ENHANCED IMMUNOMODULATORY APPLICATIONS OF NUCLEIC ACID ENCAPSULATING LIPOSOMES

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BY

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AUGUST 2009

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ABSTRACT

ENHANCED IMMUNOMODULATORY APPLICATIONS OF NUCLEIC ACID ENCAPSULATING LIPOSOMES

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Recent studies have demonstrated that innate immune system has great ability to discriminate self from non-self through the action of innate immune receptors. The most extensively studied innate immune receptor family is the Toll-like receptors (TLRs). Endosomal/intracellular TLR3, TLR7/8 and TLR9 recognize dsRNA, ssRNA and unmethylated CpG DNA respectively. Upon activation following recognition of nucleic acids by endosomal TLRs, B cells secrete IL6, dendritic cells and macrophages secrete type I IFNs, IL12 and NK cells secrete IFNγ which yields Th1 type immune response. Modulating the immune response to mount such an immune response by TLR ligands are harnessed in medical applications such as anticancer, antiviral, antibacterial therapies, anti-allergen, as vaccine adjuvant and as immunoprotective agents.

Promising clinical applications of TLR ligand nucleic acids are hampered due to their premature *in vivo* digestion by endonucleases and rapid clearance via serum protein absorption leading to limited stability and bioavailability. A powerful tool to overcome this problem can be achieved by encapsulating TLR ligands within liposomes, which increase *in vivo* stability as well as augment targeting and internalization to relevant innate immune cells.

In this study we aimed to establish the most immunostimulatory liposome type encapsulating or coencapsulating CpG ODN and pIC. Five different liposomes possessing different physicochemical properties were prepared and their immunostimulatory potential when nucleic acid TLRs are loaded, were assessed. Following stimulation of splenocytes with combinations of these liposome types we have observed that neutral, anionic and stealth liposome encapsulating D-ODN, led to a dose dependent significantly higher IFNγ production over free counterpart. Stealth liposome encapsulating pIC induced both IL6 and IFNγ 10 and 250 fold respectively over free pIC. Neutral and anionic liposome coencapsulating D-ODN with pIC were very strong type 1 IFN as well as Th1 cytokine inducers both *in vitro* and *ex vivo*. Then, we immunized B6 mice with anionic liposome coencapsulating D-ODN and OVA to establish the immuno-adjuvant properties of liposome formulations *in vivo*. We assessed primary and secondary anti-OVA IgG subclass responses of mice. Results strongly implicated that even after primary immunization, we could obtain significantly higher anti-OVA IgG and IgG2a response over OVA mixed D-ODN group. After booster injection, 22 fold more IgG, 26 fold more IgG1 and 13 fold more IgG2a were obtained compared to free group. Our findings demonstrated that when simultaneous delivery of adjuvant (D-ODN) and antigen (OVA) within a proper depot system is given to a host, very potent antigen specific immunity is achieved. This knowledge will pave the way to design of novel effective vaccine adjuvants.

Keywords: TLR, CpG DNA, dsRNA, liposome, vaccination, cytokine, IgG

ÖZET

NÜKLEİK ASİT İÇEREN LİPOZOMLARIN GELİŞMİŞ İMMÜNMODÜLATÖR UYGULAMALARI

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Ağustos 2009

Son çalışmalar, bağışıklık sisteminin doğal bağışıklık sistemi reseptörleri yoluyla kendinden olan ve olmayan varlıkların ayrılmasında çok başarılı olduğu göstermiştir. Doğal bağışıklık sistemi reseptörleri arasından üzerinde en çok çalışılan Toll-benzeri reseptörlerdir (TLR). Endozomlarda bulunan TLR3, TLR7/8 ve TLR9 sırasıyla dsRNA, ssRNA ve metillenmemiş CpG DNA'yı tanır. TLR tarafından algılanma sonucu aktifleşen B hücreleri IL6, dendritic hücreler ve makrofajlar Tip I IFN ve IL12,NK hücreleri ise IFNγ salgılar ki bu sitokinler Th1 tipi bağışıklık tepkisi verilmesini sağlar. TLR ligantlarını kullanarak bağışıklık sistemine Th1 tipi tepki verdirmenin kansere ve alerjiye karşı tedavilerde, aşı adjuvanı şeklinde veya viral ve bakteriyel enfeksiyonlara karşı koruyucu ajan olarak uygulamaları olabilir.

TLR ligantı nükleik asitlerin etkin uygulamaları, malzemenin sağlamlığını ve etkinliğini etkileyen kan içinde hızla parçalanması ve serum proteinleri tarafından adsorbe edilmesi nedeniyle sekteye uğramaktadır. Bu problem TLR ligantlarının *in vivo*'da yapısını koruyan, doğal bağışıklık hücrelerine hedeflenmesi ve hücre içine alınmasını arttıran lipozomlara enkapsüle edilmesi ile çözülebilinir.

Biz bu çalışmada en güçlü bağışıklık tepkisi verdiren pIC ve CpG ODN enkapsüle eden lipozomun belirlenmesine çalışıldı. Farklı fizikokimyasal özelliklere sahip TLR ligantı nükleik asit içeren lipozomlar hazırlandı ve immün uyarıcı potansiyelleri tayin edildi. Dalak hücrelerinin lipozom kombinasyonları ile uyarılması sonucunda yüksüz, negatif yüklü ve stealth lipozomun içine enkapsüle D-ODN'in, serbest haline göre IFNγ salgılanmasında doza bağlı güçlü bir artış görüldü. pIC içeren stealth lipozomun serbest pIC'ye göre IL6 salgılanmasını 10 kat, IFNγ'yı ise 250 kat arttırdığı tespit edildi. D-ODN ve pIC'yi beraber enkapsüle eden yüksüz ve eksi yüklü lipozomun tip I IFN ve Th1 tipi sitokin salgılanmasını *in vitro* ve *in vivo*'da güçlü bir şekilde teşvik ettiği gözlemlendi. Daha sonra D-ODN ve OVA antijenini beraber enkapsüle eden eksi yüklü lipozomun immün-adjuvant özelliğini *in vivo*'da göstermek için B6 fareleri lipozom formülasyonları ile aşılandı. Farelerin birincil ve ikincil aşılama sonucu verdikleri anti-OVA IgG alt sınıflarının tepkilerini tayin edildi. İlk aşılamadan sonra bile D-ODN'in ve OVA'nın serbest halde aşılandığı gruba göre IgG ve IgG2a titrasyonunda kayda değer artış gözlendi. İkincil aşılamadan sonra ise IgG miktarında 22 kat, IgG1 miktarında 26 ve IgG2a miktarında 13 kat artış tespit edildi. Bulgularımız, adjuvanın (D-ODN) ve antijenin (OVA) uygun bir depo sistemi ile konağa verilmesi sonucu çok etkili antijene özel immün tepkisi elde edildiğini gösteriyor. Bu bilgi yeni ve etkili aşı yardımcıları geliştirilmesinin önünü açacaktır.

Anahtar kelimeler: TLR, CpG DNA, lipozom, aşılama, sitokin, IgG

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ABBREVIATIONS

APC	Antigen presenting cell
AVA	Anthrax vaccine adsorbed
bp	Base pairs
BCG	Bacille Calmette Guerin of Mycobacterium bovis
BCR	B-cell receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freund's adjuvant
CMV	Cytomegalovirus
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
FBS	Fetal Bovine Serum
HBV	Hepatitis-B Virus
HIV	Human Immunodeficiency Virus
HN	Hemagglutinin and neuraminidase
HPV	Human papillomavirus
Ig	Immunoglobulin
IκK	Inhibitor kappa B kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IFN	Interferon

IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
MALP	Mycoplasmal lipopeptide
MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
mDC	Myeloid dendritic cells
MSR	Macrophage scavenger receptor
MyD88	Myeloid Differentiation Primary Response gene 88
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pIC	polyinosinic acid:cytidylic acid
PLG	Polylactide-co-glycolide

PNPP	Para-nitrophenyl pyro phosphate
PRR	Pattern recognition receptors
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT	Reverse transcriptase
SA-AKP	Streptavidin Alkaline-phosphatase
SLE	Systemic Lupus Erythematosus
SPG	β-(1 →3)-D-glucan schizophyllan
SSCL	Sterically stabilized cationic liposomes
ssRNA	Single-stranded RNA
TCR	T-cell receptor
Th	T-helper
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated factor
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain containing adaptor inducing IFN-β

1. INTRODUCTION

The most effective way to fight against infectious diseases is vaccination. Nevertheless vaccines are not available for numerous infectious diseases such as malaria and HIV/AIDS or effectiveness of available vaccines are controversial. For instance BCG vaccine was developed in early twentieth century by French scientists Albert Calmette and Camille Guerin consisting of an attenuated strain of Mycobacterium bovis. It has been used more than 70 years and its efficacy is somewhat controversial. Although meta-analysis of literature yields theoretical efficacy rate of 50% no matter the age of vaccinated individual (Brewer 2000), it is suggested that BCG fails to protect against pulmonary TB in adults, which is the primary source of dissemination. (Kaufmann 2000)

Safety profile of vaccine that will substitute BCG is very important, because this vaccine will obviously be applied to HIV positive patients. The disadvantage of attenuated vaccines is that the attenuated strains have risk to develop disease in immunodeficient patients. For instance, WHO does not suggest application of BCG vaccine to HIV-positive children (Reece *et al.* 2008). Subunit vaccines are more favorable at this point.

Subunit vaccines are composed of antigen specific for the pathogen, which the vaccine is designed against for. The antigen would be protein component of capsids of a virus or secreted protein of bacteria. However antigens are not immunogenic. Therefore subunit vaccines contain adjuvant (e.g. aluminum hydroxide). According to the nature of adjuvants, the response of immune system against antigens differs. That's why, while designing an effective vaccine against an infectious disease, the nature and infecting strategy of the pathogen should be considered. The adjuvant included in vaccine must trigger the correct immune response, which will be effective against the disease.

To eradicate the infectious diseases and stop infectious disease related deaths, or at least reduce the number of deaths to the level of interpretations at 2030, effective and attainable vaccines against every infectious disease should be discovered. Formulating a strong vaccine adjuvant is the first step of vaccine discovery process. In this project we tried to reveal novel and effective vaccine adjuvant. The formulas we are offering contains synthetic form of unmethylated DNA containing cytosine-phosphate-guanine (CpG) motif, which is called CpG oligodeoxynucleotide (CpG ODN) and synthetic form of double

stranded RNA (dsRNA), pIC. These are ligands for TLR3 and TLR9, which are members of diverse innate immune receptors. The delivery system we are utilizing is liposomes. We analyzed immunostimulatory activities of different liposome-ODN complexes and came up with several promising formulas.

1.1. The immune system

Colonizing microbes that are detrimental to host are called pathogens. Mammalian host provide several niches to pathogens such as skin, intestine, upper and lower respiratory tract, urogenital tract and internal organs. These niches provide optimum living conditions, such as rich nutrients and optimum temperature for pathogens to colonize. However hosts need to fight off the invaders. Otherwise damage given by pathogens would be fatal. The system that prevents microbial colonization is called the immune system.

All living organisms are continuously exposed to foreign entities throughout their life such as microorganism, poisonous substances, self metabolites and food. If they can successfully pass the first line of defense (skin, mucosa) they are encountered with second line of defense which is the innate (natural) arm of immune system. The foreign, infiltrated entities need to be discriminated from safe-self entities and they should be eliminated from the body before they invade the body and damage the host. After their foreignness is identified by innate immune system, the elimination is mainly performed by adaptive (acquired) immunity, which is considered as third line of defense.

It was previously known that while response of adaptive immunity is highly specific to antigens, innate immunity gave relatively less specific immune response. However it was revealed that innate immune system has greater specificity than previously thought. It was discovered that innate immune cells can differentiate self from non-self. While innate immunity remains unresponsive to self entities, it initiates pro-inflammatory response by involving in antigen presentation and adaptive immunity priming process.

1.1.1. The innate immunity

The existence of innate immunity was recognized by Russian immunologist Elie Metchnikoff, who is the discoverer of macrophages. He realized that many microorganisms are engulfed and digested by phagocytic cells.

Innate immune system is composed of several modules; mucosal epithelia, phagocytes, acute phase proteins, complement system, inflammasomes, natural killer (NK) cells, type-I IFNs and IFN-induced proteins, eosinophils, basophils and mast cells are members of modules (Medzhitov 2007).

Mucosal epithelial and skin is the main interface between host and pathogen world. Mucosal epithelial cells and keratinocytes produce antimicrobial proteins that limit pathogens' viability and proliferation. Epithelial cells at mucosal surfaces produce mucins, which prevent pathogens to attach and enter (Medzhitov 2007).

Phagocytosis is crucial for clearance of both intracellular and extracellular pathogens. It is carried out by macrophages and neutrophils and facilitated by antibodies and opsonins, which are host products of acute phase response and complements system. Acute phase proteins are secreted by hepatocytes upon exposure to pro-inflammatory cytokines, IL1β, IL6 and TNFα. The main function of these proteins are opsonizing pathogens for phagocytosis and activating complement system (Murphy *et al.* 2008).

Inflammasomes are protein complexes that activate proinflammatory caspases such as caspase-1,-5,-4,12 (in human). Activated caspases processes IL1 family cytokines, IL1β, IL18 and IL33. These cytokines have diverse functions in host defense such as initiating inflammation and acute phase response (Meylan *et al.* 2004).

NK cells are considered as specialized cells in defense against intracellular pathogens. They force infected cells to get into apoptosis and produce large amount of IFNγ (Bancroft *et al.* 1987; Scharton *et al.* 1993). They express activating and inhibitory receptors. The ligands of these receptors are expressed by infected cells. If the activating ligand overrides, NK cell kills the infected cell, otherwise, the target cell can survive (Lanier 2005).

Type I IFNs (IFNα/β) are produced in response to viral activation. They have critical roles in defense against viruses. Their production induces more than 100 genes, which have diverse role in antiviral response. Type I IFNs will be discussed in previous sections in more detail.

Eosinophils and basophils have role in host defense against multicellular parasites such as helminthes. They are recruited to the site of infection from blood circulation. They contain granules with enzymes and toxic proteins. Mast cells reside in mucosal tissues and

connective tissues. They are believed to have role in protecting the internal surfaces of the body from pathogens and parasitic worms. Mast cells release granules upon activation. Eosinophils, basophils and mast cells are involved in allergic reactions (Murphy *et al.* 2008).

The main difference between innate and adaptive immune system is the way they are recognizing pathogens. Adaptive immune system mediates recognition by antigen receptors. Somatic recombination of the gene segments expressing antigen receptors enables the generation of a diverse repertoire of receptors (Schatz *et al.* 1992). Innate immune recognition is mediated by germline encoded pattern recognition receptors.

1.1.1.1. Pattern recognition receptors

Pattern recognition receptors (PRR) recognize molecular structures that are unique to microorganisms. These structures are called pathogen associated molecular patterns (PAMPs) although they are also found on non-pathogenic microorganisms. These molecules are invariant among microorganisms. They are products of pathways that are unique to microorganisms. This feature enables self-non-self discrimination. Additionally these structures have vital roles for microorganism. Microorganisms cannot evade innate and adaptive immune system by changing structure of PAMPs through evolutionary process (Medzhitov 2007).

Examples of bacterial cell wall PAMPs are lipopolysaccharide (LPS), which is found only on gram negative bacteria, peptidoglycan, lipoteichoic acids and cell-wall lipoproteins. β-glucan is PAMP of fungal cell wall. Unmethylated DNA containing cytosine-phosphate-guanine (CpG) motif in bacterial commonly found on bacterial genome, viral double stranded RNA (dsRNA), single stranded (ssDNA) are another examples of PAMPs.

Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (NALP1, NALP3, NAIP5, IPAF, ASC, NOD1 and NOD2) and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) (DAI, RIG1, MDA5, LGP2) are cytosolic receptors. They are involved in recognition of bacterial and viral PAMPs. While NLRs recognize viral and bacterial components, defined RLRs only recognize viral DNA and RNA. C-type lectin receptors (CLRs) (FcγR, collectins, mannose receptor, DC-SIGN, Dectin-1 Dectin-2, CARD9) are membrane-bound receptors, which recognize viral, bacterial and fungal components. Most

studied PRRs are Toll-like receptors (TLRs). 11 different TLR, TLR1-TLR11 was defined to date. Every TLR recognize different microbial components. (Table 1**)**

Table 1: Pattern recognition receptors and their ligands. Adopted from (Ishii *et al.* 2008).

	Microbial Signature	**TLRs**	**RLRs**	**NLRs**	**CLRs**
Viruses	Structural proteins (capsid, envelope proteins)	TLR2, TLR4			
	DNA	TLR9			FcγR
	RNA	TLR3 (dsRNA), TLR7, TLR8 (ssRNA)	RIG-1, MDA5 , LGP2	NALP3	FcγR
Bacteria	Cell wall components, LPS, PGN, lipoteichoic acid, lipoproteins	TLR2/1, TLR2/6, TLR4		NOD1, NOD2, NALP1, NALP3	Collectins (MBL)
	Flagellin	TLR5		IPAF, NAIP5	
	Perotoxins			NALP3	
	DNA	TLR9		ASC	
	RNA			NALP3	
Protozoan parasites	GPIs	TLR2, TLR4			
	Malaria hemozoin	TLR9			
	Proteins (*T. cruzi* Tc52, profilin)	TLR2, TLR11			
	DNA	TLR9			
Helminths	Lipids	TLR2			
	RNA	TLR3			
Fungi	Cell wall components (GlcNAc, mannan, β-glucan)	TLR2, TLR4, TLR6			Mannose receptor, DC-SIGN, Dectin-1, Dectin-2, CARD9
	DNA	TLR9			

The production of antimicrobial molecules by mucosal epithelia and keratinocytes, is induced by engagement of TLRs and NOD receptors. During acute phase response, secreted PRRs, collectins, ficolins and pentraxins are secreted from hepatocyte. Cell autonomous viral recognition and subsequent NK cell activation ligand expression is mediated by direct TLR3 activation or by recognition of pathogenic entities by cytoplasmic PRRs, RIG-1 or MDA5 or by cell-autonomous detection of excessive cellular stress. NK cell activation is also controlled by plasmacytoid dendritic cells, which were activated through TLRs and

expressing IL15 upon viral infection. Type I IFN production is controlled either by intracellular PRRs or by endosomal TLRs, TLR3, TLR7 and TLR9. Mast cells can be directly activated by TLR engagement. (Medzhitov 2007) It is obvious that PRRs have direct influence on control of innate immune response.

1.1.1.1.1. Toll like receptors

Toll was initially discovered in Drosophila melanogaster as an essential receptor for embryonic patterning. Subsequently it was understood that Toll is critical component of host defense against fungal and bacterial infections. (Leulier et al. 2008) Thereafter its human homolog has been defined. (Medzhitov et al. 1997) TLRs are a group of evolutionarily conserved proteins belonging to the IL-1R superfamily, characterized by an extracellular LRR and an intracellular Toll/IL-1 receptor like (TIR) domain. TIR domain of Toll proteins is a conserved protein-protein interaction module, which is also found in a number of transmembrane and cytoplasmic proteins in animals and plants have a role in host defense. (Medzhitov 2001)

1.1.1.1.2. TLRs in innate and adaptive immunity

TLRs in the innate immune system serve an essential role not only in recognition of pathogen, but also in directing the course and type of innate immune response generated following exposure to foreign antigen. (Takeda *et al.* 2003) TLRs have been demonstrated to have a wide array of functions including initiation of proinflammatory responses and antiviral responses, up-regulation of costimulatory molecules on antigen presenting cells (APC), release of chemokines to induce migration of responder cells to the site of infection, and induction cross-priming of T cells by DCs (Takeda *et al.* 2005). TLRs have emerged as essential not only in innate immune responses but also in shaping adaptive immune responses to pathogen. The signals for activation of adaptive immunity are mostly provided by DCs. TLR-mediated recognition of pathogens by DCs induces the expression of costimulatory molecules such as CD80/CD86 (which provides a costimulatory signal necessary for T cell activation and survival) and production of inflammatory cytokines such as IL-12 (Iwasaki *et al.* 2004) DCs subsets can induce either Th1 and Th2 responses. Activation of TLR9 in DCs induces production of IL-12, thereby changing the naïve $CD4^+$ T cell differentiation toward

Th1 type (Iwasaki *et al.* 2004). LPS stimulates TLR4 signaling pathway and DCs to support Th1 and Th2 cell differentiation (Kaisho *et al.* 2002).

1.1.1.1.3. TLR family members

Mammalian TLRs comprise of a large family consisting of at least 11 members. TLRs play important roles in recognizing specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses. It is expressed in a variety of somatic cell types (Zarember *et al.* 2002), most predominantly in the cells of the immune system, including, lymphocytes, macrophages and DCs (Iwasaki *et al.* 2004; Kabelitz 2007). TLRs can be subcategorized according to their localization in the cells. TLR1, 2, 4, 5, 6 and 10 which are seemed to specialized in the recognition of mainly bacterial products; are located on the plasma membrane, whereas TLR3, 7, 8 and 9 that are specialized in viral and intracellular bacteria detection and nucleic acids, are located in the intracellular endosomal and/or ER compartments (Iwasaki *et al.* 2004; Latz *et al.* 2004).

1.1.1.1.3.1. TLR1, TLR2 and TLR6

TLR2 responds to various microbial products such as lipoproteins, bacterial PGN and LTA, lipoarabinomannan of *mycobacteria*, glycosylphosphatidylinositol anchors of *Trypanosoma cruzi*, a phenol-soluble modulin of *Staphylococcus epidermis* and zymosan of fungi (Takeda *et al.* 2005). One of the aspects proposed for the wide spectrum recognition of microbial components TLR2 recognizes, is that TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2. The studies done with the TLR6 or TLR1 deficient mice showed no inflammatory response to mycoplasma-derived triacyl and diacyl lipopeptides respectively. TLR2 defective mice did not show any response to both triacyl and diacyl lipopeptides. (Takeda *et al.* 2002) This proves that TLR1 and TLR6 functionally associate with TLR2 and have role in discriminating diacyl or triacyl lipopeptides. In addition to that TLR2 has been shown to functionally collaborate with distinct types of receptors such as dectin-1, a CLR family receptor for the fungal cell wall component β-glucan. It was reported that TLR2 triggers TNF-α and MIP-2 secretion from macrophages through the MyD88 signaling pathway with yeast C. albicans. (Gil *et al.* 2006) It has been revealed that PGN could also be delivered to

the cytosol for NOD1 recognition from extracellular sites or from phagocytosed bacteria (Chamaillard *et al.* 2003). Therefore we can suggest that for the recognition of PGN, TLR and NLR could act together.

1.1.1.1.3.2. TLR3

The discovery of double-stranded (ds) RNA as the ligand for endosomal located TLR3 helped recognize that TLRs may have a key role in the host defense against viruses by enhancing NF-κB and interferon (IFN)-regulatory factor 3 (IRF3) pathways (Alexopoulou *et al.* 2001; Matsumoto *et al.* 2003). As exceptions, inflammatory cell of lamina propria express TLR3 on cell surface during active Crohn's disease (Cario *et al.* 2000). In addition to dsRNA viruses, most viruses during their replication synthesize and induces the production of type I interferons (IFNα/β), which exert anti-viral and immunostimulatory activities. NK cells are the major players in the antiviral immune response and express TLR3 and are activated directly in response to synthetic dsRNA, polyinosinic acid:cytidylic acid (pIC) (Schmidt *et al.* 2004). While viral nucleic acid recognition by TLR7/8 and TLR9 preferentially expressed on plasmacytoid dendritic cells induces high amount of type I IFNs, TLR3 expressed myeloid dendritic cells and macrophages induces high amount of type I IFN secretion and IL12 in response to pIC (Siegal *et al.* 1999; Kadowaki *et al.* 2001; Colonna *et al.* 2004). TLR3 in DCs recognize viral infection in phagocytosed dying cells (Schulz *et al.* 2005). Moreover TLR3 interacts with CD14, which binds dsRNA and facilitates the uptake of dsRNA into endosome (Lee *et al.* 2006). Upon TLR3 activation by dsRNA, PI3K is recruited to phosphorylated tyrosine residues of TLR3 and supports the activation of IRF3 which induces transcription of Type I IFNs. Besides, TRIF associating with TBK1 through TRAF3 and NAP1 phosphorylates and activates IRF3, which in turn activates activation of IFNα/β. Moreover tyrosine kinase c-Src also associates with TLR3 to activate IRF3. However the precise role of c-Src could not be fully understood yet. (Honda *et al.* 2006) Please see Figure 1B for TLR3 mediated Type I IFN induction pathways.

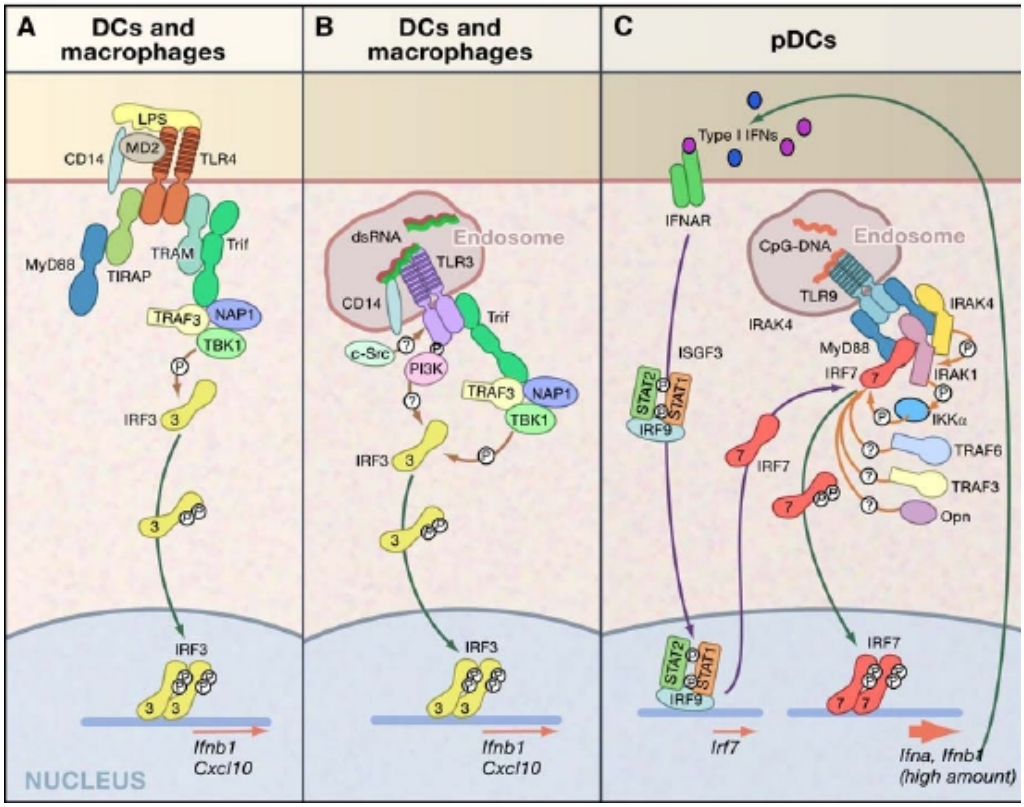


Figure 1A-C: TLR mediated Type I IFN Induction Pathways. Adapted from (Honda *et al.* 2006).

