IMMUNOMODULATORY EFFECTS OF TLR LIGANDS AND POLYSACCHARIDE COMBINATIONS: STRATEGIES TO AUGMENT INNATE IMMUNE RESPONSE

A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

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SEPTEMBER 2007

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ABSTRACT

IMMUNOMODULATORY EFFECTS OF TLR LIGANDS AND POLYSACCHARIDE COMBINATIONS: STRATEGIES TO AUGMENT INNATE IMMUNE RESPONSE

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M.Sc. in Molecular Biology and Genetics
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September 2007, 90 Pages

Microbial infection initiates multiple TLR ligand mediated signaling cascade on innate immune cells. While some TLRs trigger a Th1 biased immune activation, others may lead to a Th2 dominant immune response. Extracellular (TLR1, 2, 4, 5, 6, 10, and 11) vs endosome-associated TLRs (TLR3, 7/8, and 9) display differential immune activation and cytokine milieu. Understanding contrasting and synergistic behaviors of these TLR subclasses when mixed together may lead to more potent formulations for immunotherapy. Delivery and retaining the stability of nucleic acid based labile TLR ligands to the site of immunologically relevant cells is also a challenge.

In the first part of the thesis, optimum TLR combinations with differential immune effects will be brought into light. Next, immunomodulatory effect of a natural polysaccharide (PS) will be characterized. Finally the ability of a PS carrier to form complex with ligands of nucleic acid sensing TLRs and its potential as a controlled delivery vehicle to stimulate the immune cells will be documented.

In brief, our results suggest that different PS types extracted from various mushroom sources are immunostimulatory and are targeted to TLR2/6 for delivery of other relevant stimulants. Moreover, certain TLR ligand combinations can be harnessed to induce more robust immune activation compared to their stand alone counterparts.

This knowledge will pave the way for establishing an effective PS based carrier of DNA/RNA ligands thus, more effective immunotherapeutic strategies for treating infectious and other local or systemic diseases be possible.

Keywords: TLR, polysaccharide, cooperation, innate immunity, vaccine, immunotherapy

ÖZET

TOLL-BENZERİ ULAK VE POLİSAKKARİT BİRLEŞİMLERİNİN BAĞIŞIKLIK DÜZENLEYİCİ ETKİLERİ: DOĞAL BAĞIŞIKLIK SİSTEMİNİ SAĞLAMLAŞTIRMAK İÇİN STRATEJİLER.

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Moleküler Biyoloji ve Genetik Yüksek Lisans
Tez Yöneticisi: Doç. Dr. İhsan Gürsel
Eylül 2007, 90 Sayfa

Mikrobiyal enfeksiyonlar birçok TLR ulağının, doğal bağışıklık hücrelerini yönlendirmiş olduğu sinyal yolaklarını başlatır. Bazı TLR'lar Th1 eğilimli bağışıklık cevabını harekete geçirirken, diğerleri Th2'nin baskın olduğu bağışıklık yanıtına neden olabilirler. Hücre yüzeyi (TLR1, 2, 4, 5, 6, 10, ve 11) ve endozomlara bağlı TLR'lar (TLR3, 7/8, ve 9) farklı bağışıklık tepkileri ve sitokin salımları gerçekleştirebilir. Bu TLR alt sınıflarını birbirleriyle beraber karıştırıp kullanarak, sinerjistik ve karşıtık etkilerini anlayarak daha etkin immün tedavi formülasyonları elde edilebilir. Bazı kararsız TLR ulaklarını bağışıklıkla ilgili hücrelere iletilene kadar kararlı tutup, salacak taşıyıcıları tasarlamak da başa çıkılması gereken bir sorundur.

Tezin ilk kısmında, bağışıklık hücreleri üzerine en çarpıcı fark yaratan TLR bileşenleri ortaya çıkarılıp etkin dozları tayin edilecektir. Sonra, mantar kökenli polisakkaridlerin (PS) doğal bağışıklığı uyarıcı özellikleri karakterize edilecektir. Son olarak da en etkin PS taşıyıcısıyla nükleik asit ulaklarının kompleksleri oluşturulup kontollu salım aracı olarak bağışıklık hücrelerini uyarma şiddeti dökümanlanacaktır.

Özetle, bulgularımız farklı mantarlardan saflaştırılan değişik PS tiplerinin bağışıklığı etkinleştirici ve diğer ilgili uyarıcı ulakların salımı için TLR2/6 almacına hedeflenerek uyardığını göstermektedir. Ayrıca, değişik ulak karışımları, TLR ulaklarının tek tek kendi başlarına yaptıkları immün etkiye göre, birleşimlerin bu etkiyi oldukça çok arttırdığı da saptanmıştır.

Bu bilgiler, çeşitli bulaşıcı ya da lokal ve sistematik hastalıkların tedavisi için, PS temelli etkin DNA/RNA ulaklarını taşıyabilen daha güçlü etki gösterebilen immünterapi yaklaşımlarının yolunu açacaktır.

Anahtar kelimeler: TLR, polisakkarit, karışım, doğal bağışıklık, aşı, immün tedavisi

To my parents Sevtap, Teoman and my sister Ezgi who have encouraged me and have made this possible with their constant help and guidance both during my time as a M. Sc. candidate and M. Sc. studies.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor, Assist. Prof. Dr. İhsan Gürsel, for the opportunity he extended to me in agreeing to be my supervisor and allowing me to become a part of his "fresh" laboratory. I would additionally like to thank him for his invaluable guidance, support, teaching and patience during my studies.

I would like to thank to my laboratory mates, Fuat, Hande, Rashad and one of the nicest senior student, Seda for their help, precious friendship and support in the lab and in my experiments.

I would also like to thank to The Scientific and Technological Research Council of Turkey (TÜBİTAK) for their financial support throughout my experiments.

Thanks to Prof. Dr. Oktay Erbatur and his group, from Çukurova University, for kindly providing the polysaccharide extracts for my studies.

Moreover, I would like to thank to my dearest friends Zeynep, Çiğdem, Esen, Koray and Rasim for their support and for always being there whenever I needed.

I wish to thank to, Bâlâ, Sevgi, Elif and Tolga for their friendship and being there with all knowledge whenever I had dilemma with my studies.

My sincere thanks go to MBG Family for their guidance, companionship and assistance.

I would like to thank to Ezgi Karasözen for boosting my morale in times of difficulty.

Without my family, none of the exceptional things in my life would have been possible, and I would like to express my love and gratitude for their everlasting support in life.

Finally, I would like to thank to Yasin who always trusted and supported me in every moment.

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ABBREVIATIONS

AFM	Atomic Force Microscopy
APC	Antigen presenting cell
AVA	Anthrax vaccine adsorbed
bp	Base pairs
BCG	Bacille Calmette Guerin of Mycobacterium bovis
BCR	B-cell receptor
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freund's adjuvant
CMV	Cytomegalovirus
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
HBV	Hepatitis-B Virus
HEK	Human embryonic kidney
HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus
Ig	Immunoglobulin
IκK	Inhibitor kappa B kinase
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IFN	Interferon
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LBP	LPS-binding protein
LPS	Lipopolysaccharide

LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
MALP	Mycoplasmal lipopeptide
MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
moDC	Myeloid dendritic cells
MSR	Macrophage scavenger receptor
MyD-88	Myeloid Differentiation Primary Response gene 88
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PA	Protective-antigen
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pI:C	Polyriboinosinic polyribocytidylic acid
PLG	Polylactide-co-glycolide
PNPP	Para-nitrophenyl pyro phosphate
PRR	Pattern recognition receptors
PS	Polysaccharide
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RIP	Receptor-interacting protein

RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT	Reverse transcriptase
SA-AKP	Streptavidin Alkaline-phosphatase
SLE	Systemic Lupus Erythematosus
SPG	β-(1 $\rightarrow$3)-D-glucan schizophyllan
SSCL	Sterically stabilized cationic liposomes
ssRNA	Single-stranded RNA
STF	Soluble tuberculosis factor
TCR	T-cell receptor
T_H	T-helper
TIR	Toll/IL-1 receptor
TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF-associated factor
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain containing adaptor inducing IFN-β
UV	Ultraviolet

1. INTRODUCTION

All kinds of vertebrates from birth to death face debilitating and potentially life-threatening infectious agents varying from viruses to fungi. Survival of the infected organism depends on its ability to recognize infectious pathogens and to respond with an appropriate defense system. “Immunity” which is derived from the Latin word *immunis*, meaning exemption from military service, tax payments or other public services in Roman Empire, refers protection from disease especially infectious disease almost for the last 300 years.

A reaction to foreign substances “antigens”, including bacteria, viruses, as well as to macromolecules such as proteins or polysaccharides, alerts the immune system and the physiological response begins. This response is divided into two subcategories known as innate and adaptive immunity. Innate immunity (also called natural and native) is the first line of host defense against pathogens and consists of cellular and biochemical defense mechanisms that are in place even before infection. Physical and chemical barriers such as epithelia, antimicrobial peptides, lysozymes, phagocytic cells; neutrophils, macrophages, natural killer (NK) cells, complement blood proteins, other types of proteins called cytokines which coordinate and regulate many of the mechanisms of the cells, are the main components that build up the innate immune system (Janeway, *2004*). Despite innate immunity, adaptive immunity – also called as specific or acquired immunity – is mediated by clonally expanded T and B lymphocytes and characterized by specifity and memory.

The innate immune system recognizes microorganisms via germline-encoded pattern recognition receptors (PRR). Among the members of PRRs that recognize pathogen-associated molecular patterns (PAMPs) are Toll-like receptors, which are evolutionarily conserved from *C. elegans* to highly organized mammals (Janeway *2002*). There are at least 13 members of the TLR family characterized to date (Akira, *2005*).

In humans ten functional Toll-like receptors (TLR) can be subdivided according to their subcellular localization. TLR1, 2, 4, 5, 6 and 10 are expressed on

the cell surface, however TLR3, 7, 8 and 9 are expressed in intracellular compartments, principally in endosomes and the endoplasmic reticulum (ER) (Kanzler, *2007*) (Table 1.1). They can occur as dimers, for instance TLR1 and TLR2 heterodimerizes as they recognize bacterial triacylated lipopeptides, TLR2 and TLR6 heterodimerization results in the recognition of diacylated bacterial lipopeptides (O'Neill, *2007*).

Table 1.1. Ligands specifity, immunological fate and cellular specifity of the Toll-like receptors.

<table>
<tr><th>TLR</th><th>Selected Ligands</th><th>Role in Immunity</th><th>Localization</th></tr>
<tr><td>TLR1</td><td rowspan="3">PGN
Zymosan
Lipoproteins</td><td rowspan="3">Antibacterial and Antifungal</td><td rowspan="10">Dendritic Cells,
Macrophages,
T Cells,
B Cells,
Epithelium</td></tr>
<tr><td>TLR2</td></tr>
<tr><td>TLR6</td></tr>
<tr><td>TLR4</td><td>LPS</td><td rowspan="2">Antibacterial</td></tr>
<tr><td>TLR5</td><td>Flagellin</td></tr>
<tr><td>TLR 11</td><td>Toxoplasma</td><td>Antibacterial/Parasite</td></tr>
<tr><td>TLR3*</td><td>dsRNA</td><td rowspan="4">Antiviral and Antibacterial</td></tr>
<tr><td>TLR7*</td><td>ssRNA</td></tr>
<tr><td>TLR8*</td><td>ssRNA</td></tr>
<tr><td>TLR9*</td><td>ss/ds DNA</td></tr>
<tr><td>TLR10</td><td>?</td><td>?</td></tr>
</table>

**Nucleic acid sensing TLRs are endosome associated, others are expressed at the cell surface.*

In addition to hetero-homo dimerization, TLRs also affect the immune system as by acting synergistically. TLR2 and TLR4 synergistically act on macrophages synergize to induce production of inflammatory cytokines (Sato, *2000*). Costimulation with TLR4 and TLR2 or TLR9 induces synergistic release of interferon-gamma (IFN-γ) and tumor necrosis factor- alpha (TNF-α) (Equils, *2003*). TLR3 and TLR9 promotes enhanced antitumor and cytokine activity (Whitmore, *2004*). Some of the combinations such as TLR3 and TLR9 were also searched if they could be used as vaccine adjuvants, combined with cationic liposomes (Zaks, *2006*).

TLR4, TLR5, TLR7/8 and TLR9 agonists have potential to therapeutic vaccination for cancer and chronic viral infections, including human-immuno deficiency virus (HIV) and hepatitis-B virus (HBV) (Kanzler, *2007*). Conjugating of HIV Gag protein (HIV vaccine candidate) to TLR7/8 agonist (Yarovinsky, *2006*) and AVA (Anthrax Vaccine Adsorbed, the licensed anthrax vaccine for human use) to TLR9 ligand CpG substantially enhances the immune response (Klinman, *2006*). Not only single TLR ligand but also the combination of more than one TLR ligand are and could be used as vaccine adjuvants with a liposomal delivery systems, mixed with licensed vaccines. The variety of TLR ligands may contribute significantly to inflammation, and appropriate agonists may represent a new class of therapeutic agents for diseases including; Hepatitis, Influenza, cancer and Human papillomavirus (HPV).

Mushrooms, *Ganoderma lucidum* and *Shiitake* have been investigated for their medicinal benefits, most notably their anti-tumor properties in laboratory mice. These studies have also identified the polysaccharide lentinan, a (1-3) β-D-glucan, as the active compound responsible for the anti-tumor effects (Kim, *1999*). Balachandran et. al. (*2006*) showed *Spriluna* (microalgae rich in protein) polysaccharides showed TLR-2 dependent immune activation through monocytes. However this is one of the unique example that reveals the relationship between non-bacterial derived polysaccharides and TLR, so relationship polysaccharide and TLR-mediated immune response should be established for usage of polysaccharides as immuno-therapeutic or immuno-carrier agent.

1.1 The Immune System

Immunity meaning “the ability of an organism to resist infection” can be mainly divided into two subcategories such as; innate and adaptive immunity. Adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection and pathogen-specific adaptor proteins, but this type of immune response is also responsible for allergy, autoimmunity and the rejection of tissue grafts (Janeway, *2002*). The defining characteristics of adaptive immunity are unique specificity for distinct molecules and ability to remember and respond more vigorously to repeated exposures to the same microbe. Therefore,

contributions of adaptive immune systems to pathogen elimination and vaccine design have been extensively studied (Medzhitov, *1997*). Innate immunity was formerly thought to be a nonspecific immune response characterized by engulfment and digestion of microorganisms and foreign substances by phagocytic cells such as macrophages and lymphocytes (Akira, *2001*). However, recent studies of host defense against microbial pathogens, have demonstrated that the type of effector response generated is strongly dependent on the strength of innate immune response initiated (Medzhitov, *1997*). Indeed, the innate immunity help to shape the final adaptive immunity therefore, they act as hand in hand. The importance of innate immunity was fueled by the discovery of pathogen-associated molecular patterns (PAMP) which are recognized by the pattern-recognition receptors (PRR). The role, PRRs play in the elimination of pathogen and activity as adjuvant has reverted the interest in the importance of the initially ignored field of innate immunity.

1.1.1 Induction of Immune System upon Exposure to Pathogens

Defense against infections by both adaptive and innate immune system cells start with the recognition of a pathogen through binding of a PAMP to a PRR through either by antigen-presenting cells (APC) such as macrophages, NK cells, dendritic cells (DC), B-cells found at the site of infection like skin and mucosal epithelia that expresses PRRs and produce antimicrobial chemicals. DCs which were distributed throughout the body, encounters pathogen at different sides of the body such as the mucosal surfaces or the skin, phagocytose then process and present the major histocompatibility complex (MHC) I or MHC II complexed antigenic epitopes to T and B cells. Moreover, activated DCs produce cytokines and chemokines that will be act on pathogen and also alert and recruit other immune cells to the site of insult/infection (Lee, *2007*). Activated T and B cells expressing T cell receptor (TCR) and B cell receptor (BCR) will migrate to the infected site upon chemokine and cytokine production (Luster, *2002*). These cells rapidly differentiate into effector cells whose main function is to control ongoing infection. Therefore, the innate immune system can instruct the adaptive immunity about the nature and location of pathogenic infection.

1.2. Innate Immune System

The innate immune system detects the presence of and the nature of infection, provides the first line of host protection, and controls the initiation and determination of the effector class of the adaptive immune response (Medzhitov, *2001*). The components of innate immunity recognize the structures that are shared by various classes of microbes and are not present in host cells (self/non-self differentiation). The innate immune system is composed of epithelia, which provide barriers to infection, cells in the circulation and in the tissues, and several plasma proteins, such as members of the complement system. The other component of the innate immune system is phagocytes, (i.e. neutrophils, monocytes/macrophages, DCs and B cells). Both types of immune cells recognize microbes in blood and extravascular tissues by surface receptors that are specific to microbial products such as Toll-like receptors. The recognition of pathogens by these phagocytic cells leads to engulfment of the microbes and activation of the phagocytes to kill the ingested agent (Bancroft, *1994*). NK cells are a class of lymphocytes that respond to Interleukin-12 (which is produced by macrophages) (IL-12), kills microbe infected cells and produces Interferon-γ (IFN-γ) that activates the other components of immune system. In addition to IL-12, NK cells' effector functions are induced by a range of cytokines including IL-15, IL-18 and type I IFNs (produced by DCs). NK cells have a crucial role in anti-viral immunity, by recognizing and eliminating Cytomegalovirus (CMV), Hepatitis C and HIV infected cells (Hammerman, *2005*). In response to microbes, macrophages and other cells secrete proteins called cytokines and chemokines. These chemoattractants can mediate many of the cellular reactions of innate immune cells. The main cytokines/chemokines appear during the onset of innate immune activation are; TNF-α, IL-1α/β, IP-10, macrophage inflammatory protein-1α (MIP-1α), MIP-3α, monocyte chemoattractant protein (MCP) and Regulated upon activation, normal T-cell expressed, and secreted (RANTES). These mediators can induce fever, apoptosis, neutrophil activation, recruitment of T and B cells and induction of inflammation as well as regulating the trafficking of immune effector cells to the site of infection.

Other indispensable cytokines such as; IL-12, (which directs T-helper 1 (T_H1) differentiation), Type I IFNs (IFN-α and IFN-β; important for anti-viral response), IL-6 (stimulates and promotes B cell proliferation), IL-15 and IL-18 (helps NK and T cell proliferation) and IL-10 (that is known to induce inhibitory/stimulatory effect on other immune cells) are involved in the orchestral activation/regulation of innate immunity.

1.2.1. Pathogen Recognition Receptors

Pathogen recognition receptors are able to discriminate self from non-self. They evolved to recognize special non-self pathogen-associated signature structures not present on the host (Medzhitov, *1997*). They can be expressed on the cell surface, in intracellular compartments (i.e. endosomal organelles or ER) or secreted into bloodstream and tissue fluids, such as opsonins. Opsonins (enhancement of the process of phagocytosis) include: mannan-binding lectin, C-reactive protein and serum amyloid proteins are the secreted molecules produced by the liver (Fraser, *1998*). PRR functions include opsonization, activation of proinflammatory signaling pathways, induction of apoptosis and phagocytosis. Several pattern recognition receptors are expressed in the cytosol where they detect these intracellular pathogens and induce responses that block their replication. The protein kinase (PKR) activates, nuclear factor-kappa B (NF-κB) and mitogen-activated protein (MAP) kinase signaling pathways upon binding to dsRNA, which leads to the induction of the antiviral type-I IFN genes (Clemens, *1997*). PKR also inhibits viral spread by inducing apoptosis in infected cells (Williams, *1999*).

Another group of proteins likely involved in intracellular pattern recognition is the family of Nucleotide-binding oligomerization domain protein-like receptors (NLR). The full range of ligands recognized by NOD proteins is currently unknown, but both NOD1 and NOD2 are reported to activate NF-κB in response to LPS, presumably through binding to their leucine-rich repeats (LRR) regions. Besides their common ligand, NOD1 recognizes a molecule called meso-DAP, that is a peptidoglycan constituent of only Gram negative bacteria and NOD2 proteins

recognize intracellular muramyl dipeptide (MDP), which is a peptidoglycan constituent of both Gram positive and Gram negative bacteria (Inohara, *2001*).

1.2.2. Toll-like Receptors

The best characterized PRRs, Toll-like receptors were identified in mammals as a family of type I transmembrane receptors, that are homologous to the *Drosophila* Toll receptor (Medzhitov, *1997*). TLRs are a group of evolutionarily conserved proteins belonging to the IL-1R superfamily, characterized by an extracellular LRR and an intracellular Toll/IL-1 receptor like (TIR) domain. TIR domain of Toll proteins is a conserved protein-protein interaction module, which is also found in a number of transmembrane and cytoplasmic proteins in animals and plants have a role in host defence (Medzhitov, *2001*). Ten TLRs are identified to date in mammals. They differ from each other in ligand specificities, expression patterns, and presumably in the target genes they can induce.

1.2.1.1 TLRs in Innate and Adaptive Immunity

TLRs in the innate immune system serve an essential role not only in recognition of pathogen, but also in directing the course and type of innate immune response generated following exposure to foreign antigen (Takeda, *2003*). TLRs have been demonstrated to have a wide array of functions including initiation of proinflammatory responses and antiviral responses, up-regulation of costimulatory molecules on antigen presenting cells (APC), release of chemokines to induce migration of responder cells to the site of infection, and induction cross-priming of T cells by DCs (Takeda, *2005*). TLRs are responsible for the adjuvant activity that is required to initiate immune responses both in natural infection and in vaccine responses (Lien, *2003*). TLRs have emerged as essential not only in innate immune responses but also in shaping adaptive immune responses to pathogen. The signals for activation of adaptive immunity are mostly provided by DCs. TLR-mediated recognition of pathogens by DCs induces the expression of costimulatory molecules such as CD80/CD86 (which provides a costimulatory signal necessary for T cell activation and survival) and production of inflammatory cytokines such as IL-12

(Akira, *2001*). DCs subsets can induce T_H1 and T_H2 responses. Activation of TLR9 in DCs induces production of IL-12, thereby changing the Th cell differentiation toward T_H1 type (Sousa, *2001*). LPS stimulates TLR4 signaling pathway and DCs to support T_H1 and T_H2 cell differentiation (Kaisho, *2002*). In addition to that some pathogen-derived adjuvants such as Complete Freund's Adjuvant (CFA), Bacille Calmette Guerin of Mycobacterium bovis (BCG) are recognized by TLRs; TLR9 and TLR2, TLR4 respectively, which may explain the involvement of TLRs in adaptive immunity (Akira, *2003*).

1.2.1.2. The TLR Family Members

Mammalian TLRs comprise of a large family consisting of at least 13 members. TLRs play important roles in recognizing specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses. Human TLR4 was the first characterized mammalian Toll (Poltorak, *1998*). It is expressed in a variety of cell types, most predominantly in the cells of the immune system, including macrophages and DCs (Medzhitov, *1997*). TLRs can be subcategorized according to their localization in the cells. TLR1, 2, 4, 5, 6 and 10 which are seemed to specialized in the recognition of mainly bacterial products; are located on the plasma membrane, whereas TLR3, 7, 8 and 9 that are specialized in viral and intracellular bacteria detection and nucleic acids, are located in the intracellular endosomal and/or ER compartments (Iwasaki, *2004* and Latz *2004*) (Figure 1.1).

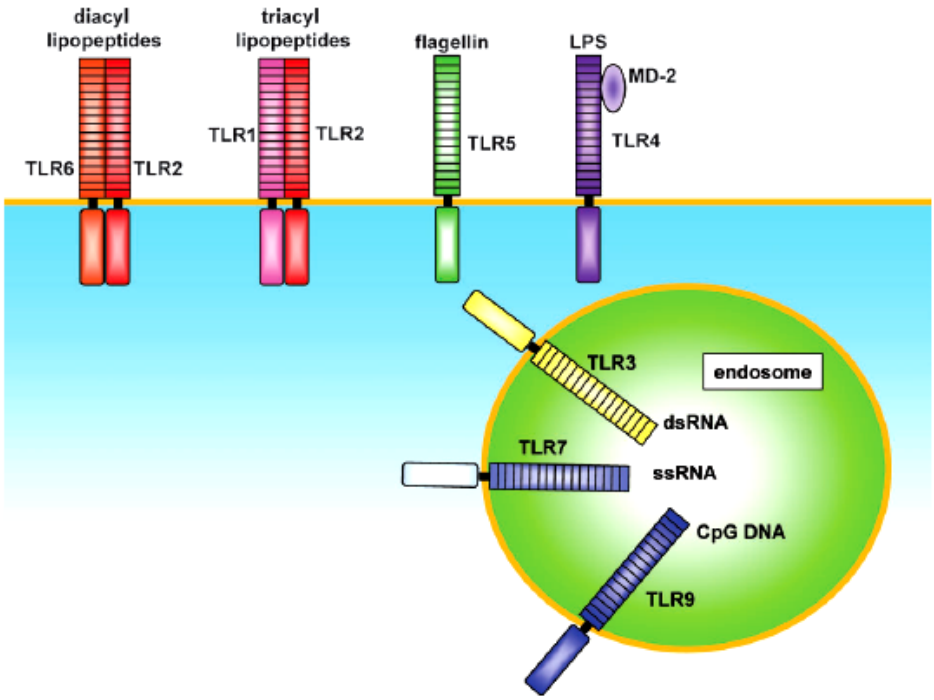


Figure 1.1. The TLR family members recognize specific patterns of microbial components. TLR1 and TLR6 cooperate with TLR2 to discriminate differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for bacterial LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 recognizes viral-derived ssRNA. TLR5's ligand is bacterial flagellin. (Adopted from Takeda, *2005*, Nature).

