INVESTIGATION OF IMPROVED IMMUNOSTIMULATORY ACTIVITY OF D AND K TYPE CpG ODNS IN LIPOSOMES

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MASTER OF SCIENCE

By

İhsan Dereli

August, 2013

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. İhsan Gürsel(Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Rengül Çetin Atalay

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Uygar Tazebay

Approved for the Graduate School of Engineering and Science:

Prof. Dr. Levent Onural
Director of the Graduate School

ABSTRACT

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İhsan Dereli
M.S. in Molecular Biology and Genetics
Supervisor: Assoc. Prof. İhsan Gürsel
August, 2013

CpG ODNs are potent immunotherapeutic agents. In human, two major classes of CpG ODNs were shown to induce differential immune activation. D ODNs are strong IFNα inducers, thus promising antiviral agents, whereas K ODNs are effective against bacterial infections. However, their effects cannot be combined. When K and D type ODNs are used simultaneously, K ODN cancels D specific effect, a phenomenon known as K and D ODN dichotomy. The prime reason for this counter acting K ODN action was subcellular compartmentalization of K type CpG ODNs upon internalization. Besides, CpG ODNs have labile nature. When investigated in clinical trials, these nucleic acid based ligands are eliminated upon administration and displayed limited bio-availability due to nuclease digestion. Hence, efforts to protect *in vivo* performance, and increase stability and accumulation near target cells became a crucial task. Liposome technology offers a simple and mild approach to harbor these ODNs within membrane bilayers and protect them. We also reasoned that, if we use liposomes that alter subcellular fate of K and D ODNs, we can retain both K and D effect when liposomal ODNs are co-administered and the breadth of immunotherapeutic spectrum could be improved. This thesis was designed to understand and characterize different types of CpG ODNs loaded into different liposomes and aimed to determine their activities in different *in vitro* and *in vivo* settings. Our results revealed that when two different classes of clinically important CpG ODNs were encapsulated within proper liposome types, it is possible to recapitulate both K and D type ODN effect in PBMCs. Furthermore, in a vaccine model against *H. felis*, although initially did not induce significantly higher anti *H.felis* immunity, liposomal CpG ODNs improved persisting antibody levels for extended periods compared to free counterparts. Collectively, our results demonstrate that this platform allows more effective *in vivo* utilization of CpG ODNs and can be formulated to develop more efficient means to combat several health problems, ranging from cancer to allergy.

Keywords: Liposome, CpG ODNs, *Helicobacter*, Vaccine, D and K dichotomy.

ÖZET

LİPOZOMLARA YÜKLENMİŞ K VE D CpG ODNLERİN ARTAN İMMÜN UYARICI ETKİLERİNİN ARAŞTIRILMASI

İhsan Dereli
Moleküler Biyoloji ve Genetik, Yüksek Lisans
Tez Yöneticisi: Assoc. Prof. İhsan Gürsel
Ağustos, 2013

CpG ODNler etkili immünoterapötik ajanlardır. İnsanda iki ana ODN sınıfının ayrık immün aktivasyonları tetiklediği gösterilmiştir. D ODNler güçlü IFNα indükleyicileri olarak önemli antiviral ajanlar iken, K ODNler bakteriyel enfeksiyonlara karşı öne çıkar. Fakat, bu ODNlerin birlikte aynı anda uygulanması çalışmaları D ODNe özgü etkilerin yok olduğunu göstermiştir. Bu olgu K ve D ODNlerin zıt etkisi olaraka bilinir. Bu zıt etkinin en önemli sebebi K ve D s ODNlerin içe alımı sırasında hücre içinde farklı kompartmanlara konuşlanmasıdır. Bundan bassa, kırılgan bir doğaya sahip olan CpG ODNler , bulaşıcı hastalıklar ve alerjik semptomlar gibi belirli durumlara karşı klinik denemelerde nükleazlar tarafından parçalandıkları için sınırlı etki göstermektedirler. *In vivo* etkisini korumak , dayanıklılığını, hedef hücreler tarafından alınımını artırmak için bu ajanları uygun taşıyıcalara yüklemek önem arz etmektedir. Lipozom teknolojisi bu ODNleri çift zar tabakası içinde korumak için basit ve zararsız bir yaklaşım sunmaktadır. Bundan başk a, K ve D sınıfı ODNLerin hücre içindeki akıbetlerini değiştirebilen lipozomlara ayrı ayrı yüklenerek birlikte uygulanması ile K ve D ODN etkisinin birleştirilebileceğini öngördük. Bu tez farklı lipozomlara yüklenmiş değişik CpG ODNleri daha iyi anlamayı, karakterize etmeyi, in vitro ve in vivo ortamlarda uygulamayı amaçlamaktadır. Bizim sonuçlarımız göstermiştir ki K ve D sınıfı ODNleri uygun lipozomlara yükleyip iki tip ODN etkisini de insan kan periferik kan hücrelerinde görmek mümkündür. Bundan başka lipozomal CpG ONDNler *H. felis* aşı modelinde, başta daha iyi performans gösteremeseler de, daha uzun süreli antikor üretimi sağlamıslardır. Dolayısıyla, nükleik asit tabanlı TLR ligandlarının *in vivo* etkisini klinik ortamda iyileştiren bu platform, kanserden alerjiye bir çok sağlık problemine karşı daha etkili tedaviler geliştirilmesi için kullanılabilir.

Anahtar sözcükler: Lipozom, CpG ODN, *Helikobakteri*, Aşılama, K ve D karşıt etkileri.

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Without my family, I wouldn't be born from the beginning, but their support and love had much more important reasons for my accomplishments than that.

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ABBREVIATIONS

APC	Antigen presenting cell
bp	Base pairs
BCR	B-cell receptor
Cag	Cytotoxin associated gene
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
FBS	Fetal Bovine Serum
Ig	Immunoglobulin
IκK	Inhibitor kappa B kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IFN	Interferon
IP	Interferon induced protein
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
MALP	Mycoplasmal lipopeptide
MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein

MSR	Macrophage scavenger receptor
MyD88	Myeloid Differentiation Primary Response gene 88
NF-κK	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pIC	polyinosinic acid:cytidylic acid
PNPP	Para-nitrophenyl pyro phosphate
PRR	Pattern recognition receptors
RIG	Retinoic acid inducible gene
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SA-AKP	Streptavidin Alkaline-phosphatase
SSCL	Sterically stabilized cationic liposomes
ssRNA	Single-stranded RNA
TCR	T-cell receptor
Th	T-helper
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated factor

TRAM	TRIF-related adaptor molecules
TRIF	TIR domain containing adaptor inducing IFN-γ

Chapter 1

Introduction

1.1 The Immune System

History of earth and the course of evolution have been and are being influenced by continuing competition between organisms. As such, one of the most striking and interesting race happens between pathogens and their hosts. Pathogens persistently and relentlessly try to insult host organisms. Therefore, all living organisms developed means to protect themselves from pathogens. Those defence systems are usually multilayered and interrelated and most of the time complexity differs from each other. Even certain bacterial species have developed complex defense system against phage infection [1] . In higher organisms, collection of multiple defense mechanisms is called the immune system.

In mammals, although the exact content may vary, protection against pathogens starts with a natural barrier the skin, which is followed by mucosa. Mucosa do not only form a simple physical barrier, but is a crucial element of immune system, as it harbours a special site for mucosal immunity. Mucosal immunity has particular importance, as it covers the largest area in body that continuously encounter with gut or respiratory pathogens and therefore mucosal

immunity is an important branch of the innate immune system [2]. Innate immune cells are the second barrier that pathogens must breach. Working principle of innate immunity is generic, and a very unique one. Innate immunity exploits the one of the oldest mechanisms that organisms developed to survive; discriminating between self and non-self by recognizing unique molecular structures. Charles Janeway introduced the pattern recognition model for immune activation in his historical talk [3]. As mentioned above, there is a constant race between hosts and pathogens. Since pathogens has an advantage in terms of rate of evolution, mammalian innate immune system learned to differentiate evolutionary conserved Pathogen Associated Molecular Patterns (PAMPs), such as lipopolysccharide (LPS) lipoteichoic acid (LTA) or certain peptidoglycans (PGN) that are not expressed by self but only expressed by pathogens via germline encoded pathogen recognition receptors (PRRs) [4].). A few years later, Polly Matzinger extended Janeway's postulation by proposing the ability of the innate immun system to discriminate between normal and pathological host sites [5], namely, The Danger Theory. Her model includes PRR recognition of Damage Associated Molecular Patterns (DAMPs), which are found in hosts normally and initiates immune reaction in disease conditions [6]. Once innate immunity is activated, an inflammatory response is ensued to eliminate the persisting infection.

Adaptive immune system, or acquired immune system function cooperatively with innate immune system to eliminate persisting pathogens despite of initial attempts of innate immunity. In fact, adaptive immunity needs to be activated by innate immunity. When innate immunity is activated by pathogens, antigen-presenting cells (APCs) of the innate immune system, which resides within peripheral tissues in immature state, quickly phagoctose microbes or infected cells. Fragmented microbial peptides are loaded onto major histocompatibility complex (MHC) class I or II molecules and displayed on surface of APC cells. MHC class I and MHC class II molecules presents those microbial peptides to CD8 and CD4 T cells, respectively At the same time co-stimulatory proteins CD80 and CD86 on surface of APCs are upregulated, which enables naive T cell activation in the lymph node [7]. A scheme covering basics of antigen presentation is showed in

Figure 1.1.). As innate immunity cope with conserved structures of pathogens to overcome infection while developing no specific memory, adaptive immunity requires diversity in order to deal with vast range of pathogenic repertoite which are already exposed to innate system. In the naive state of T and B cells, upon encountering an antigenic determinant, a series of somatic recombination of genes that encode antigen receptors; T cell receptor (TCR) and B cell receptor (BCR) is initiated. This system allows generation of vast numbers of different receptors, specific to each epitope, with different specificity. When an antigen receptor is stimulated by an APC, the cell that receptor belongs to undergoes clonal expansion, rapid proliferation and commitment. This state is known as the formation of effector T and/or B cells, and at this stage, they are out of their naive state. B cells, however, cannot be activated de novo by APCs. They require a sub-population of T cells called T-helper cells for activation. TCRs and soluble BCRs (antibodies) specific for a peptide epitope portion of microbial antigen eventually help elimination of the insulting organisms. Adaptive immunity is specific and long lasting, due to establishment of B and T cells memory.

1.2 Innate Immune System

Innate immunity is the first line of defense of the immune system and composed of several different systems and cell types. As mentioned above, mucosal immune system covers the largest area that body encounters with pathogens or harmful substances. Some major components worth mentioning includes intestines, bronchial airways, mouth and mucosal surfaces. Moreover, epithelial tissues secretes mucosa that forge a natural barrier and antimicrobial peptides against invading pathogens [10]. Mucosal immune system also hosts several specialized immune cell types, such as mucosal-associated invariant T lymphocytes (MAIT) [11]. Thus, innate immunity provides first physical and chemical barriers against pathogens [12].

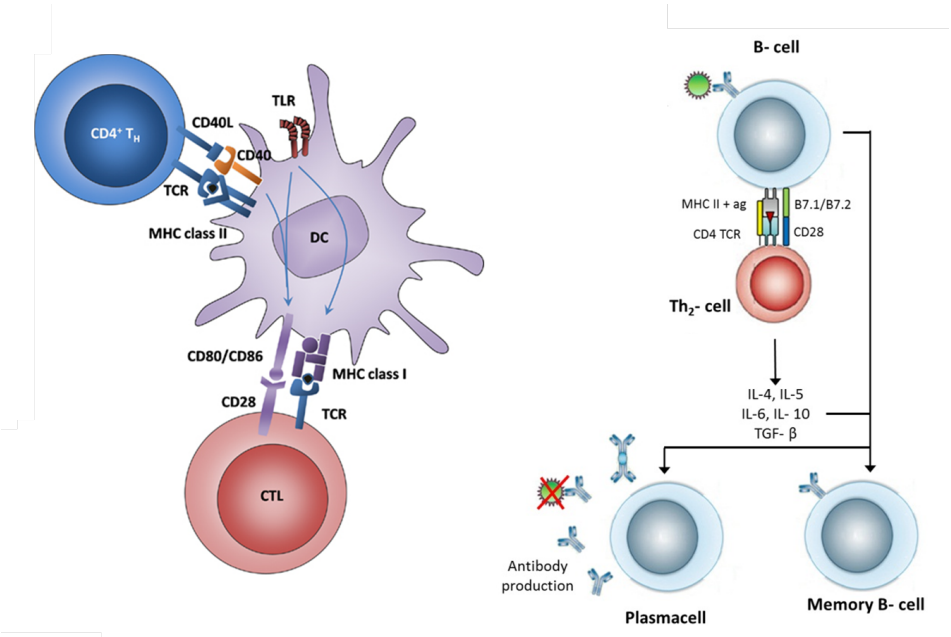

Figure 1.1: Activation of DC upon Ag internalization and presentation of epitopes to T and B cells. Adapted from [8, 9]

Innate immune system cells are challenged by diverse range of pathogens and it must have ability to deal with different complications. Therefore, immune system has cell types that are able to accomplish different tasks in case of an infection. Major cell types of innate immune system are phagocytes (i.e. neutrophils, monocytes/macrophages, DCs), natural killer (NK) cells, mast cells, eosinophils and basophils.

NK cells destroy cells that lack MHC I molecule on their surface. All host cells normally express MHC I molecule on their surface, which prevent NK cells to attack host cells. In case of viral infection [13], or tumor growth [14, 15], MHC I molecule is downregulated and along with additional signals, NK cells recognizes and kills those altered host cells.

Mast cells, eosinophiles and basophiles are characterized by granules in their cytoplasm, and they are commonly called as granulocytes. Those granules are

histamine and heparin rich, along with chemokines and toxins. Upon infection, the contents of granules are released into environment. Histamine causes vasodilation and recruits other immune cells to site of the infection. Chemokines are also important for recruitment of other immune cells. Toxins are effective measures against pathogens. However, granulocytes are double edged swords of immune system, as they have active roles in allergic diseases [16, 17, 18]

Neutrophiles, monocytes/macrophages and dendritic cells are defined under common term phagocytes, due to their very alike working mechanisms when they encounter with pathogens. Neutrophils are the first cells that arrive the site of infection, and they help elimination of pathogens by releasing toxins or generating reactive oxygen species, such as hydrogen peroxide (H_2O_2), a process known as respiratory burst). Macrophages are mature monocytes and both cell types utilize a similar system to eliminate pathogens. Macrophages differ from blood circulating monocytes as they reside on tissues. Arguably, macrophages are the most efficient type of the phagocytes, due to their ability to ingest large number of microbes. Dendritic cells are also digest microbial particles or infected cells. But their main role is not elimination of infection, rather antigen presentation to T cells, thereof mature dendritic cells are called the sentinel antigen presenting cells (APCs). Immature dendritic cells circulate through body. When an infection occurs, they migrate to site of infection, phagocytose pathogens or remnants of infected cells. They process microbial particles so that TCRs can bind them, remove them to their surfaces and present to T cells. Along with co-stimulatory molecules, this entire procedure ensures proper activation of T cells, consequently activation of adaptive immune system.

One major element of the innate system is the complement system. Complement system is actually a cascade of soluble proteins. Once this cascade is activated by presence of pathogens, complement system proteins are sequentially cleaved. Final product forms a pore structure in the membranes of pathogens, causing rapid lysis of the pathogens. Marking the pathogens via opsonization and attracting macrophages and neutrophils via chemotaxis are other crucial functions

of complement system.

Apart from cells and complement system, innate immune system is characterized by initiation of inflammation, a rapid and strong mechanism that prevents spread of infection. Inflammation is started by recognition of pathogens, through their PAMPs PRRs. PAMPs are reliable source to identify pathogens, due to their evolutionary conserved structures, presumably because they are vital for pathogens survival or function. Inflammation can be described as, and much precisely, concerted activation of immune system through communication of immune cells via chemokines and cytokines. Chemokine and cytokine secretion leads to migration of granulocytes, macrophages and dendritic cells, along with other immune cells, to the site of infection. Thereafter, immune cells try to eliminate the infection. However, once more, everything starts from recognition of pathogens by innate immune cells..

1.2.1 PAMPs and PRRs

As already mentioned numerous times, PAMPs of pathogens are recognized by PRRs of immune cells. PAMPs are evolutionary conserved and invariant structures among broad range of organisms belong to microbial world. PRRs are germline-encoded relatively small group of receptors that are highly expressed by immune cells.

Lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acids and cell-wall lipoproteins, fungal cell wall product B-glucan, unmethylated cytosine-phosphate-guanine (CpG) motif rich bacterial genomic DNA, yeast zymosan, viral single and double stranded RNAs (ssRNA and dsRNA), are among widely studied PAMPs [4, 12, 19].

Some PRRs are secreted to body fluids. Mannan-binding lectin, C-reactive protein (CRP), and serum amyloid protein (SAP) are well known examples [20]. A group of PRRs are classified as cytoplasmic PRRs. Nucleotide binding

oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG)-like helicases (RIHs) are cytoplasmic PRRs [4]. NLRs are mainly responsible for detecting bacterial or viral components, while RLRs s and TLRs can recognize variety of PAMPs [20, 21]. A summary of PAMPs and PRRs is given in Table 1.1

Table 1.1: Pattern recognition receptors and their ligands. Adapted from Ishii et. al. [19]

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

TLR: toll like receptors, RLR: RIG like receptors, NLR: NOD like receptors, CLR: C-type Lectin like receptors

TLRs are the most abundantly and extensively studied PRR family. Toll was originally identified in Drosophila (fruit fly) as an important molecule for

embryonic development [22]. Later on, it was revealed that a group of molecules which are associated with immune activation have surprising homology with Toll [23]. Not long after, immunogenic role of Toll was discovered and termed as Toll-Like Receptors (TLRs) in humans and mice have arisen as important immune system factors [24] . TLRs are members of IL1R superfamily and they contain ectodomain leucine-rich repeats (LRR) that mediate the recognition of PAMPs, a transmembrane region, and intracellular Toll-IL-1 receptor (TIR) domains that mediate downstream signaling pathways. They can reside on cell surface, as well as intracellular compartments (Figure 1.2).

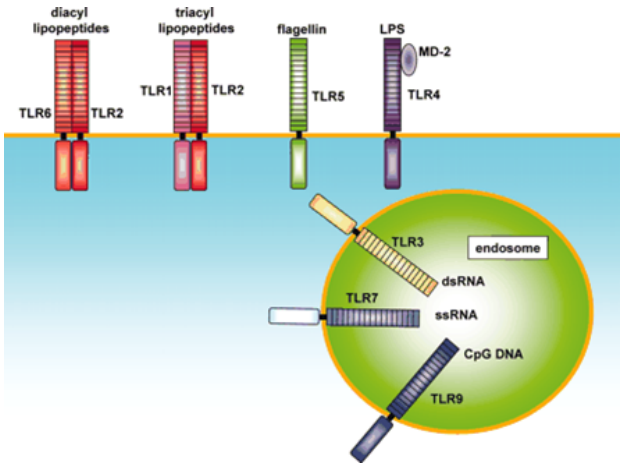

Figure 1.2: Cell surface and endosome associated TLRs with their representative ligands. Note that some TLRs requires dimerization for their activation via their ligands. Adapted from Takeda and Akira [25]

1.2.1.1 Cell Surface Toll-Like Receptors

TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 (only in mice) are positioned on the cell surface. They are mainly responsible for recognizing microbial cell membrane molecules. TLR2 is usually forms heterodimers with TLR6 or TLR1 [26]. Therefore, those heterodimers and TLR2 itself has ability to recognize PAMPs that belong to diverse range of species [26]. In general, Triacylated lipopeptides signal

through TLR1-TLR2 heterodimers, while diacylated lipopeptides signal through TLR2-TLR6 heterodimers, although it is also suggested that not only lipid moiety but also peptide part is responsible for determining ligand-receptor interaction in hosts [27]. Ligands of TLR2 include LTA and PGN from Gram-negative bacteria, Zymosan from *S. cerevisae* and lipoarabinomannan from *Mycobacteria* or the hemagglutinin protein from measles virus [19].

TLR4 is one of the earliest identified TLRs and co-receptor for LPS [28]. It was demonstrated that TLR4 mediates the LPS responsiveness in TLR4 deficient mice, which were hyporesponsive to endotoxin challenge [25, 29].). Later studies discovered that, TLR4 actually is a co-receptor and LPS mediated signaling requires activation and collaboration of other molecules as well, such as CD14 and LPS binding protein (LBP) [30].

TLR5 recognizes bacterial flagellin. Flagellin is an important protein for bacterial motility [31]. Indeed, TLR5 is expressed on the basolateral side of the epithelia, but not on the apical side, which explains why only invading microbes, not commensal bacteria induce an immune response [32].

TLR11 is not present in humans, but present in mice and have a role in recognizing bacteria that infects bladder and kidney as it is highly expressed in bladder in kidney [33]. Additionally, protozoan profilin-like protein (i.e. toxoplasma gondii) is one of the major ligands for TLR11 [4, 34].

1.2.1.2 Intracellular Toll-Like Receptors

TLR3, TLR7 and TLR8 (functional only in human), TLR9 and TLR10 [25] are found in intracellular compartments (REFS). TLR3 recognizes dsRNA and ssRNA which are common and important intermediate products of viral replication [35]. Since TLR3 is positioned on intracellular compartments, it is also evident that TLR3 has important role against viral infection [36].The synthetic dsRNA analog polyinosinic-polycytidylic acid (polyIC) is an important candidate antiviral

reagent, due to its ability to activate TLR3 signaling [37].

TLR7 and TLR8 recognize ssRNA. Genomes of many RNA viruses are ssRNA and they were shown to be activated by RNA virus infection [36]. TLR7 is highly expressed in pDCs and upon activation of TLR7 signaling pathway, expression of antiviral type IFNs, most notably IFNa is induced in pDCs [38]. A prominent immune modifier and anti-viral reagent, resiquimod R848 (guanine nucleotide analog), is in fact a synthetic ligand of TLR7 [39]

TLR9 recognizes unmethylated CpG motifs. In addition to CpG suppression, mammalian genome contains methylated CpG residues. Of note, bacterial genome represent 25-30 fold more unmethylated CpG dinucleotides compared to mammalian genome. [40]. While physiological role of TLR9 is certainly significant, most of the literature is related to the immunotherapeutic applications of TLR9. Certain types of synthetic CpG ODNs can induce type I IFN secretion, while other CpG types are potent pro-inflammatory cytokine inducers [41]. It should be noted that many factors affect the final outcome, such as pH, compartmentalization, and sequence of CpG ODN along with their structure. [42, 43, 44]

1.2.1.3 TLR Signaling Pathway

TLRs share many common elements in their signaling pathway. However, due to minor but essential differences, each TLR has specific response. TLRs can alter gene expression profile of host cells dramatically, via activating transcription factors such as NF-κB and IRFs. One of the earliest TLR downstream effector proteins was myeloid differentiation primary response gene 88 (MyD88). Later studies conceived that TLR3 signaling is MyD88-independent-TRIF dependent pathway and TLR4 signaling is possible through both pathways.

1.2.1.3.1 MyD88 Dependent Pathway: M MyD88 associates with the TIR domain of TLRs. Its importance for TLR signaling has been shown in MyD88

deficient mice, which are unresponsive to various TLR ligands [45, 46]. MyD88 is an adaptor protein that recruits IL-1 receptor associated kinases (IRAK) IRAK-1, IRAK-2, IRAK-M and IRAK-4 [47]. IRAK-2 and IRAK-M are inactive (36). IRAK-4 phosphorylates IRAK-1 [48] and activated IRAK1 interacts with TRAF6. Both AP-1 transcription factors and NF-κB transcription factor are activated by TRAF6 through distinct pathways. AP-1 TFs activation is induced by MAP kinases in which TRAF6 initiates the signal cascade. Concurrently, TRAF6 is involved in IKK mediated degradationof IKB, and NF-κB nuclear localization as a consequence, via activation of TAK1-TAB complex.

1.2.1.3.2 MyD88 Independent-TRIF Dependent Pathway: Possibility of a MyD88 independent TLR pathway was arisen when NF-κB nuclear localization was not halted but delayed in MyD88 deficient mice in response to LPS; although no cytokine expression was observed [45]. It seemed that TLR4 was able to induce signaling pathway regardless of MyD88, a defective one though. Further studies revealed that TLR3 also has a MyD88 independent pathway, but unlike TLR4, TLR3 completely relies on MyD88 independent pathway [49]. Instead of MyD88, TRIF and related protein TIRAP act as adaptor protein and mainly induces type I IFN expression via activation of IRF3. NF-KB signal was also affected by TRIF-dependent pathway.

1.3 Synthetic CpG ODNs

Immunostimulatory properties of CpG containing bacterial DNA was discovered in 1995 [51]. Later on, immunomodulatory effects of CpG ODNs are widely studied. CpG ODNs can be used as effective vaccine adjuvant, with relatively low adverse effect profile [52]. Consequently, two distinct classes of ODNs, Class A or D and Class B or K type ODNs were identified with differential activation of immune system. Regardless of their differences, distinct classes of CpG ODNs share some

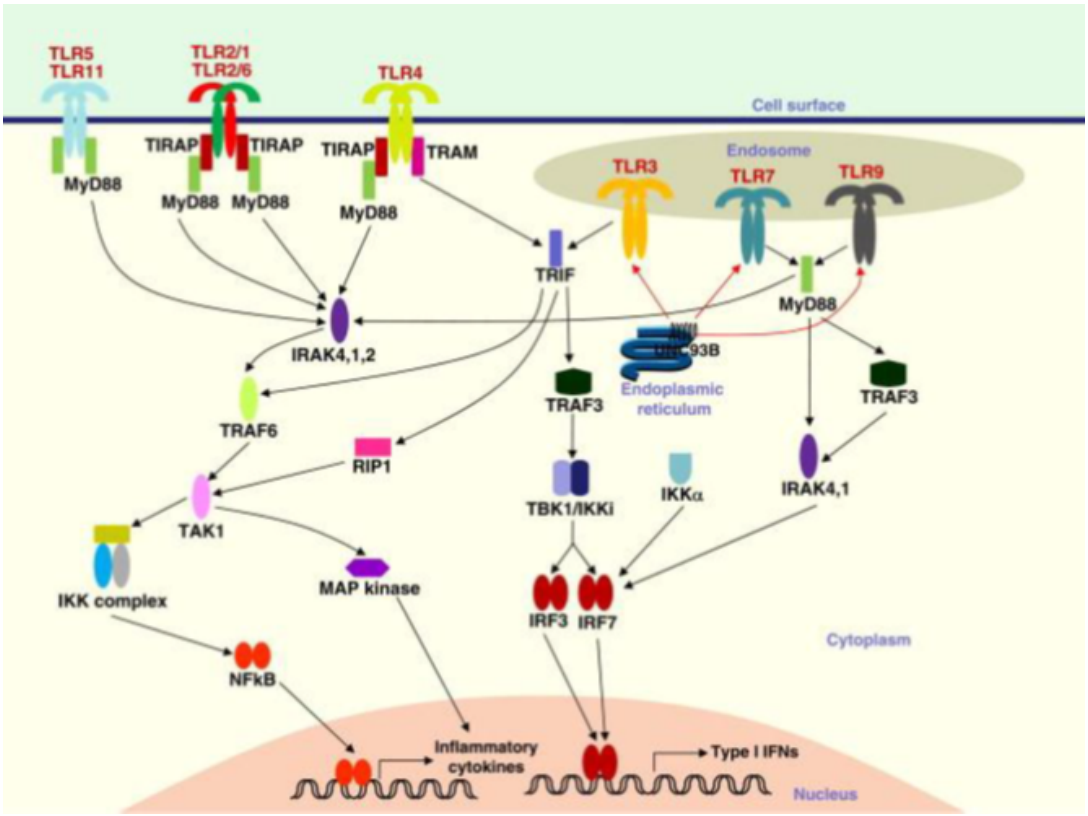

Figure 1.3: Initiation of MyD88 and TRIF dependent signaling cascade upon TLR-ligand interaction. Adapted from [50]

common properties; minimum required length for immune stimulation is 8 bp, in optimum sequences, unmethylated CpG nucleotides are flanked by PuPu and PyPy bases in mice and PuPy and PuPy in humans, despite significant homology between murine and human TLRs (over 75 % homology exists). Finally, partial or complete modified backbone to reduce nuclease attack is necessary for specific classes to induce an immune activation.

