

***IN VIVO* APPLICATIONS OF LIPOSOMAL VACCINES
ENCAPSULATING SINGLE OR DUAL PATHOGEN-
ASSOCIATED MOLECULAR PATTERNS**

A DISSERTATION SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN MOLECULAR BIOLOGY AND GENETICS

By
Banu Bayyurt Kocabaş
March, 2017

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March, 2017

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

IN VIVO APPLICATIONS OF LIPOSOMAL VACCINES ENCAPSULATING SINGLE OR DUAL PATHOGEN- ASSOCIATED MOLECULAR PATTERNS

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Ph.D. in Molecular Biology and Genetics
Advisor: İhsan Gürsel
March 2017

Nucleic acid-based pattern recognition receptor (PRR) agonists are promising adjuvants and immunotherapeutic agents. Combination of PRR ligands potentiates immune response by providing synergistic immune activity via triggering different signaling pathways and may impact antigen dependent T-cell immune memory. However, the duration of short circulation due to nuclease attacks is hampering their clinical performance. Liposomes enable protein and nucleic acid based compounds to have high encapsulation efficiency. Herein, we aimed to develop liposomal carrier systems that co-encapsulating single TLR9 or combinations with TLR3 or STING ligands and assess their potential as adjuvants and immunostimulatory agents in *in vivo* applications. Liposomal dual nucleic acid formulations induced synergistic innate immune activation, enhanced cytokine production along with internalization capacity of ligands. In anti-cancer vaccine study, CpG ODN and poly(I:C) co-encapsulation significantly increased OVA-specific Th1-biased immune even after eight months post-booster injection. Challenge with OVA-expressing tumor cell line, E.G7, demonstrated that mice immunized with liposomes co-encapsulating CpG ODN and poly(I:C) had significantly slower tumor progression dependent on OVA-specific cytotoxic memory T-cells. In our second *in vivo* application, liposomal CDN and TLR9 therapy led to 80% remission of established melanoma tumor. Increased IgG2c/IgG1 ratio in mice treated with liposomal formulations indicating the development of antigen specific Th1-biased immunity was observed. Furthermore, along with the treatment, IFN- γ producing CD8⁺ T-cells significantly increased and M2-type macrophages decreased at the tumor bed. In conclusion, co-encapsulating

dual ligands into liposomes enhanced the anti-tumor activity of single ligands. In the third part, immunization with CpG ODN loaded liposomal formulations together with antigens increased antigen-specific humoral response against FMDV and *Helicobacter*. In addition, the liposomal CpG ODN reduced bacterial gastric colonization by antigen-dependent Th1 and Th17 immune responses after *helicobacter* challenging.

Keywords: Innate immunity, liposomes, TLR, STING, CpG ODN, poly(I:C), cGAMP, cancer immunotherapy, vaccine, adjuvant.



ÖZET

TEK VEYA İKİLİ PATOJEN İLİŞKİLİ MOLEKÜLER KALIPLARLA YÜKLÜ LİPOZOMAL AŞILARIN *IN VIVO* UYGULAMALARI

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Nükleik asit esaslı kalıp tanıma reseptörü (PRR) agonistleri umut verici adjuvanlar ve immünoterapötik ajanlardır. PRR ligandlarının kombinasyonu, farklı sinyal yollarını tetikleyip sinerjik immün aktivite sağlayarak bağışıklık tepkisini güçlendirmektedir ve antijene bağımlı T-hücre bağışıklık hafızasını etkileyebileceği düşünülmektedir. Bununla birlikte, nükleaz ataklar nedeniyle kısa dolaşım süresi, klinik performanslarını sınırlamaktadır. Lipozomlar, protein ve nükleik asit esaslı bileşikler yüksek yükleme verimliliğine sahiptir. Bu çalışmada, bağışıklık uyarıcı ajanlar olarak kullanılmak üzere TLR9 ve TLR3 veya STING ligandlarıyla birlikte yüklenmiş lipozomal taşıyıcı sistemler geliştirmeyi ve *in vivo* uygulamalarda adjuvan ve immünostimülatör ajan olarak potansiyellerini değerlendirmeyi amaçladık. Lipozomal ikili nükleik asit içeren formülasyonların, sinerjistik doğal immün aktivasyonunu indüklemiş, ligandların hücre içine alım kapasitesini ve sitokin üretimini arttırmıştır. CpG ODN ve poly(I:C) birlikte yüklü lipozomlarla birlikte ovalbumin (OVA) yüklü kanser aşısı çalışmalarında, OVA'ya özgü Th1'e özgü bağışıklık cevapları ikinci enjeksiyondan sekiz ay sonra bile belirgin şekilde yüksek olduğu anlaşılmıştır. Aşılanmış hayvanlara OVA ifade eden tümör hücre hattı E.G7 verildiğinde, CpG ODN ve poly(I: C) birlikte yüklü lipozomal aşılar enjekte edilmiş farelerde, OVA'ya özgü sitotoksik hafıza T-hücrelerine bağlı olarak önemli derecede tümör büyümesinin yavaşladığı gösterilmiştir. İkinci çalışmamızda, lipozomal CDN ve TLR9 tedavisi ile fareye yerleştirilmiş melanoma tümörünün %80 oranında büyümesinin azaldığı ortaya çıkmıştır. Lipozomal formülasyonlarla tedavi edilen farelerde, antijene özgü Th1-yanlı bağışıklığın göstergesi olan IgG2c/IgG1 oranını arttırmıştır. Ayrıca, tedavi ile birlikte IFN- γ üreten CD8⁺ T hücrelerin önemli ölçüde

yükseldiđi ve M2 tipi makrofajların tümör yatađında azaldıđı gösterilmiřtir. Sonuç olarak, çift ligandların birlikte lipozoma yüklenmesiyle, tekli ligandların anti-tümör etkinliğini arttırmıřtır. Üçüncü bölümde, antijenlerle birlikte CpG ODN yüklü lipozomal formülasyonlar ile FMDV ve *Helicobacter pylori*'e karşı ařılama, antijene özgü humoral yanıtın arttıđı anlařılmıřtır. Ayrıca, lipozomal CpG ODN ile ařılanmıř fareler helikobakter ile enfekte edildiklerinde, antijen varlıđına bađlı Th1 ve Th17 bađıřıklık yanıtları ile bakteriyel mide kolonizasyonunu azaltmıřtır.

Anahtar sözcükler: Dođal bađıřıklık, lipozom, TLR, STING, CpG ODN, poly(I:C), cGAMP, kanser immünoterapisi, ařı, adjuvan.





To my precious family...

Acknowledgement

Foremost, I would like to express my gratitude to my advisor Prof. Dr. İhsan Gürsel for his guidance, continuous support, encouragement and in particular his patience during my thesis studies. Without his contribution, I would not be able to carry my scientific vision to this degree. He was not only a scientific mentor but also a life-mentor.

I would like to express my deepest appreciations to my PhD dissertation committee members, Prof. Dr. Mehmet Öztürk and Assoc. Prof. Dr. Ayça Sayı Yazgan for their contribution in this thesis through their helpful suggestions. Moreover, I would like to thank Assoc. Prof. Özlen Konu Karakayalı and Asst. Prof. Murat Alper Cevher accepting to become members of my thesis jury and appreciated for their valuable suggestions.

I am really grateful to the past and present members of Gürsel Group, Fuat, Tamer, Gizem, Kübra, Defne, Yusuf, Merve, Arda, Mehmet, İhsan, Begüm, Gözde, Hakan, Elif, Muzaffer, Özlem, Gizem, and Havva for their tremendous support and understanding during my research. I would like to give special thanks to Gizem Tincer König and Kübra Almacıoğlu who helped me in my all researches but more than this for their friendship, moral and endless support. I specifically thank Defne Bayık Watson who always motivated me to continue my PhD without losing my enthusiasm. I would also like to thank all the seniors and intern students, especially Cansu, Begüm, Burcu and Beril, who took part in this project with me.

It was a privilege to work in the same group with Prof. Dr. Mayda Gürsel, thanks for her enthusiastic and resourceful scientific assistance. I would like to offer my special thanks to Mayda Gürsel Group members, Bilgi, Esin, Soner, Naz, Ersin and Cihan

for helping me in my research. Without Bilgi and Esin's friendship, I could not survive in the first years of my PhD.

I want to share my thanks to MBG veterinarian Gamze Aykut for her help and guidance in animal experiments and technicians Abdullah Ünnü and Turan Dastandır for their support in technical issues.

I am truly indebted to all of my dearest friends in MBG family, Şahika, Pınar, Buket, Pelin, İnci, Alican, Aslı, Nilüfer, Verda, Erdem, Seda, Zeynep and especially Büşra for giving me full support and morale during this long and rough journey. I specifically thank Özlem Tufanlı for her friendship, faith in me and all supports in every struggle.

Without my family, none of the outstanding things in my life would have been achievable. I would like to express my deepest love and thankfulness to my mother Timsal for her endless love, my father Yalçın for his wise advices, my sister Bengü for her all supports, my brother-in law Uğur and my niece Leyla for their invaluable and everlasting support with love.

Once for all, I would like to thank my dearest husband Murat for cheering me up whenever I need and supporting throughout this tough period with his eternal love and patience. I feel lucky to have a great family, amazing colleagues and lifelong friends.

This work was supported by TÜBİTAK (SBAG-113S207) and SANTEZ Grant (1414 STZ.2012-1).

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Abbreviations

Ab	Antibody
AIM	Absent in melanoma
AFM	Atomic force microscopy
AGS	Aicardi- Goutières Syndrome
ALF	Army Liposome Formulation
AP-1	Activator protein 1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
AS01	Adjuvant System 01
BM-DM	Bone marrow-derived macrophages
BM-DC	Bone marrow-derived dendritic cells
bp	Base pairs
BSA	Bovine serum albumin
c-di-AMP	cyclic adenosine monophosphate
c-di-GMP	Cyclic diguanylate monophosphate
CARD	Caspase activation and recruitment domain
CCK-8	Cell counting kit-8
CD	Cluster of differentiation
CDN	cyclic dinucleotide
cDNA	Complementary Deoxyribonucleic Acid
Chol	Cholesterol
cGAMP	Cyclic GMP-AMP

cGAS	Cyclic GMP-AMP synthase
CHEMS	cholesteryl hemisuccinate
CLR	C-type lectin receptors
CpG	Unmethylated cytosine-guanosine motifs
CTL	Cytotoxic T cells
CXCL	CXC-chemokine ligand
DAMP	Danger-associated molecular pattern molecules
DC	Dendritic cell
DC-Chol	3β-[N-(N',N'-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride
DDA	Dimethyldioctadecylammonium
ddH ₂ O	Double distilled water
DDX41	DEAD-Box Helicase 41
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DLS	Dynamic light scattering
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
ELISA	Enzyme linked-immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot
EMA	European Medicines Agency
ER	Endoplasmic reticulum

EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth disease virus
FU	Fluorouracil
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine triphosphate
h	Human
hp	<i>Helicobacter pylori</i>
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
IP-10	Interferon gamma-induced protein 10
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
IκB	Inhibitor kappa B
IKK	Inhibitor kappa B kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic Acid
m	Murine
mAb	Monoclonal antibody

MALP	Macrophage-activating lipopeptide
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen activated protein
MAVS	Mitochondrial antiviral signaling protein
MCP	Monocyte chemoattractant protein
MD-2	Myeloid differentiation protein-2
MDA	Melanoma differentiation-associated protein
MHC	Major Histocompatibility Complex
MLV	Multi-lamellar vesicles
moDC	Monocyte-derived dendritic cells
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response gene 88
M ϕ	Macrophage
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like receptors
NLRP	NOD-like receptor protein
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerization Domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine

PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PDI	Polydispersity index
PD-L1	programmed death-ligand 1
PEC	Peritoneal exudate cells
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PGN	Peptidoglycan
poly(I:C)	Polyinosinic-polycytidylic acid
PNPP	p-nitrophenyl phosphate
PO-	Phosphodiester backbone
PRRs	Pattern recognition receptors
PS-	Phosphorothioate backbone
PS	Phosphatidylserine
RES	Reticuloendothelial system
RIG	Retinoic acid-inducible gene
RLR	RIG-I-Like Receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase PCR
RT	Room temperature
SA-ALP	Streptavidin-alkaline phosphatases
SAVI	STING-associated vasculopathy with onset in infancy
s.c.	Subcutaneous
SLE	Systemic Lupus Erythematosus

siRNA	Small interfering RNA
SSCL	Sterically stabilized cationic liposomes
ssDNA	Single stranded DNA
ssRNA	Single-stranded RNA
STING	Stimulator of interferon genes
SUV	Small unilamellar vesicle
TAK	TGF- β activated kinase
TBK	TANK-binding kinase
TCR	T-cell receptor
TDA	Trehalose 6,6'-dibehenate (TDB)
Th	T-helper
TIR	Toll/IL-1 receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing IFN- β
Treg	Regulatory T cells

Chapter 1

Introduction

1.1 The immune system

The immune system is the most important system that protects our body from bacteria and protects the body's own cells from non-self insults. Lower level organisms such as plants and invertebrates protect themselves against pathogens by innate immune system while higher organisms like mammals have more specific arm known as the adaptive immune system on addition to innate immune system [1,2].

The innate immune system is the first line of defense mechanism; it is fast and most effective line of defense to wane of intruders. The receptors involved in this system are germ-line encoded and invariant. While it is non-specific to antigen, it is compromised as pathogen-specific. Sensors of innate immune system are highly conserved molecules and collectively known as the pattern-recognition receptors (PRRs). They are specialized to recognize pathogen-associated molecular patterns (PAMPs) [3,4]. However recently, a new concept was introduced. Against already established dogma that innate immunity does not rely on memory is debated and based on recent evidences indeed innate immune cells could maintain a memory. This is termed as “trained memory” or “innate immune memory”. As discovered,

innate cells also remember and respond in a higher magnitude when they are re-infected with same pathogen [5,6].

Adaptive immunity is the second line of defense and is highly antigen-specific. Expansion of antigen-specific clones of T- and B-cell development is slower than the response triggered by innate immunity. The random rearrangement of V, D and J segments constitutes highly specific and unlimited number of B- and T- cell receptor repertoires. Most importantly, it develops protection mechanism against re-infection known as immunologic memory [1]. The adaptive immune response is based on activation of cellular responses, which is activation of $CD4^+$ T helper cells (Th1 and Th2), and cytotoxic T cells (CTLs), and humoral responses as production of antigen-specific antibody secretion from B cells [3]. Expression of peptides bound on major histocompatibility complex class I (MHC-I) are recognized by CTLs which kill infected cells or tumor cells that are on response to abnormally expressed peptides. Antigen presentation is maintained by antigen presenting cells (APCs) via presenting peptides on MHC-II to T cells. During antigen presentation, up-regulation of co-stimulatory molecules is required for T cell activation. Activated cells differentiate into subtypes of T cells and produce specific cytokines and help B cells to sense antigen and produce antibodies [7].

1.2 The innate immunity

The first line of defense is the innate immunity that composes of complement system, phagocytic and antigen presenting cells. It reacts promptly and aims to prevent spreading of infection at the onset of invasion. After the neutralization of the insult, antigen presenting cells, particularly dendritic cells (DCs) instruct cells of the adaptive immune system to become antigen specific effector cells. They process pathogen-specific proteins and epitopes are presented to T- and B-cells establishing antigen-specific immunity [8,9].

1.2.1 Pattern recognition receptors (PRRs)

Innate immune cells such as dendritic cells and macrophages recognize invading pathogens by detecting specific conserved patterns that belongs to microbial world via PRRs. Generally, the components of microorganisms detected by PRRs are known as PAMPs [9]. Lipopolysaccharide (LPS), lipoteichoic acid, lipoproteins on bacterial and fungal cell wall components, or single stranded or double stranded RNA, as well as unmethylated CpG motif containing synthetic oligodeoxynucleotide are well-known PAMPs [7,10].

PRRs can also recognize damage-associated molecular patterns (DAMP) that are products of damaged cells, tissues or necrotic/apoptotic cells. DAMP are immunostimulatory products that alert immune system and have a crucial role in tissue remedy as well as defense of body [11].

The well-known PRRs are Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) -like receptors (RLRs), nucleotide-binding oligomerization domain (NOD) like receptors (NLR) and C-type lectin receptors (CLR) together with cytosolic DNA and RNA sensors. While CLRs are found on the cell membrane, RLRs that are cytoplasmic RNA helicases such as RIG-I and melanoma differentiation-associated protein-5 (MDA-5) sense intracellular pathogen-associated compounds within cytosol. They are known to sense viruses and lead to secretion of interferons. NLRs are also found in cytosol and are specialized to recognize bacterial components such as peptidoglycan [12].

1.2.2 Nucleic acid sensors

Endosomal and cytosolic nucleic acid sensors detect different molecular patters like unmethylated CpG motifs or cyclic dinucleotides and initiates strong innate immune activation. Recently, it was debated that nucleic acid based adjuvants are more potent and safe to be included within vaccine formulations [13,14].

1.2.2.1 Toll -like receptors (TLRs)

Innate immune cells recognize microbial (bacterial and viral) components via pattern recognition receptors (PRRs) through pathogen-associated molecular patterns (PAMPs) and differentiate self from non-self [1,2]. Toll-like receptors (TLRs) are the most extensively studied PRRs (Figure 1.1) [15].

TLRs are type I transmembrane proteins and member of IL-1R superfamily. They have first discovered in fruit fly, *Drosophila melanogaster* and named Toll receptor that regulates antifungal immune response [16]. Homologous form was then identified in mammalian innate immune system by Medzhitov and Janeway in 1997 and named as Toll-like receptors [17].

They contain an ectodomain leucine-rich repeats (LRRs), and Toll-IL-1 receptor (TIR) domains. While LRRs promote PAMP recognition, TIR domain is required for maintaining the downstream signaling pathway of TLRs. There are 12 type (TLR1-9 and TLR11-13) of TLR in mice and 10 (TLR1-10) in human. Some of these TLRs (1, 2, 4, 5, 6, and 11-12) are located at cell surface to detect compounds such as lipopolysaccharides (LPS) of extracellular pathogens before invading into cell, while some (3, 7/8, 9) reside in intracellular vesicles like endosomes, lysosomes or endolysosomes to detect nucleic acids (DNA and RNA) of pathogens [3,12,14].

The downstream signaling pathway of TLRs are initiated after recruiting two adaptor proteins: TIR-domain-containing adaptor-inducing interferon- β (TRIF) and myeloid differentiation primary response gene 88 (MyD88) (Fig. 1.1). All TLRs use MyD88-dependent pathway except TLR3 that recruits TRIF adaptor protein only. TLR4 uses both pathways. Mainly MyD88-dependent signaling pathway activates nuclear factor kappa B (NF- κ B) and mitogen activated protein (MAPK). N-terminal death domain of MyD88 is reacted with death domain of IL-1R associated kinase-4 (IRAK-4) that phosphorylates IRAK1 and IRAK2. These molecules activate tumor necrosis factor-receptor-associated factor6 (TRAF6) that activates TGF- β activated kinase 1 (TAK1). TAK1 phosphorylates I κ B kinase (IKK) and it activates MAPKs. IKK

phosphorylates I κ B that degrades the interaction between NF- κ B. As a consequence, these factors promote expression of pro-inflammatory cytokines such as IL-6 and IL-12 [3]. However, recruitment of TRIF initiates interferon secretion as an anti-viral defense by interacting with TRAF3 and TRAF6 rather than inflammatory cytokines. Same in MyD88 dependent signaling, TRAF6 activates NF- κ B and MAPKs leading expression of pro-inflammatory cytokines. TRAF3 activates interferon regulatory factor 3 (IRF3) leading secretion of interferons following TLR3 activation [18].

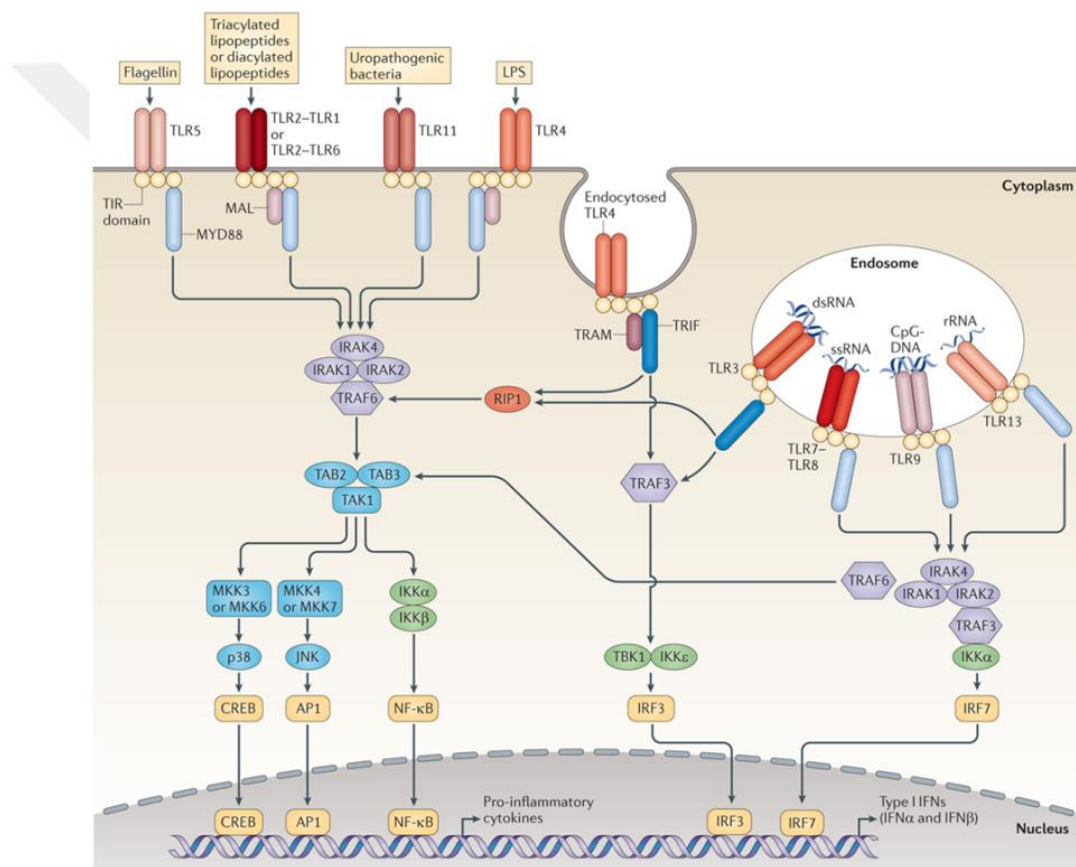


Figure 1.1. Signaling pathway of Toll-like receptors [19].

TLRs have potential to recognize various pathogens such as virus, parasites, and bacteria (Table 1.1). Even though, TLRs and innate immune activation is important

to elevate adaptive immune system, it has been shown that in the absence of TLR expression adaptive immune system still recognize pathogens and respond perfectly [18].

Table 1.1. TLRs and their agonists [10,13,20].

Types	Cellular localization	Pathogens	Agonists
TLR1/2	Membrane	bacteria	Lipoprotein, LTA, PGN
TLR2	Membrane	Virus, fungus, parasites	Structural protein, mannan, mutin
TLR3	Endosome	dsRNA, ssRNA, dsDNA viruses	dsRNA, poly(I:C), polyU
TLR4	Membrane	Bacteria, virus, fungus, parasites	LPS, structural protein, mannan, glycoinositolphospholipids
TLR5	Membrane	Bacteria	Flagellin
TLR2/6	Membrane	Bacteria, fungus	Lipoprotein, LTA, PGN, zymosan, b-glucan
TLR7/8	Endosome	ssRNA viruses, bacteria, fungi, protozoan parasites	GU-rich ssRNA, Imidazoquinolines (R848, imiquimod, 3M001/2), guanosine analogues
TLR9	Endosome	ds DNA viruses, bacteria, protozoan parasites	DNA, CpG ODNs, hemozoin

1.2.2.2 Endosomal nucleic acid sensors

Extracellular pathogens are generally recognized by immune cells and phagocytosed through endosomal pathway. Intracellular TLRs following internalization and subsequent pathogen degradation/digestion in the late endosome binds to nucleic acid motifs upon microbial cargo leakage into endosome [18]. Not only viruses but also other microbes internalized by endosomes would be sensed by nucleic acid sensing TLRs.

TLR family members are sub-divided into cell membrane-associated and endosome-associated receptors. Strikingly, endosomal TLRs are specialized to sense microbial

specific nucleic acids. While TLR3 and TLR7/8 recognizes double and single-stranded RNA, TLR9 recognizes bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs (CpG ODNs) [3,21–23].

1.2.2.2.1 TLR3

TLR3 senses double stranded RNA (dsRNA) and its analog polyinosinic-polycytidylic acid (poly(I:C)) [21]. While TLR3 recognizes RNA viruses like reovirus, they would also sense single stranded RNA (ssRNA) viruses such as West Nile virus and dsDNA viruses like herpes simplex virus through expression of dsRNA during transcription [24]. TLR3 reside at early endosome as acidification is important for receptor-ligands interaction and endoplasmic reticulum (ER) is not necessary for trafficking of TLR3 unlike TLR7/8 and TLR9 proteins [18].

Poly(I:C), a synthetic analog of dsRNA initiates signaling cascade through TLR3 and induces type I IFNs along with pro-inflammatory cytokines mediated by NF- κ B, mitogen activated protein kinase (MAPK) and IRF3 via TRIF-dependent (MyD88-independent) pathway [3,21,25]. Type I IFNs are known as an important cytokine in linking innate and adaptive immunity. They induce and increase the expression of costimulatory molecules, differentiation of monocytes to dendritic cells, priming of T cells, cross-priming of CD8⁺ T cells and extending the survival during antigen-driven clonal expansion. Type I IFNs inhibits secretion of Th2-biased cytokines such as IL-4 and IL-5 and stimulates type II IFN production and prolong the survival of CD4 T cells. Because poly(I:C) can also bind to the cytoplasmic RNA helicase, MDA-5 and activates the IRF-3 pathway by TLR independent manner, type I IFN is produced by DCs, macrophages and also by non-hematopoietic cells [10]. Poly(I:C) was found to mimic the viral infections rather than bacterial or parasitic. Off note, it does not require IL-12 for the induction of Th1 immunity.

1.2.2.2.2 TLR7 and TLR8

TLR7 and TLR8 recognizes ssRNA and its synthetic analogs such as imiquimods or resiquimods and guanine analogs [22,26,27]. Like HIV and influenza, some viruses have ssRNA and some bacteria release their RNA into endosome [28,29]. In addition, TLR7 would recognize some siRNAs [30]. Imiquimod is Food and Drug Administration (FDA) approved TLR7 agonist. It is now topically used to treat neoplasias, lentigo maligna, and basal cell carcinoma treatments that induces pro-inflammatory cytokine and chemoattractant secretion, and provides Th1-biased immunity [31]. Imidazoquinoline resiquimod (R848) is both activating TLR7 and TLR8 which means in human it elevates both sensors and excessive cytokine release would be the reason of its side effects in clinical use [32].

1.2.2.2.3 TLR9

One of the most studied TLRs is TLR9 recognizing CpG ODNs, which are found frequently in bacterial and viral genome but rare in mammalian DNA due to CpG methylation and suppression [33,34]. The immunostimulatory effects of CpG ODN have been first discovered during studies investigating oligodeoxynucleotides on B cell proliferation by Krieg et al. [35]. When CpG motif is transformed into GpC or CpG ODN is methylated, the immunostimulatory effect was abolished [33]. However, the mechanism through recognition by TLR9 has been discovered five years later by Hemmi et al. [36]. Activation of TLR9 enhances innate immune response and Th1-biased adaptive immunity through recruiting MyD88 adaptor protein and activation of NF- κ B and IRFs [37]. CpG ODN can trigger production of pro-inflammatory cytokines, antigen presentation, DC maturation, and elevates adaptive and humoral immune responses when administered together with antigen.

Even though TLR9 is programmed to detect non-self DNA by controlling the unmethylated motif of ODN and CpG motif presence as a sequence dependent manner, it might detect self DNA in some circumstances. When DNA is mixed with LL37 or N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate

(DOTAP) and they enter cell by endosomal uptake. At this circumstance, endosomal DNA activates TLR9 [38,39].

Contrary to mice, in humans, there are three main types of CpG ODN classes; D-type CpG ODNs (also known as CpG-A type) have polyG tail at both 5' and 3' tails with phosphorothioate (PS) links and contain one CpG motifs (purine/ pyrimidine/ CpG/ purine/ pyrimidine) in the center with phosphodiester (PO) linkages. D-ODNs with mixed backbone form G-tetrads due to its poly-G tails and cause aggregated structure with high-order complexation of tails. They are recognized at early endosome and recruits MyD88. They trigger production of redundant amounts of interferon- α from plasmacytoid dendritic cells (pDCs) and IFN- γ from natural killer (NK) cells through NF- κ B and IRF7 via MyD88-dependent signaling pathway, however; they stimulate merely B cells [34,40]. To initiate activation CXCL16 is required as a co-receptor that acts for recruiting D-type CpG ODN within TLR9 expressing early endosomes [41].

The most suitable ODN type for clinical use is K-type CpG ODN (also known as CpG-B). Unlike D-type CpG ODN, K-type CpG ODNs have more than one CpG motifs with phosphorothioate backbone and they have no polyG tails. They are recognized at late endosome and activates NF- κ B transcription factor. While D-type CpG ODN induces IFN- α secretion from pDCs, K-type secretes tumor necrosis factor- α (TNF- α). In addition, they promote pro-inflammatory cytokine secretion, B cell maturation; however, they inefficiently stimulate pDCs to secrete interferons [37,40].

C-type CpG ODN is the third class that combines the properties of D- and K-type CpG ODN. Unlike K-type ODN, they can promote interferon secretion from pDCs, however amounts are less than initiated by D-type CpG ODN. Besides, they can trigger B cell maturation [42,43].

Design and modification of CpG ODN is very important to elicit proper innate immune response through TLR9 activation [44]. The different immune activations by

these two CpG ODNs with different structures are suggested to be due to intracellular recognition by early or late endosome [45]. Besides, high interferon secretion by pDCs treated with D-type ODN is associated with interaction of CpG ODN with CXCL16 scavenger receptor, which is not found in B cells [41] and not a prerequisite co-receptor for K-type CpG ODN. Moreover, cell types expressing TLR9 is different between mice and human that makes it complex to understand most effective CpG ODN in clinical use (Table 1.2).

Table 1.2 Cellular localization of endosomal TLRs [46,47].

	Mouse				Human			
	TLR3	TLR7	TLR8*	TLR9	TLR3	TLR7	TLR8	TLR9
pDC		+		+		+		+
XCR1⁻ DCs		+		+			+	
XCR1⁺ DCs	+				+			
Monocytes	+	+		+	+		+	
B cells		+		+	+	+		+
Neutrophils		+					+	

1.2.2.3 Cytosolic nucleic acid sensors

One of the most important pathogen associated molecular patterns is nucleic acid and particularly, DNA. While immune cells detect pathogen-derived DNA, hematopoietic or non-hematopoietic cells could also recognize DNA leak into cytosol as a danger signal (damage-associated molecular pattern, DAMP). Detection of DNA either as pathogen-specific or damage-associated within the cytosol, elevates production of inflammatory cytokines and especially interferons (IFNs) [14,48].

RIG-I and MDA-5 recognize dsRNA according to its length and chemical structure. mRNA is differentiated between pathogenic or damage-associated forms through its first base pair that has 2'-O-methylation. These sensors both have two main

components, caspase activation and recruitment domain (CARD) and helicase domain to activate mitochondrial antiviral signaling protein (MAVS) [46]. Another cytosolic sensor is absent in melanoma 2 (AIM2). It recognizes cytosolic dsDNA and activates inflammasome that leads to secretion of IL-1 β and IL-18 [13,48].

1.2.2.3.1 cGAS and STING

Cytosolic dsDNA binds to cyclic GMP-AMP (cGAMP) synthase (cGAS) enzyme and modifies the structure of cGAS. DNA sensed by cGAS has a wide range of recognition that is independent of the cell source. While B-form DNA from dead cells are recognized within cytosol by cGAS, microbial DNA ranging from viruses (such as herpes simplex virus, vaccinia virus, adenovirus, retrovirus) to bacteria (such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Listeria monocytogenes*) is also sensed by this enzyme [49–51]. The association of DNA/cGAS leads to activation of cGAS and subsequently converts cytosolic GTP and ATP into 2'3' cGAMP (Figure 1.2). cGAMP functions as endogenous second messenger and binds to stimulator of interferon genes (STING) adaptor protein bound to ER [52–56]. 2'3' cGAMP is the non-canonical STING ligand.

STING is also known as TMEM173/MPYS/ MITA/ERIS, which is a highly conserved and ER-bound adaptor protein [54,57,58]. Bacteria-derived secondary metabolites collectively known as cyclic dinucleotides (CDNs hereafter) such as cyclic diguanylate monophosphate (c-di-GMP) and 3'3' cGAMP are sensed directly by STING, which is known as the canonical STING ligand. STING upon interaction with its cognate ligand recruits tank-binding kinase-1 (TBK1) and activates IRF3 upon phosphorylation. IRF3 is translocated to nucleus to activate the expression of IFN β . In addition, IKK is recruited by STING and phosphorylates IK κ B α . This interacts with p65/p50 heterodimer of NF- κ B and then translocate into nucleus to transcribe the expression of pro-inflammatory cytokines [59–62].

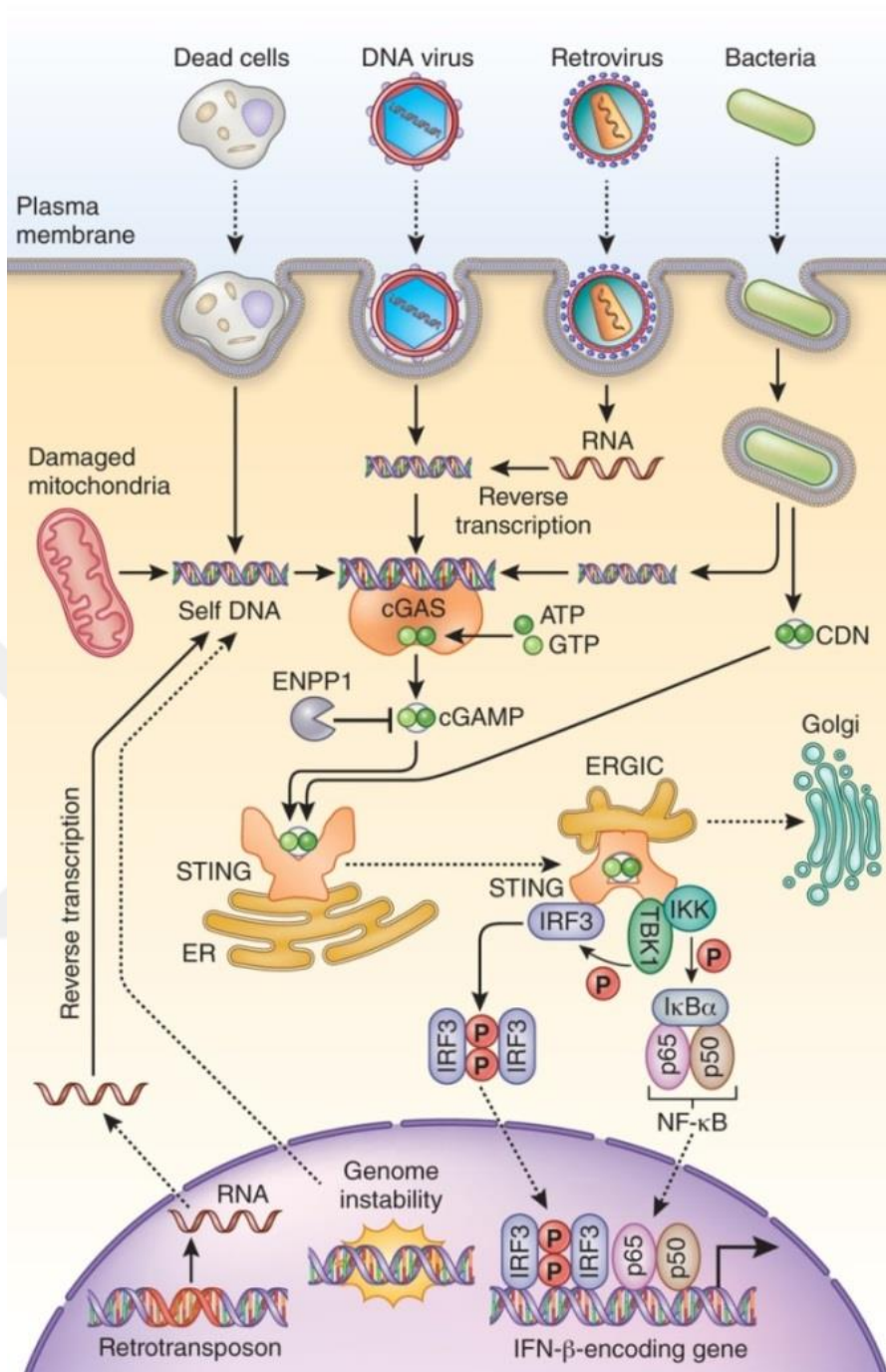


Figure 1.2. Schematic illustration of cGAS and STING pathway [56].

pDCs recognize CDNs such as cGAMP and activates cGAS-STING pathway, thereby inducing type I IFN secretion [63]. Besides pDCs, human monocyte-derived DCs and macrophages also constitutively express STING and cGAS [64].

While the activation of STING and cGAS ensures protection of host from infections, excessive interferon production in cases where there are deficiencies in DNase activity such as TREX1 (a DNase III) or DNase II deficiencies leading to uncontrolled accumulation of self-DNA within cytosol elicits immune dysfunctions and leads to severe autoimmune or autoinflammatory conditions. One of the worst severe forms of such STING associated problem is the STING-associated vasculopathy with onset in infancy (SAVI). Excessive systemic interferon alpha is also seen in Aicardi- Goutières syndrome (AGS) and in Systemic Lupus Erythematosus (SLE) [65–68].

Recent studies confirmed that DNA released by tumors exists in circulation. Scientists speculated that antigen presenting cells could internalize tumor derived DNA, and this is sensed by cGAS and consequently activates STING pathways [56].

1.2.3 Cross-talk of PRRs

Innate immune system during a pathologic insult activates multiple signaling cascades. This activation is dependent on simultaneous engagement of different ligands to their corresponding receptors (either exists on the cell surface or present on endosomes or even expressed within the cytosol). In some cases, PRRs might cross talk with each other and could either boost or suppress the overall resultant pathogen specific innate immune response [69],[70].

1.2.3.1 Cooperation between TLRs

One of the well-studied cooperation was established between TLRs. During a pathogen invasion, due to the expression of multiple PAMP in/on the pathogen multiple signaling cascades were activated due to the engagement of multiple TLRs with their agonists. TLRs recognize various PAMPs such as nucleic acids, lipids, and proteins with distinct patterns (Table 1.1). The localization of TLRs promotes the recognition of diverse type of molecules. Therefore, TLRs effectively distinguish different pathogen types such as bacteria, virus, parasites and fungus based on their

signature PAMPs. The activation of different TLRs result cross-talk between each other that dictates the magnitude and sustainability of immune response.

One way of leading cooperation of TLRs is the engagement of multiple agonists. The invasion of ssRNA virus would activate TLR7 and then dsRNA coming from viral replication trigger TLR3 pathway. Infection by *Mycobacterium tuberculosis* is recognized by TLR2, TLR4 and TLR9 through sensing lipoarabinomannan, phosphatidylinositol mannosides and its nucleic acid, DNA, respectively [71]. Molecular patterns of fungal species such as *C. albicans* and *A. fumigatus* is sensed by innate immune cells via TLR2, TLR4, TLR6, and TLR9 through sensing β -glucan, chitin, mannan and DNA, respectively [72,73]. The recognition of parasites by immune system, leads to several TLR sensing such as membrane glycolipids by TLR2 and TLR4, prolin-like molecules by TLR11 and hemozoin-like molecules by TLR9 [74–76].

The activation of different TLRs results cooperation between TLRs and would lead the elevation of different cytokines [77] (Table 1.3). Dual combinations of macrophage-activating lipopeptide-2 (MALP-2) or poly(I:C) or CpG ODN ligands enhanced the quantity of IL-12p70 production from APCs while triple ligands including MALP-2, poly(I:C) and CpG ODN enhanced both the expansion of T cell clones and increased IL-15 secretion [78]. In a study, mouse dendritic cells stimulated with TLR3 and TLR9 ligands secreted high levels of IL-12 and IL-6 than incubation with single ligands [79]. Similarly, combination of TLR3 or TLR4 and TLR7/8 or TLR9 agonists acted synergistically in a dose dependent manner on DC activation through IL-12 production [80]. In addition, crucial protective role of presence of both TLR3 and TLR9 activation has been demonstrated in cytomegalovirus infection in which defense mechanism depends on synergistic type I IFNs and IL-12 production [81].

In order to understand the cytokine elevation with multiple TLR activation the mechanism of synergy should be studied. According to literature, MyD88 and TRIF adaptors merge synergistic activation since simultaneous activation of MyD88- and

TRIF-dependent pathways leads to superior immune activation [82]. Most of the ligands of these pathways lead synergism. For instance, between TLR3/TLR2; TLR3/TLR7; TLR3/TLR9; or TLR4/TLR2; TLR4/TLR7; TLR4/TLR9 [83].

Table 1.3. Cytokine synergy induced by cross-talk between TLR ligands modified from [84]

Cell type	Combination	Cytokines	Mechanism	Refs
m-BM-DCs	TLR2, 3 and 9	IL-12 and IL-15	NA	[78]
m-BM-DCs	TLR3 and 9	IL-12	Autocrine type I IFN	[79]
m-BM-DM	TLR4 and 9	TNF and IL-6	JNK	[85]
m-Mϕ	TLR3 and TLR9	NO, IL-6, IL-12 and TNF- α	NA	[86]
m-PEC	TLR3/4 and 7/9	IL-12	IRF5	[83]
m-PEC	TLR2 and TLR4	TNF- α	NA	[87]
m/h pDCs	TLR4 and TLR2 or TLR7	IL-6 and IL-12	NF- κ B, PI3K.	[88]
h-moDCs	TLR 3, 4 and 8	IL-12, IL-6, and IL-10	p38, NF- κ B, PI3K	[80,89,90]
h-pDC	TLR3 and TLR7	IFN- α	NA	[91]
h-PBMC	TLR2 or TLR4 and TLR7/8 TLR8	IFN- γ	NA	[92]
h-PBMC	TLR3 and TLR2, 5, 7/8, or 8	IFN- α /- β	NA	[92]
h-PBMC	TLR5 and TLR3 or TLR9	IL-1 β	NA	[93]

BM-DM, bone marrow-derived macrophages. BM-DC, bone marrow-derived dendritic cells. M ϕ : macrophages. PEC: peritoneal exudate cells. moDC: monocyte-derived dendritic cells. pDC: plasmacytoid dendritic cells. PBMC: peripheral blood mononuclear cells. NA: not available.

1.2.3.2 Cooperation between TLRs and STING

Synergistic immune response due to activation of MyD88 dependent and independent pathways were also studied by using combinations of STING and TLR9 agonists [94,95] both in mice and on human PBMCs. Cytosolic DNA induces STING-mediated immune activation initiated by CDNs and whereas CpG ODN in endosome leads to TLR9 mediated immune response. STING agonist (3'3'-cGAMP) prone to trigger Th2-biased immune response and K-type CpG ODN has low effects on type I IFN secretion. The combination of ligands enhanced type I IFN, IL-6, IP-10 and TNF- α , secretion and efficiency of agonists as an immunotherapeutic agent against tumor regression [95]. The synergistic activity of K3 plus c-di-GMP or cGAMP has also been reported by Temizoz *et al.* In this study, cGAMP and K3 combination had an anti-tumor activity with synergistically increased type-II IFNs from particularly NK cells [94].

Recently, another cross-talk between TLR and STING pathway has been discovered. Invasion of *Neisseria* was observed to induce type I IFN secretion through TLR4/MD-2 and activating cGAS and consequently STING/TBK1/IRF3 axis. Both recognition of lipooligosaccharides and cytosolic DNA provided augmented IFN- β secretion. This cooperation between TLR4 and cGAS pathways was demonstrated on both human and mouse macrophages [96].

1.3 Immunotherapeutic applications of nucleic acid-based PRR agonists

Conventional vaccines include attenuated or inactivated pathogens. Administration of killed or attenuated vaccine elicits strong humoral and cell mediated immune response possibly by multiple ligand/receptor engagements yielding protective immune response. However, the safety concerns of the conventional vaccines necessitate the development of much safer and easy to produce modern vaccines. Synthetic or subunit vaccine even though they are easy to prepare and much safer, their potency is weak and requires inclusion of potent adjuvants in order to mimic the

response of conventional vaccines. Therefore, design and testing of new adjuvants is an important research field in vaccinology [8].

Adjuvants are being also used in conventional vaccines to maintain long-lasting adaptive immunity. Although aluminum salts (alum) has been used since mid-1920s in human vaccines as an adjuvant, the mechanism of action was recently being explored. Recently, in addition to Alum, oil emulsions such as MF59, ASO series (detoxified LPS) are already licensed adjuvants [97]. In addition natural and synthetic PAMPs like CpG ODN and poly(I:C), liposomes (CAF01) are being tested in clinical phase trials. The mode of adjuvant action of Alum was found years after licensing [98]. However, now we have extensive knowledge about how to activate immune response with PRR agonists. Basically, immunization with vaccines containing PAMPs leads to DC maturation, inflammatory cytokines and interferon secretion, activation of different types of T-helper cells and CTLs, B cell differentiation into plasma and memory cells and finally antigen-specific antibody and cytokine production (Figure 1.3). Therefore, researchers are focused on the studies to investigate the immunogenic potency of PAMPs as adjuvants and immunotherapeutic agents.

Nucleic acid-based TLR ligands are promising candidates as Th1-biased vaccine adjuvants [34,47], anti-cancer [99] or anti-allergic therapeutic agents [100]. Nucleic acid sensors are very important initiators of interferons which have important roles in antiviral defense system. They would also lead the adaptive immune response. There are mainly three types of interferons. Type I IFNs include IFN- α (13 subtypes) and IFN- β , IFN- ϵ , IFN- κ , and IFN- ω , while type II IFNs consist of IFN- γ and type III consists of IFN- λ (4 types). IFNs initiate antiviral immune response by binding into IFN-stimulated genes (ISGs). Besides direct effects of interferons on viral replication, type I IFNs contribute to adaptive immune response maturation including generation of cytotoxic T cells and Th1-biased immune responses [46].

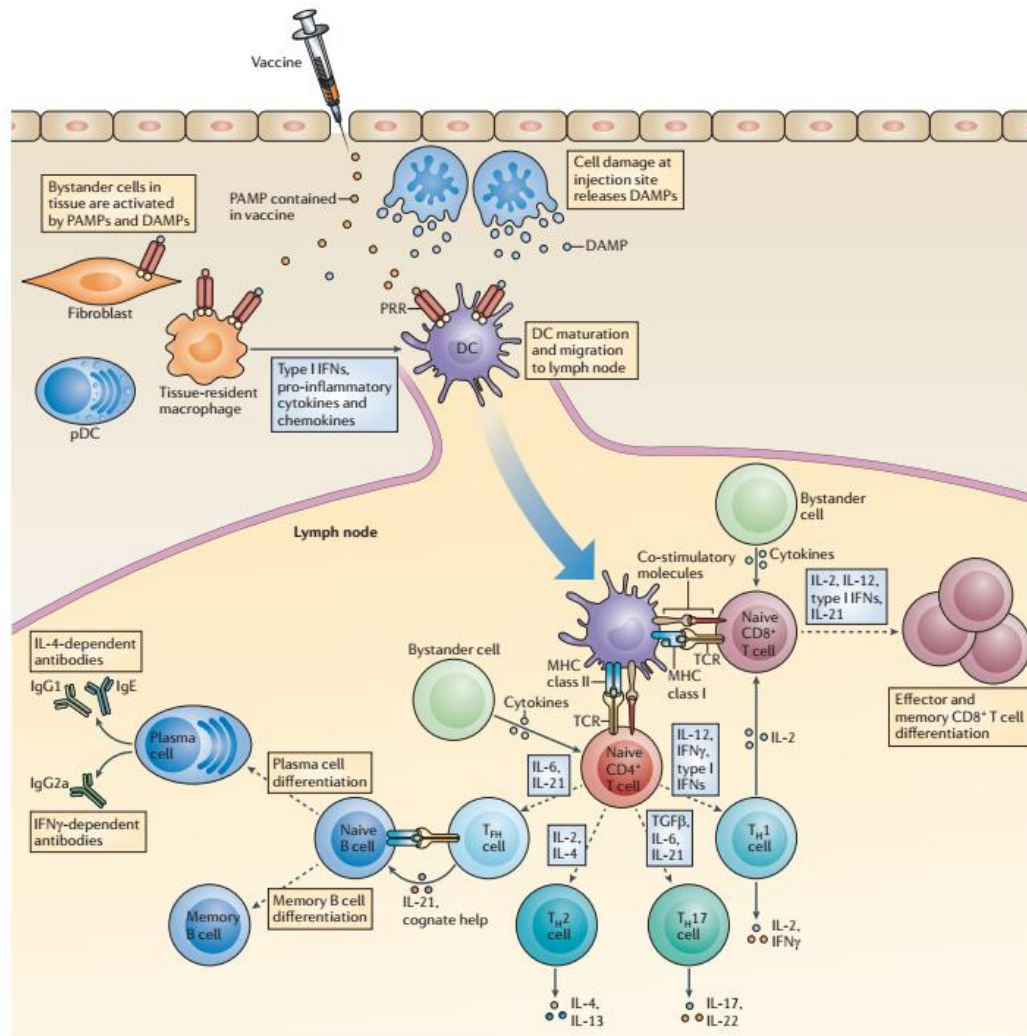


Figure 1.3. PRR induced adaptive immune response after vaccination [13].

Synthetic CpG ODN can be used as adjuvants in vaccines against various types of pathogens. However, many studies failed to boost immune response in human even though they were so active in rodents and mice models [101]. In a clinical study, the treatment capacity of C-type CpG ODN was tested in patients infected with Hepatitis C virus. While viral RNA levels in blood decreased following therapy, no antiviral response was observed [102]. Among other CpG types K-type is most promising CpG ODN, however, IFN induction capacity is still limited. Due to the immunostimulatory effects of K-type CpG ODN on B cell activation, it was

suggested that K-type could be developed as a prophylactic vaccine rather than immunotherapeutic agent. In prophylactic vaccines against anthrax and hepatitis B infections, the adjuvant potency was demonstrated. Studies proved that when K-type CpG ODN was used, immune protection after immunization was developed [103]. However, their potential use is hampered in clinic due to *in vivo* degradation by nucleases or rapid clearance by serum protein adsorption. To improve the stability of TLR9 agonist in the body one approach was to prevent nuclease attack. To do that, PO bonds was modified with PS linkages in synthetic ODNs [33]. Here, an oxygen atom in phosphate backbone is replaced by sulfur. While increasing insufficiently the stability of CpG ODN by protecting from nuclease attacks, the affinity to TLR9 and consequently efficiency of CpG ODN as an adjuvant is reduced [104]. PS-CpG ODN might cause some side-effects such as splenomegaly and arthritis [105,106] following treatment in dose and sequence dependent-manner [107]. In addition, indirectly activation of complement system would result cardiovascular diseases due to PS backbone [108]. Therefore, free ODNs still require the protection against nucleases that stabilize its immunostimulatory activity. While it gains resistance to nucleases, there are some disadvantages such as poor cellular uptake and toxicity. Liposomes increases the stability of ODNs as adjuvants and protects them from digestion but also enhances their immunostimulatory and immunotherapeutic breadth [109,110]. Besides, when CpG ODNs with PS and PO backbone were compared following liposome encapsulation, liposomal formulations PO- and PS backbone did not differ as immune response in a leishmaniasis model [111].

