activated with 4T1/Her2 exosome and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC surface markers and then with anti-IFN γ -APC antibodies. The frequency of IFN- γ producing cells within CD4 $^{+}$ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean $\pm$ SEM (n = 4). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: p > 0.05, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

As seen in 3.12, similarly intracellular cytokine staining assay revealed that when splenocytes were stimulated overnight with 4T1/Her2 exosome in culture, only Exo (CpG ODN + p(I:C)) group had significantly augmented IFN γ production from CD8 $^{+}$ T-cells compared to the control group (P=0.0127).

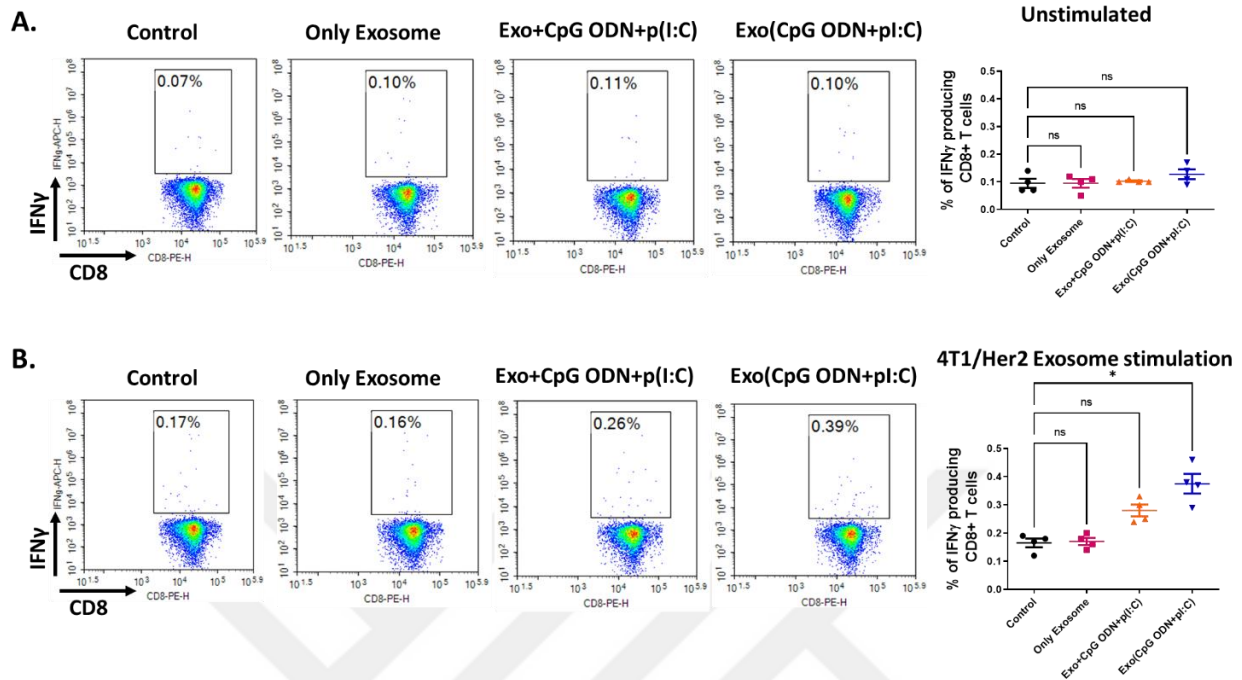


Figure 3.12. Intracellular cytokine production assay on splenocytes of mice immunized two times with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN + poly(I:C). **A)** 14 days later after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD8-PE surface markers and then with anti-IFN γ -APC antibodies. **B)** 14 days later after the booster, isolated splenocytes of different groups of vaccinated mice were activated with 4T1/Her2 exosome and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD8-PE surface markers and then with anti-IFN γ -APC antibodies. The frequency of IFN- γ producing cells within CD4 $^{+}$ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean $\pm$ SEM (n = 4). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: p > 0.05, *:p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

4T1/Her2 specific memory T-cell is one of the major contributing cell populations to eliminate Her2 expressing tumor cells, therefore, we analyzed the immunized mice splenocytes to assess the antigen-specific CD4 $^{+}$ memory T cell percentage. We determined that mice immunized with Exo (CpG ODN+p(I:C)) generated significantly higher IFN γ producing CD4 $^{+}$ memory T cell compared

to the control group ($p=0.016$) (Fig 3.13A). When immunized mice cells were incubated with 4T1/Her2 expressing exosome, IFN γ producing CD4 $^{+}$ memory T cells expanded significantly in Exo (CpG ODN+ p(I:C)) treated group compared to the control group ($p=0.0042$) (Fig 3.13B).

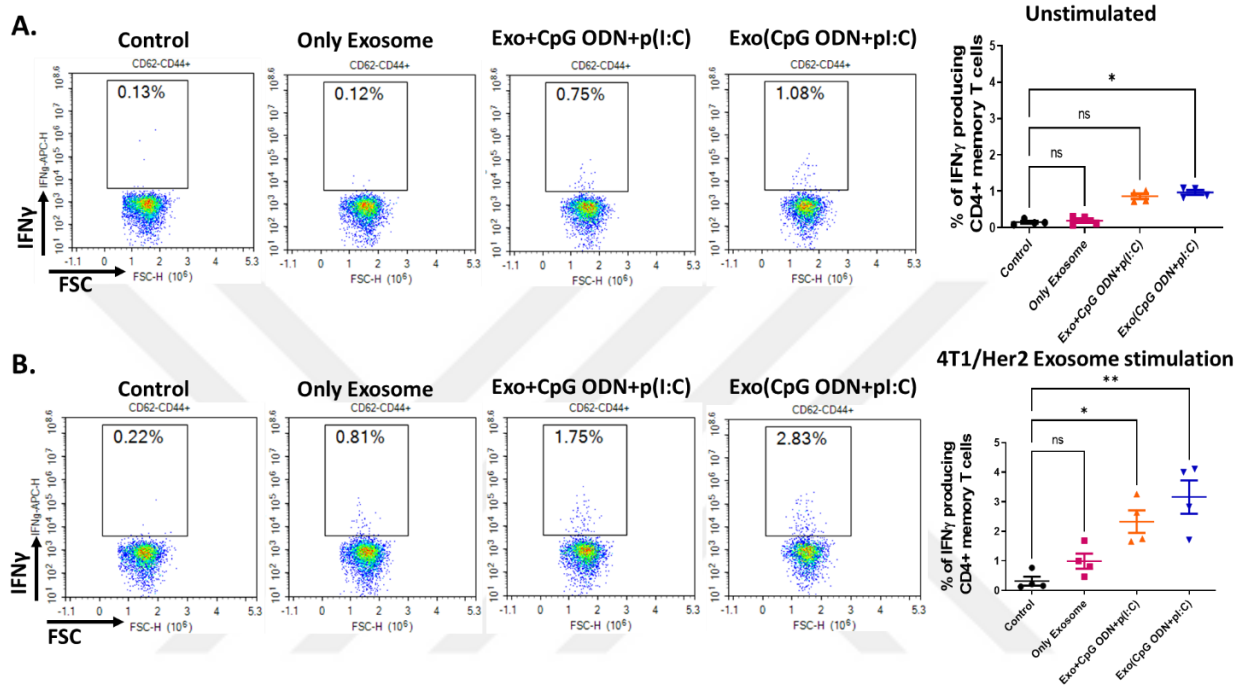


Figure 3.13. IFN- γ producing CD4 $^{+}$ memory T cell assay on splenocytes of mice immunized two times with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN + poly(I:C). **A)** 14 days later after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC, CD44-APC/Cy7, CD62L-BV605 surface markers and then with anti-IFN γ -APC antibodies. **B)** 14 days later after the booster, isolated splenocytes of different groups of vaccinated mice were activated with 4T1/Her2 exosome and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC, CD44-APC/Cy7, CD62L-BV605 surface markers and then with anti-IFN γ -APC antibodies. CD4 $^{+}$ memory T cell identified as CD45 $^{+}$ CD3 $^{+}$ CD4 $^{+}$ CD8 $^{-}$ CD44 ^{high} CD62 ^{low} . The frequency of IFN- γ producing memory T cells within CD4 $^{+}$ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean $\pm$ SEM ($n = 4$). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

3.2.5. Administration of only 4T1 tumor-derived sEVs loaded with CpG ODN and poly(I:C) to animals regress tumor growth in 4T1 tumor-bearing mice.

Considering the strong cellular and humoral immune responses in vaccinated mice, it was expected that the immune responses would lead to antitumor activity in an established 4T1-luc tumor model. To evaluate if the cellular and humoral immune responses induced by the Exo (CpG ODN+p(I:C)) vaccination were potent enough to induce an in vivo antitumor effect, the mice were injected with 4T1-luc tumor cells orthotopically into the right mammary fat pads. Following after tumor inoculation, animals were intraperitoneally injected with Exo (CpG ODN+p(I:C)) and were observed for tumor regression for 36 days post tumor formation. As shown in Fig 3.14A, during the days 10–36, the group of mice that received Exo (CpG ODN+p(I:C)) regressed tumor development significantly in comparison with untreated control group ($P=0.0045$ for day 32 and $P=0.0024$ for day 36). Also, The survival time was significantly prolonged in Exo (CpG ODN+p(I:C)) treated groups in comparison with the control group receiving PBS ($P = 0.0229$). All mice belonging to control group were dead by day 49 whereas 3 out of 5 mice treated with Exo (CpG ODN+p(I:C)) were alive by day 60. Furthermore, we analyzed the $IFN\gamma$ secretion, helper T cell and cytotoxic T cell population from splenocytes of animals that treated with the PBS and CpG ODN+ pI:C loaded exosomes at day 0, day 3, day 6, day 10, day 17, day 24 and day 31 after tumor inoculation. As shown in Fig 3.14B, Splenocytes from animals that treated with TLR ligands loaded formulation secretes significantly higher level of $IFN\gamma$. Also, we found that the percentage of $CD4^+$ and $CD8^+$ T cells in splenocytes are significantly higher than mice treated with TLR ligands loaded exosomes compared to control group. ($P= 0.0079$). The anti-tumor effect of Exo (CpG ODN+p(I:C)) group was evaluated by measurement using an IVIS imaging system. We measured bioluminescence activity every 10 days (day 0, day 10, day 20 and day 32 after tumor inoculation). As shown in the bioluminescence imaging results at day32, 1 out of 5 mice treated with Exo (CpG ODN+p(I:C)) had no luciferase signal (Fig 3.14C) and exosome treated group had significantly lower luciferin activity over time (Fig 3.14D).

Collectively, our findings implied that a combination of poly(I:C) and CpG ODN ligands within exosomal formulations induced stronger and persistent humoral and cellular anti-4T1/Her2 specific immune response sufficient to regress tumor growth.

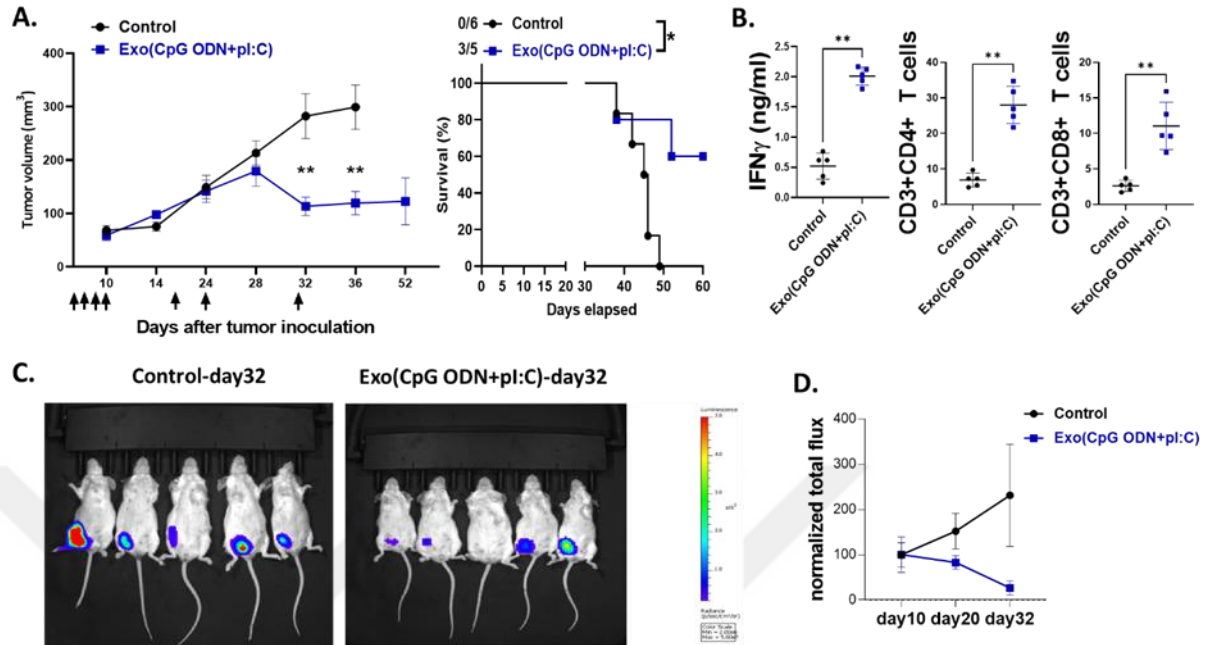


Figure 3.14. The therapeutic effect of vaccination with PBS and exo(CpG ODN+p(I:C)) in BALB/c mice against a 4T1-Luc in vivo established tumor mice model **A)** 4T1-luc tumor development is suppressed by immunization with exosome co-loaded with CpG ODN+p(I:C) (30ug each, arrows indicates i.p. injections of exosome vaccine formulation) and the effect of vaccination on the survival time of mice (6 for control group and 5 for vaccinated group) was observed during a 60-day period post tumor inoculation. **B)** IFN γ assay, Helper T cell and Cytotoxic T cell staining on ex vivo expanded splenocytes of mice treated with PBS, and exosome loaded formulation **C)** In vivo luciferase images of 4T1-luc tumors in control and vaccinated group at day 32. (IVIS Spectrum). **D)** Luciferin signal activity normalized to day 1. Tumor volume plot is collected from 2 independent experiments (n=5 in each group). Mann-Whitney test was used for statistical analysis. Statistical significance for tumor volume and survival time was analyzed by Šídák's multiple comparisons test and Log-rank (Mantel-Cox) test, respectively. Normalized total flux was analyzed by Bonferroni's multiple comparisons test. Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$.