1.3.1 A or D Type ODN

This class of ODNs (D ODN hereafter) has been characterized with partially modified backbone and polyG runs at the ends (i.e. PolyG-PS/PO/PS-PolyG). Palindromic sequences that are located before and after CpG nucleotides cause secondary structure formation. Assembly of two or more D type ODN via Hogstein Base pairing of Poly G caps also leads to formation of heterogeneous higher order structures. Of note, these concatamers are necessary for the D-ODN activity. It was also shown that palindromic sequences and polyG runs are crucial for D type ODN activation [53]. Macromolecular aggregation of D ODNs is recognized by scavenger receptor CXCL16 and causes localization of D type ODNs into early endosomes. Thereafter, D type ODNs directly promote strong and prolonged IFN and IFN expression from pDCs [54]. D-ODN also indirectly induce IFN and IP-10 from PBMC and contribute to NK cell-mediated cytolytic activity [55]. Activation of costimulatory molecules CD86 and CD80 somewhat moderate, an D type ODNs are weak inducers of pDC maturation [54].

1.3.2 B or K type ODNs

B or K type ODNs (K ODN hereafter) have fully phosphorothioated backbone. They do not have internal palindromic sequences or polyG tails. Usually 2-3 CpG motifs are required for optimum activity [56]. Unlike D type ODNs known as a strong type 1 IFN inducer, K type ODNs can induce very little if any IFN expression. However, they are very potent inducers of proinflammatory cytokines IL6 and IL12 from mouse spleen cells [57]. They also promote rapid maturation of pDCs. In physiological conditions, K type ODNs remain linear and do not aggregate.

1.3.3 Other Types of CpG ODNs

Two main CpG ODN types have different immune stimulatory properties. Many novel CpG ODNs were defined that try to recapitulate both D type and K type specific immune activation. For example C type ODN can induce both TNFa and IFNa from albeit lower than K ODN and D ODN respectively [58]. It has structural similarities with both type of ODNs; fully phosphorothioated backbone like K type ODNs and internal palindromic sequences like D type ODNs [59]. C type ODN captures properties of both K and D type ODN, but not at desirable rate. Many other novel CpG ODN types are defined, such as Y shaped ODN [60], however, their exact immunomodulatory natures yet to be cleared.

1.3.4 Competitive Action of Mechanisms of D and K Type ODNs

As mentioned above, K type and D type ODNs display a dichotomy in terms of immune stimulatory activities. Due to its ability to form higher order structures, recognition of D type ODNs through TLR9 requires CXCL16 involvement, a scavenger receptor. D type ODNs localize to early endosomes after CXCL6 mediated recognition, which leads to co localization of MyD88 and interferon regulatory factor-7 (IRF7) [61]. Subsequent events promote IFN secretion from pDCs, and IFN secretion from mouse spleen cells, which are inducers of cell mediated immune response. K ODNs preserve their linear structure in physiological conditions. Therefore, their recognition through TLR9 is CXCL16 independent. K ODNs are quickly localizes to late endosome and induces TNFa secretion from pDCs and leads to pDC maturation [62], eventually humoral immune activation [7]. Even though both CpG ODN types hold promising vaccine adjuvant properties, it is desirable to capture both types of immune regulatory properties in one single formula. Unfortunately, simultaneous administration of D and K type ODNs are not possible. Although they do not inhibit their binding,

uptake and localization kinetics, K type ODN inhibits D type ODN dependent activation [62, 63]. While K type ODN acts on both B-cells and pDCs, D type ODN only act on pDCs. D type ODN localizes to early endosome and induces IFNa expression. K type ODN localizes to late endosome induces TNFa secretion and promotes pDC maturation, thereby prevents IFNa secretion from pDCs [62, 63].

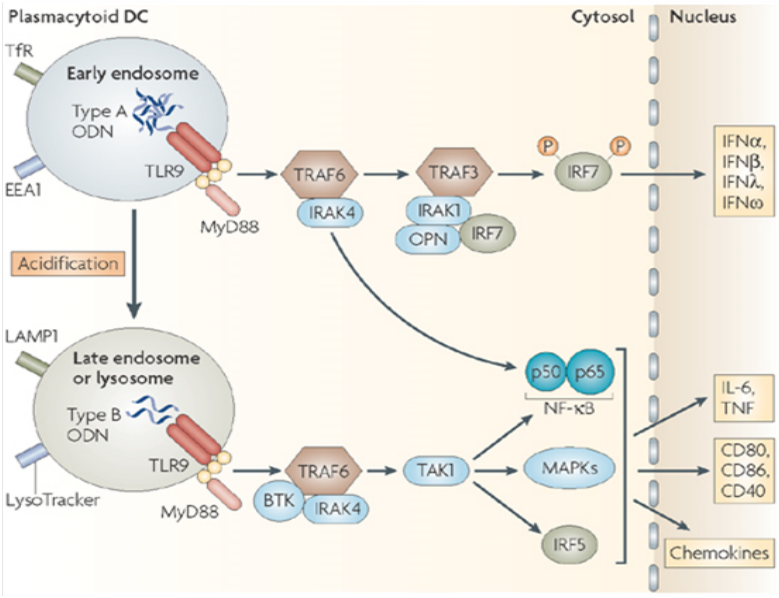

Figure 1.4: Differential immune activation of human pDC following D and K triggered signaling cascade. Adapted from [64]

K type ODN entered many clinical trials; however, D ODN entered few. D ODN has ability to spontaneously form heterogeneous high ordered structures. This uncontrollable nature of D ODN prevents its GMP production and FDA permission. Only administration via particles that can stabilize D type ODN can lead to clinical trials [65]. Therefore, new approaches have been trying to overcome this problem. One possible method is designing K-like ODNs that can have D type effects, as mentioned in previous section. Another method would be administration of K type ODNs embedded cationic lipid particles, which can helps localization of K type ODNs to early endosomes instead of late endosomes and IFN production [55].

1.4 Helicobacter Vaccine

Helicobacter is a helix shaped gram negative genus of bacteria. Some species have been found in gastrointestinal track of mammals and birds. It is estimated that 45-50 % human population was infected with *Helicobacter pylori* (*H. pylori*), major infectious species in humans [66]. Although 85 % percent of the cases are reported to be asymptomatic, in the remaining cases colonization in the gastric lumen causes persistent inflammatory response, which eventually leads to gastritis (64). Non-pathogenic strains have been found to lack Cag pathogenicity island in their genome [67]. Helicobacter can survive in low acidic environment of gastric lumen by producing large amounts of urease and neutralizing acid [68]. Urease breaks down the urea to carbon dioxide and ammonia, which is a basic molecule and helps helicobacter survival in such extreme condition [69]. *H. pylori* is also able to use chemotaxis to move away from low pH lumen into mucosal surface of epithelia, which has considerably more neutral pH [70] owing to multiple flagella [71].*H. pylori* flagellin is able to evade TLR5 [72].

Treatment of *H. pylori* associated gastritis was aimed by combined antibiotic and proton pump inhibitor treatment in mid 1990′s [73], since then this treatment modality is still used in the clinic [74]. In case *H. pylori* and complications are not eradicated, stronger antibiotics are used [74]. However, increasing antibiotic resistance of *H. pylori* strains became a major problem in recent years and infection has proved difficult to cure [75]. Besides, patient compliance and recurrent infections are often causes problems [76]. Therefore, development of vaccine against *H. pylori* is currently taking a lot of scientific attention.

1.4.1 *Helicobacter* Infection

Developing vaccine against *H. pylori* brings unique challenges. Unlike blood borne pathogens or viral infections, *H. pylori* infection and subsequent inflammation should be studied in mucosal immunity [77]. Due to its distinct dynamics, vaccine

development related to mucosal immunity should be handled separately [78, 79]. This is a further obstacle for development of efficient vaccine formulations against *H. pylori*. Another difficulty is lack of a proper animal model. Until Lee *et al.* introduced *Helicobacter felis*, a *Helicobacter* species isolated from cat, there were no gold standard mouse model for studying *in vivo H. pylori* infection [80, 81], although it is now possible to use H. pylori in mouse model [**?**, 81]. Furthermore, finding a proper and non-toxic mucosal adjuvant, efficient route of administration is another great challenge [82]. Nowadays, those adversities have been fairly overcome. Clinically, it was shown that *H. pylori* infected individuals were able to produce IgG antibody against *H. pylori* antigen [83]. IgA class antibodies are prime antibody type in mucosal linings and have critical role in mucosal immunity [84]. Indeed, oral delivery of microparticles encapsulating *H. pylori* lysates can induce IgA and IgG production in mice [85]. Using live vectors that express *H. pylori* related genes is another approach. This method uses a non-pathogenic bacteria as a vector and is reported to induce significant IgA production and reduced *H. pylori* colonization after challenge [86]. The above mentioned two methods have serious drawbacks, as there is no reliable data regarding to protective effect of the vaccine for the former and non-specific immune response due to live vector for the latter. There are also studies with conventional adjuvants, such as cholera toxin [87], but they are not feasible options for humans. CpG ODNs are highly effective adjuvants with minimal adverse effects. It is not surprising that last couple of years CpG ODNs are included in efforts to develop vaccine against *H. pylori* [88, 89, 90]

1.5 Liposome Technology

Liposomes are artificial vesicles composed of one or more lipid bilayers, which enclose aqueous compartments between bilayers and core aqueous phase [91]. Interior sides of lipid bilayers and inner aqueous compartments have hydrophobic and hydrophilic nature, respectively. This reciprocal feature of liposomes and

versatility in manufacturing are prime reasons for developing liposome-based technologies. Following seminal observation by Alec Bangham in mid 1960s; that lipids can form spherical structures in aqueous environments [92], Gregoriadis and Ryman for the first time successfully loaded these vesicles with proteins and injected in vivo and observed their fates [93].

Liposomes can exists in varying sizes, lipid composition, net charge and structure. Changing lipid composition can dramatically alter physicochemical properties of liposomes [94]. Lipid bilayers can be single or more, in case of single bilayer, liposome is called unilamellar, and multilamellar in case of latter. Liposomes ranging 0.5 uM and 4-5 uM is called large vesicles, bigger and smaller vesicles are called giant vesicles and small vesicles, respectively. Small unilamellar vesicles (SUVs) and large multilamellar vesicles (MLVs) are commonly used liposomes for pharmaceutical purposes, [95] whereas giant unilamellar vesicles are perfect model lipid membranes [96, 97]. SUV on the other hand are suitable either as transfection reagents or as imaging agents [98].

Liposomes are biocompatible vesicles and can entrap both hydrophobic and hydrophilic molecules in lipid bilayers and in aqueous compartments, respectively. This gives a broad range of materials that can be entrapped into liposomes. Unlike free administered drugs, liposomal drugs localize inside the cells and even inside cellular compartments more efficiently [99]. Moreover, liposomes protect their biological cargoes from nuclease and protease degradation [100, 101, 102]. Engineering liposomes is easy and intended to overcome possible problems during drug delivery. One such modification of liposomes is attaching polyethyleneglycol (PEG) to the liposomes, via either covalent bonding to lipids or embedding PEG containing molecules into lipid bilayers using hydrophobic moieties. PEGylated liposomes are called stealth liposomes since they are less susceptible to elimination from serum and elimination by reticuloendothelial system (90, 91). It is also possible to manufacture targeted liposomes by attaching in order to increase liposomal drug accumulation to the desired tissue or cell type [98, 103] (FIG-LASIC). Targeted liposomes are manufactured by attaching

molecules that are able to recognize specific sites in target tissue or cell, Igs most of the times. It is also possible to profit both stealth and targeted liposome delivery by covalently attaching antibody fragments that lack Fc portion to the PEG chains [101].

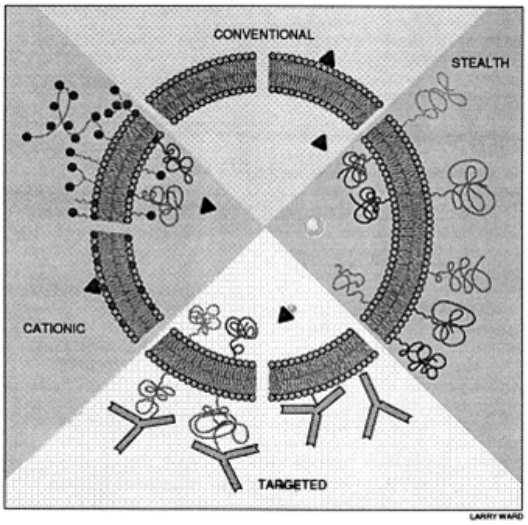

Figure 1.5: Frequently used liposomes for therapeutic purposes; conventional, cationic, stealth and targeted liposomes. Adapted from Lasic (1997) [104]

1.5.1 Liposomal Vaccines

Liposomal vaccine formulations are capable of combining two signals in one package that is a necessary step to achieve therapeutic vaccination (i.e. antigen plus adjuvant). In fact, liposomes can have their adjuvant effects in certain formulations [105, 106]. Hence, liposomes are among promising candidates for new generation vaccine formulations [107]. A phase IIB clinical trial for liposomal L-BLP25 peptide, which is a fragment of a highly expressed glycoprotein in common cancers, against non-small lung cancer showed that liposomal vaccine formulation significantly increased median survival rate [108]. It is also worth mentioning, co-encapsulation of the antigen and adjuvant into single liposomal compartment

can boost the performance of vaccine formulation [109]

TLR ligands were also used as liposomal adjuvants [110, 111], nucleic acid based TLR ligands in particular, due to ease of their incorporation into cationic liposomes. Cationic liposomes that co-encapsulated poly(I:C) and synthetic cord factor is shown to induce CTL dependent tumor regression [112]. In another study, co-encapsulation of different TLR ligands (poly(I:C) and CpG ODN) into anionic liposomes was proven to induce TH1 biased immunity and robust response against antigen ovalbumin, despite possible steric hindrance [**?**]. Hence, liposomal vaccine formulations containing nucleic acid based TLR ligands as adjuvants clearly demonstrated encouraging results, which support further studies.

Chapter 2

Aim of The Study

CpG ODNs are potent immunotherapeutic agents and already appeared in clinical trials for variety of purposes. Liposomal delivery systems are also extensively utilized. In the first part of this study, we aimed to investigate in vitro performance of different types of liposomes that encapsulated CpG ODNs in a mouse macrophage cell line and spleen cells. Best performing formulations were tested in a *H. felis* vaccine model. Up to date, there is no other study that tries to combine liposomal vaccination methods and CpG ODNs.

K and D type ODNs have distinct immune stimulatory properties. A good immunenotherapeutic reagent would have both ability. However, differential immune activation mechanisms of K and D type ODNs due to their subcellular fates are major hamper for their simultaneous administration. Despite efforts to overcome this problem, there are no successful solution. In the second part of this study, our purpose was to alter subcellular localization of D and K types ODN by encapsulating them into liposomes in order to to recapitulate their individual effect on human PBMCs when they were administered simultaneously.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 ELISA Reagents

Mouse cytokine ELISA reagents, including monoclonal catching antibody, biotinylated reporter antibody, recombinant cytokines and streptavidin-alkaline phosphatase (SA-AKP) were purchased from BioLegend (USA). SA-AKP substrate p- nitrophenyl phosphate disodium salt (PNPP) was purchased from Thermo (USA). Mouse Immunoglobulin ELISA reagents; goat anti-mouse IgG, IgG1, IgG2a monoclonal antibodies conjugated with alkaline phosphatase (AKP) were obtained from Southern Biotech (USA). Human cytokine ELISA reagents for IL6 and TNFα were from Biolegend (USA). For IFγ and IFN-a2α, reagents were purchased form BD (USA) and MabTech (USA) respectively.

3.1.2 TLR Ligands

CpG ODNs (TLR9 ligand) were either synthesized by Alpha DNA (Montreal, Canada). or synthesized in Therapeutic ODN Research Lab (THORLab) (Bilkent, Ankara, Turkey) with a MerMade6 Oligonucleotide Synthesizer. Peptidoglycan (PGN) (isolated from B.subtilis) was from Fluka (Switzerland), lipopolysaccharide (LPS) which was isolated from E. coli was from Sigma (USA). Detailed informaiton on CpG ODNs were given in Table 3.1.

Table 3.1: List of Oligonucleotides that are used in this thesis. Capital letters denote a nucleotide with phosphorothioate backbone and those in lower cases show phosphodiester backbone. K3, K23 and 1555 are K type ODNs whereas D35, 1466 and D3CG are D type ODNs

K3 (20mer)	5′-ATCGACTCTCGAGCGTTCTC-3′
K3 Flip (20mer)	5′-ATGCACTCTGCAGGCTTCTC-3′
D35 (20mer)	5′-GGtgcatcgatgcaggggGG-3′
D35 Flip (20mer)	5′-GGtgcatgcatgcaggggGG-3′
K23 (12mer)	5′-TCGAGCGTTCTC-3′
1555 3cg (20mer)	5′-GACGTTGACGTTGACGTTGG-3′
1466 Acore MB (16mer)	5′-TCaacgttgattcaAA-3′
D3CG MB (20mer)	5′-GGtcgatcgatcgaggggGG-3′

3.1.3 Lipids

Cholesterol was purchased from Sigma Aldrich (USA). L-α-Phosphatidylcholine (PC), 3α-[N-(N',N'-Dimethylaminoethane)-carbamoyl] Cholesterol Hydrochloride (DC-Chol), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N- [Methoxy(Polyethylene glycol)-2000] (Ammonium Salt)(PEG-PE), 1,2-Dioleoyl-sn- Glycero-3-Phosphoethanolamine (DOPE) were all from Avanti Polar Lipids (USA).

3.2 Methods

3.2.1 Cell Line Maintanence

RAW 264.7 cell line, murine macrophage-like cells were cultured in 10% FBS supplemented complete RPMI-1640 media. Medium was changed every 2-3 days. Upon reaching 80% confluency, cells were passaged with 1:3 ratio or used for in vitro stimulation.

3.2.2 Stimulation of RAW 264.7 cells for Gene Expression Analysis

2.5×10^6 cells were transferred to 15 ml falcons and each tube was completed to 900 μl wth 5% FBS supplemented RPMI-1640 media. Stimulants were added in 50 μl 5% FBS supplemented RPMI-1640 media. Tubes were kept in tilted position with loosened caps until termination of the experiment. Incubation periods for gene expression studies were 2 and 4 hours.

3.2.3 Spleen Single Cell Suspension Preparation

Mice were sacrificed by cervical dislocation and spleens were removed into 35 mm petri dish that contains 2 ml ice cold 2% FBS supplemented complete RPMI-1640 with sterile surgical equipment. Then spleens were smashed with back of a sterile syringe plunger with circular movements in order to obtain single cell suspension. Media was transferred to 15 ml falcon with sterile pasteur pipette leaving connective tissue and fat clumps in the petri dish. Then whole cell suspension is completed to 10 ml and washed twice at 375 g for 5 minutes. After the final wash step, cells were counted and adjusted to appropriate concentration for further use.

3.2.4 Cell Counting by Hemocytometer

After the final wash step, cells were resuspended in 1 ml 5% FBS containing complete RPMI-1640. Then 20 μl cell suspension were diluted 1:50 with 980 μl media. Another 15 μl diluted cell suspension is mixed 15 μl Trypan blue (1:1) and counted with Neubaer cell counting chamber. 16 cells from each corner were counted and average cell number per 1 mm^2 was founded under light microscope. Each 4×4 cell at corners hold 0.1 mm^3 liquid as depth between coverslip and chamber is 0.1 mm and they have 1 mm^2 area. Therefore, total cell number in 1 ml can be calculated by formula;

Average cell number × 2 × 10^4 × 50(dilution factor) = total cell number.

3.2.5 Cell Counting by Flow Cytometer

After single cell suspension were prepared in 1 ml, 10μl of cell suspension was added to 990 μl of isotonic solution in 5 ml polystyrene round bottom tubes (BD, Ref no: 352052). Then particles in 20 μl of resulting suspension were counted in Accuri C6 Flow Cytometer. Only live and nucleated cells were gated and counted, apoptotic, dead or non-nucleated blood cells were ignored. Final number was multiplied with 5.000.

3.2.6 In vitro Splenocyte Stimulation

For stimulation in 96 well cell culture plates with lid (CellStar), 100 μl or 125 μl of 4×10^6 cells/ml suspension were conveyed to each well(400.000 cells/well and 500.000 cells/well respectively). Each specific reagent was added in 50 μl 5% FBS supplemented RPMI-1640 media. Finally, each well was completed to 200 μl or 250 μl with 5% FBS supplemented RPMI-1640 media in order to have 2×10^6 cells/ml. Stimulations were performed in at least duplicate wells and each

experiment was repeated twice at different times. Supernatants were collected after 24-36 hours depending on the secretion of specific cytokine to be examined. For gene expression studies, 2.5×10^6 cells were transferred to 15 ml falcons and each tube was completed to 900 μl. Stimulants were added in 50 μl 5% FBS supplemented RPMI-1640 media. Tubes were kept in tilted position with loosened caps until termination of the experiment. Incubation periods for gene expression studies were 2 and 4 hours.

3.2.7 Human Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood samples from healthy donors were collected into vacutainer tubes that contains EDTA as anti-coagulant (BD Vacutainer, purple cap). 15 ml ficoll (Lymphocyte Separation Medium, Lonza) was placed into 50 ml falcon tube and 22.5 ml. blood from anti-coagulant tubes was gently layered onto the ficoll reagent. Samples were centrifuged at 500 g for 30 min at room temperature. It is important to set break off to keep separated layers intact. The buffy coat that PBMCs reside was collected using sterile pasteur pipette. Cells were washed twice with 50 ml 2% FBS supplemented RPMI-1640 media in a new 50 ml falcon tube (500 g at RT) to ensure no ficoll reagent was left. At the end of the last wash, pellet was resuspended in 1 ml 5% FBS supplemented RPMI-1640, samples from same donors were polled together and cells were counted.

3.2.8 In vitro PBMC stimulation

In vitro PBMC stimulation experiments were done as follows, cells were collected, isolated and counted as described above (Section 2.2.7). Then, 2.5×10^5-3×10^5cells in 100 μl were layered into wells of 96 well plates (Preferred cell number for each well was 3×10^5, however, depending on total cell number and experimental groups,

2.5×10^5/well was also used). Different ODN formulations were added in 50 μl and each well was then completed to 250 μl, yielding 1×10^6-1.2×10^6 cells/ml. 220-230 μl of supernatants were collected from each well after 36 hours of incubation.

3.2.9 Liposome Preparation

Phospholipids and cholesterol were prepared as 10 mg/ml or 20 mg/ml stock solutions in chlorofrom. Stock solutions were stored at $-20\,^{\circ}$C. Various phospholipids and cholesterol mixed in different ratios in round bottom flasks to obtain liposomes as shown in Table 3.2 . Chloroform was evaporated using a rotary evaporator (ILVAC, Germany) in which relatively uniform thin lipid film was obtained. A brief nitrogen stream was applied onto thin lipid film (30-40 sec) to remove residual chloroform. Round bottom flasks were sealed with glass caps in order to prevent oxygen saturation to increase following nitrogen streaming, presumably minimizing lipid peroxidation. 1 ml of PBS were added for each 20 μmol thin lipid film. If less than 20 μmol lipid was used, up to 10 μmol, PBS amount was adjusted accordingly, but if lipid film contained less than 10 μmol total lipid, no less than 500 μl PBS was used. 20-30 glass beads were added to each flask and lipid film was disrupted by moving flasks rotationally. This step generated large empty multilamellar vesicles. Resulting milky solution was collected using 1 ml disposable micropipette tip. 100 μl PBS were added again onto glass beads to ensure most of the liposomes that were entrapped on glass beads were collected. In order to generate small unilamellar vesicles (SUVs), which arguably most suitable form of liposomes for encapsulation, liposomes were sonicated 5 times for 30s with an amplitude of 40% at $+4\,^{\circ}$C and 5 times for 30s with an amplitude of 70% (VibraCell, SONICS, USA). Liposomes were kept on ice for 10 seconds in between sonications to prevent excessive heating. For 20 μmol lipid, 1 mg ODN was added to SUVs. If there were less than 20 μmol or SUVs were aliquoted, ODN amount was adjusted properly (1mg ODN/20 μmol lipid). ODN-SUV mixture was then snap frozen in liquid nitrogen and lyophilized (Benchtop K, Virtis, USA). Dehydrated

ODN-liposome mixtures appeared as powders after this step and encapsulation was achieved during controlled rehydration step, as previously reported (referans). Briefly, if the sample volume was more than 300 μl, 1:10 volume of DNase RNase free ddH_2O of the final volume prior to lyophilization was added. (e.i. 50 μl DNase RNase free ddH_2O was added for 500 μl liposome-ODN mixture). If it was less than 300 μl, 30 μl DNase RNase free ddH_2O was added onto powder and vortexed for 15 s every 5 min for 5 times at RT. Same amount of PBS was added to mixture and vortexed for a few seconds. Then final volume was adjusted to yield 1μg/μl ODN. Liposome preperations were stored at +4 °C until use.