TLR3, a sensor of poly(I:C), which is a synthetic analog of dsRNA, is expressed in different cell types including immune cell, brain cells and tumor cells. In a study on the treatment of melanoma cells, a direct apoptotic and anti-proliferative effect of poly(I:C) through TLR3 on tumor cells was observed [112]. The direct effect of poly(I:C) on tumor cells also leads indirect anti-tumor effect by immune cells that phagocytoses damaged tumor cell components [113]. Besides, poly(I:C) has a direct adjuvant effect that activate DCs, CTLs and NK cells and promote cross-presentation and NK-DC interaction by inducing IFN- β and pro-inflammatory cytokines. However, due to instability in the body, it failed to present its potential in clinical

studies. Although there is a more stable form of poly(I:C), known as “poly(ICLC)”, with increased stability with resistant to hydrolysis by serum proteins and elevated interferon secretion, unfortunately severe side-effects like fever and hypotension [114] were observed when used in clinical trials, therefore clinical development of this form is not preferred. Encapsulating poly(I:C)/(ICLC) into particulate systems such as liposomes is now being considering as more promising alternative since this will allow to obtain more stable form of TLR3 agonist and hopefully promote increased adjuvant activity [115–117].

The adjuvant potential and immunostimulatory property of c-di-GMP was first demonstrated in a study by Gray *et al.* [118]. It was considered as a potent vaccine adjuvant against bacterial infections by triggering specifically bacterial immune responses [119]. In a vaccination study, even though, c-di-GMP showed no immunogenic effect, combination with poly(I:C) and anti-CD40 antibody increased CD8⁺ T cell activity [120]. In addition, c-di-GMP has been used in the protective pertussis vaccine and increased T cell activation [121]. In a treatment study, it was used as immunotherapeutic agent in metastatic breast cancer [122]. c-di-GMP has poor cellular uptake due to its circular structure. In addition, uptake by lymph nodes is not sufficient to elicit immune activation. Furthermore, activation of c-di-GMP administered together with immunogenic antigens such as ovalbumin (OVA) has been observed to activate adaptive immunity while more relevant epitopes such as influenza antigen has been insufficient to elevate antigen-specific responses at low doses [123–125]. In another study on hepatitis, combining hepatitis B surface antigen and c-di-GMP at high doses (70-290 µg) elicited humoral responses but they caused systemic inflammation [118]. Therefore, some studies used c-di-GMP together with CpG ODN or loaded into pH liposomes to enhance their cellular uptake and adjuvant potency by reducing site effects [95,126,127].

Another CDN, cGAMP, has an adjuvant potential that has been shown to increase antigen-specific antibody production and IFN-γ production, and consequently activates CTLs and NK cells. After the discovery of cGAMP and its impact on the immune system, several studies started to investigate its potential as therapeutic

agent and adjuvant. In a vaccine study, 3'3' cGAMP was administered intranasal and elevated antigen-specific adaptive immune response with Th1, Th2 and Th17 biased immune activations [128]. As a cutaneous vaccine adjuvant, when 2'3'-cGAMP was administered intradermal, it enhanced cellular and humoral immune responses against influenza with no side effects or irritation on skin [129]. The combinational therapy of cGAMP with anti-cancer agent, fluorouracil (5-FU) elicited higher regression of colon cancer and reduced the toxic effects of cancer agent. In addition to interferon production capacity, cGAMP induces some anti-tumor cytokines such as IL-2, monocyte chemoattractant protein-1 (MCP-1) and IL-12, elevated DC maturation and cross-priming. Besides, cGAMP has no cytotoxicity on tumor cells but it activates STING pathway to provide anti-tumor immune responses [130].

Combinational therapy with synthetic CDNs and radiotherapy have been confirmed to control pancreatic tumor through activated antigen-specific CD8 T-cells [131]. In a study, the combination of cGAMP and programmed death-ligand 1 (PD-L1) monoclonal antibody was used as immunotherapy against melanoma tumor. They have shown the importance of cGAS pathway in tumor microenvironment as in deficiency leading less responsive to PD-L1 treatment. cGAMP and PD-L1 combinational treatment leads enhanced activity of DCs and cross-presentation resulting elevated anti-tumor immunity [132,133]. Additionally, STINGVAX a cell-based vaccine prepared using CDNs treated GM-CSF secreting cells combined with PD-1 treatment and suppressed tumor development [134].

Studies on adjuvant development have been also benefited from the synergistically activation of immune response by the combinations of TLR ligands and used as adjuvants to elicit higher magnitude innate immune responses. In a tuberculosis vaccine, the synergy between TLR4 and TLR9 pathways enhanced the protection against *M. tuberculosis* infection [135]. Synergism between TLR2/6 and TLR9 has been used in the protective treatment against influenza pneumonia [136]. Combination of TLR2/6, TLR3, and TLR9 ligands provided protection against viral infection by eliciting high quality antigen-specific T cell responses with high avidity and immunological memory [78,135].

1.4 Liposomes as a drug delivery system

Drug delivery systems are designed to improve the effects of therapeutic molecules such as chemotherapeutic agents or adjuvants without any side effects. These effects are multiple depending on the application, and include i) increasing stability of molecules, ii) maintains longer circulation, iii) enhances cellular uptake, and iv) providing site-specific targeting to their effector cells. Liposomes, which are spherical nano-vesicles with lipid bilayers, have been developed and tested for decades for several biomedical applications and were suggested to be the most promising drug delivery system in clinical use.

Liposomes were first discovered in 1965 [137,138]. Then, in 1971, it was first used as a drug delivery system by Gregoriadis *et al.* [139]. Lipids have a hydrophilic head and a hydrophobic tail and self-assembled lipid-bilayer form in certain circumstances. Core of liposomes are hydrophilic which enables encapsulation of various hydrophilic molecules while lipophilic molecules would be entrapped into the lipid bilayer [140]. Now, liposomes are widely used in cosmetics and pharmaceutical industries. Doxil[®], which is sterically stabilized PEGylated liposome loaded with Doxorubicin (a chemotherapeutic agent), is a licensed product for the treatment of various cancer types such as metastatic breast and ovarian cancers. Ambisomes[®] is another liposomal product which is loaded with amphotericin B to treat fungal infections.

Liposomes have some advantages like their biosafety, biocompatibility and stability in the body. Liposomes with different physicochemical characteristics can be prepared by changing lipid types and ratios and modification of surface. Cationic lipids such as DOTAP and 3 β -[N-(N',N'-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), phosphatidylcholine (PC), cholesterol (Chol), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) are the most widely used lipids for the liposomes preparation with different characteristics such as pH-dependent release or stronger affinity to load anionic DNA molecules [141].

1.4.1 Cytosolic delivery by pH-sensitive liposomes

Intracellular transport of drugs can be maintained by pH-dependent release by liposomes [142,143]. It can be used to deliver nucleic acids into the cytosolic sensors or deliver antigens into cytosol to elicit processing for presentation on MHC-I/II system.

The release mechanism of pH-sensitive liposomes can be mainly in three ways. In acidic environment, pH-sensitive lipids destabilize liposome structure and consequently cause pore formation in endosome or cargo would leak into cytosol through endosomal membrane after destabilization of liposome structure. Lastly the fusion of liposome with endosome membrane to release its cargo into cytosol could be a third mechanism [143–145].

DOPE, which has a fusogenic property and is one of the pH-sensitive lipids, destabilizes the endosome in acidic pH. The structure of the lipid in acidic environment turns into hexagonal from bilayer and causes endosome break down [142]. It can be combined with cholesteryl hemisuccinate (CHEMS) without losing its pH sensitivity but it would be unresponsive to pH change when combined with phosphatidylglycerol (PG), phosphatidylserine (PS) or PC. In a study, DC-Chol: DOPE (1:1) was shown to be taken up cholesterol dependent macropinocytosis pathway and escaped from lysosomal compartment probably by destabilizing the lysosome [146].

CHEMS: DOPE is the most known pH-dependent liposome formulation however it is rapidly removed from the circulation by reticuloendothelial system (RES). Unfortunately, PEGylation that would longer the circulation duration was observed to reduce pH sensitivity of this liposomal formulation [147,148].

Oleic acid: DOPE and succinylated poly(glycol):PC or 3-methyl-glutarylated poly(glycidol):PC lipid combinations are other liposomal formulations that cause fusion of liposomes with endosomes in acidic environment [149]. DOPAQ, DODAT, DOMPAQ, DOMPAT, DOBAQ, DOBAT, DOAAQ and oleic acid are other

zwitterionic lipids that are anionic or neutral lipids at physiologic pH but turn into cationic lipid at lower pHs at which pH is lower than the pKa of lipid. Cholesterol, PC and PEG is combined with these liposomes to make them stable [150]. As mentioned, most of the pH sensitive liposome formulations have an instability problem in the body. However, improved blood circulation could be achieved by the incorporation of stealth character upon PEG incorporation into lipid moieties, except for CHEMS containing liposomes. The enhancement of liposome stability without losing pH sensitivity improves targeting efficiency of drugs at the tumor side due to decreased pH in the tumor micro-environment [151]. DOPE is a pH-sensitive lipid that has a fusogenic property and destabilizes the structure of liposome in an acidic environment by changing lipid bilayer into hexagonal structure. The structure change in liposome causes the breakdown of endosome membrane. In a study, DOPE addition to PLGA nanoparticles destabilized lysosomes and leaked into cytosol in a time dependent manner [142].

1.5 Immunostimulatory properties of liposomes

After couple of years that liposomes were discovered, Allison and Gregoriadis found the adjuvant property of liposomes. It was suggested in these years that incorporation of *Mycobacterium tuberculosis* or any products that known to have adjuvant property would enhance liposomes' potential use in vaccines [152]. Now, there are many immunogenic liposomes incorporating immunogenic lipids, or loaded or modified with adjuvants or antigens.

The investigations are now focused on the potential applications of modified liposomes conjugating or encapsulating antigens. There are many liposomal formulations in vaccine and therapeutic applications [153]. One of the immunogenic cationic liposomes, CAF01, is composed of glycolipid trehalose 6,6'-dibehenate (TDB), which is mycobacterial core factor, and dimethyldioctadecylammonium (DDA) [154,155]. Another immunostimulatory liposomes are Army Liposome Formulation (ALF) that contains monophosphoryl lipid A (MPLA) together with neutral and anionic phospholipids and Adjuvant System 01 (AS01) containing MPLA and saponin [156]. However some clinical studies are investigating their

adjuvant properties in human, they have still safety concerns and requires detailed investigation of immunogenic properties.

Adjuvant potential of nano-carriers are dependent on size, charge and administration site [157]. Physicochemical properties of liposomes such as composition and charge would also affect the immunostimulatory properties of adjuvant even though lipids themselves are non-immunogenic [158].

1.5.1 Liposome-loaded nucleic acids-based PRR agonists

According to Guideline on Adjuvants in Vaccines for Human Use by The European Medicines Agency (EMA), the meaning of adjuvant is described as: “a component that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses.”. It is addressed that more than one adjuvant could be included into vaccine harboring a single or multiple antigens in free form or conjugated with adjuvant. There are two liposome-based adjuvants employed in six approved or about to be approved products, while several liposomal adjuvants are still tested in the clinical phase trials for their potency [154].

Nucleic acid-based PRR ligands such CpG ODN are initiating strong innate immune activation, however, they have instability problems that are rapidly removed from the circulation due to the serum adsorption and nucleases. Liposomes increases the shelf-life, resistance to nucleases and long-term circulation in the body and also improves the adjuvant potential of CpG ODN [109,110]. Liposomal CpG ODNs have a wide range of applications such as anti-viral, anti-bacterial, and anti-cancer vaccine adjuvants or immunotherapeutic agents. In mucosal vaccine, cationic liposomes containing DOTAP and DC-Chol together with antigen induces Th2-type immune responses and elevates antigen-specific mucosal immunity with increased humoral response but fairly low IgA response. When K-type CpG ODN was loaded into cationic liposome, a Th-1 biased immune activity was elicited with high IgA production which is vital for mucosal defense [159]. In parasite vaccines, combination therapy with anti-leishmanial drug and liposome-encapsulated CpG

ODN 2006 was shown to inhibit parasite proliferation and decreased Th2-biased cytokine production such as IL-10 [160]. As a protective vaccine against *Burkholderia pseudomallei*, cationic liposome containing DOTAP encapsulating CpG ODN was used and was reported to achieve 100% protection for a duration of 30 days post-vaccination [161]. In the tumor treatment, liposome encapsulated CpG ODN demonstrated enhanced Th1-immune response and DC activation. Anti-tumor activity was based on T-cell and NK cell infiltration into tumor and decreased population of regulatory T cells (Treg) in tumor side [162]. For the late stage tumor treatment, combinational therapy with cisplatin and liposome-loaded CpG ODN was used in the treatment of melanoma. Therapy induced high levels of Th1-biased cytokine levels, apoptosis of tumor cells and CTL activity. However, full tumor eradication was not observed [163]. In a breast cancer vaccine, cationic liposome encapsulating p53 HER-2/neu derived peptide and CpG ODN elevated T-helper 1 and cytotoxic T-cell responses and inhibited tumor development after vaccination of animals [164].

As mentioned earlier sections, poly(I:C) is a synthetic analog of dsRNA sensed by TLR3. Although poly(I:CLC) is more stable than poly(I:C), it is not considered suitable and safer for clinical applications, primarily due to many undesirable side-effects such as activation of inflammasome and interferon pathways [165]. Liposomes are suitable carriers to entrap poly(I:C). Loading is also increased its immunostimulatory properties while sparing ligand usage. In a study, the injection of liposomal poly(I:C) to mice increased neutrophil migration into lymph nodes, upregulated expression of inflammatory and anti-viral genes in skin tissue and activated CTLs. More importantly, no side effects were demonstrated. Moreover, liposomal poly(I:C) reduced the local toxicity of free poly(I:C) [166]. In a study, cationic liposome-encapsulated poly(I:C) used as anti-tumor adjuvant. Data revealed that tumor growth was reduced mainly due to augmentation of tumor-specific CTLs and IFN- γ expression. In addition, in this study, liposomal poly(I:C) activated TLR3 signaling pathway more than free version possibly due to effective internalization [167]. In another study, cationic immunogenic liposome, CAF09 entrapping poly(I:C) was administered to mice. Intraperitoneal administration of CAF09

increased CD8⁺ T cells compared to other administration routes [168]. Like free version, the apoptotic effect of liposomal poly(I:C) was demonstrated in xenograft human gastric adenocarcinoma cells [169]. Hansen *et al.* also used CAF05 liposomes entrapping poly(I:C) in the anti-tumor treatment. This cationic liposomal formulation enhanced CD8⁺ T cell activity and decreased tumor size in melanoma and TC-1 tumor models [116].

STING ligands have just discovered and the effects on the immune system have begun to move to clinical practice. However, they have also similar limitations with CpG ODN and poly(I:C), namely, premature elimination upon administration. In addition, the cellular uptake of these CDNs with circular structures is very limited, as mentioned earlier. Therefore, *in vivo* use of CDN agonists could be promoted upon encapsulation within suitable carriers alleviating the need for high dose administration to obtain appreciable amounts of immune activation. There is only limited number of studies employing nano-carriers to enhance the immunostimulatory effects of CDNs. Generally, CDNs are being used in *in vitro* studies by complexing with transfection reagents. A study compared the immune response elicited from the delivered cGAMP by lipofectamine and cationic polyethylenimine/hyaluronic acid hydrogel. While delivery systems were significantly increased interferon secretion and uptake by macrophages, hydrogel entrapping was better than lipofectamine-cGAMP complex [170]. Drug delivery system to enhance the immunostimulatory of c-di-GMP was investigated in a study using PEGylated liposome. In that study, liposomes enhanced the lymphatic uptake of agonist without toxic effect, furthermore, APC activation, antigen- specific Th-1-biased immunity was attained even though the dose was reduced by 30-fold compared to free ligand administration. Interestingly, encapsulation altered the immunostimulatory property of c-di-GMP. Liposomal c-di-GMP increased TNF- α levels but the immune activity was independent of interferon production [124]. Another efficient cytosol delivery of c-di-GMP was tested upon loading the ligand into pH-sensitive liposome containing YSK05 lipid with high fusogenic property. Liposome loaded c-di-GMP increased redundant amount of IFN- β . It activated NK

cells, judged by elevated CD69 expression and provided anti-tumor effect on lung metastasis of B16-OVA cells by recruiting NK cells to the tumor site [126,127].

1.6 Outline and aim of the study

Nucleic acid-based PRR agonists are promising immunotherapeutic agents that activate innate immune system either sensed by endosomal TLRs or sensed by ER-resident cytosolic nucleic acid sensor STING. Upon nucleic acid ligand recognition, immune cells such as dendritic cells and macrophages are activated. They secrete interferons and inflammatory cytokines. Consequently, humoral and cellular immune cells become effector in an antigen-specific manner. Besides, multiple PRRs trigger synergistic immune response with the activation of different signaling pathways upon multiple ligands engagement mimicking pathogen invasion. Incorporation of these biological adjuvants either alone or in combination could enhance the vaccine potency when they are tested in human vaccines. However, clinical applications of nucleic acid-based ligands are significantly hampered either due to pre-mature *in vivo* degradation via nucleases or rapid clearance via serum protein adsorption. Liposome encapsulation, which is a safer and easy way of manufacturing depot drug delivery system proved to increase *in vivo* stability of nucleic acids, promoted the internalization of its cargo by relevant innate immune cells, and acted as a depot release system preventing premature wasting of the loaded cargo.

One of the main purposes of this study was dedicated to enhance synergistic immune response elicited by TLR9, TLR3 or STING agonists by co-encapsulating them within liposomes. In order to address this, we encapsulated CpG ODN and poly(I:C) into neutral liposome and in another study CpG ODN and CDNs (2'3'-cGAMP or c-di-GMP) were encapsulated within pH sensitive sterically stabilized PEGylated liposomes. The physicochemical characterization such as size, polydispersity and encapsulation efficiency (EE) using dynamic light scattering (DLS), atomic force microscopy (AFM) and spectrofluorometry of the liposomal delivery systems were carried out. Similarly, *in vitro* stimulation assays in culture was conducted to analyze the benefit of encapsulation over free ligand usage.

The immunostimulatory effects of encapsulated multiple agonists was tested in i) mouse splenocytes, ii) BM-DMs, iii) BM-DCs, and iv) human PBMCs. Interferon and pro-inflammatory cytokine productions were determined by enzyme-linked immunosorbent assay (ELISA) and type-I IFN reporter cell line. Up-regulation of co-stimulatory molecules (i.e. CD80, CD86, MHC-I and MHC-II) was analyzed by flow cytometry following staining of surface markers with corresponding fluoro-chrome-conjugated antibodies of immune cells.

The adjuvant potential of vaccines including liposome-loaded multiple PRR agonist and model antigen OVA was tested in two independent animal models against melanoma and T-cell thymoma.

In anti-cancer vaccine studies, C75BL/6 mice was i.p. immunized with neutral liposome co-encapsulating CpG ODN and/or poly(I:C) together with OVA. Anti-OVA specific immunoglobulins (IgG) subtypes were determined from mouse sera by ELISA. After 8th month of booster immunization, tumor challenge was performed by inoculating four millions of E.G7 thymoma cells expressing OVA into immunized mice. Tumor size was recorded by measuring with electronic caliper following 14 days. Cellular response was investigated in splenocytes of immunized mice harboring tumors by IFN- γ Enzyme-Linked ImmunoSpot (ELISPOT) at the end of the study.

In the treatment of established tumors, B16-OVA (1 million) cells were inoculated in the right flank of C57BL/6 mice. When tumor sizes reach ca. 50-75 mm³, mice was treated with free or co-encapsulated CpG ODN and cGAMP formulations. Following ten days, antigen specific IgGs was determined from sera of sacrificed mice. Cellular activation against antigen was determined by ELISA and ELISPOT assays. Infiltrated immune cells to tumor micro-niche, population of immune cells in splenocytes and lymph nodes of immunized mice were analyzed following staining cells with fluoro-chrome-conjugated antibodies by flow cytometry.

Finally, efficiency of vaccines composed of liposomal K and D-type CpG ODNs was investigated co-encapsulating anti-viral and anti-bacterial antigens or extracts against foot-and-mouth disease (FMD) virus or *helicobacter pylori*, respectively. According to the immunization schedules, antigen-specific IgG was performed from collected sera. In helicobacter vaccine, colony formation in stomach was determined after challenged with *H. pylori* cells.



Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Cytokine antibody pairs (monoclonal unlabeled and biotinylated antibodies listed in Table 2.1) and recombinant proteins (Table 2.2) for ELISA were purchased from Biolegend (USA), Mabtech (Sweden) and BD (USA).

Table 2.1. Monoclonal unlabeled and biotinylated antibodies used throughout the thesis studies.

Product	Catalog#	Clone	Company/Country
Anti-mouse IL-4	MM450C	1D11	Endogen/USA
Anti-mouse IL-6	504502	MP5-20F3	Biolegend/USA
Anti-mouse IL-10	504902	JES5-2A5	Biolegend/USA
Anti-mouse IL-12	505202	C15.6	Biolegend/USA
Anti-mouse IL-17	506902	TC11-18H10.1	Biolegend/USA
Anti-mouse IFN- γ	3321-3	AN18	Mabtech/Sweden
Anti-mouse TNF- α	510802	6B8	Biolegend/USA
Biotin anti-mouse IL-4	MM450DB	24G2	Endogen/USA

Biotin anti-mouse IL-6	504602	MP5-32C11	Biolegend/USA
Biotin anti-mouse IL-10	505004	JES5-16E3	Biolegend/USA
Biotin anti-mouse IL-12	505302	C17.8	Biolegend/USA
Biotin anti-mouse IL-17	507002	TC11-8H4	Biolegend/USA
Biotin anti-mouse IFN- γ	3321-6-250	R4-6A2	Mabtech/Sweden
Biotin anti-mouse TNF-	506312	MP6-XT22	Biolegend/USA
Anti-human IP-10	555046	4D5/A7/C5	BD/USA
Anti-human IFN- α	3425-1a-20	N/A	Mabtech/Sweden
Biotin anti-human IP-10	555048	6D4/D6/G2	BD/USA
Biotin anti-human IFN- α	3425-1a-20	N/A	Mabtech/Sweden

Streptavidin-alkaline phosphatases (SA-ALPs) were obtained from Mabtech (Sweden). Goat anti-IgG, IgG1, IgG2a, IgG2c, and IgA monoclonal antibodies conjugated with alkaline phosphatase (ALP) were purchased from Southern Biotech (USA) and used in IgG ELISA. Substrate for alkaline phosphatase, p-nitrophenyl phosphate disodium hexahydrate (PNPP), was purchased from Thermo Fisher Scientific (USA).

Table 2.2. Recombinant Cytokines/Growth factors used throughout the thesis studies.

Product	Catalog#	Company/Country
Mouse IL-4	RMIL4I	Endogen/USA
Mouse IL-6	575702	Biolegend/USA
Mouse IL-10	575804	Biolegend/USA
Mouse IL-12	573102	Biolegend/USA
Mouse IL-17	577802	Biolegend/USA
Mouse IFN- γ	3321-1A-20	Mabtech/Sweden
Mouse GM-CSF	RGMCSF10	Thermo/USA
Mouse TNF- α	575202	Biolegend/USA
Human IP-10	551130	BD/USA
Human IFN- α	3425-1a-20	Mabtech/Sweden

Fluorochrome-labeled antibodies (Table 2.3) were obtained from Tonbo biosciences or Biolegend (USA). B16-Blue™ IFN- α/β cells that allow the detection of bioactive murine type I IFNs were obtained from Invivogen (France).

Table 2.3. Fluorochrome-conjugated antibodies for staining cell surface molecules.

Product	Catalog #	Company/Country
Anti-mouse B220	103208	Biolegend/USA
Anti-mouse CD3	100206	Biolegend/USA
Anti-mouse CD4	100406	Biolegend/USA
Anti-mouse CD8	100711	Biolegend/USA
Anti-mouse CD11b	50-0112/ 101206	Tonbo/ Biolegend/USA
Anti-mouse CD11c	50-0114/ 117306	Tonbo/ Biolegend/USA
Anti-mouse CD19	115506	Biolegend/USA
Anti-mouse CD45	80-0451	Tonbo/USA
Anti-mouse CD69	104510	Biolegend/USA
Anti-mouse CD80	104706	Biolegend/USA
Anti-human CD83	305322	Biolegend/USA
Anti-mouse CD86	105016	Biolegend/USA
Anti-mouse CD206	141716	Biolegend/USA
Anti-mouse F4/80	123110	Biolegend/USA
Anti-mouse Gr1	108408	Biolegend/USA
Anti-human HLA-DR	307608	Biolegend/USA
Anti-mouse H2-Kd (MHC-I)	116607	Biolegend/USA
Anti-mouse I-A/I-E (MHC-II)	107608	Biolegend/USA
Anti-mouse NK1.1.	20-5941	Tonbo/USA
Anti-mouse TCR- β	109226	Biolegend/USA

2.1.2 Pattern-recognition receptor ligands and antigens

In *in vitro* and *in vivo* experiments, TLR and STING ligands (cyclic dinucleotides; CDNs) were used for stimulations. Lipopolysaccharides (isolated from *E.coli*; LPS; TLR4 ligand) were purchased from Sigma (USA) and poly(inosinic acid: cytidylic acid) (poly(I:C); TLR3 ligand) were obtained from Amersham (UK). Fluorescein-conjugated poly(I:C) was purchased from Invivogen (France). FITC- or Cy5-labeled or unlabeled CpG ODN ODNs (TLR9 ligands) were synthesized by Alpha DNA (Montreal, Canada) or was kind gift from Dr. Dennis M. Klinman (NCI/NIH, USA) and Dr. Ken J. Ishii (IFReC, Japan). Sequences used in the experiments were listed below. All ODNs were endotoxin free. Bases shown in capital letter have phosphorothioate linkage and those in lower case have phosphodiester linkages. CpG motifs are underlined.

D35 (20 mer): 5'-GGTgc⁺atcgatgcaggggGG-3'

K3 (20 mer): 5'-ATCGACTCTCGAGCGTTCTC-3'

1555 (15mer): 5'-GCTAGACGTTAGCGT-3'

The c-di-GMP and 2'3'-cGAMP were purchased from Invivogen (France) and 5-carboxyfluorescein (FAM) conjugated c-di-GMP and 2'3'-cGAMP were obtained from Biolog (Germany).

Chicken ovalbumin was obtained from Sigma (USA) and Imject OVA™ was from Thermo Scientific (USA). FMD antigen, serotype O, and commercial FMD vaccine were kind gift from FMD Institute (Ankara, Turkey). FMD vaccine was monovalent vaccine formulation that contains FMDV O/TUR/07 inactivated antigen in double oil emulsion with montanide ISA 206 (Seppic, France) *Helicobacter pylori* (SS1) and its crude extract was kind gift from Dr. Ayça Sayı Yazgan (ITU, MBG Dept. Istanbul, Turkey). In infection studies, bacteria were prepared and colony counting was done by her graduate students.

2.1.3 Phospholipids and lipids used to prepare liposomes

Cholesterol (Chol) was purchased from Sigma Aldrich (USA). L- α -phosphatidylcholine (PC), L- α -phosphatidylserine (PS), 3 β -[N-(N',N'-Dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (DC-Chol), 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine-N-[methoxy(Polyethylene glycol)-2000] (Ammonium Salt) (PEG-PE), 1,2-dioleoyl-sn glycerol-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (USA).

2.1.4 Cell culture media and standard buffer solutions

DNase/RNase free water, DPBS (Dulbecco's Phosphate-Buffered Saline, 1x), RPMI-1640 (Roswell Park Memorial Institute medium) and high glucose DMEM (Dulbecco's Modified Eagle Medium), Na-pyruvate, HEPES, L-glutamine, penicillin/streptomycin, non-essential amino acid solution, were from Gibco (USA). Fetal bovine serum (FBS) was obtained from Biowest (USA). Components of various culture media and different buffers were given in detail in Appendix A.

2.2 Methods

2.2.1 Mouse strains

Wild type adult male or female BALB/c, C57BL/6 mice and T-cell deficient athymic nude mice (6-12 weeks old) were housed in animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22 ± 2 °C) regulated with 12-hour light and 12-hour dark cycles. They were provided with unlimited access of 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 as well as isolating primary spleen cells, generating bone marrow-derived dendritic cells and macrophages for *in vitro* stimulations throughout this thesis.

2.2.2 Liposome preparation

Various phospholipid stocks were prepared in chloroform at 10-20 mg/ml stock solutions and stored at -20 °C until use as described earlier [110]. Lipids were mixed in different ratios (Table 2.4) in a round bottom flask to prepare liposomes via dehydration-rehydration method. Mixtures in chloroform were evaporated using rotary evaporator (ILMVAC, Germany). The solvent-free dry lipid film was purged with nitrogen to eliminate residual chloroform and oxygen, thereby preventing lipid peroxidation. Empty multi-lamellar vesicles (MLV) were generated by adding glass beads and 1 ml PBS into 20 μ mole dried lipid film and was swirled until all lipid layer is dissolved into solution. Sonication was performed to MLVs with ten cycles of 30 s ON and 10 s OFF at 4 °C using a Vibra Cell sonicator (Sonics and Materials, Danbury, CT, USA) to generate the small unilamellar vesicles (SUV).

SUV were mixed with different reagents ranging from nucleic acids to proteins alone or with their respective combinations that was intended for encapsulation. Then, mixture was snap-frozen in liquid nitrogen, and freeze-dried overnight (VirTis benchtop K, Germany). Encapsulation was achieved during rehydration step: i) sterile DNase/RNase free dH₂O (1/10 of total initial snap-frozen volume) was added to the dehydrated liposome powder and vigorously vortexed for 15 s every 5 min for total 30 min at room temperature (RT), ii) equal volume of PBS (4.5/10 of total volume) was added to the mixture at the end of 30 mins and incubated for 10 mins at RT to allow equilibration of the tonicity. iii) PBS (4.5/10 of total volume) was added to the mixture, generally yielding a final liposome concentration of 20 μ M lipid/mg encapsulated molecule(s). Liposome formulations were stored at 4 °C until for further use.

Table 2.4. Lipid composition (molar ratios) and charges of different liposome types.

Liposome types	Charges	Lipid Ratios					
		PC	Chol	PS	DC-Chol	DOPE	PEG-PE
Neutral	no	1	1	-	-	-	-
Anionic	-	1	-	0.25	-	0.5	-
Cationic	+	6	-	-	4	0.06	-
Stealth	no	-	4	-	-	6	0.06
SSCL	+	-	-	-	4	6	0.06

PC: phosphatidylcholine; Chol: cholesterol; PS: phosphatidylserine; DC-Chol: dimethylaminoethanecarbamol-cholesterol; DOPE: dioleoylphosphatidylethanolamine; PEG-PE: polyethylene glycol-phosphatidylethanolamine; SSCL: sterically stabilized cationic liposomes.

2.2.2.1 Liposome characterization

2.2.2.1.1 Size measurement

To analyze the size of liposome particles by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern, UK), liposomes (10 μ l, of 1 μ mol) were diluted in 1 ml PBS and triplicate readings of samples were recorded.

2.2.2.1.2 AFM

Liposomes were diluted in DNase/RNase free water (1/500) and were deposited on silicon wafer. Following complete drying under hood, images were taken by using AFM in non-contact mode (PSIA, XE-100E, Korea).

2.2.2.1.3 pH sensitivity analysis by calcein leakage

SSCLs were prepared with different composition to investigate their pH sensitivity with respect to the changes in DOPE amount (Table 2.5). 10 μ mol lipid film was

hydrated with 1 ml calcein solution (80 mM) [171]. Glass beads were used to generate multilamellar vesicles (MLV) as explained above (Section 2.2.2.). MLVs were sonicated for 30 s for 10 times to prepare SUVs and centrifuged at 16000 x g for 1 h to remove excess amount of calcein.

Liposomes (100 µL) were diluted 10x with 1x PBS (900 µl) at different pHs (4, 5, 6 and 7.4). They were incubated at 37 °C for 1 h. After incubation, 500 µl were collected from samples. 5 µl Triton X 100 (10%, v/v) was added to the remained solution (500 µl) and vortexed vigorously. Total calcein concentration was calculated from the Triton X-100 treated samples. Samples were analyzed in spectrofluorometer (ex: 495 nm; em: 515 nm; BioTek Synergy^{HT}, USA).

The calcein leakage was calculated using the equation below:

$$\% \text{ Calcein} = [(C_{\text{pH}} - C_0) / (C_{\text{total}} - C_0)] \times 100$$

C_0 = Concentration of calcein in the buffer (pH: 7.4)

C_{pH} = Concentration of calcein at different pHs

C_{total} = Concentration of calcein after Triton X-100 treatment

Table 2.5. Lipid composition (molar ratios) of SSCLs.

Liposome Type	Charges	Lipid Ratios		
		DC-Chol	DOPE	PEG-PE
SSCL	+	5	5	0.06
SSCL	+	4	6	0.06
SSCL	+	3	7	0.06

SSCL: sterically stabilized cationic liposome.

2.2.2.1.4 Encapsulation efficiency

Liposomes encapsulating FITC-labeled CpG ODN and/or fluorescein-labeled poly(I:C) or FAM-labeled cGAMP and/or cold OVA were centrifuged with 1x PBS

(pH: 7.4) at 16000 g for 1h. Fluorescent non-encapsulated ligands were detected using spectrofluorometer (excitation at: 495 nm; emission at: 515 nm; BioTek, Model: Synergy HT). Encapsulation efficiency (EE) was calculated using the equation below:

$$\% EE = (C_i - C_0) / (C_i) \times 100$$

C_i : initial amount; C_0 : non-encapsulated amount.

2.2.3 Cell culture

2.2.3.1 Splenocyte isolation

Splenocytes were isolated from surgically removed spleen of 8-12 wks. old female BALB/c or C57BL/6 mice. Single cell suspensions were prepared by mechanical dissociation of spleen using back of the sterile plunger of a 5 mL syringe. Cells were washed twice with 2% FBS containing complete RPMI-1640 (2% RPMI, hereafter) and centrifuged at 300 x g for 5 min. Pellet was re-suspended with 5% FBS containing complete RPMI-1640 (5% RPMI, hereafter). Cell counting was performed by hemocytometer or flow cytometry as explained in Section 2.2.3.6.

2.2.3.2 Bone marrow-derived dendritic cell (BM-DC) generation

Female C57BL/6 mice were euthanized by cervical dislocation and bones (femurs and tibias) were aseptically removed. After immersing bones into 70% EtOH for 2 min, both ends of each bone were cut with a scissor and bone marrow was isolated by flushing out with medium using a syringe (25G). Cells were washed twice with 2% RPMI and centrifuged at 300 x g for 5 min. Pellet was re-suspended with 10% RPMI [172]. The cell counting was performed by hemocytometer (Section 2.2.3.7.). The cell concentration was adjusted to 1×10^6 cell/ml and plated into petri dishes in 10% RPMI containing 20 ng/ml rm-GM-CSF and 10 ng/ml rm-IL-4. At days 3 and 6, half of the media was removed and replenished with fresh media including 20

ng/ml rm-GM-CSF and 10 ng/ml rm-IL-4. At day 8, cells were incubated with 50 mM sterile EDTA in 1x PBS to detach slightly attached immature BM-DCs, and then was centrifuged at 300x g for 5 min and immature BM-DCs were recovered as pellet. In addition, cell counting was performed by hemocytometer as explained in Section 2.2.3.7. CD11b and CD11c double positivity of cells were analyzed following staining of BM-DCs by flurochrome-conjugated mAbs and analyzed via flow cytometry.

2.2.3.3 Bone marrow-derived macrophage (BM-DM) preparation

As described in Section (2.2.3.2) cells were prepared from mouse bones, washed, centrifuged and counted by hemocytometer. The cell concentration was adjusted to 1×10^6 cell/ml and plated into sterile petri dishes supplemented with 30% (v/v) L-929 conditioned medium (L-929-CM, supernatant of confluent L-929 cells) [173]. After incubating cells for 6 days, they were collected by centrifugation and expression levels of F4/80 and CD11b of BM-DMs were analyzed following staining with flurochrome-conjugated respective mAbs and analyzed via Accuri C6 flow cytometry. Cell counting was performed by hemocytometer as explained in Section 2.2.3.7.

2.2.3.4 Human peripheral blood mononuclear cell (hPBMC) isolation

Human peripheral blood mononuclear cells (hPBMCs) were collected from healthy donors in EDTA containing vacutainers and purified using Ficoll-Paque, density gradient centrifugation [40]. Briefly, lymphocyte separation medium (10 ml, Lonza) was added onto collected blood (15 ml) and centrifuged at 600x g for 30 min at RT in the break off setting. After centrifugation, phase separation occurs and interphase layer (cloudy ring) was transferred into a new falcon tube by using a sterile Pasteur pipette and washed twice with 2% RPMI and centrifuged at 300x g for 10 min. After washing, pellet was re-suspended with 10% RPMI and counted by hemocytometer or by flow cytometry as explained in Section 2.2.3.7.

2.2.3.5 Cell lines

2.2.3.5.1 B16-OVA

B16-OVA cells are derived from B16-F10 (ATCC® CRL-6475™) cells, a mouse melanoma cell line. B16-OVA were stably transfected with plasmids that carry a copy of chicken OVA. Cells were cultured in 10% RPMI media. Media was refreshed in every 2-3 days. When cells were reached around 80% confluency, cells were passaged 1:10 ratio following trypsinization.

2.2.3.5.2 B16-Blue IFN- α/β

B16-Blue IFN- α/β cell line was purchased from Invivogen (France). It is stably transfected with a plasmid containing SEAP reporter gene under the control of the IFN- α/β inducible ISG54 promoter. The SEAP activity was determined via PNPP or Quanti Blue to determine IFN- α/β levels in supernatants of cells. Cells were cultured in 10% RPMI media containing 100 $\mu\text{g/ml}$ Normocin and 100 $\mu\text{g/ml}$ Zeocin. Media was refreshed in every 2-3 days. When cells were reached around 80% confluency, cells were passaged 1:10 ratio following trypsinization.

2.2.3.5.3 E.G7-OVA

E.G7-OVA are derived from EL4 (ATCC, CRL-2113™) cells, a mouse lymphoma cell line. E.G7 was stably transfected with a plasmid (pAc-neo-OVA) that carry a copy of chicken OVA and neomycin resistance gene. Cells were cultured in 10% RPMI media supplemented with 1 mg/ml G-418 (Hyclone, USA). When cells were reached around 80% confluency, cells were passaged 1:5 ratio.

2.2.3.5.4 L-929

L-929 (NCTC clone 929 [L cell, L-929, derivative of Strain L] (ATCC® CCL-1™)) is a mouse fibroblast cell line. It is cultured with 10% DMEM medium. Media was

refreshed in every 2-3 days. When cells were reached around 80% confluency, cells were passaged 1:5 ratio following trypsinization.

2.2.3.5.5 RAW 264.7

RAW 264.7 (ATCC® TIB-71™) cell line is a murine monocyte/macrophage. It is cultured with 10% RPMI medium. Media was refreshed in every 2-3 days. When cells were reached around 80% confluency, cells were passaged 1:5 ratio by gently scraping.

2.2.3.6 Cell counting and layering

After centrifugation and precipitation of cells, pellets were re-suspended in 1 ml of their respective culture medium. In cell counting by hemocytometer, cells were diluted 50 fold with media (20 μ l from cell suspension into 980 μ l media) and 2 fold with trypan blue (10 μ l from 50x diluted cell suspension into 10 μ l trypan blue) to discriminate dead cells during counting on hemocytometer. 10 μ l of diluted cell suspension was added onto hemocytometer and cells in red areas (4x4) were counted under the light microscope (Figure 2.1).

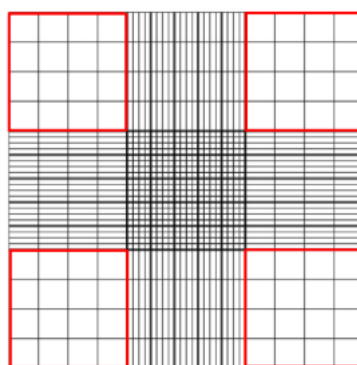


Figure 2.1. Representative grids of hemocytometer observed under the light microscope.

Volumes of the grids were 0.1 mm^3 and total cell number was calculated according to the formula below:

$$\text{Total cell \#} = (\text{all counted cell \# in red grids}/4) \times 10^4 \times 10^2 (\text{dilution factor}) \times 1 (\text{ml})$$

In cell counting using flow cytometer (BD Accuri™ C6, San Jose, USA), cells were diluted by adding 20 μl cell into 10 ml Isoton II Diluent buffer. 20 μl from the total volume was counted and event number in the live cell gate excluding the apoptotic cells and cell debris according to SSC and FSC alignment of cells and then were multiplied by dilution factors (x25 000) to calculate Cell #/mL.

For cytokine ELISA and surface marker experiments via flow cytometry, $2 \times 10^6/\text{ml}$ splenocytes, $1 \times 10^6/\text{ml}$ PBMC, $1.25 \times 10^6/\text{ml}$ PEC, $0.5 \times 10^6/\text{ml}$ RAW or BM-DMs and $0.5 \times 10^6/\text{ml}$ BM-DCs were plated into 96-well plate or 15 ml falcons in 200-250 μl or 1 ml 5% RPMI media. For gene expression experiments, 10×10^6 splenocytes and 2×10^6 BM-DCs were seeded in 15 ml falcon or in 12-well plates, respectively, in 1 ml 5% RPMI media.

2.2.4 Stimulation of immune cells with free or liposome loaded ligands

In stimulation assays, cells (splenocytes, RAW, BM-DC, BM-DM and PBMCs) were incubated with different concentrations of free and liposome loaded TLR3 (poly(I:C); 0-100 $\mu\text{g}/\text{ml}$), TLR4 (LPS; 1-10 $\mu\text{g}/\text{ml}$), TLR7 ligand (R848; 1-10 $\mu\text{g}/\text{ml}$) STING (2'3' cGAMP and c-di-GMP; 0-16 μM) and TLR9 ligands (1555, D35, K3; 0.1-3 μM) for 24-36h at 37 °C in CO₂ incubator. In dual ligand stimulations, free or separately or co-encapsulated D35 plus poly(I:C) at 1:1 (wt ratios) combination and or K3 plus c-di-GMP/ 2'3' cGAMP at 1:3 or 1:9 (μM ratios) combinations were used.

2.2.5 Cell viability assay

After stimulation of splenocytes or BM-DMs, supernatants were collected to new 96 well-plates. Cell Counting Kit-8 (CCK-8; Dojindo, Japan) solution was prepared by mixing with complete medium at 1: 10 (v:v) ratio. CCK-8 solution (100 $\mu\text{l}/\text{well}$) was

added on stimulated cells. Cells were incubated at 37 °C for several hours (1-4). Then, ODs were detected by microplate ELISA reader at 450 nm (μQuant, BioTek).

2.2.6 Uptake and binding of ligands and liposomes by immune cells

In vitro uptake and binding of free or liposome loaded fluorescein-labeled poly(I:C) (2 μg/ml), FITC-labeled 2'3' cGAMP (3 μM), Cy5- or FITC-labeled D35 (2 μg/ml), Cy5- or FITC-labeled K3 ligands (1 μM) were analyzed via flow cytometry in a dose and time dependent manner. Uptake of molecules was assessed by quenching fluorescence of molecules on cell membrane via trypan blue.

2.2.7 *In vivo* experiments

2.2.7.1 Immunization protocols

2.2.7.1.1 Prophylactic immunization

In prophylactic immunization, C57BL/6 mice (female, 6-8 month-old, n=8) were injected intraperitoneally (i.p.) with free or liposome encapsulated CpG ODN (D35) or poly(I:C) (10 μg each/animal) or OVA (5 μg/animal), or their 1:1 combinations. After two weeks of first injection, tail vein primary bleedings were conducted and collected blood samples were kept in glass containers. Blood was incubated at 37 °C for 1-2h. Sera in the supernatant was collected into 1.5 ml Eppendorf and centrifuged at 8 000 rpm for 5 min at RT. Sera were collected into new 96-well plate and stored at -20 °C until use. Next day, booster injections were performed to animals. IgG subtypes were detected by ELISA method from collected sera samples (Section 2.2.8.2). After 8th month, mice were challenged with E.G7 thymoma cells (Section 2.2.7.2) and following 15 days mice were euthanized by cervical dislocation. IFN-γ ELISPOT was performed using splenocytes of immunized mice (Section 2.2.9.).

2.2.7.1.2 *Helicobacter pylori* vaccination

H. pylori (a kind gift by Assoc. Prof. Dr. Ayca Sayi Yazgan, ITU-Istanbul, Turkey) was grown on solid medium on horse blood agar containing 4% Columbia agar base (Oxoid; Basingstoke), 5% defibrinated horse blood (HemoStatLabs), 0.2% β -cyclodextrin, 5 μ g/ml trimethoprim, 8 μ g/ml amphotericin B, 10 μ g/ml vancomycin, 5 μ g/ml cefsulodin, and 2.5 U/ml polymyxin B sulfate (all from Sigma-Aldrich) at 37°C for 2 days under microaerophilic conditions. Bacteria extracts were prepared with sonication.

In *Helicobacter pylori* vaccination, C57BL/6 mice (female, 6-8 month-old, n=5) were injected i.p. with 15 μ g/mice free or liposome loaded TLR3 (poly(I:C)) or TLR9 (K-ODN: 1555 or D-ODN: D35) ligands together with *Helicobacter pylori* SS1 extract (15 μ g/mice) in total 100 μ l sterile 1xPBS. Formulations were administered a week intervals three times. Blood samples were collected from their tail veins before each injection and sera was isolated as explained above. IgG subtypes were detected by ELISA method from collected sera of each animal (Section 2.2.8.2).

After 28 days, mice were challenged with *Helicobacter pylori* SS1 strain. Infections were performed by feeding mice with oral gavage in 100 μ l of suspension of bacteria. 15 days following challenge, mice were euthanized by cervical dislocation. IFN- γ and IL-17 ELISPOT was performed using splenocytes of immunized mice (Section 2.2.8). Bacterial colonization was assessed by colony count assay. Briefly, one-sixth of each stomach was transferred to a tube containing Brucella broth and homogenized with an homogenizer. Serial dilutions were plated on horse blood plates to determine bacterial loads.

2.2.7.1.3 FMD vaccination

In FMD vaccination, BALB/c (male, 6-8 month-old, n=5) was i.p. injected twice with two week intervals with 5 μ g/mice free or liposome loaded D35 or K3 together with 0.5 μ g/mice FMD serotype O antigen in total 100 μ l volume. Commercial FMD

vaccines (0.5 and 3 $\mu\text{g}/\text{mice}$) were injected to mice as positive controls. Bleedings were performed each month after first injection for a period of six months. Sera was prepared as explained previously and used in IgG ELISA studies (Section 2.2.8.2.).

2.2.7.2 Thymoma tumor challenge

E.G7 thymoma cells were cultured in 10% RPMI supplemented with 1 mg/ml G-418 as explained in Section 2.2.3.6.3. Mycoplasma negative E.G7 cells were harvested and prepared for challenge experiment when they were 60% confluent. After 8th month of booster injection of CpG ODN or poly(I:C) (10 μg each/animal) or OVA (5 $\mu\text{g}/\text{animal}$), or their 1:1 combination, animals were subcutaneously (s.c.) inoculated with 4×10^6 E.G7 thymoma cells expressing OVA (100 $\mu\text{l}/\text{mice}$ in 1x PBS) to right flank of mice (female, 6-8 month old, n=8). Daily tumor development was measured by an electronic caliper. Tumor size was calculated by formula: (length) x (width) x (height) and recorded for 14 days and represented as mm^3 . Then animals were euthanized by cervical dislocation. Splenocytes were isolated and IFN- γ -producing CD8⁺ T-cells were determined by ELISPOT assay (Section 2.2.9.).

2.2.7.3 Established melanoma model

B16-OVA ($1 \times 10^6/\text{mice}$) cells were s.c. injected to right flank of C57BL/6 mice (female, 6-8 month-old, n=5). Tumor size were measured via an electronic caliper and recorded each day. Tumor volume was calculated by the formula used in section 2.2.6.2. They were treated with free or co-encapsulated K3 (5 $\mu\text{g}/\text{dose}/\text{mouse}$) and cGAMP (1.7 $\mu\text{g}/\text{dose}/\text{mouse}$) plus OVA (5 $\mu\text{g}/\text{dose}/\text{mouse}$) on days 7 and 9 (single doses of each molecules) and 12 and 14 (double doses of each molecules) administered intraperitoneally.

At the end of the experiment, blood was collected from tail vein of mice and IgG subtypes were analyzed by ELISA (section 2.2.7.2.) from collected sera with proper titrations of each sample. Tumors and spleens were weighed and photographed. Single cell isolated from tumor by incubating with liberase (Sigma) were stained with flurochrome-labeled mAbs to determine percentages of infiltrating cells as

explained in flow cytometer section (Section 2.2.9.). Splenocytes were incubated with OVA or SIINFEKL to detect antigen-specific IFN- γ production by cytokine ELISA (Section 2.2.7.1.) or ELISPOT (Section 2.2.8.), respectively.

2.2.8 Enzyme linked immunosorbent assay (ELISA)

2.2.8.1 Cytokine ELISA and SEAP reporter assay

After stimulation assays as explained in Section 2.2.4., supernatants were collected and transferred into new plate after centrifugation of cells in 96-well plate at 300x g for 5 min. High binding polysorb (Nunc, Denmark) or Immulon 2 HB (Thermo Scientific, USA) plates were coated with anti-cytokine antibodies (50 μ l/well) for 4h at RT or overnight (o/n) at +4 °C and then blocked with 200 μ l blocking buffer (Appendix A) for 2 h at RT or at +4 °C for o/n. Wells were washed with wash buffer (Appendix A) for 5 times for 5 min intervals and rinsed with ddH₂O. Samples and 2-fold serially diluted recombinant proteins as standards were added into wells (50 μ l/well) and incubated for 4 h at RT or o/n at +4 °C. Cytokine amount was detected incubating with biotinylated anti-cytokine Abs (1:2000 diluted in T-cell buffer; 50 μ l/well) for 2 h followed by phosphatase-streptavidin (1:3000 diluted in T-cell buffer; 50 μ l/well) for 1h. At the end of each step, plates were washed as previously indicated. Finally, p-nitrophenyl phosphate (PNPP, Thermo) substrates were added into wells (50 μ l/well) and OD values were detected by ELISA plate reader at 405 nm (BioTek, μ Quant). According to the standard curve, concentration of secreted cytokines was calculated.

For detection of murine type I IFNs, B16-Blue™ IFN- α/β cell line was used. B16-Blue IFN- α/β cells ($7-8 \times 10^4$ /well in 180 μ l) were plated into 96-well plate and either directly stimulated with ligands or incubated with supernatants (20 μ l) collected from stimulated cells. After 24 h, supernatants were (20 μ l/well) incubated with PNPP (50 μ l/well) in new 96-well plate. Likewise in cytokine ELISA protocol, OD values were detected by microplate ELISA reader at 405 nm (μ Quant, BioTek).