PART-III: The potential use of SPION loaded exosomes for therapeutic cancer vaccine

3.3.1. Characterization of SPION loaded exosomes isolated from RAW 264.7 cell line

Since we show that exosomes were loaded with biomolecular ligand such as TLRs ligand and model antigens, in this part of thesis, we postulated that inorganic compounds such as magnetic iron nanoparticles (SPION) could also be encapsulated within exosomes using our approach. Also, the in vivo delivery of exosome to tumor site using an external stimulus such as magnetic field would expand the breadth of EV-based immunotherapy in the clinic.

Initial experiments were planned to characterize the SPION loaded exosomes isolated from Raw 264.7 cell line. Exosomes were analyzed by i) surface marker expression by flow cytometry, ii) exosome-specific protein expression by immunoblotting iii) SPION detection by transmission electron microscopy (TEM), iv) morphological analyses by Scanning electron microscopy (SEM) and iv) size distribution by DLS. (Fig 3.15)

Anti-CD81 conjugated latex bead-captured exosomes were stained for expression of CD81 and CD9 surface marker proteins and analyzed using flow cytometry. (Fig 3.15A). As expected, exosomes express their cardinal surface marker. Alix protein expression were detected by immunoblotting. (Fig 3.15B). TEM analysis showed that SPION were co-existed with exosomes. (Fig 3.15C). SEM studies revealed that exosomes had spherical shape. (Fig 3.15D). DLS analysis indicated that exosome an average size around 150-180nm. (Fig 3.15E). Collectively, these results indicate that exosomes were successfully isolated from RAW 264.7 cell line and can be loaded with inorganic compounds by lyophilization technique.

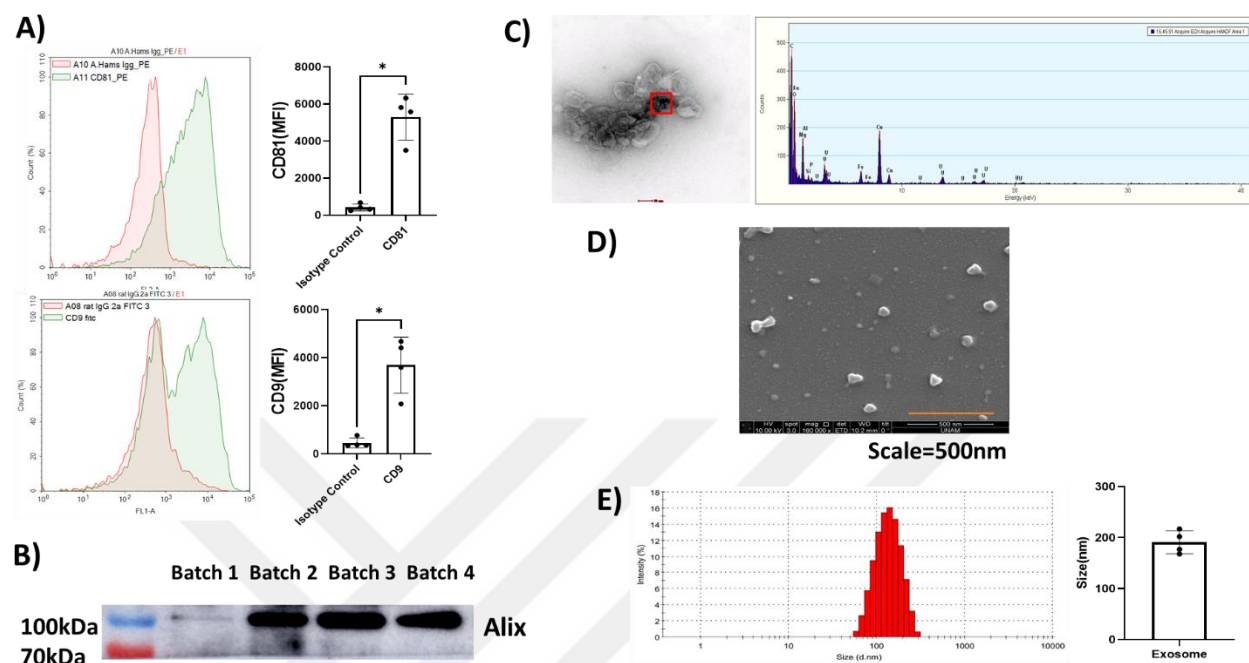


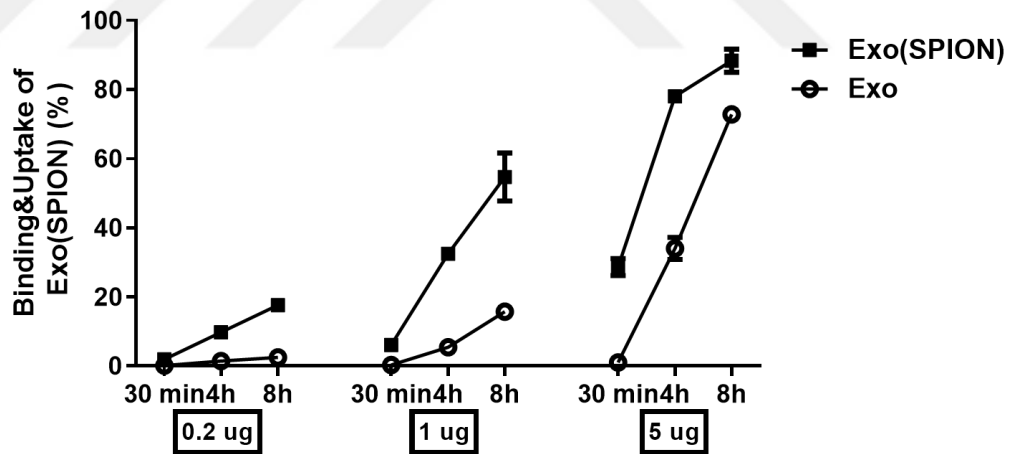
Figure 3.15. Characterization of SPION loaded exosomes isolated from RAW 264.7 cell line.

A) Exosomes coupled to anti-CD81 conjugated beads were phenotyped using flow cytometry analysis. Bar graphs represent the average MFI values of specific antibodies to isotype control staining of 4 separate exosome batches. **B)** Western blot analysis of exosomal marker Alix in RAW 264.7 cell derived exosome. **C)** TEM photomicrographs of SPION loaded RAW 264.7 cell derived exosomes. **D)** A representative scanning electron microscopy (SEM) image of RAW264.7 cell-derived exosome. Scale bar represents 500 nm. **E)** Dynamic Light Scattering (DLS) analysis showing average size distribution of RAW 264.7 cell-derived exosome (n=3 independent batches). Statistical significance was analyzed by t-test. Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$.

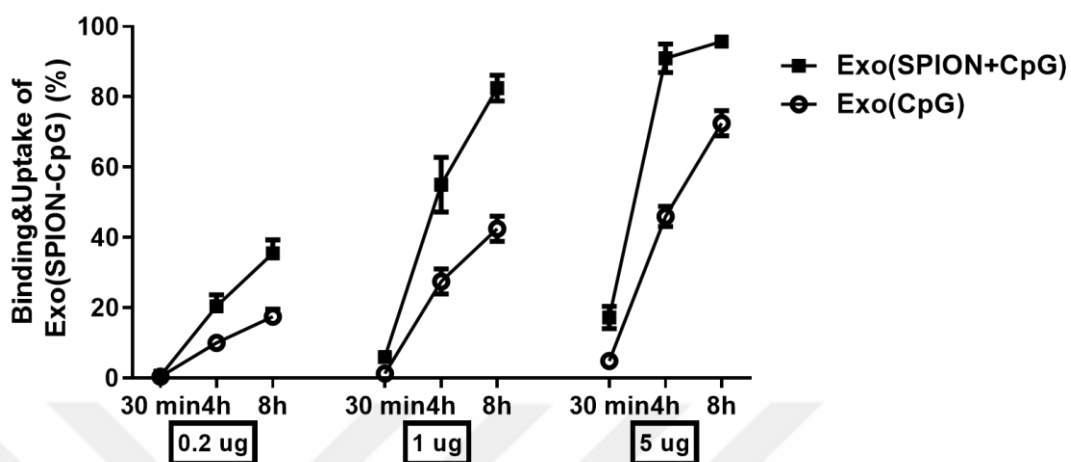
3.3.2. Internalization capacity of CpG ODN and SPION loaded exosomes by RAW 264.7 cell line and splenocytes

Next, we wanted to investigate the effect of SPION loading on internalization capacity of exosomes. For this, RAW 264.7 cells were incubated with different doses of exosomal form of SPION (4 μ g exosome:1 μ g SPION ratio) and exosomal form of SPION and CpG ODN (4 μ g exosome:1 μ g SPION:1 μ g Ligand ratio) for 30 minutes to 8 hours. We found out that time- and dose-dependent binding and uptake of exosomes encapsulating with SPION or SPION-CpG ODN by RAW 264.7 cells were significantly higher compared to unloaded exosomes. (Fig 3.16A and Fig 3.16B). Also, entry of SPION loaded exosomes into i) F4/80⁺ macrophages, ii) CD19⁺ B-cells, iii) CD11c⁺ dendritic cells, and iv) CD3⁺ T-cells were much higher compared to CpG loaded exosomes. (Fig 3.16C). When these results were taken together, loading SPION enhanced the in vitro delivery of exosomes within immune cells.

A)



B)



C)

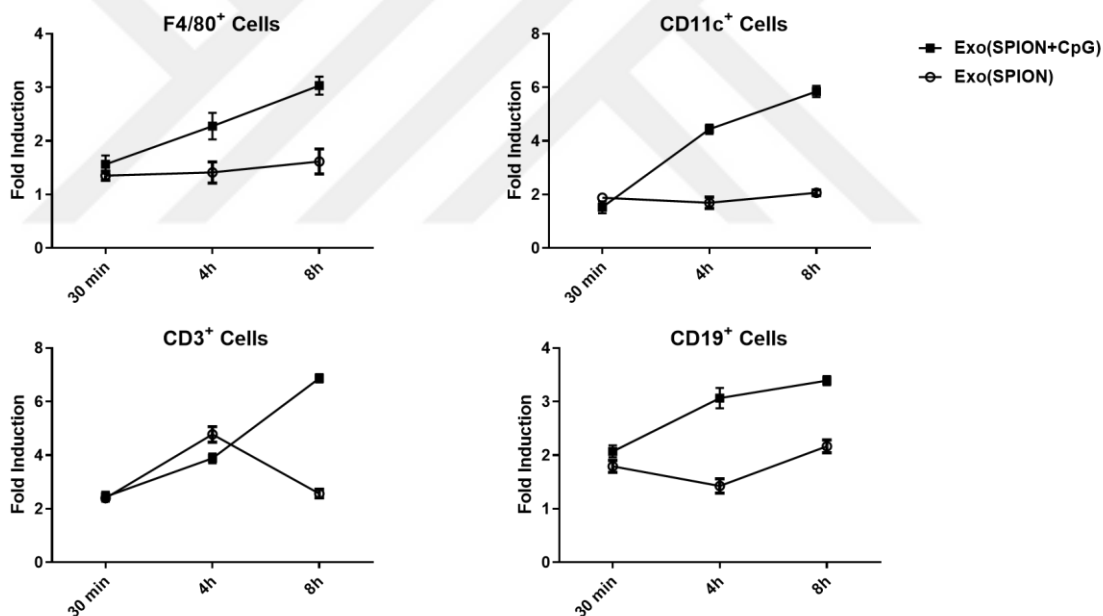


Figure 3.16. Co-encapsulation of CpG ODN and SPION within exosomes increases ODN internalization into RAW264.7 cells and splenocytes cells A) RAW 264.7 cell line were incubated with different doses of exosomal form of SPION (4µg exosome:1µg SPION:1 µg Ligand ratio) for 30 minute-8 hours. B) RAW 264.7 cell line were incubated with different doses of exosomal form of CpG ODN and SPION (4µg exosome:1µg SPION:1 µg Ligand ratio) for 30 minute-8 hours. C) Splenocytes isolated from wild type C57BL/6 mice were incubated with different doses of exosomal form of CpG ODN and SPION (4µg exosome:1µg SPION:1 µg Ligand

ratio) for 30 minute-8 hours. Line graphs shows uptake of SPION loaded exosomes by F4/80+ Macrophage cells, CD11c+ DC cells, CD3+ T cells, and CD19+ B cells

3.3.3. IFN γ and IL-12 induction capacity of CpG ODN and SPION loaded exosomes from splenocytes

After we investigated that loading SPION improved exosome binding and uptake capacity by immune cells, we next want to explore how this enhanced internalization effect on the IFN γ cytokine secretion, which is important in tumor clearance. For this purpose, splenocytes isolated from wild type C57BL/6 mice were stimulated with free CpG ODN, exosome-encapsulated CpG ODN or exosome-encapsulated CpG ODN together with SPION (1 μ g/ml per ligand; 4ug exosome/1ug ligand) for 12 hours for IL12 and IFN γ . We found out that spleen cells incubated with exosomes encapsulating SPION + CpG ODN induced significantly higher levels of IL-12 (Fig 3.17A) and IFN γ (Fig 17B) secretion.

