# LIPOSOME ENCAPSULATION OVERCOMES D-TYPE AND K-TYPE CpG ODN DICHOTOMY AND INDUCES SYNERGISTIC IMMUNE ACTIVATION

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MASTER OF SCIENCE

By

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August, 2014

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## ABSTRACT

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M.S. in Molecular Biology and Genetics
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Liposomes are one of the best candidates for the encapsulation of labile bioactive agents due to their safety and high entrapment efficiency. In human, two structurally distinct classes of CpG ODN are capable of activating different signaling pathways, leading to differential immune activation. While K-type ODN triggers plasmacytoid dendritic cells (pDCs) to mature and produce TNFα, D-type ODN leads to IRF-7 dependent IFNα secretion. Strikingly, when K-and D-type ODN are co-incubated in their free forms, K-ODN masks the D-ODN specific immune activation. Identifying proper delivery vehicles that provide both ODN types to display their superior features upon stimulation is of great clinical importance. In this study, first we investigated the synergistic effects of K- and D-ODN upon encapsulating them within five different liposome types. Then with the selected potential liposome combinations, we identified synergistic activation capacities both on human PBMCs and on mice splenocytes. In PBMC cytokine results revealed that D-ODN loaded in all five liposome types stimulated more IFNα than free D-ODN. Similarly, liposomal K-ODN triggered more TNFα than free K-ODN type. While incubation of free K and D- type ODN as expected, abrogated D-specific IFNα production from PBMC, simultaneous incubation with neutral or anionic D-ODN loaded liposomes plus cationic liposomes loaded with K-ODN significantly increased K-specific as well as D-specific effect rather than masking it (i.e. more production of TNFα and IFNα specific for K and D, respectively). This improved synergistic immune activity for both D and K ODN observed with ND+CK combination in 100% of individuals (TNFa) and 90% of individuals for IFNa. Additionally, intracellular cytokine staining findings supported improved TNFα and IFNα, from pDC population of PBMCs. Co-stimulatory molecule expressions and APC activation also significantly upregulated compared with free treatment. In mice contrary to ND+CK combination, sterically stabilized cationic liposome encapsulated K-ODN combined with i) neutral, ii) anionic, iii) cationic or iv) stealth encapsulated D-ODN increased IL6, IL12 and IFNγ levels, when stimulated simultaneously. Moreover, ex vivo experiments showed that cellular uptake and pro-inflammatory cytokine gene expressions significantly increased with combined liposomal formulations. This study established that by selecting proper liposome type(s) we reverse antagonistic action of K-ODN on D-ODN and induce a synergistic effect leading to a more robust immunostimulatory activity in both human and mice. This approach could broaden the immunotherapeutic application of these two important CpG ODN classes in clinic.



**Keywords:** Liposomes, TLR9, CpG ODN, Synergism, Immune Response

# ÖZET

## LİPOZOMA YÜKLENMİŞ D-TİPİ VE K-TİPİ ODN'LERİN SİNERJİK İMMUN AKTİVASYONU

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Yüksek yükleme verimi ve güvenliği sayesinde, lipozomlar ; kararsız biyoaktif ajanların taşınmasında en iyi adaylardan biridir. İnsanda, yapı olarak farklı gruplandırılan CpG ODN'ler farklı sinyal yolaklarını aktive ederek, değişik immün cevaplara yol açarlar. K-tipi ODN plazmasitoid dendiritik hücrelerin (pDC) olgunlaşması ve aktifleşmesi sonucu TNFα salgılanmasına sebep olurken, D-tipi ODN IRF'ye bağımlı sinyal yolaklarını aktive ederek IFNα üretilmesine neden olur. Çarpıcı bir şekilde, K-tipi ve D-tipi ODN'ler serbest hallerinde hücrelere verildiklerinde, K-tipi D-tipi etkisini bloke eder ve sadece K-tipine özel cevap gözlemlenir. Her iki tip ODN için de en uygun ve en önemli özelliklerini kaybetmeyecekleri bir taşıyıcı sistemin geliştirilmesi klinik uygulamalar için çok önemlidir. Bu çalışmada, öncelikle K- ve D- tipi ODN'leri 5 farklı lipozoma yükleyerek sinerjik etkilerini araştırdık. Bu lipozomlara yüklü ODN'lerin insan periferik kan mononükleer hücreleri ve fare dalak hücreleri üzerinde sinerjik bir etki gösterebilme kabiliyeti en yüksek olan potansiyel gruplarını seçtik. İnsan periferik kan hücreleri üzerinde yapılan sitokin ELİSA deneyleri sonucunda, kullanılan 5 lipozomal kombinasyonun da serbest ODN'lere göre daha fazla IFNα ve TNFα ürettiğini gözlemledik. Serbest haldeki K-ve D-tipi ODN'ler beraber inkübe edildiklerinde beklendiği gibi D-spesifik olan IFNα salımında azalma gözlemlenmiştir. Nötral ve anyonik lipozoma yüklü D-tipi ODN (ND ve AD) ile katyonik lipozoma yüklü K-tipi ODN (CK) beraber hücreye verildiklerinde ise, K-tipi D-tipini engellemek yerine, hem K-tipine özgü etkiyi arttırmakta hem de D-tipine spesifik etkiler göstermektedir. Bu gelişmiş sinerjik etki, ND+CK grubunda TNFα salımı için kullanılan donörlerin %100'ünde gözlemlenirken, IFNα için %90'ında gözlemlenmektedir. Bunlara ek olarak, hücre içi sitokin boyama deney sonuçlarına göre TNFα ve IFNα sekresyonu özel olarak insan pDC'lerinden artmaktadır. Yardımcı uyaran molekül ifadeleri ve antijen sunucu hücrelerin de lipozomal kombinasyonlarda, serbest ODN'lere göre belirgin bir şekilde arttığı gözlemlenmiştir. Fare deneylerinde, insan deneylerinden farklı olarak ND+CK grubu yerine, K-tipi ODN yüklü sterik olarak stabilize edilmiş katyonik lipozom (SSCL) ile D-tipi ODN yüklü i) nötral, ii) anyonik, iii) katyonik ve iv) stealth kombinasyonlarında sinerjik etkiler görülmüştür (IL6, IL12 ve IFNγ üretiminde artış). Ex-vivo deneyleri sonucunda, lipozomal kombinasyonların CpG ODN'lerin hücre içine alımını ve proinflamatuar sitokinlerin gen ifadelerini arttırdığını ortaya çıkarmıştır. Bu çalışmada hem insan, hem de farede doğru lipozom tipi seçimiyle K-tipi ODN'in D-tipi ODN'e karşı gösterdiği antagonist etkiyi, sinerjik bir etkiye çevirebileceğimizi kanıtladık. Yaklaşımımız bu önemli iki tip ODN'in immunoterapötik uygulamalarda kullanımını arttırabilecek niteliktedir.

**Anahtar Kelimeler**: Lipozom, TLR9, CpG ODN, sinerjizm, immün cevap

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*DEDICATED*

*to my mother Esin and my brother Cahit*

# Content

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## Abbreviations

| | |
|---|---|
| A | Anionic liposome |
| APC | Antigen presenting cell |
| Bp | Base pairs |
| cDNA | Complementary deoxyribonucleic acid |
| C | Cationic liposome |
| CpG | Unmethylated cytosine-phosphate-guaniosine motifs |
| CXCL | CXC-chemokine ligand |
| DC | Dendritic cell |
| DNA | Deoxyrobinucleic acid |
| dsRNA | Double-stranded RNA |
| ELISA | Enzyme linked-immunosorbent assay |
| FBS | Fetal bovine serum |
| Ig | Immunoglobulin |
| IκK | Inhibitor kappa b kinase |
| IL | Interleukin |
| IFN | Interferon |
| IP | Interferon induced protein |
| IRAK | IL1 receptor-associated kinase |
| IRF | Interferon-regulatory factor |
| LPS xii | Lipopolysaccharide |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MyD88 | Myleoid differentiation primary response gene 88 |
| N | Neutral liposome |
| NF-κB | Nuclear factor-kappa b |
| NK | Natural killer cell |
| NLR | Nucleotide-binding oligomerization domain like proteins or receptors |
| NOD | Nucleotide-binding oligomerization domain |
| ODN | Oligodeoxynucleotide |

| | |
|---|---|
| PAMP | Pathogen associated molecular patterns |
| PBS | Phosphate buffer solution |
| PCR | Polymerase chain reaction |
| pDC | Pasmacytoid dendritic cell |
| pIC | Polyionosinic acid:cytidylic acid |
| PNPP | Para-nitrophenyl pyro phosphate |
| PRR | Pathogen recognition receptor |
| RIG | Retinoic acid inducible gene |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute |
| RT | Reverse transcriptase |
| S | Stealth Liposome |
| SA-AKP | Streptavidin Alkaline-phosphatase |
| SSCL | Sterically Stabilized Cationic Liposome |
| ssRNA | Single-stranded RNA |
| TIR | Toll/IL1 receptor |
| TIRAP | Toll/IL1 receptor-associated protein |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRAF | TNF-associated factor |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TIR domain containing adaptor inducing IFNγ |

# CHAPTER 1

# 1. Introduction

## 1.1. The Immune System

A powerful and efficient defense mechanism is operational for the discrimination between self and infectious/pathogenic non-self among organisms of different genera. The system that has highly effective weapons awaiting any intruders is called the immune system [1]. The immune system consists of effector cells and molecules, which protects the body from infectious agents and their damages. Immunity can be divided into two main categories; innate and adaptive immunity.

Adaptive (acquired) immunity is a specific immune response against pathogens, which provides recognition and antigen production, and is developed throughout the lifetime of an individual [2]. Adaptive immune system is only present in vertebrates and cartilaginous fish.

The innate immune system on the other hand, is the first line of defense against pathogens [3]. Innate immunity consists of a fixed number of germ-line encoded receptors that recognizes components of microorganisms and viruses [1]. Phagocytic cells and complement system mediate innate immune system. Upon infection, cellular mediators of innate immunity; macrophages, neutrophils and natural killer cells migrate towards to the infection leading to the activation of inflammatory signaling cascades.

It is accepted that innate immune signals play an important role for the activation of adaptive immunity. Upon the activation of innate immunity, dendritic cells are maturated and migrated in to lymph nodes where they are capable of activating naive $CD4^+$ T cells. Activated of T cells differentiates into Th1 or Th2

phenotypes depending on the pathogen. Th1 cells support the cell-mediated immunity whereas Th2 cells shape humoral immunity. [1], [4].

Table 1.1 Innate vs Adaptive immunity (Adapted from Albertz et al., 2002)

| Innate Immune System | Adaptive Immune System |
|---|---|
| Response is non-specific | Pathogen and antigen specific response |
| Exposure leads to immediate maximal response | Lag time between exposure and maximal response |
| Cell mediated and humoral components | Cell mediated and humoral components |
| No immunological memory | Exposure leads to immunological memory |
| Found in nearly all forms of life | Found only in jawed vertebrates |

## 1.2. Innate Immunity

### 1.2.1. Pathogen Recognition Receptors

The innate immune system has a limited specific recognition system for pathogens, which relies on germline-encoded-pathogen-recognition receptors (PRRs) [3]. PRRs recognize typical repeating patterns that are expressed by microorganisms. These molecular structures are called pathogen-associated molecular patterns (PAMPs) [2]. Recently, it was revealed that, PRRs also recognize endogenous molecules released from damaged cells, called damage/pathogen associated molecular patterns (D/PAMPs).

PRRs have common features. They recognize PAMPs, that are important for the microorganism survival, and because of that it is difficult for microorganism to alter. Secondly, PRRs are expressed in the host regardless of their life-cycle stage. Thirdly, PRRs are germline encoded, nonclonal and expressed in all cells without a memory. Different PRRs recognize distinct PAMPs, and activate different signaling pathways, which leads to specific immune responses. Mechanisms of recognition are highly conserved from plants and fruit flies to mammals [3].

PRRs can be divided into 2 groups on basis; cytoplasmic and signaling PRRs. Cytoplasmic PRRs can be grouped into 3 families; interferon (IFN)-inducible proteins, caspase-recruiting domain (CARD) helicases, and nucleotide-binding

oligomerization domain (NOD)-like receptors (NLRs). Toll-like receptors (TLRs) and C-type lectin receptors belong to signaling PRR family [6] (Table 2).

Table 1.2: Pattern recognition receptors in mammals and their ligands (Adapted from Lee,2007a).

| Family | Member(Major Ligand) |
|---|---|
| **TLRs** | TLR1(Triacyl lipopetides), TLR2 (LTA, zymosan, lipopetides), TLR3 (dsRNA, pI:C), TLR4(LPS), TLR5(flagellin), TLR6(diacyl lipopetides), TLR7 (ssRNA, R848), TLR8(ddRNA, R848), TLR9(CpG-DNA), TLR11(profillin-like molecule) |
| **C-type lectin receptors** | Mannose receptor(ligands bearing mannose, fucose or N-acetyl glucosamine), DC-SIGN(ICAM-2/3 HIV gp120, Mycobacterium tuberculosis ManLAM), Dectin-1 (zymosan, B-glucans from fungi) |
| **Scavenger receptors** | Scaenge receptor A(modified LDL, apoptotic cells), CD36 (oxidized LDL, apoptotic cells), MARCO (modififed LDL) |
| **Complement receptors** | Integrins [CR3 (iC3b, B-glucan fibrinogen), CR4 (iC3b, b-glucan, fibrinogen)],gC1qR (C1q), C5aR (C5a) |
| **IFN-inducible proteins** | PKR(dsRNA), OASs(dsRNA) |
| **CARD helicases** | RIG-I (uncapped 5'triphosphate RNA), MDA5(pI:C, dsRNA from EMCV) |
| **NOD-like receptors** | NOD1(iE-DAP), NOD2 (MDP), 14NALPs[NALP1 (cell rupture), NALP1b (anthrax lethal toxin), NALP3 (bacterial mRNA, R848, extracellular ATP, uric acid crystals)], IPAF (Salmonella flagellin), NAIP5 (Legionella flagellin |
| **Complement** | C3 (carbohydrates and proteins on microbial surfaces), C1q (immune complexes, apoptotic cells) |
| **Pentraxins** | SAP(LPS,C1q, apoptotic cells), CRP (PC, C1q, apoptotic cells), PTX3(galactomannan, C1q, zymosan, apoptotic cells) |
| **Collectins** | MBL(LPS, LTA, HIV gp120 |

Various non-professional immune cells express these PRRs including macrophages and DCs. PRRs upregulate the transcription of genes involved in many inflammatory responses by PAMPs or DAMPs [7]. Innate immunity is activated through these of proinflammatory molecules, which are triggered by PRRs [8],[67].

### 1.2.2. Toll-like Receptor Family

Toll-like receptors are major and most studied class of PRRs, which are conserved through the worm *Caenorhabditis elegans* to mammals [9], [10], [11], [12]

TLRs are integral glycoproteins, containing leucine-rich repeat (LRR) motifs that mediate the recognition of PAMPs and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains which activate the downstream signaling pathways by using adaptor molecules such as MyD88, TIRAP, TRIF, and TRAM [13],[74]. 10 TLRs have been identified in human that recognize different PAMPs derived from viruses, bacteria and fungi [8].

Depending on the cellular localization and PAMP ligand types, TLRs are

divided into two subgroups [13]. TLR1, TLR2, TLR4, TLR5, and TLR6 are found on the cell surface and typically recognize ligands that are usually bacterial in origin. Endosomal TLRs; TLR3, TLR7, TLR8, and TLR9 are responsible for recognizing nucleic acids including double-stranded (ds) RNA and CpG DNA [14] (Figure 1.1).

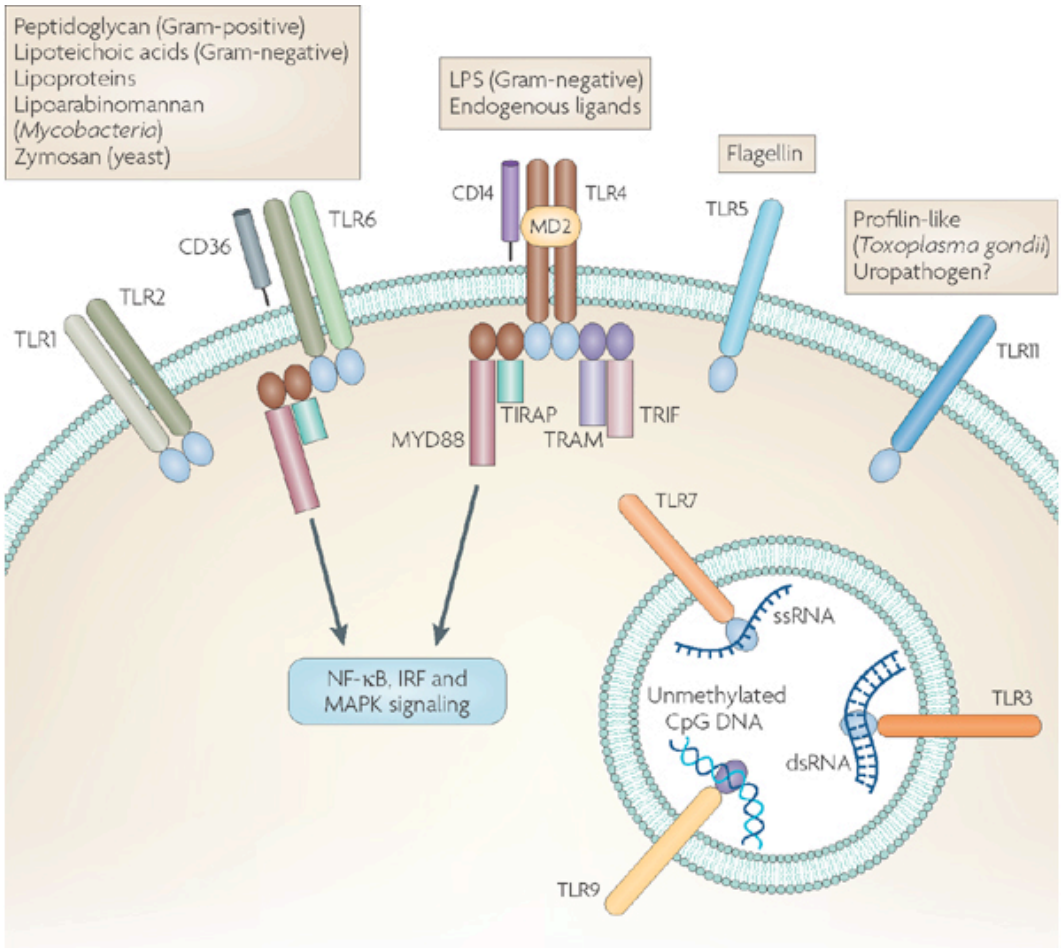



Figure 1.1: Toll-like receptors and their ligands (Adapted from Rakoff-Nahoum, 2009).

### 1.2.2.1. TLR1, TLR2 and TLR 6

TLR2 recognizes a wide spectrum of microbial components such as; lipoproteins, lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphospatidylionsitol anchors from Trypanosoma cruzi, a phenol-soluble modulin from Straphylococcus epidermis, zymosan from fungi and glycolipids from Treponema maltophilum [9]. In general,

TLR2 forms heterodimers with TLR1 and TLR6. By heterodimerization TLR2 gains the ability to recognize different microbial components. TLR2- TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma, where as TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma. Studies have proved that the structure caused by heterodimerization provides recognition of different PAMPs by TLR2 [13]. Upon recognition, TLR2 activates the adaptor molecules TRAP and MyD88 , which initiates a MyD88 dependent signaling pathway leading inflammatory cytokine production [6].

#### 1.2.2.2.TLR3

TLR3 recognizes, polyinosinic-polycytidylic acid (poly (I:C)), which is a synthetic analog of double stranded RNA (dsRNA) and mimics viral infection by inducing antiviral immune responses. Type I interferon and inflammatory cytokine productions are promoted by TLR3 activation through TRIF-dependent pathway [13],[6] deficient mice are impaired in their response do dsRNA but not abolished completely. Which indicates that TLR3 is not the only receptor for dsRNA, there are additional sensors exist such as; CARD helicases [16].

#### 1.2.2.3. TLR4

TLR 4 is essential for protection against gram-negative bacteria in both human and mice. Lipopolysaccharide (LPS) is the major component of the outer layer of gram-negative bacteria [17]. Recognition of LPS by TLR4 activates two major intracellular signaling pathways; MyD88- dependent and TRIF- dependent [18]. TLR4 is the only TLR which can initiate both MyD88 and TRIF-dependent pathways by using all four adaptors [13]. The MyD88-dependent pathway is activated by the recruitment of MyD88 to cytoplasmic TIR domain of TLR4, TRIF-dependent pathway is activated similarly by the recruitment of TRIF to cytoplasmic TIR domain of TLR4 on the plasma membrane [18]. Inflammatory cytokine expressions such as; IL6, IL12 and TNFa are induced by the MyD88-dependent pathway, whereas type I IFN expressions are upregulated by the TRIF-dependent pathway. TLR4 can also

activate other signaling molecules including phosphatidylinositol-3 kinase (PI-3K) and MAP3Ks such as MEKK3, TPL2, and ASK1A. Recently it was reported that CD14, a co-receptor, helps the diversification of TLR4 ligand repertoire and activation of downstream signaling pathways [6]. Additionally TLR4 recognizes various substances from viruses, fungi and mycoplasma. Danger associated molecular patterns (DAMPs) also activates TLR4 [19].

#### 1.2.2.4. TLR5

TLR5 is responsible for the recognition of flagellin, a protein component of bacterial flagella. Mostly epithelial cells, monocytes and immature DCs especially in the small intestine express TLR5. Mouse TLR11, a relative of TLR5, expression is high in the kidney and bladder. Uropathogenic bacterial components are recognized by TLR11. Additionally TLR5 is highly expressed in lungs and they are important in the defense against respiratory tract pathogens [13],[3].

#### 1.2.2.5. TLR7 and TLR8

TLR7 and TLR8 are phylogenetically and structurally closely related subgroup of TLR superfamily. Upon recognition of ssRNA by TLR7 and TLR8, DCs are activated. TLR7 is mostly expressed in plasmascytoid DCs (pDCs) myeloid DCs (mDCs) expression of TLR7 is still controversial [75]. Mostly TLR7 expression is not detected in human monocyte-derived DCs [20]. TLR7 and TLR8 activate innate immune responses upon viral infections; ssRNA sequences containing GU-rich or poly-U sequences can stimulate both receptors. Recently synthetic compounds of imidazoquinoline have been identified as an agonist as well [21]. Recognition activates MyD88-dependent signaling pathway and leads to recruitment of interleukin-1 receptor-associated kinase family [78]. This results in the activation of the transcription factor nuclear factor NF-κB, which controls the expression of proinflammatory cytokine genes [22],[80].

#### 1.2.2.6. TLR9

Bacterial genomic DNA is recognized by TLR9, its stimulatory effect is

caused by the presence of unmethylated CpG dinucleotides [3].The frequency of CpG motifs are highly reduced and the cytosine are mostly methylated which leads to abrogation of the immunostimulatory activity [9]. Synthetic oligonucleotides that contain CpG motifs mimic bacterial DNAs immunostimulatory activity [3],[73]. There are two types of CpG DNA; A/D-type and B/K-type, which have different immune responses. B/K-type CpG DNA induces inflammatory cytokines such as; IL12 and TNFα, whereas A/D-type CpG DNA stimulates greatly pDCs to secrete IFNα but have less ability to induce IL12. TLR9 is essential for both types of CpG DNA recognition by the activation of MyD88-dependent signaling pathway. Upon recognition DCs, macrophages and B cells are directly activated and a strong Th1 immune response is observed [9],[70]. TLR9 also recognizes insoluble crystal hemozoin, a byproduct of the detoxification process after digestion of host hemoglobin by *Plasmodium falciparum* [13],[69].

### 1.2.2.7. TLR Signaling Pathways

Recognition of PAMPs and DAMPs by TLRs triggers the activation of several signaling cascades, leading to upregulation of related gene transcriptions. Dimerization and conformational changes of TLRs are required for TIR-domain containing adaptor molecule recruitment to the TLR [3]. The TIR domain is conserved through all TLRs except for TLR3 [23],[65]. There are four adaptor molecules namely MyD88, TIR-associated protein (TIRAP)/MyD88- adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM1) [3]. TLR signaling is divided into 2 distinct pathways on the usage of the adaptor molecules; MyD88 and TRIF [7]. Activation of distinct signaling pathways, leads to production of different pro-inflammatory cytokines and type IFNs (Figure 1.2) [7],[72].

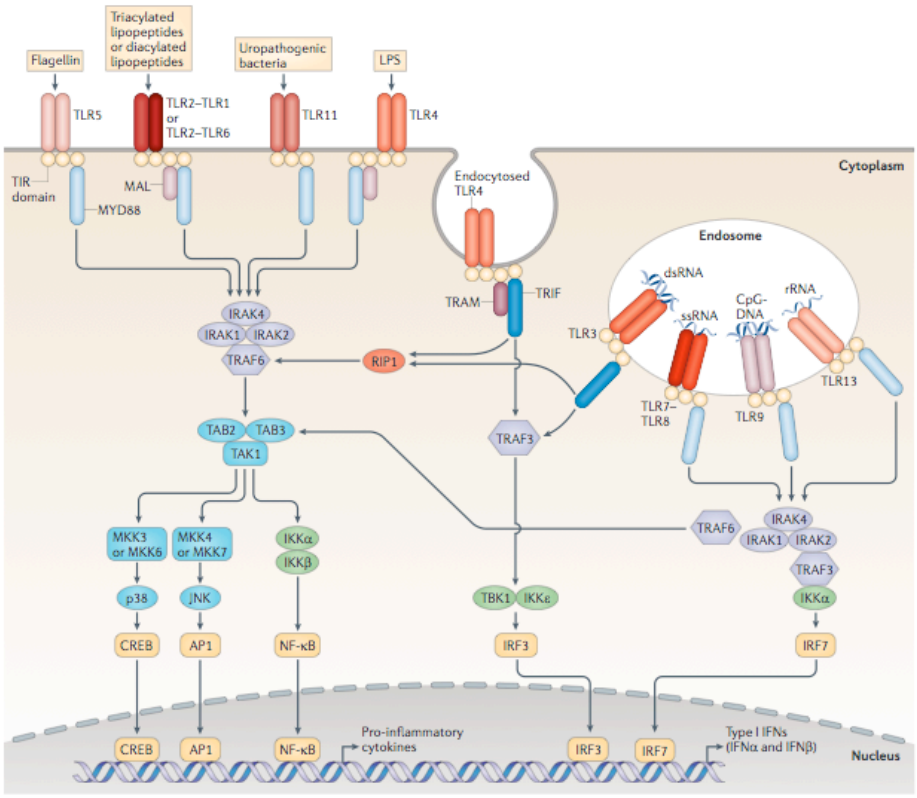



Figure 1.2 TLR signaling pathways (Adapted from O'Neill, 2013).

### 1.2.2.7.1. MyD88 Dependent Pathway

MyD88 was first defined as a myeloid differentiation primary response gene in 1990 [77]. Afterwards, the homology between MyD88 and the cytoplasmic domains of Drosophila Toll and mammalian IL1 receptors were discovered, MyD88 was defined as a signal transduction molecule that has an important function in the activation of the immune system [25], [26]. MyD88 is critical for signaling from all TLRs [23]. Upon stimulation, MyD88 recruits IL1 receptor associated kinases; IRAK4, IRAK1, IRAK2 and IRAKM [13]. IRAK4 mediates phosphorylation of IRAK1, which leads to association of TRAF6 and activates 2 distinct signaling pathways. First pathway activates AP-1 transcription factors through MAP kinases. And the other pathway activates TKA1/TAB complex that leads to enhanced activation of IκB kinase (IKK) complex and finally transcription factor NF-κB is activated. Other structures related to MyD88 also are essential for the TLR signaling

pathways such as; TIR domain contacting molecule TIRAP. TIRAP/Mal plays an important role for the activation of TLR2 and TLR4 via the MyD88-depent pathway [9].

#### 1.2.2.7.2. MyD88 Independent/Trif Dependent Pathway

TLR3 and TLR4, upon recognition, activate another pathway by recruitment of another adaptor protein TRIF [7]. In MyD88- deficient macrophages, stimulation with TLR4 ligands activates NF-KB however production of inflammatory cytokines are not induced, which indicates that production of inflammatory cytokines through TLR4 depends on MyD88-dependent pathway, whereas NF-κB does not. Studies have showed that, TLR4 stimulation activates IRF-3 and leads to NF-κB activation by TRIF dependent pathway. The TRIF-dependent pathway activates booth IRF3 and NF-κB, by the recruitment of TRAF6 and the activation of TAK1 for NF-κB. RIF forms a complex with TRAF6, TRADD, Pellino-1 and RIP for the activation of TAK1 and leads to NF-κB and MAPK pathway activation [13]. Hence, TLR3 and TLR4 signal through the MyD88-independent pathway to induce IFN-β [9].

## 1.3. Synthetic CpG ODNs

TLR9 recognizes bacterial DNA that contains unmethylated CG dinucleotides and triggers immune responses. The immunostimulatory effects of bacterial DNA are mimicked by synthetic oligodeoxynucleotide (ODN) sequences expressing unmethylated CpGs [27]. Immunostimulatory effects of these ODNs were first reported in 1992 [28]. Minimum ODN length for stimulatory effect has been shown as 8 bp. Optimally, stimulatory ODN contain an unmethylated CpG dinucleotide flanked by two 5' purines (Pu) and two 3' pyrimidines (Py) in mice [79]. However, immunostimulatory effects of CpG ODN differ between species. Human PBMC's immune responses to ODN that are highly stimulatory in mice are poor [29]. TLR9 is highly expressed on professional antigen presenting cells in human such as; B cells and plasmacytoid dendritic cells (pDCs) [30]. Four classes of synthetic CpG ODN have been described (Table 1.3), that have different structural properties leading to different types of cytokine and chemokine releases through different pathways

[27].CpG ODN boost up the efficacy of vaccines against bacterial, viral and parasitic pathogens in both mice and human [31].

Table 1 3: Comparasion of different types of ODN. Adapted from Bode et al., 2011.

| ODN Type | Representative sequence | Structural characteristics | Immune effects |
|---|---|---|---|
| D- Type | GGTGCATCGATGCAGGGGGG | Mixed PS-PO backbone | Induces strong pDC IFNα secretion |
| | | Single CpG | APC maturation |
| | | Poly-G tail at 3'end | |
| K-Type | TCCATGGACGTTCCTGAGCGTT | PS Backbone | Induces strong B-cel activatinl |
| | | Multipe CpG | pDC maturation |
| | | 5' motif most stimulatory | Induces TNFα and IL6 production |
| C-Type | TCGTCGTTCGAACGACGTTGAT | PS Backbone | Induces B cell and pDC proliferation |
| | | Multiple CpG | Stimulates IL6 and IFNα production |
| | | TCG dimer at 5'end | |
| | | CpG motif in a central palindrom | |
| P-Type | TCGTCGACGATCGGCGCGCGCCG | PS Backbone | Stimulates pDC and B cells |
| | | Two plaindromes | Strong IFNα secretion |
| | | Multiple CpG | |

### 1.3.1. D or A Type ODN

D-type ODNs (also referred to as A type) have a mixed, phosphodiester/phosphorothioate backbone, contain a single CpG and have poly G tails at the 3' ends. The poly G tails interact with each other, forming 'G tetrads' and ODN clusters [32]. This type of ODN interacts with CXCL16 that is expressed on pDCs but not on B cells. D-type ODN activates pDCs to secrete IFNα, however it has weak affects on B cells [33]. They are retained in the early endosome for long time of period, where they interact with MyD88/IRF-7 complexes. This interaction triggers a signaling pathway leading to IFNα production eventually [27].

