

**SELF-NANOPARTICLE FORMING IMMUNOSTIMULATORY
DNA: STRUCTURE-FUNCTION RELATIONSHIP STUDIES**

**A THESIS SUBMITTED TO
THE MATERIAL SCIENCE AND NANOTECHNOLOGY PROGRAMME
OF THE INSTITUTE OF ENGINEERING AND SCIENCES
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE**

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JANUARY 2009

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ABSTRACT
SELF-NANOPARTICLE FORMING IMMUNOSTIMULATORY DNA:
STRUCTURE-FUNCTION RELATIONSHIP STUDIES

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M.Sc. in Material Science and Nanotechnology
Supervisor: Assoc. Prof. Dr. Ihsan Gürsel
January 2009, 107 pages

Toll-like receptors (TLRs) are one the most critical and widely studied members of the family of pattern recognition receptors expressed on innate immune cells. They recognize microbial signatures, such as bacterial/viral DNA, LPS, from gram negative bacteria, peptidoglycan from gram positive bacteria, zymosan from yeast, lipopeptides or profilin protein from parasites, and even single or double stranded RNA of viruses. Among several members of TLR family, TLR9, that recognizes microbial unmethylated dinucleotide motifs on DNA initiate a robust Th1-biased inflammatory response. Synthetic oligodeoxynucleotides expressing unmethylated CpG motifs, mimic bacterial DNA effect and can be harnessed for the treatment of health problems ranging from infectious diseases to cancer, or to allergy/asthma as well as stand alone immunoprotective agents and also as a vaccine adjuvants that improve protection against pathogens. To date, various classes of CpG ODNs have been identified and were shown to induce differential immune activation in mice and man. Distinct structure-function relationship analyses revealed that these single-stranded linear ODNs alter the immune milieu as they are formulated to form complex multimeric DNA aggregates. Recently, Guanosine-rich D type CpG ODNs has been reported to form complex aggregates, that are differentially regulating immune cells to mount an anti-viral immunity. However, the clinical trials of this type are hampered mainly due to batch to batch variation during large-scale synthesis. To the best of our knowledge, there is no report on self-nanoparticle forming DNA except G-rich sequences. This thesis project was designed to generate stable, self-nanoparticle forming, G-run free, CpG expressing ODNs.

In this thesis, we designed a new generation CpG ODN, then characterized their structural and immunological properties. Our results suggest that dendrimeric structure confers higher immunostimulatory potential unparallel to conventional ODNs. Following four hours of *in vivo* ODN administration into mice indicated that nanoparticle-forming CpG ODNs initiated substantially high spleen and peritoneal exudate cell activation as evidenced by IFN γ and IL-12 production from culture medium. In order to shed light on the uptake and binding mechanisms, blocking experiments revealed that at least one type of scavenger receptor is critical for nanoparticle ODN internalization. Collectively, these data suggested that the improved stability to nucleases along with significantly higher binding to immune cells (no additional ODN formulation is required) seem to be the critical factors contributing to the nanoparticle CpG ODN mediated immune activation. The *in vitro* and *in vivo* performances implicated that these next generation immune stimulatory DNA molecules are promising candidates for various clinical applications.

Keywords: Innate immunity, TLR9, scavenger receptors, nanoparticulate and dendrimeric CpG ODN, immunotherapy

ÖZET

KENDİ KENDİNE NANOPARÇACIK OLUŞTURAN BAĞIŞIKLIK UYARICI DNA: YAPI-FONKSİYON İLİŞKİSİ ÇALIŞMALARI

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Ocak 2009, 107 sayfa

Toll-benzeri reseptörler (TLR) doğal bağışıklık hücrelerinde ifade edilen patern tanıyıcı reseptörler ailesinin en çok çalışılan ve en kritik üyesidir. Bu reseptörler bakteri/virüs DNA'sı, gram negatif bakterilerdeki lipopolisakkaritler (LPS), gram pozitif bakterilerdeki peptidoglikan, maya mantarlarındaki zimosan, parazitlerdeki lipopeptidler veya profilin proteinleri, hatta virüslerdeki tek veya çift sarmallı RNA'lar gibi mikrobiyal işaretleri tanırlar. TLR ailesinin birçok üyesi arasından, mikrobiyal DNA üzerindeki metillenmemiş dinükleotid motifleri tanıyan TLR9 reseptörleri güçlü bir Th1 etkili enflamatuvar yanıt başlatırlar. Metillenmemiş CpG motifleri taşıyan sentetik oligodeoksinnükleotidler, bakteri DNA'sını taklit ettiklerinden bulaşıcı hastalıklardan kansere, alerji/astıma kadar birçok sağlık problemlerinin tedavisinde kullanılabilir olmalarının yanında bağışıklık koruyucu ajan ve patojenlere karşı korunmayı geliştirmek için aşı adjuvanı olarak da kullanılabilirler. Şimdiye kadar birçok CpG ODN sınıfı tanımlanmış, bunların fare ve insanlarda bağışıklık sistemini farklı şekillerde aktive ettiği gösterilmiştir. Yapı-fonksiyon ilişkisi analizleri göstermiştir ki tek zincirli lineer ODN'ler kompleks multimerik DNA kümeleri şeklinde formüle edildiklerinde farklı yönde bağışıklığa yol açmaktadırlar. Son zamanlarda Guanozilerce zengin CpG ODN'lerin kompleks kümeler oluşturup bağışıklık hücrelerini farklı şekilde yönlendirerek anti-viral bağışıklık oluşturduğu bildirilmiştir. Ancak bu çeşit ODN'lerin klinikte kullanılması büyük ölçekli üretim esnasında üretimden üretime farklılıklar olması nedeniyle engellenmiştir. Bizim bilgimiz dahilinde, Guanozin zengini diziler haricinde kendi kendine nanoparçacık oluşturan DNA'lar bulunmamaktadır. Bu tez projesi dayanıklı, kendi kendine nanoparçacık oluşturan, G-yoksunu, CpG taşıyan ODN'ler ortaya çıkarmak için tasarlanmıştır.

Bu tez çalışmasında yeni jenerasyon CpG ODN tasarlanmış olup yapısal ve bağışıklık sistemi üzerinde etkileri karakterize edilmiştir. Sonuçlarımız dendrimerik yapıların bilinen diğer ODN'lere kıyasla çok daha yüksek oranda bağışıklık uyarıcı potansiyelinin olduğunu önermektedir. Farelere *in vivo* ODN uygulamasından dört saat sonra, nanoparçacık oluşturan CpG ODN'lerin, kültür ortamındaki IFN γ ve IL-12 üretiminden, dalak ve periton immün hücrelerini oldukça yüksek düzeyde aktive ettiği anlaşılmıştır. Hücreye alım ve bağlanma mekanizmalarını aydınlatmak için yapılan bloklama deneyleri en az bir çeşit skavenger (çöpçü) reseptörünün nanopartikül ODN'lerin hücreye alımında kritik rolü olduğunu ortaya çıkarmıştır. Sonuç olarak, bu verilerin ışığında nükleazlara karşı dirençli ve bağışıklık hücrelerine daha fazla bağlanmaları (ek bir ODN formülasyonu gerektirmeden) nanopartikül oluşturan CpG ODN kaynaklı bağışıklık aktivasyonuna katkıda bulunan faktörler olduğu söylenebilir. *In vitro* ve *in vivo* performansları bu yeni jenerasyon bağışıklık uyarıcı DNA'ların birçok klinik uygulama için güçlü adaylar olduğunu vurgulamıştır.

Anahtar kelimeler: Doğal bağışıklık, TLR9, scavenger reseptörleri, nanoparçacıklı ve dendrimerik CpG ODN, immünterapi

Dedicated to all dear members of my family who have supported me throughout my life and showed heart to heart affection and love which helped me not to feel alone...

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. Ihsan Gursel who had given me a chance to study in his lab and work on this very interesting project. His guidance helped me to focus on subject and not get destructed. I learned many from his skeptical approach to a subject which was very beneficial to make progress on my project. His patience will be a reference for me throughout my life.

I have special thanks to my labmates, Gizem, Fuat, Kutay, Erdem, Tamer and ex-labmate Hande, who were aware of their responsibilities and contributed to this thesis by minimizing routine technical problems in the lab. They were there whenever I needed them.

I would like to acknowledge Institute of Material Science and Nanotechnology (UNAM) administration, as they provided me opportunity to be an MSc student and supplied part of my stipend and health insurance. Dr. Aykutlu Dana and members of his group from UNAM, Burkan Kaplan and Musa Kurtuluş Abak, provided great assistance in AFM imaging studies, so I thank to them.

I appreciate Dr. Mayda Gürsel for her useful comments about my research which were very helpful.

Thanks to Dr. Ali O. Gure who boosted my interest to immunology in his Molecular Immunology course.

I would like to thank to administration of Molecular Biology and Genetics department, as they opened their labs to my use and let me to perform my experiments there. Moreover, I appreciate graduate students and technicians of Molecular Biology and Genetics department for the assistance they provided in experiments.

Life wouldn't be so interesting without my friends. They were with me in my hard times. Thanks to all of them.

My parents Irshad, Ezize and my sister Arzu deserves one of the bigger parts of acknowledgements as they supported me everytime and were very respectful to my decisions, especially one about being a molecular biologist. I have very special thanks to my grandparents who had great contributions to me, especially my grandfather who stimulated my interest to science subjects.

I have very sincere thanks to my dear lady Büşra. Most intense part of the experiments were coincided with first months of our marriage. Besides her patience, she supported me morally and made me to feel better in stressful conditions.

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ABBREVIATIONS

AFM	Atomic Force Microscopy
APC	Antigen presenting cell
AVA	Anthrax vaccine adsorbed
BP	Base pairs
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CS	Chondroitin sulfate
CpG	Unmethylated cytosine-phosphate-guanosine motifs
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOTAP	1,2- dioleoyl-3-trimethylammonium-propane
DS	Dextran sulfate
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
FBS	Fetal Bovine Serum
G-run	Guanosine run
HBV	Hepatitis-B Virus
HEK	Human embryonic kidney
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
I κ K	Inhibitor kappa B kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IFN	Interferon
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor

LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic Acid
MALP-2	Macrophage-Activating Lipopeptide-2
MCP	Monocyte Chemoattractant Protein
MDP	Muramyl dipeptide
Mf	Macrophage
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MyD-88	Myeloid Differentiation Primary Response gene 88
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pI:C	Polyribonucleosinic polyribocytidylic acid
PLG	Poly(lactide-co-glycolide)
PNPP	Para-nitrophenyl pyro phosphate
PRR	Pattern recognition receptors
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT	Reverse transcriptase
SA-AKP	Streptavidin Alkaline-phosphatase
SLE	Systemic Lupus Erythematosus
ssRNA	Single-stranded RNA
SR	Scavenger Receptor
STF	Soluble tuberculosis factor
TCR	T-cell receptor
Th	T-helper
TIR	Toll/IL-1 receptor
TLR	Toll-like Receptor
TNF α	Tumor Necrosis Factor α

1. INTRODUCTION

Infectious diseases are one of the main causes of death worldwide. Although there is a significant decrease in mortality rate due to diseases caused by infectious agents, millions of people still die every year due to AIDS, respiratory infections, malaria, tuberculosis and diarrheal diseases (World Health Organization, 2008). More effective vaccines and biodrugs are needed to provide effective protection to these afflictions and decrease current mortality rate. In achieving this, a continued research effort is required in order to better understand how mammalian immune system recognize and discriminate non self “pathogens” and mount a response that leads to effective pathogen eradication before it causes any harm to the host. Developing novel strategies in order to accelerate this early immune response as well as to increase its magnitude and duration will help us to pave the way for better curative treatments during combat with these and newly arising deadly pathogenic diseases.

Immune system while discriminating self from non-self, relies on a very simple rule. This dogma is known as the “Danger Signal” and is based on the recognition of specific signature molecules expressed on pathogens, and are missing on our cells. With this, innate arm of the immune system mount a robust orchestral activation in order to contain and furthermore eradicate the invading infectious organisms. This first line of defence not only contains the invading intruders but instructs the formation of a strong adaptive immune response first by migrating to the local lymph nodes and there attains the function of an antigen presenting cell that interacts with the T helper and B cells. This interaction controlled and initiated by the “Innate Immunity” leads to “Adaptive Immunity”.

1.1. Innate Immune System

Innate immune system has many arms which are so organized that they form several layers of barriers (that varies with their sophistication and complexity) in order to prevent the access of infecting organisms into the body (i.e. skin, mucous, cilia, tears, soluble proteins etc are some examples of this complex network). Infectious microorganisms, although rarely, find access to reach internal parts of our body, and due to rich nutrient sources, and availability of an optimum growth environment, they start multiplying inside our body at an unprecedented rate. At this stage, several ‘danger’ signals are wandering around and are recognized by one or many members of the innate immune system cells. Cells of the innate immune system

includes mucosal epithelia, dendritic cells (DC), macrophages (Mφ), B-cells, natural killer (NK) cells, eosinophils, basophils and mast cells. Soluble components of the innate immune system includes, acute-phase proteins, complement system proteins, opsonins, polyreactive immunoglobulins (Ig) and mediators of immune response such as cytokines and chemokines. Mucosal epithelia, which is found in all metazoans, is the main barrier between host and microorganisms. Epithelial cells and keratinocytes secrete antimicrobial peptides to decrease colonization of microorganisms (Medzhitov R, 2007). Microorganisms that pass epithelial barrier meet with phagocytes (macrophages, dendritic cells and neutrophils) in local tissues. Resident macrophages recognize acute-phase and complement proteins, host plasma proteins that are already bound to bacterial surfaces (opsonization), and engulf microorganism with further initiation of bactericidal mechanisms - such as acidification, degrading enzymes, nitric oxide and antimicrobial peptide production which destroy microbes. Mφ and DC induce a number of early acting cytokines and chemokines (please see Table 1.1. and Table 1.2.) which recruit neutrophils and monocytes to site of infection. Monocytes generally mature further into DC and Mφ at infection site. NK cells are specialized cells to sense especially viral infections (already infecting a host cell) and induce death of these host cells (Janeway CA, 2005). Eosinophils, basophils and mast cells are mainly involved in protection from multicellular parasites, while mast cells, also can sense microbial byproducts (Medzhitov R, 2007). Resident immature dendritic cells, which express numerous pathogen-recognition receptors (PRR) including Toll-Like Receptors (TLR) and Scavenger Receptors (SR) (these will be discussed in more detail in the subsequent sections), recognize certain microbial signatures and furthermore phagocytose them. Immature DC becomes mature and can no longer continue phagocytosis. They become Antigen Presenting Cells (APC) and migrate to draining lymph nodes. They are involved in further degradation and processing of microbial proteins for 'antigen presentation'. This process is the sentinel stage that shapes adaptive immunity. The T and B cells are instructed and converted to antibody producing B-Cells, and effector T cells (Janeway CA, 2005).

Table 1.1. Cytokines affect behaviour of target cells (Adopted from Janeway CA, 2005).

Cytokine	Produced by	Function
IL-1β	Mf, epithelial cells	Fever, T cell and Mf activation
TNFα	Mf, NK and T cells	Local inflammation and endothelial activation
IL-6	B, T cells, Mf, endothelial	T and B cell growth and differentiation, fever
IL-12	Mf and DC	Activate NK cells, induce CD4 T-cells to differentiate into Th1
IL-15	Many non-T cells	CD8 memory T cell survival, stimulates NK and T cell growth
IL-18	Activated Mf	Induce IFN γ secretion via NK and T cells, favors Th1 immunity
IFNα	DC	Antiviral, increased MHCI expression
IFNγ	T cells, NK cells	Suppress Th2 immunity, Mf activation, increased expression of antigen processing components

Mf: Macrophage, T: T-cells, DC: Dendritic cells, NK: Natural Killer cells

Table 1.2. Chemokines induce chemotaxis of target cells to infection sites (Janeway CA, 2005)

Chemokines	Produced by	Attracted cells	Major effect
MIP-1α	Monocyte, T, Mast cell, fibroblast	Monocyte, NK, T, basophil, DC	promotes Th1, antiviral defense and competes with HIV-1
MIP-1β	Monocyte, Mf, Neutrophil, endothelial	Monocyte, NK, T, DC	competes with HIV-1
MCP-1	Monocyte, Mf, fibroblast, keratinocyte	Monocyte, NK, T, basophil, DC	promotes Th2, activate Mf and histamine release from basophils
IP10	T, fibroblast, endothelial, monocyte, keratinocyte	Resting T, NK, monocyte	promotes Th1, antiangiogenic, immunostimulant

1.1.1. Pathogen Recognition Receptors (PRRs)

Microorganisms have shared structures, which are necessary for their survival like LPS (lipopolysaccharide), PGN (peptidoglycan) including single-stranded and double stranded genome of viruses or bacteria. Most of these molecules are recognized by host immune system and named as pathogen-associated molecular patterns (PAMPs). Certain properties of PAMPs fit very well with innate immune recognition of host (Medzhitov R, 2007):

- 1) PAMPs are similar among different classes of microorganisms.
- 2) PAMPs are molecules unique to microorganism world, thus immune system cells use to discriminate self and non-self.
- 3) PAMPs have critical roles in microbial physiology, thus, these molecules can not be altered throughout the course of adaptive evolution.

Specific innate immune receptors sensing these diverse structures is known as the pathogen-recognition receptors (PRRs) (Medzhitov R, 1997). Similar to PAMPs, PRRs also share common properties (Akira S, 2006):

- 1) Each PRR recognizes a specific PAMP, activates signaling pathway and distinct immune response against pathogen
- 2) PRRs are expressed constitutively by host immune system and detect the pathogens regardless of their life-cycle stage.
- 3) PRRs are germline encoded, nonclonal, expressed on all cells of a given type, and independent of immunologic memory.
- 4) They are highly conserved structures from plants and fruit flies to mammals

PRRs can be classified as cytosolic PRRs which include nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and RIG-I-like helicases (RLHs) and membrane-bound PRRs which include Toll-like receptors (TLRs) and Scavenger Receptors (SR) (Kumagai Y, 2008, Mukhopadhyay S, 2004).

PRRs collectively are divided into two broad groups: i) non-signaling PRRs, such as Scavenger Receptors and ii) signaling PRRs, such as the famous family of Toll like receptors. This section will summarize the features of these major players of immunity.

1.1.1.1. Scavenger Receptors

Scavenger receptors are large family of receptors expressed primarily by myeloid cells, but also expressed on selected endothelial cells (Mukhopadhyay S, 2004). They recognize various endogenous ligands such as phosphatidyl serine (apoptotic cells), low density lipoproteins (LDL) and its modified forms (acetylated LDL, oxidized LDL), modified proteins and also molecular patterns from foreign organisms. Scavenger receptors are divided into 8 major classes, mainly due to their molecular structure (Figure 1.1.). Among their diverse physiological roles, in accordance with the aim of this thesis, only the roles of scavenger receptors in recognition of microbial molecular signatures and their contribution to shape innate immunity will be covered .

Class A : Scavenger Receptor class-A (SRA)- I/II, Macrophage Receptor with Collagenous structure (MARCO), Scavenger Receptor class A member 5 (SCARA5) and SR with C type Lectin-I /Collectin from Placenta receptor-I (SRCL-I/CL-PI) are members of this class which have roles in pathogen recognition. These receptors recognize different species of Gram-positive and Gram-negative bacteria (Greaves DR, 2008) and modified LDL. Some of them also specialized in yeast (*Saccharomyces cerevisiae*) recognition such as SRCL-I (Mukhopadhyay S, 2004). SRCL-I was also shown to be involved in zymosan recognition and phagocytosis of fungi by human vascular endothelial cells (Jang S, 2008). Two well studied and known members i) SRA and ii) MARCO will be covered briefly.

SR-A I/II : SR-A, which is a trimeric type II transmembrane glycoprotein, has 3 isoforms - SR-AI, SR-AII and SR-AIII – that are alternative splice variants of same gene. SR-A was found to be expressed in myeloid cells (except monocytes), mast cells, specific subpopulations of bone marrow-derived and splenic dendritic cells (Plüddemann A, 2007). It binds to LPS (Hampton RY, 1991) and to LTA (Dunne DW, 1994) of Gram-negative and Gram-positive bacteria, respectively. Recently it is also reported that SR-A plays a role in recognition and internalization of dsRNA (Limmon GV, 2008). However, SR-A was found to be not essential for uptake and immunostimulatory activity of K-type CpG ODN and *E.coli* DNA (Zhu FG, 2001, please note that “D-type” was not used in this study). SR-A^{-/-} mice are more susceptible to *Listeria monocytogenes* (Suzuki H, 1997), *Staphylococcus aureus*

(Thomas CA, 2000) and *Streptococcus pneumoniae* (Arredouani MS, 2006) which shows critical role of SR-A in recognition of these pathogens.

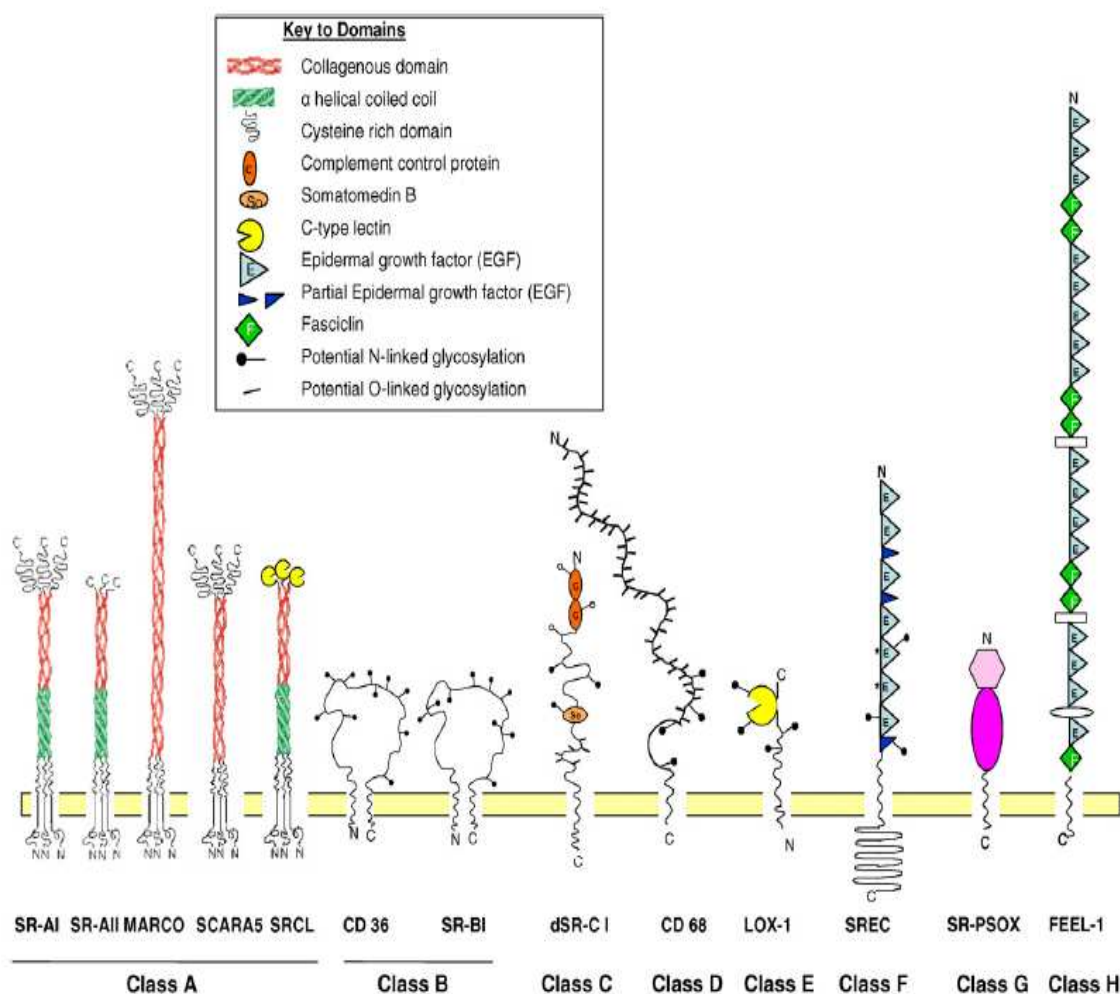


Figure 1.1. Classification of scavenger receptors. Each domain is indicated in the key above. (adopted from Plüddemann A, 2007)

MARCO: MARCO is constitutively expressed by peritoneal and spleen marginal zone macrophages and also by medullary cord macrophages of lymph node (Kraal G, 2000). However, low level MARCO expression can be upregulated via TLR-mediated induction in macrophages derived from other sources (Doyle SE, 2004). CpG ODN (K-type) binding to peritoneal macrophages from MARCO^{-/-} mice was unchanged, while CpG-induced IL-12 production was severely impaired (Jozefowski S, 2006). MARCO recognizes LPS, LTA (Greaves DR, 2008), Gram-positive bacteria such as *Streptococcus pneumoniae* (Arredouani M, 2004) and Gram-negative bacteria such as *Neisseria meningitides* (Mukhopadhyay S,

2006). MARCO^{-/-} mice infected with murine model of pneumococcal pneumonia, were unable to clear bacteria from lungs and showed decreased survival, while it induced increased cytokine release (Arredouani M, 2004).

Class B : CD36 and Scavenger Receptors class B I (SR-BI) are the members of this family involved in innate immunity.

CD36: CD36, type III transmembrane receptor, is expressed in microvascular (but not large vessel) endothelium, adipocytes, skeletal muscle, dendritic cells, epithelia of the retina, breast, and intestine, smooth muscle cells, and hematopoietic cells, including erythroid precursors, platelets, monocytes/macrophages, and megakaryocytes (Febbraio M, 2001). In addition to TLR2/6 heterodimer, studies showed that CD36 is indispensable for recognition of microbial diacylglycerides such as R-enantiomer of MALP-2 (a diacylated bacterial lipopeptide) as well as lipoteichoic acid (Hoebe K, 2005). CD36 was found to be critical for recognition and uptake of *Staphylococcus aureus* (Stuart LM, 2005). Finally, CD36 recognizes parasitic infection-induced membrane changes and mediates nonopsonic phagocytosis of *P. falciparum*-infected erythrocytes which is critical for malaria infected erythrocyte clearance (Febbraio M, 2001). CD36 has also been shown to have critical role in phagocytosis of apoptotic cells by macrophages (Fadok VA, 1998).

SR-BI: SR-BI, which has 2 alternative splice variants - SRBI & SRBII, is expressed on monocytes, macrophages and dendritic cells (only in humans), and is also found on hepatocytes, adipocytes and adrenal glands (Plüddemann A, 2007). SR-BI and SR-BII mediate uptake of *Mycobacterium fortuitum* by non-phagocytic cells such as HEK-293 and might have a role in non-opsonized uptake of several bacterial species (Philips JA, 2005). Human SR-BI binds LPS (Vishnyakova TG, 2003) and E2 glycoprotein of Hepatitis C virus (HCV) (Scarselli E, 2002) and mediates their internalization (Barth H, 2008).

Class C: Drosophila Scavenger Receptor-CI (dSR-CI), type I transmembrane, plays a role in phagocytosis of gram-positive and gram-negative bacteria and the recognition of microbial β -glucans in Drosophila (Mukhopadhyay S, 2004; Plüddemann A, 2007)

Class D: CD68 is not reported for pathogen recognition (Greaves DR, 2008).

Class E: Lectin-like oxidised LDL receptor-1 (LOX-1), a type II membrane glycoprotein, is the only member of the class E (Plüddemann A, 2007). CHO-K1 cells expressing LOX-1 stably have been shown to bind Gram positive and negative bacteria, which is Lox-1-dependent (Shimaoka T, 2001). Moreover, it has a contribution to antigen cross-presentation by recognizing Hsp70 (Delneste Y, 2002). Recently, a link between TLR9 and LOX-1 has been reported. Activation of TLR9 via CpG motifs increased LOX-1 expression (Lee JG, 2008). CpG type used in this study wasn't mentioned, while it was ROS-inducer from macrophages, so probably it was K-type CpG ODN.

Class F: Scavenger Receptor (SR) expressed by Endothelial Cells-I (SREC-I) and SREC-II, type I transmembrane receptors, were not yet reported for any pathogen recognition (Plüddemann A, 2007; Greaves DR, 2008).

Class G: Scavenger Receptor that binds Phosphatidylserine and Oxidized lipoprotein (SR-PSOX), which is also known as CXC chemokine ligand 16 (CXCL16), is type I transmembrane molecule. SR-PSOX is expressed on macrophages and dendritic cells and in many organs (Plüddemann A, 2007). It can also perform function as chemokine ligand for an orphan G protein-coupled receptor Bonzo/CXCR6, when it is cleaved with ADAM10 – metalloproteinase (Mukhopadhyay S, 2004; Abel S, 2004). SR-PSOX/CXCL16 can recognize and phagocytose Gram-positive and negative bacteria, and maintain adherence of cells to CXCR6 expressing T cells (Plüddemann A, 2007). Chemokine form might have critical role in inflammatory valvular heart disease by recruiting CD8⁺ T cells to inflamed tissues and enhance IFN γ production (Yamauchi R, 2004). CXCL16 on plasmacytoid dendritic cells has been shown to recognize D-type CpG ODN and play critical role in its activity (Gursel M, 2006) (see section 1.2.2. for more details).

Class H: Fasciclin, EGF-like, laminin-type EGF-like and Link domain-containing scavenger receptor-1 and 2 (**FEEL-1 and FEEL-2**) are members of type I transmembrane proteins and are expressed on endothelial cells of the spleen, liver and lymph nodes. However, only FEEL-1 is expressed on monocytes and macrophages (Plüddemann A, 2007). These receptors also

increase uptake of Gram positive and negative bacteria when expressed in non-phagocytic cells such CHO (Mukhopadhyay S, 2004).

1.1.1.2. Toll Like Receptors (TLRs)

Among PRRs, probably the most widely studied and famous member is the TLR family that includes 12 murine (TLR1-9, TLR11-13) and 10 human (TLR1-10) receptors identified so far (Takeda K, 2007). While some TLRs (TLR1, 2, 4, 5, 6 and 10) are located on the plasma membrane of the innate immune cells, others such as TLR 3, 7, 8 and 9 are located in the intracellular endosomal and/or ER compartments. TLR 10-13 are not well characterized. Only TLR11 among them is known to recognize protozoan profilin (*Toxoplasma gondii*) and uropathogenic bacteria in mice. In humans they are non-functional due to stop codon inside the gene (Takeda K, 2007). Because of special focus of this thesis, TLR9 receptor for CpG motifs will be discussed in details as a separate section (please see 1.1.1.3.).

1.1.1.2.1. Extracellular TLRs

TLR1, 2 and 6 : TLR2 differs from other TLRs. It can be activated by a heterotypic interactions either with TLR1 or TLR6. TLR2/TLR6 and TLR2/TLR1 were known to recognize diacylated and triacylated lipopeptides, respectively (components of bacterial PGN layer). CD14 and CD36 are necessary accessory molecules required for TLR2/TLR6 activation (Miyake K, 2007). TLR2 is also triggered by zymosan from yeast and several glycolipids expressed by other specific bacteria (Takeda K, 2007).

TLR4: It is the first identified TLR member (Poltorak A, 1998). TLR4, senses endotoxin (lipopolysaccharide LPS) found in outer membrane of Gram-negative bacteria. Lipid A portion of LPS is responsible for its toxicity. LPS binding protein (LBP) and CD14 are accessory molecules that have a role in recruiting LPS and transfer and load onto TLR4/MD-2 complex (Miyake K, 2007). MD-2 is an accessory molecule, and its absence abolishes TLR4 signaling.