Table 3.2: Lipid composition and molar ratios for different liposomes, taken from another study by Gursel et al. [115]

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

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

3.2.10 Enzyme Linked ImmunoSorbent Assay (ELISA)

3.2.10.1 Cytokine ELISA

When desired time point was reached, 96 well plates were spun at 400g for 5 minutes. Supernatants (170-220 μl) were collected into new 96 well plates and either immediately used or stored at -20 until another use. PolySorb Nunc Immuno Plates were coated with monoclonal antibodies against mouse or human cytokines (50 μl/well) and incubated for 4 hours at RT or for ON at +4 °C. As each antibody against different cytokines optimized at different concentrations,

working concentrations for coating antibodies were given in Table 3.3. Coating antibody solution was discarded and wells were blocked with 200 μl 5% w/v BSA-1X PBS 0.025% Tween-20 at RT for 2 hours. Plates were washed with PBS-Tween 20 (0.05% Tween) 5 times. PBS-Tween 20 was kept in wells in between and plates were rinsed with ddH_2O 3 times. Plates were dried by tapping. Washing was repeated after subsequent steps. 50 μl supernatants and recombinant cytokine standards were added into the wells. Starting concentrations for cytokine standards (duplicate) were given in Table 3.4. Standards were serially diluted 2 fold with 50 μl 1X PBS 7 or 11 times. Two wells were left with 50 μl 1X PBS for blank measurement. Samples and standards were incubated ON at +4 °C. Plates were washed and Biotin conjugated antibodies against human and mouse cytokines that were diluted (1:1000) in %5 v/v FBS-1X PBS %0.025 Tween-20 and 50 μl added into each well and incubated at RT for 3 hours. During this incubation, Streptavidin-Alkaline Phosphatase (SA-AKP) was diluted (1:3000) in the same buffer as biotinylated antibodies, since SA-AKP works best when prepared at least 1-2 hours beforehand. After washing of plates, 50 μl diluted SA-AKP were added to each well. After 1 hours of incubation at RT, Plates were washed and PNPP substrate was added (50 μl/well). For one plate, one PNPP tablet was dissolved in 4 ml ddH_2O and 1 ml 5X buffer was added afterwards. Until 2X serially diluted recombinant proteins generate an S-shape standard curve, ODs at 405 nm were read after each 30 min intervals with an automated plate reader (BioLabs).

Table 3.3: Working concentrations for coating antibodies

Anti mouse IL6	2 μg/ml
Anti mouse IL12	4 μg/ml
Anti mouse IL10	7.5 μg/ml
Anti human IL6	2 μg/ml
Anti human TNFα	3 μg/ml
Anti human IFNγ	5 μg/ml
Anti human IFNα	5 μg/ml

Table 3.4: Starting concentrations for recombinant cytokines

Recombinant mouse IL6	1000 ng/ml
Recombinant mouse IL12	500 ng/ml
Recombinant mouse IL10	50 ng/ml
Recombinant human IL6	1000 ng/ml
Recombinant human TNFα	1000 ng/ml
Recombinant human IFNα	250 ng/ml

3.2.10.2 IgG ELISA

IgG ELISA was very similar to cytokine ELISA except a few changes. Washing procedure was identical and incubation times were very similar. PolySorb Nunc 96 well plates were coated with 50 μl 5 μg/ml soluble part of total textith. felis cell extract, which was a kind gift of Ayca Sayı Yazgan (ITU) and incubated ON at +4 °C. Wells were blocked with same blocking buffer for 2 hours at RT as cytokine ELISA and washed. Sera from mice was diluted as follows in different 96 well U bottom plates; First row was prepared by mixing 40 μl serum with 240 μl 1X PBS in order to obtain 1/7 titration. 210 μl 1X PBS were added to rows beneath. 70 μl from the rows above were added to rows below, in which samples were titrated 1:4 at each row. Samples were diluted one time 1:7 and seven times 1:4. 50 μl of serum titrates were added to each well and incubated ON at +4 °C. Plates were washed. Antibodies that were raised against heavy chain of mouse antibody subclasses or against total IgG directly linked to alkaline phosphatase (AKP). Antibodies against total IgG, IgG2a and IgG1 were diluted 1:2000 in 5% v/v FBS-1X PBS 0.025% Tween-20 whereas antibodies against IgA were diluted 1:1000. 50 μl diluted AKP-linked antibody were added to wells and incubated at RT for 2-3 hours. Plates were washed again and PNPP substrate was added. OD measurements at 405 nm were taken in every 30 minutes after PNPP addition.

3.2.11 Total RNA Isolation

As stimulations for gene expression studies were done in 15 ml falcon tubes, samples were directly centrifuged at 500 g for 5 minutes. All centrifugations were done at +4 °C and all steps were completed on ice during RNA isolation. Supernatants were discarded and pellet was resuspended with ice cold PBS, centrifuged again at 500 g for 5 minutes. Supernatant was again discarded and 1 ml Trizol reagent (Invitrogen) directly added onto pellet. Mixate was homogenized by repetitive pipetting and transfered to 1.5 ml eppendorf tube. In order to reach critical 5:1 phenol chloroform ratio in phenol-chloroform extraction method, 200 μl of chloroform (Sigma) added to 1 ml homogenate and tubes were vigorously shaken for 10-15 seconds. Samples were incubated 3 minutes at RT before centrifugation at 16.000 g for 17 minutes. Approximately 450-500 μl of clear upper phase (aqueous phase) was transferred to new 1.5 ml eppendorf tubes and 500 μl isopropanol was added and tubes were gently inverted to ensure proper mixation, followed by incubation for 10 minutes at RT. Samples were spun at 16000 g for 15 minutes. Supernatants was discarded and 1 ml 75% ethanol was added. At this points, in order to remove trace amounts of isopropanol, samples were vortexed just long enough to move pellet. Samples were spun at 6000g for 8 minutes and gently washed with >99.9% ethanol. Samples were centrifuged once more at 6000g for 8 minutes, supernatant was discarded, and samples were dried in a petri dish in tilted position. Dried pellets were dissolved in 20 μl DNAse RNAse free water. RNA quality and quantity was assessed by OD measurements at 260 and 280 nm, which were obtained by NanoDrop ND1000 spectrophotometer. Isolated RNA samples were expected to have a 260/280 ratio between 1.8-2.0, which is an indicator minimal DNA, protein and organic solvent contamination. For samples that did not have proper 260/280 ratio, ethanol washing steps were repeated, until acceptable values were reached.

3.2.12 cDNA Synthesis

cDNA was synthesized from total RNA with ProtoScript First Strand cDNA Synthesis Kit (NEB) according to manufacturers instructions. Briefly, 1 μg of total RNA was mixed with 100 ng oligo(dT)15 primer (1 μl) and each sample was completed to 8 μl with DNAse RNAse free water. After a pre-denaturation step (5 min at +65 °C),10 μl RT Buffer (including dNTP mix and 10 mM MgCL2) and 2 μl M-MuLV RNase H+ reverse transcriptase (includes RNase inhibitor) were added to tubes. samples were spun down quickly. Reaction conditions were as follows; 1 hour at +42 °C, 5 minutes at +85 °C. cDNAs were stored at −20 °C for further use.

3.2.13 PCR

Primers specific to mouse genes were designed NCBI′s primer designing tool (Primer BLAST) which uses Primer3 for primer designing and NCBIs database along with BLAST and global alignment algorithms to screen primer pairs availability. All primers were further analyzed via online UCSC in Silico PCR tool to test primer pairs with different databases and algorithms. Detailed information on mouse primers and their reaction conditions were given in Table 3.5 and Table 3.6 respectively.

PCR reaction mixtures were prepared with Quick-Load Taq 2X Master Mix (NEB) which loading dye was included in MAster Mix directly. Final volume was 25 μl for each primer pair which contained 12.5 μl Master Mix, 1.5 μl cDNA solution, 1 μl from forward primer and 1 μl from reverse primer and 9 μl ddH_2O.

Table 3.5: Designed mouse primers and amplicon sizes

Primer		Sequence	Amplicon Size
mIL1β	Forward	5′-GCTGCTTCCAAACCTTTGAC-3′	431 bp
	Reverse	5′-GGCCACAGGTATTTTGTCGT-3′	
mIL6	Forward	5′-TGGAAATGAGAAAAGAGTTGTGC -3′	120 bp
	Reverse	5′-CAGTTTGGTAGCATCCATCATT -3′	
mMIP1α	Forward	5′-ACCATGACACTCTGCAACCA-3′	238 bp
	Reverse	5′-AGGCATTCAGTTCCAGGTCA-3′	
mIP10	Forward	5′-GCCGTCATTTTCTGCCTCAT-3′	127 bp
	Reverse	5′-GCTTCCCTATGGCCCTCATT-3′	
mTLR9	Forward	5′-AGTGTCACTTCCTCAATTCTCT -3′	118 bp
	Reverse	5′-TTCGACGGAGAACCATGTTG -3′	
mGAPDH	Forward	5′-AGCTCATTTCCTGGTATGACA -3′	128 bp
	Reverse	5′-CTCTCTTGCTCAGTGTCCTT -3′	

Table 3.6: PCR conditions for mouse primers

	Gene Names	
PCR Steps (Temp; Time)	mIL6, mTLR9 & mGAPDH	mIL1β,mMIP1α & mIP10
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′

3.2.14 Agarose Gel Electrophoresis

Agarose gel was prepared with 2% (w/v) agarose in 150 ml 1X TAE buffer. Ethidium bromide was added as 1 μg/ml. Lanes were loaded with 10 μl PCR product. 3 μl 50-500 bp or 100-1000 bp DNA ladders (BioLabs, USA) were used as markers. Gels were ran at 110V for 50-55 minutes and visualized under UV transilluminator (Vilber Lourmat, France).

3.2.15 Immunization Protocol

Five adult female C57/BL6 mice per group were tail bled prior to immunization (d=-1) and one day later (d=0) they were injected with liposomes that co-encapsulated 15 μg *s*oluble whole ... H. felis extract and 15 μg ODN subcutaneously. Two weeks later, booster injection was done (d=14). Just one day before booster injection (d=13), animals were tail bled and sera were collected. Two weeks (w=4) and six weeks (w=8) after booster injection, animals were tail bled and sera were collected again. Timeline for immunization protocol was given in Figure 3.1

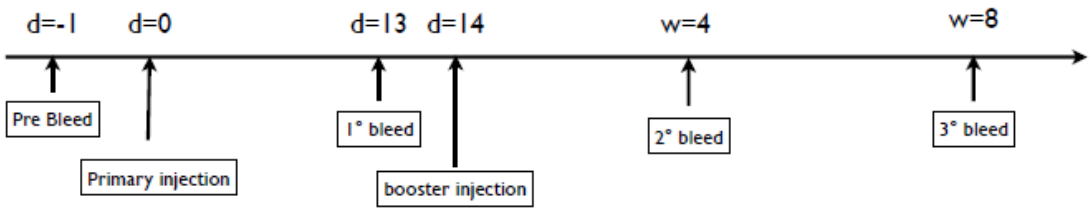

Figure 3.1: Timeline for immunization of mice with CpG and H. felis cell extract or liposomes co-encapsulating CpG and H. felis cell extract

3.2.16 Sera Collection from Immunized Mice

Mice were bled prior to immunization, one day before booster injection, 2 weeks after booster injection and six weeks after booster injection. Blood was obtained from tail veins of animals and collected to round bottom borosilicate glass test tubes. Blood samples were incubated at +37 °C for 2 hours. After incubation, clot and remaining liquid part can be separated. Liquid part from each sample was collected to 1.5 ml eppendorf tubes, centrifuged at 6000 g for 5 min at RT and cell and cell debris free supernatants were collected to 96 well plates and samples were stored −20 °C until use.

3.2.17 Maintenance of Animals

For immunization, only adult female BALB/c mice (8-12 weeks old) were used. Adult female or male BALB/c or C57/BL6 mice were used for isolation of spleen cells. Animals were housed in the animal holding facility of the Bilkent University Department of Molecular Biology and Genetics. Animals were kept in monitored and controlled environment at $+22 \pm 2\,^{\circ}\mathrm{C}$ with 12 hour light 12 hour dark cycles. Animals had *ad libitum* access to food and water. All experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC)

3.2.18 Statistical Analyses

Two tailed Student's t test was used to understand statistical difference between treatment groups. $p<0.01$ was accepted as statistically significant difference.

Chapter 4

Results

4.1 Effects of Liposomes Encapsulating D-ODN and K-ODN on RAW 264.7 Gene Expression

First, we determined to compare the immunostimulatory effects of different classes of free form of CpG ODNs to that of liposomes encapsulating CpG ODNs on specific gene message transcript upregulations of several pro-inflammatory cytokines (IL6 and IL1β), chemokines (MIP1α and IP10) and TLR9 using RAW 264.7 cells. K and D type CpG ODNs, namely K3 and D35, respectively along with their control sequences K3-flip and D35-flip were used during these comparison studies. Based on our previous experience, K3 was encapsulated within neutral liposomes (NL) and D35 was encapsulated within anionic liposomes (AL) ([113]). Cells were stimulated with different treatments for 2 or 4 hours. After cells were pelleted and total RNA was purified, specific gene transcripts were studied by RT-PCR. Of note, PGN and LPS were used as positive controls throughout these analyses. Figure 4.1 shows agarose gel electrophoresis images of analyzed gene transcripts following RT-PCR.

Pro-inflammatory cytokines IL1β and IL6 mRNA levels were not visible without any stimulation. Furthermore, neither K3 nor neutral liposomes containing K3 could improve the upregulation of these genes. Moreover, D35 alone also failed to induce any IL1B and IL6 expression. However, D35 in anionic liposomes not only induced higher expression levels of both IL6 and IL1β, it showed much longer lasting activation effect, since LPS mediated upregulation subsided by 4h compared to 2 h stimulation AL-D35 band intensities remained unchanged.

Chemokines (MIP1α and IP10 have a basal level of expression. Similar to cytokine expression, neither K3 nor NL-K3 could increase further than the basal level. In fact, it seems that NL induced a slight decrease for both chemokines. Similar to IL6 and IL1β case, free D35 also did not increase any chemokine expression. AL containing D35, however, increased expression of MIP1α and IP10 at 2 hours. Unlike cytokines IL1β and IL6, chemokine expression levels at the end of 4 hours in vitro stimulation did not subside. BothMIP1α and IP10 expressions continued to increase at 4 hours when cells were stimulated with AL-D35.

TLR9 is a receptor evolved to sense unmethylated CpG motifs. When we checked whether free or liposomal CpG ODNs could induce any increase in the expression level of this receptor whereas only D35 within liposomal formulation, led to an increase of TLR9 expression.

When taken together these results suggested that Anionic Liposomes loaded with D35 CpG ODN induces much stronger and persisting cytokine/chemokine upregulation than other treatments.

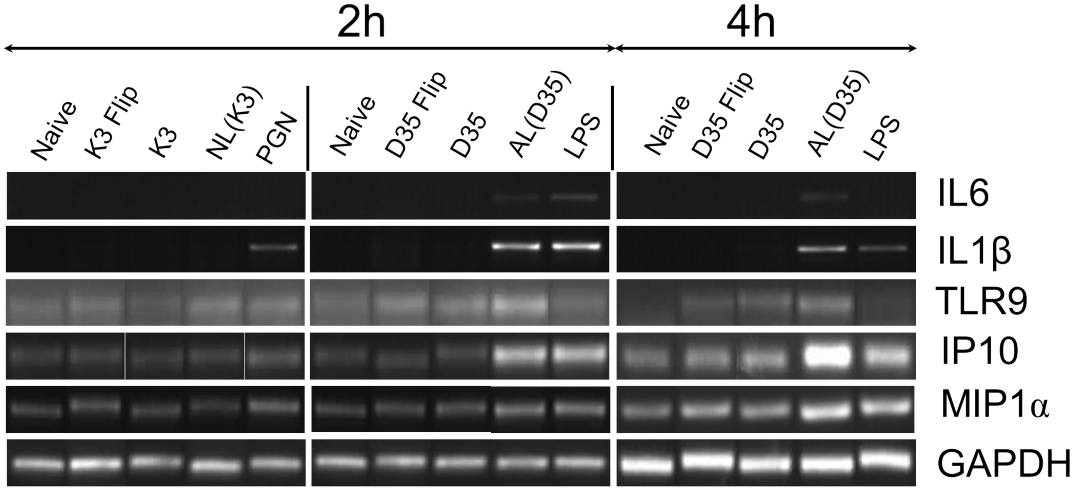

Figure 4.1: Gel photomicrographs of several gene products following RT-PCR obtained from total RNA of RAW 264.7 cells following stimulation with free K3 or within neutral liposome (NL), free D35, or within anionic liposomes (NL) along with their control sequences. PGN (2.5 g/ml) and LPS (1.0 g/ml) were used as positive controls.

4.2 In Vitro Stimulation of Splenocytes with Liposomal CpG ODN

One of the major aims of this thesis was to develop a vaccine formulation that could be effective against *Helicobacter* infections and furhermore could be tested in a mice model. To this end, candidate ODNs were tested in an in vitro assay before mice experiemnts were initiated. Splenocytes were stimulated either with liposomes that co-encapsulated K23 and K3 (1:1 w/w or 5:3 molar ratio) or 1466 and D3CG (1:1 w/w). Co-encapsulation is a powerful feature enabled by dehydration-rehydration method for preparing cargo loaded liposomes. While K23 and K3 had complete phosphorothioated backbone (i.e. PS), 1466 and D3CG had mix-backbone (PS/PO/PS) In our in vitro assays we intended to co-encapsulate these ODN pairs within five different type of liposomes. These formulations then

diluted serially by 3 fold to determine the lowest active dose in vitro. Cells were following 36 hours of in vitro incubation were harvested and supernatants were assessed for IL6, IL12 and IL10 productions by ELISA.

Free or liposomal formulations of K3 and K23 or their free counterparts stimulated much higher IL6 secretion than 1466 Acore MB and D3CG MB CpG ODNs (Figure 4.2 and Figure 4.3). This was an expected finding, because K23 and K3 were both members of K type ODN and these ODNs are generally known to be strong IL6 inducers. D3CG is a D type ODN and 1466 is modified to become a D-like ODN too. Of note, D type ODNs are relatively weak IL6 inducers.

As seen in Figure 4.2 different mixtures of K23 and K3 (only at higher doses) did not perform better when they were coencapsulated into liposomes than when they were used alone or together. As expected, at suboptimal doses, such as 9 fold or 27 fold dilution, K23 and K3 mixture in liposomes were much more potent (Figure 4.2). Interestingly, K23 alone showed the greatest stimulation almost at all dilutions in vitro. Still, we focused on K23 and K3 mixture and their liposomal counterparts as it is vital to use several sequences together as a mixture in vivo or in clinical trials, since individuals show different response to different sequences and a mixture yields the best response ([114]). Among five different liposome types, cationic liposomes at high doses were similar to that of free ODN responses. As it was titrated down, they displayed more potent immunostimulatory effects, at low doses. This might be due to toxicity of cationic lipids and when dose was reduced, toxicity decreased and cationic liposomes induced IL6 secretion comparable to other liposome types. In general, at low doses all liposome formulations stimulated splenocytes to secrete IL6 better than free K23 and K3 mixture.

In addition to IL6 secretion, IL12 production from splenocytes followed by stimulation with free or liposomal CpG ODN was assessed. Both K23/K3 mixture and 1466/D3CG mixtures led to similar amounts of IL12 when ODNs were at high doses (Figure 4.4 and Figure 4.5). For IL6, K23/K3 mixture had an obvious upper hand. Consistent with IL6, IL12 levels were higher at low doses when liposomal

formulations were used to stimulate spleen cells. Notably, at the lowest dose, anionic liposomes co-encapsulationg K23 and K3 mixtures induced pronounced IL12 secretion (Figure 4.4). Importantly, IL12 levels remained relatively stable with regard to serial dilutions of the ODN mixture. It is important to note that at low doses, mixing K23 and K3 made a positive contribution to immunostimulatory capacity of the ODNs supporting the view that a CpG ODN cocktail instead of using one specific type of CpG ODN sequence improves in vivo performance and breadth of activation. Unlike K23 and K3 stimulation, we observed a dose-dependent reduction in the levels of IL12 after stimulation with 1466 and D3CG mixture (Figure 4.5). Of note, cationic lipid containing formulations performed better for IL12 induction. Consistently in our hands, these formulations were the most potent liposomal formulations for IL6 secretion.

Finally, IL10 is an immunomodulatory cytokine, supporting Treg development. When we checked the IL10 production levels of different ODN mixtures, 1466 Acore MB and D3CG MB did not induce any detectable IL10 secretion. K23 and K3 induced IL10 secretion, however, there was no significant difference between liposomal formulations and free CpGs (Figure 4.6).

All these activities were CpG motif dependent, since control ODN or empty liposomes were inactive. Of note, amount of lipids were even higher than the lipids administered along with the highest CpG ODN formulations, but there was no notable difference from untreated spleen cells after stimulation with free liposomes.

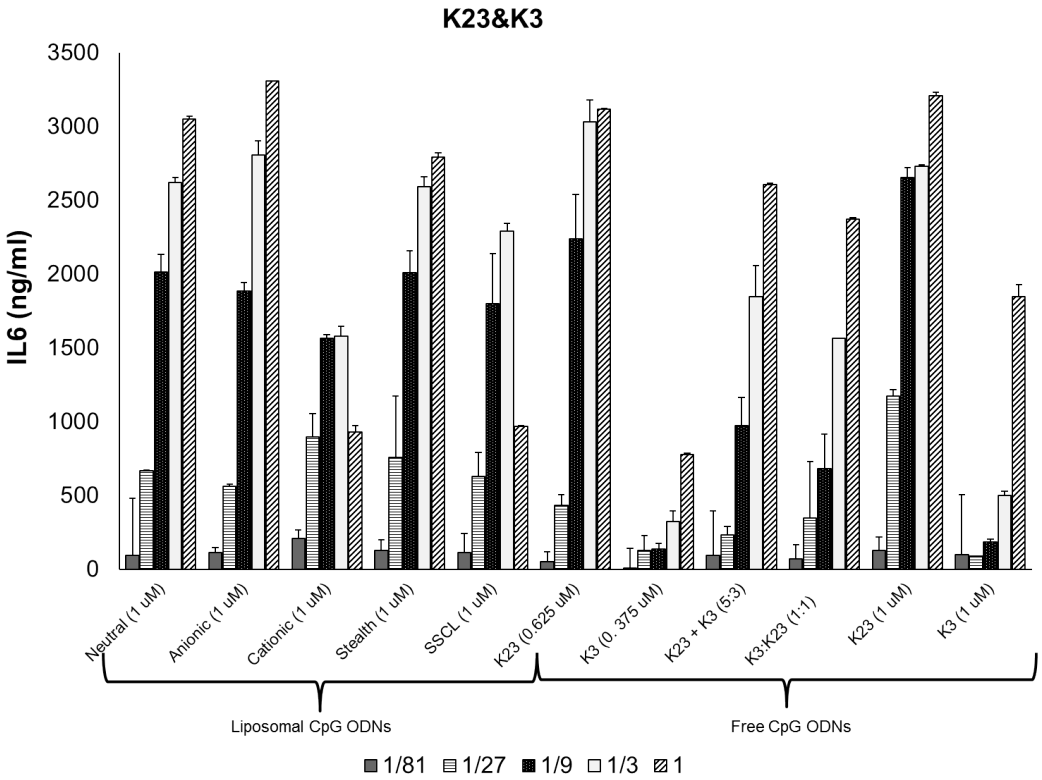

Figure 4.2: Dose dependent IL6 secretion profiles of different liposome formulations co-encapsulating K23, and K3. Spleen cells ($2X10^6$ cells/ml) were treated with different doses of liposome formulations in culture. Starting doses corresponded to 1 μmol/ml ODN and diluted 3 fold at each subsequent steps, for four times. 36h later supernatants were collected and IL6 levels were detected by Cytokine ELISA. $p<0.01$ for liposomal vs free K23 + K3 mixture at 1/9 and 1/27 dilutions

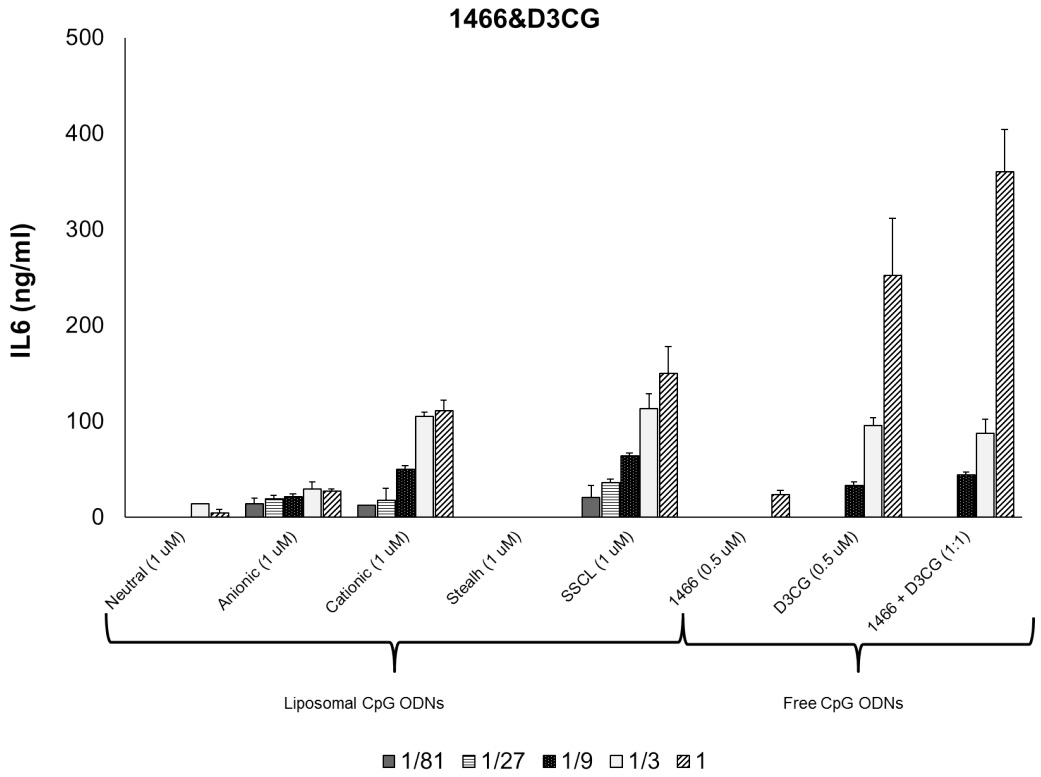

Figure 4.3: Dose dependent IL6 secretion profiles of different liposome formulations co-encapsulating 1466 Acore MB, and D3CG MB. Spleen cells ($2X10^6$ cells/ml) were treated with different doses of liposome formulations in culture. Starting doses corresponded to 1 μmol/ml ODN and diluted 3 fold at each subsequent steps, for four times. 36h later supernatants were collected and IL6 levels were detected by Cytokine ELISA. $p<0.01$ for SSCL and cationic liposomal vs. free 1466 + D3CG mixture at 1/9 dilution

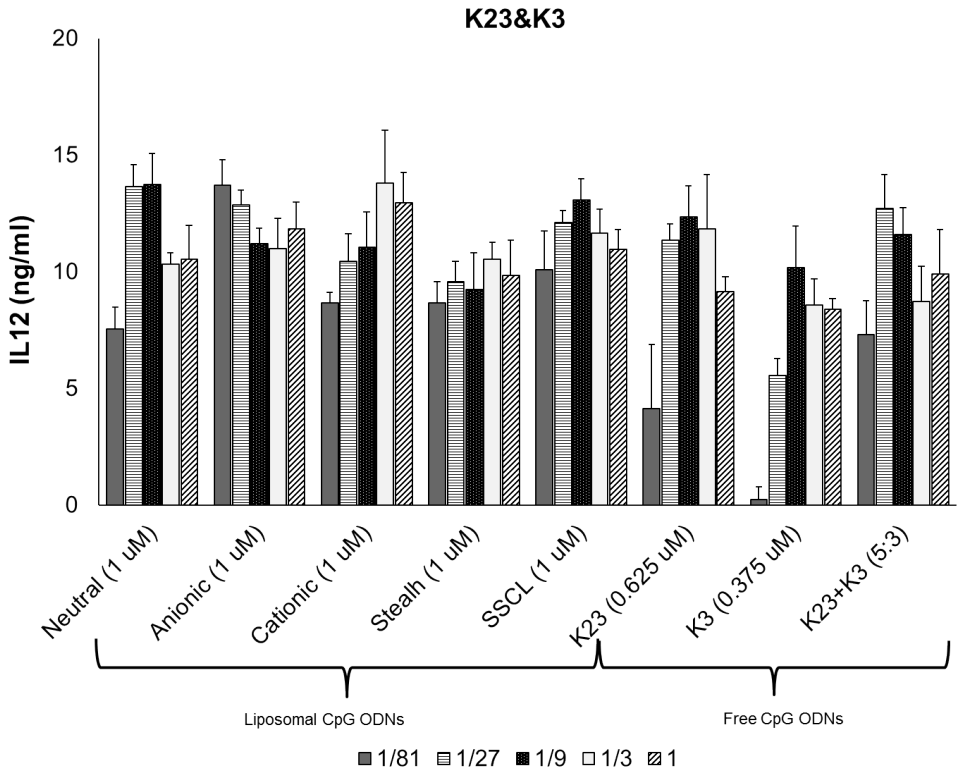

Figure 4.4: Dose dependent IL12 secretion profiles of different liposome formulations co-encapsulating K23, and K3. Spleen cells ($2X10^6$ cells/ml) were treated with different doses of liposome formulations in culture. Starting doses corresponded to 1 μmol/ml ODN and diluted 3 fold at each subsequent steps, for four times. 36h later supernatants were collected and IL6 levels were detected by Cytokine ELISA.