### 1.3.2. K or B Type ODN

Typical K/B-type ODNs have a complete phosphothioate backbone, which decreases its susceptibility to DNAse digestion, resulting in a longer in vivo half-life than ODN with phosphodiester backbone [32]. Unlike D/A-type ODN, it does not form higher ordered structures and also lacks poly-G tails. K/B-type ODN is used in most studies with CpG ODNs, because of its stable structure [33], [31]. K/B-type ODN expresses multiple CpG motifs [34]. The K/Btype ODN also promotes the maturation and activation of pDCs, however it does not induce IFNα secretion but stimulates TNFα production because this type of CpG ODN is rapidly transported

into late endosomes through early endosomes (Figure 1.3). It stimulates IL6 secretion, induces B cells to proliferate and secrete IgM [29].

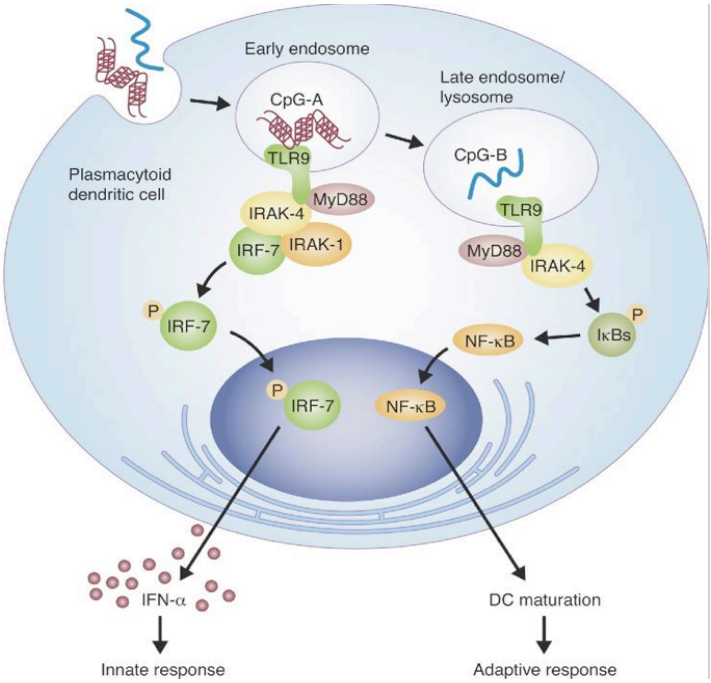



Figure 1.3: Subcellular location of D- and K-type CpG ODNs into late and early endosomes in pDCs (adapted from Williams, 2006).

### 1.3.3. C-type CpG ODN

The immunostimulatory effects of C-type ODNs are intermediate between D and K classes [33]. It also have an entire phopsphorothioate backbone and expresses TCGTCG at the 5'end, it resembles to D-type ODN because of its palindromic CpG motifs [32]. This class of CpG ODN stimulates B cells to secrete IL6 and induces IFNα production from pDCs. It is found in both early and late endosome, thus expresses both D and K- type properties [27].

### 1.3.4. Other Types of CpG ODNs

There is several more CpG ODNs, which do not fit to any other class described above. One of them is, P-type ODN, they contain two palindromic

sequences that enable them to form higher ordered structures. P-Type ODNs, stimulate B cells and pDCs to secrete IFNα [27].

Another type is Y-shaped DNA (Figure 1.4), they are composed of using 3 30-base ODNs. Y-type ODN induces TNFα and IL6 [35].

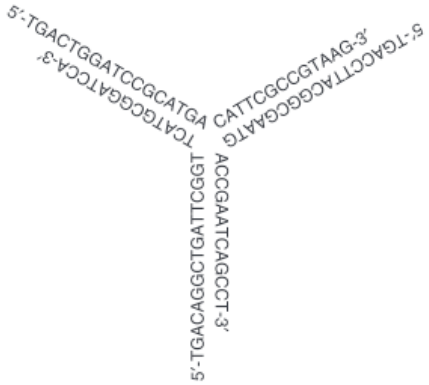



Figure 1.4: Y-shaped CpG ODN (Adapted from Nishikawa, 2008).

### 1.3.5. The Dichotomy of D-and K-Type ODNs

The CpG ODN classes described above have different responses in human pDCs as mentioned. The response mechanisms of these ODNs are poorly understood and still being investigated. Studies revealed that both types of ODNs enter the same immune cells, however, their localization differs leading to different immune responses [30].

In mice, it was established that D- and K- type ODNs trigger different signaling pathways that involves IRF-7 or NF-κB. Depending on the physical forms of the molecules one is localized to early endosomes, where the other to late endosomes. Sequence and backbone structures are important for their localization. Localization differentiation causes the activation of different signaling pathways [36]. K-type ODNs have been shown to localize in early endosome where it interacts with TLR9 directly, activating a signaling cascade that involves serine kinase interleukin-1 receptor-associated kinase interaction with the adaptor protein tumor necrosis

receptor-associated factor 6. NF-ϰB signaling pathway is activated as well as activated protein-1 transcription family members Jun and Fos, causing the transcription of immune response genes such as IL6 [30]. However there is no evidence that D-type ODN interacts with TLR9. It has been established that, D-type ODNs bind to the chemokine and scavenger receptor CXCL16 that pDCs express on their surfaces. This binding leads D-type ODN to accumulate in early endosome for much longer period, where TLR9-MyD88-IRF7 signaling pathway is activated. Eventually IFNα secretion is induced from pDCs [37].

Moreover, to their different immune responses, when these two types of ODNs, D- and K-type, are introduced simultaneously to human monocytes, K-type cross inhibits D-type ODN's response. Studies showed that D-type ODN was unable to trigger monocytes to differentiate and secret IFNα in the presence of K-type ODN. This dichotomy is not caused because of any competing uptake mechanisms, they do not block one another's entry to cell, moreover they are found in different locations in the cell; D-type in endosome and K-type in lysosomes [30]. An explanation to their dichotomy is; lysosomal TLR9 signaling by K-type ODN inhibition to the induction of IFNα secretion by endosomal TLR9 [38].

To overcome this dichotomy, new CpG ODNs were described or modifications were made to K-type ODN in order to stimulate pDCs to secrete IFNα. A third CpG ODN described was the C-type ODN, as mentioned above, that has the ability of both B-cell activation leading IL6 secretion and IFNα induction from pDCs [69].K-type ODNs are the only CpG ODN that are being used in clinical trials. Due to their ability to from G quadruplex based structures that complicated their manufacturing made it difficult for the D-type ODNs to progress into the clinic.

Another developed strategy for using IFNα inducer CpG in clinical trials, is converting a conventional K-type ODN into a IFNα inducer CpG, by multimerizing them. Multimerized CpG ODN is retained in early endosome causing the activation of MyD88/IRF-7 signaling pathway, which leads to IFNα secretion. In a recent study, a nanoring with Tat peptide and a K-type ODN was formed, which induced IFNα production similar to D-type ODN did [34]. Another study was done with K3 and

Schizophyllan (SPG), a soluble B-glucan derived from *Schizophyllum commune*. K3 forms a nanostructure with SPG that shows characteristics of both K-and D-type ODNs at the same time. It has been reported that K3-SPG induces PBMC into secreting large amounts of both type I and II IFN. By nanostructure formation with SPG, K-type ODN does not lose its K-type activity rather it gains D-type activity at the mean time [39].

## 1.4. Drug Delivery with Nanoparticles

There are many issues facing delivery of drugs to target sites, such as; high toxicity, poor solubility, aggregation, non-specific targeting, instability in vivo and short half –lives. A proposed method to overcome many physical and degradative barriers is encapsulating drugs into nanoparticles. Encapsulation of drugs into nanoparticles, protects the drug from degradation and improves cellular uptake, in many cases reduces toxicity [40]. Solid lipid nanoparticles, dendrimers, polymers, silicon or carbon materials, magnetic nanoparticles and liposomes are the examples of nanoparticles for drug delivery systems (Figure 1.5) [41]. Liposomes has been the most successful candidate in clinical applications, there are many currently approved liposomal drugs are being used and numerous in late-stage clinical development in the United States [42], [43]. Doxorubicin encapsulated Sterically stabilized PEG`ylated liposome, Doxil™, is approved for the treating of metastatic breast cancer, ovarian cancer, and Kaposi's sarcoma. Chitosan nanoparticles are also effective against tumor growth [44]. Poly(lactic-co-glycolic acid) (PLGA) NPs are being used in cancer therapy, causing enhanced binding due to their surface modifications leading to more efficient anti-tumoral effect [45]. In addition to synthetic particles, natural polymers are also being used for nanoparticular formations in drug delivery [66].

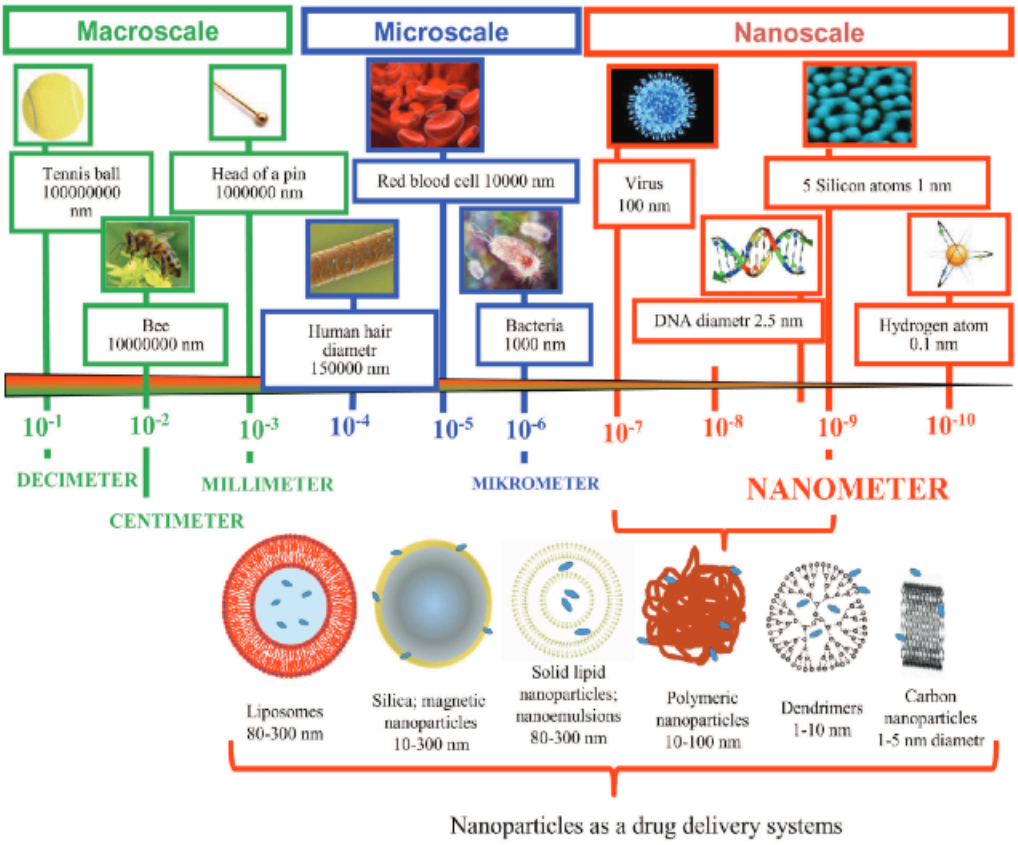



Figure 1.5: Nanoparticle drug delivery systems (adapted from Wilczweska, 2012).

Liposomes are small vesicles, which are made of cholesterol and natural non-toxic phospholipids. There are different types of vesicles; large multilamellar vesiscles, (LMV) ranges in size between 500nm to 5um, small unilamellar vesicles (SUV) they are formed by single bilayer and around 100nm in size, large unilamellar vesicles are formed by single bilayer and range in size between 200nm to 800 nm [46].

Late Alec Bangham and colleagues published the first description of a swollen phospholipid system in 1965. Later, Gregory Gregoriadis, one of the early pioneers in liposome field, first established the concept of liposomes. Gregoriadis and colleagues were the first to entrap drugs into liposomes and describing a novel drug delivery system in 1971 [47], [68]. The name liposome, was derived from two Greek words; 'lipos' means fats and 'soma' means body [48].

Liposomes (Figure 1.6) are promising delivery systems, due their sizes, their hydrophobic and hydrophilic character. Moreover, most drug toxicities decrease when encapsulated into liposomes and drugs that are loaded into liposomes are protected from degradation leading to longer circulation time of the drug through the body [46]. Additionally, with liposomal loading, drugs can be targeted to the cells of interest by surface functionalization of the carriers. The most common surface modification used is polyethylene glycol (PEG), by which liposome gains immunogenicity along with prolonged half time and accumulation to the preferential target tissue by enhanced permeation retention (EPR) effect [49]. Both amphiphilc and lipophilic molecules can be loaded into them, due to their phospholipid bilayers [50]. According to their lipid composition, surface charge, size and the method of preparation, they have different properties. Their bilayer components determine the rigidity and fluidity. Liposomes are greatly used in cosmetic and pharmaceutical industries [47].

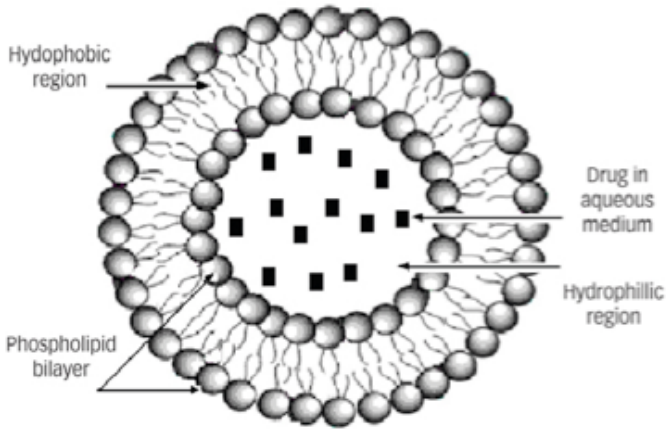



Figure 1.6: Representative diagram of liposomes (Adapted from Dhandapani, 2013).

### 1.4.1. Delivery of CpG ODN in liposomes

Even though, nucleic acids have been shown to have great immunostimulatory effects, their in vivo and in vitro applications require delivery vehicles [43]. The uses of free CpG ODNs have several disadvantages. Free ODN lacks specifity for target cells; poor cellular uptake and most importantly, they are sensible to nuclease degradation [52].

Lipidic delivery systems are being extensively used due their advantages such

as targeting, simplicity and protection like described above [53]. Liposomes can be administered in vivo by different ways, including intravenous, intraperitoneal, aerosol or direct injection [54]. Moreover, encapsulation of CpG ODN into liposomes enhances delivery to target cells when compared with free forms[71]. Most importantly, liposomes protect ODNs from nuclease degradation thus allowing a prolonged circulation time. Therefore, liposomal delivery systems enhance immunostimulatory activity of CpG ODNs [52].

# Chapter 2

# 2. Aim of this study

CpG ODNs are promising candidates to be used as adjuvants in the vaccine development. However, they also have some disadvantages like mentioned before. Studies have been still going on to overcome these disadvantages. D- and K- type ODNs have different immune responses. K-type ODN, which has progressed into clinical trials as adjuvants, strongly stimulates B-cells and pDC that leads to secretion of IL6 and TNFα, as an antibacterial response. Whereas, D-type ODN induces pDCs to secrete IFNα causing an antiviral immune response. Moreover manufacturing process of D-type ODN is complicated due to its quadruplex structures, and D-type ODN shows batch-to-batch variation, which makes it difficult for D-type ODN to enter any clinical trial. Researchers are still keen to develop a system to combine D- and K- effects in a single CpG ODN or in a multimerized system.

Liposomes are extensively used as drug delivery systems. Along with many features it provides to the drug, most importantly it brings protection. CpG ODNs are highly sensitive to DNase degradation, and encapsulation in liposomes protects CpG from degradation, thus provides a longer circulation time. Additionally, when D- and K- type ODN are introduced to cell in their free forms, K-type ODN blocks D-type response, and only K-type specific response is observed.

To overcome all of these disadvantages and to develop a system that will combine both CpG ODN effect in a universal system, we will encapsulate both of these CpG ODNs to different kinds of liposomes, and introduce them to human PBMC and murine splenocyte to observe whether we will see a combined effect. The main aim of this study is to overcome the dichotomy of D- and K- type ODN by encapsulating them into liposomes and to identify proper liposome combinations encapsulating D- and K- that leads to expression of both D- and K- superior activity.

# Chapter 3

# 3. Materials and Methods

## 3.1. Materials

### 3.1.1.ELISA Reagents

Mouse cytokine ELISA reagents, including monoclonal antibodies for cytokines, recombinant antibodies for IL6 and IL12 were purchased from Biolegend (USA), whereas reagents for IFNg and streptavidine-alkaline phosphatase (SA-AKP) were purchased from MabTech (USA). P-nitrophenyl phosphate disodium salt (PNPP)-substrate for alkaline phosphates was purchased from Thermo (USA).Human cytokine ELISA reagents for TNFa and IFNg were purchased from BioLegend (USA) , IP10 reagents were from BD and reagents for pan IFN-a were from MabTech (USA).

### 3.1.2.TLR Ligands

D- and K-Type CpG Oligodeoxynucleotides (ODNs) (TLR9 ligand) and their CG dinucleotides flip forms (i.e. control ODNs) were synthesized either by Alpha DNA (Montreal, Canada) or on an in-house machine (Mermade[6], ODN Synthesizer, USA). their sizes and base sequences are listed in Table 3.1.

Table 3.1: Sequences of ODN used. Upper case letters: Phosphorothoiate linkage between bases, lower case letters: Phosphorodiester linkage between bases

| **K-type ODN** | |
|---|---|
| K3(20mer) | 5’- ATCGACTCTCGAGCGTTCTC-3’ |
| K23(12mer) | 5’-TCGAGCGTTCTC-3’ |
| 1555’(15mer) | 5’-GCTAGACGTTAGCGT-3' |
| 1555-3CG(20mer) | 5’-GACGTTGACGTTGACGTTGG-3' |
| 1466-pG6-PS(16mer) | 5’-CAACGTTGAGGGGGG-3' |
| 1466-pT6-PS(16mer) | 5’-TCAACGTTGATTTTT-3' |
| | |

| **D-type ODN** | |
|---|---|
| D35 (20mer) | 5’-GGtgcatcgatgcaggggGG-3' |
| D35-3CG (20mer) | 5’-GGtcgatcgatcgaggggGG-3' |
| pG6-1466-PO | 5’-ggggggtcaacgttga-3' |
| | |
| **Control ODNs** | |
| 1471-PS | 5'-TCAAGCTTGA-3' |
| 1466-Acore-PS | 5'-TCAACGGTTGAGGGGGG-3' |
| 1466-Acore-PO | 5'-tcaacggttgattcaaa-3' |
| 1466-Acore-MB | 5'-tcaacggttgattcaaa-3' |

### 3.1.3. Lipids

Cholesterol was purchased from Sigma Aldrich (USA). L-a-Phosphatdylcholine (PC), 3a-[N-(N’,N’-Dimethylaminoethane)-carbamoyl] Cholesterol Hydrochloride (DC-Chol), 1,2-Dioleoyl-sn-GLycero-3-Phosphoethanolamine-N-[MEthoxy(Polyethyleneglycol)-2000] (Ammonium Salt) (PEG-PE), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE), L-a-Phosphatdylserine (PS) were all purchased from Avanti Polar Lipids (Alabama, USA).

## 3.2. Methods

### 3.2.1. Cell Culture

#### 3.2.1.1. Spleen and Lymph Node Single Cell Suspension Preparation

Mice (C57/BL6 or BALB/C strains) were sacrificed by cervical dislocation, their spleens and Lymph nodes were removed and transferred into 3 ml of 2% FBS supplemented complete RPMI-1640 media. Single cell suspensions were obtained, by smashing the spleens with back of a sterile syringe plunger with circular movements. Media was collected into 15 ml falcons, leaving the tissue clumps and connective tissues in the Petri plate, and washed twice with 10 ml of fresh 2% FBS supplemented complete RPMI-1640 media followed by centrifugation at 540 g for 10 minutes. At

the end of the last washing, cells were re-suspended in 1ml of 5% FBS supplemented complete RPMI-1640 for counting by Flow Cytometry.

#### 3.2.1.2. Peritoneal Exudate Cell Preparation

Mice were injected intraperitoneally (ip) with 10 ml of 2% FBS supplemented complete RPMI-1640 and peritoneal exudate cells (PECs) containing macrophages were collected back into the syringe (usually 8-9ml was recovered). Cells were washed twice with fresh media followed by centrifugation at 540 g for 10 minutes. After the washing steps, cells were resuspended in 1 ml of 5% FBS supplemented complete RPMI-1640 for counting by Flow Cytometry.

#### 3.2.1.3. Human Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood samples were collected from healthy donors into vacutainer tubes that contained EDTA as anti-coagulant (BD Vacutainer, purple cap). 22.5 ml blood was slowly layered onto, 15 ml of ficoll (Lymphocyte Separation Medium, Lonza). Samples were centrifuged at 540 g for 30 minutes with the break set to off for keeping layers separated. The cloudy layer that was settled over the ficoll, (which contained PBMCs), was collected carefully using a sterile Pasteur pipette. Recovered PBMCs were washed with fresh media twice followed by centrifugation at 540 g for 10 minutes. After the washing steps, the pellet was re-suspended in 1 ml of 5% FBS containing complete RPMI-1640 and counted by Flow Cytometry.

### 3.2.2. Cell Lines

#### 3.2.2.1. RAW 264.7

RAW 264.7 (*Mus musculus*), macrophage/monocyte cells (ATCC) were cultured with 10 % FBS supplemented complete RPMI-1640. Cells were passaged every 3-4 days when they reached 80-90% confluency.

### 3.2.3. Time Lapse detection of Labeled Liposome Uptake within RAW 264.7 cells

Differential internalization of D-and K-Type liposomes within cells were followed by labeling two different populations of liposomes with two different dyes that excitates at different wavelengths by Nikon Fluorescent (Nikon Eclipse Ti) microscope with an attachments of $CO_2$ chamber and live camera recording.

While D-type ODN were encapsulated in Neutral Liposome (labeled with FM1-43 dye), K-type ODN were encapsulated in Cationic Liposome (labeled with SPDiOC Dye). Initial experiments were conducted first with RAW cells that were incubated with one type of liposome. Images, as well as live video recordings were recorded for 15 mins, with 15-second intervals. Next, D- and K-Type CpG ODN loaded two different liposome populations were co-incubated with RAW 264.7 cells ($2x10^5$ cells/well) and early stage simultaneous uptake/internalization kinetics of liposomes by cells were recorded by fluorescence microscope (Nikon Eclipse Ti). As done before, images were recorded for 15 mins, with 15-second intervals. Later proper images were converted into a video file.

### 3.2.4. Cell Counting by Flow Cytometer

20 µl of single cell suspensions, which were prepared in 1 ml (dilution factor 1ml/20µl ; 1000/20=50), were added into 10 ml of isotonic solution . 20 µl from the prepared 10 ml solutions (dilution factor 10ml/20µl; 10000/20=500) were counted in Accuri C6 Flow Cytometer. Live and nucleated cells were gated, apoptotic, dead or non-nucleated blood cells were ignored. Counted number was multiplied with 25000 (multiplication of dilution factors; 50x500=25000).

### 3.2.5. In Vitro Stimulation of Murine Cells

For stimulation in 96-well plates, from cell culture stock ($2x10^6$ cells/ml, 150 µl fractions) of i) lymph node and ii) PECs were layered ($3x10^5$cells/well). D-and K-Type CpG ODNs as stimulants in either free form (0.2-3.0 $\mu$M) or different liposome

loaded form were added in 50 µl RPMI-1640 media to adjust the final well volumes to 250 µl. Stimulations were done in duplicate wells for each treatment groups. Supernatants were collected after 24 hours. Each experiment, at least repeated with 3-5 independent mice done on at least 2-3 separate occasions.

### 3.2.6. In Vitro Stimulation of PBMCs

PBMCs are isolated and counted as described above (Section 3.2.2). Afterwards, $3x10^5$ cells in 150 µl were layered to 96-well cell culture plates. Stimulants (as mentioned in Section 3.2.3.) were added in 50 µl of 5% FBS supplemented RPMI-1640, each well were prepared as duplicates and the final volume was set to 250 µl. Supernatants were collected after 24 hours of stimulation.

### 3.2.7. Liposome Preparation

All phospholipid and cholesterol stock solutions were prepared as 10 mg/ml or 20 mg/ml in chloroform and stored at -20°C. Phospholipids and cholesterol were mixed according to the ratios in Table 3.2 to obtain different liposomes with different physicochemical characteristics. Chloroform was evaporated with rotary evaporator (ILVAC, Germany) and a thin uniform film was formed. Nitrogen gas was streamed through the round bottom flasks to prevent remaining oxygen initiate peroxidation of the lipids. Next, onto lipid film, 1 ml of PBS was added and to obtain large multilamellar vesicles, films were disrupted by adding glass beads and shaking the flasks rotationally, which resulted in a milky solution. In order to generate small unilamellar vesicles (SUVs), liposomes were sonicated 10 times for 30s durations with amplitude of 50% (VibraCell, SONICS, USA). 150 mg of ODN was added to each 4,5 µmol of liposomes. The mixture was snap frozen in liquid nitrogen and lyophilized overnight (Benchtop K, Virtis, USA) to obtain lipid/ODN powder.

Encapsulation of ODNs within liposomes was achieved by controlled re-hydration method; 20 µl of DNase RNase free $ddH_20$ was added to dehydrated liposomes and vortexed for 15s every 5 minutes for 25 minutes. Same amount of PBS was added to the mixture and vortexed for a few seconds and let stand on the bench

for another 10mins. Finally, 110 µl of PBS was added to the mixture to have a final volume of 150 µl bringing the ODN concentration within liposomes to 1$\mu$g/$\mu$l of lipozomes. Liposome formulations were kept at 4°C until for further use.

Table 3.2: Lipid Compositions of different liposome types

| **Liposome** | **Compostion of Lipids (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) |

#### 3.2.7.1. Labeling Liposomes with SP-DiOC and FM1-43

Cationic liposomes were labeled with 1uM of SP-DiOC, anionic and neutral liposomes were labeled with 200 µg/ml of FM-143. Labeled liposomes were kept at dark for 30 minutes at RT, washed with 1 ml of PBS by centrifugation at 15000g for 30 minutes (Eppendorf Minifuge; Model: 5415R). The supernatant was discarded and the pellet was resuspended at 150 µl of PBS.

### 3.2.8. Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

After 24 hours of stimulation, 96 well plates were centrifuged at 540 g for 5 minutes. Approximately 230 µl of supernatants were collected and transferred into a new 96 well plate for immediate use or storing at -20°C for further use. 2HB plates were coated with monoclonal antibodies against mouse or human cytokines (50 µl/well) according to working concentrations listed in Table 3.3 and incubated overnight at +4°C. Wells were blocked with Blocking Buffer (200 µl, 5% w/v BSA-1X PBS, 0.025% Tween-20) at RT for 2 hours after coating antibody solutions were discarded. Plates were washed with washing buffer (PBS-Tween 20) for 3 minutes 5 times. Cell Supernatants and appropriately diluted standard recombinant cytokine antibodies (Table 3.4) were added (50 µl/well) and incubated overnight at +4°C.

Plates were washed as previously mentioned. Biotinylated anti-cytokine antibodies (50$\mu$l/well) were added to wells, in T-cell buffer with 1:1000 dilutions and incubated for 2 hours at RT. Streptavidin alkaline-phosphatase (SA-AKP) 1:1000 diluted was added again as 50 µl/well portions, following the washing steps and incubated for 1 hour at RT. For SA-AKP detection PNPP substrate was prepared according to the product`s protocol sheet and pipetted over each well at 50 $\mu$l portions. Yellow color formation was followed by reading the plates in automated ELISA Reader (Molecular Devices, USA) at OD set to 405nm.Concentrations of the culture supernatants were calculated from the S-Shaped curve of each standard recombinant cytokine.

Table 3.3 : concentrations of coating antibodies for mouse and human cytokine ELISA experiments (50ul/well transferred)

| | |
|---|---|
| Anti mouse IL6 | 2 µg/ml |
| Anti mouse IL12 | 4 µg/ml |
| Anti mouse IFNg | 1 µg/ml |
| Anti human TNFa | 2 µg/ml |
| Anti human IFNa | 4 µg/ml |
| Anti human IFNg | 4 µg/ml |
| Anti human IP10 | 2 µg/ml |

Table 3.4: Starting concentrations and detection limits for each recombinant standard cytokines (each cytokine was diluted by 11X by 1/2 dilutions)

| Cytokine Name | Initial Conc | Detection limits |
|---|---|---|
| rm IL6 | 2000 ng/ml | 976pg/ml |
| rm IL12 | 500 ng/ml | 244pg/ml |
| rm IFNg | 40 ng/ml | 19 pg/ml |
| rh TNFa | 1000 ng/ml | 488pg/ml |
| rh IFNa | 25 ng/ml | 12pg/ml |
| rh IFNg | 500 ng/ml | 244pg/ml |
| rh IP10 | 100 ng/ml | 48pg/ml |

### 3.2.9. Ex-Vivo Experiments

C57/BL6 mice were injected i.p. either with free 20 µg D + 20 µg K-Type CpG ODN, or 20 µg of FM1-43 labeled D ODN neutral liposome + SP-DiOC labeled

K ODN loaded SSCL,. In another experiment, Neutral liposome was replaced by anionic liposome and mixed with K-Type CpG ODN loaded SSCL. Also labeled stealth liposome that was loaded with D ODN was again mixed and injected with SP-DiOC labeled K ODN loaded SSCL. All formulations were suspended in 200 µl of 1X PBS. 6 hours later mice were sacrificed. Spleen, lymph nodes and PEC were collected. Single cell suspensions were prepared as described previously for cytokine ELISA. Cells were also used to assess surface marker expression levels by FACS analyses. Additionally, LN, PEC and splenocyte cells were used to purify total RNA, and study various cytokine and chemokine gene expression levels by PCR.

### 3.2.10. Determination of the Gene Expression

#### 3.2.10.1. Total RNA Isolation

Untreated or ODN treated cells (5-10x$10^5$/ml) from sacrificed mice were centrifuged at 540xg for 10 minutes, supernatants were discarded and pellets were resuspended in 1 ml of Trizol (Invitrogen, USA). Samples were either stored at -80°C for further use or in some cases directly continued to extract total RNA as described below. Of note, all steps during RNA isolation including centrifugations were performed on ice and at +4°C.