TLR5: Flagellin, a monomeric protein of bacterial flagella, is recognized by TLR5 receptor. *Salmonella typhimurium* does not induce inflammatory response in TLR5 knockout mice.

However, these mice were highly resistant to infections by this pathogen, where TLR5-mediated inflammation seems to have role in microbial invasion of host (Takeda K, 2007).

1.1.1.2.2. Endosomal/Intracellular TLRs (nucleic acid-sensing TLRs)

TLR3: Double-stranded RNA (dsRNA) appears during viral replication and induces type I IFN production. TLR3^{-/-} mice have defective immune response against dsRNA (Alexopoulou L, 2001). TLR3 knockout mice do not show high susceptibility to infection with most of viruses (Takeda K, 2007).

TLR7/8: These two members are highly homologous and both of them recognize synthetic compounds, imidazoquinolines, which are clinically used for treatment of genital warts. That is associated with viral infection in humans and mice (mouse TLR8 is non-functional) (Takeda K, 2005; Takeda K, 2007). Moreover, TLR7 and human TLR8 recognize viral guanosine or uridine-rich single-stranded RNA (ssRNA) which is found in human immunodeficiency virus, vesicular stomatitis virus and influenza virus (Takeda K, 2005).

1.1.1.3. Toll Like Receptor 9 (TLR9)

1.1.1.3.1. Patterns Recognized by TLR9 as Stimulus

TLR9 is the only known member of TLR family that can recognize specific DNA motifs. Hemmi et al., five years after the identification of CpG motifs, reported that TLR9 is responsible for recognizing the CpG DNA in mice. They showed that splenocytes, lymph node cells, dendritic cells, and macrophages from TLR9 null mice do not respond to CpG motif expressing ODNs (CpG ODN), as evidenced by the loss of pro-inflammatory cytokine production or cell surface maturation marker downregulation (please see table 1.2. for TLR9 expression profile). Moreover, D-galactosamine (D-GalN) sensitized-mice are resistant to lethal effects of CpG ODN (Hemmi H, 2000). Then, it was shown that human TLR9 also is prerequisite for bacterial DNA/CpG DNA-dependent immunostimulation in both primary cells (B-cells and CD123⁺ DC) and TLR9 transfected cell lines (Bauer S, 2001; Takeshita F, 2001). Later findings challenged the idea that recognition of foreign DNA is restricted to CpG motifs. Non-CpG phosphodiester ODNs (PO-ODNs), which were delivered into endosomes after complex formation with DOTAP (Yasuda K, 2006), and self chromatin DNA/IgG autoantibody complexes (Boule MW, 2004) have been shown to be recognized via TLR9 and

stimulate innate immunity. Recently, TLR9 has been shown to recognize 2'-deoxyribose sugar backbone (base-free) of phosphodiester DNA and activate cytokine secretion, but not phosphorothioate (PS) modified 2'-deoxyribose, PS or PO modified 2'-ribose backbones (Haas T, 2008a). Immunostimulatory activity and TLR9 affinity is increased further, when bases and CpG motifs were added to this backbone. PolyG addition (24 extra guanosines at 3'end) or DOTAP complexations were used here to target sugar backbone or PD-ODN into endosomes. PS modified sugar backbones were highly affine to TLR9 and TLR7 (significantly higher than PO-modified ligands) by PD-ODNs *in vitro and in vivo* (Haas T, 2008a).

Table 1.3. Variations in TLR9 expression among mice and human (adopted from Krieg AM, 2006).

	B-cells	pDC	mDC	Monocyte/Mf
Human	yes	yes	no	no
Mouse	yes	yes	yes	yes

pDC: plasmacytoid DC, mDC: myeloid DC. Mf: macrophage

1.1.1.3.2. Mechanism of TLR9 Activation

In non-stimulated cells TLR9 is localized to endoplasmic reticulum, which is transported to early endosomes and then lysosomes upon CpG stimulation to interact with CpG (Latz E, 2004). Kim et. al, suggested that the transfer from ER to endosomes is mediated by UNC93B1 protein (Kim Y, 2008). Two independent studies, revealed that the ER to endosome docking was mediated by endosomal localization motifs present on TLR9 which are conflicting on region of these motifs at TLR9 protein (CA Leifer, 2006; GM Barton, 2006). There is also other contradicting ideas on movement of TLR9 from ER to endosomes: Latz et al. reported that this is maintained by non-secretory pathway (Latz E, 2004), while Chockalingam et al. reported recently that secretory pathway is involved and TLR9 passes through Golgi on the way to endosomes (Chockalingam A, 2008). In endosomes, TLR9 exists

as homodimers, ligand unbound form is the inactive conformation of the receptor. When ligand (CpG DNA) binds, separate TIR domains get closer and recruit MyD88 for initiation of signaling cascade (Latz E, 2007). Fitting with this idea, it was shown that only aggregated, multimeric forms of ODNs are stimulatory. As concentration of multimeric forms increase in ODN solution, it becomes more immunostimulatory (Wu CC, 2004). Multimeric ligand seems to have more advantage to stimulate homodimer receptor, otherwise two monomeric receptor should be activated separately with 2 different monomeric form of ODN at the same time in endosomes/lysosomes.

Interaction of TLR9 with bacterial/viral DNA in endosomes of wild-type cells seems to prevent recognition of self-DNA, since artificially expressing TLR9 as chimeric protein (TLR9/TLR4) on the cell surface makes mammalian DNA stimulatory. Viral DNA becomes nonstimulatory in this case possibly due to existing protecting coat around the DNA (GM Barton, 2006). Mammalian DNA with phosphodiester backbone showed parallelism with PO-ODNs, which become more stimulatory against surface expressed TLR9, in sensitivity to DNase. (GM Barton, 2006). This finding challenged previous notion accepting CpG motifs as a determinant of self/non-self discrimination. Lastly, TLR9 has been shown to get activated after proteolytic cleavage of carboxy terminal fragment at endosomes via cathepsin proteases (Ewald SE, 2008; Park B, 2008), which is concordant with previous data that TLR9 signaling is blocked by inhibition of endosomal acidification (Ahmad-Nejad P, 2002). This acidification is required for optimal activity of TLR9-cleaving proteases. These recent findings strengthened the hypothesis that TLR9 functionality at endosomes is mainly for self/non-self discrimination. Since to get activated, it should have to go to endosomes where self DNA does not have access.

1.1.1.3.2.1. Accessory Molecules Involved in TLR9 Induction

LL37: LL37 is an endogenous antimicrobial peptide which is expressed highly in psoriatic skin, which is an autoimmune disease of skin. During physical injury, disease condition worsens, self-DNA release and pDC activation occurs. LL37 form complexes with self-DNA to be delivered to early endosomes of pDC and stimulate IFN α production. Otherwise, self-

DNA is inactive because it can't colocalize with TLR9 in endosomes to stimulate it (Lande R, 2007).

HMBG1 and RAGE: HMBG1 (High-mobility group box 1) protein is a nuclear protein which binds DNA and is involved in bending of double-helix to increase affinity of DNA-transcription factor interactions. In inflammatory cytokine stimulation, it is released from dendritic cells and regulates IFN α stimulation in an autocrine manner. Recent work shows that HMBG1 complexation with A/D-type ODN (but not B/K-type ODN) increases its B cell activating and IFN α -inducing potential from pDC. Moreover, this additional stimulation depends on interaction of RAGE (receptor for advanced glycation end-products), which is multi-ligand receptor of immunoglobulin superfamily, with HMBG1-TLR9 complex. They also showed that HMBG1 and RAGE have critical role in IFN α induction with DNA-containing immune complexes in lupus patients (Tian J, 2007).

Cathepsin-K: Cathepsin-K is an osteoclast-specific protease which is involved in degradation of bone matrices. It is seen as a therapeutic target to treat diseases, such as osteoporosis and autoimmune arthritis, in which osteoclast activity abnormally increases. Recently its role in CpG-induced TLR9 activation has been delineated (Asagiri M, 2008). Specific inhibition of Cathepsin-K by pharmacological inhibitor in dendritic cells, dose-dependently reduced TLR9-mediated, but not TLR2 or TLR4-mediated, production of IL-12, IL-23 and upregulation of maturation markers such as CD40, CD80 and CD86 (Asagiri M, 2008). Moreover, cathepsin K^{-/-} mice were refractive to stimulation with CpG ODNs but not with LPS, regarding IL-6 and IL-12 production from dendritic cells.

UNC93B1: Missense mutation in *Unc93b1* gene causes impaired signalling via TLR3, 7, 9 (called as '3d mutation' due to defects in 3 different signal pathway) and exogenous presentation of MHCII. Mice with these mutations show increased susceptibility to murine cytomegalovirus (MCMV) infection (Tabeta K, 2006). This impaired signaling is caused by defects in delivering these TLRs from endoplasmic reticulum to endosomes, which is mediated by UNC93B1 protein. TLR9 artificially expressed on plasma membrane is not affected from '3d' mutation and responds to CpG motifs (Kim Y, 2008).

1.1.1.3.2.2. Signaling Through TLR9

After 2h stimulation of TLR9-expressing cells with CpG ODN, number and size of CpG-containing vesicles increases significantly, some of which co-stain with TLR9. Colocalization of TLR9 with CpG in same endosomes have been shown to depend on presence of intracellular ‘Toll/IL-1R homologous region’ (TIR) domain of TLR9. (Takeshita F, 2004) CpG-TLR9 interaction recruits MyD88, which is an adaptor protein involved in signalling through most of TLRs (for clear depiction please look Figure 1.2.). MyD88 deficiency completely impairs TLR9 signaling, which indicates that it does not use other adaptor molecules like several TLRs. MyD88 has two domains: TIR domain to interact with TIR of TLR9 and death domain (DD) to interact with interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-4. IRAK-4 phosphorylates IRAK-1, which in turn upregulates its kinase activity, which leads to recruitment of tumor necrosis factor associated factor 6 (TRAF6)- E3 ubiquitin ligase (Klinman DM, 2004b; Ishii KJ, 2006, Cao W, 2007; Kumagai Y, 2008b). TRAF6 stimulates the protein kinase TAK1, which in turn phosphorylates I κ B to induce nuclear translocation of NF- κ B and initiates signaling by the mitogen-activated protein kinase (MAPK) kinase family. Interferon production in pDC, is initiated at the early endosomes, IRF7 translocates to nucleus, which is mediated by phosphorylation of IRAK1 and TRAF6, and starts type I interferon (IFN) expression (Cao W, 2007; Kumagai Y, 2008b). Recently it has been shown that when rapamycin sensitive PI(3)K-mTOR-p70S6K pathway is blocked through mTOR or downstream mediators, IRF7 activation and type I IFN production is also impaired due to inhibited interaction between TLR9 and MyD88 (Cao W, 2008). IRF5 is also an important transcription factor activated by TRAF6 and together with transcription factors NF κ B and AP1 (activated by MAPK) it induces the expression of pro-inflammatory cytokines, chemokines and costimulatory molecules (Cao W, 2007).

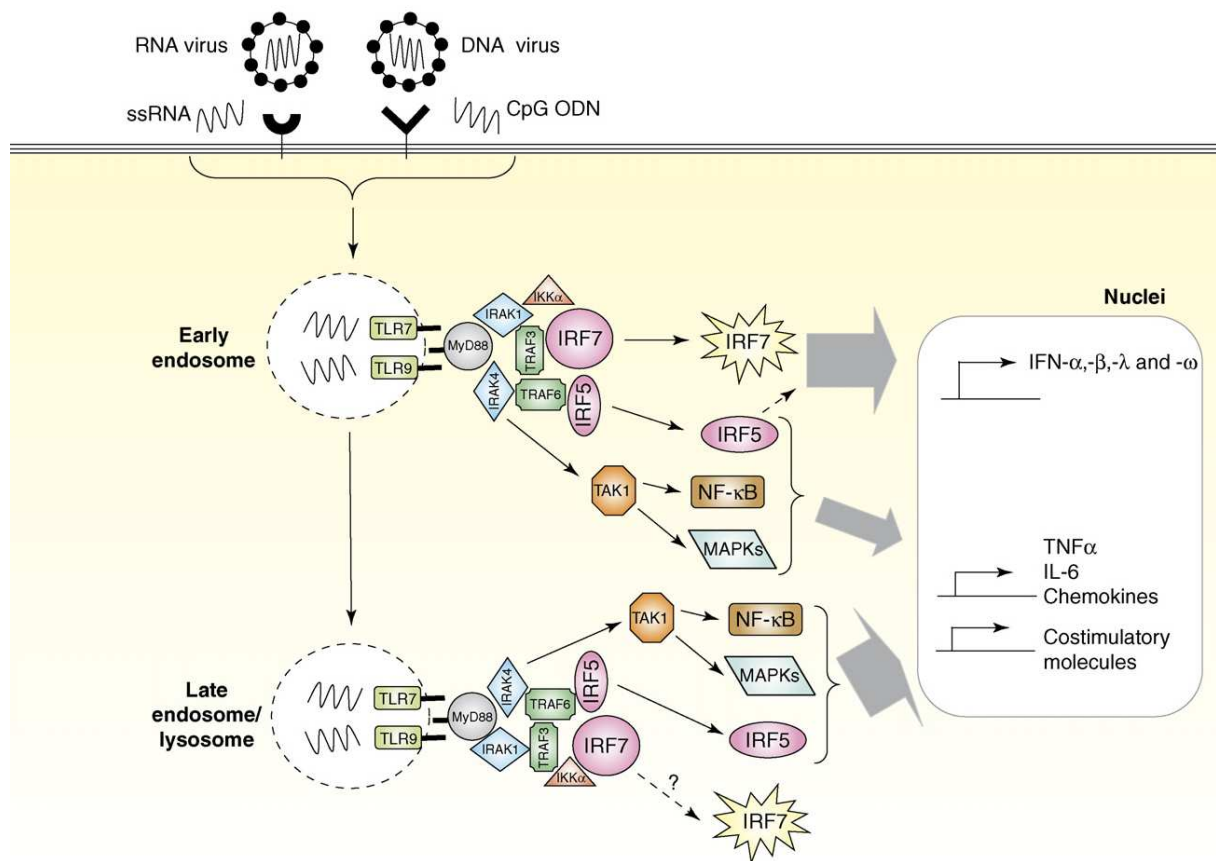


Figure 1.2. Signaling pathway activated through TLR7 and TLR9 (Cao W, 2007)

1.1.1.3.3. Cross-talk and Cooperation of TLR9 with other TLRs

When a microbial infection takes place, cells of the immune system recognize the pathogen through interacting with multiple TLR ligands present on the pathogen surface. While it achieves this recognition and mounts a response necessary for pathogen clearance, the response it generates must not harm self tissues. To achieve this, co-operation and cross-talk is necessary between different TLRs and other members of immune system. Recent evidence strengthen this hypothesis that sometimes TLRs synergize and induce an immune response which is quantitatively more than additive of individual TLR response. In other cases, one TLR member antagonizes the other and brings down the response generated.

Several *in vitro* and *in vivo* studies have been performed and demonstrated that this cooperation does exist. Synergistic cooperation of TLR9 and TLR3 led to a more pronounced activation of TLR genes in, and proinflammatory cytokine production from mouse

macrophages (Tincer G, 2007 and Trinchieri G, 2007). During the course of *Trypanozoma cruzi* infection, TLR9 and TLR2 cooperates to resist infection. TLR2, TLR3, and TLR9 contributes to control murine cytomegalovirus (MCMV) infection (Trinchieri G, 2007). During the *Mycobacterium tuberculosis* infection TLR9 and TLR2 cooperates to yield much stronger IL-12 (Th1 cytokine) induction. The double-knockout mice for TLR2 and TLR9 revealed that IL-12 production is completely impaired, and none of the animals could survive after bacterial aerosol challenge (Bafica A, 2005).

TLR9 along with other TLRs were also studied to establish whether there is a cross-talk between them. It was shown that TLR9 along with either TLR5 or TLR7, showed antagonistic character to certain TLR9-mediated immune effects. Substimulatory concentrations of TLR7 ligands such as R848, loxoribine or ssRNA significantly antagonized IFN α production by A/D-ODN and C-ODN from pDC, when both ligands (TLR9 and TLR7) given together (Berghöfer B, 2007). Moreover, when TLR5 and TLR9 were used together for stimulation, CpG-induced IFN α production and subsequent NK cell-mediated lysis were suppressed (Merlo A, 2007).

1.2. Therapeutic Potential of TLR Ligands with Emphasis on TLR9

1.2.1. CpG ODNs

Oligodeoxynucleotide (ODN) sequences designed for antisense therapy were first shown to have immunostimulatory effect in 1992 by Yamamoto et al. They observed that some of antisense ODNs containing palindromic sequences induced strong type I and/or type II IFN production and led to profound interferon mediated NK cell killing activity. Later, it was understood that unmethylated CpG motifs, which are nearly 20 times more frequently expressed in microbial DNA than mammalian DNA, are responsible for this immune activation (Krieg AM, 1995). CpG motif expressing ODNs stimulated murine B-cell proliferation and IgM production significantly, whereas control ODNs in which cytosines are methylated or CpG is inverted to GpC didn't have any stimulatory effect. Minimum ODN length for stimulatory effect was 8 bp. Location of CpG was also important that methylation of 5' CpG completely abolished while methylation of CpG at 3' end didn't reduce

immunogenicity of ODN (Krieg AM, 1995). CpG ODNs stimulate BALB/c mouse spleen cells to produce IL-6, IL-12 and IFN γ . If a sequence contains multiple CpG motifs over its single strand, it increases its stimulatory effect. Number of cytokine secreting cells peaked after 12h stimulation which then declined gradually (Klinman DM, 1996). CpG ODN stimulates human PBMC also to release TNF α , IL12, IL-6 and upregulate monocyte and B-cell activation markers. (Bauer M, 1999). They induce polyclonal activation and differentiation into plasma cells of memory B cells without BCR (B-cell receptor) signaling or T cell help which makes them powerful adjuvant for induction of humoral immunity. However, additional BCR signaling is needed for activation of naive B cells (Bernasconi NL, 2002). Overall response to CpG ODNs by innate immune system is Th1-polarized. The immunization of mice with CpG ODN plus an antigen, induces IFN γ , IL-12, antigen specific IgG2a (all are Th-1 associated), and suppress IL-5 (Th-2 associated) production in response to specific antigen (Chu RS, 1997). So far, specificity of sequences flanking CpG motifs were studied by many scientists. Central hexameric sequence should be PuPuCpGPyPy for mouse and PuPyCpGPuPy for humans to get optimal immune activation that substituting a Purine (Pu) for a Pyrimidine (Py), or vice versa, significantly reduces or eliminates ODN activity (Krieg A, 1995; Verthelyi D, 2001). However, further sequences can be modified so that resulting ODNs elicit distinct immune profile. CpG ODNs designed up to now can be grouped into i) A/D-type CpG, ii) B/K-type CpG, and iii) C-type CpG which are discussed below. Moreover, additional less well established sequences, which do not fit to any of these three groups will be also covered.

1.2.1.1. A/D type CpG ODNs

This group of CpG ODNs is named as A-ODN by some groups and D-ODN by others. It will be referred to as D-ODN hereafter. D-ODNs have a mixed backbone of phosphodiester and phosphorothioate linkages. Generally, a single CpG motif is present within the middle of a palindromic region with phosphodiester (PO) backbone. This palindrome is capped by G (guanosine)-runs at both ends where the linkages between the guanosine pairs at 3' and 5' ends are phosphorothioate (PS) which confers resistance to exonuclease digestion. Minimum length of an active D-ODN is 18 bp. Representative sequences are given below:

1. D19: 5'-GGtgcatcgatgcagGGGG-3'
2. D29: 5'-GGtgcacggtgcagGGGG-3'
3. D35: 5'-GGtgcatcgatgcaggggG-3'
4. D no poly(G): 5'-GGtgcttcgatgcaaaaaA-3'
5. D3CG: 5'-GGtcgatcgatcgaggggG-3'
6. ODN 2216 : 5'-GGgggacgatcgtcggggG-3'

Please note that lowercase letters indicate phosphodiester linkages between bases, whereas uppercase letters indicate phosphorothioate linkages between bases. Bold bases are CpG motifs, and underlined bases represent unmethylated CpG dinucleotides.

Inversion, replacement, or methylation of the CpG abolishes the stimulatory activity completely. The polyG ends (minimum 4 G at 3' end is required for optimal activity) and central palindromic region of the ODN also contribute to D-type activity significantly (Verthelyi D, 2001). D-ODN has a high tendency to spontaneously assemble into higher-order structures, heterogeneous particles mainly composed of globular (~50nm size) and linear structures (~100nm size), under physiological concentration and this structure formation depends on the presence of poly(G) motifs and on the palindromic region (Costa LT, 2004). The model for this multimerization was suggested by Kerkmann et.al., and according to this, two monomeric D-ODN form a duplex via Watson-Crick base pairing at their palindromic regions. Moreover, four poly(G) ends of the two duplexes are linked together via a non Watson-Crick base pairing. This is an interaction established between Guanosines and is known as "Hoogsteen Base Pairing". The interaction between the planar four Gs is called "G-tetrads" and multiple forms of these G-tetrads are named as G-Quadruplexes (Figure 1.3.). Exposing to high temperatures generally breaks down G-tetrads to their monomeric molecules (Kerkmann M, 2005).

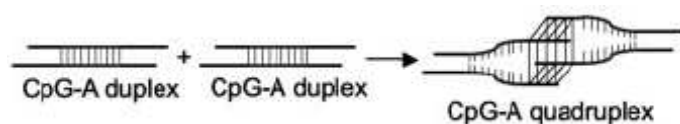


Figure 1.3. Model for higher-order structure formation. G-tetrad links 4 D-ODN strands. (Kerkmann M, 2005)

D-ODN induces high amounts of IFN- α/β directly from plasmacytoid dendritic cells (pDCs) and IFN- γ from PBMC (peripheral blood mononuclear cells). Maximum activity is reached at 3 μ M. They also indirectly induce IFN- γ , IP-10 from PBMC and NK cell activation strongly. NK cell-mediated IFN- γ production by D-ODN is IFN α/β -dependent, and IL12-independent (Krug A, 2001; Verthelyi D, 2001). They induce moderate levels of costimulatory molecules such as CD80, CD86 and HLA DR on pDC and B cells which are lower than K/B type CpG ODNs (Krug A, 2001). D-ODN stimulates monocytes to mature into CD83⁺/CD86⁺ dendritic cells which is IFN- α -dependent (Gursel M, 2002b). NK cell-mediated cytolytic activity, which is a very critical part of innate immunity against intracellular pathogens, was also increased considerably when PBMC (not pure NK cells themselves) were incubated with this type of CpG ODN (Krug A, 2001). D-type ODN stimulates mouse splenocytes to produce moderate IL-6, IL-12 and high levels of IFN- γ . Interestingly, IFN- γ induction by D-type ODN reaches maximal activity at 1 μ g/ml and declined as dose increased further (Vollmer J, 2004). Totally, these ODNs drive CD4 T cells to Th-1 direction. The clinical future of this class of ODN is hampered by the fact that G-rich containing sequences are problematic during synthesis. Poly dG-based oligonucleotides lack pharmaceutical attributes due to unpredictable secondary structure formation depending on the experimental conditions, nonsequence-specific protein binding, including immune stimulation (Wang D, 2008).

1.2.1.2. K/B-type CpG ODNs

This CpG ODN class is also called differently by different groups, B or K-type ODN. It will be referred to as K-ODN hereafter. These are full phosphorothioate backbone ODNs with at least one CpG motif, which is highly active as it is placed closer to 5' terminus of the ODN, and at least one more base upstream of the 5'-CpG motif is required. Thymidine in the

immediate 5' position is most favorable for humans (ODN2006). For mouse active CpG ODNs, CpG motif doesn't have to be at the very 5' end (ODN1555, ODN1826). Minimum length of ODN should be 12 bases for sustained immune activation, where ODNs with base number less than this have reduced activity on immune cells (Verthelyi D, 2001). This type doesn't contain any polyG sequences and form any particulate complex structure under physiological conditions, it is believed to remain single stranded linear sequences (Costa LT, 2004). Optimal K-ODN sequences for human and mouse are given below:

1. ODN1826: 5'-TCCATGACCGTTTCCTGACCGTT-3'
2. ODN1555: 5'-GCTAGACCGTTAGCGT-3'
3. ODN2006: 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'
4. K23: 5'-TCGAGCGTTCTC-3'

K-ODNs trigger IL-6 and IgM secretion from B-cells and their proliferation. They also activate CD19⁺ B cells by upregulating CD69 (early activation marker) and CD25 (late activation marker) (Gursel M, 2002a). These ODNs are superior for maturation of pDCs and induction of proinflammatory cytokines from pDCs (Krug A, 2001). These ODNs can synergize with GM-CSF and activate the maturation markers of dendritic cells; MHC II, CD40, and CD83 (Hartmann G, 1999). Poor IFN α induction from pDCs is also characteristic of this type of ODNs, whereas it is reported that even this trace amount of IFN α is enough to induce MHC-I cross presentation in dendritic cells (Gray RC, 2007). K-ODN induced IFN γ , IP10 secretion from PBMC is little also if compared to other ODN types. However, splenocytes are stimulated better, regarding IFN γ response if mouse-active sequence is used. Spleen cells also secrete very high amounts of IL-6 and IL-12 in response to K-type ODNs (Krug A, 2001; Verthelyi D, 2001; Vollmer J, 2004). Mouse-active K-type ODN induces very high levels of nitric oxide (NO) from mouse-macrophage cell line (RAW264.7), whereas D-ODN induces significantly lower NO amount from these cells (Utaisincharoen P, 2002).

1.2.1.3. C-type CpG ODNs

C-ODNs have fully phosphorothioate backbone with 5' CpG sequences ('TCGTCG motif') and 12-16 bp palindromic sequence at 3' end for most optimal activity (Vollmer J, 2004). TCG at 5' end is necessary for ODN activity as shifting it to 3' end reduces IFN α inducing capacity. Nevertheless, 2-O-methyl (RNA-like) modification within the 5'-CpG motif led to complete loss of IFN α secretion. Immunostimulatory activity of C-ODN depends on the ODN length, the base content and a 5'-TCG. Further nucleotides from the palindromic region at 3' end do not have significant additive effect on stimulatory capacity, unless length of sequence is shorter for optimal stimulatory activity. Minimum length for optimal activity is 22 bp, where adding polyT to 3' end of shorter sequences significantly increases their activity (Jurk M, 2004). Palindromic sequence, which is believed to allow duplex formation in an endosomal environment (Fig 1.4.), is vital for immunostimulatory activity of C-ODN (Krieg AM, 2006). Since similar sequences without palindromic property have severely reduced immunostimulatory effect, especially in the context of IFN α induction.



Figure 1.4. Duplex formation through palindromic sequences by 2 monomeric C-ODNs (ODN2395). (Krieg A., 2006)

These ODNs were reported to combine the immunostimulatory activity of D-type and K-type ODNs. They directly activate human B cells with similar strength as K-ODNs (IL-6 secretion, proliferation and upregulation of activation markers such CD86) and induce IFN α production from pDCs weaker than D-ODN but stronger than K-ODN. They induce also higher levels of IFN γ , IP-10 from PBMC and upregulate activation markers on NK cells better than K-ODN. B-cell activation with this type of ODN is pronounced in whole human PBMC stimulation which is not the case for K type CpG ODN. IFN α production from pDC seems to have an additive effect to B-cell activation in whole PBMC stimulation. Depleting pDC from PBMC

lowers B-cell activation by C-type while it does not effect output of K-type stimulation of B-cells, significantly (Hartmann G, 2003).

Same sequences active in humans induce strong IL-6 and moderate IL12 and IFN γ from BALB/c splenocytes. Moreover, mice immunized with mixture of recombinant HbsAg (surface antigen of hepatitis-B virus) and C-ODN induced several times higher HbsAg specific IgG2a isotype response, which is a clear demonstration of developing a Th1-biased immune response (Vollmer J, 2004).

To sum up, C-ODNs are somewhere between D-ODNs and K-ODNs both structurally and immunologically. They are not linear as K-ODN but have tendency to form duplexes in body fluid, inspite of not forming highly multimeric structures as D-ODN. Immune profile is also somewhat mixture of 'D' and 'K' that they induce lower levels of interferon than D-type, whereas induce B cells directly as K-ODNs.

1.2.1.4. Other Types of CpG ODNs

In order to better modulate immune system, several different CpG ODN were designed other than 'K', 'D' and 'C' types. One of them is Y-shaped ODN which is formed by 3 linear CpG ODNs which have complementary regions with each other (Figure 1.5). This ODN have fully phosphodiester backbone and induces higher levels of IL-6 and TNF α from RAW264.7 cells, from mouse macrophage cell line, than single- and double-strands of same ODNs when used with same amounts. It is claimed in that report than this potency in cytokine release is related with efficient uptake into macrophages (M. Nishikawa, 2008).

Another work reported that 3' extension of phosphodiester linear (CpG or non-CpG) ODNs with 24 guanine nucleotides turns these sequences into an effective IFN α inducer. Further extension doesn't improve activity, whereas shorter polyG addition reduces. CpG motif has an additive effect to this stimulation. They showed that polyG addition confers complexation and efficient uptake into immune cells, like same sequences were mixed with DOTAP (complexation agent) (Haas T, 2008b).

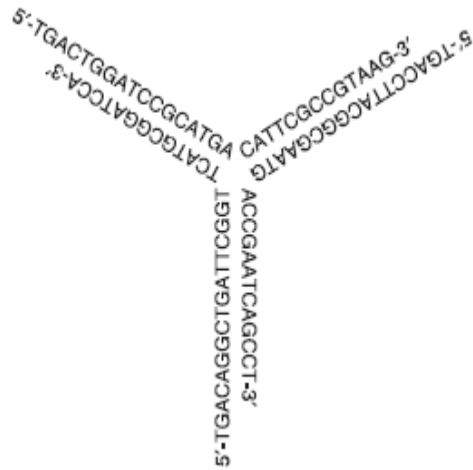


Figure 1.5. Y-shaped CpG ODN (Nishikawa M, 2008).