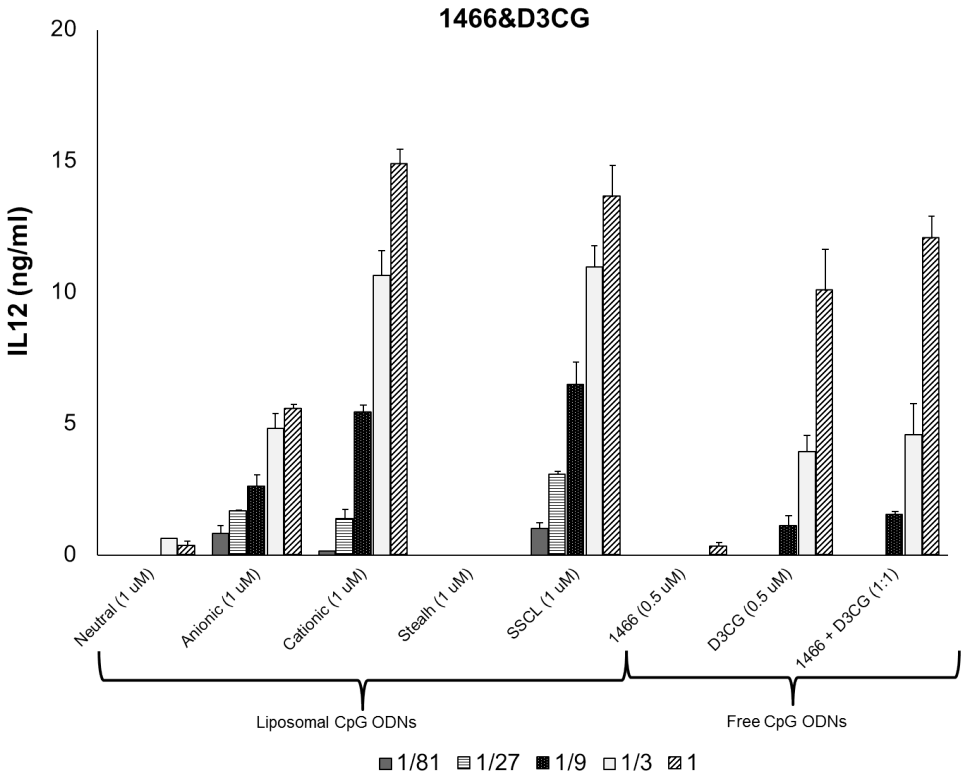

Figure 4.5: Dose dependent IL12 secretion profiles of different liposome formulations co-encapsulating 1466 Acore MB, and D3CG MB. Spleen cells ($2X10^6$ cells/ml) were treated with different doses of liposome formulations in culture. Starting doses corresponded to 1 μmol/ml ODN and diluted 3 fold at each subsequent steps, for four times. 36h later supernatants were collected and IL6 levels were detected by Cytokine ELISA. $p<0.01$ for SSCL and cationic liposomal vs. free 1466 + D3CG mixture at 1/3, 1/9 and 1/27 dilution

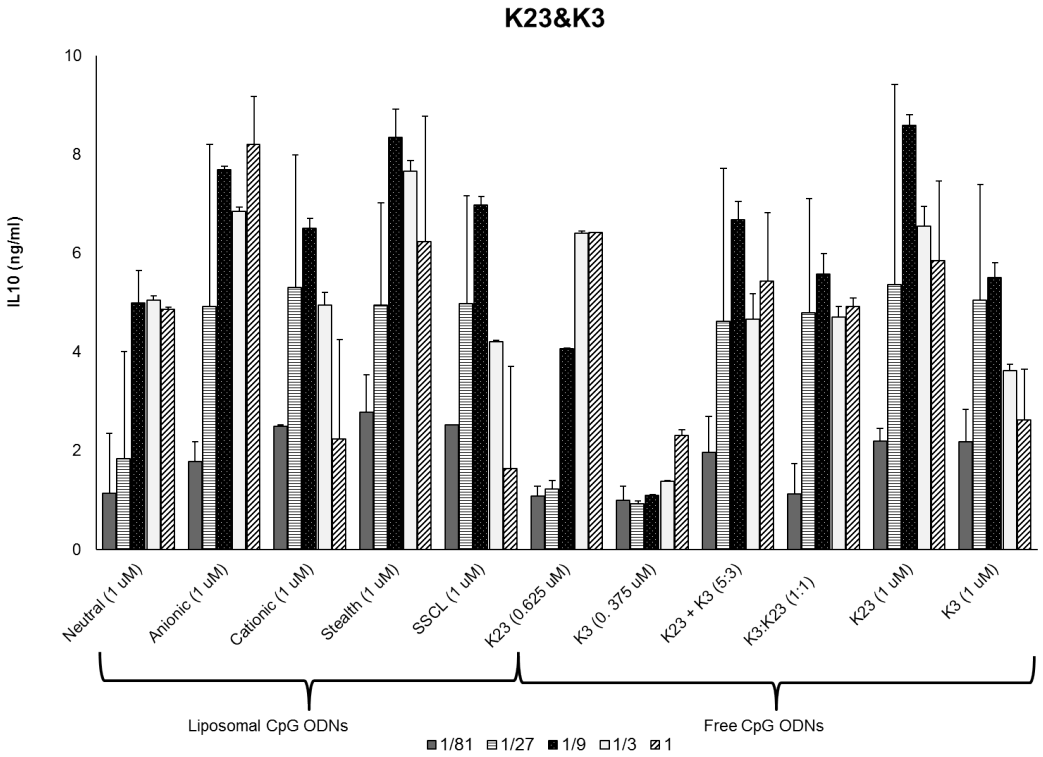

Figure 4.6: Dose dependent IL110 secretion profiles of different liposome formulations co-encapsulating K23, and K3. Spleen cells (2X10^6 cells/ml) were treated with different doses of liposome formulations in culture. Starting doses corresponded to 1 μmol/ml ODN and diluted 3 fold at each subsequent steps, for four times. 36h later supernatants were collected and IL6 levels were detected by Cytokine ELISA.

4.3 Immunization with Liposomes Co-Encapsulate CpG ODN and *H. felis* Total Cell Extract

After in vitro studies, the most potent candidates were selected for *H. felis* immunization in mice. Anionic liposomes for K23 and K3 co-encapsulation and liposomes with cationic lipids for 1466 and D3CG co-encapsulation were arguably the most prominent formulations. Although cationic liposomes were as prominent for 1466 and D3CG, we thought that SSCL that contain PEG along with cationic lipid were also suitable for immunization study. Finally, we also included 1555 in cationic liposomes for *H. felis* immunization, since this formula was shown to be a strong immune stimulant in another study [113].

6-8 weeks old female BALB/c mice were immunized on day 0 and day 14 with *H. felis* total cell extract along with CpG ODN or their liposome encapsulated forms. One day before booster injection (d=13), two weeks after booster injection and 6 weeks after booster injection mice were tail bled, sera were collected, titrated and IgG, IgG1 and IgG2a levels were analyzed by ELISA. IgG2a/IgG1 ratio was calculated to determine whether cell mediated immunity or humoral immunity was the outcome after immunization.

Many conventional vaccines require a booster injection after primary injection to promote sufficient immune protection. This is also very important in mice vaccination studies. However, we decided to include comparison of IgG levels after the primary injection, because we postulated that *H. felis* extract naturally contains many immunomodulatory molecules and they might contribute along with CpG ODN to boost a robust anti-*H.felis* immunity even after single vaccination. As mentioned above, *H. felis* is a gram negative bacteria and mammalian immune system has more than one mechanism to recognize components of gram negative bacteria. This might have been also a drawback; since injection of total cell extract could mask the effect of co-encapsulation of *H. felis* antigen along with CpG adjuvants within liposomes.

Bleeding two weeks after primary injection revealed that *H. felis* total cell extract is sufficient to provoke immune system to produce IgG against *H. felis* antigen (Figure 4.7). In fact, much to our surprise at first hand, two of PBS injected mice also had high levels of IgG (Figure 4.7, also see Appendix for IgG2a and IgG1 graphs for 1st bleed). Under certain circumstances, however, this is not as surprising as it first seems. Our facilities are not pathogen free and it is likely that those animals have encountered gram negative bacteria before.

Regarding free CpG ODNs and their liposomal encapsulated forms in terms of total IgG production, there were not essential difference between them, but there were slight improvement of free CpGs over liposomal formulations (Figure 4.7.A). This is also true for IgG2a subtype (Figure 4.7.B). In terms of IgG1 subtype, no conclusive inference can be done as IgG1 readings were not proportional to dilution, which probably means they were below proper detection limit (Figure 4.7.C)

After the booster injection, liposomal formulations were still not performing better than free CpGs in terms of total IgG. Free 1466 Acore MB and 1555 were better at the selected dilution (1/7168) and only Anionic K23&K3 had a slight advantage over free K23&K3 (Figure 4.8.A). IgG subclasses IgG1 and IgG2a, however, gave mixed results. Liposomal 1466 indeed induced more IgG2a secretion than its free form, whereas other formulations induced more IgG2a secretion when they were injected in free forms (Figure 4.8.B). IgG1 secretion was more for Anionic K23&K3 and SSCL 1466. Liposomal 1555 and free 1555 induced approximately the same levels of IgG1. (Figure 4.8.C).

IgG levels for each mouse and each group after second bleeding were as expected gave higher titers than the first bleeding (Figure 4.8). IgG levels of animals that were immunized with *H. felis* cell extract alone also had high levels of IgG in their sera (Figure 4.8), even higher than some liposomal groups (i.e. Cationic 1555). Nevertheless, CpG groups had still more effective for IgG2a and IgG1.

Third bleed was important to judge the persistance of antibody titers agains *H.felis*. As this bleeding was 6 weeks after the last injection, we thought that

IgG levels of mice that were injected with *H. felis* cell extract alone would start to subside down to normal levels. We also expected that liposomal CpG formulations would have induced higher IgG than free CpGs since they would affect more immune cells due to co-encapsulation and relatively slow serum clearance. Our initial assumptions were partially true. Total IgG levels of mice immunized with cell extract alone were lower, but CpG injected groups were also lower (Figure 4.9). Besides, liposomal CpGs were again not better than free CpGs (Figure 4.9.A). However, IgG2a and IgG1 showed stronger anti *H. felis* titers. Anionic K23&K3 clearly induced more IgG2a and IgG1 than those of free CpG and *H. felis* cell extract alone (Figure 4.9.B and Figure 4.9.C). SSCL 1466 and cationic 1555 also caused more IgG1 secretion (Figure 4.9.C).

We finally compared IgG2a and IgG1 levels of immunized mice. IgG2a/IgG1 ratio is used to determine whether antibody response led to a Th1-biased (cell mediated) or humoral immune response. If the ratio is higher than 1, it means Th1 response is dominant and if it is smaller than 1 antibody mediated response is dominant. Among treatment groups, only free K23&K3 were able to have a ratio of IgG2a/IgG1 higher than 1 (Figure 4.10), which was lost 4 weeks later. It was considerable that free CpG groups have all higher ratio than liposomal groups, that quickly turned just the opposite at the end of 6 weeks post booster injection. It is tempting to speculate that liposomal groups caused more stable immune reaction than free CpG groups, however, we did not have means to test this possibility. These animals are still under investigation and *H.felis* challenge will be done to these mice at the end 10 weeks. The time frame of this thesis unfortunately goes beyond these assays.

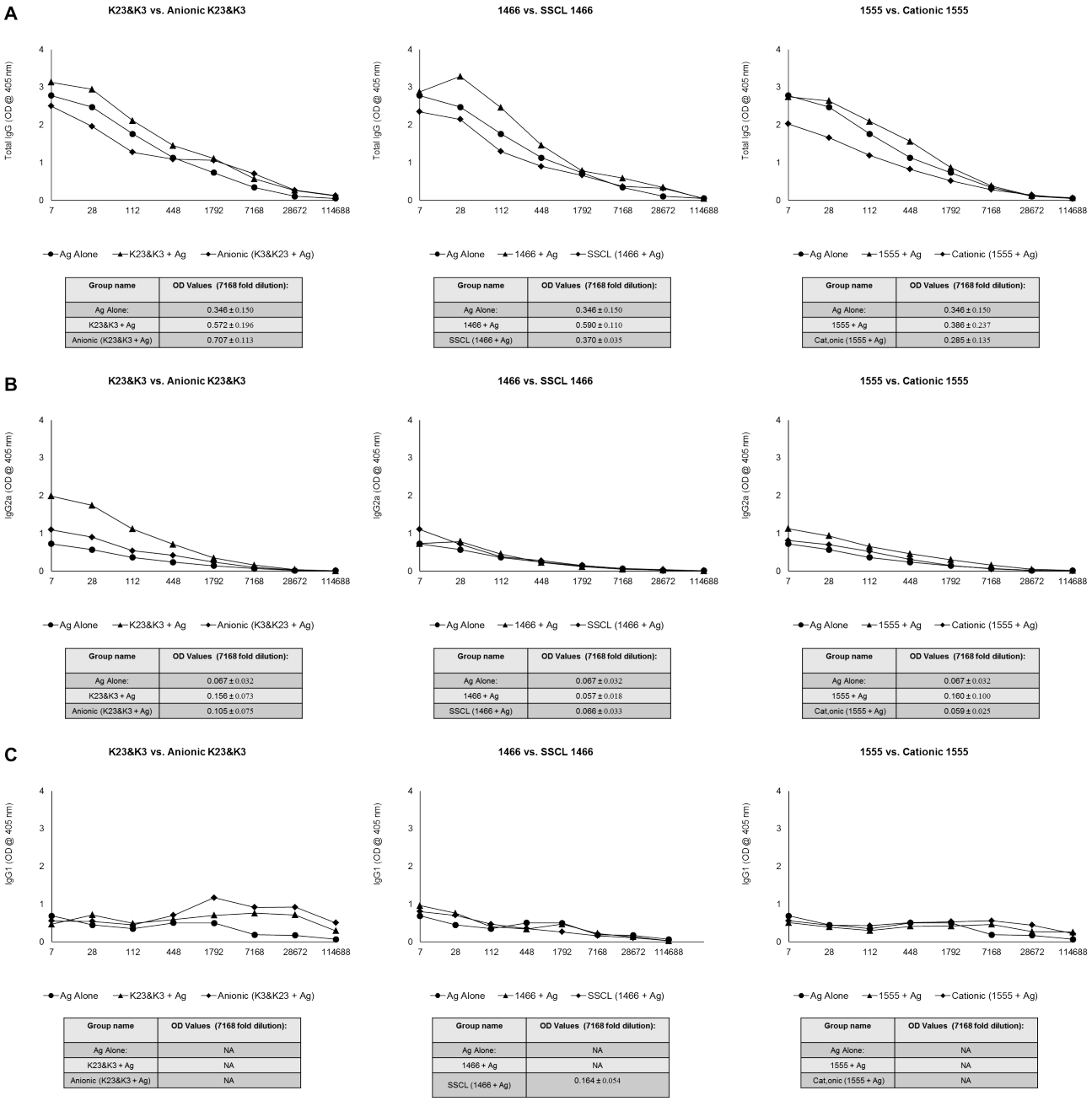

Figure 4.7: Comparison of IgG levels of immunized mice against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA. A. Total IgG levels. B. IgG2a levels. C. IgG1 levels. For individual responses and means, see Figures A.1- A.4.

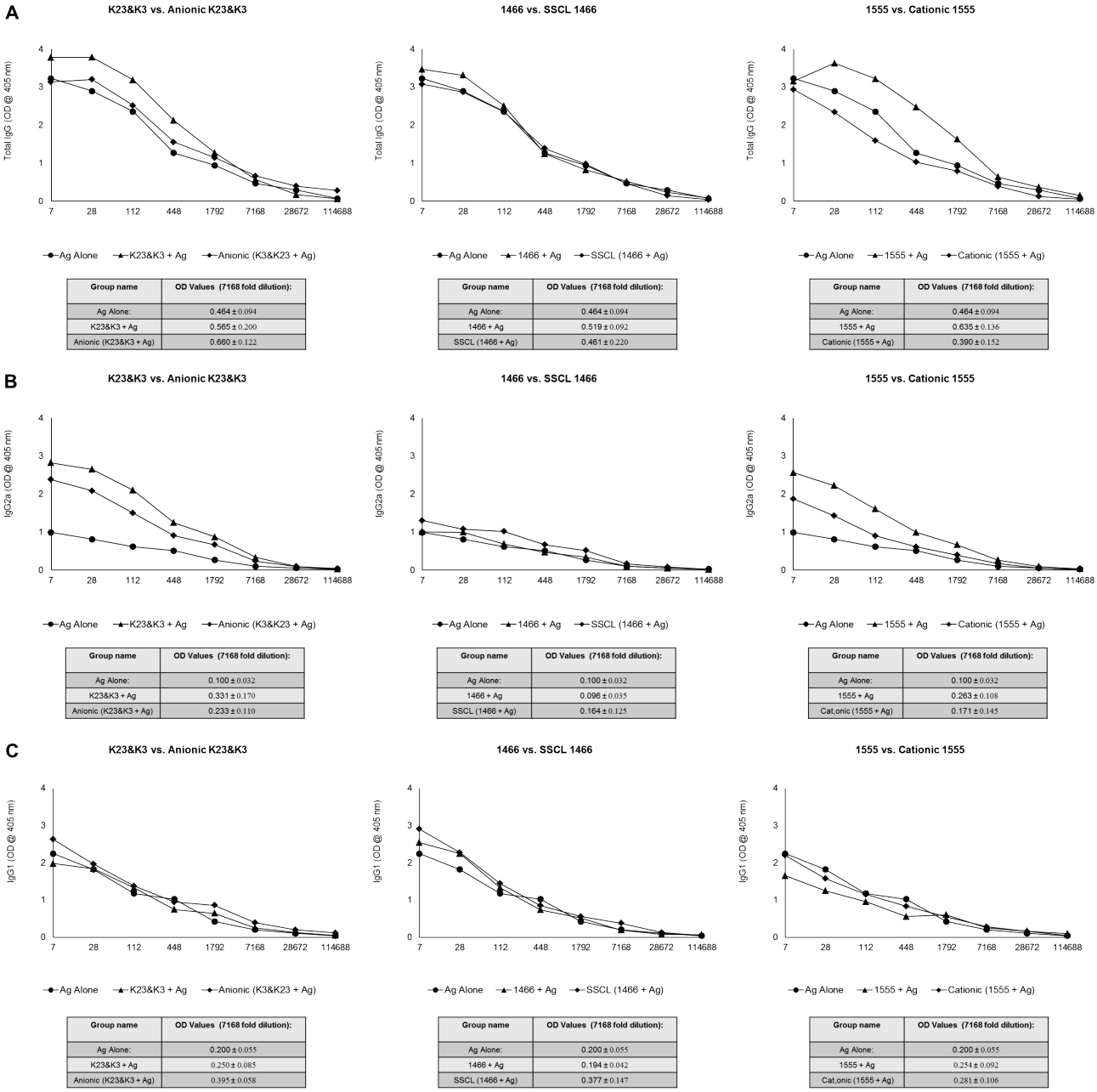

Figure 4.8: Comparison of IgG levels of immunized mice against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA. A. Total IgG levels. B. IgG2a levels. C. IgG1 levels. For individual responses and means, see Figures A.7- A.10.

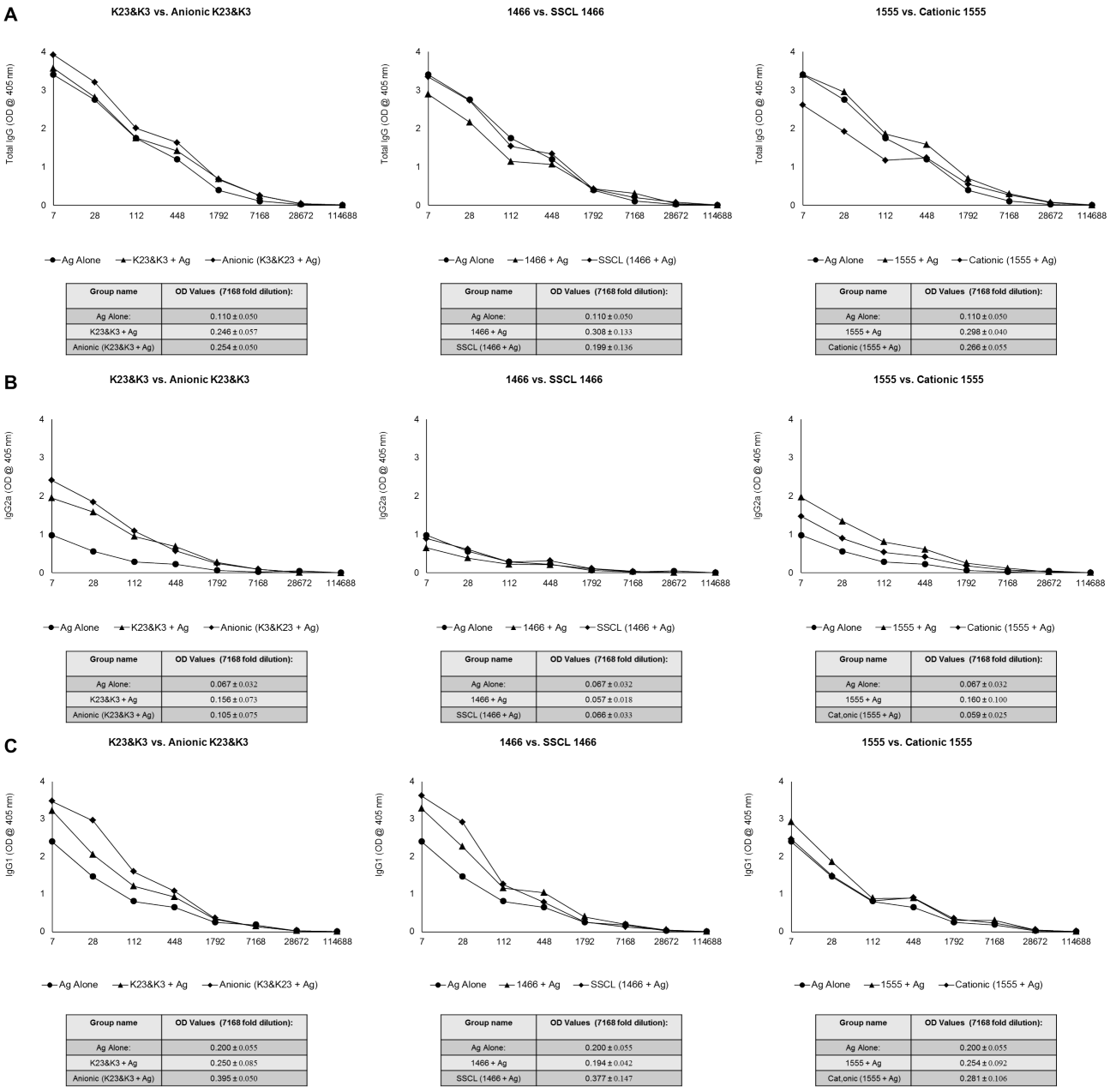

Figure 4.9: Comparison of IgG levels of immunized mice against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA. A. Total IgG levels. B. IgG2a levels. C. IgG1 levels. For individual responses and means, see Figures A.13- A.16.

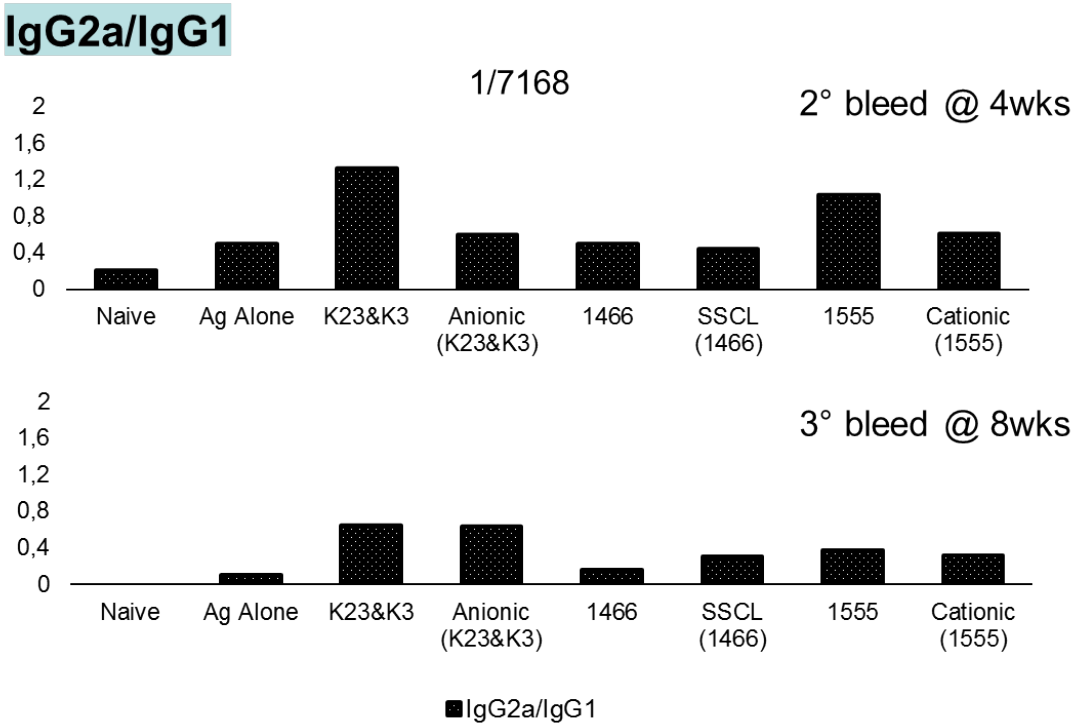

Figure 4.10: Persistence of anti-*H.felis* responses among different treatment groups.