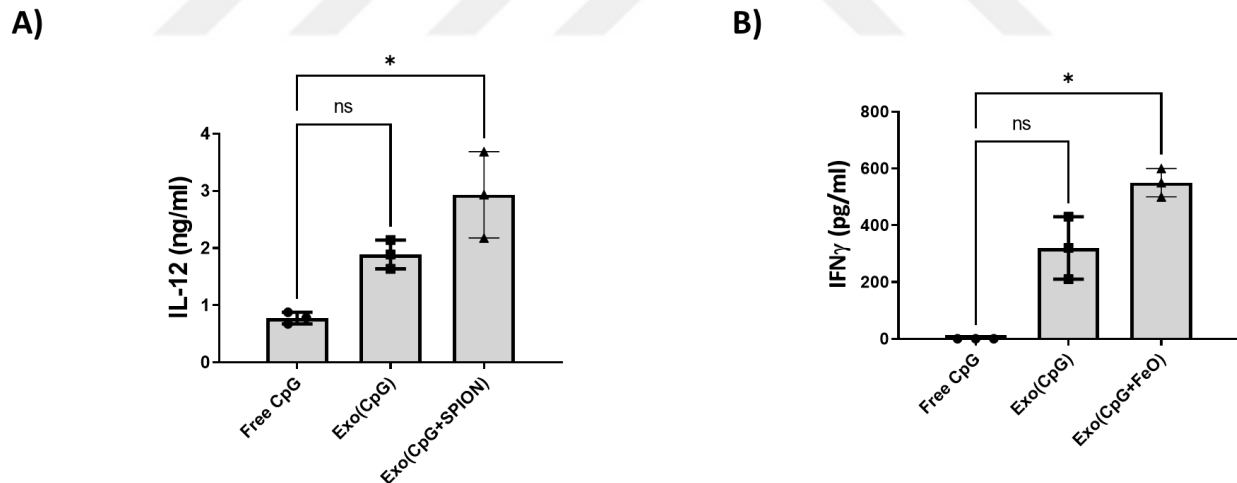


Figure 3.17. Co-encapsulation of CpG ODN and SPION within exosomes treated splenocytes secretes higher amounts of IL-12 and IFN γ . Splenocytes isolated from wild type C57BL/6 mice were stimulated with free CpG ODN, exosome-encapsulated CpG ODN or exosome-encapsulated CpG ODN together with SPION (1 μ g/ml per ligand; 4ug exosome/1ug ligand) for 12 hours for IL12 and IFN γ . Bar graphs represent **A)** IL12 and **B)** IFN γ production detected by ELISA from cell supernatants. Statistical significance was analyzed by Kruskal Wallis test. *, p<0.05, **

3.3.4. In vivo application of TLR ligands and SPION loaded exosomes in HCC tumor model in athymic mice

After, observing this improved immune activation of SPION loaded exosomes, we wished to test in vivo performance. Following xenograft transplantation of HUH7 cells (n=8/group) at the back of nude mice, animals were followed for 7-9 days to observe the formation of palpable tumors. They were then injected intraperitoneally 4 times with the RAW 264.7 cell derived exosomes that was loaded with i) aTLR3L; pI:C, ii) a TLR9L; CpG ODN and iii) SPION. Changes in tumor volumes were recorded daily by an electronic caliper (Fig 3.18A). Notably, at day=43 after tumor inoculation, Exo(p(I:C) +CpG ODN+ SPION) therapy led to significant tumor regression compared to the other groups. (Fig 3.18A). Similarly, extracted tumor weights of Exo(p(I:C) +CpG ODN+ SPION) treated group were significantly smaller than unencapsulated p(I:C) +ODN+SPION mixture treated groups (Fig 3.18C). In another experiment, aiming to gauge the benefit of the SPION loaded exosome therapy, animals (n=10/group) were followed until all control mice succumbed to tumor. At the end of two months, while all PBS treated mice died, 9/10 mice that had 4 times injections of Exo(p(I:C) +CpG + SPION) survived. Interestingly, 40% of mice that had p(I:C) +CpG + SPION mixture was alive (Fig 3.18B). This data strongly implied that when TLR3 and TLR9 ligands were effectively encapsulated with SPION loaded exosomes, a more pronounced innate immune activation was elicited, and this collective activation helped to regress tumors and improve survival rate of treated mice.

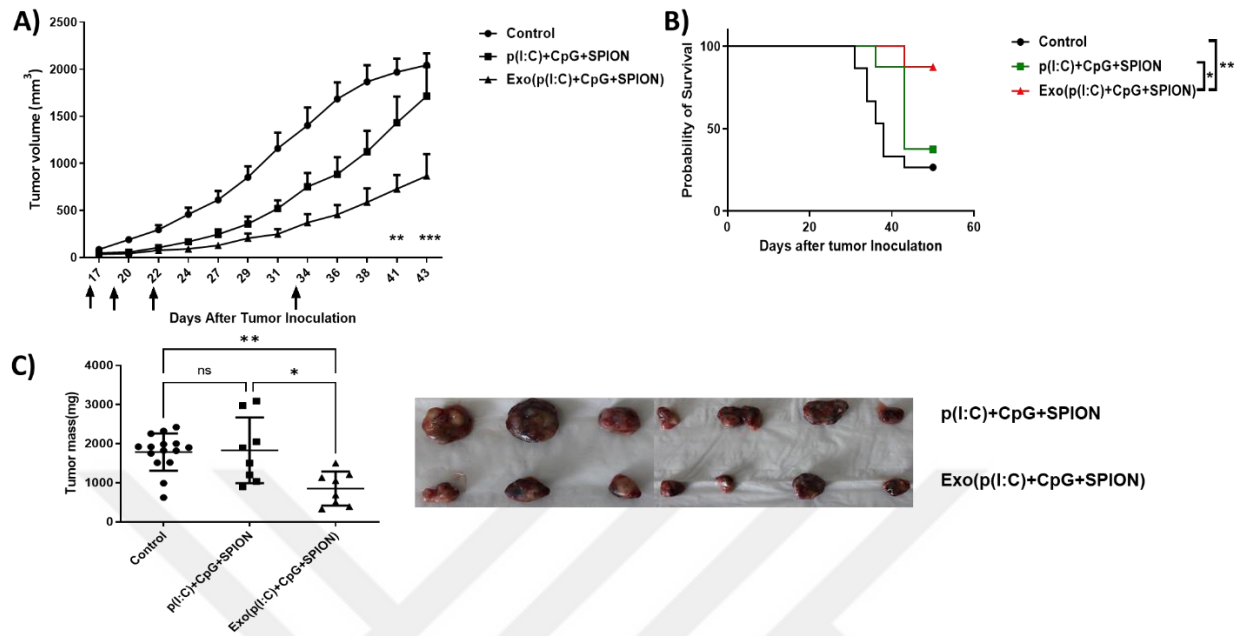


Figure 3.18. HUH7 Tumor-bearing animals that were treated with Exo(p(I:C) +CpG + SPION) (4-time injection) significantly regressed tumor development compared to other groups at time of sacrifice after tumor inoculation. A) Line graphs shows average tumor volumes. **B)** Percent survival of mice treated with Exo (pI:C + CpG + SPION) and other combinations **C)** Representative pictures showing differences in tumor sizes between groups at the time of sacrifice (day 43 after tumor inoculation). Two-way ANOVA with Bonferroni correction was used for Kinetic Tumor Volume. Statistical analysis represents the significance of Exo(p(I:C + CpG +SPION) group to p(I:C) +CpG+ SPION group. ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

CHAPTER 4

DISCUSSION

4.1. Application of EVs as a cancer immunotherapy

Immunotherapy has become a powerful clinical strategy to boost the activation of the host immune system against tumors that evade immune surveillance and induce tolerance. These cancer therapies enhance antitumor immune responses with fewer off-target effects than traditional treatments, such as chemotherapy and radiation that lack tumor selectivity and have severe side effects [258]. Tumor antigen-based cancer immunotherapy has gained significant attention to elicit antigen-specific strong T and B cell responses. However, the purification of particular tumor antigens is challenging [259,260]. Therefore, identifying tumor-specific nano-structures that naturally harbor endogenous tumor antigens and further engineering these structures to co-deliver immune-stimulating adjuvants yielding potent anti-tumor immunity is of great benefit during the management of established tumors. A depot system that permits the simultaneous presentation of tumor antigen and initiating tumor-specific immune activation could offer effective tumor targeting while reducing undesirable toxicity and pave the way for patient-specific anti-cancer immunotherapy [261,262].

In this part of the thesis, we use the lyophilization method and exosomes as a depot system to permit the external loading of exosomes with various organic or inorganic ligands. The lyophilization-based loading method was initially designed to achieve high levels of entrapment in liposomes [263,264]. The technique involved mixing small unilamellar vesicles (SUVs) with an equal volume of an aqueous molecule of interest, followed by freeze-drying of the mixture under

reduced pressure into a lyophilized powder. Controlled powder rehydration with a small volume of distilled water enables vesicle reformation and high-yield entrapment of the active agent [265,266]. Considering that exosomes are lipid vesicles similar in size to SUVs, we hypothesized that the lyophilization approach could be extended to exosomes to enable ligand encapsulation. This approach provides several advantages over existing exosome loading/delivery systems by surmounting the major drawback of batch-to-batch variations attained by conventional cell-feeding methods [138]. Such external feeding-based methods do not allow encapsulation of synthetic ligands such as immunostimulatory CpG ODNs without changing the physiological state of the cells intended for exosome loading and purification. In contrast, the present technique warrants external exogenous ligand loading into purified exosomes. Similarly, our lyophilization-based loading method is advantageous over the electroporation-based method, which critically compromises exosome integrity. It, at best, accomplishes loading efficiencies of ~0.05 % for siRNA and ~2% for linear DNA[267,268]. Considering that other available methods, such as “the engineered producer cell approach,” can only express specified nucleic acids (i.e., siRNA or miRNA) but not synthetic ones (i.e., CpG ODN) in the lumen to enclose the cargo within the secreted exosomes [269], our strategy provides the flexibility of encapsulating both natural and synthetic nucleic-acid based therapeutics [270,271].

We carefully investigated the effect of dehydration-rehydration on exosome integrity. Importantly, we demonstrate that the vesicular integrity and content remain unaffected upon lyophilization and reconstitution (Figure 3.1A-3.1C-3.1D). Notably, reconstitution and inclusion of ligand(s) increase exosome average size distribution (Figure 3.1B), suggesting that reconstitution might initiate the formation of biomolecule-encapsulating multilamellar exosomes, similar to the process observed with dehydration-rehydration vesicles [272]. Furthermore, injecting only exosome and CpG-loaded exosomes into mice confirmed that exosomes alone do not induce an immune response, whereas CpG-loaded exosomes significantly induce an immune response (Figure 3). Moreover, when we analyzed the biodistribution of exosomes encapsulated with model antigen and immunostimulatory ligands CpG ODNs, we showed that these exosomes mainly were taken up by antigen-presenting cells (Figure 2). This data indicated that activating antigen-presenting cells is the first requirement of the developing antigen-specific adaptive immune response to regress tumor development.

The cell-free exosome-based immunotherapy concept utilizes cell line or patient-derived exosomes as a novel cancer immunotherapy strategy in several clinical trials with limited success. The first exosome-based anti-cancer therapeutic was reported two decades ago [273]. After that, interest in dendritic cell-based exosomes led to the realization that immature DCs secrete a copious number of exosomes that lack immune stimulatory potential[274], whereas mature DCs have limited exosomes secretion capacity[275], but the secreted EVs possess potent immunostimulatory activity [30,276]. Here, we used the lyophilization method to bypass all these deficiencies and extend the outcomes of these studies simply by utilizing cell culture-derived exosomes and loading them with an immunostimulatory agent of interest, following their purification.

α -GalCer is a natural glycolipid recognized by CD1d restricted iNKT cells. α -GalCer-activated iNKT cells have a protective role against tumors, autoimmune diseases, and infectious diseases [233,234,235]. Activation of iNKT cells by α -GalCer induces full maturation of DCs. α -GalCer increases the expression of co-stimulatory molecules, including CD40, CD80, and CD86, and MHC class II molecules on DCs [236]. Therefore, our study used α -GalCer to boost the anti-tumor activity of CpG-loaded macrophage-like cell-derived exosomes along with model antigen OVA via lyophilization. Our findings showed that loading triple ligands into exosomes improved the tumor regressing capacity of immunostimulatory ligands in the B16F10-OVA melanoma tumor-bearing mice and significantly extended the survival rate of tumor-bearing mice (Figure 4).