1.2.2.2.1. TLR 2, TLR1 and TLR6

TLR2 responds to various microbial products, including lipoproteins, Gram-positive bacterial PGN and LTA, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from a protozoan *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis,* Zymosan from fungi (Takeda, *2005*). One of the aspects proposed for the wide spectrum recognition of microbial components TLR2 recognizes, is that TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2. The studies done with the TLR6 or TLR1 deficient mice showed no inflammatory response to mycoplasma-derived triacyl and diacyl lipopeptides respectively. This proves that TLR1 and TLR6 functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides. In addition to that TLR2 has been shown to

functionally collaborate with distinct types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component β-glucan. The bacterial components which were mentioned above, act on immune system from monocytes and macrophages to produce inflammatory cytokines such as TNF-α and IL-6 (Sato, *2000*). Gil et. al. (*2006*) reported that TLR2 triggers TNF-α and MIP-2 secretion from macrophages through the MyD88 signaling pathway with yeast *C. albicans*. In *2003*, it has been found that PGN, could also be delivered to the cytosol for NOD1 recognition from extracellular sites or from phagocytosed bacteria (Chamaillard, *2003*). Therefore we can suggest that for the recognition of PGN, TLR and NLR could act together.

1.2.2.2.2. TLR3

The discovery of double-stranded (ds) RNA as the ligand for endosomal located TLR3 helped recognize that TLRs may have a key role in the host defense against viruses by enhancing NF-κB and interferon (IFN)-regulatory factor 3 (IRF3) pathways (Alexopoulou, *2001*). dsRNA is produced by most viruses during their replication and induces the synthesis of type I interferons (IFNα/β), which exert anti-viral and immunostimulatory activities. NK cells are the major players in the antiviral immune response and express TLR3 and are activated directly in response to synthetic dsRNA, polyriboinosinic polyribocytidylic acid (poly I:C) (Schimdt, *2004*). Also myeloid DCs mainly produce IL-12 and IFN-β on TLR3 stimulation (Ito, *2002*).

1.2.2.2.3. TLR4

As mentioned above TLR4 is the first identified mammalian Toll. This extracellular TLR is expressed in variety of cell types, most predominantly in macrophages and DCs (Medzhitov, *1997*). TLR4 functions as the signal-transduction for signal-transducing receptor for lipopolysaccharide (LPS) which is a major component of the outer membrane of Gram-negative bacteria (Hoshino, *1999*). Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS is first bound to a serum protein, LPS-binding protein (LBP), which functions by transferring LPS monomers to CD14 (Wright, *1999*). CD14 is a high-affinity LPS

receptor that can either be secreted into serum, or expressed as a glycophosphoinositol (GPI)-linked protein on the surface of macrophages. Another component of the LPS receptor complex is MD-2 (Shimazu, *1999*). Although its precise function is not known, MD-2 is required for LPS recognition (Schromm, *2001*). In addition to LPS, TLR4 is involved in the recognition and is considered to be an accessory protein other ligands, including LTA, and a heat-sensitive cell-associated factor derived from *Mycobacterium tuberculosis* (Li, *2001*). Interestingly, TLR4 and CD14 were also shown to trigger a response to the fusion (F) protein of respiratory syncytial virus (RSV). Since it is not clear yet whether the F protein of RSV represents an example of a viral PAMP, an alternative possibility is that the RSV evolved the ability to stimulate TLR4 for its own benefit (Kurt-Jones, *2000*).

1.2.2.2.4. TLR5

TLR5 recognizes flagellin, the protein subunits that make up bacterial flagella. TLR5 is expressed on the basolateral side of the intestinal epithelium, where it can sense flagellin from pathogenic bacteria, such as *Salmonella*. Flagellin induces lung epithelial cells to induce inflammatory cytokine production (Hawn, *2003*).

1.2.2.2.5. TLR7 and TLR8

Both of these TLRs are structurally highly conserved proteins, and recognize the same ligand in some cases. Although both TLRs are expressed in mice, mouse TLR8 appears to be nonfunctional (Akira, *2006*). It has been revealed that murine and human TLR7 (but not murine TLR8) recognizes synthetic compounds, imidazoquinolines (R848), which are clinically used for treatment of genital warts associated with viral infection (Hemmi, *2002*). Murine TLR7 and human TLR8 recognize guanosine or uridine-rich single-stranded RNA (ssRNA) from viruses such as HIV, vesicular stomatitis virus and influenza virus. ssRNA is abundant in host, but usually host-derived ssRNA is not detected by TLR7 or TLR8. This might be due to the fact that TLR7 and TLR8 are expressed in the endosome, and host-derived ssRNA is not delivered to the endosome (Lund, *2004*).

1.2.2.2.6. TLR9

One of the most popular TLR, TLR9 is the receptor for unmethylated bacterial genomic DNA which are primarily expressed on B cells, NK cells and DCs to proliferate, mature and secrete various cytokines (IL-12, IFN-γ, IL-6), chemokines or immunoglobulins (Ig) (Krieg, *2000*). A single nucleotide substitution or methylation of a cytosine residue within the CpG motif completely abrogates the immunostimulatory property of bacterial DNA (Krieg, *1995*). Because bacteria lack cytosine methylation, and most CpG is methylated in the mammalian genome, CpG motifs might signal the presence of microbial infection. There are at least two types of synthetic CpG DNA, termed A or D-type CpG DNA and B or K-type CpG DNA (Klinman, *2004*). B/K-type CpG DNA is made up of phosphorothioate backbone and possesses >1 CpG motifs on a single backbone, and is a potent inducer of inflammatory cytokines such as IL-12, IL-6 and TNF-α, B cell proliferation and IgM secretion. A/D-type CpG DNA is structurally different from B/K CpG DNA, which are phosphodiester/phosphorothioate mixed backbone, and G-runs at 3'–5' ends, and a single CpG motifs has a greater ability to induce IFN-α production from pDCs, but inability to induce B-cells (Gursel, *2006* and Verthelyi, *2001*). TLR9 has been shown to be essential for the recognition of both types of CpG DNA (Hemmi, *2003*). In addition to bacterial CpG DNA, TLR9 has been shown to recognize viral-derived CpG DNA in pDC such as Mouse cytomegalovirus MCMV (Krug, *2004*). While TLR9 is essential for CpG mediated effect the mechanism of the observed dichotomy between K and D type CpG-ODN on human cells was elusive. Recently, Gursel et. al. revealed that pDC but not B cells expresses a co-receptor known as CXCL16 and IFN-α induction by pDC trigerred by D-type ODN is significantly dependent on the CXCL16 expression. In addition to bacterial and viral CpG DNA, TLR9 is presumably involved in pathogenesis of autoimmune disorders. The immunoglobulin-G_{2a} (IgG_{2a}) is bound and internalized by the B cell receptor, and the chromatin, including hypomethylated CpG motifs, is then able to engage TLR9, thereby inducing rheumatoid factor. Chloroquine is clinically used for treatment of autoimmune diseases such rheumatoid arthritis and systemic lupus erythematosus (SLE) (Boule, *2004*). Since chloroquine blocks TLR9-dependent signaling (Hacker, *1998*), it act as an anti-inflammatory agent by inhibiting TLR9-dependent immune response. More

than a dozen of human clinical trails have been initiated utilizing TLR9 agonists. It seems likely that the targeted activation of TLR9 using CpG ODN will enhance the treatment of cancer and infectious diseases, as well as showing new hopes for reducing the harmful inflammatory responses such as, asthma and other allergic diseases (Krieg, *2006*).

1.2.2.3. TLR Signaling Pathways

Activation of TLRs by PAMPs leads to induction of various genes that involved in host defense, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase (iNOS) and antimicrobial peptides, which can directly eliminate microbial pathogens. Although both TLRs and IL-1Rs rely on TIR domains to activate NF-κB and MAP kinases and can induce some of the same target genes, a growing body of evidence points to several differences in signaling pathways activated by individual TLRs (Thoma-Uszynski, *2001*). Besides, activation of specific TLRs lead to slightly different patterns of gene expression profiles. For example, activation of TLR3 and TLR4 signaling pathways results in induction of type I IFNs, (Doyle, *2002*) but activation of TLR2- and TLR5-mediated pathways does not (Hoshino, *2002*). In addition to TLR3 and TLR4, TLR7, TLR8 and TLR9 signaling pathways also lead to induction of type I IFNs but in a different manner (Ito, *2002*). Although myeloid differentiation primary response gene (88) (MyD 88) is common in all TLR pathways. It has been revealed that there are MyD88-dependent and MyD88-independent/TRIF dependent signaling (Figure 1.2).

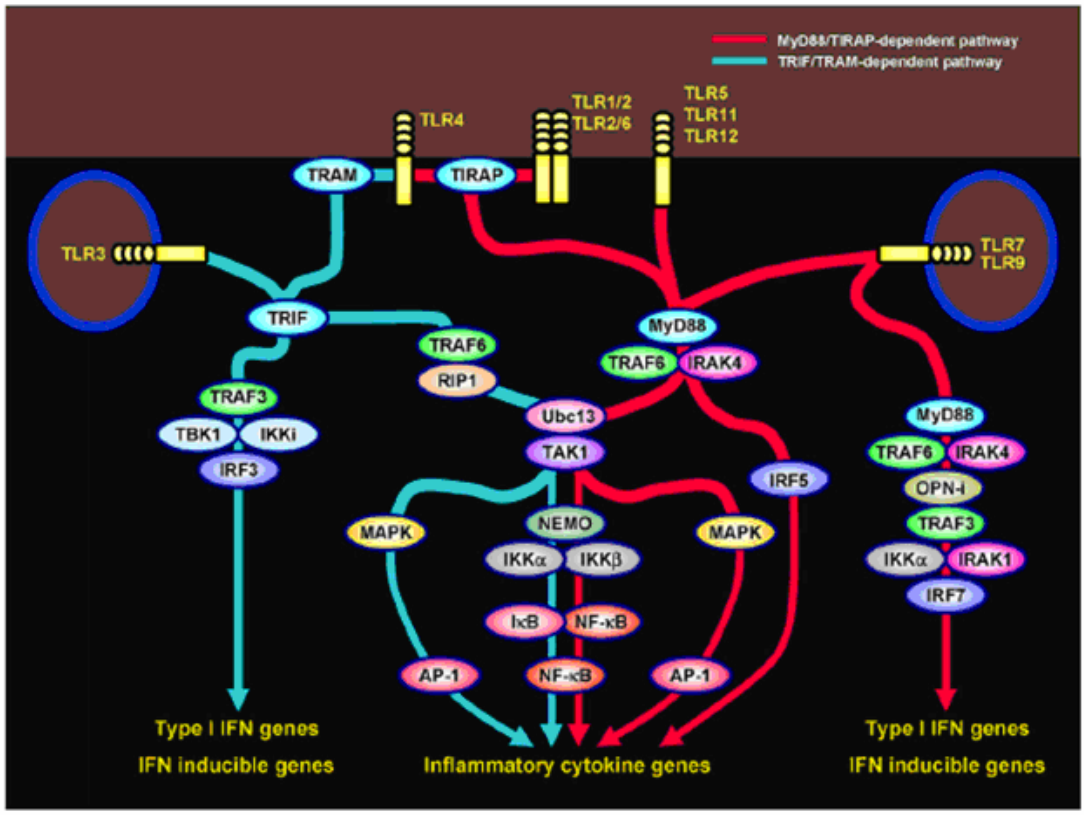


Figure 1.2. The MyD88-dependent and MyD88-independent TLR signaling pathways (Adopted from Akira, *2005*).

1.2.2.3.1. *MyD88-Dependent Pathway*

The role of Toll-mediated recognition in the control of MyD88 protein was studied using MyD88-deficient mice. A MyD88-dependent pathway is analogous to signaling pathways through the IL-1 receptors. MyD88, including a C-terminal TIR domain and an N-terminal death domain, joins with the TIR domain of TLRs. After stimulation, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs by the interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6, leading to the activation of two distinct signaling pathways. One pathway leads to activation of AP-1 transcription factors through activation of MAP kinases. Another pathway activates the TAK1/TAB complex, which enhances activity of the Inhibitor kappa B kinase (IκK) complex. Once activated, the IκK complex induces phosphorylation and subsequent degradation of IκB, which leads to nuclear

translocation of transcription factor NF-κB (Takeda, *2004* and Klinman, *2004*). MyD88-deficient mice do not show production of inflammatory cytokines such as TNF-α and IL-12p40 in response to all TLR ligands (Takeuchi, *2000* and Klinman, *2004*). This once again proves that MyD88 is essential for inflammatory cytokine production through all TLRs. MyD88-deficient macrophages, show impaired inflammatory cytokine production in response to TLR4 and TLR2 ligands in contrast to TLR3, TLR5, TLR7 and TLR9 ligands (Yamamoto, *2002*).

1.2.2.3.2. *MyD88-Independent/TRIF Dependent Pathway*

TLR4 ligand-induced production of inflammatory cytokines is not observed in MyD88-knock-out macrophages; on the other hand, delayed NF-κB expression is observed. This shows that although TLR4 signaling depends on MyD88-dependent pathways, a MyD88-independent component exists in TLR4 signaling. TLR4-induced activation of IRF-3 leads to production of IFN-β. IFN-β in turn activates Stat1 and induces several IFN-inducible genes, like TLR3 (Yoneyama, *1998* and Alexopoulou, *2001*). TRIF-deficient mice generated by gene targeting showed impaired expression of IFN-β- and IFN-inducible genes in response to TLR3 and TLR4 ligands (Yamamoto, *2002*). Studies with the other TRIF-related adaptor molecules (TRAM)/TICAM-2 showed that TRAM is involved in TLR4-mediated, but not TLR3-mediated, activation of IRF-3 and induction of IFN-β and IFN-inducible genes (Yamamoto, *2003*), so TRAM is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway. Key molecules that mediate IRF-3 activation have been revealed to be non-canonical IκKs, Tank binding kinase-1 (TBK1) and IκKi/IκKe (Fitzgerald, *2003*). It has been recently reported that, complete MyD88 and TRIF expression is required for the effective cooperation, resulting in the induction of IL-12, IL-6, and IL-23 but not of TNF-α and IP-10 upon MyD88- and TRIF-dependent TLR stimulation. Downstream of MyD88, TRIF and IRF5 were identified as an essential transcription factor for the synergism of IL-6, IL-12, and IL-23 gene expression (Ouyang, *2007*). Since TRAF6 is critically involved in TLR mediated NF-κB activation, and TRAF6 associates the N terminal portion of TRIF (Gohda, *2004*) and the association of C-terminal portion of TRIF with Receptor-interacting protein-1 (RIP1) (Meylan, *2004*) leads to NF-κB activation.

1.2.2.4 TLR Cooperation

Because TLR-family members can collectively recognize biomolecules such as lipid, carbohydrates, peptides and even nucleic-acids that are broadly expressed by different groups of microbes, (Table 1.2) recently scientists are trying to understand the synergistic/antagonistic relationships between TLRs.

Table 1.2. Types of ligands that pathogens expressed, for multiple TLRs (Adopted from; Trinchieri, *2007*).

Pathogen	Toll-like receptor (TLR)	TLR ligand
Mycobacterium tuberculosis	TLR2	Lipoarabinomannan
	TLR4	Phosphatidylinositol mannosides
	TLR9	DNA
Salmonella typhimurium	TLR2	Bacterial lipoprotein
	TLR4	Lipopolysaccharide
	TLR5	Flagellin
Neisseria meningitidis	TLR2	Porin
	TLR4	Lipopolysaccharide
	TLR9	DNA
Haemophilus influenzae	TLR2	Lipoprotein
	TLR4	Lipopolysaccharide
Candida albicans	TLR2	Phospholipomannan
	TLR4	Mannan
	TLR9	DNA
Murine cytomegalovirus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Herpes simplex virus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Influenza virus	TLR7, TLR8	Single-stranded RNA
	TLR3	Double-stranded RNA
	TLR4	Not determined
Respiratory syncytial virus	TLR3	Double-stranded RNA
	TLR4	Envelope F protein
Trypanosoma cruzi	TLR2	Glycosylphosphatidylinositol anchor
	TLR4	Glycoinositolphospholipid-ceramides
	TLR9	DNA
Toxoplasma gondii	TLR2	Glycosylphosphatidylinositol anchor?
	TLR11	Profilin

The early reports for TLR cooperation are shown that, there is a synergism between TLR2 and TLR4. Sato et. al. (*2000*) and Beutler et. al. (*2001*), showed that a TLR2 agonist: Mycoplasmal lipopeptide (MALP-2) or MDP and TLR4 ligand: LPS

synergistically act on peritoneal macrophages and induce production of inflammatory cytokines such as; TNF-α and IL-6. However, the induction of cross-tolerance between the two receptors and the use of MDP as a putative TLR2 ligand, which has recently been formally identified as a ligand for NOD2 (Girardin, *2003*). In one of these studies complicates the interpretation of these early reports. A subsequent study showed that stimulation of mouse macrophages with both polyI:C and CpG DNA induced more-than-additive levels of TNF, IL-6 and IL-12 p40 which confirmed that cooperation between certain TLRs does exist (Whitmore, *2004*). Equils et. al. in *2003*, in addition to their early reports that shows, TLR4 mediates LPS induction of HIV-Long terminal repeat (LTR) trans-activation through IL-1R signaling molecules and NF-κB activation, TLR2 ligand; soluble tuberculosis factor (STF) with the combination of TLR9 ligand plays a central role in HIV-LTR transactivation. Also costimulation with TLR4 and TLR2 or TLR9 elevates synergistic release of Th1 cytokines, IFN-γ and TNF-α in HIV-1 transgenic mouse spleen cells. In human and mouse DCs, TLR3 and TLR4 potently acted in synergy with TLR7, TLR8 and TLR9 in the induction of a selected set of genes. Synergic TLR stimulation increased production of IL-12 and IL- 23 from DCs However, the expression of a few genes, were also downregulated in a synergistic manner by the combined TLR stimulation (Napolitani, *2005*). Cytokine production can also be negatively regulated by simultaneous signalling through certain TLRs. Especially, the production of IL-10 after TLR2 stimulation was shown to block the expression of IL-12 p35 and CXC-chemokine ligand 10 (CXCL10; also known as IP10) by human DCs in response to either TLR3 or TLR4 ligands (Re, *2003*). Stimulation of mouse or human DCs with the TLR7 and TLR8 ligand R848 and either polyI:C or the TLR4 ligand LPS results in higher amounts of IL-12 p70 than the amounts induced by the individual TLR ligands (Roelofs, *2005*). However, when a TLR2 ligand was combined with any other TLR ligand the synergy for IL-12 p70 production was low of absent. Only a low-level synergy for IL-12 p40 (induced by MyD88 pathway), TNF-α and IL-6 production was observed when a TLR2 ligand was combined with a TLR3, TLR4 or TLR9 (Bekeredjian-Ding, *2006*). In addition to these works, CpG (TLR9) and LPS (TLR4) can cooperate in a functional manner. The synergistic effect on cytokine production from DCs was restricted to IL-12p40 and IL-12p70, but not IL-6, TNF-α or IL-10, and required a time window of about 4h pretreatment with CpG before LPS (Theiner,

2007). An example of the synergism between TLRs is that, pretreatment of mouse macrophages with the TLR9 and TLR7 ligands results in substantial decrease in the secretion of IL-6 and TNF-α in response to *B. antracis* infection of macrophages (Sabet, *2006*). This result indicates that combination of TLR ligands could be used as vaccine adjuvants for the treatment of bacterial diseases.

In addition to the cooperation between different ligands of TLRs, synergistic induction of cytokine production has also been observed for DCs or macrophages activated by a TLR ligand combined with ligands for other PRRs. Especially NOD1 and NOD2 can synergize with many TLR ligands, including TLR2 ligands, for the induction of TNF and IL-12 p40 production (Tada, *2005*). Because of the degradation of bacterial PGNs into different compounds that can activate NOD proteins, the synergy between TLR- and NLR family receptors can boost the response not only to a single pathogen but also to a single component of a pathogen (Girarin, *2005*). Besides, a TLR5 ligand flagellin could induce the immune system via both TLR and NLRs (Franchi, *2006*).

To evaluate the TLR cooperation *in vivo*, various double knock-out mice were studied. *Tlr2*$^{-/-}$ or *Tlr9*$^{-/-}$ mice at high doses of aerosol-challenge of *M. tuberculosis* are clearly susceptible to infection than are their wild-type counterparts (Bafica, *2005*). This indicates that under high levels of infectious stress, the function of each TLR involved in pathogen recognition becomes more crucial for the control of microbial growth, an explanation with possible TLR cooperation in host defence. *Tlr2* and *Tlr4* double-knockout mice are more susceptible to infection than either of the *Tlr2*$^{-/-}$ or *Tlr4*$^{-/-}$ single knock-out parental strains (Weiss, *2004* and Reiling, *2002*). During *T. cruzi* and *M. tuberculosis* infection, as judged by the bacterial load, the *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$ double-knockout mice were clearly less susceptible to infection than *Myd88*$^{-/-}$ mice, therefore indicating that other MyD88-dependent signaling events, in addition to TLR2 and TLR9 signaling, are involved in host resistance to these pathogens (Trinchieri, *2007*).

The idea that more than one TLR–ligand interactions are required for the induction of effective host resistance to pathogens has important implications for the design of superior vaccines and immunotherapy against infectious diseases. Individual

TLR7, TLR8 and TLR9 agonists have already been used successfully as adjuvants to improve CD4+ and CD8+ T-cell responses, to candidate microbial vaccine antigens. These agonists seem to be particularly effective when they are covalently conjugated to the immunogens (Krieg, *2006*).

Because of the results as mentioned above we aimed to study the cooperation of TLR ligands in dose-specific manner for the induction of immune response in a synergistic way. The determination of appropriate TLR ligands combination will guide and teach us how to include of more than one TLR ligand into vaccine formulation that induces stronger immune response, thus avoid combinations that will lower the overall immune response.

1.2.2.5. Delivery of TLR Ligands

Starting from 1990's the use of liposomes as carriers of peptide, protein, and DNA vaccines requires simple, easy-to-scale-up technology capable of high-yield vaccine entrapment. Liposomes are vesicles consisting of one or more concentric bilayers alternating with aqueous compartments. They are usually made up of phospholipids or other amphiphiles such as nonionic surfactants (Gregoriadis, *1999*). Gursel et. al. showed a technique that has been developed for the entrapment of live microbial vaccines into giant liposomes under conditions which retain their viability in 1995. They indicated that these kinds of liposomes (containing microbial vaccines and other soluble antigens or cytokines if required) could be used as carriers of vaccines in cases. Even though more stable backbone is used to synthesize CpG-ODN (a phosphorothioate modified form) when used *in vivo* still eliminated rapidly from the circulation due to the adsorption onto serum proteins and degradation by serum nucleases (Barry, *1999*), prolonging the bioavaliabilty and duration of CpG ODN by liposomal capsulation can improve their therapeutic efficiency. Pioneering studies by Gursel et. al. (*2001*) revealed that, sterically stabilized cationic liposomes (SSCL) contain positively charged and hydrophilic elements can efficiently encapsulate CpG ODN and significantly enhance DNA uptake by cells of the immune system. The immunostimulatory activity of SSCL-encapsulated ODN significantly exceeded that of free ODN *in vitro* and *in vivo*. In particular, coencapsulation of CpG ODN with a model Ag ovalbumin (OVA) increased Ag-specific IFN-γ production (10-fold) and

IFN-γ-dependent IgG_{2a} anti-OVA antibody production (40-fold), consistent with the preferential induction of a T_H1-biased immune response. In addition to that in *2005*, Xie et. al. confirmed that co-administrating CpG ODN polylactide-co-glycolide (PLG; another cationic microparticle that improves the uptake and processing of immune adjuvants) with the licensed anthrax vaccine, "AVA", resulting in a more rapid and stronger anti-protective antigen (PA; the core of anthrax vaccine) antibody response; IgG, than immunization with AVA alone *in vivo*. Not only CpG ODN but also pI:C a TLR3 ligand has been co-administrated with cationic liposomes and thereby elevated the type I IFN, IFN-α production and have a unique effective on $CD8^+$ T cell responses *in vivo* (Zaks, *2006*). Besides synthetically produced liposomes, the CpG delivery could be achieved by natural carriers, such as a *β*-(1→3)-D-glucan schizophyllan (SPG) polysaccharide from a fungus called *Schizophyllan commune*. SPG when modified with other peptides and cholesterol, and when the phosphorothioate CpG ODN complex made then exposed to macrophages, dramatic enhancement in the secretion of cytokines; like IL-6 and IL-12 secretion is observed (Mizu, *2004*).