1.1.1.1.3.3. TLR4

Human TLR4 was the first identified mammalian Toll (Poltorak *et al.* 1998). This extracellular TLR is expressed in variety of cell types, most predominantly in macrophages and DCs (Medzhitov *et al.* 1997). TLR4 functions as the signal-transduction for signal-transducing receptor for lipopolysaccharide (LPS) which is a major component of the outer membrane of Gram-negative bacteria (Hoshino *et al.* 1999). Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS is first bound to a serum protein, LPS-binding protein (LBP), which functions by transferring LPS monomers to CD14 (Wright *et al.* 1989). CD14 is a high-affinity LPS receptor that can either be secreted into serum, or expressed as a glycophosphoinositol (GPI)-linked protein on the surface of macrophages. Another component of the LPS receptor complex is MD-2 (Shimazu *et al.* 1999). Although its precise function is not known, MD-2 is required for LPS recognition

(Schromm *et al.* 2001). In addition to LPS, TLR4 is involved in the recognition and is considered to be an accessory protein other ligands, including LTA, and a heat-sensitive cell-associated factor derived from Mycobacterium tuberculosis (Li *et al.* 2001). Interestingly, TLR4 and CD14 were also shown to trigger a response to the fusion (F) protein of respiratory syncytial virus (RSV). Since it is not clear yet whether the F protein of RSV represents an example of a viral PAMP, an alternative possibility is that the RSV evolved the ability to stimulate TLR4 for its own benefit (Kurt-Jones *et al.* 2000). TLR4 is the only TLR that can induces Type I IFN secretion. It is regulated through Trif-dependent pathway. TBK1 associating with TRIF through NAP1 and TRAF3 phosphorylates and activates IRF3. Activated IRF3 localize to nucleus and activates expression of IFNα/β genes. TLR4 utilizes the same pathway for Type I IFN induction with TLR3 mediated type I IFN induction (Figure 1a,b).

1.1.1.1.3.4. TLR5

TLR5 recognizes flagellin, the protein subunits that make up bacterial flagella from gram positive and gram negative bacteria (Hayashi *et al.* 2001). Flagella is recognized by the innate immune system of mammals(Wyant *et al.* 1999) and plants (Gomez-Gomez *et al.* 2000). It induces TNFα and IL6 secretion through MyD88 dependent pathway (Hayashi *et al.* 2001).

1.1.1.1.3.5. TLR7/8

Both of these TLRs are structurally highly conserved proteins, and recognize the same ligand in some cases. Although both TLRs are expressed in mice, mouse TLR8 appears to be nonfunctional (Akira *et al.* 2006). It has been revealed that murine and human TLR7 (but not murine TLR8) recognizes synthetic compounds, imidazoquinolines (R848), which are clinically used for treatment of genital warts associated with viral infection (Hemmi *et al.* 2002). Murine TLR7 and human TLR8 recognize guanosine or uridine-rich single-stranded RNA (ssRNA) from viruses such as HIV, vesicular stomatitis virus and influenza virus. Unless host-derived ssRNA is presented to endosomes, they are not detected

by TLR7 or TLR8. This might be due to the fact that TLR7 and TLR8 are not engaging with ssRNA (Lund *et al.* 2004).

1.1.1.1.3.6. TLR9

The most extensively studied TLR is TLR9. It recognizes unmethylated CpG dinucleotides which are common in bacterial and viral genome but suppressed and methylated in vertebrate genomes. The reason of the epigenetic difference between bacterial or viral genome and vertebrate genome is that bacteria lack cytosine methylation mechanism. Thus genome of pathogens is good marker for host to distinguish self and non-self. TLR9 is primarily expressed on B cells, NK cells and DCs After encountering of TLR9 with its ligand, TLR9 expressing immune cells proliferate, mature and secrete various cytokines (IL-12, IFN-γ, IL-6), chemokines or immunoglobulins (Ig) (Krieg 2000). A single nucleotide substitution or methylation of a cytosine residue within the CpG motif completely abrogates the immunostimulatory property of bacterial DNA (Krieg *et al.* 1995).

There are at least two types of synthetic CpG DNA, termed A or D-type CpG DNA and B or K-type CpG DNA (Klinman 2004). B/K-type CpG DNA is made up of phosphorothioate backbone and possesses more than one CpG motifs on a single backbone, and is a potent inducer of inflammatory cytokines such as IL-12, IL-6 and TNF-α, B cell proliferation and IgM secretion. A/D-type CpG DNA is structurally different from B/K CpG DNA, which are phosphodiester/phosphorothioate mixed backbone, and G-runs at 3'–5' ends, and a single CpG motifs has a greater ability to induce IFN-α production from pDCs, but inability to induce B-cells (Verthelyi *et al.* 2001; Gursel *et al.* 2002). TLR9 has been shown to be essential for the recognition of both types of CpG DNA (Hemmi *et al.* 2003). In addition to bacterial CpG DNA, TLR9 has been shown to recognize viral-derived CpG DNA in pDC such as herpes simplex virus (Krug *et al.* 2004).

Although both type of ODN are recognized by TLR9 there is dichotomy between K and D type CpG ODN on human cells. It was revealed that the the reason of differential immune activation might be membrane bound scavenger receptor known as CXCL16 expressed on pDC. Higher ordered structure (G-tetrad) of D-ODN have role in recognition of D-ODN by CXCL16. D-ODN most probably binds to CXCL16 by the help of G-tetrads

(Gursel *et al.* 2006). Recognition of D-ODN by CXCL16 might sequester it into early endosomes for a long time while K-ODN immediately localize to lysosomal vesicles. After recognition of K-ODN in late endosome, MyD88 and IRF5 colocalize with TLR9 and induce TNFα secretion (Asselin-Paturel *et al.* 2005). Following D-ODN recognition by TLR9 in early endosome of pDCs, MyD88 and interferon regulatory factor (IRF)-7 colocalize with TLR9 and subsequent downstream signaling induces IFNα production. However in conventional dendritic cells, D-ODN is rapidly transferred to lysosomal vesicles (Figure 2) (Honda *et al.* 2005). This spatiotemporal regulation of MyD88-IRF7 pathway in DCs might be the reason of high interferon secretion by pDCs. Prolonged signaling allows continuous activation of the positive feedback system and phosphorylation of de novo synthesized IRF7 to induce robust type I IFN production (Asselin-Paturel *et al.* 2005; Honda *et al.* 2006). Please see Figure 1c.

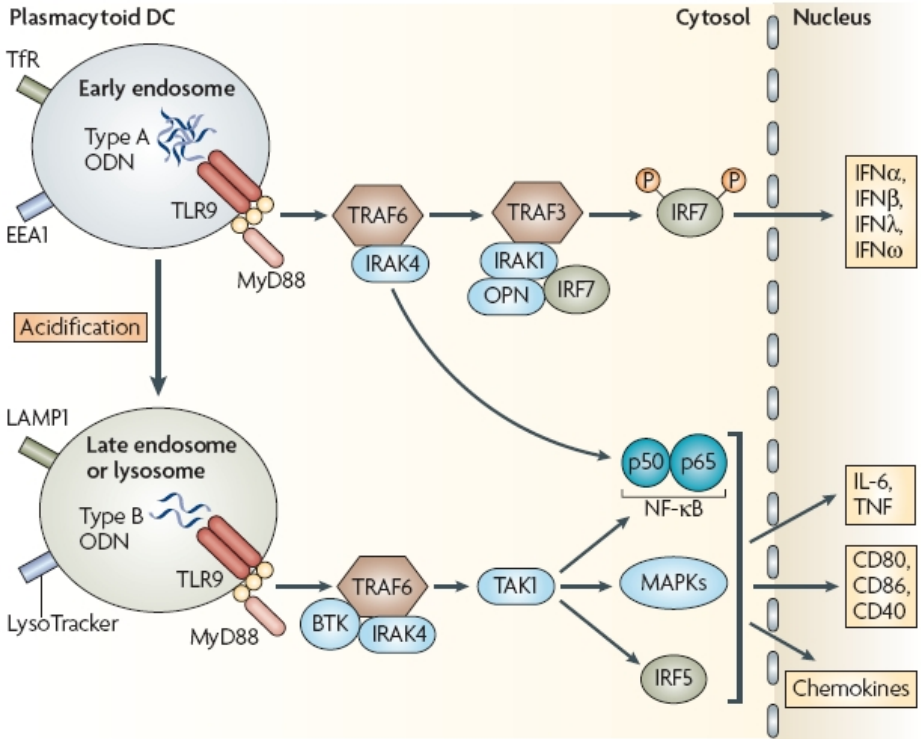


Figure 2: Signaling of D and K-ODN classes in different subcellular compartments of plasmacytoid dendritic cells. Adapted from (Gilliet *et al.* 2008)

Bacterial and viral CpG DNA and TLR9 are presumably involved in pathogenesis of autoimmune disorders. For instance, immunoglobulin-G2a (IgG2a) might bind to autoantigens such as intact nucloesomes, DNA or histones. The immune complexes are recognized by B cell receptor (BCR), its internalization is facilitated. Nucleososmes including hypomethylated CpG motifs, is then able to engage TLR9, thereby inducing autoimmune diseases such as systemic lupus erythematosus (Viglianti *et al.* 2003). More than a dozen of human clinical trails have been initiated utilizing TLR9 agonists. It seems likely that the targeted activation of TLR9 using CpG ODN will enhance the treatment of cancer and infectious diseases, as well as showing new hopes for reducing the harmful inflammatory responses such as, asthma and other allergic diseases (Krieg 2006).

1.1.1.1.4. TLR signaling pathways

Activation of TLRs by PAMPs leads to induction of various genes that involved in host defense, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase (iNOS) and antimicrobial peptides, which can directly eliminate microbial pathogens (Thoma-Uszynski *et al.* 2001). Although both TLRs and IL-1Rs rely on TIR domains to activate NF-κB and MAP kinases and can induce some of the same target genes, a growing body of evidence points to several differences in signaling pathways activated by individual TLRs. Besides, activation of specific TLRs lead to slightly different patterns of gene expression profiles. For example, activation of TLR3 and TLR4 signaling pathways results in induction of type I IFNs, (Doyle *et al.* 2002) but activation of TLR2- and TLR5-mediated pathways does not (Hoshino *et al.* 2002). In addition to TLR3 and TLR4, TLR7, TLR8 and TLR9 signaling pathways also lead to induction of type I IFNs but in a different manner (Ito *et al.* 2002). It has been revealed that there are MyD88-dependent and MyD88-independent/TRIF dependent signaling.

1.1.1.1.4.1. MyD88 Dependent Pathway

The role of Toll-mediated recognition in the control of MyD88 protein was studied using MyD88-deficient mice. A MyD88-dependent pathway is analogous to signaling pathways through the IL-1 receptors. MyD88, including a C-terminal TIR domain and an N-

terminal death domain, joins with the TIR domain of TLRs. After stimulation, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs by the interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6, leading to the activation of two distinct signaling pathways. One pathway leads to activation of AP-1 transcription factors through activation of MAP kinases. Another pathway activates the TAK1/TAB complex, which enhances activity of the Inhibitor kappa B kinase (IκK) complex. Once activated, the IκK complex induces phosphorylation and subsequent degradation of IκB, which leads to nuclear translocation of transcription factor NF-κB (Takeda *et al.* 2004). MyD88-deficient mice do not show production of inflammatory cytokines such as TNF-α and IL-12p40 in response to all TLR ligands (Takeuchi *et al.* 2000; Klinman 2004). This once again proves that MyD88 is essential for inflammatory cytokine production through all TLRs. MyD88-deficient macrophages, show impaired inflammatory cytokine production in response to TLR4 and TLR2 ligands in contrast to TLR3, TLR5, TLR7 and TLR9 ligands (Yamamoto *et al.* 2002). For brief summary, please see Figure 3.

1.1.1.1.4.2. MyD88-independent-TRIF dependent pathway

TLR4 ligand-induced production of inflammatory cytokines is not observed in MyD88-knock-out macrophages; on the other hand, delayed NF-κB expression is observed. This shows that although TLR4 signaling depends on MyD88-dependent pathways, a MyD88-independent component exists in TLR4 signaling. TLR4-induced activation of IRF-3 leads to production of IFN-β. IFN-β in turn activates Stat1 and induces several IFN-inducible genes, like TLR3 (Yoneyama *et al.* 1998; Alexopoulou *et al.* 2001). TRIF-deficient mice generated by gene targeting showed impaired expression of IFN-β- and IFN-inducible genes in response to TLR3 and TLR4 ligands (Yamamoto *et al.* 2002). Studies with the other TRIF-related adaptor molecules (TRAM)/TICAM-2 showed that TRAM is involved in TLR4-mediated, but not TLR3-mediated, activation of IRF-3 and induction of IFN-β and IFN-inducible genes (Yamamoto *et al.* 2003), so TRAM is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway. Key molecules that mediate IRF-3 activation have been revealed to be non-canonical IκKs, Tank binding kinase-1 (TBK1) and IκKi/IκKe

(Fitzgerald *et al.* 2003). It has been recently reported that, complete MyD88 and TRIF expression is required for the effective cooperation, resulting in the induction of IL-12, IL-6, and IL-23 but not of TNF-α and IP-10 upon MyD88- and TRIF-dependent TLR stimulation. Downstream of MyD88, TRIF and IRF5 were identified as an essential transcription factor for the synergism of IL-6, IL-12, and IL-23 gene expression (Ouyang *et al.* 2007). Since TRAF6 is critically involved in TLR mediated NF-κB activation, and TRAF6 associates the N terminal portion of TRIF (Gohda *et al.* 2004) and the association of C-terminal portion of TRIF with Receptor-interacting protein-1 (RIP1) (Meylan *et al.* 2004) leads to NF-κB activation. For brief summary please see Figure 3.

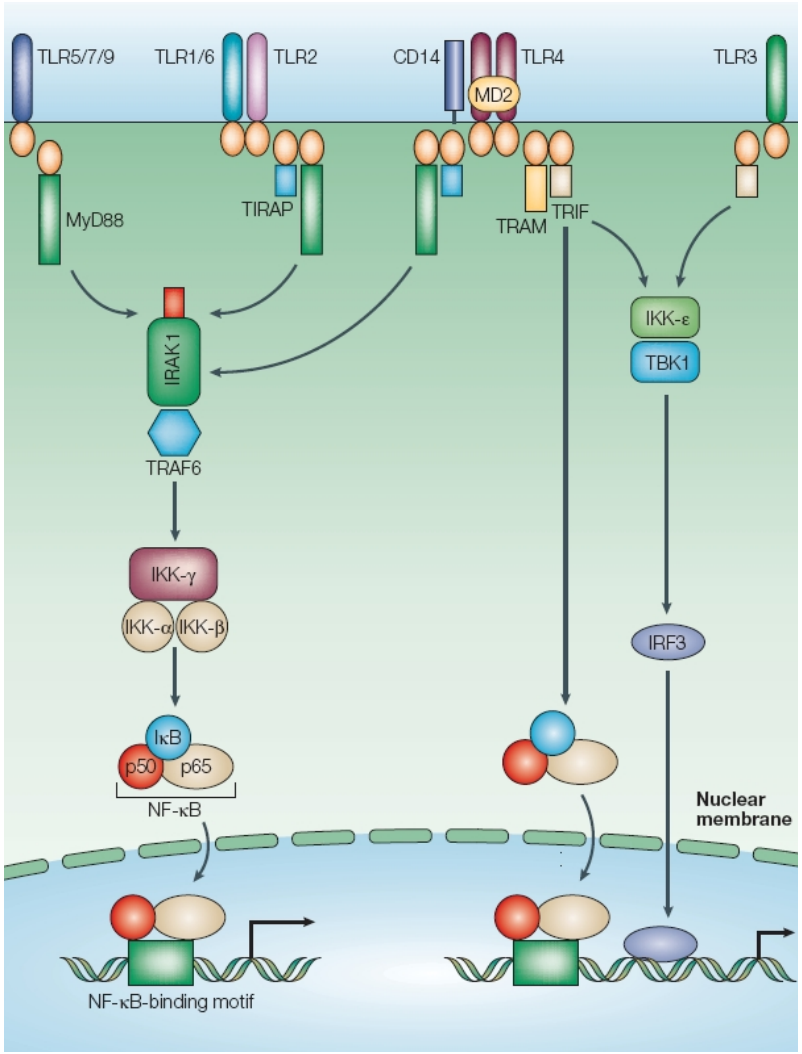


Figure 3: TLR Signaling Pathways. Adapted from (Akira *et al.* 2004)

1.2. Liposomes as delivery system

1.2.1. Liposomes: a historic brief

Since its invention at 1964 by Dr. Alec D. Bangham, hundreds of studies have been performed to make liposomes appropriate for biomedical applications such as drug delivery systems, transfection reagents, red blood cell substitutes, vaccine delivery systems and vaccine adjuvants. Use of liposomes as carriers of peptide, protein, and DNA vaccines requires simple, easy-to-scale-up technology capable of high-yield vaccine entrapment. Last 20 years eventually have resulted in the approval of several liposomal drugs and biomedical products and technologies involving liposomes (Torchilin 2005).

Liposomes are spherical structures formed by several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. They form self closed structures in water due to hydrophilic and hydrophobic interactions between phospholipids and water. There are different types of vesicles; Large multilamellar vesicles (LMV) range in size from 500nm to 5μm and consist of several bilayers. Small unilamellar vesicles (SUV) consist of single bilayer and they are around 100nm in size. Large unilamellar vesicles range in size from 200nm to 800nm and formed by single bilayer. Long circulating liposomes are modified in a way that they could not be cleared rapidly by reticuloendothelial system (RES). The modification is usually a surface grafting with certain polymers such as polyethylene glycol (PEG). Immunoliposomes carry antibodies attached to their surface and able to accumulate in the area within the body where the attached antibody recognizes its antigen. Long circulating immunoliposomes are kind of liposomes with combined properties of long circulating liposomes and immunoliposomes. They are not cleared from blood circulation rapidly and can accumulate at their target efficiently (Torchilin 2005)

1.2.2. Advantages and disadvantages of liposomes

Liposomes are biocompatible materials. They can entrap both water-soluble and insoluble pharmaceutical agents in their aqueous compartment and into membranes respectively. Liposomes protect liposome-incorporated pharmaceuticals from uptake by cells of RES or other inactivating effects such as cleavage of nucleic acids or proteins by nucleases and proteases in blood or rapid clearance due to serum protein absorption.

Liposomes can deliver pharmaceuticals into cells or even inside individual cellular compartments (Torchilin 2005). Liposomes do not accumulate appreciably in tissues such as hearth, kidneys and gastrointestinal tract. Therefore side effects of encapsulated drugs which are toxic to these organs could be lowered (Gregoriadis 1984). Besides size, charge and surface properties of liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation and or by variation of preparation methods. (Torchilin 2005)

Liposome technology has some disadvantages beside remarkable advantages. Full physicochemical characterization of liposomes is needed in early stages, which can be used to obtain regulatory approval for the liposome product. Nevertheless poor characterization of diverse array of liposomes complicates the optimization steps and delays their clinical usage. For a pharmaceutical product a minimum shelf life of two years, preferably without refrigerator cooling is a requirement. Liposomes are not that much stable. Sterile preparation of liposomes could be done by autoclaving. This is not a realistic option if labile drugs are being utilized. Moreover organic components such as ethanol or chloroform that are used during manufacturing of liposomes should be removed completely. Residual organic solvents might be carcinogenic during chronic usages (Crommelin *et al.* 2003).

1.2.3. Delivery of TLR ligands in liposome

Rapid clearance of TLR ligand nucleic acids such as CpG ODN and pIC, hampers their biomedical applications. To prevent premature clearance of CpG ODN by nucleases found in the blood, more stable backbone is used to synthesize CpG-ODN (a phosphorothioate modified form). However when they were used *in vivo,* they were still eliminated rapidly from the circulation due to the absorption onto serum proteins and degradation by serum nucleases (Barry *et al.* 1999). Prolonging the bioavaliabilty and duration of CpG ODN by liposomal encapsulation can improve their therapeutic efficiency. Hopefully it was shown that, sterically stabilized cationic liposomes (SSCL) contain positively charged phoshoplipid and polyethylene glycol can significantly enhance DNA uptake by cells of the immune system. The immunostimulatory activity of SSCL-encapsulated ODN significantly exceeded that of free ODN *in vitro* and *in vivo*. In particular, coencapsulation of CpG ODN with a model Ag ovalbumin (OVA) increased Ag-specific

IFN-γ production (10-fold) and IFN-γ-dependent IgG2a anti-OVA antibody production (40-fold), consistent with the preferential induction of a TH1-biased immune response (Gursel *et al.* 2001; Klinman *et al.* 2004). Many article in the literature reports enhanced immunostimulatory activity of CpG ODN encapsulated or coencapsulated with an antigen into various types of liposomes (Li *et al.* 2003; Suzuki *et al.* 2004; Jaafari *et al.* 2007; Badiee *et al.* 2008; Wilson *et al.* 2009). Moreover there are some studies showing that CpG ODN encapsulating liposomes can be utilized in anticancer therapies (Ishii *et al.* 2003; de Jong *et al.* 2007; Hamzah *et al.* 2009).

In addition to that co-administrating CpG ODN in polylactide-co-glycolide (PLG; another cationic microparticle that improves the uptake and processing of immune adjuvants) with the licensed anthrax vaccine, "AVA", resulting in a more rapid and stronger anti-protective antigen antibody response; IgG, than immunization with AVA alone *in vivo (*Xie *et al.* 2005*)*. Not only CpG ODN but also pIC has been co-administrated with cationic liposomes and thereby elevated the type I IFN, IFN-α production and have a unique effective on $CD8^+$ T cell responses *in vivo* (Zaks *et al.* 2006). Besides, CpG delivery could be achieved by natural carriers, such as a *β*-(1→3)-D-glucan schizophyllan (SPG) polysaccharide of a fungus called *Schizophyllan commune*. When SPG is modified with other peptides and cholesterol and complexed with CpG ODN induces dramatic enhancement in secretion of cytokines such as IL-6 and IL-12 by macrophages (Mizu *et al.* 2004).

1.2.4. Nucleic acid encapsulation methods in liposomes

There are several ways of encapsulating nucleic acids in liposomes. The first approach is called preformed vesicle approach (PFV). It employs incubation of large unilamellar vesicle (LUV) containing a cationic lipid and PEG with oligo or polynucleic acids in the presence of ethanol. The use of membrane destabilizing agent, ethanol, in conjunction with PEG-lipid enables controlling the interaction between negatively-charged polyelectrolytes and cationic liposomes and result in the encapsulation of nucleic acids in liposomes. After incubation time for encapsulation is over, ethanol is removed by dialyzing (Gregoriadis 2007).

Second way of nucleotide encapsulation in liposomes is detergent dialysis procedure. In contrast to PFV approach, the detergent dialysis procedure starts with a micellar system and the encapsulation is obtained after removal of detergent by dialysis. Nucleic acids are encapsulated into unilamellar vesicles called stabilized plasmid-lipid particles (SPLP) (Gregoriadis 2007).

An alternative method of nucleic acid encapsulation is called dehydration-rehydration protocol. The first step of the protocol is generating LUV by suspending mixture of lipids in water. If desired, LUV can be turned into SUV by sonicating the liposomes. After nucleic acid or protein solution is added onto liposomes, the mixture is freeze dried. At this step, aqueous compartment of liposomes is emptied and bilayers collapse on themselves (dehydration). Then water is added onto liposomes. Water dissolves the materials at the periphery of liposomes and starts to diffuse into liposomes. This is the time when the encapsulation occurs (Gregoriadis *et al.* 1999).

We have preferred to use dehydration-rehydration protocol rather than PFV or detergent dialysis procedure. Because the materials we have used (CpG ODN, pIC, OVA) are labile molecules. Ethanol used in PFV approach or detergent used in detergent dialysis procedure would alter their structure which would affect their activities. Besides, encapsulation ratios obtained by dehydration-rehydration protocol is higher than other methods (Gregoriadis *et al.* 1999) (Please see Table 7 in the Results Section).

2. AIM OF THE STUDY

Without doubt TLR ligands will be in therapeutics in near future. They are candidate of immunotherapeutic agents for anticancer, antiviral and antibacterial therapy. They might be used to treat allergy or utilized as vaccine adjuvant. Moreover, they can be harnessed as stand alone immunoprotective agents to provide instant protection against pathogenic insults where proper vaccines are not available.

Nucleic acids with bacterial and viral origins (and their synthetic forms) are ligands of endosomal TLRs. They are potential immunotherapeutic agents however their clinical applications are hampered due to premature *in vivo* digestion by endonucleases and rapid clearance via serum protein absorption leading to limited stability and activity. This problem can be overcome by encapsulating TLR ligands in liposomes, which increase *in vivo* stability as well as augment targeting and internalization to relevant innate immune cells.

The major plan is to assess the immunostimulatory activities of five different liposome encapsulating pIC and two types of CpG ODN, K-ODN and D-ODN both *in vitro*, *ex vivo* and *in vivo* animal model. Since liposomes possess different physicochemical properties, we anticipate that this will be translated into a differential immunostimulatory activity mediated by the liposomes encapsulating same cargo. Therefore, we needed to try each of them separately. In order to improve the in vivo bioavailability coupled with stability and internalization/targeting of the labile nucleic acid TLR ligands pIC and CpG ODN were selected as the major cargo components throughout this study. Moreover, pIC and CpG ODN are known to trigger Th1 type cytokine secretion such as IFNγ, IL6, IFNα/β, and IL12 which are critical mediators of defense against viruses and bacteria. The necessity for designing such a formulation will also allow us to co-encapsulate antigen of interest in the same liposome vesicles, thus ensuring their simultaneous presentation to relevant immune cells in a depot fashion.

Following the initial screening and potency determination experiments, the final stage of this thesis will be dedicated to utilize the most promising candidate formulation in a model vaccination experiment in mice.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Reagents

All cell culture media components were from Hyclone (USA). Cytokine ELISA reagents; i) monoclonal unlabeled and biotinylated antibodies against IL6 and IFNγ were purchased from Thermo Scientific (USA) and Endogen Pierce (USA) respectively. ii) Recombinant cytokines, iii) streptavidine-alkaline phosphatase (SA-AKP) were also bought from Endogen (USA). iv) *p*-nitrophenyl phosphate disodium salt (PNPP) - substrate for alkaline phospahatase, was purchased from Thermo Scientific, (USA). Immunoglobulin ELISA reagents; goat anti-mouse IgG, IgG, IgG2a, IgG3b monoclonal antibodies conjugated with alkaline phosphatase (AP) were obtained from Southern Biotech (USA). Ovalbumin (OVA) was obtained from "Imject OVA" kit of Pierce (USA).

DNase/RNase free water was obtained from Hyclone (USA). TRI Reagent (Trizol) for RNA isolation was from Invitrogen (USA). *DyNAmo*^{TM} cDNA Synthesis kit, *DyNAzyme™ II PCR Master Mix* for PCR was obtained from Finnzymes (Finland).

10-150 bp DNA ladder was from Fermentas, and 100-1000 bp DNA ladder was from Jena Bioscience.

L-α-Phosphatidylcholine (PC) was purchased from Sigma Aldrich (USA). Cholesterol (Chol), 3ß-[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-Glycero-3-Phosphoethanolamine (DOPE) were all from Avanti Polar Lipids (USA). Heidolph Laborota Collegiate Rotary Evaporator (Germany) and Maxi Dry Lyo, Heto-Holten (Denmark) freeze dryer were used during liposome preparation.

3.1.2. TLR Ligands

TLR ligands for stimulation assays were as follows and supplied from several vendors: peptidoglycan (PGN) isolated from *B.subtilis*; (Fluka, Switzerland), lipopolysaccharide (LPS) (isolated from *E.coli*; Sigma, USA), poly inosinic acid: cytidylic acid (pIC) (Amersham, UK) and CpG and control GpC ODNs (please see Table 2 for details)

were synthesized by Alpha DNA (Montreal, Canada), and was kind gift by Dr. Dennis M. Klinman (NCI/NIH, USA). All ODNs were free of endotoxin and protein. Sequences of CpG ODNs are summarized at the table below. Bases shown in capital letter have phosphorothioate and those in lower case have phosphodiester backbone. CpG or flip (GpC) motifs are underlined.

Table 2. CpG ODNs that are used in stimulation experiments.