T helper cells are crucial to activating antigen-specific effector cells to develop immune responses against tumors and infectious diseases [277,278]. Antigen-presenting cells or MHC class II-expressing tumors can activate tumor antigen-specific Th cells. The delivery of antigens limits tumor cell migration via APC to lymph node T cells in the early stage of the disease. Melanomas typically express a high level of MHC class II, whereas most tumor cells do not express MHC class II. Tumor antigen-specific CTLs are directly activated by antigen primed Th cells. Researchers indicated that Th cells enhanced tumor-specific CTL function through co-stimulatory molecules present on the surface of the CTL, such as MHC class II, CD134, and CD27 [279]. Furthermore, Th cells release $\text{IFN}\gamma$ to activate APC, thus enhancing CTL activity indirectly [280]. $\text{IFN}\gamma$ producing Th1 cells activate tumor macrophages to produce nitric oxide and superoxide. Nitric oxide and superoxide are crucial mediators in killing tumor cells [281]. Consequently, Th cells express many factors that impact the tumor antigen-specific CTL response. For these reasons, when

we analyzed the nature of the anti-OVA response in the context of Th-based immune development, our findings showed that analysis of IgG2c/IgG1 ratio of triple ligands loaded exosome generated Th1 biased immune response. This Th1 biased immune response increased the amount of OVA-SIINFEKL specific CD8⁺ cytotoxic T cells and regressed melanoma tumors in mice (Figure 5).

Activating T cells or NK cells is essential in recognizing and eliminating tumor cells in cancer immunotherapies. However, the tumor microenvironment has a wide variety of factors that limit the activity of effector cells. Myeloid-derived suppressor cells (MDSC) emerged as one of the major mechanisms blocking immune effector cells' function in cancer. MDSC are largely immature myeloid cells that display potent immune-suppressive activity [282]. MDSC increases tumor progression by promoting tumor cell invasion and tumor angiogenesis. MDSC has also contributed to the formation of pre-metastatic niches [283]. Therefore, we investigated how triple ligand-loaded exosomes affect the tumor microenvironment's immune cells. Our findings demonstrated that MDSC and M2-like macrophages, tumor-promoting cells, were not significantly different in triple-loaded exosome immunized animals compared to other groups. Triple ligand-loaded exosomes improved the tumor repressing immune cell types like CD4⁺ T cells, CD8⁺ T cells, and NK cells (Figure 6).

Our study demonstrates that adjuvanted lyophilized exosomes of non-dendritic cell origin can mount sufficient cancer-antigen dependent humoral and cell-mediated immune responses. This activity is potent enough to eradicate solid tumors in a preventive cancer vaccine study and is also capable of significantly regressing established B16 melanoma in a therapeutic vaccine study. These therapeutic effects are independent of MHC-mismatch [284]. In conclusion, we concluded that the lyophilization-based exosome loading methodology would help expand the therapeutic utility of exosome-based immunotherapies.

4.2. Application of TEXs in cancer immunotherapy

Tumor-derived exosomes (TEXs) are promising candidates for the next generation of immunotherapeutic vaccines against cancers. Besides being a good reservoir of various tumor antigens, TEXs play an active role in the communication of tumor cells in all stages of cancer development and regulate immune responses through both immunosuppressive and immunostimulatory functions [285]. Due to this differential role of TEXs, and their limited immunogenicity, their modifications into an effective immuno-adjuvant delivery system that can stimulate a strong humoral and cell-mediated immune responses remains challenging. In the second part of this thesis, we modified 4T1/Her2 cell-derived exosomes by loading with two potent adjuvants, CpG ODN and poly(I: C). Modified exosomes co-encapsulating CpG ODN and p(I: C) have shown immunostimulatory properties by activating antigen-specific primary and memory T cell responses. Furthermore, our exosome-based adjuvant vaccine stimulated robust Th1 type immune responses via the elevated secretion of IgG2a antibody and IFN γ , resulting in tumor regression in 4T1 tumor-bearing mice.

A combination of TLR adjuvants like CpG ODN and p(I: C) triggers synergistic immune responses more than their standalone counterparts when given within a carrier system [286]. Therefore, we evaluated the immunogenicity of modified TEXs co-encapsulated with the combination of both adjuvants. As expected, administration of only tumor-derived exosomes to the mice did not generate statistically significant responses. In contrast, exosomes loaded with CpG ODN and p(I: C) significantly increased CD4⁺ and CD8⁺ T cell responses and the production of antigen-specific antibodies (Figure 3.8-3.13). Since co-encapsulating CpG ODN and p(I: C) was more promising than other treatment groups, we investigated its potential therapeutic effect against the established 4T1 tumor model in mice. Our exosome-based adjuvant formulation significantly regressed tumor growth (Figure 3.14). Furthermore, the simple co-administration of adjuvant with TEXs in clinical trials resulted in poor anti-tumor immunity due to inefficient simultaneous delivery of tumor antigen and adjuvant to the same antigen-presenting cells (APCs) [287]. In the present study, we successfully encapsulated two adjuvants into TEXs carrying endogenous antigens and have shown that compared to the co-administration mixture of (Exo + CpG ODN+p(I: C)), co-encapsulation Exo(CpG ODN + pI: C)) strategy yielded i) stronger antigen-specific Th1-biased humoral

immunity, ii) higher levels of IFN γ secretion and iii) more IFN γ producing CD4 $^{+}$ and CD8 $^{+}$ T cells (Figure 3.8-3.12).

Induction of tumor antigen-specific cytotoxic T lymphocytes and humoral immune responses generated by efficiently delivering tumor antigens to APCs are essential for effective cancer immunotherapy [288]. For this reason, vaccines against cancers focus on identifying immunogenic antigen epitopes that selectively stimulate the induction of robust T helper cells, B cells, and cytotoxic T cells [289]. However, only a few tumor-associated antigens have been identified for many cancer types. They can be poorly immunogenic or may not be found in many patients [290]. Moreover, the identification and purification of new epitopes that can be able to activate T and B cells are incredibly challenging. Using experimental approaches to screen immunodominant epitopes is quite expensive, and its complexity has required the support of bioinformatics-based predictions. Therefore, we proposed to harness tumor-derived exosomes isolated from the tumor biopsy or a tumor cell capable of expressing all biologically relevant antigens (including the neo-antigens) as an efficient cancer vaccine carrier, thereby excluding the need to identify these epitopes. Her2 is considered an ideal target for cancer vaccine development due to its 100-fold higher overexpression in patients with breast cancer and correlates with poor clinical outcomes [291]. E75 is a 9-amino acid human leukocyte antigen– (HLA-) restricted peptide located in the Her2 extracellular domain, which is currently under clinical trials [292]. Studies have shown that the E75 peptide vaccine improves CTL and CD4 $^{+}$ Th1 responses [293]; however, this vaccine can be used in a limited patient population due to HLA class I restriction. Using TEXs from HER2-overexpressing cell lines as an adjuvant delivery system overcomes this restriction. It activates a broader immune response since these exosomes express a full-length Her2 molecule (Figure 3.7) as the whole panel of tumor-related antigens. Therefore, TEXs are potent delivery systems that can stimulate a breadth of T cell clones by presenting multiple antigenic epitopes. Besides promoting strong Th1 type immune responses via the elevated production of IgG2a antibody against 4T1/Her2-derived exosomes (Figure 3.8), our 4T1/Her2-derived exosome-adjuvant formulation efficiently activated humoral immune responses against E75 peptide as well (Figure 3.9). Based on these findings, using tumor-derived exosomes as a delivery system for cancer immunotherapy is a more convenient and suitable approach, as it applies to all cancer types, where biopsies are available from the patient.

In conclusion, we demonstrated that modified TEXs with CpG ODN and p(I: C) co-encapsulation generated humoral and cell-mediated immune responses and regressed tumor growth in 4T1 tumor-bearing mice as well as extended survival (Figure 3.14). The combination of adjuvants with tumor antigens inherently expressed by tumor-derived exosomes (without inserting additional antigen epitope) exerted more robust anti-tumor immune responses. These findings propose a functional TEX-based adjuvant delivery system as a promising cell-free vaccine and are of great interest for future vaccine design experiments in clinical practice.

4.3. Utilization of SPION encapsulated exosomes in cancer therapy

Nanotechnology is one of the areas to develop effective therapeutic nanomaterials. These nanomaterials are specifically designed to make the desired response in a biological system. Superparamagnetic iron oxide nanoparticles have arisen as one of the most studied particles for cancer therapy. Their superparamagnetism provides contrast agents with magnetic resonance imaging to monitor the specific tissue and organ in biological systems. Furthermore, their biodegradability is an essential feature for in vivo applications. Therefore, SPIONs hold great promise in successfully diagnosing and treating aggressive cancers [294,295]. However, SPIONs should be carefully optimized to serve as multifunctional therapeutic agents in diseases.

One of the most critical challenges to using therapeutic SPIONs in cancer treatments is to overcome biological barriers. These biological barriers in the body must be bypassed to extend the blood's half-life and increase the probability of reaching target cells. The cell membrane is a negatively charged phospholipid bilayer embedded with glycoproteins, glycolipids, and cholesterol. Therefore, the cell membrane can act as a physical barrier for SPION uptake. For these reasons, researchers try to optimize the SPIONs to enable the uptake by the cell through endocytosis or transcytosis across the cell membrane. In this part of the thesis, we used macrophage-like cell line-derived exosomes as a carrier system to bypass the cell membrane barriers of SPIONs uptake since exosomes can be easily taken up by cells because of their similarity to the cell membrane. We carefully investigated the effect of SPION loading on exosome integrity. Our findings showed that the vesicular integrity and content remain unaffected upon SPION loading into exosomes (Figure

3.15). Notably, TEM analysis indicated that SPION could either load or interact with exosomes without disrupting exosome structure (Figure 3.15C).

SPIONs can be used as antigen carriers and thereby modulate the immune system. Combining SPIONs with exosomes in cancer therapy can increase its uptake and payload to the antigen-presenting cells. Thus, it stimulates the immune system and ensures that cancer cells are killed more efficiently [296]. For this purpose, we analyzed how SPIONs and CpG loading into exosomes affects the uptake and binding capacity of exosomes by immune cells. Our results demonstrated that loading SPION enhanced the in vitro delivery of exosomes and CpG-loaded exosomes within antigen-presenting cells and lymphocytes. (Figure 3.16). Notably, Encapsulating SPIONs and CpG ODN within exosomes enhanced in vitro stimulatory capacity of CpG ODN, as seen by the increased pro-inflammatory cytokine release and IFN γ secretion from splenocytes, which are essential mediators in tumor clearance (Figure 3.17).

These results encouraged us to test the SPION-loaded exosomes with CpG ODN and poly (I: C) in the therapy of established hepatocellular carcinoma on athymic mice, which is a challenging experiment set up because of the lack of an adaptive immune system in athymic mice. We recorded daily changes in tumor volumes by an electronic caliper. SPION loading into exosomes led to significant tumor regression compared to the free form of exosomal vaccine formulation at day=43 after tumor inoculation. Similarly, tumors extracted from animals treated with SPION loaded exosomes were significantly smaller than the control or unencapsulated mixture-treated groups. Another experiment showed that SPION loading into exosomes extended the survival rate of mice (Figure 3.18). This data proposed that when TLR3 and TLR9 ligands were effectively delivered to immune cells through SPION loaded exosomes, they stimulated more pronounced innate immune activation in athymic mice. This enhanced activation helped control tumor growth and the extended survival of treated mice.

The long-term goals of nanotechnology are to create SPION-based cancer immunotherapy that delivers magnetically targeted immune cells and cancer cells in vivo. Therefore, our preliminary data propose that SPIONs systems can be further improved by magnetic field-guided exosomes explicitly targeting cancer cells and immune cells.

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APPENDIX



WESTERN BLOTTING Buffers

RIPA buffer (Radio Immuno Precipitation Assay Buffer)

- 150 mM Sodium chloride
- 1.0% NP-40 or Triton X-100
- 0.5% Sodium deoxycholate
- 0.1% SDS
- 50 mM Tris, pH 8.0

Buffer was prepared freshly, kept at +4 °C, and used immediately.

Laemmli buffer(4x)

- 4% SDS
- 10% 2-mercaptoethanol
- 20% Glycerol
- 0.004% Bromophenol blue
- 0.125 M Tris-HCl

Buffer pH was adjusted to 6.8 and stored at -20 °C.

Running buffer(10x)

- 25 mM Tris base
- 195 mM Glycine
- 0.1% SDS

Its pH was adjusted to 8.3 and stored at RT. It was diluted to 1X before use.

Transfer buffer

- 25 mM Tris base
- 195 mM Glycine
- 20% Methanol

Its pH was adjusted to 8.3 and stored at +4 °C.

Blocking and antibody incubation buffer

- 50 ml PBS-T
- 2.5 g BSA

The solution was mixed well and stored at +4 °C.

Resolving gel (12%)

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 3.96 ml 30% Acrylamide
- 100 µl 10% APS
- 10 µl TEMED
- 3.44 ml ddH₂O

The solution was mixed well by vortexing and used immediately.

Resolving gel (8%)

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 2.64 ml 30% Acrylamide
- 100 µl 10% APS
- 10 µl TEMED

- 4.76 ml ddH₂O

The solution was mixed well by vortexing and used immediately.

Stacking gel (5%)

- 2 ml 0.5 M Tris (pH 6.8)
- 80 µl 10% SDS
- 1.3 ml 30% Acrylamide
- 80 µl 10% APS
- 16 µl TEMED
- 4.5 ml ddH₂O

The solution was mixed well by vortexing and used immediately.

Phosphate Buffered Saline (10x)

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

After adjusting pH to 6.8, it was stored at RT

PBS-T

- 100 ml of 10X PBS
- 900 ml ddH₂O
- 1 ml Tween20

The solution was mixed well and stored at RT.

ELISA buffers

Blocking buffer

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

The mixture was stored at -20°C.