2.2.8.2 IgG and IgA ELISA

Immulon 1B microtitre plates (Thermo Scientific, USA) were coated with 50 µl/well specific immunized protein (7.5-10 µg/ml OVA) or extracts (5 µg/ml *Helicobacter pylori* SS1) and were incubated for o/n at +4 °C and then blocked with 200 µl blocking buffer for 2 h at RT. Serially diluted sera collected from tail vein of naive and immunized mice were added to plates for o/n at 4 °C. Bound immunoglobulin subclasses (IgG, IgG1, IgG2a, IgG2c and IgA) were detected using 1:3000 diluted streptavidin-conjugated anti-immunoglobulin antibodies (Southern Biotech, USA) in blocking buffer for 3 h at RT and following PNPP addition (50 µl/well). Washing steps were same as cytokine ELISA (Section 2.2.8.1). OD values were detected by ELISA plate reader at 405 nm (BioTek, µQuant).

In FMD specific IgG ELISA, Immulon 1B plates were coated with 1:2000 diluted Rb anti-mSerO Ab (50 µl/well) and incubating for 4h at RT or o/n at +4 °C. Blocking and washing steps were same as indicated above. 1:20 diluted FMD serotype O in blocking buffer was added into wells and incubated at RT for 2h. Sera were diluted in 1:500 Rb serum and incubated o/n at +4 °C. FMD specific IgGs were detected by incubating with 1:3000 streptavidin-conjugated anti-immunoglobulin antibodies and PNPP, as explained above.

2.2.9 ELISPOT

Anti-IFN-γ (2 µg/ml, Mabtech) and IL-17 mAb (4 µg/ml, Biolegend) coated (50 µl/well) Immulon 2 HB plates were blocked with blocking buffer same as done for cytokine ELISA plates and were kept in a sterile environment until use. Splenocytes were 4-fold serially diluted starting from 1×10^6 cells/well and seeded to pre-coated Immulon 2 HB plates. Cells were incubated with 50 µl/well OVA (10 µg/ml), SIINFEKL (257-264 peptide, Anaspec Inc., San Jose, CA; 7.5 µg/ml), *Helicobacter pylori* SS1 extract (5 µg/ml) for 14-18 h at 37 °C in a 5% CO₂ incubator. Cells secreting cytokines were detected using biotinylated anti-cytokine mAbs followed by phosphatase-streptavidin incubation step, as described in cytokine ELISA section

(Section 2.2.8.1). However, wash buffer used in first washing step was prepared using only ddH₂O and Tween-20 to remove cells from the assay plates. Then, all washing steps were same as explained in cytokine ELISA section. Low melting agarose (Prona, Reducta, Poland)/ BCIP (1/4, v/v) solutions (70 µl/well) were added after SA-ALP addition. Spots were counted under binocular stereo microscope next day.

2.2.10 Flow cytometry analysis

2.2.10.1 Staining of cell surface molecules

Cells were fixed via Fix & Perm Medium A (Invitrogen, USA) or in some assays studied alive. They were resuspended with 100 µl FACS buffer (Appendix A) containing 1 µg/ml of flurochrome-conjugated antibodies (Table 2.3) and incubated for 30 min either at RT or at 4 °C in dark. When tumor cells were stained, cells were first Fc blocked by incubating them with TruStain fcX™ (anti-mouse CD16/32) for 5 min prior to staining. In *in vivo* experiments, cells were stained with Zombie Aqua Fixable Viability Kit (Biolegend). Then, cells were washed twice with 2 ml FACS buffer and centrifuged at 300x g for 10 min. Stained cells were re-suspended in 1x PBS and analyzed on BD Accuri C6 or Beckman CytoFLEX flow cytometer.

2.2.11 Determination of gene expression

2.2.11.1 Total RNA isolation

Cells (2.5-10 X 10⁶) were centrifuged at 300x g for 10 min at 4 °C and supernatants were discarded without disrupting the cell pellet. Cells were lysed with 1 ml TRIzol (phenol and guanidinium thiocyanate solution; Life Technologies) by gently pipetting until clear pink solution is visible. The lysates could either be stored at -80 °C for a week or total RNA could be isolated immediately. 200 µl of chloroform was added onto cell lysates. Tubes were shaken vigorously for 15 s and incubated at RT for 3 min. Then, samples were centrifuged at 16000 x g for 17 min at 4 °C. Approximately 500-600 µl of the clear upper aqueous phase of centrifuged solution

was collected to a clean Eppendorf and 500 µl of isopropanol was mixed gently. Samples were incubated for 10 min at RT and then centrifuged 16000x g for 10 min. the supernatants were discarded without distrusting the pellet. Pellets were washed twice, first with 1 ml of 75% EtOH and then with 1ml >99.9% EtOH. Centrifugation was performed at 9600xg for 7 min in the each washing step. Pellet was left to air-dry in tilted position under laminar flow cabinet. Purified total RNA were dissolved according to the pellet size with RNase/DNase free ddH₂O (~20-30 µl). DNA, protein, polysaccharides and phenol contamination was checked via measuring OD values of samples by NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 260 and 280 nm. Total RNA samples should be around 1.8/2 ratio at A260/280. RNA samples were then stored at -80°C until further use.

2.2.11.2 cDNA synthesis

cDNAs were synthesized from total RNA samples by cDNA synthesis kit (NEB, USA) as per manufacturers' protocol. Briefly, 1 µg total RNA was mixed with 2 µl of Oligo(dT) and total volume was completed to 8 µl with RNase/DNase free H₂O. Samples were pre-denatured at 70 °C for 5 min in an MJ Mini thermocycler (BIO-RAD, USA). After spinning down, 10 µl reaction mix (buffer containing dNTP mix and MgCl₂) and 2 µl enzyme mix (RNase inhibitor plus reverse transcriptase) were added to each sample. Total volume of the tubes were brought to 20 µl by the addition of ddH₂O and tubes were incubated at 42 °C for 60 min and at 80 °C for 5 min. cDNA samples were stored at -20°C for further use.

2.2.11.3 Semi-quantitative RT-PCR

PCR mixtures were prepared according to the protocol in Table 2.6.

Table 2.6. RT-PCR mixtures.

Ingredients	Volume
OneTaq Quick-Load 2X Master Mix with Standard Buffer (NEB)	12.5 μ l
DNase/RNase free H ₂ O	9.5 μ l
Forward primer	1 μ l (10 pmole)
Reverse primer	1 μ l (10 pole)
cDNA	1 μ l
Total	25 μl

For each gene, specific primer pairs were used (Table 2.7). According to the PCR conditions listed in Table 2.8, PCRs were set and products were kept at 4 °C until they were loaded onto 1.5-2% agarose gel. Intensities of genes were calculated using ImageJ program (NIH) and they were normalized according to the intensities of the house-keeping gene (β -actin).

Table 2.7. Primer pairs of cytokines and chemokines.

Primers		Sequences	Product size
m β -actin	Forward	5'-GTATGCCTCGGTCTGTACCA-3'	450 bp
	Reverse	5'-CTTCTGCATCCTGTCAGCAA-3'	
mIL-1 β	Forward	5'-GCTGCTTCCAAACCTTTGAC-3'	481 bp
	Reverse	5'-GGCCACAGGTATTTTGTCGT-3'	
mIL-15	Forward	5'-CATCCATCTCGTGCTACTTGTGTT-3'	127 bp
	Reverse	5'-CATCTATCCAGTTGGCCTCTGT-3'	
mIP-10	Forward	5'-GCCGTCATTTTCTGCCTCAT-3'	127 bp
	Reverse	5'-GCTTCCCTATGGCCCTCATT-3'	
mMx1	Forward	5'-GTGCCCTATCAAGGGAATGA-3'	485 bp
	Reverse	5'-TTGTAAGGGTGGAAACCCAAG-3'	
mTLR3	Forward	5'-GGGGCTGTCTCACCTCCAC-3'	250 bp
	Reverse	5'-GCGGGCCCCGAAAACATCCTT-3'	
mTLR9	Forward	5'-GATGCCCACCGCTCCCGCTATGT-3'	430 bp
	Reverse	5'-TGGGGTGGAGGGGCAGAGAATGAA-3'	
mTNF- α	Forward	5'-CCACCACGCTCTTCTGTCTAC-3'	189 bp
	Reverse	5'-ACTCCAGCTGCTCCTCCACT-3'	

Table 2.8. RT-PCR conditions.

PCR Step	m β -actin/ IL-15/ IP-10/ TLR3/ TLR9/	mTNF- α / IL-1 β / Mx1
Denaturation	94 °C ; 30''	94°C ; 30''
Annealing	55 °C ; 30''	60°C ; 30''
Extension	72 °C ; 1'	72°C ; 30''
Number of cycles	34	40
Final extension	72 °C ; 10'	72°C ; 5'

2.2.12 Statistical Analysis

Statistical significant differences between groups were determined using one-way or two-way ANOVA following Tukey's or Bonferroni post-hoc comparison and Student's t-test analysis using GraphPad Prism software (version 5, San Diego, CA, USA). $P < 0.05$ were considered as significant.

Chapter 3

Results

3.1 Physicochemical properties and immunostimulatory effects of liposomes

3.1.1 Size distribution analysis of AFM in dry state

Liposomes were prepared following the protocol explained in Section 2.2.2. The size characterization of neutral and SSCL liposomes in dry state were performed using AFM and in aqueous dispersed state were performed by DLS analysis.

The AFM studies revealed that the size of neutral liposome was larger than SSCL while their spherical shapes were comparably similar (Figure 3.1.).

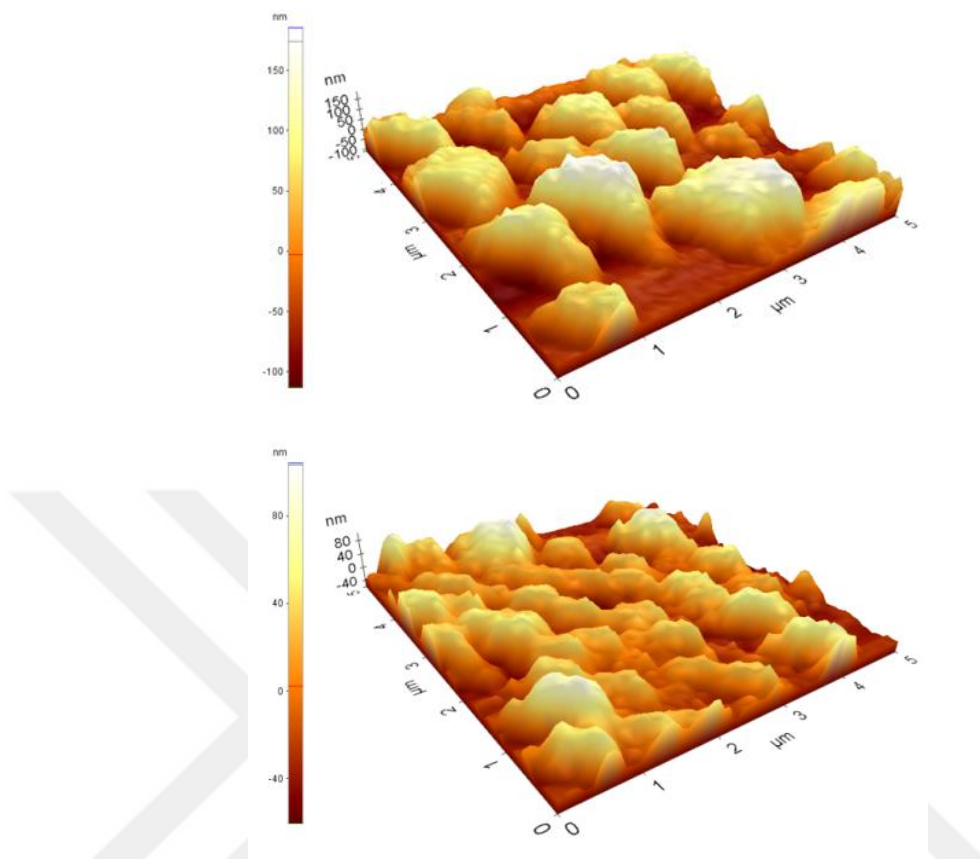


Figure 3.1. AFM micrographs of empty/ unloaded neutral (upper panel) and SSCL (lower panel) liposomes.

3.1.2 Size and polydispersity index analyses in aqueous state by DLS

Encapsulation of TLR ligands within liposomes did not significantly change the size of the neutral liposomes; however, in SSCL, loaded liposomes (401 ± 14 nm) were notably higher than unloaded liposomes (e.g. K3 loaded; 754 ± 45 nm) (Table 3.1). PDI indicates the homogeneity of particles. When PDI value increases it means that polydispersity or heterogeneity increases. $PDI < 0.7$ is accepted as particles being relatively monodispersed. PDI of empty neutral liposomes was 0.824 and decreased

by loading TLR ligands into neutral liposome to around 0.600. When compared to empty neutral liposomes (PDI=0.824), SSCL was more monodisperse considering their low PDI value (PDI=0.262). Parallel to neutral liposomes, the PDI value of SSCL further decreased when ligand loaded liposome size distribution was measured, implying that the PDI of both liposome types reached near to monodispersed upon ligand loading.

Table 3.1. Size (mean $\pm$ SD) and PDI of empty and loaded liposomes

	Size (nm)	PDI
Empty Neutral L.	638 $\pm$ 30	0.824 $\pm$ 0.086
(CpG ODN)Neutral L.	794 $\pm$ 115	0.676 $\pm$ 0.039
(poly(I:C)) Neutral L.	489 $\pm$ 47	0.605 $\pm$ 0.047
(CpG ODN+poly(I:C) Neutral L.	681 $\pm$ 35	0.688 $\pm$ 0.065
(CpG ODN+poly(I:C+OVA) Neutral L.	492 $\pm$ 84	0.638 $\pm$ 0.055
Empty SSCL	401 $\pm$ 14	0.262 $\pm$ 0.008
(K3)SSCL	754 $\pm$ 45	0.148 $\pm$ 0.016
(cGAMP)SSCL	636 $\pm$ 43	0.036 $\pm$ 0.015
(K3+cGAMP)SSCL	674 $\pm$ 74	0.209 $\pm$ 0.011

3.1.3 Assessment of pH sensitivity of sterically stabilized cationic liposomes

We planned to prepare pH-sensitive liposomes that would disrupt its lipid bilayer at lower pH following internalization within immune cells and release its cargo when trafficked to late endosome thereby leak the encapsulated cytosolic ligand into cytosol and thus allow the interaction of cytosolic sensor with the ligand. To find out a proper lipid combination that will be stable during cellular uptake while its structure destabilizes at lower pH (similar to that of late endosome) and secrete out its cargo, we prepared SSCL with different DOPE lipid ratios. DOPE has a fusogenic property and destabilizes the endosome in acidic pH [145]. The structure of the lipid in acidic environment turns into hexagonal from bilayer, causes endosome break

down. Calcein is a fluorescent dye that has a self-quenching property at high concentrations. It was entrapped into SSCL by hydrating lipid films with calcein solution during liposome preparation. Then, liposomes were suspended in 1x PBS at different pH (4-7) and incubated at 37 °C for 1 h. Rate and degree of calcein leakage in solution having different pH was detected using spectrofluorometer and will indicate the pH sensitivity character of each tested liposome preparation.

SSCLs with different DOPE lipid ratios were all pH-sensitive (Figure 3.2). Their structure was destabilized and leaked calcein. This leakage was faster when pH decreased. Neutral liposomes that contain Chol and PC were used as negative control. Calcein leakage was similar at all pHs in neutral liposomes, while SSCLs leaked calcein in pH-dependent manner. It was significantly correlated with the increase in the DOPE content presented in the liposome formulation. Liposome containing the highest DOPE lipid ratio (3:7:0.06) demonstrated the highest calcein leakage in a pH-dependent manner ($p < 0.001$, two-way ANOVA, Bonferroni post hoc test). As a conclusion, we selected SSCL containing highest DOPE ratio (3:7:0.06) to entrap K3-a ligand interacting with the endosome membrane associated TLR9 and cGAMP-a ligand interacting with the cytosolic STING sensor, for further experiments. We hypothesized that it would be stable at neutral pHs ($5.6 \pm 4.6\%$ calcein leakage at pH 7) while it would release its cargo at acidic environment due to destabilization of its structure (53.2 ± 10.2 at pH 4).

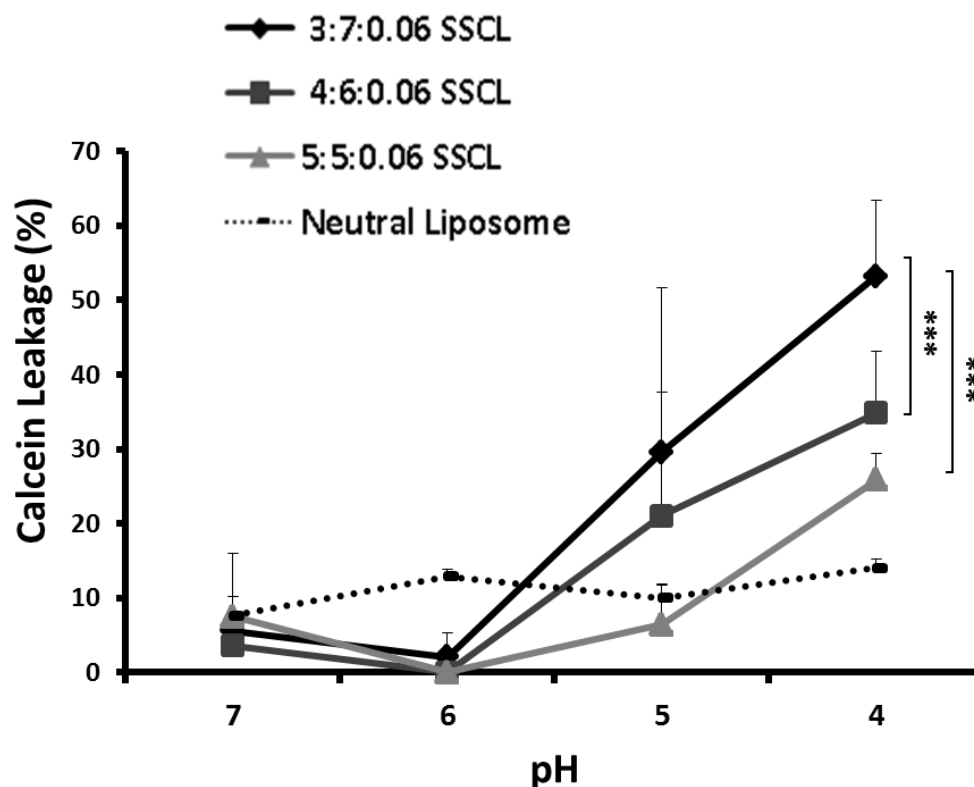


Figure 3.2. Calcein leakage from SSCL containing different lipid molar ratio of DOPE and neutral liposome at different pHs.

Calcein, a fluorescent dye that has a self-quenching property at high concentrations was entrapped into liposomes by hydrating lipid films with calcein solution. pH dependent release mechanism of liposomes were investigated incubating them in 1 X PBS at different pHs: 7.0 – 4.0 in a shaker (100 rpm, 37 °C) for 1 h. Dequenching of calcein was detected via spectrofluorometry (ex: 495 nm, em: 515 nm). The calcein leakage percentage was calculated as $[(CpH-C0) / (C100-C0)] \times 100$. C0 = concentration of calcein in the buffer before incubation. CpH = concentration of calcein at different pHs after incubation. C100 = total concentration of calcein after Triton X-100 treatment of samples. Statistical significance was determined by two-way ANOVA followed by Bonferroni (HSD) test. ***p<0.001; ns: non-significant.

3.1.4 Encapsulation efficiency of ligands loaded into liposomes

The encapsulation efficiency of liposomes was analyzed as described in Section 2.2.2.1.4 (Table 3.2). When we compare the CpG ODN encapsulation efficiency of liposomes, all neutral liposomes encapsulating CpG ODN and/or poly(I:C) and/or OVA was similar and approximately loaded with ~70% CpG ODN. Surprisingly, poly(I:C) encapsulation efficiency significantly increased when poly(I:C) was loaded

within liposome together with CpG ODN either with OVA or alone. K3 encapsulation was similar in (K3)SSCL and (K3+cGAMP)SSCL forms.

Table 3.2. Encapsulation efficiency of CpG ODN and poly(I:C) or cGAMP in liposomes loaded with single, dual or triple molecules.

	CpG ODN	poly(I:C)/cGAMP
(CpG ODN)Neutral L.	74±9	-
(polyI:C) Neutral L.	-	61±3
(CpG ODN+poly(I:C) Neutral L.	73±11	89±3
(CpG ODN+poly(I:C)+OVA) Neutral L.	73±2	82±6
(K3)SSCL	81.8±0.3	-
(K3+cGAMP)SSCL	85.6±0.4	-

3.2 Co-encapsulation of PRR ligands within liposomes

3.2.1 Synergism between TLR3 and TLR9 ligands

Our main aim was to combine and enhance the synergistic immunostimulatory activity of the nucleic acid-based TLR3 (poly(I:C)) and TLR9 (CpG ODN) ligands known to trigger MyD88 and TRIF dependent signaling cascades. To achieve this goal, we co-encapsulated them in neutral liposomes thereby achieving simultaneous ligand delivery to innate immune cells. Before testing this hypothesis, we investigated the dose-dependent synergistic/antagonistic immune response that was elicited by simultaneous administration of poly(I:C) and CpG ODN in non-encapsulated forms. Mouse splenocytes were stimulated with either single poly(I:C) and CpG ODN or combination of ligands at different concentrations (0-18 µg/ml) for 36 h. IL-6, IFN-γ and IFN-α/β levels were detected by ELISA or IFN-α/β reporter cell line (B16-Blue™ IFN-α/β Cells) from cell supernatants. Cytokine production was presented as fold induction with respect to naive group (Figure 3.3A). The standalone poly(I:C) stimulation induced only IFN-α/β secretion while CpG ODN

increased IL-6 and IFN- α/β levels. Simultaneous stimulation of splenocytes with poly(I:C) and CpG ODN at different concentrations elevated IL-6, IFN- γ and IFN- α/β levels more than that of splenocytes stimulated with single ligands or more than sum of cytokine levels secreted from splenocytes stimulated with either CpG ODN or poly(I:C) at the same doses with their combination (cytokine levels of sum of single ligands/combination < 1 = synergism; >1 = antagonism; Figure 3.3B). Combination of CpG ODN at 18 $\mu\text{g/ml}$ with different doses of poly(I:C) led synergy in secretion of all tested cytokines. Surprisingly, splenocytes incubated with the highest dose of poly(I:C) with the combination of CpG ODN at different concentrations had lower levels of IFN- α/β than the sum of cytokine levels detected from splenocytes stimulated with single ligands. High dose of poly(I:C) acted as an antagonist of CpG ODN. Therefore, we considered to co-encapsulate the lowest doses of ligands that we used (2 $\mu\text{g/ml}$) into liposomes to achieve synergistic immune response.

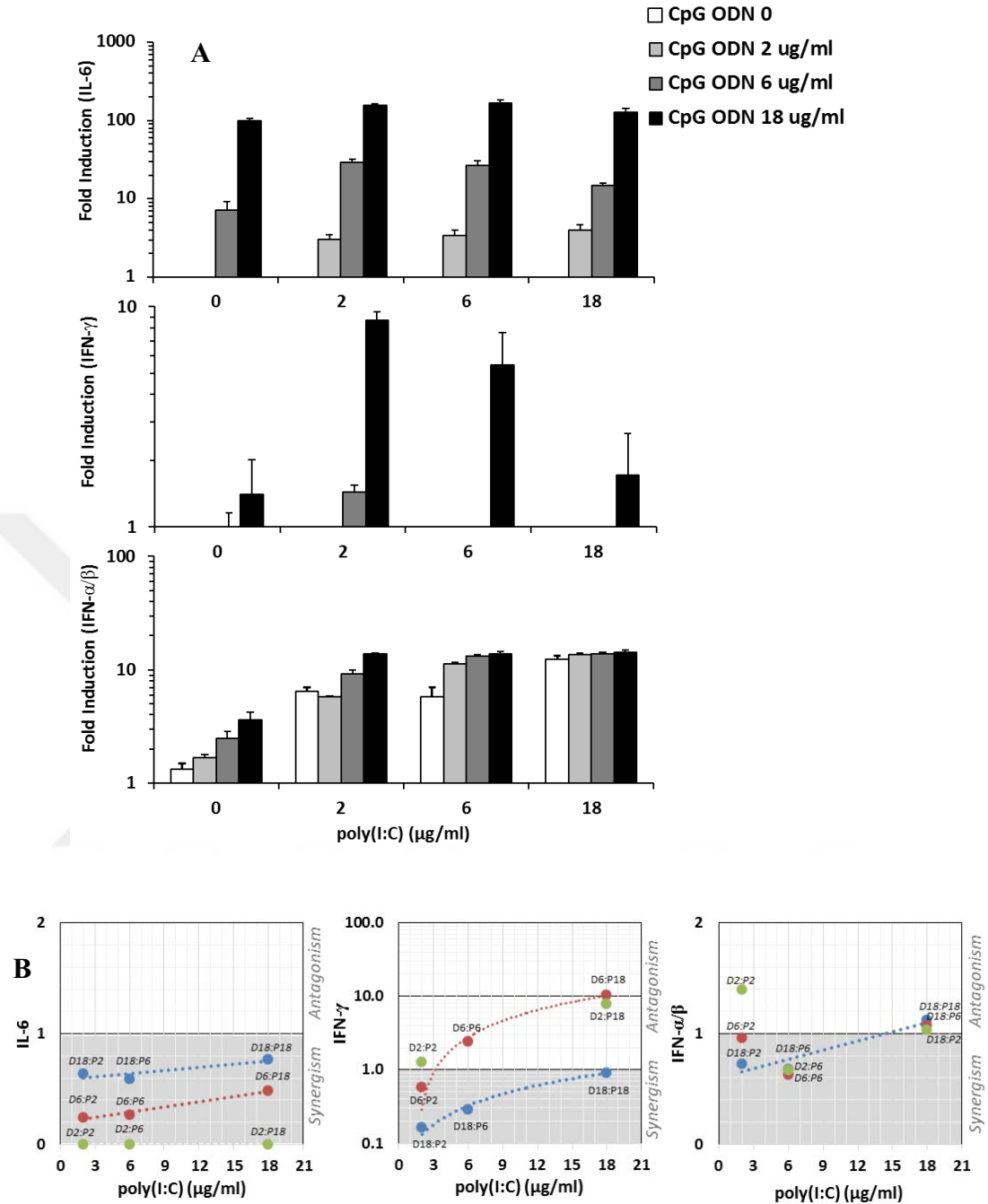


Figure 3.3. *In vitro* immunostimulatory effect of simultaneously administration of non-encapsulated CpG ODN and/or poly(I:C) in splenocytes.

Splenocytes (2×10^6 cell/ml) were stimulated with CpG ODN or poly(I:C) or their combinations at different doses (0-18 $\mu\text{g/ml}$) for 36h. Supernatants were collected and used to detect IL-6 and IFN- γ via ELISA and IFN- α/β via IFN- α/β reporter cell line (B16-Blue™ IFN- α/β Cells). (A) Fold induction was determined with respect to naive group. (B) Synergism and antagonism was assessed by comparing cytokine levels using an equation. C_x : Amount of cytokine detected in supernatant of splenocytes after X stimulation.

$$(C_{\text{CpG ODN}} + C_{\text{poly(I:C)}}) / C_{\text{CpG ODN+poly(I:C)}} < 1 = \text{Synergism}; > 1 = \text{Antagonism}$$

Additionally, the expression levels of pro-inflammatory cytokine TNF- α , interferon induced Mx1 and IP-10 and TLR3 and TLR9 were investigated after stimulation of splenocytes with CpG ODN (1.2 or 6 $\mu\text{g/ml}$) or poly(I:C) (1.2 or 6 $\mu\text{g/ml}$) or their 1:1 combinations for 2 h via RT-PCR.

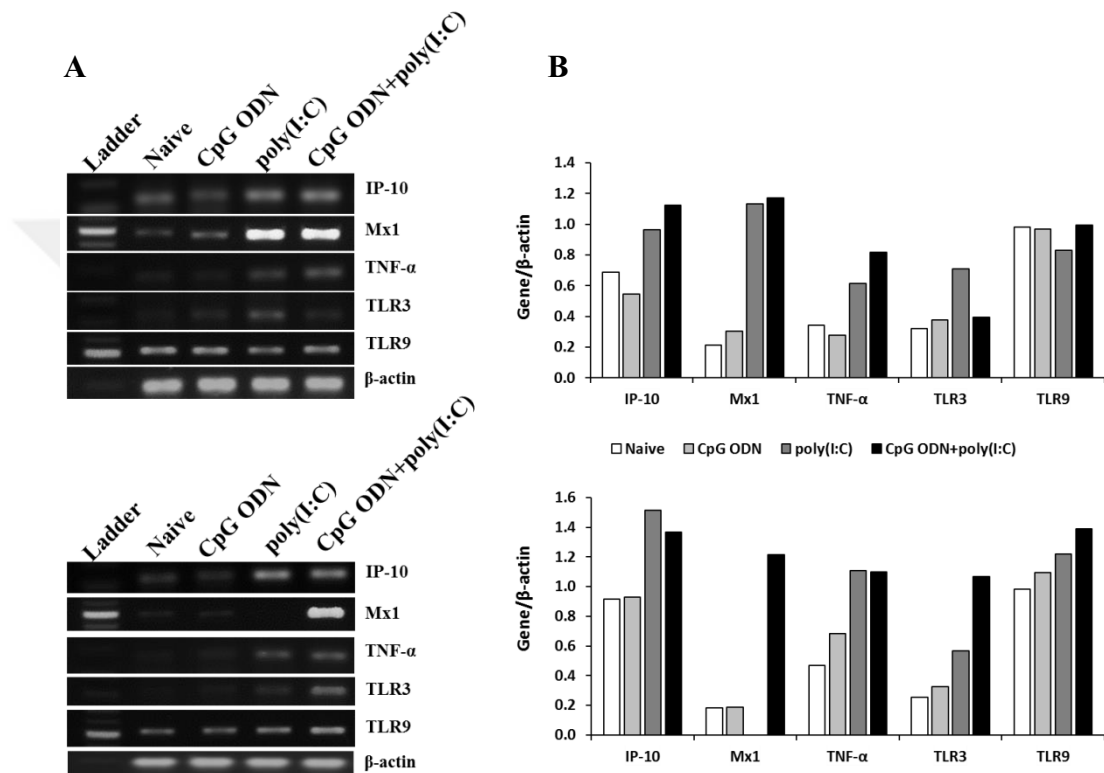


Figure 3.4. Expression levels of cytokine-related genes and TLRs in splenocytes after CpG ODN and/or poly(I:C) stimulation.

Splenocytes ($10 \times 10^6/\text{ml}$) were stimulated with CpG ODN or poly(I:C) or their 1:1 combination at different doses (upper panel: 1.2 $\mu\text{g/ml}$ / each ligand; lower panel: 6 $\mu\text{g/ml}$ / each ligand). After 2 h, cells were collected and total RNA was isolated using TRIzol according to phenol: chloroform extraction method. PCRs were studied using cDNA synthesized from total RNA of samples. (A) PCR products were visualized in agarose gel. (B) Intensities of bands were measured using ImageJ and normalized according to intensity levels of house-keeping gene, β -actin, of each group.

The assay was conducted using cDNA from total RNA isolated by phenol: chloroform method. ImageJ was used to analyze the intensities of band of primer products on agarose gel. Low doses (1.2 µg/ml) of CpG ODN and poly(I:C) increased TNF- α and similar effects with poly(I:C) were observed as expected. At high doses (lower panel), combination increased Mx1, TLR3 and TLR9 expressions (Figure 3.4).

3.2.1.1 *In vitro* immunostimulatory effects of liposomes co-encapsulating TLR3 and TLR9 ligands

In the previous section, simultaneous administration of two ligands, namely, CpG ODN and poly(I:C) boosted IL-6, IFN- γ and IFN- α/β production in a dose dependent manner from splenocytes. Strikingly, higher amount of poly(I:C) did not synergistically activated innate immune cells but rather reduced the level of cytokines. We hypothesized that the synergy would be established only when low doses of the ligands were used to co-encapsulate into liposomes. The prospect of this approach was assessed by incubating splenocytes either with free or encapsulated poly(I:C) and CpG ODN at 2 µg/ml dose of each ligand for 36 h. Supernatants of splenocytes were used to detect IL-6 and IFN- γ via ELISA and IFN- α/β by IFN- α/β reporter cell line. To understand the impact of co-encapsulation, splenocytes were also stimulated with free combination, CpG ODN + poly(I:C), separately encapsulated liposomal formulation, (CpG ODN)L + (poly(I:C))L and co-encapsulated formulation, (CpG ODN + poly(I:C))L. Data implied that, co-encapsulating poly(I:C) and CpG ODN significantly increased IL-6, IFN- γ and IFN- α/β levels over either free single ligands, non-encapsulated dual combined or separately encapsulated ligands ($p < 0.05$, one-way ANOVA, Tukey's HSD post hoc test, Figure 3.5A-C). Influence of co-encapsulation was remarkable considering that separately encapsulated ligands did not induce any significant elevation in cytokine production compared to free or encapsulated single and dual ligands.

Activation of NF- κ B pathway by liposome co-encapsulated TLR ligands would promote survival and proliferation of immune cells. This might be the reason of

elevated cytokine production from splenocytes stimulated with co-encapsulated ligands. To understand the effect of ligands on the proliferation of splenocytes, Cell Counting Kit-8 was used to detect the proliferative capacity of liposomes following stimulation of splenocytes with indicated groups. Relative cell viability was calculated via normalizing cell viability of groups with naive group. Results revealed that there was no significant difference between groups with respect to their cell viability (ns, one-way ANOVA, Tukey's HSD; Figure 3.5D).

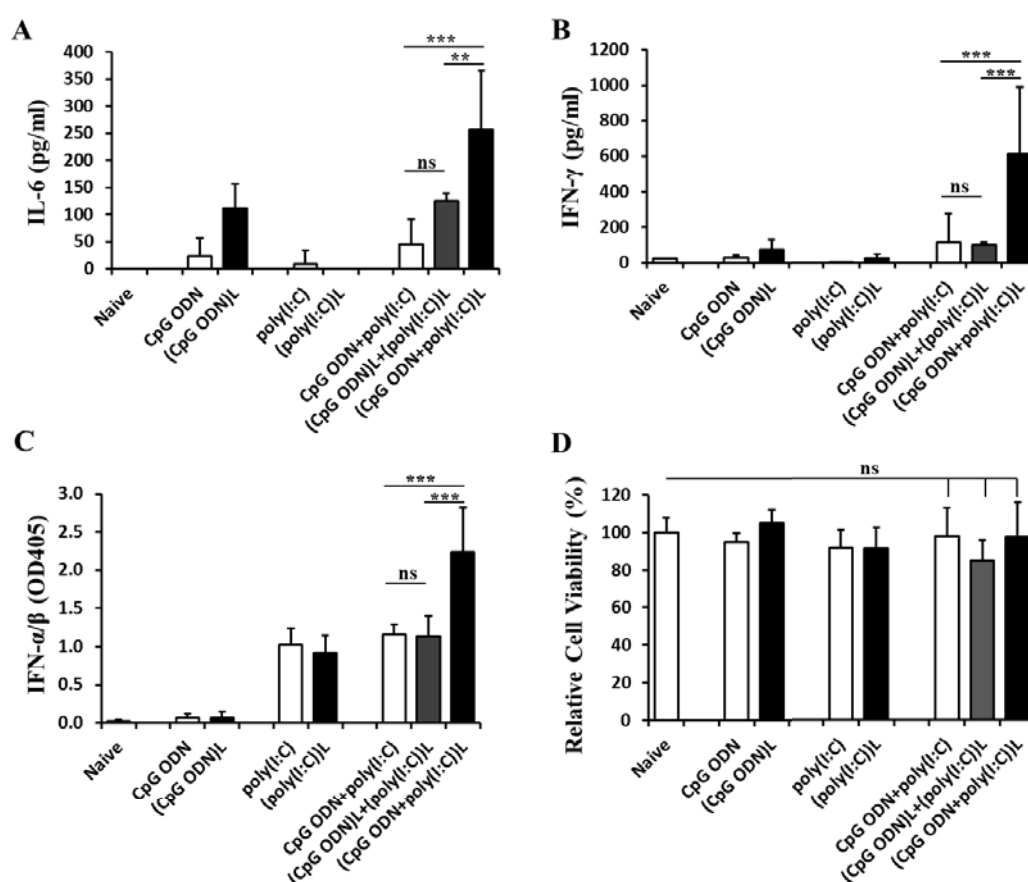
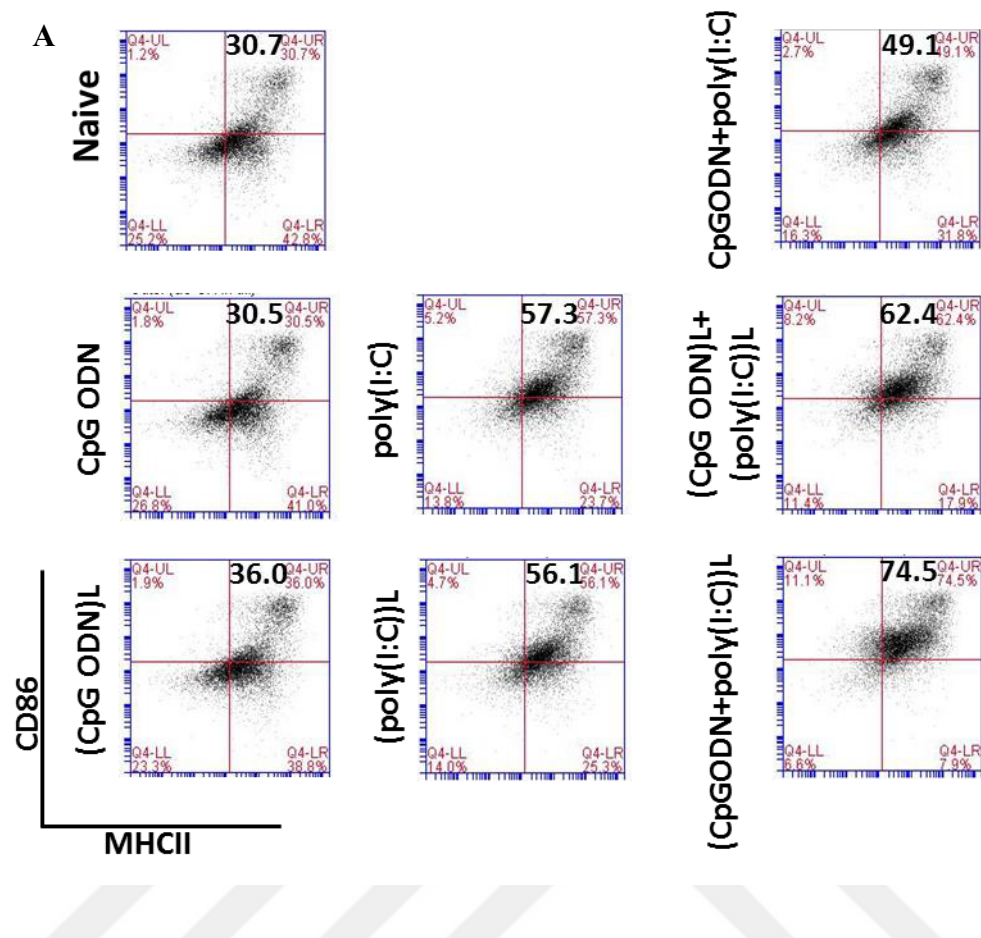


Figure 3.5. *In vitro* immunostimulatory effect of liposomes co-encapsulating CpG ODN and poly(I:C).

Splenocytes were stimulated with free or liposome-encapsulated CpG ODN and/or poly(I:C) (2 μg/ml from each ligand) for 36h. Supernatants were collected to detect (A) IL-6 and (B) IFN-γ cytokine levels by ELISA and (C) IFN-α/β level via reporter cell line (B16-Blue™ IFN-α/β Cells). (D) Cell viability was detected using Cell Counting Kit-8 and absorbance was measured at 450 nm. Relative cell viability was determined according to naive group. Data were presented as mean±SD. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001.

We next assessed the effects of liposomes co-encapsulating poly(I:C) and CpG ODN on APC activation and maturation. Thus, BM-DCs were generated in the presence of GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) for 6 days in culture as explained in Section 2.2.3.3. CD11b and CD11c double positive cells were analyzed via flow cytometry to determine purity of cell population. They were 80% CD11b⁺/CD11c⁺ positive (Appendix B). Following harvest, they were incubated with free or loaded TLR ligands (2 µg/ml per each ligand) for 24h. Then, supernatants were collected to detect IL-12 secretion by ELISA and IFN- α/β via IFN- α/β reporter cell line. Cells were stained to determine the number of CD11c⁺ BM-DCs positive for MHC-I, MHC-II, CD80 or CD86 by flow cytometry. Our cell gating strategy throughout these investigations is presented in Appendix B. No significant difference was observed with respect to surface marker upregulation levels between non-encapsulated and liposome-loaded single CpG ODN or poly(I:C) groups (Appendix C).

MHC-II⁺/CD86⁺ double positive CD11c⁺ BM-DCs was higher in co-encapsulated group (74.0±2.0%) than non-encapsulated or liposome-loaded poly(I:C) (57.5±2.6% and 55.0±1.5%, respectively) or non-encapsulated or liposome-encapsulated CpG ODN (30.5±2.0% and 35.0±2.0%, respectively) or their separately encapsulated combination (62.5±3.5%) (Figure 3.6A). While MHC-I and MHC-II expression levels were slightly higher in co-encapsulated group, (CpG ODN + poly(I:C))L, than free CpG ODN plus poly(I:C) combination. The expression levels of co-stimulatory molecules (i.e. CD80 and CD86) were significantly increased for co-encapsulated group treated BM-DCs, compared to free or liposome loaded single or dual ligand combination groups (Figure3.6B-E).



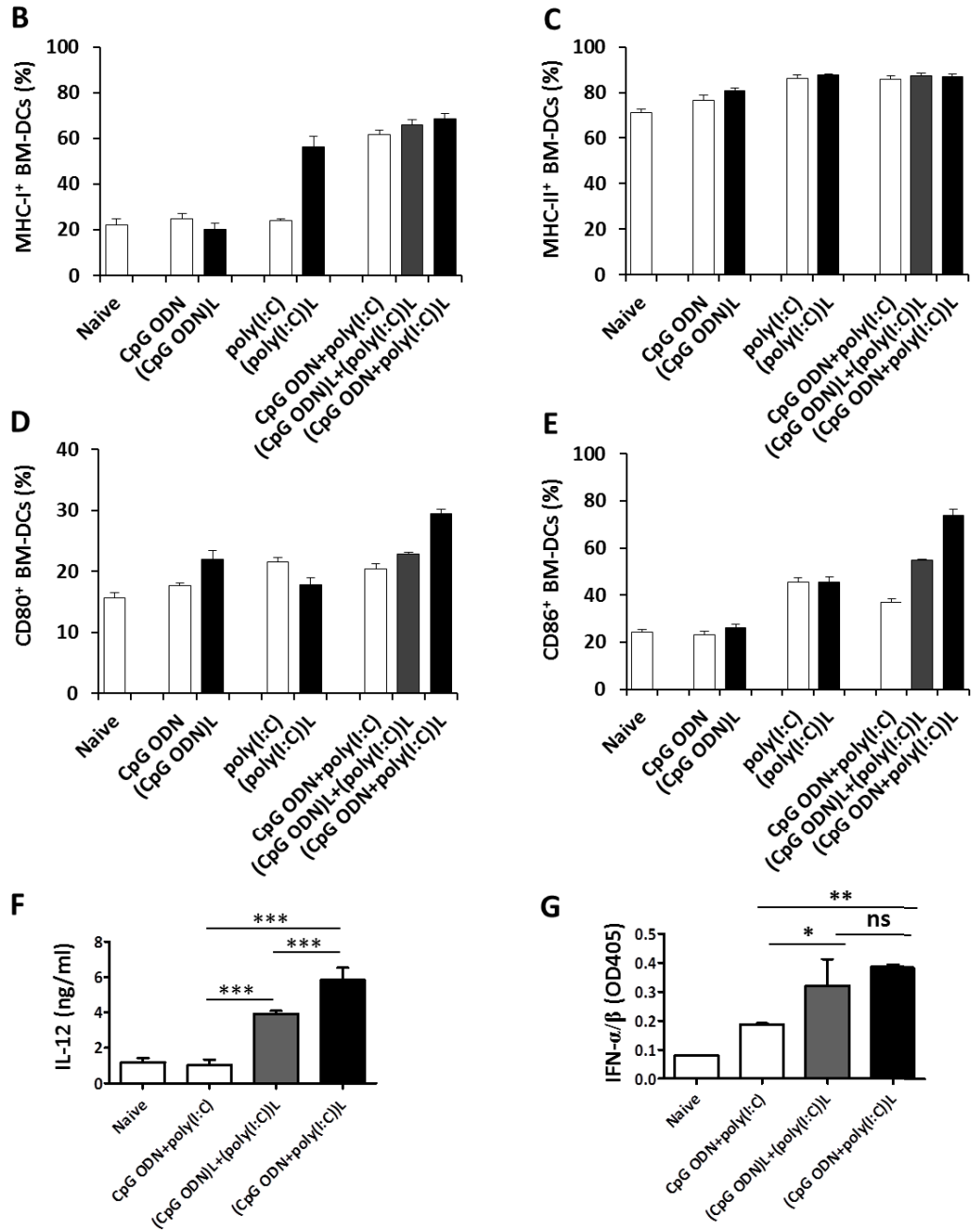


Figure 3.6. Maturation of BM-DCs by incubating with liposomes co-encapsulating CpG ODN and poly(I:C).

Immature CD11c⁺ BM-DCs were incubated with non-encapsulated or liposomal CpG ODN and/or poly(I:C) (2 μ g/ml from each ligand) at 37 °C for 24h. Expression levels of CD86 and MHC-II were analyzed by flow cytometry: (A) representative dot plots. (B) MHC-I, (C) MHC-II, (D) CD80 and (E) CD86 positivity of CD11c positive BM-DCs. Following stimulation, supernatants were collected to determine (F) IL-12 levels by ELISA and (G) IFN- α/β levels by reporter cell line. Statistical significance was determined by ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

When IL-12 production was analyzed from BM-DC supernatants after treatment, we detected that dual ligand co-encapsulated group generated six fold more IL-12 from the supernatants, whereas separately encapsulated ligands yielded only ~4 fold more IL-12 over non-encapsulated ligand combination treated groups (Figure 3.6F). Type I IFN secretion from BM-DCs treated with co-encapsulated CpG ODN plus poly(I:C) was found to be ~2.0 fold and ~0.7 fold more compared to non-encapsulated combination treated group and separately encapsulated CpG ODN and poly(I:C) group, respectively (Figure 3.6G).

To understand whether the results are correlated with death or proliferation of BM-DCs, cell viability was analyzed using PI staining at the end of the stimulation and no significant difference between groups were observed (ns, one-way ANOVA, Tukey's HSD; Figure 3.7).

Additionally, expression of cytokine-related genes (IP-10, IL-1 β , TNF- α , Mx1, and IL-15) were assessed in BM-DCs (2×10^6 /ml) stimulated with free or encapsulated CpG ODN and/or poly(I:C) (2 μ g/ml per each ligand) for 4 h by PCR using cDNA from total RNA of samples. The expression of interferon-related gene IP-10, pro-inflammatory cytokines IL-1 β and TNF- α slightly increased in co-encapsulated ligand containing liposome treated group when compared to free and separately encapsulated CpG ODN and poly(I:C) (Figure 3.8).

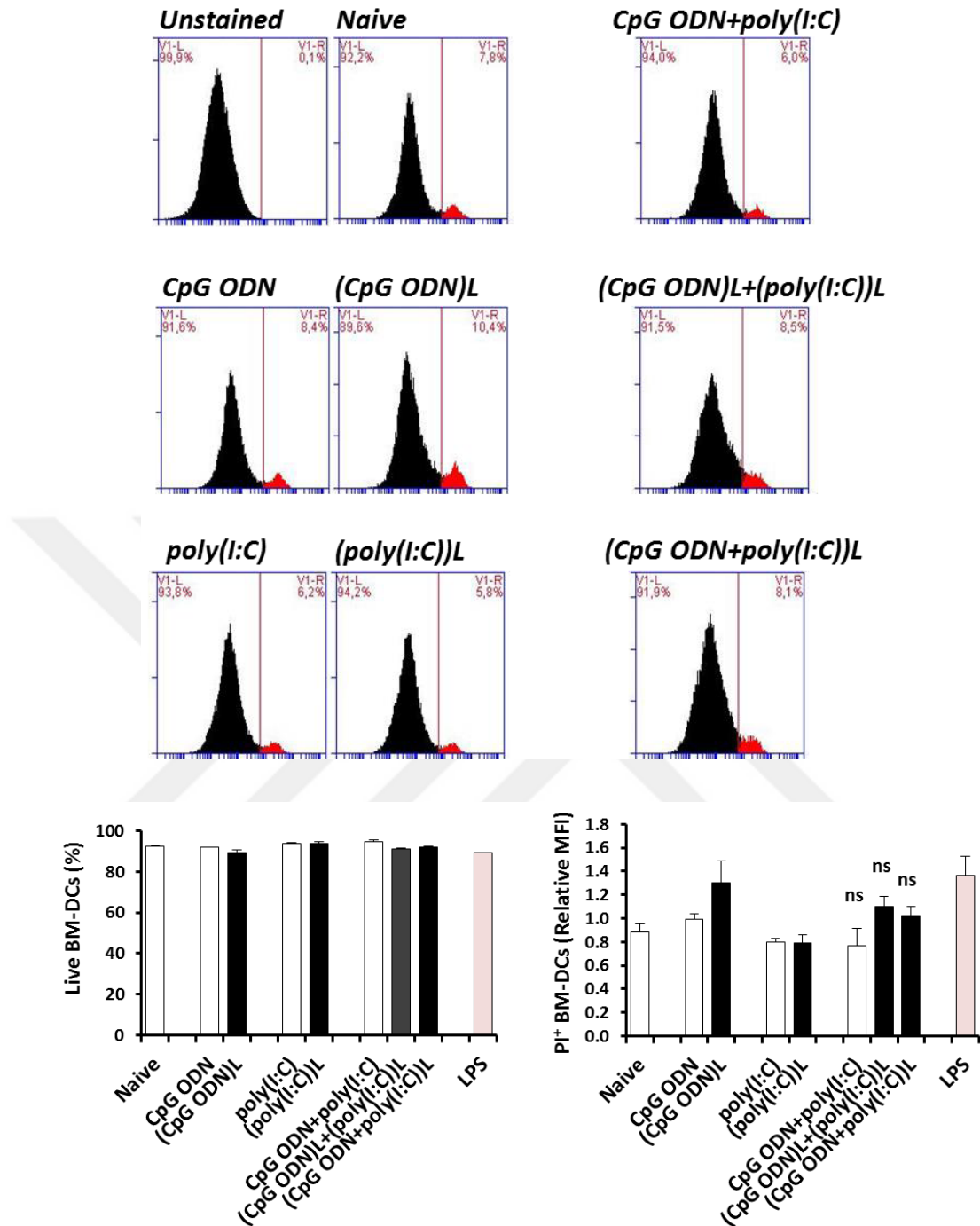


Figure 3.7. Cell viability of BM-DCs treated with free and liposome-loaded CpG ODN and/or poly(I:C).

After stimulation, BM-DCs were stained with propidium iodide (PI) and analyzed via flow cytometry. Representative plots were presented at left side while percentage and relative MFI (with respect to unstained group) of PI⁺ BM-DCs were shown at right side. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test.