4.4 Simultaneous Stimulation of PBMCs with liposomes encapsulating D35 or K3

In humans, K type ODNs and D type ODNs have different immune stimulatory properties. K type ODN stimulates TNFα secretion from pDCs, whereas D type ODN induces IFNα secretion. K type ODN, therefore initiates a pro-inflammatory cascade against invading pathogens (mostly effective against bacterial infections), D type ODN, however, leads to mainly antiviral immune response via type I interferon signaling. A good protective reagent would have ability to induce both type of immune reactions against diverse range of pathogens. Unfortunately, simultaneous administration of K and D type ODNs is not an option in their free forms, as mentioned in Section 1.3.4, this is mainly due to counter action of these ODNs on immune cells. When K-ODN is internalized by pDC, due to accumlation of K-ODN in late endosomes, it leads to maturation of pDCs along with TNFα secretion, therby D-ODN effect is abolished. Several groups have tried to develop new CpG ODN classes (such as C-type ODNs, or P-Type ODNs), but none of those efforts successfully recapitulated D ODN-specific effects (i.e. these new ODN classes are still more close K-type ODN activity).

We tried to overcome this problem by encapsulating D and K type ODN within different liposomes and tried to analyze these different liposomes loaded with K or D type ODNs in a synergistic fashion. In a preliminary experiment, we aimed to identify candidate liposome types loaded with different ODNS. We asked the question whether there are synergistic liposome formulations. Here the idea was to identify different liposome combinations where D and K ODNs does not cancel each other‘s activity when loaded within liposomes. PBMCs were isolated from blood of two healthy subjects and stimulated in culture simultaneously with K and D type ODNs (free or liposome encapsulated forms). We tested all five type of liposomes for K type ODN K3 encapsulation and neutral and anionic type liposomes for D type ODN D35 encapsulation. We also used two different concentrations of CpG ODNs (0.3 uM and 1.0 uM) to mix optimum and sub-optimum ODN

concentrations as this might have been the key for ODN synergism.

Figure 4.11 shows IL6 profile of PBMCs that were stimulated with K3 and/or D35 encapsulated into different liposomes. IL6 secretion did not show noteworthy additive or synergistic effect of K3 and D35 when PBMCs were treated with high doses of K3 (1.0 uM K3). We were able to see higher induction of IL6 secretion when 0.3 uM K3 was mixed with 0.3 uM or 1.0 uM D35 than either D35 or K3. TNFα secretion was also increased positively by simultaneous administration of K3 and D35 at lower doses, albeit there were some additive effects of K3 and D35 at higher doses (Figure 4.12). Although both K3 and D35 were reported as good immune stimulants for PBMCs, we did not see any IL6 secretion when CpG ODNs were used without liposomes. PBMCs from subject #1 (S1 hereafter) were also not stimulated by free CpG ODNs to secrete TNFα. Only S2 had very little TNFα secretion. This situation is a proof that liposomes can boost the effect of CpG ODNs. It is also important to note that empty liposomes did not induce any cytokine or interferon induction indicating that the activation is CpG motif dependent (data not shown).

IFNγ production by K3 and D35 somewhat affected different from IL6 and TNFα. D35 is a natural inducer of IFNγ. Yet rates of IFNγ were not detectable even when liposomal D35 was used (Figure 4.13). Low dose K3 also did not produced any interferon. However, when 1 uM K3 in liposomes was used, PBMCs started to secrete detectable levels of IFNγ. Among different liposomes, stealth liposomes encapsulated K3 were the most successful type. Interestingly, when only liposomal K3 was used, IFNγ production was superior. Only SSCL liposomes encapsulating K3 showed a synergism with liposomal D35. This effect was subject specific. Only in S2, IFNγ production was detectable, unfortuantley in S1 it was completely absent. This was a good demonstration of discrepancy in human responses. Nevertheless, it was clear that 1.0 μM K3 in high doses were able to induce IFNγ, when delivered in specific liposome formulation. This is an indication of the alteration of the subcellular distribution of K3 when given in liposomes. It was also worth mentioning that in our hands, D35 mediated cytokine production

is dose independent.

Figure 4.14 shows IFNα induction ability of the PBMCs. This figure contains only groups that were treated with cationic K3 and free or liposomal D35 since regardless of liposome type, when free or liposomal encapsulated K3 was used along with D35, D35 lost much of its IFNα induction activity. When PBMCs were treated with cationic liposomes that encapsulate K3 along with liposomal D35 cells were able to secrete IFNα. Alone D35 at high dose induced higher IFNα in S2 but not in S1. K3 completely abolished this effect in S2. Both PBMCs from two subjects secreted high amounts of IFNα when neutral or anionic liposomes containing D35 were used. When taken together, the cytokine profiles of PBMCs from two subjects suggested that suboptimal doses (0.3 uM) of K and D type ODNs in liposomes are indeed important for synergistic stimulation of immune cells. This is probably due to alteration of subcellular fate of K3 when delivered in liposomes thus cancelling their competitive induction pathways.

We, therefore, planned a follow up study to investigate in depth the beneficial effect of combining two types of ODNs within liposomes. For this, we have chosen a single K3 dose (0.3 uM) in liposomes, along with either 0.3 or 1.0 uM D35. Based on the preliminary study only cationic lipid containing liposomes (cationic and SSCL) were chosen for K3. K3 in those liposomes did not exhibit considerable disparity for cytokines IL6. TNFα and IFNγ was perniciously affected by those liposomes. In fact, IFNγ secretion was almost none when cationic liposomes encapsulating K3 were given to PBMCs, however, other liposomes has done the same thing for IFNα. Consequently, cationic liposomes and SSCL were our prime candidates for K3 encapsulation. We deduced that neutral and anionic liposomes were not enough for D35 encapsulation, henceforth we included stealth liposomes for the next study.

Figure 4.15 and 4.16 shows IL6 and TNFα secretion following stimulation of PBMCs from four different healthy subjects with simultaneous treatment of liposomes encapsulating K3 and D35, respectively. Among liposomal K3 groups,

SSCL performed better for those cytokines in this study. Although exact cytokine amounts differ between patients, the general trend was strikingly the same. SSCL-K3 consistently stimulated IL6 and TNFα secretion. This effect of SSCL-K3 was synergized with Anionic-D35 and partially abolished with Stealth-D35 or Neutral-D35. Neither free K3 nor free D35 reproduced this effect when IL6 and TNFα were considered.

Unfortunately, only one subject$'$s PBMCs secreted IFNγ (Figure 4.17). SSCL-K3 was again the most successful one. IFNα secretion profile of subjects exhibited intriguing discrepancy (Figure 4.18). Free D35 induced IFNα secretion in a dose dependent manner, which was lost when K3 added as the second stimulant. For two out of four subject (S1 and S4), as such Cationic-K3 but not SSCL-K3 or liposomal D35 formulations stimulated IFNa secretion, whereas for one subject only the liposomal D35 induced IFNα secretion. (S1). For the remaining subject (S2), liposomal D35 and Cationic K3 were both active, although liposomal D35 alone induced higher IFNα level. Furthermore, the groups that cationic liposomes synergized with D35 encapsulating liposomes were different.

In conclusion, it was apparent that when K and D type CpG ODNs were encapsulated within different liposomes and administered simultaneously, it was possible to induce secretion of different cytokines and interferons that were normally not likely to be secreted in other forms. However, we still lack conclusive evidence for which types of liposomes have superiority over others to consistently and reproducibly activate both K and D ODN specific signaling pathways.

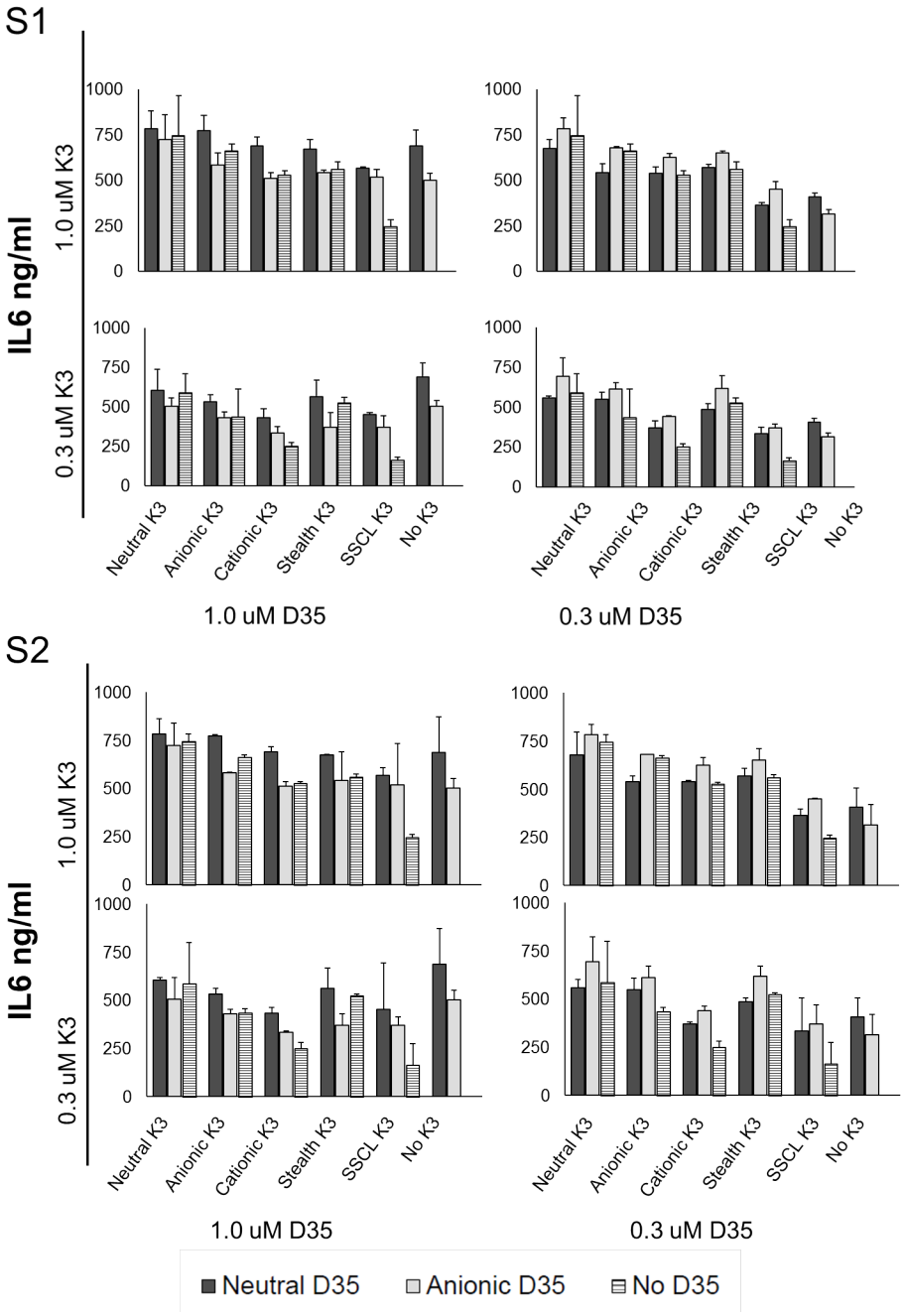

Figure 4.11: Dose and Liposome type dependent IL6 production by K and D type CpG ODNs from human PBMCs

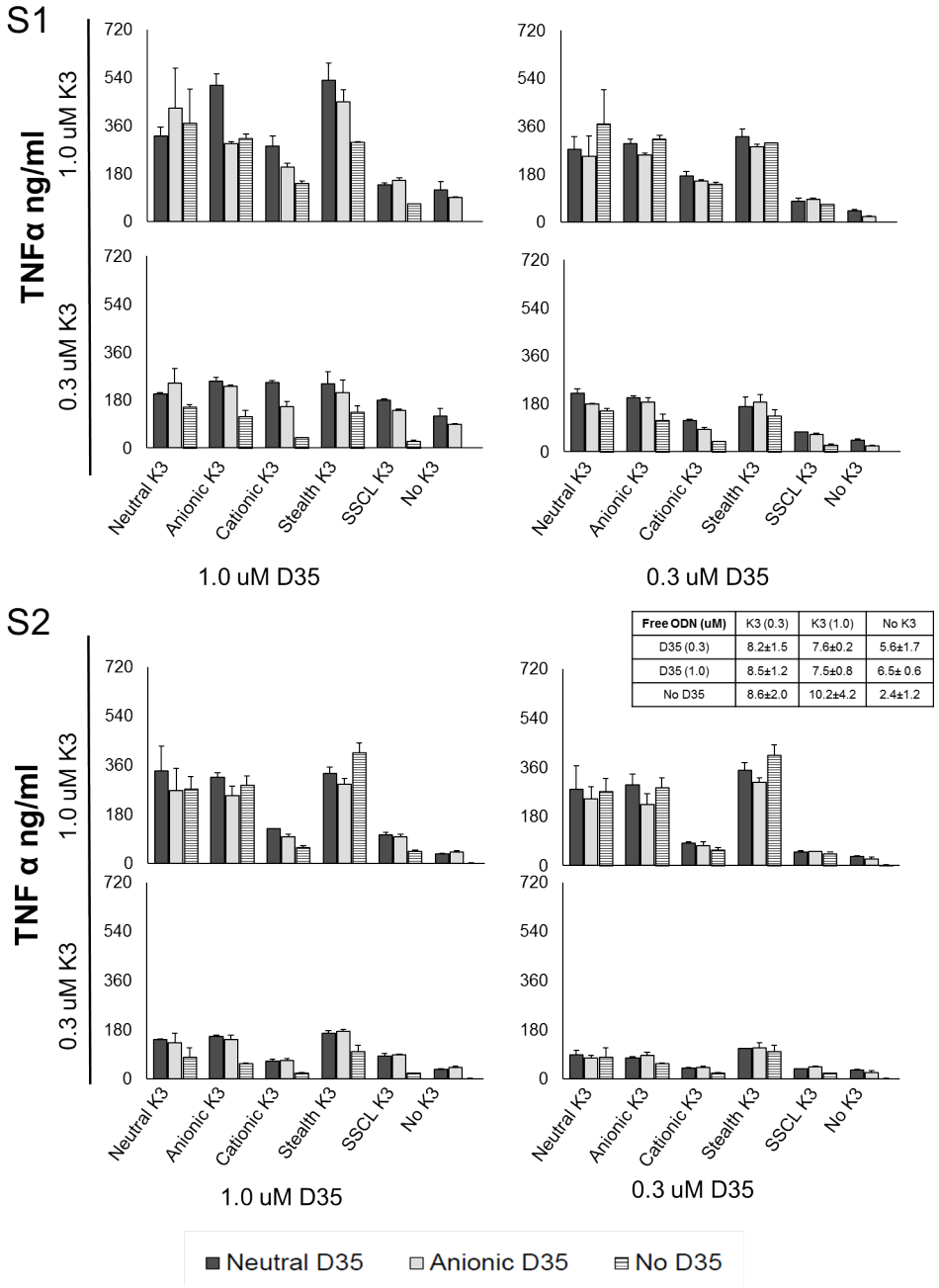

Figure 4.12: Dose and Liposome type dependent TNFα production by K and D type CpG ODNs from human PBMCs

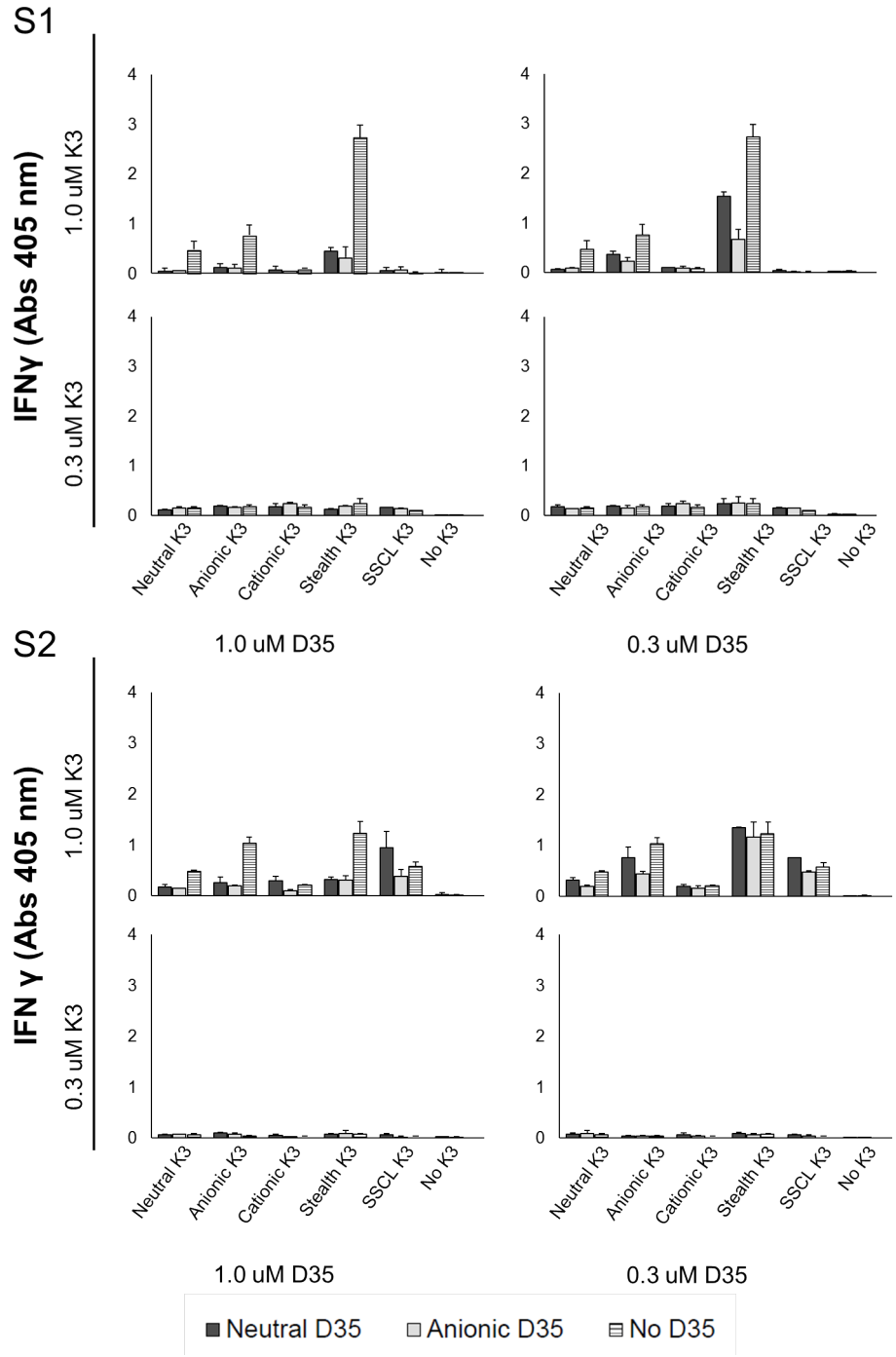

Figure 4.13: Dose and Liposome type dependent IFNγ production by K and D type CpG ODNs from human PBMCs

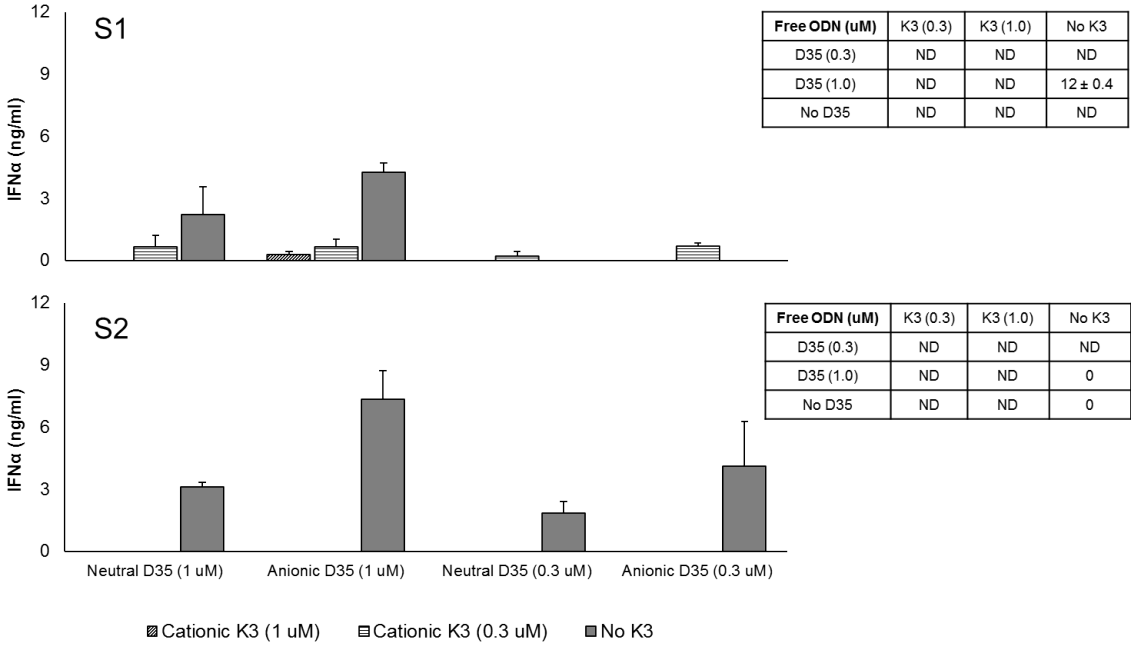

Figure 4.14: Dose and Liposome type dependent IFNα production by K and D type CpG ODNs from human PBMCs

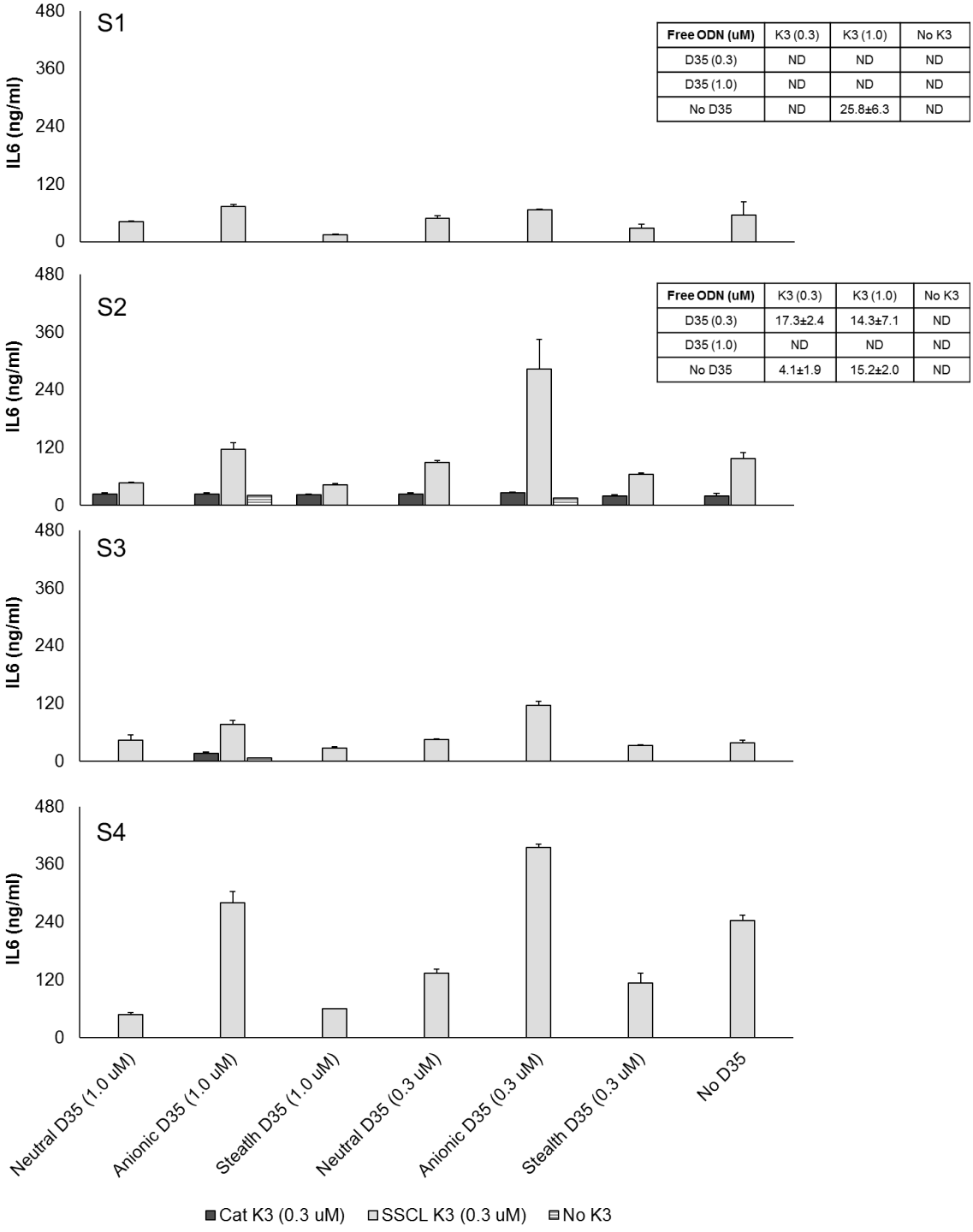

Figure 4.15: IL6 production after simultaneous administration of liposomal K and D to human PBMC