Wash buffer

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

PBS (10x)

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

After adjusting pH to 6.8, it was stored at RT.

Curriculum Vitae and Publication

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Article Publication

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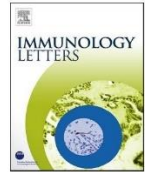
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TLR ligand loaded exosome mediated immunotherapy of established mammary Tumor in mice

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ABSTRACT

Tumor-derived exosomes (TEXs) could be harnessed as an immunotherapeutic cancer vaccine. These nano-vesicles are inherently possess rich tumor antigen reservoirs. Due to their undesirable features such as poor or limited immunogenicity as well as facilitation of cancer development via mediating communication between tumor cells TEXs could be transformed into an effective immune adjuvant delivery system that initiates a strong humoral and cell-mediated tumor-specific immune response. Engineering TEXs to harbor immunostimulatory molecules still remains a challenge. Previously, we demonstrated that nucleic acid ligand encapsulated liposomes could trigger synergistic strong humoral, and cell mediated immune responses and provokes tumor regression to that of their standalone counterparts. In this study, we evaluated to immunogenicity of 4T1/Her2 cell-derived exosomes upon loading them with two potent immuno adjuvant, a TLR9 ligand, K-type CpG ODN and a TLR3 ligand, p(I:C). Engineered TEXs co-encapsulating both ligands displayed boosted immunostimulatory properties by activating antigen-specific primary and memory T cell responses. Furthermore, our exosome-based vaccine candidate elicited robust Th1-biased immunity as evidenced by elevated secretion of IgG2a and IFN γ . In a therapeutic cancer model, administration of 4T1 tumor derived exosomes loaded with CpG ODN and p(I:C) to animals regress tumor growth in 4T1 tumor-bearing mice. Taken together this work implicated that an exosome-based therapeutic vaccine promoted strong cellular and humoral anti-tumor immunity that is sufficient to reverse established tumors. This approach offers a personalized tumor therapy strategy that could be implemented in the clinic.

1. Introduction

Immunotherapy has become a powerful clinical strategy to boost the activation of host immune system against tumors that evade immune surveillance and induce tolerance. These cancer therapies enhance antitumor immune responses with fewer off-target effects than traditional treatments, such as chemotherapy and radiation that lack tumor selectivity and have severe side effects [1]. Tumor antigen-based cancer immunotherapy has gained significant attention to elicit antigen-specific strong T and B cell responses, however, the purification of particular tumor antigens is challenging [2,3]. Therefore, identification of tumor-specific nanostructures that naturally harbors endogenous tumor antigens and further engineering these structures to co-deliver immune stimulating adjuvants yielding potent anti-tumor immunity is

of great benefit during the management of established tumors. A depot system that permits simultaneous presentation of tumor antigen as well as initiating tumor-specific immune activation could offer effective tumor targeting while reducing undesirable toxicity could pave the way for patient specific anti-cancer immunotherapy [4,5].

Exosomes with a size between 30 and 150 nm are small extracellular vesicles secreted (any small sized EVs irrespective of their biogenesis) by all type of mammalian cells [6]. Hereafter, the term “exosomes” refers to small EVs in this work. They mediate a novel mode of intercellular communication through their bioactive cargos such as lipids, nucleic acids, metabolites, and proteins, which can be delivered to the target cells [7]. Using their natural delivery capabilities, exosomes have been successfully served as immunotherapeutic nanocarriers in cancer treatment [8]. Furthermore, they are attractive as a delivery system because

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of their stability in circulation, biocompatibility, and low toxicity [9,10,11].

Tumor derived exosomes (TEXs) are appropriate tumor specific antigen sources to induce immunity against tumor cells since they are capable of even presenting endogenous tumor-specific neoantigens [12]. They can transfer a wide spectrum of immunogenic tumor antigens expressed in parental tumor cells to DCs and elicit tumor-specific cytotoxic T lymphocytes [13, 14]. As well as their modification by different methods to enrich tumor antigens, immunostimulatory molecules and microRNAs, TEXs can be loaded with therapeutic cargo [15,16]. These engineered TEXs can increase their uptake by antigen presenting cells, upregulate the expression of cancer-specific antigenic epitopes, repress immunosuppressive targets in the tumor microenvironment, improve DC maturation and enhance tumor cell elimination [17,18,19]. Moreover, the feasibility to isolate TEXs from patients' sera enables their use in personalized immunotherapy [20]. Taken together, TEXs as a modified carrier for immunostimulatory agents along with tumor antigens can be a novel candidate for tumor vaccine.

Among immunostimulatory agents, nucleic acid-based adjuvants are safe and effective inducer of innate immune effector functions [21,22]. CpG oligonucleotides (CpG ODNs) recognized by Toll-like receptor 9 (TLR9) is one of the most extensively used adjuvants in the clinic to stimulate immune response characterized by the production of Th1 and pro-inflammatory cytokines [23,24]. K type CpG ODN activates B cells and trigger plasmacytoid dendritic cells (pDCs) to secrete tumor necrosis factor- α (TNF- α) [25]. Polyinosinic-polycytidylic acid (p(I:C)), a TLR3 agonist, is another potent adjuvant that has been widely studied as an anti-tumor drug in cancer patients [26]. p(I:C) is shown to induce type I and II interferons (IFN) and the activation of CD4⁺ and CD8⁺ T cell responses overcoming immunosuppressive function of regulatory T cells in the tumor microenvironment [27, 28, 29, 30].

The simultaneous delivery of tumor cell-derived exosomes and adjuvants to the same immune cells could effectively induce a potent anti-tumor immune response. Herein, we co-encapsulated CpG ODN and p(I:C) into 4T1/Her2 cell-derived exosomes. These 4T1 breast cancer cells were modified to overexpress Her2 tumor antigen that are present within TEXs. Our results revealed that dual adjuvant encapsulating exosome vaccine induced a strong Th1-biased Her2-specific immune response and increased the production of type II IFNs. We also demonstrated that dual adjuvant co-encapsulating exosome vaccine generated Her2-specific CD4⁺ memory T cell. Moreover, when used as a therapeutic vaccine, CpG ODN and p(I:C) loaded exosomes significantly regressed 4T1 tumor growth in mice. Taken together, we present an exosome-based therapeutic vaccine candidate that could promote strong cellular and humoral anti-tumor immune responses that offers an effective personalized tumor therapy strategy in the clinic.

2. Materials and methods

2.1. Materials

TLR9 ligand, CpG-ODN GACGTTGACGTTGACGTTGG was purchased from Alpha DNA (Canada). TLR3 ligand, p(I:C) was from Amersham (Piscataway, NJ, USA). Her2/E75 peptide was purchased from Immunostep (Spain). All cell culture media components were from Gibco (NY, USA) and Lonza (Walkersville, MD, USA). Cytokine ELISA reagents: recombinant cytokine, monoclonal unlabeled and biotinylated antibody against m-IFN γ were purchased from Mabtech (Cincinnati, OH, USA). P-nitrophenyl phosphate disodium salt substrate (PNPP) was purchased from Thermo Scientific (USA).

Immunoglobulin ELISA reagents: goat anti-mouse total IgG, IgG1, IgG2a monoclonal antibodies conjugated with alkaline phosphatase (AP) were from Southern Biotech (Birmingham, AL, USA). FACS antibodies conjugated to fluorescent chromophores were obtained from Biolegend. Rabbit monoclonal antibody against TSG101 and rabbit monoclonal antibody against Her2/Erbb2 were from Abcam

(Cambridge, UK) and Cell Signaling (Massachusetts, USA) respectively. Cytofix/Cytoperm and Perm/wash buffer were purchased from BD (New Jersey, USA).

2.2. Mice and cell lines

Adult Balb/c mice (female, 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.

4T1-luc and 4T1/Her2 cell lines were cultured in Dulbecco's Modified Eagle Medium, (DMEM) with 10% fetal bovine serum (FBS). GFP tagged HER2 expressing mouse breast cancer cell line (4T1-HER2) was kindly provided by Prof. Michael Kershaw (Cancer Immunology Program, Peter MacCallum Cancer Center, Victoria, Australia) [41,42]. 4T1-luc cell line is originally developed by G Gary Sahagian [43].

2.3. Isolation of tumor derived exosomes from cell culture supernatant

Exosomes were isolated from 4T1/Her2 and 4T1-luc cell lines via differential ultracentrifugation method. Following supernatant collection from cultured cells, they were centrifuged at 1500 x g for 10 min at RT to discard the cell debris. Supernatants were transferred to ultracentrifugation tubes (Beckmann Coulter) and centrifuged at 10,000 x g for 10 min at +4 °C to remove any large particles, followed by a second centrifugation at 30,000 x g for 30 min. This generated a pellet consisting of medium sized EVs and an upper aqueous phase containing the exosomes. The supernatant was collected and passed through a 0.2 μ m filter to ensure removal of larger particles. To sediment exosomes, filtered supernatant was centrifuged at 100,000 x g for 90 min at +4 °C. The pellet was washed once with PBS at 100,000 x g for another 90 min at +4 °C. Following washing, supernatants were aspirated, and the exosome pellet was resuspended in an appropriate volume of Ca²⁺/Mg²⁺ free 1x PBS and used in further studies.

2.4. Exosome characterization

Forty μ L of carboxyl modified latex beads (Life Technologies) were incubated with 40 μ g of LEAF purified anti-mouse CD81 antibody (Biolegend) at RT for 30 min. Then, the volume was adjusted to 500 μ L with 1x PBS and bead-antibody mix was incubated overnight at RT by continuous rotation. Following incubation, beads were blocked with 5% BSA containing 1x PBS for 4 h at RT by rotation. To analyze the specific molecules on exosomes, two μ g of exosomes were mixed with anti-mouse CD81 conjugated beads and incubated at RT overnight. Following washing by centrifugation at 13,200 x g, bead-associated exosomes were incubated separately with 2 μ g/mL anti-CD9-FITC, anti-CD81-PE antibodies in sodium azide containing FACS buffer or with their corresponding isotype controls for 30 min at RT. The exosome-bead complexes were washed and analyzed with Novocyt D3000 (Agilent, California, USA).

Total cellular and exosomal proteins were extracted using a RIPA lysis buffer and samples were vortexed and sonicated, respectively. Protein concentration was determined by BCA Protein Assay Reagent Kit (Thermo Scientific). Twenty five μ g of cell lysate, medium sized EVs lysate or exosome lysate were separated on 10% SDS-polyacrylamide gel for 90 min at 120 V following 10 min run on 5% SDS-polyacrylamide resolving gel and then were transferred to PVDF membranes (Thermo Scientific) by applying 100 V for 90 min. Membranes were blocked with 5% non-fat dry milk containing 1x PBS-T for 2 h at RT. Then, blocked membranes were incubated with primary antibodies against TSG101 (Abcam) or Her2 (Cell Signaling) prepared in 5% non-fat dry milk containing 1x PBS-T at a 1:1000 dilution at +4 °C overnight. Following primary antibody incubation, membranes were washed and incubated

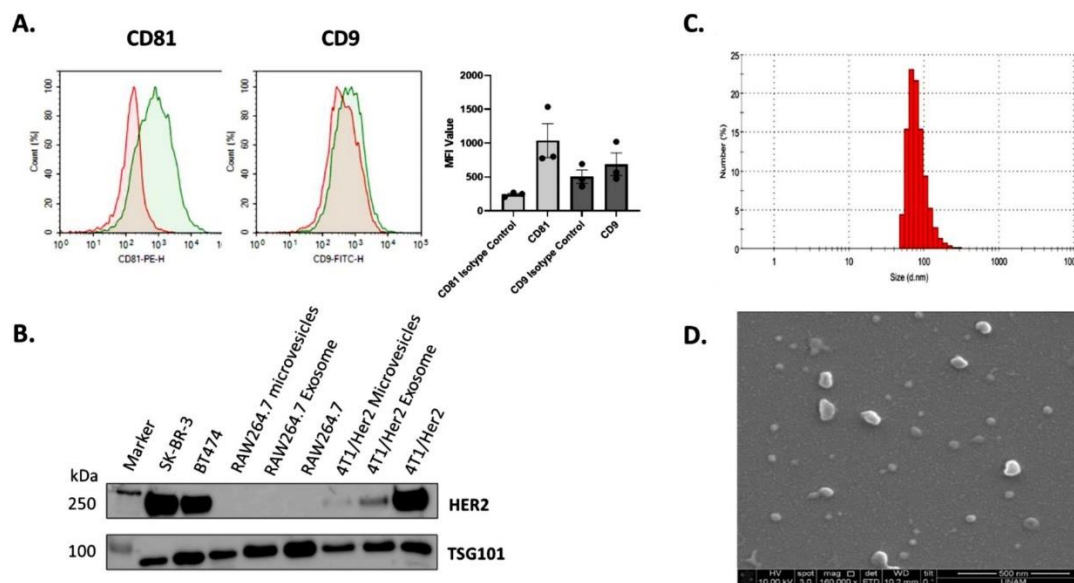


Fig. 1. Characterization of exosomes isolated from 4T1/Her2 cell line. **A)** Exosomes coupled to anti-CD81 conjugated beads were phenotyped using flow cytometry analysis. Bar graphs represent the average MFI values of specific antibodies to isotype control staining of 3 separate exosome batches. **B)** Western blot analysis of exosomal marker TSG101, and Her2 expression in total cell extracts and cell-derived exosomes. (SK-BR-3 and BT474 cell lines were used as positive controls and RAW264.7 cell line was used as a negative control) **C)** Dynamic Light Scattering (DLS) analysis showing average size distribution of 4T1/Her2 cell-derived exosomes ($n = 3$ independent batches) **D)** A representative scanning electron microscopy (SEM) image of 4T1/Her2 cell-derived exosomes. Scale bar represents 500 nm.

with 1:5000 diluted secondary antibodies, HRP conjugated horse anti-mouse IgG (Cell Signaling) for Tsg101 and Her2 in 5% non-fat dry milk supplemented 1x PBS-T at RT for 1 h. Membranes were developed using the chemiluminescent detection kit, ECL+ from Amersham according to the manufacturer's protocols and visualized by Amersham Chemiluminescence Imager 600 (GE, Amersham).