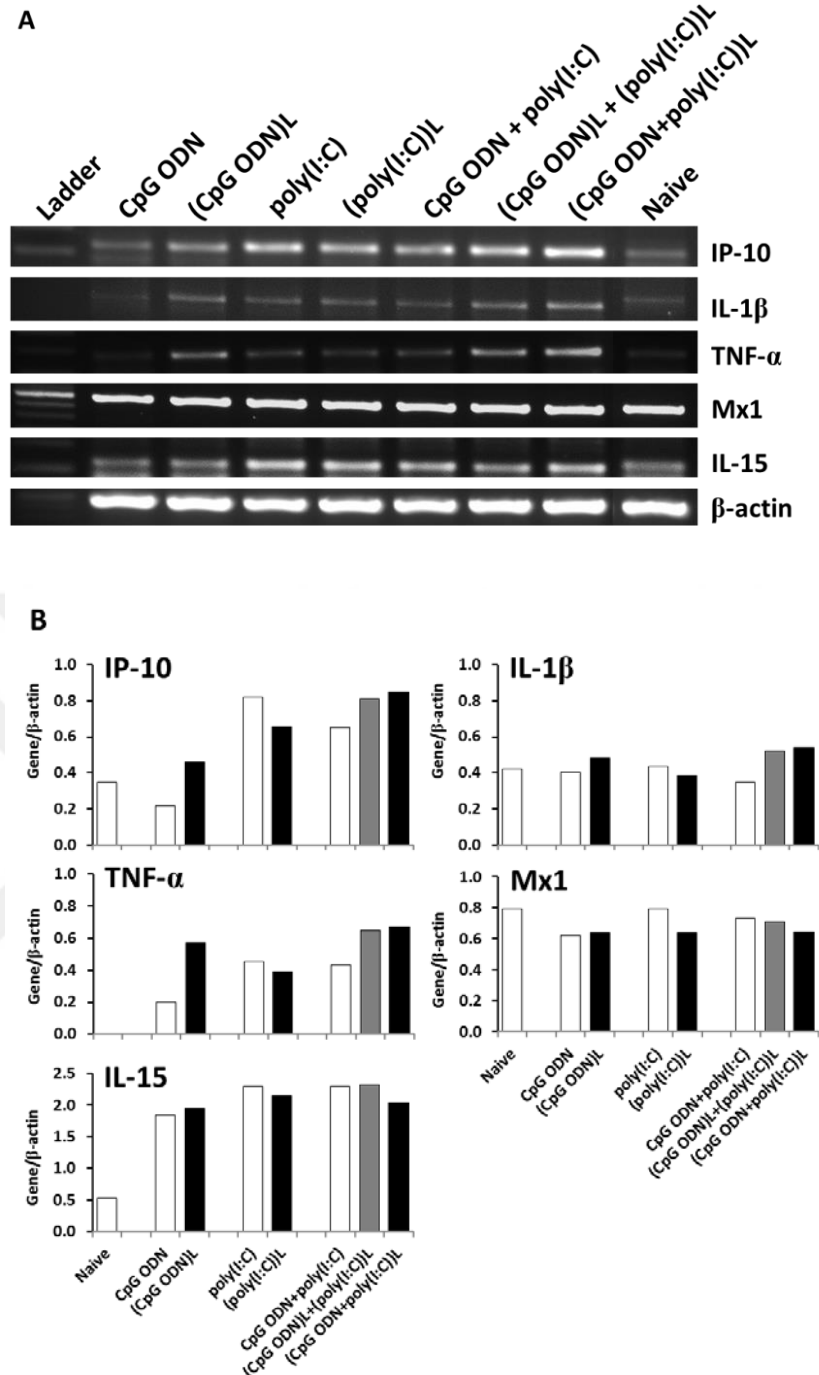


Figure 3.8. Expression of cytokine-related genes in BM-DCs after stimulation with free or encapsulated poly(I:C) and CpG ODN.

BM-DCs ($2 \times 10^6/\text{ml}$) were stimulated with free or encapsulated CpG ODN or poly(I:C) or their 1:1 combination ($2 \mu\text{g}/\text{ml}$ / each ligand). After 4 h, cells were collected and total RNA was isolated using TRIzol according to phenol: chloroform extraction method. IP-10, IL-1 β , TNF- α , Mx1, and IL-15 expression levels were determined by PCR using cDNA synthesized from total RNA of samples. (A) PCR products were visualized in agarose gel. (B) Intensities of bands were measured using ImageJ and normalized according to intensity levels of house-keeping gene, β -actin, of each group.

Next, we generated BM-DMs using 30% L-929-conditioned medium. After incubating them in cell culture for 6 days, cells were collected and F4/80 and CD11b double positivity of cells were analyzed by flow cytometry (Appendix D). The BM-DMs were >95% CD11b and F4/80 double positive implicating that they were converted to macrophages. Then, cells ($0.5 \times 10^6/\text{ml}$) were stimulated with free and encapsulated CpG ODN or poly(I:C) or their 1:1 combinations at various concentrations. Supernatants were used to detect IL-12 and nitric oxide (NO) via ELISA and Griess method, respectively. IL-12 levels were increased upon ligand co-encapsulation within liposome, when compared to free CpG ODN and poly(I:C) except the highest dose (18 $\mu\text{g}/\text{ml}$; Figure 3.9A). However, NO levels were significantly increased in co-encapsulated group at 18 and 6 $\mu\text{g}/\text{ml}$ (Figure 3.9B). In addition, IFN- α/β levels were found to be non-significant between groups (Figure 3.9C).

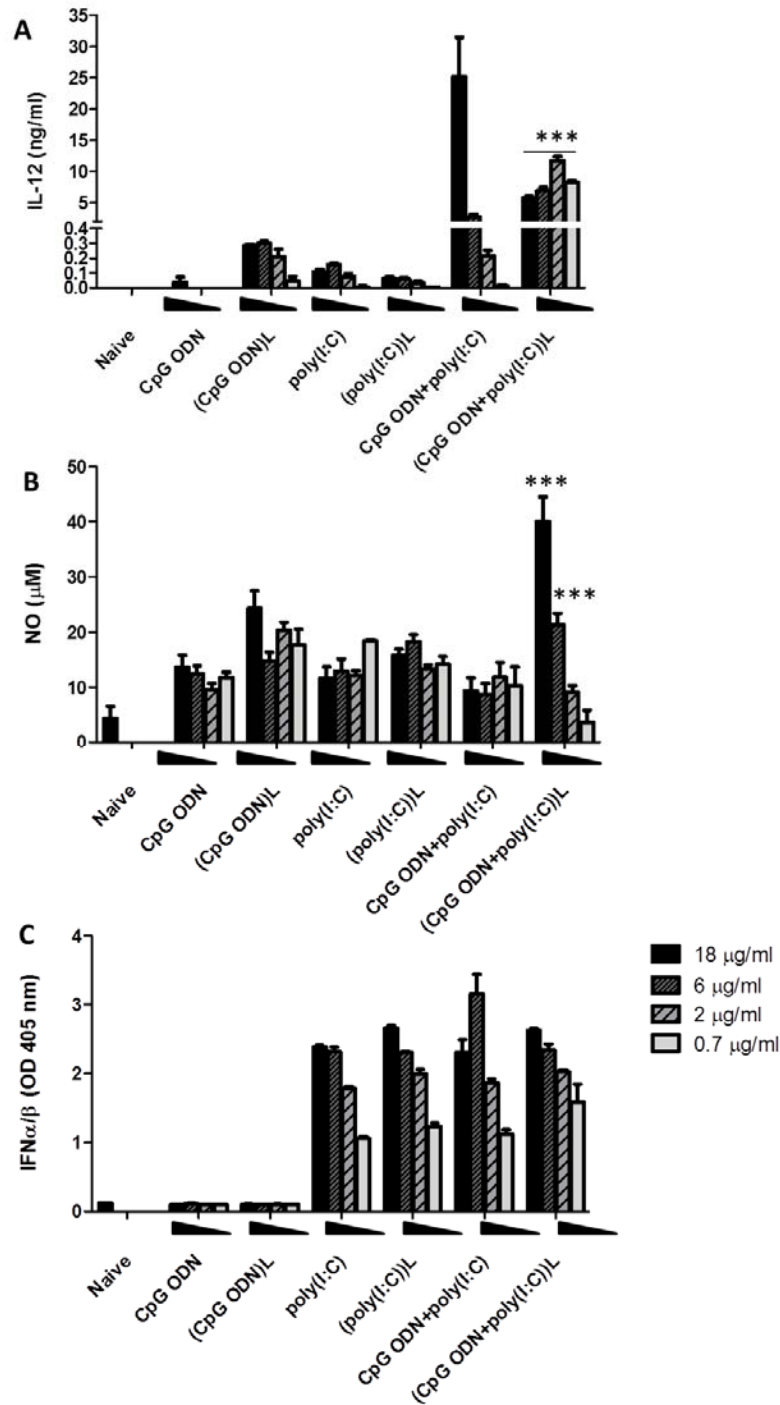


Figure 3.9. Activation of BM-DMs with free or liposome-loaded CpG ODN and/or poly(I:C) after 24 h stimulation.

BM-DMs were incubated with free or liposomal CpG ODN and/or poly(I:C) (2 μ g/ml/ each ligand) for 24 h. Supernatant were used to determine IL-12 via ELISA, NO by Griess reagent and IFN- α/β levels via reporter cell line. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ***p<0.001 (free vs co-encapsulated group).

Moreover, RAW 264.7, macrophage cell line, was incubated with free or loaded CpG ODN and/or poly(I:C) for 24 or 48 h. TNF- α level was detected from supernatant of cells by ELISA. Two and 5-fold more TNF- α production was detected from (CpG ODN+poly(I:C))L treated cells than free dual ligand treated group (Figure 3.10A).

To address effective antigen processing by APCs stimulated with liposomal ligands, DQTM-OVA which is an ovalbumin protein conjugated to a pH insensitive self-quenching dye, was simultaneously administered with free CpG ODN+poly(I:C)+DQTM-OVA, separately encapsulated, (CpG ODN)L+(poly(I:C))L+DQTM-OVA, or co-encapsulated (CpG ODN+poly(I:C))L+free DQTM-OVA or co-encapsulated into liposomes loaded with dual ligands, (CpG ODN+poly(I:C)+DQTM-OVA)L. Upon internalization, fluorescence could be detected when ovalbumin is hydrolyzed (detected @ FL1 channel) and accumulated (detected @ FL2 channel) in the cell. We assessed antigen processing and accumulation of DQTM-OVA by flow cytometry after stimulation of RAW 264.7 cells with indicated groups for 6 h. All data was normalized to degradation level of each treatment group incubated at 4 °C. We found that fluorescence signals indicating both antigen processing and accumulation were significantly higher in cells treated with ligand plus DQTM-OVA co-encapsulated group than free or liposome-loaded dual ligands administered simultaneously with free DQTM-OVA ($p < 0.05$, one-way ANOVA, Tukey's HSD; Figure 3.10B and C).

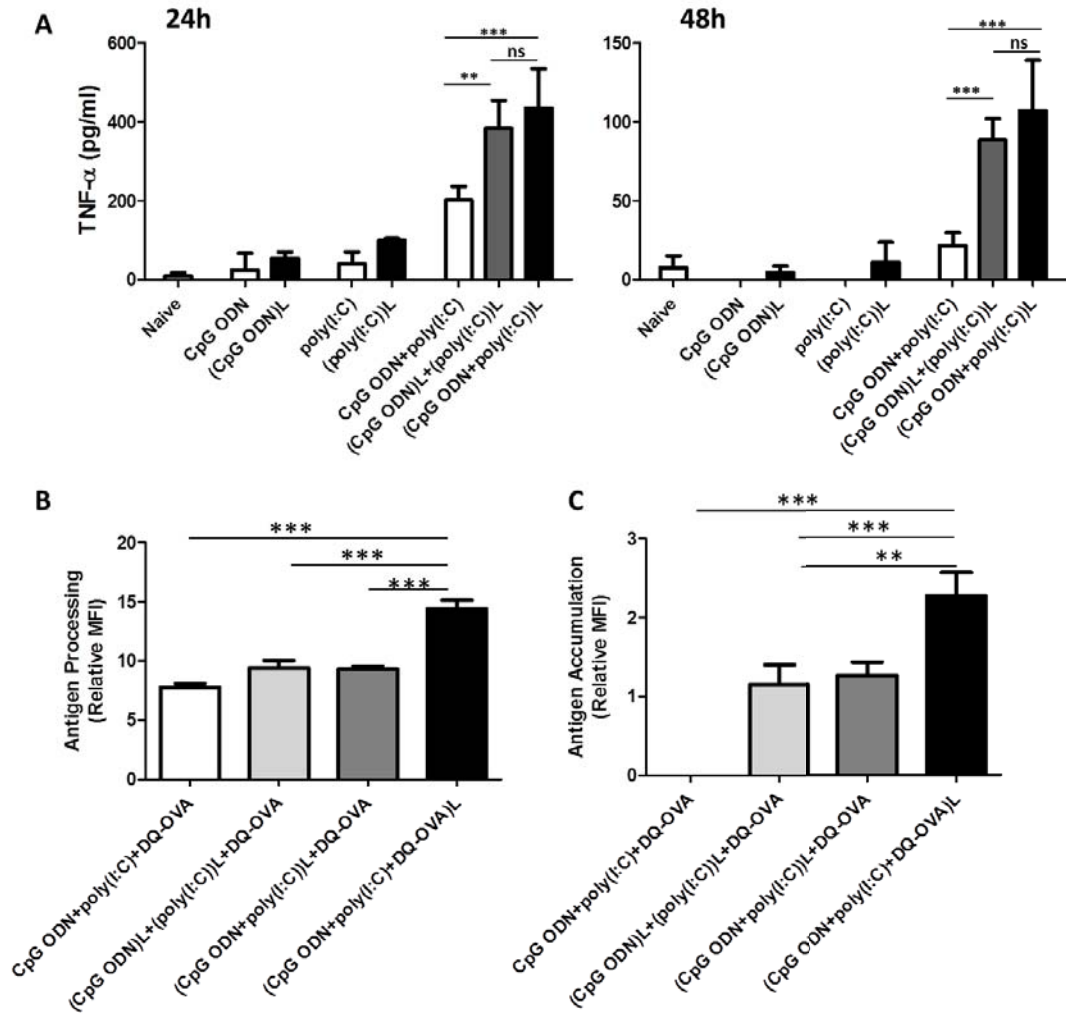


Figure 3.10. TNF- α secretion and antigen processing of macrophage cell line, RAW 264.7, after stimulation with CpG ODN and/or poly(I:C) and DQ™-OVA. RAW cell line was incubated with free or loaded CpG ODN and/or poly(I:C) for 24 or 48 h. Supernatants were used to detect TNF- α via ELISA. Separately, cells were also incubated with free, separately encapsulated or co-encapsulated CpG ODN and poly(I:C) together with free DQ™-OVA or liposome-loaded with CpG ODN, poly(I:C) and DQ™-OVA for 6h. Antigen processing (FL1) and accumulation (FL2) were analyzed via flow cytometry using of self-quenching property of DQ™-OVA and hydrolysis of protein into peptide release fluorescence signal at FL1 and accumulation of signal in the cell detected at the FL2 channel.

3.2.1.2 Uptake of TLR3 and TLR9 ligands loaded into liposomes by splenocytes

The activity of ligands enhanced by co-encapsulation was not correlated with the cell proliferation of immune cells as previously shown. Another possibility of this mechanism would be due to effective uptake and binding of ligands when encapsulated into liposomes. To assess this hypothesis, splenocytes were incubated with non-encapsulated and liposomal forms of Cy5-labeled CpG ODN and/or fluorescein-labeled poly(I:C) for 2h. Fluorochrome positivity of cells was analyzed via flow cytometry (Figure 3.11). Free dual ligand treated cells had 5.2% and 1.3% (for CpG ODN and poly(I:C) signal, respectively) positivity; whereas, dual ligand co-encapsulated treatment gave 12.5% and 6.5% CpG ODN and poly(I:C) positivity, respectively (Figure 3.11A). When relative MFI values of the treatments were analyzed, this improvement in ligand binding was more pronounced. Consequently, co-encapsulation by liposomes provided ~2.5 fold more CpG ODN and ~5 fold more poly(I:C) binding (Figure 3.11B). When we analyzed double positivity (Cy5⁺ fluorescein⁺) of fluorochrome in the cell, co-encapsulating them increased cellular uptake and binding more than its free combination or separately encapsulated ligands into liposomes (Figure 3.11C).

Additionally, splenocytes were stimulated with free or liposome-loaded labeled CpG ODN and/or poly(I:C) for 24 h. APC types internalizing ligands were analyzed using flow cytometry after staining with fluorochrome-conjugated anti-CD11b, CD11c and CD19 antibodies (macrophages, Dendritic and B-cells, respectively). No significant difference was observed between cell types internalizing ligands after analyzing surface markers of APCs and fluorochrome positivity of ligands (Figure 3.11D). In all investigated cases the degree of ligand uptake whether or not liposomes were involved was as follows: Macrophages>DCs>B-cells.

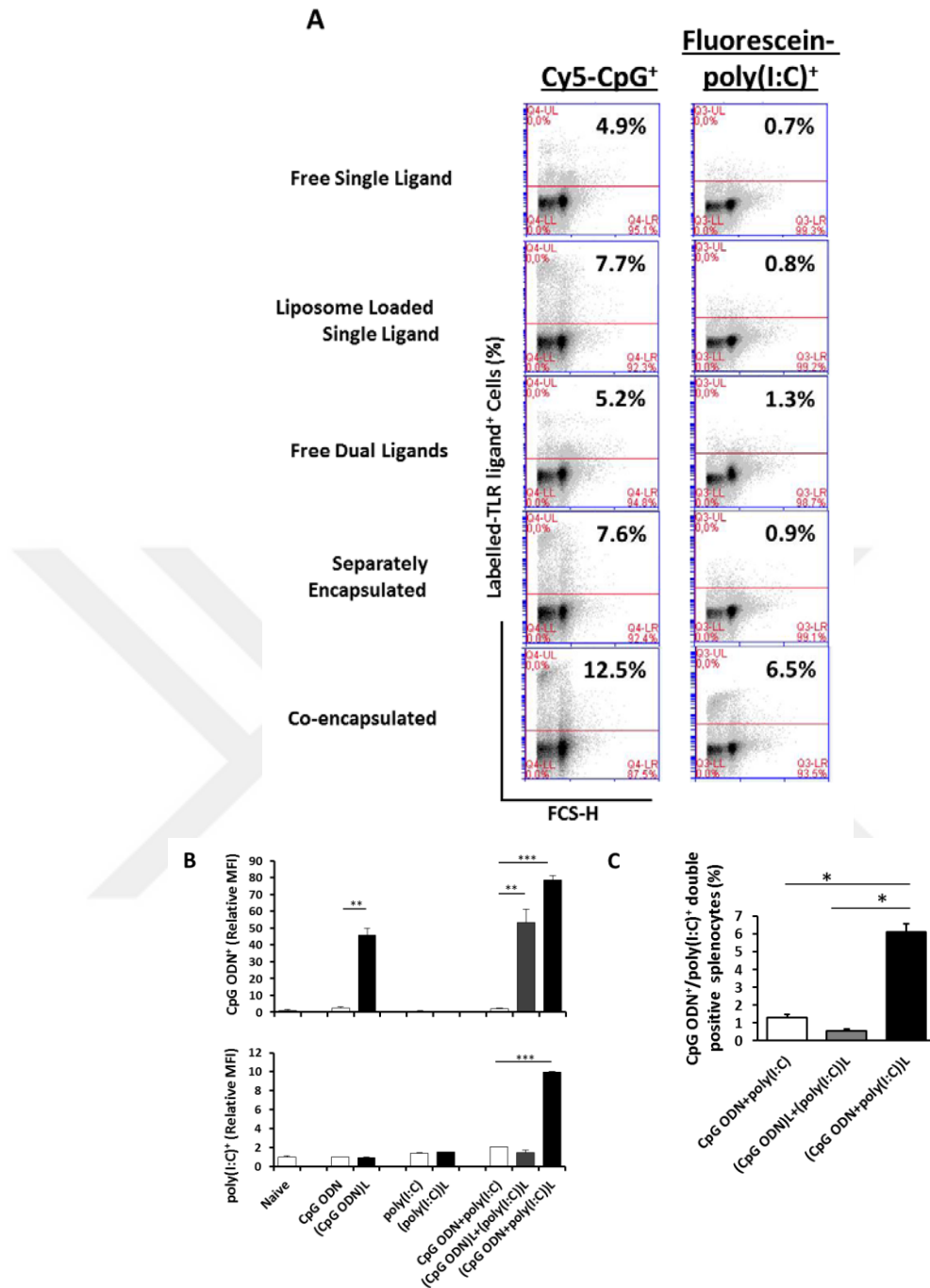


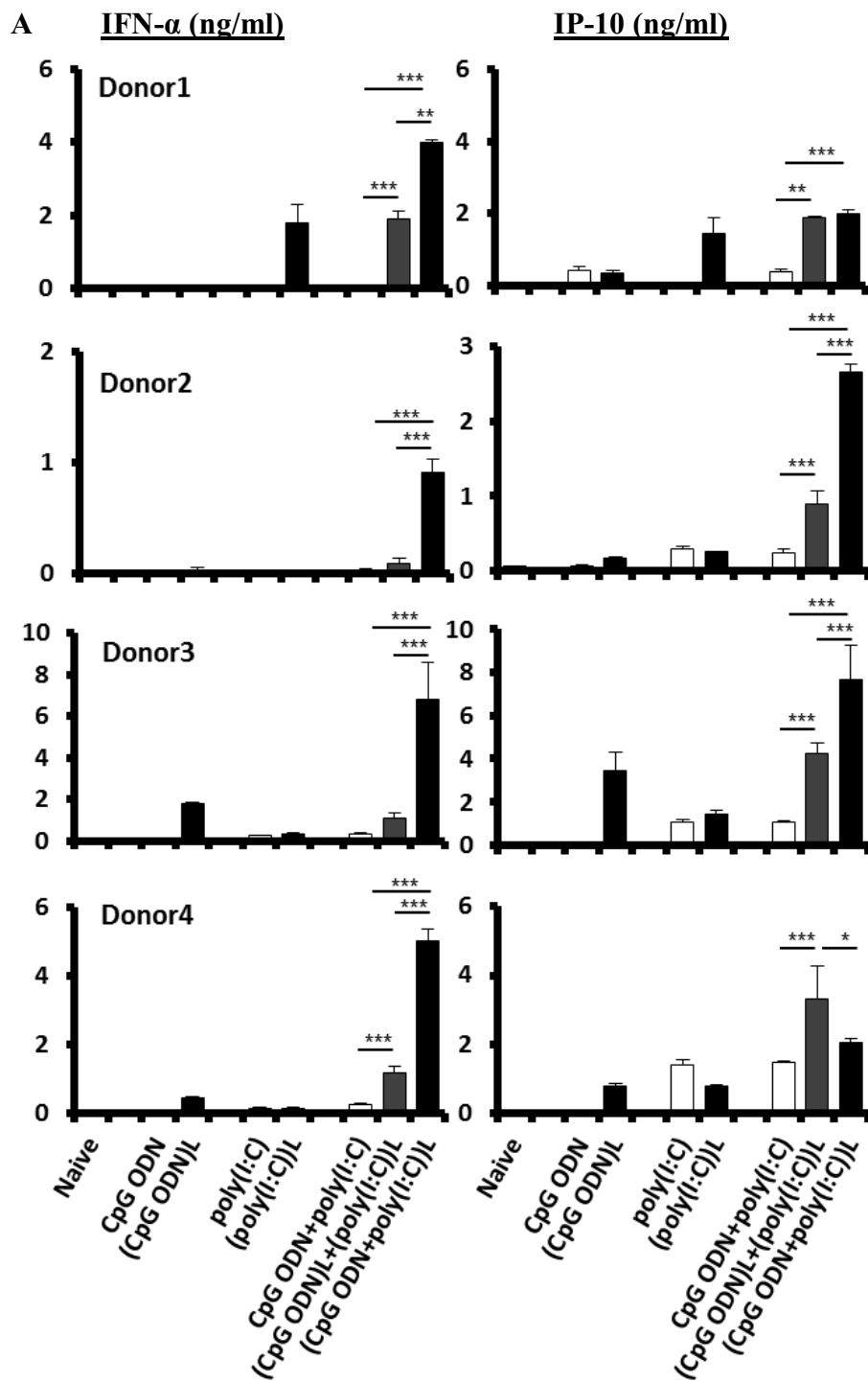
Figure 3.11. Cellular uptake and binding of liposomes co-encapsulating CpG ODN plus poly(I:C) by splenocytes.

Splenocytes were incubated with free or liposome-encapsulated Cy5-labelled CpG ODN and/or fluorescein-labelled poly(I:C) (2 µg/ml) for 2h at 37 °C. (A) Representative dot-plots and (B) relative MFI values, (C) double positive cell percentages were presented. (D) Splenocytes were stained with anti-CD11b (macrophages), CD11c (dendritic cells) and CD19 (B cells) after 24h of stimulation with fluoroconjugated ligands and double positivity of ligands and cell surface markers were presented. (Student's t-test; *p<0.05; **p<0.01; ***p<0.001 between groups).

3.2.1.3 Human PBMC activation by liposomes co-encapsulating TLR3 and TLR9 ligands

In mice, we demonstrated that immune cells secrete more type I and II IFNs when stimulated with co-encapsulated CpG ODN and poly(I:C) than stimulated with free dual ligands. Additionally, maturation of APC was higher considering the increased up-regulation of surface markers and cytokine production. This strong immune response was probably due to increased binding and uptake of dual ligands when co-administered within liposomes. We next sought to establish whether this formulation triggers hPBMCs to the same extent that we observed in mice. Therefore, hPBMCs were collected from healthy donors and stimulated with free or loaded CpG ODN or poly(I:C) or their 1:1 combination for 24h. All hPBMCs produced significantly higher levels of IFN- α (Figure 3.12A, left panels) when treated with (CpG ODN+poly(I:C))L group. Similarly, the IP10 production from monocytes was more in co-encapsulated liposomal group compared to free combination (Figure 3.12A, right panels).

In addition, HLA-DR and CD83 surface markers expression of hPBMCs were found to be significantly higher in cells that was stimulated with co-encapsulated dual ligands in liposomes (one-way ANOVA, Tukey's HSD, $p < 0.05$; Figure 3.12B).



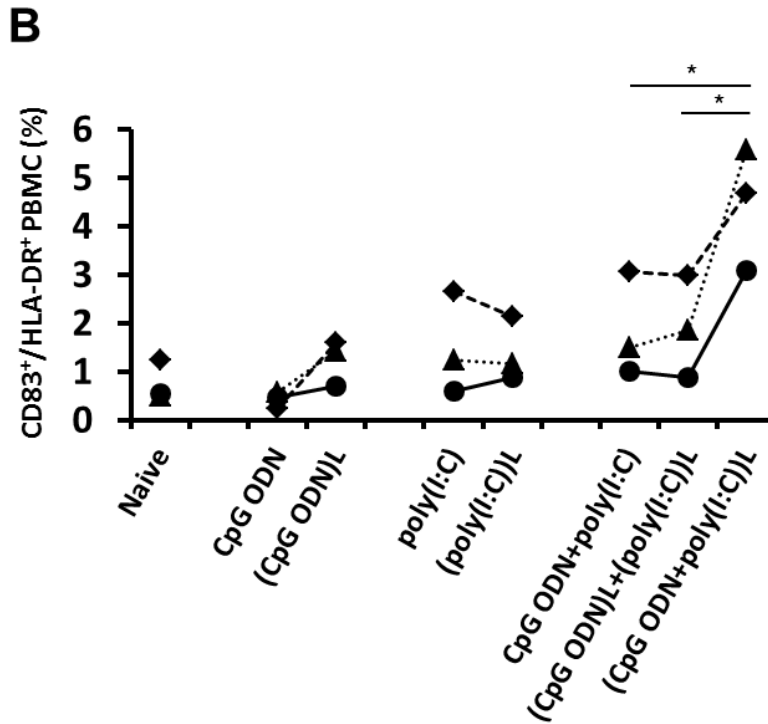


Figure 3.12. *In vitro* immunostimulatory effects of liposomes co-encapsulating CpG ODN and/or poly(I:C) on hPBMCs.

hPBMCs were isolated from healthy donors' blood and stimulated with free or liposome encapsulating CpG ODN and/or poly(I:C) for 24h. Supernatants were used for (A) IFN- α and IP-10 cytokine ELISA. (B) CD83 and HLA-DR positive PBMCs were analyzed via flow cytometry. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2.2 Synergism between TLR9 and STING ligands

Recently, a synergistic immune activity between STING and TLR9 ligands has been reported [94,95]. These studies suggested that more pronounced immune activation could be obtained from immune cells when simultaneously stimulated with CDNs and CpG ODN since it leads the activation of both IRF3 and NF- κ B pathways which resulting higher production of both pro-inflammatory cytokines and type I IFNs. However, this induction was dependent if high doses of c-di-GMP or cGAMP were used for stimulation probably to overcome low level cellular uptake. The

internalization of STING ligands is significantly less than TLR9 ligands because of their circular structure.

We sought to bypass the internalization problem of STING ligands by co-encapsulating them with CpG ODN in liposomes. Preliminary experiments were planned to assess the dose dependent synergism between these ligands, thus, we incubated splenocytes with STING (c-di-GMP and cGAMP) and TLR9 (K-type CpG ODN) ligands at different concentrations for 24 h. We demonstrated that simultaneous administration of STING (c-di-GMP and cGAMP) and TLR9 (K3) ligands increased IL-6 and IL-12 productions compared to their standalone single stimulation levels (Figure 3.13). Consistent with previous studies by others, the STING ligands must be included in the mixture at high doses compared to CpG ODN dose. When immune cells were stimulated at high combinations of CDNs (i.e. 1:16, 1:8 and 1:4; CpG:CDN) there were significantly higher cytokine secretion from immune cells (Figure 3.13). To enhance the synergistic activity at lower doses that free ligands do not activate cytokine production we encapsulated both ligands into liposomes.

As mentioned earlier, pH-sensitive liposomes were selected for co-encapsulation. At low pH, these liposomes become unstable and release its cargo into late endosome and cytosol. CDNs should be delivered to cytosol and TLR9 ligand, K3, should be released at late endosome. In previous studies, SSCL were shown to enhance the immunostimulatory activity of K3 [110]. Therefore, we prepared modified pH-sensitive SSCL as presented at Section 3.1.2.

Our hypothesis was the following: the pH-sensitive SSCL following internalization will decompose in late endosome due to low pH. K3 will be released and interact with TLR9 at late endosome. The DOPE component of the SSCL will then destabilize the endosome in acidic pH.

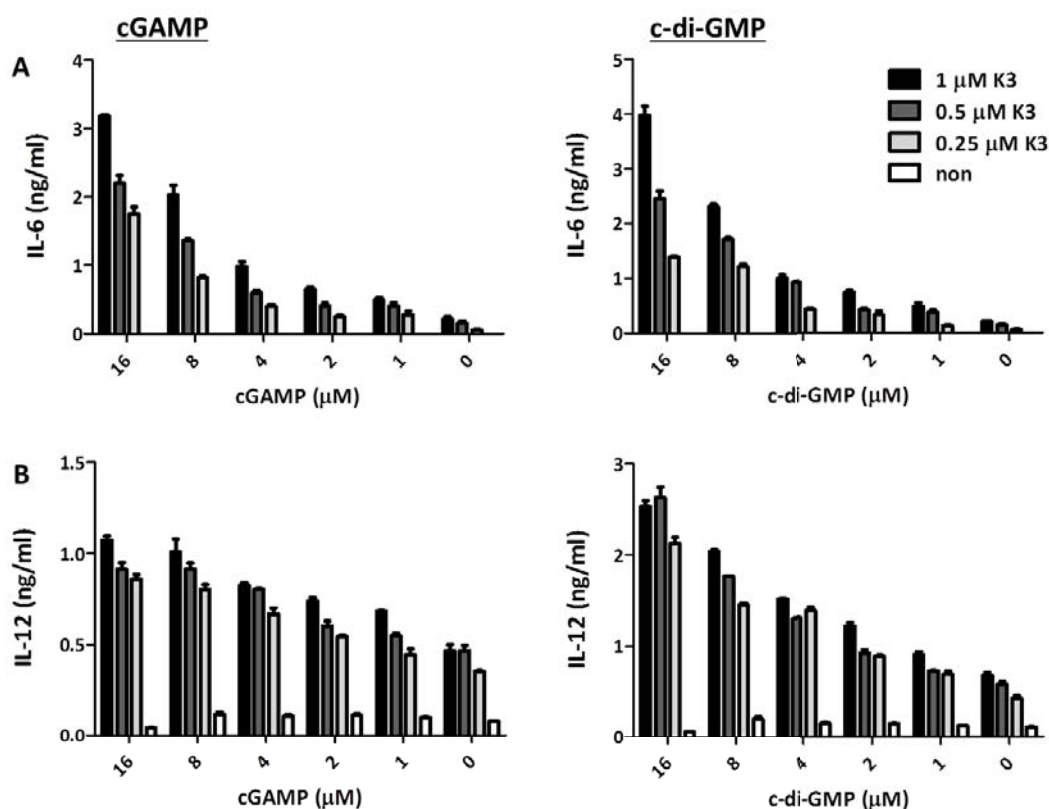


Figure 3.13. IL-6 and IL-12 levels from splenocytes stimulated with free K3 and/or c-di-GMP or cGAMP.

Splenocytes were stimulated with K3 (0-1 μ M) and/or c-di-GMP or cGAMP (0-16 μ M) for 24h. (A) IL-6 and (B) IL-12 was detected from supernatants of splenocytes via ELISA. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

The structure of the lipid in acidic environment turns into hexagonal from bilayer, causes endosome break down. Thus, the decomposition results a pore formation at the late endosome facilitating the leakage of CDNs into cytosol (explained in the Figure 3.14). Co-delivery of both K3 and CDNs will trigger the activation of both anti-bacterial and anti-viral responses.

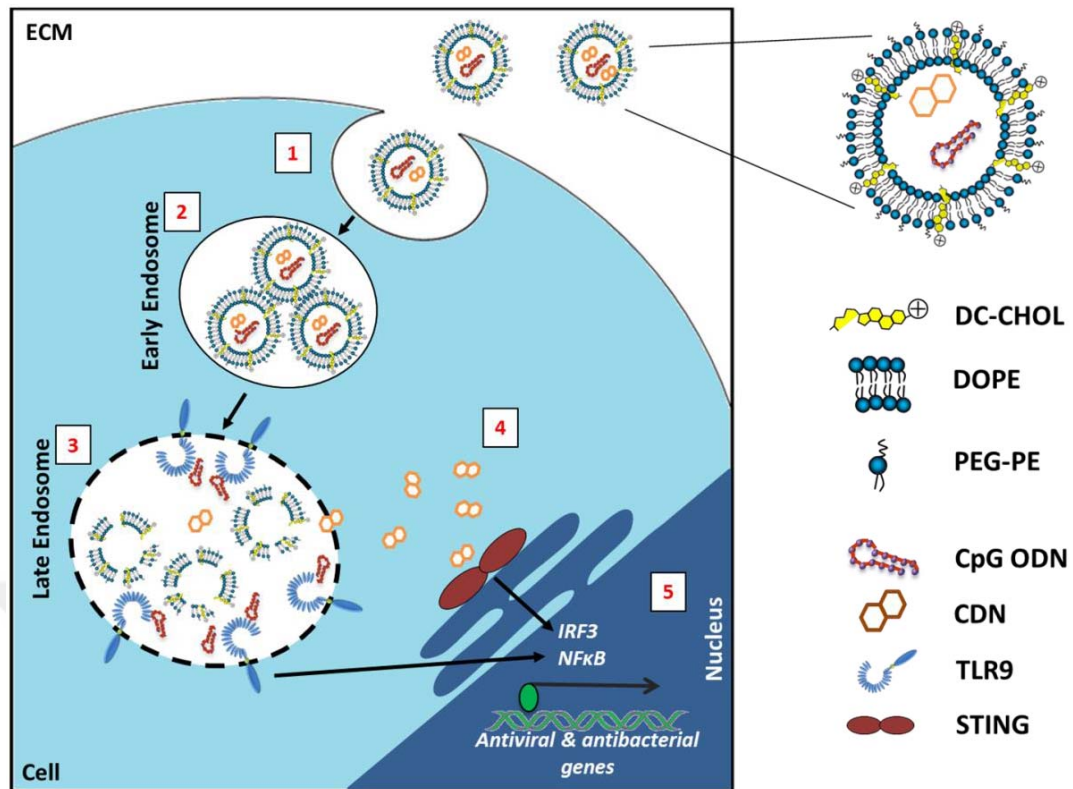


Figure 3.14. Proposed delivery mechanism of TLR9 ligand, CpG ODN, and STING ligands, cyclic dinucleotides (CDNs), co-encapsulated pH sensitive liposome.

1) CpG ODN and CDN co-encapsulated liposomes are internalized by cells. 2) Liposomes remain stable in early endosome (pH: 6.5). 3) Lipid bilayer is destabilized in late endosome/lysosome (pH: 4.5-5.5) due to decrease in pH and CpG ODNs released from liposomes interacts with TLR9. Structural change of lipid bilayer effects membrane stability of late endosome and pore formation occurs on it. 4) Destabilization of liposome causes small CDNs to leak out from pores of late endosome and release into cytosol, there, they interact with STING. 5) Interaction of ligands with TLR9 and STING activates NF-κB and IRF3 transcription factors, respectively, which leads expression of antiviral and antibacterial genes. ECM: extracellular matrix, CDN: cyclic di-nucleotide, ODN: oligodeoxynucleotides.

3.2.2.1 *In vitro* immunostimulatory effects of pH sensitive cationic liposomes co-encapsulating STING and TLR9 ligands

K3 and CDNs were co-encapsulated into modified pH-sensitive SSCL at 3:1 or 9:1 (w/w) ratios. Immunostimulatory potency of pH-sensitive SSCL co-encapsulating K3 and cGAMP/c-di-GMP was investigated by incubating splenocytes either with free or encapsulated K3 and/or cGAMP/ c-di-GMP at different concentrations for 36 h. After stimulation, supernatants were collected and IL-6, IL-12, IFN- γ and IFN- α/β were detected via ELISA and reporter cell line, B16-Blue™ IFN- α/β cells.

Simultaneous stimulation with free K3 and cGAMP or c-di-GMP increased the production of IL-6 and IL-12 than free single ligands, confirming previous observations. Strikingly, co-encapsulation of K3 and cGAMP or c-di-GMP either at 1+3 μ M each or at 1+1 μ M each did not enhance the synergism compared to free combinations when IL-6 and IL-12 production level was investigated (Figure 3.15). However, when CDN concentration was reduced to 0.3 or 0.1 μ M and K3 was kept at 0.3 or 0.1 μ M concentration based on IL-6 induction, they surpassed free or liposome loaded single ligand induction levels ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test, Figure 3.15A-B). Interestingly, based on IL-12 secretion, loading c-di-GMP at 0.3 μ M dose with 0.1 μ M K3 into liposomes had any significant effect compared to free or liposome loaded single ligand induction levels (Figure 3.15C-D).

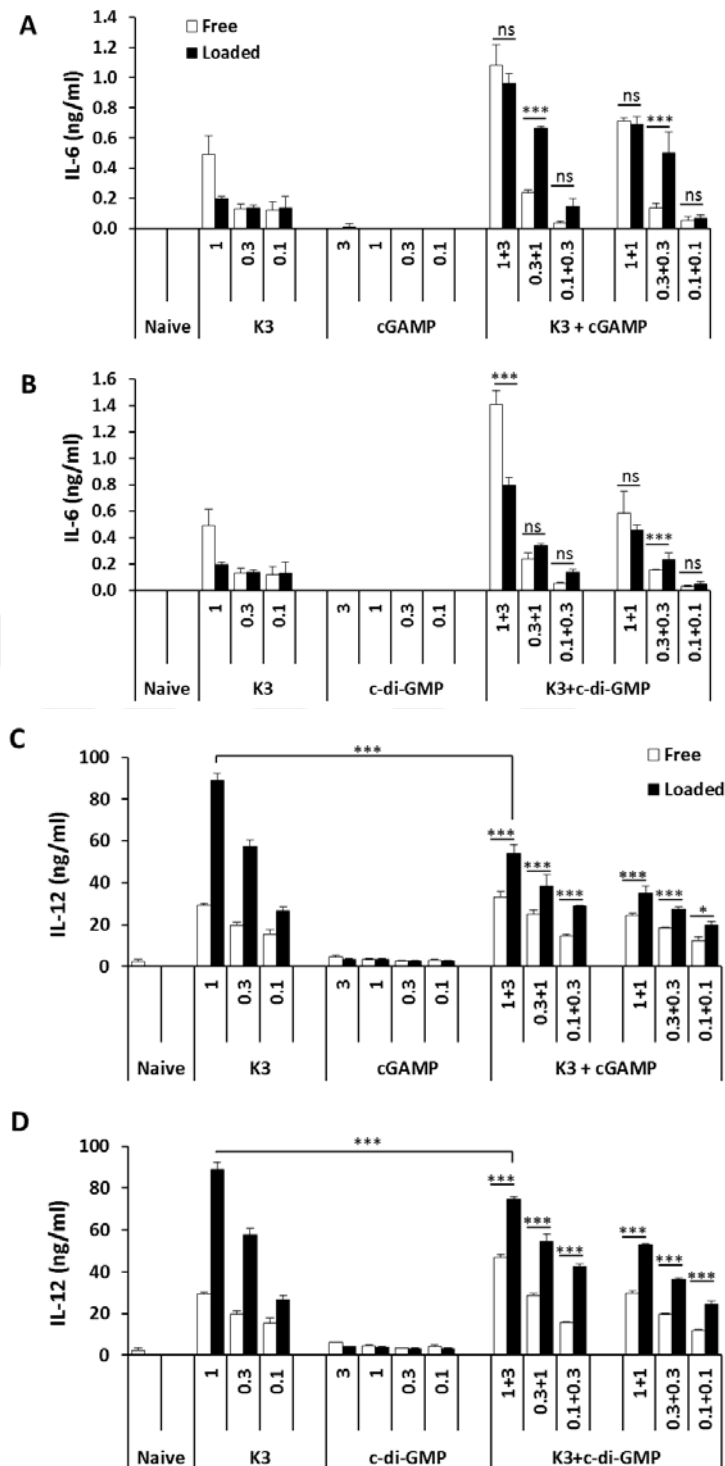


Figure 3.15. IL-6 and IL-12 production from splenocytes stimulated with free or loaded K3 (0-1.0 μ M) and/or cGAMP or c-di-GMP (0-3.0 μ M). Splenocytes were stimulated with free or loaded K3 or cGAMP/ c-di-GMP or their 1:3 or 1:1 combinations for 36h. Supernatants were used to detect IL-6 (A and B) and IL-12 (C-D) by ELISA. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The surprising effect of co-encapsulation of these two ligands within liposomes was demonstrated when type-I and type-II interferon secretions were analyzed. At 1+3 μ M concentrations of combinations of free K3 plus cGAMP or c-di-GMP led barely detectable IFN- γ (0.423 ± 0.009 or 0.144 ± 0.013 ng/ml, respectively) and IFN- α/β (0.325 ± 0.060 or 0.057 ± 0.025 ng/ml, respectively) production compared to single ligands at the same concentration ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test). Interestingly, encapsulating either single cGAMP or c-di-GMP in liposomes showed nearly no effect in the improvement of both IFN inductions.

When K3 and cGAMP/ c-di-GMP co-encapsulated into SSCL at different ratios that no synergistic activation was observed in free single and dual ligands, approximately 10-60 fold more IFN- γ secretion ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test; Figure 3.16A-B) and 4-6 fold more IFN- α/β secretion was obtained compared to free dual ligand combinations ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test; Figure 3.16C-D).

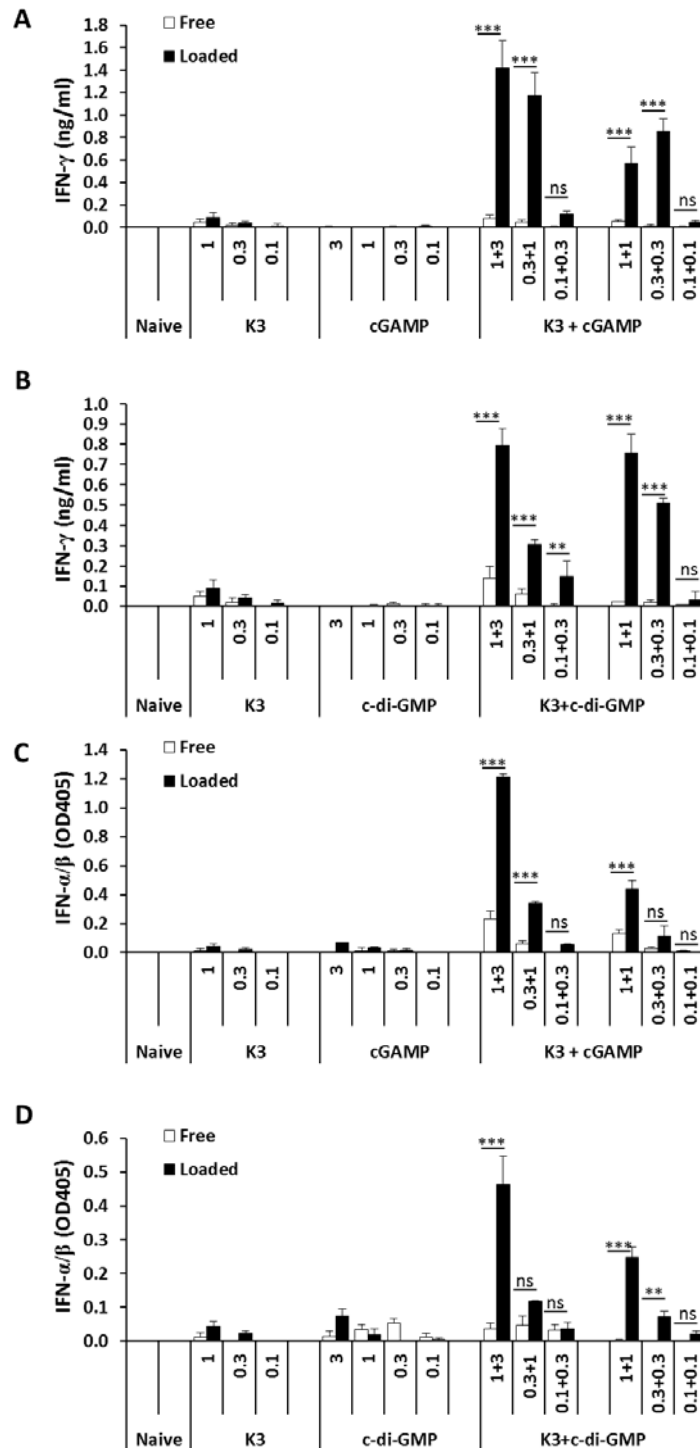


Figure 3.16. Type I and II IFN production from splenocytes stimulated with free or loaded K3 (0-1.0 μ M) and/or cGAMP or c-di-GMP (0-3.0 μ M).

Splenocytes were stimulated with free or loaded K3 or cGAMP/ c-di-GMP or their 1:3 or 1:1 combinations for 36h. Supernatants were used to detect IFN- γ (A and B) and IFN- α/β (C-D) by ELISA and reporter cell line (B16-Blue™ IFN- α/β Cells), respectively. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

APCs interaction with T-cells promotes T-cell differentiation and determines the polarization of immune system together with antigen processing capacity. To investigate the cytokine profile of bone marrow derived dendritic cells (BM-DCs) that were generated by incubating with GM-CSF and IL-4 for 6 days were treated with free or liposome encapsulating K3 or cGAMP/ c-di-GMP or their combination (@ 3:1 ratio, either 0.3 or 1.0 μ M K3 mixed with 1.0 or 3.0 μ M CDNs) for 24 h. Supernatants were used to detect IL-6 and IL-12 via ELISA. Co-encapsulated liposomes, (K3+cGAMP)SSCL and (K3+c-di-GMP)SSCL, increased the cytokine production of ligands when their induction levels were compared with free single and dual ligands and loaded single ligands ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test; Figure 3.17) except the free combination of K3 and c-di-GMP at 1+3 μ M. These results confirmed that incubating CpG ODN, K3 with CDNs within liposomes but not in their free combinations improve synergistic immune activity of BM-DCs.

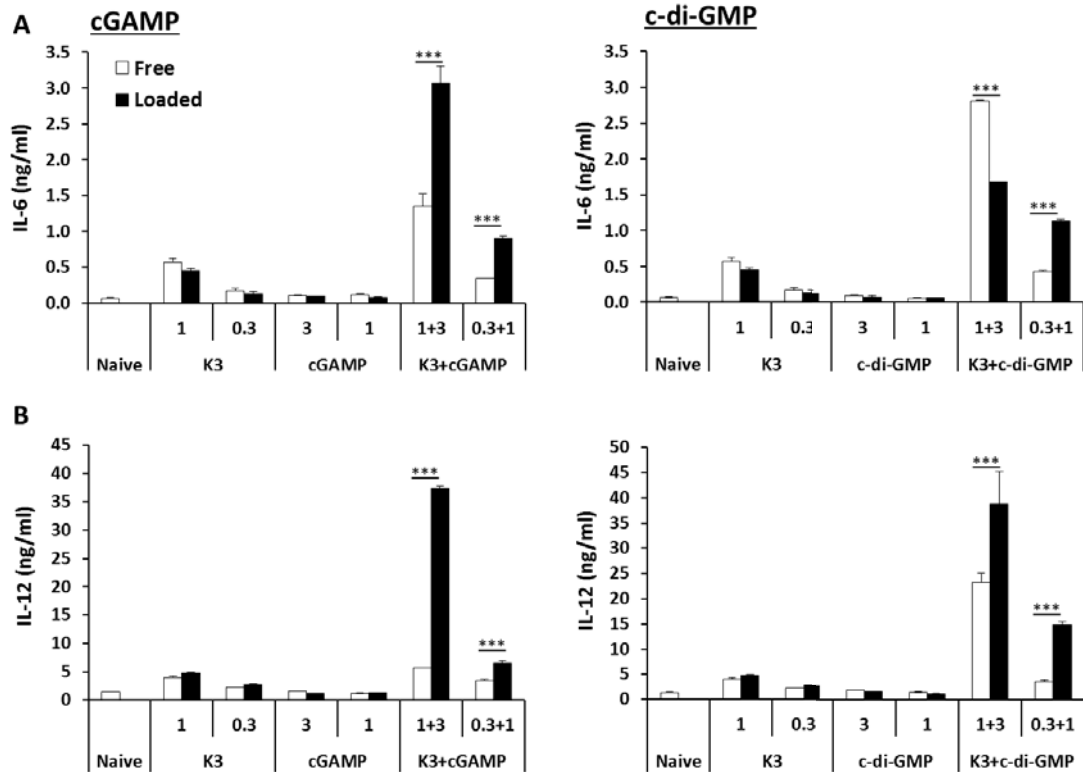


Figure 3.17. IL-6 and IL-12 production from BM-DCs stimulated with free or loaded K3 and/or cGAMP or c-di-GMP.