Samples were transferred into Eppendorf tubes and 200 µl chloroform was added into each. After the tubes were shaken vigorously for 10-15 seconds, samples were centrifuged at maximum speed for 18 minutes (Eppendorf Minifuge; Model: 5415R, @14000 rpm). At the end of centrifugation, 450-500 µl of upper clear phase was collected into new Eppendorf tubes. 500 µl isopropanol was added in order to precipitate RNA, incubated at RT for 10 minutes and centrifuged again @ 14000rpm for 13 minutes. The pellet was washed with the addition of 1 ml 75 % EtOH and centrifuging at 8000 rpm for 8 minutes. Washing step was repeated with 99,8 % EtOH. The pellet was left under laminar flow hood to air dry. Purified RNA was resuspended in 20 µl of DNase/RNase free $H_2O$. The OD of the samples were measured at 260 nm with the NanoDrop ND-1000 spectrophotometer (NanoDrop

Technologies, USA). The absorption ratios of the samples at 260/280 were expected to be in 1.8-2.0. RNA samples were then either stored at -80°C for further use or continued for PCR protocols.

### 3.2.10.2. cDNA Synthesis

cDNA synthesis kit (NEB, USA) ProtoScript First Strans cDNA was used to transcribe total RNA samples.1 µg of total RNA was mixed with 100 ng of Oligo (dT) 15 mer promer (1 µl) and each sample was completed with DNase/RNase free $H_2O$ to 8 µl. Samples were pre-denatured at 65°C for 5 minutes. 10 µl RT Buffer ( dNTP mix and 10mM $MgCl_2$) and 2 µl M-MuLV RNase H+ reverse transcriptase ( includes RNase inhibitor) were added to samples. After spinning down the samples quickly, were incubated at 42° C for one hour and at 85° C for 5 minutes. cDNA samples were stored at -20°C for further use.

### 3.2.10.3. PCR

All primers (Table 3.5) except mIL18 were adopted from Guiletti et al 2001, mIL18 was designed using Primer3 Input 0.4.0 program (http://frodo.wi.mit.edu/primer3/input.htm). PCR reaction was prepared with Quick-load Taq 2X Master Mix (NEB, USA). Final volume of the reaction was 25 µl, containing 12.5 µl Master Mix, 2 µl cDNA, 1 µl of each primer (forward and reverse) and 8.5 µl dd $H_2O$ (Table 3.6).

Table 3.5: Mouse primer sequences and their expected product sizes.

| **Primer** | **Direction** | **Sequence** | **Product Size** |
|---|---|---|---|
| mIP10 | Forward<br>Reverse | 5'-GCCGTCATTTTCTGCCTCAT-3<br>5'-GCTTCCCTATGGCCCTCATT-3' | 127bp |
| mIL18 | Forward<br>Reverse | 5'-GATCAAAGTGCCAGTGAACC-3'<br>5'-ACAAACCCTCCCGACCTAAC-3' | 491bp |
| mIL15 | Forward<br>Reverse | 5'-CATCCATCTCGTGCTACTTGTGTT-3'<br>5'-CATCTATCCAGTTGGCCTCTGT-3' | 126bp |
| mCD40 | Forward<br>Reverse | 5'-GTCATCTGTGGTTTAAAGTCCCG-3'<br>5'-AGAGAAACACCCCGAAAATGG-3' | 91bp |

| | | | |
|---|---|---|---|
| mGAPDH | Forward<br>Reverse | 5'-AGCTCATTTCCTGGTATGACA-3'<br>5'-CTCTCTTGCTCAGTGTCCTT-3' | 128bp |

Table 3. 6: PCR conditions for mouse primers.

| | **Gene Names (mouse)** | |
|---|---|---|
| **PCR Steps (Temp;Time)** | *gapdh* | *ip10, il18, il15, cd40* |
| Initial Denaturation | 94° C; 5' | 94° C; 5' |
| Denaturation | 94° C; 30" | 94° C; 30" |
| Annealing | 60° C; 30" | 55° C; 30" |
| Extension | 72° C; 40" | 72° C; 40" |
| Cycle # | 30 | 35 |
| Final Extension | 72° C; 5' | 72° C; 5' |

### 3.2.10.4. Agarose Gel Electrophoresis

2 % agarose gel was prepared (w/v) with 150 ml of 1X TAE buffer. Ethidium bromide was added as 1$\mu$g/ml. Each lane was loaded with 10 µl PCR product and 5µl of 100 or 50 bp DNA ladders (NEBiolabs, USA) as markers. Gels were run at 100V for 30 minutes and visualized under UV transilluminator (Vilber Lourmat, France). The gel images of different samples were then pooled and transcript level comparison was done on combined gel image.

## 3.2.11. Flow Cytometry

### 3.2.11.1. Fixation of Cells

After single cell preparations and treatments of cells, they were centrifuged and pellets were resuspended in 50 µl of fixation solution (4% paraformaldehyde Invitrogen, USA) while vortexing. Cells were incubated at RT for 15 min and 20x more PBS-BSA Azide (1ml, FACS Buffer) was added and cells were centrifuged at 1800 rpm for 10 minutes. Supernatants were discarded and the cells were resuspended in 1ml of FACS Buffer and stored at +4°C for further use.

### 3.2.11.2. Ex-vivo FACS Analyses of cells to determine Liposomal CpG ODN Internalization

Six hours after injection with labeled D- or K-Type CpG ODN loaded liposomal formulations, animals were sacrificed. Spleen, lymph node and PEC single cells were recovered fixed after washing and centrifugation as described previously and analyzed by BD Accuri C6 Flow Cytometer. From FL1 channel for SP-DiOC signal and FL2 channel for FM-143 signal two different liposome population`s level of internalization were assessed.

### 3.2.11.3. Intracellular Cytokine Staining (ICCS) of IFNα and TNFα

#### 3.2.11.3.1. IFNα Secretion Assay

PBMCs were stimulated with different free or liposomal CpG ODN formulations for 5 hours. To detect secreted IFNα, an IFN-α Secretion Assay Kit was used and protocol from the product data sheet was followed (Cell Enrichment and Detection Kit (PE) MACS, Miltenyi Biotec). The experiment was done on ice and centrifuges were set to 4°C. After the washing steps, cells were incubated with IFNα catch reagent on ice for 10 minutes before the addition of cell supernatant medium and further incubated at 37°C for additional 20 minutes for achieving the capture of the secreted form of IFNα to the supernatants. IFNα which were bound by pDCs were stained with i) anti-IFN-α detection (PE-labeled), ii) anti-BDCA-2 (FITC labeled, Biolegend, USA) and iii) anti-CD123 (PE/Cy5 labeled, BD, USA) for 30 minutes at dark. FACS analyses were performed after washing and re-suspending cells in PBS.

#### 3.2.11.3.2. TNFα ICCS

For the detection of TNFα, cells were stimulated for 4 hours in the presence of Brefeldin-A (in order to store the secreted TNFα inside the cells). After stimulation, cells were washed fixed as described above Section and stained with anti-CD123-

FITC (Biolegend) and and anti-TNFα-PE/Cy7 (Biolegend) for 30 minutes at dark. FACS analyses were performed after washing and re-suspending cells in PBS.

### 3.2.12. Statistical Analysis

Statistical significance between untreated (or control) and treated groups was evaluated using Student's t-test. $p<0.05$ set as statistically significance between the analyzed groups.

# Chapter 4

# 4. Results

Previous studies demonstrated that D and K ODNs induce differential immune activations [30]. Moreover, when D and K-ODNs were co-incubated with human PBMCs D-specific immune activation was lost and only K-specific cardinal cytokine production could be determined (i.e. primarily TNFα secretion). The major theme of this thesis was to establish that when suitable liposomal formulations encapsulating D and K-type CpG ODNs were used it leads to simultaneous cytokine secretion specific to both CpG ODN types.

In order to identify the most potent ODN sequence from both ODN classes, we tested three potential CpG ODN candidate sequences from each CpG Classes.

## 4.1. Determination of the most potent immunostimulatory ODN-liposome formulation

Immunostimulatory effects that were the most important feature for vaccine adjuvant development of TLR9 ligands could be enhanced by encapsulating them within liposomes [55]. To demonstrate that this is the case, 5 different liposome formulations were used with total of 6 different K- and D-type ODNs sequences as mentioned in the Materials and Methods Section (Section 3.1.2, Table 3.1). Splenocytes and PBMC were stimulated with high (1μM) and low doses (0.2μM) of these CpG ODNs. Cells were incubated for 24 hours and while IL6, IL12, IFNγ secretions from splenocytes (Section 4.1.1) were determined, IFNα, TNFα, IFNγ and IP10 productions from human PBMCs (Section 4.1.2) were assessed by cytokine ELISA from cultured cell supernatants.

### 4.1.1. Mouse Spleen cell stimulation Assays

IL6 secretion levels (Figure 4.1) and IL12 (Figure 4.2) secretion levels showed a similar trend in the repeat experiment (Appendix Figure A1-2).

Liposomal formulations of CpG ODNs showed higher secretion of IL12 than free forms of CpG ODNs even in the low doses of stimulation in splenocytes (Figure 4.2 A-F ). Cationic and SSCL encapsulated 1466-pT6-PS, 1555 and 1555-3CG showed approximately 2 fold higher IL12 amounts irrespective of their doses (Figure 4.2 B-D-E). 1466-pG6-PS, D35-3CG and pG6-1466-PO CpG ODNs stimulated almost 3 fold higher IL12 levels when these ODNs were encapsulated within anionic liposomes (Figure 4.2 A-C-F ).

All liposome encapsulated D35-3CG (except the cationic liposome formulation), augmented higher than 10 fold of IFNγ when used at low dose (Figure 4.3 C). Only SSCL encapsulation of all the other CpG ODNs stimulated IFNγ production. 1466-pG6-PS and 1555 induced almost 4 fold higher levels whereas, 1466-pT6-PS, 1555-3CG did not cause any increase in IFNγ production levels (Figure 4.3 A-F).

These immune activity was CpG ODN specific, since stimulation with empty liposome or control ODNs encapsulated in liposomes did not induce any detectable IL6, IL12 or IFNγ production.

According to IL6, IL12 and IFNγ cytokine ELISA results, liposomal D35-3CG and 1555 were chosen as D- and K- type candidate ODNs. Further splenocyte experiments were performed using these sequences.

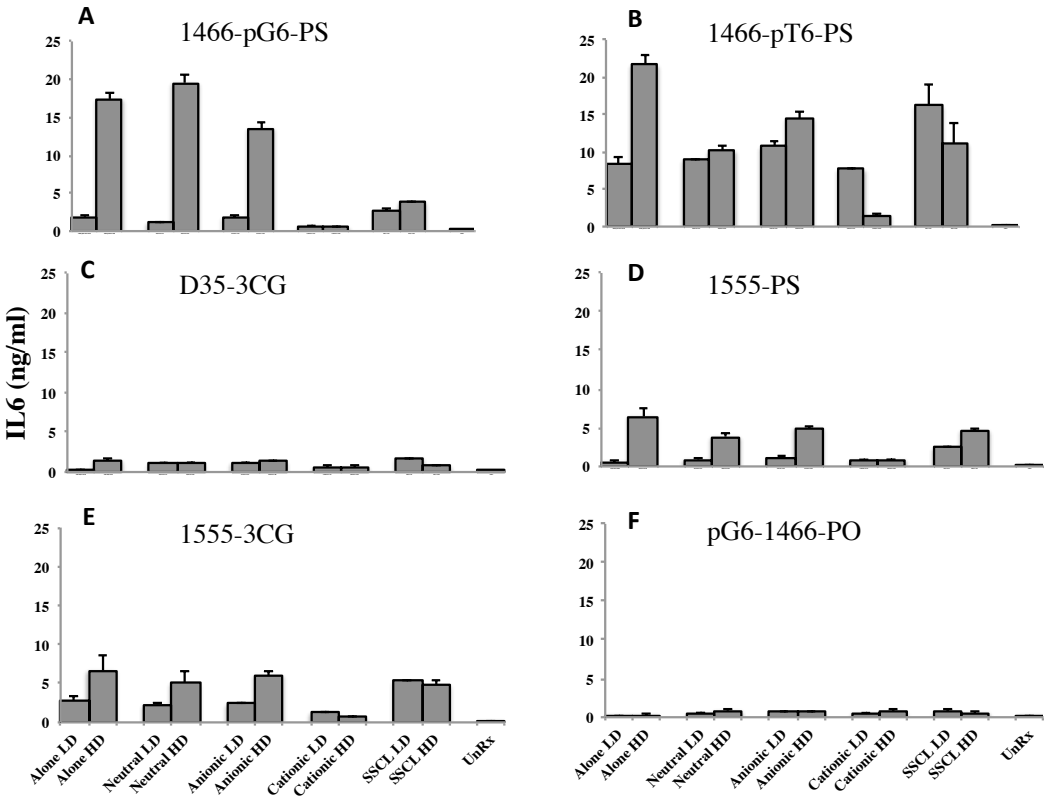



Figure 4.1 A-F : Dose dependent IL6 secretion of different liposome formulations and alone forms of candidate CpG ODNs from mouse splenocytes. HD corresponds to 1μM of encapsulated or alone CpG ODN, whereas LD represents 0.2μM ODN concentrations. Experiment samples were run in duplicates and IL6 levels were detected by cytokine ELISA from cell supernatants after 24h of stimulation.

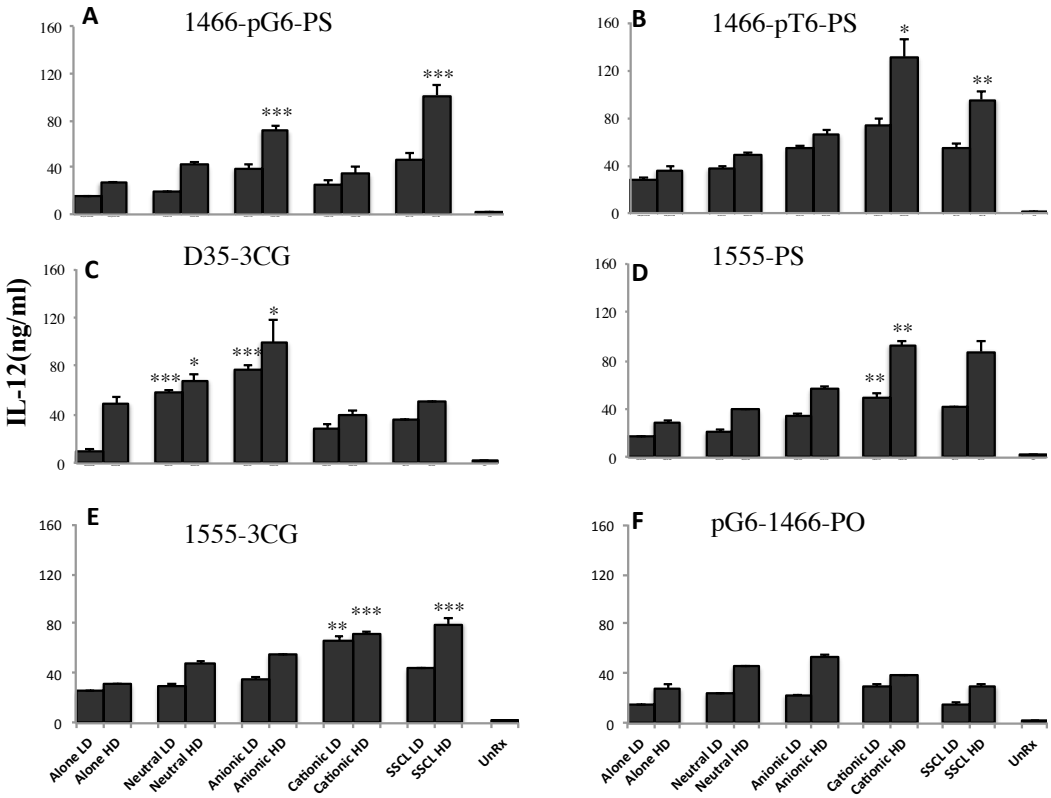



Figure 4.2 A-F: Dose dependent IL12 secretion of different liposome formulations and alone forms of candidate CpG ODNs from mouse spleen cells. HD corresponds to 1μM of encapsulated or alone CpG ODN, whereas LD represents 0.2μM ODN concentrations. Experiment samples were run in duplicates and IL12 levels were detected by cytokine ELISA from cell supernatants after 24h of stimulation. Paired t-test was done between alone and liposomal forms of CpG. ( * $p$<0.05, ** p<0.01, *** p <0.005).

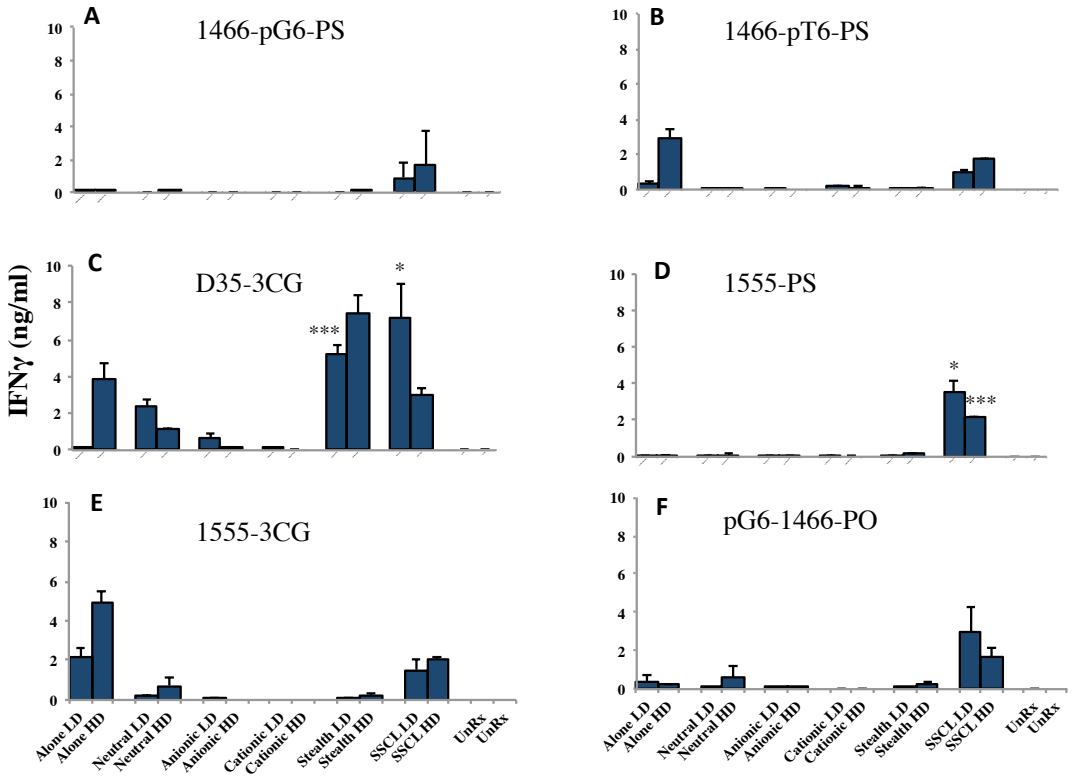



Figure 4.3 A-F: Dose dependent IFNγ secretion of different liposome formulations and alone forms of candidate CpG ODNs from mouse spleen cells in the repeat experiments. HD corresponds to 1μM of encapsulated or alone CpG ODN, whereas LD represents 0.2μM ODN concentrations. Experiment samples were run in duplicates and IL6 levels were detected by cytokine ELISA from cell supernatants after 24h of stimulation. Paired t-test was done between alone and liposomal forms of CpG. ( * $p$<0.05, ** p<0.01, *** p <0.005).

### 4.1.2. Human PBMC stimulation Assays

When 1466-pT6-PS and 1555 were used in their free forms during cell stimulation, they did not trigger any detectable IFNγ (Figure 4.4 B- D) production from human PBMCs. However, upon SSCL encapsulation, D35-3CG, significantly augmented IFNγ production compared with their alone forms (Figure 4.4 C p<0,01). In addition to SSCL, neutral and anionic encapsulated 1555-3CG stimulation yielded increased levels of IFNγ (i.e. approximately 10 fold with 1555-3CG, p<0,05).

TNFα (Figure 4.5A-F) production increased mostly (2-8 folds) with neutral liposome encapsulated D35-3CG and 1555-3CG compared with alone forms (Fig.

4.5C and E). 1466-pG6-PS showed 2-4 folds of increased TNFα secretion with SSCL and anionic liposomal forms (Fig. 4.5A ). Notably, liposomal 1466-pT6-PS and 1555 did not show any significant augmentation in TNFα levels (Fig. 4.5B and D). K-type ODNs activates pDC and causes TNFα production within 4 hours of stimulation, however, along with TNFα many upregulated cytokine gene expression decreases within 24 hours post-stimulation [56]. As a result of this suppression, cytokine ELISA results of K-type ODN stimulation did not yield any appreciable amounts of TNFα production.

Unlike K-type ODNs, D-type ODNs stimulate pDCs to produce IFNα. As expected, only D35-3CG showed detectable levels of IFNα (Figure 4.6) production from PBMC. Especially neutral liposome encapsulated D35-3CG augmented IFNα for up to 8-folds, whereas anionic liposomal counterpart induced only 3-fold higher, and finally SSCL liposome type led to 4-fold higher secretion.

Similar to splenocyte results, empty liposomes did not cause any detectable cytokine secretions, implicating that the effect was dependent only on CpG ODN.

For further experiments with PBMC, based on their enhanced immunostimulatory effects D35-3CG and 1555-3CG were chosen as the most potent D- and K-type ODN candidates, respectively.

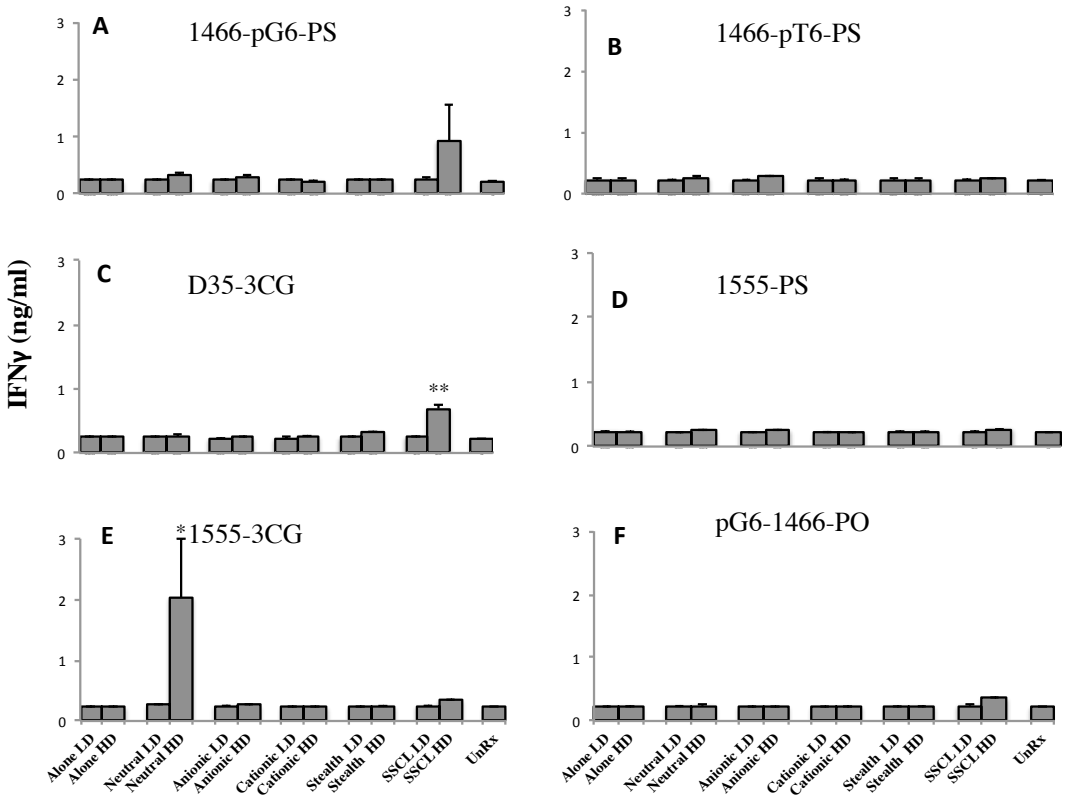



Figure 4.4 A-F: Dose dependent IFNγ secretion levels from stimulated PBMC supernatants, after incubation of 24h were assessed by cytokine ELISA. Experiments were run in duplicates. 0.2μM for low dose and 1μM for high dose of liposomal and alone forms of CpG ODNs were used for stimulation. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

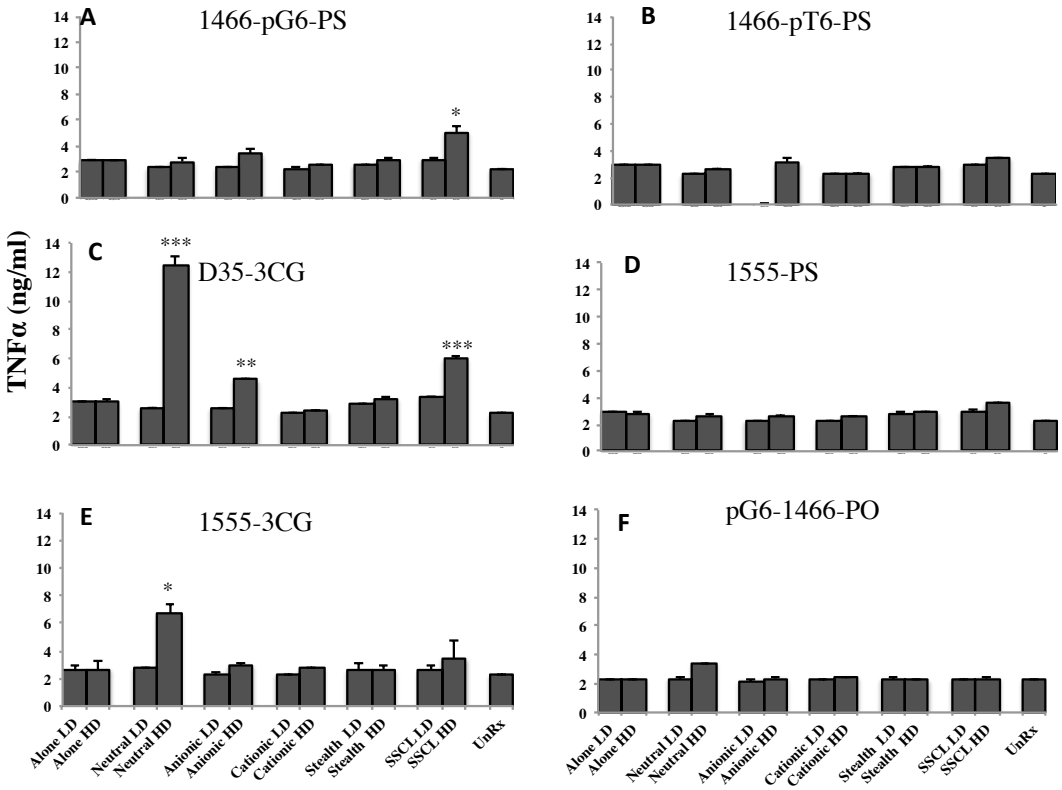



Figure 4.5A-F:Dose dependent TNFα secretion levels from stimulated PBMC supernatants, after incubation of 24h were assessed by cytokine ELISA. Experiments were run in duplicates. 0.2μM for low dose and 1μM for high dose of liposomal and alone forms of CpG ODNs were used for stimulation. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

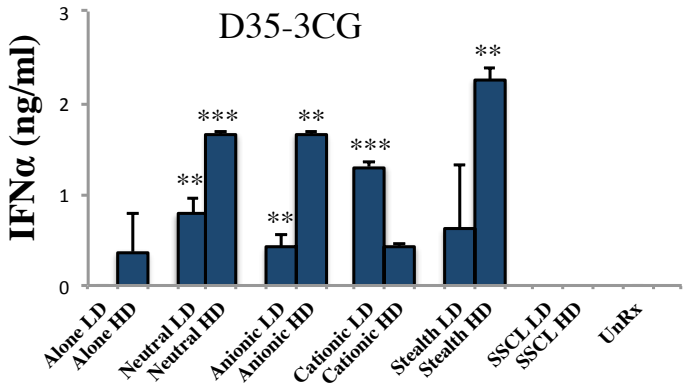



Figure 4.6 : D35-3CG was the only liposomal and alone form of CpG ODN that showed detectable IFNα secretion from stimulated PBMC in a dose dependent manner. Cells were stimulated for 24h and supernatants were collected for cytokine ELISA. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

## 4.2 . Delineation of synergistic immunostimulatory effects of liposomal D- and K- type CpG ODNs in Mice and in Man

The major aim of this thesis was to bypass the dichotomy and contrasting features exist between D- and K- type ODN and induce a synergistic effect when proper liposome formulations were used to load these ODN classes. When introduced simultaneously, K- type ODN blocks D-type specific response and only K-type response was observed [30].To overcome this problem, chosen D- and K- type ODNs, were encapsulated into 5 different liposomes. Splenocytes and PBMCs were stimulated simultaneously with liposome encapsulated D- and K- type ODNs.

### 4.2.1. Studies with Mouse Spleen cells

Splenocytes were stimulated with 2 doses (LD:0.2μM, HD: 1μM) of free or liposomal ODNs and after 24h cytokine ELISA of IL6, IL12 and IFNγ were assessed from cell supernatants.

Almost all combinations of liposomal formulations showed enhanced production of IL6 (Figure 4.7) and IL12 (Figure 4.8) in splenocytes, except cationic liposome encapsulated D plus cationic liposome encapsulated K (CD+CK) and SSCL encapsulated D plus SSCL encapsulated K (SSCLD +SSCLK) did not show any increase in IL6 secretion for both doses compared with single liposome encapsulated forms.

However, IFNγ (Figure 4.9) production, which was primarily mediated by D-ODN, was observed only in specific combinations. Most combinations of neutral liposome encapsulated D-type ODN showed enhanced effects when compared with neutral liposome encapsulated D-ODN alone counterpart, which was an expected outcome, since best liposomal formulations of D-type were neutral and anionic liposomal formulations. Additionally, stealth liposome encapsulated D ODN in addition to SSCL encapsulated K augmented IFNγ levels.

Importantly, simultaneous introduction of D- and K- ODN within proper liposomal formulations on to immune cells, demonstrated significantly higher levels

of cytokine production even at the lowest doses of both D- and K-ODNs, rather than suppressing each other's activity as reported before. Combination formulations were reduced to 4 main combinations for splenocytes for further experiments (Table 4.1). Based on the cytokine ELISA results of all combinations, these paired liposome groups were selected; i) ND+SSCLK, ii) AD+SSCLK, iii) CD+SSCLK, iv) SD+SSCLK. Combinations were chosen based on their ability to induce superior levels of IL6, IL12 and IFNγ compared to either alone liposomal formulations or free ODN counterparts. These preliminary results obtained from mouse data, not only revealed that liposomes provide individual D- and K activity to be induced in culture, but also proper pairings of liposomal D and Liposomal K further triggers a synergistic activation and simultaneous production and detection of both cardinal cytokines (i.e. IFNα-D-specific and TNFα: K-specific) in culture.