To analyze the size of exosomes by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern, UK), exosomes were diluted in PBS and triplicate readings of samples were recorded.

Purified exosomes were verified by scanning electron microscopy (SEM). Ten μ L of the exosome solution were deposited on silica surfaces. After evaporation, samples were sputter-coated with 8 nm of Au/Pd alloy using a precision coating system (Gatan Inc, USA).

2.5. Loading of exosomes with CpG ODN and p(I:C)

Thirty μ g of exosome were mixed with 30 μ g CpG ODN and 30 μ g pI:C. The mixture was snap-frozen in liquid nitrogen and lyophilized overnight in VirTis Bench Top K Freeze Dryer (SP Industries, PA, USA). The exosome-ligand powder mixture was rehydrated in a controlled fashion by addition of nuclease free ddH₂O. Then, this rehydrated mixture was vortexed for 15 s every 2 min for a period of 20 min. Next, 1 x Ca²⁺ /Mg²⁺ + free PBS was added, and the mixture was incubated at RT for another 10 min. Then, the mixture was vortexed for 15 s and the controlled rehydration process was completed with the addition of 1x Ca²⁺/Mg²⁺ free PBS to adjust the desired concentration of ligand encapsulated exosome solution. As such prepared exosome formulations were maintained at +4 °C until further use.

The encapsulation efficiency was performed from three independent batches of each exosome preparations. To detect CpG ODN loading, 30 μ g Cy5-labeled CpG ODN and 30 μ g unlabeled pI:C were mixed with 30 μ g of exosome. Following reconstitution as described above, the Cy5-signal was followed both from the exosome pellet and from the unencapsulated fraction of supernatant using fluorometer. Next, 30 μ g

unlabeled CpG ODN and 30 μ g FITC-labelled pI:C were mixed with 30 μ g of exosome, and same procedure was performed to identify the pI:C loading. For each ligand the encapsulation efficiencies were found to be $88.8 \pm 9.2\%$ for the CpG ODN and $94.1 \pm 7.3\%$ for pI:C.

2.6. Immunization of balb/c mice and splenocyte isolation

6–8 weeks-old female Balb/c mice (4 per group) were vaccinated 2 times in 2-week intervals intraperitoneally (I.P) with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exosome loaded with CpG ODN+p(I:C). Two weeks after the last booster dose, all mice were sacrificed and their splenocytes and blood serum were collected aseptically for the detection of cellular immune responses.

2.7. In vitro stimulation

Splenocytes (2×10^6 cell/mL in 250 μ L) were stimulated with 10 μ g/mL 4T1/Her2 cell-derived exosome for 72 h at 37 °C in a 5% CO₂ incubator. After stimulation, the supernatants were collected and used for IFN γ cytokine ELISA.

2.8. Enzyme-linked immunosorbent (ELISA) assay

Immulon 2 HB microtiter plates (Thermo Scientific, San Jose, CA, USA) were coated with Her2 E75 peptide (40 μ g/mL), 4T1/Her2 cell lysate (10 μ g/mL), 4T1/Her2 exosome lysate (10 μ g/mL) and anti-mouse IFN γ (2 μ g/mL) respectively, and then blocked with PBS-BSA. Serially diluted standards and culture supernatants or serially diluted mouse sera were added to plates for overnight at 4 °C. Cytokine was detected using biotinylated anti-mouse IFN γ (1 μ g/mL) antibody followed by phosphatase-streptavidin whereas bound IgG subclasses were detected using alkaline phosphatase streptavidin conjugated anti-mouse IgG (1:1000), anti-mouse IgG1 (1:1000) and anti-mouse IgG2a

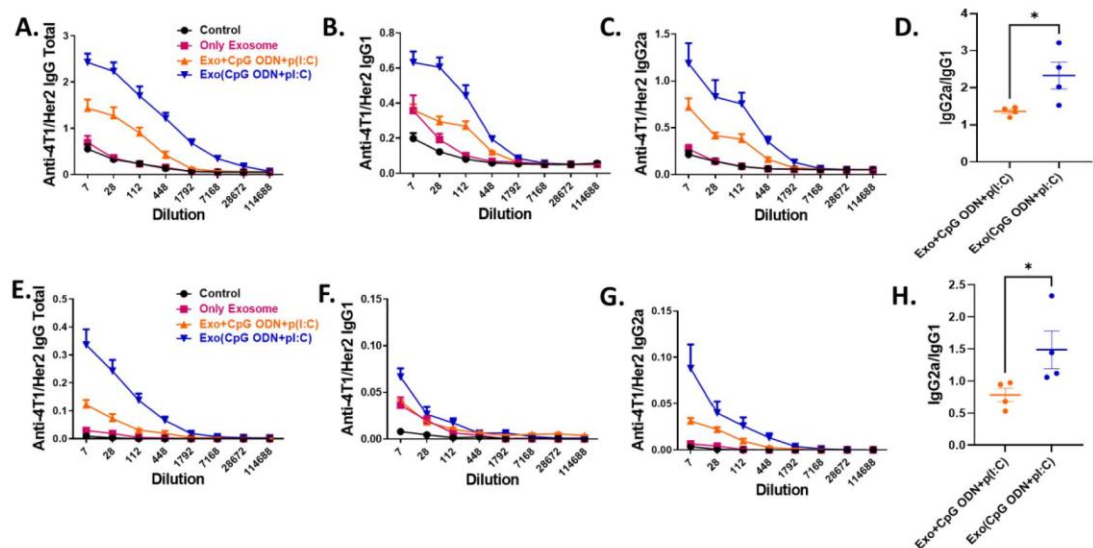


Fig. 2. 4T1/Her2 cell-derived exosomes loaded with CpG ODN and p(I:C) immunization generates sustained and magnified anti-4T1/Her2 immune responses. Mice ($n = 4$ mice/group) were injected i.p. with PBS, only 30 μ g exosomes, free form of 30 μ g exosomes+30 μ g CpG ODN and 30 μ g p(I:C) or exo(CpG ODN+p(I:C)) on days 0 and 14. Mice were sacrificed on day 28. **A–C)** Sera of immunized mice were investigated for systemic anti-4T1/Her2 antibody production against 4T1/Her2 cell lysate. End-point average titers of anti-4T1/Her2 total IgG, IgG1 and IgG2a responses of mice were shown. **D)** IgG2a/IgG1 ratio of individual mouse either immunized with free or immunized with exosomal formulation at 2 weeks after booster injection (at 1:120 dilution against 4T1/Her2 cell lysate). Mann-Whitney test was used for statistical analysis. **E–G)** Sera of immunized mice were investigated for systemic anti-4T1/Her2 antibody production against protein extracts of 4T1/Her2 cell-derived exosomes. End-point average titers of anti-4T1/Her2 total IgG, IgG1 and IgG2a responses were shown. **H)** IgG2a/IgG1 ratio of individual mouse either immunized with free or immunized with exosomal formulation at 2 weeks after booster injection (at 1:15 dilution against protein extracts of 4T1/Her2 cell-derived exosomes). Mann-Whitney test was used for statistical analysis. The data indicate the mean $\pm$ SEM ($n = 4$). Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$. Exo: exosome.

(1:1000) antibodies and developed by the addition of PNPP substrate.

2.9. Intracellular cytokine assay via flow cytometry

10^6 cells/mL of splenocytes in medium containing Brefeldin A (5 μ g/mL, was included in the last 6 h) was stimulated with 4T1/Her2 derived exosomes (10 μ g/mL) for 24 h at 37 $^{\circ}$ C. After stimulation, cells washed two times with stain buffer (2% FCS in PBS). Cells were stained with 1 μ g/mL anti CD45-PE/Cy7, anti CD3-Pacific Blue, anti CD4-FITC, anti CD8-PE, anti CD44-APC/Cy7, anti CD62L-BV605 for 30 min at 4 $^{\circ}$ C. The cells were washed with stain buffer and fixed with Cytofix/Cytoperm solution. Fixed cells were washed two times with Perm/Wash buffer and then stained with 1 μ g/mL anti IFN γ -FITC antibody for 30 min at 4 $^{\circ}$ C. The cells were washed with Perm/Wash buffer and suspended in 300 μ L stain buffer for flow cytometric analysis with Novocyte D3000 (Agilent, California, USA).

2.10. The therapeutic model of 4T1-luc tumor mice model

6–8 week-old female Balb/c mice were injected with 2×10^5 4T1-luc tumor cells into right mammary fat pads orthotopically with 5–6 mice per group. Tumor-bearing mice were then intraperitoneally injected with a PBS and 30 μ g exosome loaded with 30 μ g CpG ODN and 30 μ g p(I:C) at day =, 1, 4, 7, 10, 17, 24 and 31 after tumor inoculation. Tumor growth was monitored by measuring the tumor volume with a caliper after tumors become palpable. Tumor volumes were calculated as length $\times$ width $^2/2$.

For IVIS spectrum, Caliper Lifesciences Lumina III series was used to visualize tumor growth and each mouse received 150 mg Luciferin/kg according to the datasheet suggestion.

2.11. Statistical analysis

One-way ANOVA with Kruskal Wallis and Mann–Whitney tests were employed to assess the significance of differences among the groups. Survival analysis was performed using log-rank test (GraphPad Prism version 9, San Diego, California). $P < 0.05$ was statistically significant.

3. Results

3.1. Characterization of exosomes

Exosomes were isolated from the 4T1/Her2 cell culture supernatants using differential ultracentrifugation method as described in Material and Methods. Isolated exosomes were subjected to analysis of i) surface marker expression by flow cytometry, ii) exosome-specific protein and Her2 expression by immunoblotting, iii) size distribution by DLS and iv) morphological analysis by SEM. Exosomes were verified by detecting the expression of positive protein markers CD81, CD9 (Fig. 1A) and TSG101 (Fig. 1B). Tumor cell-derived exosomes are a good source of cellular components that stimulate the immune response, such as tumor-associated antigens. To check whether the isolated exosome pools carry antigen, we confirmed that purified exosomes of 4T1/Her2 cells also express Her2 whereas isolated exosomes of RAW264.7 as a negative control does not (Fig. 1B). The size of exosomes abundantly ranged from 40 to 190 nm (Ave. Mean Size: 70 ± 20 nm, Fig. 1C). Under scanning electron microscopy (SEM), the purified exosomes exhibited a typical round-shaped structures (Fig. 1D). Collectively, these findings support that extracellular nanovesicles derived from 4T1/Her2 cell line displays convincing exosome features.

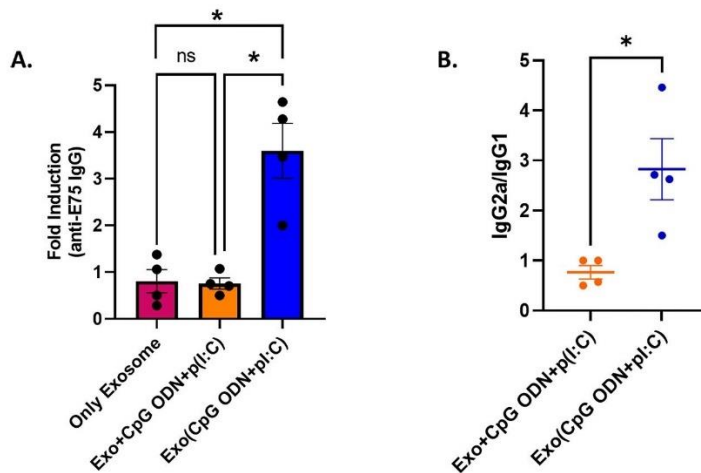


Fig. 3. 4T1/Her2 cell-derived exosome loaded with CpG ODN and p(I:C) immunization magnified anti-E75 immune responses. Mice ($n = 4$ mice/group) were injected i.p. with PBS, only 30 μ g exosome, free form of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or exo(CpG ODN+p(I:C)) on days 0 and 14. Mice were sacrificed on 14 day after the booster injection **A)** Bar graphs showing fold induction over control group of sera of immunized mice for systemic antibody production against Her2/E75 peptide. **B)** IgG2a/IgG1 ratio of individual mouse either immunized with free or immunized with EV formulation at 2 weeks after booster injection (at 1:30 dilution against Her2/E75 peptide). Mann-Whitney test was used for statistical analysis. The data indicate the mean $\pm$ SEM ($n = 4$). Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$. Exo: exosome.