BM-DCs generated by incubating GM-CSF and IL-4 for 6 days were incubated with free or encapsulated K3 (0.3 and 1.0 μ M) and/or cGAMP or c-di-GMP (1.0 and 3.0 μ M) for 24 h. Supernatants were used to detect IL-6 and IL-12 levels via ELISA. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

In a parallel experiment, bone marrow derived macrophages (BM-DMs) were stimulated with free and encapsulated single dual ligands for 24 h. Polarization of macrophages under the influence of the delivered ligand(s) from M0 phenotype to either M1- or M2-phenotype is important during anti-cancer or adjuvant therapy or during vaccination. M1-type is more inflammatory and M2-type macrophages are more suppressive in character. To that end, we wished to delineate the fate of BM-DMs upon liposome treatment. To evaluate the effect of K3 (@ 1.0 μ M) and cGAMP (either @ 1.0 or @ 3.0 μ M) co-encapsulated liposomes on macrophage polarization, IL-10 and IL-12 levels were detected from supernatants of stimulated

BM-DMs. Cytokine productions (ng/ml) along with IL12/IL10 ratio from treated BM-DMs were presented in Table 3.3. IL12/IL10>1 implicated that a M1-phenotype is dominant whereas IL12/IL10<1 implicated that a M2-phenotype is dominant within the macrophage population upon ligand treatment. Based on the findings presented in Table 3.3, pH-sensitive SSCL co-encapsulating K3 and cGAMP or c-di-GMP secreted higher amounts of IL-12 than IL-10 indicating the development of M1 type macrophages. IL-12/IL-10 ratio was 4 and 4.7 for K3 plus cGAMP at 1:1 and 1:3 μ M ratio, respectively, while it was 2.9 and 4.5 for K3 plus c-di-GMP at 1:1 and 1:3 μ M ratio, respectively.



Table 3.3. Cytokines (ng/ml; mean±SD) produced by BM-DM stimulated with free or liposome loaded K3 (1.0 µM) and/or cGAMP/ c-di-GMP (1.0 and 3.0 µM).

		IL12	IL10	IL12/IL10
K3 1 µM	Free	3.0±0.2	23.7±0.5	0.1
	Loaded	5.9±0.1	9.3±3.6	0.6
cGAMP 3 µM	Free	0.0±0.0	0.0±0.0	0
	Loaded	0.0±0.0	0.0±0.0	0
c-di-GMP 3 µM	Free	0.0±0.0	0.0±0.0	0
	Loaded	0.0±0.0	0.0±0.0	0
K3 + cGAMP 1+3 µM	Free	26.1±3.0	22.5±4.5	1.2
	Loaded	153.1±1.9	38.8±1.0	4.0
K3 + cGAMP 1+1 µM	Free	10.3±0.2	13.8±1.6	0.7
	Loaded	160.4±7.2	34.4±0.4	4.7
K3 + c-di-GMP 3+1 µM	Free	3.6±0.6	0.0±0.0	0
	Loaded	95.8±0.8	21.2±1.2	4.5
K3 + c-di-GMP 3+1 µM	Free	2.6±0.4	9.2±4.6	0.3
	Loaded	42.3±1.3	14.6±0.0	2.9

BM-DM: bone marrow-derived macrophages; cGAMP: cyclic GMP-AMP.

Increased activation of immune response after stimulation with K3 and cGAMP would induce proliferation of immune cells. Besides, toxicity of SSCL on cells should also be explored. To assess this, splenocytes and BM-DMs were incubated with Dojindo kit reagent (CCK-8) for 2h after stimulation either/or with CDNs and K3 CpG ODN. OD was detected at 450 nm by ELISA reader. Relative cell viability was calculated with respect to untreated cells. While cell viability increased in splenocytes stimulated with K3 and K3+cGAMP (1+3 μ M) groups, other groups had no proliferative or toxic effect on splenocytes ($p < 0.05$, one-way ANOVA, Tukey's post-hoc test; Figure 3.18A). In addition, incubation of both free and liposome-loaded K3, K3+cGAMP combinations with BM-DMs significantly increased the relative cell viability (Figure 3.18B, $p < 0.001$) while cGAMP alone formulations had no detrimental or proliferative effect on cells.

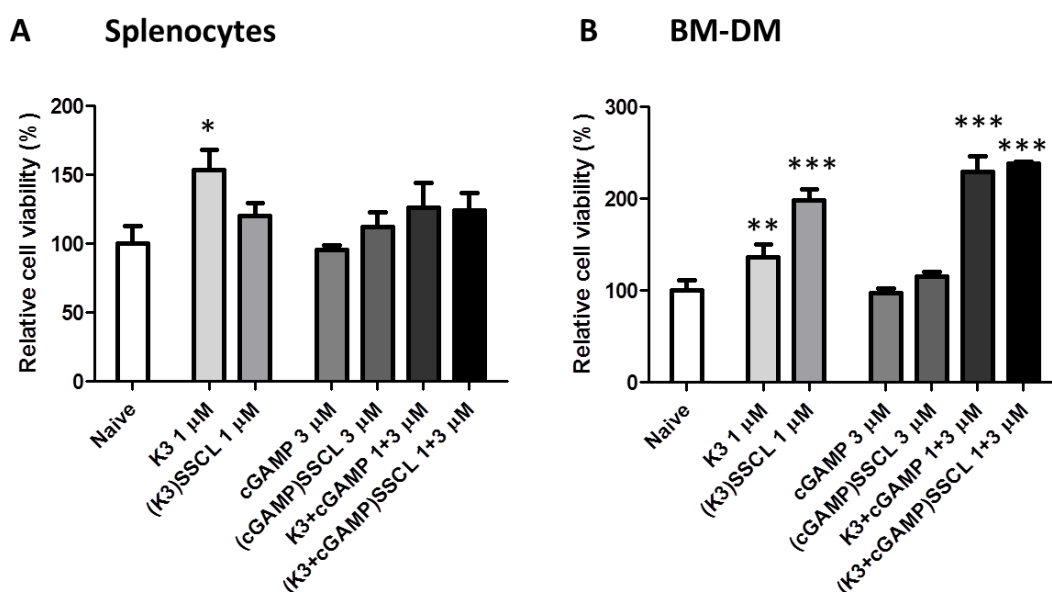


Figure 3.18. Relative cell viability of splenocytes and BM-DMs treated with free and liposome-loaded K3 and cGAMP.

After stimulation, viability of (A) splenocytes and (B) BM-DMs was detected using Cell Counting Kit-8 (CCK-8) and absorbance was measured at 450 nm. Relative cell viability was determined according to naive group. Data were presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test (Naïve vs groups). ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2.2.2 Uptake of STING and TLR9 ligands loaded into liposomes by splenocytes

Liposome protects its cargo and increases cellular uptake of small molecules such as ODNs and CDNs. Previously we showed that immune cells (i.e. spleen cells, BM-DMs or DM-DCs) were significantly activated when they were treated with pH-sensitive SSCL co-encapsulating K3 and cGAMP. To investigate whether elevated cellular uptake of K3 and cGAMP combination is mediated upon encapsulating them within liposomes, splenocytes were incubated either with free or liposome loaded Cy5-labeled K3 and FAM-labeled cGAMP (or c-di-GMP) combinations for 1 h and specific fluorescence positivity was analyzed by flow cytometry (Figure 3.19A-B). In addition, splenocytes were incubated with dual free or co-encapsulated ligands for 24 h to investigate which APCs are internalizing these ligands following staining with PE-labeled anti-CD11b and anti-CD11c antibodies (Figure 3.19C-D).

After stimulation with co-encapsulated K3 and cGAMP, K3 uptake ($39.8 \pm 1.6\%$) was higher than free dual ligands ($13.4 \pm 0.4\%$) but less than (K3)SSCL ($52.9 \pm 7.5\%$). In c-di-GMP combination, it was $59.5 \pm 6.2\%$ in co-encapsulated K3+c-di-GMP and $11.8 \pm 1.5\%$ in free dual ligands ($p < 0.05$, one-way ANOVA, Tukey's post-hoc test, 3.19A). However, cellular uptake of single loaded K3 was higher than K3 plus c-di-GMP co-encapsulated liposomes. The similar trend was observed in cGAMP uptake by splenocytes. After stimulation with single loaded liposome, cGAMP uptake was $17.8 \pm 5.3\%$ and it was $11.0 \pm 2\%$ in K3 plus cGAMP co-encapsulated SSCL while it was $0.5 \pm 0.02\%$ in free dual ligands. However, c-di-GMP uptake increased when encapsulated into liposome ($5.3 \pm 0.6\%$) and combined with K3 ($16.3 \pm 4.1\%$) ($p < 0.05$, one-way ANOVA, Tukey's post-hoc test; Figure 3.19B).

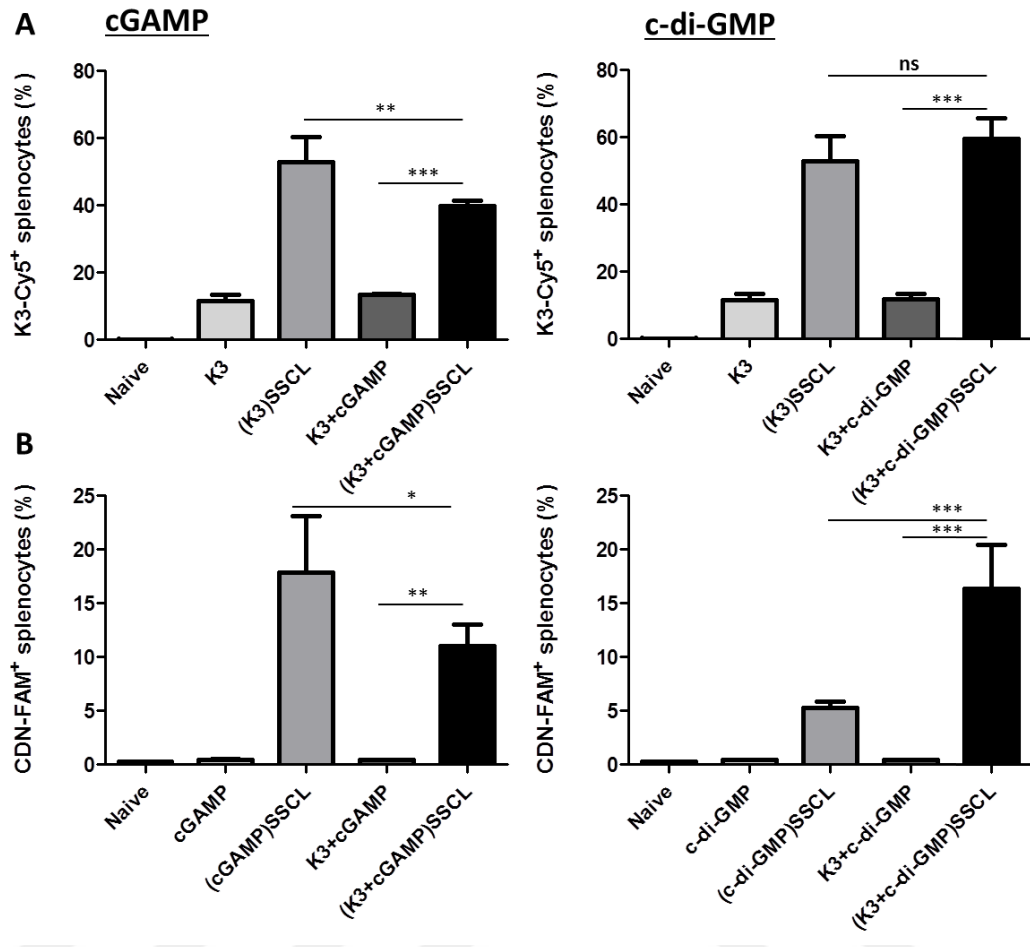


Figure 3.19. Cellular uptake and binding of liposomes co-encapsulating K3 plus cGAMP/ c-di-GMP by splenocytes.

Splenocytes were incubated with free or liposome-encapsulated Cy5-labeled K3 (1 μ M) and/or FAM-labeled cGAMP/ c-di-GMP (3 μ M) for 1h at 37 °C. (A) K3 and (B) cGAMP/ c-di-GMP uptake was analyzed using flow cytometry. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

3.3 *In vivo* Applications of Liposomal Constructs

We wished to harness the superior feature of loading different ligand(s) within liposomes. For this, we chose four *in vivo* model systems:

- a) Effect of liposomal adjuvant as a vaccine adjuvant for improved FMDV vaccine potency.
- b) Development of *H. pylori* liposomal vaccine harboring TLR9 adjuvants
- c) Development of preventive cancer vaccine against thymoma and melanoma tumors co-encapsulating TLR3 and TLR9 ligands.
- d) Development of therapeutic vaccine against established melanoma tumors involving i) TLR3 plus TLR9 ligands or ii) TLR9 plus CDN ligands

Unifying feature of the following studies was to demonstrate that when the activities of the ligand(s) of interest were identified *in vitro* assays, it is significantly efficient to test their *in vivo* potency within a liposomal carrier system. Moreover, where necessary, antigen of interest could be co-loaded within this depot delivery system. Boosting antigen dependent humoral and cell mediated immunity for better host protection following therapy could be achieved.

3.3.1 Adjuvant effects of liposomal Foot and Mouth Disease Virus vaccine formulations co-encapsulating antigen and CpG ODN

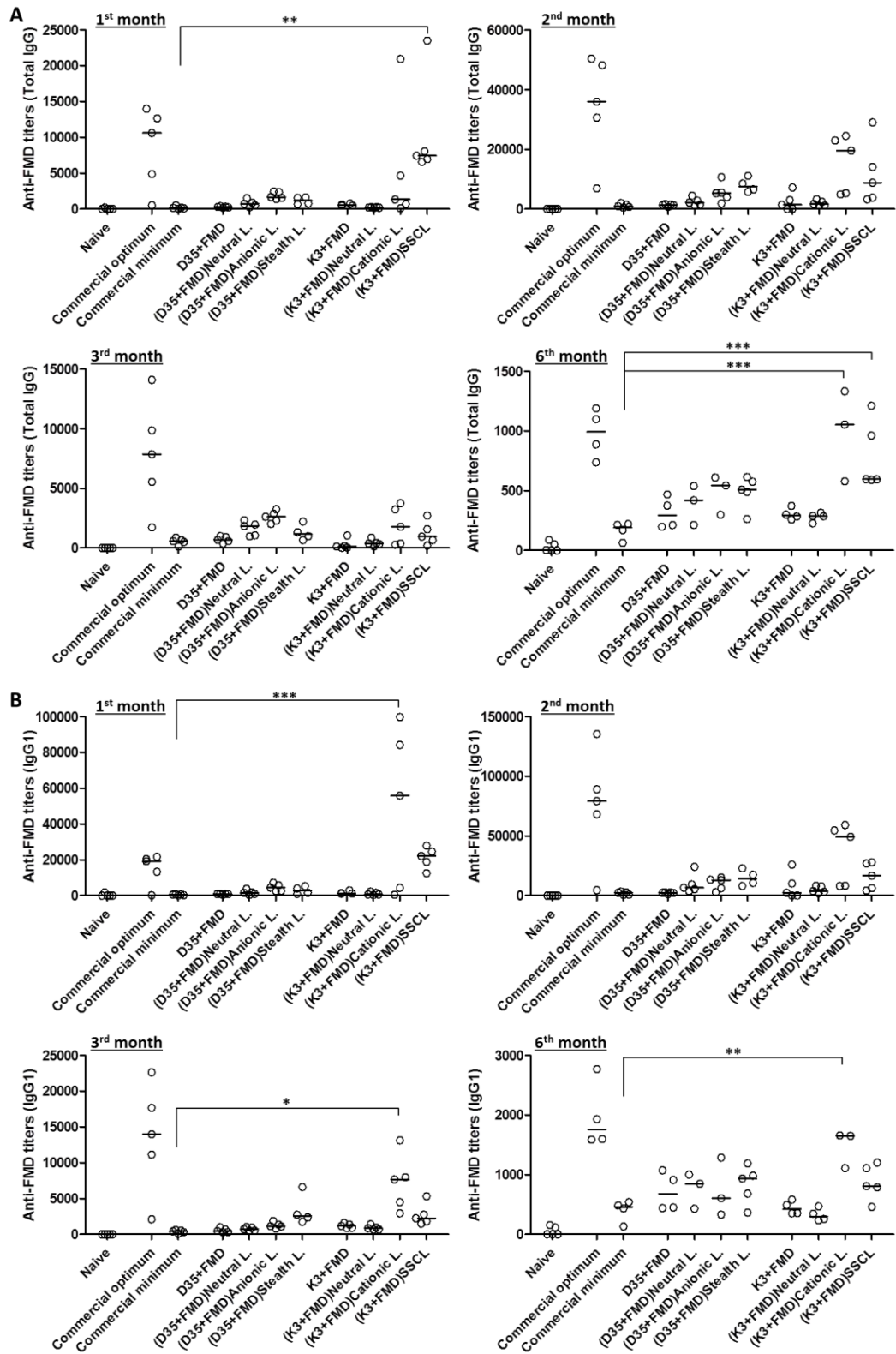
In Turkey, Foot and mouth disease virus (FMDV) is an epidemic among ovine and bovine livestock. The local FMDV vaccine contains inactivated virus extract. The vaccine potency, however, is limited requiring repeated immunization. Moreover, the total vaccine Ag extracted by SAP institute permits preparation of only 11million doses for the nationwide market. The total annual vaccine dose, however, is around 45 million doses. With the total antigen at hand it is impossible to vaccinate all

livestock. We import the rest of the vaccine from outside sources. Around 180 million TL is spent every year on this vaccine alone.

More protective, long lasting formulations would help protect nationwide livestock. Importantly, we will spare millions of dollars for other expenses. Our aim was to design a liposomal FMD vaccine using liposomes co-encapsulating TLR ligands and FMD antigen, where we reduce Ag dose and improve the number of bottled vaccine without increasing the required antigen amount. For this we diluted the antigen up to 6 fold and used within liposomal CpG ODN formulation as an improved FMDV vaccine.

To find out the most effective liposomal formulation, mice (BALB/c; n=5) were injected twice with two week intervals with commercial vaccine containing low dose (LD; 0.5 µg antigen/mice) and optimum dose (OD; 3 µg antigen/mice) antigen, and free, and liposomes (1 µmole/mice) encapsulating TLR9 (D- or K-ODNs; 5 µg/mice) ligands together with FMD serotype O antigen (0.5 µg/mice). Sera were collected from tail vein of mice each month after first injection and IgG (Total IgG, IgG1, and IgG2a) levels in serum were detected via IgG ELISA.

We found that cationic liposome and SSCL formulations increased total IgG titers more than commercial vaccine at minimum dose (i.e. 6 fold diluted FMDV Ag dose) and it was at similar levels with commercial vaccine optimum that had 6 fold more antigens in it at the end of the 6th month (Figure 3.20A). Besides, IgG1 and IgG2a levels were higher at cationic liposomes co-encapsulating K3 and 6 fold less FMDV antigen. ($p < 0.05$, one-way ANOVA, Tukey's post-hoc test, Figure 3.20B and C).



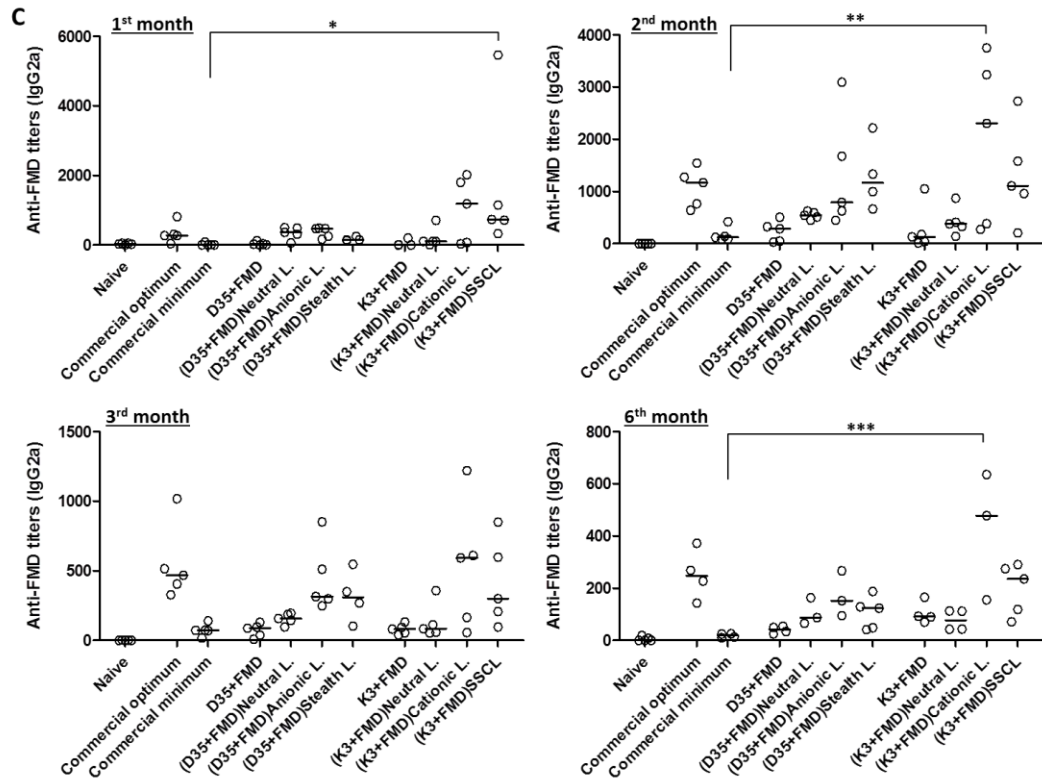


Figure 3.20. *In vivo* antibody response of mice immunized with commercial vaccine or free or liposome loaded D/K-ODN together with FMD serotype O antigen.

BALB/c (n=5) mice were immunized twice with two week interval by i.p. injection with commercial FMD vaccine (3 and 0.5 µg/mice) or free or liposome loaded D- or K-ODN (5 µg/mice) together with FMD serotype O antigen (0.5 µg). Sera were collected from tail vein of mice each month after first injection. Serum (A) Total IgG, (B) IgG1 and (C) IgG2a were detected by ELISA. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. *p<0.05; **p<0.01; ***p<0.001.

Liposomes not only acted as a depot system but also spared antigen and adjuvant. Furthermore, it provided longer immunostimulatory effect upon administration and led to a more pronounced FMDV Ag-specific humoral immunity. Co-encapsulating K-type or D-type CpG ODN together with 6 fold less FMDV antigen elicited immune response more than the commercial FMD vaccine. Therefore, this approach could allow more vaccine vials to be produced with the same Ag amount at hand.

In other words, if Turkey produces a total of 10 million to Ag dose formulate, in the light of our findings this Ag stock could be formulated into 60 million doses of FMDV vaccine with the addition of proper CpG ODN based adjuvant, an amount much higher than the national vaccine reserve.

3.3.2 Adjuvant effects of liposomal TLR ligands against *Helicobacter pylori* (SS1)

H. pylori (Hp) is a gram negative, spiral microaerophilic bacterium and responsible for gastritis, peptic ulceration, and gastric cancer (70% of all gastric cancers) [174]. It infects 50% of the global population, 10% develop peptic ulcers, 1% gastric malignancies. Although, the host elevates *H. pylori* specific IgG and IgA eradication is rarely observed and might even impair host inflammatory responses and facilitate chronic bacterial colonization. Vaccines could only protect transiently, there was no complete *H. pylori* eradication and even with vaccination reoccurrence could not be prevented (i.e. recolonization of *H. pylori*). Our aim was to design a liposomal vaccine formulated with strong Th1 dominant adjuvants. Therefore, we co-encapsulated TLR ligands and *H. pylori* SS1 extract against *H. pylori* infection. Of important note in these studies, D-type CpG ODN was encapsulated in Anionic liposomes and as mentioned before K-type CpG ODN was loaded within SSCL.

C57/Bl6 mice were injected three times with a week interval with liposomal and free TLR9 (D- and K-type CpG ODNs) ligands. Then 4 weeks after first injection, three of immunized mice/group was infected with *helicobacter pylori* SS1 strain using a feeding needle. Animals were killed and stomach and spleen were collected after two weeks of pathogen inoculation. Sera were collected from tail vein after second week post-injection. Serum (i) Total IgG, (ii) IgG1 and (iii) IgG2c were detected by ELISA and (iv) IgG2c/IgG1 ratio was calculated. Splenocytes were incubated with SS1 extract or medium alone for 24h and supernatants were used in IFN- γ ELISA. Ag-specific IL-17 and IFN- γ ELISPOT were also performed using splenocytes incubated with SS1 extract or medium. Stomachs were used to detect bacterial colony formation and IgA via ELISA.

After two weeks of first injection, Total IgG and IgG2c levels in sera of mice immunized with liposomes loaded with D-ODN or K-ODN together with *H. pylori* SS1 extract was higher than mice immunized with non-encapsulated ligands ($p<0.001$, one-way ANOVA, Tukey's post-hoc test; Figure 3.21A-B). The IgG1 levels were lower than IgG2c levels in all groups (Figure 3.21C). More importantly, the IgG2c/IgG1 was found to be >1 , implying that a Th1-biased anti-SS1 immune response was raised in liposome-loaded D- and K-ODN than free ligands (Figure 3.21D).

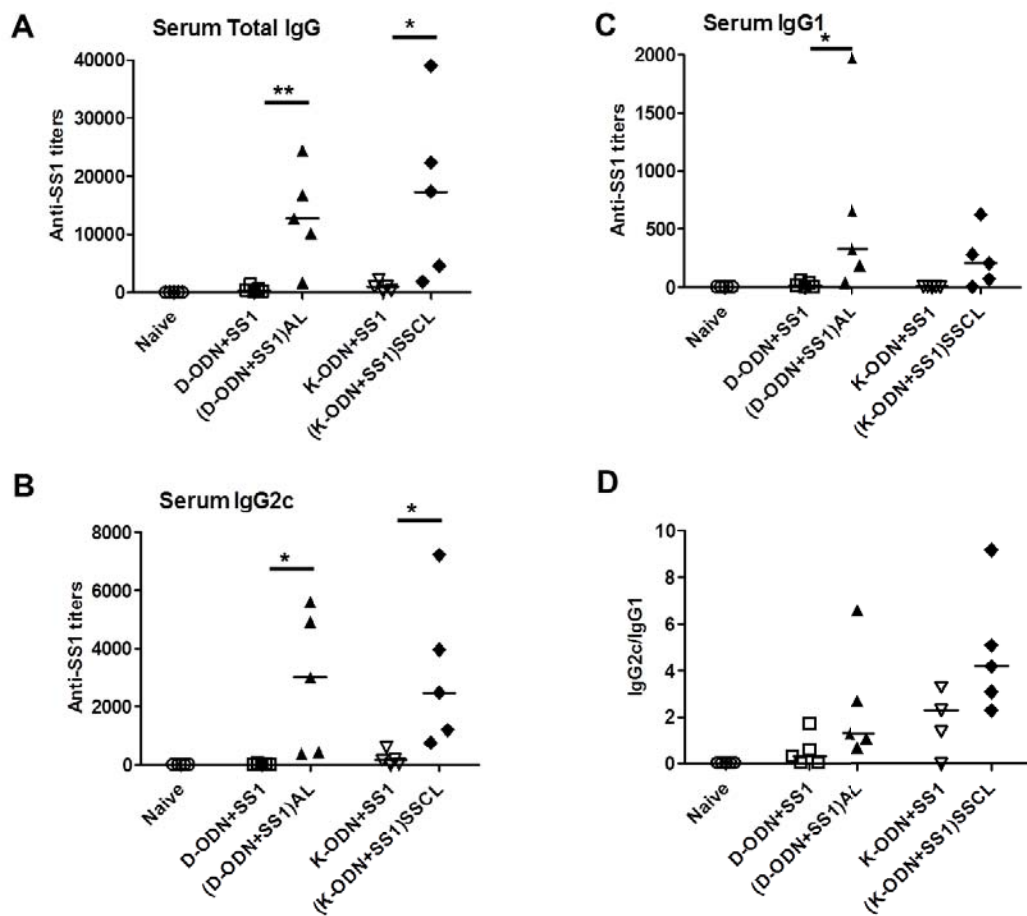


Figure 3.21. *In vivo* antibody response of mice immunized with free or liposome loaded D/K-ODN against *H. pylori* SS1 extract.

C57BL/6 (n=5) mice were immunized three times with a week interval by i.p. injection with free or liposome loaded D- or K-ODN (15 μ g/animal) together with *helicobacter pylori* SS1 extract. Sera were collected from tail vein of mice after second week post-injection. Serum (A) Total IgG, (B) IgG1 and (C) IgG2c were detected by ELISA and (D) IgG2c over IgG1 ratio was calculated. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

The increase in the antibody response suggested that liposome-loaded ODNs were more effective than free ligands. However, the protection potential was not correlated with antibody response. Therefore, we challenged and fed mice with *H. pylori* SS1 cells after a month of first injection. Following 15 days, mice were euthanized by cervical dislocation and organs (spleen and stomach) were collected. The colony formation unit was calculated after spreading stomach tissue in agar plate. The CFU was lower in liposome-loaded K-ODN than other groups (Figure 3.22).

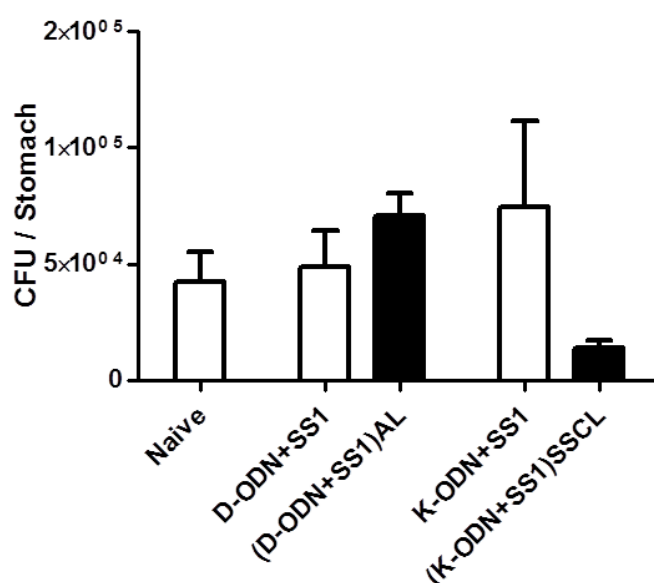


Figure 3.22. Infection of immunized mice with *H. pylori* SS1 strain.

4th week after first immunization, C57BL/6 mice (n=3) were challenged with *H. pylori*. Gastric bacterial colony formation was determined by colony count assay from stomach tissue after euthanizing mice 15 days post-infection.

Neutralizing antibody relevant to mucosal sites is IgA. Therefore, we next investigated the anti-SS1 specific serum IgA levels before and after challenge with bacteria. There was no difference between IgA levels of mice immunized either with free and liposome-loaded K- or D-ODNs before challenge. Even though, the IgA levels in sera were similar in all groups before and uninfected groups, in infected mice, IgA levels increased in liposome-loaded K- and D-ODN formulations when we compared with levels in mice immunized with free formulations. Additionally, IgA

levels in protein extracted from stomach was higher in mice immunized with K-ODN loaded liposomes after infection (Figure 3.23).

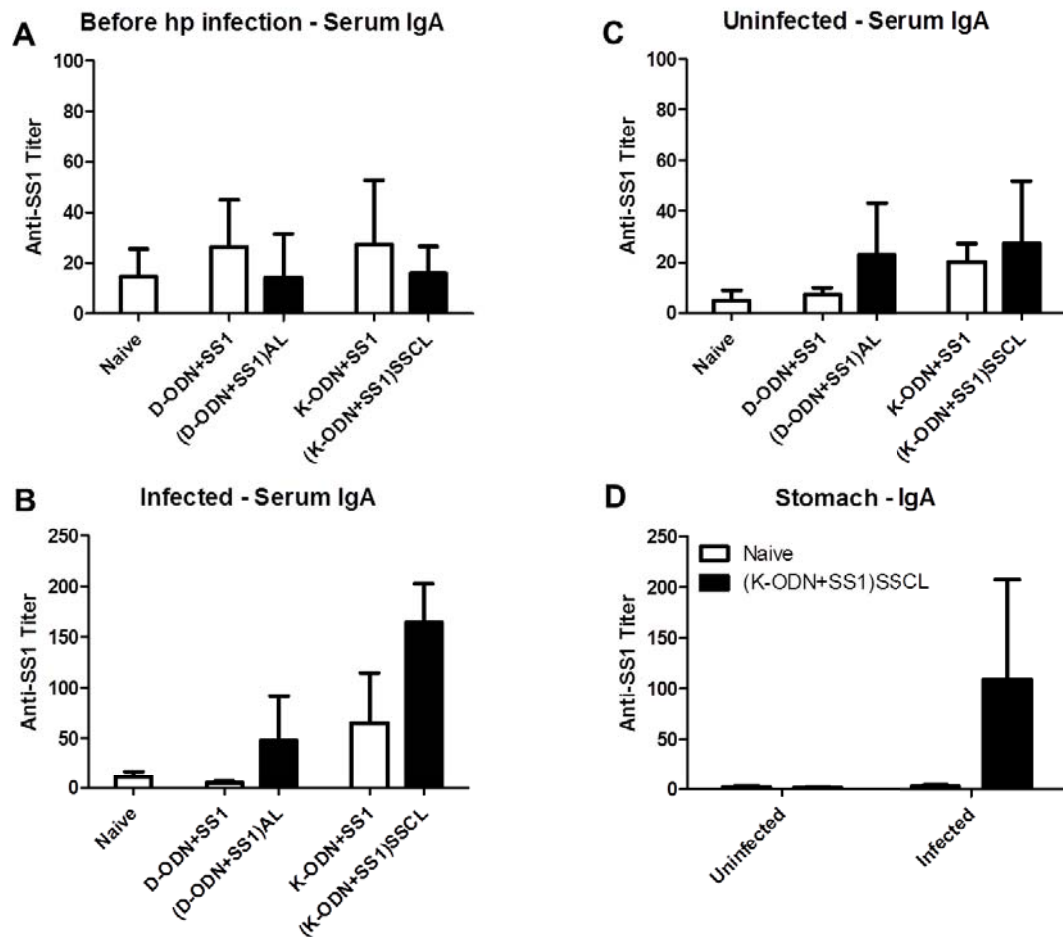


Figure 3.23. Serum and stomach IgA production of immunized mice before and after challenged with *H. pylori*.

Serum IgA were detected from collected sera from tail vein of immunized mice (A) before and (B,C) after challenged with *H. pylori* SS1. After euthanizing immunized C57BL/6 mice, (D) stomach IgA level was determined via ELISA from protein isolated from stomach tissues.

The cellular immune response that provided the protection from helicobacter infection after immunized with liposome-loaded K3 was investigated by incubating splenocytes with SS1 extract to detect antigen specific IL-17 and IFN- γ levels for 24h. IL-17 and IFN- γ levels were elevated especially in liposome-loaded K3 (Figure 3.24). In conclusion, K3-ODN encapsulated liposomes together with SS1 extract protected mice from helicobacter infection by increasing sera IgG2c/IgG1 ratio, IgA

in sera and stomach and activating Th1 and Th17 cell populations as increasing antigen specific IL-17 and IFN- γ levels.

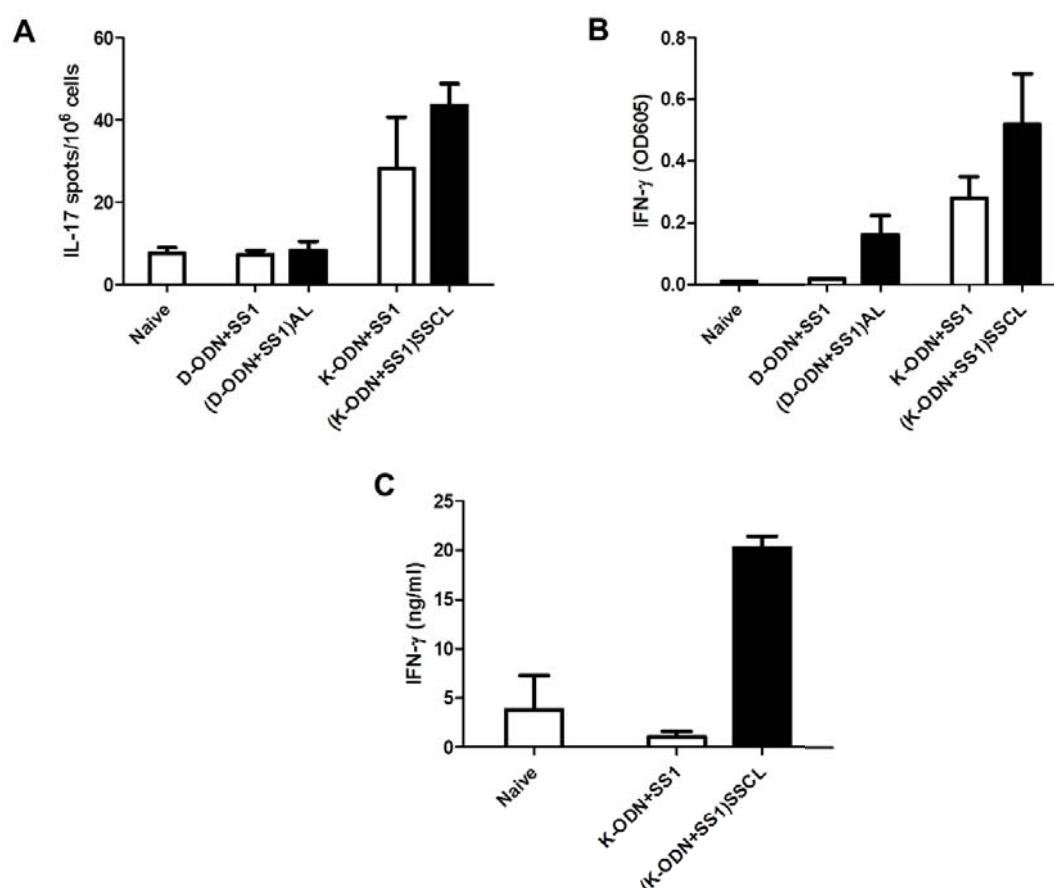


Figure 3.24. Helicobacter specific immune responses induced by immunization with free or liposome loaded D or K-ODN after challenged with *H. pylori* SS1 feeding.

Splenocytes isolated from immunized and challenged mice were used to determine (A) IL-17 and (B) IFN- γ via ELISPOT and (C) ELISA assays by incubating with helicobacter extract for 24 h.

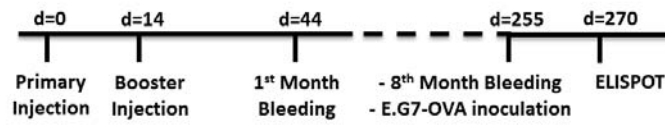
3.3.3 Immunization with liposomes co-encapsulating TLR3 and TLR9 ligands against model antigen OVA

In vitro studies were discussed in Section 3.2.1. After observing that poly(I:C) and CpG ODN co-encapsulated within liposomes boosted Th1-biased immune activation and improved APC function (Section 3.2.1.), we investigated the potential of this

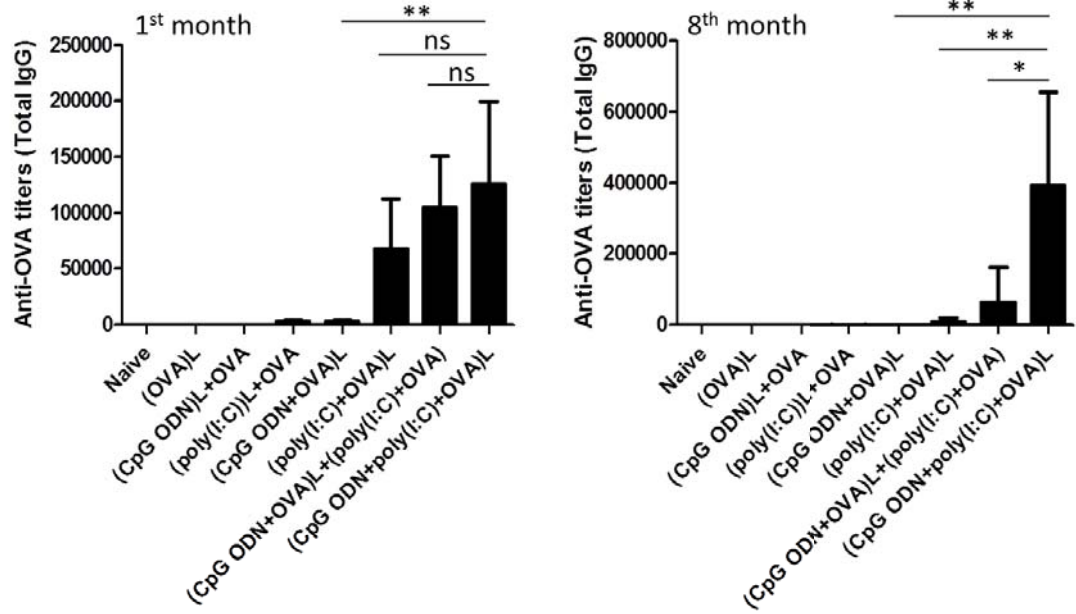
system as a preventive vaccine (Figure 3.25A). C57/Bl6 mice were injected i.p. twice with CpG ODN (10 µg/mice) and/or poly(I:C) (10 µg/mice) and/or OVA (5 µg/mice) formulations with two wks. interval. Sera were collected from animals following tail bleedings. Anti-OVA IgG levels were detected by end-point ELISA. Serum titrations for total IgG, IgG1 and IgG2c subtypes were analyzed following 1st and the 8th month post-booster injections (Figure 3.25B-D). Co-encapsulation of OVA and TLR ligands, except (CpG ODN+OVA)L, gave the highest IgG titers at the end of eight months post-booster injection. At the end of eight months of antibody titer, (CpG ODN+poly(I:C)+OVA)L group gave the highest IgG2c titers with lower IgG1 titer.

In conclusion, co-encapsulation of antigen and dual adjuvants provided antigen-specific long-lasting IgG production.

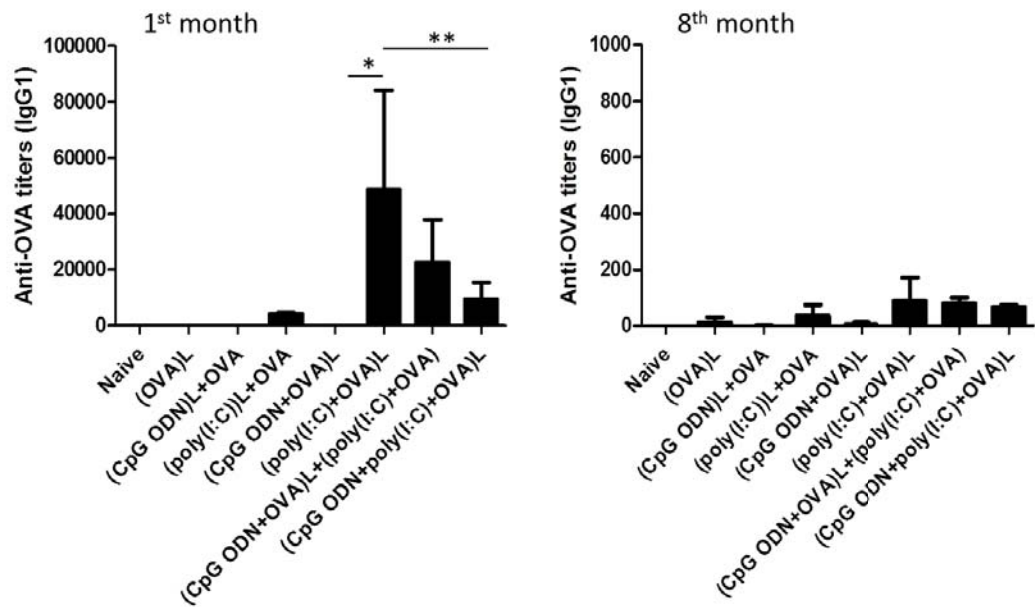
A



B



C



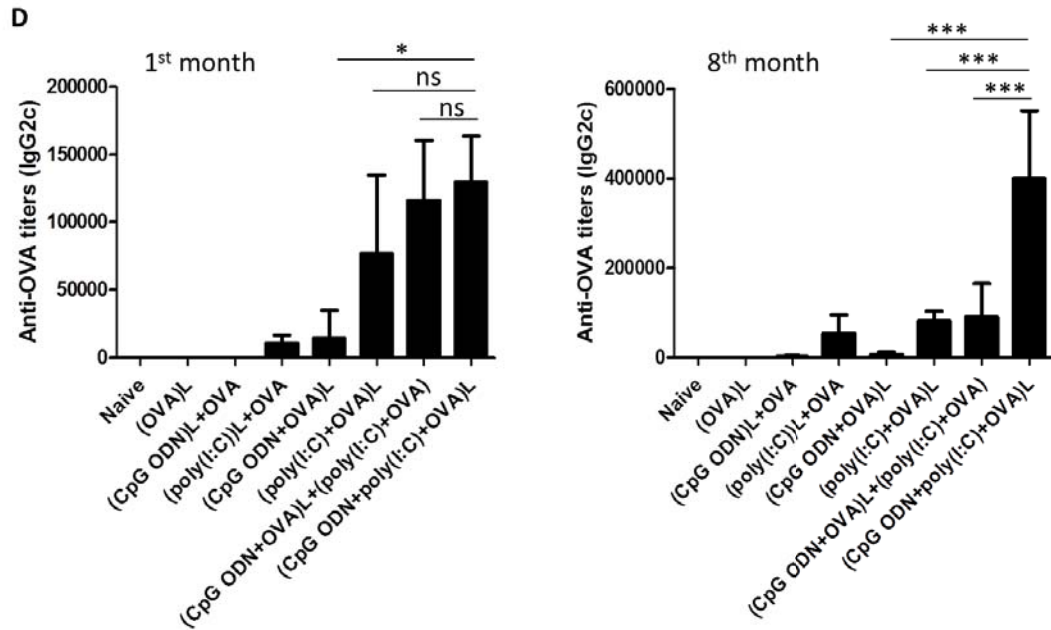


Figure 3.25. Immunization with liposomes co-encapsulating CpG ODN, poly(I:C), and/or OVA promotes Th-1 biased immunity.

(A) Immunization schedule. C57BL/6 female mice (8/group) were immunized twice with 14 day intervals with liposome encapsulated CpG ODN (10 μ g/animal) and/or poly(I:C) (10 μ g/animal) or their combinations with OVA (5 μ g/animal) and sera was collected from tail vein 1st and 8th month post-booster injection. (B) Diluted sera were used for OVA specific (B) total IgG, (C) IgG1 and (D) IgG2c by end-point ELISA (mean $\pm$ SD). Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p <0.05; ** p <0.01; *** p <0.001.

3.3.4 Cancer vaccine studies

3.3.4.1 Vaccination of C57BL/6 mice with TLR3 and TLR9 against thymoma

We then sought whether OVA immune animals could effectively control or eradicate tumor inoculation. At the end of eight month post-booster injection, immunized mice were s.c. inoculated with 4×10^6 E.G7 cells (EL4 cells transduced to express OVA) and tumor development were followed for 14 days. The individual tumor development was followed daily with electronic caliper. In all animals that were immunized with saline, tumor formation was observed 4 days post-E.G7 inoculation.

The median of tumors in naive group reached 402.5 mm³., the tumor sizes measured at day 14 was presented in Figure 3.26. Animals immunized with liposomal formulations showed reduced tumor sizes. After challenge, mice immunized with both CpG ODN and poly(I:C) gave 75% (n=6/8) tumor free animals at the end of 14 days. All groups were significantly different than naive group (one-way ANOVA, $p < 0.01$).

OVA specific CD8⁺ memory T-cells are one of the major contributing cells to eliminate OVA-expressing tumor cells. The tumor specific CD8⁺ T-cell numbers were analyzed by IFN- γ ELISPOT assay. The splenocytes from immunized animals were treated with MHC-I specific OVA epitope (SIINFEKL) for 18h. The data revealed that co-administration of ligands plus OVA to mice either with separately encapsulated or co-encapsulated liposomes significantly increased IFN- γ ⁺ CD8⁺ T cell numbers (Fig. 3.26B). (CpG ODN+poly(I:C)+OVA)L vaccine treated animals generated similarly higher CD8⁺ memory T-cells compared to (CpG ODN+OVA)L+ (poly(I:C)+OVA)L group.

Collectively, our findings implied that a combination of poly(I:C) and CpG ODN adjuvants in liposomal formulations induced stronger and persistent humoral and cellular anti-OVA specific immune response sufficient to eradicate tumors.

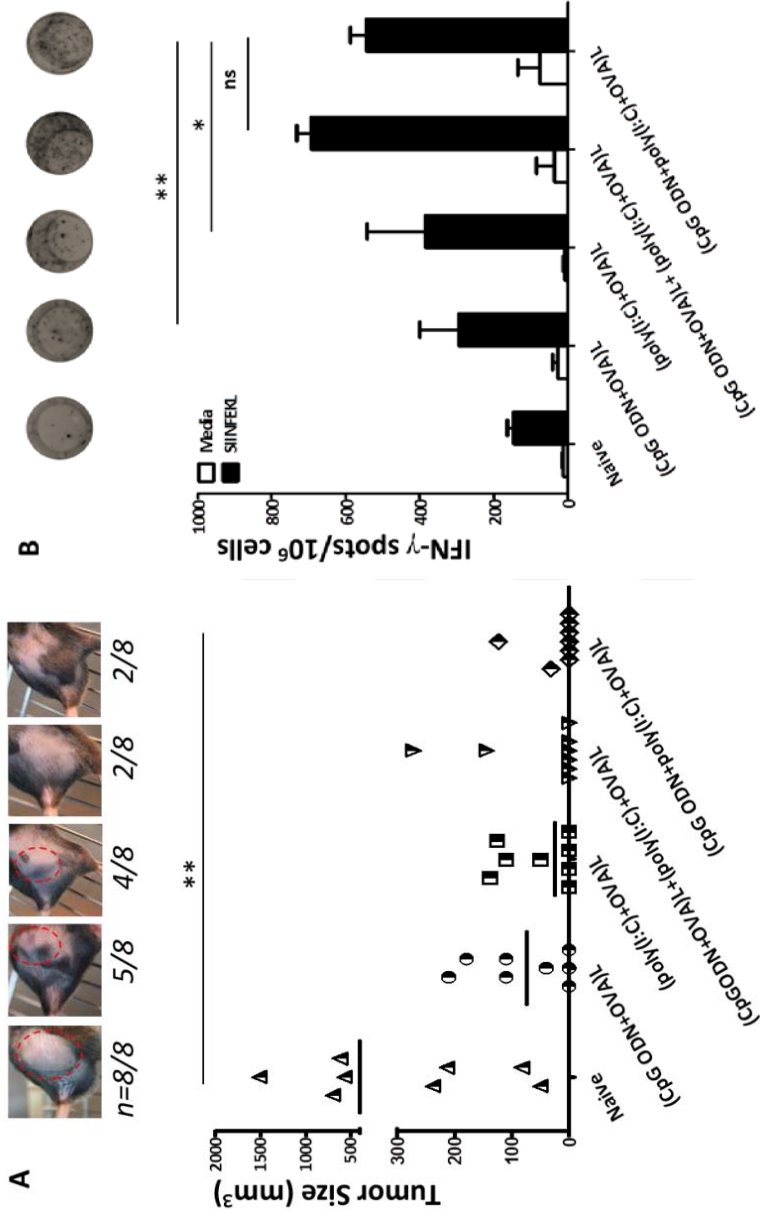


Figure 3.26. Protective effect of vaccination with liposomes co-encapsulating CpG ODN and/or poly(I:C) together with OVA against OVA expressing E.G7 thymoma tumor cell challenge.