ODN Name and Size	Sequence
1555 (15mer)	GCTAGACGTTAGCGT
K23 (12mer)	TCGAGCGTTCTC
D35 (20mer)	GGtgcatcgatgcaggggGG
D3CG (20mer)	GGtcgatcgatcgaggggGG
1612 (15mer)	GCTAGAGCTTAGGCT
I-127 (20mer)	GGtgcatgcatgcatgcaggggGG

Nucleotides shown in capital letter have phosphorothioate backbone and those in lower case have phosphodiester backbone.

3.1.3. Standard Solutions, Buffers, and Culture Media

See appendix A.

3.2. METHODS

3.2.1. Maintenance of animals

Adult male or female BALB/c or C57/BL6 mice (8-12 weeks old) were used for the *in vivo* experiments as well as generating primary spleen cells for *in vitro* stimulations throughout this thesis. The animals were kept in the 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).

3.2.2. Liposome Preparation

Cholesterol and various phospholipids (Avanti Polar Lipids, Alabaster, AL) were combined in different ratios as shown in Table 3. Lipid stocks were prepared in chloroform at stock solution 10mg/ml and were stored at -40°C until use.

Chloroform was evaporated in a round bottom flask using a rotary evaporator at 37°C. (Heidolph, Laborota, Germany) The solvent free lipid film was purged with argon or nitrogen to eliminate residual chloroform and oxygen, thereby preventing lipid peroxidation. To generate empty multilamellar vesicles 1ml of PBS was added to each 20μmol dried lipid film. The mixture was sonicated for 30 s each time for 5 times at 4°C using a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT) The small unilamellar vesicles were then mixed with 1mg/ml ODN, frozen in liquid nitrogen and freeze dried overnight. (Heto-Holten, Maxi-Dry Lyo, Denmark) ODN encapsulation was achieved during rehydration step. DNase/RNase free dH2O was added to dehydrated ODN/liposome powder and vortexed for 15 s every 5 min for 30 minutes at room temperature. PBS was added to the mixture yielding a final liposome concentration of 20μM lipid/mg DNA. Liposome formulations were stored at 4°C until use.

Table 3: Lipid composition and molar ratios for different liposome types

Liposome Type	Liposome Composition (molar ratio)
Neutral	PC:Chol (1:1)
Anionic	PC:DOPE:PS (1:0.5:0.25)
Cationic	DC-Chol:PC:DOPE (4:6:0.06)
Stealth	Chol:DOPE:PEG-PE (4:6:0.06)
Cationic-stealth (SSCL)	DC-Chol:DOPE:PEG-PE (4:6:0.06)

PC, phosphatidylcholine; Chol, cholesterol; DOPE, dioleylphosphatidylethanolamine; PS, phosphatidylserine; DC-Chol, dimethylaminoethanecarbamol-cholesterol; PEG-PE, polyethylene

Representations of five types of liposomes encapsulating ODN is shown in Figure 4.

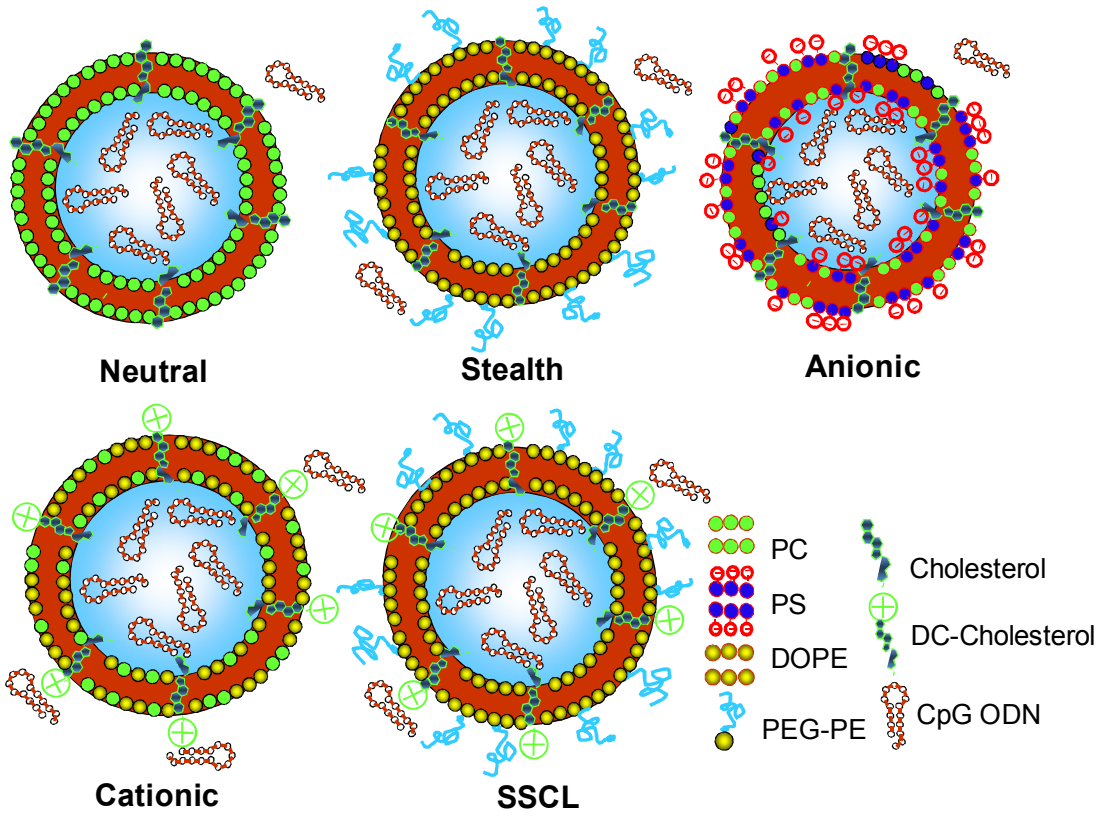


Figure 4: Schematic diagrams of produced liposomes

3.2.3. Determination of ODN encapsulation

50µl of every liposome-ODN formulation was centrifuged for 1hour at 16100xg. Supernatant was collected into eppendorf tubes. Non-encapsulated ODN concentration in the supernatant was determined by OD measurements at 260 and 280nm wavelengths with the spectrophotometer NanoDrop® ND-100 (NanoDrop Technologies, USA). Amount of ODN encapsulation was determined by subtracting the amount of non-encapsulated ODN from the original input amount and dividing it to the original input ODN amount that was initially mixed with empty SUVs before freezing and drying.

3.2.4. Cell Culture

3.2.4.1. Single Cell Suspension Preparation

Mice were euthanized by cervical dislocation. Spleens were removed with sterile instruments and put into 2 ml 2% FBS supplemented regular RPMI media in 6-well plates. In

sterile cell culture conditions, single cell suspensions were obtained by smashing spleens with the back side of a sterile syringe in media with circular movements. Homogenous part of media was collected by using sterile plastic pasteur pipettes while cell clumps belonging to fibrous or connective tissue were left. Cells were centrifuged at 1500 rpm for 10 minutes at room temperature. Medium was sucked cell pellet was gently resuspended in 10 ml fresh media and centrifuged at the same conditions. These washing steps were repeated twice to remove remaining tissue debris. At the end of last washing step, cells were resuspended in 10 ml 5% FBS supplemented regular RPMI and counted.

3.2.4.2. Cell Counting and Layering

After cells are resuspended in 10 fresh media as indicated above, 10 µl from these cell suspensions was taken and diluted 10 fold with 90 µl medium to reduce the cell number in the final 100 µl solution. 10 µl from diluted solution was placed on Neubaer cell counting chamber. Cells in 4 corners (composed of 16 small squares) with 1mm^2 area were counted under light microscope (Figure 5).

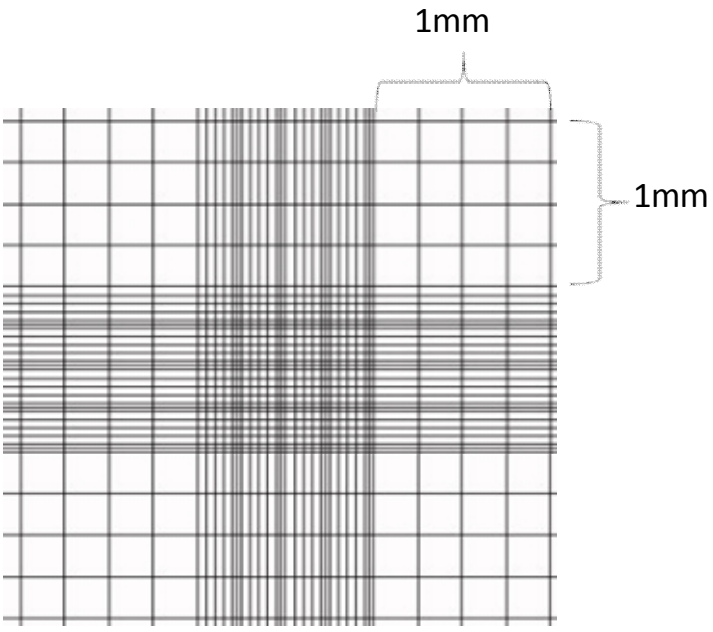


Figure 5: Neubaer cell counting chamber

The depth between coverslip and cell counting chamber is 0.1 mm. Thus, each 1 mm^2 area holds a volume of 0.1 mm^3. So, the number of cells/ml can be obtained from the equation given below:

$$\left(\frac{\text{Total Cell Number in 4 1mm squares}}{4}\right) \text{x Dilution factor x } 10^4 = \text{number of cells per ml}$$

Number of cells per $0.1mm^3$

After total cell number was determined, original cell stock was centrifuged and resuspended in an appropriate volume of 5% FBS supplemented oligo medium if cells were to be stimulated with ODNs. If stimulation with ODNs was not to be performed, cells were resuspended in 5% FBS supplemented regular medium. Working cell concentration was adjusted to $4x10^6$ cells/ml for ELISA experiments, $1x10^7$cells/ml for RNA extraction and $2x10^6$ cells/ml for FACS analysis unless otherwise stated.

3.2.5. Stimulation Protocols

3.2.5.1. Cell Stimulation

For stimulation in 96-well cell culture plates, 100 µl of $4x10^6$ cells/ml stock (400,000 cells) were transferred to 96-well plates. Total volume was completed to 200 µl with ODN solution in 100 µl, 5% FBS supplemented oligo medium. Unless otherwise stated, final concentration of CpG ODNs was from 0.3 to 3.0 µM, and pIC was between 1µg/ml to 25µg/ml. Stimulations are performed in triplicate wells for each indicated treatment.(Gursel *et al.* 2001) Supernatants are collected after 24-48h for analysis of secretion of various cytokines. For FACS analysis, 1ml of original cell stock ($2x10^6$ cells) was transferred to 15 ml falcon tube. For gene expression studies 500µl of original cell stock ($1x10^7$ cells) was transferred into 15 ml falcon in 5% FBS oligo medium. Total volume was completed to 1 ml with specific ODNs in 500µl 5% FBS supplemented oligo medium. Final oligo concentration was 1µM, unless otherwise stated. For gene expression studies, incubation periods were 2-8

hours while incubation periods were 6-72 hours for FACS analysis depending on the marker to be examined. Falcon tubes were left in tilted position with loosened caps to allow airflow and lids of 96-well plates were closed during the incubation period in CO_2 incubator.

3.2.6. *In vivo* experiments

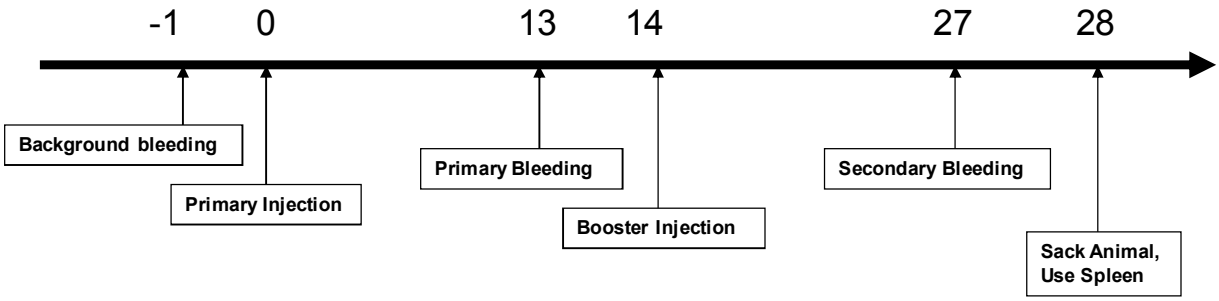
3.2.6.1. Injection of animals with different liposomal ODN or pIC or D35 plus pIC co-encapsulating different types of liposomes.

BALB/C mice was injected ip with either 25μg or 50μg of free D35 or free pIC or their free combinations (for 25μg, 12.5 μg of each stimulant was mixed and injected or for 50μg, 25 μg of each stimulant mixed and injected together). Mice were also ip injected with 10μg or 20μg D35 encapsulating 5 different types of liposome formulations. Third set of mice were injected with 10μg or 20μg pIC encapsulating 5 different types of liposome formulations. The last set of animal cohort was ip injected with 10μg or 20μg co-encapsulating D35 plus pIC within 5 different liposome formulations (here, each stimulant was adjusted to encapsulate 1:1 (wt/wt ratio) of D35 and pIC. 4 hours later mice were sacrificed and their spleens were extracted and split into two fractions. One half of the spleen was used to extract total RNA for PCR studies. From the other half, $4x10^5$ cell/well were incubated for 48 hours in 96 well plates. After incubation, the *ex vivo* stimulation was monitored and at this stage there are no further stimulations. The cell supernatants were collected and layered into IL6 and IFNγ monoclonal antibody coated ELISA plates. Additionally, $2x10^6$ cells from each treated animals were incubated in 15ml falcon tubes. After 24 hours of incubation cells were fixed and stained for anti-CD86 expression.

3.2.6.2. Immunization protocol with specific ODNs and OVA

Three to five adult male C57/BL6 mice per group were injected intraperitoneally (ip) with 15 μg of D35, D35 encapsulated anionic liposomes or control ODN and 7.5 μg of OVA. Fourteen days later, booster injection was performed ip with the same ODN and OVA formulations. Animals were tail bled one day before each injection and on the twenty-seventh day. Blood was incubated (to obtain mouse sera) at 37°C for 1.5 hours, the pelleted clot was discarded and then the remaining part was spun at 13200 rpm for 1 minute. The serum was collected and stored at -20°C for further use. Animals were sacrificed on day twenty-eight

and their spleens were removed. Half of the spleen was used to obtain single cell splenocyte suspension and were incubated to compare IFNγ secretion between different treatment groups and the rest of the spleen cells were used to generate total RNA and studied for the expression of certain cytokines at mRNA level.



Immunization Groups

1. Untreated
2. Control ODN + OVA
3. D3CG + OVA
4. Anionic Lipo(Control ODN+OVA)
5. Anionic Lipo(D3CG+OVA)

•D3CG:15µg/mouse;
Control ODN: 15µg/mouse;
OVA: 7.5 µg/mouse;
•Free or liposome encapsulating above adjuvant plus Ag were injected twice (d-1 & d-14), IP, after bleeding. At the end of d-28 mice were sacked and spleens were removed and studied for IFNγ and recall Ag response.
•From Serums, anti-OVA Ig subtypes were analysed by ELISA
•3 mice/group

Figure 6: The schedule of immunization protocol.

3.2.7. Fluorescence Activating Cell Sorting (FACS)

3.2.7.1. Cell Surface Marker Staining

Cells were centrifuged at 1500 rpm for 7 minutes at the end of the 24 hour incubation period. Supernatant was sucked. The protocol was slightly modified from earlier studies (Gursel *et al.* 2002; Gursel *et al.* 2006). Briefly, pellet was disturbed by using a pin rack holder. If cells were to be stained and analyzed later, cells were fixed in 100 µl fixation medium (Caltag, Austria) and transferred to 1.5 ml eppendorf tubes. Cells were incubated in dark at room temperature for 15 minutes. 1 ml PBS-BSA-Na azide was added into each tube

to wash cells. Cells were spun at 2000 rpm for 5 minutes. Supernatant was discarded and the washing step was repeated. At the end of the second washing step, PBS-BSA-Na azide was discarded and cells were incubated in fresh 50 µl PBS-BSA-Na azide containing 3 µl of FITC-associated monoclonal antibody against CD86 (BD Pharmingen). If cells were to be stained and analyzed immediately, fixation was not required. The cells can be stained in 50 µl PBS-BSA-Na azide containing 2-6 µl of fluorochrome-associated cell surface marker. The remaining steps are similar but all steps should be performed on ice if cells are not fixed. Cells were washed twice, resuspended in 500 µl PBS-BSA-Na azide, transferred to FACS tubes and analyzed in FACSCalibur (BD, USA).

3.2.8. Enzyme Linked Immunosorbent Assay (ELISA)

3.2.8.1. Cytokine ELISA

At the end of the incubation periods of cells stimulated in 96-well tissue culture plates, plates were spun at 300xg for 6 minutes and 170 µl supernatant was collected from each plate. Supernatants can be stored at -20°C. The ELISA protocol is a modified version reported by Gursel, I, *et al.*(Gursel *et al.* 2003). Briefly, 96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) or Immulon 2HB plates (Thermo Labsystems, USA) were coated with monoclonal antibodies against mouse cytokines IFNγ, IL-6 and IL4. 50 µl from antibody solution (10 µg/ml for monoclonal antibodies against mouse IFNγ and IL-6) was added to each well. Plates were incubated at room temperature for 5 hours or at 4°C for overnight. Coating antibody was spilled. Wells were blocked with 200 µl blocking buffer for 2 h at room temperature. Plates were washed with ELISA wash buffer 5 times with 5 minute incubation intervals after each wash and then rinsed with ddH2O for 3 times. Plates were dried by tapping (same washing procedure was repeated in the subsequent steps). Supernatants of cultured cells and recombinant proteins were added (50 µl/well from both). Starting concentration for mouse IL6 and mouse IFNγ recombinant cytokines were 2000ng/ml and 500 ng/ml, respectively. Recombinant cytokines were serially two-fold diluted serially with 50 µl 1X PBS. Plates were incubated for 2-3 hours at room temperature or overnight at 4°C. Plates were washed as explained above. Then, 50 µl from 0.5 µg/ml biotinylated-secondary antibody solution (original biotinylated antibody stock was 1/1000

diluted in T-Cell Buffer, please see Appendix A for details) was added to each well. Plates were incubated with biotinylated antibodies overnight at 4°C and were washed as previously described. 50 µl from 1:2500 diluted streptavidin-alkaline phospahatase solution (SA-AP in T-cell buffer must be prepared one day prior to its use and kept at 4°C) was added to each well. SA-AP was further incubated for 1 hour at room temperature. The washing step was repeated. 1 PNPP tablet was added to 4 ml ddH2O and 1 ml PNPP substrate for each plate and 50 µl of PNPP substrate was added to each well. Yellow color formation was followed and OD at 405 nm was analyzed with ELISA reader for several readings until recombinant cytokine standards reach a four parameter saturation and S-shaped curve was obtained. Each sample was layered as triplicate wells in order to obtain reproducible readings during the study of each cytokine by ELISA. Average concentration for each group was determined by the generated cytokine specific standard curve.

3.2.8.2. Anti-OVA IgG ELISA

IgG ELISA was very similar to cytokine ELISA except for a few steps. All incubation times and washing steps are similar with cytokine ELISA (please see above section for details). For the IgG subclasses instead of Immunlon 2HB, Immulon 1B plates (Thermo Labsystems, USA) were used. Each well was coated with 50 µl of 10 µg/ml OVA (Pierce, USA) diluted in 1X PBS. Blocking and washing steps are same as cytokine ELISA. Instead of cell supernatants, mouse serum from each animal was layered on the first row in 1/7 titration (10 µl serum was diluted with 60 µl 1X PBS). All wells except for the 1st row were pipetted with 52.5 µl, 1X PBS and 4-fold serial dilution was performed on vertical axis by transferring 17.5 µl from above well to bottom well. Antibodies against mouse IgG, IgG1, IgG2a, IgG2b were directly linked with alkaline phosphatase (AP). AP-linked IgG antibodies (1 mg/ml) were 1:3000 diluted in T cell buffer and again prepared one day prior to their use. After the addition of PNPP, yellow color formation was followed and OD at 405 nm was obtained with ELISA reader for several readings. Titrations at which similar ODs can be observed were used to compare the level of IgGs between different treatment groups.

3.2.9. Determination of gene expression at transcript level

3.2.9.1. Total RNA Isolation

Cells were centrifuged at 1500 rpm for 7 minutes at 4°C. Supernatant was discarded and cells were lysed with 1 ml Trizol, a mono-phasic solution of phenol and guanidinium thiocyanate (Invitrogen). Cell lysates can be stored at -80°C or can be transferred into 1.5 ml eppendorf tubes for further RNA purification steps. All subsequent steps were performed on ice and all centrifugation steps were done at 4°C. To each ml of Trizol used, 200 µl chloroform solution was added to each tubes and were immediately shaken vigorously for 15 seconds. After they were incubated at room temperature for 3 minutes, samples were centrifuged at 13200 rpm for 15 minutes at 4°C. Following centrifugation, 550-600 µl of the clear upper aqueous phase was transferred to a new eppendorf and 500 µl isopropanol was added to each tube. The samples were gently inverted by up and down motion to allow homogeneous mixing. Then, tubes were incubated at room temperature for 10 minutes and centrifuged at 13200 rpm for 15 minutes. Supernatant was sucked and the pellet was gently washed with 1 ml of 75% ethanol. Samples were centrifuged at 8000 rpm for 7 minutes. Again, supernatant was discarded and pellet was gently washed with 1ml >99.9% ethanol. After centrifugation at 8000 rpm for 7 minutes, ethanol was discarded and pellet was dried under asceptic conditions under laminar flow hood in a tilted position. Dry pellets were dissolved in 20 µl RNase/DNase free ddH2O. The OD measurements at 260 and 280 nm wavelengths were obtained with the spectrophotometer NanoDrop® ND-1000 (NanoDrop Technologies, USA). Purified total RNA samples were expected to have A260/A280 value between 1.8 to 2.0 ratios which indicates minimal contamination with DNA, protein, polysaccharides or phenol. Samples above or below this ratio were either discarded or subjected to a repeated RNA purification procedure. All samples were stored at -80°C for further use.

3.2.9.2. cDNA synthesis

cDNAs were synthesized from total RNA samples with the cDNA synthesis kit (Finnzymes) according to the manufacturers' protocol. 1µg total RNA was mixed with 1 µl of Oligo (dT) 15-mer (100 ng) primer and total volume was completed to 8 µl with RNase

DNase free H2O in 0.2 ml PCR tubes. Tubes were pre-denatured at 65°C for 5 minutes in MJ Mini thermocycler (BIO-RAD, USA) and then spinned down. 10µl RT Buffer (includes dNTP mix and 10 mM MgCl2) and 2µl M-MuLV RNase H+ reverse transcriptase (includes RNase inhibitor) were added to the mixture. Tubes were incubated at 25°C for 10 minutes, 40°C for 45 minutes and 85°C for 5 minutes. cDNA samples were stored at -20°C for further use.

3.2.9.3. Semi-quantitative RT-PCR

Designed primers were developed with Primer3 Input v.0.4.0 program (http://frodo.wi.mit.edu/primer3/input.htm) and Primer Designer Version 2.0 using the cDNA sequences of human and mouse genes available at the Ensembl™ database. Each primer pair was blasted (http://www.ncbi.nlm.nih.gov/BLAST/) against the mouse genome. Other primer sequences were obtained from literature. All reagents were mixed and subjected to optimal PCR conditions for specific primer set (Table 4) in MJ Mini thermocycler (Biorad). PCR mixtures were prepared according to the protocol in Table 5. Running conditions were given in Table 6. PCR products were kept at 4°C until they were loaded on agarose gel.

Table 4: Mouse primer sequences

Primer		Sequence	Product Size
m β-actin	Forward Reverse	5'-GTATGCCTCGGTCGTACCA-3' 5'-CTTCTGCATCCTGTCAGCAA-3'	450 bp
m TLR-3	Forward Reverse	5'-GGGGCTGTCTCACCTCCAC-3' 5'-GCGGGCCCGAAAACATCCTT-3'	250 bp
m TLR-7	Forward Reverse	5'-TTAACCCACCAGACAAACCACAC-3' 5'-TAACAGCCACTATTTTCAAGCAGA-3'	700 bp
m TLR-9	Forward Reverse	5'- GATGCCCACCGCTCCCGCTATGT-3' 5'-TGGGGTGGAGGGGCAGAGAATGAA-3'	430 bp
m TNF-α	Forward Reverse	5'-CCACCACGCTCTTCTGTCTAC-3' 5'-ACTCCAGCTGCTCCTCCACT-3'	189 bp
m IL-4	Forward Reverse	5'-CCAAGGTGCTTCGCATATTT-3' 5'-TTTCAGTGATGTGGACTTGGAC-3'	161 bp
m IL-6	Forward Reverse	5'-TGTGCAATGGCAATTCTGAT-3' 5'-CTCTGAAGGACTCTGGCTTTG-3'	226 bp
m IFNγ	Forward Reverse	5'-GCGTCATTGAATCACACCTG-3' 5'-ATCAGCAGCGACTCCTTTTC-3'	195 bp

m IL-15	Forward Reverse	5'-CATCCATCTCGTGCTACTTGTGTT-3' 5'-CATCTATCCAGTTGGCCTCTGT-3'	126 bp
m IL-18	Forward Reverse	5'-GATCAAAGTGCCAGTGAACC-3' 5'-ACAAACCCTCCCCACCTAAC-3'	384 bp
m IP-10	Forward Reverse	5'-GCCGTCATTTTCTGCCTCAT-3' 5'-GCTTCCCTATGGCCCTCATT-3'	127 bp
m CD40	Forward Reverse	5'-GTCATCTGTGGTTTAAAGTCCCG-3' 5'-AGAGAAACACCCCGAAAATGG-3'	91 bp
mIFNα 6/8	Forward Reverse	5' TCAAGTGGCATAGATGTGGAAGAA 3' 5' TGGCTCTGCAGGATTTTCATG 3'	384 bp

Table 5: PCR Reagents

Reaction Ingredients	Volume
2x DyNAzyme™ II PCR Master Mix[A]	12,5 µl
DNAse/RNAse free H_2O	9 µl
Forward primer	1 µl (10pmol)
Reverse primer	1 µl (10pmol)
cDNA	1,5 µl
Total	25 µl

[A] *2x DyNAzyme™ II PCR Master Mix includes 0.04 U/µl DyNAzyme™ II DNA Polymerase, 20 mM Tris-HCl (pH 8.8 at 25°C), 3 mM MgCl2, 100 mM KCl, stabilizers and 400 µM of each dNTP.*

Table 6: PCR Conditions

	Gene Names			
PCR Steps (Temp ; Time)	mTNFα, mIFNγ, mIL15, mIL6	mCD40	mβactin, mIL18, mIP10, mTLR3, mTLR7	mIFNα 6/8, mTLR9
Initial Denaturation	-	95°C ; 3''	-	94°C ; 2'
Denaturation	94°C ; 30''	95°C ; 30'	94°C ; 30''	94°C ; 30''
Annealing	60°C ; 30''	62°C ; 30'	55°C ; 30''	64,3°C ; 30''
Extension	72°C ; 30''	72°C ; 45'	72°C ; 30''	72°C ; 1'
Repeat (Cycle #)	40	34	34	40
Final Extension	72°C ; 5'	72°C ; 7'	72°C ; 5'	72°C ; 10'

' denotes mins; '' denotes secs

3.2.9.4. Agarose gel electrophoresis and quantification of band intensities

1.5% agarose gel was prepared with 1X TAE buffer and final concentration of ethidium bromide was 1 μg/ml. 2 μl loading dye was mixed with 10 μl PCR product. 10 μl of mixture was loaded to each well. 5 μl of 105 ng/μl DNA ladder with 100-1000bp range or low range DNA ladder with 50-1000bp range (Jena Biosciences) was loaded on an empty well and used as a marker. 100V is applied for 45 minutes and the gel was visualized under UV transilluminator (Vilber Lourmat, France). The gel exposure time was kept fixed at 0.1 second for each run. Band intensity analysis was performed with BIO-PROFILE Bio-1D V11.9 sofware. Band intensity for each product was measured and background intensity was subtracted from this value. Blanked intensities for each product were divided by blanked house-keeping gene (β-actin) values to normalize resulting quantitative data or directly compared.