1.2.2. Differential Immune Response Mediated by Particulate and Linear CpG ODN.

Although both D-type and K-type ODNs contain CpG motifs and initiate their effect by triggering through TLR9 receptor, and even if they stimulate same cell types (B-cell and pDCs in human) they end up inducing quite distinct immune activation from each other. Understanding mechanism behind this dichotomy is very critical, since it will help to modulate immune response by administering K- or D- type according to the desired therapy. Firstly, D-ODNs have been reported to localize to different vesicles than K-ODNs which is claimed to be due to poly-G motifs of D-ODNs. Moreover, D-ODN was taken up four fold more efficiently than K-ODN by PBMC, where excess concentrations of each ODN did not inhibit binding or uptake of the other (Gursel M, 2002a). Collectively these data suggest that these ODNs use different mechanisms of uptake. Poly-G tail, which confers particulate nature to D-ODNs, is possibly responsible in efficient uptake of D-ODNs. Higher efficiency of uptake of particulate ODNs into immune cells such as mouse macrophages and DCs (De Jong S, 2007; Anderson RB, 2008) and human pDC (Kerkmann M, 2006) has been confirmed by other studies. Furthermore, ‘Interferon Regulatory Factor (IRF)-7’ was found to be critical for IFN α induction from pDC by D-ODN, as pDC from IRF7^{-/-} mice is unresponsive to D-ODN, while cDC (conventional dendritic cells) release cytokines such IL-12 and IL-6 upon D-ODN stimulation. K-ODN-mediated stimulation of pDC or cDC from this IRF7-null mice is not affected (Honda K, 2005a). Two recent works helped to explain this unexpected feature of D

and K ODN differential activity. Firstly, Honda K. et. al. reported that induction of different cytokines from pDCs by two types of ODNs is caused by different localization patterns by K or D-type ODNs in pDCs. D-type CpG oligodeoxynucleotides are retained for longer periods in the early endosomal vesicles of plasmacytoid dendritic cells, whereas K-type ODNs accumulate in vesicles with lysosomal markers (Honda K, 2005b). According to this model, D-ODN interacts with MyD88–IRF-7 complex in early endosomes, which results with IRF7-mediated IFN α production. While, K-ODN activates MyD88-IRAK4/1-TRAF6 complex which activate NF- κ B and IRF-5-mediated pro-inflammatory cytokine production (Asselin-Paturel C, 2005). Retention of D-ODNs in early endosomes is facilitated possibly due to their higher-order structure, since artificial targeting of K-ODNs to early endosomes is achieved through making their complexes with DOTAP (Honda K, 2005b). These particulate forms of K-ODN induce IFN α comparable with D-ODN from pDC (Kerkmann M, 2005). Recruitment of D-ODN to early endosomes and their IFN α -inducing capacity seems to result from its interaction with CXCL16, a scavenger receptor only expressed on pDCs but not on B-cells (Gursel M, 2006). *Gursel M. et al.* showed that CXCL16 is necessary for localization of D-ODN to early endosomes and initiate IFN α induction. Moreover, ODNs without poly-G tail, but with same sequence as D-ODN are unable to interact with CXCL16, which shows that interaction between CXCL16 and D-ODN depends on poly-G sequences (Figure 1.6.). Identification of specific receptor for D-type ODN might explain also higher efficiency of artificially multimerized ODNs, regarding uptake and immune stimulation (Shirota H, 2001; Haas T, 2008). To date, there is no convincing evidence showing that particulate forms of K-ODN depends on additional recognition by cell surface receptor for immune stimulation. However, high potential for immune stimulation of nanoparticle CpG at doses where free CpG ODN is not active strengthens this hypothesis (Gursel I, 2001; Diwan M, 2004; Anderson RB, 2008).

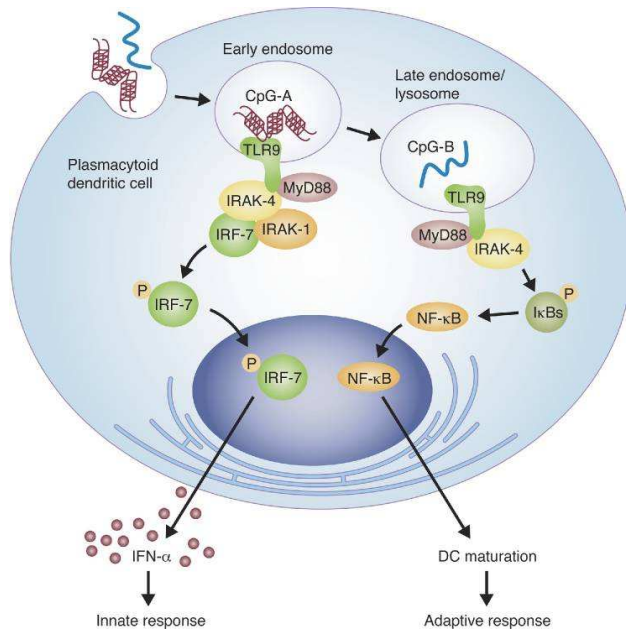


Figure 1.6. Dichotomy of different CpG ODN types delivered to early or late endosomes induce innate or adaptive immune responses, respectively, in pDCs (Williams R, 2006).

1.2.3. CpG ODNs as Therapeutics

Critical cellular target for CpG ODN therapy are pDCs. Mice strains that do not possess pDC, couldn't be protected against pathogenic infections. Moreover, pDCs from CpG-treated donors were able to mount protection in naive recipients. Another TLR-9⁺ cell type is B-cell, where CpG induces polyclonal activation of immunoglobulins that help to eradicate pathogen. NK cells and macrophages are indirectly activated by cytokines released upon CpG treatment from pDCs (Klinmann DM, 2004b).

In an attempt to define the best optimum sequence effectively work on numerous outbreed human populations, several hundred sequences covering broad range of motif sequences were synthesized and tested on PBMC isolated from over 100 donors. It has been observed that no single ODN is optimal for all tested individuals. Two strategies were suggested to overcome this polymorphism. One is synthesizing single ODN with different CpG motifs or using mixtures of multiple ODNs each expressing different CpG motifs (Leifer CA, 2003). This should be taken into consideration in therapeutic approaches with CpG ODNs, which are shown to be effective against infectious diseases, cancer, allergy/asthma.

Infectious diseases : CpG ODNs increase resistance even, of immunosuppressed, newborn and pregnant animals, to pathogenic infection. For example, in SIV (retrovirus like HIV)-infected macaques D-type CpG ODN reduces parasitic (leishmania) load 1000 fold (Klinmann DM, 2004b). However, CpG treatment increases SIV load in macaques, probably by activation of infected T cells. As HIV infection doesn't interfere with innate immunity until late stages of disease, CpG ODN therapy may be used to protect patients from other infections (Klinmann DM, 2004b).

Moreover, induction of T_H1 immunity by CpG ODN suggests that they can be used as vaccine adjuvants. CpG ODN + antigen immunization of mice has been shown to induce cellular and humoral immunity against antigens more powerfully than antigen alone. Chemical linkage between antigen and CpG ODN boosts antigen-specific response further (Klinmann DM, 2004b).

K-type ODN and vaccine mixture increases anti-HB (hepatitis-B) IgG levels 60 fold when priming and 15 fold after boosting, to that compared of vaccine alone in cynomolgus monkeys (Verthelyi D, 2003). In another study, CPG 7909 plus vaccine induced Hepatitis B surface antigen (HBsAg)-specific antibody responses (anti-HBs) which appeared earlier and had higher titres from 2 weeks after the initial priming to 48 weeks individuals who received vaccine alone (Krieg AM, 2006).

K-type ODNs have been shown not only to increase magnitude, but also accelerate development of antigen-specific immune response. They increased specific antibody response induced by AVA (the licensed vaccine against anthrax) alone 6-fold, when administered together with AVA (Klinmann DM, 2004).

Cancer : CpG ODNs encapsulated in sterically stabilized cationic liposome and recombinant interleukin-13 Pseudomonas exotoxin (IL-13-PE) were significantly effective against human neck and head cancer as xenografts in mice. CpG activity was dependent on NK cell activity, IL-13-PE increased this activity (Ishii KJ, 2003). In another study it was shown that K-type CpG ODN effectively suppresses metastasis of cancer cells, especially if they are administered after surgical removal of the primary colon tumor cells (Kim HA, 2008). For

small tumors CpG ODN seems to be effective alone, while for larger tumors CpG therapy should be combined with other therapies like chemotherapy, radiotherapy and etc. (Krieg A, 2006). For now, four different CpG ODNs are in clinical trials for treatment of 11 different cancers such as breast cancer and melanoma (Krieg A, 2008). Possible scenario of CpG anti-tumor activity is illustrated at Figure 1.7. CpG ODNs activate NK cells via IFN α induction. Then NK cells induce death of tumor cells which causes tumorigenic antigen release to immune milieu. After processing antigens and getting matured, DC initiates antigen specific T-cell response against the tumor.

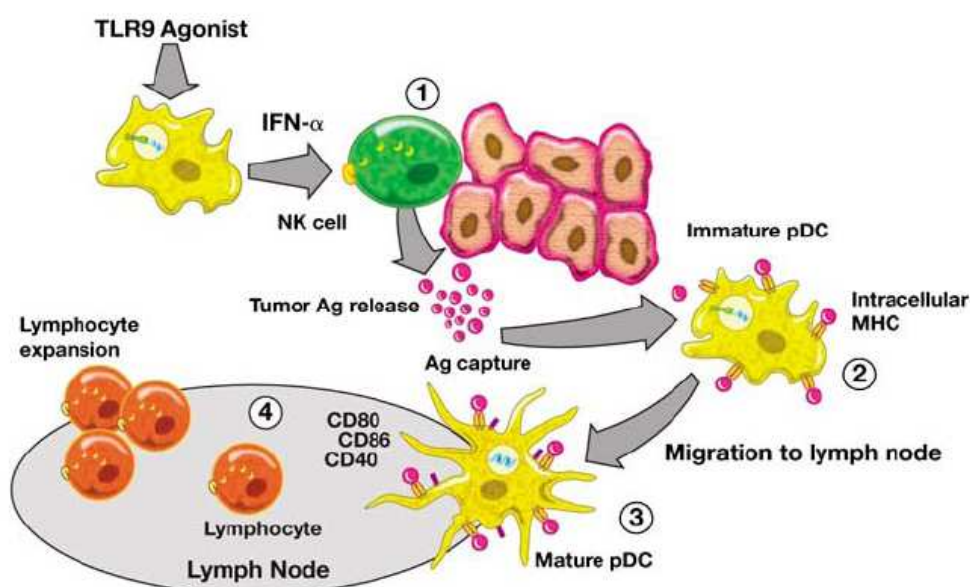


Figure 1.7. Hypothetical effect of CpG ODN on tumorigenic tissue (Krieg AM, 2008)

Allergy/Asthma : Exact mechanism for therapeutic effect of CpG ODN on asthma is not clear. T_H1 cytokines is not necessary for this (Kline JN, 2008). It is also reported that CpG ODNs conjugated with ragweed allergen have been shown in human clinical trials that redirect significantly allergy-associated T_H2 immunity to non-allergic T_H1 (Krieg A, 2006).

2. AIM AND HYPOTHESIS

CpG ODNs are immune stimulating DNA based novel group of immunotherapeutics. Accumulating evidence both from preclinical and clinical studies indicated that CpG ODNs are effective against treating infectious diseases, asthma/allergy and cancer as well as improving the vaccine potency when included as an immunoadjuvants. Three major classes of CpG ODNs have shown to be effective in humans, the well studied class is the linear single stranded multiple CpG motif expressing K-type. The D-type CpG ODNs are unique due to several reasons, it stimulates a very special DC subclass known as the interferon factory, pDC. This differential activation of D type but not K-type is mainly due to the formation of multimeric aggregates of D-type (Kerkmann M, 2005). So far, the mechanism for this dichotomy between D and K-ODNs were reported to be the differential recognition by plasmacytoid dendritic cells. They expresses a surface scavenger receptor and D but not K-ODN interact with this receptor, D-ODN then moves to a different subcellular compartment and possibly due to its multimeric nature it is retained for longer periods in the early endosomes (Gursel M, 2006 ; Honda K, 2005b). The signaling cascade initiated via TLR9, where scavenger receptors possibly have a role involves a transcription factor IRF7, which directs IFN α production by pDC (Wu CC, 2004; Honda K, 2005a; Guiducci C, 2006; Gursel M, 2006; Haas T, 2008).

Antisense ODN complexations with polymeric dendrimers have been studied in the past by several groups (Kang H, 2005 ; Juliano RL, 2006 and Akhtar S, 2007). The major goal was to deliver ODNs within complexed polymeric nanoparticles (Santhakumaran LM, 2004). So far, no one have attempted to synthesize CpG motifs expressing ODNs on a dendrimeric structure. We have reasoned that this will confer more effective internalization as well as resistance to nuclease digestion and due to its nanoparticle nature will be stable and active until reaches to the site of the target immune cells. In this context, the present MSc Thesis project is carried out to explore the followings;

- 1) To synthesize a dendrimeric CpG ODN and characterize its structural/physical properties, and understand the nanoparticle forming nature.
- 2) To evaluate these dendrimeric CpG ODNs as a distinct class of new generation immunostimulants.

3) Finally, to understand the possible mechanism of dendrimeric ODN internalization by immune cells and its *in vivo* performance.

3. MATERIALS and METHODS

3.1. MATERIALS

3.1.1. Reagents

All oligodeoxynucleotides (ODNs) and PCR primers were synthesized at AlphaDNA (Canada). Lower cases represent phosphodiester linkages whereas upper cases are phosphorothioate linkages for the ODN sequences.

ODN 2006	(24mer)	: 5`-TCGT <u>CGT</u> TTTTGT <u>CGT</u> TTTTGT <u>CGT</u> -3`
ODN1466	(10mer)	: 5`-TCGAC <u>CGT</u> TGG-3`
ODN1471	(10 mer)	: 5`-TCGAG <u>GCT</u> TGG-3`
ODN1555	(15mer)	: 5`-GCTAGAC <u>CGT</u> TAGCGT-3`
ODN1612	(15mer)	: 5`-GCTAGAT <u>GTT</u> TAGCGT-3`
K23	(12mer)	: 5`-T <u>CGAG</u> <u>CGT</u> TCTC-3`
D35	(20mer)	: 5`-GgtgcatcgcgatgcaggggGG
ODN420	(70mer)	: 7x 1466, (linked via symmetric bifunctional linker ; Scheme 3.1 A)
ODN421	(70mer)	: 7x 1471, (linked via symmetric bifunctional linker; Scheme 3.1 B)
ODN422	(90mer)	: 9x 1466, (linked via symmetric bifunctional linker and then via trifunctional symmetric linker (Scheme 3.2 A)
ODN423	(90mer)	: 9x 1471, linked via symmetric bifunctional linker and then via trifunctional symmetric linker (Scheme 3.2 B)

ELISA reagents; i) paired antibodies for cytokines, ii) recombinant cytokines, iii) streptavidine-alkaline phosphatase (SA-AKP), iv) *p*-nitrophenyl phosphate disodium salt (PNPP) - substrate for alkaline phosphatase, were purchased from Endogen/Perbio, Pierce Biosciences (USA).

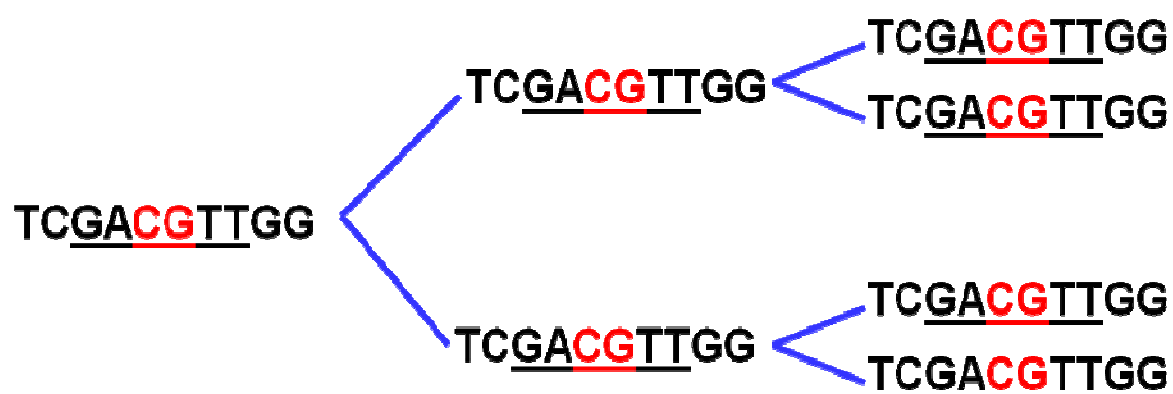
DNase/RNase free water, RPMI1640 culture media and Na-Pyruvate, HEPES, Glutamate Penicillin/Streptomycin were all from Hyclone (USA).

TRI Reagent for RNA isolation was obtained from Molecular Research Center (USA). *DyNAmoTM* cDNA Synthesis kit, *DyNAzymeTM II PCR Master Mix* for PCR were obtained from Finnzymes (Finland).

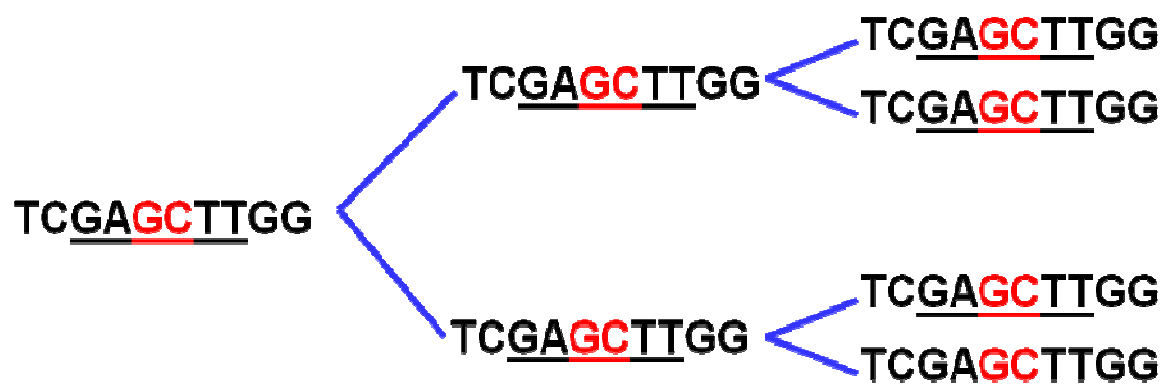
DNase I and its buffer were purchased from Roche (Switzerland).

Stains-All dye, scavenger receptor ligands - dextran sulfate and fucoidan, control polysaccharide-chondroitin sulfate were all purchased from Sigma-Aldrich (USA).

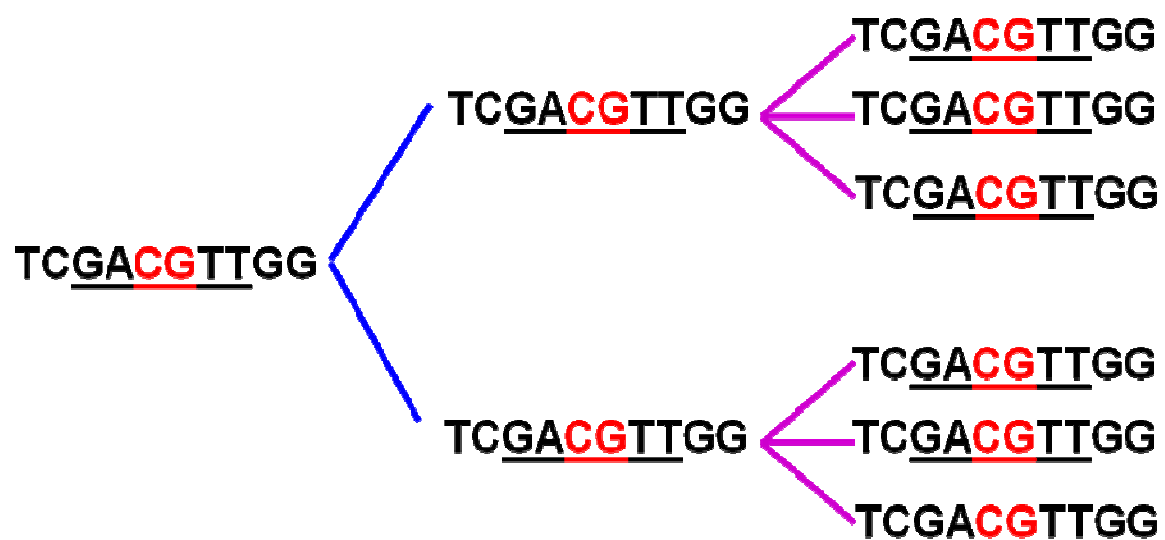
10-150 bp DNA ladder was from Fermentas, and 100-1000 bp DNA ladder was from Jena Bioscience.



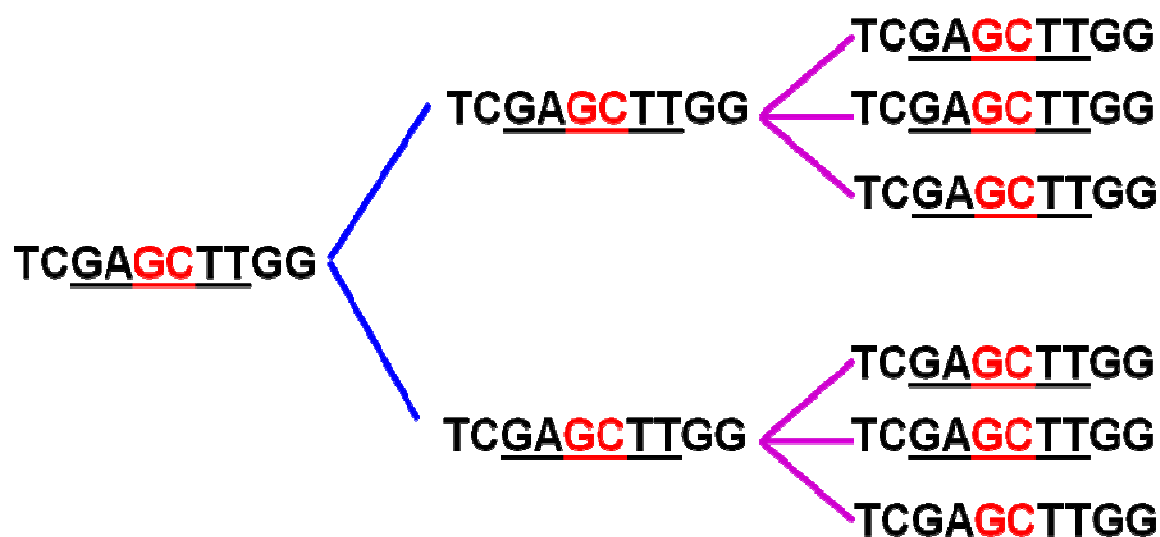
SCHEME 3.1 A. ODN420: Schematic representation of the ODN420 formation via the use of bifunctional linkers at the 3'-end of ODN 1466 sequences. Synthesis was terminated at G=3.



SCHEME 3.1 B. ODN421: Schematic representation of the ODN421 formation via the use of bifunctional linkers at the 3'-end of ODN 1471 sequences. Synthesis was terminated at G=3 (third generation).



SCHEME 3.2 A. ODN422: Schematic representation of the ODN422 formation via the use of bifunctional linker for G=2 and trifunctional linkers for G=3 at 3' ends of ODN 1466 sequences. Synthesis was terminated at G=3.



SCHEME 3.2 A. ODN423: Schematic representation of the ODN423 formation via the use of bifunctional linker for G=2 and trifunctional linkers for G=3 at 3' ends of ODN 1471 sequences. Synthesis was terminated at G=3.

3.1.2. Buffers, Solutions, Culture Media

For details of various culture media, different buffers solutions, and other assay solutions used throughout the thesis is presented in the Appendix I.

3.2. METHODS

3.2.1. Atomic Force Microscopy (AFM)

10 μ l of ODNs with different concentrations were deposited on glass coverslips and dried at room temperature under aseptic conditions. Images were taken at Non-Contact mode using of PSIA AFM (XE-100E model, Korea). Multi75Al model tips were from Budget Sensors. Tips' resonance frequency and force constant were 75 kHz and 3 N/m, respectively. Areas were scanned with 1-1.2 Hz rates (3 and 5 μ m²). Images were analyzed by using XEI 1.6 software. For detection of the nanoparticle size and the size distribution following AFM study, at least 3 images were taken into account for each sample. At least 150-200 individual

particles were analysed during the computation of the average size and size distribution for each studied sample.

3.2.2. Polyacrylamide Gel Electrophoresis (PAGE)

ODNs were run in 20% polyacrylamide gel at 75V for 15min followed by 50V for 45min. 10 µl from 10-150 bp range DNA ladder was used in the study. To visualize DNA bands, gel was incubated in 0.005 % (w/v) Stains-All dye as it is suggested by manufacturer. After 2-3 hr incubations (when bands become visible), gel was rinsed in ddH₂O and exposed to UV or direct sunlight for a few minutes until the background gel staining was disappeared. The gel photo is captured under white light using (Vilber Lourmat, France) .

3.2.3. Animals

Adult female Balb/c (8-12 weeks old) were used for most of the animal experiments. Age matched C57Black/6 mice were used for strain comparison experiments. The animals were maintained in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22° C ±2) regulated with 12 hour light and 12 hour dark cycles. They were provided with unlimited access of food and water. Our experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC).

3.2.4. Cell culture

3.2.4.1. Preparation of single cell splenocyte suspension culture

Mice were euthanized by cervical dislocation. Spleens were removed by using sterile instruments and put into 2% regular RPMI media before starting cell culture experiment. In aseptic cell culture conditions, spleens were made single cell suspensions by squeezing them in a 6 cm petriplates using a back side of a sterile syringe in media. Homogenous part of media were collected by using sterile plastic pasteur pipettes avoiding cell clumps that are composed of fibrous or connective tissue. Cells were precipitated at room temperature (r.t.) by centrifugation at 1500 rpm for 8 to 10 mins using a Beckman centrifuge. Pellet was resuspended and washed twice with fresh media and used for further studies.

3.2.4.2. Cell counting & distribution

After spleen cells were washed with a fresh media as indicated above, pellet were resuspended with 5% Oligo Media. 10 μ l from these cell suspensions were taken and diluted 10 fold with PBS to reduce cell number in the final 100 μ l solution, (otherwise it would be very crowded to count the cells). 10 μ l from diluted solution were micropipetted into Neubaer cell counting chamber. Cells in 4 corners (composed of 16 small squares) with 1mm² area were counted under light microscope (Figure 2.1.).

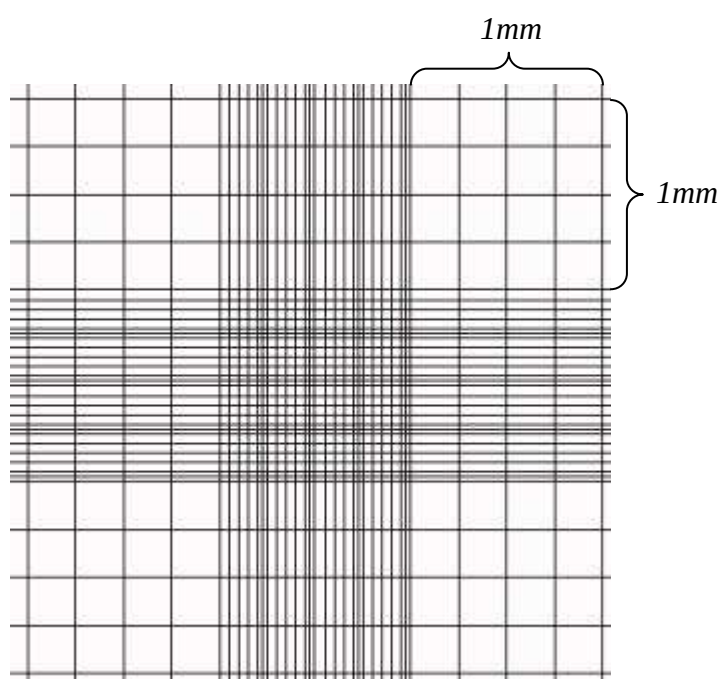


Figure 2.1. Neubaer cell counting chamber

Each 1 mm² area has 0.1 mm³/0.1 μ l (depth between coverslip and counting chamber is 0.1 mm) liquid capacity. So, number of cells/ml was obtained from the equation given below:

$$\left[\frac{\text{Total cell number in 4 squares}}{4} \right] \times 10 \times 10^4 = \text{number of cells per ml.}$$

$\uparrow$
 dilution factor

number of cells per 0.1 mm³

After determination of total cell number of the original suspension, for most of the experiments (unless otherwise stated) cell concentration was adjusted to 2×10^6 /ml by diluting with 5% Oligo media. 200 μ l from this suspension was added to each well of 96-well plate (4×10^5 cells/well). Cells were used for further treatments. For mRNA analyses experiments, cell concentration was adjusted to 10^7 /ml.

3.2.4.3. Stimulation Assay Protocols

Splenocytes in 96-well plates were stimulated with titrating doses of several CpGs, control or D or K ODNs as well as dendrimeric ODNs during dose dependent-response assay. In other experiments stimulating concentration of ODNs were mainly adjusted to 0.5 μ M. For the most part of the study, ODNs were freshly diluted with the proper cell culture media and adjusted to be 50 μ l/well. In cases where, equal amount of ODN stimulation was used, ODN mixture was adjusted to 8 μ g/well for each ODN type regardless of their individual molecular weights. Stimulations were performed in triplicate wells for each indicated treatment. Supernatants were collected after 48h for further analysis. For gene expression studies, cells were stimulated in 15 ml falcons with 0.5 μ M of ODNs (depending on the type of ODN) for 1 to 8 hours. During bulk cell stimulations to assay for mRNA analyses by RT-PCR the caps of the falcon tubes were left loose to allow airflow to the cells. Falcons were left in tilted position while plates with the lids on were incubated in CO₂ incubator for various time points depending on the type of the experiment.

3.2.4.4. Blocking Assays in the Presence of Scavenger Receptor Ligands

Splenocytes in 96-well plates were treated with different concentrations of freshly prepared chondroitin sulfate (control), dextran sulfate and fucoidan (scavenger receptor blockers) for 30 min. The cells were then stimulated with 8 μ g/ml of each ODN for 48h. Supernatants were collected for cytokine analysis.

3.2.4.5. Experiments with ODN Pre-incubation

Murine splenocytes were cultured as 4×10^5 cells/well in 250 μ l final volume of culture media (in 5% oligo media) in 96-well plates. After cells were pre-incubated with various ODNs for different incubation periods (from 1 to 8 h), they were spun at 200 g for 3-5 min. Cells were

washed twice with 250 µl warm fresh regular media to remove excess free ODNs remained in the culture (that were not taken up or bound at the surface of the cells at the end of the pre-incubation period). Fresh cell culture medium was added over cells. Plates were incubated at 37°C, under 5% CO₂ up to 48h without further ODN stimulation. Supernatants were collected at several time points (6 to 48 h) and stored at -20°C for further analysis.

3.2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

96-well PolySorp plates (F96 Nunc-Immunoplate, NUNC, Germany) were coated with monoclonal antibodies against mouse cytokines i) IFN γ , ii) IL-6 and iii) IL-12. 50 µl from antibody solution (10 µg/ml for IFN γ and IL-6 or 5 µg/ml for IL-12) were added to each well. Plates were incubated at room temperature for 5 hours or at 4°C for overnight. Solution in wells were discarded. Then wells were blocked with 200 µl blocking buffer for 2 h at RT or at 4°C for overnight. Plates were washed with ELISA wash buffer 5 times with 5 min incubation intervals after each wash. Plates were dried by tapping (same washing procedure was repeated in upcoming steps). Supernatants of cultured cells and recombinant proteins were added (50 µl/well from both). Starting concentration for IL6, IFN γ and IL-12 recombinant cytokines are 4000ng/ml, 500ng/ml and 500ng/ml, respectively. Recombinant cytokines were diluted serially (two fold with 1X PBS). Plates were incubated 3-4 hours at RT or overnight at 4°C. Plates were washed as explained above. After washing and blotting, 50 µl from 0.5 µg/ml biotinylated-secondary antibody solution (already 1/1000 diluted in T-Cell Buffer, please see Appendix I for details) were added to each well. Biotinylated anti-cytokine incubation was kept for another 2 h at RT or overnight at 4°C. Plates were washed 5 times using identical procedure as explained earlier in this section and dried by blotting. 50 µl from 1:5000 diluted streptavidin-alkaline phosphatase solution (SA-AKP in T-cell buffer must be prepared ahead of time at least one hour before pipeting) was added to each well. SA-AKP was further incubated for an hour at RT or overnight in the fridge. The washing step was repeated and 50 µl of PNPP substrate was added to each well. Yellow color formation was followed and detected by several optical density measurements with (Biotek, Model: Microquant, USA, @ 405 nm) until recombinant cytokine standards reached a four parameter saturation (S-Curve is obtained). All ELISA assays were performed in triplicate repeats for each treatment groups.