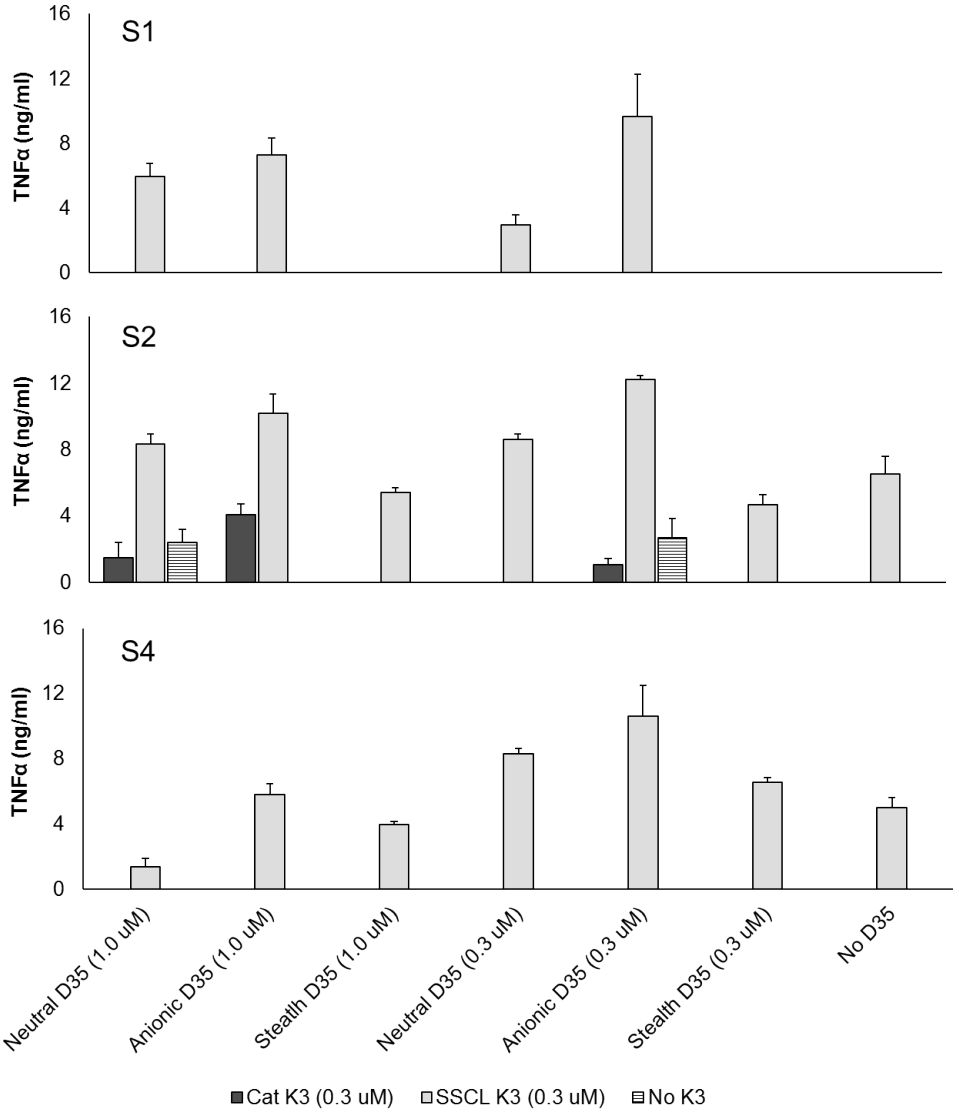

Figure 4.16: TNFα production after simultaneous administration of liposomal K and D to human PBMC

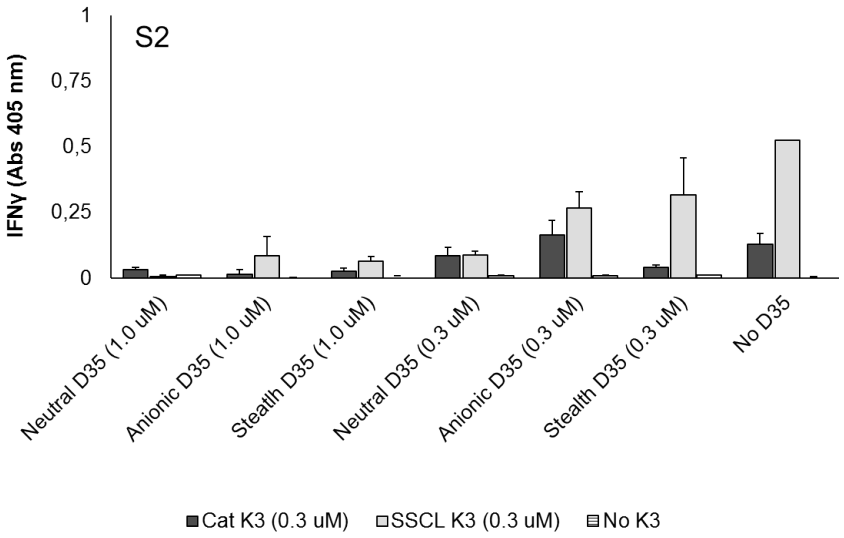

Figure 4.17: IFNγ production after simultaneous administration of liposomal K and D to human PBMC

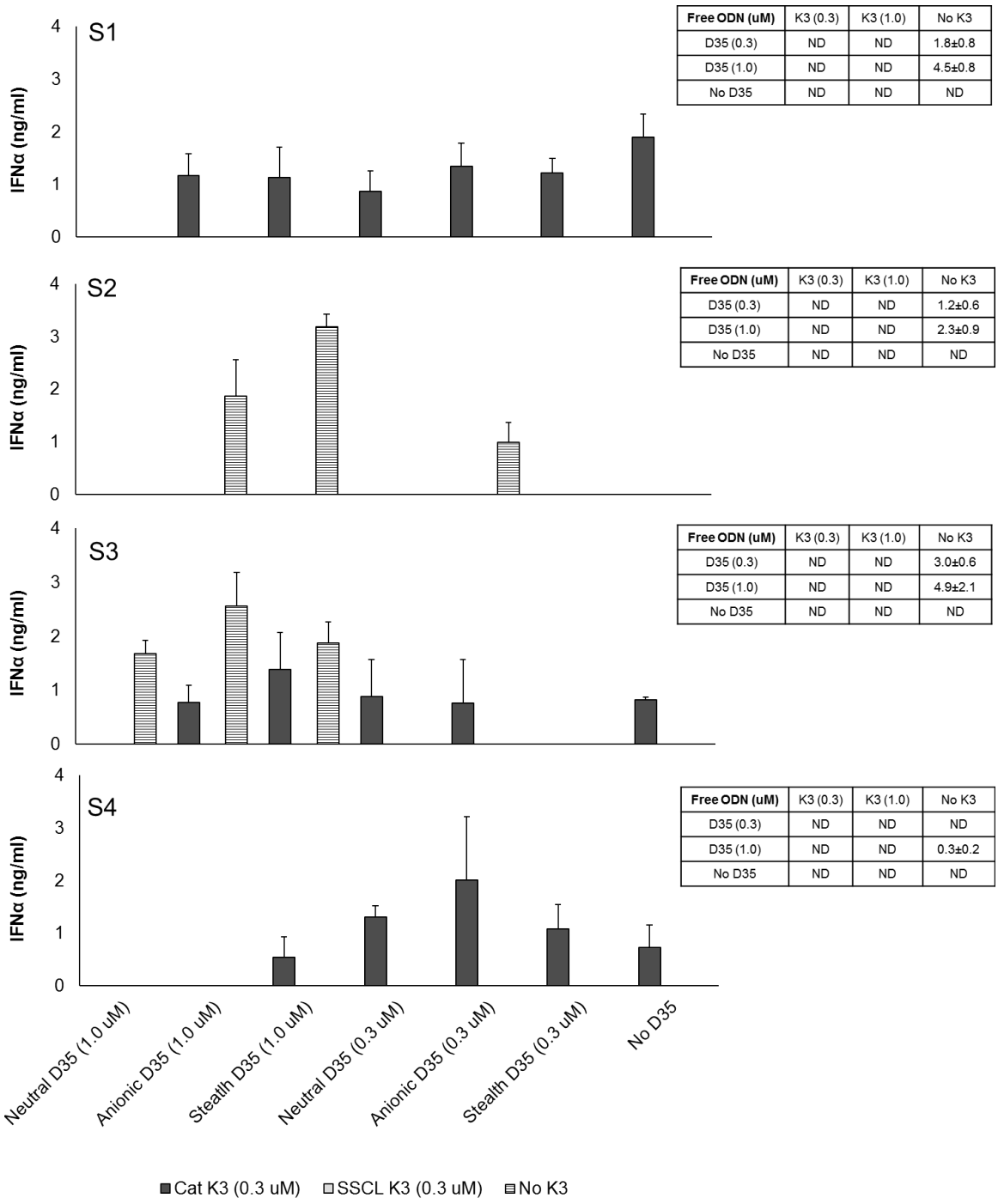

Figure 4.18: IFNα production after simultaneous administration of liposomal K and D to human PBMC

Chapter 5

Discussion

In this thesis, immune stimulatory properties of K and D type ODNs in liposomes were investigated in two different contexts.

It was previously shown that encapsulation of CpG ODNs into liposomes can improve both immunostimulatory activity and uptake by immune cells in vitro and in vivo [115]. Besides, liposome encapsulated CpG ODNs had promising adjuvant effects [115, 113]. Hence, we aimed to analyze effects of liposomal introduction of CpG ODNs to RAW 264.7 cells on critical proinflammatory cytokine genes IL1β and IL6, as well as chemokines IP10 and MIP1α and TLR9, receptor evolved for sensing unmethylated CpG motifs. We choose these readouts because earlier studies reproducibly proved that ODN treatment triggers pro-inflammatory and inflammatory response as well as TH1 biased immunity [116]. We have used anionic liposomes for D35 and neutral liposomes for K3 based on a previous study in our lab [113]. We were able to show that anionic liposome encapsulated D35, but not control sequence or non-encapsulated D35 can induce prolonged and pronounced cytokine expression (Figure 4.1). This effect was clearly CpG specific and liposomes contributed the augmentation of all these parameters tested on this cell line. Besides, chemokine expression and TLR9 expression was boosted by AL containing D35 (Figure 4.1). We were not able to capture those effects on

NL encapsulating K3. In accordance with our findings, in 2001 Verthelyi et. al supported our results as they demonstrated that optimum sequence specificity is another element for this enhanced activity [53].

Our utmost aim for the first part of this thesis was testing augmented vaccine adjuvant effects of CpG ODNs when they were coencapsulated with helicobacter antigen into liposomes against helicobacter vaccine development to control widespread infections. Consequently, after obtaining preliminary data on liposomal CpG formulations, we then proceeded to in vitro splenocyte stimulation assays with liposomal CpG ODNs co-encapsulating different type of CpG ODNs in a dose dependent manner. Earlier studies suggested that coencapsulation of different TLR ligands into one liposomal compartment might cause synergism between them [113]. Moreover, due to discrepancy in human responses, which is fairly reasonable since humans are out breed species, it is not realistic to expect one type of CpG ODN can yield protective immunity against pathogens in the majority of a cohort. Therefore, Klinman et. al. have used a cocktail of CpG ODNs to immunize macaques against anthrax [117] and demonstrated the benefit of administering three different type of CpG rather than using one. Thus, we also co-encapsulated two different CpG ODNs into five different types of liposomes. Co-encapsulated CpG ODNs were K23 along with K3, two K type ODNs, both are strong inducers of proinflammatory cytokine expression in splenocytes [118], and D3CB MB, a D type ODN along with 1466 A-core MB, D-like ODN, with modest proinflammatory cytokine induction effects yet they are type I IFN inducers [113]. Furthermore, we serially diluted CpG ODNs to find the lowest dose that was active in vitro. Our results, as expected, revealed that, at high doses, liposome incorporation did not contribute to IL6 and IL12 production. However, as we titrated the ODN concentration, liposomal CpG ODN mixtures yielded reliable amounts of IL6 and IL12 where free counterparts lost their effect (Figures 4.2, 4.3, 4.4 ad 4.5). This phenomenon was also observed by Erikci et. al. [113]. IL6 secretion at low doses was most pronounced when K23&K3 mixture in anionic liposomes was given to splenocytes. For the 1466&D3CG mixture, best performing liposomal formulations were cationic and SSCL. Consistently,

anionic liposomes and cationic liposomes in addition to SSCL were the most potent formulas for IL12 secretion, for K23&K3 and 1466&D3CG, respectively. IL10 was among tested cytokines. There was a correlation between high levels of IL10 secretion and H. pylori induced gastritis [119], which is probably due to Treg activity [120, 121]. We found that liposomal CpG ODNs did not increase IL10 levels significantly compared to free counterpart irrespective of ODN type tested (Figure 4.6).

Consequently, we tested our most prominent liposomal CpG ODN formulations against *H. felis*. CpG ODNs and *H. felis* antigen were co-encapsulated into liposomes and mice were immunized with these formulas. Total cell lysate was used as antigen. Primary response, probably due to presence of several TLR ligands in *H. felis* antigen, indicated that, all formulations induced an Ig response (Figure 4.7). Secondary response after booster injection analyses revealed that Ag + ODN combination were as effective as liposomal counterparts (Figure 4.8). This unexpected result probably was due to the Ag and CpG dose that we selected. In this study, the amounts were 15 μg each for Ag and CpG per mouse. This was considerably higher that doses that were used in a previous study [122]. There, cholera toxin + CpG + Ag were used (10μg + 15 + 10μg per mouse). In our study we have decided 15 + 15 μg for Ag and CpG (free and liposomal). After seeing our results, it would have been more appropriate to reduce Ag and CpG dose. Only then we would be able to detect the effect of liposome encapsulation. This study is still ongoing. We are following the persistence of the antibody response against *H. felis* and our results indicated that free CpG + Ag dependent anti *H. felis* response tends to drop more faster than liposomal formulations (Figure 4.9). At this stage, it is fair to say that including liposomes improves antigenicity against *H. felis* compared to free combinations.

Final part of this thesis focused to overcome contrasting activity of simultaneous K and D CpG ODNs usage on PBMC. After the demonstration of unique features of D and further understanding of batch to batch variation problem that led to abandoning D ODN specific clinical trials [123], researchers attempted

to design new ODN classes resembling to D sequences, such as Y ODN, C type ODN or P ODN [58, 60, 55]. Yet their simultaneous usage failed to recapitulate the dual effect (i.e. K + D ODN effect). Alternatively, Honda et al used cationic liposomes complexed with K ODN in 2005 and demonstrated IFNa secretion from PBMCs [61]. Even this attempt did not broaden the spectrum of K + D ODN usage, since it only mimicked D ODN-like effect. In our novel approach, we tried to utilize both ODNs encapsulated in different liposomes and tested them simultaneously on peripheral blood. Our data strongly suggest that putting D35 ODN in anionic liposomes and co-incubating with liposomal K3 improved K ODN specific effect and putting K3 in cationic liposomes and coincubating with liposomal D35 improved D ODN effect. In two different trials, TNFa and IL6 secretion was increased when K in liposomes combined with D35 in anionic liposome (Figures 4.12 and 4.11 for first trial and Figures 4.16 and 4.15 for second trial). We also succeeded to recapitulate D ODN specific effect and even augment it when K3 in cationic liposomes and D35 in liposomes were simultaneously given to human PBMC (Figures 4.14 and 4.18) It was known that K and D ODNs have differential immune stimulatory activities as K ODN localizes to late endosome and rapidly promotes pDC maturation, whereas D ODN localizes to early endosomal compartments in where it induces IFNa expression [63, 62]. In case of simultaneous administration to PBMCs, despite uptake kinetics are not effected, maturation pDCs cancels D ODN specific effect [63, 62]. Or data suggests that we were able to change intracellular fate of D and K type ODNs by encapsulating them into specific liposome types and successfully overcome D and K ODN dichotomy.

Chapter 6

Future perspectives

In this study, the first time liposomal CpG formulations were tested against H. felis in an vaccination study, after conferring best in vitro performing formulations. Yet, efficacy of liposomal formulations did not surpass free counterparts. However, liposomal formulations tend to induce more stabile response. The more prolonged study was required to further confirm this results. Moreover, we lacked proper reagents to check IgA levels; a major antibody type for mucosal immunity. An assay to determine IgA levels would increase confidence of our results. Besides, in order to test potential of liposomal CpG ODNs as protective reagents, a pathogen challenge could be done to immunized mice some time after booster injection.

In the second part, we were able to capture both K type CpG ODN specific and D type CpG ODN specific effects when we combine proper liposomal K and D type ODNs (i.e. anionic D35 and cationic K3). We speculated that this effect was due to altered subcellular compartmentalization of K type ODN K3. As a follow up study, one can test this hypothesis in vitro and in vivo, by using appropriate dyes to monitor uptake and internalization of liposomal K3 and free K3 ODN along with liposomal D35 ODN and free D35 ODN. This would allow to confirm liposomes can change subcellular fates of K and D type CpG ODNs and their effects can be combined for broader range of immune stimulation. Working K and D CpG ODN dichotomy in mice with suitable reagents that we lack would be another option to develop our results.

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Appendix A

Supplementary Data

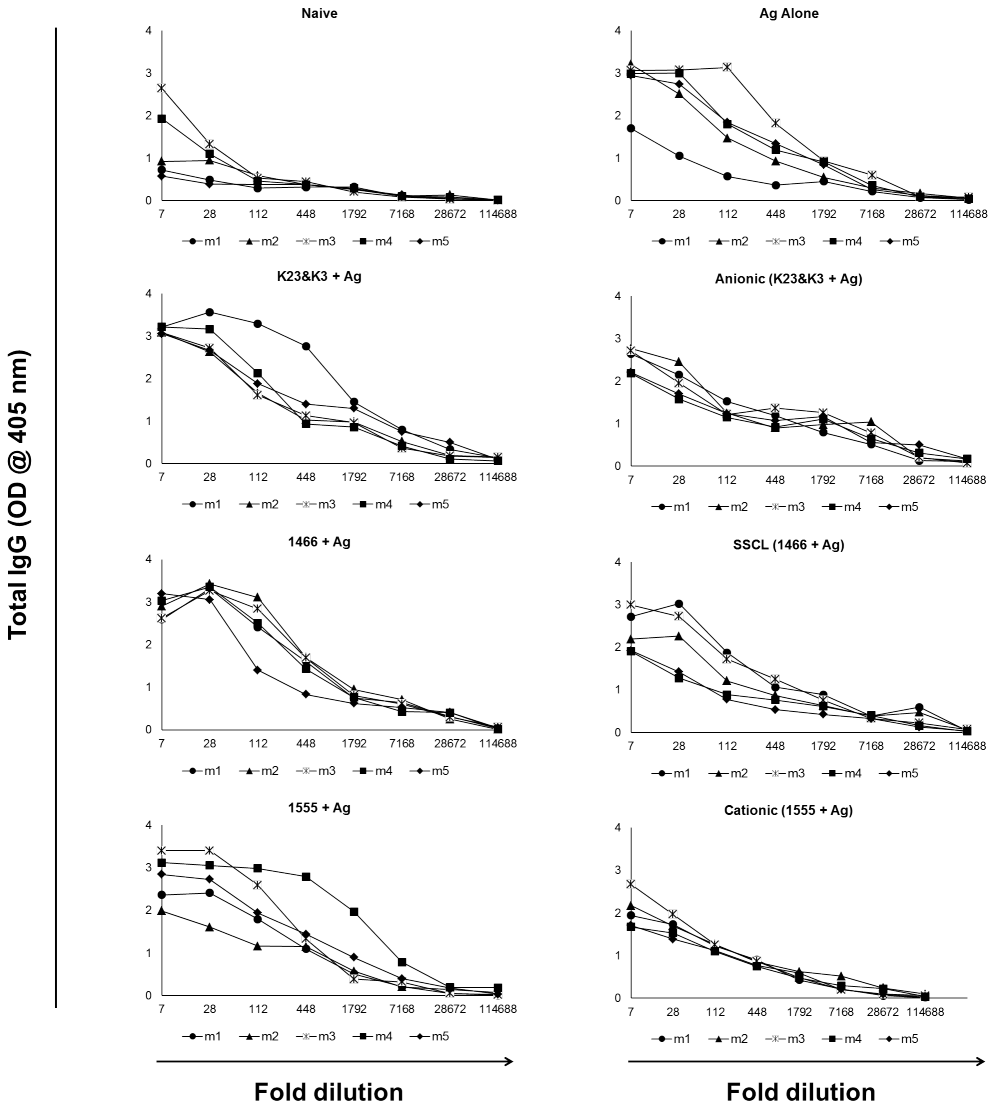

Figure A.1: IgG levels of individual mouse immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

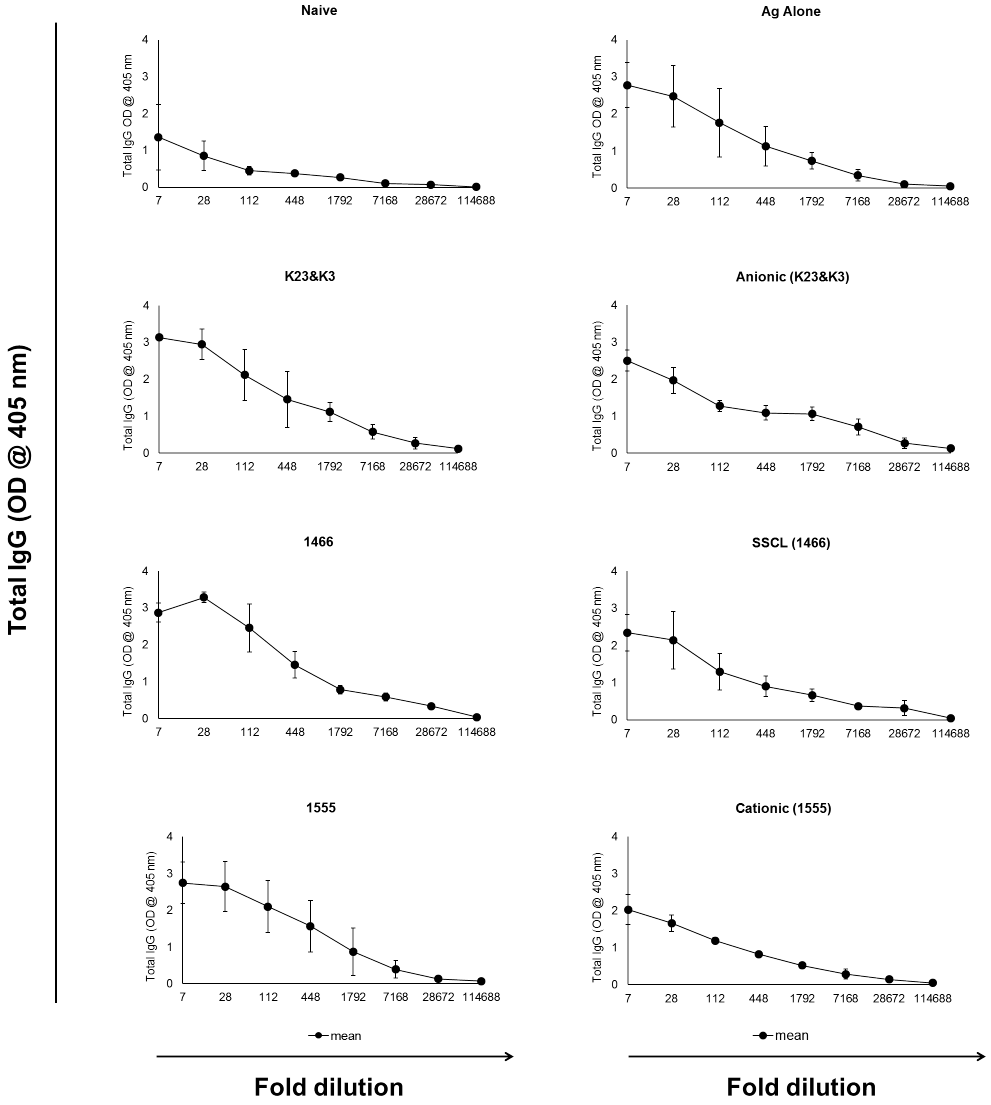

Figure A.2: Mean IgG levels of mice immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

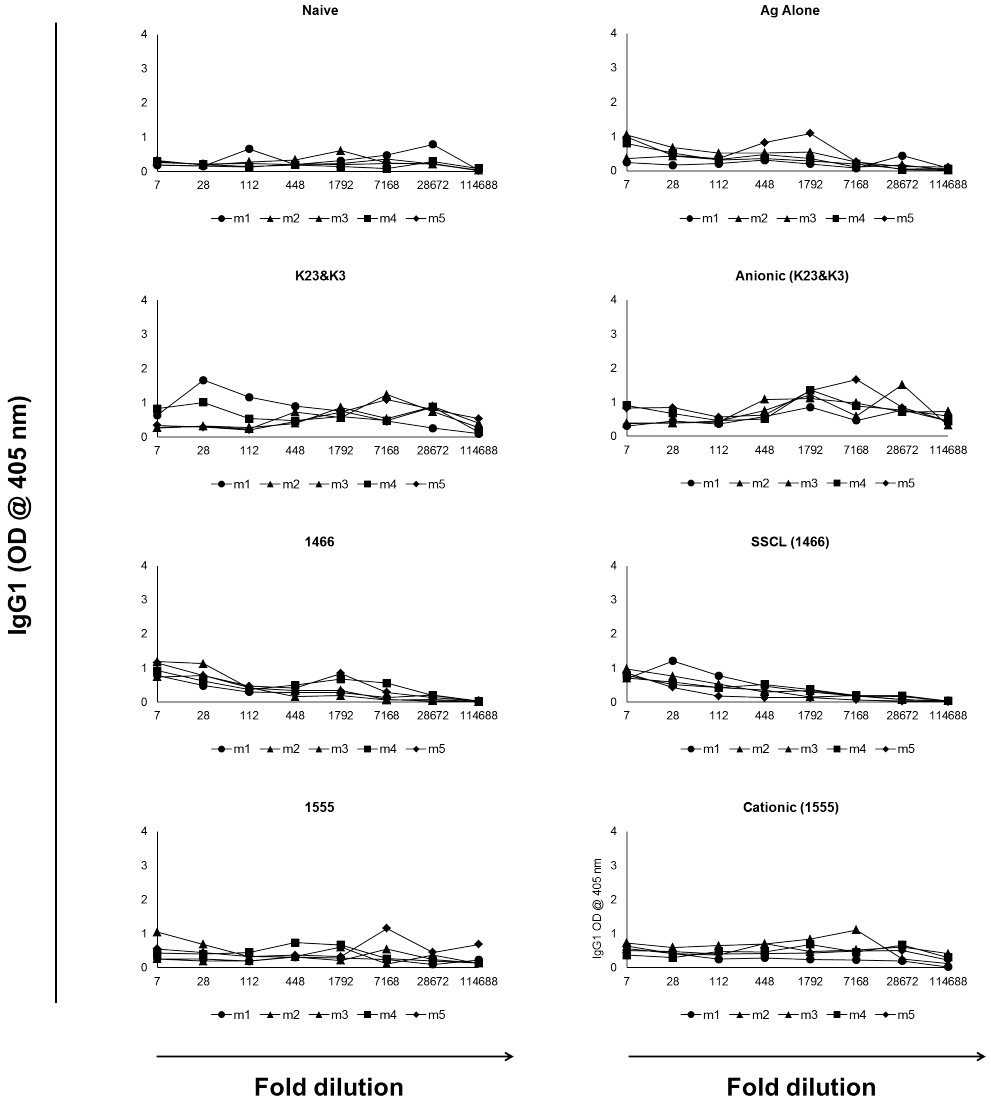

Figure A.3: IgG1 levels of individual mouse immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