3.2.10. Statistical Analysis

Statistical analysis was performed in SigmaSTAT 3.5 software. Student's t-test was used to understand if differences between untreated groups, groups treated with control-ODNs and ODN-treated groups were statistically significant.

4. RESULTS

4.1. Studies to reveal the most potent immunostimulatory ODN-liposome formulation

Modulating the immunostimulatory activities of TLR ligands is critical to design effective vaccine adjuvants. Previous studies have shown that nucleic acid-based intracellular TLR ligands are very labile and their immunostimulatory activity can be enhanced by encapsulating them into liposomes (Barry *et al.* 1999; Gursel *et al.* 2001). For this, five different types of liposomes varying in size, lamellarity and surface net charge was prepared as mentioned in Materials and Methods section. These TLR ligands were encapsulated either alone or with their 1:1 combinations into these liposomes. Our first effort was to determine which liposome type encapsulated which TLR ligand at the highest efficiency, since this will be correlated with the improved cytokine response following splenocyte stimulation *ex vivo*. The cytokine responses from splenocytes were determined by ELISA and RT-PCR (plese see below sections).

4.1.1. Determining the liposome encapsulation efficiency

Due to different physicochemical characteristics of liposomes, their ability to encapsulate different nucleic acid based ligands was expected to vary. After the liposomes were spun down from PBS solution where the liposomes were suspended in, the concentration of ODNs in the solution (i.e. unencapsulated fraction) was determined by UV spectroscopy (NanoDrop®). As the total amount of ODNs initally mixed with the SUV before the encapsulation step was known (1.0 mg CpG or pIC was mixed with 20 μmole lipid), it was possible to calculate the amount of ODN entrapped within the liposomes. The Table 7 summarizes the percent ODN encapsulation within different liposomes as well as controlled release profiles from different formulations. It was already established that the ability of positively charged liposomes to encapsulate ODN is far better than neutral, stealth or negatively charged liposomes (Gursel *et al.* 2001). The reason for improved entrapment in cationic liposomes is due to the strong electrostatic interaction between positively charged cationic lipid with negatively charged ODNs.

Table 7: The encapsulation efficiency and ODN release of K and D-type ODN encapsulating liposomes.

Liposome Type	K-Type ODN Loading (%)[A]	D-Type ODN Loading (%)	Percent ODN Released Over Time[B] (D and K-Type averaged)				
			12h	1d	2d	4d	8d
Neutral	67.4±5.6	84.9±3.3	5.3±3.3	7.7±4.7	12.7±3.3	13.1±4.2	21.7±5.7
Anionic	71.5±4.2	89.5±4.6	12.5±2.9	19.6±3.8	23.5±7.2	32.5±3.0	40.2±5.1
Cationic	93.1±7.7	97.1±2.4	3.1±1.7	5.1±0.7	8.1±2.8	15.1±3.6	20.1±6.1
Stealth	78.9±2.0	88.9±3.8	8.9±2.0	10.8±3.3	14.9±3.1	16.1±2.6	18.9±3.8
SSCL	85.4±11.2	98.7±3.5	2.4±1.2	5.4±1.7	7.4±2.2	10.1±1.2	13.9±4.2

All ODN measurements were done using 1555 (K-Type) and D35 (D-Type) CpG ODNs, run in triplicates from at least three independent liposome preparations are reported. Data represents Ave ± SEM.

[A] OD260nm from supernatants were used to calculate loading efficiencies after obtaining the liposome pellet.

[B] Release of ODN @ 37°C into PBS was followed by OD readings from supernatants after obtaining the liposome pellet.

The ODN encapsulation efficiency with D-type CpG was much higher than that of K-Type regardless of different liposome preparations (see Table 7, left panels). As seen in Table 7, liposomes were assayed for duration of 7d and ODN release was followed every 12h intervals. Three different liposome types gave ~20% release of their cargo at the end of one week. However, the lowest release level was seen with SSCL liposomes (13.9±4.2) and anionic liposomes gave the highest ODN release (40.2±5.1), meaning SSCL are the most stable and anionic is the least stable to retain ODN.

The storage stability (i.e. shelf-life) is critical for liposome formulations. We have investigated the storage stability of these liposomes five months after their production date. The more a liposome is stable the more it retains its cargo.

Table 8: Percent encapsulation of different type of CpG ODNs encapsulated or coencapsulated with pIC in 5 types of liposomes following five month of storage in the fridge.

	1555	K23	1612	D35	I-127	K23+pIC	D3CG+pIC	I-127+pIC
Neutral	12.8	0	3.2	84.6	58.0	0	0	38.0
Anionic	23.0	0	24.0	82.7	58.0	0	0	0
Cationic	95.0	75.0	79.6	97.9	92.8	70,6	82,4	92.2
Stealth	8.6	N.D	40.8	85.4	N.D	N.D	N.D	N.D
SSCL	95.1	84.8	85.6	98.3	95.0	89.8	87.3	92.4

N.D. not determined

As summarized in Table 8, the encapsulation efficiency measurements 5 months after the production date revealed that SSCL and cationic liposomes are quite stable upon storage at 4°C. Regardless of the type of the nucleic acid TLR ligands used during liposome preparation, SSCL retained over 85% and cationic over 70% of its cargo at the end of 5 months. Strikingly, D35 encapsulating liposomes (regardless of their properties) held over 80% of the initial input amount at the end of this storage period (please check lane 4 with others, Table 8).

4.1.2. Cytokine ELISA after *in vitro* stimulations of splenocytes with K-ODN and D-ODN encapsulating liposomes.

In order to understand the cytokine secretion profile upon stimulation with TLR ligand encapsulating liposomes we collected supernatants of spleen cell cultures after 36 hours of incubation. IL6 and IFNγ levels were checked by ELISA. Initial studies were done with 1555 ODN. When CpG ODN 1555 is encapsulated into cationic and SSCL liposomes it induced approximately 20 and 100 ng/ml IFNγ, while no IFNγ secretion could be detected upon stimulation with free ODN at lowest dose (0.3μM) (Figure 8). Similarly, but specifically at low ODN concentration, IL6 response is augmented by 3 fold when 1555 is encapsulated into SSCL liposome (a rise from 90±16.4 to 266.8±23.8 ng/ml) (Figure 7). These activations were strictly CpG motif specific. When Control ODNs were encapsulated or empty liposomes themselves were treated with spleen cells, no appreciable amounts of IFNγ or IL6 was detected from their cell supernatants which is a strong indication that the observed immunogenicity is CpG motif dependent (Figure 13 and Figure 14).

The heterogenicity in liposome/1555 response was observed. When 1555 is loaded within neutral, stealth and anionic liposomes it substantially lost its stimulatory activity. (Figure 7 and Figure 8)

Stimulation with various D35-liposome complexes also yielded heterogeneous responses. Results indicated that IFNγ and IL6 production from mouse splenocytes were significantly increased when D-type ODN was encapsulated in neutral, anionic, or stealth liposomes. Surprisingly, and contrary to what we have observed for 1555 ODN, D-ODN activity was completely lost when loaded within vesicles where positively charged lipid is included in the formulation (Figure 11 and Figure 12).

Alternative design of K-ODN, K23, was also tested throughout this study. Of note, the most distinguishable features of K23 from 1555 are, K23 is shorter (12mer vs 15mer) and it contains two CpG motifs in its sequence whereas 1555 is 15-mer and has only one CpG motif. To our surprise, following stimulation with K23-liposome formulations, the cytokine production levels (IFNγ and IL6) from spleen cells, resembled more to D-Type ODN-liposome complex stimulations rather than 1555-liposome formulation stimulations. At the lowest dose of K23 (0.3μM) that is encapsulated into neutral and anionic liposomes, 3 fold more IL6 was induced (Figure 9). Although free K23 could not stimulate any detectable IFNγ secretion, K23 encapsulated within neutral and anionic liposome induced approximately 5 and 10 fold more IFNγ secretion. Considering the lowest stimulation dose, while 1555 induces the strongest IFNγ and IL6 secretion when they are encapsulated into SSCL liposome, K23 and D35 yielded robust proinflammatory cytokine responses in either anionic or neutral liposomes. As the dose of K23 increased, the IFNγ secretion increased correspondingly (Figure 10).

The substantial loss of IFNγ secretion with certain liposomes encapsulating CpG ODNs (D or K-Types), prompted us to determine whether a Th2-dominant cytokine secretion is mediating these suppressions. Earlier studies reported that IL4 (a Th2-biased cytokine) is the cardinal cytokine that can suppress IFNγ production (Tanaka *et al.* 1993). To rule out this possibility, we have checked IL4 levels from the liposome encapsulating K- or D-type ODN treated mouse spleen cells and found out that there was no detectable IL4 from these sups

(data not shown). This finding implied that the suppression was not due to a Th1 to Th2 shift in immunity (that is mediated by different liposome types). Collectively these results indicate that K-ODN and D-ODN induces differential cytokine production when they are encapsulated into liposomes with different physicochemical characteristics.

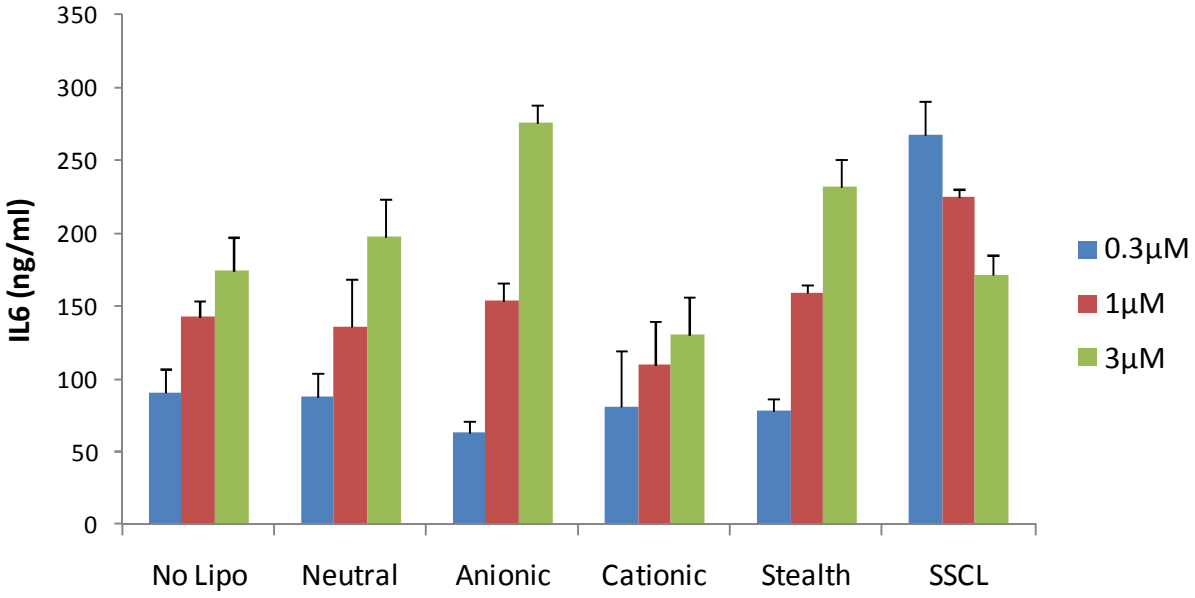


Figure 7: Dose-dependent IL6 production from splenocytes following 36 hours of stimulation with free (No Lipo) or various liposomes encapsulating 1555 (0.3-3.0 µM), IL6 production by splenocytes was detected from cell supernatants by ELISA. Experiment samples were run in triplicates and results are reported as the average of at least two independent studies. Results (mean concentration ± SEM) are combination of two independent experiments. $p<0.001$ for 0.3µM and 1µM, p=NS for 3µM (SSCL vs No Lipo paired t-test analyses comparisons).

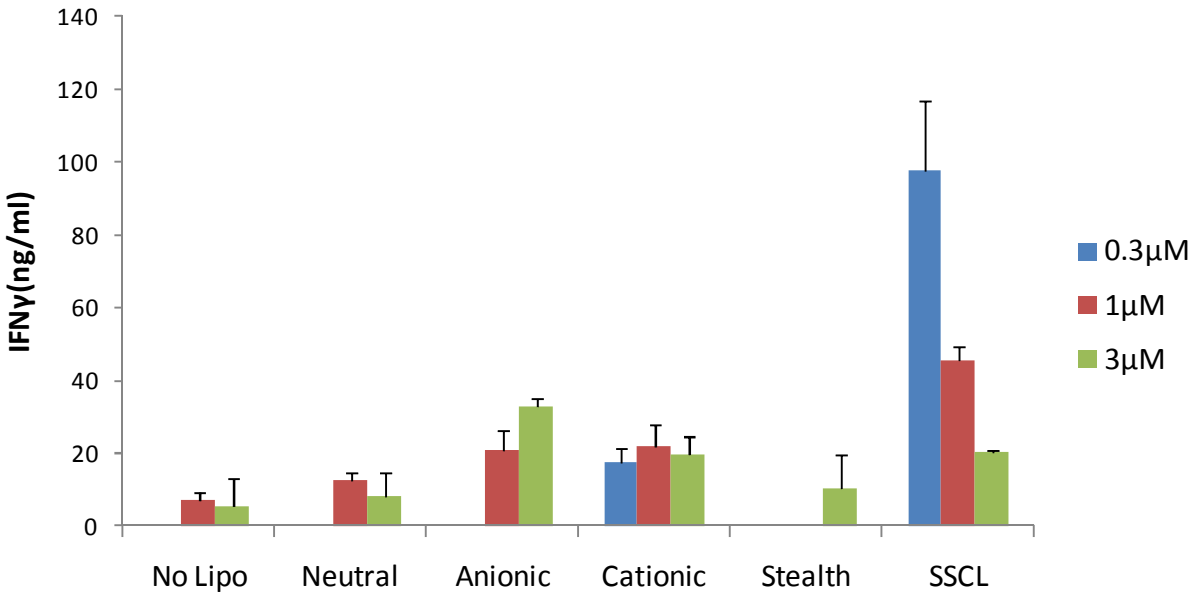


Figure 8: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating 1555. IFNγ production by splenocytes was detected from cell supernatants by ELISA. Experiment samples were run in triplicates and results are reported as the average of at least two independent studies. $p<0.01$ for 0.3 and 1µM (Cationic vs No Lipo) and $p<0.0001$ for 0.3 and 1µM (SSCL vs No Lipo, paired t-test analyses comparisons).

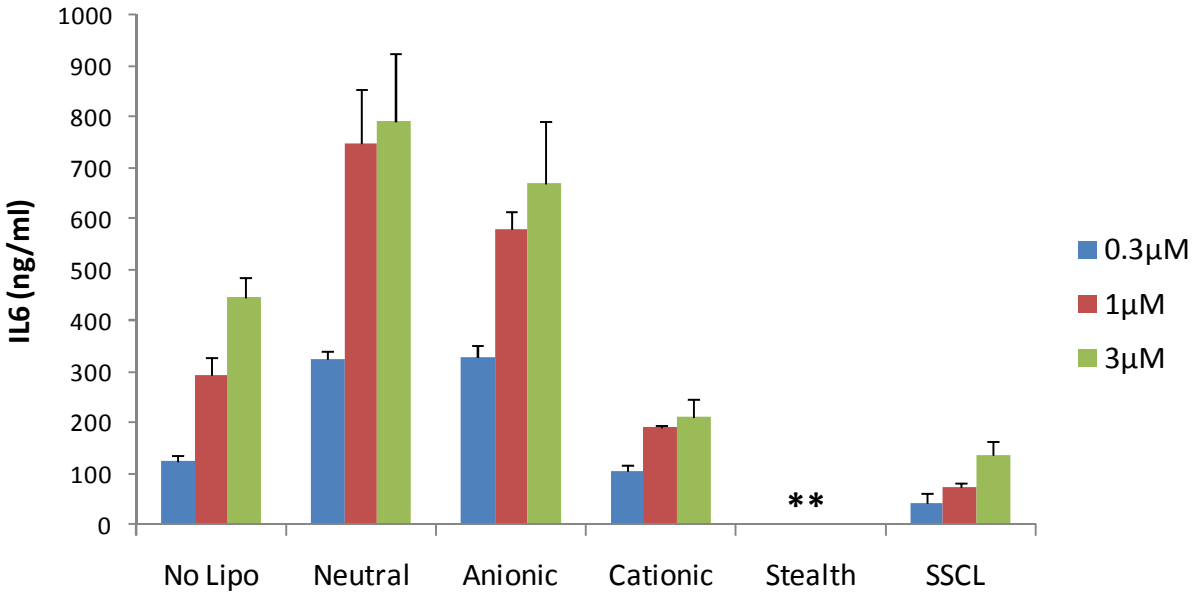


Figure 9: Dose-dependent IL6 production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating K23. (For more information please see Figure 7 legend) $p<0.001$ for 0.3µM, 1µM and 3µM (Neutral vs No Lipo and Anionic vs No Lipo paired t-test analyses comparisons). **; not determined.

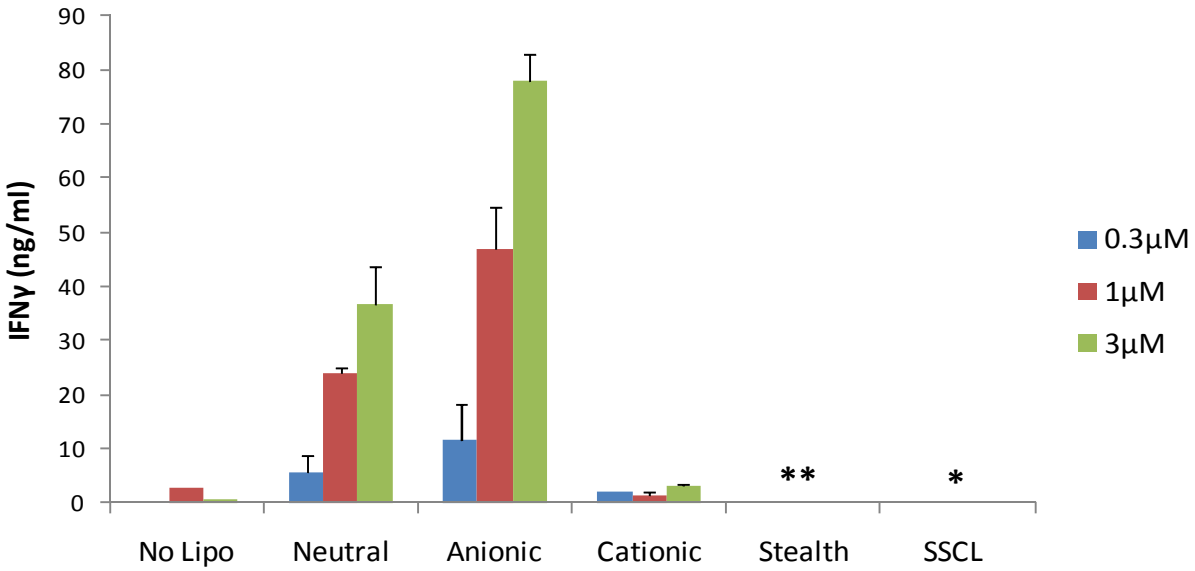


Figure 10: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating K23. $p<0.01$ for 0.3 µM (Neutral vs No Lipo and Anionic vs No Lipo) and $p<0.0001$ for 1µM and for 3 µM (Neutral vs No Lipo and Anionic vs No Lipo paired t-test analyses comparisons). **; not determined, *; not detectable. (For more information please see Figure 8 legend)

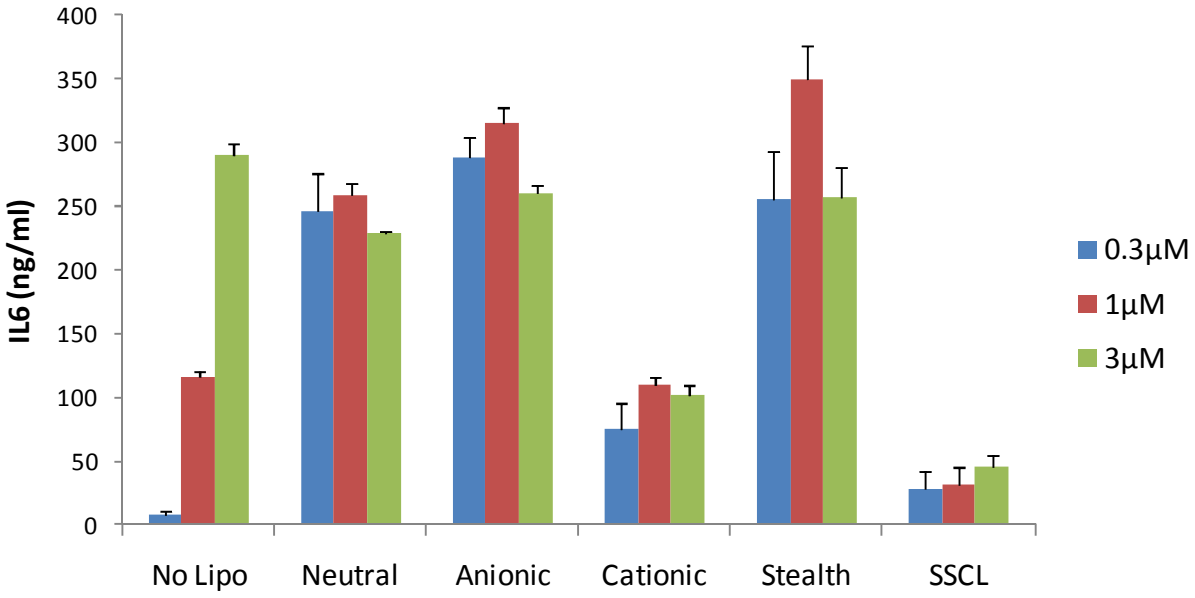


Figure 11: Dose dependent IL6 production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating D35 (For more information please see Figure 7 legend). $p<0.001$ for 0.3µM, and 1µM (Neutral vs No Lipo, Anionic vs No Lipo and SSCL vs No Lipo paired t-test analyses comparisons).

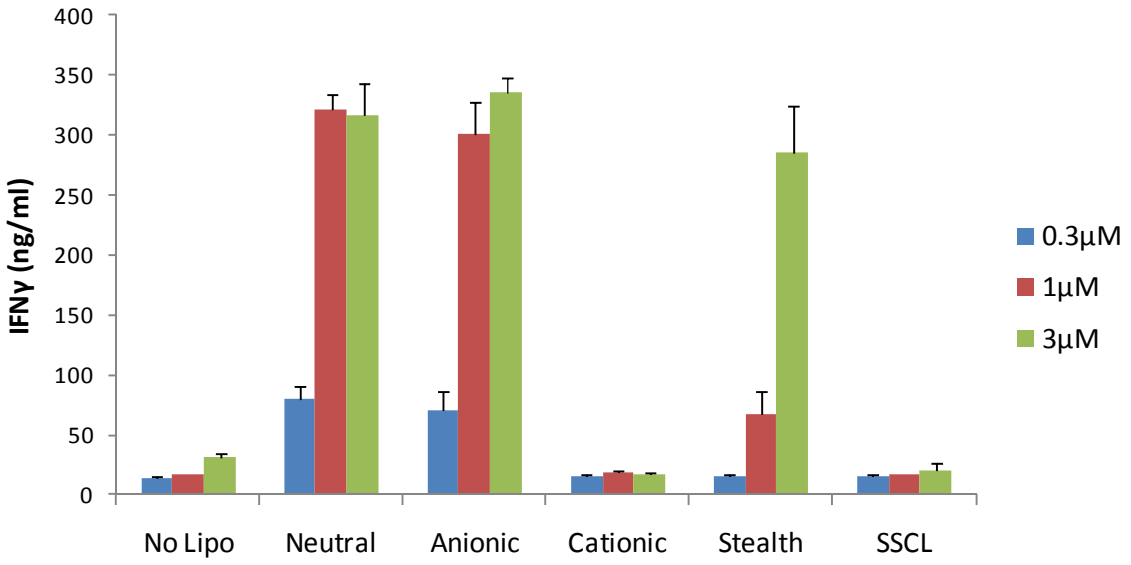


Figure 12: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating D35. (For more information please see Figure 8 legend) p=0.06 for 0.3µM, p<0.001 for 1µM and 3µM (Neutral and Anionic vs No Lipo paired t-test analyses as well as SSCL vs No Lipo for 3 µM analyses comparisons). Cationic and SSCL vs No Lipo t-test was NS

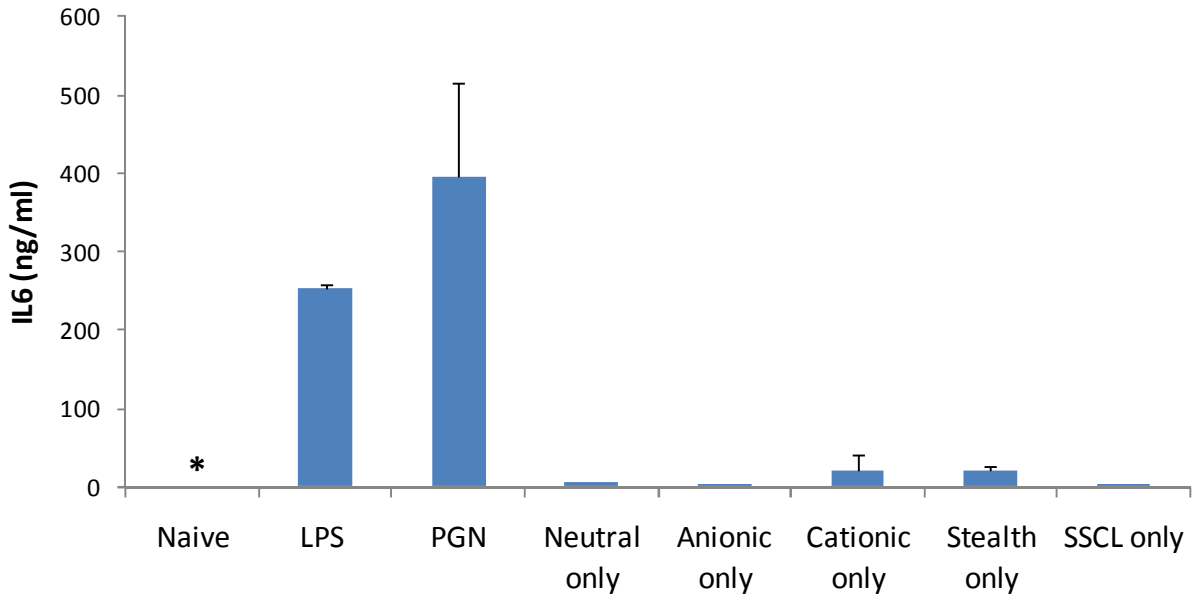


Figure 13: IL6 production from splenocytes following stimulation with 0,86µM liposomes that do not encapsulate any TLR ligand, 5µg/ml PGN and 5µg/ml LPS as positive control (For more information please see Figure 7 legend) *; not detectable.