Since some polysaccharides could be used as carrier molecules of CpG DNA. We postulated that polysaccharides from different types of edible mushrooms could reproduce the same effect thus, could serve as a novel delivery agent for nucleic acid ligands such as: CpG ODN, R848, pI:C. The enhanced *in vivo* action would implicate that these nucleic acids were protected from agile biological milieu targeted naturally to the cells of the immune system via TLR2/6 or TLR1/2 systems.

1.2.2.6. Therapeutic Implications of TLRs

The discovery of TLRs has opened up a whole new range of therapeutic possibilities, for infectious, autoimmune diseases, sepsis and cancer. (Table 1.3) Preclinical studies suggest that TLR3, TLR4, TLR7/8 and TLR9 agonists have potential to enhance therapeutic vaccination for cancer and chronic viral infections, HIV and HBV (O'Neill, *2003*). Advantage of therapeutic applications of TLR ligands compared to current vaccines are; TLR agonists enhances $CD8^+$ T-cell (kill cells that are infected with viruses) responses to protein antigens and overcoming tolerance to

self-antigens, probably necessary for generating responses to tumor-associated antigens (Hodi, *2006*).

Table 1.3. Potential role of TLRs as therapeutic targets in disease. (Adopted from O'Neill, *2003*).

TLR	Target diseases
TLR1/2	**Bacterial/fungal diseases** **Gram-positive sepsis**
TLR3	**Viral diseases**
TLR4	**Bacterial diseases** **Gram-negative sepsis** **Chronic inflammation** **Autoimmune diseases** **Vaccines** **Cancer** **Atherosclerosis**
TLR5	**Bacterial diseases**
TLR2/6	**Mycobacterial diseases**
TLR7	**Viral diseases**
TLR8	**Viral diseases**
TLR9	**Bacterial and viral diseases** **Autoimmune diseases** **Vaccines** **Cancer** **Allergy**

Since TLR3 is a key initiator of anti-viral host defense, stimulating TLR3 would be predicted to have an anti-viral adjuvant effects, in the other hand blocking TLR3 might be useful in limiting viral virulence. Targeting of TLR3 with Ampligen (with a synthetic mismatched dsRNA,) for HIV is currently in phase II trials (Hemispherx). Other viral TLR7/8 ligands, imiquimod and resiquimod have been studied in cutaneous disorders, like basal cell carcinoma and moles that are caused by HPV and shown that they induce cytokine production and elevate cutaneous immune responses (Licenced by 3M Pharma company).

The probable key driver of TNF during sepsis, TLR4 have been targeted mainly in allergic diseases. Blocking of TLR4 is of use in the prevention of over-exuberant immune response induced in sepsis and autoimmune diseases such as Familial Mediterranean Fever and uveitis. Indeed TLR 4 antagonists have been in

phase III clinical trials for the treatment of severe sepsis (Takeda Pharmaceutical and Eisai).

TLR9 ligands; CpG ODNs has been in clinical trials for treatment of cancers, like melanoma, breast cancer, leukemia, non-small-cell lung, renal and colorectal cancers (Krieg, *2007*). Conjugation of hepatitis B virus plus CpG ODN developed 15-fold higher anti-hepatitis B antibody titers than did animals immunized with vaccine alone (Klinman, *2004*). Consequently Hepsilav, a candidate HBV vaccine is in phase III trials (Dynavax Technologies). Also VaxImmune (CpG B class ODN) for anthrax and influenza antigens with CpG ODN for influenza are in clinical phase studies. In monkey models, CpG ODN are capable of inhibiting airway hyper responsiveness, eosinophilia and even features of airway remodeling (Fanucchi, *2004*). Therefore, four different CpG DNAs are targeted to suppress asthma (Dynavax Technologies, Coley Pharmaceuticals, Idera Pharmaceuticals).

TLR4 and TLR9 agonists are being developed for the cure of allergic rhinitis because of their ability to induce strong Th1 responses (Racila, *2005*)

Many companies with preclinical antagonists to the intracellular TLRs 7, 8 and 9 have shown efficiency in models of SLE (Dynavax Technologies, Coley Pharmaceuticals and Idera Pharmaceuticals).

In conclusion, variety of TLR agonists or antagonists for the most severe diseases are undergoing preclinical and various stages of clinical trials.

Since more than one TLR ligand could induce more powerful immune activation, we propose that introduction of two or more TLR ligands in vaccination could provide more efficient, robust and long-lasting immune-response.

2. AIM OF STUDY

Studying the immunobiology of the Toll-like receptors is one of the most popular fields in basic and applied immunology in recent years. There is no doubt-that TLR therapeutics soon will be in the clinics. The TLR ligand(s) are strong candidate drugs as an immunotherapeutic agent against diseases like cancer, allergy, and infectious diseases (including viral, parasitic and bacterial infections) as well as vaccine adjuvant or immunoprotective agent where there is no available vaccine. Recently, accumulating data indicate that two or more TLR ligands can cooperate to mount a suitable immune response in order to contain a pathogenic insult. We proposed that including more than one TLR ligands in vaccine formulations would provide an added benefit and help to establish a more efficient and stable immune response.

Because of this, the first part of this study was dedicated to reveal the antagonistic/synergistic cooperation of several candidate TLR ligands. These were the most promising, and potent extracellular and endosomal-associated TLR ligands members planned for the clinical trials (i.e. TLR2/6, TLR3, TLR4, TLR7/8 and TLR9).

The second part of the study is dedicated to establish a novel natural delivery depot system especially designed for the targeted delivery of candidate nucleic acid based TLR ligands (because of their lability in biological milieu as well as low level accumulation within relevant immune cells). For this, we have selected candidate polysaccharides extracted from different mushrooms and first characterized their stand alone immunostimulatory potential and then prepared complexes with the candidate RNA and DNA ligands and tested their synergistic effect on spleen cells.

3. MATERIALS AND METHODS

3.1. MATERIALS

All cell culture media components were from Hyclone (USA) unless otherwise stated. Cytokine pairs and recombinant proteins for ELISA were from Endogen (USA) unless otherwise mentioned. TLR ligands; for stimulation assays were as follows and supplied from several vendors: peptidoglycan (PGN) (isolated from *B.subtilis*; Fluka, Switzerland), pI:C (Amersham, UK), lipopolysaccharide (LPS) (isolated from *E.coli*; Sigma, USA), Zymosan (isolated from *S.cerevisiae*; Invivogen, USA), phosphorothioate backbone modified synthetic CpG ODN 1555 (15mer) (GCTA*GACGTT*AGCGT), CpG ODN 2006 (24mer) ('TCGTCGTTTT*GTCGTTT*TGTCGTT or CpG ODN K3 (20mer) (*ATCG*ACTC*TCGAGCGT*TCTC) as control ODN; and 1612 ODN (GCTAGATGTTAGCGT) (Alpha DNA, Canada) K3 Flip ODN (ATGCACTCTGCAGGCTTCTC), R848 or gardiquimod (Invivogen, USA). Four different polysaccharide extracts were kind gift from Prof. Dr. Oktay Erbatur (Cukurova University, Chem Dept., Adana, Turkey). They were isolated from *Shiitake* and the different strains (Balcali and Alata) of *Ganoderma lucidum*, under subcritical water conditions at 100°C (AROsm, BROsm) and 150°C (AR150, BR150). For RNA isolation and for cDNA synthesis, obtained from TRIdity G (AppliChem, Germany) and DyNAmo^{TM} cDNA Syntesis kit (Finnzymes, Finland) respectively and were used according to the manufacturer's protocol. HEK 293 and RAW cells were transfected with Fugene6 (Roche, Germany) or Lyovec (Invivogen, USA) and Luciferase acitivity was detected with using Promega kit. Several plasmids were expanded in house and purified using Endotoxin free plasmid isolation kit from Qiagen (Germany).

3.1.1. Polysaccharides

For the coding of four different polysaccharide extracts, see appendix A

3.1.2. Standard Solutions, Buffers, Media

See appendix B

3.2. METHODS

3.2.1. The Maintenance of the Animals

Adult male or female BALB/C mice (8-12 weeks old) were used for the experiments. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22^{o} 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).

3.2.2. Cell Culture

3.2.2.1. Spleen Cell Preparation

Spleens were removed from the BALB/C female mice after cervical dislocation. Single cell suspensions were obtained by smashing of spleens with the back of the sterile syringes by circular movements suspended in the 2% FBS supplemented regular RPMI media. The cells were washed 2-3 times at 1500 rpm for 10 mins. The cell pellet was gently dislodged with fresh media, the tissue debris was removed and finally the splenocyte suspension was counted and adjusted to 2-4x10^{6}/ml unless otherwise stated.

3.2.2.2. Cell Lines

3.2.2.2.1. RAW 264.7

Macrophage / monocyte like RAW 264.7 (*Mus musculus*) cells (ATCC) were cultured with RPMI 1640 plus 5% regular FBS. Adherent RAW 264.7 cells were passaged in every 3-4 days when they reached >90% confluency with fresh media, following washing (2% FBS containing RPMI).

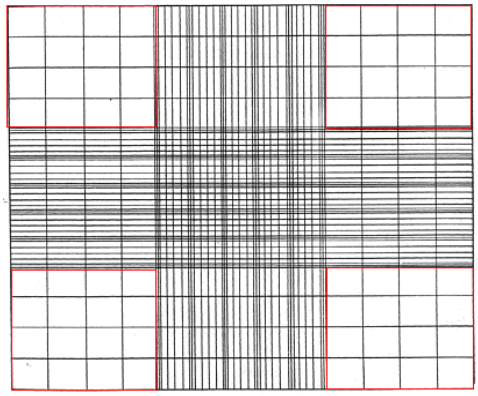
3.2.2.2.2. HEK 293 hTLR2/6

Adherent HEK cells, that stably expresses *hTLR2/6* genes (Invivogen) were sustained in a High-glucose DMEM media with 5% or 10% regular FBS (Hyclone) supplemented with 10µg/ml Blasticidin S (Invivogen). Cells were passaged by scraping in every 3-4 days intervals as they get over 90% confluency.

3.2.2.3. Cell Number Detection with Thoma Cell Counter

After the spleen cells, RAW cells, HEK 293 hTLR2/6 were pooled, washed and precipitated, they were suspended in 10 ml of 5% regular RPMI-1640 media. Cells were diluted 10 fold and micropipetted on a hemocytometer.

The number of cells in the chamber was determined by counting under the light microscope from these gridlines as indicated with red areas:



The cell number was calculated according to the following formula:

$$\left[\frac{\text{Cell number}}{4}\right]\left[10^6\right] = \text{Total cell number in 10 ml media}$$

3.2.2.4. Cell Distribution

For NO Assay and Cytokine ELISA; 2-4x10^6/ml cells were distributed into 96 well plates with a final volume of 200µl or 250µl media per well. After 6 to 42 hours stimulation, supernatants were collected from the plates and stored at -20°C. Supernatants were layered on the 96 well plates with or without dilution for two assay as previously mentioned.

Spleen cells (6-8 x 10^6) were splitted into 6 well plates or 15 ml falcons with a final concentration 2-3 ml, for RNA isolation after stimulation with TLR ligands for 2 and 4 hours.

For the transfection assay 2-4x10^6/ml HEK 293 hTLR2/6 cells were added to 24 well plates, in a 500 µl/ well media final volume.

3.2.3. Stimulation with Ligands and Polysaccharides

Spleen cells were stimulated with the combination of two TLR ligands in various doses (i) PGN; 5 µg/ml, (ii) pI:C; 20 µg/ml, (iii) LPS; 1 µg/ml, (iv) R848; 1 µg/ml and (v) CpG ODN 1555, including control either K3 Flip ODN or 1612 ODN; 1 µM. These ligands were diluted in 5x, 25x, 125x, 625x doses and used for stimulations.

For the stimulation assays including polysaccharides, RAW and HEK 293 hTLR2/6 cells were incubated with PS-1, PS-2, PS-3 and PS-4 for concentration ranges between 20 µg/ml to 0,002 µg/ml. PGN; 20 µg/ml- 0,002 µg/ml or Zymosan 20 µg/ml- 0,02 µg/ml and LPS 5 µg/ml- 0,5 µg/ml including control ODN; 3 µg/ml- 0,3 µg/ml.

Cells were incubated with 5% oligo FBS RPMI-1640 or 5% oligo FBS DMEM when stimulated with ODNs and cultured with 5% regular RPMI-1640 or 5% regular FBS supplemented DMEM as they stimulated with other TLR Ligands and polysaccharides.

3.2.4. Delivery of TLR Ligands with Polysaccharides

Sonicated (at PMA 48%, 1 min.) PS-4 was mixed PS:ligand (1:1 w/w) with CpG 2006 ODN, CpG, K3 Flip ODN, pI:C (pI:C was mixed at 1:5 ratio as well), and R848 seperately and these five different mixtures were incubated over night at 4°C prior to stimulation of RAW cells for 24-42 hours with a starting concentrations of 0,5μM; CpG ODN 1555, 0,5μM; K3 Flip ODN, 20 μg/ml and 4 μg/ml; pI:C, 2 μg/ml; R848, 2 μg/ml; PS-4 for NO Assay and Cytokine ELISA. These stimulants were further diluted up to 625x and used for cell stimulation.

3.2.5. Enzyme Linked-Immunosorbent Assay (ELISA)

Cytokine ELISA

Polysorp (F96 Nunc-Immunoplate, NUNC, Germany) plates were coated with anti-cytokine IL-4, IL-6, IL-12, IFNγ, TNFα monoclonal antibodies (Pierce, Endogen); 5 μg/ml, 10 μg/ml, 5 μg/ml, 5 μg/ml, 5 μg/ml respectively for 4-5 hours at room temperature or overnight at +4°C. The wells were blocked with 200 μl blocking buffer for 2 hours at room temperature and washed with wash buffer for 5 minutes, 5 times and rinsed with ddH_2O at the end of the washes. Supernatants and serially diluted recombinant proteins of IL-4 (1000 ng/ml), IL-6 (2000 ng/ml), IL-12 (100 ng/ml), IFNγ (2000 ng/ml), TNFα (2000 ng/ml) were added and incubated for 2 hours at room temperature or overnight at +4°C. Plates were washed as previously described. For the detection of cytokine levels; biotinylated anti-cytokine antibodies (Pierce, Endogen) were prepared in a T-cell buffer, 1:1000 dilution, added to the wells and incubated for 2 hours at room temperature or overnight at +4°C, followed by washing as described earlier. 1:5000 diluted Streptavidin Alkaline-phosphatase (SA-AKP) was prepared in T-cell buffer and added to the plates for 1 hour at room temperature. After washing the plates; PNPP substrate (Pierce) was added and after color formation, multiple optical density readings at target 405 nm, were recorded on an ELISA plate reader (BioTek, μQuant).

Concentrations of the cytokines from cell supernatants were determined by constructing 4-parameter standard curves, from recombinant proteins as mentioned above.

3.2.6. NO_2 Detection

Griess Assay

For the calorimetric detection of Nitrate (NO_2) level, that was produced by macrophages, NO Assay using Griess Reagent (Sigma) was performed. Supernatants were added, either diluted or as such, to the microtiter plates. For the linear standard curve; 100µM $NaNO_2$, which is dissolved in NO free H_2O, with 2x dilution, prepared. Equal volumes of Greiss Reagent and supernatants were mixed. After 15 min. plates optical densities at 540 nm were measured on a plate reader.

3.2.7. Transfection of Cells

Stably transfected HEK 293 cells with hTLR2/6 were transiently transfected with cationic lipid molecule FuGENE 6 as decribed in manufacturer's protocol. Briefly, 6µl FuGENE 6 is added to 94 µl (serum and penicillin/streptomycin free media) in an eppendorf. Mixture was vortexed for 3-5 secs, and then incubated at room temperature for 5 min. 2µg p5xlucNF-κB Luciferase plasmid was added to the mixture and further incubated for 15-30 min. The transfection mixture was distributed 20 µl/well in a drop wise manner as a control GFP expressing plasmid or mock plasmid was used for transfectin. Wells were swirled to ensure distribution over the entire plate surface. Cells were incubated for 24-36h prior to specific stimulations.

3.2.8. Luciferase Assay

After 12-24 hours stimulation, media was removed, each well was washed gently with warm 1x PBS. 100 µl/well Luciferase substrate reagent was added for cell lysis. Plates were left on a rocking shaker for 15 min. at room temperature.

Supernatants were transferred into a new white 96 well plate and relative light signal from cell extracts were recorded on a Reporter Machine™ (Turner Biosystems, USA).

3.2.9. Determination of the Gene Expression

3.2.9.1. Total RNA Isolation from the Cells

After incubating cells (spleen, RAW) with combinations of TLR Ligands and polysaccharides, for 2 and/or 4 hours, total RNAs were extracted. The cells were removed and centrifuged at 2500 rpm for 5 min. in cold media. Then the media was removed and 10^7 cells were extensively mixed and lysed by a 1 ml of mono-phasic solution of phenol and guanidinium thiocyanate: TRItidy G (Applichem, Germany). 200 µl of chloroform for every 1ml of TRItidy G was used and tubes were shaked vigorously for 15 seconds and incubated at room temperature for 2-3 min. followed by a centrifugation for 15 min. at 13.900 rpm at 4°C. The clear aqueous phase was transferred to a fresh tube. Total RNA was precipitated by adding 500 µl of 2-propanol for every 1ml of TRItidy G, incubated at room temperature for 10 min. and centrifuged for 10 min. at 13.900 rpm. Next the supernatant was removed and the pellet was washed with 1 ml of 75% EtOH for every 1 ml of TRItidy G used. Tubes were vortexed and centrifuged at 8000 rpm for 7 min. in order to remove 2-propanol from the pellet. Supernatant was discharged and pellet was washed with >99.9% EtOH, vortexed and centrifuged as previously described. After centrifugation, the alcohol was removed and pellet was dryed under laminair flow hood, and dissolved with 20-30 µl RNase/DNase free H_2O. The OD measurements were taken at 260/280 nm wavelengths with the spectrophotometer NanoDrop® ND-1000 (NanoDrop Technologies, USA). The expected value of the A_{260}/A_{280} ratio in order to determine if there is a phenol, protein or DNA contamination in the RNA samples was between 1.8-2.0. The isolated RNA was stored at -80°C for further use.

3.2.9.2. cDNA Synthesis and PCR

3.2.9.2.1. cDNA Synthesis

The cDNAs were synthesized from the total RNA samples with the cDNA synthesis kit according to the manufacturers' protocol. 1-2µg RNA was mixed with 1µl of Oligo(dT) primer and completed to a total volume of 12 µl with RNase DNase free H_2O (Hyclone). They were pre-denatured at 65°C for 5 min. then chilled on ice for 3-5 min. 15µl RT Buffer (includes dNTP mix and 10 mM $MgCl_2$) and 3µl M-MuLV RNase H^+ reverse transcriptase (includes RNase inhibitor) were added to the mixture and incubated at 25°C for 10 min., 40°C for 45 min., 85°C for 5 min. and on ice (+4°C) for 10 min. respectively. cDNA's were runned on 2% Agarose gel for 45-50 min, at 80V and visualized under transilluminator (Gel-Doc BIO-RAD, USA and Vilber Lourmat, France) for 1 sec exposure time. The cDNA's were stored at -20°C.

3.2.9.2.1.2. PCR

3.2.9.2.1.2.1. Primers

Primers such as; *il-18*, *cxcl16*, *mip-3α* 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 data available in the literature and references were cited at their proper primer set (Table 3.1).

Table 3.1. The sequences, the sizes and the sources of the primers used.

Primer		**Sequence**	**Product Size (base pair)**
β-actin	Forward Reverse	:5'-GTATGCCTCGGTCGTACCA -3' :5'-CTTCTGCATCCTGTCAGCAA -3'	450 bp

mTLR-1	Forward	:5'-TTTGGGGGAAGCTGAAGACATC -3'	410 bp
	Reverse	:5'-CTTCGGCACGTTAGCACTGAGAC-3'	
mTLR-2	Forward	:5'-TCTCTGGGCAGTCTTGAACATTTG -3'	320 bp
	Reverse	:5'-CGCGCATCGACTTTAGACTTTG- 3'	
mTLR-3	Forward	:5'-GGGGCTGTCTCACCTCCAC-3'	250 bp
	Reverse	:5'-GCGGGCCCGAAAACATCCTT- 3'	
mTLR-4	Forward	:5'-TGCCGTTTCTTGTTCTTCCTCT -3'	240 bp
	Reverse	:5'-CTGGCATCATCTTCATTGTCCTT - 3'	
mTLR-5	Forward	:5'- TGGGGCAGCAGGAAGACG -3'	380 bp
	Reverse	:5'- AGCGGCTGTGCGGATAAA- 3'	
mTLR-6	Forward	:5'- GCCCGCAGCTTGTGGTATC - -3'	650 bp
	Reverse	:5'- GGGCTGGCCTGACTCTTA - 3'	
mTLR-7	Forward	:5'- TTAACCCACCAGACAAACCACAC -3'	700 bp
	Reverse	:5'- TAACAGCCACTATTTTCAAGCAGA - 3'	
mTLR-9	Forward	:5'- GATGCCCACCGCTCCCGCTATGT - 3'	430 bp
	Reverse	:5'- TGGGGTGGAGGGGCAGAGAATGAA - 3'	
mIP-10[*]	Forward	:5'-GCCGTCATTTTCTGCCTCAT- 3'	127 bp
	Reverse	:5'- GCTTCCCTATGGCCCTCATT- 3'	
mTNF-α[*]	Forward	:5'- CATCTTCTCAAAATTCGAGTGACAA - 3'	175 bp
	Reverse	:5'- TGGGAGTAGACAAGGTACAACCC - 3'	
*mIL-15**	Forward	:5'- CATCCATCTCGTGCTACTTGTGTT - 3'	126 bp
	Reverse	:5'- CATCTATCCAGTTGGCCTCTGT - 3'	
*mIL-6**	Forward	:5'- GAGGATACCACTCCCAACAGACC - 3'	141 bp
	Reverse	:5'- AAGTGCATCATCGTTGTTCATACA - 3'	
mIL-18[¶]	Forward	:5'-ACAACTTTGGCCGACTTCAC - 3'	491 bp
	Reverse	:5'- ACAAACCCTCCCCACCTAAC - 3'	
mMCP-1	Forward	:5'- AGGTCCCTGTCATGCTTCTG - 3'	249 bp
	Reverse	:5'- TCTGGACCCATTCCTTCTTG - 3'	
mCXCL16[¶]	Forward	:5'- CGCTGGAAGTTGTTCTTGTG - 3'	384 bp
	Reverse	:5'- GGTTGGGTGTGCTCTTTGTT - 3'	
mMIP-3α[¶]	Forward	:5'- CGTCTGCTCTTCCTTGCTTT - 3'	237 bp
	Reverse	:5'-CCTTTTCACCCAGTTCTGCT - 3'	
*mCD40**	Forward	:5'-GTCATCTGTGGTTTAAAGTCCCG - 3'	91 bp
	Reverse	:5'- AGAGAAACACCCCGAAAATGG - 3'	

¶ Designed primer

* Adopted from Giulietti et. al., *2001*

3.2.9.2.1.2.2. Semi-Quantitative RT-PCR

For the comparison of the mRNA expression levels of the samples, semiquantitative reverse-transcriptase PCR (MJ Mini, BIO-RAD, USA) was performed. Quantification of the band intensities was performed using MultiAnalyst and Bio1D softwares. The quantitated values for the samples were normalized by the division of the quantitated values of–corresponding *β-actin* sample intensities. The reaction ingredients used in PCR reactions are shown in Table 3.2 and the condition of PCR reactions are shown in Table 3.3.

Table 3.2. PCR reaction composition

Reaction Ingredients	Volume
cDNA	1 µl
DyNAzyme TM^{II} Master Mix (Finnzymes)	12,5 µl
Forward Primer (Alpha DNA)	1 µl (10 pmol)
Reverse Primer (Alpha DNA)	1 µl (10 pmol)
RNase DNase free H_2O	9,5 µl
Total	**25 µl**

Table 3.3. PCR running conditions

94 °C, 30 sec
55 °C, 30 sec
72 °C, 1 min.
} **35 cycles**

Final Extension: 10 min. at 72 °C

3.2.9.2.1.2.3. Agarose Gel Electrophoresis

2% agarose gel was prepared with 1X TAE buffer and 1 mg/ml ethidium bromide solution. Lanes were loaded by mixing 5µl agarose gel loading dye with 10µl

of cDNA sample. For every gel, the low range DNA ladder (Jena Biosciences) was used as a marker and the gels were visualized as previously mentioned. Comparisons of the cDNA band intensities for the analysis were done with BIO-PROFILE Bio-1D V11.9 sofware.