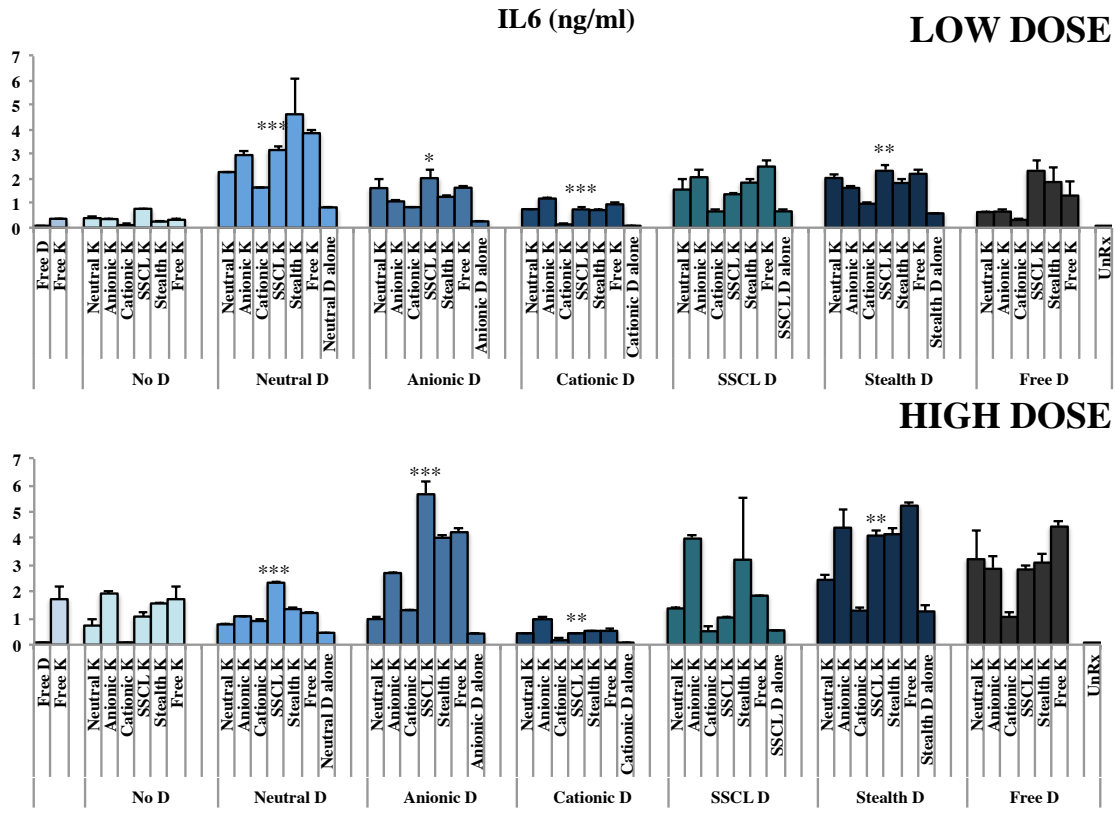



Figure 4.7: IL6 cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with splenocytes. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

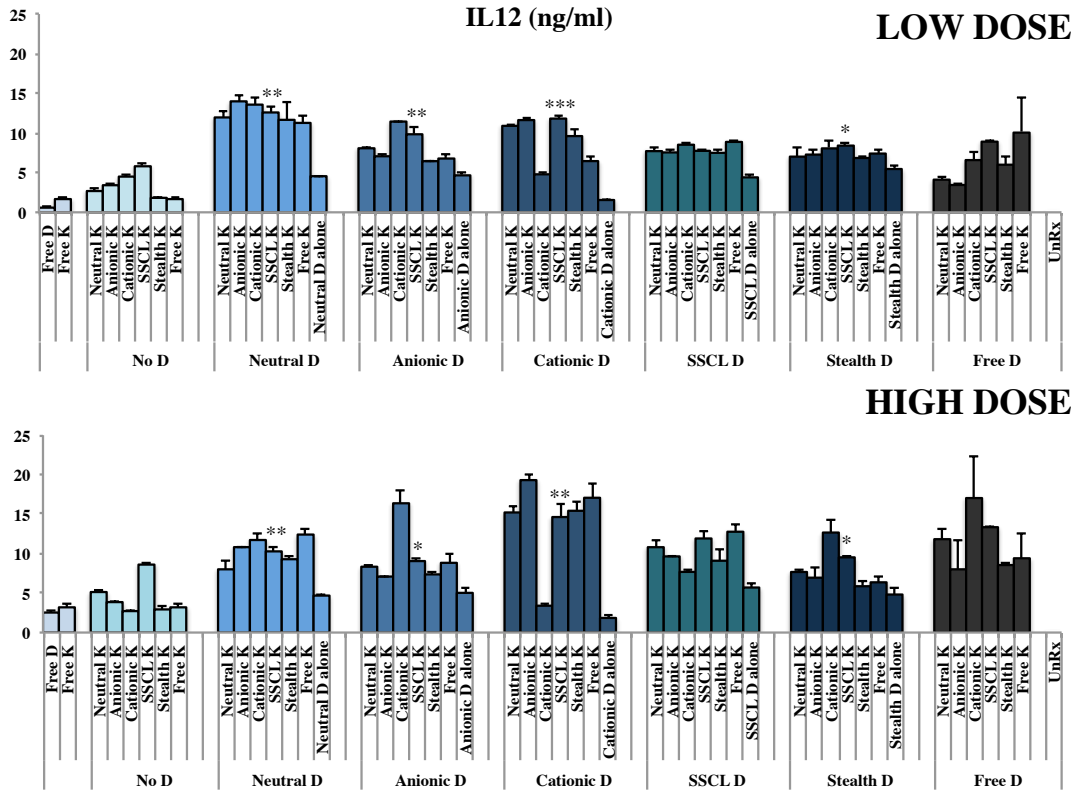



Figure 4.8: IL12 cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with splenocytes. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

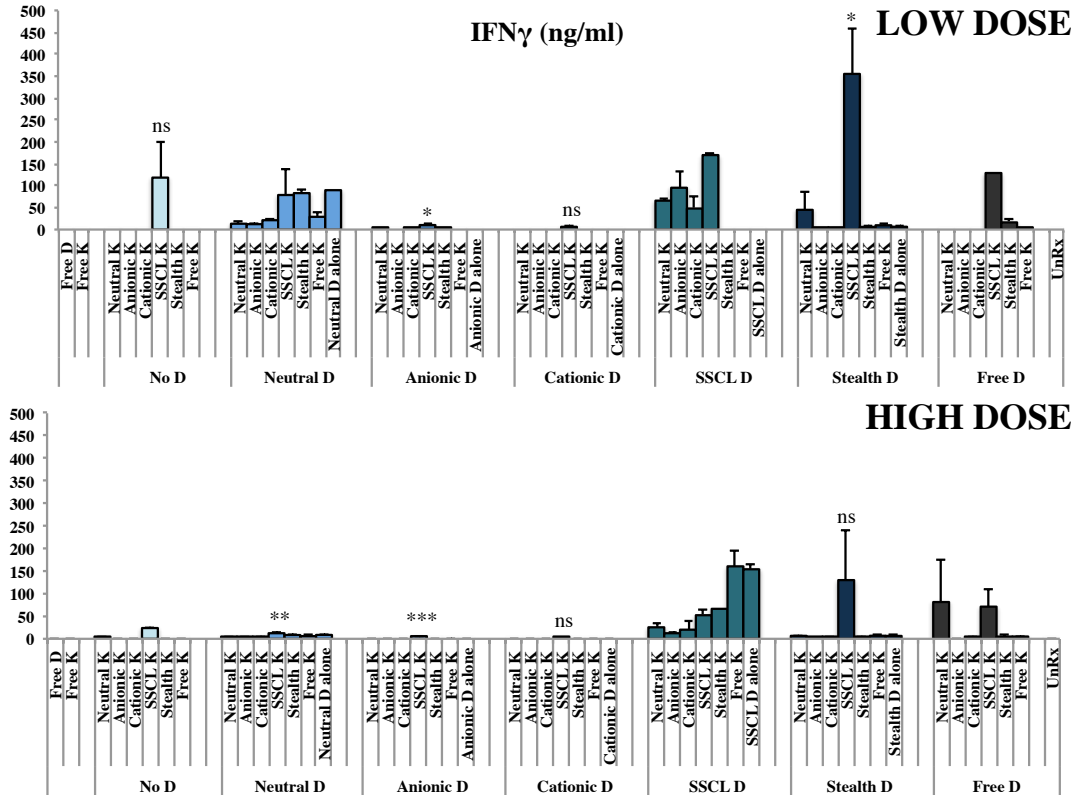



Figure 4.9: IFNγ cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with splenocytes. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG. ( * $p$<0.05, ** p<0.01, *** p <0.005).

Table 4.1: General outlook of formulation dependent cytokine specific upregulation for splenocytes. (+;increased secretion of cytokine compared to free D+K, -; decreased or same level of secreted cytokine compared to free D+K).

| | IL6 | IL12 | IFNγ | | IL6 | IL12 | IFNγ |
|---|---|---|---|---|---|---|---|
| | LD | | | | HD | | |
| ND+NK | + | + | - | | + | + | - |
| ND+AK | + | + | - | | + | + | - |
| ND+CK | + | + | - | | + | + | - |
| ND+SSCLK | + | + | + | | + | + | + |
| ND+SK | + | + | - | | + | + | - |
| AD+NK | + | + | - | | + | + | - |
| AD+AK | + | + | - | | + | + | - |
| AD+CK | + | + | - | | + | + | - |
| AD+SSCLK | + | + | + | | + | + | + |
| AD+SK | + | + | - | | + | + | - |
| CD+NK | + | + | - | | + | + | - |
| CD+AK | + | + | - | | + | + | - |
| CD+CK | - | + | - | | - | + | - |
| CD+SSCLK | + | + | + | | + | + | - |
| CD+SK | + | + | - | | + | + | - |
| SSCLD+NK | + | + | + | | + | + | - |
| SSCLD+AK | + | + | + | | + | + | - |
| SSCLD+CK | - | + | - | | - | + | - |
| SSCLD+SSCLK | + | + | + | | + | + | - |
| SSCLD+SK | + | + | - | | + | + | - |
| SD+NK | + | + | - | | + | + | - |
| SD+AK | + | + | - | | + | + | - |
| SD+CK | + | + | - | | - | + | - |
| SD+SSCLK | + | + | + | | + | + | + |

### 4.2.2. Studies with human PBMCs

PBMC were stimulated same as done with mouse splenocytes, after 24 hours of incubation with liposomal and alone combinations of D- and K- type ODNs TNFα, IFNα and IFNγ levels were measured by cytokine ELISA from cell supernatants.

Data revealed that, IFNα (Figure 4.10) production augmented only when anionic and neutral liposome encapsulating D-Type CpG ODN was mixed along with cationic liposome encapsulating K-type CpG ODN (i.e. AD+CK or ND+CK). Along with these 2 combinations, all combinations of cationic D and liposomal K-type ODN increased IFNα, compared to alone form of cationic D-type ODN stimulation with PBMC.

TNFα (Figure 4.11) secretion levels were enhanced in all combinations of neutral and stealth liposome encapsulating D-type ODN along with addition of liposomal K-type ODN compared to alone forms of either neutral or stealth D-type ODN. Addition to those groups, strikingly, anionic liposome encapsulating D ODN plus cationic liposome encapsulating K-Type CpG ODN (i.e. AD+CK) gave up to 4-fold higher TNFα production.

Simultaneous stimulation of neutral liposome encapsulated D with cationic (ND+CK), or SSCL D-ODN and stealth liposome encapsulated K ODN (SSCLD+SK) increased IFNγ (Figure 4.12) levels almost 10-fold compared to either free or neutral D alone counterparts.

As seen in Table 4.2, similar to splenocyte experiments, Liposome mixing experiments indicated superior combination groups. Based on initial PBMC screening experiments there were five combinations demonstrated augmented ability to simultaneously induce both TNFα, (a K-type ODN specific response) and IFNα, (a D-type ODN specific response) compared to either alone liposome formulations or free ODN counterparts. these combinations were; i) ND+CK, ii) AD+CK, iii) SD+CK, iv) CD+AK, v) CD+SSCLK (Table 4.2).

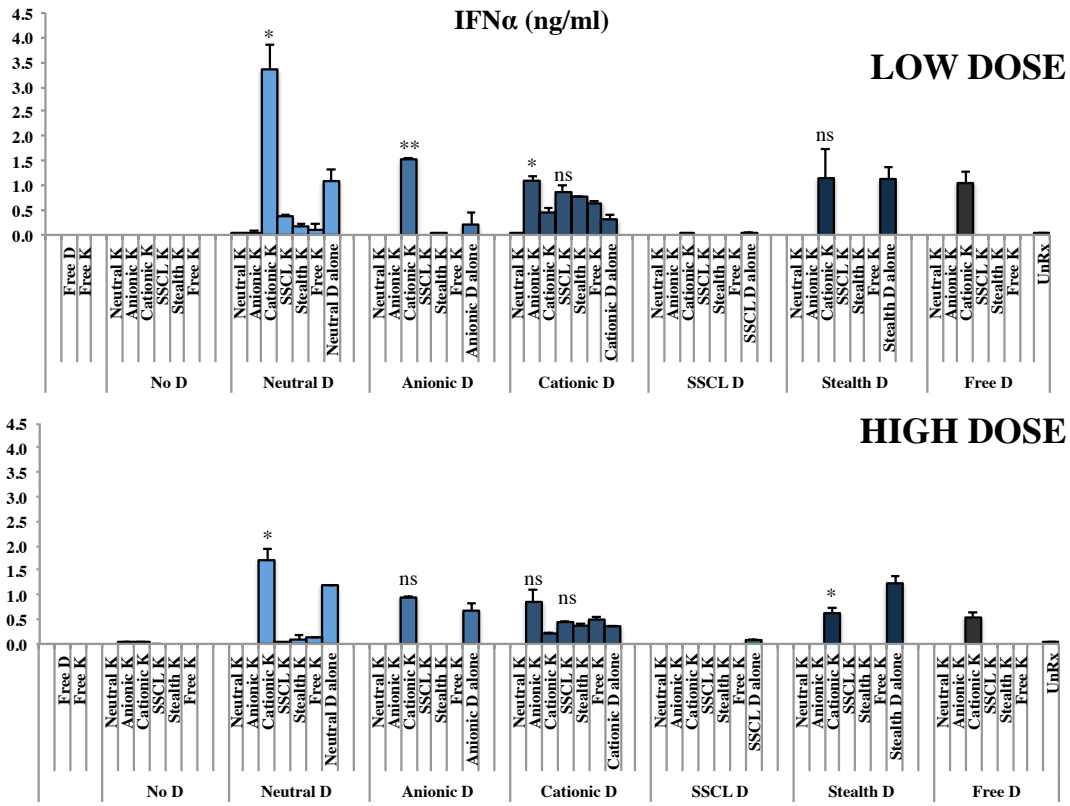



Figure 4.10: IFNα cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with PBMC. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

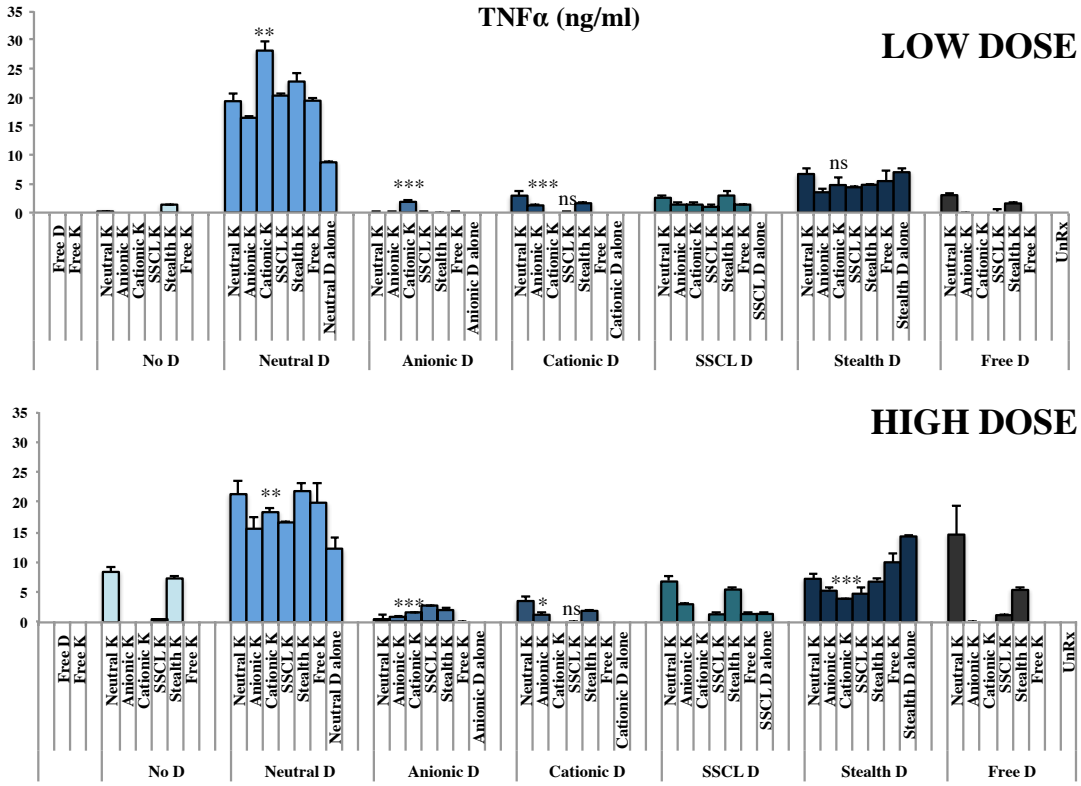



Figure 4.11: TNFα cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with PBMC. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

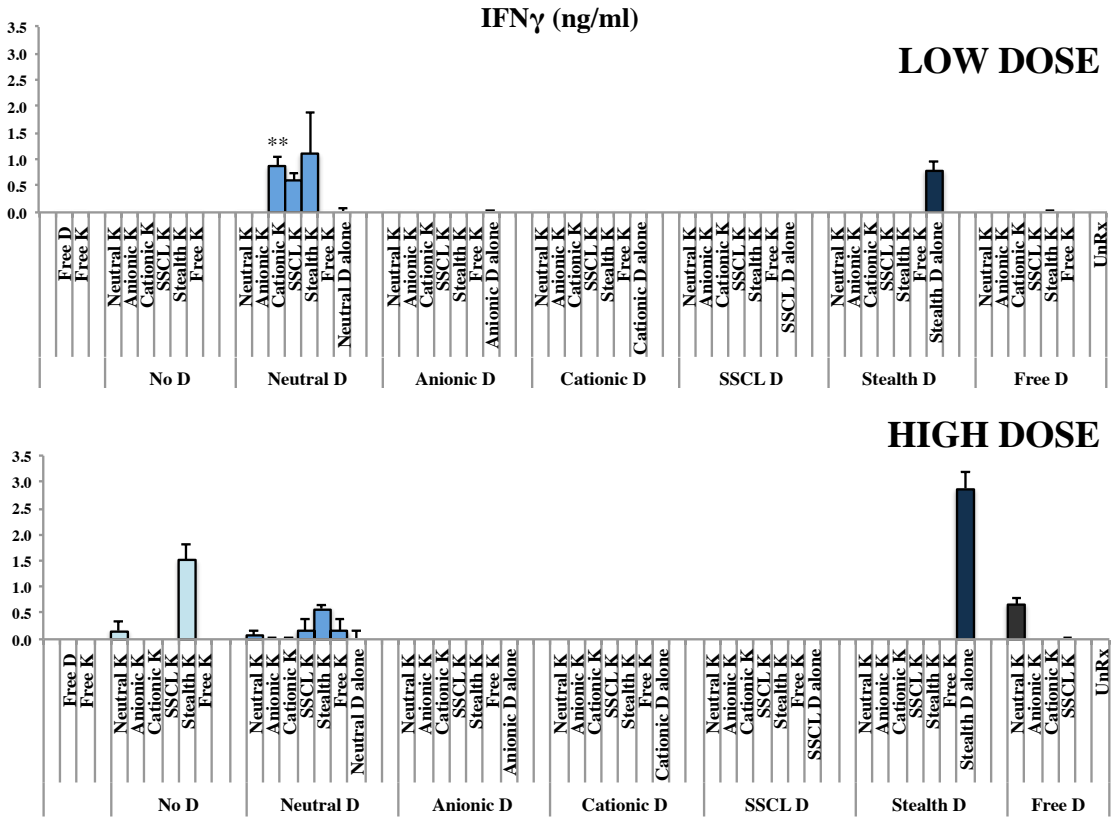



Figure 4.12: IFNγ cytokine ELISA results for low and high doses of simultaneous stimulation of liposomal D- and K- type ODN with PBMC. Experiment was run in duplicates; 0.2μM for low dose and 1μM for high dose of both liposomal and alone CpG ODN were used. Paired t-test was done between alone and liposomal forms of CpG .( * $p$<0.05, ** p<0.01, *** p <0.005).

Table 4.2: General outlook of formulation dependent cytokine specific upregulation for PBMC. (+;increased secretion of cytokine compared to free D+K, -; decreased or same level of secreted cytokine compared to free D+K).

| | TNFα | IFNα | IFNγ | | TNFα | IFNα | IFNγ |
|---|---|---|---|---|---|---|---|
| | LD | | | | HD | | |
| ND+NK | + | - | - | | + | - | - |
| ND+AK | + | - | - | | + | - | - |
| ND+CK | + | + | + | | + | + | - |
| ND+SSCLK | + | - | + | | + | - | + |
| ND+SK | + | - | + | | + | - | - |
| AD+NK | - | - | - | | + | - | + |
| AD+AK | - | - | - | | + | - | - |
| AD+CK | + | + | - | | + | + | - |
| AD+SSCLK | - | - | - | | + | - | - |
| AD+SK | - | - | - | | + | - | - |
| CD+NK | + | - | - | | + | - | - |
| CD+AK | + | + | - | | + | + | - |
| CD+CK | - | + | - | | - | - | - |
| CD+SSCLK | - | + | - | | + | - | - |
| CD+SK | + | + | - | | + | - | - |
| SSCLD+NK | + | - | - | | + | - | - |
| SSCLD+AK | + | - | - | | + | - | - |
| SSCLD+CK | + | - | - | | - | - | - |
| SSCLD+SSCLK | + | - | - | | - | - | - |
| SSCLD+SK | + | - | - | | + | - | - |
| SD+NK | + | - | - | | - | - | - |
| SD+AK | - | - | - | | - | - | - |
| SD+CK | - | + | - | | - | - | - |
| SD+SSCLK | - | - | - | | - | - | - |

## 4.3. Establishing the benefit of selected liposome combinations in healthy donors

Previous studies revealed that because human race is an out-bred population, an immune response to a single ODN displays huge variations in magnitude primarily due to genetic background disparities among individuals [57]. Another factor leading to variation in response between mice and man, is the breadth of TLR9 expression. TLR9 is only restricted to B-cells and pDCs in human compared to mice (in addition to B-cells, and pDCs, monocytes, dendritic cells and macrophages also express TLR9 in mice) thus, response against TLR9 ligands is different and shows vast variations among individuals whereas it is more reproducible in an inbred mice strain. To assess whether this problem is still valid with liposome encapsulated ODNs, we compared the response of free D and K type CpG ODN to that of liposome loaded counterparts

with PBMCs isolated from 10 healthy individuals. Throughout these assays, along with TNFα, (a K-specific cardinal cytokine) and IFNα (a D-specific cardinal cytokine), we also determined IFNγ and IP10 levels by cytokine ELISA from PBMC supernatants after 24h of stimulation.

Of 10 patients, Figure 4.13 shows the panel of cytokine production of only 2 representative PBMC profiles in response to 13 different CpG ODN containing formulations., whereas, Figure 4.14 shows the fold inductions liposomal CpG ODNs over mixture of free D+K type ODNs for all patients` responses for only TNFα and IFNα (Appendix Figure A-3-4-5-6). The major question to address throughout these assays was to detect whether certain liposome types induce synergy between D and K CpG ODNs rather than antagonize each other, in addition to understand the existence of disparities among individuals.

As seen in Figure 4.13, even we used 0.2μM of each ODN in liposomes, most of the formulations surpassed the immune activity levels of either Free K or free D mediated cytokine production (with the exception of IP10 levels). The most potent combination for TNFα, IFNα, and IFNγ induction was found to be ND+CK. Results revealed that regardless of the liposome type, all combinations of D- and K-type ODNs induced higher TNFα, IFNα, and IFNγ production compared to their free D+K counterparts (either at 0.2μM or 1.0 μM for free K-ODN or 3.0 μM for free D-ODN).

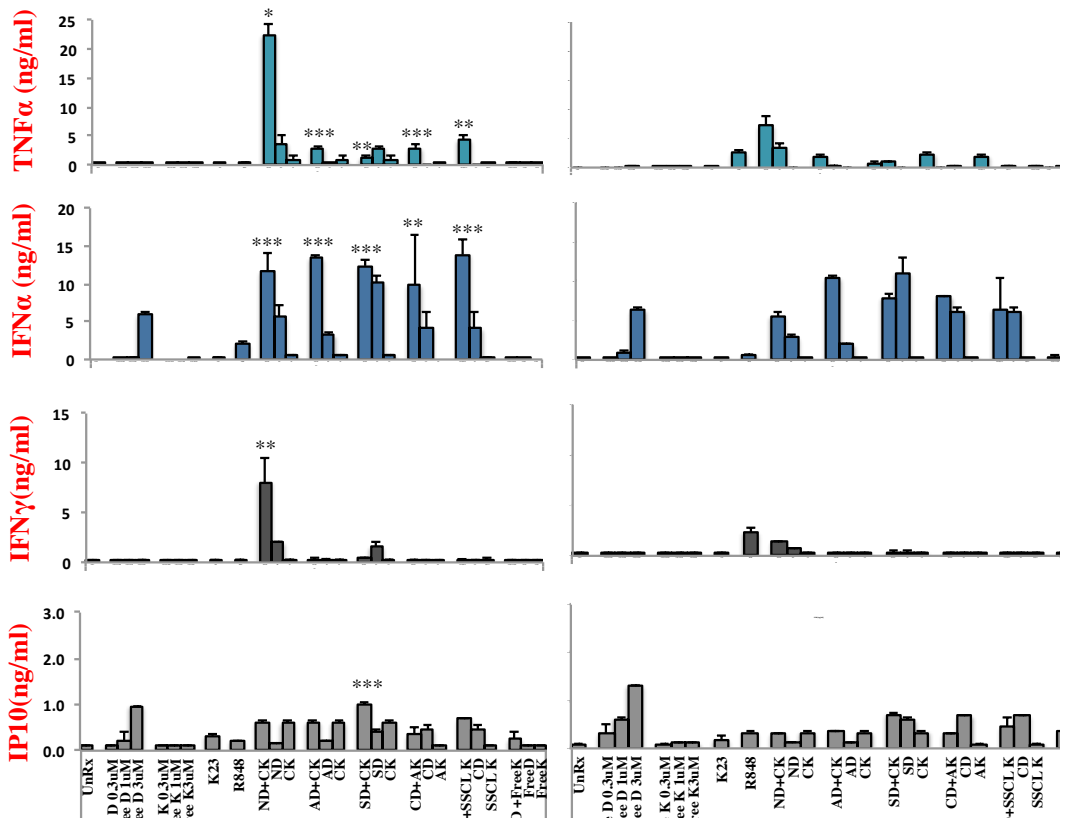
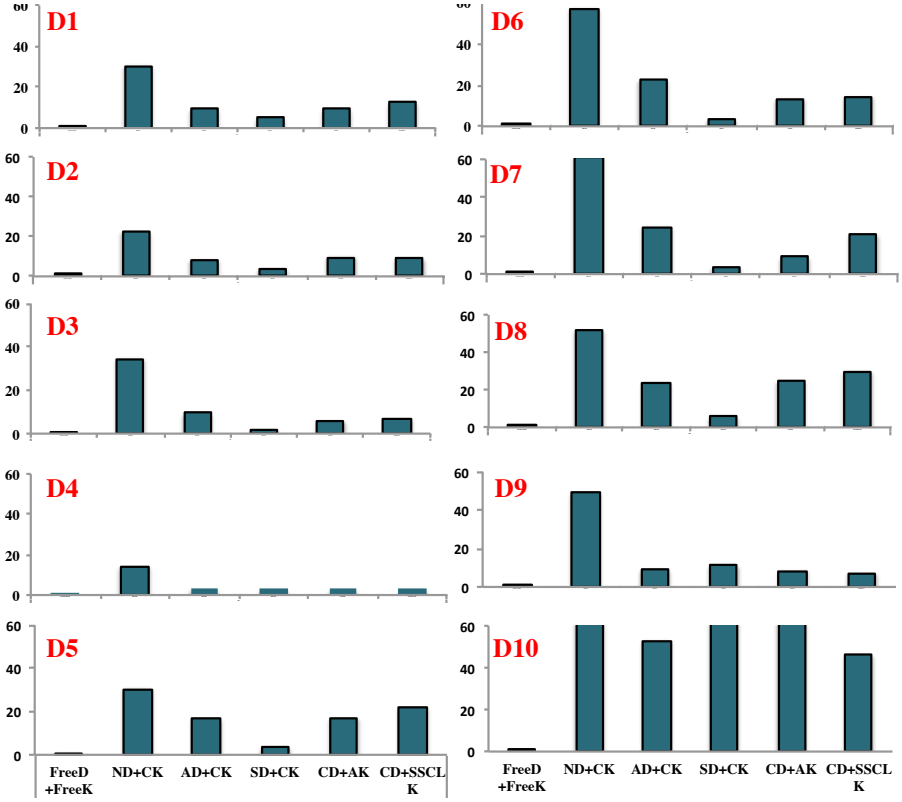



Figure 4.13: Representative cytokine ELISA results for TNFα, IFNα, IFNγ, IP10 with chosen 5 different liposomal formulations for 2 different donors out of 10. Paired t-test were done between free D+K and liposomal combinations of CpG. ( * $p$<0.05, ** p<0.01, *** p <0.005).

Figure 4.14 A-B summarized the healthy donor responses against liposomal CpG ODN to that of their free mixed counterparts. Here, only the D specific and K-specific cardinal cytokines were plotted (Fig. 4.14) with the aim to demonstrate that there is synergism between certain liposome associated D and K rather than antagonistic action that is seen only when given in mixture.