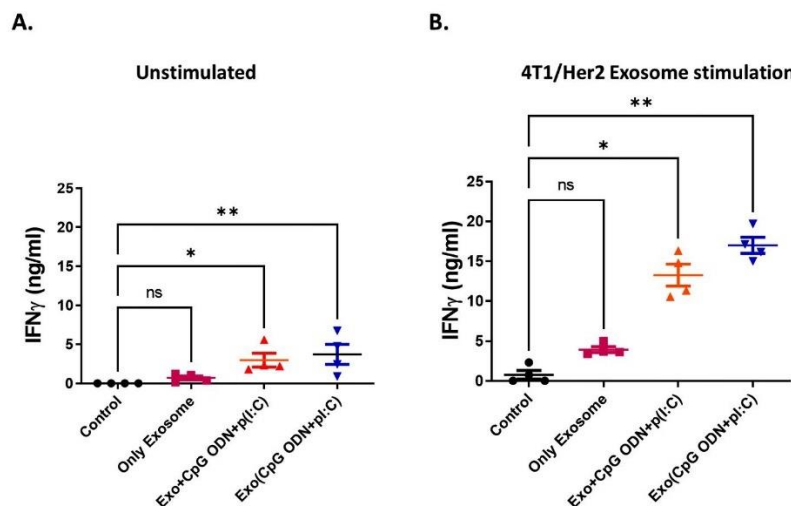


Fig. 4. IFN γ assay on ex vivo expanded splenocytes of mice immunized two times with PBS, unencapsulated 30 μ g exosome, mixture of 30 μ g Exosome + 30 μ g CpG ODN and 30 μ g p(I:C) or exosome co-loaded with (CpG ODN+p(I:C)) **A)** Fourteen days after the booster, the spleens of mice from each group were harvested and immune responses were evaluated using an IFN γ ELISA assay. **B)** Fourteen days after the booster, spleens of mice ($n = 4$) were harvested and incubated with 4T1/Her2 exosome in culture for 72 hrs and the secreted IFN γ levels were then evaluated using IFN γ ELISA kit. The data indicate the mean $\pm$ SEM ($n = 4$). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$. Exo: exosome.

3.2. Humoral immune responses

We intended to develop an exosomal carrier system co-encapsulating two nucleic acid-based TLR ligands as immunoadjuvants naturally expressing tumor-associated antigen Her2 and analyzed its potency as a prophylactic and therapeutic vaccine. Previous studies have shown that tumor derived exosomes have a dual effect on the immune system, immunosuppressive or immunostimulatory effects. To combine and enhance the synergistic immunostimulatory activity of TLR3 (p(I:C)) and TLR9 (CpG ODN) ligands, we co-encapsulated them within 4T1/Her2 cell-derived exosomes thereby aimed to achieve simultaneous antigen and adjuvant delivery to relevant antigen presenting cell. Next, we assessed the humoral responses from sera of mice collected at day 28 following twice (@Day = 0 and 14) i.p. injection to Balb/c mice. 4T1/Her2-specific total IgG, IgG1 and IgG2a titers against 4T1/Her2 total cellular protein extract or 4T1/Her2 total exosome extract were analyzed by ELISA. As expected, there was no 4T1/Her2-specific antibody production in the control group and only exosome injected animals raised strong antibody titers. Besides, the level of 4T1/Her2 specific total IgG, IgG1 and IgG2a in sera of mice immunized with dual ligand-loaded exosomal group was significantly higher than those animals

that was immunized with free combination of antigen containing exosome and TLR ligands (Fig. 2A–C and Fig. 2E–G). We also detected significantly elevated IgG2a/IgG1 ratio after immunization with exosomes co-encapsulating p(I:C) and CpG ODN indicating the development of antigen specific Th1-biased immunity ($p = 0.0286$ for both antigens) (Fig. 2D and Fig. 2H).

Furthermore, we analyzed whether exosome loaded with CpG ODN and p(I:C) produce Her2 specific antibodies. To assess this, we used Her2 specific E75 peptide. As shown in Fig. 3A, mice immunized with dual ligand loaded exosomes produced significantly higher Her2/E75 specific total IgG antibody compared to mice immunized with only exosome and free form of exosomal combination ($p = 0.0286$). We detected significantly elevated IgG2a/IgG1 ratio after immunization with CpG ODN plus p(I:C) loaded exosomes ($p = 0.0286$ for both antigens, Fig. 3B). Collectively these results pointed out that CpG ODN and p(I:C) loaded exosomes elicited anti-4T1/Her2 and Her2/E75 specific Th1-biased immunity.

3.3. Induction of specific IFN γ from splenocytes

To evaluate the type of immune response generated, splenocytes

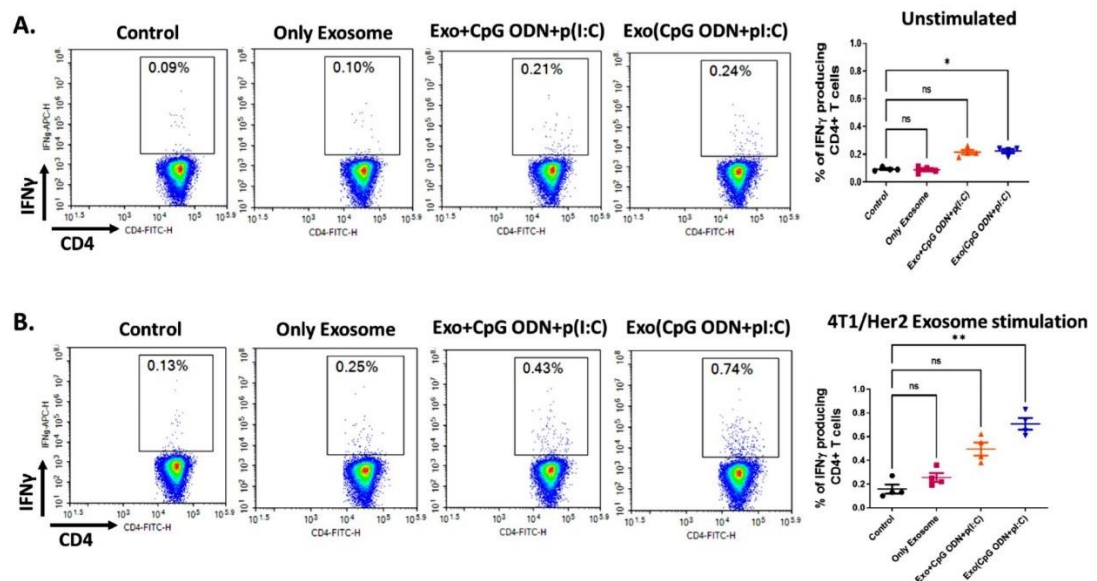


Fig. 5. Intracellular cytokine production from splenocytes of mice immunized two times with PBS, unencapsulated 30 µg exosome, mixture of 30 µg exosome plus 30 µg CpG ODN and 30 µg p(I:C) or exosome co-loaded with (CpG ODN+p(I:C)). A) Fourteen days after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC surface markers and then with anti-IFN-γ-APC antibodies. B) Fourteen days later the booster, isolated splenocytes of different groups of vaccinated mice were stimulated with 4T1/Her2 exosome for 24 hrs in culture and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FITC surface markers and then with anti-IFN-γ-APC antibodies. The frequency of IFN-γ producing cells within CD4+ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean ± SEM (n = 4). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$. Exo:exosome.

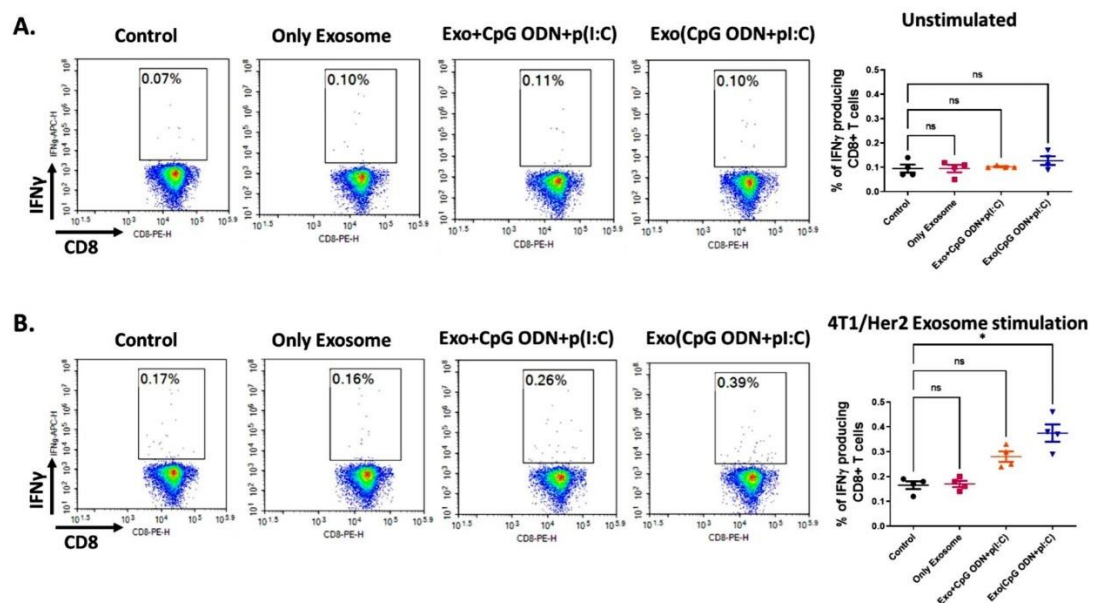


Fig. 6. Intracellular cytokine production assay on splenocytes of mice immunized two times with PBS, unencapsulated 30 µg exosome, mixture of 30 µg exosome + 30 µg CpG ODN and 30 µg p(I:C) or exosome co-loaded with (CpG ODN+p(I:C)). A) Fourteen days later after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD8-PE surface markers and then with anti-IFN-γ-APC antibodies. B) Fourteen days later after the booster, isolated splenocytes of different groups of vaccinated mice were incubated with 4T1/Her2 exosome for 24 hrs and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD8-PE surface markers and then with anti-IFN-γ-APC antibodies. The frequency of IFN-γ producing cells within CD8+ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean ± SEM (n = 4). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: $p > 0.05$, * $p < 0.05$. Exo:exosome.

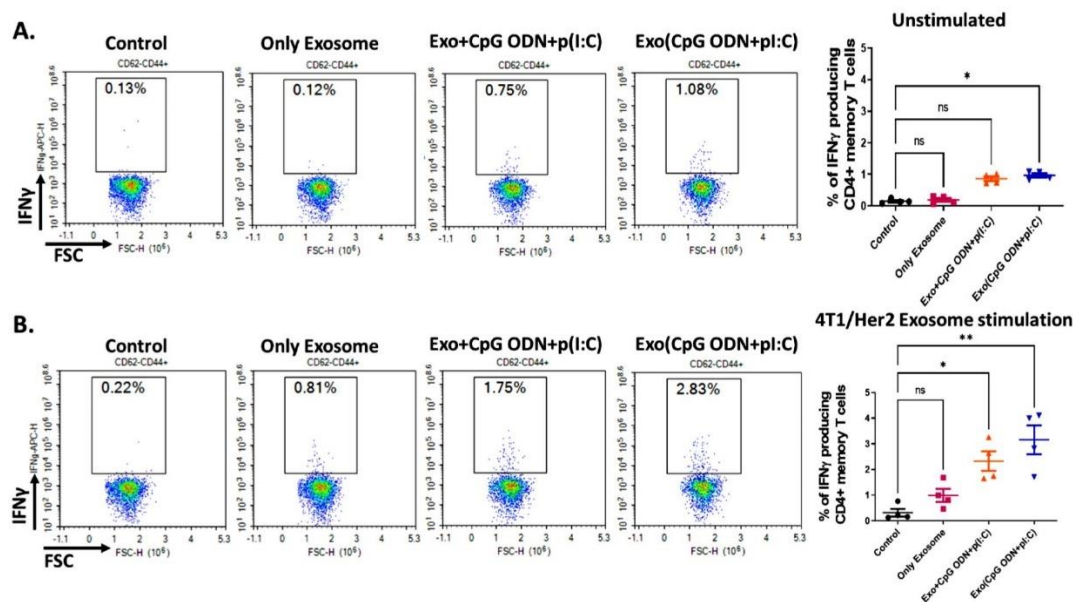


Fig. 7. IFN- γ producing CD4 $^{+}$ memory T cell assay on splenocytes of mice immunized two times with PBS, unencapsulated 30 μ g exosome, mixture of 30 μ g exosome+30 μ g CpG ODN and 30 μ g p(I:C) or co-loaded with (CpG ODN+p(I:C)). **A)** Fourteen days later after the booster, isolated splenocytes of different groups of vaccinated mice (4 mice from each group) were stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FTTC, CD44-APC/Cy7, CD62L-BV605 surface markers and then with anti-IFN γ -APC antibodies. **B)** Fourteen days later after the booster, isolated splenocytes of different groups of vaccinated mice were stimulated with 4T1/Her2 exosome in culture for 24 h and stained with CD45-PE/Cy7, CD3-Pacific Blue, CD4-FTTC, CD44-APC/Cy7, CD62L-BV605 surface markers and then with anti-IFN γ -APC antibodies. CD4 $^{+}$ memory T cell was identified as CD45+CD3+CD4+CD8-CD44^{high}CD62L^{low}. The frequency of IFN- γ producing memory T cells within CD4 $^{+}$ population were determined by Novocyte 3000 Cell analyzer. The data indicate the mean $\pm$ SEM ($n = 4$). Statistical significance was analyzed by Kruskal Wallis test. Statistically significant differences designated as follows: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$. Exo:exosome.

were isolated two weeks after the last booster injection and IFN γ production was analyzed by ELISA assay. As shown in Fig. 4A, we detected that both non-encapsulated combination and dual ligand co-encapsulated exosome group generated more IFN γ compared to control group ($p = 0.0143$, $p = 0.0089$, respectively). Furthermore, Fig. 4B shows that when the splenocytes of immunized mice were activated with 4T1/Her2 exosomes, specific IFN γ productions of non-encapsulated combination and co-encapsulated exosome group were boosted significantly compared to control groups ($p = 0.0347$, $p = 0.0019$, respectively).