3.2.10. Atomic Force Microscopy (AFM)

Polysaccharides diluted in DNase/RNase free H_2O were deposited on silicone wafer. Following complete drying images were taken by using non-contact mode XE-100E model AFM (PSIA with XEI 1.6 software incorporated) with a 0.73-0.79 Hz scanning rate. The scanning area sizes were in range from 3µm x 3µm to 0.5µm x 0.5µm. AFM images were taken with the help of Dr. Aykutlu Dana's group.

3.2.11. Statistical Analysis

Statistical significance was determined between unstimulated group (or control ODN stimulated) versus stimulated groups using SigmaSTAT software via Student's t- test analysis.

4. RESULTS

4.1 Studies to Establish the Benefit of Using More Than One TLR Ligand Combinations in Immunotherapy

Recent findings suggest that more than one TLR members can collectively recognize "non-self" lipid, carbohydrate, peptide (or combinations thereof) and nucleic-acid structures that are broadly expressed by various infectious microorganisms. This concerted activation is the key to control pathogen attack and spread. Understanding the synergistic and/or antagonistic interaction of more than one TLR ligand during the initial induction on immune cells is critical and may contribute to develop more effective vaccine adjuvants or immunotherapeutic agents. The prime aim of this project is to first delineate these interactions by systematically combining different ligands and determining how these combinations alter the immune response (i.e. determine whether ligand combinations increase or decrease the immune activation of the spleen cells compared to a single ligand activation).

4.1.1 Determining the Most Adequate Combination of TLR Ligands

Preliminary optimization assays were undertaken to establish the optimum and sub-optimum doses of different ligands required for murine splenocyte stimulation. For that, varying ligand concentrations were tested on the mouse spleen cells in culture for 42 h. The cell supernatants were collected and stored at -20°C and then were subjected to cytokine ELISA. Initial experiments revealed that two different doses could be used for further experiments. We have named these concentrations (based on their stimulation potency) as optimum and sub-optimum ligand doses. Table 4.1 summarizes the ligands and their combinations used throughout the rest of the study. We have decided to carry out the rest of the experiments using spleen cells rather than isolated single cell type. The major reason for this is that when these agents are given *in vivo*, many cell types are influenced by these ligands (either

directly or upon co-stimulation) so, to see overall activation effect we believed that using a mixed cell population (such as spleen cell population) rather than looking at a single cell type is going to be more informative and conclusive as well as close to *in vivo* setting. Therefore, we have conducted our further ligand cooperation studies using freshly isolated single cell suspension of BALB/C mice splenocytes.

Table 4.1. The list of TLR ligands which were used for the stimulation of spleen cells

Group #	TLR Ligands	Stimulated TLR	Optimum Concentration	Suboptimum Concentration
1	Naïve	*None*	Only media	Only media
2	Control ODN (1612)	*None*	1µM	0,2µM
3	Peptidoglycan (PGN)	TLR2/6	5 µg/ml	1 µg/ml
4	pI:C	TLR3	20 µg/ml	4 µg/ml
5	Lipopolysaccharide	TLR4	1 µg/ml	0,2 µg/ml
6	R848 (Imiquimod®)	TLR7/8	1 µg/ml	0,2 µg/ml
7	CpG ODN (1555)	TLR9	1µM	0,2µM
8	Control ODN + PGN	— + TLR2/6	1µM + 5 µg/ml	0,2µM + 1 µg/ml
9	Control ODN + pI:C	— + TLR3	1µM + 20 µg/ml	0,2µM + 4 µg/ml
10	Control ODN + LPS	— + TLR4	1µM + 1 µg/ml	0,2µM + 0,2 µg/ml
11	Control ODN + R848	— + TLR7/8	1µM + 1 µg/ml	0,2µM + 0,2 µg/ml
12	Control ODN + CpG ODN	— + TLR9	1µM + 1µM	0,2µM + 0,2µM
13	CpG ODN + PGN	TLR9 + TLR2/6	1µM + 5 µg/ml	0,2µM + 1 µg/ml
14	CpG ODN + pI:C	TLR9 + TLR3	1µM + 20 µg/ml	0,2µM + 4 µg/ml
15	CpG ODN + LPS	TLR9 + TLR4	1µM + 1 µg/ml	0,2µM + 0,2 µg/ml
16	CpG ODN + R848	TLR9 + TLR7/8	1µM + 1 µg/ml	0,2µM + 0,2 µg/ml
17	R848 + PGN	TLR7/8 + TLR2/6	1 µg/ml + 5 µg/ml	0,2 µg/ml + 1 µg/ml
18	R848 + pI:C	TLR7/8 + TLR3	1 µg/ml + 20 µg/ml	0,2 µg/ml + 4 µg/ml
19	R848 + LPS	TLR7/8 + TLR4	1 µg/ml + 1 µg/ml	0,2 µg/ml + 0,2 µg/ml
20	pI:C + PGN	TLR3 + TLR2/6	20 µg/ml + 5 µg/ml	4 µg/ml + 1 µg/ml
21	pI:C + LPS	TLR3 + TLR4	20 µg/ml + 1 µg/ml	4 µg/ml + 0,2 µg/ml
22	PGN + LPS	TLR2/6 + TLR4	5 µg/ml + 1 µg/ml	1 µg/ml + 0,2 µg/ml

4.1.1.1 Cytokine ELISA

In order to understand the nature of the immune response with these ligand combinations i) trigger a Th1-biased (judged by IL-6, TNF-α or IFN-γ production) or ii) a Th2 (IL-4 secretion) dominated immune activation, in addition to a pronounced cytokine production upon more than TLR addition to the cell culture media, we determined IL-6, TNF-α, IFN-γ and IL-4 cytokine secretion from culture supernatants following cell stimulation.

One of the main proinflammatory cytokine, IL-6 which is mainly secreted by T-cells B-cells and macrophages; mainly in response to a bacterial infection was

highly secreted in the combinations of CpG either with PGN (TLR2), pI:C (TLR3), or LPS (TLR4) increased IL-6 production by about 2-3 fold independent of the tested ligand concentrations (Fig. 4.1, and Fig. 4.2). When a TLR3 ligand pI:C plus R848 (TLR7/8) was used, IL-6 production from spleen cells was significantly increased (Fig. 4.1 and Fig. 4.2) compared to either of the single ligand stimulation. Similar trend but substantially at a lower level PGN mixture was also increased IL-6 level (Fig. 4.1 and Fig. 4.2). When IL-6 profile is collectively assessed, data suggest that endosome-associated TLRs act synergistically and exceed IL-6 production seen by a single TLR ligand, whereas, pI:C plus LPS did not show any improvement in the cytokine level.

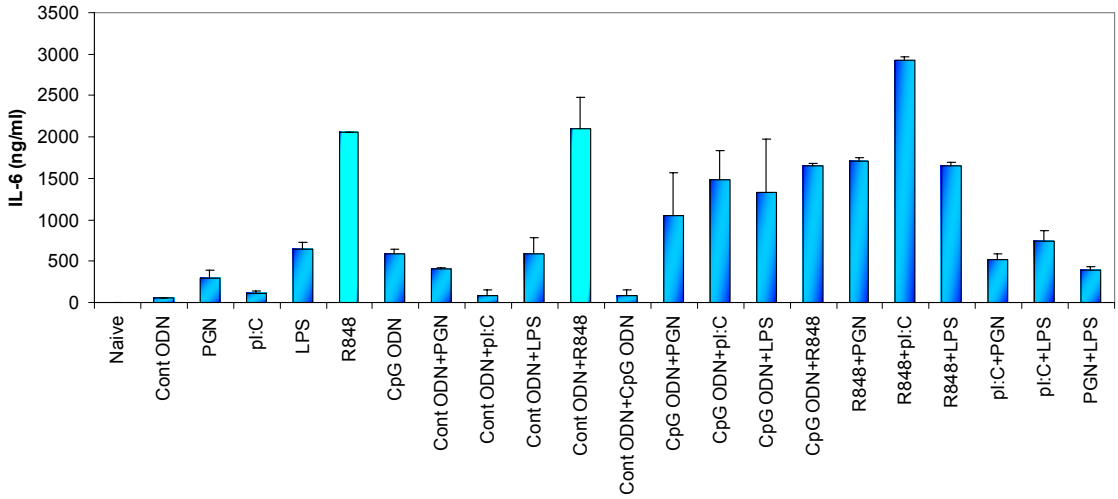


Figure 4.1. IL-6 production from spleen cells following stimulation with various TLR ligand combinations set at optimum induction doses. *(Cont ODN; Control ODN).* $p <$ 0,02 (compared with unstimulated or control group).

Stimulation of spleen cells with CpG ODN plus PGN or LPS synergistically enhanced the IFNγ mediated cytokine production. However, LPS, PGN or CpG ODN plus R848 showed no substantial improvement (or inhibition) of IFNγ production (Fig. 4.3 and Fig. 4.4).

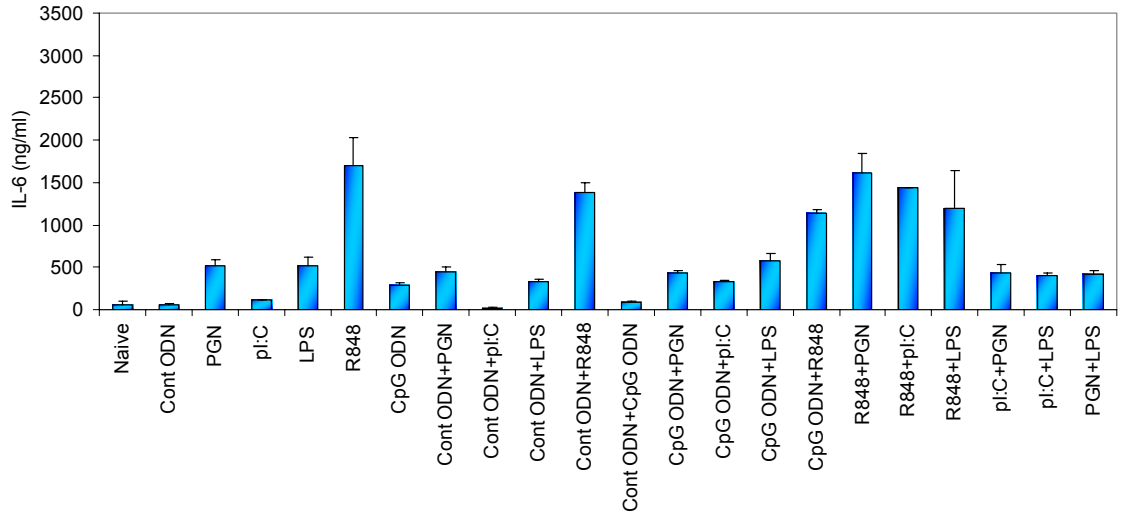


Figure 4.2. IL-6 production from spleen cells following stimulation with various TLR ligand combinations set at suboptimum induction doses. $p < 0{,}04$ (compared with unstimulated or control group).

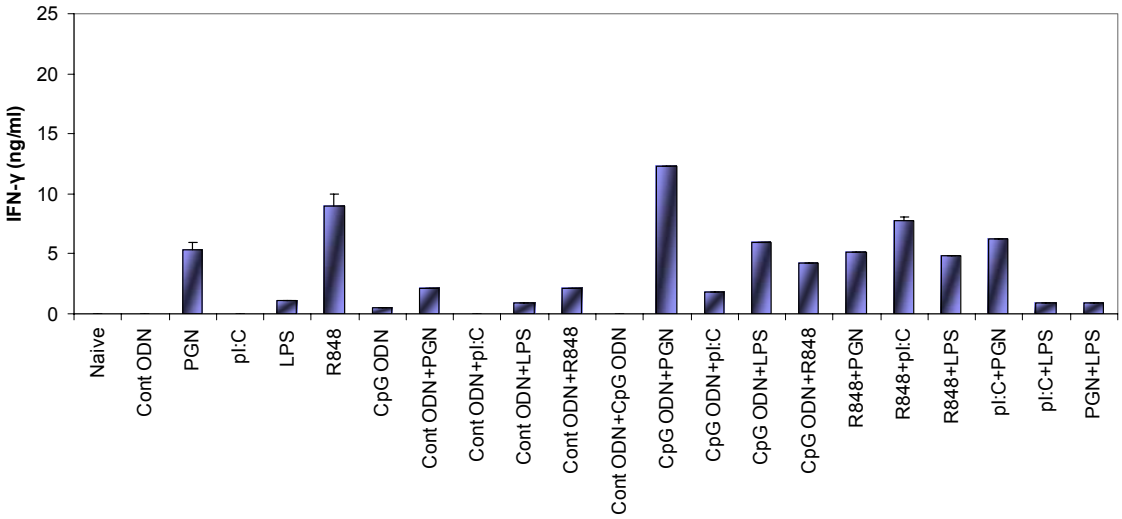


Figure 4.3. IFN-γ production determined by ELISA from spleen cells which were stimulated with optimum doses of TLR ligands for 42 hours. $p < 0{,}001$ (compared with unstimulated or control group).

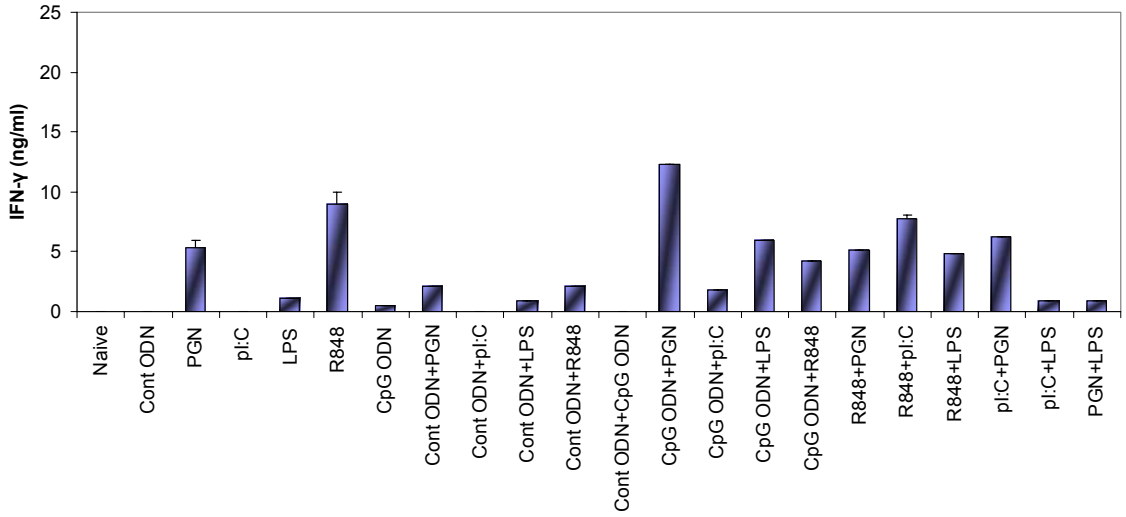


Figure 4.4. IFN-γ production determined by ELISA from spleen cells which were stimulated with suboptimum doses of TLR ligands for 42 hours. $p < 0{,}001$ (compared with unstimulated or control group).

Tumor necrosis factor-alpha (TNF-α) is another cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction with IL-6. ELISA results of TNF-α secretion with the co-stimulation of TLR ligands resulted as; R848 plus CpG, LPS and Control ODN raised >2 fold in both optimal (Fig. 4.5) and suboptimal (Fig. 4.6) doses. Also, at all doses, cooperation of pI:C with PGN significantly elevated the TNF-α level in spleen cells. TLR3 ligand pI:C or TLR2 ligand PGN with CpG ODN antagonistically acted on the TNF-α production after 42 hours stimulation, similar result was seen with Control ODN plus pI:C group (at two different doses).

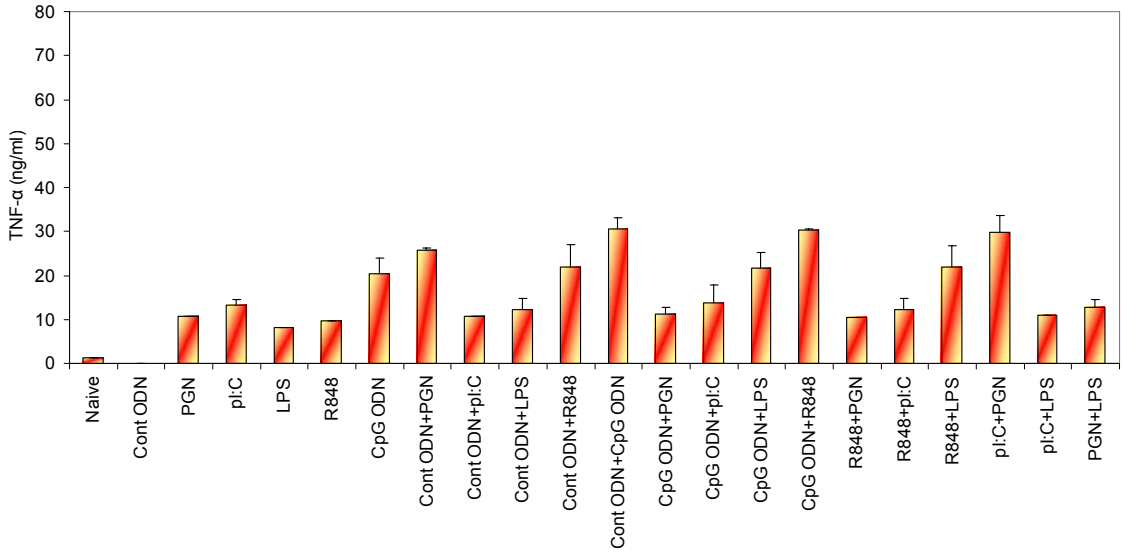


Figure 4.5. TNF-α production from spleen cells following stimulation with various TLR ligand combinations set at optimum induction doses. $p < 0{,}001$ (compared with unstimulated or control group).

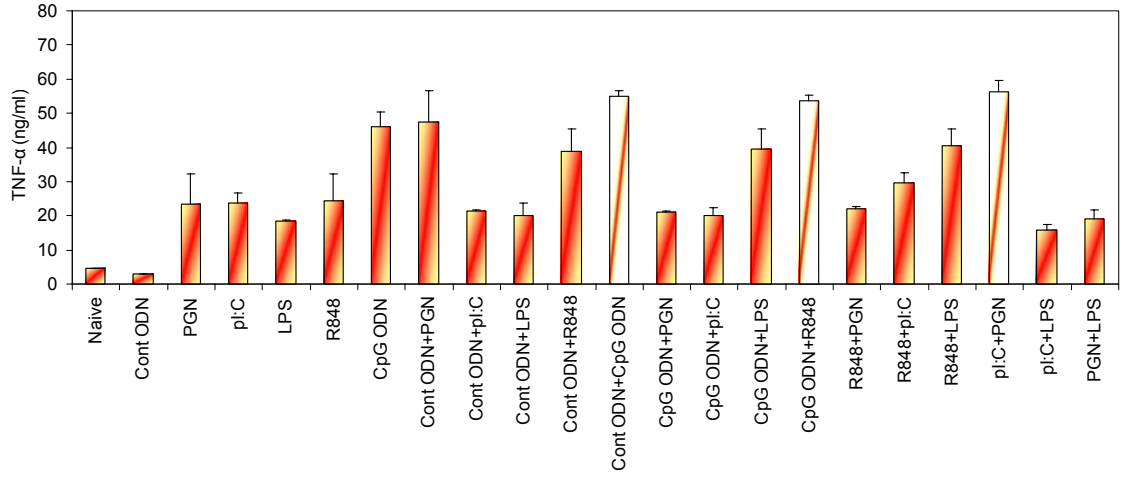


Figure 4.6. TNF-α production from spleen cells following stimulation with various TLR ligand combinations set at suboptimum induction doses. $p < 0{,}001$ (compared with unstimulated or control group).

IL-4 ELISA was also performed to establish if any of these combinations trigger a Th-2 biased inflammatory response (Fig. 4.7, Fig. 4.8). Combination of TLR4 ligand LPS either with PGN or pI:C significantly surpassed LPS only IL-4 level by spleen cells. Also similar results were seen with R848 plus pI:C or PGN or with PGN plus CpG ODN groups (Fig. 4.7 and Fig. 4.8).

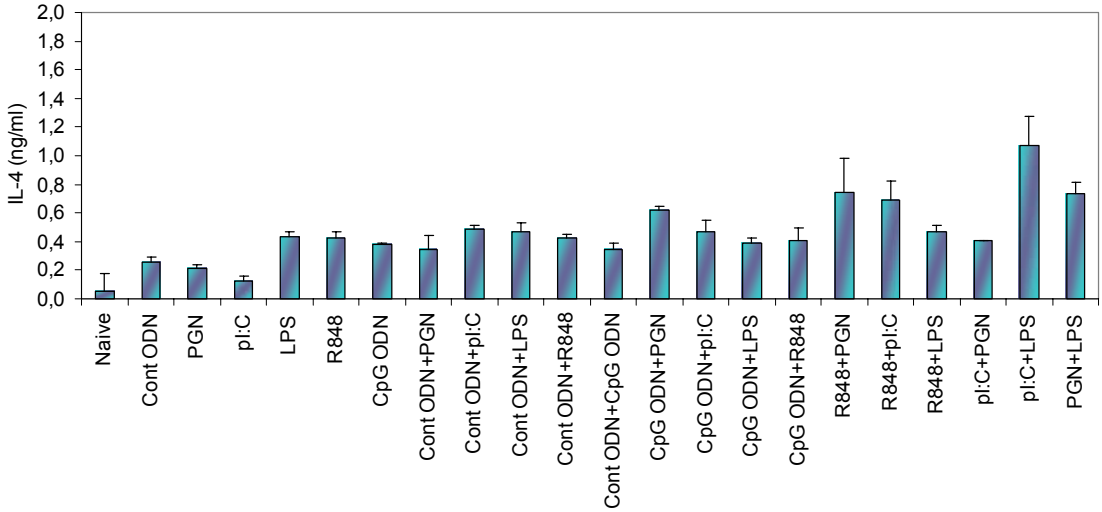


Figure 4.7. IL-4 production from spleen cells following stimulation with various TLR ligand combinations set at optimum induction doses. $p < 0{,}001$ (compared with unstimulated or control group).

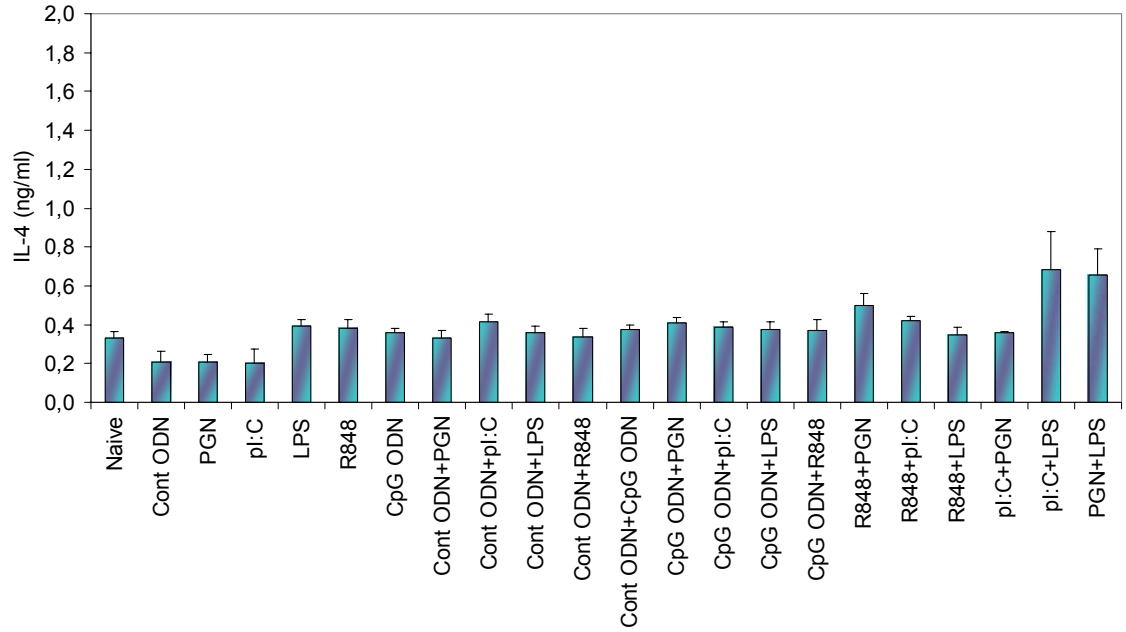


Figure 4.8. IL-4 production from spleen cells following stimulation with various TLR ligand combinations set at suboptimum induction doses. $p < 0{,}03$ (compared with unstimulated or control group).