**A)TNFα Fold Inductions**




**B) IFNα Fold Inductions**

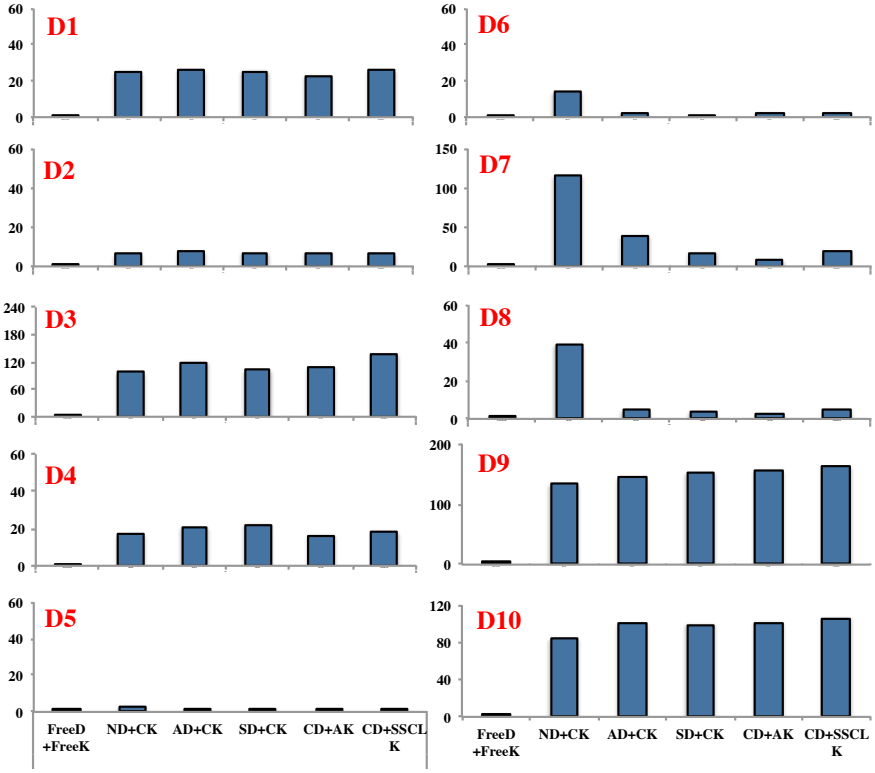



Figure 4.14 A-B: Fold Inductions of TNFα and IFNα for 10 healthy donors

As seen below more globally, in the Table 4.3 and 4.4, of five selected different liposome combinations harboring D-and K-CpG ODN, there are substantial increase in the levels of synergistic immune activation of both D-specific and K-specific cytokine productions. More strikingly, our data revealed that among the liposome combinations tested only ND+CK formulation induced both TNFα and IFNα (Tables 4.3 and 4.4). The response rate of donors were 100% of the cases more TNFα and 90% of the tested cases more IFNα from PBMCs.

Table 4.3 : Overall response of 10 healthy donors to selected liposome combinations loaded with D and K-Type CpG ODNs

| | TNFa | IFNa | IFNg | IP10 |
|---|---|---|---|---|
| ND+CK | 10/10 | 9/10 | 8/10 | 5/10 |
| AD+CK | 7/10 | 7/10 | 0/10 | 5/10 |
| SD+CK | 2/10 | 3/10 | 2/10 | 8/10 |
| CD+AK | 6/10 | 4/10 | 0/10 | 3/10 |
| CD+SSCLK | 6/10 | 4/10 | 0/10 | 6/10 |

Table 4 4 : Overall patient-to-patient immune response to liposomal CpG formulations

| | | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TNFa | ND+CK | + | + | + | + | + | + | + | + | + | + |
| | AD+CK | + | + | - | - | + | + | + | + | - | + |
| | SD+CK | - | - | - | - | - | - | - | - | + | + |
| | CD+AK | + | + | - | - | + | + | + | + | - | - |
| | CD+SSCLK | + | + | - | - | + | + | + | + | - | - |
| | | | | | | | | | | | |
| IFNa | ND+CK | + | + | + | + | - | + | + | + | + | + |
| | AD+CK | + | + | + | + | - | - | + | - | + | + |
| | SD+CK | + | - | + | + | - | - | - | - | - | - |
| | CD+AK | + | + | - | - | - | - | - | - | + | + |
| | CD+SSCLK | + | - | + | - | - | - | - | - | + | + |
| | | | | | | | | | | | |
| IFNg | ND+CK | + | + | - | + | + | - | + | + | + | + |
| | AD+CK | - | - | - | - | - | - | - | - | - | - |
| | SD+CK | - | - | - | - | - | - | - | - | + | + |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD+AK | - | - | - | - | - | - | - | - | - | - |
| | CD+SSCLK | - | - | - | - | - | - | - | - | - | - |
| | | | | | | | | | | | |
| IP10 | ND+CK | - | - | + | - | + | + | + | + | - | - |
| | AD+CK | - | - | + | - | + | + | + | + | - | - |
| | SD+CK | + | + | + | + | + | + | + | + | - | - |
| | CD+AK | - | - | - | - | + | - | + | - | + | - |
| | CD+SSCLK | + | - | - | - | + | + | + | - | + | + |

Collectively, our findings indicate that when a K-type ODN is loaded on a proper liposome, and mixed with another liposome type that was loaded with a D-ODN and incubated with human peripheral blood cells, they both exert their immune stimulatory action in a synergistic fashion and trigger both cytokines from that donor cells (i.e. simultaneous TNFα and IFNα). The accumulated knowledge so far, implicated that K ODN is superior over D-ODN when they were co-incubated together with immune cells. Therefore, the resultant immune response is solely K-Specific. Here, we reproducibly demonstrated in healthy donors that this is not the case only if one chooses to use proper liposome types encapsulating both D and K-CpG ODNs.

## 4.4. Stimulation of PBMC with different D- and K- type ODNs sequences retain liposome based benefits

As mentioned in Section 4.3 and demonstrated experimentally that liposome encapsulation override differential and contrasting immune activation of D-and K-specific immune activity, we then asked whether different types of D and K sequences still reproduce these effects on human healthy donor PBMCs. For this, three different healthy donor PBMCs were studied. D35 was used as D-type ODN along with K3 and K23 as K-type ODNs. As presented in Figures 4.15 and 4.16, replacing a K-ODN sequence 1555-3CG with two different K-Type sequences (i.e. K3 or K23) or similarly, replacing a D-ODN sequence D35 with D-3CG sequence reproduced the original synergistic immune activation in these new donors` PBMCs. Here, as it is seen clearly, two D-ODN encapsulating liposomes and one K-ODN encapsulating liposome, namely, Neutral, Anionic, and Cationic liposomes,

respectively, helped both CpG ODN types to induce their superior immune activation leading to both TNFα and IFNα (Figures 4.15 and 4.16).

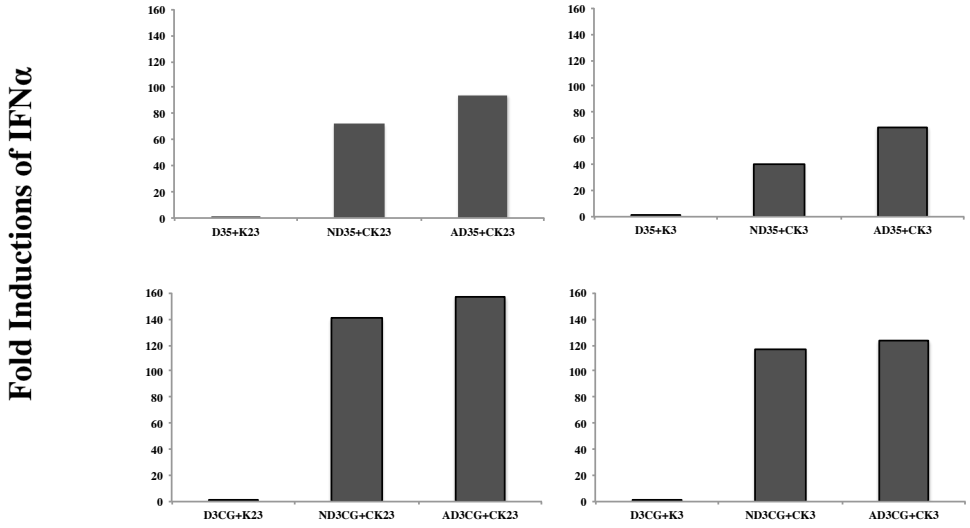



Figure 4.15: Fold Inductions of IFNα secretion obtained from cytokine ELISA results. D35 was used as D-type ODN instead of D35-3CG, K3 and K23 were used as K-type ODN instead of 1555-3CG. Experiments were run in duplicates. Three healthy donors were used; supernatants were analyzed by cytokine ELISA after 24h of stimulation.

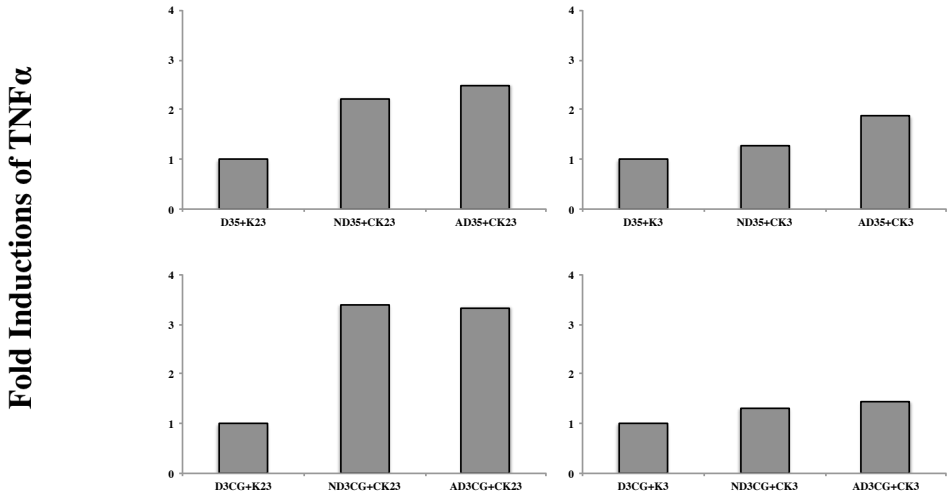



Figure 4.16: Fold Inductions of TNFα secretion obtained from cytokine ELISA results. D35 was used as D-type ODN instead of D35-3CG, K3 and K23 were used as K-type ODN instead of 1555-3CG. Experiments were run in duplicates. 3 healthy

donors were used; supernatants were analyzed by cytokine ELISA after 24h of stimulation.

These findings support the idea that liposome encapsulation can overcome the dichotomy of D-and K-type ODN and also creates a synergism, causing increased D- and K- type specific response with PBMC stimulation.

## 4.5. Intracellular Detection of TNFα and IFNα upon PBMC stimulation

PBMC were isolated from 2 healthy donors, and stimulated with liposomal and free formulations of CpG ODNs. D35-3CG and 1555-3CG were used as D- and K- type ODN sequences, respectively. Cells were incubated for 4 hours with stimulants and Brefeldin A for intracellular cytokine staining of TNFα was added at the time of incubation. For detection of IFNα, cells were incubated for 5 hours and IFNα secretion assay was used to detect IFNα from pDCs.

Cells were stained with CD123-FITC and TNFα-PE/Cy7 to distinguish the TNFα secretion specifically from pDCs. CD123-FITC positive cells were gated and analyzed for TNFα-PE/Cy7 (Appendix Figure A-7) positivity, fold inductions were calculated comparing liposomal formulations with free forms of D + K ODN (Figure 4.17). AD+CK and SD+CK increased TNFα secretion up to 1.6 fold. Alone form of D-type ODN also promoted TNFα secretion as expected since we are comparing 3μM D+1μM K in free form vs 0.2 μM (D+K each in liposome forms).

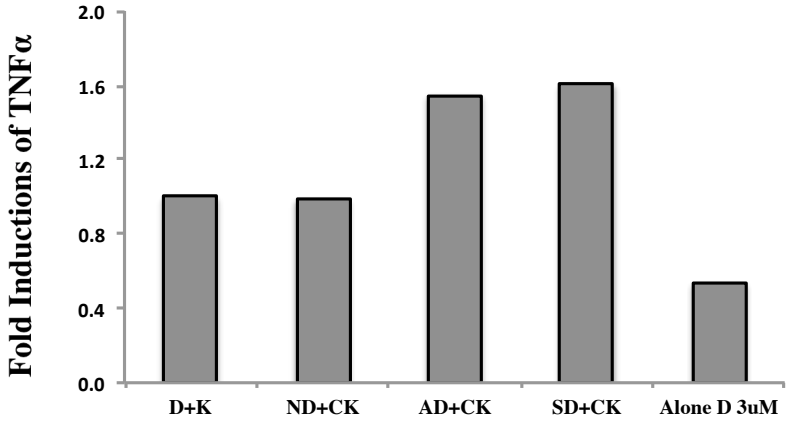



Figure 4.17: Fold induction of TNFα secretion from pDC. pDCs were defined by CD123-FITC positive cell gating. D35-3CG and 1555-3CG were used as D-and K-type ODNs.3μM of D-type and 1μM of K-type ODN were used in D+K combination. 0,2μM of each ODN were used in liposomal combinations.

While five different treatment groups were used for TNFα experiment, only 4 treatment groups were chosen for stimulation to measure IFNα production, these were; i) UnRx, ii) D+K, iii) ND+CK and iv) AD+CK. Intracellular IFNα was analyzed with IFNα secretion assay; cells were stained with BDCA-2-FITC and IFNα-PE. BDCA-2-FITC positive cells were gated in order to analyze pDC specific secretion of IFNα (Appendix Figure A-8) (Figure 4.18). Fold inductions of IFNα secretion were calculated based on IFNα secretion from cells, which were stimulated only with free forms of (D+K, 3uM +1uM). Liposomal formulations (0.2 uM each ODN) stimulated ~20 fold higher IFNα compared free forms of D-and K-type ODNs (D+K) (Figure 4.18).

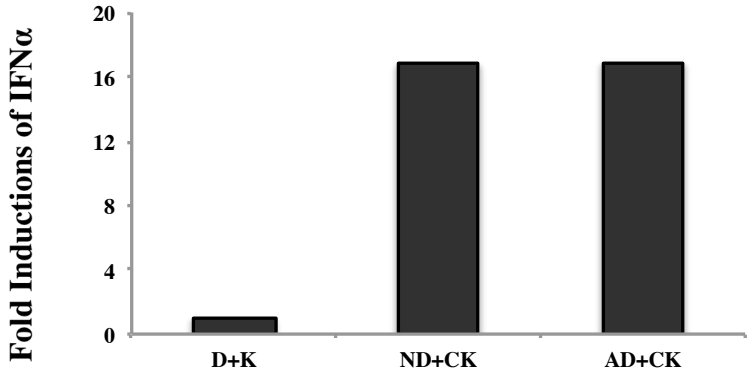



Figure 4.18: Fold induction of IFNα secretion from pDC. pDCs were defined by BDCA-2-FITC positive cell gating. D35-3CG and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K combination. 0.2μM of each ODN were used in liposomal combinations.

IFNα secretion assay was repeated, but with a different D-type ODN; D35. Same experimental groups were used. Cells were stained with BDCA-2-FITC and positive cells were gated, IFNα secretion specifically from pDC was analyzed (Figure 4.19, Figure 4.20). In this set of experimnts, two healthy donors blood were used. Fold inductions were calculated (Figure 4.21). According to FACS analyses, there was a significant increase in the percent of pDC population, when cells were stimulated with liposomal forms of CpG ODNs instead of their free forms. pDC population increased up to 6-7 % with ND+CK or AD+CK treated groups from % 0.8 in UnRx or D+K groups (Figures 4.19 and 4.20). Addition to that, IFNα-PE positive cells were around 80% with liposomal groups, where D+K stimulated cells only had 4.5 % IFNα-PE positivity (Figures 4.19 and 4.20). IFNα secretion increased almost 25 fold with liposomal combinations (ND+CK, AD+CK), compared with free D+K ODNs (Figure 4.21).

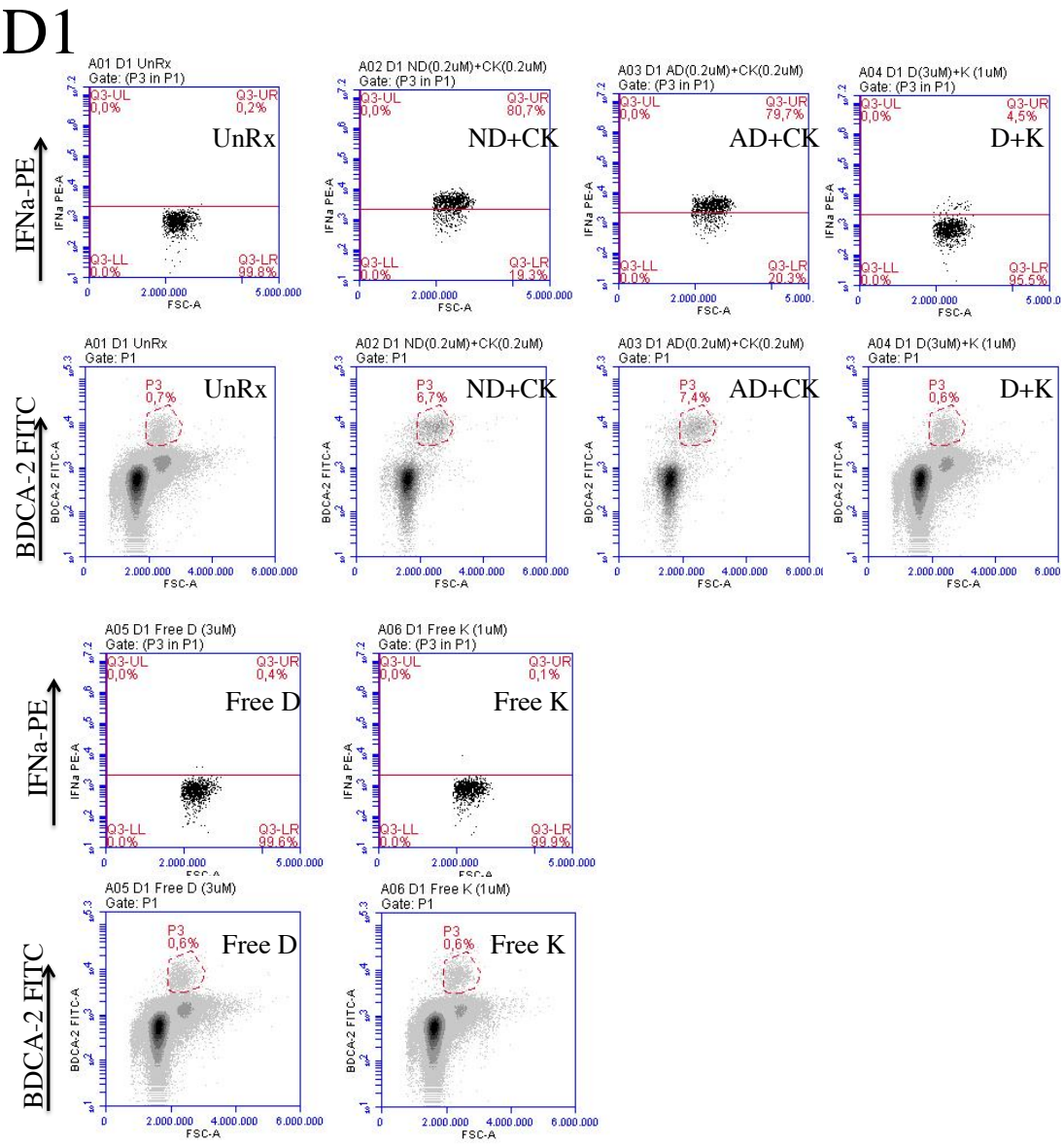



Figure 4.19:FACS analysis of IFNα secretion from PBMC isolated from healthy donor 1. pDC were chosen by gating BDCA-2-FITC positive cells. D35 and 1555-3CG were used as D-and K-type ODNs.3μM of D-type and 1μM of K-type ODN were used in D+K; 0,2μM of each ODN were used in liposomal combinations.

# D2

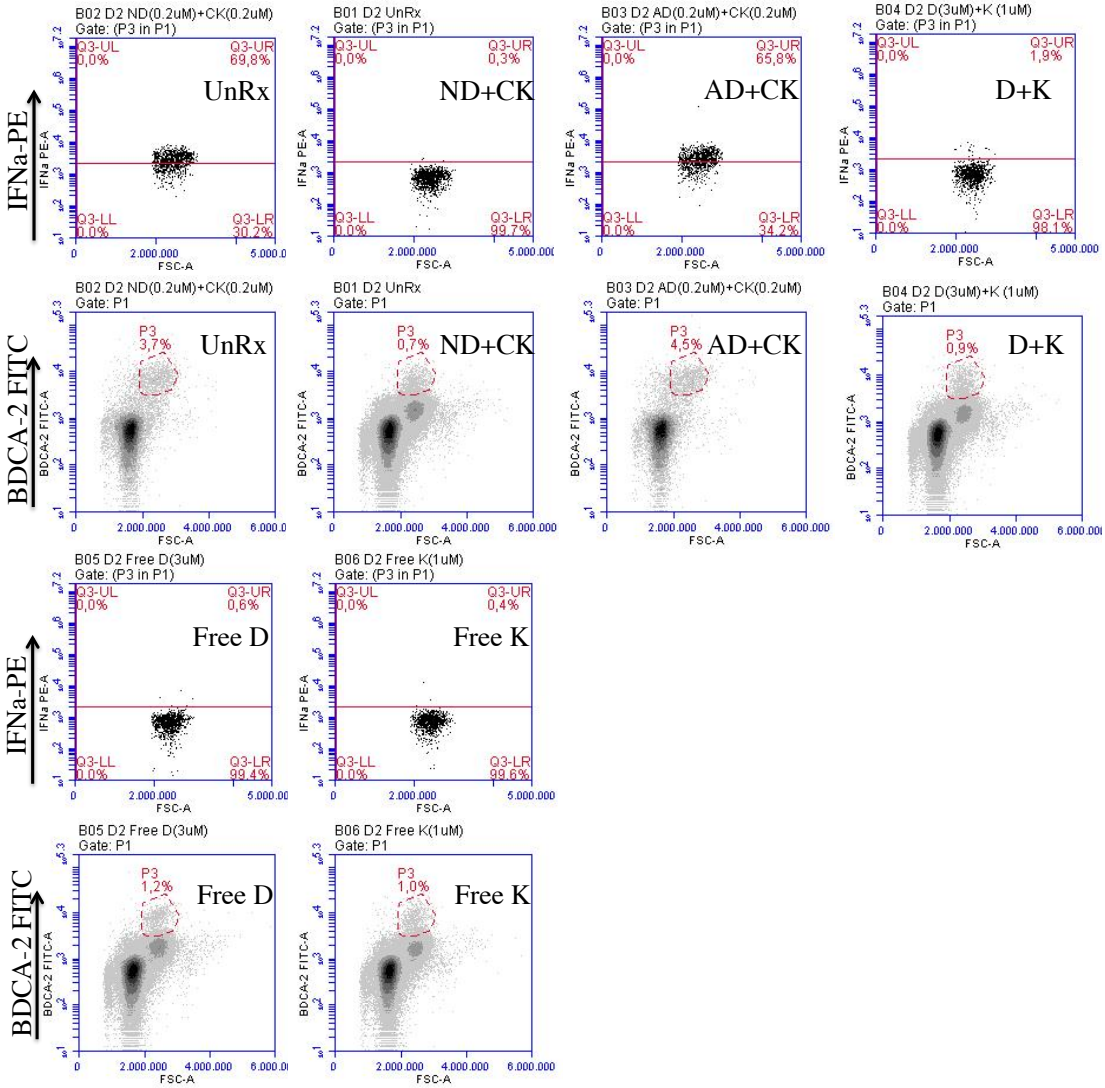



Figure 4.20: FACS analysis of IFNα secretion from PBMC isolated from healthy donor 2. pDC were chosen by gating BDCA-2-FITC positive cells. D35 and 1555-3CG were used as D-and K-type ODNs.3μM of D-type and 1μM of K-type ODN were used in D+K; 0,2μM of each ODN were used in liposomal combinations.

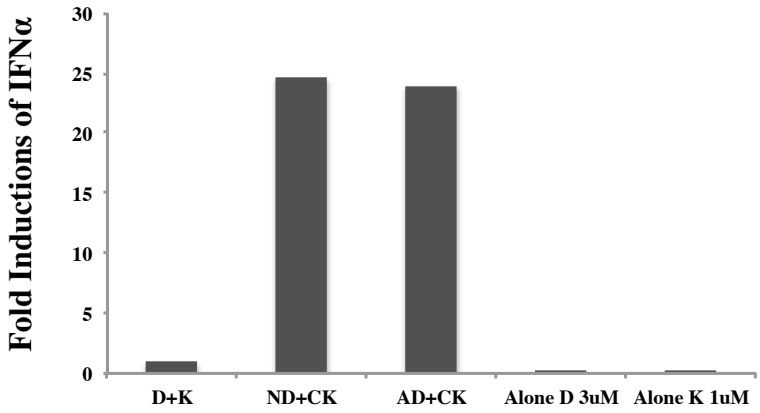
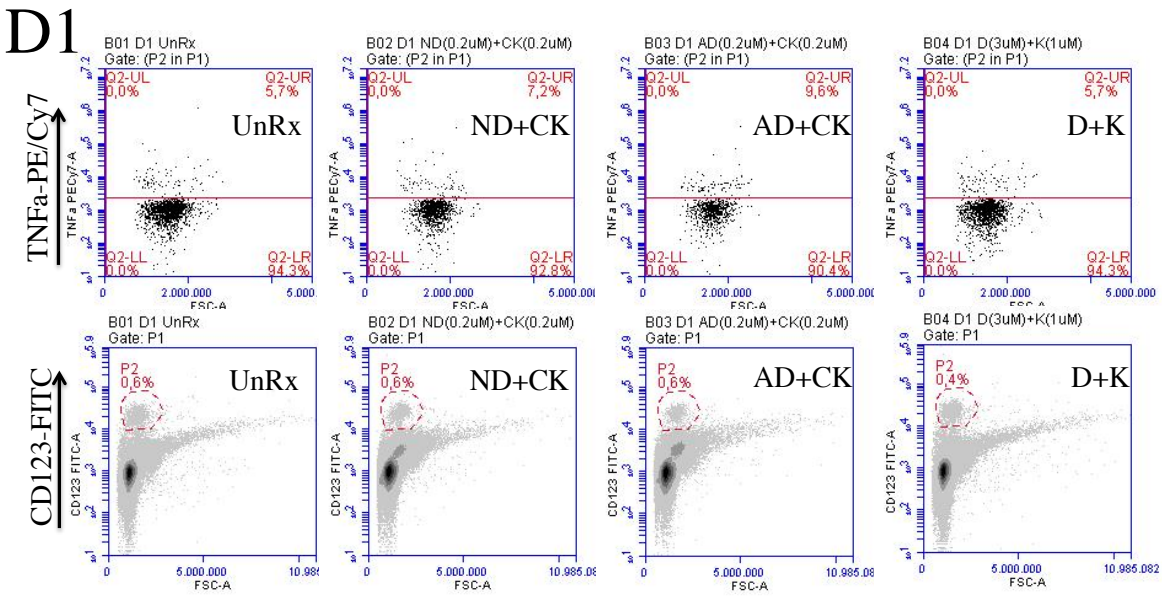



Figure 4.21: Fold induction of IFNα secretion from 2 healthy donors pDCs. pDCs were defined by BDCA-2-FITC positive cell gating. D35 and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K combination. 0.2μM of each ODN were used in liposomal combinations.

TNFα intracellular cytokine staining was repeated as well with D35 instead of D35-3CG as the D-type ODN (Figure 4.22-Figure 4.23). Fold inductions were calculated based on TNFα secretion as mentioned earlier (Figure 4.24). Here, as we observed before similar TNFα intracellular staining levels were observed. The most potent treatment group in augmenting TNFα secretion was AD+CK group.



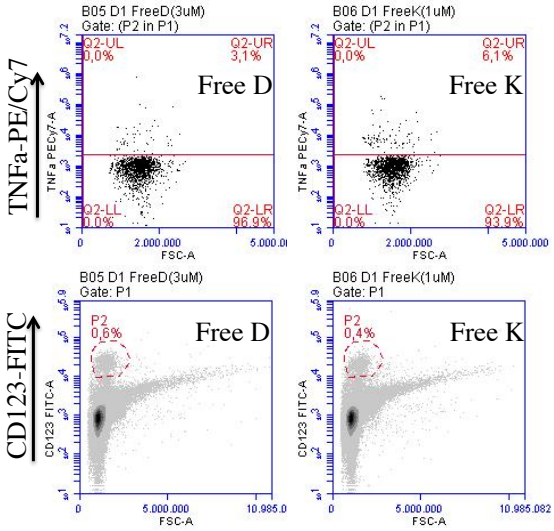
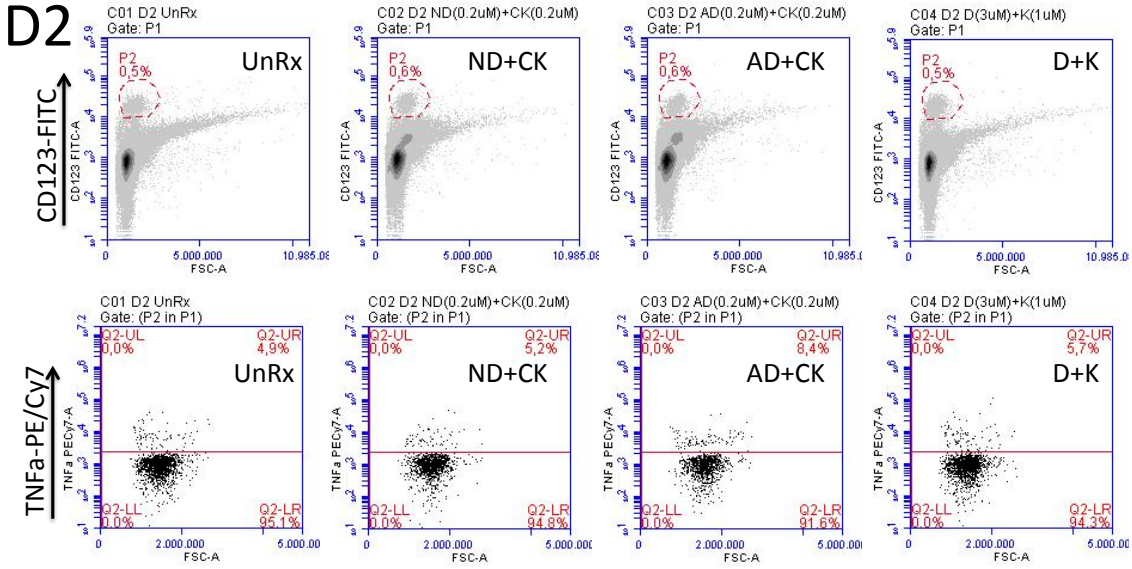



Figure 4.22: FACS analysis of TNFα secretion from PBMC isolated from healthy donor 1. pDC were chosen by gating CD123-FITC positive cells. D35 and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K; 0.2μM of each ODN were used in liposomal combinations.