3.2.6. Quantification of Gene Expression by PCR Analyses

3.2.6.1. Total RNA Isolation

After stimulating splenocytes with various ODNs for 1h and 6h, 3-4 ml cold 2% regular media was added to each falcon and centrifuged at 1500 rpm for 8 min at 4°C. The media were sucked and pellet was resuspended with 4 ml cold media. After next centrifugation with same conditions, supernatant was sucked again. Pellet was dissolved with 1 ml TRI reagent, mono-phasic solution of phenol and guanidinium thiocyanate, and cells were lysed. Cell lysates were either stored at -80°C till RNA isolation or immediately transferred into 1,5 ml eppendorf tubes for further RNA purification steps. All centrifugation steps were done at 4°C. For 1ml of TRI reagent was used in first step, 200 µl of chloroform was added to each eppendorf and then tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 min. Then eppendorfs centrifuged for 17 min. at 13.200 rpm. The clear upper aqueous phase without touching the interphase were transferred into a new clean eppendorf tube. 500 µl of isopropanol was added for every 1ml of TRI reagent and mixed by inverting gently up/down to allow homogen mixing without shearing RNA. Then tubes were incubated at room temperature for 10 min. and centrifuged for 12 min. at 13.200 rpm. In the next step the supernatant was removed and the pellet was gently washed with 1 ml of 75% ethanol (inverting the tube up/down for 6-8 times) in order to exchange isopropanol and other remaining aqueous reagents with ethanol. Supernatant was discarded and pellet was gently washed with 1ml >99.9% ethanol and centrifuged at 8000 rpm for 8 min. After centrifugation, ethanol was removed and pellet was let to dry at RT under lamin-air flow hood in a tilted position. Dry pellets were dissolved with 20-30 µl RNase/DNase free ddH₂O. The OD measurements were taken at 260 and 280 nm wavelengths with the spectrophotometer NanoDrop[®] ND-1000 (NanoDrop Technologies, USA). Purified total RNA samples were expected to have A_{260}/A_{280} value between 1.8 to 2.2 ratio, which indicates minimal contamination with DNA, protein, polysaccharides or phenol. The RNA samples were stored at -80°C for further use.

3.2.6.2. Semi-Quantitative RT-PCR

To detect relative mRNA expression level of TLR family genes, as well as pro-inflammatory and inflammatory cytokine and chemokine genes, semi-quantitative RT-PCR protocol was performed for the isolated total RNA samples from treated splenocytes as described in previous section. Briefly, a semi-quantitative RT-PCR assay includes cDNA synthesis from RNA, PCR by using specific primers for desired gene, gel electrophoresis of PCR products, measurement and normalization of gel band intensities against a house keeping gene.

3.2.6.2.1. cDNA Synthesis

cDNA was synthesized from total RNA isolated from splenocytes (see Section 3.2.6.1. for details) according to manufacturer's protocol. 1 µg from total RNA samples were mixed with 1 µl Oligo(dT)15 primer (100 ng) and completed to 8 µl with DNase/RNase-free water. Samples were incubated at 65°C for 5 min in MJ Mini thermocycler (BIO-RAD, USA). After being chilled on ice for 2-3 min, samples were mixed with 2 µl reverse transcriptase (includes RNase H⁺ inhibitor) and 10 µl reverse transcriptase buffer (includes dNTP mix and 10mM MgCl₂). cDNA reaction was run in same thermocycler at these conditions: 10 min. @ 25°C, 45 min. @ 40°C, 5 min. @ 85°C and then cooled down to 4°C. cDNA products were stored at -20°C unless they were used shortly after synthesis. Otherwise, they were kept at 4°C to prevent repeated freezing and thawing.

3.2.6.2.2. Polymerase Chain Reaction (PCR)

3.2.6.2.2.1. Primers for PCR

Primers for *il-6*, *il-18*, *cxcl16*, *ifnγ* genes were designed using Primer3 Input 0.4.0 program (<http://frodo.wi.mit.edu/primer3/input.htm>) and Primer Designer 3.0 program with the cDNA sequences of the mouse homologues of these genes which are available at the Ensembl database. Each primer pair was blasted (<http://www.ncbi.nlm.nih.gov/BLAST/>) against mouse genome. Other primer sequences were obtained from literature (Table 3.1.)

Table 3.1. The primer names, sequences, and expected product sizes of the primer pairs used throughout PCR assays.

Primer		Sequence	Product Size (base pair)
<i>β-actin</i>	Forward	:5'-GTATGCCTCGGTCGTACCA -3'	450 bp
	Reverse	:5'-CTTCTGCATCCTGTCAGCAA -3'	
<i>tlr-1</i>	Forward	:5'-TTTGGGGGAAGCTGAAGACATC -3'	410 bp
	Reverse	:5'-CTTCGGCACGTTAGCACTGAGAC-3'	
<i>tlr-2</i>	Forward	:5'-TCTCTGGGCAGTCTTGAACATTTG -3'	320 bp
	Reverse	:5'-CGCGCATCGACTTTAGACTTTG- 3'	
<i>tlr-3</i>	Forward	:5'-GGGGCTGTCTCACCTCCAC-3'	250 bp
	Reverse	:5'-GCGGGCCCGAAAACATCCTT- 3'	
<i>tlr-4</i>	Forward	:5'-TGCCGTTTCTTGTTCCTCCT-3'	240 bp
	Reverse	:5'-CTGGCATCATCTTCATTGTCCTT-3'	
<i>tlr-5</i>	Forward	:5'-TGGGGCAGCAGGAAGACG-3'	380 bp
	Reverse	:5'-AGCGGCTGTGCGGATAAA-3'	
<i>tlr-6</i>	Forward	:5'- GCCCGCAGCTTGTGGTATC-3'	650 bp
	Reverse	:5'-GGGCTGGCCTGACTCTTA-3'	
<i>tlr-7</i>	Forward	:5'-TTAACCCACCAGACAAACCACAC-3'	700 bp
	Reverse	:5'-TAACAGCCACTATTTTCAAGCAGA-3'	

<i>tlr-9</i>	Forward	:5'- GATGCCCACCGCTCCCGCTATGT - 3'	430 bp
	Reverse	:5'-TGGGGTGGAGGGGCAGAGAATGAA- 3'	
<i>ip-10*</i>	Forward	:5'-GCCGTCATTTTCTGCCTCAT- 3'	127 bp
	Reverse	:5'- GCTTCCCTATGGCCCTCATT- 3'	
<i>tnf-α*</i>	Forward	:5'-CATCTTCTCAAAATTCGAGTGACAA-3'	175 bp
	Reverse	5'-TGGGAGTAGACAAGGTACAACCC-3'	
<i>il-1β</i>	Forward	:5'-GCTGCTTCCAAACCTTTGAC- 3'	431 bp
	Reverse	:5'-GGCCACAGGTATTTTGTCGT- 3'	
<i>il-6</i>	Forward	:5'-TGTGCAATGGCAATTCTGAT - 3'	226 bp
	Reverse	:5'-CTCTGAAGGACTCTGGCTTTG- 3'	
<i>il-15*</i>	Forward	:5'- CATCCATCTCGTGCTACTTGTGTT - 3'	126 bp
	Reverse	:5'- CATCTATCCAGTTGGCCTCTGT - 3'	
<i>il-18</i>	Forward	:5'-ACAACTTTGGCCGACTTCAC - 3'	491 bp
	Reverse	:5'- ACAAACCCTCCCCACCTAAC - 3'	
<i>mcp-1</i>	Forward	:5'- AGGTCCCTGTCATGCTTCTG - 3'	249 bp
	Reverse	:5'- TCTGGACCCATTCTTCTTG - 3'	
<i>cxcl16</i>	Forward	:5'- CGCTGGAAGTTGTTCTTGTG - 3'	384 bp
	Reverse	:5'- GGTTGGGTGTGCTCTTTGTT - 3'	
<i>mip-1α</i>	Forward	:5'- ACCATGACACTCTGCAACCA- 3'	238 bp
	Reverse	:5'- AGGCATTCAGTTCCAGGTCA- 3'	

<i>mip-1β</i>	Forward	:5' - CCAGCTCTGTGCAAACCTAA- 3'	250 bp
	Reverse	:5' - CTGTCTGCCTCTTTTGGTCA- 3'	
<i>ifnα 6/8</i>	Forward	:5'-TCTGATGCAGCAGGTAGAGATAC- 3'	384 bp
	Reverse	:5' - CAAATGGTGCAGATACAAAAGTGG- 3'	
<i>ifnγ</i>	Forward	:5' - GCGTCATTGAATCACACCTG- 3'	195 bp
	Reverse	:5' - ATCAGCAGCGACTCCTTTTC- 3'	

3.2.6.2.2.2. PCR Protocol

All reagents were mixed and subjected to optimal PCR conditions for specific primer set in MJ Mini thermocycler (Table 3.2). Running conditions for *ifn α 6/8* were given at Table 3.3.a., for *tlr9*, *ifn γ* , *il-6*, and *il-15* at Table 3.3.b and for the rest of the genes at Table 3.3.c. PCR products were kept at 4°C until run on agarose gel, otherwise stored at -20°C until for further use.

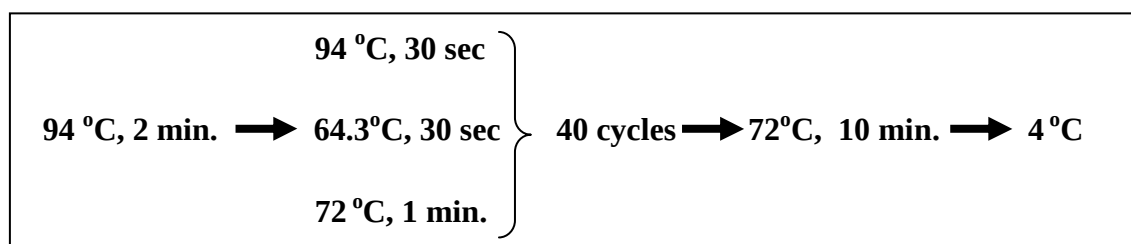
Table 3.2. PCR reactants

Reaction Ingredients	Volume
2x DyNAzyme™ II PCR Master Mix *	12,5 μ l
DNAse/RNAse free H ₂ O	9 μ l
Forward primer	1 μ l (10pmol)
Reverse primer	1 μ l (10pmol)
Cdna	1,5 μ l
Total	25 μl

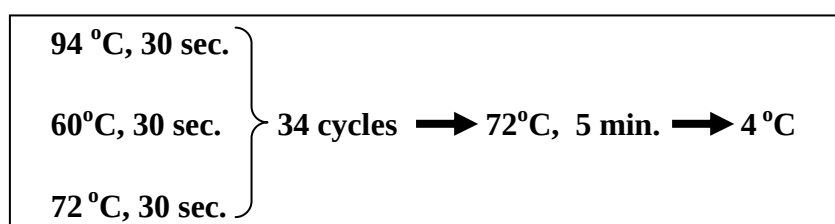
*2x DyNAzyme™ II PCR Master Mix includes 0.04 U/ μ l DyNAzyme™ II DNA Polymerase, 20 mM Tris-HCl (pH 8.8 at 25°C), 3 mM MgCl₂, 100 mM KCl, stabilizers and 400 μ M of each dNTP.

Table 3.3. PCR Running Conditions

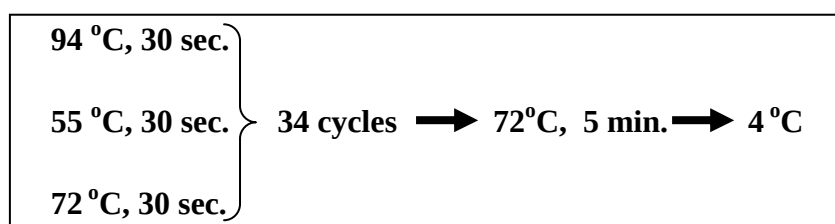
a)



b)



c)



3.2.6.2.3. Agarose Gel Electrophoresis and Band Intensity Measurements

1.5% agarose gel was prepared with 1X TAE buffer. Final concentration of ethidium bromide was 0,5 µg/ml in gel. 2 µl of agarose gel loading dye and 10 µl of PCR products were mixed and applied to each lane. 5 µl from 105 ng/µl DNA ladders with 100-1000bp range was used as a marker and the gels were visualized under UV transilluminator (Vilber Lourmat, France). The gel exposure time was kept fixed at 0.6 sec for each run, unless otherwise stated.

Band intensity analyses were performed with BIO-PROFILE Bio-1D V11.9 software. Band intensity for every gene was measured and background intensity was subtracted from this

value. Blanked values for each gene were divided by blanked β -actin values (house keeping gene) to normalize resulting quantitative data.

3.2.7. DNase Treatment of the ODNs

Dendrimeric ODNs and their monomeric forms were treated with DNase I (1,2U/ μ g DNA) according to manufacturer's protocol. In each reaction tube, there were 3 μ l DNase I (12U), 10 μ l ODN (10 μ g), 20 μ l enzyme buffer and 37 μ l ddH₂O. After incubation at 37°C for varying time intervals, 4,8 μ l from 0,1 M EDTA was added to each tube in order to chelate existing cations and to stop DNase I protein digestion in the solution. Then tubes were incubated at 95°C for 5 min to achieve DNase I inactivation. 20 μ l of this solution was stored for PAGE experiments and the rest (40 μ l) was used in stimulation of murine splenocytes. As a control, group of cells were treated only with DNase I (including its buffer) to eliminate the possibility of immunogenic contamination by DNase I or its buffer. At the end of the stimulation, supernatants were used to compare DNase treated vs non-treated groups in terms of their ability to induce cytokine production (by ELISA) and molecular integrity (by PAGE).

3.2.8. ODN treatment of Animals and Detection of ex-vivo Responses

Animals were injected intraperitoneally (ip) with 50 μ g (high dose: HD) or 10 μ g (low dose: LD) of several ODNs (1-3 animals/group). As given below:

1) Naive 2) ODN1466/HD, 3) ODN 1466/LD, 4) ODN 2006/HD, 5) ODN 2006/LD, 6) ODN420/HD, 7) ODN420/LD, 8) ODN421/HD, 9) ODN421/LD, 10) ODN422/HD and 11) ODN422/LD.

Four hours after ip injection, peritoneal exudate cells (PECs) were removed by flushing 10 ml cold (5% Oligo RPMI1640) media into peritoneal cavity of euthanized mice and immediately collecting back with 10 ml syringe (with 30 gauge needle). Cells were layered into 24-well plate as 1×10^6 cells/well. After 36 hours supernatants were collected to measure cytokine production level. Spleens were also removed and splenocytes were cultured as 10×10^6 per

well. (please see Section 3.2.4.1. for single cell suspension preparation protocol). After 12h, 24h and 48h supernatants were collected to analyze ex-vivo cytokine production.

3.2.9. Statistical Analysis

By using SigmaStat 3.5 software, statistical analysis between groups were done by pairwise two-tailed students` t-test to compare the statistical significance between naive, control and the ODN treatment groups in pairs.

4. RESULTS

Nucleic acid based immunotherapeutics utilizing a subgroup of TLR family receptors (collectively known as endosome-associated TLRs) has been the focus of considerable research interest in recent years. Among RNA and DNA based ligands, CpG dinucleotide containing oligodeoxynucleotides (hereafter CpG ODN) that are recognized by TLR9 expressing innate immune cells constitute the most promising, DNA therapeutic candidate agents that will enter into clinics in the near future. While the ability for TLR9 to be differentially regulated by nucleic acids of varying sequence classes (D/A type, K/B type, or C-type ODNs) and structures offer flexibility for a broad spectrum of immunotherapeutic design, it also necessitates a more comprehensive characterization of these agonists in terms of how these structural parameters correlate with the divergent activation of unique cellular responses. Despite the utilization of three distinct classes of CpG ODNs as a TLR9 agonists in human trials as anti-cancer, anti-allergen, anti-infective or immunoprotective agents these issues have not been adequately addressed.

Recent findings suggest that there is a dichotomy in the response between different classes of CpG ODNs (i.e. D vs K ODN types) [Verthelyi D, 2001; Gursel M, 2002a; Gursel M, 2006; Klinmann DM, 2004a; Klinmann DM, 2004b]. The studies delineating structure-function relationships demonstrate that, in addition to the number of CpG motifs on an oligodeoxynucleotide, the complex formation ability of specific sequences also affects the immunomodulatory performances of these ODN classes. G-quadruplex formation ability due to a poly G-runs at 3'ends of individual sequences as well as mixed backbone linkage chemistry between bases (i.e. phosphodiester plus phosphorothioate linkages expressed on a single strand), were reported to trigger TLR9-mediated signaling cascade leading to type I interferon production by dendritic cells (as in the case of D/A type ODNs). Whereas pure phosphorothioate backbone modified sequences that may even express multiple CpG motifs on a single strand, initiates cell maturation, and Th1 dominant cytokine/chemokine production by innate immune cells (i.e. K/B Type ODN) in human and mice (Krug A, 2001, Klinmann DM, 2004b).

In this study, we attempted to design a novel multimeric CpG motif expressing ODNs-namely “Dendrimeric CpG ODNs” that are capable of forming complex higher order structures. The

original postulation was that these novel ODN structures will have spontaneous nanoparticle forming potential. The nanoparticle structure will offer several advantages over previously designed sequences. These novel sequences will induce i) superior immunostimulatory potential over conventional D and K type ODN classes, mainly due to ii) increased nuclease stability, iii) higher uptake level, iv) and yield reduced premature digestion/elimination when given *in vivo*. This thesis is designed to address these issues as well as molecular characterization of these complex structures by physical techniques such as atomic force microscopy.

4.1. Synthesis of Dendrimeric ODNs

A 10-mer single CpG motif expressing ODN sequence (ODN 1466) was used as the starting monomeric sequence in order to generate dendrimeric ODN structures. The ODN420 had a bifunctional symmetric linker added at the 3'- end of the ODN 1466. On to this bifunctional linker, two more ODN 1466 (please see section 3.1.1. for the sequence details) was further synthesized (this step constituted the first generation synthesis). On to each of these two strands again bifunctional linkers were attached, and ODN synthesis was continued from these linker ends (i.e each had two more ODN 1466 strands). This completed the second generation synthesis. In short, overall seven ODN1466 sequences were linked together (by the help of three bifunctional symmetric linkers), therefore, ODN 420 is generated. It had a base length of 70 mer oligonucleotide (US provisional patent pending, 2008).

The dendrimeric ODN (ODN421, Scheme 3.1 B) was synthesized using identical strategy explained above for the ODN420 as a control sequence lacking active CpG motifs. In that, ODN 1471 sequence was used as the starting monomeric sequence. Please note that the only difference between ODN1466 and ODN 1471 is the CG dinucleotide. It is flipped to GC in 1471 sequence (See section 3.1.1. for the sequences).

To synthesize ODN422 while first generation was completed by the use of a symmetric bifunctional linker, in the second generation synthesis a tri-functional symmetric linker was used to link 3 ODN1466 sequences to the G1 strands. ODN422 yielded a nine sequences linked to each other and had a length of 90-mer oligonucleotide having a nine CpG motifs

linked on a dendrimeric strand (Scheme 3.2 A). Control sequence to ODN422 was synthesized in a similar fashion but here ODN 1471 was the starting monomeric sequence, thus, lacked active CpG motifs. (Scheme 3.2 B).

4.2. Atomic Force Microscopy (AFM) Imaging of Dendrimeric ODNs

The first and foremost feature of these novel sequences was to be able to display higher-order complex structures leading to DNA nanoparticle formations. To observe whether this was achieved, dendrimeric oligos were investigated with Atomic Force Microscopy (AFM) (please see section 3.2.1. for details). Different areas were scanned by the cantilever probe at non-contact mode and images were recorded for each dendrimeric ODN. Detailed analyses of the AFM micrographs revealed that ODN420 demonstrated uniform-sized nanoparticulate conformations (Figures 4.1A to D). While cantilever oscillations gave significant phase changes, the topography showed substantial altitude differences ranging from 20 to 30 nm individual peaks, indicating depth shifts among individual oligo sequences. The phase contrast AFM recording gives an idea about the solid existence of DNA particles on the glass coverslip, whereas topography data provide evidence on the individual heights of these solid entities that are scattered homogenously over the glass coverslip. Investigation of the specimen area at 3x3 μm surface revealed that when ODN420 and ODN421 dried on a silicone base they formed nanoparticles having an average size distribution of 35 ± 10 nm. Whether these colloidal structures are aggregates of several individual sequences or they are organized in singlets are not fully understood under these conditions. However, ODN422 particles, differently from ODN420, appeared with high aggregation tendency. It seemed that individual particles can stick together to form bigger aggregates although individual particles are visible they are more sticky coalesced than ODN420 (Figure 4.2). To address the issues regarding aggregation status of ODNs, additional experiments were designed. One plausible approach was to run these oligos on Polyacrylamide gels (PAGE).

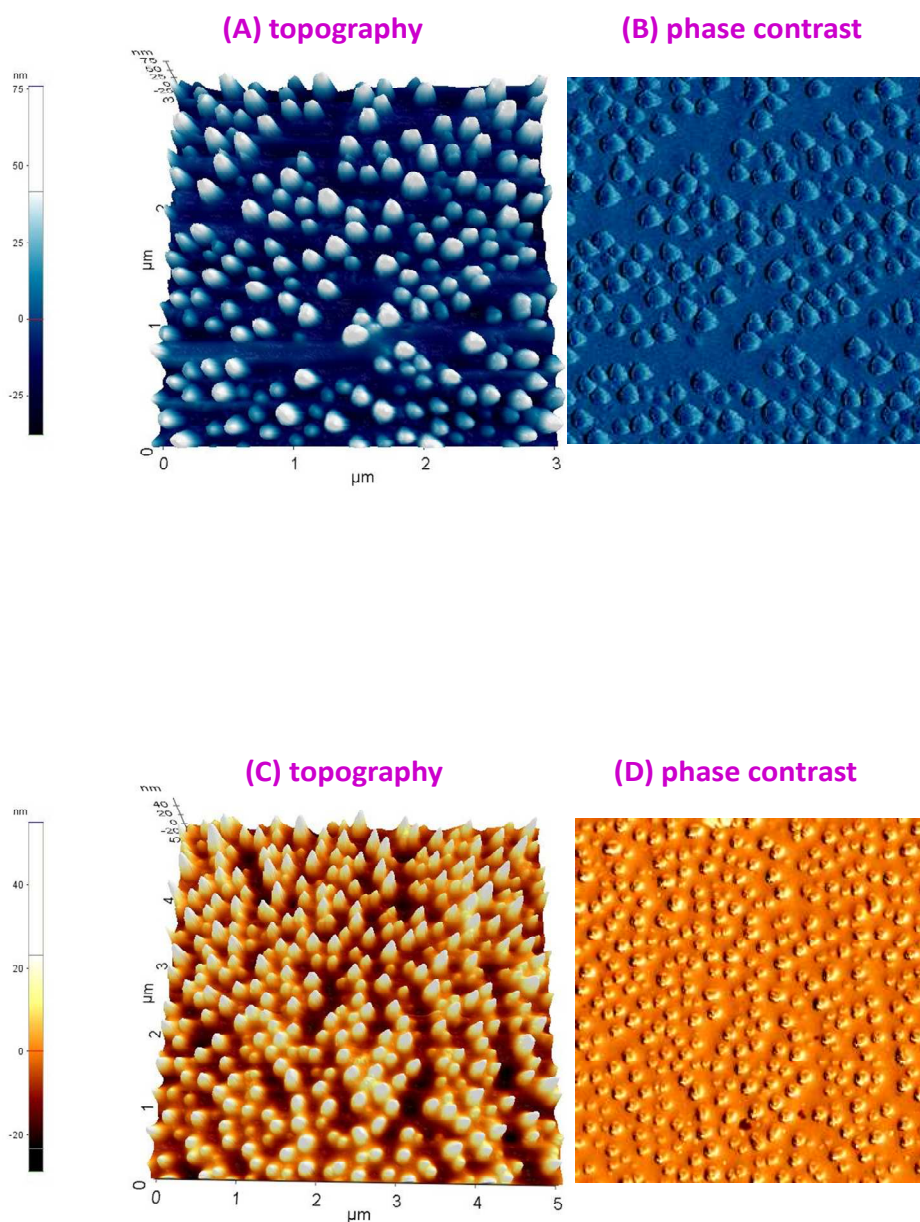


Figure 4.1. Bifunctional dendrimeric ODNs spontaneously form uniform-sized nanoparticles. 10 μ l aliquots from 1 μ M ODN420 solution was deposited onto glass coverslips. Non-contact mode AFM was set to investigate the topography (A and C) and phase contrast (B and D) images. Scanned surface areas were 3 μ m² (for A and B) and 5 μ m² (for C and D).

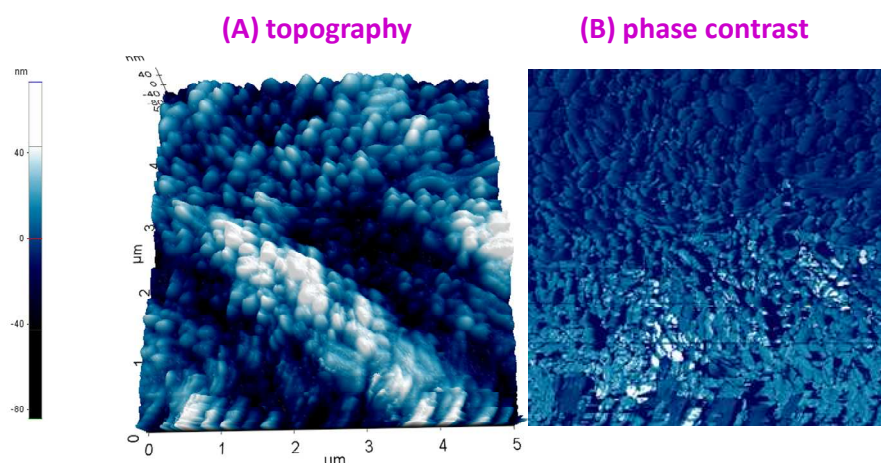


Figure 4.2. Trifunctional dendrimeric ODNs tend to form heterogeneous aggregates. 10 μ l aliquots from 1 μ M ODN422 solution was deposited onto glass coverslips. Non-contact mode AFM was used to get topography (A) and phase contrast (B) images. Scanned surface area was 5 μm^2 .

4.3. PAGE Analysis of Dendrimeric and Conventional ODNs

After observing that dendrimeric ODNs can yield homogeneous nanoparticles, the effort was channelled to understand whether these structures are the result of single dendrimeric unit or several dendrimers are aggregating to yield a nanoparticle structure. As seen in Figure 4.3 along with dendrimeric ODNs, several D and K type conventional ODNs as well as the monomeric ODN1466 were run on a polyacrylamide gel. The lanes were loaded with 10 μ g of each ODN. The staining showed that ODN420 and ODN421 is suspended in the solution as a single monomeric unit as they aligned at the corresponding weight marker (at 70mer location). When the electrophoresis data and AFM images are taken together it is fair to speculate that the nanoparticles observed under AFM (for ODN420) belongs to a single dendrimeric unit rather than oligo aggregates whereas ODN422 is more heterogenous than

ODN420 and thus the size distribution of nanoparticular aggregates under AFM appeared more dispersed. Gel image shows random multimerization of D35, as it appeared as a smear band on the gel. Removing G-rich sequences from D35 (D35-noPolyG) abrogates multimerization, which is observed as a single band on the gel. ODN2006, clinical grade K-type ODN, is observed as single pure band.

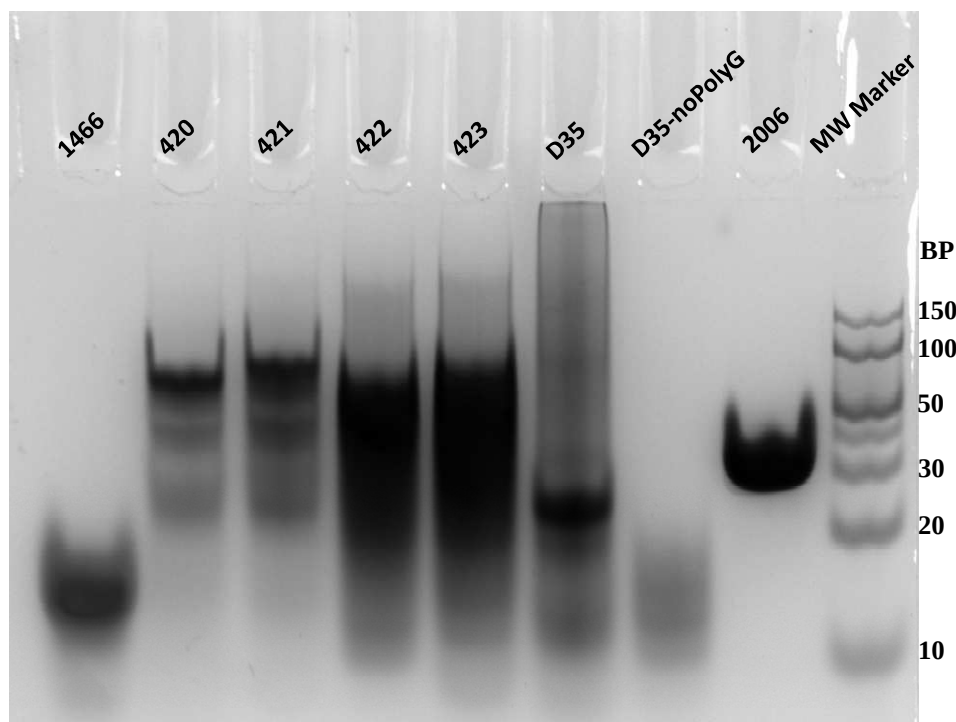


Figure 4.3. Polyacrylamide gel electrophoresis of different types of ODNs. Several dendrimeric and linear ODNs were run on 20% polyacrylamide gel. To visualize the individual ODN bands, PAGE gel was incubated overnight in Stains-ALL solution at room temperature under dark. The gel was rinsed several times with ddH₂O and exposed to UV or direct sunlight for few minutes. After bands became visible and the background was clear from the stain, the gel picture was taken by a digital camera under white light. 1466: 10mer, 2006: 24mer , D35: 20mer, D35-noPolyG: 20mer, 420/421: 70mer, 422/423: 90mer. (BP: Base pair)

4.4. *In vitro* Stimulation Assays Using Mouse Spleen Cells

4.4.1. IL-6 Production Mediated by Different CpG ODNs: Dose-dependent Treatment at Equimolar Concentrations

After the encouraging AFM and PAGE studies implicating that the newly synthesized dendrimeric sequences formed nanoparticles, a series of experiments were designed to investigate the immunostimulatory activity of these ODNs along with conventional CpG ODN classes. The expectation was that the CpG ODNs capable of forming complex structures should yield superior immunostimulatory performance over conventional linear single stranded CpG ODN classes. Single cell suspensions of female Balb/c mouse spleen cells were prepared in warm fresh culture media, and plated at $2 \times 10^6/\text{ml}$ ($4 \times 10^5/\text{well}$) final concentrations in 96 well plates. The cells were stimulated in triplicates with different oligos, at indicated titrating doses as given in Figure 4.4. Following incubation for 48 h, the culture supernatants were collected and stored for IL-6 cytokine detection with ELISA (please see section 3.2.5. for details).