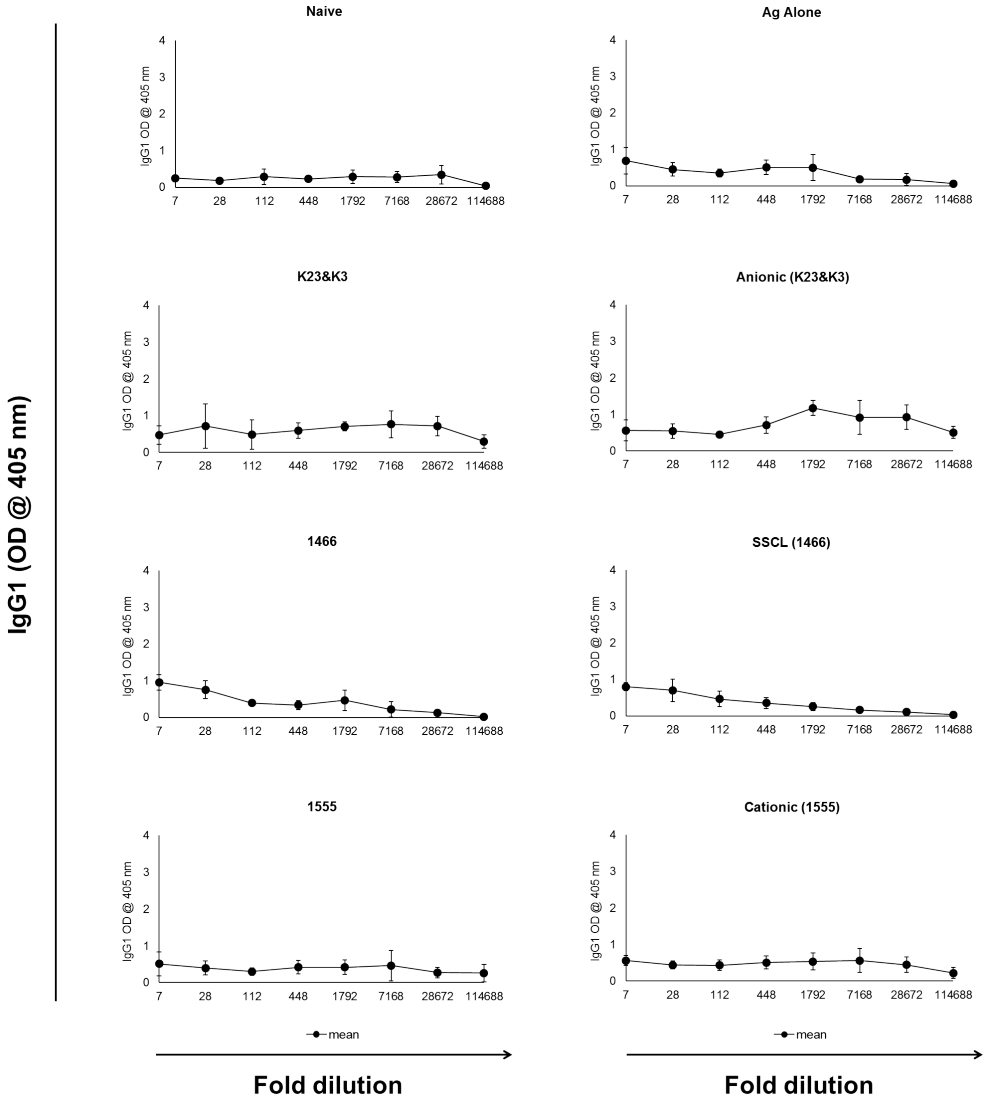

Figure A.4: Mean IgG1 levels of mice immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

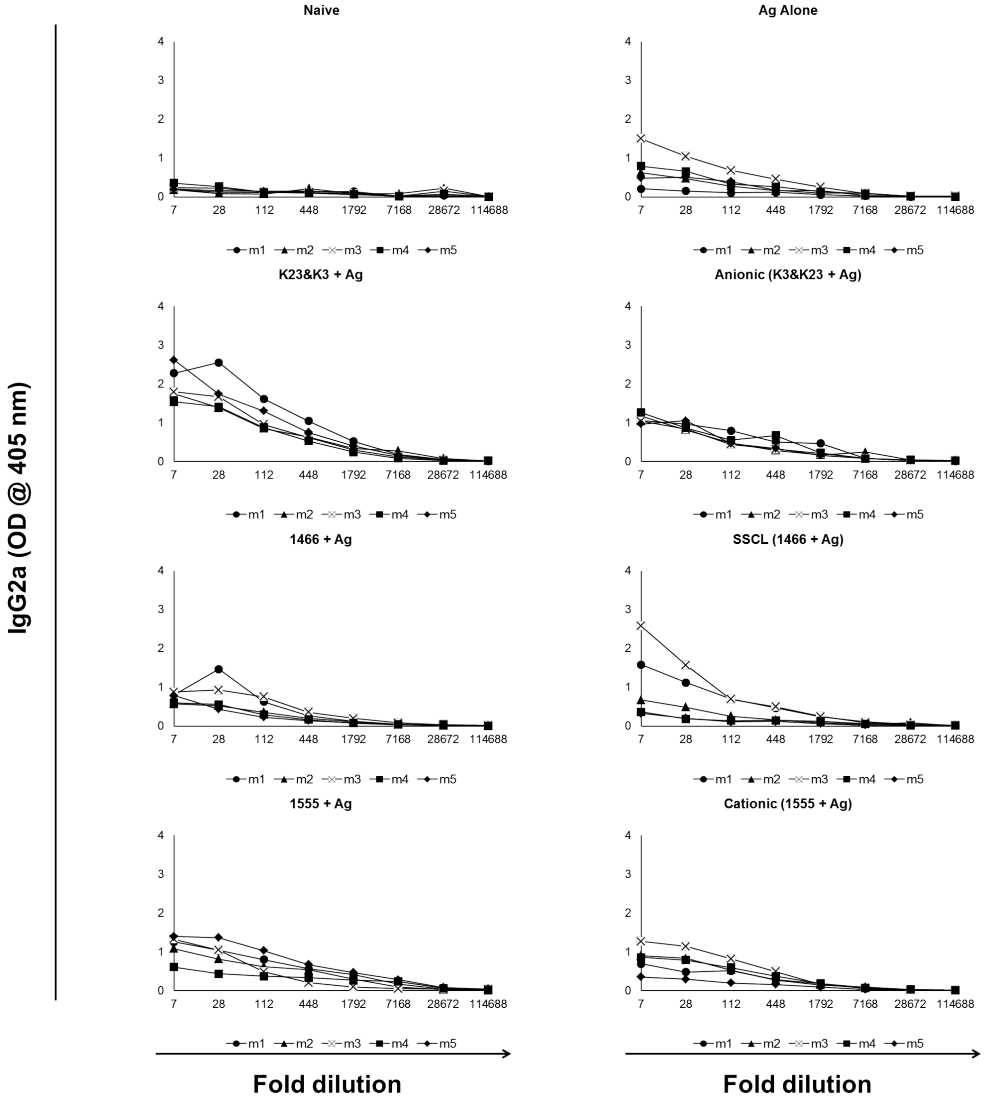

Figure A.5: IgG2a levels of individual mouse immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG2a levels were detected with IgG2a ELISA.

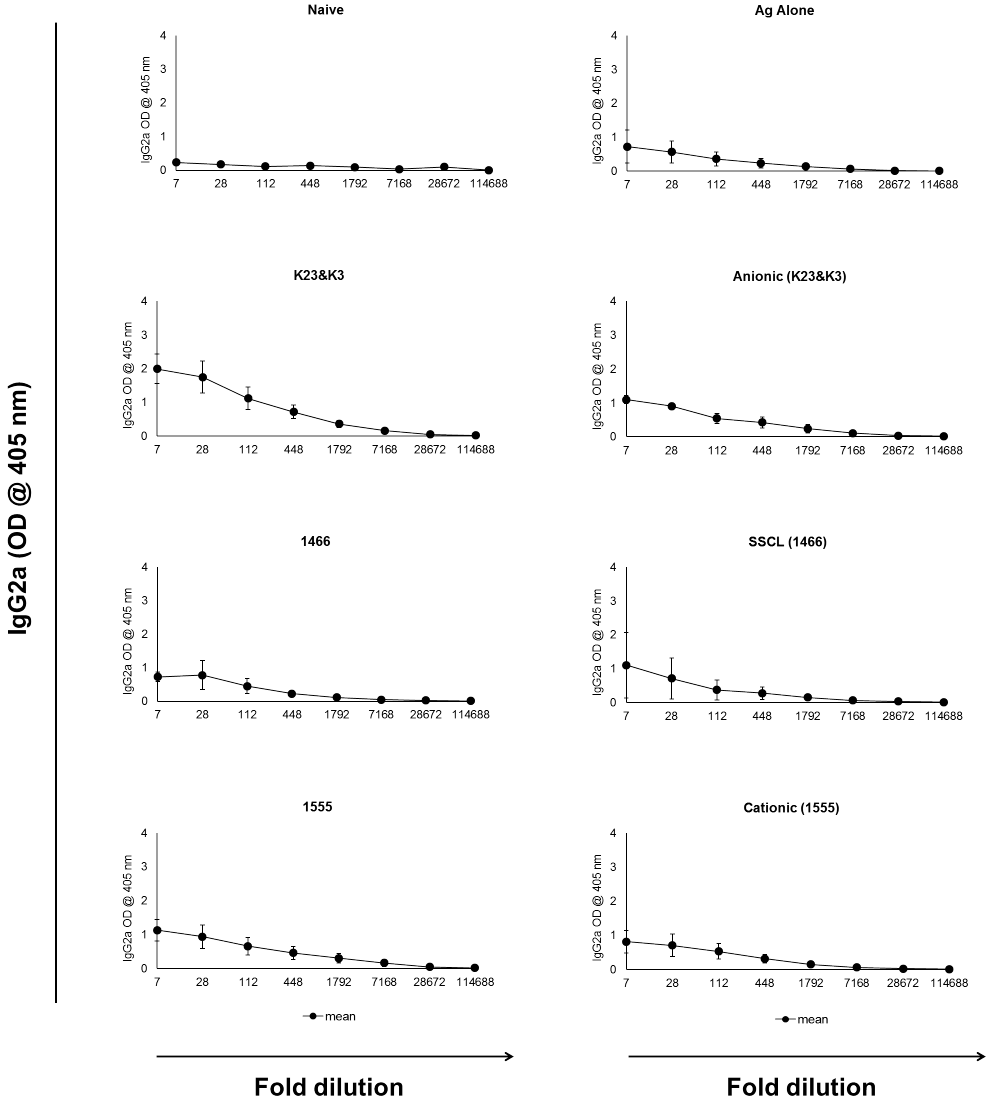

Figure A.6: Mean IgG2a levels of mice immunized against *H. felis* 2 weeks after 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG2a levels were detected with IgG2a ELISA.

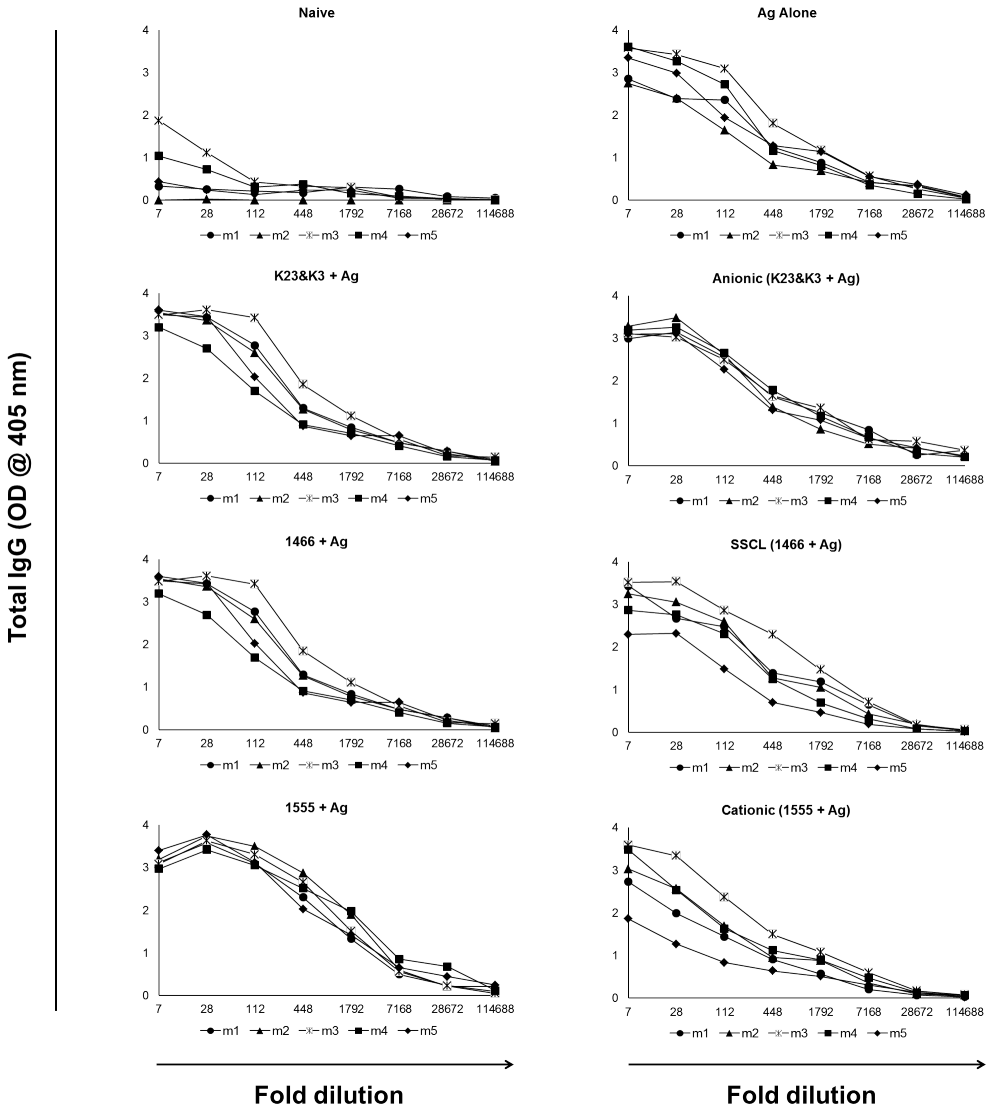

Figure A.7: IgG levels of individual mouse immunized against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

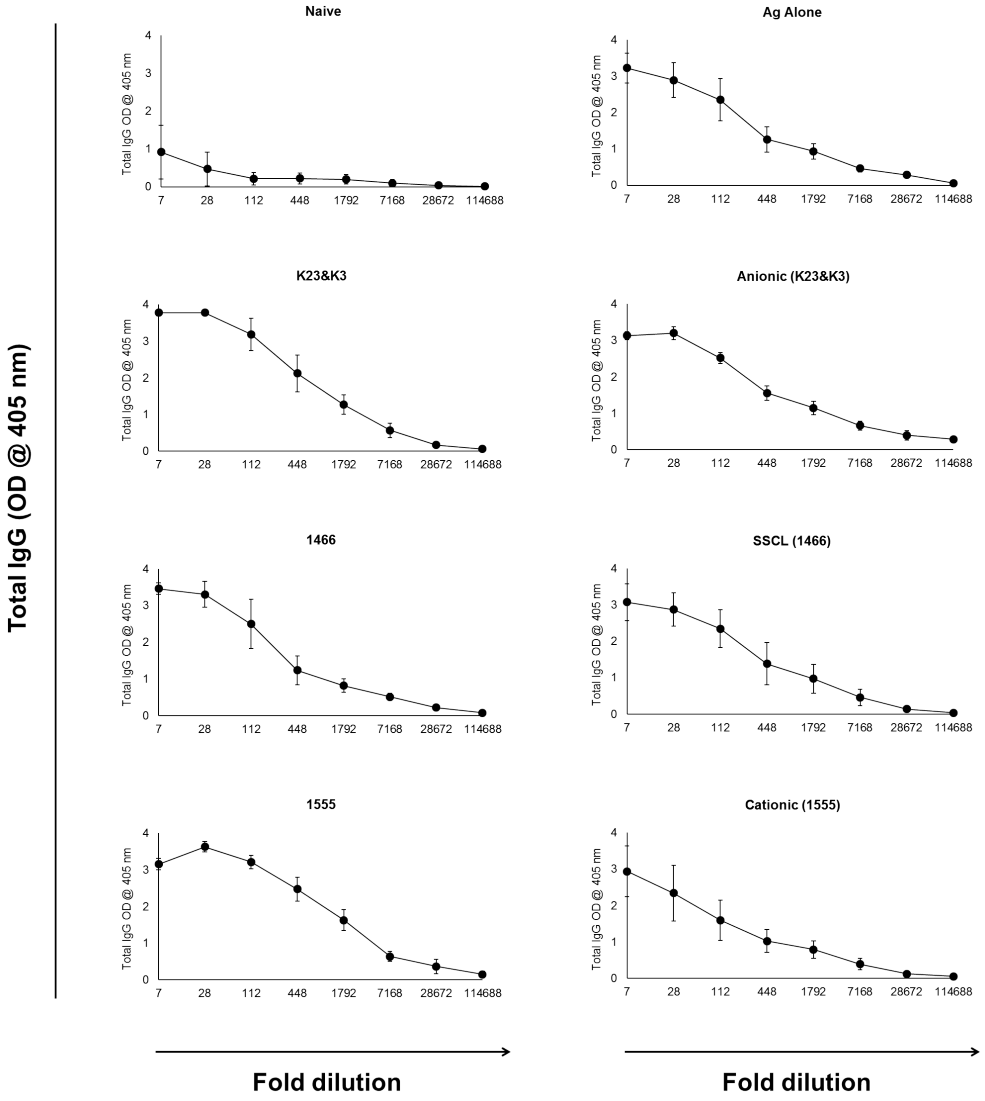

Figure A.8: Mean IgG levels of mice immunized against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

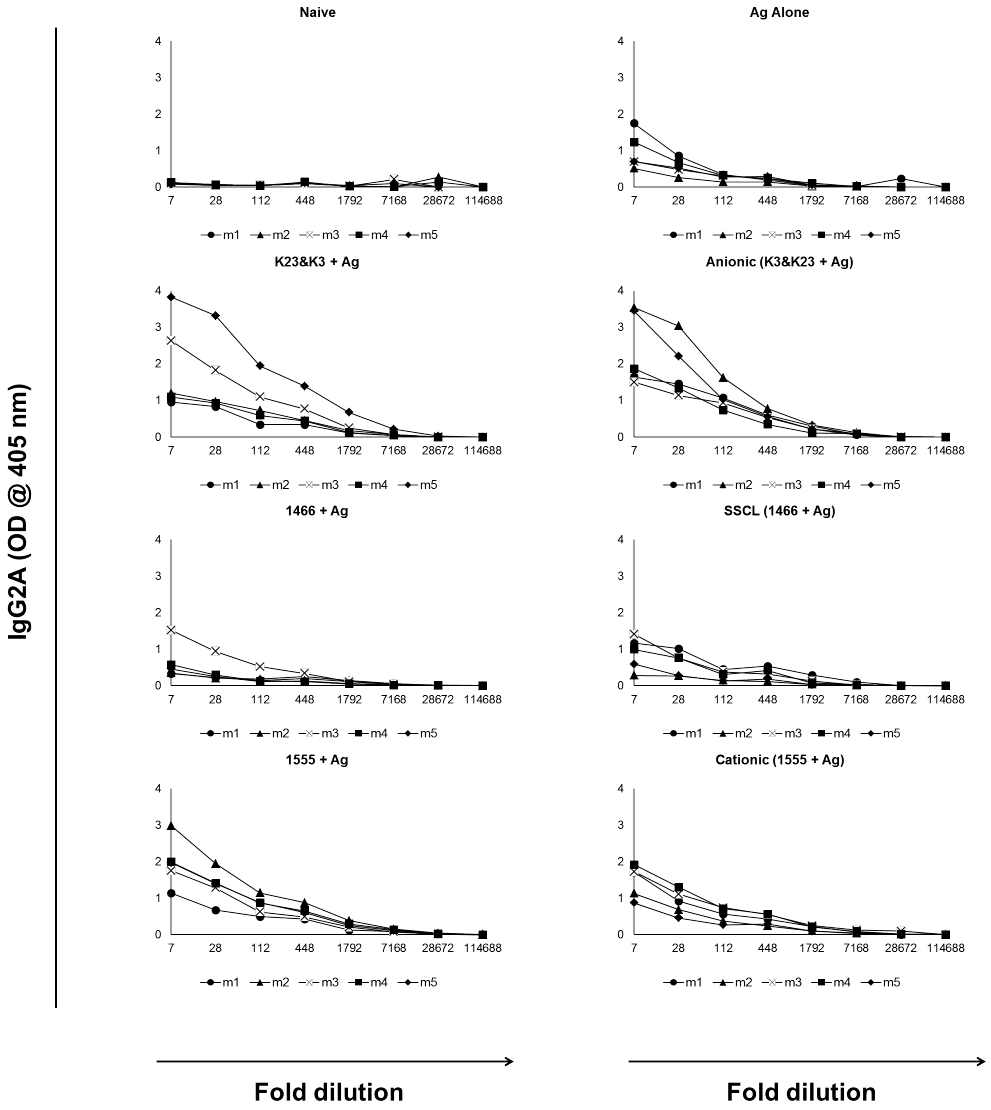

Figure A.9: IgG1 levels of individual mouse immunized against *H. felis* 2 weeks booster 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

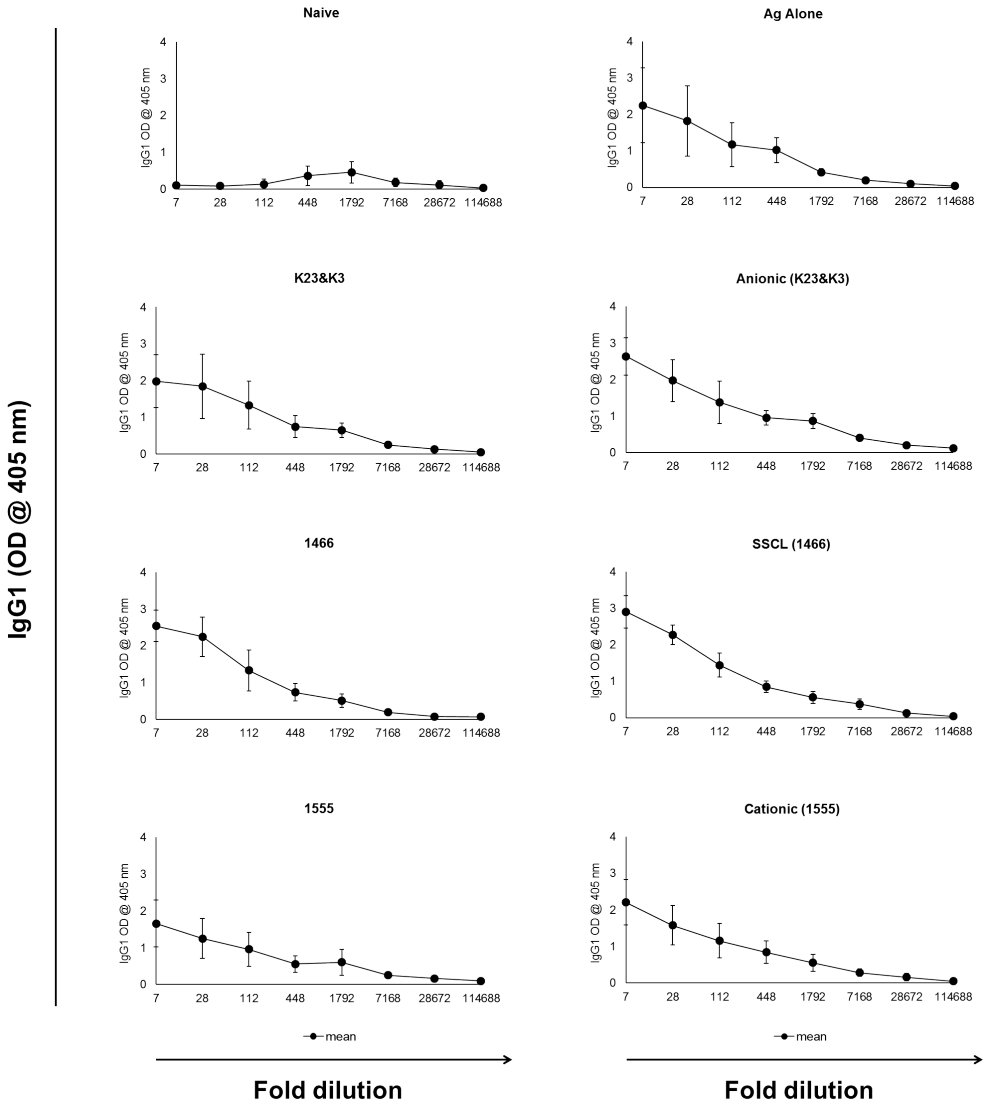

Figure A.10: Mean IgG1 levels of mice immunized against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

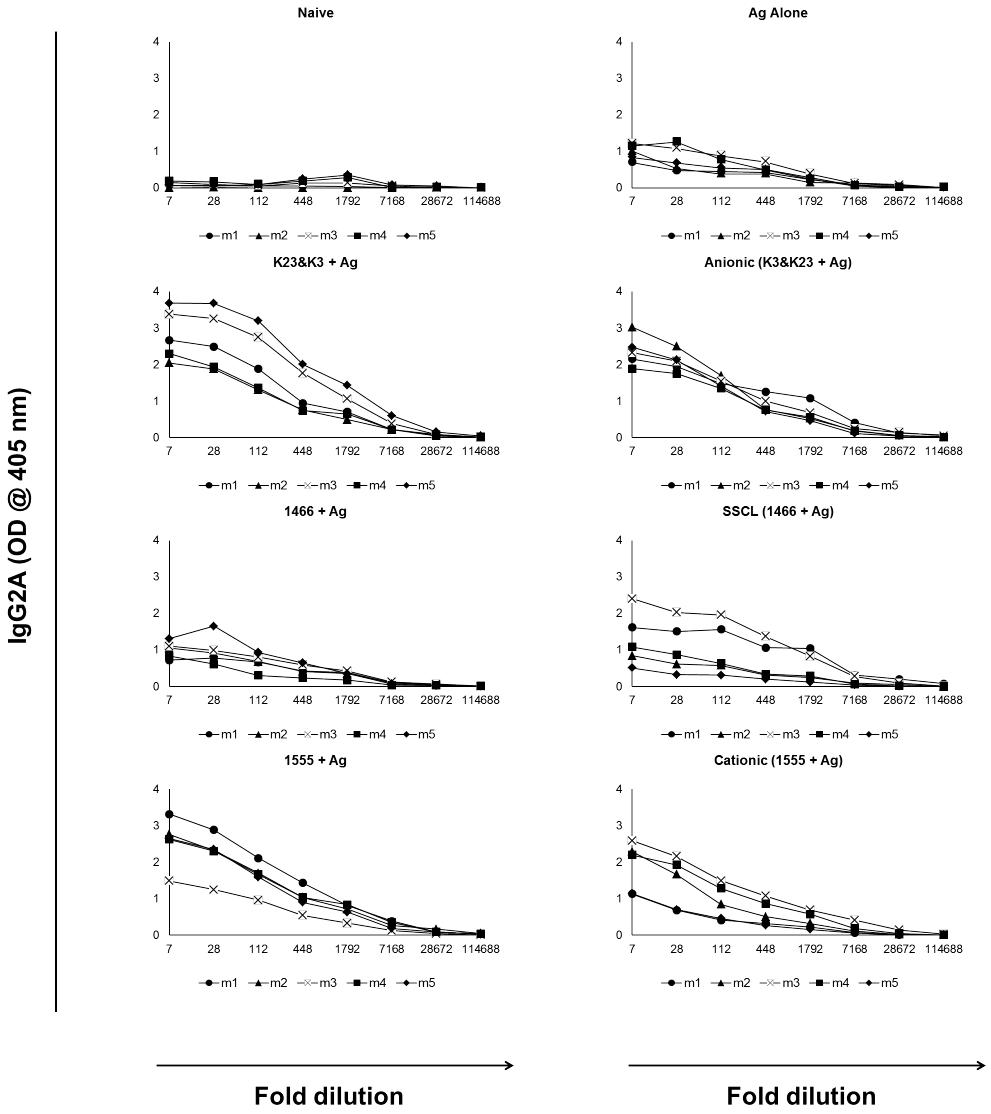

Figure A.11: IgG2a levels of individual mouse immunized against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