Table 4.2 summarizes four cytokine ELISAs and demonstrates which treatment group up (+) or down (–) regulated these cytokines. In general, the combination of R848 with TLR ligands such as: pI:C, PGN, CpG ODN and

combination of CpG ODN plus PGN, pI:C, LPS gave synergistic immune activation for at least two different Th1 type cytokines. It is worth to note that the LPS combinations either with pI:C, PGN or R848 had very little or no positive effect on the overall Th1 biased immune response and significantly increased Th2 response, therefore it is of caution to combine these ligands if the aim is to boost the Th1 immunity, but on the other hand if the idea is to boost Th2 biased immune response then it could prove beneficial. As expected there was no significant change of cytokine levels when Control ODN was mixed with these TLR ligands (Fig 4.1 to 4.8 and Table 4.2).

Table 4.2. Upregulated and downregulated cytokine profiles compared to single TLR ligand induction level. (***red circles****; antagonism,* ***green circles****; synergism*).

	ID	TNFα		IFNγ		IL-6		IL-4	
		Opt.	Subopt	Opt.	Subopt	Opt.	Subopt	Opt.	Subopt
1	Naive	- -	- -	- -	- -	- -	- -	-	+/-
2	Cont	- -	- -	- -	- -	- -	- -	+/-	-
3	PGN	+/-	+/-	+	+ +	-	+/-	-	-
4	pI:C	+/-	+/-	- -	-	- -	- -	-	-
5	LPS	-	-	-	+	+/-	+/-	+	+/-
6	R848	-	+/-	+ +	+	+ +	+	+	+/-
7	CpG	+	+	-	-	+/-	-	+/-	+/-
8	Cont + PGN	+	+	-	+	-	-	+/-	+/-
9	Cont + pI:C	+/-	+/-	- -	-	- -	- -	+	+/-
10	Cont + LPS	+/-	+/-	-	-	-	-	+	+/-
11	Cont + R848	+	+	-	+/-	+	+	+	+/-
12	Cont + CpG	+ +	+ +	- -	- -	- -	- -	+/-	+/-
13	CpG + PGN	+/-	+/-	+ + +	+ + +	+	-	+	+/-
14	CpG + pI:C	+/-	+/-	+	-	+	-	+	+/-
15	CpG + LPS	+	+	+/-	+	+	+/-	+	+/-
16	CpG + R848	+ +	+ +	+/-	+/-	+ +	+	+	+/-
17	R848 + PGN	+/-	+/-	+	+	+	+	+ +	+
18	R848 + pI:C	+/-	+	+	+	+ + +	+ +	+ +	+/-
19	R848 + LPS	+	+	+	+	+ +	+	+	+/-
20	pI:C + PGN	+ +	+ +	+	+	-	-	+/-	+/-
21	pI:C + LPS	+/-	-	-	+/-	+/-	-	+ + +	+ +
22	PGN + LPS	+/-	-	-	+/-	-	-	+ +	+ +

4.1.1.2. mRNA Expression Profile of TLRs

To establish the changes in TLR message level upon stimulation with TLR ligand combinations we have performed reverse transcriptase PCR of *β-actin*, *tlr2*, *tlr3*, *tlr4*, *tlr5*, *tlr6*, *tlr7* and *tlr9* genes after stimulation of spleen cells for 4 hours with single TLR ligand or combination of the two TLR ligands at optimum doses (Fig. 4.9).

We compared the band intensities of stimulated groups with unstimulated (naive) group. We considered the band intensity of naïve group as "1" and calculated the expression levels of other groups. Figure 4.10 shows the fold induction graphs of all *tlr* genes that were analyzed with RT-PCR.

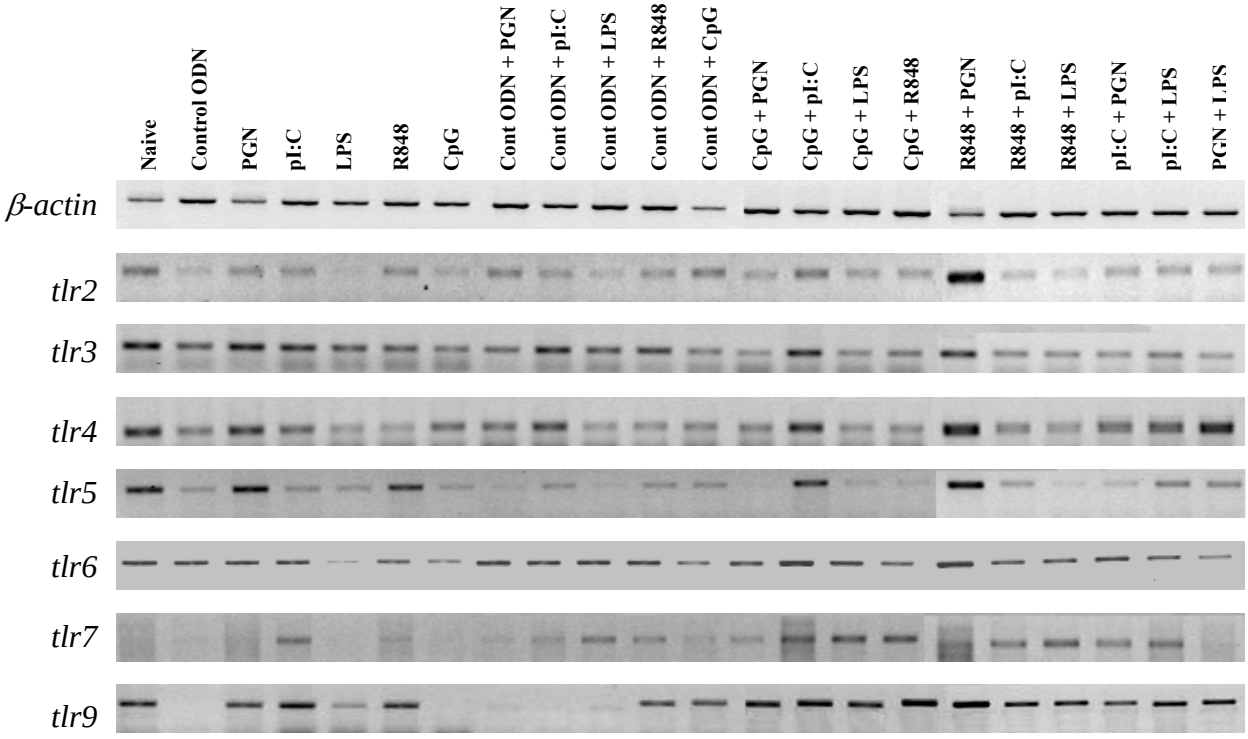
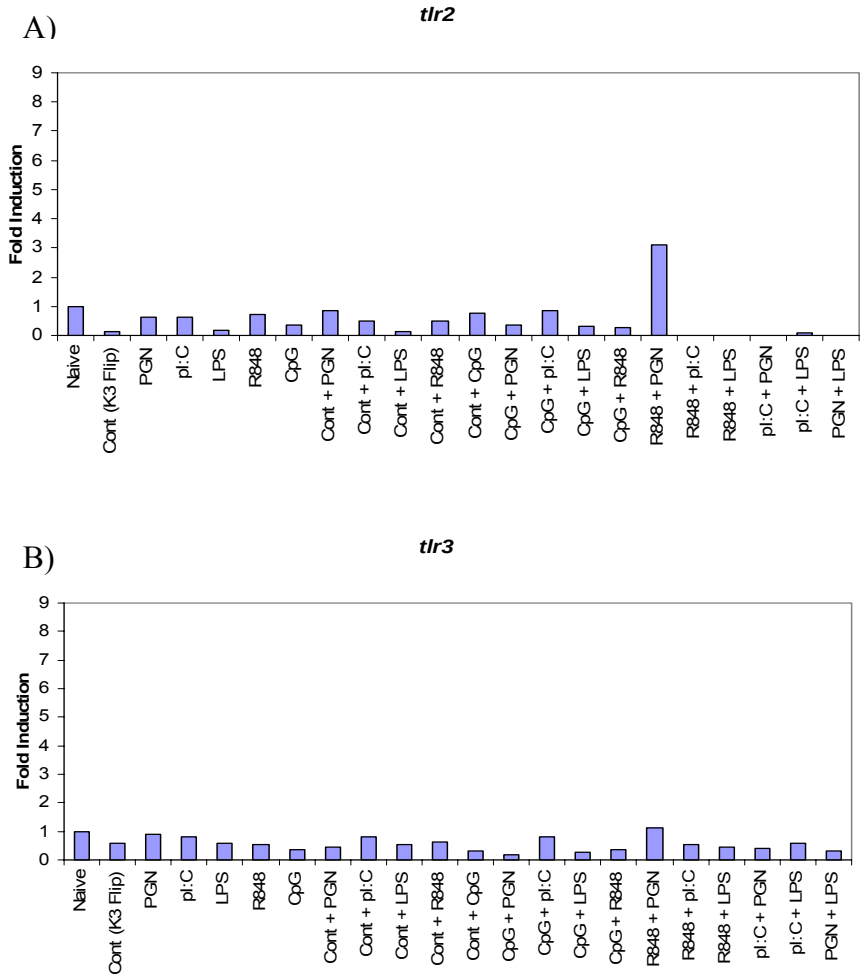


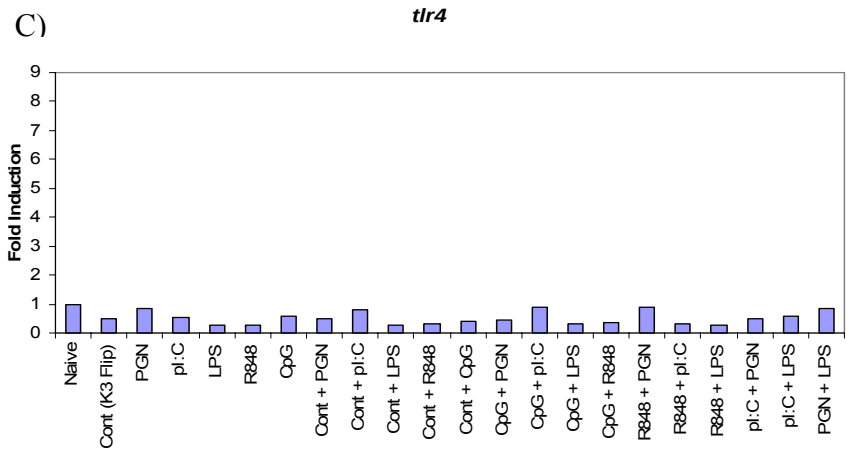
Figure 4.9. The agarose gel picture of the RT-PCR products of the *tlr* genes. The spleen cells were stimulated with TLR ligands in optimum doses for 4 hours.

Tlr-2 gene expression was significantly increased almost 3 fold and *tlr-3* gene was slightly increased with the stimulation of R848 plus PGN when compared with unstimulated group. (Fig 4.10*A*) The combination of CpG ODN and pI:C also upregulated the *tlr2*, *tlr3* (Fig 4.10*B*), *tlr4, tlr5* expression. The mRNA levels of *tlr4*

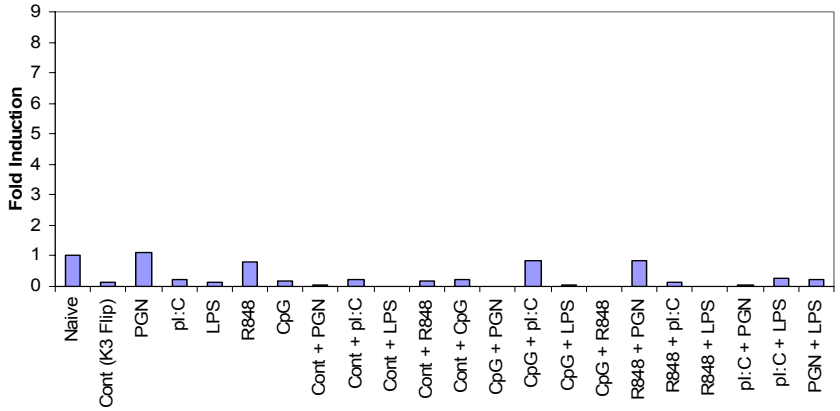
(Fig 4.10*C*), *tlr6* (Fig 4.10*E*) weren't changed or slightly down-regulated with the combinations of TLR ligands when compared with the alone TLR ligand mRNA levels. Cooperation of R848 with other TLR ligands up regulated the *tlr7* and *tlr9* expression levels 2-5 fold at optimum concentrations but down regulated *tlr5* expression (Fig 4.10*D*). The combinations of two ligands each other such as; TLR2 ligand PGN, TLR3 ligand pI:C and TLR4 ligand LPS also resulted higher expression levels of *tlr7* (Fig 4.10*F*) and *tlr9* genes (Fig 4.10*G*). Control ODN either with LPS or R848 elevated the *tlr7* expression, but decreased the *tlr9* gene expression. CpG ODN plus pI:C or LPS substantially increased *tlr7* gene expression. Consistent with previous ELISA data, R848 plus other TLR ligand combinations elevated the message intensity of *tlr2*, *tlr3*, *tlr7* and *tlr9* genes. Table 4.3 summarizes the gene regulation status of mouse TLR panel after treatment with multiple ligand combinations.



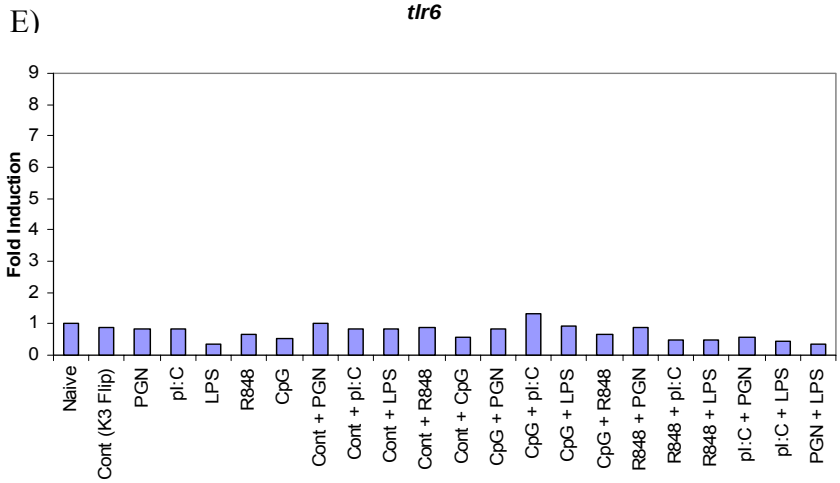
C)


tlr4
Fold Induction
0
1
2
3
4
5
6
7
8
9
Naive
Cont (K3 Flip)
PGN
pI:C
LPS
R848
CpG
Cont + PGN
Cont + pI:C
Cont + LPS
Cont + R848
Cont + CpG
CpG + PGN
CpG + pI:C
CpG + LPS
CpG + R848
R848 + PGN
R848 + pI:C
R848 + LPS
pI:C + PGN
pI:C + LPS
PGN + LPS

D)


tlr5
Fold Induction
0
1
2
3
4
5
6
7
8
9
Naive
Cont (K3 Flip)
PGN
pI:C
LPS
R848
CpG
Cont + PGN
Cont + pI:C
Cont + LPS
Cont + R848
Cont + CpG
CpG + PGN
CpG + pI:C
CpG + LPS
CpG + R848
R848 + PGN
R848 + pI:C
R848 + LPS
pI:C + PGN
pI:C + LPS
PGN + LPS

E)


tlr6
Fold Induction
0
1
2
3
4
5
6
7
8
9
Naive
Cont (K3 Flip)
PGN
pI:C
LPS
R848
CpG
Cont + PGN
Cont + pI:C
Cont + LPS
Cont + R848
Cont + CpG
CpG + PGN
CpG + pI:C
CpG + LPS
CpG + R848
R848 + PGN
R848 + pI:C
R848 + LPS
pI:C + PGN
pI:C + LPS
PGN + LPS

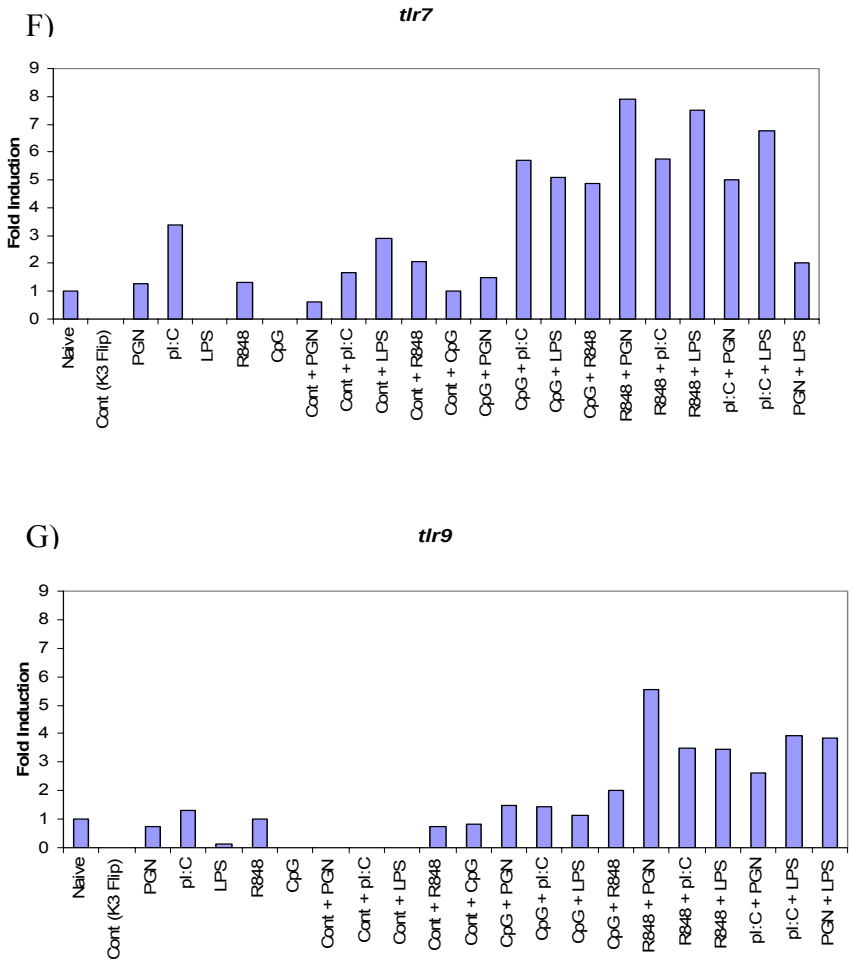


Figure 4.10. The fold induction graphs of *tlr* genes. *A)* The expression profile of *tlr2* gene of stimulated groups versus unstimulated group. *B)* The expression profile of *tlr3* gene. *C)* The expression profile of *tlr4* gene. *D)* The expression profile of *tlr5* gene. *E)* The expression profile of *tlr6* gene. *F)* The expression profile of *tlr7* gene. *G)* The expression profile of *tlr9* gene.

Table 4.3. Upregulated and downregulated mRNA message, based on band intensities of single TLR Ligand.

	Gene Expressions						
	TLR2	**TLR3**	**TLR4**	**TLR5**	**TLR6**	**TLR7**	**TLR9**
Cont ODN + PGN		--	--	----		--	--
Cont ODN + pI:C			+			--	---
Cont ODN + LPS			-	---	++	++	
Cont ODN + R848	-		-	----			
Cont ODN + CpG ODN	++						
CpG ODN + PGN	-	---					
CpG ODN + pI:C		++	+	++++	++	+	
CpG ODN + LPS		--	--	---	++	+++	
CpG ODN + R848		-	-	-----		++++	+
R848 + PGN	++++	+	+	-		++++	+++++
R848 + pI:C	--		--	----	--	++	++
R848 + LPS	--			-----	-	++++	+++
PGN + pI:C	--	--	-	-----	--	++	++
pI:C + LPS	--	-			-	++	+++
PGN + LPS	--	---		---	--	+	++++

4.1.1.3 Cytokine ELISA of IL-6 and IFN-γ with suboptimum doses

Since suboptimal concentrations of TLR ligand combinations gave almost the same cytokine production levels as observed for optimal doses (especially the groups having R848 were too strong), we were curious to delineate the degree of spleen cell stimulation by these ligands when they were further titrated (i.e. up to 125 fold dilution was monitored during the stimulation assays). This data provide insight and help us to investigate whether these cooperative effects are still retained at very low level ligand doses (which were neglected in the preliminary phases of the study). In Table 4.4, the regulatory action of these ligand combinations is presented for IFN-γ and IL-6 production. These findings suggest that, as observed earlier, R848 still strongly synergize with several other ligands and induce strong cytokine induction even at very low dose of ligand combinations. In contrast to that; LPS plus pI:C or PGN decreased IL-6 and IFN-γ cytokine levels.

Table 4.4. The fold induction of TLR ligand combinations based on the overall, IL-6 and IFN-γ cytokine level of all groups. (***red boxes***; *antagonism*, ***green boxes***; *synergism*). $p < 0{,}01$ (compared with unstimulated groups).

ID	IFN-γ			IL-6		
	5x	25x	125x	5x	25x	125x
Naive	- -	- -	-	- -	- -	- -
Cont	- -	- -	-	- -	- -	- -
PGN	+ +	-	-	-	-	- -
pI:C	- -	- -	-	- -	- -	- -
LPS	-	-	+/-	-	-	- -
R848	+ +	+ +	-	+ + +	+ +	- -
CpG	- -	- -	-	-	-	- -
Cont + PGN	+/-	- -	-	-	-	- -
Cont + pI:C	- -	- -	-	- -	-	- -
Cont + LPS	- -	-	+ +	-	-	- -
Cont + R848	+ +	+ +	+/-	+ + + + +	+ +	- -
Cont + CpG	- -	- -	-	- -	-	- -
CpG + PGN	+/-	-	-	-	+/-	- -
CpG + pI:C	- -	- -	+/-	-	-	- -
CpG + LPS	-	-	+/-	-	-	- -
CpG + R848	+ +	+ +	-	+ + + +	+ +	- -
R848 + PGN	+ +	+ + +	-	+ + + +	+ + + +	+
R848 + pI:C	+ + +	+ + +	+/-	+ + + + +	+ + +	+
R848 + LPS	+ + +	+ + +	+ + +	+ + + +	+ + +	+
pI:C + PGN	+/-	-	-	-	-	+
pI:C + LPS	- -	-	+/-	-	-	- -
PGN + LPS	- -	-	+/-	-	-	- -

4.2 Immunomodulatory Effects of Polysaccharides Extracted from *Ganoderma lucidum* and *Shiitake* Mushrooms

After establishing the contrasting and synergistic effects of several ligand combinations on splenocytes, we were curious to develop a depot delivery system that will protect the TLR ligands and also help to improve the uptake by the immune cells and at the same time induce synergistic effect by acting as a co adjuvant to the TLR ligand incorporated with it. Our ligand mixture experiments suggested that among several ligand candidates TLR2/6 ligand PGN is inducing a robust immune activation when combined with CpG ODN especially for IFN-γ induction (which is an indication of not only the humoral immunity but another important arm the cell mediated

immunity is also elevated). We aimed to identify a candidate which can mimic PGN and yet will be cheap and easy to formulate with our nucleic acid TLR ligands (as well as non nucleic acid ones too).

Accumulating data in the literature strongly suggest that extracts from certain mushrooms (*Ganoderma* and *Shiitake* mushrooms) can induce i) anti cancer and ii) immunomodulatory activities (Paterson, *2001*). These polysaccharides are amphiphilic in nature and can easily form complexes with the nucleic acids or other small immune response modifiers, such as R848. In this part of the study we will demonstrate that, four different polysaccharides (named as PS1, 2, 3, and 4) (See appendix A) extracted and purified from *Ganoderma* and *Shiitake* mushrooms can activate innate immune system through TLR2 and TLR6 when used as such on splenocytes or macrophage cell line and furthermore can be harnessed as a controlled delivery store system for our target nucleic acid based TLR ligands. This PS:Nucleic acid combinations can induce synergistic effect and can further improve Th1 mediated cytokine production from mouse spleen cells.