Following 8th month, immunized C57BL/6 mice with liposomes encapsulating CpG ODN and/or poly(I:C) or their combinations together with OVA were inoculated with OVA expressing E.G7 thymoma cells (4 x 10⁶ cells/animal). (A) Tumor volume was measured daily for a duration of 14 days by caliper after observing palpable tumor. (B) At the end of tumor follow-up mice were euthanized and splenocytes were isolated and IFN-γ⁺ CD8⁺ T cells were detected stimulating serially diluted splenocytes with OVA 257-264 peptide (SIINFEKL) for 18 h by ELISPOT method. Data were presented as mean±SD. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; *p<0.05; **p<0.01.

3.4 Application of liposomal CpG ODN formulations as an immunotherapeutic agent

3.4.1 Therapeutic vaccine study with liposomes co-encapsulating CpG ODN and poly(I:C) against established melanoma tumor

TLR ligands can be used in cancer treatment as immunotherapeutic agents. To investigate the effect of co-encapsulated CpG ODN and poly(I:C) formulation in the treatment of tumor, melanoma model, B16-OVA (1×10^6) cells, were established on the right flank of C57BL/6 mice ($n=5$ / group) by s.c. injection. When tumor size reached to $\sim 500 \text{ mm}^3$, one group of mice were injected with CpG ODN and poly(I:C) co-encapsulated liposomes ($50 \text{ }\mu\text{g/dose}$) either together with OVA ($15 \text{ }\mu\text{g/dose}$; on days 2 and 6) or as such (on days 0 and 4). The tumor size was recorded each day after measuring with electronic caliper.

The tumor size was significantly higher in naive, untreated mice, than in mice treated with (CpG ODN+poly(I:C))L (Student's t-test, $p=0.0134$; Figure 3.27A). The tumor weight of naive mice was larger than treated mice (Figure 3.27B).

After treatment, humoral response of mice was investigated by analyzing OVA-specific IgG titers in serum. Antigen specific total IgG, IgG1 and IgG2c levels were higher in sera of mice treated with CpG ODN plus poly(I:C) co-encapsulated liposomes (Student's t-test, $p=0.0024$, $p=0.0070$, $p=0.0040$, respectively; Figure 3.27C).

After euthanizing mice, splenocytes were incubated with SIINFEKL and OVA to determine IFN- γ and IL-4 secreting antigen-specific CD8⁺ T cells via ELISPOT. While IFN- γ producing antigen-specific CD8⁺ T cells increased in mice treated with co-encapsulated liposomes (Student's t-test, $p=0.0034$), IL-4 producing cells were lower than naïve group (Student's t-test, $p=0.0047$; Figure 3.27D).

We have shown that, in addition to long-term protective property of co-encapsulated CpG ODN plus poly(I:C) in liposomes acted as a preventive cancer vaccine (as

presented in Section 3.3.3), the therapy of established tumors in a therapeutic vaccine model of very large tumors ($\sim 500 \text{ mm}^3$), liposomes significantly regressed the tumor burden of mice, even though the treatment was started at a very late time point following melanoma tumor inoculation.

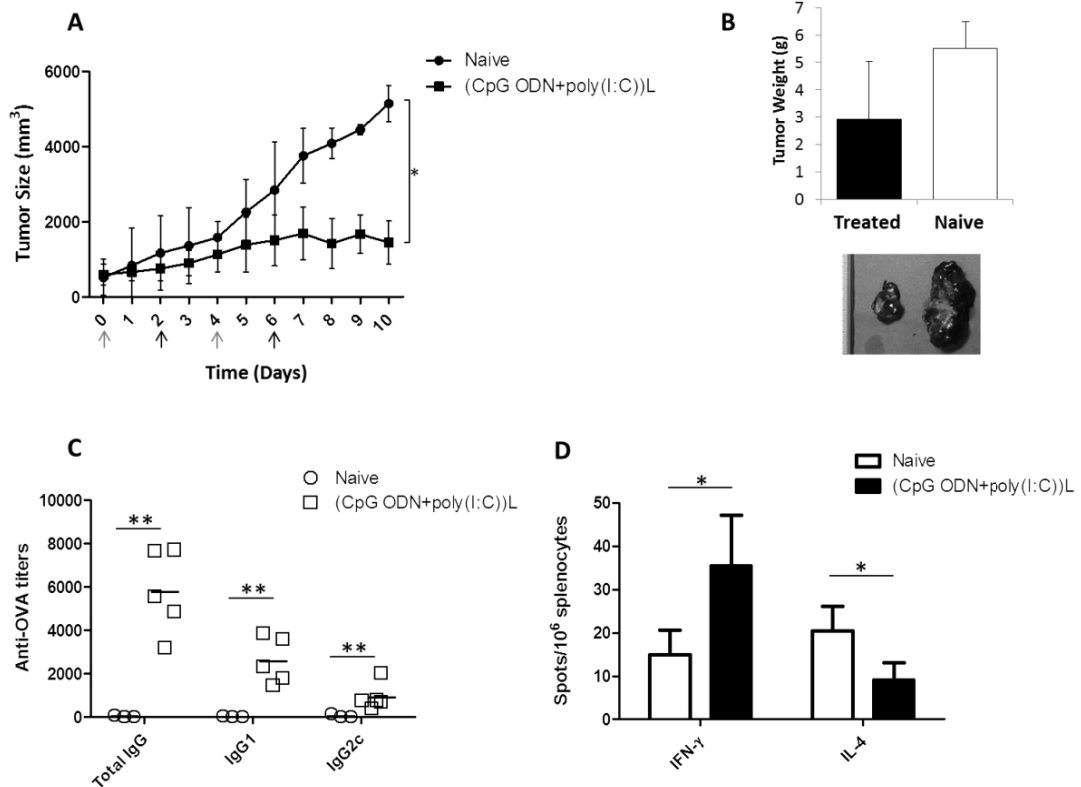


Figure 3.27. CpG ODN and poly(I:C) co-encapsulated liposome in tumor immunotherapy.

(A) C57BL/6 mice ($n=5/\text{group}$) were inoculated subcutaneously with B16-OVA ($1 \times 10^6/\text{mice}$). When tumor volume was around 500 mm^3 , they were treated twice (@ 0 and 4d) with co-encapsulated CpG ODN plus poly(I:C) ($50 \mu\text{g}/\text{dose}$) and therapy was repeated together with OVA ($15 \mu\text{g}/\text{dose}$; on days 2 and 6, post-therapy date). (B) At the end of the experiment, tumors were isolated and weighted and photographed. (C) Anti-OVA specific IgGs (total IgG, IgG1 and IgG2c) were determined via ELISA using collected sera. (D) $\text{IFN}\gamma^+\text{CD8}^+$ spots and IL4^+ splenocytes were determined incubating splenocytes with SIINFEKL and OVA, respectively for 24 h and using ELISPOT assay. Statistical significance was determined by Student's t-test; $*p<0.05$, $**p<0.01$.

3.4.2 Therapeutic vaccine study with pH sensitive liposomes encapsulating cGAMP and K3 ligands against established melanoma tumor

Synergy between CDNs and ODNs demonstrated that dual administration of ligands onto splenocytes increased type-I and type-II IFNs when encapsulated into pH-sensitive liposomes (mentioned in Section 3.2.2). In addition, we have demonstrated that pH sensitive liposome encapsulation of these ligands yielded significant increase in the cytokine secretion from APCs (i.e. from BM-DCs and BM-DMs). Overall, cGAMP was more promising than c-di-GMP when we considered both mice and human results. Therefore, we used cGAMP and K3 combination to assess their *in vivo* potential in the treatment of established tumor as an immunotherapeutic agent together with antigen.

First, we inoculated B16-OVA melanoma cells (1×10^6) into right flank of mice. Tumor size was measured with electronic caliper. When it was around $\sim 100 \text{ mm}^3$, five mice per group were treated either with free or with liposome co-encapsulated K3 (5 $\mu\text{g}/\text{dose}$) plus cGAMP (1.7 $\mu\text{g}/\text{dose}$) together with OVA (5 $\mu\text{g}/\text{dose}$) by i.p. administration on days 7 and 9 (single doses of each molecules), and 12 and 14 (double doses of each molecules). Following a week post-first treatment (at day 14), a significant drop was observed in the tumor growth rate in mice treated with liposomes. After this reduction, tumor volume was retained at the same size until the end of the experiment. At the end of the experiment (at day 18), 80% reduction in the size of tumor was observed in co-encapsulated group, (K3+cGAMP+OVA)SSCL, when tumor sizes were compared to naïve (untreated) or free ligands, K3+cGAMP+OVA, treated mice (two-way ANOVA, Bonferroni test, $p < 0.05$). Tumor size of mice treated with free ligands and naïve group was almost same (Figure 3.28). At the end of the experiment, tumor and spleen were collected, weighted and photographed. Tumor weight was lower in SSCL group. Spleen weight and size was increased in SSCL group than free combinations and naïve mice

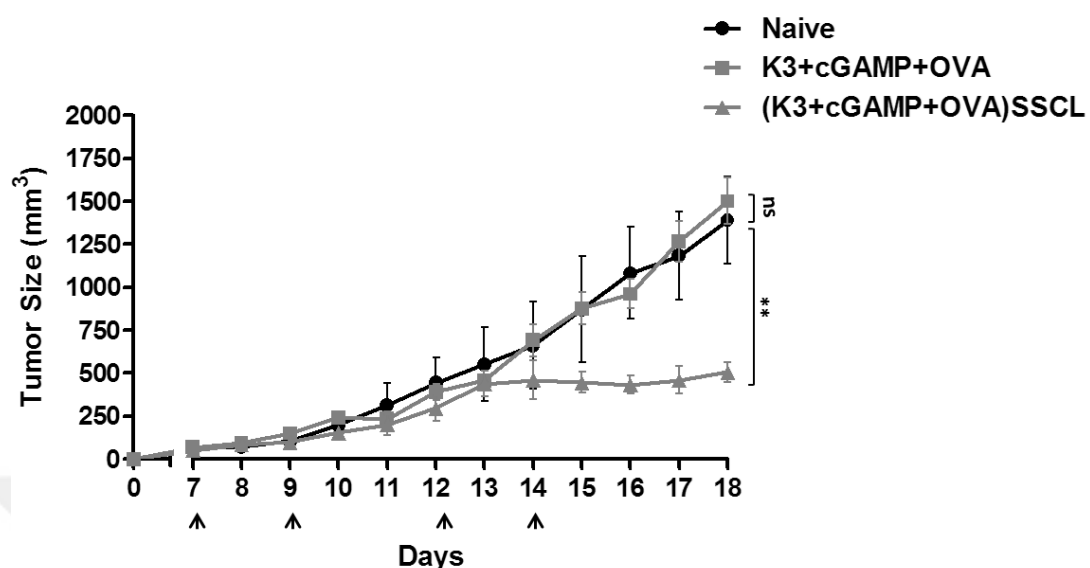


Figure 3.28. pH-sensitive K3 and cGAMP co-encapsulated SSCL in tumor immunotherapy.

C57BL/6 mice (n=5/group) were inoculated subcutaneously on day 0 with B16-OVA-luc (1×10^6 /mice). When tumor volume was around 50 mm³, they were treated with free or co-encapsulated K3 (5 µg/dose) and cGAMP (1.7 µg/dose) plus OVA (5 µg/dose) on days 7 and 9 (single doses of each molecules) and 12 and 14 (double doses of each molecules) administered intraperitoneally. P value was determined by two-way ANOVA followed by Bonferroni (HSD) test comparing treatment groups with untreated (naive) group, **p<0.01.

Sera were collected from tail vein of each animal to determine humoral response. OVA specific IgGs were detected by ELISA. Total IgG and IgG2c levels of liposome treated group animals were higher than other treatment groups. Mice treated with co-encapsulated liposomes as expected yielded higher IgG1 titers, which was similar with group treated with free dual ligands. To analyze whether the therapy promoted a Th1 biased antigen dependent immunity, IgG2c/IgG1 ratio was plotted and data revealed that for liposome treated group it was higher than one, confirming that a Th1-biased immune response was established (one-way ANOVA, Tukey's test, p<0.05; Figure 3.29A-B).

At the end of the experiments, splenocytes were isolated and incubated with OVA, in order to check antigen specific IFN- γ production levels from T-cells. Consistent with reduced tumor volume, IFN- γ secretion was higher in dual liposomal formulation treated animals than free ligand treated or naive mice (Figure 3.29C). In addition, splenocytes were incubated with SIINFEKL and IFN- γ production was analyzed via ELISPOT. Similar with OVA treatment, SSCL co-encapsulating K3 and cGAMP significantly elevated IFN- γ producing CD8⁺ T cells ($p < 0.001$, one-way ANOVA, Tukey's post-hoc test; Figure 3.29D).



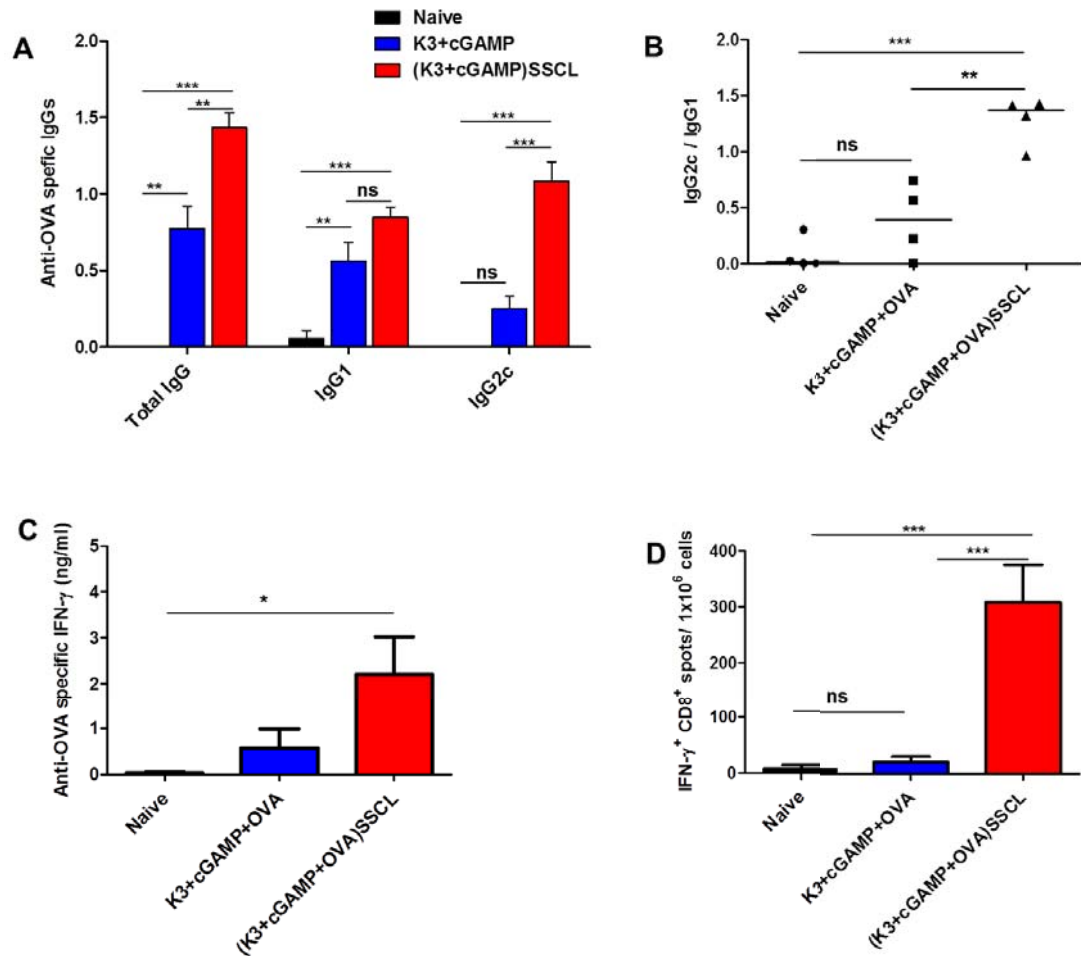


Figure 3.29. Humoral and cellular immune response elicited from mice treated with free or co-encapsulated K3+cGAMP after tumor inoculation.

At the end of the experiment, sera (n=4/group) was collected and (A) anti-OVA specific IgGs (total IgG, IgG1 and IgG2c) were determined via ELISA. OD@405 nm values were expressed as mean $\pm$ SD and (B) IgG2c over IgG1 ratio was calculated. Spleen were collected from immunized mice and isolated splenocytes were incubated with OVA protein or SIINFEKL peptide for 24 h. (A) Anti-OVA specific IFN γ levels and (B) IFN γ ⁺CD8⁺ spots were analyzed via ELISA and ELISPOT methods, respectively. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; *p<0.05; **p<0.01; ***p<0.001.

We next asked whether the suppressive tumor microenvironment was altered upon liposome therapy and tumor associated macrophage polarization was reversed (from M2 biased to M1 biased). To investigate tumor infiltrating immune cells, we collected tumors and incubated with liberase to isolate single cells. Cell type percentages in tumors were evaluated via staining of isolated single cells from

tumors by flow cytometer (Figure 3.30). No significant change was observed with respect to M0, M1, TAM, MDSC and granulocyte percentages in tumors between groups. However, dual ligands loaded into SSCL significantly decreased M2 macrophages when compared to naive group and free dual ligands ($p<0.05$, one-way ANOVA, Tukey's post-hoc test; Figure 3.30).

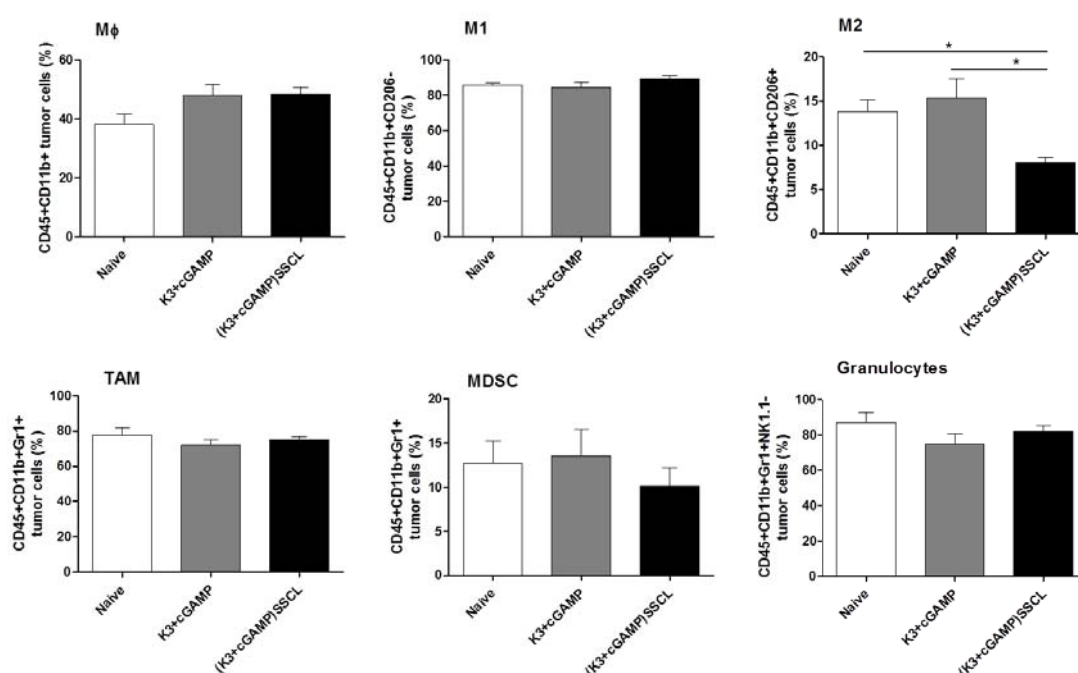


Figure 3.30. Infiltrated cells isolated from tumor of mice treated with free or co-encapsulated K3+cGAMP or left untreated.

Collected tumors were treated with liberase and filtered from cell strainer. Tumor cells were stained with flurochrome-conjugated mAbs to analyze macrophage, MDSCs and granulocyte population in the tumor tissue via flow cytometry. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Additionally, there was no significant difference between free and co-encapsulated dual ligands according to other cell types in tumor, MLN and splenocytes (Appendix E-G).

Chapter 4

Discussion

Nucleic acids that are sensed by endosomal TLRs or cytosolic nucleic acid sensors are the most promising immunoadjuvants for clinical applications. They are being tested either as a standalone or in combination therapies in phase I/II or phase III clinical trials as potent immunomodulatory agents against viral and bacterial diseases, as well as cancer immunotherapeutic agents. Surprisingly, several clinical trial settings demonstrated that even though nucleic acids have a huge potency to elevate immune response against specific antigen, they are rapidly cleared from the body due to nuclease digestion or serum protein adsorption upon *in vivo* administration. This short circulation time that was coupled with premature elimination before reaching target cell site significantly hampered clinical development of nucleic acid ligand mediated therapies [175]. Recently, scientists acknowledging these potentials embarked on to develop next generation therapeutic constructs harboring these ligands. Their prime aim was to develop carrier systems that would generate stable ligands as well as improve the breadth of activation *in vivo*. Previously, we have demonstrated that encapsulation of CpG ODN into liposomes with different physicochemical properties such as net surface charge and PEG modification of the membrane phospholipids significantly enhanced *in vitro* and *in vivo* immune activity of CpG ODNs. We further reported that different CpG ODN types loaded within different liposomes differentially augmented cellular

uptake and boost innate immunity and significantly improved antigen-dependent adaptive immunity. For instance, D-type CpG ODN showed enhanced immune activity, if only, they were encapsulated into either neutral or anionic liposomes. However, improved immunostimulatory activity of K-type CpG ODN observed only when they were loaded within cationic or SSCL liposomes [109,110]. Herein, based on our previous studies, we developed liposome-loaded single or dual nucleic acid ligands and aimed to provide more effective adjuvants that elicit ligand accumulation to relevant immune cells, thereby eliminating limited bioavailability, providing more efficient ligand internalization and boosting *in vivo* immune activity.

During the natural course of pathogenic infection multiple innate sensors are being triggered. This leads to orchestrated activation of multiple signaling cascades helping host to control infection [71,84]. In a study, infection of virus has been observed to activate NF- κ B through activation of TLR2, TLR3 and TLR9 [176]. Similarly, in cytomegalovirus infection, both TLR3 and TLR9 activation was essential to elevate protective immune response dependent on synergistic type I IFNs and IL-12 production [81]. In *Neisseria* infection, TLR4/MD-2 and activating cGAS augmented IFN- β secretion [96]. Another aim of this thesis was to mimic the natural course of infection using multiple PAMPs to activate innate immune system. Krummen *et al.* demonstrated that the synergistic immunostimulatory action between TLR ligands was more pronounced than their standalone counterparts when MyD88 and TRIF-dependent signaling was combined [79]. Therefore, we utilized the synergistic immune response by the simultaneous activation of MyD88-dependent and independent pathways by co-encapsulating CpG ODN and poly(I:C) or cGAMP/c-di-GMP within liposomes and elicit elevated and sustained immune response.

TLR3 and TLR9 agonists mimic the simultaneous infection of RNA and DNA viruses which triggers the production of type I IFNs. CpG ODN and poly(I:C) combination have been shown to elevate type I IFNs in rat pDCs and increased B cell activation [177]. In addition they induce both anti-viral and anti-bacterial immune responses. In one study by Tross *et.al.*, upon simultaneous administration of dual TLR3 and TLR9 ligands to macrophage cell line RAW264.7, it was reported that the

immune-related gene expression profiles were upregulated more effectively than either of the standalone single ligand treatment [172]. IL6, IL1A, IFNA6, IFNB1, CSF3, IFNA6T, TSLP and INF5 were synergistically elevated genes after 4 h stimulation. We extended these observations in the first part of this thesis by loading CpG ODN plus poly(I:C) within liposomes to achieve both production of type I IFNs and pro-inflammatory cytokine production that contribute to generate more potent cytotoxic T cell activity (Figure 4.1). First, we demonstrated that while low doses of single or dual CpG ODN and poly(I:C) ligands in free forms were ineffective to induce appreciable amounts of IL-6, IFN- γ and IFN- α/β , their liposomal forms were capable to secrete these cytokines. Moreover, co-encapsulated form synergistically increased all those cytokines. This effect was independent of the mitogen effect of the ligands that induce proliferation of the cells upon ligand internalization, but possibly more dependent on the triggering downstream signaling pathways controlled by TRIF and MyD88 adaptor proteins.

The potent adjuvant should activate innate immune system by increasing cytokine production in addition to maturing antigen presenting cells with up-regulation of co-stimulatory molecules such as CD80 and CD86. In a study, mouse dendritic cells stimulated with TLR3 and TLR9 ligands secreted high levels of IL-12 and IL-6 than incubation with single ligands [79]. Similarly, combination of TLR3 and TLR9 agonists acted synergistically in a dose dependent manner on DC activation through IL-12 production [80]. Additionally, *in vivo* administration of ligand combinations elevated MHC-I expression on splenic DCs [86]. Consistent with these findings, we demonstrated that dual ligands in neutral liposome increased surface markers (CD80, CD86, MHC-I and MHC-II) more than free single and dual ligands but also than single or separately-encapsulated liposomal formulations. Similarly, type I IFN and IL-12 secretion was significantly elevated by co-encapsulation when compared with non-encapsulated or separately encapsulated dual ligands. Supporting our findings, it was shown that, IL-12 production from BM-DCs was synergistically increased with non-encapsulated combination [79,178]. However, in those studies, non-encapsulated poly(I:C) plus CpG ODN could not achieve to synergistically increase neither CD86 surface marker nor type I IFN secretion.

Synergy between CpG ODN plus poly(I:C) was also demonstrated on enhancement of bactericidal property of monocytes/macrophages by elevating NO, IL-12, TNF- α , IL-6 and type I IFNs [86]. Similar immune responses were observed when we treated BM-DMs with dual liposomes which enhanced the secretion of IL-12, TNF- α and NO but not type I IFNs more than free combinations. Especially, synergistically TNF- α secretion from macrophages were observed as similar with previous study in macrophage stimulated with sequential delivery of CpG ODN and poly(I:C) [82]. Additionally, co-encapsulation of dual ligands with antigen (DQ-OVA) increased the antigen processing and possibly loading efficiency of OVA peptide onto MHCs by macrophages than free or liposomal dual ligands simultaneously administered antigen formulations.

Liposomes act as a depot delivery system and improve cellular uptake of the ligands. Previously, the uptake of CpG ODN by immune cells was increased when it was encapsulated within SSCL [110]. In a study, lipofectamine mixed phosphorothioate-modified CpG ODN plus poly(I:C) was demonstrated to block poly(I:C) uptake by tumor cells [179]. In this study, we used neutral liposomes to load these ligands, and furthermore we showed that uptake of either of the CpG ODN or poly(I:C) by immune cells significantly increased when administered together within liposomes. Rather than blockage, the dual uptake of ligands in co-encapsulated liposomes amplified six-fold more than non-encapsulated or separately encapsulated dual ligands if neutral liposomes are used instead of cationic counterpart [179].

Elevated IL-12 and type-II IFNs from APCs would trigger Th1-biased humoral response as judged by the ratio of IgG2c/IgG1>1. Interestingly, simultaneous administration of separately loaded CpG ODN or poly(I:C) adjuvant together with OVA was effective to induce higher antigen presentation than delivery of non-liposomal forms. In a vaccine study, co-encapsulation of OVA with CpG ODN increased IgG2 and decreased IgG1 titers when compared to free combinations (Bal et al. 2011). We have observed that after injection of co-encapsulated liposomal dual ligands together with antigen, IgG2c production was higher than mice immunized with single or separately encapsulated agonists following 8 months.

We observed that Th1-biased immune response and antigen specific IFN- γ producing CD8⁺ T cells inhibited tumor development following EG.7 inoculation 8th month post immunization. In literature, with free combinations antigen dependent CTLs and protective adaptive immunity was reported to be elevated. In a HIV vaccine study, adjuvants targeting DEC205 combined with synergistic TLR ligands, CpG ODN and poly(I:C), enhanced the *in vivo* CTL responses through increasing antigen-specific IFN- γ and CD107a expressions [183]. Moreover, in another study, combination of TLR2, TLR3 and TLR9 induced highly functional CTLs and production of high levels of IFN- γ [78]. Here, we employed much lower doses of TLR3 and TLR9 ligands within liposomes and extended the challenge time to 8th months following immunization. It is important to note that our immunization led to formation of long lived antigen specific memory CD8⁺ T-cells sufficient to clear OVA expressing tumor cells following incubation to mice.

Schlosser *et al.* have demonstrated that co-encapsulation of OVA with CpG ODN or poly(I:C) in PLGA microparticle enhanced cross-presentation and consequently CTL activity more than mixed formulations containing micro-particles loaded with antigen and adjuvant (Schlosser et al. 2008). However, Kasturi *et al.* indicated that when antigen and adjuvant was separately encapsulated into PLGA nanoparticles induced stronger antibody response than co-encapsulated formulations [184]. We showed that even though half the amount of antigen was present in (CpG ODN+poly(I:C)+OVA) co-encapsulated group, the formulation demonstrated higher anti-OVA IgG titers than mixed liposome formulation (i.e. equal mixtures of CpG ODN+OVA and poly(I:C)+OVA liposome populations). However, no tumor formation was observed in both co-encapsulated and separately encapsulated groups and CTL activity was higher in separately encapsulation than co-encapsulation.

In a similar study, DDA:TDB liposome-loaded CpG ODN and poly(I:C) were investigated on cellular and humoral responses according to size and structural changes in delivery vehicle. However, total IgG and Ag-specific T cell responses were activated in multilamellar vesicles (MLV) but not in SUV, they used high amounts of adjuvants and antigen in every injection (250 μ g DDA:DSPC, 50 μ g

TDB, 20 µg CpG, 50 µg poly(I:C) and 20µg OVA) [185]. Our non-immunogenic formulation is more effective since we used lower amounts of adjuvant and antigen. Moreover, this study is the first study demonstrating enhanced anti-tumor activity of CpG ODN and poly(I:C) when co-encapsulated into non-immunogenic neutral liposomes over the course of 8 months, and successfully protects immunized mice to develop tumors.



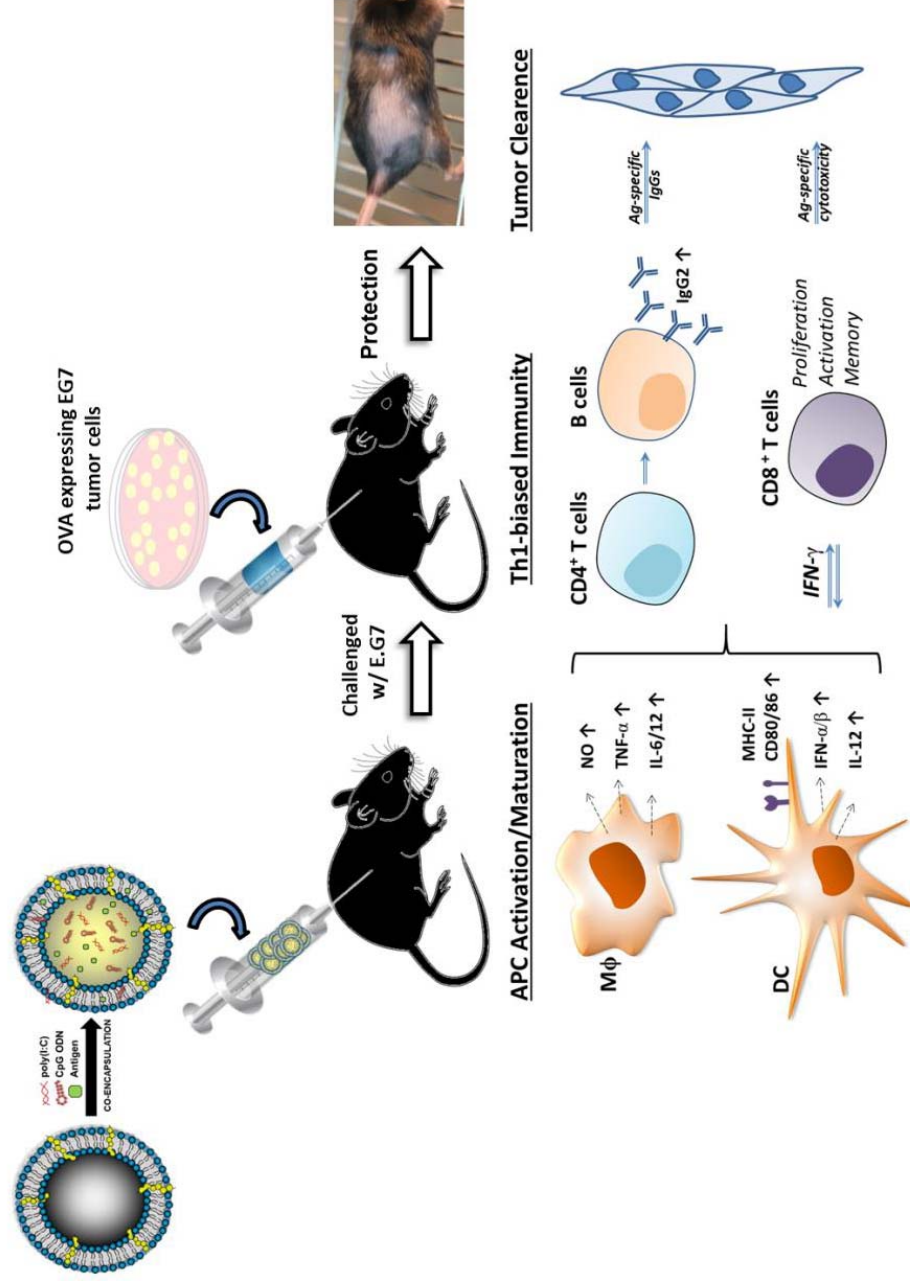


Figure 4.1. Schematic illustration of action mechanism of CpG ODN plus poly(1:C) co-encapsulated neutral liposome in anti-cancer vaccine as adjuvant [186].

In the second part of the thesis, we aimed to combine the properties of TLR9 and STING ligands by loading into pH-sensitive liposomes which would eliminate the disadvantages of these agonists and design an adjuvant appropriate to the clinic.

K-type ODN is the most potent CpG ODN in clinical use but induce only secretion of low levels of interferons. New strategies are being developed to improve potential of K-ODN by combining or conjugating with different molecules. In one study, Gungor *et.al.* reported that they developed nanorings of K-type CpG ODN with 11-mer TAT peptide. This resulted not only more immunogenic CpG ODN vaccine adjuvant but also generated a stable complex capable of inducing IFN- α following stimulation of either human and mouse immune cells [187]. In another study, K-type CpG ODN was complexed with immunogenic nonagonistic Dectin-1 ligand schizophyllan (SPG) which influenced the type I IFN productions like D-type ODN and keeping K-type properties such as B cell maturation. Furthermore, the influenza split vaccine including nanoparticulate adjuvant increased antigen-specific humoral response and protectivity against influenza in mice and nonhuman primate model [188].

In addition to nanoparticulate forms of K3, combination with interferon inducers such as STING agonists can enhance its immunostimulatory properties. Previously, we have demonstrated that co-administration of K3 and c-di-GMP activate synergistic immune responses through secretion of IL-6, IL-12 and TNF- α , upregulated CD86 positivity in MHC-II positive splenocytes and led type-I IFNs secretion in mice to complete adjuvant property of K3 [95]. In addition, K3 activity can be increased by combining with another STING agonist cGAMP. In back to back publications, the properties of cGAMP plus K3 have been demonstrated in human PBMCs. The combination elevated both type I IFNs and IFN- γ production, especially from NK cells [94,95]. However, similar with CpG ODNs, CDNs have similar problems in *in vivo* applications like poor cellular uptake, insufficient migration to lymph nodes due to their low molecular weights and rapid clearance from the body [124]. Therefore, we co-encapsulated K3 and CDNs (cGAMP or c-di-GMP) into pH sensitive liposomes which would provide the endosomal delivery of

K3 and cytosolic release of CDNs to activate both TLR9 and STING signaling cascades. We tested their immunostimulatory properties in splenocytes. Co-encapsulated K3 and CDNs into modified SSCL, type-I and type-II IFNs were synergistically increased in splenocytes while IL-12 and IL-6 production was elevated in BM-DCs more than free combinations.

By encapsulating CDNs into pH-sensitive SSCL, we predicted elevated immune responses after stimulations of immune cells. However, free or encapsulated CDNs did not induce innate immune activation at lower than optimal doses even though CDN uptake was highly increased (approximately 20 fold for cGAMP and 5 fold for c-di-GMP). Conflicted to our findings, naturally packaging of cGAMP by viral particles led the activation of innate immune system through STING signaling pathways in virus infections. Interestingly, entrapping into virus-like particles also enhanced immune responses [189,190]. Additionally, in a study by Miyabe *et al.*, c-di-GMP was encapsulated into pH sensitive liposome using YSK05/POPE/CHOL (40/25/35) lipids (c-di-GMP/YSK05-Lip). Liposomal c-di-GMP showed higher IFN- β secretion than free and Lipofectamine2000 transfected groups [126]. In addition, the anti-tumor activity of c-di-GMP/YSK05-Lip was tested in lung metastasis generated by melanoma. Treatment activated many cytokines IL-6, TNF- α , IFN- γ and IFN- β production at early time point and enhanced NK activity to eliminate metastasis [127].

Adjuvant should provide antigen presentation and induce cytokine release from APCs. In a study, the delivery of cGAMP incorporated within hydrogels magnified macrophage dependent interferon- β secretion [170]. Moreover, particulate form of c-di-GMP activated APCs though elevating co-stimulatory molecules [124]. Different than previous publications, in our study, triggering both STING and NF- κ B pathways elevated IL-12 and IL-6 secretions from BM-DCs and BM-DMs. M1-type macrophages were generated in dual liposomal formulations than M2-type as judged by elevated IL-12/IL-10 ratio after stimulation of BM-DMs. Surprisingly, while liposomal dual ligands had no impact on cell viability of splenocytes, they promoted proliferation of macrophages.

In some cancers, STING pathways are shut down by tumor virus oncogenes [191]. This suggested that anti-tumor immune responses would be activated using STING agonists. In a study by Corrales *et al.*, intratumoral effects of CDNs were investigated considering that tumor-resident DCs secrete IFN- β through STING activation and prime spontaneously T-cells. High doses (25 – 50 μ g/injection) of synthetic CDNs proved to regress established tumor when administered intratumorally [192]. We believed that with the help of liposomes more extensive immune activations could be generated and this boosted immune milieu would overcome suppressor environment existing in the tumor micro-niche and would generate an inflammatory environment supporting tumor regression following therapy.

Even though, liposomal K3 plus c-di-GMP showed similar immunostimulatory properties with liposomal K3 plus cGAMP, noncanonical cGAMP was demonstrated to be much more potent to induce IFN- β , than the canonical c-di-GMP ligand. Besides, c-di-GMP had lower affinity to bind both mouse and human STING and lower potency to induce type I IFN production than cGAS triggered ligands. In a study, anti-tumoral activity of cGAMP was tested. Data suggested that activated antigen dependent humoral immune system by inducing IgG1 production and subsequent induction of antigen specific IFN- γ and IL-2 production by T cells were critical to control tumor [193]. Additionally, cGAMP was found to be a safer and potent adjuvant in cutaneous vaccine against influenza [129]. However, formulations in these studies used excessive amounts of cGAMP to elicit sufficient immune responses and could pose serious side effects. So, we suggested that we would enhance the anti-tumor activity of cGAMP at lower doses and decrease the possible side-effects by our liposomal formulation co-encapsulating K3 and cGAMP into SSCL.

We used free or co-encapsulated K3 and 2'3' cGAMP to increase antigen-specific adaptive immune response and regress the established melanoma tumor. We have demonstrated ~80% reduction in tumor sizes, boosted Th1-biased humoral response (IgG2c/IgG1>1), increased IFN- γ producing CD8⁺ T cells and reduced M2-type

macrophage at the tumor bed. In a recent study, intratumoral administration of cGAMP led to migration of macrophages into tumor side. They had phagocytic activity and high expression levels of interferon related genes. Similar to our finding, it was observed that anti-tumor activity of CDNs was dependent on macrophages and CD8⁺ T cells [194].

In a study, the combination of cGAMP and PD-L1 monoclonal antibody was used as immunotherapy to melanoma tumor. They have shown the importance of cGAS pathway in tumor microenvironment as in deficiency leading less responsive to PD-L1 treatment. cGAMP and PD-L1 combination treatment leads enhanced activity of DCs and cross-presentation resulting elevated anti-tumor immunity [132]. We propose that combination of CDN with K3 would replace the need to block PD-L1 via monoclonal antibody and would deviate the suppressor micro environment to an inflammatory by our liposomal formulation.

6-dimethylxanthenone-4-acetic acid (DMXAA or vadimezan) that is another potent synthetic agonist of STING pathway. However, it failed in clinical trials as has no effect on human STING (hSTING) [195,196]. DMXAA has been shown to elicit re-education of M2-type macrophages in a metastatic tumor and 2'3'-cGAMP was observed to have similar effects [197]. Therefore, our data strongly suggests that a noncanonical 2'3' cGAMP together with K3 in liposomes would be more potent immunotherapeutic agent in clinics.

In the last part of this thesis, the potential of single liposomal formulations loading K or D-type CpG ODN were also tested against anti-viral and anti-bacterial vaccine models after co-encapsulating viral antigen (FMD sero type O) and bacterial extract (*H. pylori* SS1). Due to B cell maturation property, K-type ODN was more effective to provide Th1-biased immunity in both prophylactic vaccine studies when we compared the antigen-specific antibody titrations of mice immunized with liposomal CpGs. It was already mentioned that different types of K-ODN is in clinical trials for many different prophylactic human vaccine studies [101]. Supporting our findings,

cationic and SSCL liposomes showed significant improvement on immunostimulatory effects of K-ODN.

In FMD vaccine studies, we decreased the antigen use in each injection 6-fold than commercial vaccine when cationic liposome-loaded K-type CpG ODN was used. In Turkey, biannual vaccination (trivalent vaccine containing O, A and Asia-1 sero type antigens) is performed routinely when cattle are older than 2 months. Multiple doses of vaccines are required during the life of an animal. Moreover, doses of vaccine should be arranged to facilitate protection against FMDV at the first vaccine. Generally, it should be two doses by month, however, due to available antigen limitations and effect-cost problems only one dose is injected in Turkey [198–200]. Considering these limitations, we suggested that our liposomal vaccine formulations would have higher potential to elevate antigen-specific antibodies and would be used at the required immunization regimes.

Vaccination is important to take cautions before *H. pylori* induce several diseases such as gastric ulcer and cancer. Unfortunately, *H. Pylori* can develop drugs resistance and consequently cannot be eradicated. The idea of vaccination against *H. pylori* started in 1990 [201] soon after *H. pylori* discovered at 1983. While bacteria are found in 50% of world population, it is categorized as carcinogen [202]. In helicobacter vaccine studies, we significantly decreased CFU in stomach in mice immunized with SSCL loaded K-type CpG ODN after challenge with *H. pylori* SS1 feeding. It is important and challenging to eradicate bacterial colony from the body. Using specific antigen but not bacterial cell extract would increase the specificity of vaccine. We activated both Th1 and Th17 immunity after vaccination that is also crucial for protection even though there is still conflicted observations in the literature as to which cytokine cocktail or immune response is crucial for protection [203–206]. Although deserves more detailed analysis and in vivo experiments, these results suggested us that liposomal formulations containing CpG ODN would be a suitable formulation both for prophylactic vaccine and for therapy against *H. pylori* infections, which will be one of our future plans.

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APPENDICES

APPENDIX A

Blocking Buffer (ELISA)

- 500 ml 1x PBS
- 25 grams BSA (5%)
- 250 µl Tween20 (0.025%)

T-cell Buffer (ELISA)

- 500 ml 1x PBS
- 25 ml FBS (5%)
- 250 µl Tween20 (0.025%)

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2.5 ml Tween 20
- 4.5 l dH₂O

Phosphate Buffered Saline (PBS) (10 x)

- 80 g NaCl
- 2 g KCl
- 8.01 g Na₂HPO₄ · 2H₂O
- 2 g KH₂PO₄
- 1 l ddH₂O

pH= 6.8; autoclaved and diluted to 1 X prior to use.

Thioglycollate Solution (4%)

- 4 grams brewer thioglycollate into 100 ml ddH₂O

High Glucose DMEM and RPMI-1640

- 2%: 10 ml FBS (inactivated at 55 °C)
- 5%: 25 ml FBS
- 10%: 50 ml FBS
- 5 ml penicillin/streptomycin (50 µg/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES, (10 mM final concentration from 1M stock)
- 5 ml Na Pyruvate (0.11 mg/ml final concentration from 100mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

APPENDIX B

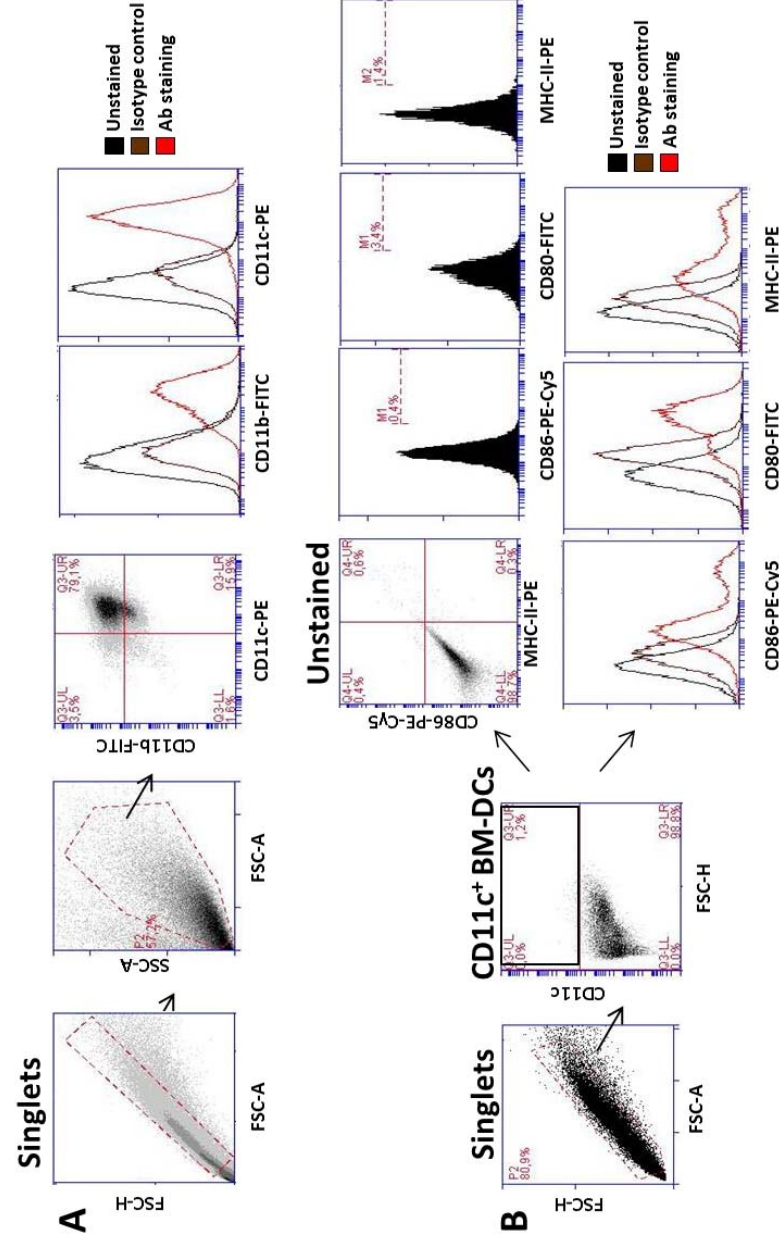


Figure B. (A) CD11b and CD11c double positivity of immature BM-DCs and isotype control of staining were checked before each generation by flow cytometry. Positivity of MHCII+, CD80+ or CD86+ of CD11c+ BMDCs generated in the presence of GM-CSF and IL-4 were determined and (B) gating strategy and isotype controls of staining were presented.

APPENDIX C

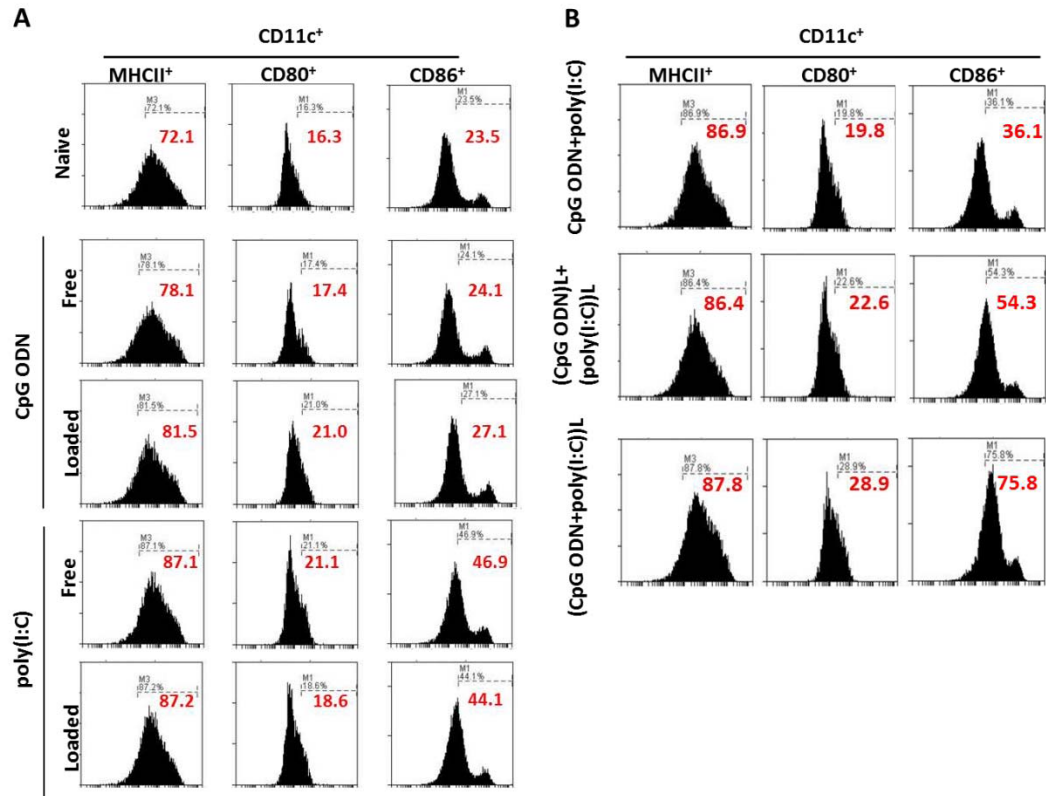


Figure C. BM-DC maturation by free or loaded CpG ODN or poly(I:C). Positivity of MHCII⁺, CD80⁺ or CD86⁺ CD11c⁺ BMDCs generated in the presence of GM-CSF and IL4 were determined after 24h stimulation with (A) free or loaded single ligands or (B) their free or liposomal combinations via flow cytometry.