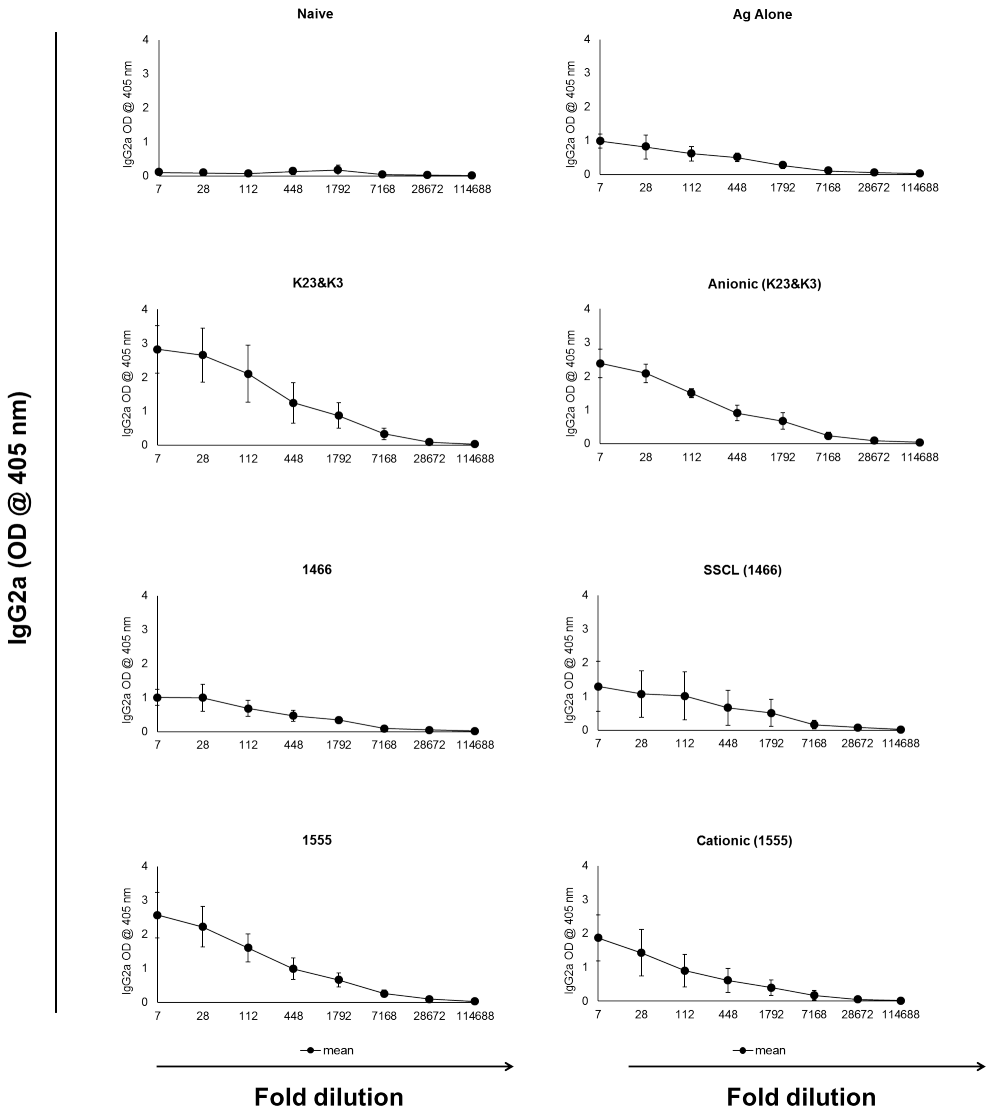

Figure A.12: Mean IgG2a levels of mice immunized against *H. felis* 2 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG2a levels were detected with IgG2a ELISA.

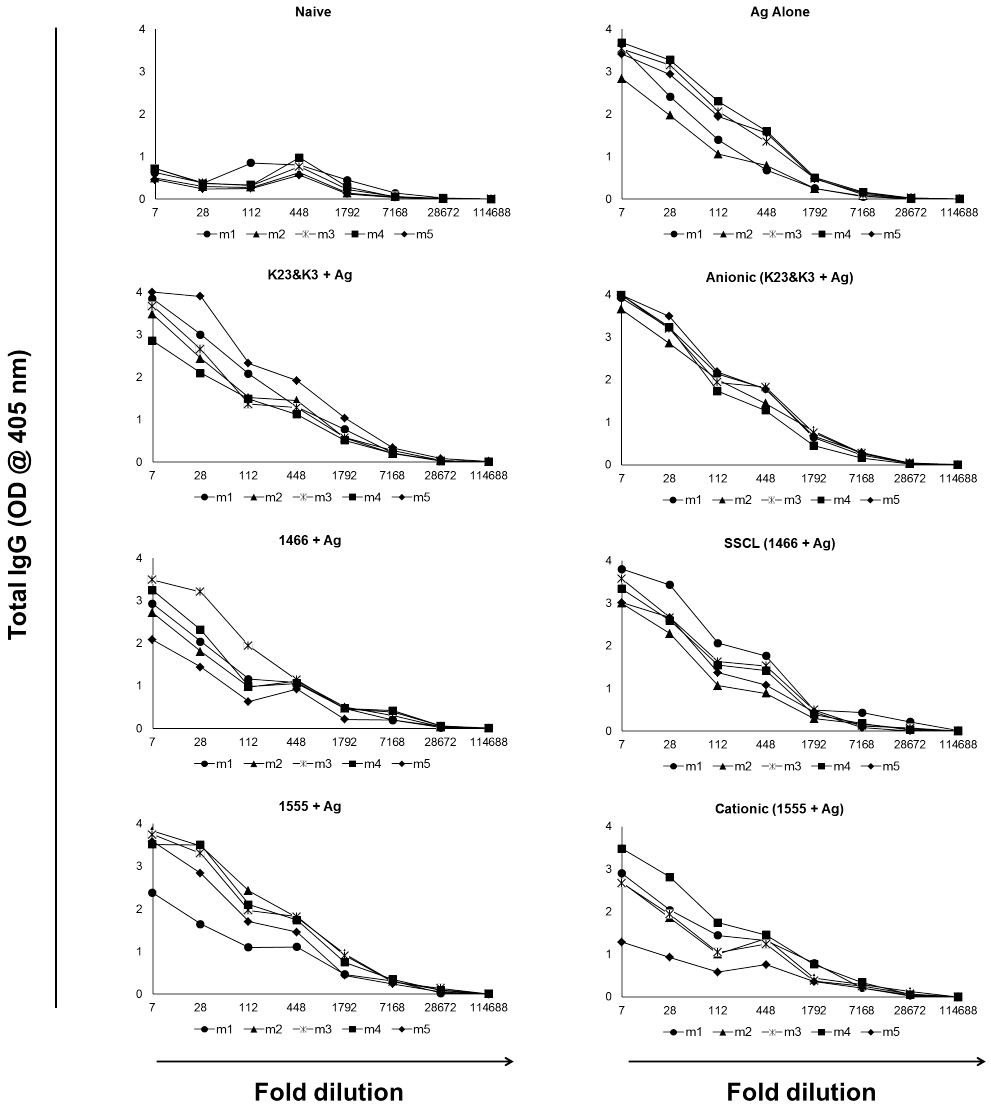

Figure A.13: IgG levels of individual mouse immunized against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

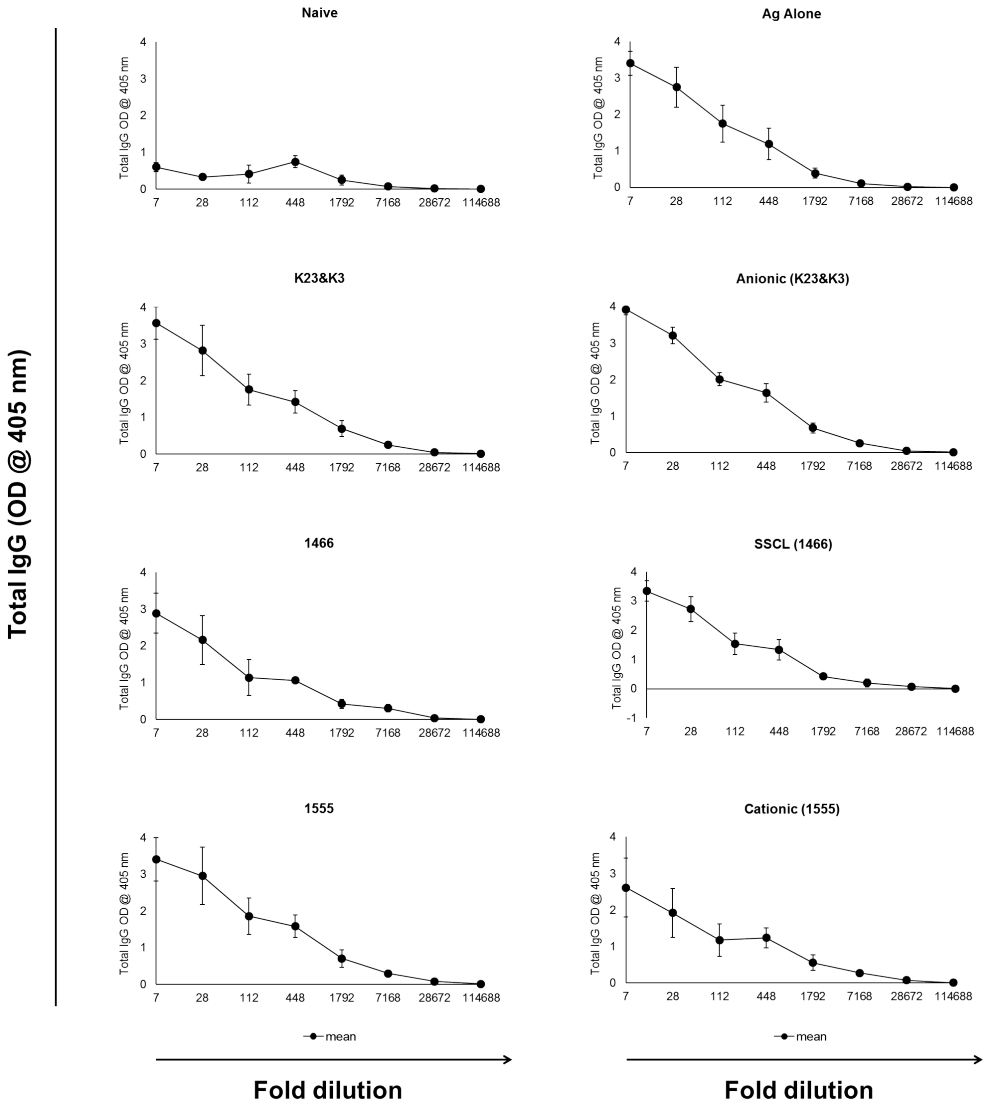

Figure A.14: Mean IgG levels of mice immunized against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG levels were detected with IgG ELISA.

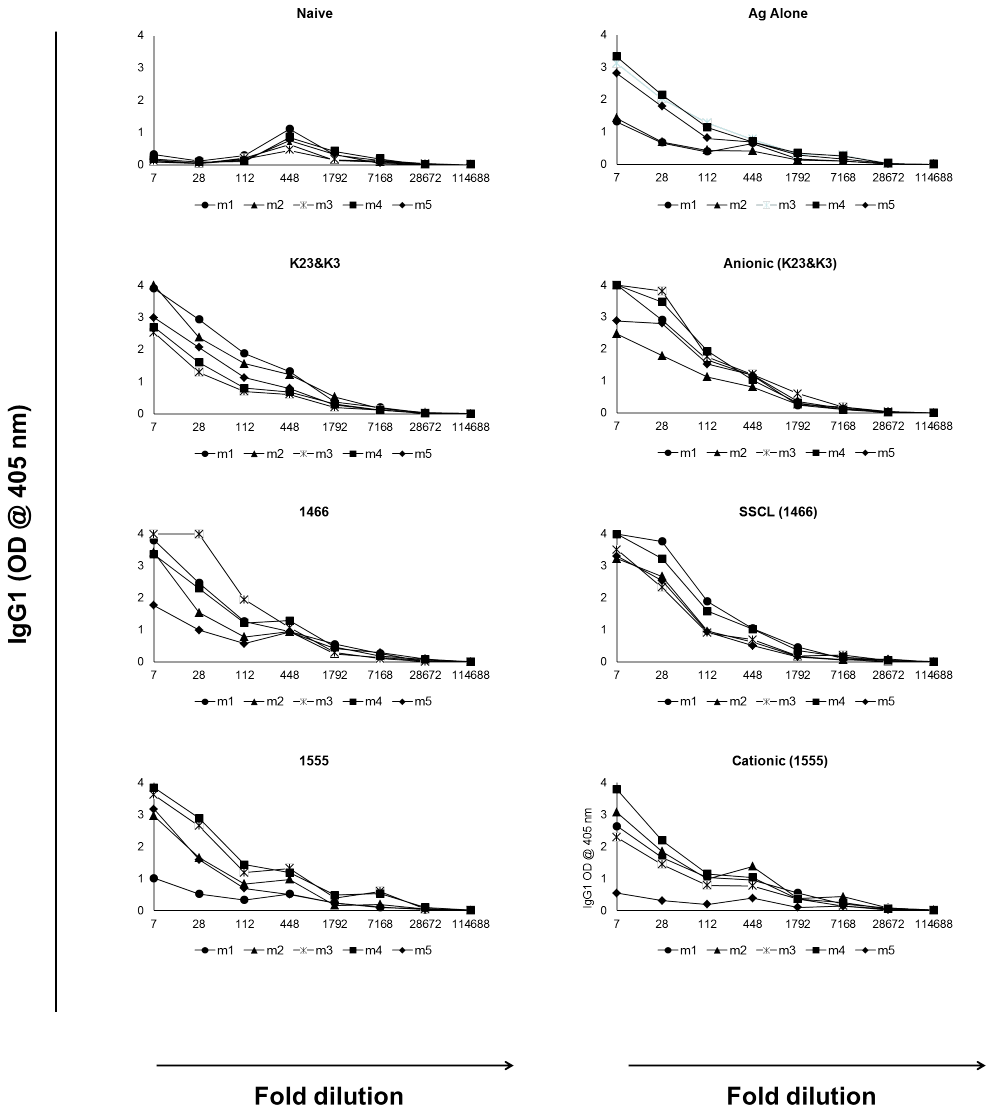

Figure A.15: IgG1 levels of individual mouse immunized against *H. felis* 6 weeks booster 1st injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

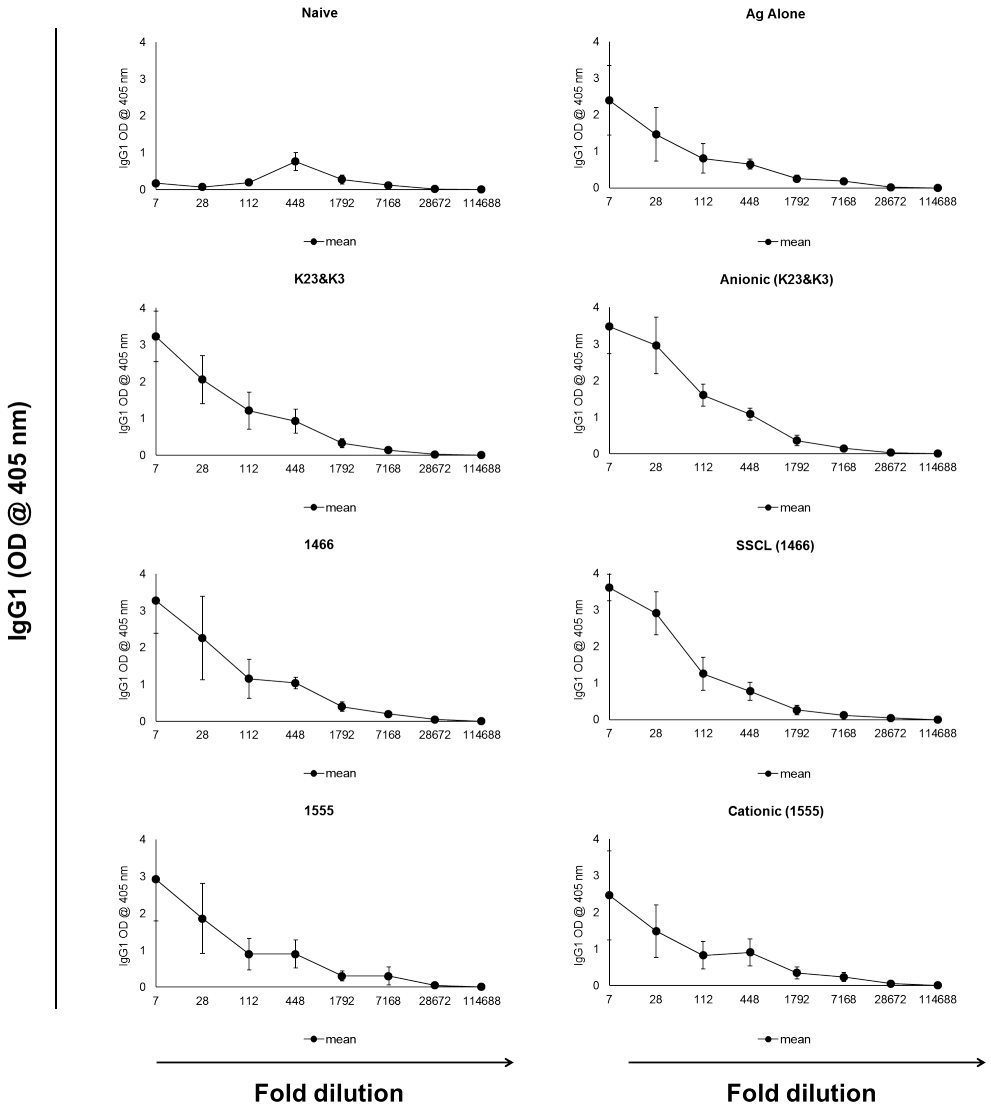

Figure A.16: Mean IgG1 levels of mice immunized against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

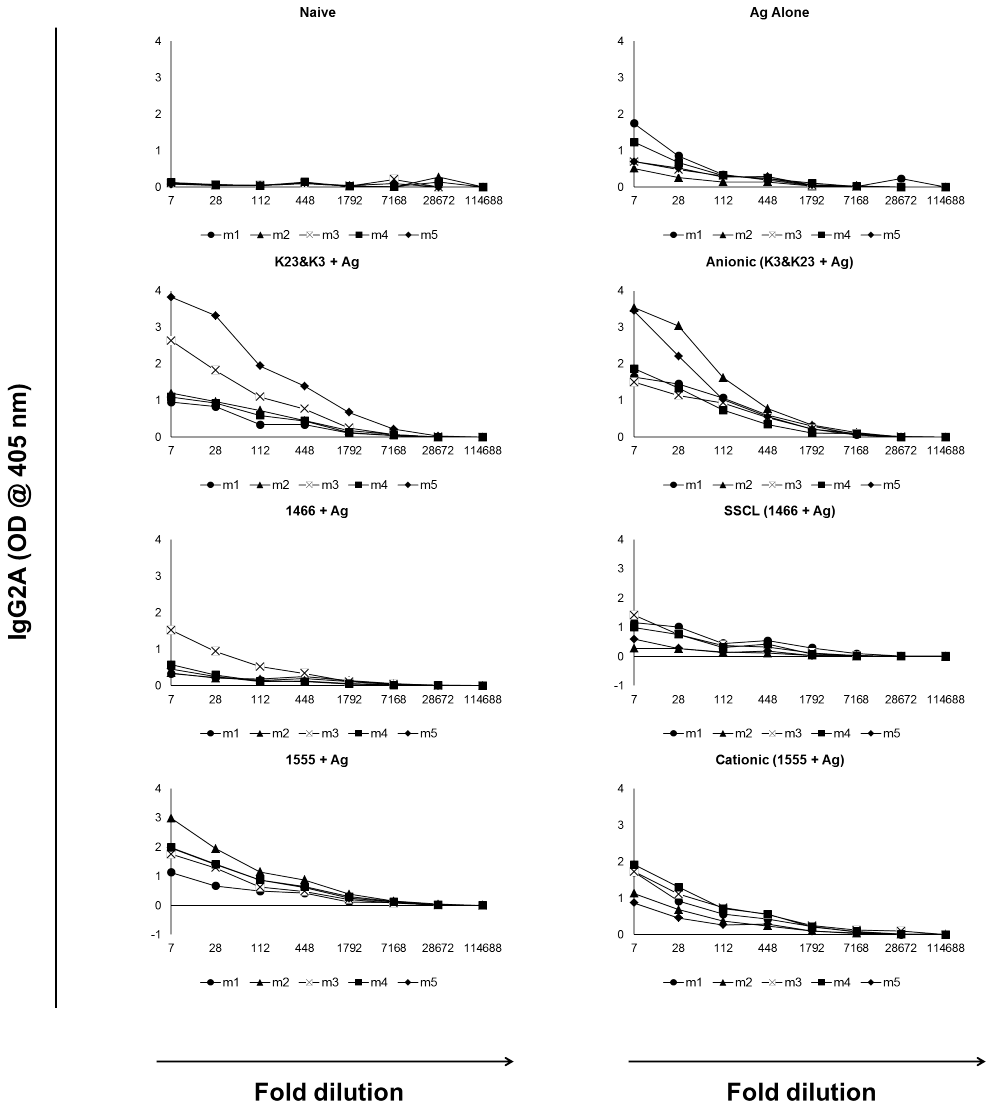

Figure A.17: IgG2a levels of individual mouse immunized against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG1 levels were detected with IgG1 ELISA.

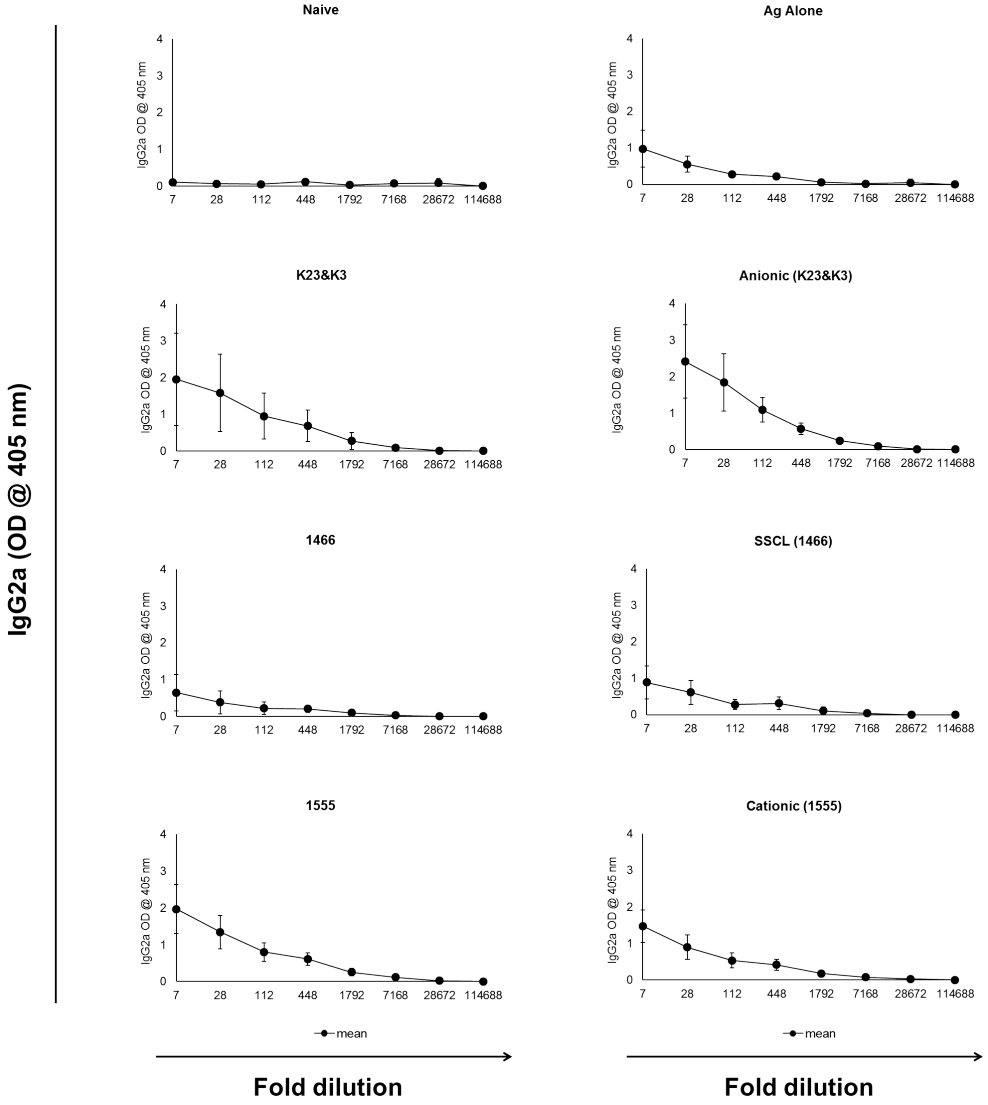

Figure A.18: Mean IgG2a levels of mice immunized against *H. felis* 6 weeks after booster injection. Mice (5 mice/group) were immunized against *H. felis* with CpG ODN and *H. felis* total cell extract or liposomes that co-encapsulate CpG ODN and *H. felis* total cell extract. IgG2a levels were detected with IgG2a ELISA.