4.2.1 Effect of Polysaccharides on Cytokine Secretion and Nitric Oxide Production by Macrophages

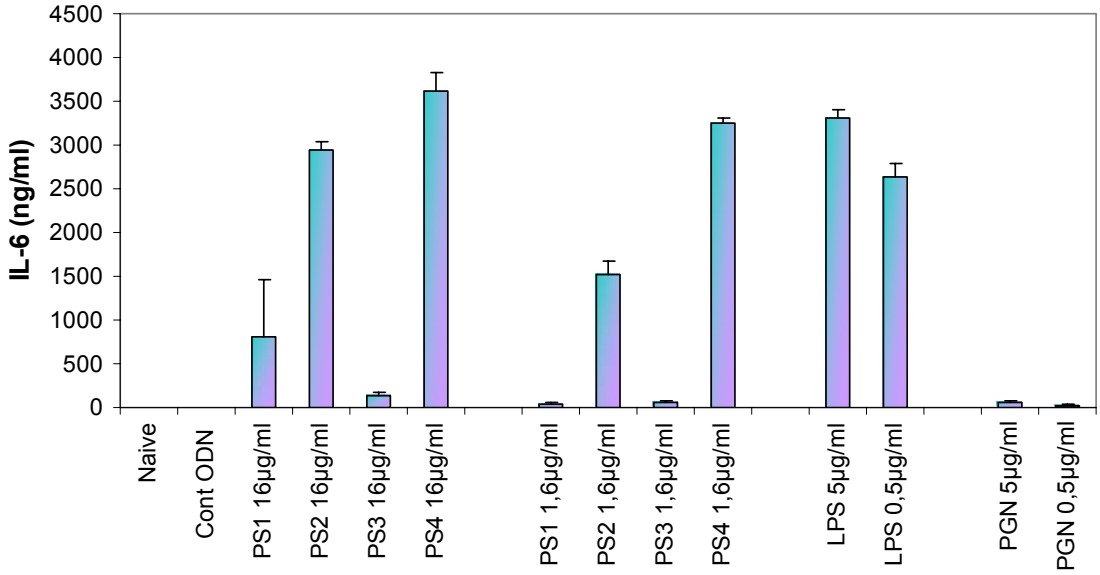
4.2.1.1 Preliminary Study of Dose and Time Dependent Cytokine ELISA and NO Assay Polysaccharides

RAW 264.7 mouse macrophage like cells were stimulated with two different doses (16 μg/ml and 1,6 μg/ml) of different types of PS. The cells were also stimulated with several positive and negative controls (TLR2/6 ligand PGN, TLR4 ligand LPS and Control ODN in 5 μg/ml, 0,5 μg/ml, 3μM concentrations respectively). After 24 hours and 42 hours incubation with these ligands the supernatants were collected and IL-6 (Fig. 4.11 *A*, *B*), TNF-α (Fig. 4.12 *A*, *B*) cytokine levels and bactericidal mediator nitric oxide (NO) levels (Fig. 4.13 *A*, *B*) were detected. Off note, Nitric oxide is a versatile, very small molecule although it is known to possess other important physiological properties (like dilation of smooth muscle and help to relieve vein pressure from sickle cell anemic patients) it is also

generated by macrophages as part of the immune response to ongoing infection. It is toxic to bacteria and other pathogens. We can measure the NO metabolite concentrations, "NO_2" for each treatment, and get an idea about the macrophage killing capacity upon stimulation with TLR ligands.

These preliminary results showed that PS2 and PS4 extracts were the most potent inducers of IL-6, and TNF-α cytokines and also they were the most potent NO inducers. When PS2 and PS4 performances were compared, upon dilution, it is clear that PS4 is more potent than PS2 (at the end of 24 h incubation). But after longer stimulations, PS2 at least for IL-6 secretion suppressed PS4. For NO secretion there is no significant difference between PS2 and PS4 albeit the magnitude of PS4 is slightly higher than PS2.

A)



B)

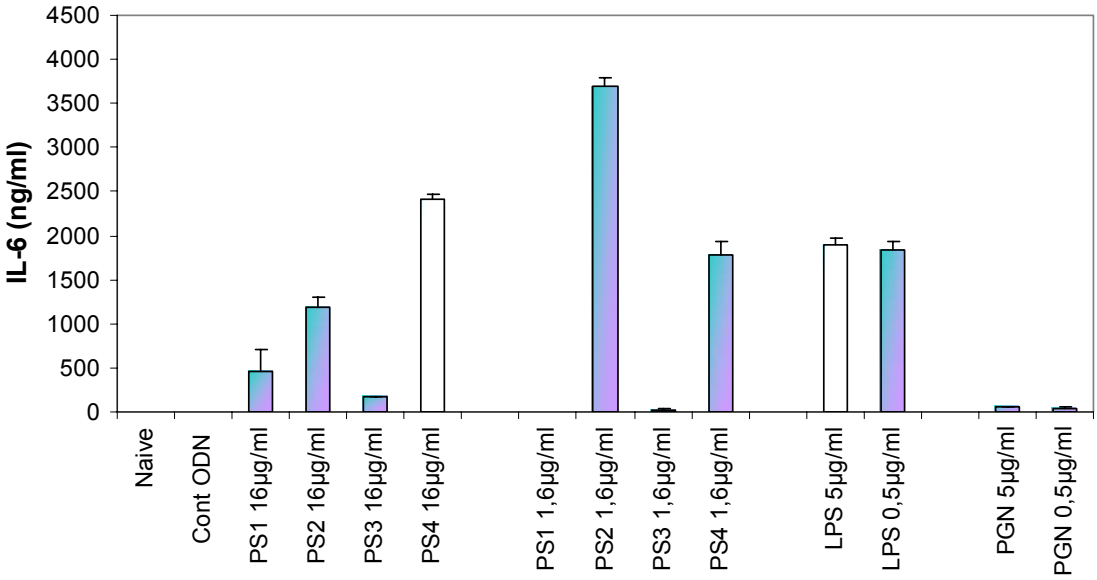
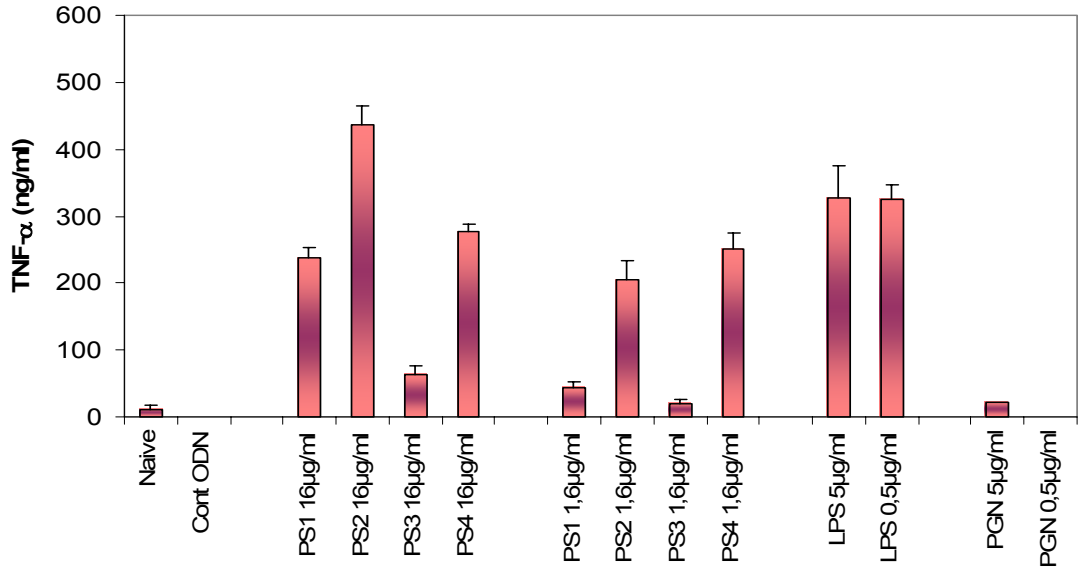


Figure 4.11. IL-6 production after 24h *(A)* or 42h *(B)* stimulation of RAW cells with different doses of PSs. $p < 0{,}01$ (compared with unstimulated or control groups).

A)



B)

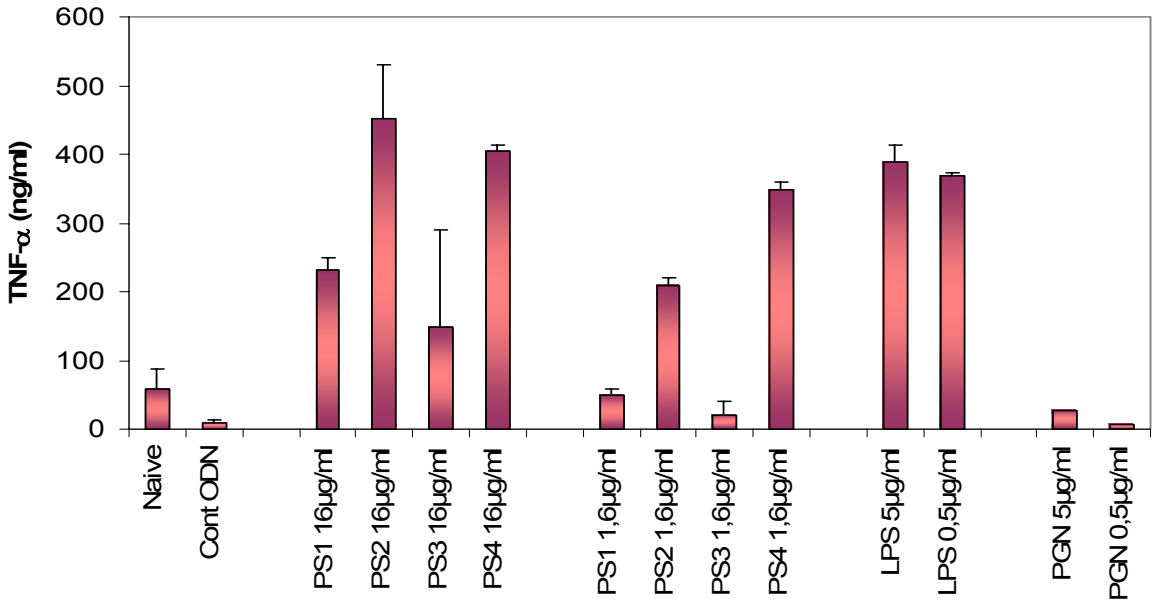
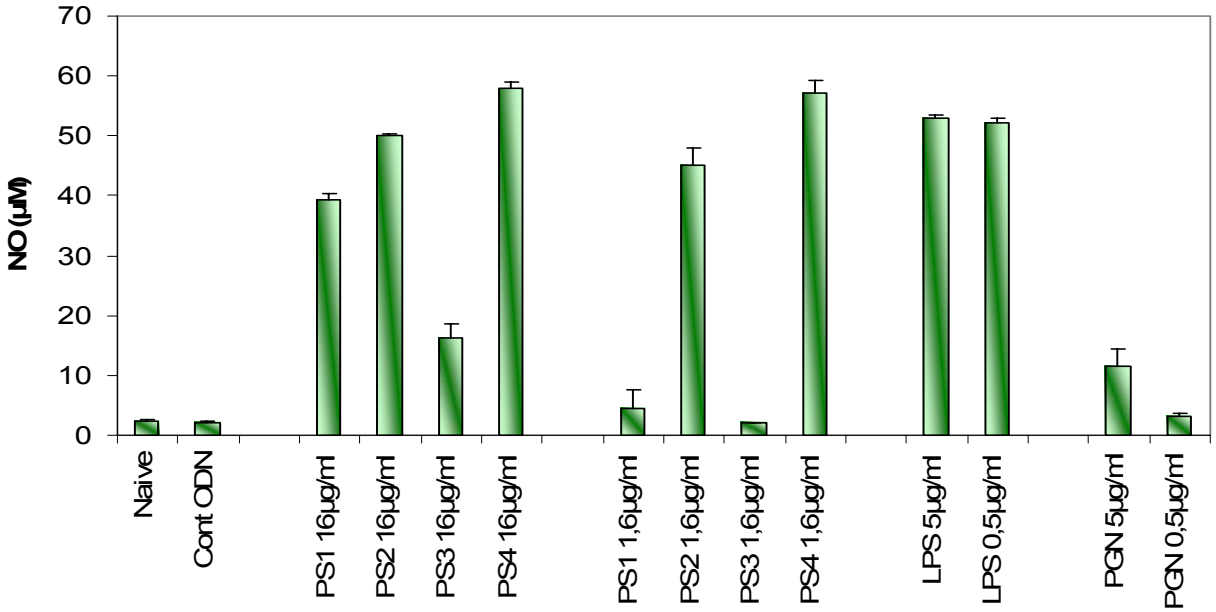


Figure 4.12. TNF-α production after 24h *(A)* or 42h *(B)* stimulation of RAW cells with different doses of PSs. $p < 0,07$ (compared with unstimulated or control groups).

A)



B)

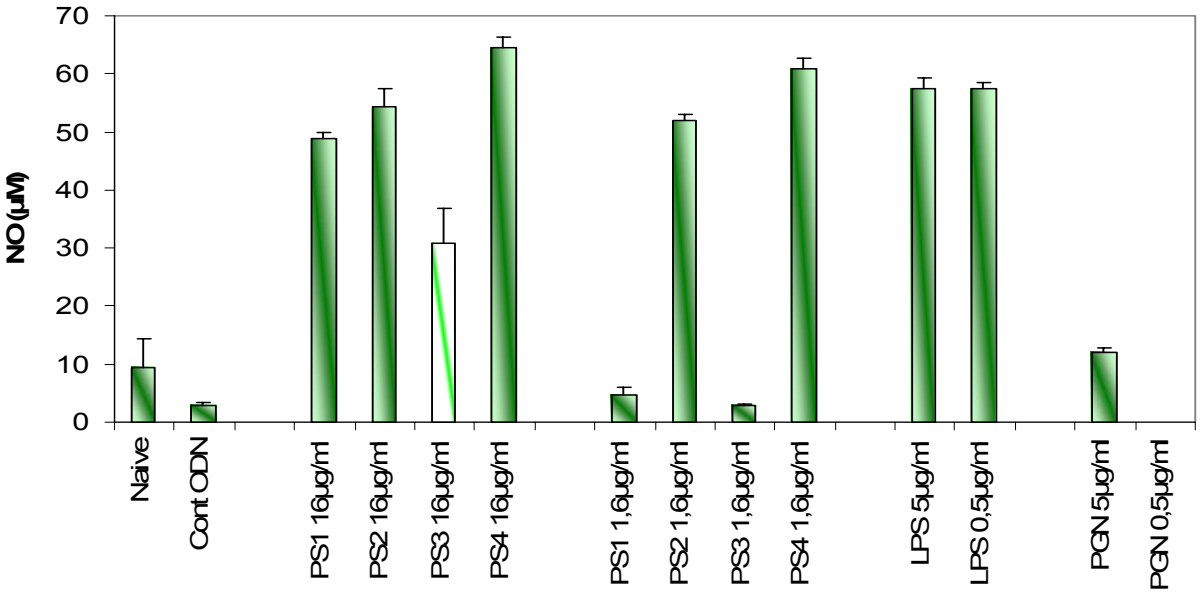
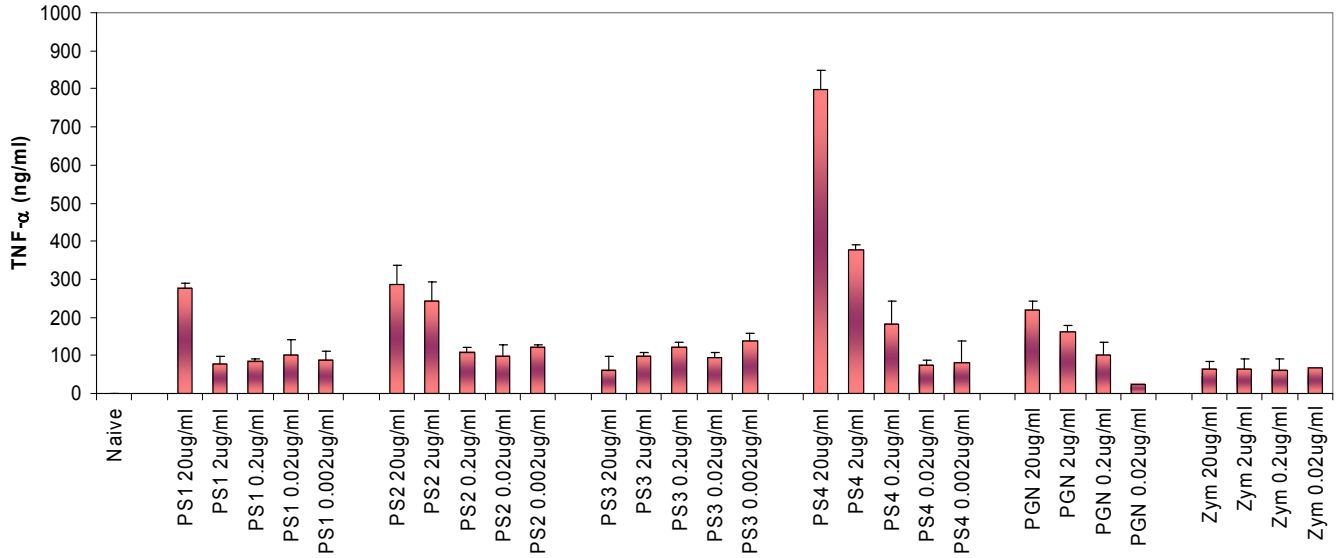


Figure 4.13. NO production by different PS extracts, PGN, LPS and Control ODN from RAW cells after 24 *(A)* or 42 hours *(B)* stimulation. $p < 0,05$ (compared with unstimulated or control groups).

4.2.1.2 Dose-dependent TNF-α and NO secretion Profiles of Polysaccharide Extracts

In order to differentiate which PS is a strong stimulant and may also serve as a depot system, we performed dose-response stimulation assay with four polysaccharides, including controls like PGN and another TLR2/6 ligand Zymosan (from 20 μg/ml to 0,002 μg/ml) for 24 hrs of culture. TNF-α level and nitric oxide responses are presented in Figs. 4.14*A* and 14.4*B* respectively. Consistent with previous preliminary study (see Figs. 4.11–4.13), PS2 and PS4 were still the most potent ones and significantly elevated TNF-α production at high PS doses. However, at lower doses PS4 once more proved that it is surpassing the rest of the test groups. Similar trend is valid for NO production (Fig. 4.14*B*). Low dose activity in culture is very informative. One can predict about the activity when these agents are given *in vivo*. Peptidoglycan gave similar stimulation pattern as PS2. Unexpectedly, zymosan showed no activity either in ELISA or in NO assays.

A)



B)

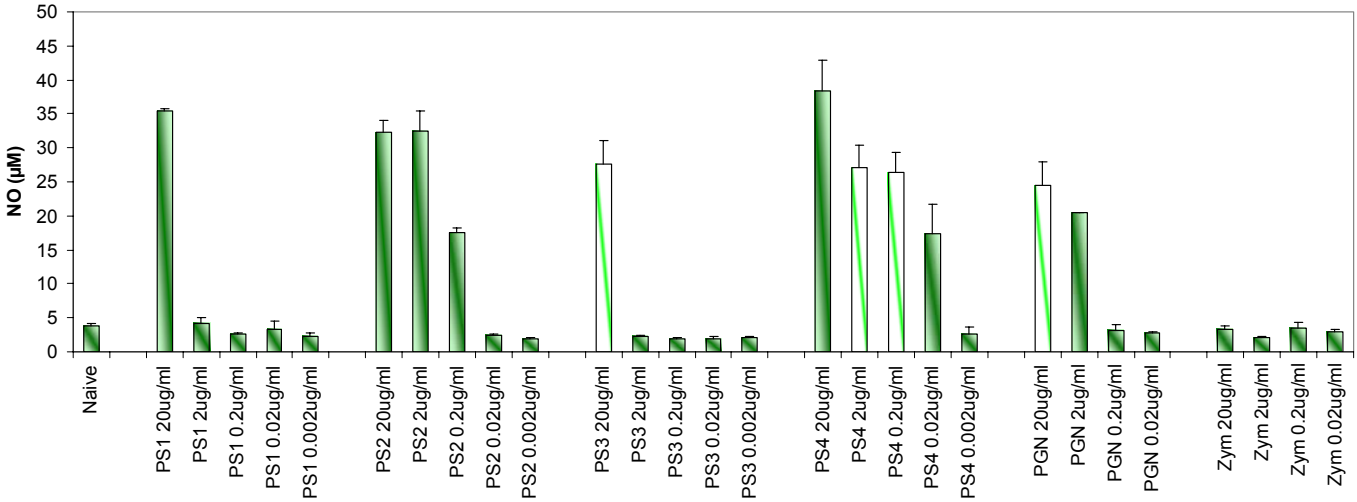
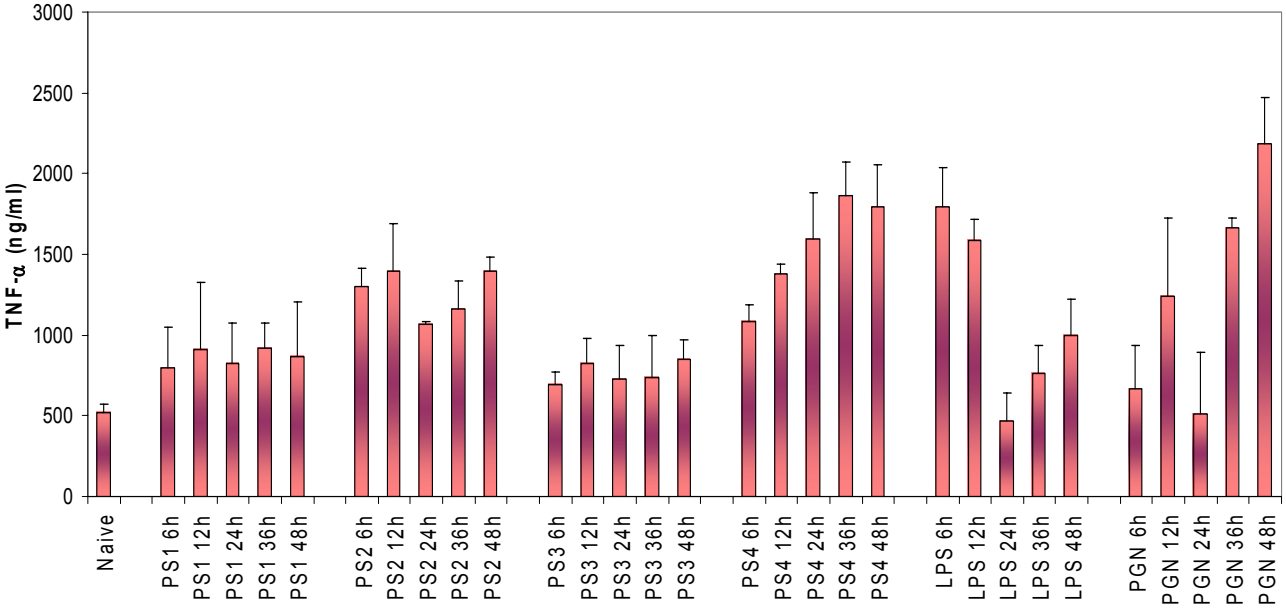


Figure 4.14. TNF-α *(A)* and Nitric oxide *(B)* production by different PS extracts, PGN and Zymosan (*Zym*) from RAW cells at different doses. $p < 0{,}03$ (compared with unstimulated or control groups).

4.2.1.3 Effect of Incubation Time on Cytokine and NO Production by Polysaccharide Extracts

To examine the time-dependent PS activity, 2 μg/ml PS doses were selected. The culture fluid was collected at 6, 12, 24, 36, and 48 hours. LPS and PGN were used as control and similar to previous assays TNF-α (Fig. 4.15*A*) and NO (Fig. 4.15*B*) levels were determined from culture supernatants. As seen in Fig. 4.15*A*, TNF-α level reached near saturation within 6hrs of stimulation independent of the PS type. Nitric oxide production showed a time-dependent production reaching near saturation after 24 hrs of incubation. Consistent with previous observations, PS2 and PS4were the most potent inducers of TNF-α 12–24h.

A)



B)

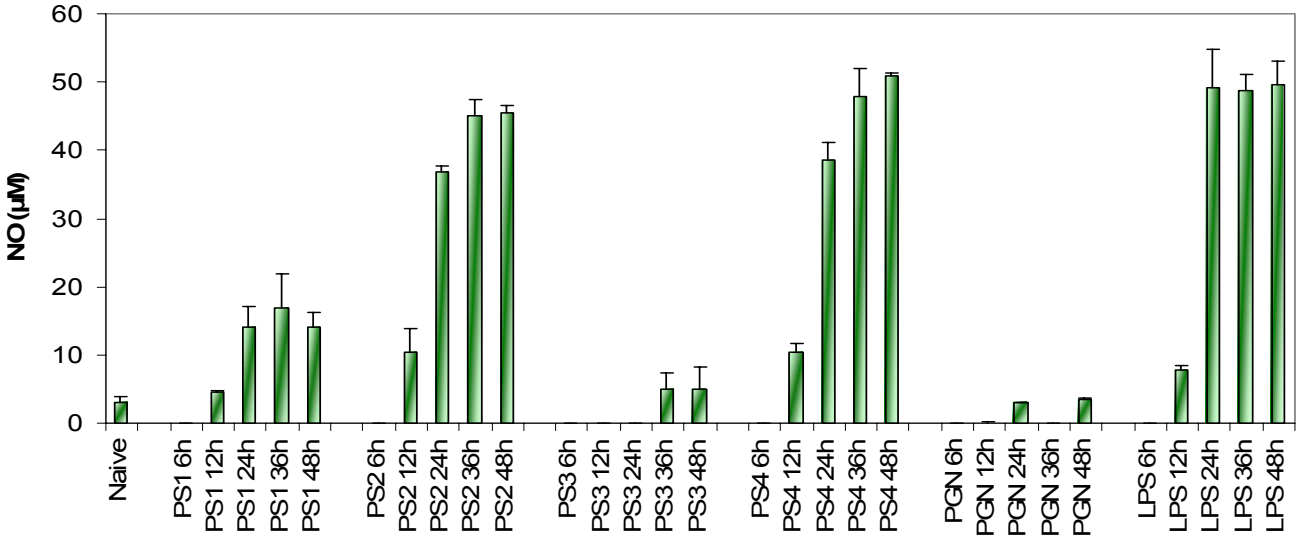


Figure 4.15. Time dependent TNF-α *(A)* and Nitric oxide *(B)* secretion by different PS extracts from RAW cells. $p < 0,07$ (compared with unstimulated or control groups).