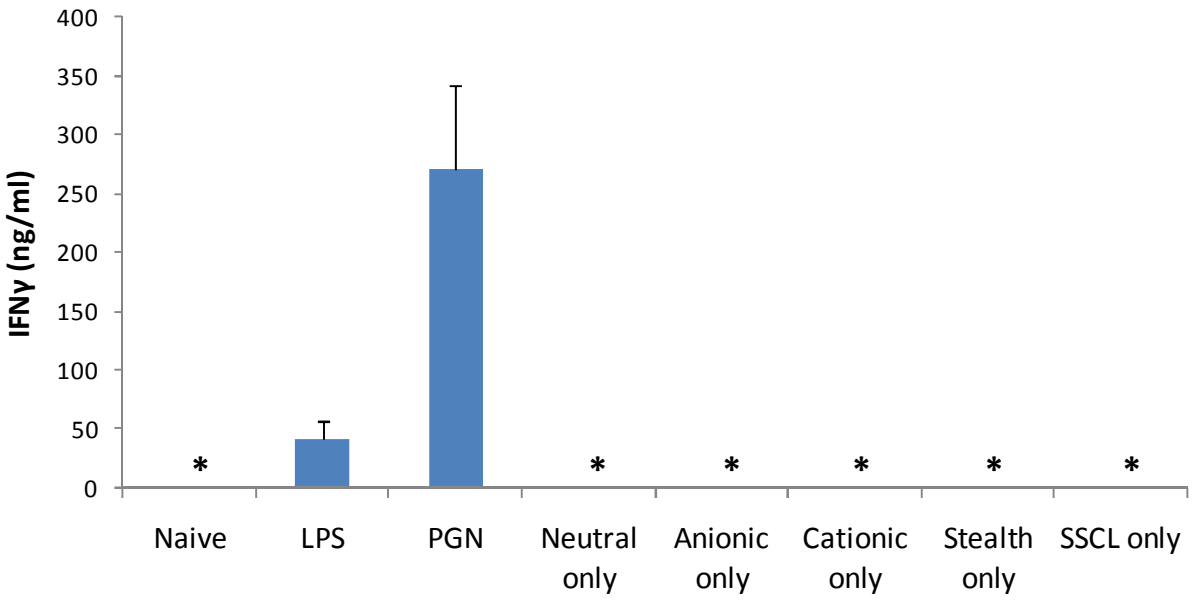


Figure 14: IFNγ production from splenocytes following stimulation with 0,86µM liposomes that do not encapsulate any TLR ligand, 5µg/ml PGN and 5µg/ml LPS as positive control (For more information please see Figure 8 legend) *; not detectable.

Table 9: Summary of relative IL6 secretion induction following stimulation with 0.3µM free or various liposomes encapsulating 1555, K23 and D35.

	1555	K23	D35
No Lipo	+	+	0
Neutral	+	++	**++++**
Anionic	+	++	**++++**
Cationic	+	+	++
Stealth	+	N.D	**++++**
SSCL	++	0	+

Lowest doses were considered. If stimulation with free ODN (No Lipo) yielded IL6 response, the amount of IL6 was marked as "+" and relative IL6 secretion inductions was calculated according to "No Lipo" group. In this case "No Lipo" is the reference group. If "No Lipo" stimulation did not yield any IL6 response, "No Lipo" group was shown as "0". Then the group that yielded lowest amount of IL6 secretion was denoted as "+" and relative IL6 secretion inductions were calculated according to that reference group. 0; no induction, +; <2 fold induction compared to reference group or, ++; between 2-3 fold induction, +++; 3-4 fold induction, ++++; 4< fold induction, N.D; not determined.

Table 10: Summary of relative IFNγ secretion induction following stimulation with 0.3μM free or various liposomes encapsulating 1555, K23 or D35

	1555	K23	D35
No Lipo	0	0	+
Neutral	0	++	**++++**
Anionic	0	**++++**	**++++**
Cationic	+	+	+
Stealth	0	N.D	+
SSCL	**++++**	0	+

Lowest doses were considered. If stimulation with free ODN (No Lipo) yielded IFNγ response, the amount of IFNγ was marked as "+" and relative IFNγ secretion inductions was calculated according to "No Lipo" group. In this case "No Lipo" is the reference group. If "No Lipo" stimulation did not yield any IFNγ response, "No Lipo" group was shown as "0". Then the group that yielded lowest amount of IFNγ secretion was denoted as "+" and relative IFNγ secretion inductions were calculated according to that reference group. 0; no induction, +; <2 fold induction compared to reference group or, ++; between 2-3 fold induction, +++; 3-4 fold induction, ++++; 4< fold induction, N.D; not determined.

The global modification in cytokine production following liposome encapsulation of various K or D-Type ODNs is summarized in Table 9 and Table 10. These tables clearly demonstrate that when different ODN classes encapsulated within different liposomes induces differential cytokine secretion from innate immune cells.

4.1.3. Effect of liposome encapsulated D-ODN or K-ODN on cytokine transcript levels.

When one of the aims of ODN incorporation within a depot system is to augment its immuno adjuvant effect, it is of great importance to detect the breadth of activation with these delivery systems. While IFNγ is a very vital cytokine to regulate and mount Th1 dominant cell-mediated immune response, IFNα is a very critical cytokine modulating anti-viral immunity. We have speculated that positively charged liposomes could alter subcellular localization of D-ODN and thus prevent it to signal from early endosome through MyD88 and IRF7. One way of analyzing this effect is to use intracellular IFNα cytokine staining by flow cytometry and another powerful approach is to use conventional ELISA. Unfortunately, none of these reagents for mouse is available at hand, thus we shifted our detection strategy

to PCR. For this, mouse spleen cells were stimulated for 1 or 8 hours, and total RNA was purified, and specific gene transcripts were studied by RT-PCR. As seen in Figure 15 and Figure 16a, eight hours after stimulations, spleen cells treated with neutral, anionic and stealth liposomes encapsulating D35 gave higher IFNα mRNA levels than positively charged liposomes encapsulating D35. As expected, 1555 did not induce any IFNα transcripts. These results indicate that positively charged liposomes interfere with D-ODN recognition by TLR9.

We could not detect difference in transcript levels of CD40 and IL18 at either time points following CpG ODN-liposome complex stimulation compared to untreated or with free ODN stimulation groups (Figure 15 and Figure 16b,c), these data collectively indicated that liposomes do not alter signaling mechanisms of these cytokines.

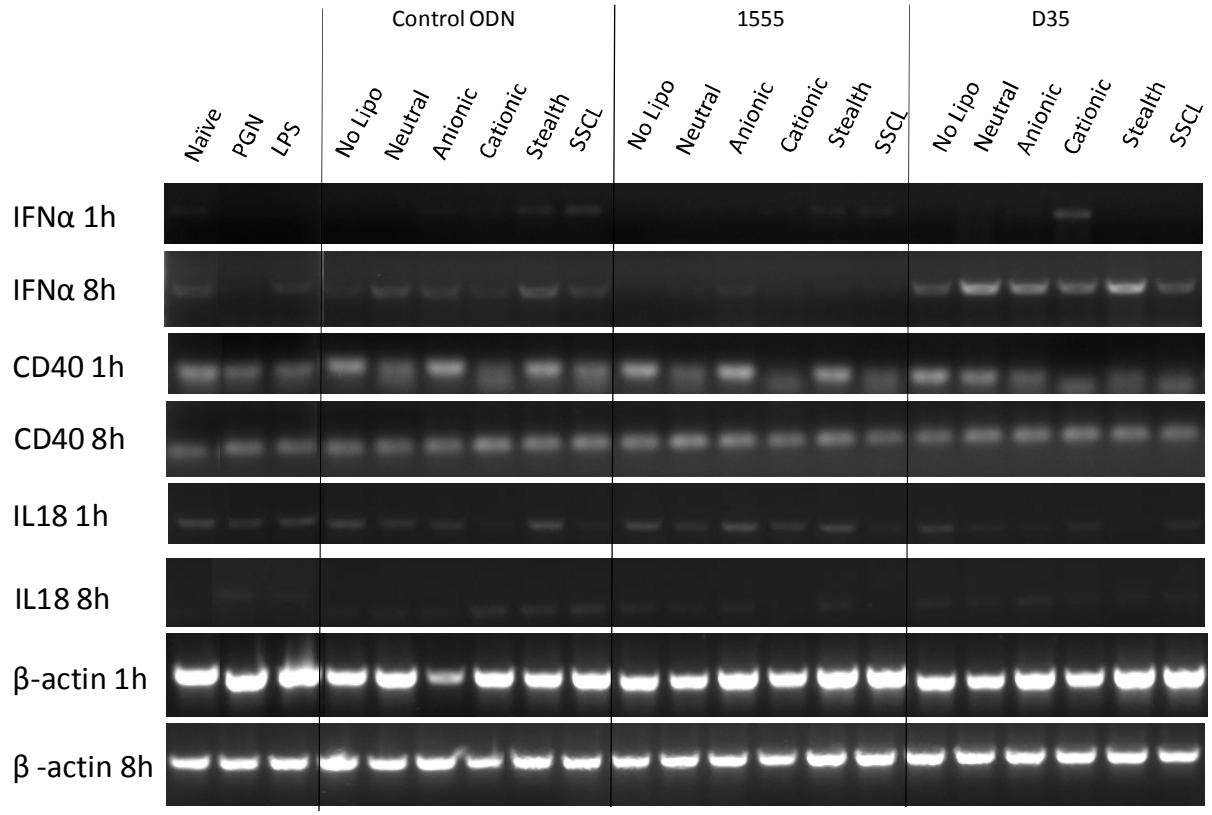


Figure 15: PCR results that was performed from total RNA of splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating 1μM control ODN, 1555 and D35. 5μg/ml PGN and LPS were used as positive control.

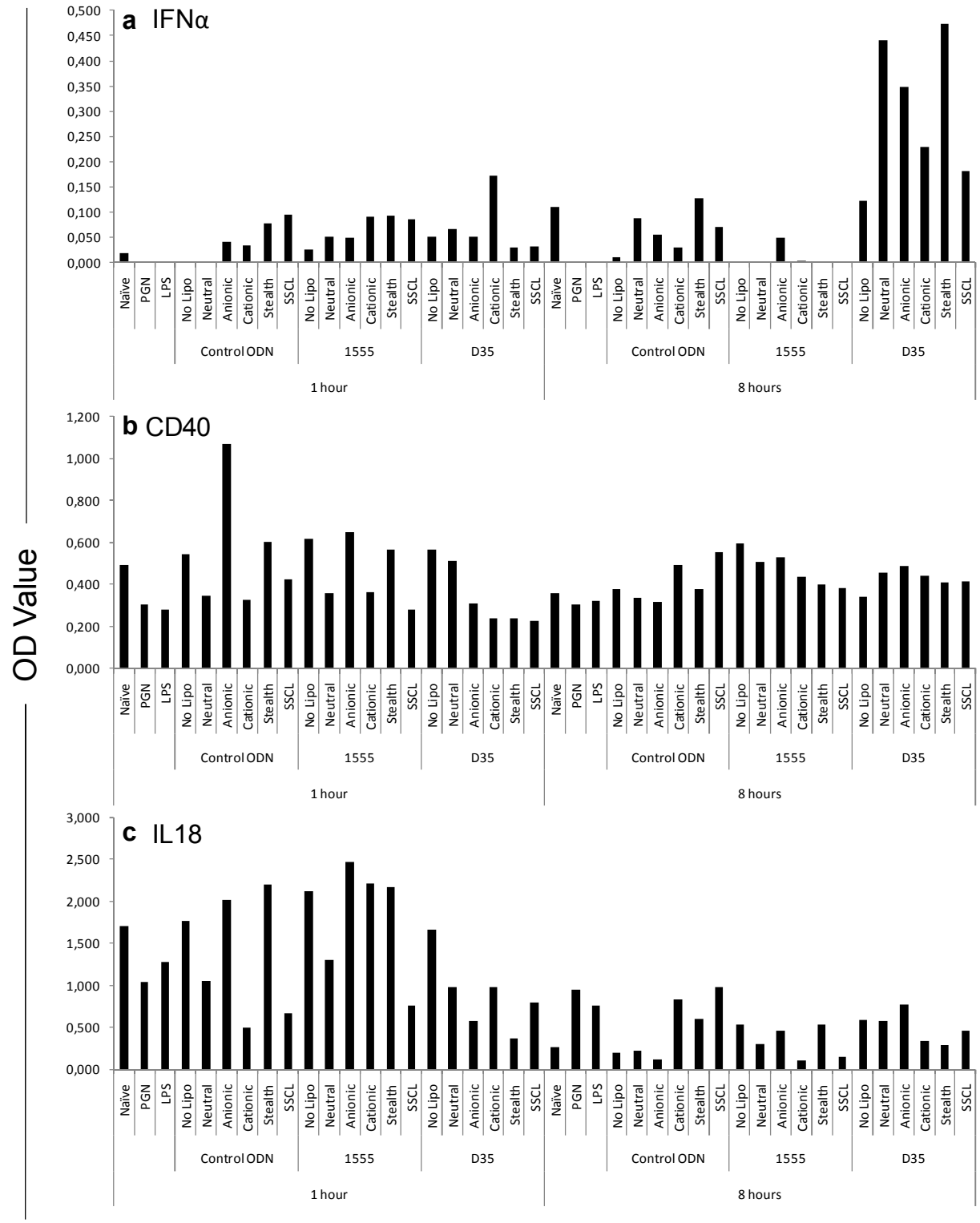


Figure 16: Density measurements of the gel electrophoresis picture in Figure 15 for IFNα (a), CD40 (b) and IL18 (c). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

4.1.4. Cytokine ELISA after *in vitro* stimulations of splenocytes with liposomes encapsulating pIC

Among nucleic acid TLR ligands, in addition to CpG DNA, dsRNA (such as synthetic pIC) triggers signaling cascade through TLR3 and is one of the strong candidates for clinical applications. Similar to CpG ODN, pIC is a very labile molecule and attains considerable efforts to improve its *in vivo* activity. We checked whether liposome encapsulating pIC offers an advantage to its free counterpart. We have checked whether we can improve cytokine production of pIC by achieving liposome encapsulation.

While 22.5μg/ml concentration of free pIC could only induce barely detectable amount of IFNγ (2ng/ml) and dose dependent substantial IL6 secretion, encapsulating pIC into neutral, anionic, cationic and stealth liposomes induced significantly high IFNγ (even at the lowest 2.5μg/ml dose where free pIC fail to induce any cytokine production) (Figure 17 and Figure 18)

The improved IFNγ with low dose stimulation is critical. When these formulations are injected *in vivo*, due to fast dilution and premature clearance by absorption onto serum proteins may facilitate ineffective accumulation and internalization level of free dsRNA leading to ineffective *in vivo* performance. Liposome encapsulation is a very potent tool to overcome this problem and thus, significantly improve the immunostimulatory activity. Data in Figure 17 and Figure 18 strongly indicate that at low dose stimulation with liposome formulations except SSCL are very strong cytokine inducers (from no induction to 350 ng/ml IFNγ with anionic liposome encapsulating pIC). At higher doses of pIC, anionic liposome induced 425ng/ml as opposed to 2ng/ml free pIC. Similarly, neutral liposomes followed the same trend. (Figure 18)

As seen in Figure 18, liposomal pIC induced substantially higher levels of IL6 for all tested liposome types, except SSCL, at all concentrations. Among all liposome types, anionic and stealth liposome encapsulating pIC gave the highest activity. Approximately, a 9 fold more induction was observed at 2.5μg/ml dose (from 30±8 to 265±31 μg/ml for pIC and anionic liposomal pIC, respectively), and at 22.5 μg/ml this trend was still retained (50±7 to 389±29 ng/ml) (Figure 17).

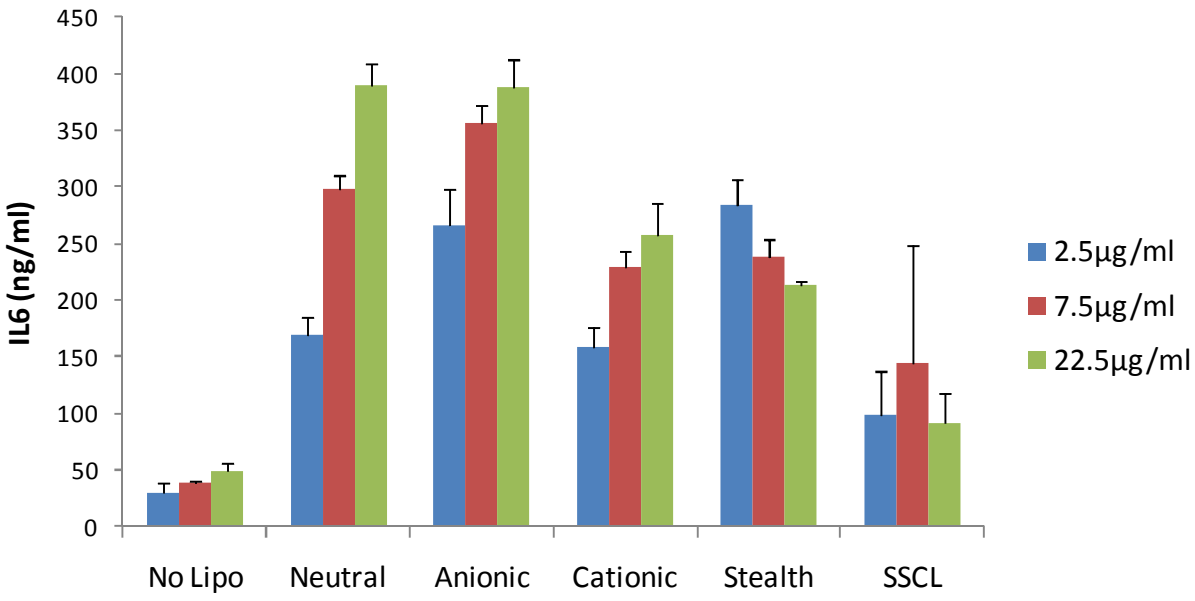


Figure 17: IL6 production from splenocytes following stimulation with free (No Lipo) various liposomes encapsulating pIC. (For more information please see Figure 7 legend) $p<0.001$ for 0.3µM, 1µM and 3µM (Neutral, Anionic, Cationic and Stealth vs No Lipo paired t-test analysis comparison). For SSCL vs No Lipo group analyses: $p=0.002$ at 2.5µg/ml and 22.5µg/ml, at 7.5µg/ml, $p=0.03$.

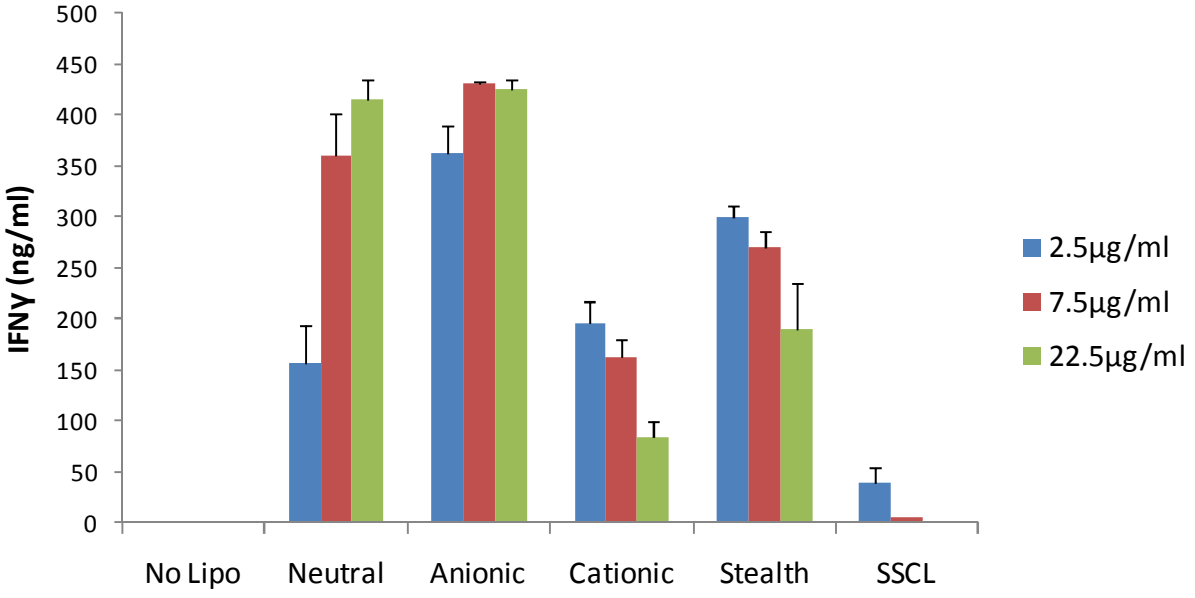


Figure 18: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating pIC. (For more information please see Figure 8 legend). $p<0.001$ for 0.3µM, 1µM and 3µM (All groups vs No Lipo paired t-test analysis comparison). At 3µM SSCL is NS

4.1.5. Effect of various liposomes encapsulating pIC on transcript level

As mentioned above, dsRNA serve as activator of innate immune cells that induce both type I IFNs and Th1 type immune response via a TRIF dependent signaling pathways. IFNα, a cardinal cytokine against anti-viral immunity is also contributing to the Th1 development. We have studied to reveal whether liposome encapsulation of either pIC alone or co-encapsulation with D-ODN improves IFNα message levels. In addition to IFNα, a co-stimulatory molecule CD40 and another Th1 related critical cytokine IL18 message from spleen cells were assessed by RT-PCR.

We have stimulated splenocytes for one and 8 hours with free or various forms of liposomes encapsulating pIC (5μg/ml). At the end of first hour, we detected basal level of IFNα and CD40 mRNA levels which is similar with untreated (naïve) group (Figure 19 and Figure 20a, b). The differential response for IL18 could be observed at the first hour. Although free pIC did not increased IL18 mRNA level, neutral and cationic liposome encapsulating pIC increased the transcript level approximately 1.5 fold. Interestingly pIC in the stealth liposome decreased the IL18 mRNA amount (Figure 19 and Figure 20c).

When pIC was loaded in stealth liposome, we have observed significant amount of IFNγ response (Figure 18). This observation is very puzzling, since IL18 is known to promote IFNγ production (Dinarello *et al.* 2003) our data imply that IFNγ induction via pIC encapsulating liposome is IL18 independent.

At the end of eighth hours of stimulation, cells stimulated with pIC encapsulating neutral or stealth liposome induced a 4 fold and an 11 fold increase in IFNα message compared to free pIC stimulation (Figure 19 and Figure 20a). Compared to 1h mRNA response, there was no change in the IL18 and CD40 transcript levels at the end of 8h of stimulation (Figure 19 and Figure 20b and c). IFNα response observed in naive group at the end of 8^{th} hour of stimulation is probably due to contamination happened in that group. Additionally, every β-actin band, which was used for normalizations, does not possess the same optical density. That's why we obtained a deceptive bar showing presence of IFNα transcript as much as obtained from stealth-pIC stimulation (Figure 19a).

Collectively these data suggests that IFNα message of pIC is augmented when it is encapsulated in either stealth or neutral liposomes. Besides IFNγ induction by pIC

encapsulating stealth liposome is independent of IL18 but cationic liposomes encapsulating pIC dependent IFNγ could be IL18 dependent.

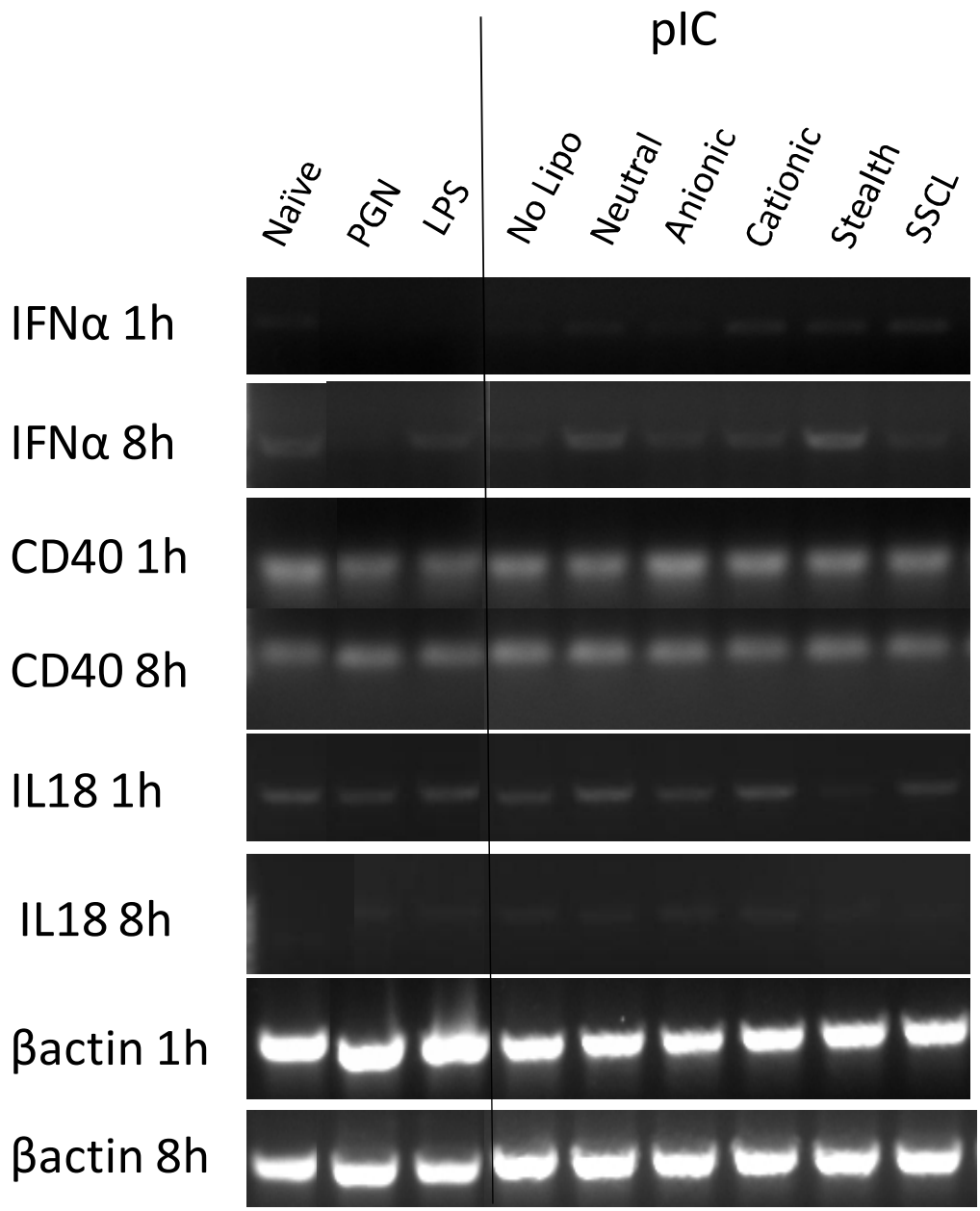


Figure 19: PCR results that were performed from total RNA of splenocytes following stimulation with free (No Lipo) or various liposomes encapsulating 5µg/ml pIC. 5µg/ml PGN and LPS were used as positive control.