3.4. CD4 $^{+}$ and CD8 $^{+}$ T cell responses

To further differentiate the contribution of TLR ligand co-encapsulation within exosomes on cellular immunity that was raised against 4T1 tumors, we performed IFN γ specific intracellular cytokine staining on splenocytes. To this end, the IFN γ + T-cell subsets following staining of CD4 and CD8 positive T cells were analyzed by flow cytometry. The data showed that before and after 4T1/Her2 exosome stimulation in culture the induction of IFN γ producing +CD4 T cells were significantly higher in mice immunized with exosomes loaded with TLR ligands compared to the control groups. ($P = 0.046$, $P = 0.0032$ respectively) (Fig. 5A, B).

As seen in Fig. 6, similarly intracellular cytokine staining assay revealed that when splenocytes were stimulated overnight with 4T1/Her2 exosome in culture, only Exo(CpG ODN+p(I:C)) group had significantly augmented IFN γ production from CD8 $^{+}$ T-cells compared to the control group ($P = 0.0127$).

4T1/Her2 specific memory T-cell is one of the major contributing cell populations to eliminate Her2 expressing tumor cells, therefore, we analyzed the immunized mice splenocytes to assess the antigen-specific CD4 $^{+}$ memory T cell percentage. We determined that mice immunized

with Exo(CpG ODN+p(I:C)) generated significantly higher IFN γ producing CD4 $^{+}$ memory T cell compared to the control group ($p = 0.016$) (Fig. 7A). When immunized mice cells were incubated with 4T1/Her2 expressing exosome, IFN γ producing CD4 $^{+}$ memory T cells expanded significantly in Exo(CpG ODN+p(I:C)) treated group compared to the control group ($p = 0.0042$) (Fig. 7B).

3.5. Therapeutic effects of exosome vaccination in 4T1-luc bearing BALB/c mice

Considering the strong cellular and humoral immune responses in vaccinated mice (Figs. 2–7), it was expected that the immune responses would lead to antitumor activity in an established 4T1-luc tumor model. To evaluate if the cellular and humoral immune responses induced by the Exo(CpG ODN+p(I:C)) vaccination were potent enough to induce an in vivo antitumor effect, the mice were injected with 4T1-luc tumor cells orthotopically into the right mammary fat pads. Following after tumor inoculation, animals were intraperitoneally injected with Exo(CpG ODN+p(I:C)) and were observed for tumor regression during 36 days post tumor formation. As shown in Fig. 8A, during the days 10–36, the group of mice that received Exo(CpG ODN+p(I:C)) regressed tumor development significantly in comparison with untreated control group ($P = 0.0045$ for day 32 and $P = 0.0024$ for day 36). Also, the survival time was significantly prolonged in Exo(CpG ODN+p(I:C)) treated groups in comparison with the control group receiving PBS ($P = 0.0229$). All mice belonging to control group were dead by day 49 whereas 3 out of 5 mice treated with Exo(CpG ODN+p(I:C)) were alive by day 60. Furthermore, We analyzed the IFN γ secretion, helper T cell and cytotoxic T cell population from splenocytes of animals that treated with the PBS and CpG ODN+p(I:C) loaded exosomes at day=1, 4, 7, 10, 17, 24 and 31 after tumor inoculation. As shown in Fig. 8B, Splenocytes from animals that treated with TLR ligands loaded formulation secretes

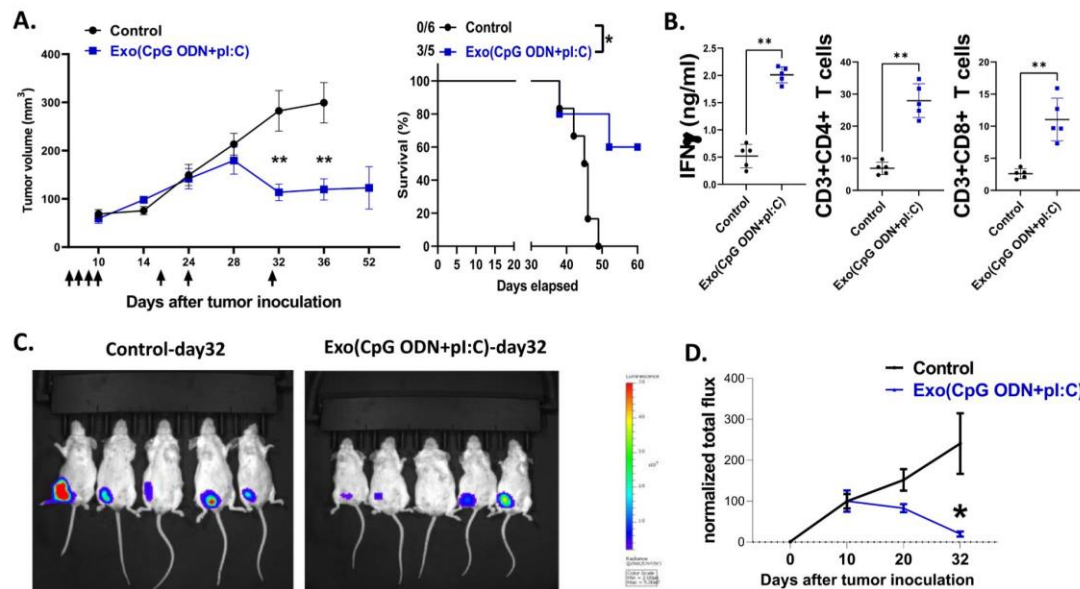


Fig. 8. Therapeutic effect of vaccination with PBS and exo(CpG ODN+p(I:C)) in BALB/c mice against a 4T1-luc in vivo established tumor mice model **A)** 4T1-luc tumor development is suppressed by immunization with exosome co-loaded with CpG ODN+p(I:C) (30 μ g each, arrows indicates i.p. injections of exosome vaccine formulation) and the effect of vaccination on the survival time of mice (6 for control group and 5 for vaccinated group) was observed during a 60-day period post tumor inoculation. **B)** IFN γ assay, Helper T cell and Cytotoxic T cell staining on ex vivo expanded splenocytes of mice treated with PBS, and exosome loaded formulation **C)** In vivo luciferase images of 4T1-luc tumors in control and vaccinated group at day 32. (IVIS Spectrum). **D)** Luciferin signal activity normalized to the signal obtained at day 1 following ortholog tumor injection. Tumor volume plot is collected from 2 independent experiments ($n = 5$ in each group). Mann-Whitney test was used for statistical analysis. Statistical significance for tumor volume and survival time was analyzed by Sidak's multiple comparisons test and Log-rank (Mantel-Cox) test, respectively. Normalized total flux was analyzed by Bonferroni's multiple comparisons test. Statistically significant differences designated as follows: ns: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$. Exo:exosome.

significantly higher level of IFN γ . Also, we found that the percentage of CD4+ and CD8+ T cells in splenocytes are significantly higher than mice treated with TLR ligands loaded exosomes compared to control group ($P = 0.0079$). The anti-tumor effect of Exo(CpG ODN+p(I:C)) group was evaluated by measurement using an IVIS imaging system. We measured bioluminescence activity every 10 days (day 0, day 10, day 20 and day 32 after tumor inoculation). As shown in the bioluminescence imaging results at day 32, 1 out of 5 mice treated with Exo(CpG ODN+p(I:C)) had no luciferase signal (Fig. 8C) and exosome treated group had significantly lower luciferin activity over time (Fig. 8D).

Collectively, our findings implied that a combination of p(I:C) and CpG ODN ligands within exosomal formulations induced stronger and persistent humoral and cellular anti-4T1/Her2 specific immune response sufficient to regress tumor growth.

4. Discussion

Tumor-derived exosomes (TEXs) are promising candidates as the next generation immuno-therapeutic vaccines against cancers. Besides being a good reservoir of various tumor antigens, TEXs take an active role in the communication of tumor cells in all steps of cancer development and regulate immune responses through both immunosuppressive and immunostimulatory functions [31]. It is of great significance to consider the safety profile of ligand loaded exosomes as less detrimental cancer antigen carriers as they initiate inflammatory responses due to its immunostimulatory nature of its cargo. We anticipate that the safety concerns could be alleviated due to its inflammatory character when harnessed as a therapeutic vaccine carrier. Due to this differential role of TEXs, and their limited immunogenicity, their modifications into an effective immuno-adjuvant delivery system that can stimulate a strong humoral and cell-mediated immune responses remains a challenge. In this study, we modified 4T1/Her2-cell-derived exosomes by loading

with two potent adjuvants, CpG ODN and p(I:C). Modified exosomes co-encapsulating both CpG ODN and p(I:C) have shown immunostimulatory properties by activating antigen-specific primary and memory T cell responses. Furthermore, our exosome-based adjuvant vaccine stimulated robust Th1 type immune responses via the elevated secretion of IgG2a antibody and IFN γ , resulting in tumor regression in 4T1 tumor-bearing mice.

Combination of TLR adjuvants like CpG ODN and p(I:C) triggers synergistic immune response more apparently than their standalone counterparts when given within a carrier system [32]. Therefore, we evaluated to immunogenicity of modified TEXs co-encapsulated with the combination of both adjuvants. As expected, administration of only tumor derived exosomes to the mice did not generate statistically significant responses while exosomes loaded with CpG ODN and p(I:C) significantly increased CD4+ and CD8+ T cell responses and the production of antigen-specific antibodies (Fig. 2-7). It is important to note that while mixing exosomes with CpG ODN and p(I:C) improves CD4+ and CD8+ T cell specific IFN γ this response is neither significant from untreated control nor exosome alone treated animals. In this respect, only encapsulation of ligands within exosomes triggered significantly higher IFN γ from these animals, although comparison of mixture treated group vs ligand loaded exosome group animals did not yield significantly different responses. Since co-encapsulating CpG ODN and p(I:C) was the most active than other treatment groups, we investigated its potential therapeutic effect against the established 4T1 tumor model in mice. Our exosome-based adjuvant formulation significantly regressed tumor growth (Fig. 8). Furthermore, the simple co-administration of adjuvant with TEXs in clinical trials resulted in insufficient anti-tumor immunity due to inefficient simultaneous delivery of tumor antigen and adjuvant to the same antigen presenting cells (APCs) [33]. In the present study, we successfully encapsulated two adjuvants into TEXs carrying endogenous antigens and have shown that

compared to co-administration mixture of (Exo+CpGODN+p(I:C)), co-encapsulation Exo(CpG ODN+p(I:C)) strategy yielded i) stronger antigen-specific Th1-biased humoral immunity, ii) higher levels of IFN γ secretion and iii) more IFN γ producing CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 2–6).

Induction of tumor antigen-specific cytotoxic T lymphocytes and humoral immune responses generated by efficient delivery of tumor antigens to APCs are essential requirements for effective cancer immunotherapy [34]. For this reason, vaccines against cancers focus on the identification of immunogenic antigen epitopes that selectively stimulates the induction of robust T helper cells, B cells and cytotoxic T cells [35]. However, only a few tumor-associated antigens have been identified for many cancer types and they can be poorly immunogenic or may not be found in a significant percentage of patients [36]. Moreover, the identification and purification of new epitopes that can be able to activate T and B cells are incredibly challenging. Using experimental approaches to screen immunodominant epitopes is quite expensive and its complexity is required the support of bioinformatic based predictions. Therefore, we proposed to harness tumor-derived exosomes isolated from the tumor biopsy or a tumor cell that are capable of expressing all biologically relevant antigens (including the neoantigens) as an efficient cancer vaccine carrier, thereby excludes the need to identify these epitopes. Her2 is considered as an ideal target for cancer vaccine development regarding its 100-fold higher overexpression in patients with breast cancer and correlates with poor clinical outcomes [37]. E75 is a 9-amino acid human leukocyte antigen–(HLA-) restricted peptide located in the Her2 extracellular domain, which is currently under clinical trials [38]. Studies have shown that E75 peptide vaccine improve CTL and CD4 $^{+}$ Th1 responses [39,40], however, this vaccine can be used in limited patient population due to HLA class I restriction. Using TEXs from HER2-overexpressing cell lines as an adjuvant delivery system overcomes this restriction and activate more broad immune responses, since these exosomes express a full length Her2 molecule (Fig. 1) as well as the whole panel of tumor related antigens. Therefore, TEXs are potent delivery systems that can stimulate a breadth of T cell clones by presenting multiple antigenic epitopes. Besides stimulating strong Th1 type immune responses via the elevated production of IgG2a antibody against 4T1/Her2-derived exosomes (Fig. 2), our 4T1/Her2-derived exosome-adjuvant formulation efficiently activated humoral immune responses against E75 peptide as well (Fig.3). Based on these findings, using tumor-derived exosomes as a delivery system for cancer immunotherapy is more convenient and suitable approach, as it is applicable to all cancer types, where biopsies are available from the patient.

In conclusion, we demonstrated that modified TEXs with CpG ODN and p(I:C) co-encapsulation generated humoral and cell-mediated immune responses and regress tumor growth in 4T1 tumor-bearing mice. as a well as extended survival (Fig. 8). The combination of adjuvants with tumor antigens inherently expressed by tumor-derived exosomes (without inserting additional antigen epitope) exerted stronger anti-tumor immune responses. These findings propose a useful TEX-based adjuvant delivery system as a promising cell-free vaccine and are of great interest for future vaccine design experiments in the clinical practice.

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Declaration of competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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