Consequently, dose-response and time-course experiments prompted us to conclude that PS2 and PS4 polysaccharides were the most potent stimulant candidates among four PSs at hand.

4.2.2 Effect of Polysaccharides on TLR and Cytokine, Chemokine Gene Expression

In order to understand whether PS triggering (via TLR2/6 pathway) changes other unrelated TLR message expression, we stimulated RAW cells with different PS extracts (10 μg/ml), up to 4 hrs in culture (PGN was used as a positive control), and checked the expression levels of whole TLR panel (TLR1-10) via RT-PCR.

4.2.2.1 Polysaccharide Stimulation Alters mRNA Expression Profile of TLRs

Results (Fig. 4.16 and Fig. 4.17) showed that at 2h stimulation PS4 gave higher and broader TLR message activity compared to PS2. When message level of naive group is compared to PS2 or PS4, it is clearly seen that PS4 mRNA message for

tlr1, *2*, *5*, *7*, and *9* highly expressed. By 4h PS2 induction level catches up the level of the PS4. If we consider the fold change in the mRNA band intensities from 2 to 4 h PS incubation, as seen in Fig. 4.17, PS2 is the strongest polysaccharide, and then comes PS4. Data indicated that only TLR2 message reduced at the end of 4 hrs of stimulation with PS2, however, in PS4 group neither TLR2 nor TLR6 levels lowered, but TLR5 message level came down. This time dependent TLR2 message level change in PS2 could contribute to the activation potency difference between PS2 and PS4 types.

Another striking finding is that PS2 and PGN behaved very similar during cytokine and NO induction experiments, here similar to previous observations, PGN and PS2 gave identical mRNA activation trend, and as seen in PS2 group, in PGN treated group, TLR2 message comes down by 4h of stimulation (Fig. 4.17). Off note, since our utmost aim is to deliver nucleic acid based TLR ligands together with PS, upregulation of TLR3, 7 and 9 messages are very promising, as it will eventually conribute to signaling through these receptors when PS plus pI:C, R848, or CpG ODN ODN is delivered to immunologically relevant cells.

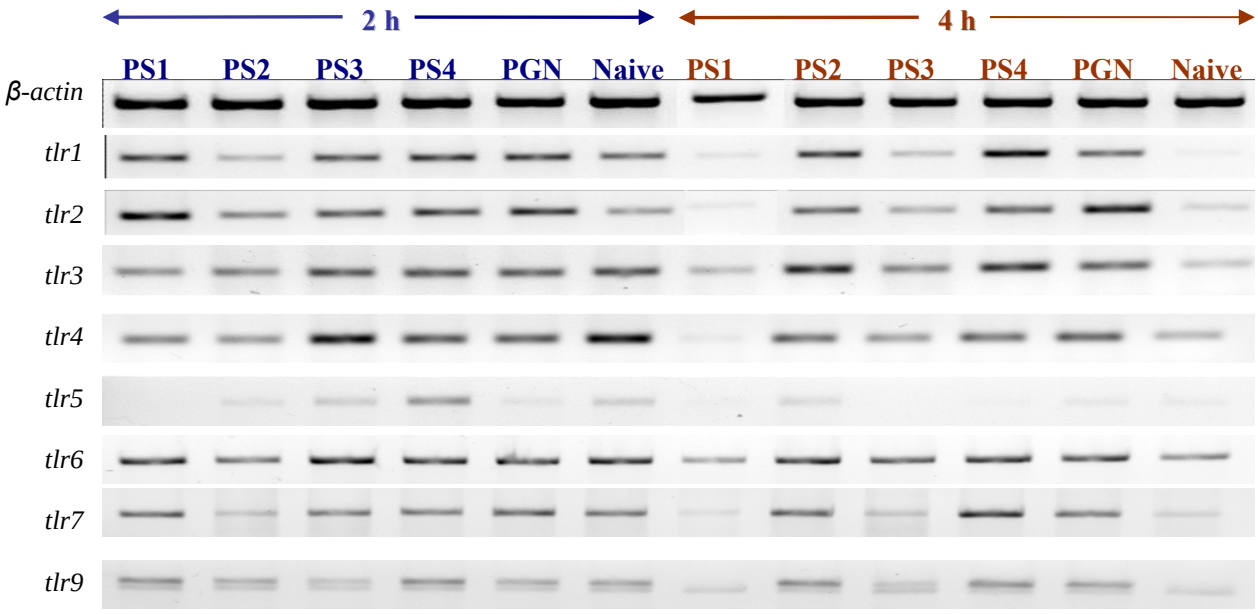
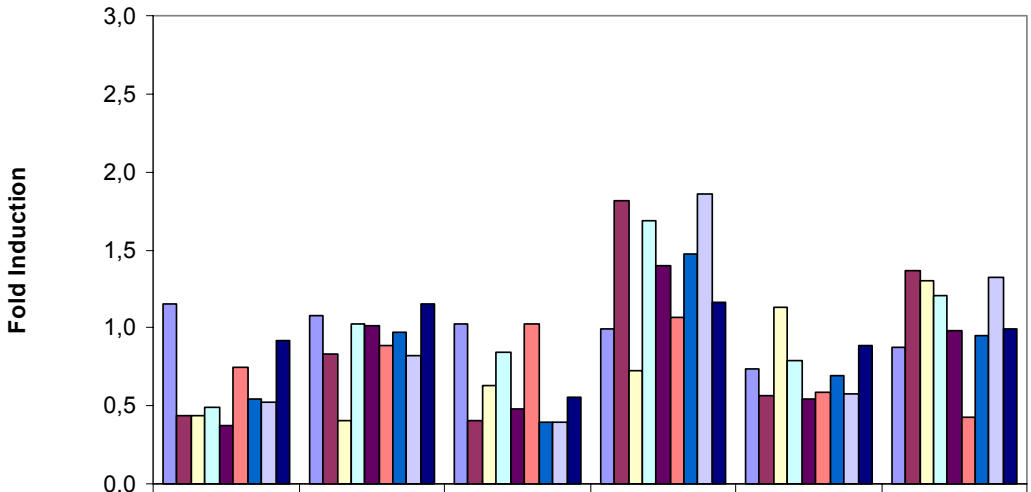


Figure 4.16. mRNA expression of TLRs upon varying PS treatment time. Total RNA from RAW cells (stimulated with PSs and positive control PGN for 2h and 4h) were subjected to RT-PCR and *tlr-1* to *tlr-9* specific-bands were visualized on agarose gels.



	Naive	PGN	PS1	PS2	PS3	PS4
β-actin	1,15	1,08	1,02	0,99	0,73	0,88
mTLR1	0,44	0,84	0,41	1,82	0,57	1,36
mTLR2	0,44	0,40	0,62	0,73	1,13	1,30
mTLR3	0,49	1,02	0,84	1,69	0,79	1,20
mTLR4	0,38	1,01	0,48	1,40	0,54	0,98
mTLR5	0,75	0,89	1,02	1,07	0,59	0,42
mTLR6	0,55	0,97	0,40	1,48	0,69	0,95
mTLR7	0,53	0,82	0,39	1,86	0,57	1,32
mTLR9	0,92	1,16	0,55	1,16	0,89	0,99

Figure 4.17. Time dependent relative expression change of TLR gene message mediated by polysaccharide stimulation. Ratio of expression level change of TLR genes from 2h to 4h is presented. Fold induction levels for each gene was calculated by dividing the value of the 2h band intensity to that of corresponding 4h intensity. (Please note that fold induction values over 1 indicate gene expression level increase over time, and the values below 1 indicate a reduction of that gene over time upon PS stimulation).

4.2.2.2 Expression Profile of Cytokines and Chemokines upon PS Treatment

Gene expressions of cytokines and chemokines which are important for the generation of innate immune response (and then to instruct an adaptive immunity) were checked with reverse-transcriptase PCR (Fig. 4.18).

When time-dependent change (from 2 to 4 h stimulation) in mRNA band intensities of these cytokines and chemokines upon PS2 or PS4 treatment are investigated (taking naive group into account too), the followings were observed:

i) There was no difference in the CXCL16 and CD40 message levels between PS2 and PS4 treatments.

ii) TNF-α and MIP-3α levels significantly increased with PS4 and slightly decreased with PS2 treatments

iii) Extending PS4 stimulation from 2h to 4h (but not PS2 stimulation) increased significantly IP10 (CXCL10), IL-15, and IL-18 message levels.

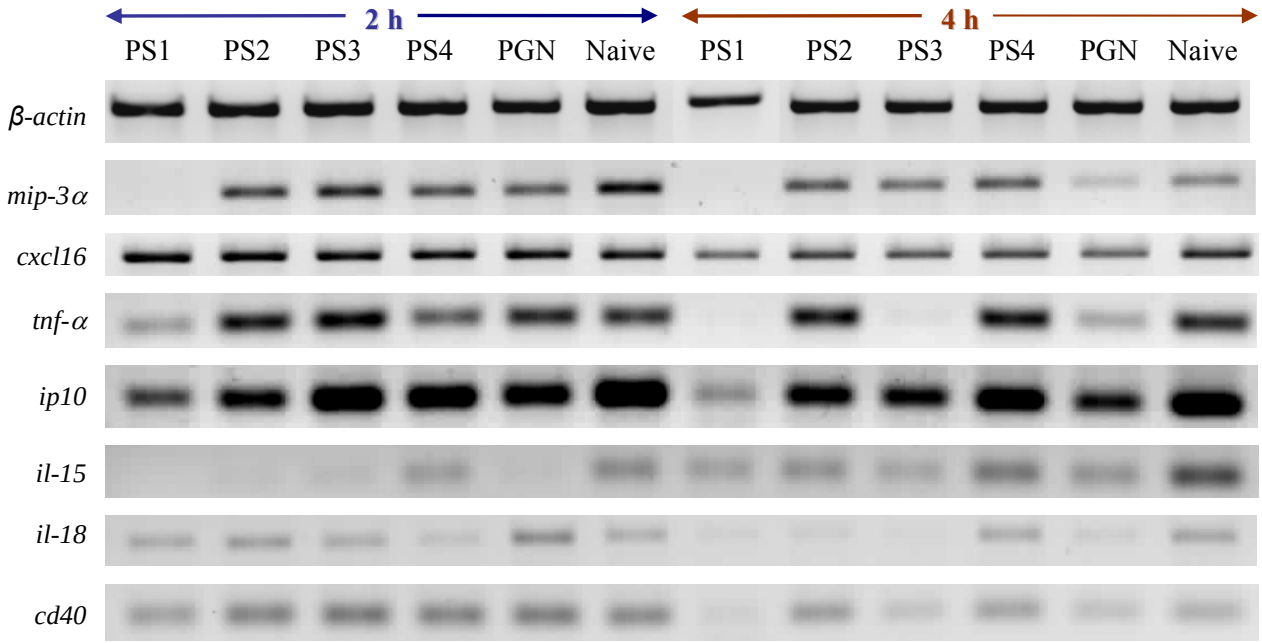


Figure 4.18. PCR analysis of PS treated RAW 264.7 cells. cDNA obtained from total mRNA was subjected PCR (including specific primer sets) to detect the message levels of various cytokines and chemokines and bands were visualized by applying the gene products on agarose gels.

4.2.3 Analysis of PS mediated NF-κB activation

We have so far analyzed the PS mediated activation using a cell line that is known to express several TLRs. But in order to convincingly demonstrate that these

cells are responding to PS via only TLR2/6 and the PS preparations are free from other contaminants like endotoxin, we designed two experiments; first we have determined the endotoxin levels of these preparations and confirmed that there is no endotoxin contamination (data not shown). Secondly, we took HEK cells stably expressing hTLR2/6 genes and transiently transfected with a plasmid controlled by a NF-κB promoter (the main transcription factor in the TLR signaling cascade) that encodes for *luciferase* gene. After different PS extracts were added to these dual transfectant HEK cells (for 12 hours) we analyzed the cell extract for the NF-κB mediated luciferase activity by using Promega`s luciferase assay kit. As seen in Figure 4.19, different PS extract induced different levels of luciferase activity. The strongest being PS type 2 and 4 (PGN showed similar activity level, as expected). These findings suggest that indeed PS extracts initiate a TLR2/6 mediated NF-κB dependent signaling cascade to produce Th1 cytokines. These results support our previous findings and are in close accordance with the previously observed TNF-α and NO values from RAW cells (see Figures 4.11 and 4.18).

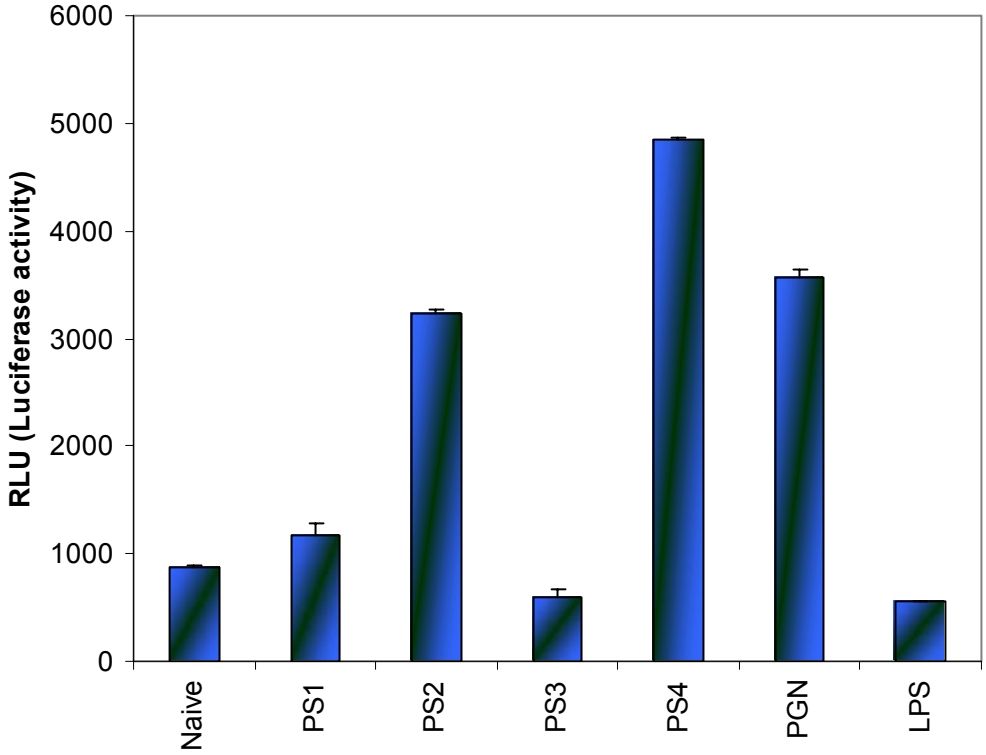


Figure 4.19. Relative luciferase activity of different PS extracts. hTLR2/6 HEKs were transfected with p5xluc NF-κB and stimulated with 5µg/ml of each PSs. 12h later cells were lysed and luciferase activity was assessed via Luciferase kit. $p < 0,05$ (compared with naive group).

4.3 Delivery of TLR Ligands with Polysaccharides

One of the goals of this study is to establish a novel carrier system that possesses immunostimulatory property, cheap, easy to obtain and suitable for targeted delivery of the labile nucleic acid TLR ligands (i.e. pI:C, R848, ss/ds RNA or CpG DNA, including plasmid DNA suitable for either transfection or gene therapy applications) to the relevant target immune cells. So far, data revealed that based on the ability to stimulate an innate immune response PS2 and PS4 consistently performed well over the rest of the candidate PS types (see Figures 4.11 to 4.19). Between PS2 and PS4, however the strongly indicated that PS4 is more potent than PS2, therefore, to pursue the rest of the formulation and delivery experiments PS4 was selected.

4.3.1. Cytokine ELISA and NO Assay

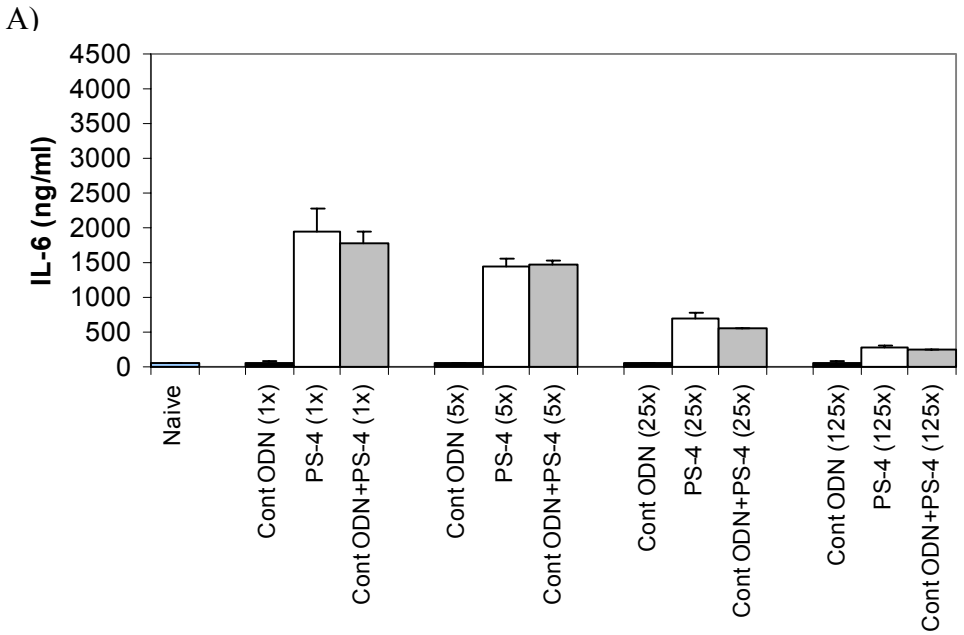
As previously mentioned in the materials and methods section, different TLR ligands plus PS combinations were first prepared. Specifically, these nucleic acid TLR ligands were selected to yield PS complexes TLR3 ligand: pI:C, TLR7/8 ligand: R848, and TLR9 ligand: CpG ODN. Since pI:C was synthesized via native PO linkage chemistry, it was anticipated that *in vivo* free pI:C will be prone to RNase digestion. Thus we have selected 2 different doses of pI:C; while achieving complexation with PS4 and hoped to observe a dose dependent increase in synergy at the end of serial dilution and stimulation studies. Later, these ligands were mixed with PS4 at a 1:1 weight ratio, and incubated at 4°C to allow adsorption/incorporation of PS with nucleic acids and then upon serial dilution (5x, 25x, 125x and 625x) were used to stimulate either mouse peritoneal macrophages (or RAW 264.7 cells) or splenocytes for 24 hours. Cell supernatants were analyzed for IL-6 and NO secretion.

As expected, mixing of PS4 with Control ODN or CpG ODN did not significantly induced CpG ODN mediated IL-6 (Fig 4.20*A*, *B*) cytokine production but synergistically increased the NO activity (Fig. 4.21 *B*) at very low doses. This activity is CpG ODN dependent since Control ODN did not produce synergistic IL-6 effect with PS4. TLR3 ligand pI:C were chosen at two different doses for PS4

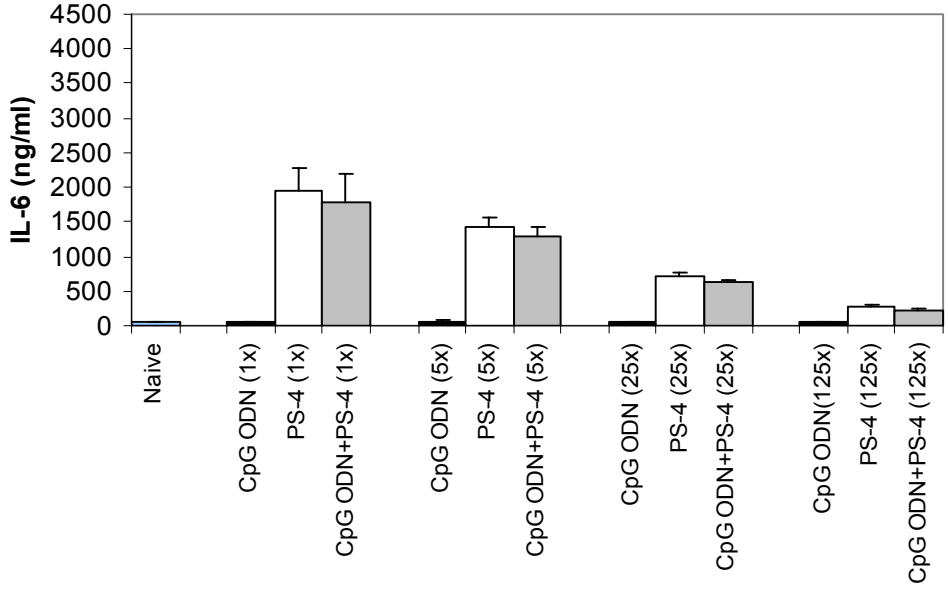
adsorption, we thought using pI:C at two different doses could be more informative since it is so sensitive to serum nucleases we can monitor the PS4 shielding better. High and low pI:C adsorbed PS4 resulted in significantly higher IL-6 levels of the either stimulant specially at 125x dilution (there was a pI:C dose dependent relation for the synergistic activity of pI:C/PS4 complexes). Please compare IL-6 values of pI:C, PS4 and PS4-pI:C complex in Fig. 4.20*C*, *D* at 625x dilution. PS4–pI:C complex also increased the NO secretion by about 2 fold compared to either pI:C or PS4 alone counterpart (compare Fig 4.21*C*, *D* at 125x dilution).

Of PS4 and nucleic acid ligand complexations and delivery studies TLR7/8 ligand "R848" showed the most significant synergistic effect with PS4 (compare Fig. 4.20*E*, and 4.21*E* at 125x dilution). At 125x dilution where both R848 alone and PS4 alone lost its stimulation ability, PS4-R848 complex induced significantly high IL-6 and NO production (Fig. 4.20*E* and 4.21*E*). NO response rose from about 12 μM to over 40 μM a rise >3 fold, while IL-6 induction rose >10 fold (ca. 200 ng/ml to >2000ng/ml).

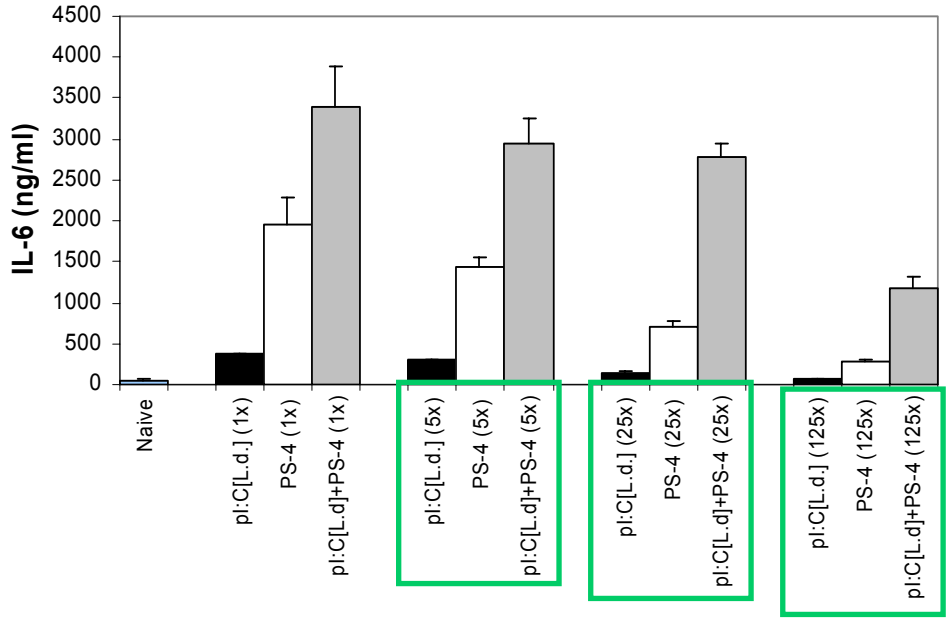
These results were critical since inclusion of TLR ligands within PS4 proved to be beneficial and may be used for in vivo stimulations in the future.