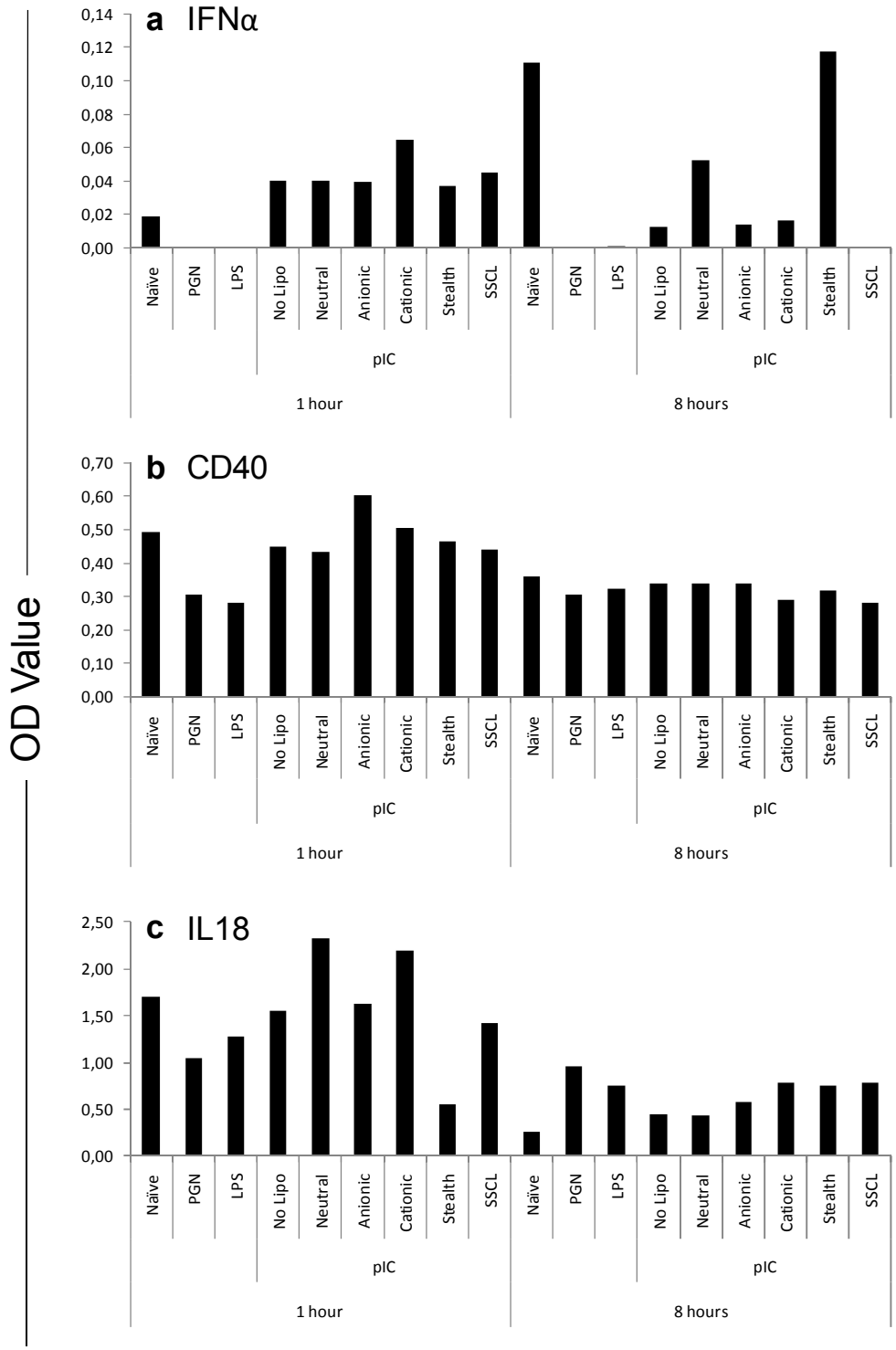


Figure 20: OD measurement results of the gel electrophoresis picture in Figure 19 for IFNα (a), CD40 (b) and IL18 (c). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

4.2. Studies to understand whether coencapsulating endosomal TLR ligands into liposomes induces a more pronounced activation

4.2.1. Cytokine ELISA after *in vitro* stimulations of splenocytes with liposomes coencapsulating K-ODN or D-ODN and pIC

Previous studies demonstrated that D class of CpG ODN (D-Type) is also a strong inducer of IFNα (Gursel *et al.* 2006). Next we have checked if we can augment effect of pIC by coencapsulating with CpG ODN into liposomes. Here, K23 and D3CG have been coencapsulated with pIC separately. Even though there was a slight increase in the IL6 secretion when K23 is coencapsulated with pIC in anionic liposome, this secretion was insignificant. However, IFNγ secretion was induced remarkably when K23 and pIC was coencapsulated both in anionic and SSCL liposome. Here it is important to note that at the lowest doses of free K23 and pIC yielded fairly low detectable amount of IFNγ (Figure 21 and Figure 22). These data revealed that when K23 and pIC is coencapsulated in anionic liposome, it synergistically induced strong IFNγ response.

D-Type ODN plus pIC in neutral or anionic liposomes boosted both IL6 and IFNγ by about 6 fold (this increase is more prominent at low doses). Surprisingly, IFNγ and IL6 secretions were suppressed at all tested doses when pIC and D3CG were loaded into cationic and SSCL liposomes (Figure 23 and Figure 24). These data suggest that in neutral or anionic liposome, pIC and D3CG showed stronger synergistic activity however when they are coencapsulated into positively charged liposomes (Cationic or SSCL), probably due to simultaneous signaling mechanisms of TLR3 and TLR9 antagonizes with each other and yield very low cytokine secretion. Collectively these findings implicated that coencapsulating TLR3 and TLR9 ligands in anionic liposome yielded the strongest Th1 type cytokine production.

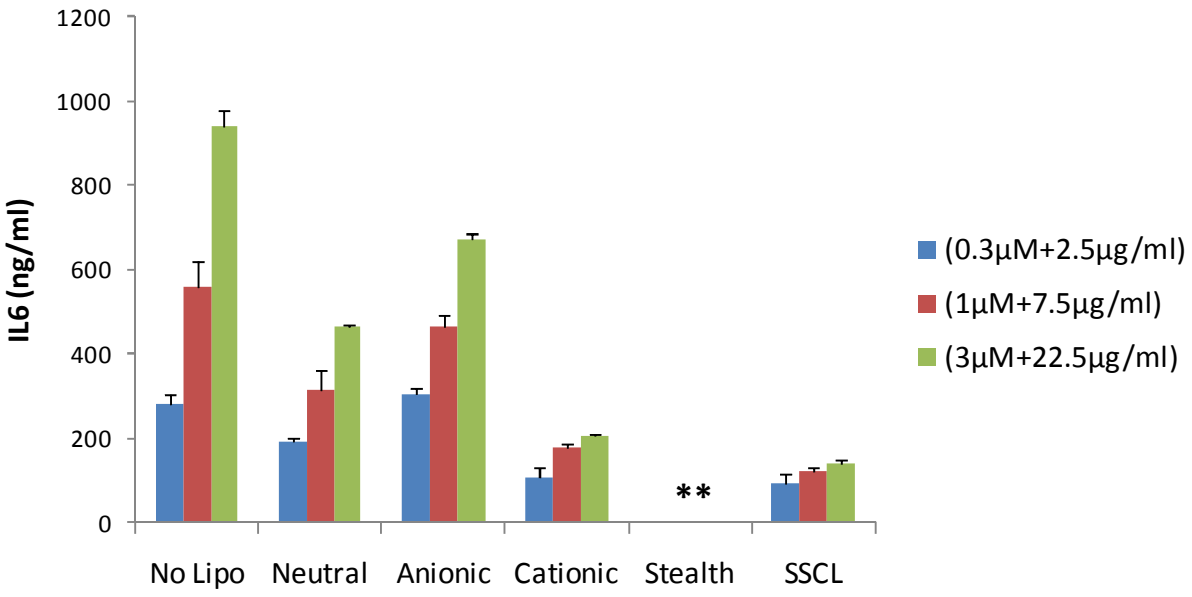


Figure 21 : IL6 production from splenocytes following stimulation with free (No Lipo) or various liposomes coencapsulating K23 and pIC (0.3-3µM and 2.5-22.5µg/ml respectively), (For more information please see Figure 7 legend).

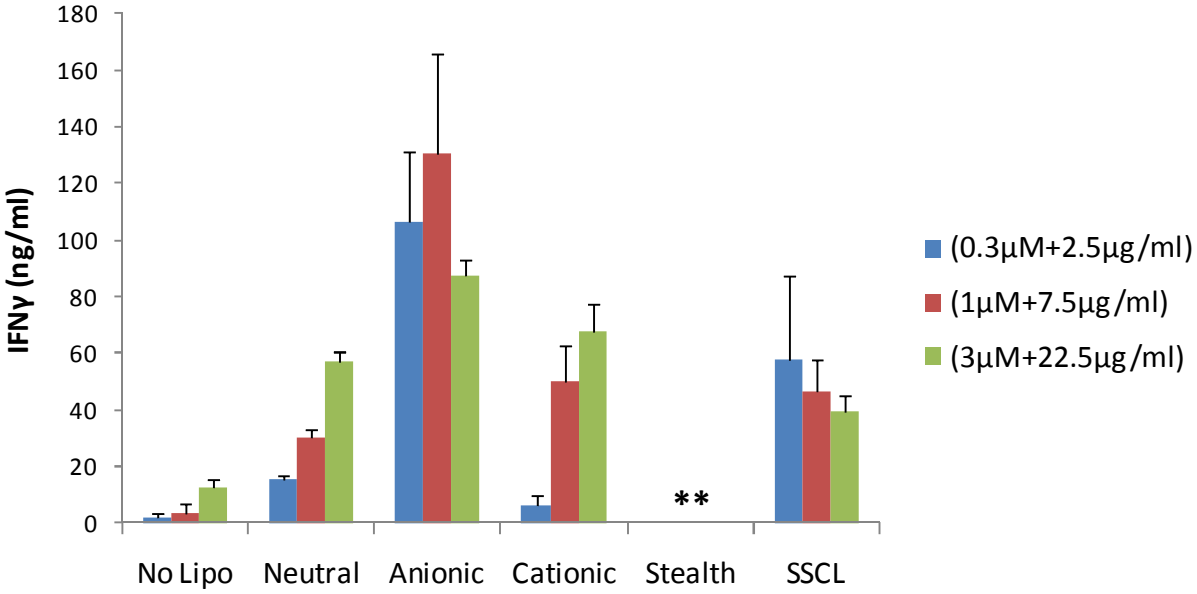


Figure 22: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes coencapsulating K23 and pIC (0.3-3µM and 2.5-22.5µg/ml respectively). (For more information please see Figure 8 legend). $p<0.01$ for 0.3µM, 1µM and 3µM of SSCL and Neutral vs No Lipo. For Anionic vs No Lipo t-test analyses, $p<0.001$ at all concentrations. For cationic group comparison: at first dose p=NS, second and third doses $p<0.01$.

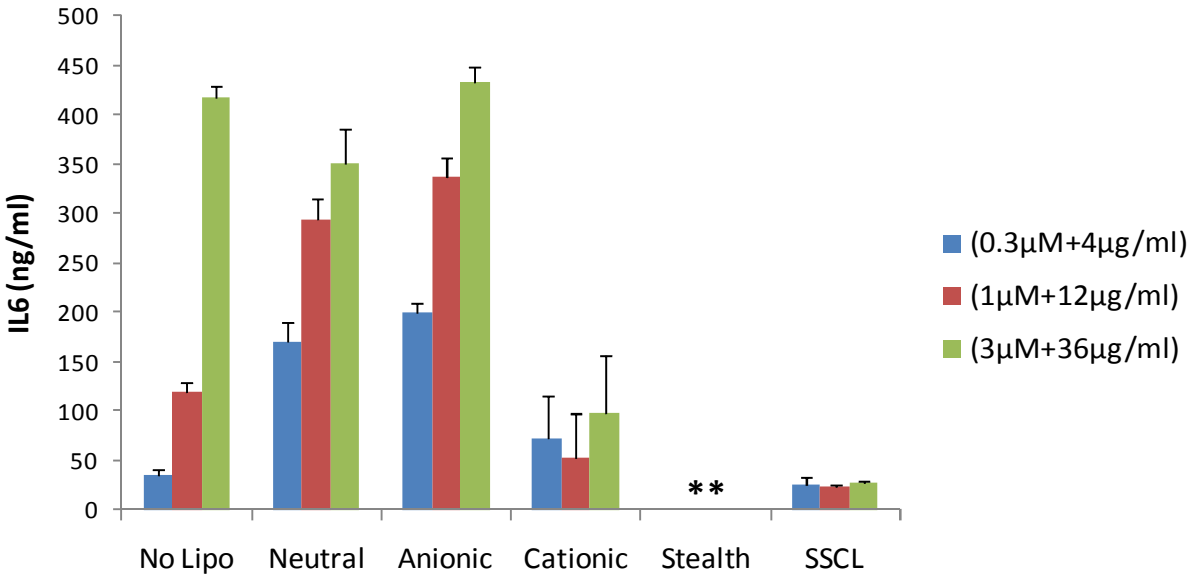


Figure 23: IL6 production from splenocytes following stimulation with free (No Lipo) or various liposomes coencapsulating D3CG and pIC (0.3-3µM and 4-36µg/ml respectively). (For more information please see Figure 7 legend). For Neutral vs No Lipo and Anionic vs No Lipo $p<0.01$ at the first 2 doses. The highest dose is NS. Cationic and SSCL groups comparisons to that of No lipo at all concentrations are NS.

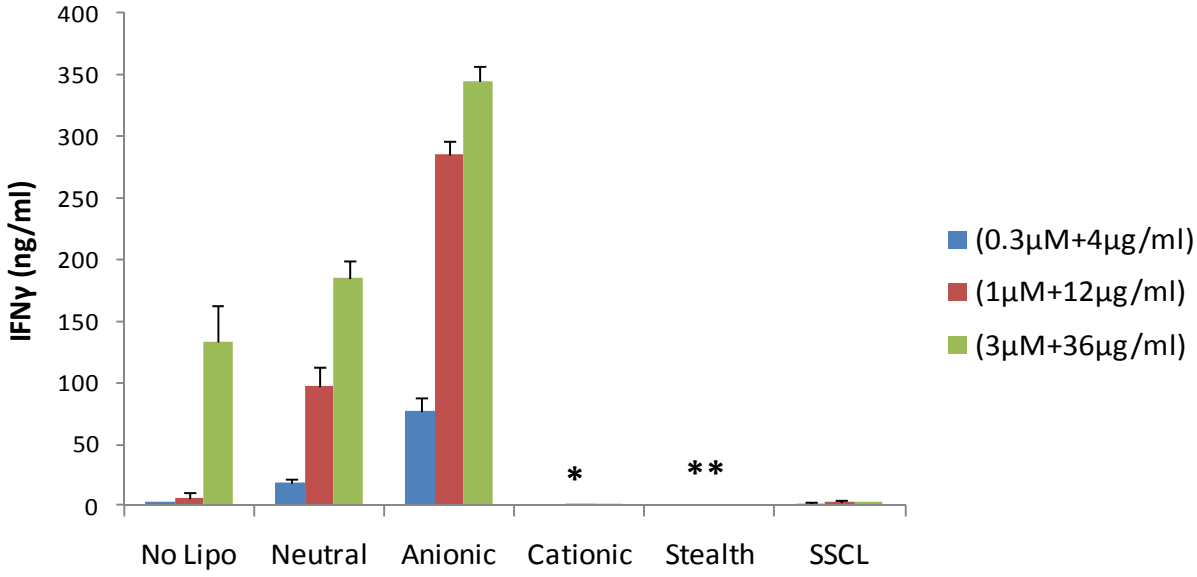


Figure 24: IFNγ production from splenocytes following stimulation with free (No Lipo) or various liposomes coencapsulating D3CG and pIC (0.3-3µM and 4-36µg/ml respectively). (For more information please see Figure 8 legend). For Neutral and Anionic vs No Lipo $p<0.001$ at the second doses. Anionic vs No Lipo, first dose is $p<0.01$, and the rest of the t-test analyses between groups are NS.

Table 11: Summary of relative IL6 secretion induction following stimulation with free or various liposomes encapsulating pIC, K23+pIC and D3CG+pIC at lowest doses

	K23	D35	pIC	K23+pIC	D3CG+pIC
No Lipo	+	0	+	+	**+**
Neutral	++	**++++**	**++++**	+	**++++**
Anionic	++	**++++**	**++++**	+	**++++**
Cationic	+	++	**++++**	+	++
Stealth	N.D	**++++**	**++++**	N.D	N.D
SSCL	0	+	+++	0	+

Lowest doses were considered. If stimulation with free ODN (No Lipo) yielded IL6 response, the amount of IL6 was marked as "+" and relative IL6 secretion inductions was calculated according to "No Lipo" group. In this case "No Lipo" is the reference group. If "No Lipo" stimulation did not yield any IL6 response, "No Lipo" group was shown as "0". Then the group that yielded lowest amount of IL6 secretion was denoted as "+" and relative IL6 secretion inductions were calculated according to that reference group. 0; no induction, +; observed induction at reference group or <2 fold induction, ++; between 2-3 fold induction, +++; 3-4 fold induction, ++++; 4< fold induction,

Table 12: Summary of relative IFNγ secretion induction following stimulation with free or various liposomes encapsulating pIC, K23+pIC and D3CG+pIC at lowest doses.

	K23	D35	pIC	K23+pIC	D3CG+pIC
No Lipo	0	+	0	+	+
Neutral	++	**++++**	+++	++	**+**
Anionic	**++++**	**++++**	**++++**	**++++**	**++++**
Cationic	+	+	+++	+	0
Stealth	N.D	+	**++++**	N.D	N.D
SSCL	0	+	**+**	+++	+

Lowest doses were considered. If stimulation with free ODN (No Lipo) yielded IFNγ response, the amount of IFNγ was marked as "+" and relative IFNγ secretion inductions was calculated according to "No Lipo" group. In this case "No Lipo" is the reference group. If "No Lipo" stimulation did not yield any IFNγ response, "No Lipo" group was shown as "0". Then the group that yielded lowest amount of IFNγ secretion was denoted as "+" and relative IFNγ secretion inductions were calculated according to that reference group. 0; no induction, +; observed induction at reference group or <2 fold induction, ++; between 2-3 fold induction, +++; 3-4 fold induction, ++++;4 < fold induction.

4.2.2. Effect of various liposomes coencapsulating CpG and pIC on IFNα, CD40, IL15 and IL18 transcript levels.

Firstly we have checked IFNα transcript levels to reveal whether liposome coencapsulation of pIC together with D3CG (alternative design of D-ODN) or K23 can increase IFNα transcript levels. Although we do not expect any IFNα response at the end of 1h of stimulation (IFNα production is under positive feedback of its own production, so 1h is too early for such kind of effect) we have detected small increase in the message level in all liposome types tested. At the end of 8h free D3CG+pIC (1μM+3,6μg/ml) showed a slight IFNα message. Consistent with previous observation regarding IFNγ and IL6 production by ELISA (please see Figure 23 and Figure 24) positively charged liposomes coencapsulating D3CG + pIC yielded a substantial lost (compared to unencapsulated counterparts) in IFNα transcript level. Neutral and anionic liposomes induced about 4-5 fold higher IFNα message (Figure 25 and Figure 26a). It is known that K-ODN does not lead to any detectable IFNα from human PBMC (Gursel *et al.* 2002). Similar to that observation, we did not observe any IFNα message induction with any liposome types tested with coencapsulating K23 and pIC (Figure 25 and Figure 26).

At the end of 1h, CD40 transcript levels did not change upon stimulation with all liposomal types. At the end of eighth hour of stimulation, cells that were stimulated with K23+pIC coencapsulating liposomes showed higher amount of CD40 mRNA compared to free form. The trend with K-ODN is reversed when D3CG + pIC message either in neutral or in anionic liposomes was investigated over time. There is an augmentation in CD40 message by 1h post-treatment and the augmentation is lost by the end of 8h (Figure 25 and Figure 26b).

IL15 mRNA levels both with pIC plus K or D-type liposomal formulations increased at eighth hour of stimulation. The exception with this cytokine is the cationic liposome coencapsulating D3CG and pIC. At 1h, none of the liposomal formulations of pIC and K23 (or D3CG) altered IL18 levels significantly (Figure 25 and Figure 26c and d). By 8h, from anionic (K+pIC) and cationic (D+pIC) liposome types, message reduction is observed.

Collectively these data reveals us that when pIC and D-ODN is coencapsulated into neutral or anionic liposome, we can obtain very strong Type I IFN cytokine response

together with augmented CD40 levels. These responses would increase protection against viral infections *in vivo*.

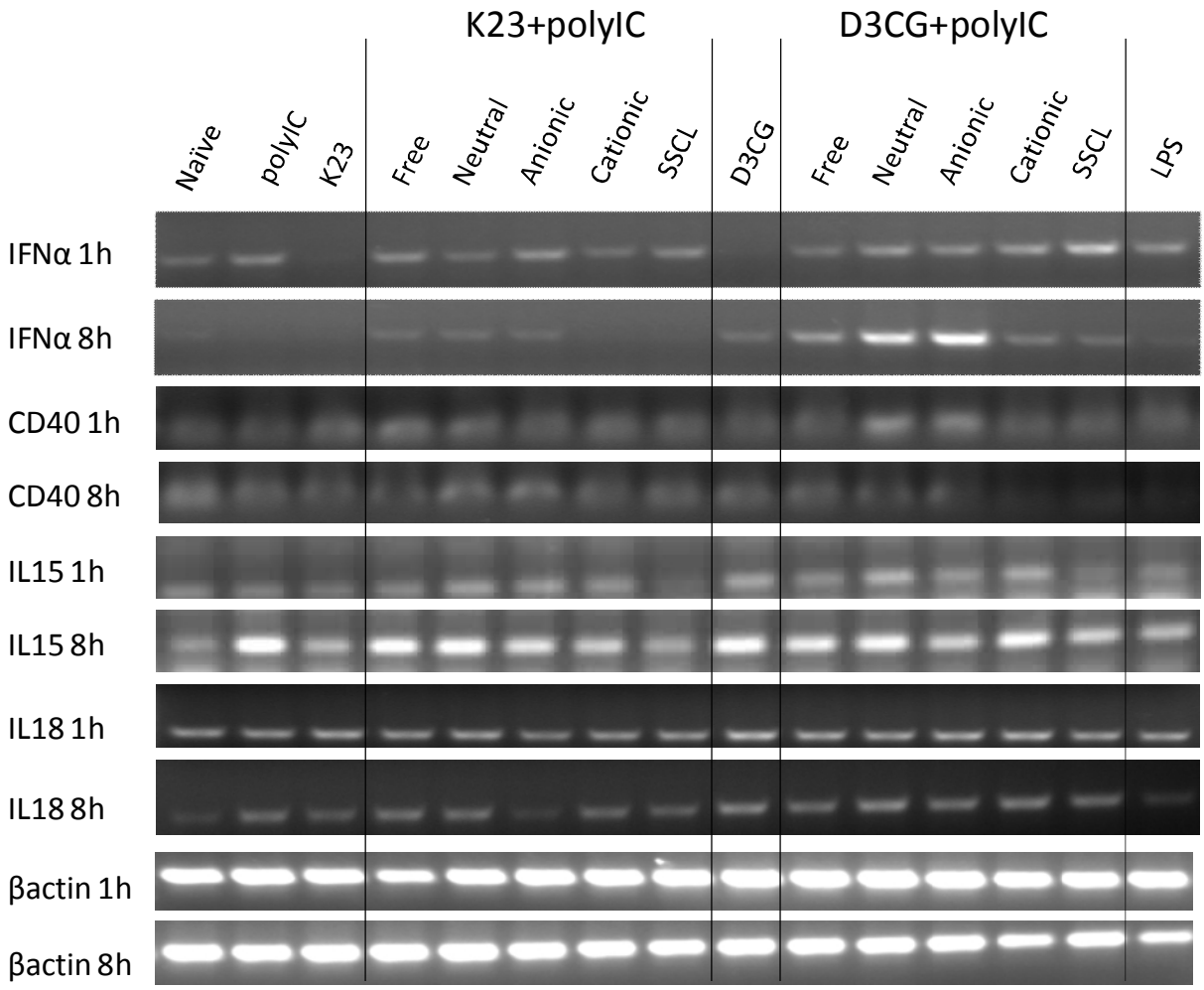


Figure 25: PCR results that were performed from total RNA of splenocytes following stimulation with various liposomes coencapsulating or free 1μM K23 or D3CG and 3.6μg/ml or 6μg/ml pIC respectively. 3,6μg/ml free pIC, 1μM free D3CG and 1μM free K23 were used as control groups.

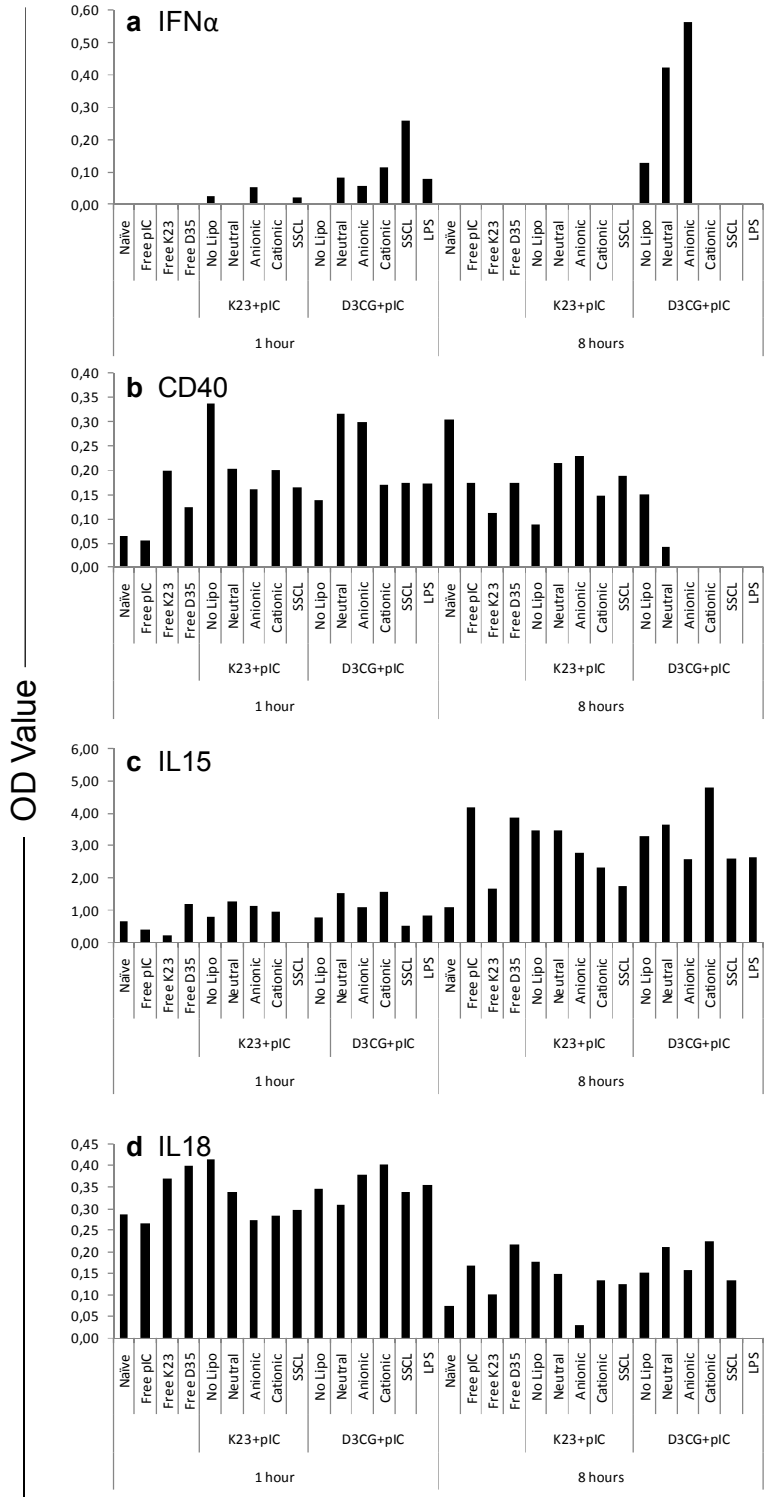


Figure 26: OD measurement results of the gel electrophoresis picture in Figure 25 for IFNα (a), CD40 (b), IL15 (c) and IL18 (d). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

4.3. Detection of activated APCs upon stimulation with nucleotide encapsulating liposomes

A naive T cell requires a second signal to proliferate and differentiate into effector T cells following encountering with an antigen. This second signal is known as co-stimulation. Co-stimulation is mediated by costimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86) expressed on mouse APCs including DCs, macrophages and B cells. The CD86 is expressed constitutively at low levels on APCs and its upregulation is initiated upon recognition either by PAMPs (i.e. through TLRs) or exposure to inflammatory cytokines such as IFNγ, IL12 or IL6. Of note, the CD80 upregulation requires longer exposure and may take days (Greenwald *et al.* 2005; Abbas *et al.* 2007). We decided to investigate the activation ability (by CD86 staining) of APCs by liposomes encapsulating or coencapsulating TLR3 and TLR9 ligands.