As seen in Figure 4.4 at 3 μM CpG dose, ODN420 (70mer) and ODN422 (90mer) were the most potent IL-6 inducers (over two fold more cytokine is secreted by ODN 420 and 422). This TLR9-mediated immune activation is strictly CpG motif dependent, since control ODNs (ODNs 421 and 423, where CG is flipped by a GC dinucleotides in these sequences) did not trigger any cytokine production in culture. The ELISA results for ODN420 and ODN422 sequences indicated that not only these sequences are active but also effectively internalized and fused with the endosomes (where TLR9 resides) of the immune cells. This initiates TLR9-mediated signaling cascade that led to the production of IL-6. Spleen cells are composed of TLR9 responsive B-cells, monocyte/macrophages and dendritic cells. Please note that T-cells also present in splenocyte culture but they do not express TLR9, thus, they are not directly stimulated by CpG DNA but rather they are induced by the cytokines/chemokines secreted by other immune cells in the culture. T cells then co-stimulated by these mediators and contribute to cytokine secretion profile at a later phase. At this stage it is not possible to comment on the contribution of the specific cell type mediating the IL-6 production.

The dose-titration experiment was specifically designed to understand the strength of the stimulus generated by dendrimeric oligos. The potency of high dose oligo under *in vitro* conditions even though it is informative, is not a prerequisite for *in vivo* success. One needs to demonstrate that very low doses of a CpG ODN in culture can lead to substantially high levels of immune activation. Figure 4.4 suggested that at 0.1 μM concentration, ODN420 is still very active and led to nearly 26 fold more IL-6 production (7,7 ng/ml and 198,5 ng/ml for ODN2006 and ODN420, respectively). Of note, the sequences used along with dendrimeric oligos, are the optimum mouse sequences (1466 and 1555) and one of the most active human specific ODN (2006) sequence (also active in mouse), but none of these sequences were effective cytokine inducers at 0.1 μM dose. This group of data revealed that ODN420 but not ODN422 is active both at high and very low ODN doses. This is a very promising information. When an oligonucleotide is administered *in vivo*, a considerable amount of DNA would be eliminated from the circulation due to adsorption onto serum proteins and DNase digestion. Hence, activity retention even at very low doses in culture provide an additional advantage to target cells that internalized these ODNs. Furthermore, if a strand, as in the case of dendrimeric oligos, is composed of several active CpG motifs, then a small amount of ODN binding would be expected to lead to sufficient activation. The *in vitro* data presented in this section strongly support this view.

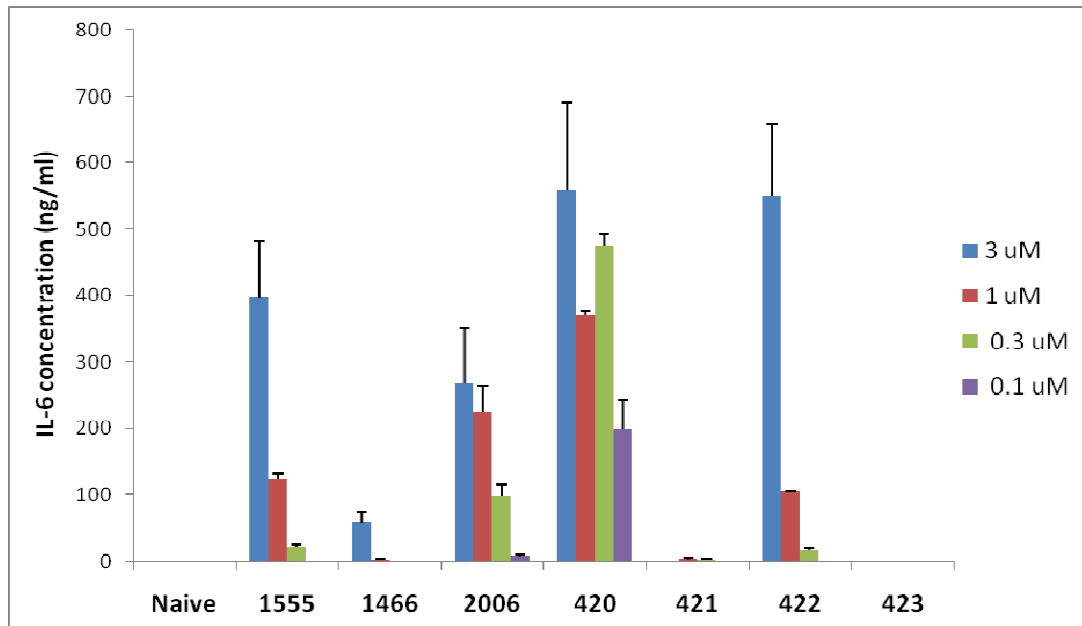


Figure 4.4. IL-6 production by murine splenocytes in response to dose-dependent CpG ODN stimulation. Varying doses of CpG and control ODNs were incubated for 48 h with spleen cells. Culture supernatants were collected and assayed for IL-6 production by sandwich ELISA. $p < 0,04$ for 3 μM ; $p < 0,004$ for 1 μM ; $p < 0,001$ for 0,3 μM ; $p < 0,003$ for 0,1 μM (420 and 2006 treated samples were compared for each dose).

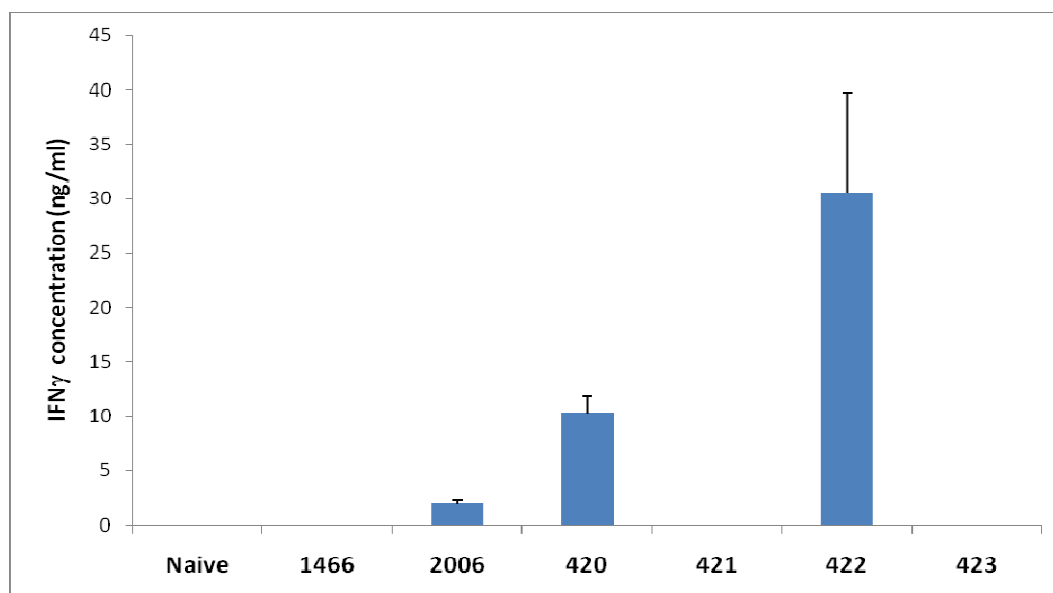
4.4.2. IFN γ and IL-6 Production Mediated by Different CpG ODNs: Treatment with equal weights of ODNs

Although dose-response studies established that dendrimeric ODN is superior over other ODN classes tested, one may argue that dendrimer possesses multiple active CpG motifs on a single strand (eg. while ODN420 had 7 active motifs, single stranded ODN1466 oligomer had only 1, and ODN2006 had 4 CpG motifs over their strands, please see section 3.1.1. for sequence details), thus, treating cells with equimolar concentrations, with equal number of ODN molecules, would favor this class over the rest of ODNs in terms of the stimulation potential.

In order to resolve this issue, murine splenocytes were stimulated with equal weights of each ODN, where dendrimeric ODNs didn't have an advantage over conventional ODNs in terms of CpG motifs. Supernatants were collected at the end of 48h stimulation with the indicated ODNs (8 μ g/ml final concentration) and IFN γ and IL-6 production were assayed by ELISA. As seen in Figure 4.5. A and B, dendrimeric ODNs consistently induced significantly higher amounts of cytokine from spleen cells. ODN 420 gave five fold more IFN γ and 2 fold more IL-6 induction than ODN2006. Surprisingly ODN422 was found to be a mediocre IL-6 inducer but a very strong IFN γ inducer in these assays. These results along with previous findings (please see Fig. 4.4) implicate that dendrimeric, nanoparticle forming ODNs are significantly superior over conventional single stranded linear CpG ODNs, and this ability is not entirely dependent on the number of CpG motifs expressed on a single strand, but rather the efficient internalization ability of these molecules.

It is very important to mention that there is no strain difference in terms of the initiated immune response. We have compared the cytokine production by Balb/C strain throughout this thesis, but to understand if there is any strain to strain difference, we have run a stimulation assay side by side with the age matched Balb/C and C57/Black6. The IL6 and IFN γ responses were quite similar in spite of the strain differences (data not shown).

A)



B)

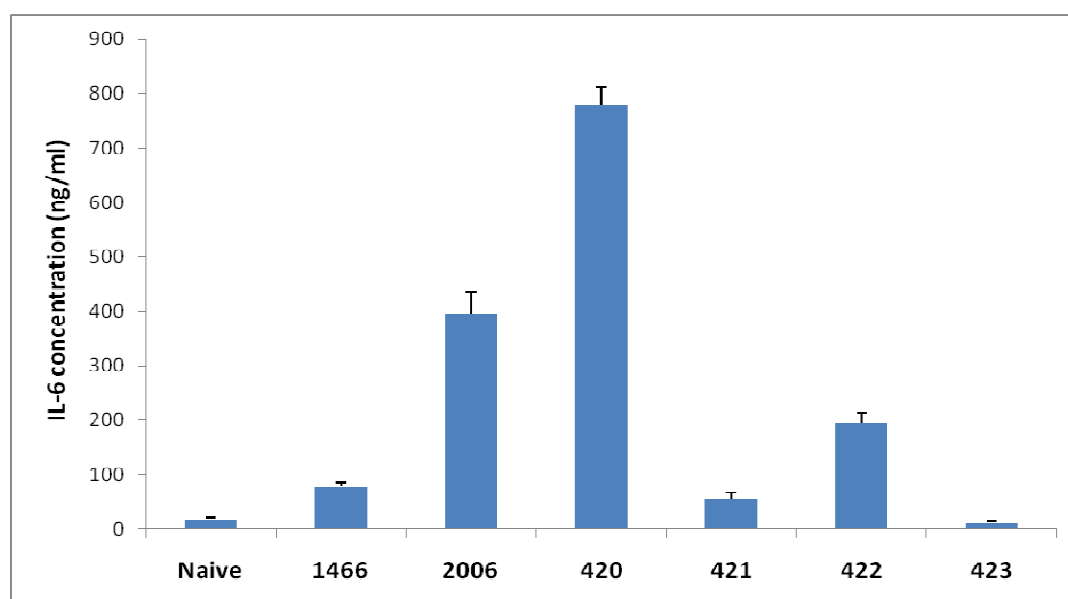


Figure 4.5. Cytokine response of murine splenocytes stimulated with equal amount of CpG/Control ODNs. Murine splenocytes were stimulated with 8 μ g/ml of each ODN for 48h. IFN γ (A) and IL6 (B) concentration at culture supernatants were detected by ‘sandwich’ ELISA method. $p < 0,007$ (between 420 and 2006), $p < 0,03$ (between 420 and 422) for IFN γ and $p < 0,001$ (between 420 and 2006) for IL-6.

4.5. Spleen Cells and ODN Mixing to Study Efficiency of Binding

In previous section, treating spleen cells with different ODN types in culture either with equal amounts or equimolar concentrations, established that nanoparticle forming CpG sequences were more potent cytokine inducers compared to conventional single stranded CpG DNA. Several possibilities may contribute to this phenomenon. One reason could be the differential internalization of these ODN species by the immune cells. One of the best ways to assess direct ODN immune cell interaction is to use chromophore-labeled ODNs. Following mixing ODN with cells, binding and internalization of different oligos reliably quantitated via flow cytometry. Unfortunately, throughout this study, due to time restrictions and problems in synthesis, we could not obtain these labeled ODN sequences on time, therefore, an alternative experiment was designed to tackle this question. As mentioned in section 3.2.4.5., cells were

pre-incubated with different oligos for a period of time and then they were extensively washed with fresh warm media and further incubated in culture (no additional ODN is present in this media) for additional periods. Culture supernatants were collected after 6, 12, 24, and 48h of post-incubation and cytokine ELISA was studied. It is important to note that the major goal was to allow ODNs to interact with the cells for a limited time, and then wash away the unbound free ODN from the culture medium. Previously, it has been demonstrated that up to 8 hours of pre-mixing of cells with ODN lead to comparable cytokine secretion, similar to the unwashed counterpart (Yamada H, 2002). In the present situation the postulation was that depending on the type of ODN, different internalization (binding plus uptake) efficiencies will lead to varying cytokine induction levels. The extent of stimulation would be monitored by collecting supernatants at different incubation times.

Several ODNs including dendrimeric ones (either at equal amount or at equimolar concentrations) were mixed with murine splenocytes for varying durations (0, 1h, 2h, 4h, and 8h). Following extensive wash, the cells were suspended in fresh media and further cultured for different incubation times (cells were left for further 6, 12, 24, and 48h in culture) to monitor the level, as well as the extent of cytokine production at specific intervals in response to the internalized ODN during mixing steps. Supernatants from each treatments were stored for cytokine detection by ELISA.

Table 4.1 summarizes the time course cytokine secretion for each CpG and control ODNs following pre-mixing with spleen cells at indicated times. While there is no detectable IL-6 from the culture fluid after 1h mixing, detectable levels of IL-6 was obtained from the culture supernatants after mixing for 2 h and onwards. ODN420 secreted the highest IL-6 level at all pre-incubation times (4-5 fold more compared to single stranded linear ODN1466 or 1555). While ODN1466 or 1555 cytokine level in culture following 12 hours post-culturing subsided and dropped to a minimum detectable levels, IL-6 induction progressively increased by the cells that was pre-mixed with ODN420. This finding suggested that, internalized ODN420 amount is sufficient to keep extended and continuous triggering of the cells and induce substantially higher levels of IL-6 secretion. The level of internalized linear ODNs probably does not reach this critical concentration inside the immune cells thus IL-6 induction remained significantly low throughout post-incubation period.

Next, an experiment was designed to confirm that short mixing time (2h mixing) does not initiate a non-CpG effect, here control dendrimeric ODNs were also included in the assay. Different from the data in Table 4.1, here equal amount of ODNs were used during mixing.

Please note that, the limited exposure time allowed to cells during ODN mixing is one indirect way of simulating binding and uptake ability of different ODNs. Following washing and post-incubation in culture to detect cytokine secretion by these pre-treated cells is a relevant assessment to understand the degree of response raised against intracellular ODN stimulation. Here, the expectation was that, the more ODN internalized by a cell, the more pronounced cytokine secretion by that cell would be achieved. Figure 4.6A, summarizes this outcome. As seen in Figure 4.6A, time-course cytokine production of spleen cells over the course of 48h post incubation was monitored. Similar to the observations mentioned above (in Table 4.1) only ODN420 consistently induced significantly higher levels of IL-6, and this effect is entirely CpG motif dependent, (the low level inductive effects of control dendrimers, ODN421, and ODN423 were undetected by 24h of post- incubation following washing)

Table 4.1. Mixing experiment: Murine splenocytes were pre-incubated with indicated ODNs for 2, 4 and 8 hours (indicated as 2h, 4h and 8h in the table). After washing, cells were further incubated with fresh media (without ODN) for 6, 12, 24, 48 hours (6, 12, 24 and 48h in the table) and supernatants were collected to perform IL-6 ELISA.

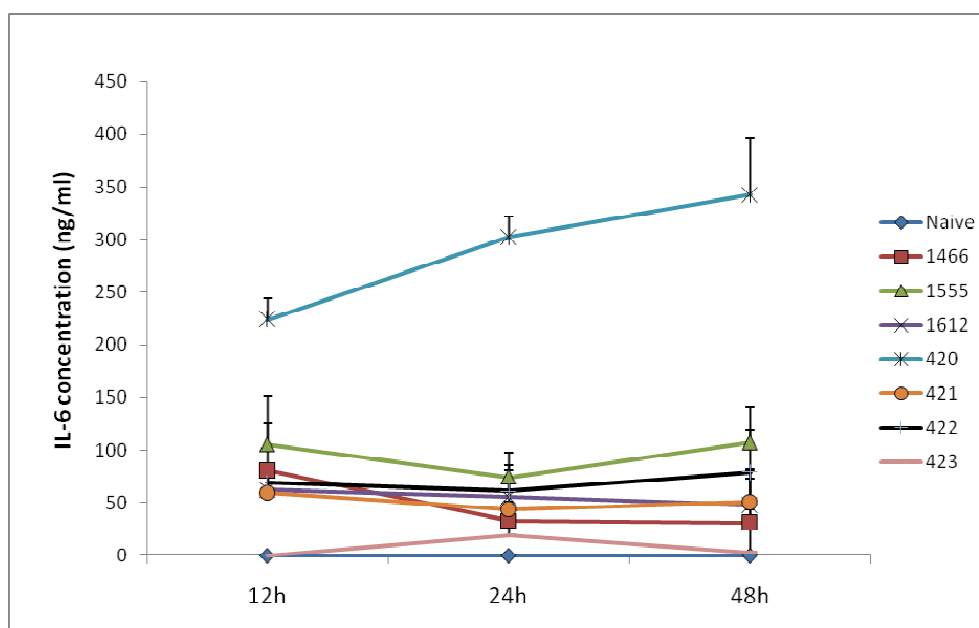
ODN-Cell mixing time	2h			
Post-incubation time	6h	12h	24h*	48h*
Naive	N.D.	N.D.	N.D.	N.D.
ODN1466	7,6 ± 5,4	69,1 ± 14,9	N.D.	19,5 ± 13,2
ODN1555	17,4 ± 5,2	6,9 ± 13,3	13,2 ± 9,7	20,4 ± 14,8
ODN420	30,6 ± 7,0	38,3 ± 16,2	74,8 ± 6,7	101,8 ± 6,1
ODN422	14,1 ± 4,1	6,5 ± 9,2	7,3 ± 9,7	14,4 ± 5,3

ODN-Cell mixing time	4h			
Post-incubation time	6h	12h	24h*	48h*
Naive	N.D.	N.D.	N.D.	N.D.
ODN1466	14,1 ± 27,4	N.D.	25,3 ± 11,1	25,8 ± 18,7
ODN1555	28,8 ± 24,7	8,9 ± 9,1	38,2 ± 10,6	34,3 ± 14,9
ODN420	7,4 ± 20,8	39,4 ± 17,5	143,8 ± 9,5	119 ± 24,4
ODN422	8,7 ± 13,5	11,8 ± 10,5	60,7 ± 9,8	29,5 ± 9,0

ODN-Cell mixing time	8h			
Post-incubation time	6h	12h	24h*	48h*
Naive	N.D.	N.D.	N.D.	N.D.
ODN1466	22,7 ± 19,9	44,1 ± 13,8	51,4 ± 3,4	10,4 ± 19,2
ODN1555	29,8 ± 24,6	33,6 ± 14,2	41,8 ± 12,8	N.D.
ODN420	47,2 ± 18,5	49,1 ± 21,5	141,8 ± 3,5	169,9 ± 19,3
ODN422	28,1 ± 21,8	84 ± 12,8	69,6 ± 3,4	50,2 ± 31,8

* p < 0,010 between 420 and 1555 treated groups. N.D.: Not detected

A)



B)

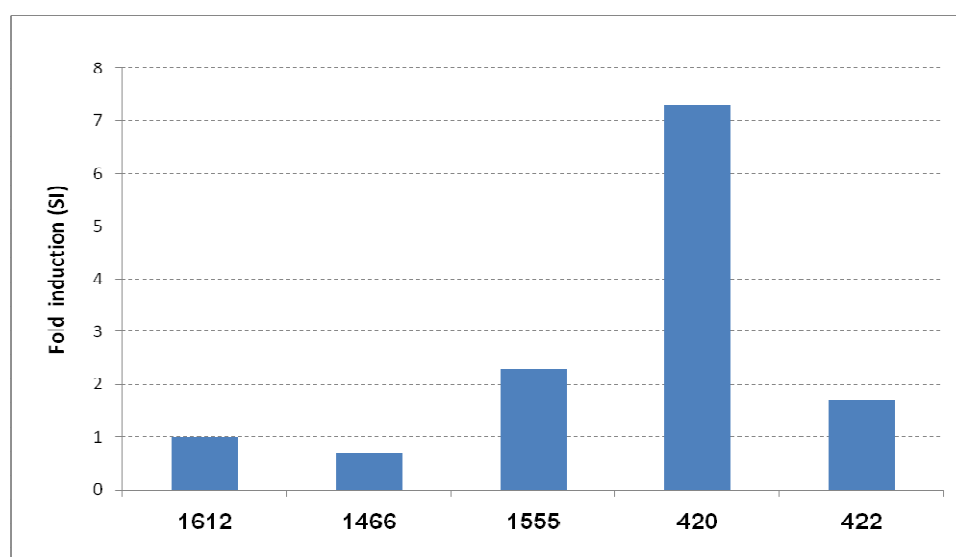
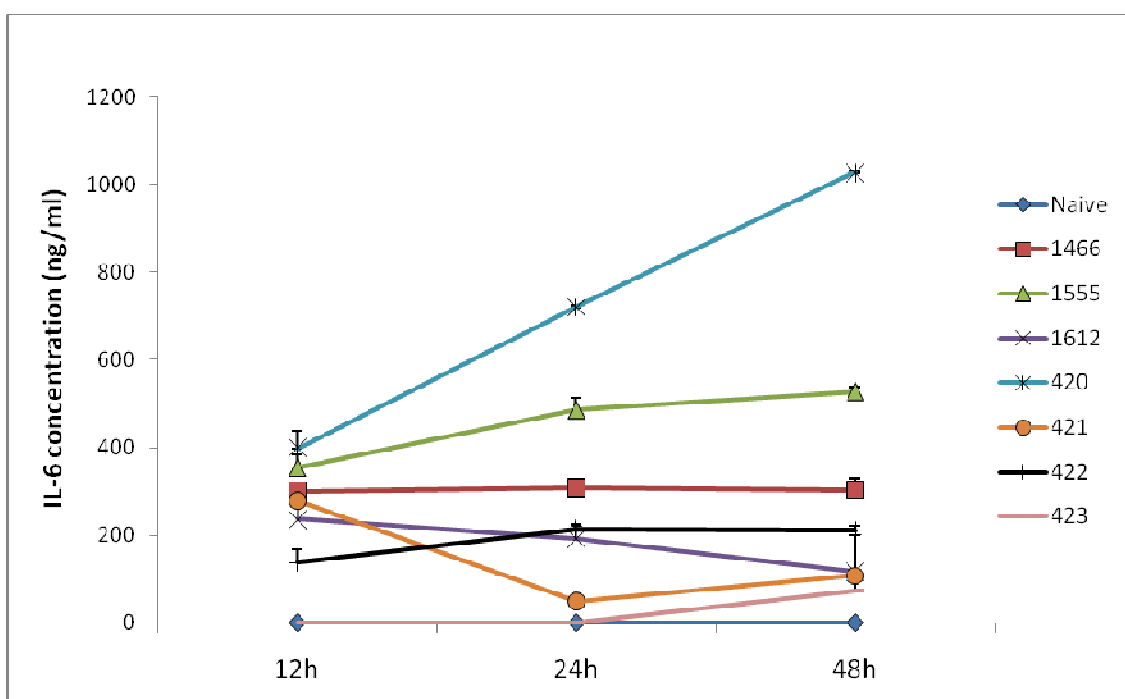


Figure 4.6. Mixing experiment. ODNs and spleen cells were mixed for 2h and washed and further incubated in fresh warm media upto 48h in culture. (A) Time-dependent IL-6 secretion profiles of ODNs from culture supernatants by ELISA. (B) IL-6 fold induction profiles of indicated ODNs. $p < 0,004$ (48h 420- and 1555-treated samples were compared)

To evaluate the maximum inductive capacity, a set of experiment was designed to monitor IL-6 production from cell supernatants. In this set-up there is no ODN mixing and washing steps, instead ODNs were left in culture as before (as in Section 4.4.2.) throughout the stimulation period. Consistent with previous findings, among the tested CpG ODNs, dendrimeric ODN420 gave the highest IL-6 production (Figure 4.7A). Not only it induced detectable IL-6 level as early as 12h post-treatment, it continued to trigger progressive cell activation and cytokine production steadily increased in culture over 48h period (please compare 12h induction level to that of 48h inductions for all ODNs in Fig 4.7 A). Figure 4.7 B presents fold induction profiles of different CpG ODNs based on control ODN IL-6 levels.

A)



B)

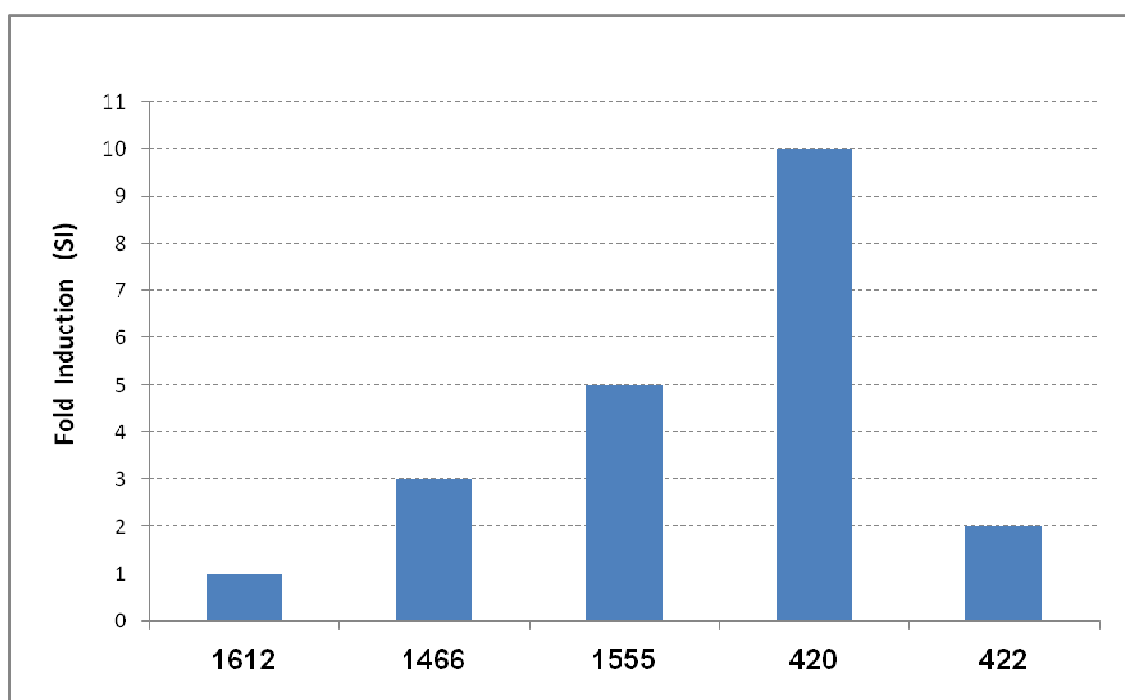


Figure 4.7. Maximal ODN activity assay (no mixing and washing is done) using 8 μ g/ml CpG ODNs and control ODNs. (A) Time-dependent IL-6 secretion from collected supernatants by ELISA. (B) Fold induction in IL-6 secretion after 48h taking control ODN induction as the base for comparison. $p < 0,001$ (only the samples at 48h, for a, and b is calculated for 420 and 1555 treated samples)

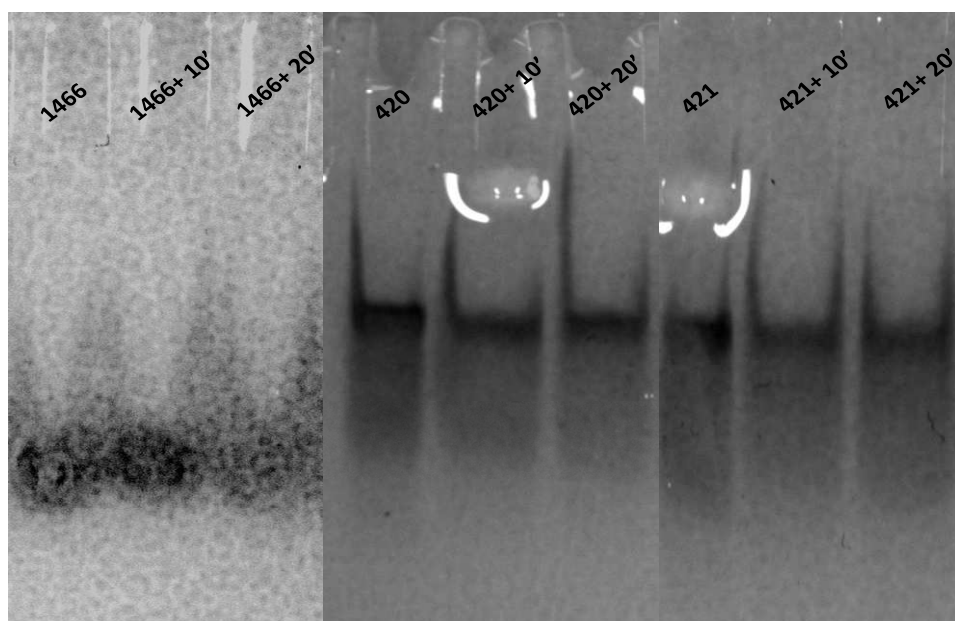
4.6. Stability Assays of the Dendrimeric ODNs: Effect of DNase Pre-treatment on CpG Mediated Immune Activation

Previous section established that enhanced activation of dendrimeric ODNs in culture could be due to efficient internalization compared to single stranded CpG ODN counterparts. The other plausible explanation for the enhanced activity of the nanoparticle forming dendrimeric ODNs independent of efficient internalization could be due to the improved stability (i.e. better DNase resistance). This option also deserves better understanding since the dendrimeric ODNs are in complex forms, it is possible that even if they are not internalized better, when

taken up by spleen cells, they are more resistant to DNA endo or exonucleases and this may lead to enhanced activity.

To understand if stability is contributing to the observed activity, series of experiments were designed. Before putting the CpG ODNs into stimulation assays, each DNA sample was subjected to DNase treatment for a specific period. ODNs were incubated for 10 or 20 minutes with sufficient amounts of DNase I (i.e. desired unit of enzyme needed to degrade specific μg of ODNs in 10 mins). As explained in the Section 3.2.7, DNase treated ODNs were subjected to PAGE analysis to visualize the level of degradation. Although a slight reduction in the band intensities was observed for nanoparticle forming dendrimeric ODN (ODN420), gel pictures revealed that monomeric ODN (1466) is nearly completely digested at the end of 20 mins of enzymatic attack (Fig. 4.8A). Moreover, when murine splenocytes were stimulated with DNase I-treated or non-treated ODNs (as a control group), DNase I-treated monomeric CpG ODN had lost its immunostimulatory capacity nearly totally (even at the end of 10 min), whereas dendrimeric ODN420 retained as much as 43 % of its original activity even after 20 mins of DNase treatment (Fig. 4.8 B and Table 4.2). Surprisingly, ODN422 was very susceptible to DNase I treatment, as evidenced by loss of gel band and cytokine induction potential.(see appendix and data not shown).