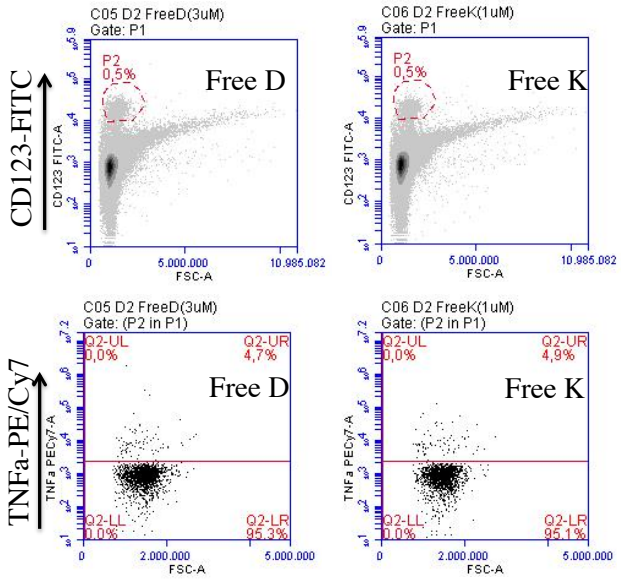



Figure 4.23: FACS analysis of TNFα secretion from PBMC isolated from healthy donor 2. pDC were chosen by gating CD123-FITC positive cells. D35 and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K; 0.2μM of each ODN were used in liposomal combinations.

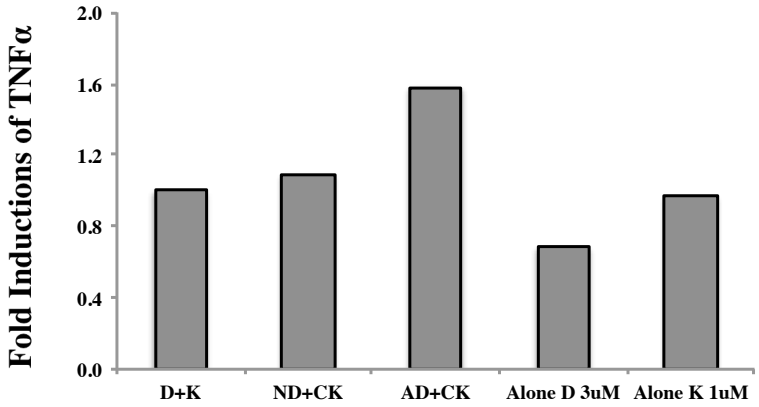



Figure 4.24: Fold induction of TNFα secretion from 2 healthy donors pDCs. pDCs were defined by CD123-FITC positive cell gating. D35 and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K combination. 0.2μM of each ODN were used in liposomal combinations.

### 4.5.1. Antigen presentation ability of PBMCs when simultaneously treated with liposomes encapsulating D and K type CpG ODNs

Major histocompatibility complex (MHC) class II are usually present only on antigen presenting cells (APC) they present processed peptides derived from extracellular proteins to $CD4^+$ T cells. There are three MHC class II isotypes; HLA-DR, HLA-DQ and HLA-DP, which consist of different α and β chains. MHC class II molecules are crucial for the development of $CD4^+$ T helper cells and activating the adaptive immune system [58]. CD80 is a stimulatory molecule found on activated B cells and monocytes, which is needed for T cell activation. We checked whether the HLA-DQ and CD80 cell surface molecules on PBMC, which were stimulated with liposomal and alone forms of D-and K-type ODNs improved their co-stimulatory molecule expressions as well as improve their APC function. Cells were fixed and stained with HLA-DQ-PE and CD80-FITC 24 hours after incubation in culture. FACS analyses were conducted and percent of double positive cells were obtained (Figure 4.25). Fold inductions were calculated based on stimulation with free D-and K-type ODNs (D+K). There was a significant increase, (i.e. 8-10 fold, in HLA-DQ and CD80) in surface marker expressions of PBMCs with liposomal formulations (Figure 4.26).

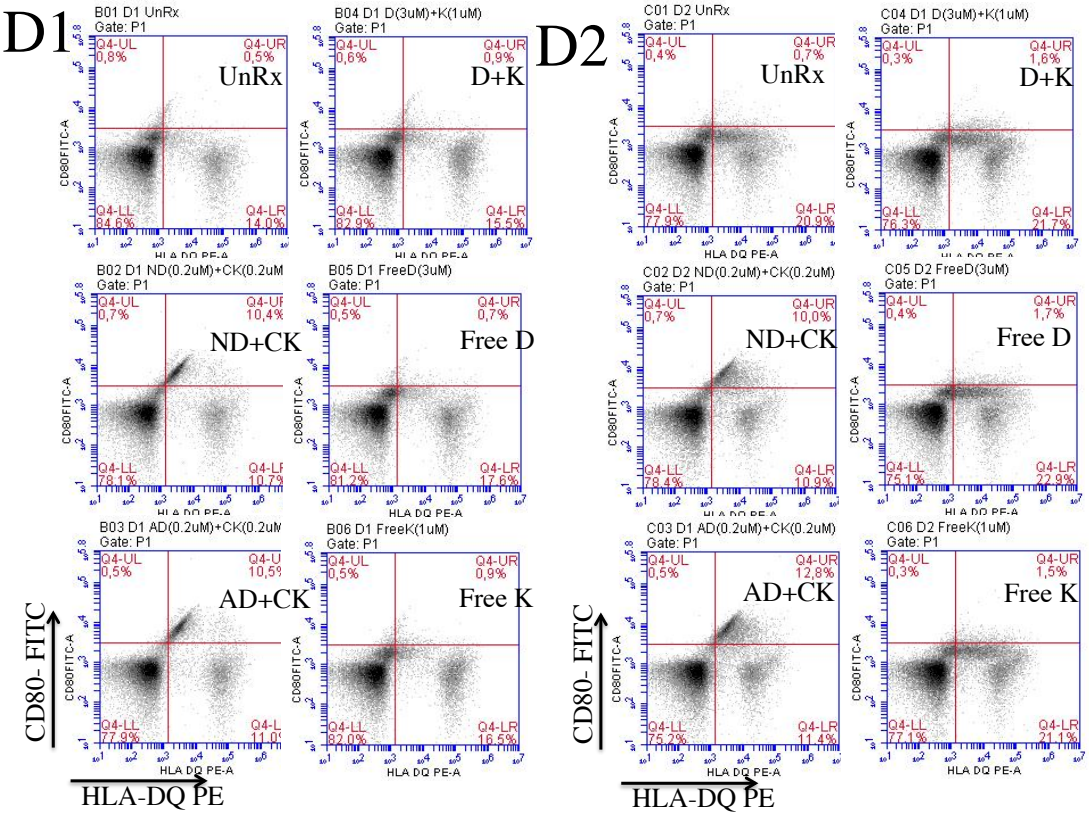



Figure 4.25: FACS results for CD80-FITC and HLA-DQ-PE expressions from PBMC. D35 and 1555-3CG were used as D-and K-type ODNs. 3μM of D-type and 1μM of K-type ODN were used in D+K; 0.2μM of each ODN were used in liposomal combinations. Cells were incubated for 24 hours and cells were fixed for FACS analysis.

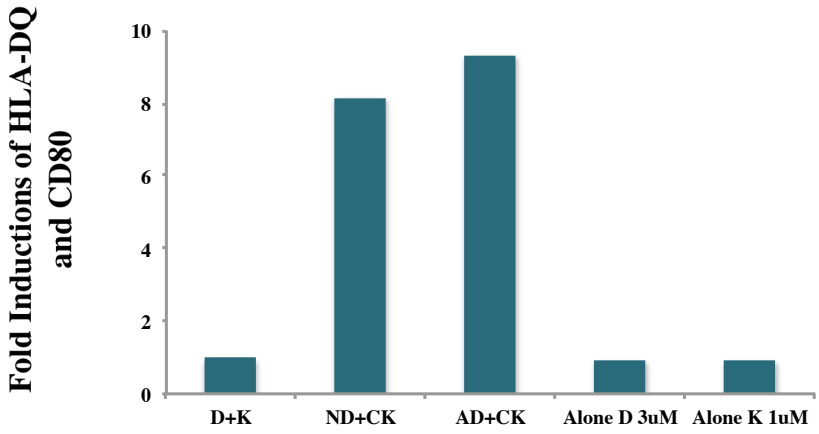



Figure 4.26: Fold inductions of HLA-DQ-PE and CD80-FITC double positive cells. 2 healthy donors were used. Cells were incubated for 24h fixed for FACS analysis afterwards. Fold inductions were calculated based on the double positive percentage of D+K.

As seen in the above sections, rather than using free forms of either D or K ODNs, which normally they do not support their simultaneous production of K or D specific signature cytokines, formulating them in different liposomes and co-incubating them with normal PBMCs, improved several features which was not possible otherwise. We established that in liposome forms, D and K ODNs improves i) TNFα, kii) IFNα, iii) Co-stimulatory molecule expression, and iv) APC function. This strategy amalgamates both CpG ODN as a potent formulation and offers a new approach to treat human subjects more effectively and efficiently.

## 4.6. Stimulation of Splenocytes does not depend on mice strain

After establishing that ND+CK or AD+CK liposome combinations are more effective than free D or K-Type CpG ODN stimulation in human setting, we revisited mouse study and reproduced matrix stimulation assays with two different strains (Balb/c and C57Bl6). Four different dose combinations were used. These were: i) LD+LD: Low Dose D-ODN + Low Dose K-ODN, ii) HD+HD: High Dose D-ODN + High Dose K-ODN, iii) LD+HD: Low Dose D-ODN + High Dose K-ODN, and iv) HD+LD: High Dose D-ODN + Low Dose K-ODN.

For liposomal formulations, we kept both ODN concentrations at 0,2μM. For the free counterparts we used optimum K and D doses along with 0.2 μM doses (i.e. 1.0 and 3.0 μM, respectively) to observe the dose effects with respect to free vs liposomal ODN inductions. As seen in the Figures 4.27 and 4.28, The pattern, which was described for human PBMCs reproduced with these two different mouse strains (in these Figures only Bl6 stain responses are presented, for Balb/c response please refer to (Appendix Figure A-9-10). However, more liposome types gave synergistic activation in addition to AD+CK, or ND+CK. Strikingly, when K-ODN was encapsulated in SSCL type of liposomes it surpassed all liposomal K activity in mice. This is primarily due to the cell types expressing TLR9 in mice. Compared to B-cells and pDCs more cells express TLR9 as reported before. SSCLK seemed to engage mouse immune cells stronger than other liposomal K formulation in this setting.

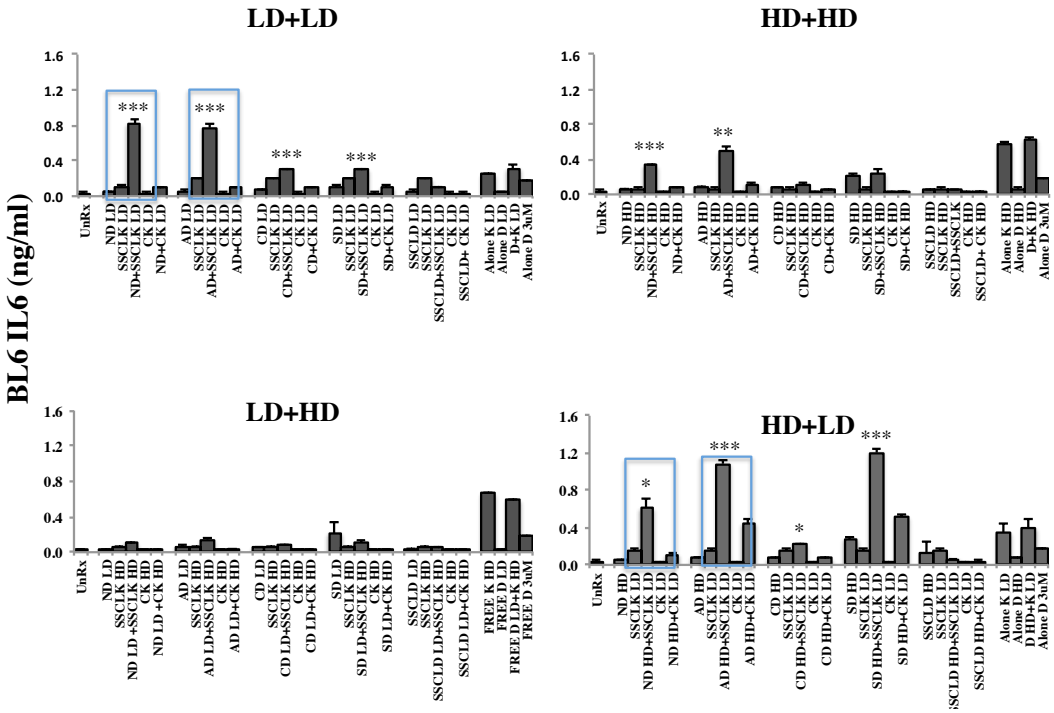



Figure 4.27: IL6 cytokine ELISA results C57/BL6 mice. Different dose combinations were used; LD+LD (0.2uM D ODN plus 0.2uM K ODN), HD+HD (1uM D ODN plus 1uM K ODN), LD+HD (0.2uM D ODN plus 1uM K ODN), HD+LD ( 1uM D ODN plus 0.2uM K ODN). Additionally 3uM of alone D-type ODN was used. Experiments were run in duplicates, supernatants were collected after 24h of stimulation. Paired t-test was done between combinations and alone forms of liposomal CpG ODN.( * $p$<0.05, ** p<0.01, *** p <0.005).

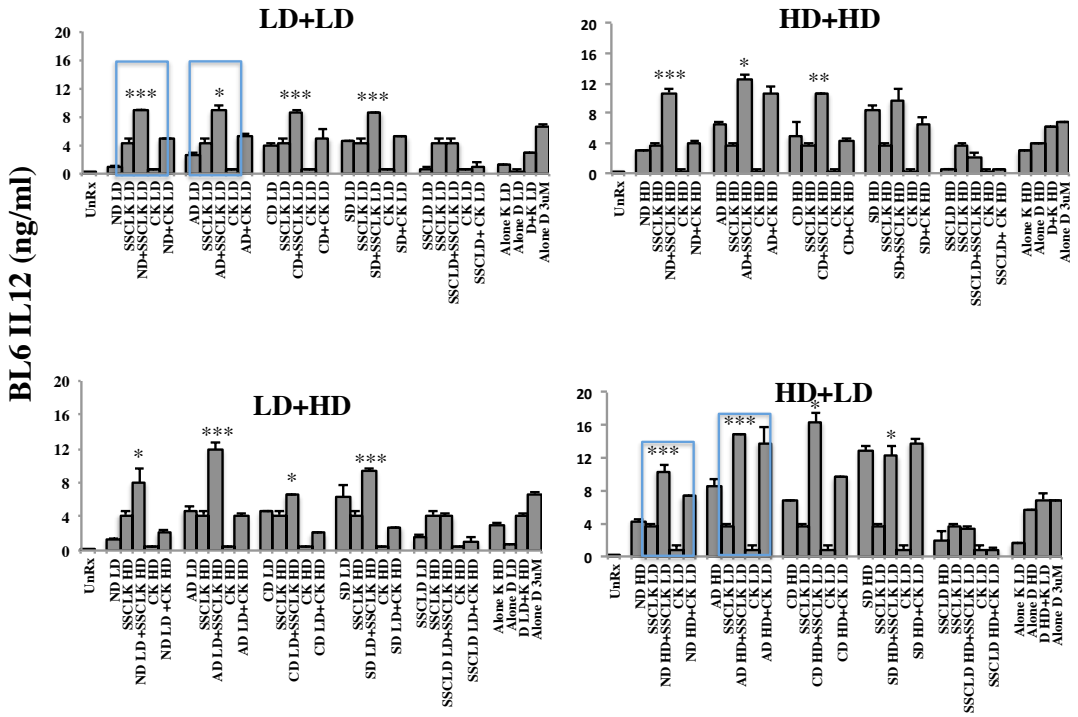



Figure 4.28: IL12 cytokine ELISA results for C57/BL6 mice. Different dose combinations were used; LD+LD (0.2uM D ODN plus 0.2uM K ODN), HD+HD (1uM D ODN plus 1uM K ODN), LD+HD (0.2uM D ODN plus 1uM K ODN), HD+LD ( 1uM D ODN plus 0.2uM K ODN). Additionally 3uM of alone D-type ODN was used. Experiments were run in duplicates, supernatants were collected after 24h of stimulation. Paired t-test was done between combinations and alone forms of liposomal CpG ODN.( * $p$<0.05, ** p<0.01, *** p <0.005).

## 4.7. Ex-vivo activity of labeled liposomes in with C57/BL6 mice

Liposomes were labeled with fluorescence molecules as described in Materials and Methods Section (Section 3.2.7.1). FM1-43 was used for labeling neutral, anionic and stealth liposomes; SP-DiOC was used to label cationic liposomes. Labeled liposomes were loaded with D- and K- type ODNs. Treatment groups were as follows; i) UnRX, ii) D+K, iii) ND+SSCLK, iv) AD+SSCLK, v) SD+SSCLK. Mice were injected i.p. with 20 μg of each liposomal or free forms of D- and K- type ODN. After 5 hours, mice were sacrificed; PEC, spleen and lymph nodes were collected. IL6 (Figure 4.29), IL12 (Figure 4.30) and IFNγ (Figure 4.31) secretions were assessed by cytokine ELISA from cell supernatants after 24 h of post ex-vivo incubation without further external stimulation with ODNs at 37°C.

While the levels of cytokine production from each immune tissue investigated gave undistinguishable levels, still it pointed out that ND+SSCLK and SD+SSCLK groups induced substantially higher levels of IFNγ response from spleen. Notably, this trend was same for IL6 and IL12 levels in spleen of treated mice (Figs 4.29-4.31). It is very important to point out that in this Ex-vivo experiment; we injected only 20 $\mu$g of ODN containing liposome, and waited only for 5 h after i.p. injection. It would be very informative to use extra animals and test different times post injection of our Liposomal ODN combinations.

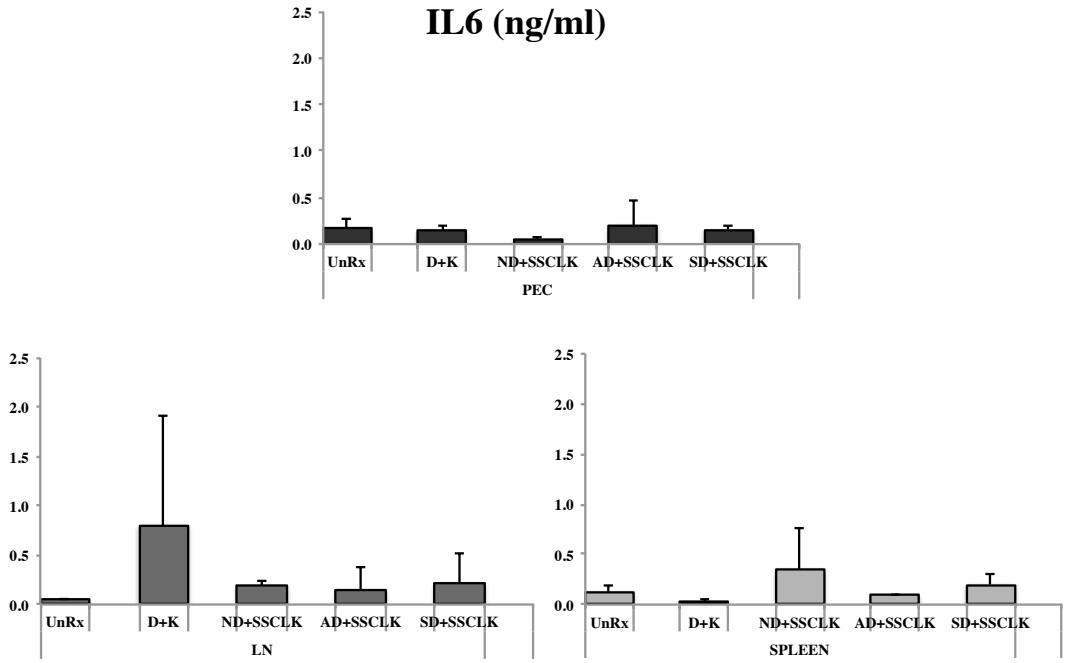



Figure 4.29: IL6 cytokine ELISA results of ex-vivo, experiment was run in duplicates. Cell supernatants were incubated for 24 hours.

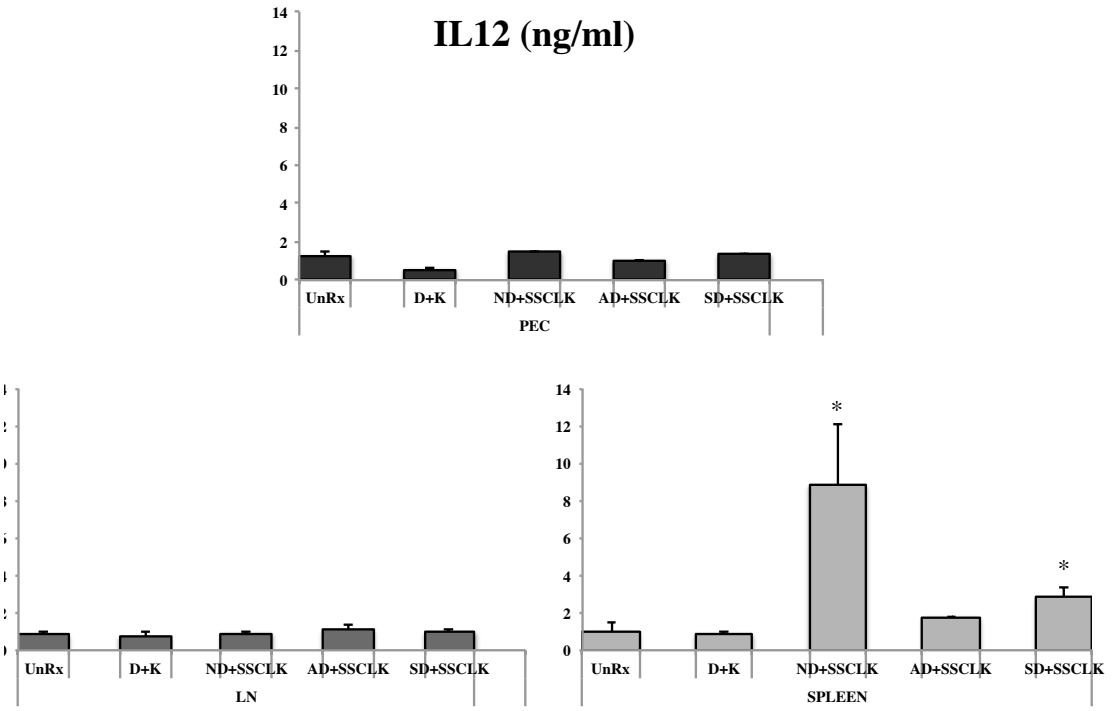



Figure 4.30: IL12 cytokine ELISA results of ex-vivo, experiment was run in duplicates. Cell supernatants were incubated for 24 hours. Paired t-test was done between liposomal combinations and free forms of CpG ODN.( * $p$<0.05, ** p<0.01, *** p <0.005).

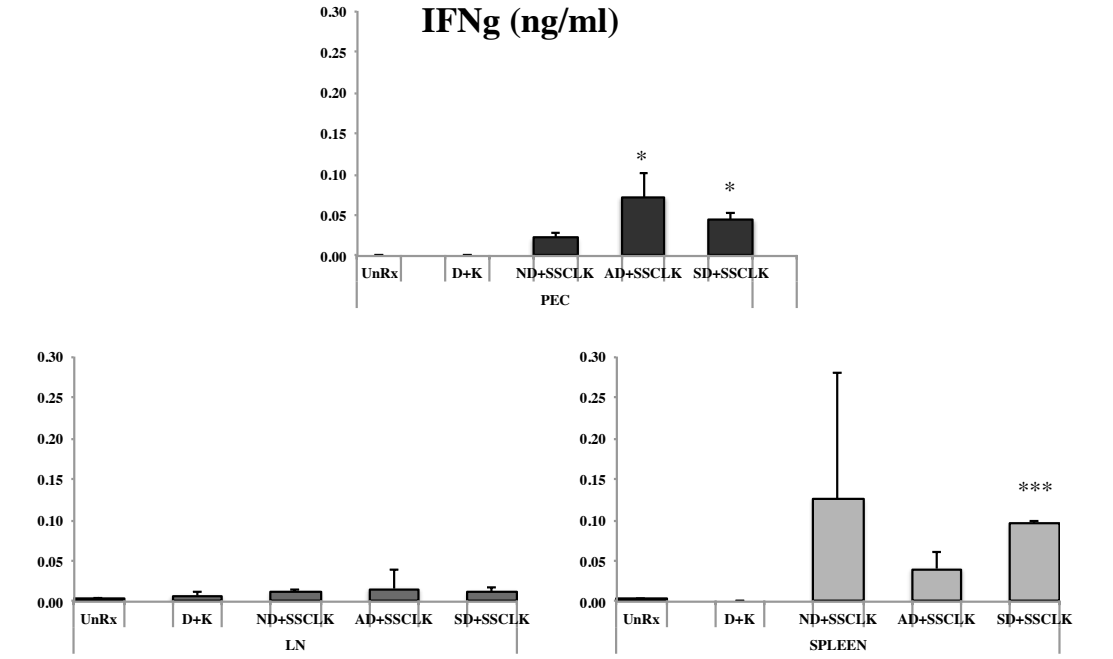



Figure 4.31: IFNγ cytokine ELISA results of ex-vivo, experiment was run in duplicates. Cell supernatants were incubated for 24 hours. Paired t-test was done between liposomal combinations and free forms of CpG ODN. ( * $p$<0.05, ** p<0.01, *** p <0.005).

As we were not very much satisfied with the ELISA results, we then obtained total RNA and checked further the gene expression profiles of several key chemokines and cytokines (i.e. *ip10, cd40, il18 and il15*). In this set of experiments, only the cells from the injection site were used for RNA isolation.

The RT-PCR results, of PECs RNA revealed that (Figure 4.32), compared with D+K, liposomal groups augmented gene upregulation of all tested candidate genes. There was no significant difference between liposomal groups in IP10, CD40 and IL15 levels. Notably, when the IL18 expression was investigated ND+SSCLK and in SD+SSCLK further improved upregulation than other treatments.

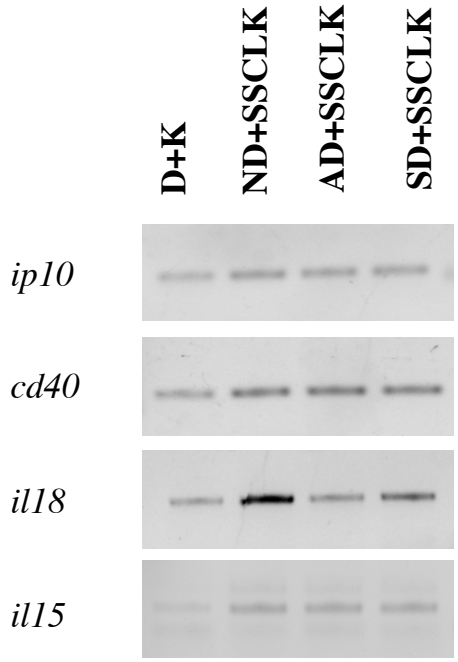
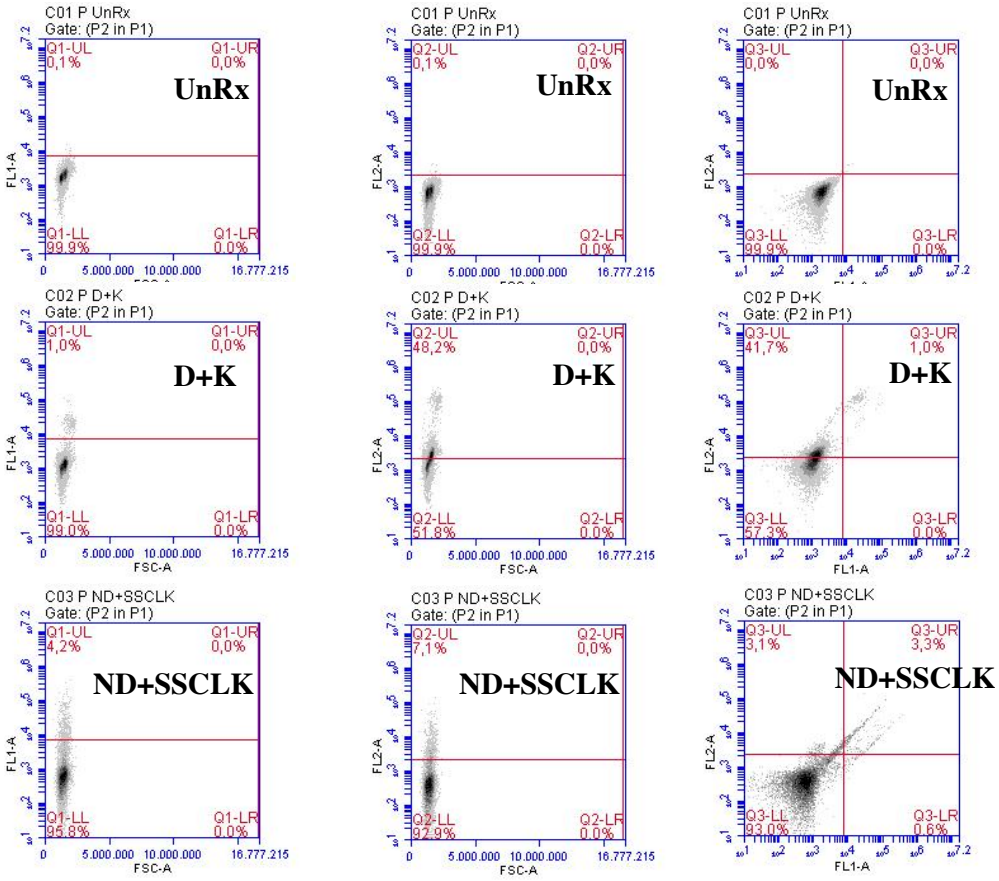



Figure 4.32: PCR products of cytokine-related gene transcripts obtained from total RNA from PEC after 5 hours of stimulation with liposomal and free forms of D- and K- type ODNs. Gene specific amplicons were run on an agarose gel (2 %).

### 4.7.1. Internalization of labeled liposomes by immune cells

Liposomes were fluorescently labeled in order to track their internalization by immune cells. After 5 h of injection, PECs were collected, fixed and analyzed by FACS (Figure 4.33). Labeled liposomes were not detected in spleen and lymph nodes (see Appendix Figure A11-12). According to FACS findings, when D- and K- type

ODNs are loaded into liposomes, while uptake of D-type ODN increased, percent uptake of K-ODN decreased. However, overall percent of the double positive cells for both liposome types increased when CpG ODNs were loaded within liposomes and introduced to cells simultaneously.