APPENDIX D

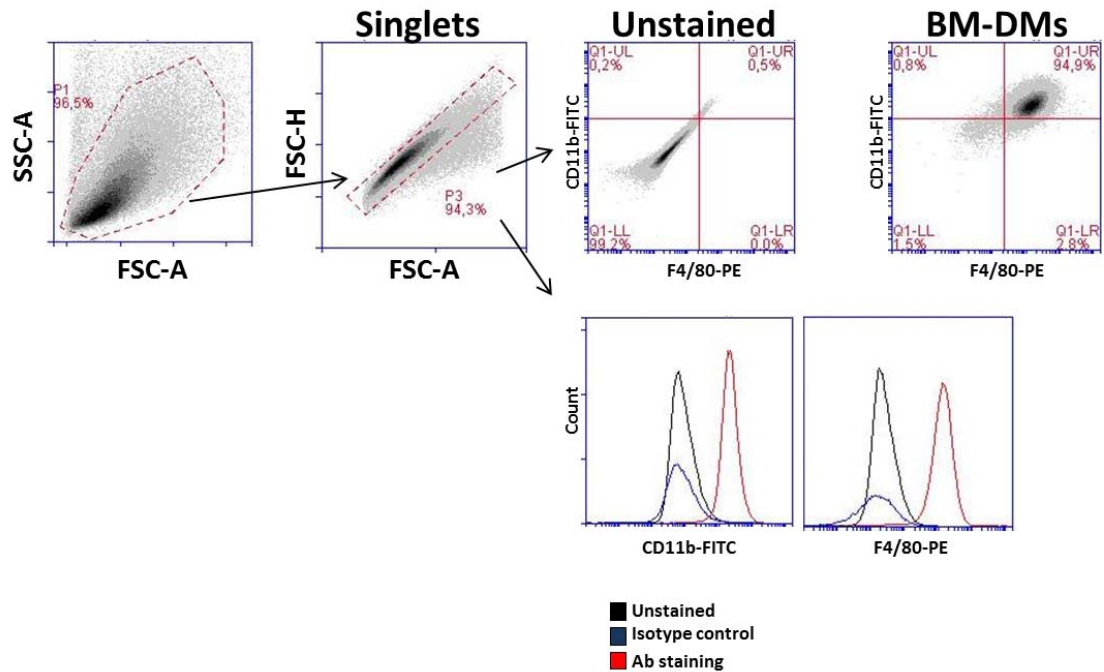


Figure D. CD11b and F4/80 double positivity of immature BM-DMs and isotype control of staining were checked before each generation by flow cytometry.

APPENDIX E

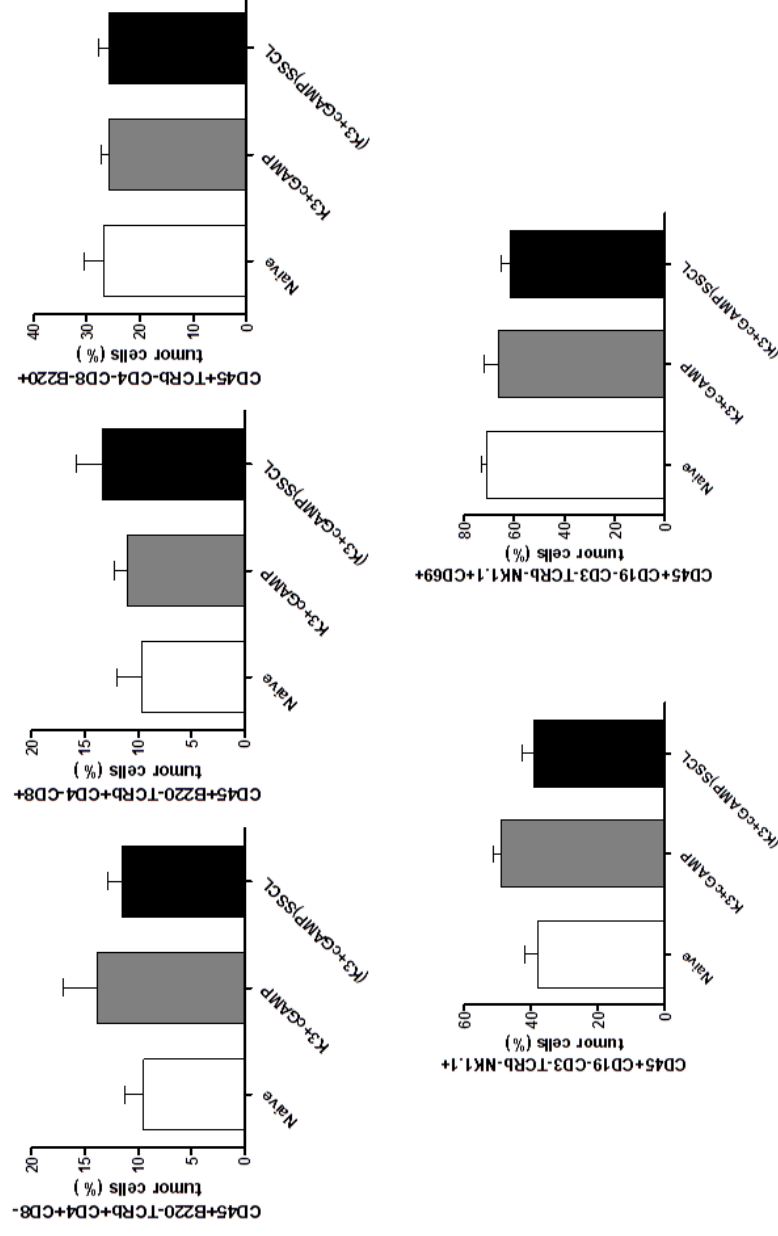


Figure E. Infiltrated cells isolated from tumor of mice treated with free or co-encapsulated K3+cGAMP or left untreated. Collected tumors were treated with liberase and filtered from cell strainer. Tumor cells were stained with fluoro-chrome-conjugated mAbs to analyze T, B and NK cells.

APPENDIX F

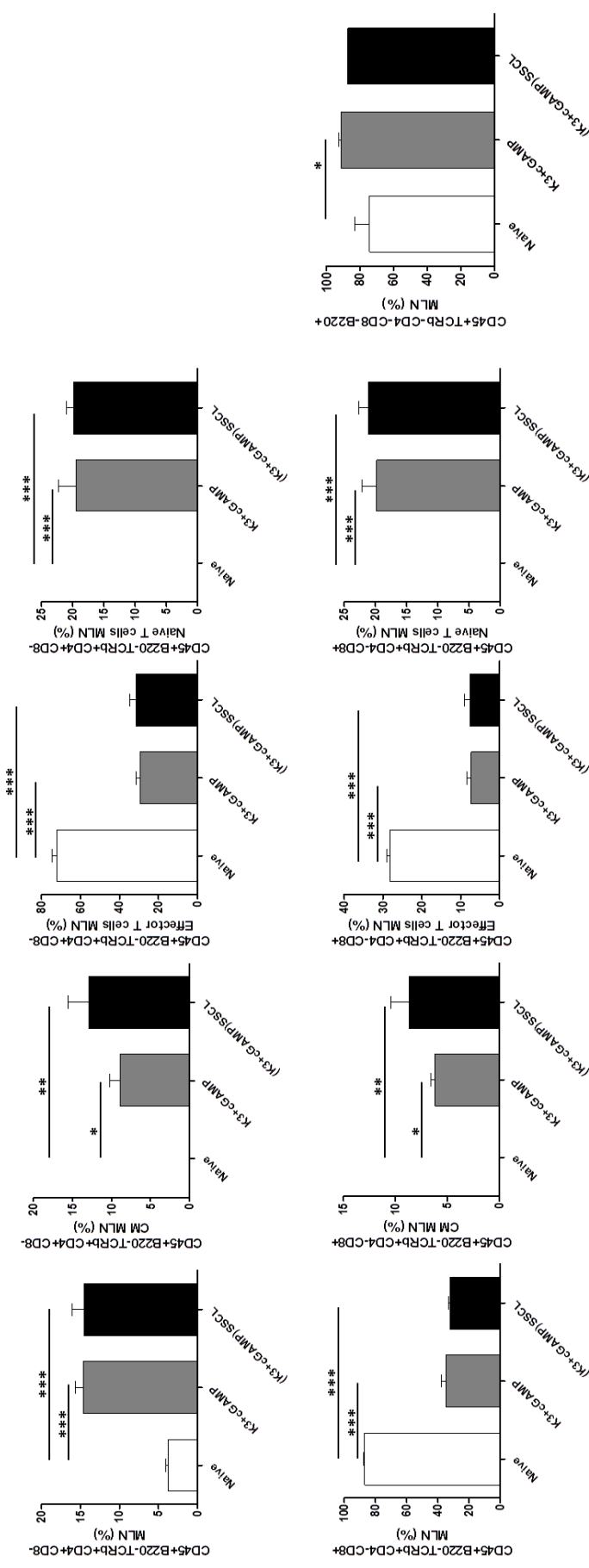


Figure F. Immune cell populations of MLNs of mice treated with free or co-encapsulated K3+cGAMP or left untreated. Collected MLNs were stained with fluochrome-conjugated mAbs to analyze immune cell population in it. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; *p<0.05; **p<0.01; ***p<0.001.

APPENDIX G

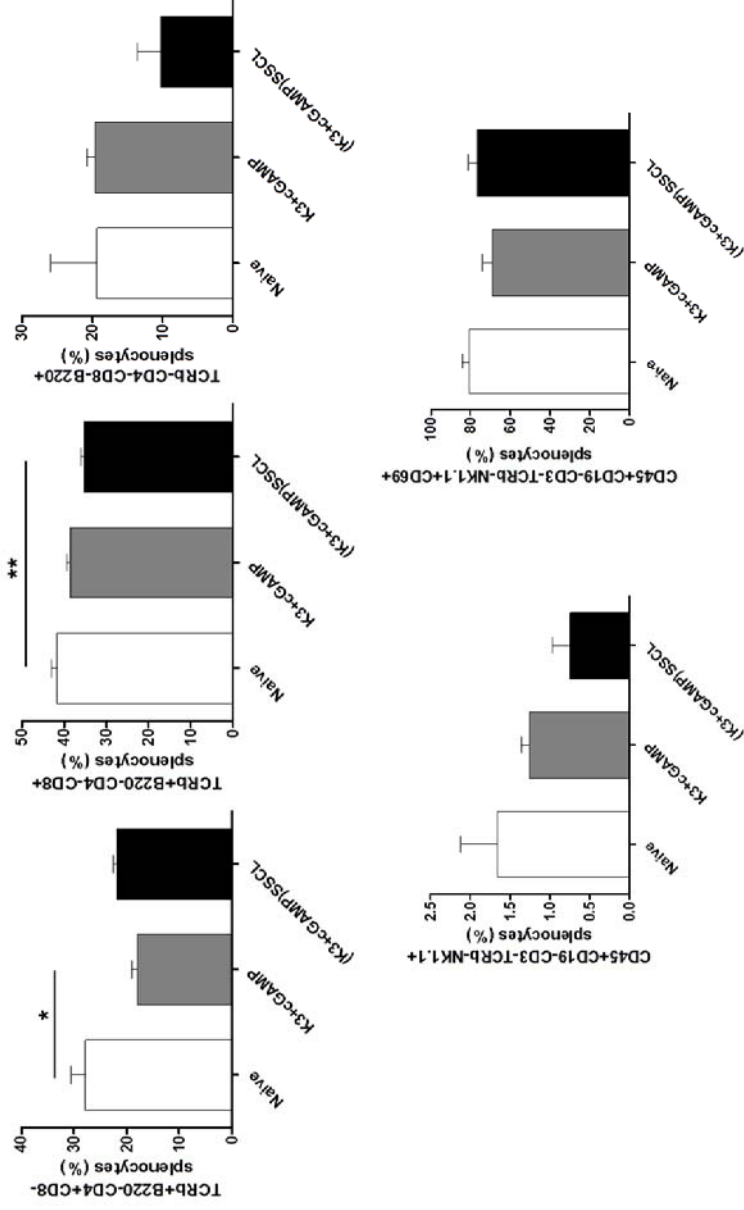


Figure G. Immune cell population in splenocytes isolated from mice treated with free or co-encapsulated K3+cGAMP or left untreated. Collected splenocytes were stained with fluochrome-conjugated mAbs to analyze percentages of T, B and NK cell populations. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Curriculum Vitae and Publications

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AWARDS & SCHOLARSHIPS

Best Poster Award: by Alkan Immunology Support Fund (ALIDEF) in International Molecular Immunology & Immunogenetics Congress (MIMIC-III), Antalya, Turkey, April 2016.

Registration and accommodation awards: by Turkish Society of Immunology for International Molecular Immunology & Immunogenetics Congress (MIMIC-III), Antalya, Turkey, April 2016.

EFIS/Immunology Letters (IL) Short-Term Fellowship: by European Federation of Immunological Societies (EFIS) for working in Prof. Christian Munz Lab. (Viral Immunobiology, University of Zurich), Zurich, Switzerland for 3 months (10 September - 02 December 2015).

Registration award: by Turkish Society of Immunology for 4th European Congress of Immunology (ECI2015), Vienna, Austria, September 2015.

Registration award: by Turkish Society of Immunology for 23rd National Immunology Congress, Antalya, Turkey, April 2015.

Conference Bursary: by Turkish Society of Immunology for International Molecular Immunology & Immunogenetics Congress II, Antalya, Turkey, April 2014.

Travel grant: by European Federation of Immunological Societies (EFIS) for 15th International Congress of Immunology, Milan, Italy, August 2013.

Project Bursary: Ministry of Science and Technology, SANTEZ project (1414 STZ.2012-1). Bilkent University, Department of Molecular Biology and Genetics, Ankara, Turkey, 2013-2015.

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RESEARCH EXPERIENCE

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TUBITAK Nanobiomat Project (TBAG, 105T508)

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2. T. Kahraman, **B. Bayyurt**, V. Ibrahimova, D. Tuncel, I. Gursel. CpG loaded fluorescent polymeric nanoparticles: a theranostic drug delivery system appropriate for TLR based applications. International Molecular Immunology & Immunogenetics Congress III (MIMIC-III), April 27-30, 2016. Antalya, Turkey (Poster).
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1. International Molecular Immunology & Immunogenetics Congress III (MIMIC-III), April 27-30, 2016. Antalya, TURKEY.
2. 4th European Congress of Immunology (ECI2015), September 6-9, 2015. Vienna, AUSTRIA.
3. Turkey-Singapore Joint Workshop on Molecular Biology, May 25-26, 2015. Ankara, TURKEY.
4. 23rd National Immunology Congress, April 26–30, 2015. Antalya, TURKEY.
5. International Molecular Immunology & Immunogenetics Congress II (MIMIC-II), April 27-30, 2014. Antalya, TURKEY.
6. 15th International Congress of Immunology (ICI2013), August 22-27, 2013. Milan, ITALY.
7. 22nd National Immunology Congress, April 27–30, 2013. Cesme, TURKEY.
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9. The Novartis International Biotechnology Leadership Camp (BioCamp), August 26-29, 2012. Basel, SWITZERLAND.
10. 10th CIMT Annual Meeting, Towards Next-Generation Cancer Immunotherapy, May 23-25, 2012. Mainz, GERMANY.
11. The EMBO|EMBL Symposium: New Perspectives on Immunity to Infection, May 19-22, 2012. Heidelberg, GERMANY.

12. International Molecular Immunology & Immunogenetics Congress (MIMIC-I), April 27-29, 2012. Antalya, TURKEY.
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14. Advances in Immunology and Cancer Biology Workshop, April 15–17, 2011, Istanbul, TURKEY.
15. 21st National Immunology Congress, April 6–9, 2011. Marmaris, TURKEY.
16. Nanobiomaterials, Tissue Engineering and Nanobiotechnology Workshop. September, 1-2, 2008. METU, Ankara, TURKEY.
17. 14th International Biomedical Science and Technology Symposium. May 03-07, 2008. Mugla, TURKEY.
18. 13th International Biomedical Science and Technology Symposium. August 26-28, 2007. Istanbul, TURKEY.
19. Expertissues Course on Surface and Biological Characterization of Materials Fundamentals of Theory and Practice (Workshop). April, 10-12, 2007. Trento University, Trento, ITALY.

SOCIETY MEMBERSHIPS

Turkish Society of Immunology

Turkish Biomaterials and Tissue Engineering Society



Encapsulation of two different TLR ligands into liposomes confer protective immunity and prevent tumor development



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ARTICLE INFO

Article history:

Received 21 September 2016

Received in revised form 30 December 2016

Accepted 4 January 2017

Available online 7 January 2017

Keywords:

TLR3

TLR9

Ligands

Immunoadjuvant

Liposomes

Immune response

Preventive cancer vaccine

ABSTRACT

Nucleic acid-based Toll-like receptor (TLR) ligands are promising adjuvants and immunotherapeutic agents. Combination of TLR ligands potentiates immune response by providing synergistic immune activity via triggering different signaling pathways and may impact antigen dependent T-cell immune memory. However, their short circulation time due to nuclease attack hampers their clinical performance. Liposomes offer inclusion of protein and nucleic acid-based drugs with high encapsulation efficiency and drug loading. Furthermore, they protect cargo from enzymatic cleavage while providing stability, and enhancing biological activity. Herein, we aimed to develop a liposomal carrier system co-encapsulating TLR3 (polyinosinic-polycytidylic acid; poly(I:C)) and TLR9 (oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs; CpG ODN) ligands as immunoadjuvants together with protein antigen. To demonstrate that this depot system not only induce synergistic innate immune activation but also boost antigen-dependent immune response, we analyzed the potency of dual ligand encapsulated liposomes in long-term cancer protection assay. Data revealed that CpG ODN and poly(I:C) co-encapsulation significantly enhanced cytokine production from spleen cells. Activation and maturation of dendritic cells as well as bactericidal potency of macrophages along with internalization capacity of ligands were elevated upon incubation with liposomes co-encapsulating CpG ODN and poly(I:C). Immunization with co-encapsulated liposomes induced OVA-specific Th1-biased immunity which persisted for eight months post-booster injection. Subsequent challenge with OVA-expressing tumor cell line, E.G7, demonstrated that mice immunized with liposomes co-encapsulating dual ligands had significantly slower tumor progression. Tumor clearance was dependent on OVA-specific cytotoxic memory T-cells. These results suggest that liposomes co-encapsulating TLR3 and TLR9 ligands and a specific cancer antigen could be developed as a preventive cancer vaccine.

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1. Introduction

Innate immune cells recognize microbial (bacterial and viral) components via pattern recognition receptors (PRRs) through pathogen-associated molecular patterns (PAMPs) and differentiate self from non-self [1,2]. Toll-like receptors (TLRs) are the most extensively studied PRRs. TLR family members are sub-divided to cell membrane-associated and endosome-associated receptors. Endosomal TLRs are specialized to sense nucleic acids. While TLR3 and TLR7/8 recognizes double and single-stranded RNA, TLR9 recognizes bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs (CpG ODNs hereafter) [3–6]. Multiple TLR triggering could synergistically activate immune response upon multiple agonists engagement [7].

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CpG ODNs, which are found frequently in bacterial and viral genome but rare in mammalian DNA, enhances innate immune response. Among different ODN classes, D-ODNs (also known as CpG-A type) synthesized as a mixed backbone ODN contain purine/pyrimidine/CpG/purine/pyrimidine motif and poly G-tail at both ends. This ODN type induces secretion of type I interferons (IFNs) from plasmacytoid dendritic cells (pDCs), and IFN- γ from natural killer (NK) cells [8,9]. It activates nuclear factor kappa B (NF- κ B) and interferon regulatory factor 7 (IRF7) via myeloid differentiation primary response gene 88 (MyD88) dependent pathway. Polyinosinic-polycytidylic acid (poly(I:C) hereafter), a synthetic analog of double-stranded RNA initiates signaling cascade through TLR3 and induces type I IFNs along with proinflammatory cytokines mediated by NF- κ B and IRF3 via TRIF-dependent (MyD88-independent) pathway [3,6,10]. Both TLR ligands trigger anti-viral and anti-bacterial immune responses and mimic viral and bacterial infections. The simultaneous sensing of these two endosomal TLR ligands by PRRs boosts innate immune response in a synergistic manner and leads to more pronounced type I IFNs [11], proinflammatory cytokines, and nitric oxide production [12] with elevated cytotoxic T

lymphocyte (CTL) activity [13,14]. When delivered together these ligands may help to improve better antigen dependent immunity and antitumor activity [15]. Nucleic acid-based TLR ligands are promising candidates as type 1T helper cell (Th1) specific vaccine adjuvants [8, 16], anti-cancer [17] or anti-allergic therapeutic agents [18], however, when given in non-encapsulated forms, their clinical performance is hampered mainly due to in vivo degradation by nucleases or rapid clearance by serum protein adsorption [19]. To overcome this problem, several strategies were proposed such as polymer-based nanoparticles [20,21], polysaccharide complexes [22], liposomes [23–25] aiming to protect these labile molecules until they reach their target cells upon administration. In our previous studies, we have demonstrated that encapsulation of TLR ligands within liposomes not only increases their stability and protects them from digestion but also enhances their immunostimulatory and immunotherapeutic breadth [24,26]. The present work, aims to extend our previous experience and planned to co-encapsulate dual TLR ligands (CpG ODN and poly(I:C)) in liposomes and test their vaccine potency leading to long-term prevention against cancer. We provide evidence that dual ligand encapsulating liposomal vaccine improved ligand internalization, enhanced APC function, promoted a strong Th1-biased antigen-specific immune response and subsequently prevented animals to develop tumors eight months after immunization. We also demonstrated that the dual ligand co-encapsulating liposomal vaccine induce a long-lasting antigen specific CD8⁺ memory T-cell immunity, critically contributing tumor clearance of immunized mice.

2. Materials and methods

2.1. Reagents

Cholesterol (Chol) and L- α -phosphatidylcholine (PC), lipids that were used in liposomes preparation was purchased from Sigma Aldrich (St. Louis, MO, USA) and Avanti Polar Lipids (Alabaster, AL, USA), respectively. TLR9 ligand, CpG ODN D35 5'-GGTgcacgatcgaggggGG-3' was kindly provided by Dr. Ken Ishii (IFReC, Japan) [27]. Bases shown in capital letter have phosphorothioate linkage and those in lower case have phosphodiester linkages. CpG motif is underlined. Cy5-labeled CpG ODN was synthesized in CBER/FDA core Facility and provided by Dr. Dennis M. Klinman TLR3 ligand poly(I:C) was from Amersham (Piscataway, NJ, USA) and fluorescein labeled poly(I:C) was from Invivogen (Toulouse, France).

All cell culture media components were from Gibco (NY, USA) and Lonza (Lonza, Walkersville, MD, USA). Cytokine ELISA reagents: recombinant cytokines, monoclonal unlabeled and biotinylated antibodies against mIL-6, mIL-12 and mTNF- α were purchased from Biolegend (San Diego, CA, USA), while streptavidin-alkaline phosphatase, hIFN- α and mIFN- γ were purchased from Mabtech (Cincinnati, OH, USA) and hIP10 was from BD (San Jose, CA, USA). *P*-nitrophenyl phosphate disodium salt substrate (PNPP) was purchased from Thermo Scientific (San Jose, CA, USA). Immunoglobulin ELISA reagents; goat anti-mouse total IgG, IgG1, IgG2c, and monoclonal antibodies conjugated with alkaline phosphatase (AP) were from Southern Biotech (Birmingham, AL, USA). FACS antibodies conjugated to fluorescent chromophores were obtained from Biolegend. B16-Blue IFN- α / β cells that allow the detection of bioactive murine type I IFNs were obtained from Invivogen and used according to the manufacturer's protocol.

2.2. Methods

2.2.1. Preparation of liposomes

Phospholipid stocks were prepared in chloroform and stored at -20°C until use. Liposomes were prepared as described earlier [24, 26]. Briefly, lipids (PC:Chol, 1:1 molar ratio, 20 μmol total lipid in 2 ml chloroform) were mixed and film was formed using rotary evaporator (ILMVAC, Ilmenau, Germany). Vesicles were generated by hydrating

film with $1 \times \text{PBS}$. These were sonicated five times for 30 s on and 30 s off at 4°C using a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT, USA) to generate smaller unilamellar vesicles (SUV).

Ligand loading within lipid vesicles were performed as described earlier [24,26]. Briefly, ligands (1 mg of each ligand) and preformed SUVs (20 μmol) were mixed and snap frozen in liquid nitrogen, and freeze-dried overnight (VirTis benchtop K, Bielefeld, Germany). Dried lipid/ODN mixture was rehydrated (1/10 initial volume) using nuclease-free ddH₂O and vortexed for 15 s every 5 min for 30 min at room temperature. Equal volume of PBS was added to the mixture to adjust the tonicity of the resulting liposomes. Liposomes were washed twice with $1 \times \text{PBS}$ (pH: 7.4) to remove the unloaded molecules and centrifuged at 16000 g for 1 h. Final concentration of liposomes is 20 μM lipid/mg ligand. Liposome formulations were stored at 4°C until use.

2.2.2. In vitro stimulation

Splenocytes isolated from wild type C57BL/6 mice by mashing spleen with syringe plunger were seeded at 2×10^6 cell/ml (250 μl) on 96-well cell culture plates and stimulated with non- or liposome-encapsulated CpG ODN or poly(I:C) or their combinations (2 $\mu\text{g}/\text{ml}$ per ligand; 20 μmol lipid/1 mg ligand) for 36 h at 37°C in a 5% CO₂ incubator. After stimulation, supernatants were collected. mIL-6 and mIFN- γ secretion was analyzed by ELISA. Additionally, IFN- α / β was assessed using B16-Blue IFN- α / β reporter cells as described previously [28]. For uptake and binding experiments, splenocytes were incubated at 37°C with labeled non-encapsulated ligands or liposomes encapsulating fluorescein-labeled poly(I:C) and/or Cy5-labeled CpG ODN for 2 h. CpG ODN and/or poly(I:C) positive cells were recorded using BD Accuri C6 flow cytometer. Cell viability was assessed using cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

All human peripheral blood mononuclear cell (hPBMC) experiments were conducted following approval from the Bilkent University Human Studies Ethical Committee (Approval #: BILHSEC NO:2014-09-02-02) and with the informed consent of all participants. hPBMCs collected from healthy donors and purified using Ficoll density centrifugation were seeded at 1×10^6 cell/ml (200–250 μl) on 96-well cell culture plates. Cells were stimulated for 24 h. After incubation, supernatants were collected and used to detect hIP-10 and hIFN- α via ELISA and cells were stained with fluorescent-labeled anti-CD83 and anti-HLA-DR, and analyzed by flow cytometry.

RAW 264.7 macrophage cell line (0.5×10^6 cell/ml) was stimulated with non-encapsulated or liposome-loaded CpG ODN or poly(I:C) or their combinations (2 $\mu\text{g}/\text{ml}$ per ligand; 20 μmol lipid/1 mg ligand) and TNF- α level was analyzed via ELISA.

Antigen processing was analyzed addition of non-encapsulated or co-encapsulating ligands (2 $\mu\text{g}/\text{ml}$ per ligand; 20 μmol lipid/1 mg ligand) together with DQTM-Ovalbumin (2 $\mu\text{g}/\text{ml}$, DQTM-OVA, Molecular Probes) into APCs (0.5×10^6 ; BM-DCs, BM-DMs and RAW cells). After 6 h incubation, fluorochrome released upon proteolytic processing were analyzed by flow cytometry

2.2.3. Generation and stimulation of bone marrow-derived dendritic cells (BM-DCs) and macrophages (BM-DM)

BM-DCs were generated from bone marrow isolated from the femur and tibia of C57BL/6 mice incubating with GM-CSF and IL-4 as described previously [29]. The 6–8-day old immature BM-DCs (1×10^6 cell/ml) were stimulated with non-encapsulated or liposomes (20 μM lipid/mg ligand) encapsulating CpG ODN (2 $\mu\text{g}/\text{ml}$) or poly(I:C) (2 $\mu\text{g}/\text{ml}$) or their 1:1 combination at 37°C in a 5% CO₂ incubator. Following 24 h of incubation, the supernatants were collected and used for measurement of cytokines by ELISA and by B16-Blue IFN- α / β reporter cells. The expression levels of CD11b, CD11c, MHC-II, CD80 and CD86 in BM-DCs was analyzed by flow cytometry. Generated BM-DCs were approximately 80% CD11b⁺/CD11c⁺ double positive (Fig. S1A). In addition, propidium iodide (PI) staining was performed to analyze cell viability of stimulated BM-DCs.

BM-DMs were generated from bone marrow isolated from the femur and tibia of C57BL/6 mice incubated with 30% L929-conditioned medium (L929-CM) for 6 days. BM-DMs (0.5×10^6 cell/ml) were stimulated with non-encapsulated or liposome-encapsulated CpG ODN or poly(I:C) or their combinations (0–18 $\mu\text{g/ml}$ from each ligand, 20 μM lipid/mg ligand) and supernatants were used for nitric oxide (NO) analysis via Griess reagent (Sigma, St. Louis, MO, USA) and cytokine ELISA.

2.2.4. Immunization study

Animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions ($22 \pm 2^\circ\text{C}$) regulated with 12 h light and 12 h dark cycles. They were provided with unlimited access of food and water. All animal studies were conducted with prior approval of the animal ethics committee of Bilkent University (Approval #: BILAEC NO:2014/32).

Female C57BL/6 mice (6–8 week old) were injected twice intraperitoneally (i.p.) two weeks apart with non-encapsulated or encapsulated CpG ODN or poly(I:C) (10 μg each ligand 0.20 μmol liposomes/injection) or their 1:1 combination plus ovalbumin (OVA, 5 μg /injection). Blood was collected from tail vein of mice at 1st and 8th month after booster injection. IgG subtypes were analyzed by end-point ELISA from collected sera with proper titrations of each samples.

At the end of eight month post-booster injection, animals were subcutaneously inoculated with 4×10^6 E.G7-OVA thymoma cells (EL4 cells stably expressing OVA, ATCC) which were cultured in complete medium containing G418 (1 $\mu\text{g/ml}$) before injection. Tumor size was measured daily by digital caliper and recorded for 14 days. Then, animals were euthanized and spleens were recovered to isolate splenocytes. IFN- γ -producing CD8 $^+$ T-cells against SIINFEKL peptide were determined by ELISPOT assay.

2.2.5. ELISA and ELISPOT assays

Immulon 2 HB or 1B microtiter plates (Thermo Scientific, San Jose, CA, USA) were coated with anti-cytokine or anti-IgG antibodies (Ab), 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-cytokine Ab followed by phosphatase-streptavidin whereas bound IgG subclasses were detected using alkaline phosphatase streptavidin-conjugated anti-IgG antibodies and developed by the addition of PNPP substrate.

Anti-IFN- γ Ab coated Immulon 2 HB plates were blocked with PBS-BSA in ELISPOT assay. Splenocytes were serially diluted and seeded to pre-coated plates either with SIINFEKL supplemented (SIINFEKL:OVA 257–264 peptide, Anaspec Inc., San Jose, CA, USA) or just media containing wells. Cells were incubated for further 18 h at 37°C in a 5% CO_2 incubator. IFN- γ $^+$ CD8 $^+$ T cell spots were detected using biotinylated anti-IFN- γ Ab followed by alkaline phosphatase streptavidin and BCIP dissolved in low melting agarose solutions (Prona, Reducta, Poland; 1/4, v/v). Spots were counted under binocular stereo microscope.

2.2.6. Statistical analysis

Statistical significant differences between groups were determined using one-way or two-way ANOVA following Tukey's or Bonferroni post-hoc comparison and Student's *t*-test analysis using GraphPad Prism software (version 5, San Diego, CA, USA). *P*-values < 0.05 were considered as significant.

3. Results

3.1. IL-6 and interferon type I/II productions were synergistically enhanced by co-encapsulating poly(I:C) and CpG ODN in neutral liposomes

Our main aim was to combine and enhance the synergistic immunostimulatory activity of the two nucleic acid-based TLR ligands known to trigger MyD88 and TRIF dependent signaling cascades by

co-encapsulating them in liposomes thereby achieving simultaneous ligand delivery to innate immune cells. The prospect of this approach was assessed by incubating splenocytes either with non-encapsulated or encapsulated poly(I:C) and CpG ODN at 2 $\mu\text{g/ml}$ dose of each ligand. Data implied that, co-encapsulating poly(I:C) and CpG ODN significantly increased both IL-6 and IFN- γ and IFN- α/β levels over either non-encapsulated alone, non-encapsulated dual combined or separately encapsulated ligands ($p < 0.05$, one-way ANOVA, Tukey's HSD post hoc test, Fig. 1A–C). To rule out the toxicity of ligand stimulation on spleen cells, proliferation assay was conducted using Cell Counting Kit-8. Results revealed that there was no significant difference between groups (one-way ANOVA, Tukey's HSD Fig. 1D).

Next, ex vivo immunostimulatory potential of dual TLR ligand co-encapsulated liposomes were checked following i.p. injection to C57BL/6 mice (Fig. 1E). Four hours after i.p. treatment, spleen cells were incubated for further 24 h in cell culture with no additional stimulation and costimulatory (CD86) molecule expressions were analyzed by flow cytometry. Even though the injected initial amounts of non-encapsulated ligands were 2.5 fold more (25 μg each ligand) than the dual ligand-loaded liposomal group (10 μg each ligand), cells that received liposomal poly(I:C) plus CpG ODN surpassed non-encapsulated combination response in terms of CD86 upregulation (Fig. 1E). Of note, 10 μg injection of each non-encapsulated ligands did not initiate any detectable CD86 upregulation. These findings revealed that only liposomes co-encapsulating both TLR ligands are potent immune inducers than their i) single or ii) dual non-encapsulated ligand counterparts both in culture and upon in vivo administration.

3.2. Liposomes co-encapsulating TLR3 and TLR9 ligands enhanced APC function

Upregulation of MHC class II as well as co-stimulatory molecules along with cytokine production is a direct evidence of improved APC activity/function. We next assessed the potential of liposomes co-encapsulating poly(I:C) and CpG ODN for cellular activation and maturation of antigen presenting cells (APCs). We found that indeed liposome formulations mediate superior effect than the non-encapsulated ligand treatments. BM-DCs were generated in the presence of GM-CSF and IL-4 for six days in culture. Following harvest they were incubated with non-encapsulated or liposome-loaded TLR ligands. After 24 h cells were fixed and the number of CD11c $^+$ BM-DCs positive for MHC-II, CD86 or CD80 was determined by flow cytometry. Our cell gating strategy throughout these investigations is presented in Fig. S1B. When the surface marker upregulation levels of non-encapsulated and liposome-loaded single CpG ODN or poly(I:C) groups were analyzed there were no significant difference (Fig. S1C). Strikingly, when TLR3 and TLR9 ligands were co-encapsulated within liposomes, it led to significantly higher levels of co-stimulatory molecule (CD80 and CD86) upregulation (Fig. 2A–B and Fig. S1D–E). According to three independent experiments, MHC-II $^+$ /CD86 $^+$ double positive CD11c $^+$ BM-DCs was higher in co-encapsulated group ($74.0 \pm 2.0\%$) than non-encapsulated or liposome-loaded poly(I:C) ($57.5 \pm 2.6\%$ and $55.0 \pm 1.5\%$, respectively) or non-encapsulated or liposome-encapsulated CpG ODN ($30.5 \pm 2.0\%$ and $35.0 \pm 2.0\%$, respectively) or their separately encapsulated combination ($62.5 \pm 3.5\%$) (Fig. 2A). This data suggested that when dual ligands were encapsulated within liposomes higher MHC-II $^+$ /CD86 $^+$ double positive BM-DCs population were obtained compared to non-encapsulated ligand combination treated BM-DCs (Fig. 2B). Of note, in order to rule out that ligand treatments induce non-specific cell death, at the end of stimulation, cell viability was analyzed using PI staining and no significant difference between groups were observed (one-way ANOVA, Tukey's HSD, $p < 0.05$; Fig. S1F).

When, IL-12 production, a cytokine known to skew immune response towards Th1 from BM-DCs in response to ligand treatments was analyzed from cell supernatants after treatment, we detected that dual ligand co-encapsulated group generated six fold more IL-12 from

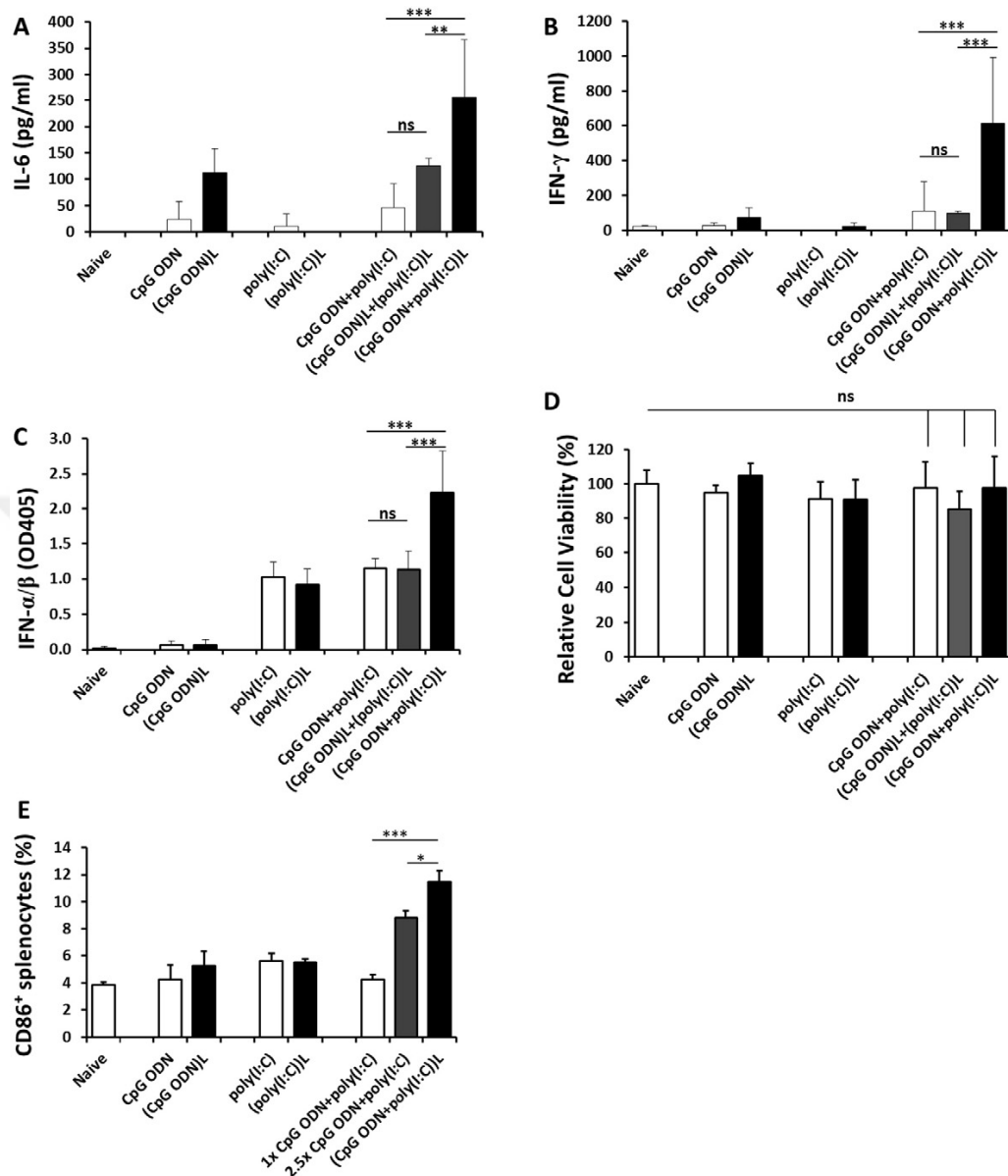


Fig. 1. Liposomes co-encapsulating CpG ODN and poly(I:C) improves in vitro and ex-vivo immunostimulatory effect. Splenocytes were stimulated with non-encapsulated or liposome-encapsulated (20 μ M lipid/mg ligand) CpG ODN and/or poly(I:C) (2 μ g/ml from each ligand) for 36 h. Supernatants were collected to detect (A) IL-6 and (B) IFN- γ cytokine levels by ELISA and (C) IFN- α/β level from reporter cell line (B16-BlueTM IFN- α/β Cells). (D) Cell viability was detected using Cell Counting Kit-8 and absorbance was measured at 450 nm. Relative cell viability was determined according to naive group. (E) Cells from mice following i.p. injection were harvested and incubated for further 24 h in cell culture. CD86 positivity was assessed by flow cytometry. Results are the average of two independent experiments done in triplicate (mean $\pm$ SD). Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * p < 0.05; ** p < 0.01; *** p < 0.001.

the supernatants, whereas separately encapsulated ligands yielded only ~4 fold more IL-12 over non-encapsulated ligand combination treated groups (Fig. 2C). Furthermore, type I IFN secretion from BM-DCs treated with co-encapsulated CpG ODN plus poly(I:C) was found to be ~2.0 fold and ~1.7 fold more compared to non-encapsulated combination treated group and separately encapsulated CpG ODN and poly(I:C) group, respectively (Fig. 2D).

In a separate experiment, bone marrow derived macrophages (BM-DM) were generated and incubated with non-encapsulated or liposomes co-encapsulating ligands for 24 h. Nitric oxide (NO), and IL-12 levels were determined by Griess reagent, ELISA, respectively. While

IL-12 production in co-encapsulating group, (CpG ODN + poly(I:C))L, was consistently higher than non-encapsulated combination at all doses except at 18 μ g/ml dose, NO production was elevated only in co-encapsulated group at 18 and 6 μ g/ml doses (Two-way ANOVA, Bonferroni; Fig. 3A and B). Moreover, 2 fold more TNF- α production was detected from (CpG ODN + poly(I:C))L treated cells (Fig. 3C).

Effective antigen presentation could only be achieved when both antigen and adjuvant simultaneously internalized by APCs. Liposomes are excellent candidates for simultaneous delivery of their cargo. To address this, DQTM-OVA, was used either with non-encapsulated or with dual ligand co-encapsulated liposomes. DQTM-OVA is an ovalbumin protein

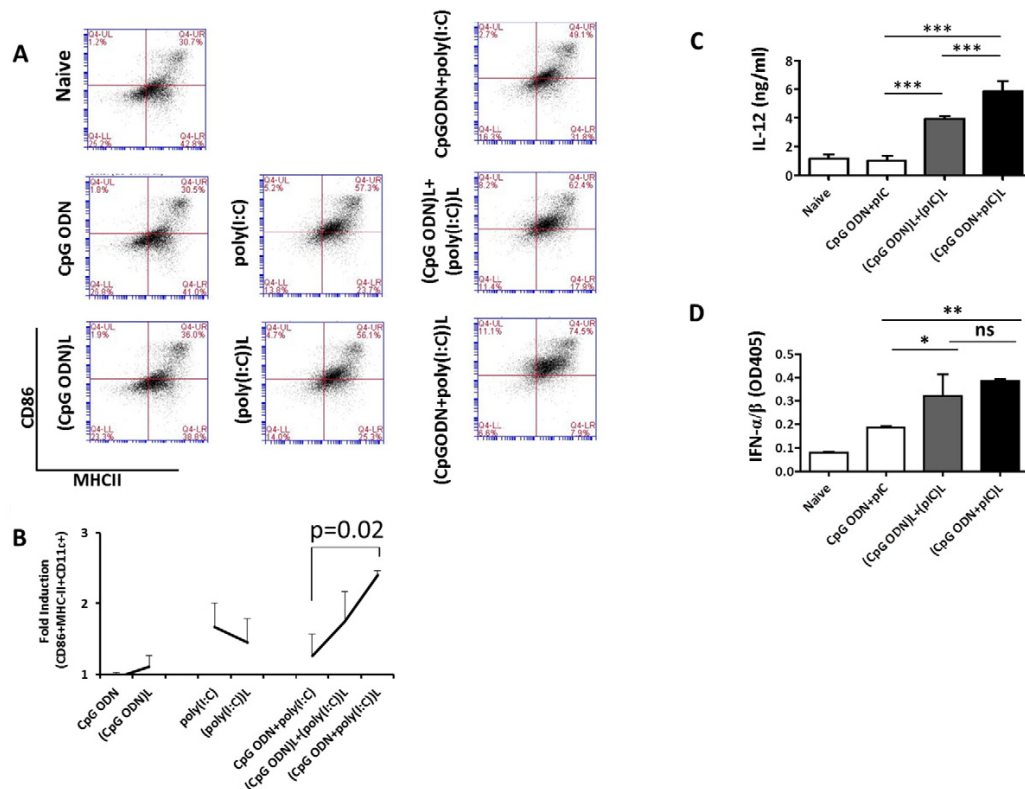


Fig. 2. Liposomes co-encapsulating CpG ODN and poly(I:C) promoted BM-DC maturation. Immature BM-DCs were incubated at 37 °C with non-encapsulated or liposomal CpG ODN and/or poly(I:C) (2 μg/ml from each ligand) for 24 h. Expression levels of CD86 and MHC-II were analyzed by flow cytometry: (A) representative dot plots. (B) Fold induction profiles of double positive cells. Average of three independent experiment are presented (Student's *t*-test, $p = 0.02$). Following stimulation, supernatants were collected to determine (C) IL-12 levels by ELISA and (D) IFN-α/β levels by reporter cell line. Results are average of two independent experiments done in triplicate (mean ± SD). Statistical significance was determined by ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

conjugated to a pH insensitive self-quenching dye. Upon internalization, fluorescence could be detected when ovalbumin is hydrolyzed. We assessed antigen delivery and processing ability of liposomal constructs following incubation with BM-DC, BM-DM and RAW 264.7 cells (Fig. 3D). All data was normalized to degradation level of each treatment group incubated at 4 °C. We found that 6 h after incubation, fluorescence signal was significantly high for liposome treated cells compared to non-encapsulated ligand treated cells (*t*-test, $p < 0.001$).

When the findings from Figs. 2 and 3 were taken together, liposomes co-encapsulating CpG ODN and poly(I:C) i) induced robust cytokine production, ii) magnified bactericidal activity, iii) upregulated costimulatory molecules, and iv) improved APC function.

3.3. Co-encapsulation of poly(I:C) and CpG ODN within liposomes augmented ligand binding by immune cells

The mechanism of the enhanced synergistic innate immune activation could be due to effective internalization of liposomal ligands by the target cells.

To verify whether augmented immune activity correlated with the improved cellular uptake, non-encapsulated and liposomal forms of Cy5-labeled CpG ODN and fluorescein-labeled poly(I:C) ligands were used to stimulate mouse immune cells and ligand specific fluorescence positivity was analyzed by flow cytometry (Fig. 4). After 2 h incubation, non-encapsulated dual ligand treated cells had 5.2% and 1.3% (for CpG ODN and poly(I:C) signal, respectively) positivity, whereas dual ligand co-encapsulated treatment gave 12.5% and 6.5% CpG ODN and poly(I:C) positivity, respectively (Fig. 4A). Consequently, co-encapsulation by

liposomes provided ~2.5 fold more CpG ODN and ~5 fold more poly(I:C) binding. When relative MFI values of the treatments were analyzed, this improvement in ligand binding was more pronounced (Fig. 4B). Thus, the improved uptake ability of both ligands via liposome encapsulation led to a robust synergistic immune activity. Furthermore, this finding revealed that rather than encapsulating TLR3 and TLR9 ligands in different liposomes, co-encapsulating them within a single carrier is more preferable (Fig. 4C).

3.4. Antigen loading together with dual TLR ligands within liposomes provides magnified and sustained antigen specific IgG response

After observing that poly(I:C) and CpG ODN co-encapsulated within liposomes boosted Th1-biased immune activation and improved APC function, we next investigated the potential of this system as a preventive vaccine (Fig. 5A). After injecting C57BL/6 mice twice (primary @ d:0 and booster @ d:14) with indicated formulations (Fig. 5B), sera were collected from animals following tail bleedings and anti-OVA IgG levels were detected by end-point ELISA. Serum titrations for total IgG, IgG1 and IgG2c subtypes were analyzed following 1st and the 8th month post-booster injections (Fig. 5B–D). The rationale of the immunization study was to identify anti-OVA antibody persistence among groups. As seen in Fig. 5B, (CpG ODN + OVA)L + (poly(I:C) + OVA)L and (CpG ODN + poly(I:C) + OVA)L groups gave the highest total IgG titers at the end of eight months post-booster injection. If one expects to raise a Th-1 biased anti-OVA immunity upon immunization, the vaccine candidate should reduce IgG1 and promote IgG2c (or IgG2a in Balb/c mice). When our results by the end of eight months of antibody titer is

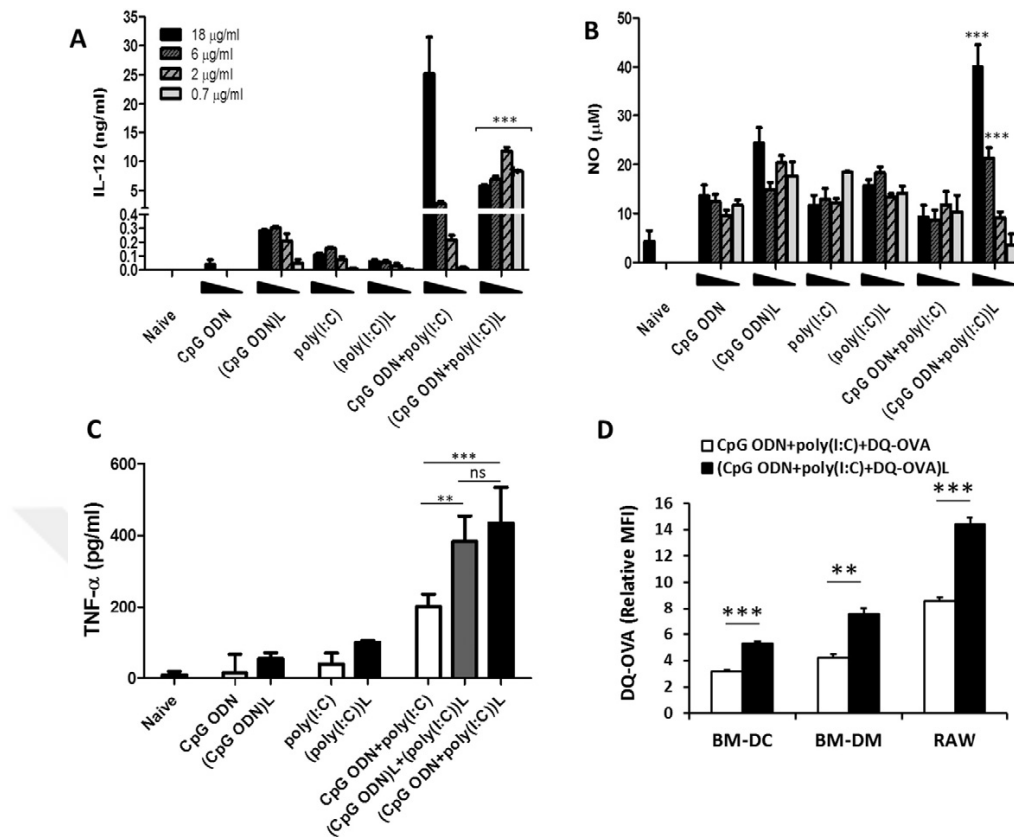


Fig. 3. Liposomes co-encapsulating CpG ODN and poly(I:C) induce pronounced macrophage activation. BM-DM were generated with 30% 1929 supplemented medium and stimulated with non-encapsulated or liposome co-encapsulating CpG ODN and poly(I:C) (0–18 µg/ml per each ligand). (A) IL-12 and (B) NO levels were determined by ELISA and Griess reagent, respectively. Statistical significance was determined by two-way ANOVA followed by Bonferroni test (non-encapsulated vs. co-encapsulated liposome). ns: non-significant; ** $p < 0.01$; *** $p < 0.001$. (C) RAW 264.7 cells were stimulated with non-encapsulated ligands or co-encapsulated CpG ODN and poly(I:C) liposomes for 24 h and TNF- α secretion was determined by ELISA (one-way ANOVA, Tukey's HSD test). (D) Antigen processing was analyzed after the addition of non-encapsulated ligands or co-encapsulated ligands (2 µg/ml from each ligand) together with DQTM-OVA (2 µg/ml) onto BM-DCs, BM-DMs or RAW 264.7 cells by flow cytometry (non-encapsulated ligands vs co-encapsulated ligands; Students t -test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Results are represented as average of two independent experiments done in triplicate (mean $\pm$ SD).

considered, Only (CpG ODN + poly(I:C) + OVA)L group gave the highest IgG2c titers and lower IgG1 titer (compare Fig. 5C and D last three bars).