A)



B)

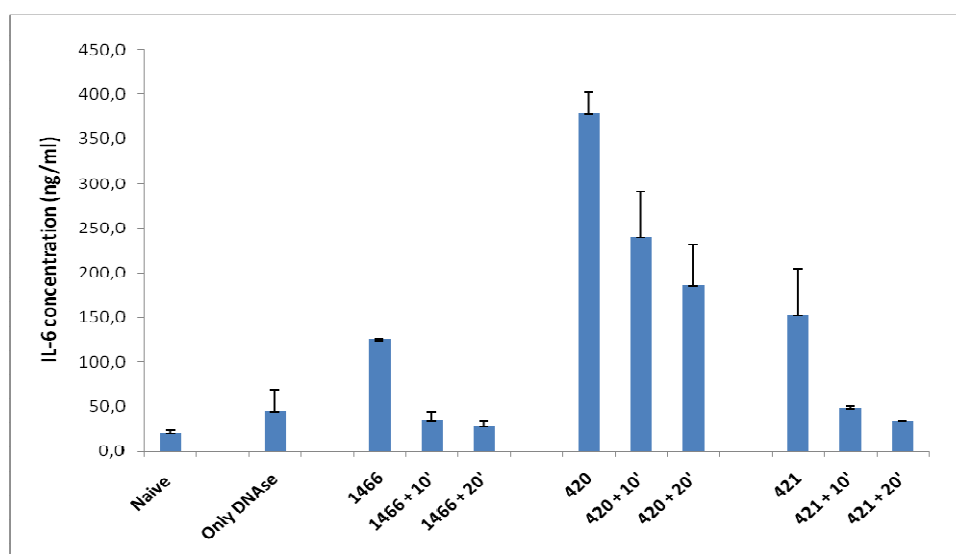


Figure 4.8. Dendrimeric nanoparticle forming ODNs (420,421) were resistant to DNase I treatment. ODN420, 421 & 1466 were treated with DNase I for 10min and 20min. Then ODN samples were analyzed by PAGE (4 μ g of each ODN/lane) (A) and furthermore used to stimulate splenocytes (8 μ g/ml) for 48h. Culture supernatants were collected and IL-6 levels were checked with ELISA (B). 10' and 20' indicates time of 'DNase treatment' for each ODN.

Table 4.2. Retained immunostimulatory potential of ODNs, in terms of IL-6 induction, after various treatment times with DNaseI.

	10 min *	20 min**
ODN 1466	7,8 $\pm$ 0,4 %	2,6 $\pm$ 0,4 %
ODN 420	56,8 $\pm$ 15,2 %	42,6 $\pm$ 13,7 %

* $p < 0,006$ ** $p < 0,04$

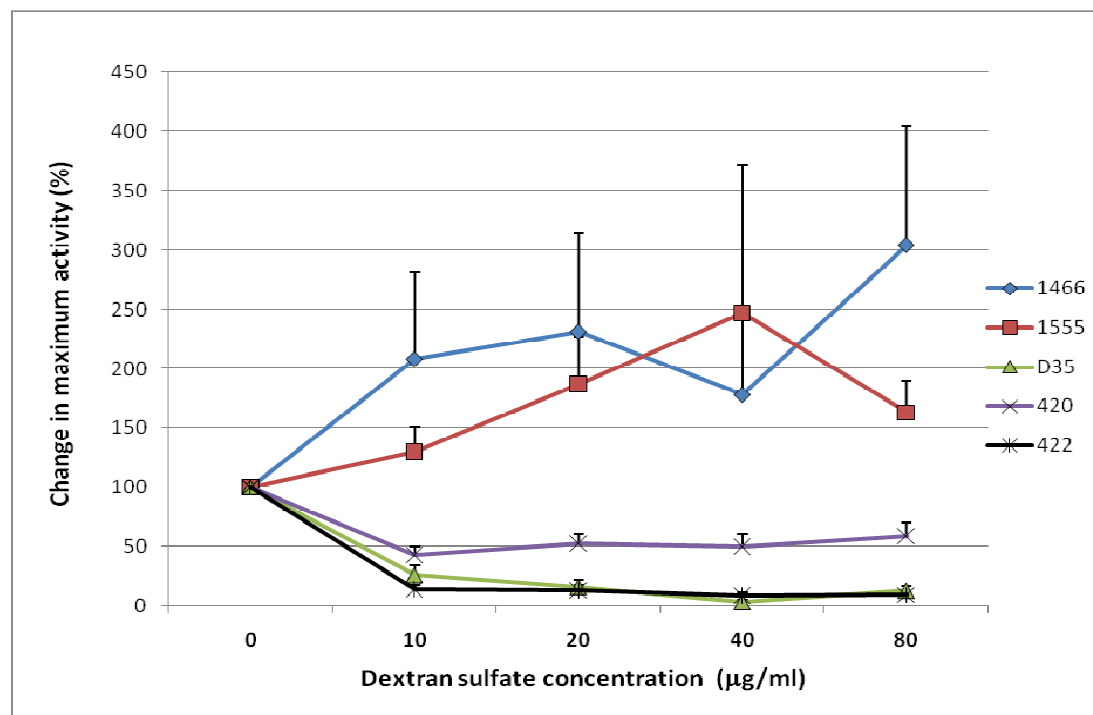
4.7. Blocking of Scavenger Receptor Interferes with Dendrimeric CpG ODN Mediated Immune Activation

The mechanism of DNA or ODN binding and uptake is not well understood. Several single and double stranded ODN binding proteins as well as receptors responsible from recognizing charged molecules may play a critical role in this complex event. However, our novel design, nanoparticle forming dendrimeric ODNs are not just oligonucleotides but also act as particles. The most well known class of extracellular surface receptors known to interact with amphiphilic particulate entities on the cell surface, is known as the “Scavenger Receptors”. This class is diverse, and most innate immune cells are known to express them on their surface.

It has been reported that specific scavenger receptors are required for the binding/uptake and recruitment to TLR9 positive endosomes when complex forming D-ODN class is interacted on the cell surface (Gursel M, 2006). To understand that if similar phenomenon is also required for the internalization, and thus, activity of nanoparticle forming ODNs we have embarked on a series of experiments. Several ligands are known to block scavenger receptors irrespective of their receptor types. Here, we have used several candidates to check whether internalization of specific ODN type are influenced by these ligands. Before the stimulations, ligands such as fucoidan and dextran sulfate were used to block cell surface scavenger receptors as it is mentioned in section 3.2.4.4. As expected in some cases fucoidan was reported to be stimulatory (Kim MH, 2008), so its blocking effect was not observed (data not shown). However, dextran sulfate and control polysaccharide – chondroitin sulfate, which is not a scavenger receptor ligand, did not show any immunogenic activity when given alone on to splenocytes. As it is shown in Figure 4.9, pre mixing cells with dextran sulfate significantly blocked immunostimulatory potential of both dendrimeric CpG ODNs (ODN420 and ODN422, 50% to 90% respectively). This inhibition is specific to avoiding ODN/Scavenger receptor interaction as there is no suppression of ODN420, or ODN422 stimulation following chondroitin sulphate (another polysaccharide that shows non-scavenger receptor specificity) pre-incubation. Even at low dextran sulphate concentration this blocking was effective. The other striking observation is that linear CpG ODN sequences, as expected were not affected from dextran sulfate pre-treatment. Unexpectedly, very high doses of the blocker slightly increased the immune response mediated by these ODNs (Figure 4.9A). As expected pre-

treatment with control polysaccharide neither as such itself, nor over oligo sequences had any (inhibitory or positive) impact on spleen cell cytokine production profile (Figure 4.9B).

A)



B)

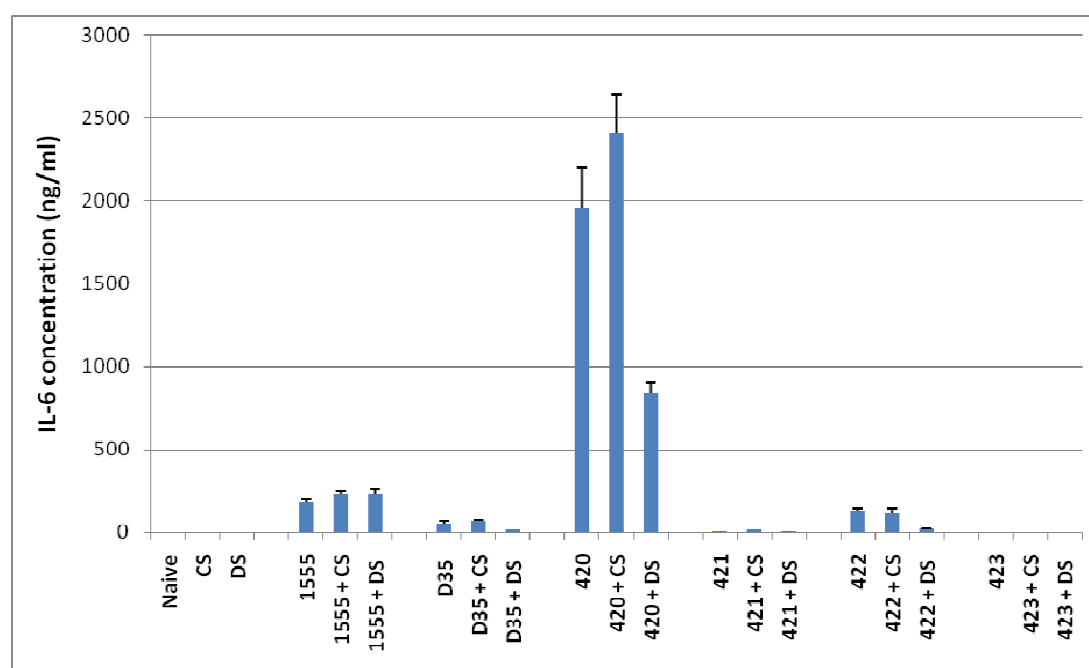


Figure 4.9. Effects of scavenger receptor ligand on CpG mediated immune activation. Murine splenocytes were pre-treated (30 minutes, at 37°C) with different doses (10, 20, 40, 80 µg/ml) of dextran sulfate (DS) and with control polysaccharide chondroitin sulfate (CS) (80 µg/ml) prior to stimulation with CpG ODNs. Culture supernatants following 48 h of incubation were analyzed for IL-6 cytokine by ELISA. Dose dependent effect of DS on maximal activity of each CpG ODN type (A). The effect of 10 µg/ml DS and 80 µg/ml CS after 30 min pre-treatment on CpG mediated IL-6 release is plotted in (B). $p < 0,006$ for 420 vs 420+DS treatments and $p = \text{NS}$ for 1555 vs 1555+DS.

4.8. Gene Expression Profile of Murine Splenocytes Stimulated with Dendrimeric CpG ODNs

In order to have more global understanding of the breadth of dendrimeric CpG ODNs` activity on the immune system, we can not use sandwich ELISA approach. As there are not available reagents for every cytokine/chemokine one wishes to study, we have decided to investigate the mRNA level of several other relevant genes including Toll-like receptor family

genes, as well as different proinflammatory, T_H1, or T_H2, and regulatory cytokine genes in addition to several proinflammatory and inflammatory chemokine transcripts from total RNA of spleen cells by PCR analyses. To differentiate between early activating and late activating genes, mouse splenocytes were stimulated for 1 or 6 hours.

4.8.1. Effects of Dendrimeric CpG ODNs on Different Toll Like Receptor Gene Expression Levels

The spleen cells were incubated for 1 or 6 h with indicated ODN, and cells were harvested and cDNA was prepared from the total RNA. In the presence of each tlr gene primer, PCR was run to evaluate the differential expression of the specific genes following ODN treatment. As seen in Fig 4.11 gel picture, dendrimeric CpG ODN420 did not induce significant TLR gene expression by 6h. But the monomeric CpG ODNs significantly upregulated expression of several TLR family genes after 6h. Similar to single stranded linear ODNs, surprisingly, ODN422 showed significant inductive effect compared to naive or its non-CpG ODN423 counterpart by 6 hours (compare band intensities for *tlr 3*, *4* and *7 genes*, Figure 4.11).

The important observation worth mentioning in this section is the unexpected feature of bifunctional ODNs. The band intensities by 6 h (including house keeping gene, β -Actin) were significantly reduced (Fig 4.11 compare Naive vs 420 and 421 intensities for β -actin gene). We have invested substantial amount of effort to understand this unexpected observation. It seems that due to strong binding of dendrimeric ODN with the cell surface, we are co-precipitating the oligo together with the total RNA during RNA purification by TRI method (see Section 3.2.6.1.), and this contaminating dendrimeric ODN fraction is masking the gene amplification during PCR protocol (see appendix IV for details). Digestion plus extensive cell washing of the cells substantially reduced contaminating dendrimeric DNA and thus improved the relevant band intensities.

But, nevertheless both ODN420 and 421 irrespective of CpG motifs, significantly reduced the expression level of the TLR family genes at all incubation times tested (1 and 6 h). (see Appendix II Relative intensity plots for individual genes)

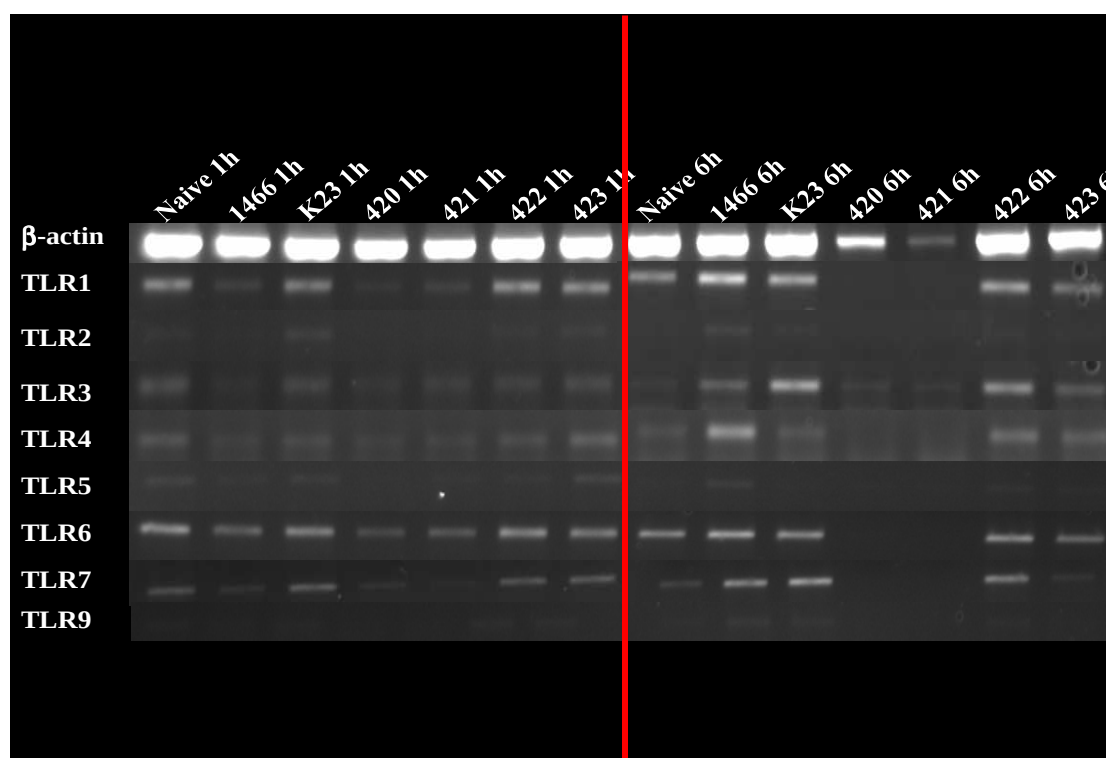


Figure 4.10. Agarose gel image of PCR products of TLRs. PCR performed with TLR family primers and products were run on 1.5 agarose gel. The image was visualized under transilluminator and captured after 0.6 second exposure with UV.

4.8.2 Effects of Dendrimeric CpG ODNs on Cytokine & Chemokine mRNA Expression Profile

In addition to different TLR family genes, a panel of cytokines and chemokines of proinflammatory, inflammatory in nature was assessed by PCR. As done in previous section different CpG DNA species were incubated with the spleen cells, and the PCR was studied from the cDNA generated from the isolated total RNA of the cells. The dendrimeric CpG ODNs interestingly, behaved differentilly to different cytokine and chemokine gene epxression when 6h stimulation was analyzed. The ODN420 gave the highest upregulation for IL-6 gene (compared to K23 and ODN422), while TNFa and IL1b levels goes to baseline ODN422, and to an extent K23 highly upregulated these genes by 6h (Figure 4.12.). This piece of evidence strongly implicate that, ODN420 and ODN422 act differentially on the immune cells.

Table 4.3. CpG ODN-mediated expression levels of TLR genes normalized with housekeeping gene (β -actin).

Gene/ β -actin	TLR1		TLR2		TLR3		TLR4	
Stimulation time	1h	6h	1h	6h	1h	6h	1h	6h
Naive	0,075	0,082	0,008	0	0,032	0,006	0,027	0,015
1466	0,021	0,163	0,001	0,013	0,005	0,05	0,01	0,101
420	0,009	0,005	0	0	0,007	0,018	0,009	0
422	0,091	0,103	0,004	0,003	0,014	0,098	0,016	0,049
Gene/ β -actin	TLR5		TLR6		TLR7		TLR9	
Stimulation time	1h	6h	1h	6h	1h	6h	1h	6h
Naive	0,012	0,002	0,091	0,083	0,032	0,027	0,005	0,002
1466	0,006	0,012	0,059	0,119	0,019	0,076	0,004	0,009
420	0,003	0,004	0,037	0,014	0,018	0,021	0,002	0,003
422	0,006	0,005	0,092	0,093	0,039	0,069	0,007	0,005

For every treatment, relative band intensities of each TLR gene were divided by the same of β -actin gene. Green boxes represent upregulation, while red boxes represent downregulation with respect to 'Naive' samples upon CpG ODN treatment.

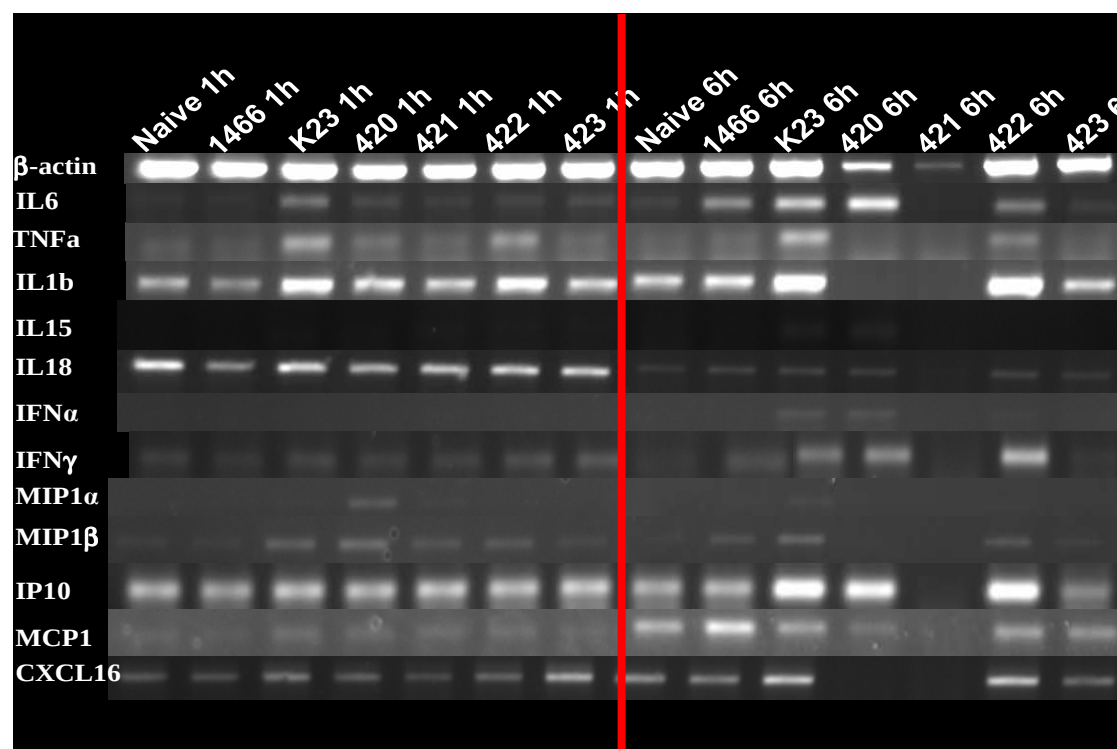


Figure 4.11. Agarose gel image of PCR products for cytokines/chemokines. PCR performed with cytokine/chemokine primers and products were run on agarose gel (for details please see Figure 4.11 legends).

When these gene activations were closely assessed by increasing incubation time, bifunctional dendrimer (ODN420) seemed to be more potent inducer of many arms of innate immune system since, expression of IL-6, IL-15, IFN α , IFN γ , and IP-10 significantly upregulated after 6 hours of stimulation. Some genes such as MIP α , MIP1 β , IL-1 β , TNF α were ‘early activating genes’ – upregulated at 1 hours (1h), but downregulated at 6h (Table 4.3.). ODN422 however, behaved slightly different as it triggered separate genes such as IL-1 β , CXCL16, IL-6, IFN α , IFN γ , IP10 after 6h. As expected, although ODN1466 were active it was significantly weaker stimulant than dendrimeric counterparts, with one exception, as it induced MCP-1 significantly higher than dendrimeric ODNs at the end of 6h (see Appendix III Relative intensity plots for individual genes).

Table 4.4. CpG ODN-mediated expression levels of cytokine/chemokine genes normalized with housekeeping gene (β -actin).

Gene/ β -actin	IL-1 β		TNF α		IL-6		IL-15	
Stimulation time	1h	6h	1h	6h	1h	6h	1h	6h
Naive	0,367	0,469	0,096	0,081	0,005	0,016	0,000	0,003
1466	0,275	0,569	0,078	0,113	0,007	0,098	0,000	0,000
420	0,608	0,042	0,169	0,113	0,026	0,367	0,000	0,047
422	0,679	0,946	0,266	0,210	0,018	0,075	0,001	0,000
Gene/ β -actin	IL-18		IFN α		IFN γ		IP-10	
Stimulation time	1h	6h	1h	6h	1h	6h	1h	6h
Naive	0,206	0,021	0,003	0,007	0,027	0,012	0,500	0,470
1466	0,107	0,033	0,006	0,006	0,020	0,036	0,471	0,483
420	0,151	0,046	0,006	0,099	0,023	0,179	0,599	1,482
422	0,183	0,030	0,006	0,018	0,044	0,163	0,509	0,862
Gene/ β -actin	MIP-1 α		MIP-1 β		MCP-1		CXCL16	
Stimulation time	1h	6h	1h	6h	1h	6h	1h	6h
Naive	0,000	0,000	0,006	0,001	0,040	0,262	0,040	0,092
1466	0,000	0,000	0,004	0,018	0,007	0,479	0,040	0,079
420	0,026	0,000	0,054	0,001	0,071	0,167	0,048	0,003
422	0,000	0,001	0,024	0,023	0,055	0,254	0,050	0,142

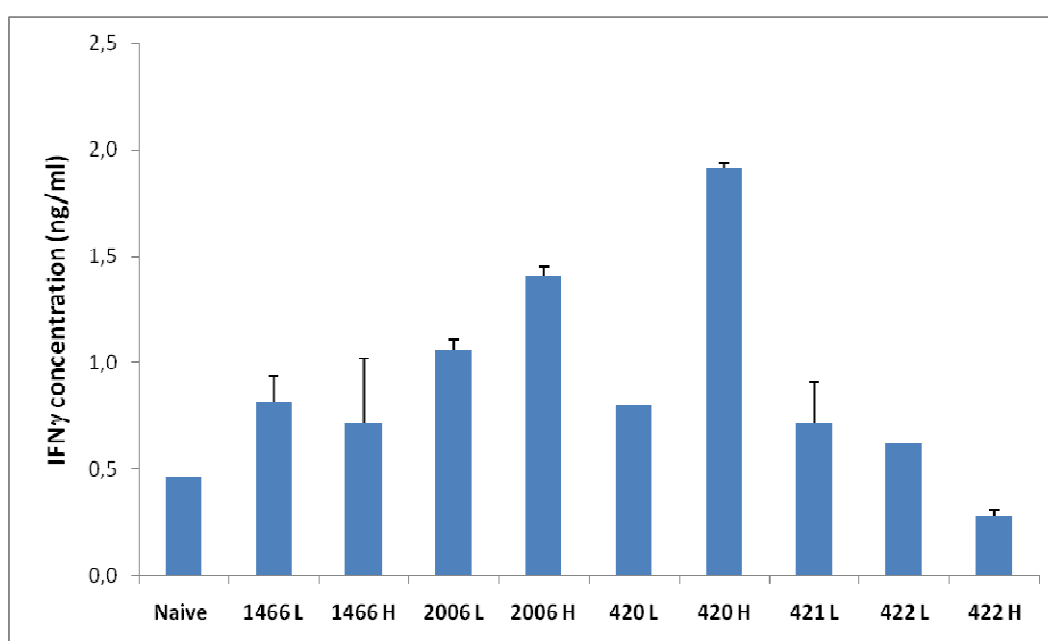
Green boxes represent upregulation, while red boxes represent downregulation with respect to ‘Naive’ samples and in some cases ‘1h’ of same gene upon CpG ODN treatment. For every treatment, relative band intensities of each cytokine/chemokine gene were divided by the same of β -actin gene.

4.9. *In vivo* Stimulatory Potential of Dendrimeric Nanoparticle Forming ODNs

So far the data indicated that dendrimeric novel ODN sequences perform much better than single stranded linear counterparts, furthermore the spectrum of activation is much broader than the conventional sequences. It is of great importance to demonstrate that this enhanced activity attained to dendrimeric nanoparticle-forming ODNs translate itself when they are injected into Balb/c mice.

To test *in vivo* efficacy of dendrimeric CpG ODNs, they were injected intraperitoneally to mice along with other conventional ODNs. Four hours after *in vivo* IP injection, cells from different sites were recovered and incubated in culture plates for further 12 to 48 h depending on the assay. Cytokine secretion profiles were analyzed from supernatants recovered either from peritoneal exudate cells (PECs) or splenocytes at indicated incubation periods (please see Figures 4.13 and 4.14). Among CpG ODNs injected to animals, ODN420 was the most active IFN γ inducer. Nearly 2,5 fold higher levels than its monomeric ODN1466 and 35% higher than ODN2006 cytokine secreted by PECs (Figure 4.13A). Similarly, IL-12 induction in PECs were the highest for ODN420. A 6-fold more cytokine production was detected compared to ODN2006. Monomeric ODN1466 induced undetectable levels of IL-12 when given *in vivo*. (Figure 4.13B).

A)



B)

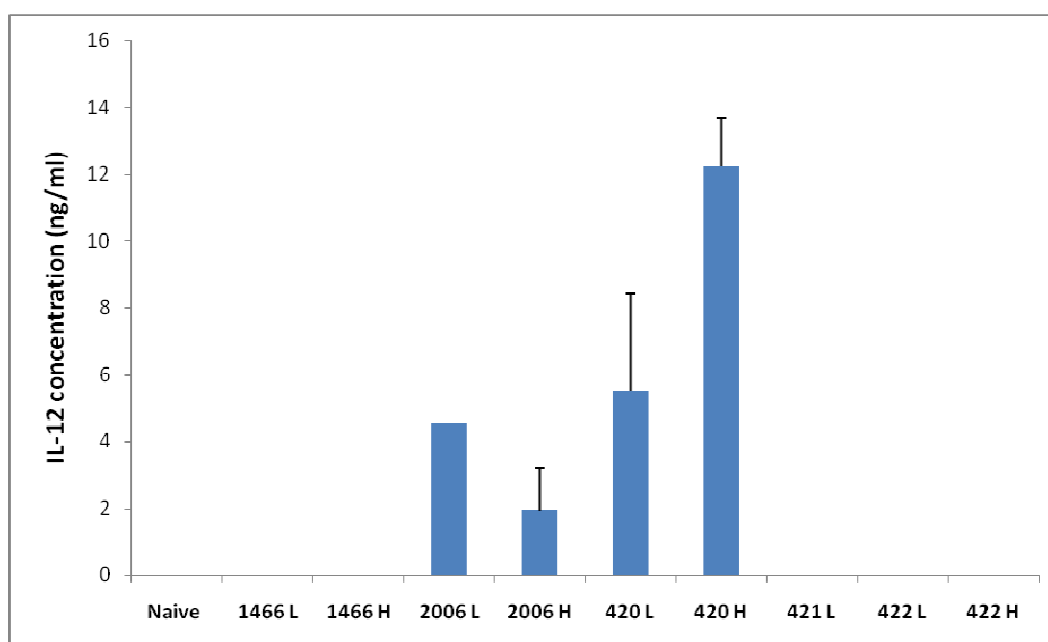


Figure 4.12. Ex-vivo cytokine secretion from murine PECs. Murine PECs were collected after 4h from high dose (H) – 50 µg and low dose (L) - 10 µg ODN injected mice. PECs were cultured ex-vivo and IFN-γ (A) and IL-12 (B) concentration were checked after 36h incubation at culture. $p = 0,004$ for IFNγ and $p = 0,021$ for IL-12 (between 420 H and 2006 H groups).

Splenocytes from ODN420 injected animal yielded the highest cytokine production, supporting the observation from PEC cells. In terms of IFNγ secretion from spleen cells over time, ODN420 stimulated nearly 2 fold more than ODN1466. 35 % difference between ODN420 and ODN2006 is not statistically significant. Quite shockingly, ODN422 was found to be less active when given *in vivo*. While it induced 3 fold more IFNγ than ODN420 at *in vitro* conditions, ODN420 could not reproduced this activity on both PEC and spleen cells after *in vivo* injection (please compare Figures 4.14 and 4.5A).