Results of CD86 staining are not supporting our previous IL6 and IFNγ ELISA data. For instance, 14% of splenocyte population that was stimulated with cationic liposome loaded with D35 was positive for CD86 (Figure 27).

A similar trend is valid for stimulations with D3CG plus pIC liposomes. Based on our previous experience, we were expecting to obtain the highest activation profile by neutral and anionic liposomes, contrary to our expectation, cationic and SSCL liposome yielded the highest CD86 upregulation. CD86 upregulation using pIC encapsulating liposomes in general gave very low activation except for SSCL type (ca. 3 fold over untreated naive group) (Figure 27).

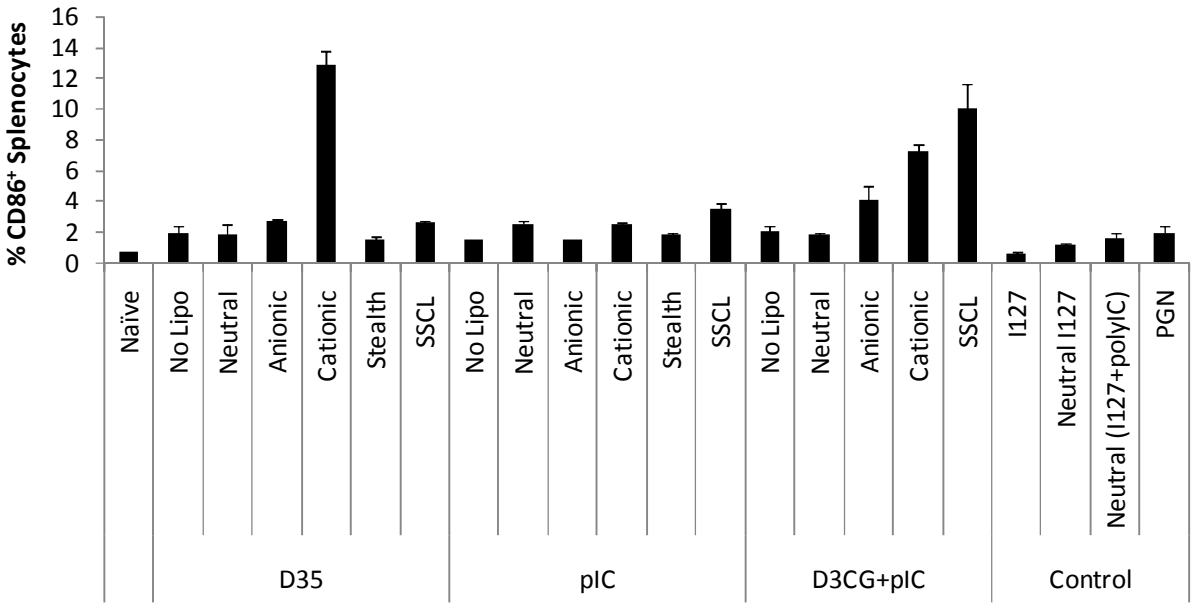


Figure 27: Percentage of CD86 expressing splenocytes upon stimulation for 4 hours with various liposomes encapsulating or free 1μM D35, 3μg/ml pIC or coencapsulated 1μM D3CG and 3μg/ml pIC. 1 μM free I-127, neutral liposome encapsulating or coencapsulated with 3μg/ml pIC and 5μg/ml PGN were used as control groups.

4.4. *In vivo* studies utilizing nucleic acid TLR ligands encapsulated in different liposome formulations

As our aim is to design a strong vaccine adjuvant, following our initial cell culture experiments we have identified (among many candidate formulations) a group of liposome encapsulating TLR ligand formulations behave more active than their free counterparts (as evidenced by increased cytokine production and message level as well as surface marker upregulation). *In vitro* studies although are important, it is not sufficient to deduce that these novel designs will perform similarly when they are injected *in vivo*. Among selected CpG ODN types, and dsRNA ligand, our observations led us to postulate that D-Type ODNs breadth of activity with or without pIC is more pronounced compared to K-Type CpG ODN when used within liposomes (increased Type I and Type II interferon, and Th1 biased cytokine secretions, co-stimulatory molecule and surface marker upregulation).

The initial experiments were designed to establish *in vivo* immunostimulatory potential of various liposomes encapsulating D-Type ODNs (D35 or D3CG) or pIC and their combinations co-encapsulated within 5 different liposomes. Mice were injected with different free or liposomal D35, pIC and combinations of D-ODN+pIC. Following ip injection spleens were extracted and split into two parts. Half of the spleen cells were incubated *ex vivo* and assayed for cytokine secretion, the other half was used to extract total RNA and was studied for i) TLR upregulation, ii) cytokine, chemokine and co-stimulatory molecule message change by PCR, and iii) co-stimulatory molecule upregulation by FACS.

4.4.1. Immunostimulatory activity of D3CG and pIC coencapsulating liposomes *ex vivo*.

To reveal the *in vivo* activity of various liposomes encapsulating only D-ODN, or only pIC and coencapsulating D+pIC, mice were ip injected with different nucleic acid-liposome formulations individually and 4h post injection, spleen cells were removed and incubated for an addition of 24h in culture (no further stimulation at this stage). Spleen cells at the end of *ex vivo* stimulation were stained with anti-CD86-FITC antibody to detect CD86+ cells.

The internalization efficiencies of the free vs liposomal formulations vary at great extend. Following ip injection, amount of free nucleic acids that is draining into spleen is maximized in 1 hour. Nucleic acids that remained in serum are digested by nucleases and thus amount of draining nucleic acids fall off sharply. However, the time, when liposomal nucleic acids draining into spleens get maximized, is later than 4th hour of injection. (Fenske *et al.* 2001; Gursel *et al.* 2001; Mutwiri *et al.* 2004). Therefore, extracting spleen of mice after 4th hour of injection can be considered as terminating the drainage of nucleic acid encapsulating liposomes prematurely although all of the free nucleic acids, which were not digested, have already been drained. This attempt is actually a way of favoring free nucleic acids over their liposomal forms. Moreover we have favored free nucleic acids for the second time by injecting 2.5 fold more D-ODN (50 μg) as opposed to 20 μg liposomal D-ODN (same trend was kept for pIC treatments). Overall, all tested liposomal formulations upregulated CD86. Among D35 group, SSCL-D35 upregulated CD86 at same level compared to those cells treated with free D35 although 2.5 fold less of CpG was used during

ip treatment of the animals (Figure 28). For pIC group, free ligand treatment gave 8.6 % CD86+ cells, Stealth liposomal pIC treatment led to about 14% CD86+ cells (again please note that free ligand is 2.5x more than liposome formulations). When D+pIC co-encapsulated group is investigated (see right panel of Figure 28), neutral and anionic liposome(D+pIC) treatments surpassed free D+pIC injected group CD86 upregulation (11.2, 9.2 vs 8.5). Please note that similar fold difference was kept throughout this group during ip injections. Additionally, it is very important to note that total amount of activated cells by neutral liposome encapsulating D35 and pIC is 1.5 and 2 fold more than neutral liposome encapsulating D35 and pIC alone respectively (please consider bars marked with "*"). Same trend is valid for anionic liposome encapsulations either (denoted as "¶"). This finding is a very clear demonstration of the augmented activity of D-ODN and pIC when they are coencapsulated in liposomes.

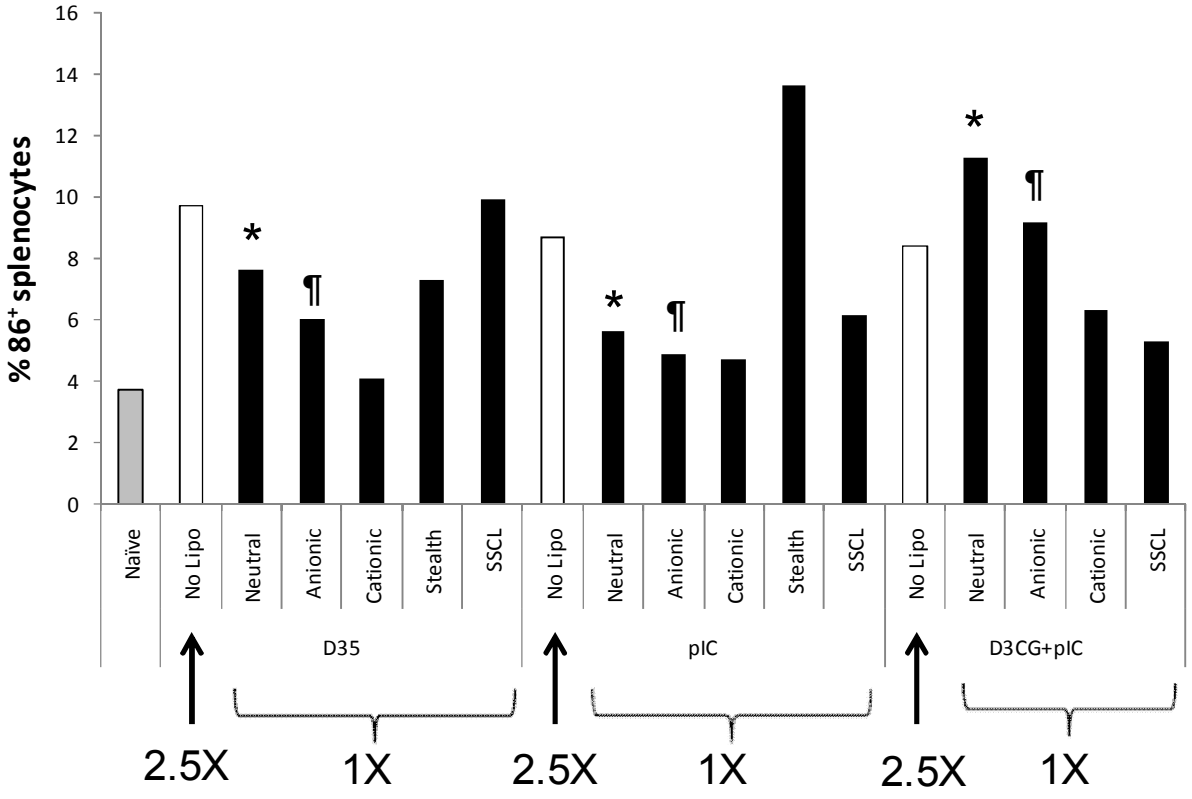


Figure 28: Percentage of CD86 expressing splenocytes extracted from mice 4 hours later immunization with 50µg/animal free D35 or pIC, 50µg/animal free D35 and pIC together (25µg/ml each), various liposome encapsulated 20 µg /animal D35, 20 µg /animal pIC and various liposome coencapsulated 20 µg/animal ligands (containing D3CG and pIC 10 µg each).

4.4.2. Effect of *ex vivo* stimulation with various liposomes encapsulating or coencapsulating D-ODN and pIC on gene message levels of spleen cells

We have checked cytokine/chemokine and TLR message upregulation levels following 4h of post ip injection.

PCR analysis from cells recovered after *in vivo* stimulation with various liposomes encapsulating or coencapsulating D35 and pIC revealed that independent of the ligand and liposome type nearly all formulations gave better gene message activity profiles compared to untreated naive animals, implying that the degree of internalization/uptake (or binding) after 4h of *in vivo* treatment with these formulations are sufficient enough to trigger an orchestral cellular activity program. As demonstrated in Figure 29 neutral and anionic liposomes coencapsulating D3CG and pIC induced higher transcript levels of all investigated parameters as shown below (please see for more details, Figure 29 right panel). These data are in good agreement with *in vitro* ELISA and PCR findings (see Figure 23-Figure 26). Similarly, pIC encapsulated stealth liposome group reproduced the *in vitro* observations. Contrary to our *in vitro* expectations, however, cationic liposome incorporating pIC did not induce appreciable degree of most gene message up-regulation (please investigate middle panel 3rd column in Figure 29, and Figures 30-32). Out of 11 different genes investigated it is fair to summarize that stealth liposomal pIC augmented both *in vitro* and *in vivo* gene message levels (please compare Figs. 17-19 and Figs. 29-32 for more details).

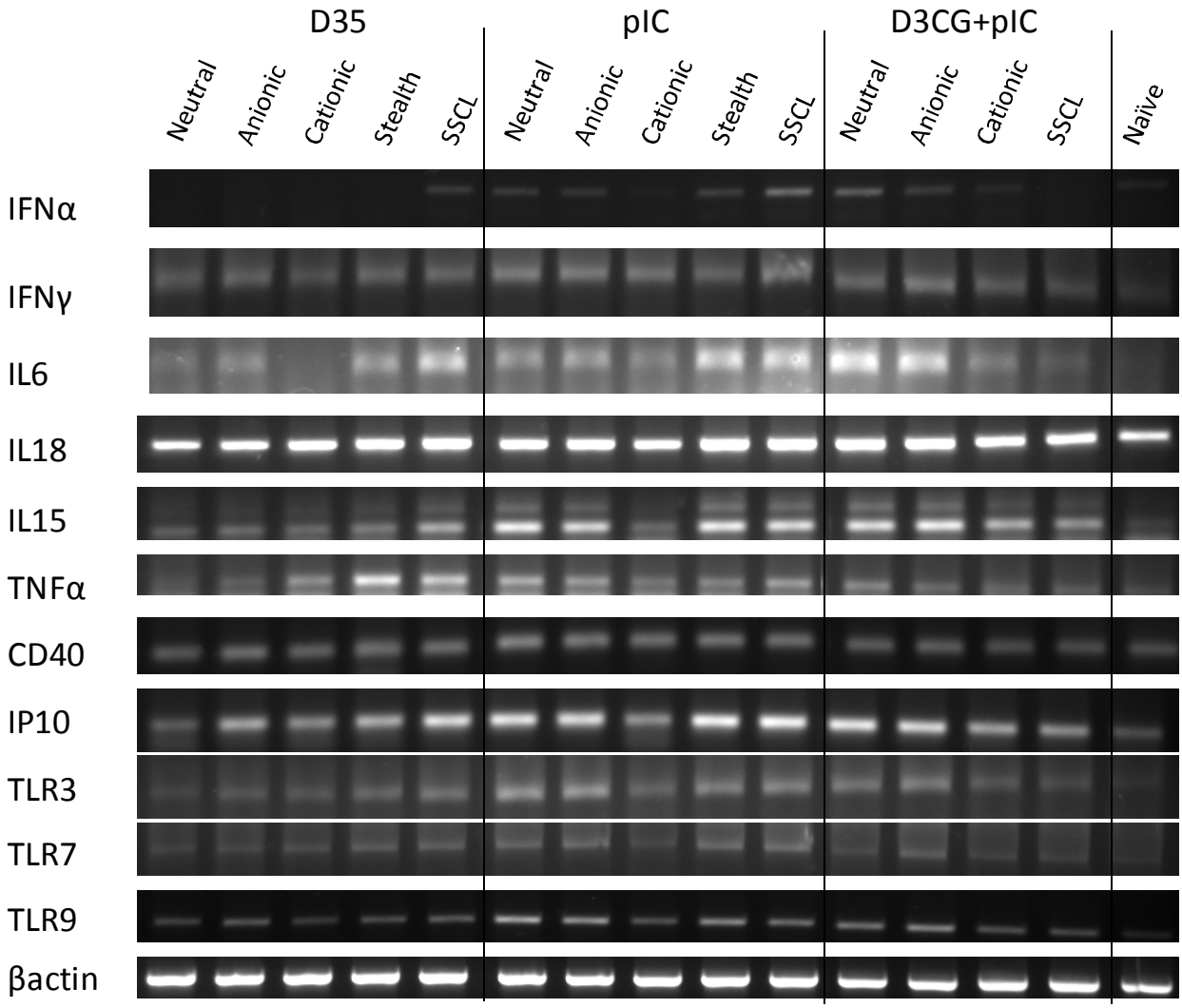


Figure 29: PCR results that were performed from total RNA of splenocytes extracted from mice 4 hours later immunization with various liposome encapsulated 20μg/animal D35, 20μg/animal pIC and various liposome coencapsulated 20μg/animal D3CG and pIC (10μg/animal each).

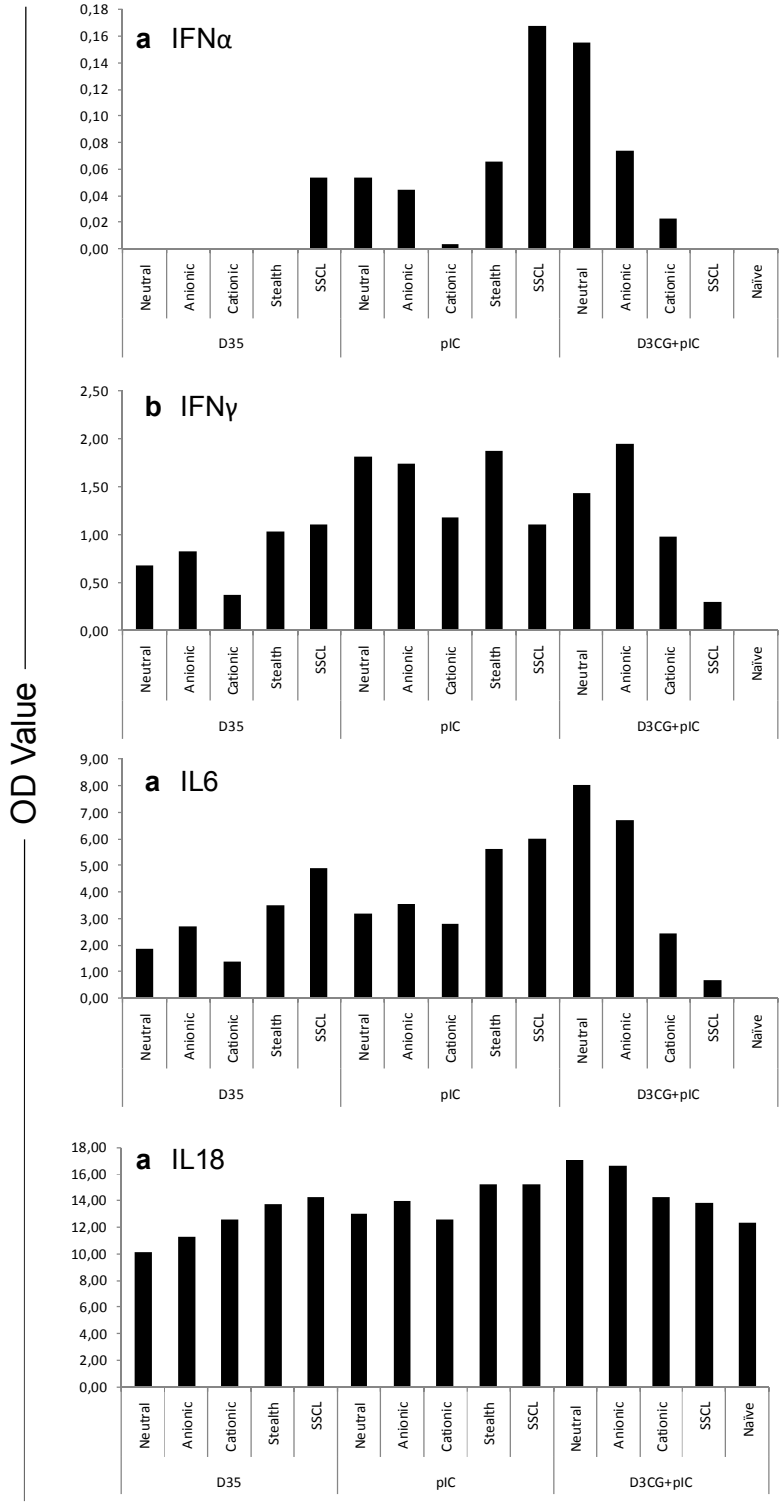


Figure 30: OD measurement results of the gel electrophoresis picture in Figure 29 for IFNα (a), IFNγ (b), IL6 (c) and IL18 (d). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

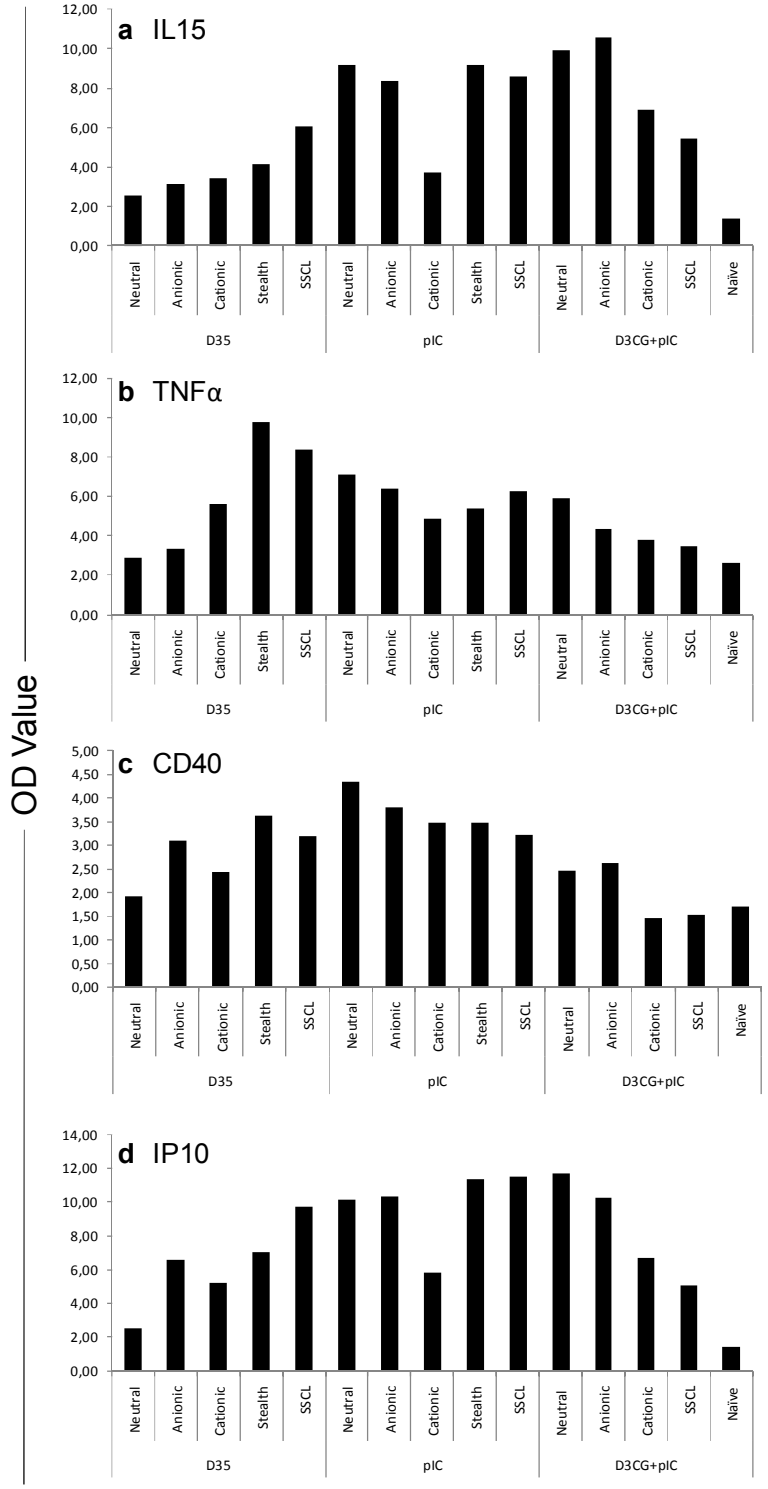


Figure 31: OD measurement results of the gel electrophoresis picture in Figure 29 for IL15 (a), TNFα (b), CD40 (c) and IP10 (d). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

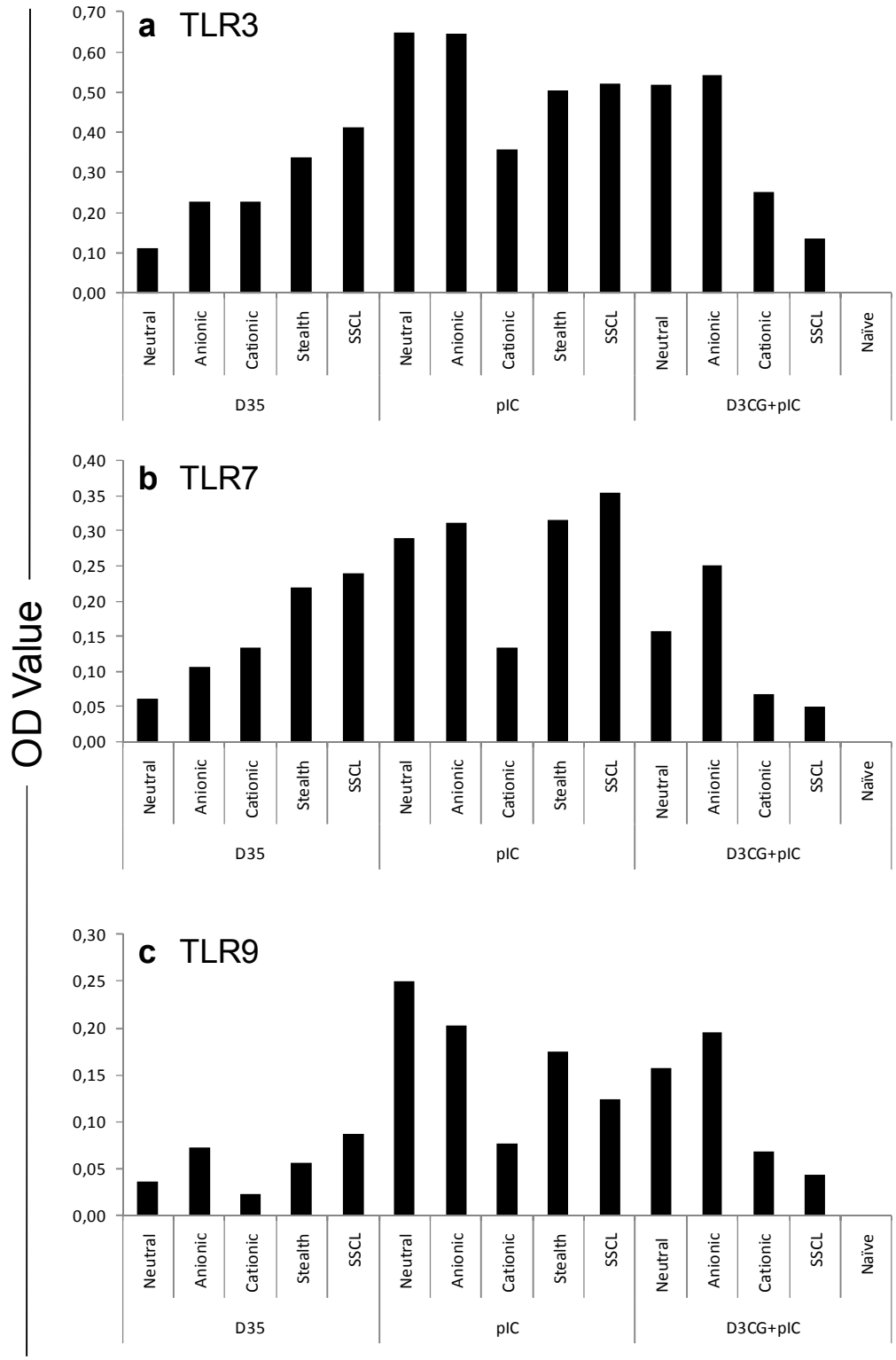


Figure 32: OD measurement results of the gel electrophoresis picture in Figure 29 for TLR3 (a), TLR7 (b) and TLR9 (c). Density measurement values of genes were normalized according to β-actin as housekeeping gene by dividing density value of gene to density value of β-actin.