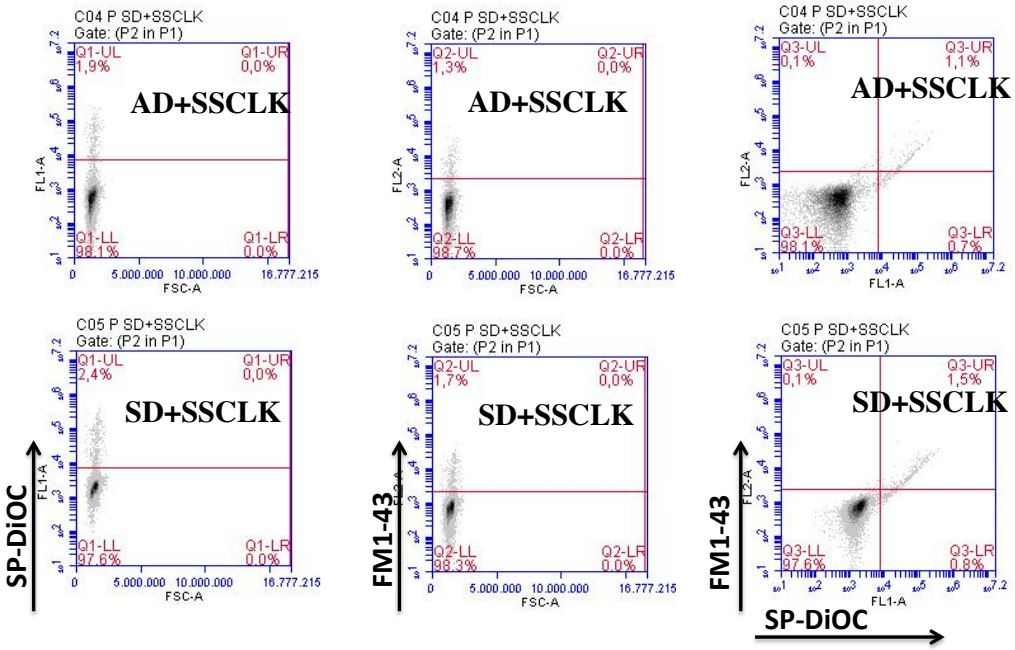
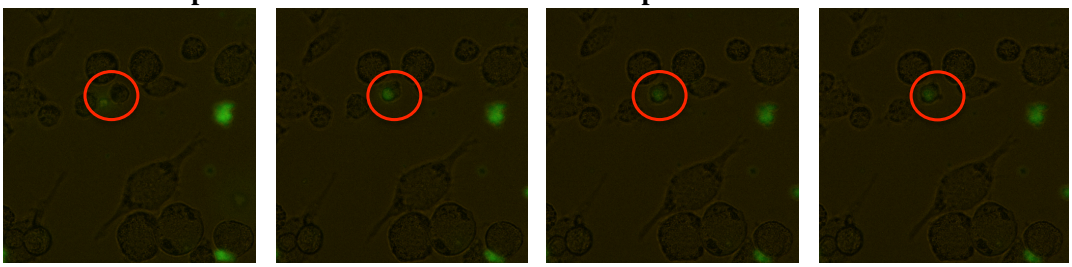



Figure 4.33: FACS analysis of labeled liposomes from PEC. Anionic, neutral and stealth liposomes were labeled with FM1-43; cationic liposome was labeled with SP-DiOC. For free forms, FITC labeled D35 and Cy3 labeled K3 were used. Mice were sacrificed after 5hours, PEC were collected and cells were fixed before FACS analysis

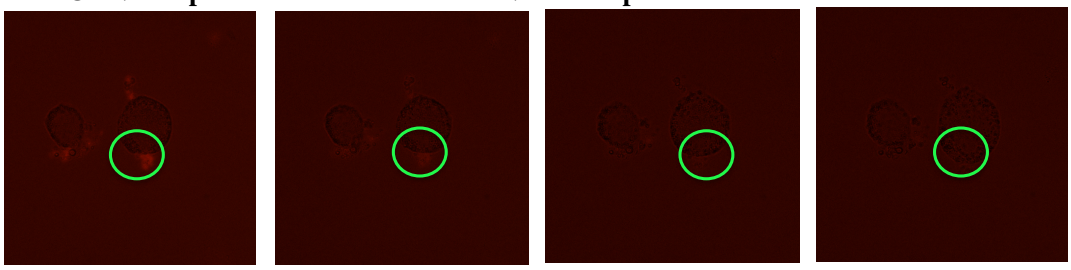
## 4.8. Screening the trajectory of labeled liposomes on mouse cell line by fluorescence microscope

5µg of each labeled liposome; FM1-43 of neutral and SP-DiOC of cationic liposome, were introduced to $1x10^5$/ml RAW cell. Live images were captured with fluorescence microsope, the images were merged, and sequenced into a video. Uptake of both labeled liposome by RAW cells were clearly visualized in less than 30 minutes. Although SP-DiOC did not bleach for the entire investigation period, unfortunately, FM1-43 bleached within minutes of exposure to laser beam. But with htis limitation in mind, it was apparent that both liposomes with an equal rate phagocytosed by RAW cells in 15 minutes.

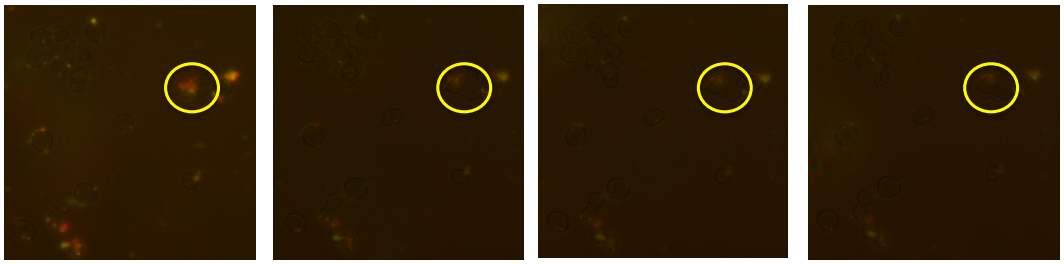
**A. K-ODN encapsulated SPDiOC Labeled Cationic Liposome:**



**B. D-ODN encapsulated FM1-43 Labeled Neutral Liposome:**



**C.SPDiOC Labeled Cationic-K Liposome+ FM1-43 Labeled Neutral-D Liposome:**



**15 seconds** **60 seconds** **105 seconds** **150 seconds**

Figure 4.34 A-C: Fluorescent microscope (Nikon Eclipse Ti) Images 40X with different laser filters for FM1-43 and SPDiOC. Every image was taken with 45 seconds apart. 10 ul of 0.2uM of each liposomal ODN were added to $1X10^5$ cells/well.

# Chapter 5

# 5.Discussion

Extensive preclinical and clinical studies have shown that CpG ODN are effective immunostimulatory candidates to be used in a variety of applications such as vaccine development as adjuvants or in allergic and autoimmune disorders. Despite their success, utility of free CpG ODN is facing many disadvantages.

When D and K- type ODN are mixed and introduced to cells in their free forms only K-specific response is observed. K-type ODN blocks D-specific response. [30],[38]. Moreover to their dichotomy, D-type ODN could not enter to clinical trials due to the difficulties with their manufacturing. Many studies are going on to develop a system that will combine the effects of K- and D- type ODN. New type of CpG ODNs were identified such as C-type, Y-shaped or P-type ODNs that would combine D& K effects, however they were not stimulatory enough compared with D-and K-type ODN [35], [31]. There are different approaches in order mimic D-specific response by multimerezing K-type, such as combining K-type ODN with SPG [39],or forming nano-rings with peptides [34].

It has been shown that no single ODN is universally active. According to Leifer, a mixture of ODN was identified as a significantly greater stimulant compared with any individual ODN. Although, along a population of donors, from a panel of ODN at least one member of ODN responded, a mixture of ODN is broadly more active than any single ODN [57].

According to many studies to improve CpG ODN, this thesis aimed to overcome the dichotomy of D- and K- type ODN and to identify universal liposome combinations encapsulating D- and K- that leads to expression of both D- and K-superior activity. Thus, our approach is to load D- and K- type ODN into different

liposomes and simultaneously stimulate cells with both to observe a combined plus synergistic effect. We used a delivery system because; liposomal encapsulation of CpG has been shown to greatly enhance the stimulatory effect compared with free forms of CpG [55], [52], [59], [60].

Liposomal CpG were used both in mice and human trials. ODN that are highly stimulatory in mice have poor effects in man [61]. The main reason for this is the difference between TLR9 expressing cells between human and mice. In humans, only pDC and B cells express TLR9 whereas in mice other DC subsets and macrophages express TLR9 as well [62]. Hence, different ODN and liposomal formulations were chosen for human and mice experiments.

We observed both D- and K-type specific response, when human and mice cells were stimulated with D- and K-type ODN encapsulated liposomes simultaneously. Liposomal encapsulation overcame the dichotomy of D and K-type ODN. Additionally, enhanced the stimulatory effects of both CpG's free forms. Different liposomal combination groups were chosen for human and mice experiments. Combinations were reduced to 4 based on their ability to induce superior levels IL6, IL12 and IFNγ compared to free forms of ODNs in mice splenocytes. And in human, combinations were reduced to 5 groups according to increased secretion of TNFα and IFNα compared to free forms of ODNs at the same time. Liposome mixing of both ODNs indicated superior combination groups for both mice and men. Liposomal encapsulation may be affecting the mechanism behind uptake of CpG ODNs. TNFα is an earlier cytokine than IFNα. When D and K type ODNs are mixed and introduced in their free forms, the secretion of TNFα may be activating a pathway that blocks IFNα secretion. If this is the case, liposomal encapsulation of K- type ODN may be causing retardation of uptake of K-ODN, causing not so early TNFα secretion. Thus, secreted TNFα would not affect IFNα secretion pathway.

When CpG ODN are separately introduced to cells in their free forms, K-type ODN localizes to lysosomes, whereas D-type ODN localizes to early endosomes. The localization difference of D-type ODN is due to a scavenger receptor CXCL16 found

only on pDC but not on B cells. Both CXCL16 and TLR9 are required for the immune activation for D-type specific response. On the other hand, CXCL16 expression has no effect on the binding or activation by K-type ODN [63]. Another change in the mechanism due to liposomal encapsulation can be the localization of D-type ODN. By liposomal encapsulation, D-type ODN may be localizing directly at the late endosome bypassing CXCL16 recognition. In order to prove this mechanism, CXCL16 positivity of pDCs, which secretes IFNα upon stimulation, can be investigated within the context of liposomal D and K ODN. To fully understand the mechanism leading to synergy by liposomal encapsulation, further research should be conducted.

According to cytokine ELISA results in human, we have observed significantly higher levels of simulataneous secretion of both IFNα (D-type ODN specific cytokine) and TNFα ( K-type specific cytokine) even at the lowest doses of each ODNs (@ 0.2uM), on human PBMC compared to higher doses of free ODNs ( D-type=3$\mu$M and K-type=1$\mu$M). Liposomal formulations managed to increase IFNα levels over 1000X fold higher and TNFα levels over 20X fold higher. TNFα secretions were lower than expected this could be due to the inability of D-ODN to induce TNFα even if we encapsulated within liposomes. After stimulation, TNFα reaches to its maximum concentration at 4 hours, afterwards many upregulated gene expressions decreases within 24 hours post stimulation [56], [64]. Table 4.4 shows that in 9 out 10 donors (90%) our combination; ND+CK resulted in synergism for both TNFα and IFNα secretions. This is a critical finding because human is out-bred population, to this date, there is no reported single universal ODN sequence that could work to trigger TLR9 optimally for everyone [57]. Based on cell differences of TLR9 expression and homology between mice and men and polymorphisms among individuals, liposomal combinations seemed to overcame the ODN differences among specific types and reduced the differences seen between mice and human experiments [62]. Consequently, we established in the present study that utilizing different D- and K- type ODN within liposomes did not affect the overall improved immune stimulatory features. Leading us to conclude that it does not depend too much on

which D- or K- type ODN sequence is used, rather correct liposome type encapsulating these ODNs matter the most.

To our surprise, intracellular cytokine staining experiments revealed that within 5h simultaneous stimulation of pDCs with liposomal D- and K- type ODN (at the lowest dose, 0.2$\mu$M), BDCA-2$^+$/CD123$^+$/IFN$\alpha^+$ fraction of pDCs population rose from 5% to 90% compared with free forms of either D- or K- type ODN (D-type ; 3$\mu$M and K-type; 1$\mu$M), or their free combinations. The 20-fold increase of IFNα secreting pDCs in only 5h post-stimulation should be further investigated, to identify other potential cell source(s) that secretes IFNα is critical since we only observed this phenotypical change only when liposomal D and K CpG ODNs were incubated with PBMCs. In intracellular staining experiments we specifically gated on pDC population to investigate the TNFα and IFNα production from those cells. while over 20X more IFNα production was seen by pDCs, we observed in the lowest dose of liposomal combinations up to 1.6 fold higher TNFα secretion either with SD+CK or with AD+CK combinations compared to optimum doses of free ODNs.

Antigen presentation ability of PBMCs were measured by staining HLA-DQ, a MHC class II isotype and CD80, a stimulatory molecule found on activated APCs. FACS analyses revealed that consistent with IFNα and TNFα secretion from pDCs, liposomal encapsulation of CpG ODNs significantly improved APC functions even at the lowest dose (i.e. 0.2$\mu$M for liposomal combinations; D-type, 3$\mu$M and K-type; 1$\mu$M at free forms). Here, we demonstrated that liposomes not only achieved CpG ODN dose sparing but also augmented immune stimulatory activity. In in vivo application this feature is of critical importance, even very little amount of liposomal formulations reach to target cell site and internalized by immune cells, liposomal cargo would be sufficient to induce APC activation.

In PBMC studies of this thesis, at the end of testing five different liposomal D- and K- type ODNs, a universally active liposomal combination (ND+CK) was identified, which induced both cardinal cytokines specific for D and K-type ODNs nearly in 100% of our tested healthy donors. We further identified that when D-type

was encapsulated in neutral liposome and K-type within cationic liposome, and incubate cells with these combinations simultaneously, not only we ended the dichotomy, but also a synergistic immune activation was yielded in human subjects.

Cytokine ELISA results revealed that in mice splenocytes different liposomal encapsulation of ODNs increase IL6, IL12 and IFNγ secretion D-and K-type specific response although dichotomy is not apparent between D and K CpG ODNs, yet a dose sparing advantage was confirmed in mouse studies, confirming human findings. It was earlier reported that encapsulating K-type ODN in sterically stabilized cationic liposome in mice (i.e. SSCLK) improves immunostimulatory action in vivo [55]. Our current data is consistent with previous studies by Gursel et al. Moreover, combination studies revealed that encapsulating D ODN in several different types of liposome formulations in a mouse strain independent and in a synergistic manner add on to K-specific immune activation as evident by cytokine results presented in Table 4.1.

With liposomal combinations i) ND+SSCLK or ii) SD+SSCLK several critical gene transcripts were augmented upon 5 h in vivo administration. FACS analyses as expected, revealed that injected liposomes were mostly internalized by the phagocytic cells residing at the injection site. Furthermore, at the injection site, uptake of encapsulated D-type ODN increases, whereas liposomal K-type ODN uptake decreases. On contrary, when both liposomal CpG were administered simultaneously, double positive cells for both liposomal formulations, increased significantly.

Mice experiments showed that introducing combination of liposomal encapsulated D- and K- type ODN enhances features of free forms of CpG ODNs. The liposomal groups identified in mice were consistent to that of human studies. This is encouraging, since our future aim is to harness these liposomes in model in vivo mouse studies. However, the underlying mechanism that induced synergy with liposomal encapsulation still deserves further investigation. Utilizing similar formulations will help us to translate those results to human clinical case.

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# 6. APPENDIX

## SUPPLEMENTARY DATA

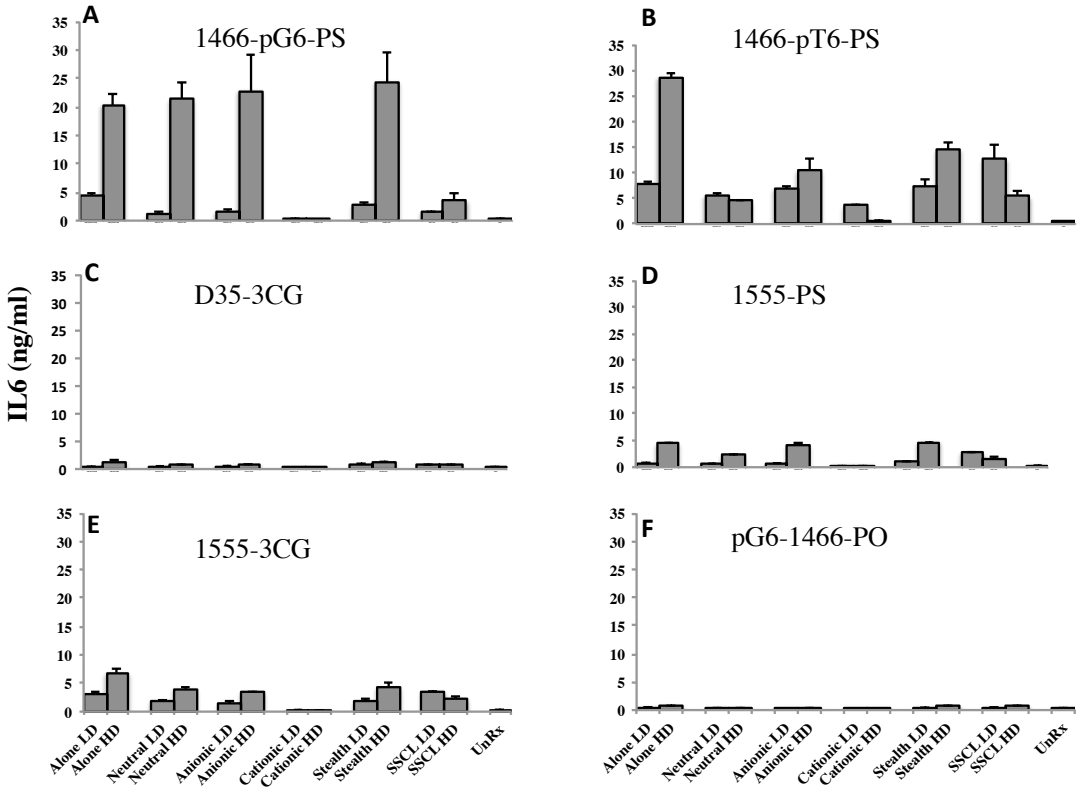



Figure A.1 A-F: Dose dependent IL6 secretion of different liposome formulations and alone forms of candidate CpG ODNs from mouse spleen cells. HD corresponds to 1uM of encapsulated or alone CpG ODN, whereas LD represents 0.2uM ODN concentrations. Experiment samples were run in duplicates and IL6 levels were detected by cytokine ELISA from cell supernatants after 24h of stimulation.

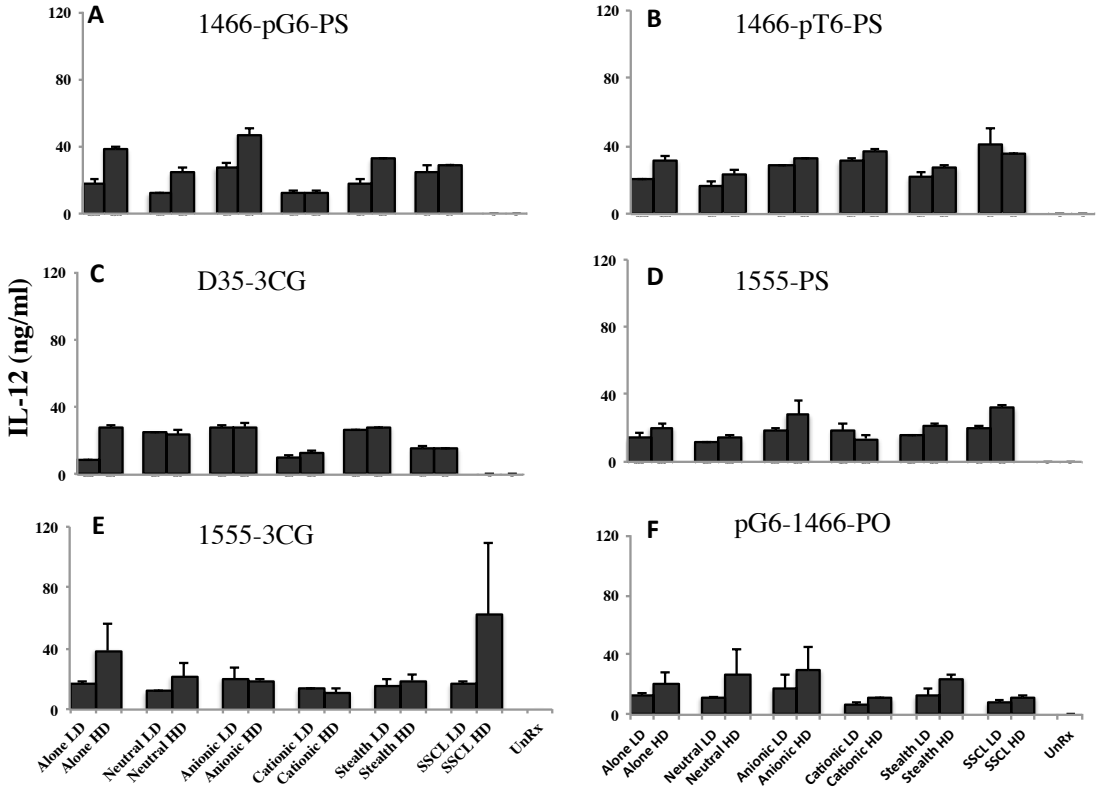



Figure A.2 A-F: Dose dependent IL12 secretion of different liposome formulations and alone forms of candidate CpG ODNs from mouse spleen cells. HD corresponds to 1uM of encapsulated or alone CpG ODN, whereas LD represents 0.2uM ODN concentrations. Experiment samples were run in duplicates and IL12 levels were detected by cytokine ELISA from cell supernatants after 24h of stimulation.

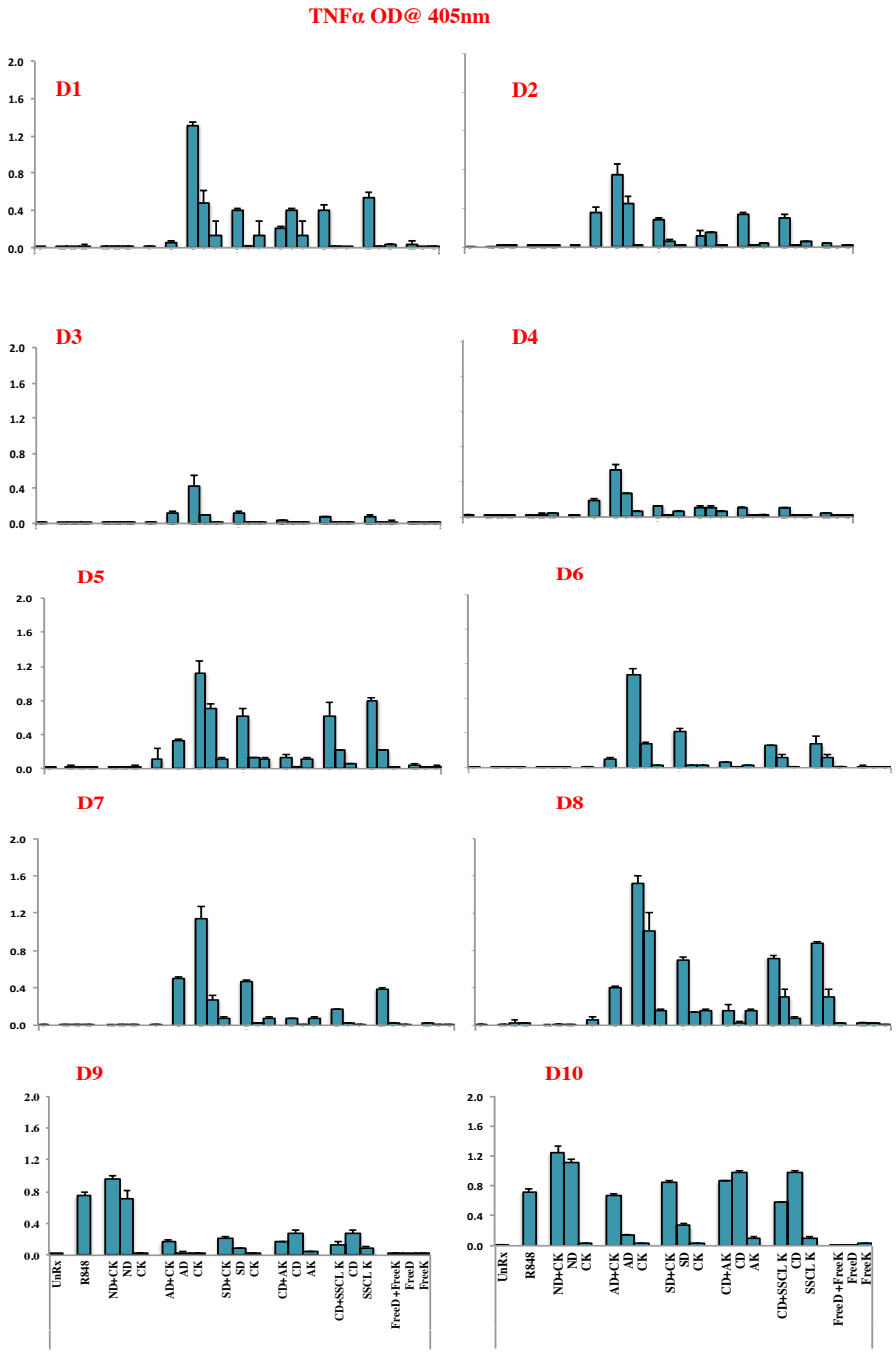



Figure A.3: Cytokine ELISA results for TNFα, with chosen 5 different liposomal formulations for 10 different donors.

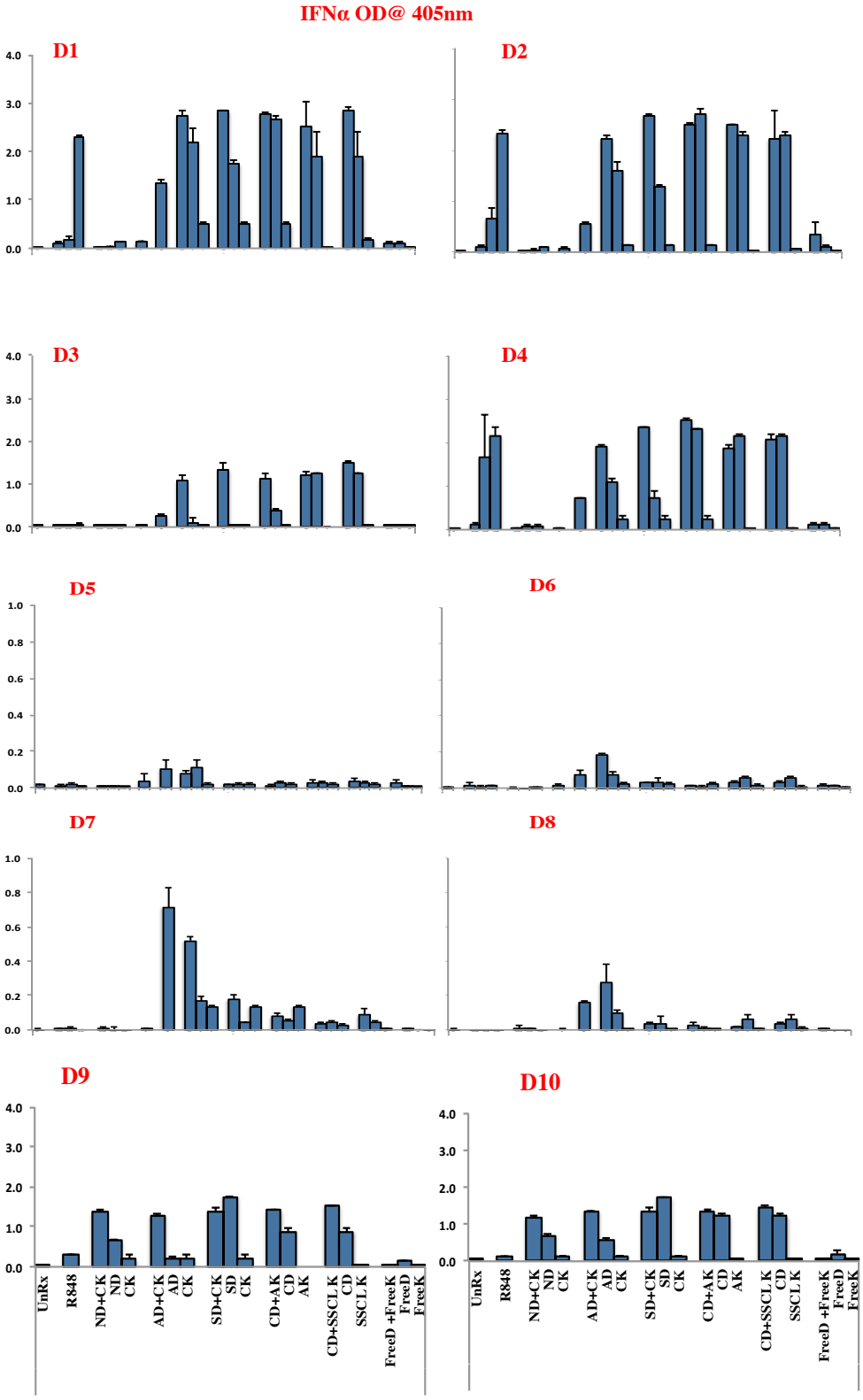



Figure A.4: Cytokine ELISA results for IFNα, with chosen 5 different liposomal formulations for 10 different donors.