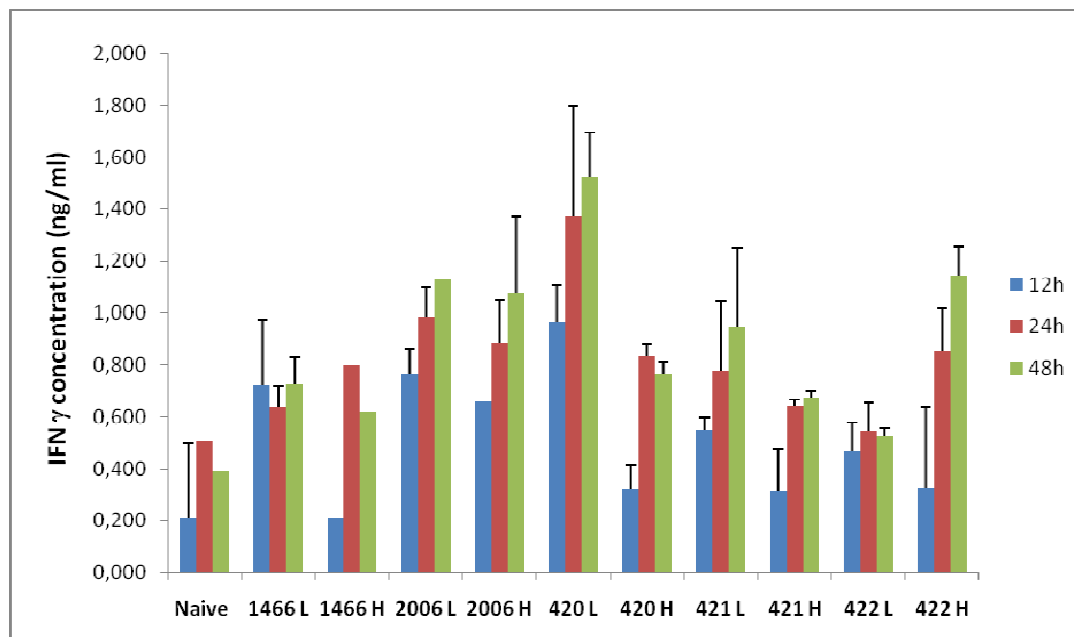


Figure 4.13. Ex-vivo cytokine secretion from murine splenocytes. Murine splenocytes were removed after 4h from high dose (H) – 50 µg and low dose (L) - 10 µg ODN injected mice. Splenocytes were cultured ex-vivo and IFN-γ concentration were checked at 12h, 24h and 48h. $p < 0,03$ (between 48h 420L and 1466L).

5. DISCUSSION

The discovery of CpG motifs and its ability to stimulate mammalian immune system sparked the oligodeoxynucleotide based immunotherapy. Since the appearance of the seminal paper in Nature journal in mid ninety several CpG based pharmaceutical companies were founded and they initiated several clinical trials utilizing synthetic CpG motif expressing DNA sequences aiming to treat diseases ranging from cancer to infectious diseases. CpG ODNs have been shown to mimic bacterial DNA and trigger innate immune cells that polarize immune milieu towards T_H1 . Establishing a T_H1 type of immunity activates cytotoxic T cells and NK cells which kill infected host cells. This type of immune response has been shown to be effective against viruses, intracellular bacteria such as *M. Tuberculosis*, allergic reactions and cancer. With the description of the intracellular receptor recognizing CpG motifs, five years after the identification of the motif (Hemmi, et.al, 2000), TLR9 based immunotherapy gained a momentum and at present there are over 40 different clinical trial ranging from phase I to III are ongoing worldwide.

In addition to the presence of CpG motifs on a strand i) backbone chemistry, (i.e. PS vs PO) ii) flanking sequences (PuPuCGPyPy or PuPyCGPuPy) iii) presence of runs of Gs at the terminal ends or iv) structural properties, of ODN sequence (i.e. presence of complementary sequences, or number of CpG motifs), and finally v) physical structure of the ODN (linear strands vs multimeric, or aggregated strands) have critical role in shaping the resultant immune response. For example, self-nanoparticle forming D-type CpG ODNs induce immune system differentially than linear K-type ODNs (main difference is type I IFN induction by D-ODNs) which is due to longer retention of formers in early endosomes of target cells. Same phenomenon was observed for linear ODNs complexed with nanoparticulate carriers such as DOTAP liposomes or polymyxin (Haas T, 2008). Moreover, nanoparticulate ODNs are recognized by scavenger receptors such as CXCL16, this confers an advantage over linear ODNs in terms of effective internalization. Another problem for linear ODN sequences seen when given *in vivo* is that the conventional CpG ODNs upon injection binds to serum proteins and degraded by nucleases before they reach to the site of target cells, and even before interacting with TLR9 in endosomes majority of the administered ODN is eliminated from circulation. Another problem seen during *in vivo* applications is the effective amount reaching to immune cells. Due to premature serum protein binding and nuclease digestion very little

amount is internalized by the immune cells leading to a limited stimulation level, that harness the immunotherapeutic potential. Previously, an alternative method was developed to improve the *in vivo* activity of these CpG ODNs. Loading them on to nanoparticulate phospholipid carriers. By this approach it was shown that liposomal ODNs stimulate immune response at much lower doses and are less susceptible to DNase attack. This make them potential candidates for improved *in vivo* applications (Gursel I, 2001). D-type ODN falls into spontaneously nanoparticulate forming structures. However, large scale synthesis of D-ODNs is not so easy because it shows batch to batch variation due to polyG runs at the ends of the ODN (Wang D, 2008). So, designing self nanoparticle forming ODNs without polyG runs which shows similar or better immune profile than D-ODN or, designing a novel ODN structure mimicking all known CpG-ODN classes but yet surpassing their *in vivo* performance is an important task.

In this study, we designed 2 different types of dendrimeric ODNs aiming to obtain self-nanoparticle forming structures, which will have higher immunostimulatory potential than linear CpG ODN counterparts. Analysis of these ODNs with Atomic Force Microscopy, which allows to investigate size and shape of molecules at nanometer resolution revealed that homogeneous DNA nanoparticles can be formed with this technology. Topography studies consistently displayed that ODN420 (using a bifunctional linker) dendrimer forms very homogenous structures reaching to a monodispersed size distribution around 35-40 nm in diameter. The ODN422 that was generated by using a trifunctional symmetric linker showed structures with heterogeneous shapes and varying sizes (20 to 55 nm). Phase contrast images also supported our topographical observations (please see Fig 4.1 and 4.2 for more details). Changes in the phase of cantilever oscillation can be attributed to any material present on the surface and does not implicate that these belong to ODN structures. However, when phase and topography images are compared, the phase shifts and topographical changes perfectly overlapped, these findings indicate that the observed nanoparticle structures are not caused by any contaminant or the roughness of the glass surface but merely belongs to the dendrimeric self nanoparticle forming ODNs deposited on the surface.

The AFM micrographs although they provided encouraging findings, could only implicate that a complexed, multimeric ODN structures are formed after using a bifunctional linker throughout ODN synthesis. Under the lights of these findings it was impossible to predict

whether these nanoparticles were folded by itself to form self complementary (one molecule) aggregates or multiple strands were intertwined over each other to form molecule aggregates with more than one ODNs . To differentiate between these possibilities we performed PAGE analysis, which helped us to separate nucleic acids accurately according to their molecular weights and/or sizes. Confirming our AFM images, PAGE gel pictures showed that ODN420 had a homogenous population of DNA structures that corresponded to 70 bp Mol.Wt. marker, exact expected weight for a single dendrimeric 420 molecule. Collectively, AFM data and PAGE gel pictures suggested that ODN420 undergoes nanoparticle formation by folding over itself, rather than undergoing multimeric aggregate formation with other ODN420 strands. Different what is observed for ODN420, the second dendrimeric structure, ODN422, not only displayed a heterogeneous appearance under AFM analyses, it gave a smear gel band after PAGE experiment, indicating that ODN422 complexation is an unstable one. The strand can interchangeably fold and unfold during complexation and the expected 90mer bp weight was not observed as a single band, but a smear was obtained in the gel pictures (please check Fig. 4.3.).

Next step was to compare the *in vitro* immunostimulatory potential of these newly designed CpG ODNs side by side with conventional mouse and/or human active ODNs. A dose-dependent stimulation assay of mouse splenocytes revealed that the dendrimeric ODNs gave the highest stimulation at the optimum dose for the conventional ODNs. Moreover, dose titration significantly reduced the potency of all of the conventional ODNs but, ODN420 was capable of stimulating the splenocytes even at doses where other ODNs had failed to induce any response. Since the Molecular Weights of dendrimeric ODNs were significantly higher than other conventional ones, conducting experiments using equimolar concentrations during dose-titration assays favors dendrimeric ODNs in order to establish the activity of different classes of ODNs, and overcome this favor, we have repeated the same experiment using equal amounts of ODNs in the stimulation assays. Here, the weight was kept same, and thus for ODN1466, (single CpG expressing linear ODN) was equal in terms of active CpG motifs compared to ODN420 but for CpG 2006, the number of CpG motifs nearly doubled the CpG motifs expressed by ODN420. Consistent what we have observed for previous assay, similar findings were obtained here. The newly designed dendrimeric ODNs gave the most potent activity on spleen cells (Please compare Figures 4.5.A and 4.5.B with Figure 4.4.). Despite

this advantage for ODN2006, ODN420 induced over two fold and eightfold more IL-6 than ODN2006 (note that this is a gold-standard CpG DNA and is a GMP lot provided by Coley Pharmaceuticals, Boston USA) and ODN1466 (monomeric type), respectively. Not only the amount of cytokines produced were higher, but the time of secretion was also extended when spleen cells were stimulated by ODN420. These results indicates that nanoparticle structures confers advantage to induce stimulation even at lower doses, which would be very important at *in vivo* applications where ODNs normally eliminated from circulation via serum protein adsorption or digestion by nucleases. IFN γ results were really surprising, ODN422 were 3-fold more active than ODN420 and 15-fold more active than ODN2006. ODN1466 didn't induce detectable level of IFN γ . ODN422 may be using additional entry mechanism to activate IFN γ secretion, which could be IFN α or IL12-dependent (please note that major IFN γ secreting cells, T cells, NK cells do not express TLR9, thus, IFN action is indirect). Aggregates with certain sizes other than ODN420 may be taken up by cells secreting either IFN α (pDC) or IL12 (macrophages) more efficiently. Collectively, these results (IL-6 and IFN γ) indicate that although CpG motif is indispensable for immune stimulation, the physical nature of the motif is critically contributing the final outcome. When stimulating cells with 7 sequences of ODN1466 expressed on the dendrimeric structure immune cells secrete robust IL-6 and IFN γ , while with same sequences present on separate strands, induce 8-fold lower IL-6 and undetectable IFN γ . These support that dendrimeric structure (possibly due its nanoparticle nature) confers significant advantage for immune stimulation.

The enhanced activity of the dendrimeric ODNs could be due to either improved internalization, or improved nuclease resistance. To differentiate between these possibilities a series of experiments were conducted.

Mixing experiments were designed to assess efficiency of binding between spleen cells and ODNs. Results of these experiments (Table 4.1, Figures 4.6 and 4.7) indicate that even short mixing time as early as 2h is enough for ODN420 to initiate long lasting stimulation and thus cytokine production (continuously increasing over 48h), while other ODNs gave immune response at basal level, which is non-detectable after 24 h post-incubation. Increasing incubation time increases response against ODNs. Immune stimulation by these dendrimers were strictly CpG motifs dependent, since GpC inverted counterparts were inactive. Mixing

experiments established that nanoparticle ODN induced far better stimulation than linear ODN even after very short term mixing of cells with the ODNs. The debate regarding the internalization vs nuclease resistance is still remained unresolved.

To test nuclease resistance of the novel dendrimeric ODNs, several ODNs were treated for 10 minutes and 20 minutes with sufficient amount of DNase I to degrade them in 10 minutes. Results showed that ODN420 is highly resistant to DNase I. While ODN1466 and ODN422 were very susceptible and digested near to completion (Figure 4.8, Table 4.2 and data not shown) ODN420 was not effected by nuclease treatment. We can deduce from these results that improved DNase resistance might be one of the mechanisms for ODN420 activation.

Mixing experiments implicated that additional receptors might play a critical role for binding and subsequent internalization of the nanoparticle forming ODN. To understand that this might be the case we blocked scavenger receptors (a plausible candidate for recognition of nanoparticulate materials) with dextran sulfate ligands. ODN422 did not seem to have advantage over ODN420 during mixing experiments, but unexpectedly, more than 90 % of its activity was lost, that also indicate a dependence on scavenger receptors for cell entry. However, about 50% of ODN420-mediated activity was blocked by scavenger receptor ligands. Although high molecular weight (m.w.) ligands block scavenger receptors more efficiently than low m.w. ligands (personal communication with Dr. Mayda Gürsel), it is encouraging to observe that even 10kDa dextran sulfate yielded over 50% ODN420 inhibition. More experiments are needed to be performed with different m.w. ligands in order to better understand ODN entry. Moreover, by using dye-tagged oligos, we could compare binding-uptake potential of novel ODNs before and after scavenger receptor blocking (Gursel M, 2006). Regarding the internalization mechanisms of ODN420 and ODN422, similar to D-type ODN, which owes its activity to CXCL16, our nanoparticle forming ODNs possibly share similar type(s) of scavenger receptors. Blocking with dextran sulfate didn't seem to affect immune-activating potential of ODN1466 and ODN1555 which are K-type, linear sequences. In fact, in some cases they even synergised with dextran sulfate to induce more pronounced cytokine secretion.

To understand the breadth of immune response as well as the impact of turning on or off other TLRs, we wanted to check expression level of TLR and several cytokine/chemokine genes at

mRNA level after stimulating splenocytes with ODNs. As expected, the nanoparticle forming ODN420 did not seem to regulate *tlr* genes, (if not suppressed), while its monomeric form strongly induced expression of nearly all TLR panel at the end of 6h. ODN422 was mediocre and induced some of the endosomal TLRs (i.e. TLR3 and 7) and TLR4. These results suggest that there might be regulatory mechanism for *tlr* genes controlled by inflammatory status of cells.

As an example to early immune related gene activation, ODN420 induced MIP1 α , MIP1 β as, IL1 β , TNF α , IL-6 levels, while , IL-15, IFN α , IFN γ , IP10 were upregulated as a late response genes. It is important to note that, MIP1 α , MIP1 β (recruiting T and NK cells) , IL-15 (activates NK cells), IFN α , (primary antiviral cytokines, activates NK cells) IFN γ , IP10 (stimulates T_H1 response) are cytokine/chemokines that are frequently upregulated during viral infection. This allow us to speculate that homogenous nanoparticle size of ODN420 may mimic DNA viruse entry and instruct immune response to get prepared for a viral infection. ODN422, however stimulated , IL1 β , TNF α , MIP1 β (early and late), IL-6, IFN α (lower amount), IFN γ , IP10, CXCL16 (late). While ODN422 didn't downregulate any of the cytokine/chemokine genes, ODN420 downregulated MIP1 α , MIP1 β , IL1 β , TNF α and CXCL16 at the end of 6h. We think these data also support our hypothesis that cells downregulate early proinflammatory cytokine/chemokine genes to block harmful inflammation after immune response reaches a peak. It is of importance to emphasize CXCL16 downregulation, which we speculate here that might be showing critical role of CXCL16 in ODN420-mediated immune activation. In the literature, there is no evidence for the CXCL16 mediated virus entry, but our hypothesis is that this receptor is act like a nanoparticle recognition receptor; upon ODN420 stimulation, cells may want to prevent more virus entry by downregulating CXCL16. This provocative idea, must be supported with further experiments.

We have promising results with regard to i) dendrimeric ODN synthesis, ii) physical characterization to establish their spontaneous nanoparticle formation, iii) the enhanced in vitro binding, uptake, nuclease resistance, that collectively translated in to very potent stimulator designed so far, also iv) the activation of multiple cytokines and chemokines at the transcript level were identified. Lastly, within the scope of this thesis, the ability of the

nanoparticle forming dendrimeric ODN when administered *in vivo* was analyzed. We compared several conventional ODNs along with our dendrimeric ones, and checked cytokine secretion (IL-12 and IFN γ) from spleens and peritoneal exudate cells (PECs) removed from ODN injected mice. Confirming our *in vitro* observations, ODN420 were significantly more potent than any other tested ODNs at inducing ex-vivo cytokine secretion. In spite of higher IFN γ secretion potential of ODN422 at *in vitro*, perhaps due to its DNase susceptibility as it was not successfully conforming nanoparticles, behaved less potent than ODN420 on both splenocyte and PEC samples (similar to the observations in mixing experiments).

To summarize, dendrimeric structure provides superior immunostimulatory properties over conventional ODNs, which might have direct relationship with spontaneous nanoparticle formation. Bifunctional dendrimers induced were potent activations both *in vitro* and *in vivo*, situations, while trifunctional ODNs seemed to be less effective (to translate their *in vitro* performance to *in vivo* settings). However, dendrimeric structure have synergistic effect with CpG motifs as CpG + dendrimer is more powerful than only CpG. ODN420 stimulates mainly anti-viral cytokines, suggesting that it might be used as antiviral agent in the future.

6. FUTURE PERSPECTIVES

The present study is the first to document that a single stranded oligodeoxynucleotides when joined together via their 5' to 3' ends using a bifunctional symmetric linker is capable of undergoing complex conformation dependent change leading to self nanoparticle forming DNA structures. These novel CpG motif expressing sequences were further characterized via biochemical and biophysical methods that they are homogeneous nanoparticles. The immunological assays further established that dendrimeric ODNs are more resistant to nuclease attack, internalize more to immune cells than conventional linear-single stranded ODNs and yield more pronounced immune activation compared to conventional ODNs as evidenced by Th1 dominant cytokines and several chemokines. This thesis work also provide evidence that nanoparticle forming CpG ODNs are entering into the immune cells via interacting with a scavenger receptor and moreover is active in vivo, inducing significantly more immune activation to that of conventional ODNs.

Our understanding of dendrimeric CpG ODN mediated immune activation must be further studied in details. First of all, it is instrumental to assess whether this approach could be applied to several other sequence motifs, and consistently improves the activation potential compared to single stranded conventional ODNs. Furthermore, the mechanism by which scavenger receptors are regulating the internalization must be studied in greater details. It would be of great interest if one could identify specific receptor(s) that are engaging with dendrimeric ODN and perhaps in a more broader sense act as a cell-surface nanoparticle DNA recognition sensor. Planned experiments that will follow this thesis are to inhibit expression of possible scavenger receptor candidates (CXCL16, SRA, SRB, CD36, MARCO and etc.) via siRNA sequences and look the effects on dendrimer-mediated immune activation. After selecting possible target receptors, to further confirm our results we will use antibody mediated blockage of scavenger receptor. Third way is to transfect stably TLR9 expressing HEK cells with plasmids i) expressing scavenger receptor genes plus ii) a luciferase reporter gene.

Moreover, we would like to evaluate via confocal microscopy and FACS analyses which type of cells in human PBMC or mouse splenocytes primarily took up the dendrimeric CpG ODN, use of labelled-ODNs is very important during such analyses.

We would like to also channel some of our efforts to study immunostimulatory potential of dendrimeric CpG ODN on human immune cells, PBMC or individual TLR9-expressing primary cells (pDCs and B-cells) and cell lines with an aim to test reproducibility our results on human immune cells. As our dendrimers are originated from optimum mouse-active sequences (PuPuCpGPyPy), we should also test dendrimers with human-active sequences (PuPyCpGPuPy).

IFN α -inducing capability of CpG ODN is very unique feature of D-Type ODNs and is a pivotal cytokine during anti-viral and anti-cancer therapy. Although we showed IFN α induction by ODN420 on mRNA-level, we should demonstrate it on protein level. Recently, it was shown that multimerized phosphodiester sequences effectively induce IFN α from human dendritic cells, while CpG motif has additive effect on induction (Haas T, 2008a, 2008b). As ODN420 folds spontaneously into multimeric structures, we plan to investigate this possibility by synthesizing phosphodiester linked ODNs along with phosphorothioate counterparts and evaluate its IFN α induction ability by mouse and human immune cells. Moreover, it is very essential to synthesize TCCA core sequence using this approach that are used to generate central palindrome of D-type ODNs (please see section 1.2.1.1.) and compare them side by side. We aim also to search exact relationship between multimerization degree and immune-activating potential. For this different dendrimers can be designed such as 3-stranded and more than 7-stranded forms. Size of individual molecule or molecular aggregate will be determined by AFM. There might be size-specific receptors on immune cells which will be checked for its role in recognition of nanoparticles with varying-size.

Consequently, *in vivo* performance of these ODNs must be performed. Mice will be pretreated with DNA nanoparticles and challenged against pathogens to establish enhanced immunoprotective potential. The present data, strongly suggest that dendrimeric CpG ODNs could be utilized in immunotherapy against infectious diseases, in anti-allergic and anti-cancer therapy, as well as a vaccine adjuvant improving the performance of already existing or novel vaccines.

7. REFERENCES

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8. APPENDICES

8.1. Appendix I

Buffers, Solutions, Culture media

Blocking Buffer (ELISA)

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

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

Loading Dye (Agarose gel)

- 0,009 grams Bromofenol blue
- 0,009 grams Xylen cyanol
- 2,8 ml ddH₂O
- 1,2 ml 0,5M EDTA
- 11 ml glycerol

After preparing, just vortex it.

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8,01 grams Na₂HPO₄ · 2H₂O
- 2 grams KH₂PO₄

complete into 1 lt with ddH₂O (pH= 6,8).

For 1X PBS's pH should be $\approx$ 7,2-7,4 and should be autoclaved prior to use.

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

- 242 grams Tris (C₄H₁₁NO₃)
- 37,2 grams Tritiplex 3 (EDTA= C₁₀H₁₄N₂Na₂O₂ · 2H₂O)

- 57,1 ml Glacial acetic acid

complete into 1 lt ddH₂O

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

T-cell Buffer [ELISA]

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

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2,5 ml Tween20
- 4,5 lt dH₂O

RPMI-1640 (Hyclone)

- 2 % : 10 ml FBS (Oligo FBS = inactivated at 65°C, Regular FBS = inactivated at 55°C)
- 5 % : 25 ml FBS
- 10 % : 50 ml FBS
- 5 ml Penicillin/Streptomycin (50 µg/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES (Biological Industries), (10 mM final concentration from 1M stock)
- 5 ml Na Pyruvate, (0,11 mg/ml final concentration from 100mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

In 500 ml media

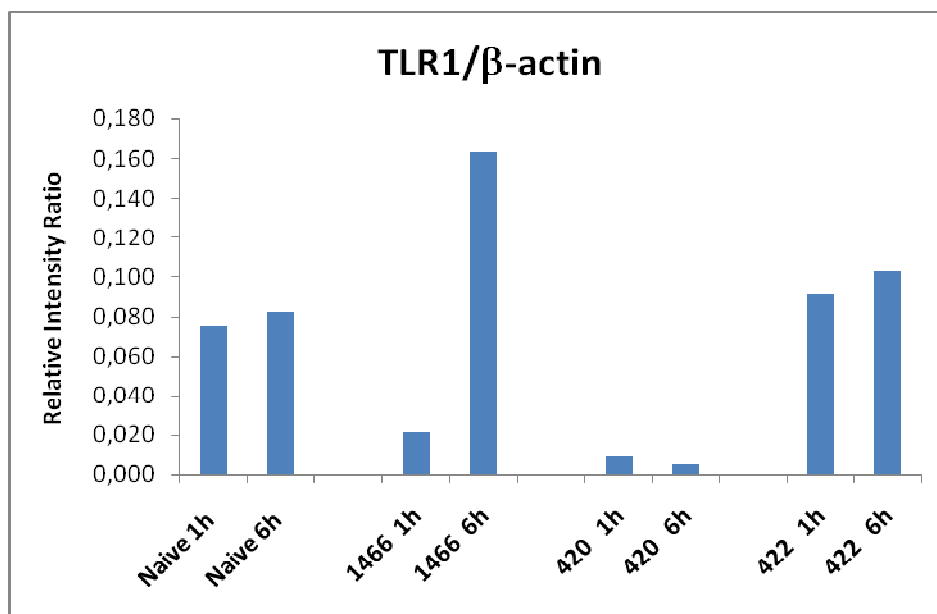
8.2. Appendix II

TLR expression profile upon treatment of murine splenocytes with CpG ODN.

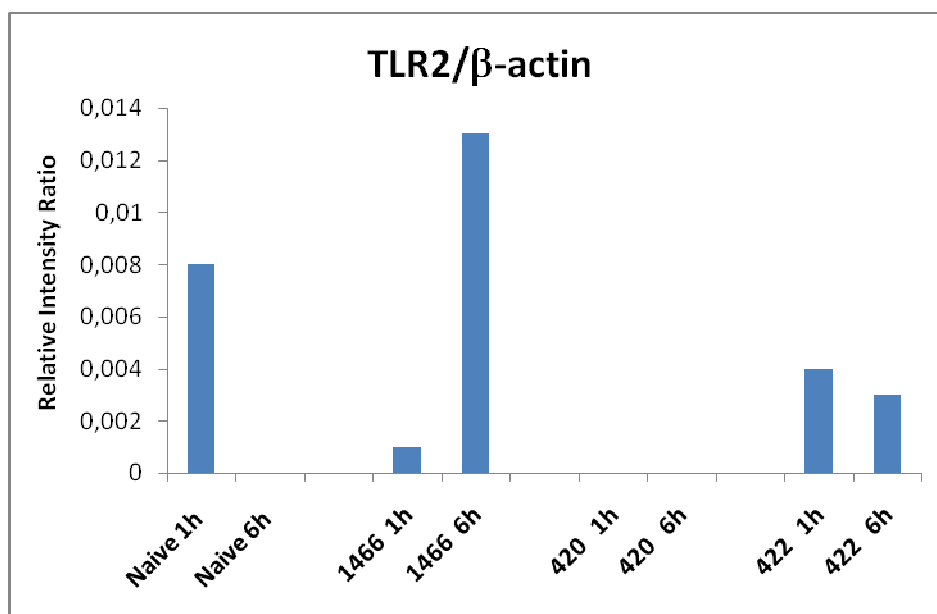
After 1h and 6h stimulation with CpG ODNs (indicated on figures) and controls (data not shown), cells were checked for expression of *tlr1* (A), *tlr2* (B), *tlr3* (C), *tlr4* (D), *tlr5* (E), *tlr6*

(F), *tlr7* (G) and *tlr9* (H) by using PCR. Agarose gel band intensities of indicated genes and housekeeping gene (β -actin) were measured. *tlr*/ β -actin ratios were calculated.

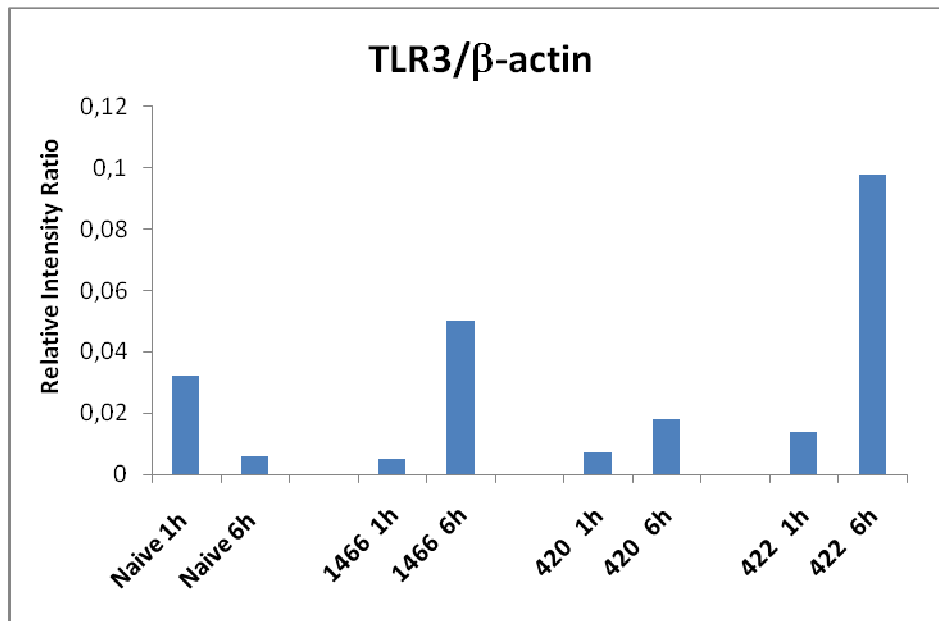
A)



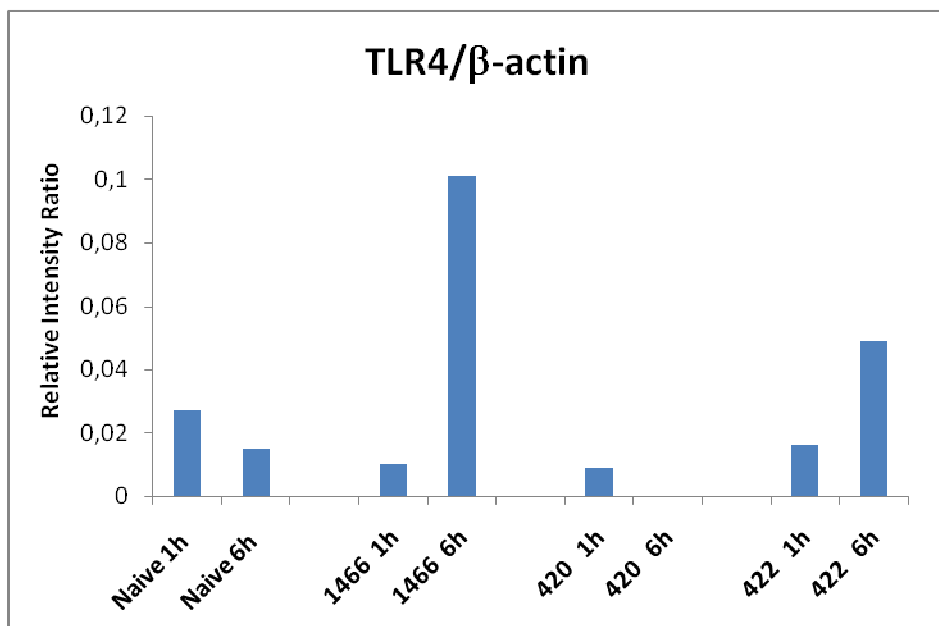
B)



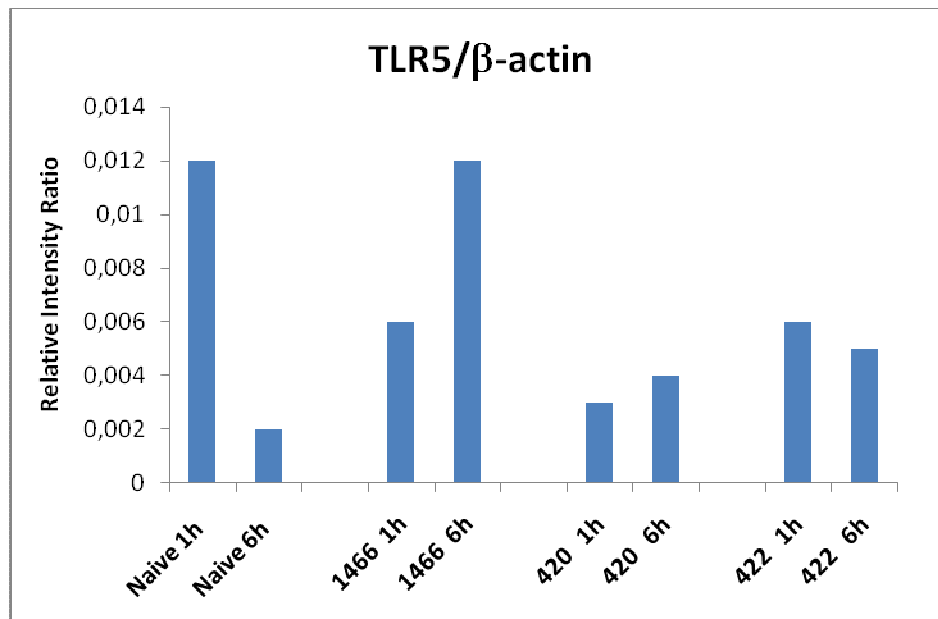
C)