Collectively this data pointed out that antigen and dual adjuvants co-encapsulated within liposomes achieved simultaneous antigen + adjuvant delivery to APC and triggered long-lasting, sustained anti-OVA specific Th1-biased immunity.

3.5. Vaccine formulations containing both CpG ODN and poly(I:C) generated persisting memory CD8⁺ T cells and prevented tumor formation

We then sought whether OVA immune animals could effectively control or eradicate tumor inoculation. At the end of eight month post-booster injection, mice were subcutaneously (s.c.) inoculated with 4×10^6 E.G7 cells (EL4 cells transduced to express OVA) and were followed for 14 days. The individual tumor development was followed daily with electronic caliper. In all animals that were immunized with saline, tumor formation was observed 4 days post-E.G7 inoculation. The median of tumors in naive group reached 402.5 mm³. The tumor sizes at day 14 post-inoculation are presented in Fig. 6A. Animals immunized with liposomal formulations showed reduced tumor sizes. Mice that received (CpG ODN + poly(I:C) + OVA)L vaccine and challenged 8 months later gave 75% ($n = 6/8$) tumor free animals at the end of $d = 14$. All groups were significantly different than naive group

(one-way ANOVA, $p < 0.01$) and the variance between co-encapsulated group and independent ligands encapsulated liposome group were significantly different (F test, $p = 0.0019$).

OVA specific memory T-cells is one of the major contributing cells to eliminate OVA-expressing tumor cells. The tumor specific CD8⁺ T-cell numbers were analyzed by IFN- γ ELISPOT assay. The splenocytes from immunized animals were treated with CD8⁺ T cell specific OVA peptide (SIINFEKL) for 18 h. The data revealed that co-administration of ligands + OVA to mice either with separately encapsulated or co-encapsulated liposomes significantly increased IFN- γ ⁺ CD8⁺ T cell numbers (Fig. 6B). Surprisingly, (CpG ODN + poly(I:C) + OVA)L vaccine treated animals did not generate significantly higher CD8⁺ memory T-cell number compared to (CpG ODN + OVA)L + (poly(I:C) + OVA)L vaccine treated group.

Collectively, our findings implied that a combination of poly(I:C) and CpG ODN adjuvants in liposomal formulations induced stronger and persistent humoral and cellular anti-OVA specific immune response sufficient to eradicate tumors.

3.6. Synergistic immune activation elucidated in hPBMCs with liposomes co-encapsulating poly(I:C) and CpG ODN

In mice, due to strong in vivo anti-tumoral immune response generated upon co-encapsulated (CpG ODN + poly(I:C) + OVA)

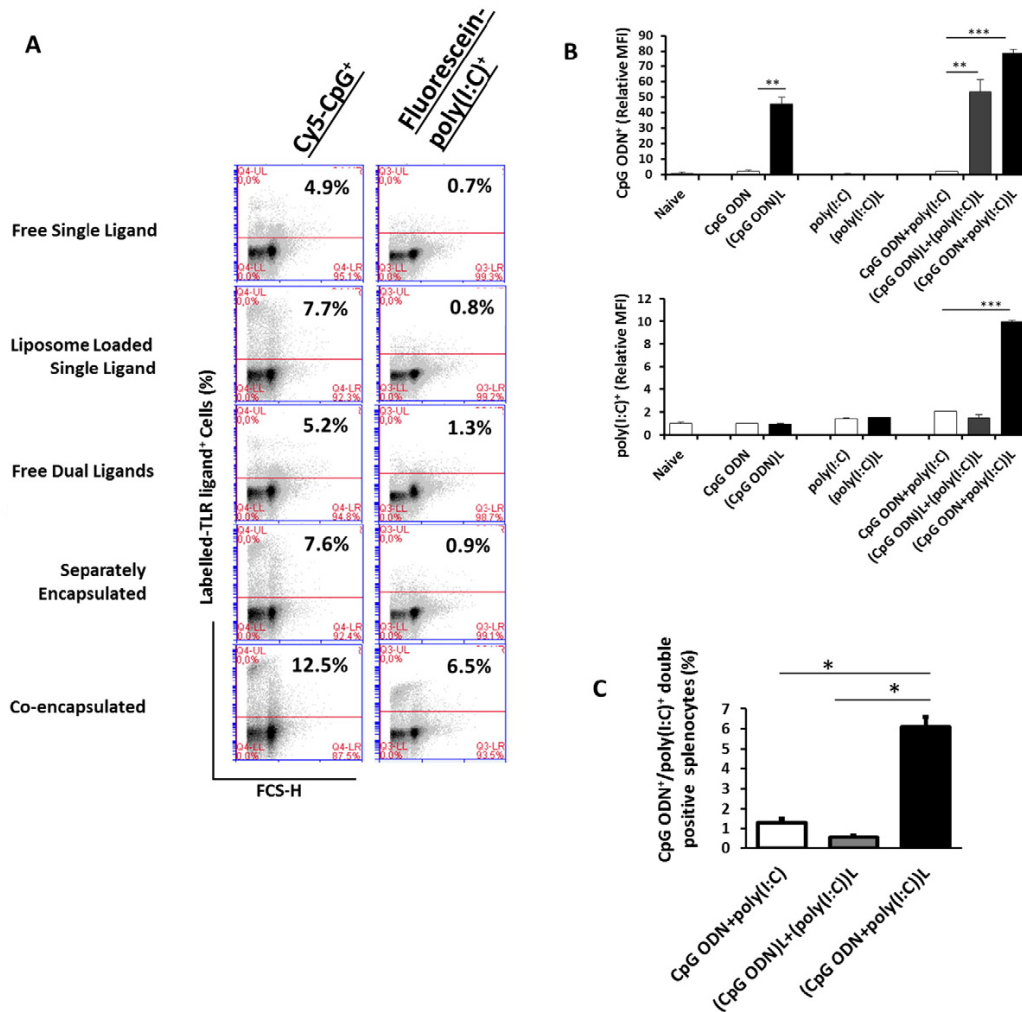


Fig. 4. Liposomes co-encapsulating CpG ODN plus poly(I:C) improves cellular uptake. Splenocytes were incubated at 37 °C with non-encapsulated ligands or liposomes encapsulating fluorescein-labeled poly(I:C) and/or Cy5-labeled CpG ODN (2 µg/ml each) for 2 h. (A) Representative dot-plots and (B) relative MFI values of uptake & binding of CpG ODN or poly(I:C). (C) Cy5-CpG ODN and fluorescein-poly(I:C) double positive cell percent after non-encapsulated ligand or liposomal ligand treated cells for 2 h (Student's *t*-test; **p* < 0.05; ***p* < 0.01; ****p* < 0.001 between groups). Data represents average of two independent experiments done in triplicate (mean ± SD).

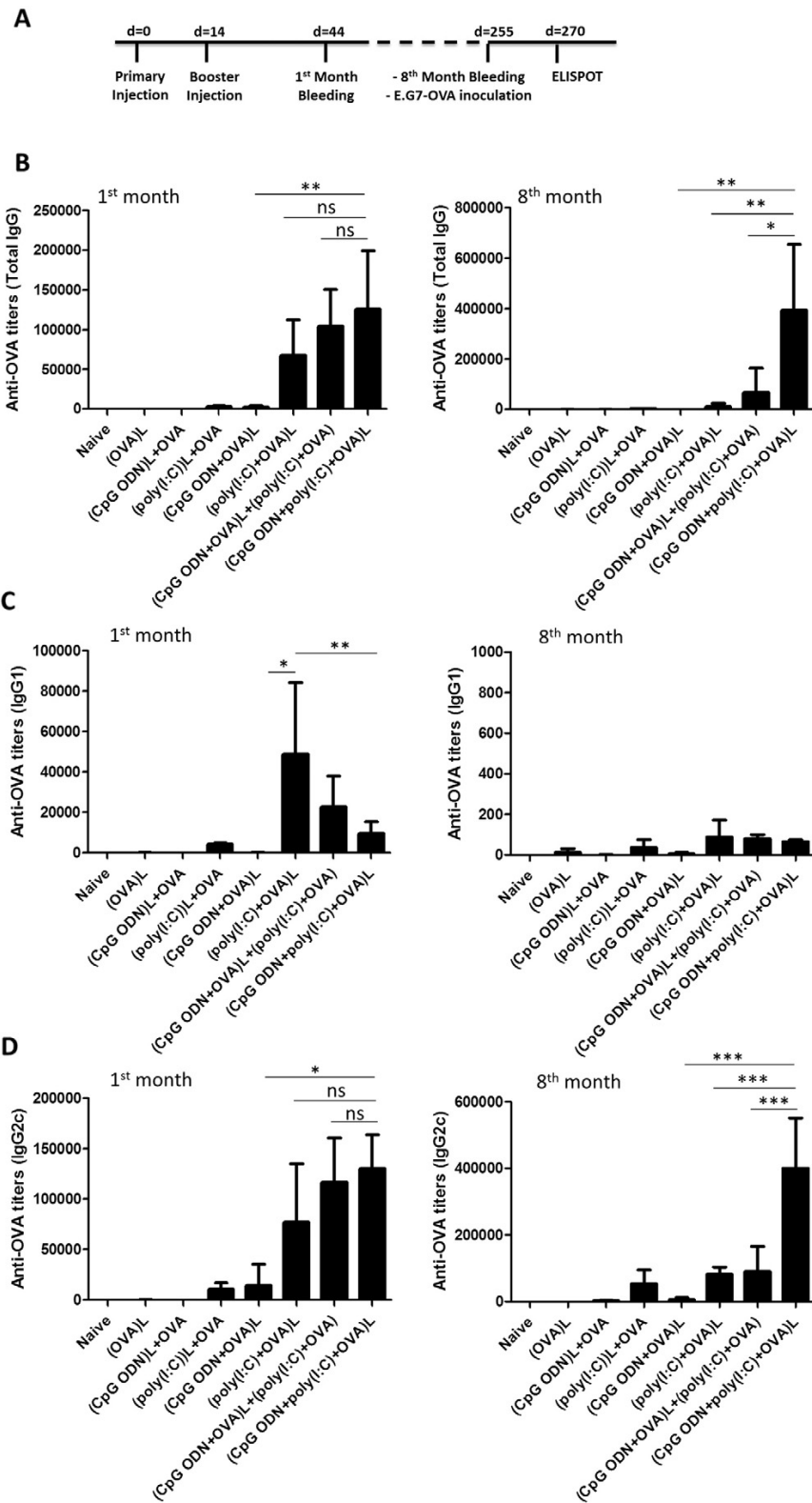
immunization, 75% of mice showed complete protection from tumor challenge. We next sought to establish whether this formulation triggers hPBMCs to the same extent that we observed in mice. Therefore, hPBMCs were collected from healthy donors and stimulated with i) non-encapsulated, ii) encapsulated and iii) co-encapsulated CpG ODN and/or poly(I:C) for 24 h. All hPBMCs produced significantly higher levels of IFN-α (A, left panels) when treated with (CpG ODN + poly(I:C))L group. Similarly, the IP10 production was more with co-encapsulated liposomal group compared to non-encapsulated or separately encapsulated ligand combinations (Fig. 7A, right panels). In addition, percentages of HLA-DR⁺/CD83⁺ hPBMCs were significantly higher in cells treated with dual ligands co-encapsulated into liposomes (one-way ANOVA, Tukey's HSD, *p* < 0.05 between non-encapsulated vs co-encapsulation, and between separately vs co-encapsulation groups; Fig. 7B).

In conclusion, co-encapsulation of poly(I:C) and CpG ODN within liposomes enhanced secretion of interferon alpha and maturation of APCs in human immune cells. A promising finding implicating that these combinations could be translated to clinical applications.

4. Discussion

There is no doubt that TLR therapeutics soon will be in the clinics. The endosomal-associated TLR ligands senses nucleic acid ligands and are one of the most promising candidate bio-drugs as immunotherapeutic agents. Clinical trials harnessing various nucleic acid TLR ligands are underway aiming to cure diseases like cancer, allergy, autoimmune or autoinflammatory and infectious diseases. One of the promising area for TLR mediated therapeutics is to utilize them as vaccine adjuvants or immunoprotective agents where there is no available vaccines

Fig. 5. Immunization with liposomes co-encapsulating CpG ODN, poly(I:C), and/or ovalbumin promotes Th-1 biased immunity. (A) Immunization schedule. C57BL/6 female mice (8/group) were immunized twice with 14 day intervals with liposome encapsulated CpG ODN (10 µg/animal) and/or poly(I:C) (10 µg/animal) or their combinations with ovalbumin (OVA, 5 µg/animal) and sera was collected from tail vein 1st and 8th month post-booster injection. (B) Diluted sera were used for OVA specific (B) total IgG, (C) IgG1 and (D) IgG2c by end-point ELISA (mean ± SD). Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.



(including viral, bacterial and parasitic infections) [8,16,18,19]. Formulating these labile nucleic acid-based agents (i.e. ds RNA or CpG DNA) in stable form within proper depot systems improve their bioavailability and bioactivity at the of the target cell sites. We previously demonstrated that liposomes enhance in vivo immunostimulatory activity of TLR ligands along with ligand sparing effect and improved shelf-life [17,24,26]. In this study, liposomes are used to co-encapsulate two important TLR ligands, expanding their breadth of synergistic immune activity, therefore, improving their immunoadjuvant effect upon co-encapsulating antigen of interest. Here, we provide evidence that these liposomes encapsulating dual TLR ligands i) upregulates costimulatory molecules, ii) magnifies proinflammatory and inflammatory cytokine secretion, iii) provides more efficient ligand internalization, iv) improves macrophage bactericidal activity v) boosts APC function of DCs and vi) promotes development of Th1 type immunity and vii) in mice provides long lasting antigen specific humoral and cell-mediated immunity capable of preventing E.G7 tumor development even after eight months post-booster injection.

Low doses of non-encapsulated single TLR ligand could be insufficient to mount an appreciable degree of immune activation however; combination of ligands even at low doses (here, 2 µg/ml of each ligand within liposomes) could trigger synergistic immune response when given within liposomes. Using MyD88 independent and dependent ligands trigger multiple signaling pathways improves the breadth of immune activation [30]. Krummen et al. demonstrated that the synergistic immunostimulatory action between TLR ligands was more pronounced than their standalone counterparts when MyD88 and TRIF-dependent signaling, like CpG ODN and poly(I:C), respectively, was combined [31]. In addition, studies showed the elevated immune response when CpG ODN is administered together with poly(I:C) [11–14,29,32]. We encapsulated these two ligands to enhance their synergistic activation and utilized them as novel form of long term effective *anti*-cancer vaccine. The demonstration of synergy between CpG ODN and poly(I:C) following co-encapsulation within liposomes were tested on murine splenocytes. Co-encapsulating CpG ODN plus poly(I:C) led to higher levels of secretion of type I and II IFNs, and IL-6 than non-encapsulated or loaded single or combinational ligands by splenocytes (Fig. 1). Co-encapsulation was stronger than separately encapsulated ligands. This improved activity suggested that co-encapsulating TLR3 and TLR9 ligands can activate immune response even better than their non-encapsulated combinations.

Additionally, type I IFN and IL-12 secretion was significantly elevated by co-encapsulation when compared with non-encapsulated or separately encapsulated dual ligands. Supporting our findings, it was shown that, IL-12 production from BM-DCs was synergistically increased with non-encapsulated combination [31,32]. However, in those studies, non-encapsulated poly(I:C) plus CpG ODN could not achieve to synergistically increase neither CD86 surface marker nor type I IFN secretion.

Liposomes act as a depot delivery system and improve cellular uptake of the ligands. Previously, we reported that uptake of CpG ODN is higher by immune cells when it is encapsulated within sterically stabilized cationic liposomes (SSCL) [26]. In this study, we showed that CpG ODN uptake was even higher when administered together with poly(I:C) into liposomes. More importantly, we provide evidence that dual uptake of ligands amplified sixfold more than non-encapsulated or separately encapsulated dual ligands (Fig. 4). In a study, lipofectamine mixed phosphorothioate-modified CpG ODN plus poly(I:C) was demonstrated to block uptake of poly(I:C) by tumor cells [34]. Here, since our ligands are encapsulated rather than complexed with the nanocarrier, we did not observe any blockage of either of the ligand uptake. Besides, the uptake increase would be explained by the decreased size of liposomes co-encapsulating dual ligands compared to unloaded liposomes (Fig. S2).

Simultaneous presentation of adjuvants and antigen plays an important role in the quality and quantity of immune response against protein

antigen [35,36]. Encapsulation of antigen with adjuvant within delivery vehicle overcomes random antigen and adjuvant uptake by APCs, furthermore it increases the internalization of exogenous antigen [20,26,35–37]. We show that, higher IgG2c production was maintained over the course of 8 months only when mice were immunized with liposomes co-encapsulating antigen and adjuvants rather than standalone counterparts (Fig. 5).

In the present work, both co- and separately encapsulated groups protected immunized mice against tumor formation. However, when the content of the separately encapsulated liposome formulation, it contained two fold more antigen than co-encapsulated formulation (i.e. (CpG ODN + OVA)L+(poly(I:C) + OVA)L vs CpG ODN + poly(I:C) + OVA)L). Therefore, it is important to note that co-encapsulated group yielded more pronounced Th1-biased *anti*-OVA immunity with 2-fold less antigen.

In a study, effect of lipid structure and size of DDA:TDB liposomes were investigated on cellular and humoral responses and CpG ODN and/or poly(I:C) were co-encapsulated or complexed to enhance immunogenicity of liposomes. Results revealed that co-encapsulating dual ligands into DDA:TDB liposomes boosted total IgG and induced Ag-specific T cell activation in multilamellar vesicles (MLV) not in SUV [37]. In that study, three immunizations with 250 µg DDA:DSPC, 50 µg TDB, 20 µg CpG, 50 µg poly(I:C) and 20 µg OVA were employed for every injection. As DDA:TDB liposomes have immunogenic effect by its own [38], Our vaccine candidate composed of 10 µg CpG, 10 µg poly(I:C), and 5 µg OVA, in neutral liposomes is the first study demonstrating enhanced synergy of CpG ODN and poly(I:C) when co-encapsulated into non-immunogenic liposomes (Fig. S3) together with antigen that with immunization, both Th1 and CTL responses were sustained over the course of 8 months, and successfully protects immunized mice to develop tumors. The aim of the present study is to develop a cancer vaccine that can protect individuals long after they are vaccinated against a specific cancer antigen. It is true that much shorter tumor challenge intervals are used for therapeutic vaccinations. However, CpG ODN and poly(I:C) is known to mediate antigen independent clearance of tumor cells. In this study, since we aimed to address the contribution of memory CD8+ T-cells, we chose to challenge our animals eight month after booster injection, therefore, TLR ligands mediated non-specific innate immune activation could not be responsible from tumor elimination.

5. Conclusion

Liposomes co-encapsulating CpG ODN and poly(I:C) boosted cellular uptake of the cargo, improved proinflammatory cytokine production along with type I/II IFN secretion, as well as co-stimulatory molecule up-regulation leading to a pronounced APC function and antigen processing. The co-encapsulation of OVA antigen into liposome vesicles created a long-lasting *anti*-cancer immune response. The heightened Th1 immunity in addition to enhanced OVA-specific memory CD8+ T cell response helped to control tumor development. Similar immune stimulatory activity was reproduced with human peripheral mononuclear cells, supporting the applicability for human use. In conclusion, the present liposomal vaccine candidate offers an effective antigen and adjuvants co-encapsulation approach and could be developed as preventive vaccine for human use against cancer or viral diseases.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

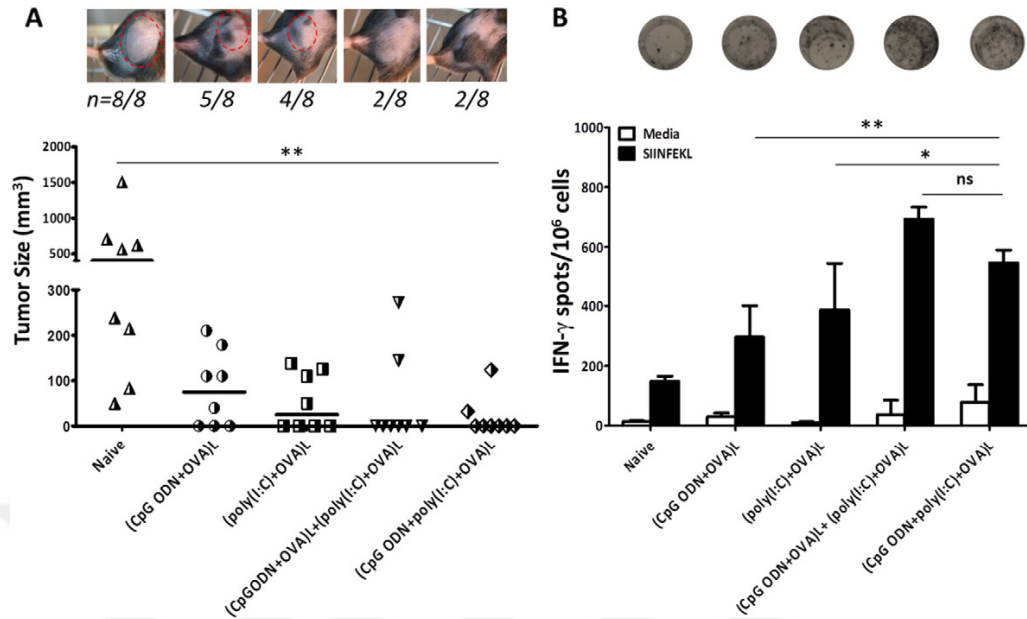


Fig. 6. Mice immunized with liposomes co-encapsulating CpG ODN + poly(I:C) + OVA resist tumor development long after booster injection. Following 8th month of booster injection, immunized animals were inoculated with OVA expressing E.G7 thymoma cells (4×10^6 cells/animal). (A) Tumor volume was measured daily for a duration of 14 days following the appearance of palpable tumor. (B) At the end of tumor follow-up mice were sacrificed and splenocytes were isolated and IFN- γ CD8 $^+$ T-cells were detected stimulating serially diluted splenocytes with OVA 257–264 peptide (SIINFEKL) for 18 h by ELISPOT method. Data were presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

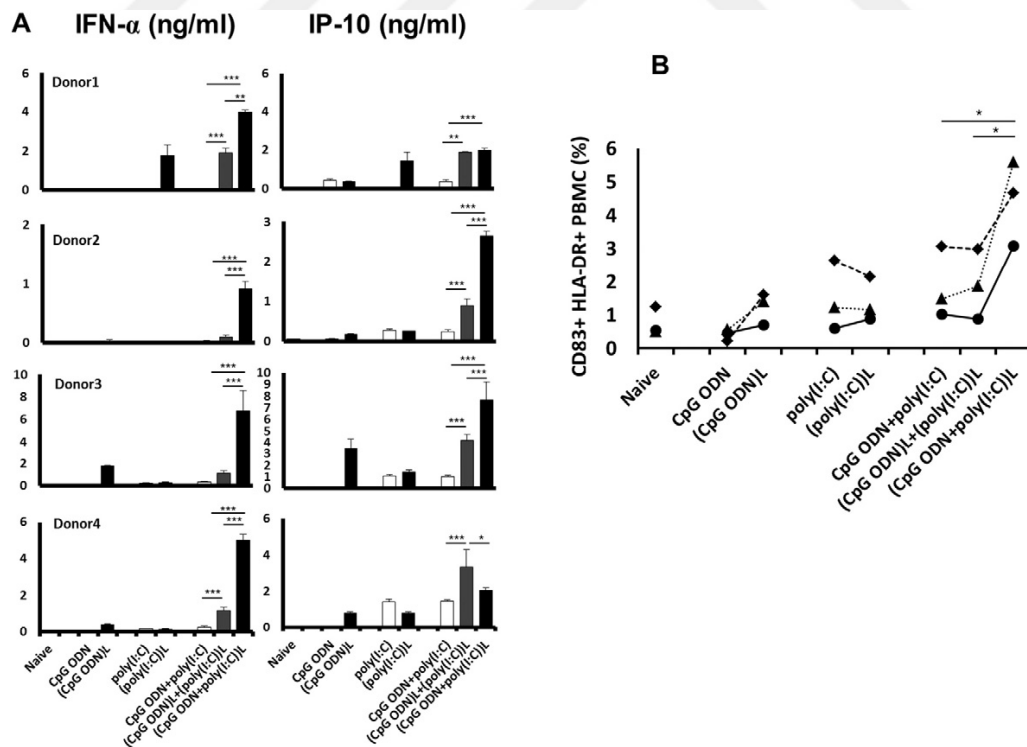


Fig. 7. Immune response of human PBMCs following treatment with liposome co-encapsulating CpG ODN plus poly(I:C). PBMCs were isolated from healthy donors' blood and stimulated with non-encapsulated or liposome encapsulating CpG ODN and/or poly(I:C) for 24 h. Supernatants were used for (A) IFN- α and IP-10 cytokine ELISA. Triplicate wells (mean $\pm$ SD) of four healthy donors are presented. (B) CD83 and HLA-DR positive PBMCs (three healthy donors) were analyzed via flow cytometry. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Acknowledgements

This work was partly supported by the Scientific and Technological Research Council of Turkey grants #: SBAG113S207 to IG.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2017.01.004>.

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SUPPLEMENTARY DATA

Supplementary Materials and Methods

AFM and DLS analyses of liposomes

Empty (non-encapsulated) liposomes were diluted in DNase/RNase free water (1/500) and were deposited on silicon wafer. Following complete drying under hood, AFM images were taken by using non-contact mode (PSIA, XE-100E, Korea). To analyze the size and polydispersity index (PDI) of empty and loaded liposomes by Zetasizer Nano ZS (Malvern, UK), liposomes (10 μ l, from 1 μ mole total lipid solution) were diluted in 1 ml PBS and measured. Triplicate readings of samples were recorded.

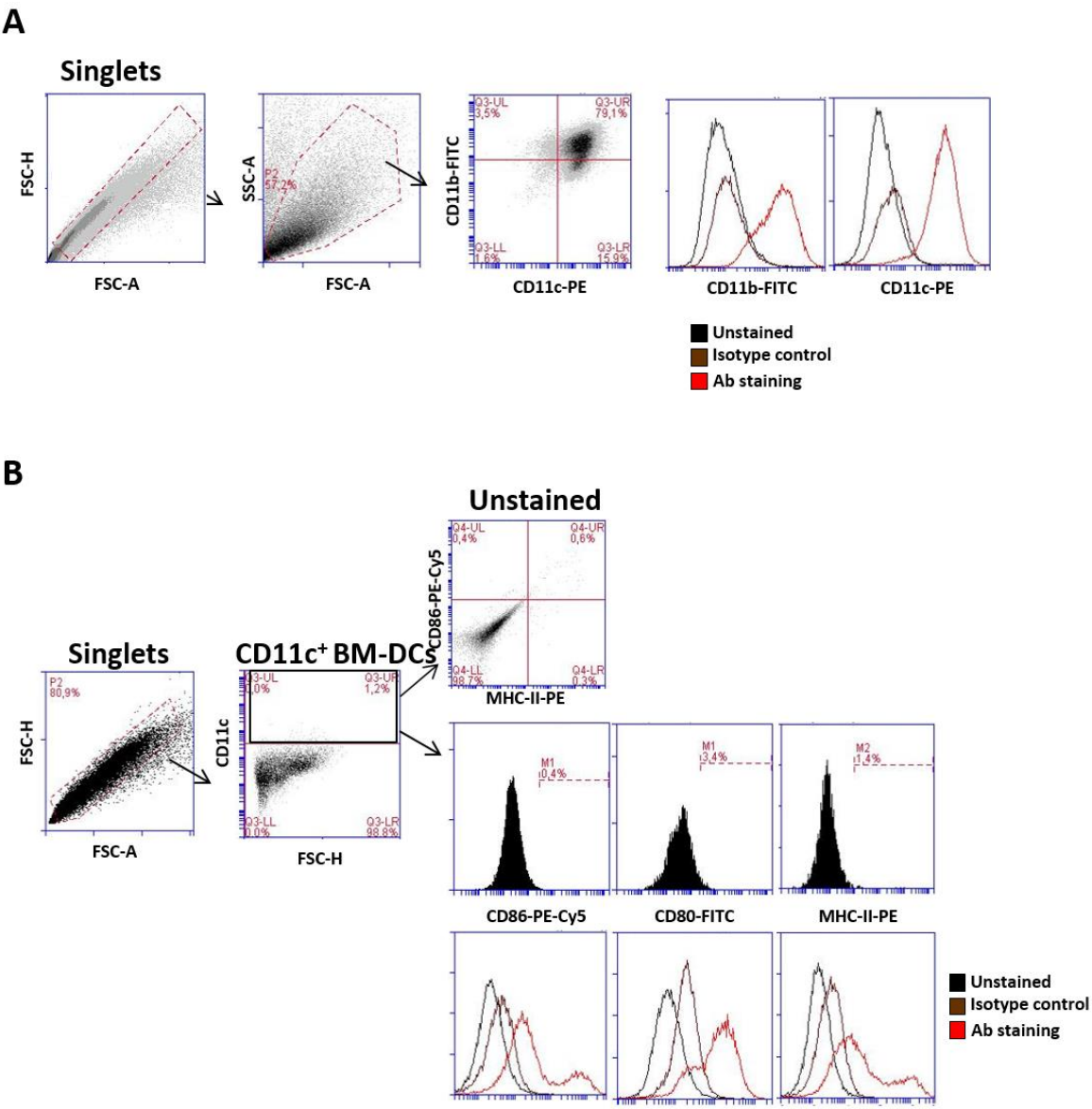
Encapsulation Efficiency of CpG ODN and poly(I:C) loaded liposomes

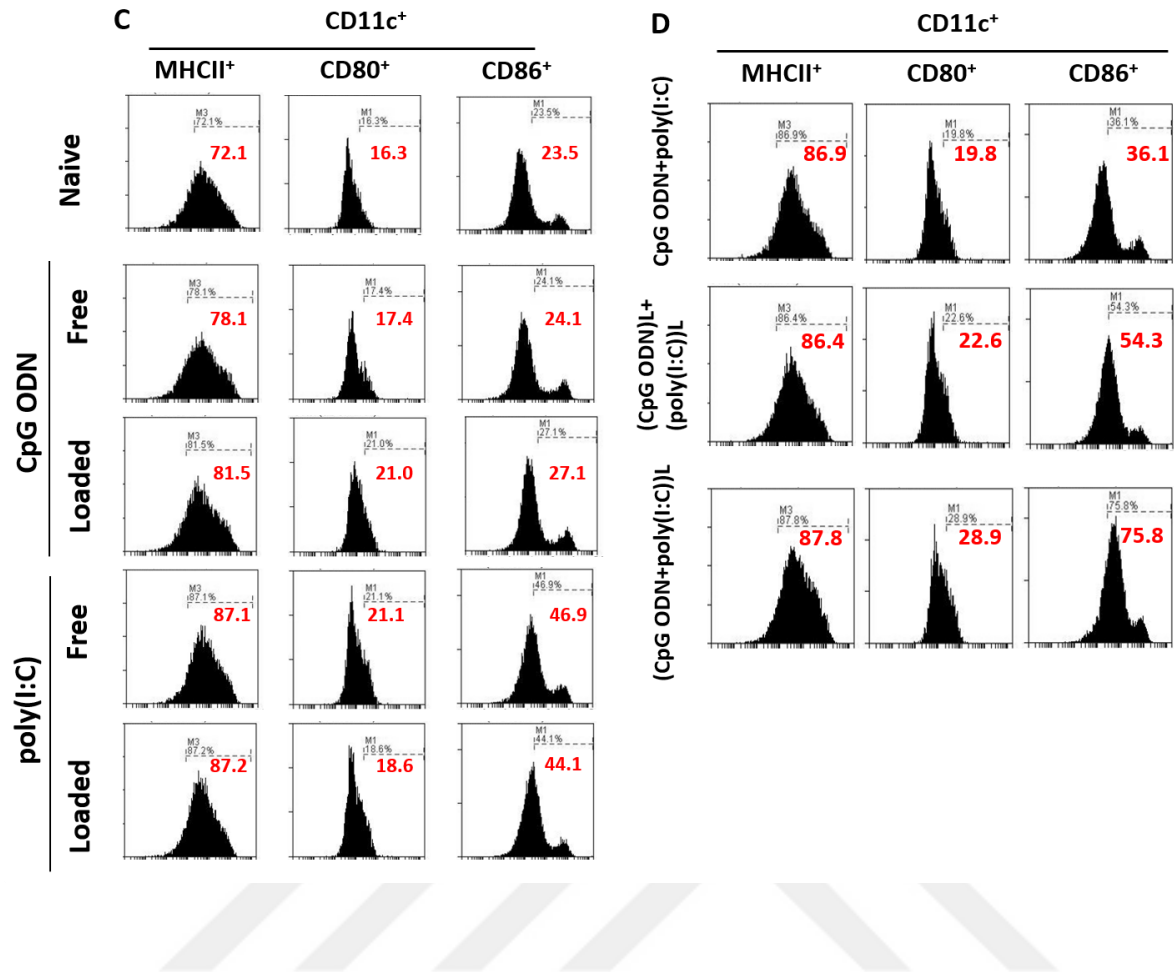
Liposomes encapsulating FITC-labeled CpG ODN and/or fluorescein-labeled poly(I:C) and/or OVA were centrifuged with 1x PBS (pH: 7.4) at 16 000 g for 1h. Fluorescent non-encapsulated ligands were detected using spectrofluorometer (excitation at: 495 nm; emission at: 515 nm; BioTek, Model: Synergy HT). Encapsulation efficiency was calculated using the equation: % encapsulation = $(C_T - C_0) / (C_T) \times 100$ (C_T : initial amount; C_0 : non-encapsulated amount).

Inflammasome activation of CpG ODN and poly(I:C) loaded liposomes

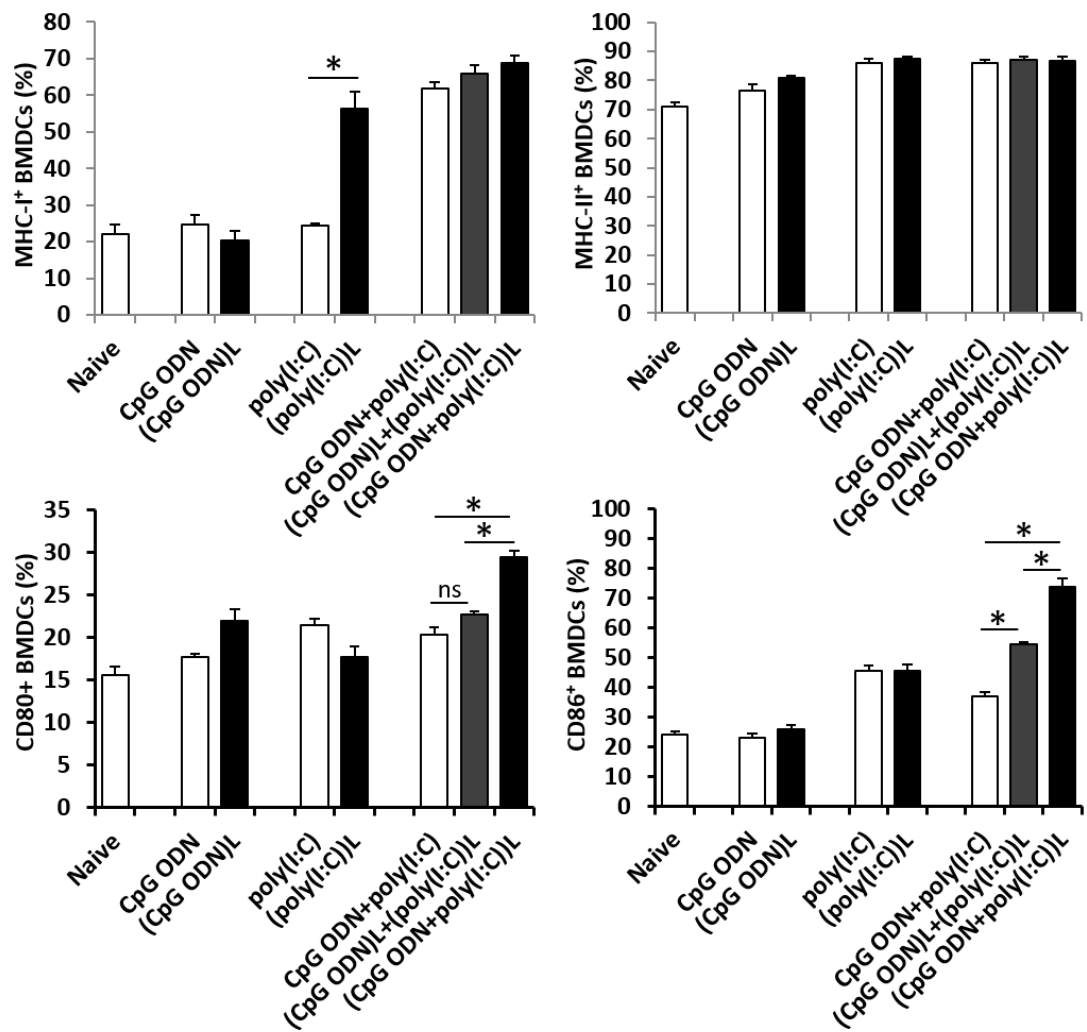
Mice were i.p. injected with 4% thioglycolate solution and 5 days later peritoneal exudate cells (PEC) were collected by washing out the peritoneal cavity with sterile medium (10 ml). 2.5×10^5 cells were seeded in 96-well cell culture plates. Cells were primed with LPS (100 ng/ml) for 4h and were then treated with non-encapsulated liposomes or liposomes encapsulated ligands for 20h. Alum (100 μ g/ml) was used as positive control throughout the inflammasome assays. IL-1 β secretion levels of treated cells was analyzed by ELISA.

SUPPLEMENTARY FIGURES





E



F

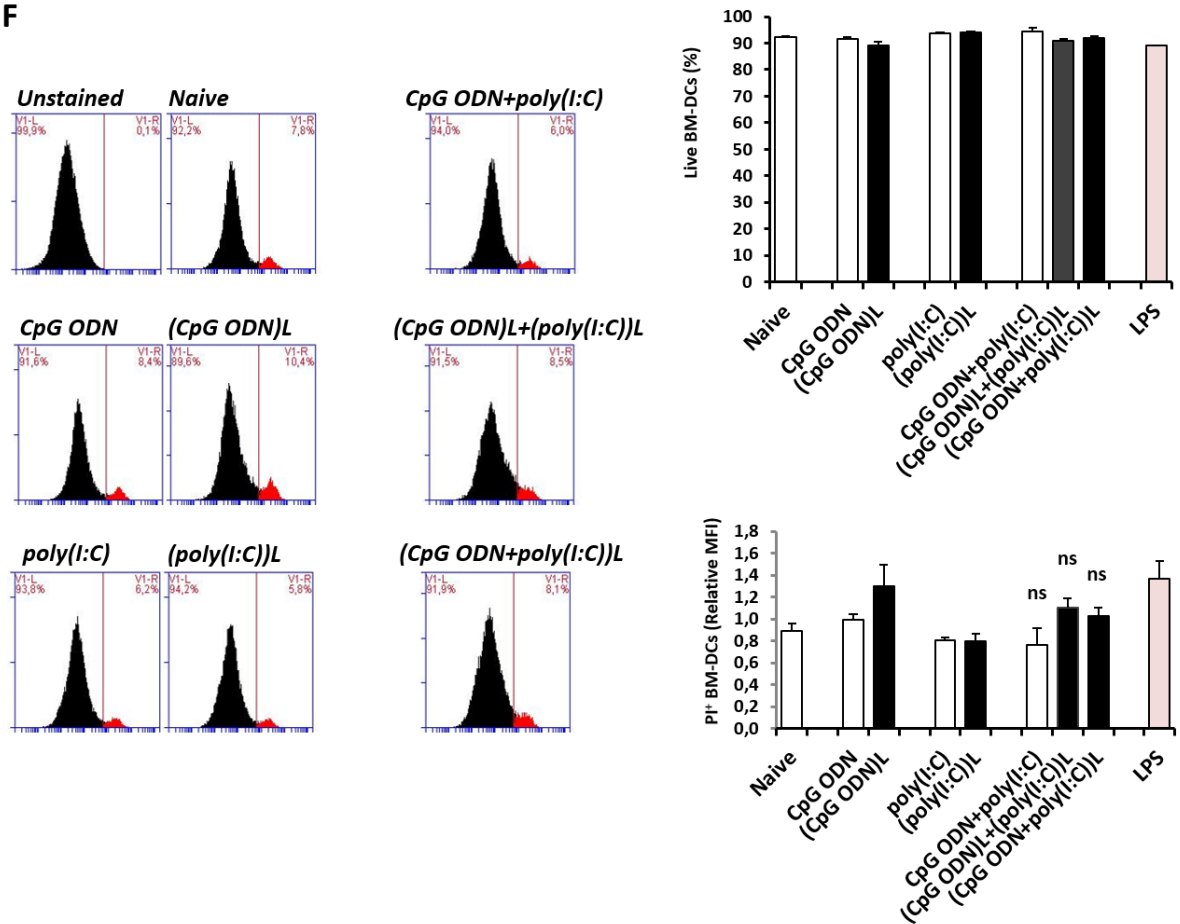


Fig. S1. BM-DC maturation by non-encapsulated or loaded CpG ODN or poly(I:C). BM-DCs were generated in the presence of GM-CSF and IL-4. (A) Double positivity levels of CD11b and CD11c expressions including isotype control staining of immature BM-DCs were checked by flow cytometry before stimulation with non-encapsulated and encapsulated ligands for 24h. (B) MHC-II⁺, CD80⁺ or CD86⁺ levels of CD11c⁺ BM-DCs were determined and gating strategy including isotype control stainings were presented. (C-D) BM-DCs that were stimulated with non-encapsulated or encapsulated ligands were analyzed by flow cytometry to determine MHCII⁺, CD80⁺ and CD86⁺ levels. (E) Percent MHC-I, MHC-II, CD80, and CD86 expression levels of BM-DC following non-encapsulated and encapsulated ligand stimulations. (F) cytotoxicity levels of non-encapsulated and encapsulated ligand(s) on BM-DCs were identified by flow cytometry following propidium iodide (PI) stainings.

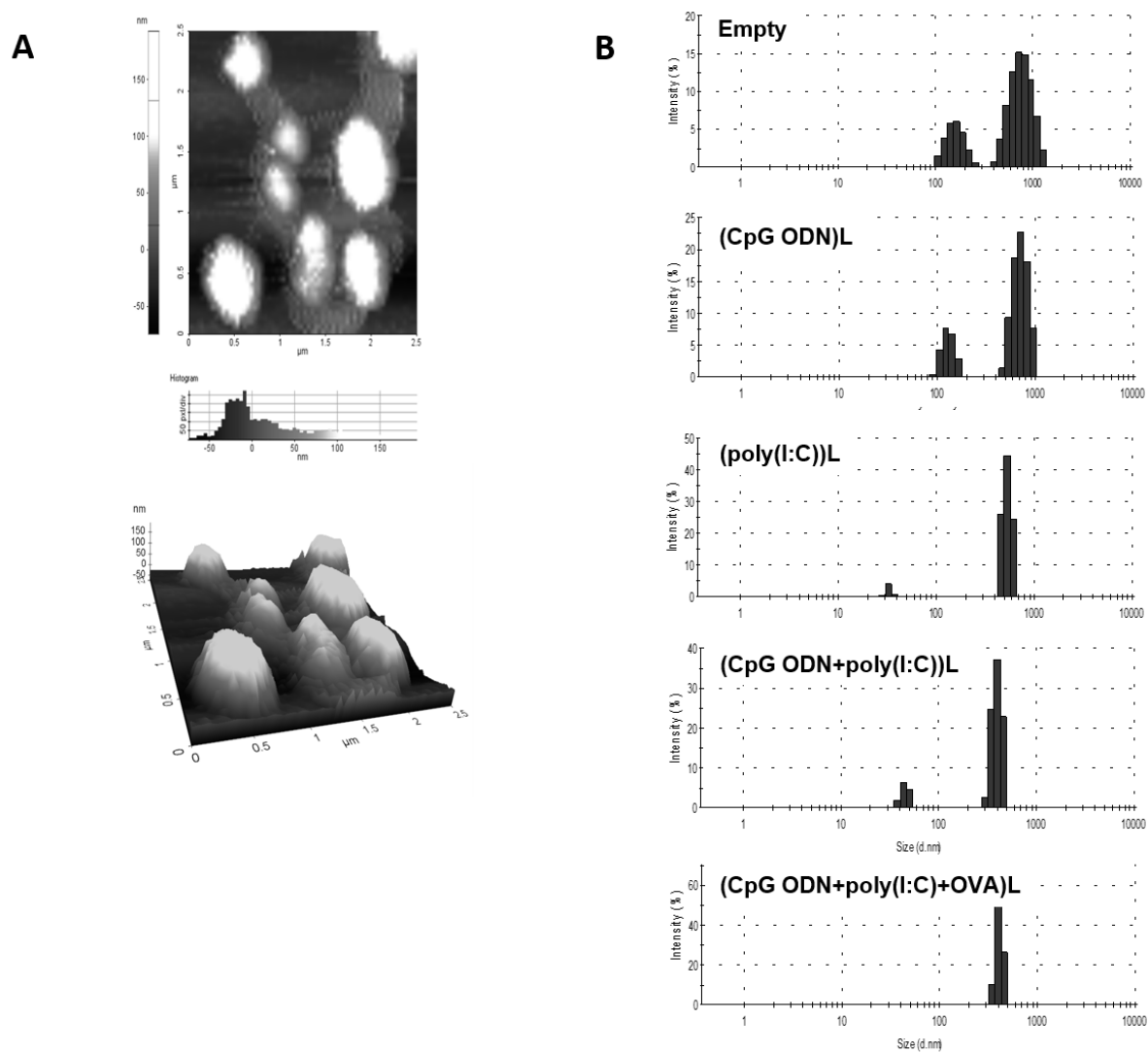


Fig. S2. Physical characterization of empty and loaded liposomes. (A) Non-encapsulated liposomes AFM micrograph was recorded by PSIA XE-100E in noncontact mode and (B) size distribution was measured by dynamic light scattering (Zetasizer Nano ZS).

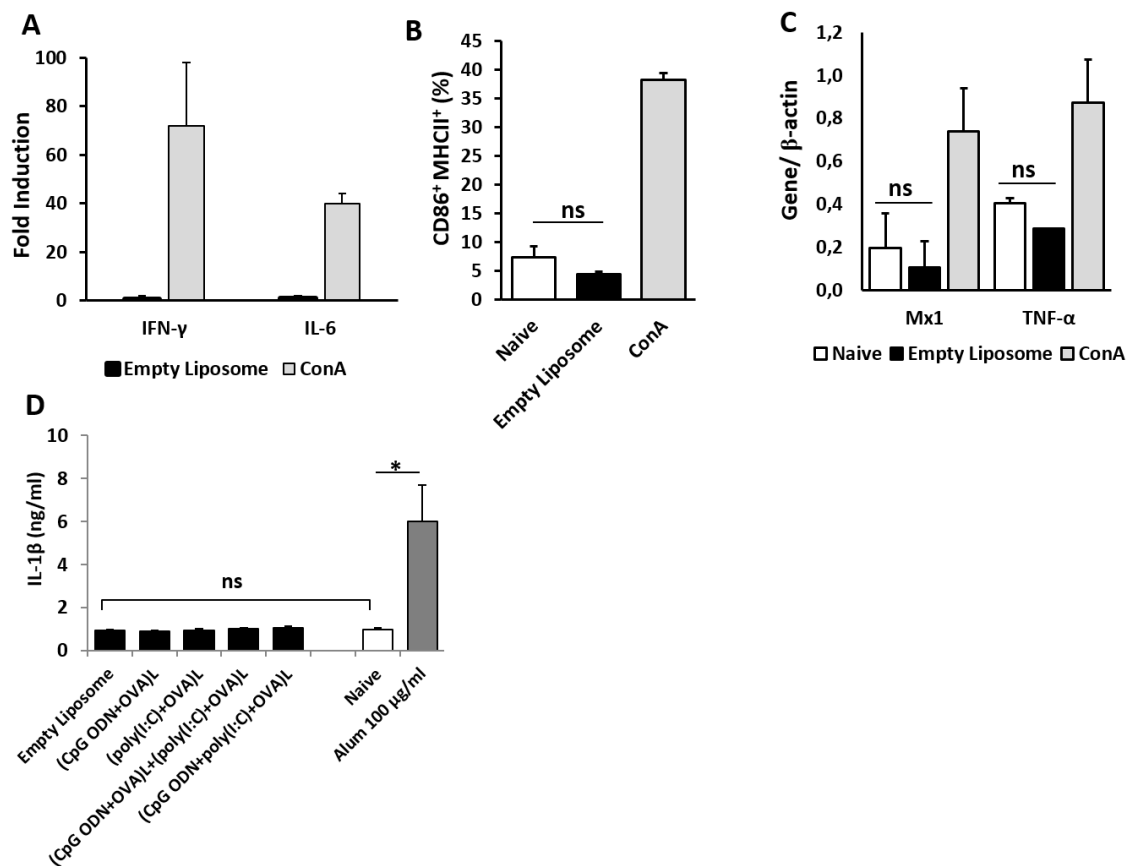
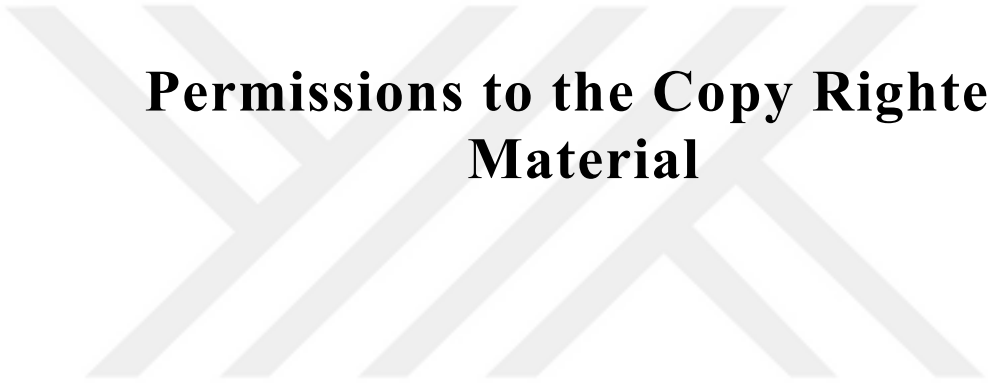


Fig. S3. Immunological properties of empty (non-encapsulated) liposomes. (A) cytokine induction capacities of non-encapsulated liposomes were assessed by incubating splenocytes with empty liposome including Concavalin A (ConA) as the positive control. Supernatants were used to detect IFN- γ and IL-6 levels and amounts were normalized to that of naive group secretion level (represented as fold induction on the figure). (B) CD86 upregulation capacity of empty liposomes following incubation with splenocytes for 24h were analyzed by flow cytometry. (C) Gene expression levels of antiviral protein Mx1 and pro-inflammatory cytokine TNF- α were measured using total RNA purified from splenocytes that was incubated with empty liposomes for 4h via RT-PCR. (D) Inflammasome activity was detected by determining IL-1 β production from peritoneal exudate cells (PECs) stimulated with empty or ligand encapsulated liposomes (Student's t-test, naive vs treatment groups).



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