4.4.3. *In vivo* Immunization Studies utilizing liposomal CpG and Ovalbumin

The utmost aim of this thesis was to investigate enhanced immunostimulatory properties of liposome encapsulating nucleic acid TLR ligands. Following *in vitro* and *ex vivo* analysis one of the most promising formulation was selected and immunization studies were planned. For this, we have selected ovalbumin (OVA) as a model protein antigen, which is 'gold standard' antigen used extensively during immunization experiments. We have injected OVA either mixed with free D-ODN or co-encapsulated form with the oligo in anionic liposomes.

Throughout this study, our findings suggested that the breadth of activation of D incorporating anionic liposomes performed far better than K-type liposomal preparations and in many instances gave better response than pIC formulations. Of note, we have not selected pIC+D combination in liposomes. The major reason for that was the problem associated with one of the lipid components (PEG2000-DOPE) during stealth liposome generation. We have yet to understand this issue, since we have no idea why we kept on failing during pIC+CpG stealth liposome preparation. It could be either due to the lipid component or the association of pIC together with the D-ODN within the liposome vesicle during the regeneration step and abolished the vesicular liposome structure entirely. We would also select neutral-D35 or stealth-pIC formulations either. The surpassing feature of these liposomes over anionic liposome encapsulating D35 is that neutral-D35 and stealth-pIC can activate more APC. However we have recognized this after we have already started to conduct immunization experiment.

Table 13: Summary of relative IL6 and IFNγ secretion inductions, IFNα transcript levels and activated APC percentages ($CD86^+$ splenocytes) following stimulation with free or various liposomes encapsulating D35, pIC and D3CG+pIC at lowest doses.

	D35	pIC	D3CG + pIC	
No Lipo	0	+	+	**IL6 ***
	+	0	+	**IFNγ ***
	+	+	+	**IFNα §**
	+	+	+	**CD86 †**
Neutral	**++++**	++++	++++	**IL6 ***
	++++	+++	+	**IFNγ ***
	++++	++++	++++	**IFNα §**
	+++	++	++++	**CD86 †**
Anionic	**++++**	++++	**++++**	**IL6 ***
	++++	++++	**++++**	**IFNγ ***
	++++	+	**++++**	**IFNα §**
	++	+	**++++**	**CD86 †**
Cationic	++	++++	++	**IL6 ***
	+	+++	0	**IFNγ ***
	++	+	0	**IFNα §**
	+	+	++	**CD86 †**
Stealth	++++	**++++**	N.D	**IL6 ***
	+	**++++**	N.D	**IFNγ ***
	++++	**++++**	N.D.	**IFNα §**
	+++	**++++**	N.D	**CD86 †**
SSCL	+	+++	+	**IL6 ***
	+	+	+	**IFNγ ***
	+	0	0	**IFNα §**
	++++	++	+	**CD86 †**

Comparing ELISA data (*) of IL6 and IFNγ and PCR data (§) of IFNα : Lowest doses were considered. If stimulation with free ODN (No Lipo) yielded IFNγ or IL6 response, the amount of cytokine was marked as "+" and relative cytokine secretion inductions was calculated according to "No Lipo" group. In this case "No Lipo" is the reference group. If "No Lipo" stimulation did not yield any IFNγ response, "No Lipo" group was shown as "0". Then the group that yielded

lowest amount of IFNγ secretion was denoted as "+" and relative IFNγ secretion inductions were calculated according to that reference group. 0; no induction, +; <2 fold induction compared to reference group, ++; 2-3 fold induction, +++; 3-4 fold induction, ++++; 4< fold induction, N.D; not determined.

<u>Comparing FACS data (†) of CD86:</u> Data from Figure 28 were considered. As concentrations of free nucleic acids were 2.5 fold more than their liposomal forms, 2.5 fold less of the % $CD86^+$ splenocyte value upon stimulation with free nucleic acids were marked as "+" and relative APC activation inductions were calculated according to the value denoted as "+". 0; no activation, +;1-2 percent more $CD86^+$ cells, ++; 2-3 percent more $CD86^+$cells,+++; 3-4 percent more $CD86^+$ cells, ++++; >4 percent more $CD86^+$ cells. N.D; not determined

As mentioned in great details (see Section 3.2.6.2) mice were divided into 5 different treatment groups. Following bleedings on day-1, mice were ip immunized the next day (at d=0) for primary response. On d=13, all animals were again tail bled and mouse sera was saved for primary anti-OVA Ig detection by ELISA. The next day, booster injections were carried out. On d=28 mice were first bled and then sacrificed, and spleens were frozen for later use to study PCR. The collected mouse primary and secondary sera were subjected to Anti-OVA IgG, IgG1, IgG2a and IgG2b ELISA following serial titrations. The Table 14, summarizes anti-ova responses of animals treated with different vaccine formulations. In conventional vaccinations, it is unlikely to detect any anti-Ag Ig response at the end of primary immunization. This is even very hard if the vaccine is a simple solution of Ag+Adjuvant. Here, (see Table 14A for details) it is clearly demonstrated that our novel vaccine formulation (Gr#5), that is, OVA Ag and CpG Adjuvant co-encapsulated within a single liposome vesicle (Anionic Liposomes) induced >150 fold more IgG over free Ag+CpG group (Gr# 3), and 14x more from Ag+Control ODN (Gr#4). In vaccine trials in addition to total IgG which is an indication of the generated humoral immunity, it is very important to mount cell mediated immune response, in which one can understand by checking the level of IgG2a titers. As seen in Table 14A the liposomal formulation co-encapsulating Ag+Adjuvant induced substantially higher IgG2a response at the end of single vaccine injection. When primary bleeding mouse sera was studied, compared to free OVA+D-ODN (Gr#3) group, over 30 fold more IgG2a from anionic liposomes co-encapsulating OVA and D-ODN (Gr#5) group was obtained (14±7 vs 428±50).

Booster injection is the cardinal phase of any vaccination since it induces both effector T and memory B cells against vaccine antigen. As seen in Table 14B, a very potent anti-OVA humoral immunity is initiated at the end of 4 weeks (Gr#5 Table 14B). A very strong improvement in total IgG (that corresponded to >22 fold more IgG) was achieved when liposomal formulation is used instead of its free solution counterpart (Gr#5 and Gr#3 titers are 28670 are 1300, respectively). IgG1 anti-OVA titers were also significantly high with liposome co-encapsulation (more than 26 fold raise between Gr#5 vs Gr#3). IgG2a titers of animals that was treated with Gr#5 was >13 fold more compared to Gr#3 animals. A significant and modest Ig titer increase was observed with liposome encapsulating control ODN+OVA. This is not an unexpected outcome, in the literature there are several reports suggesting that oligo sequences containing no CpG-motifs can induce a modest immune activation independent of both TLR9 and CpG motifs. This is primarily due to the backbone chemistry, here our Control ODN possesses PS-PO mixed backbone and this Ag dependent Ig response could be initiated due to the presence of phosphorotioate regions expressed on the control ODN. Moreover, liposome encapsulation probably contributed to this augment effect.

Our findings demonstrate that delivery of D-ODN and antigen encapsulated in anionic liposome induces stronger immune stimulation against the antigen than free D-ODN can mediate. This knowledge will pave the way to design of novel effective vaccine adjuvants.

Table 14: Primary and secondary anti-OVA IgG subclass responses of mice immunized with free or liposome encapsulating Ag and CpG ODN.

A. Primary IgG Levels

Gr #	Treatment Groups	IgG	IgG1	IgG2a	IgG2b
1	**Untreated**	7±4	7±4	7±4	7±4
2	**Control ODN+ OVA**	7±4	7±4	7±2	7±1
3	**D3CG+OVA**	28±14	14±7	14±7	7±2
4	**Anionic Lipo(Control ODN+OVA)**	300±150	35±10	7±2	7±1
5	**Anionic Lipo(D3CG+OVA)**	4250±1000*	112±12*	428±50	7±1

B. Secondary IgG Levels

Gr.#	Treatment Groups	IgG	IgG1	IgG2a	IgG2b
1	**Untreated**	7±1	7±2	7±2	7±4
2	**Control ODN + OVA**	7±2	7±4	7±2	7±1
3	**D3CG+OVA**	1300±450	828±245	112±12	112±12
4	**Anionic Lipo(Control ODN+OVA)**	5168±2590*	4355±1430*	448±20*	112±20
5	**Anionic Lipo(D3CG+OVA)**	28672±5050[a]**	21600±7150[a]**	1500±250[a]*	28±4

Female Balb/c mice (6-8 wks old, 5/group) was immunized with OVA (5 µg/ml) plus control or CpG ODN (15 µg/ml) (free or liposome encapsulated forms) at day 1 and bleed on d=13 before booster injection. Two weeks post-booster injection mice were first tail bled and then sacrificed. Sera was titrated and assayed for anti-OVA IgG subclasses and reported as 1/T for primary and secondary antibody response. Results (mean titer ± SD) are combination of two independent experiments.

a 0.001<p (Gr#4 vs Gr#5 paired t-test analysis comparison).

**0.01<p (represent the statistical significance of Gr#3 vs Gr#4 or Gr#5 paired t-test analysis comparison)*

*** 0.0001<p (Gr#3 vs Gr#4 or Gr#5 paired t-test analysis comparison)*

5. DISCUSSION

This study shows that encapsulating pIC and CpG ODN in liposomes yields differential immune activation depending on the physicochemical properties of liposome which is not possible to obtain with free ligand. It is possible to enhance activation of TLR ligand nucleic acids by liposome encapsulation both *in vitro* and *in vivo*. Antigen specific immune response obtained by triggering immune response with D-ODN can be boosted by coencapsulation of antigen and D-ODN in anionic liposome. Moreover, coencapsulation enhances the synergism of pIC and CpG ODN.

Advantages of dehydration-rehydration protocol over Ethanol extraction method approach and detergent dialysis procedure is high encapsulation ratios and providing opportunity to handle labile molecules. While the highest encapsulation ratio was encapsulation of K-ODN in neutral liposome with 67.4%, the highest encapsulation is with 98.7% of D35 in SSCL (Table 7).

ODN releasing amounts of liposomes differ according to type of the liposome and type of CpG ODN. While 40% of ODN is released from anionic liposome in 8 days and neutral, anionic and stealth liposomes lose majority of their encapsulated ODNs in 5 months, positively charged liposomes encapsulating any kind of ODN protects their stability. This is most probably due to electrostatic interaction between negatively charged ODNs and positively charged lipids. Exceptionally, D-ODNs and its control I-127 encapsulating liposomes remained their stability regardless of surface net charge. It is interpreted that positively charged groups generated after formation of G-tetrads on D-ODN interact with lipids and thus liposomes encapsulating D-ODNs can remain stable (unpublished data). Anionic liposome encapsulating D35, which we offer as vaccine adjuvant candidate keeps 82.7% of its content even after 5 months. Long shelf life with minimum cold storage requirements is preferred for pharmaceutical agents. Therefore we can say that our formulation can fulfill these requirements of medicine industry.

1555, a design of K-ODN, induces more IFNγ and IL6 secretion when encapsulated into positively charged liposomes (cationic, SSCL) compared to negatively charged (anionic) or neutral liposomes (neutral, stealth). These data are actually confirmation of previously reported data by Gursel et al.(Gursel *et al.* 2001). Interestingly alternative

design of K-ODN, K23, behaves differently in liposomes compared to 1555. It induces higher amount of IFNγ and IL6 secretion in neutral and anionic liposomes but the stimulation is repressed when it is encapsulated into positively charged liposomes. The characteristic difference between 1555 and K23 is that K23 contains an extra CpG motif and it is size is 12mer while 1555 is 15mer. It is known that structure of CpG ODNs affect activity (Krieg 2006). Additionally these data speculate that not only the physicochemical properties of liposomes but also the sequence of CpG ODN has effect on differential cytokine secretion.

We have revealed out that neutral, stealth and anionic liposomes encapsulating D35 boost IFNγ secretion compared to free form. Stimulation with lowest dose (0.3μM) of D35 in neutral, anionic and stealth liposomes augmented IL6 secretion for 4, 5 and 2 folds respectively compared to free D35. 3μM of free D35 can induce as much IL6 secretion as 0.3μM D35 encapsulated into neutral, anionic and stealth liposomes. Contrast to response of cells to 1555, stimulation with positively charged liposomes repressed both IFNγ and IL6 secretions. Therefore we can conclude that neutral or anionic liposomes encapsulating D-ODN can induce high amount of cytokine secretion even in low doses.

One of the possible explanations of the repression of immune activation upon stimulation with positively charged liposome encapsulating K23 and D35 would be induction of Th2 type immune response. However we have revealed that this is not the case (data not shown). Another hypothesis is that positively charged liposomes change the subcellular localization of CpG ODNs. We have suspected that positively charged liposomes prevents ODNs to be sequestered in early endosome where D-ODN signaling cascade is initiated. Cationic and SSCL liposomes carry CpG ODNs directly to late endosome. Since D-ODN is not active at this site the pronounced IFNγ and IL6 secretion by positively charged liposomes encapsulating 1555 is significantly higher than the liposomes encapsulating D-ODN.

Another support to this hypothesis that the positively charged liposomes change the subcellular localization of CpG ODNs comes from the IFNα transcript levels. It is known that D35 signals from early endosome and induce IFNα production through MyD88 and IRF7 in pDCs. We have demonstrated that cationic and SSCL liposomes loaded with D35 does not improve IFNα message compared to free D35 counterpart at 8h of stimulation. As hypothesized, neutral, anionic and stealth liposomes induced significantly high levels of

IFNα message. Therefore, results implicated that anionic and stealth liposomes keeps D35 at an extended period compared to free, or positively charged liposomes in the early endosome leading to more pronounced IFNα message (please see Figure 15 and Figure 16). To reproduce this finding an *in vivo* experiment was designed. Mice were injected with free or liposome encapsulating D35 and spleens were extracted at 4h post-injection to isolate total RNA and study gene specific messages by PCR (Figure 29). Please note that there is an intentional selection of the dose of free vs carrier coupled D35. Here, to compare the potency, free was 2.5 fold more than the injected dose of the Liposomal D35. Another choice was made for the spleen cell harvest from the animals. Here, we selected 4h again knowingly, to favor free ODN internalization kinetics over liposomal counterpart. Although we have expected to observe IFNα transcript after stimulation with neutral, anionic or stealth encapsulating D35 but not with positively charged liposomes groups, only SSCL-D35 lane gave a positive band. We have extracted spleen of mice after 4^{th} hour of stimulation. Accumulation rate of different liposomes to spleen is expected to be different at early time points whereas, free ODN accumulation is at its highest level. The recovered spleens were checked to identify which liposomes induced more gene transcript. As expected, K-ODN did not induce any IFNα message. Although Honda et.al reported that encapsulating K-ODN within cationic liposomes induced IFNα (Honda *et al.* 2005), in our assay system we could not detected any transcript with the liposomal forms of 1555. The discrepancy between these findings could be due to the type of the mouse strain or the type of the selected K-ODN sequence. Type I interferons are crucial in regulation of innate and adaptive immune response and they have important roles in autoimmune diseases (Theofilopoulos *et al.* 2005). Therefore controlling type I interferon production is highly important for vaccine designs or cancer therapies.

CD40 is a receptor expressed on macrophages and important in activation of infected macrophages with intracellular pathogens by CD4 T cells expressing CD40 ligand. CD40 is significantly induced by 1555 alone and 1555 encapsulating anionic and stealth liposome at first hour of stimulation. Free D35 also induces CD40 expression. Therefore we can say that K-ODN and D-ODN encapsulation into any of the liposomes do not augment its expression compared to free form.

IL18 is considered as IFNγ-inducing factor. Together with IL12, IL18 induces high amount of IFNγ expression(Dinarello *et al.* 2003). It is an important cytokine in innate(French *et al.* 2006; Srinivasan *et al.* 2007) and adaptive(Iwai *et al.* 2008) immune regulation. Stimulation with 1555 containing liposomes does not augment IL18 expression compared to free 1555 at first hour of stimulation. Except the free form, liposomal forms of D35 do not induce IL18 expression. We can conclude that IFNγ induction by liposomal forms of either D-ODN or K-ODN is not maintained by increased levels of IL18 production.

We observe IFNα, CD40 and IL18 induction upon control ODN encapsulating liposomes. These stimulations are TLR independent because the control ODN do not contain CpG motif. We speculate that high concentration of control ODN that is internalized into phagocytic cells together with liposomes trigger TLR independent signaling pathways through recognition by cytosolic DNA sensors such as DAI (Takaoka *et al.* 2007) or AIM2 (Hornung *et al.* 2009).

We have also stimulated cells with liposomes encapsulating pIC. Our data indicates us that neutral, anionic, cationic and stealth liposomes encapsulating pIC induces high amount of IFNγ and IL6 secretion even at the suboptimal doses of free pIC stimulation. For instance 2.5μg/ml of pIC encapsulated in anionic liposome yields 170 fold more IFNγ secretion than even 22.5μg/ml of free pIC. Among liposome types, only SSCL does not induce significant amount of IL6 and IFNγ secretion.

We have checked IFNα, CD40 and IL18 mRNA levels after stimulation with free pIC and its liposome encapsulated forms. When pIC is encapsulated in stealth and neutral liposomes, IFNα message level is augmented. CD40 levels do not show significant difference either 1st hour or 8th hour of stimulation. At first hour of stimulation differential IL18 levels were detected. IL18 transcript level increases after stimulation with neutral and cationic liposomes encapsulating pIC, however pIC encapsulated in stealth liposome suppresses IL18. It is known that IFNγ expression is induced by IL18 (Takeda *et al.* 1998) . We have detected high amount of IFNγ secretion when cells were exposed to neutral, anionic, cationic and stealth liposomes encapsulating pIC. Therefore we might speculated that IFNγ secretion upon stimulation with anionic and stealth liposomes is IL18 independent while neutral and cationic liposome encapsulating pIC induces IFNγ secretion via IL18.

Previous studies have shown that various combinations of TLR ligands show synergistic effect on cytokine secretion (Whitmore et al. 2004; Napolitani et al. 2005; Warger et al. 2006; Bohnenkamp et al. 2007; Trinchieri et al. 2007). Combination of synergistic TLR ligands might have implications on vaccine adjuvant design (Zhu et al. 2008). D-ODN and pIC, which are both type I IFN inducers, mediates synergism by enhancing TRIF and MyD88 dependent pathways separately, which induces proinflammatory cytokines and type I IFNs. Activated positive feedback loop of IFNα increase levels of IFNα (Prakash *et al.* 2005; Honda *et al.* 2006). We hypothesized that we can augment synergistic effect of pIC and CpG ODN and thus cytokine levels by coencapsulating them in liposomes. We have shown that it is possible to increase synergistic IFNγ and IL6 secretion of pIC and D-ODN by coencapsulating them into neutral and anionic liposomes. Interestingly, positively charged liposomes suppressed immune activation mediated by pIC and D-ODN. This data speculate us that TLR3 and TLR9 signaling interfere with each other when they are in positively charged liposomes. Moreover coencapsulation of pIC and D-ODN in neutral and anionic liposomes induces IFNα and CD40 mRNA levels at eighth and first hour of stimulation respectively. There was no significant change in either IL15 or IL18 levels after stimulation with liposomal forms. IL15 and IL18 have role in activation of NK cells which secretes high amount of IFNγ upon activation (French *et al.* 2006). Induction of IFNγ secretion by neutral and anionic liposomes encapsulating D-ODN and pIC might be IL15, IL18 and maybe NK cell independent. After an infection, until T cells are primed and differentiated into effector T cells, NK cells start to secrete IFNγ, which suppress dissemination of pathogens at early phases of the infection. After completion of naive T cell priming and differentiation process, CD4+ T cells dominates IFNγ secretion (Murphy *et al.* 2008). We speculate that upon liposomal stimulations, activity of NK cells might not be augmented, however IFNγ secretion that is mediated by CD4+ T cells might be enhanced.

Stability of liposomes effect has deep impact on their activation capacity. Change in the physical characteristic of liposomes such as size (due to aggregation or fusion of vesicles) and release of encapsulated nucleic acid would alter stimulatory effect of its cargo. Besides phospholipids containing polyunsaturated fatty acids, amino phospholipids and cholesterol (Chol) can undergo per/auto-oxidation or ester bonds in phospholipids would undergo

cleavage (Gregoriadis 1992). This is the main reason why we observed unexpected CD86 expression results after *in vitro* stimulation of splenocytes with D35 and pIC co/encapsulating liposomes (Figure 27).

Ex vivo experiments performed with liposomal D35, pIC and their coencapsulated form showed augmented immunostimulatory activities of nucleic acids in liposomes. Although 2.5 fold more free D35 and pIC were injected to mice compared to liposomal forms with neutral, stealth and SSCL liposomes encapsulating D35 induced either very close or similar amount of DC activation. Stealth liposome encapsulating pIC, neutral and anionic liposomes coencapsulating D3CG and pIC exceeded amount of APC activation mediated by free D3CG and pIC. Gursel et al. demonstrated that when 50μg of free or liposomal 1555 was injected to mice, 10 and 8 fold more 1555 were detected in group of mice's spleen that were injected with SSCL encapsulating 1555 than group acquired free 1555 at 2h and 24 hours post-injection (Gursel *et al.* 2001). This means that liposomal protection of nucleic acids from nucleases and opsonization by blood serum proteins increases amount of nucleic acid taken up by lymphoid organs. This would be the main reason of why liposomal D-ODN and pIC can yield more activation than free forms although concentration of liposomal forms are 2.5 fold diluted. Here, it is very important to note that the neutral and anionic D or pIC induced CD86 positive cells were much lower than the treatment involved coencapsulated D+pIC counterpart. This finding is a very clear demonstration of the augmentation of immunostimulatory activities of D-ODN and pIC when they are coencapsulated in liposomes. Moreover as explained earlier, we have adjusted our experimental set-up in such a way that free D-ODN possessed an advantage over the liposomal formulations, but as seen in Figure 28 (right panel) both neutral and anionic liposomes encapsulated D+pIC upregulated more CD86 compared to mice had free D+pIC mixture. It is fair to suggest that, enhanced immunostimulatory activity provided by liposomes, in addition to the antigen sparing ability will offer a powerful system against induction of protective immunity during vaccination studies.

We have obtained significantly high amount of IgG titers after immunization with liposome encapsulating antigen and CpG ODN compared to their free forms. That is probably because APCs can internalize both antigen and CpG ODN together. Therefore they

can provide 3 signals necessary for naïve T cell activation more efficiently. Actually we are mimicking a pathogen. Bacteria and viruses are closed compartment with antigens and innate immune receptor ligands are all together. Pathogens are phagocytosed by APC with its every component. Liposome encapsulated CpG ODN and antigen can be considered as a very simple pathogen.

In the literature there are some instances of studies that have used same approach. For instance Joseph et al. demonstrated that when mice were vaccinated once with liposomes (DMPC:DMPG mole ratio of 9:1) coencapsulating 25 μg CpG ODN and and 0.5μg influenza virus subunit hemagglutinin and neuraminidase (HN) antigen, they did not observed any IgG1 titer and 550 IgG2a titer 21 days post infection (Joseph *et al.* 2002). We have injected mice with anionic liposome coencapsulating 7.5μg OVA and 15μg D3CG. 13 days later we have obtained we have obtained 122 IgG1 titer and 428 IgG2a titer. We have made the Ig level measurements 8 days before Joseph et al. did, we have detected IgG1 levels and IgG2a levels were close. Although amount of CpG ODN and antigen types and amounts are different, we might speculate that the immune response obtained upon our formulation is more rapid and stronger.

Gursel et al. immunized mice with SSCL coencapsulating 10 μg 1555 and 2μg OVA. While previous study demonstrated that total IgG titer reached 19000, the present study showed 1.5 fold more IgG (a titer of 28672). Collectively, our *in vivo* experiments demonstrated that contrary to the established dogma D35 when encapsulated within a proper liposome, it performs as effective as 1555 (one of the most optimum mouse specific ODN).

In this study we established that different liposomes induced differential innate immune activation otherwise not possible to obtain with free ligand. Coencapsulation of pIC and D3CG yields synergistic immune activations both *in vitro* and *ex vivo*. Adjuvanticity D-ODN can be enhanced significantly by coencapsulating D-ODN and antigen in anionic liposome. The information from this work established an unappreciated feature of liposome-mediated immune modulation and could be harnessed to design more effective therapeutic vaccines targeting infectious diseases, cancer or allergy.

6. FUTURE PERSPECTIVE

This study for the first time established a novel property of D-ODN encapsulating liposome. In our *in vivo* experiment, enhanced immunogenicity must be translated into antigens targeting infectious disease or cancer. Had the researcher found enough time he would have performed these experiments. Data from *ex vivo* experiment also provided a very promising future for liposome coencapsulating TLR3 and TLR9 ligand. It is inevitable to compare the performance of liposomal D run parallel with D+pIC liposomes to establish the benefits of including TLR3 ligand in this scenario.

Stealth liposome loaded with D and, pIC gave very promising *in vitro* and *ex vivo* readouts ranging from cytokine production, gene expression amplification as well as surface marker upregulation. Unfortunately for the reasons we have yet to understand one of the most important components generating stealth liposome was problematic. We have tried to resolve this problem by using a new batch at hand and unfortunately we could not solve reproducible stealth liposome generation problem. Although a significant synergistic immune activation was obtained when anionic and neutral encapsulating D+pIC was used, our anticipation is that stealth liposomes loaded with D and pIC would perform as effective as neutral and anionic and these studies must be performed. All these studies mentioned above would resolve the best formulation suitable for D and pIC.

Recent literature finding suggested that varying distribution of nucleic acid ligand subcellular localization may drive differential immune responses. Our findings with regard to different liposomal/ligand formulations suggest that differential immune activation is probably mediated by localization of different liposomes within subcellular organelles. Unfortunately these studies were not completed within the time frame of this thesis. Confocal studies elaborating on this issue must be carried out in the near future and it is of utmost importance to demonstrate that different liposomes depending on their physicochemical feature deliver their cargo to different intracellular vesicles. These sets of studies for the first time provide an insight on the molecular mechanism of how different liposomes mediate differential immune activation.

Improved induction of IFNα when certain liposome types were used in stimulation assays (involved in any of the nucleic acid TLR ligands) is a very critical finding.

Unfortunately due to the unavailability of either FACS or ELISA reagents we had to rely on the PCR finding. In the future these reagents must be obtained and the IFNα production capacity mediated by these formulations must be established (both *in vitro* and *in vivo*).

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8. APPENDICES

8.1. Appendix A

Standard Solutions, Buffers, Media

Blocking Buffer (ELISA)

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

Crystal particles of BSA should be dissolved very well, with magnetic-heating stirrer for 20-30 min. The buffer should be stored at -20°C.

Loading Dye (Agarose gel)

- 0.009 grams Bromophenol blue
- 0.009 grams Xylene cyanol
- 2.8 ml ddH2O
- 1.2 ml 0,5M EDTA
- 11 ml glycerol

After preparing, just vortex it.

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8.01 grams Na2HPO4 . 2H2O
- 2 grams KH2PO4

into 1 lt ddH2O

pH= 6.8. For 1xPBS's pH should be ≈ 7.2-7.4. Should be autoclaved prior to use.

PBS-BSA-Na-Azide

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

TAE (Tris-Acetate-EDTA) [50x]

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

into 1 lt ddH_2O

Dissolves in ≈1 day. Should be autoclaved. Diluted to 1X prior to use

T-cell Buffer [ELISA]

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

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2.5 ml Tween20
- 4.5 lt dH_2O

High Glucose DMEM (Hyclone) and RPMI-1640 (Hyclone)

- 2 %: 10 ml FBS (Oligo FBS = inactivated at 65°C, Regular 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 (Biological Industries), (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 concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

In 500 ml media.