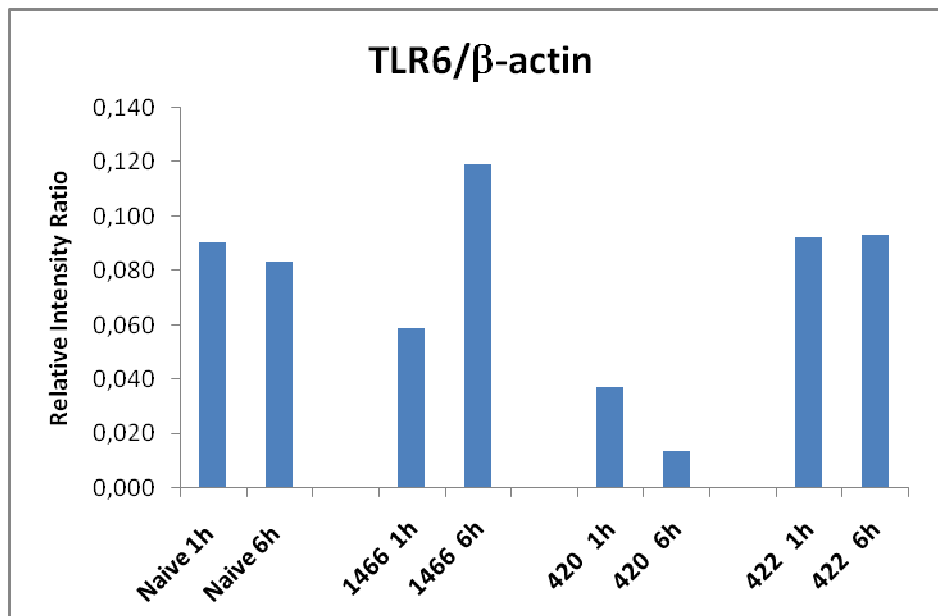
D)



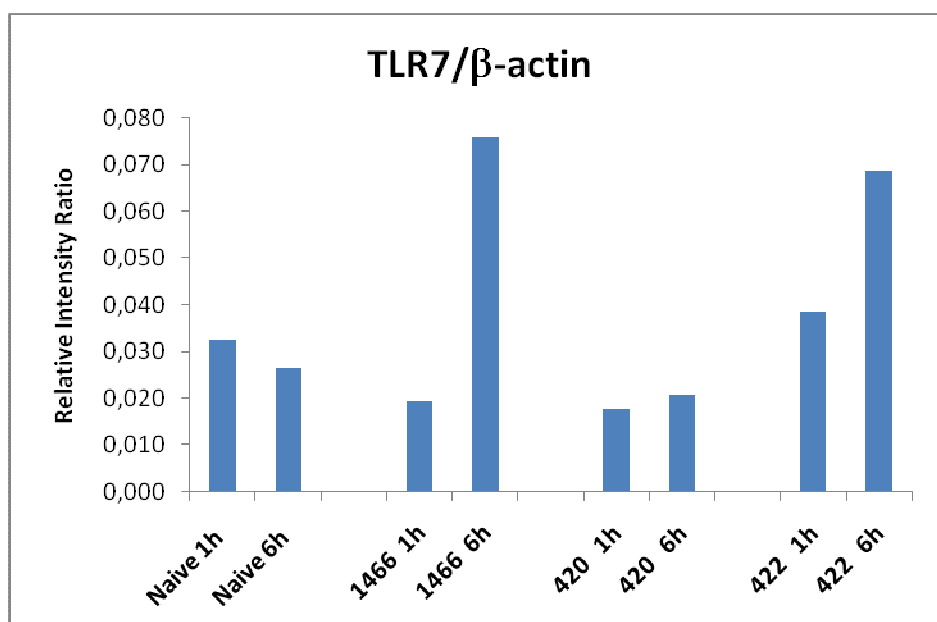
E)



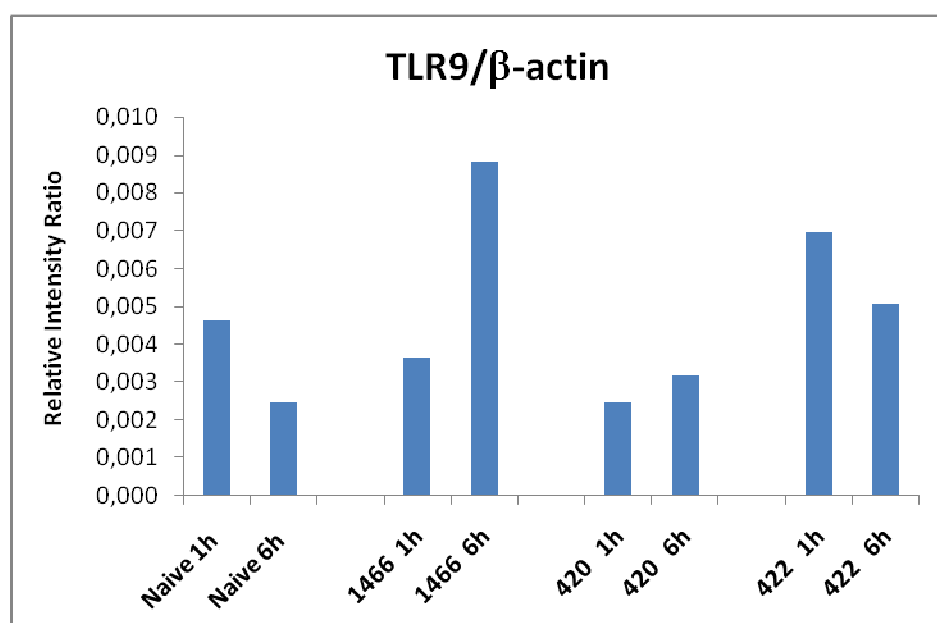
F)



G)



H)

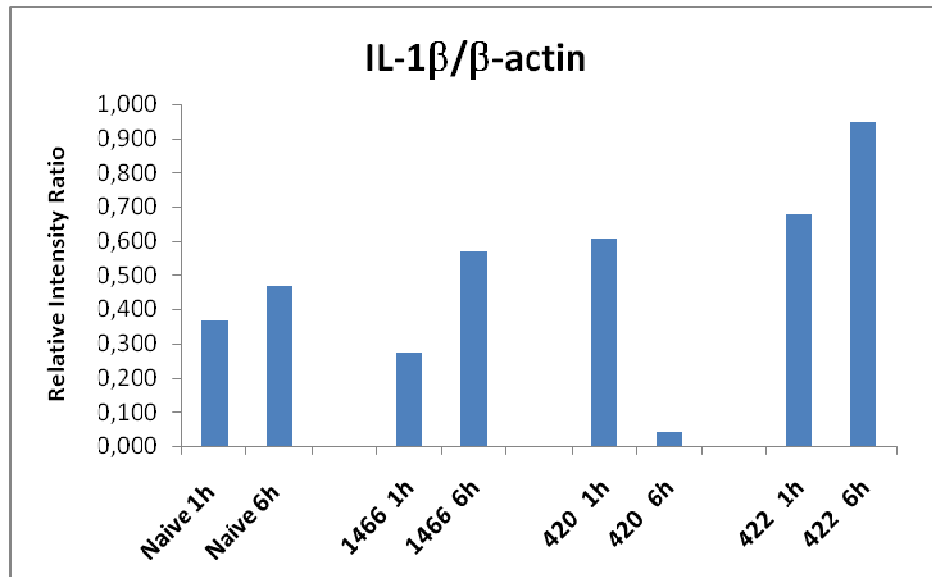


8.3. Appendix III

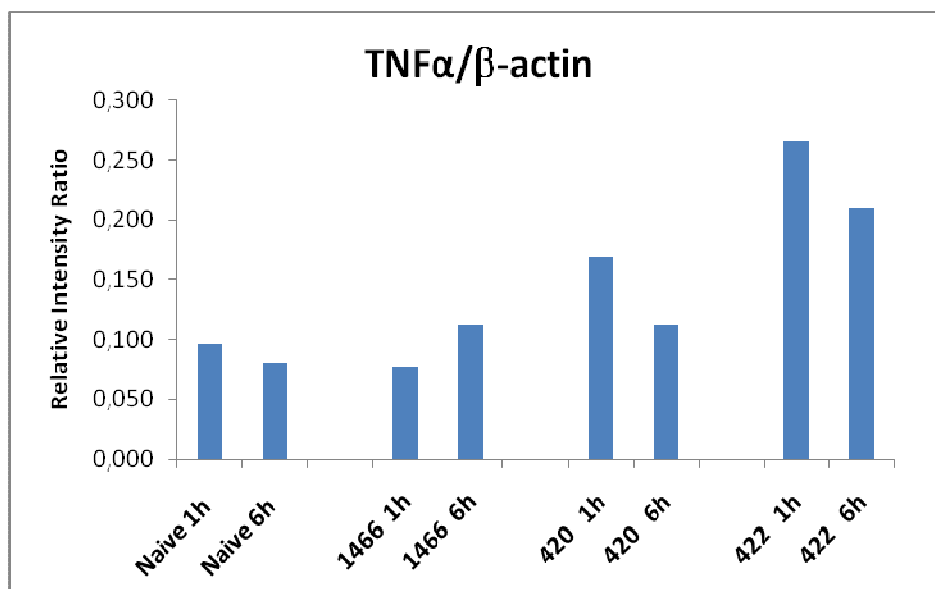
Cytokine & chemokine expression profile upon treatment of murine splenocytes with CpG ODN. After 1h and 6h stimulation with CpG ODNs (indicated on figures) and controls (data not shown), cells were checked for expression of *il-1b* (A), *tnfa* (B), *il-6* (C), *il-15* (D),

il-18 (E), *ifn α* (F), *ifn γ* (G), *ip10* (H), *mip-1 α* (I), *mip-1 β* (J), *mcp-1* (K), *cxcl16* (L) by using PCR. Agarose gel band intensities of indicated genes and housekeeping gene (β -actin) were measured. Cytokine(chemokine)/ β -actin ratio was calculated.

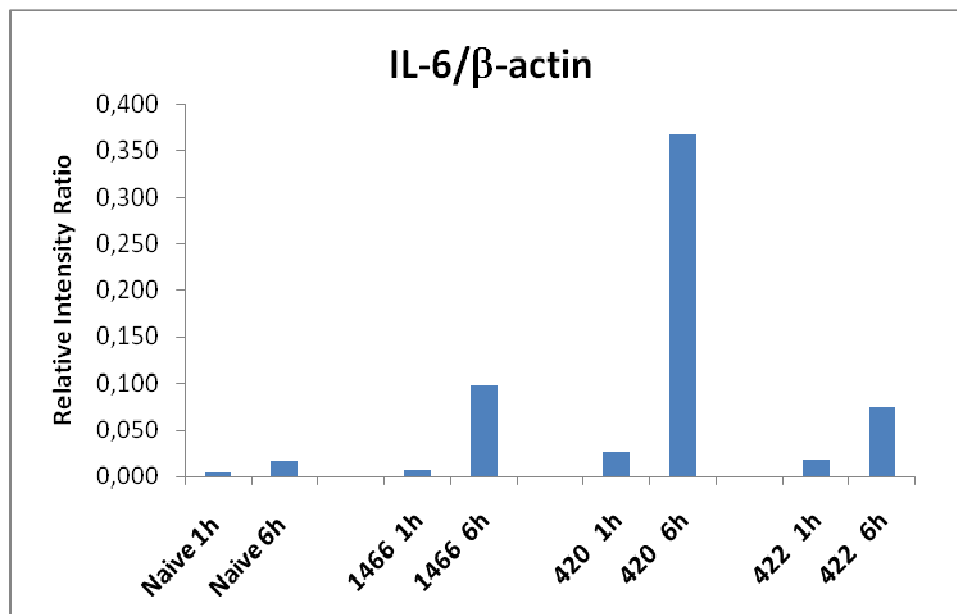
A)



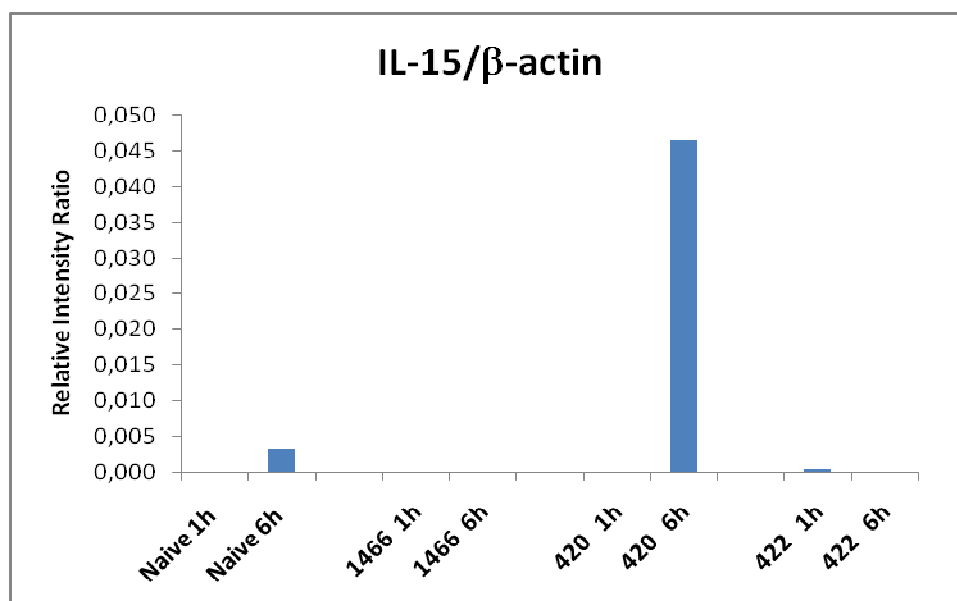
B)



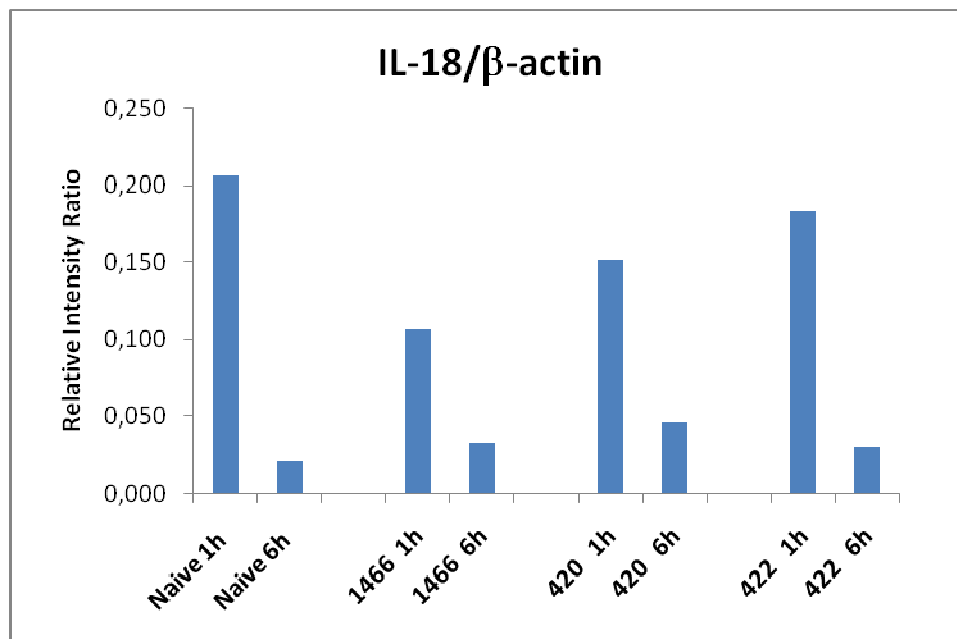
C)



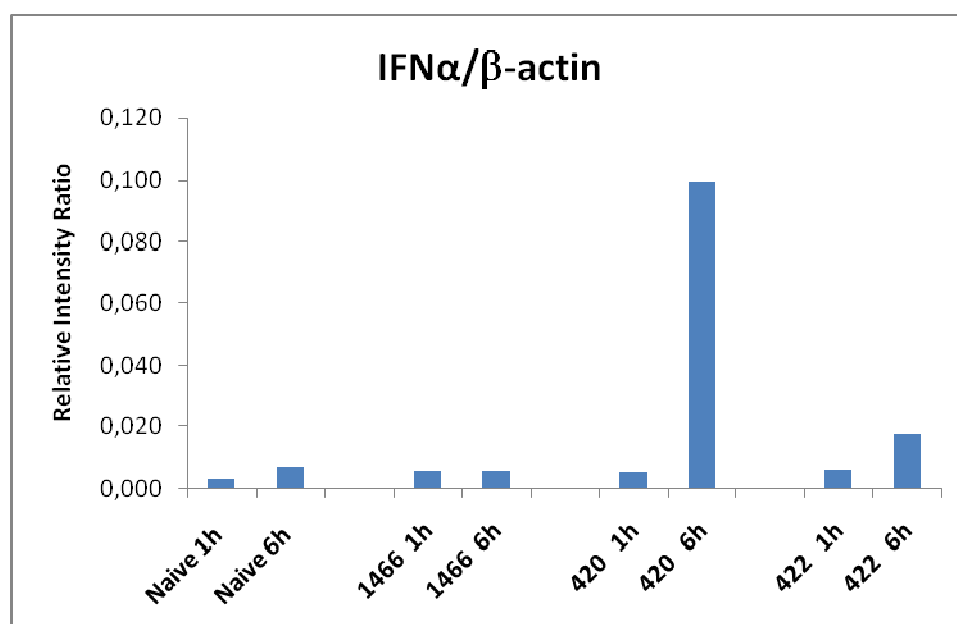
D)



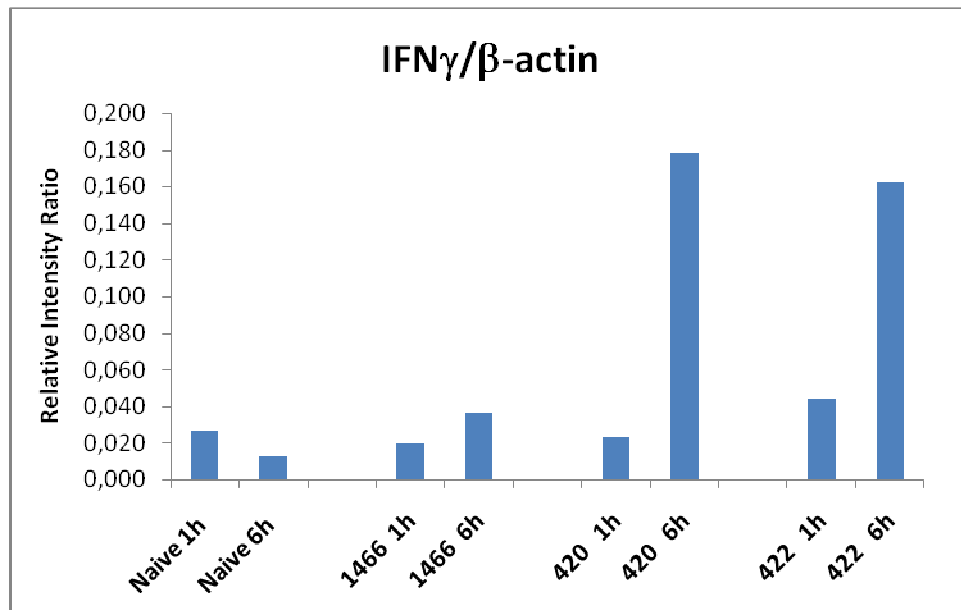
E)



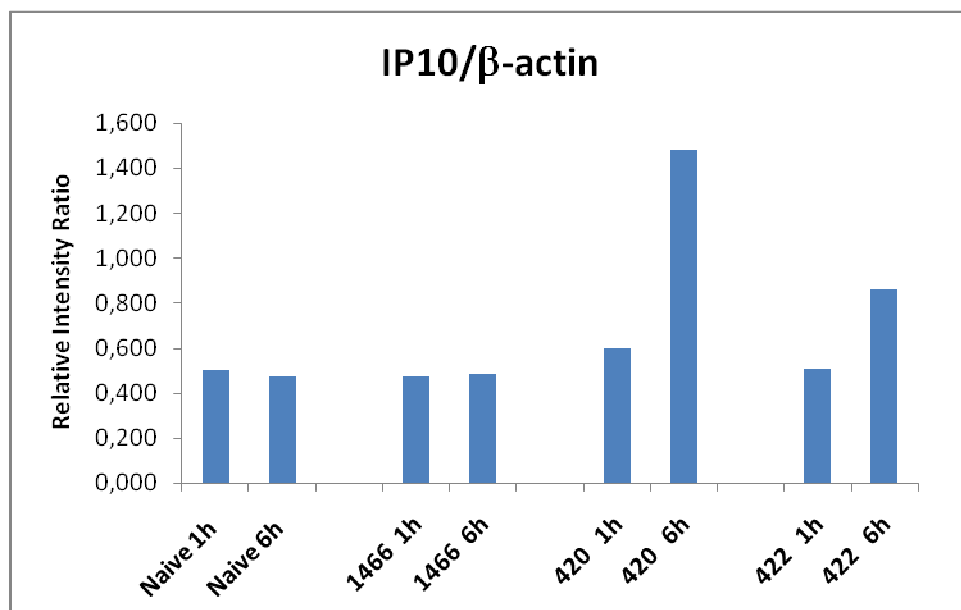
F)



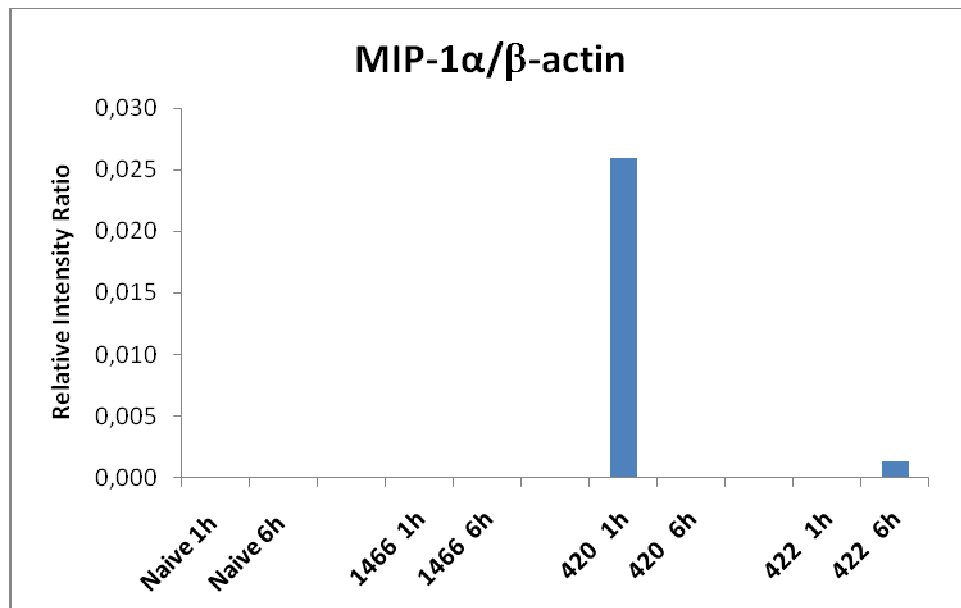
G)



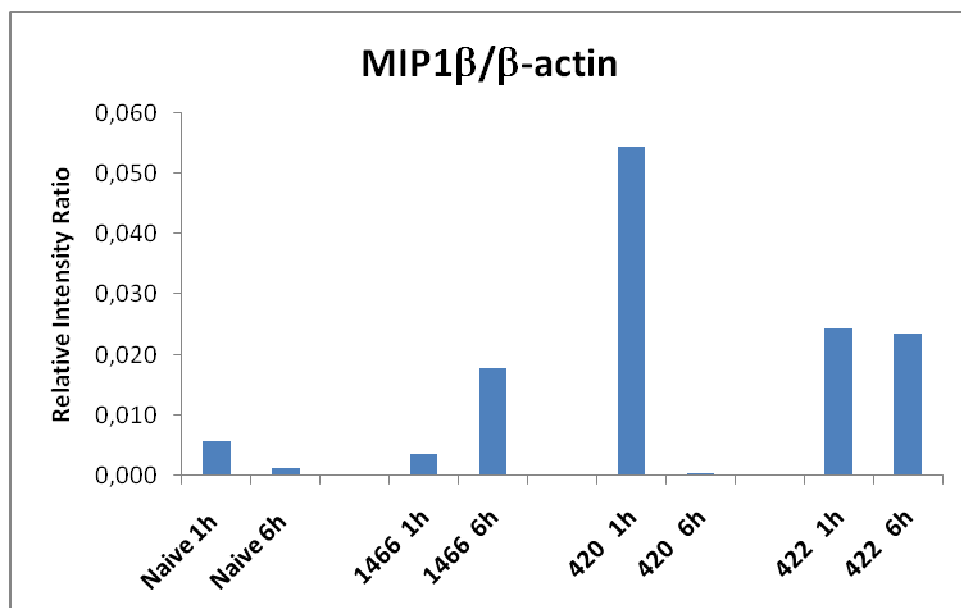
H)



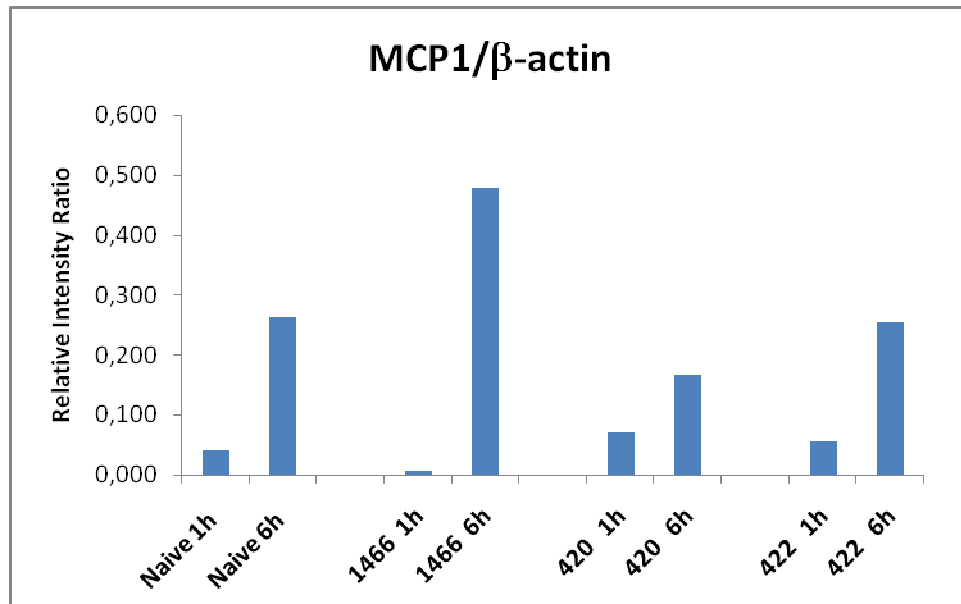
I)



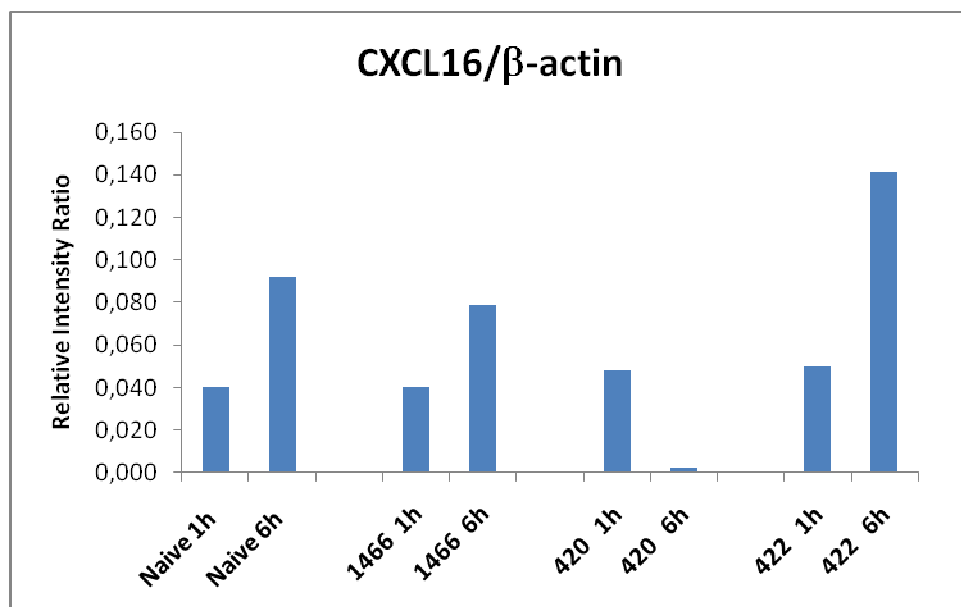
J)



K)



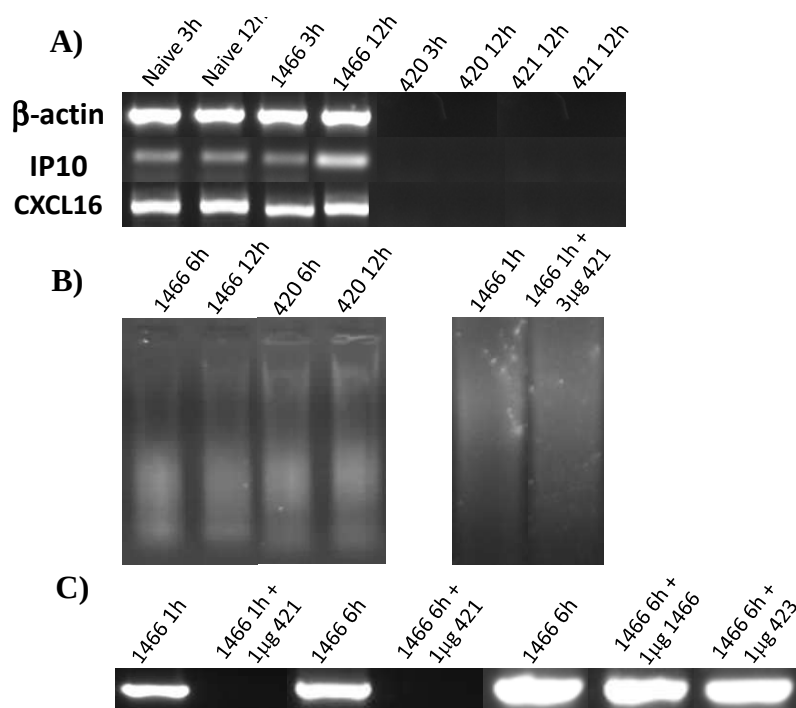
L)



8.4. Appendix IV

Dendrimer interference with PCR.

In vitro stimulation experiments showed that RNA, or cDNA from the same RNA, of bifunctional dendrimer treated samples were inhibited somehow to display bands indicating gene expression (A). We assumed that dendrimer stays in cellular extracts due to their highly stable structures (please see table 4.2.) and comes with isolated RNA. Then either inhibits cDNA synthesis or PCR from cDNA. Next experiments showed that latter was the case. Since cDNA obtained from dendrimeric ODN treated samples gave smear on gel which was indifferent from other working samples and dendrimeric ODN addition to RNA of conventional ODN treated samples didn't inhibit cDNA synthesis (B). While, even 1 μ g dendrimeric ODN addition to cDNA samples blocked PCR of otherwise working sample. Linear ODN or trifunctional dendrimeric ODN addition to cDNA samples were not effective as bifunctional dendrimer to inhibit PCR (C).



8.4. Appendix IV

ODN422 were susceptible to DNase treatment, unlike ODN420. ODN420, 421 and 422 were subjected to 30min DNase I treatment and analyzed with PAGE subsequently.