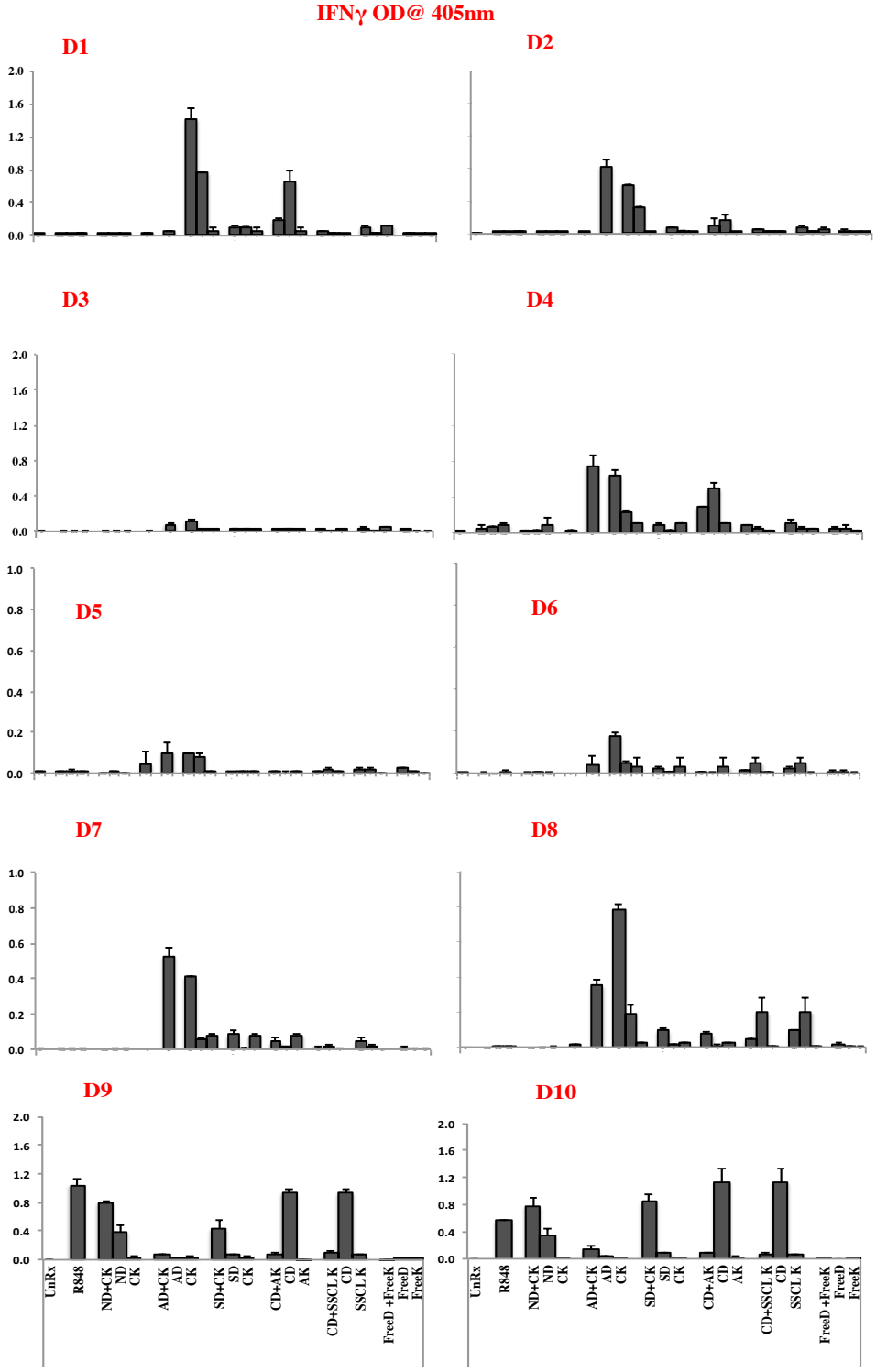



Figure A.5: Cytokine ELISA results for IFNγ, with chosen 5 different liposomal formulations for 10 different donors.

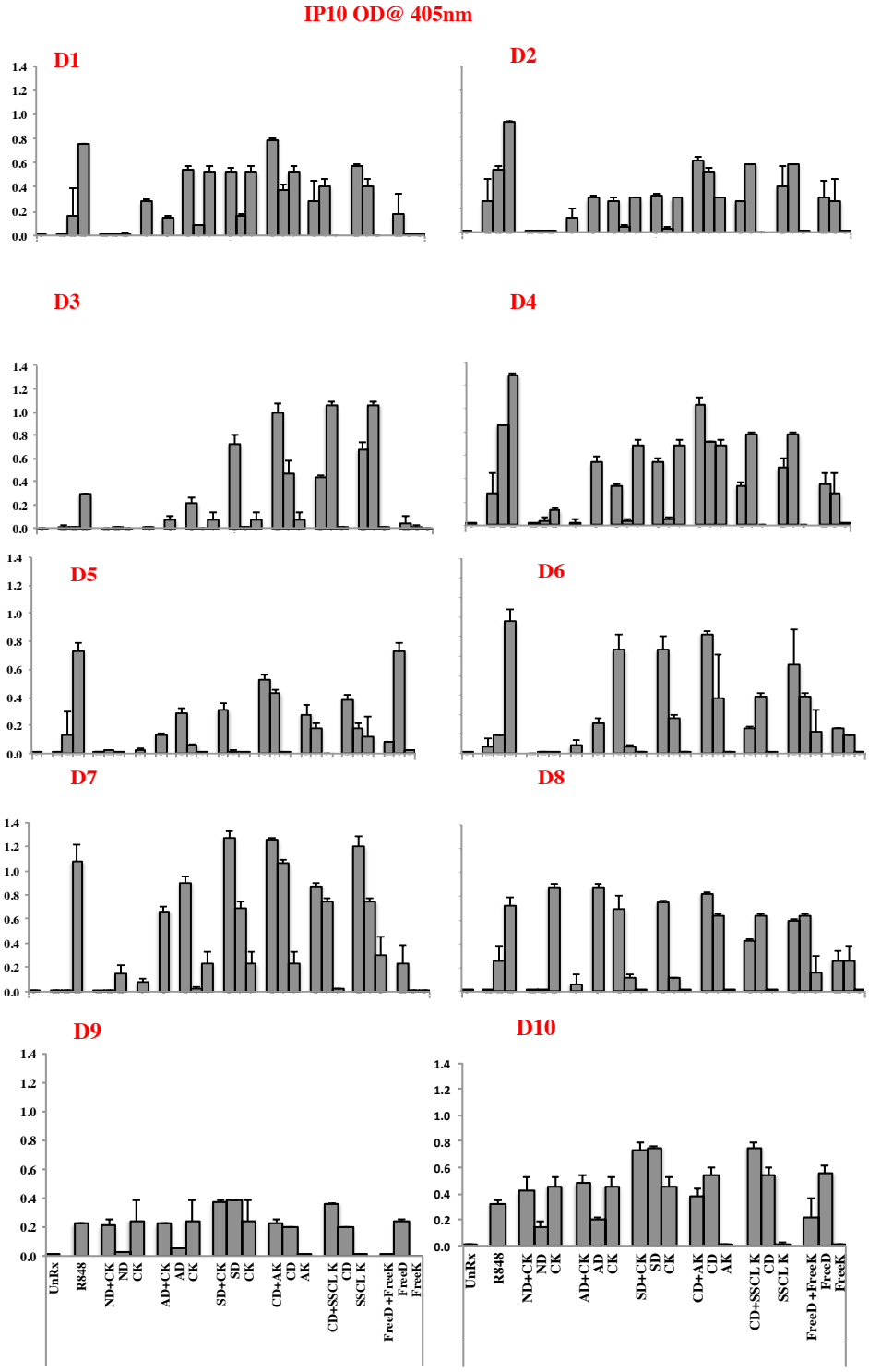



Figure A.6: Cytokine ELISA results for IP10, with chosen 5 different liposomal formulations for 10 different donors.

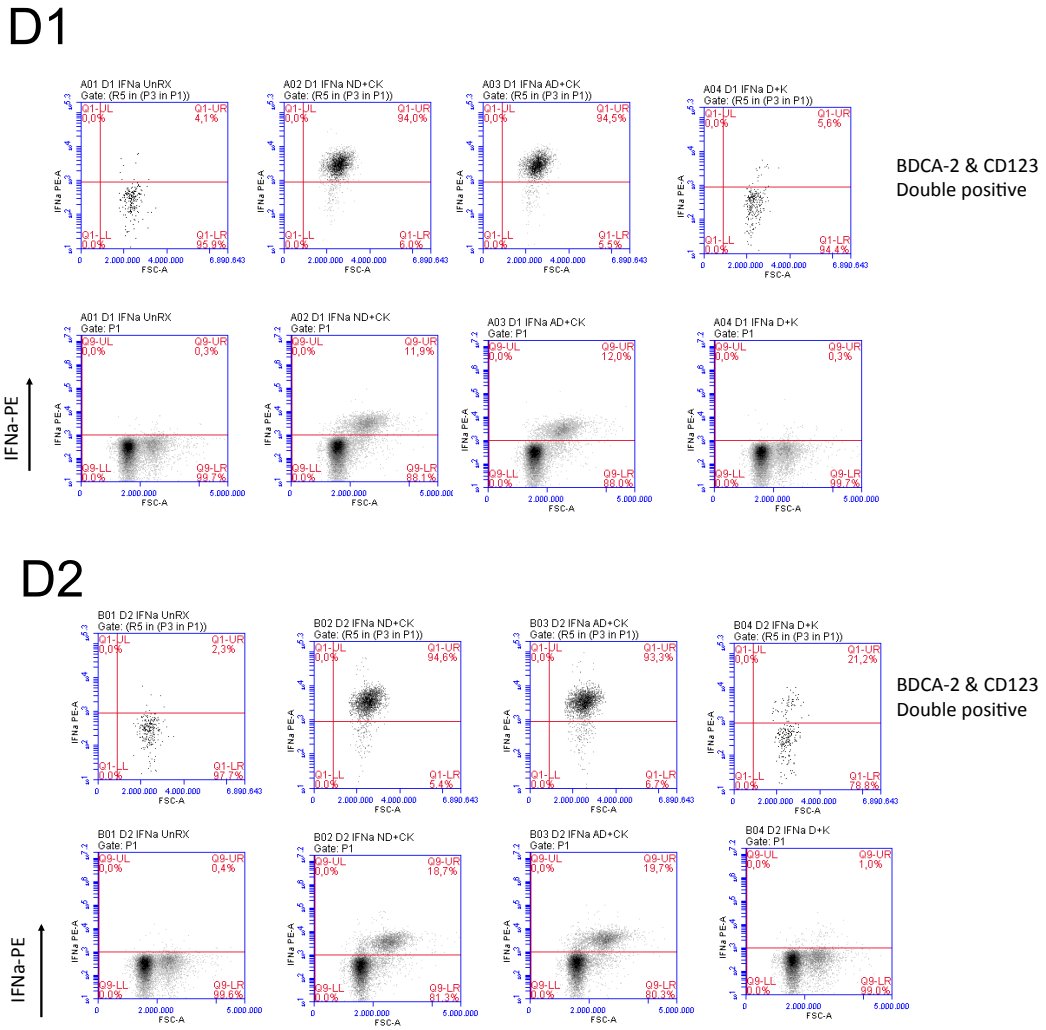
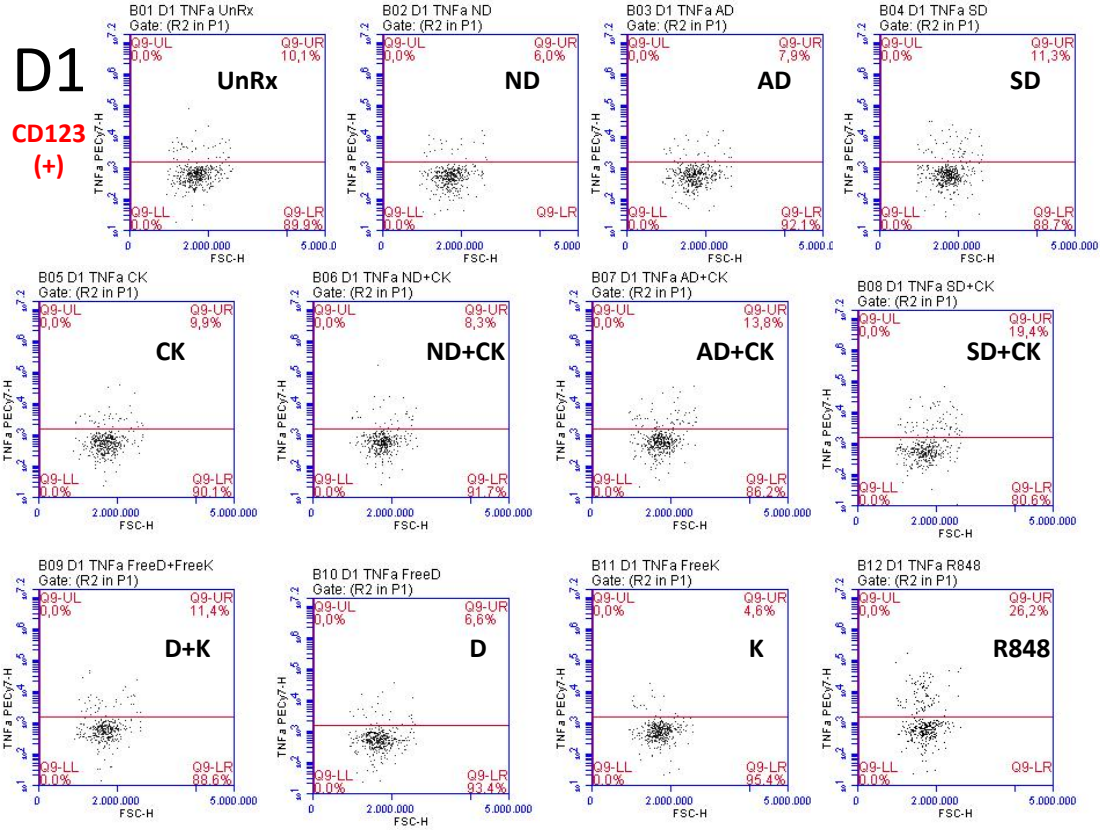



Figure A.7: FACS analysis of IFNα secretion from PBMC isolated from healthy donor 1. pDC were chosen by gating BDCA-2-FITC positive cells. D35-3CG and 1555-3CG were used as D-and K-type ODNs.3μM of D-type and 1μM of K-type ODN were used in D+K; 0,2μM of each ODN were used in liposomal combinations.


D1
CD123 (+)
B01 D1 TNFa UnRx
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 10,1%
Q9-LL 0,0%
Q9-LR 89,9%
UnRx
B02 D1 TNFa ND
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 6,0%
Q9-LL 0,0%
Q9-LR
ND
B03 D1 TNFa AD
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 7,9%
Q9-LL 0,0%
Q9-LR 92,1%
AD
B04 D1 TNFa SD
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 11,3%
Q9-LL 0,0%
Q9-LR 88,7%
SD
B05 D1 TNFa CK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 9,9%
Q9-LL 0,0%
Q9-LR 90,1%
CK
B06 D1 TNFa ND+CK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 8,3%
Q9-LL 0,0%
Q9-LR 91,7%
ND+CK
B07 D1 TNFa AD+CK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 13,8%
Q9-LL 0,0%
Q9-LR 86,2%
AD+CK
B08 D1 TNFa SD+CK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 19,4%
Q9-LL 0,0%
Q9-LR 80,6%
SD+CK
B09 D1 TNFa FreeD+FreeK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 11,4%
Q9-LL 0,0%
Q9-LR 88,6%
D+K
B10 D1 TNFa FreeD
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 6,6%
Q9-LL 0,0%
Q9-LR 93,4%
D
B11 D1 TNFa FreeK
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 4,6%
Q9-LL 0,0%
Q9-LR 95,4%
K
B12 D1 TNFa R848
Gate: (R2 in P1)
Q9-UL 0,0%
Q9-UR 26,2%
Q9-LL 0,0%
Q9-LR
R848
TNFa PECy7-H
FSC-H
0
2.000.000
5.000.000

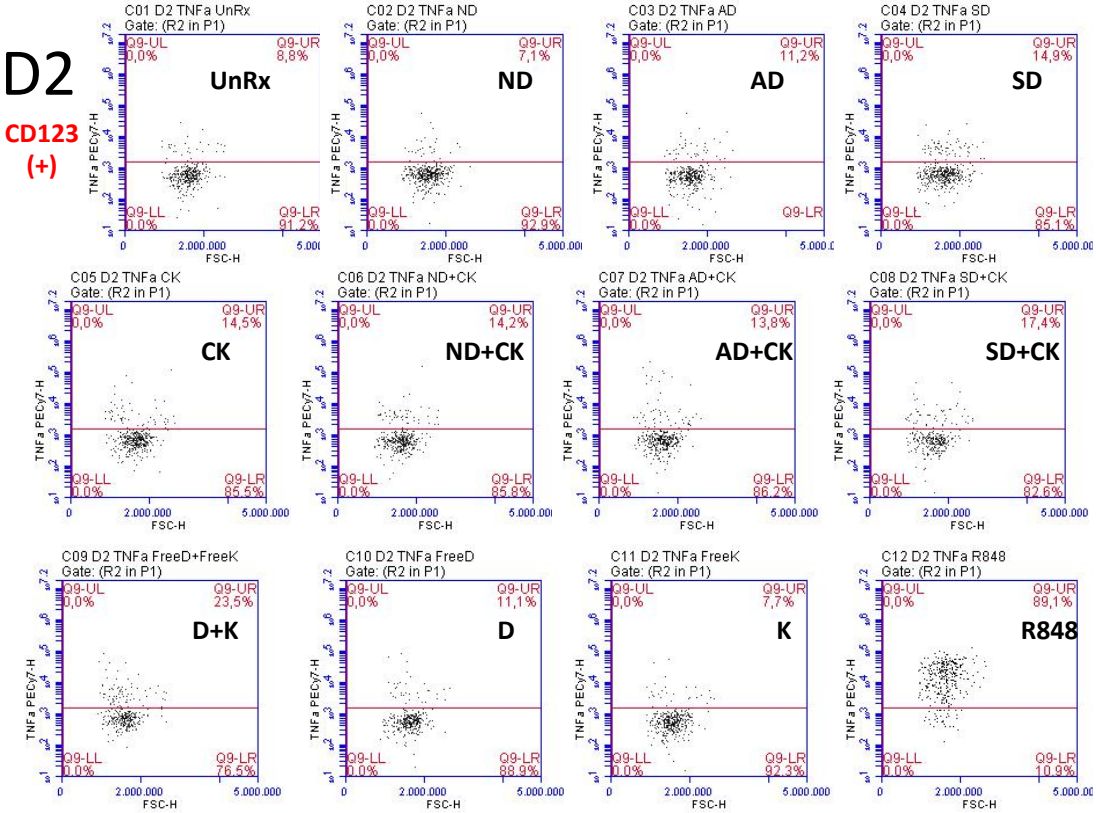



Figure A.8: FACS analysis of TNFα secretion from PBMC isolated from healthy donor 1. pDC were chosen by gating CD123-FITC positive cells. D35-3CG and 1555-3CG were used as D-and K-type ODNs.3μM of D-type and 1μM of K-type ODN were used in D+K; 0,2μM of each ODN were used in liposomal combinations.

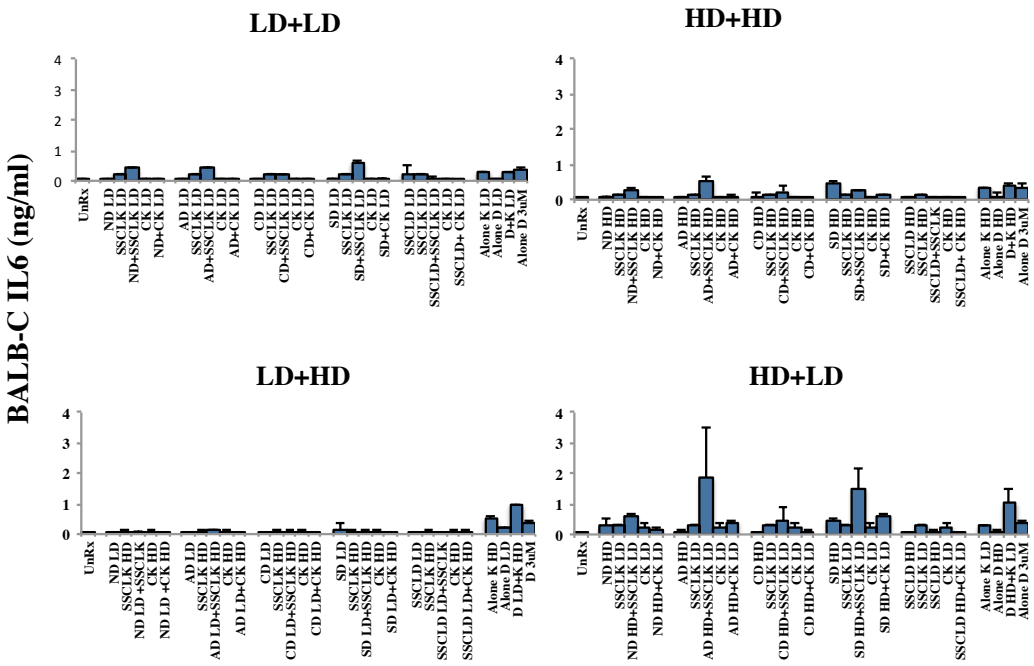



Figure A.9: IL6 cytokine ELISA results Balb-c mice. Different dose combinations were used; LD+LD(0,2uM D ODN plus 0,2uM K ODN), HD+HD (1uM D ODN plus 1uM K ODN), LD+HD (0,2uM D ODN plus 1uM K ODN), HD+LD ( 1uM D ODN plus 0,2uM K ODN). Additionally 3uM of alone D-type ODN was used. Experiments were run in duplicates, supernatants were collected after 24h of stimulation

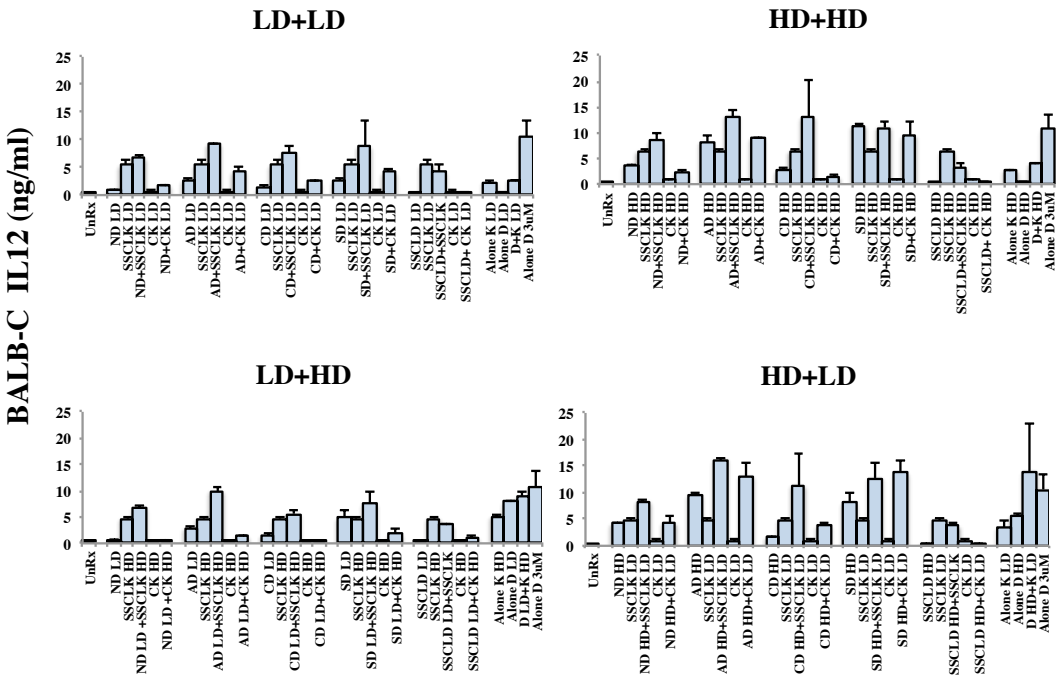



Figure A.10: IL12 cytokine ELISA results for C57/BL6 mice. Different dose combinations were used; LD+LD(0,2uM D ODN plus 0,2uM K ODN), HD+HD (1uM D ODN plus 1uM K ODN), LD+HD (0,2uM D ODN plus 1uM K ODN), HD+LD ( 1uM D ODN plus 0,2uM K ODN). Additionally 3uM of alone D-type ODN was used. Experiments were run in duplicates, supernatants were collected after 24h of stimulation.

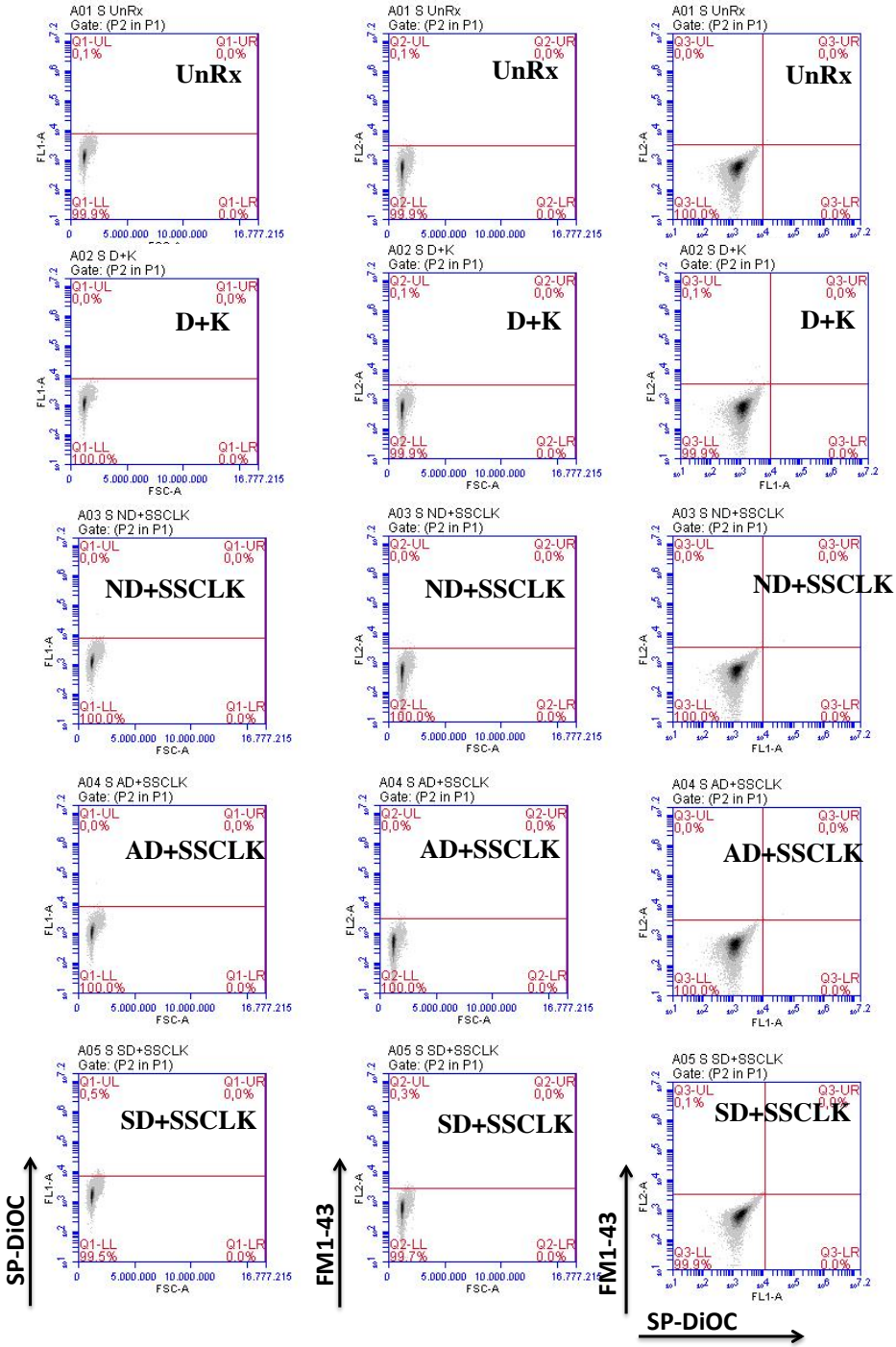



Figure A.11:FACS analysis of labeled liposomes from SPLEEN. Anionic, neutral and stealth liposomes were labeled with FM1-43; cationic liposome was labeled with SP-DiOC. For free forms, FITC labeled D35 and Cy3 labeled K3 were used. Mice were sacrificed after 5hours, PEC were collected and cells were fixed before FACS analysis

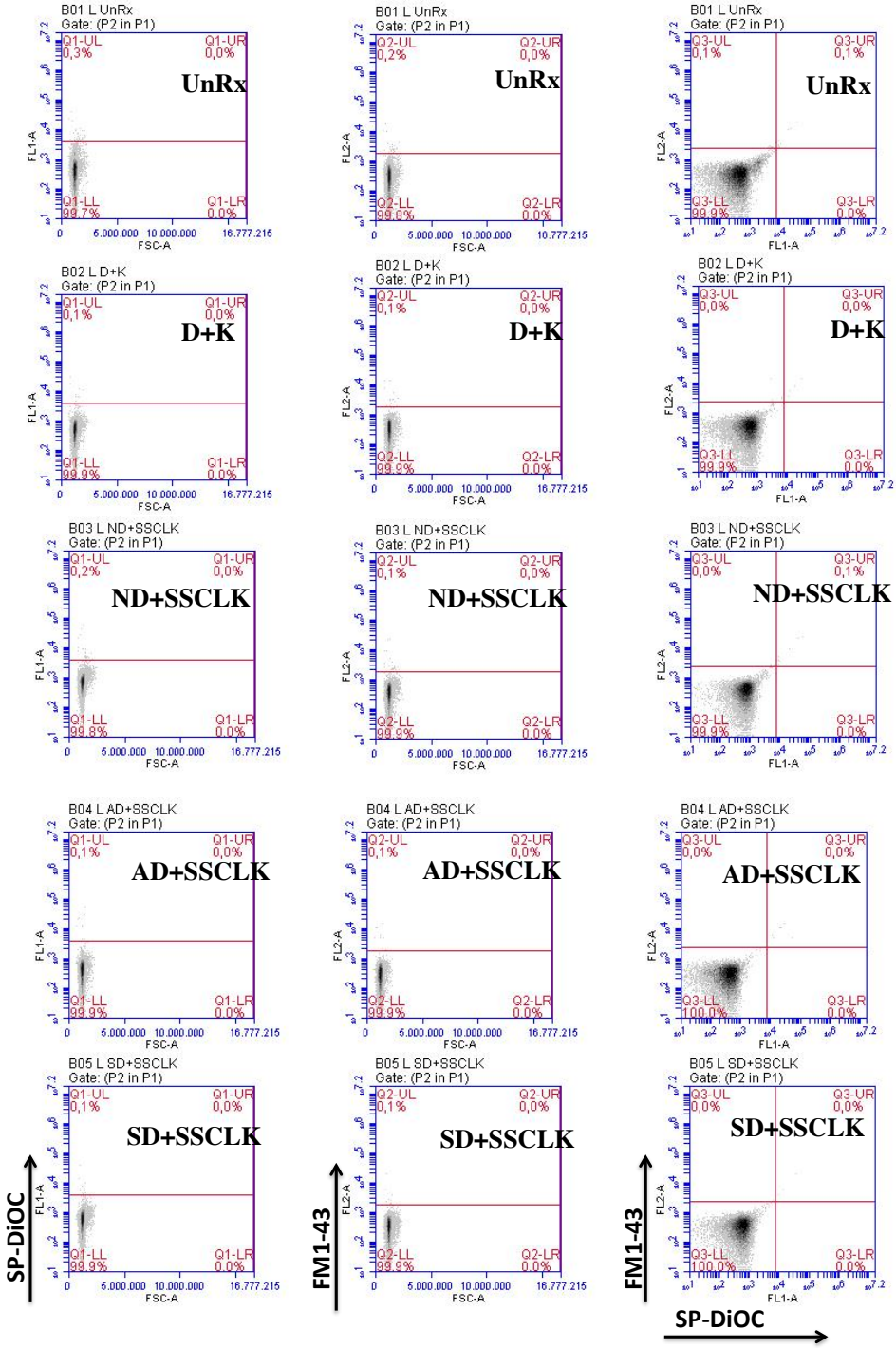



Figure A.12:FACS analysis of labeled liposomes from LYMPH NODE. Anionic, neutral and stealth liposomes were labeled with FM1-43; cationic liposome was labeled with SP-DiOC. For free forms, FITC labeled D35 and Cy3 labeled K3 were used. Mice were sacrificed after 5hours, PEC were collected and cells were fixed before FACS analysis