B)


IL-6 (ng/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Naive
CpG ODN (1x)
PS-4 (1x)
CpG ODN+PS-4 (1x)
CpG ODN (5x)
PS-4 (5x)
CpG ODN+PS-4 (5x)
CpG ODN (25x)
PS-4 (25x)
CpG ODN+PS-4 (25x)
CpG ODN(125x)
PS-4 (125x)
CpG ODN+PS-4 (125x)

C)


IL-6 (ng/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Naive
pI:C[L.d.] (1x)
PS-4 (1x)
pI:C[L.d]+PS-4 (1x)
pI:C[L.d.] (5x)
PS-4 (5x)
pI:C[L.d]+PS-4 (5x)
pI:C[L.d.] (25x)
PS-4 (25x)
pI:C[L.d]+PS-4 (25x)
pI:C[L.d.] (125x)
PS-4 (125x)
pI:C[L.d]+PS-4 (125x)

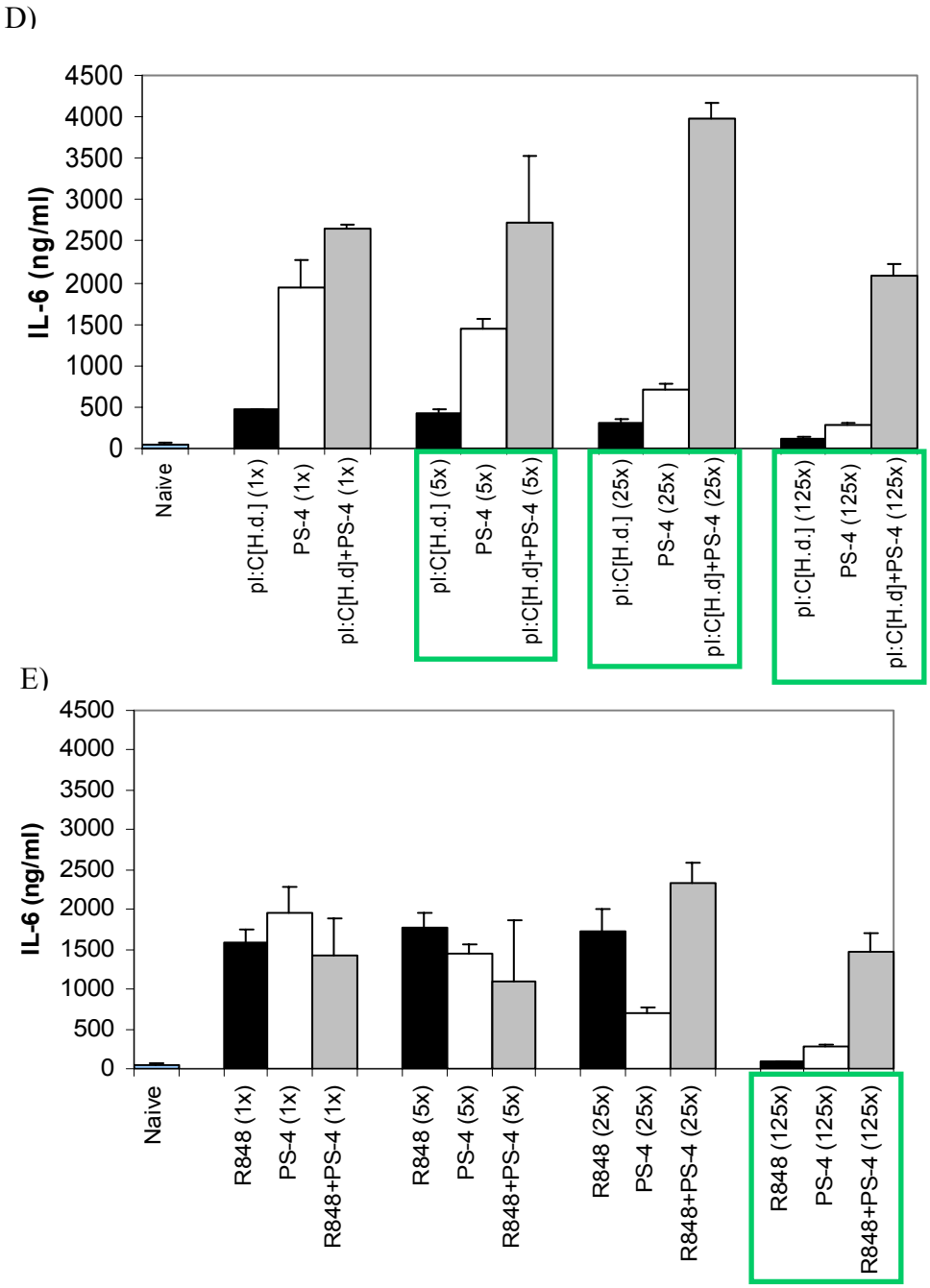
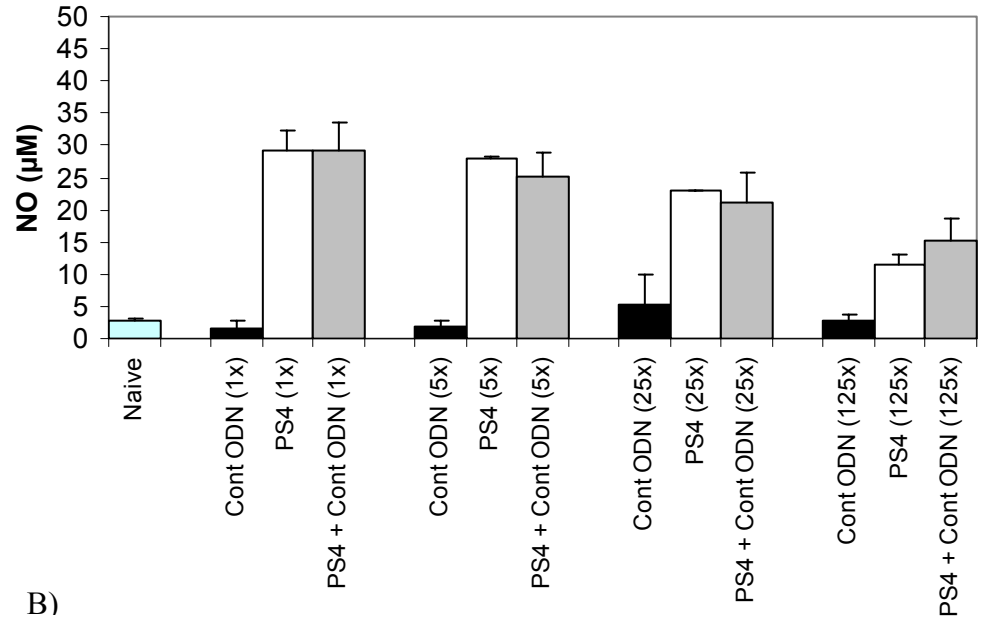
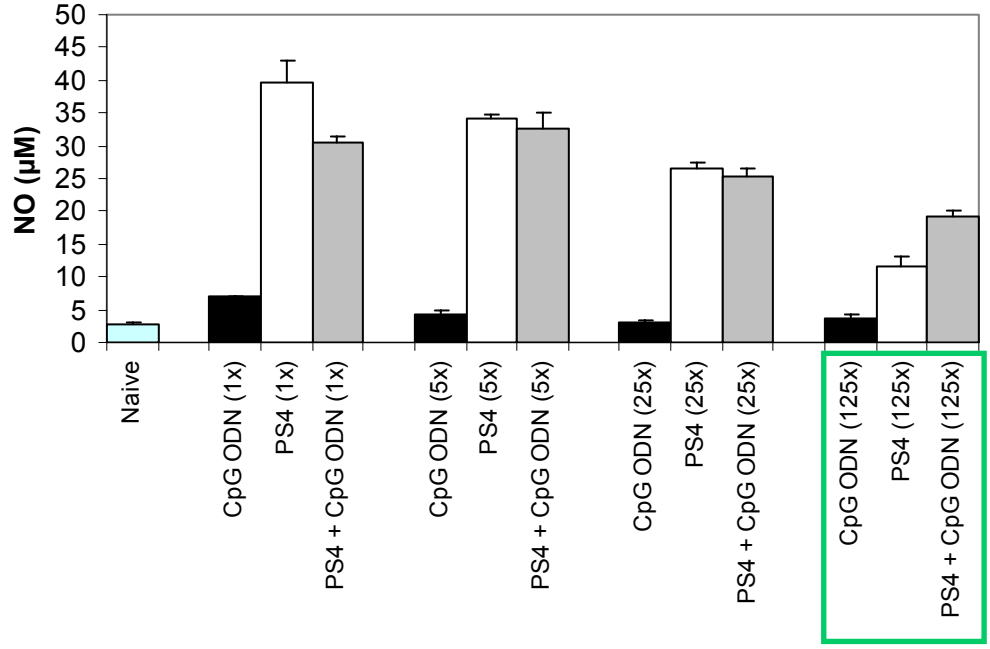


Figure 4.20. IL-6 ELISA of RAW 264.7 cells when stimulated with PS-4 only, TLR ligands only and PS-4 complexed TLR ligands. *A)* Control ODN *B)* CpG ODN (ODN1555) *C)* pI:C at low dose [0.4 µg/ml pI:C at 1x dilution] *D)* pI:C at high dose [2.0 µg/ml pI:C at 1x dilution] *E)* R848. $p < 0{,}001$ (compared to ligand only and PS only levels at 5X, 25X and 125X dilutions). ***(green boxes; synergism).***

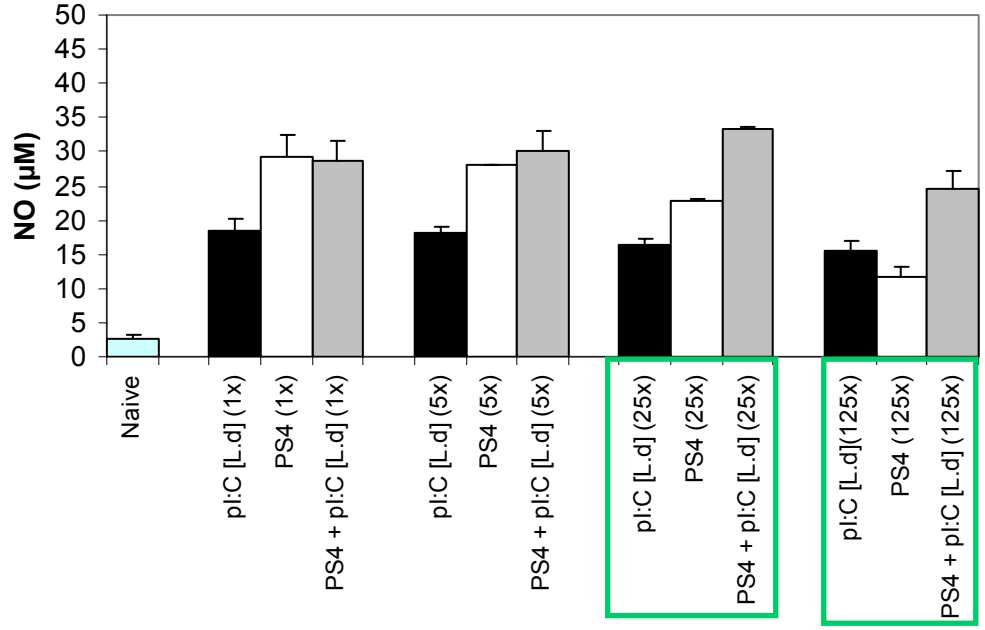
A)


NO (μM)
50
45
40
35
30
25
20
15
10
5
0
Naive
Cont ODN (1x)
PS4 (1x)
PS4 + Cont ODN (1x)
Cont ODN (5x)
PS4 (5x)
PS4 + Cont ODN (5x)
Cont ODN (25x)
PS4 (25x)
PS4 + Cont ODN (25x)
Cont ODN (125x)
PS4 (125x)
PS4 + Cont ODN (125x)

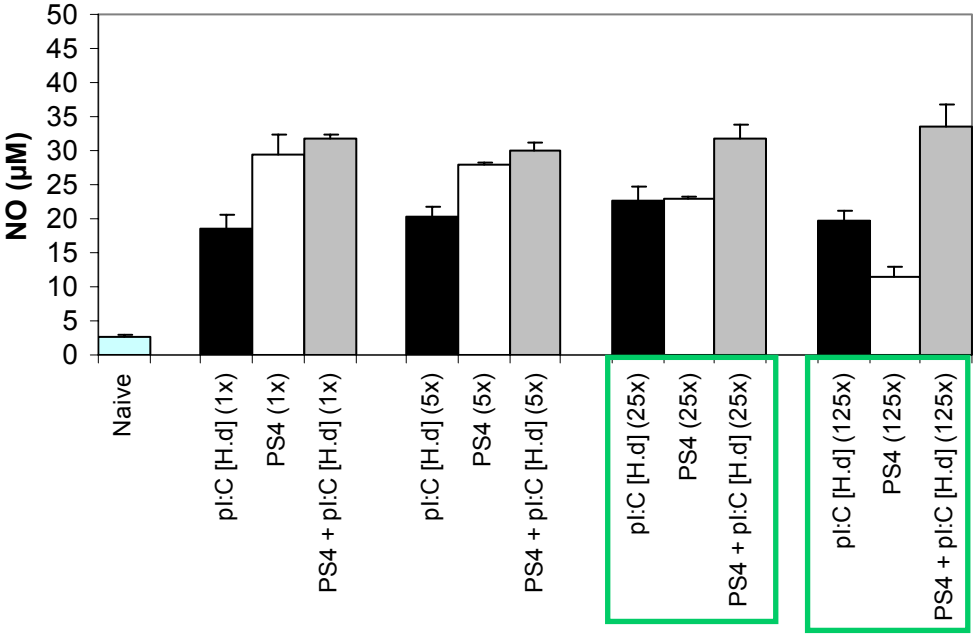
B)


NO (μM)
50
45
40
35
30
25
20
15
10
5
0
Naive
CpG ODN (1x)
PS4 (1x)
PS4 + CpG ODN (1x)
CpG ODN (5x)
PS4 (5x)
PS4 + CpG ODN (5x)
CpG ODN (25x)
PS4 (25x)
PS4 + CpG ODN (25x)
CpG ODN (125x)
PS4 (125x)
PS4 + CpG ODN (125x)

C)


NO (µM)
50
45
40
35
30
25
20
15
10
5
0
Naive
pI:C [L.d] (1x)
PS4 (1x)
PS4 + pI:C [L.d] (1x)
pI:C [L.d] (5x)
PS4 (5x)
PS4 + pI:C [L.d] (5x)
pI:C [L.d] (25x)
PS4 (25x)
PS4 + pI:C [L.d] (25x)
pI:C [L.d](125x)
PS4 (125x)
PS4 + pI:C [L.d] (125x)

D)


NO (µM)
50
45
40
35
30
25
20
15
10
5
0
Naive
pI:C [H.d] (1x)
PS4 (1x)
PS4 + pI:C [H.d] (1x)
pI:C [H.d] (5x)
PS4 (5x)
PS4 + pI:C [H.d] (5x)
pI:C [H.d] (25x)
PS4 (25x)
PS4 + pI:C [H.d] (25x)
pI:C [H.d] (125x)
PS4 (125x)
PS4 + pI:C [H.d] (125x)

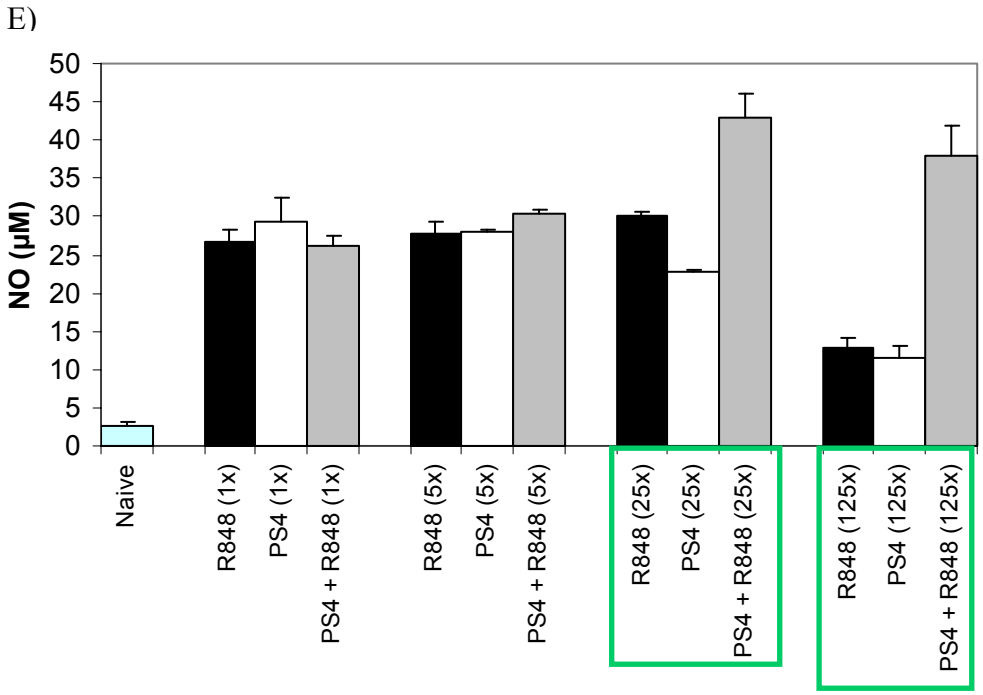
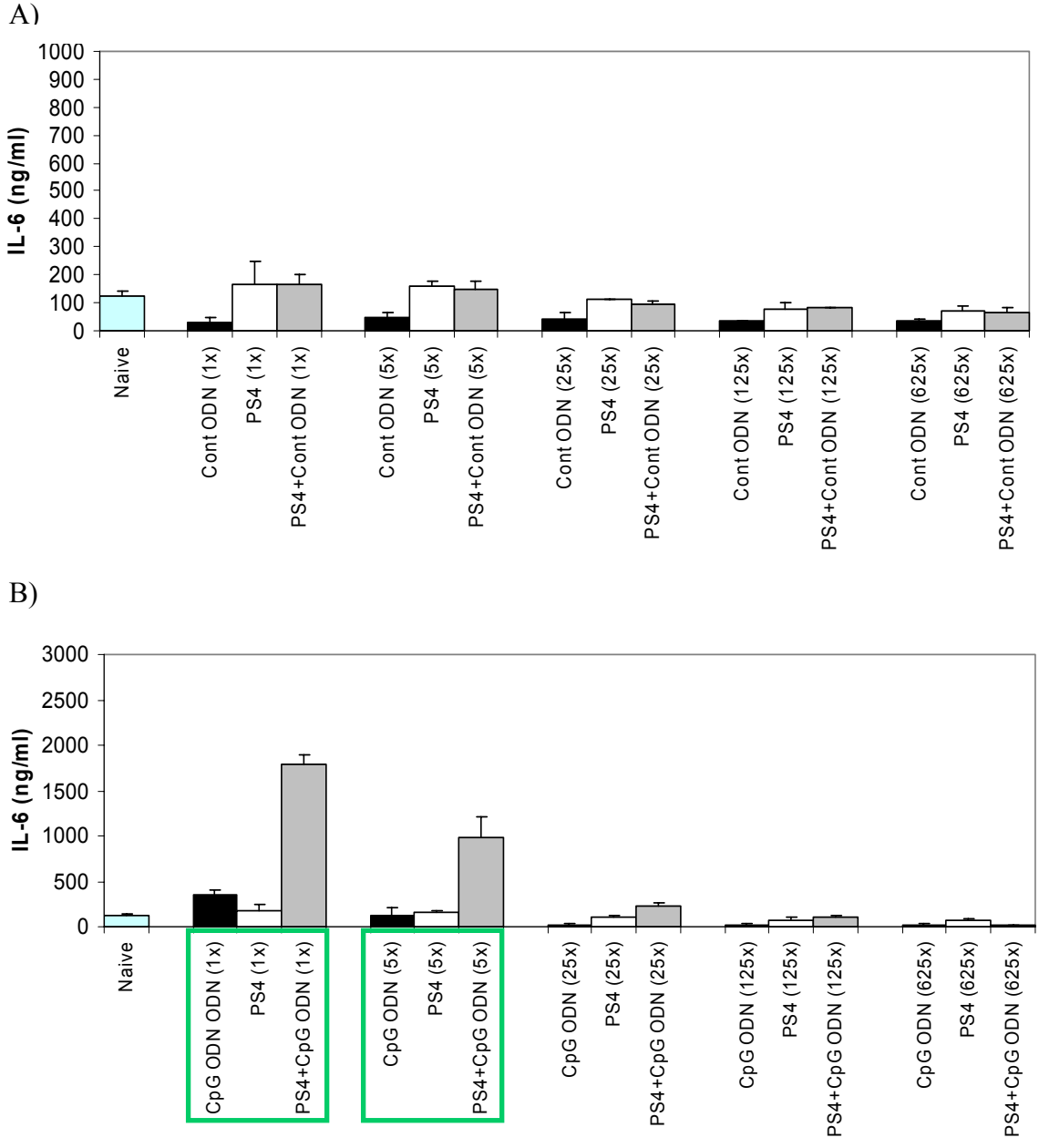


Figure 4.21. NO production of RAW 264.7 cells when stimulated with alone PS-4 only, TLR ligand only and PS-4complexed TLR ligand. *A)* Control ODN *B)* CpG ODN (ODN1555) *C)* pI:C at low dose [0.4 μg/ml pI:C at 1X dilution] *D)* pI:C at high dose [2.0 μg/ml pI:C at 1X dilution] *E)* R848. $p < 0{,}002$ (compared to ligand only and PS only levels at 25X and 125X dilutions). *(**green boxes**; synergism).*

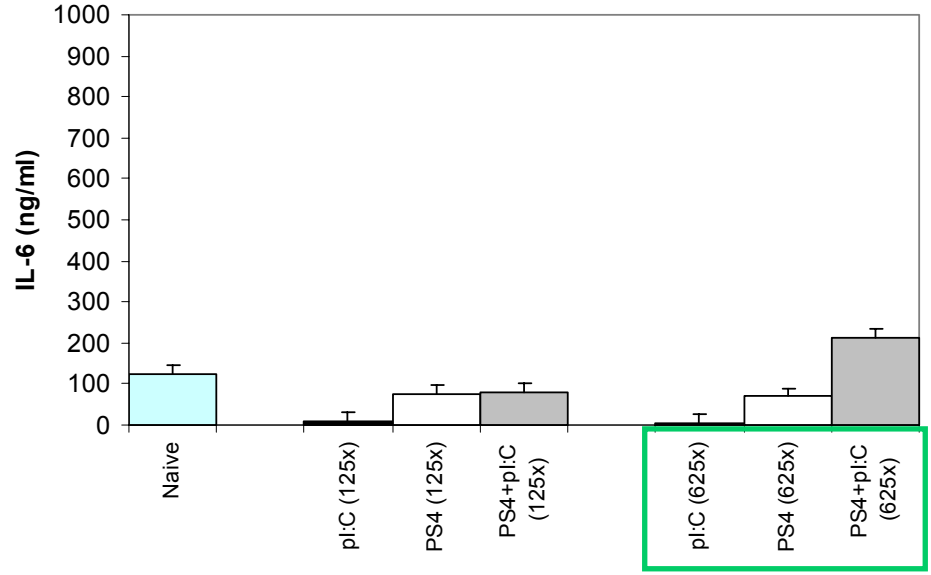
4.3.2. Stimulation of Spleen Cells with PS4 and TLR ligands

To see the effect of PS4 complexed TLR ligands on various immune cells, we stimulated spleen cells either with i) PS4 only, ii) CpG ODN only and iii) PS4-CpG ODN ODN complex (i.e. TLR ligand only, PS4 only and PS4-TLR ligand complex). Control ODN, pI:C or R848 were prepared similarly as mentioned earlier and were serially diluted and used accordingly. Splenocyte culture stimulation was extended for 42 hours. While CpG ODN group was tested at 1x to 625x dilution, TLR3 and TLR7 ligands were used only at 125x and 625x dilutions. The reason for choosing only two concentration for pI:C and R848 was due to the fact that previous experiments gave no synergistic effect for IL-6 and NO production at higher concentrations. As expected, Control ODN complexed with PS4 did not enhance IL-6 production (Fig 4.22*A*). However, at 1x and 5x dilutions, contrary to previous observation when CpG ODN (ODN 2006) complexed with PS4, a robust synergistic IL-6 induction was

observed (almost >4 fold increase compared to either CpG ODN only or PS4 only treatments (Fig 4.22*B*) this trend is seen even at 25x dilution but the magnitude is relatively small. Interestingly, at 625x dilution both pI:C and R848 complexed with PS4 showed almost 3 times higher synergistic cooperation while inducing IL-6 (Fig. 4.22*C*,*D*) .



C)



D)

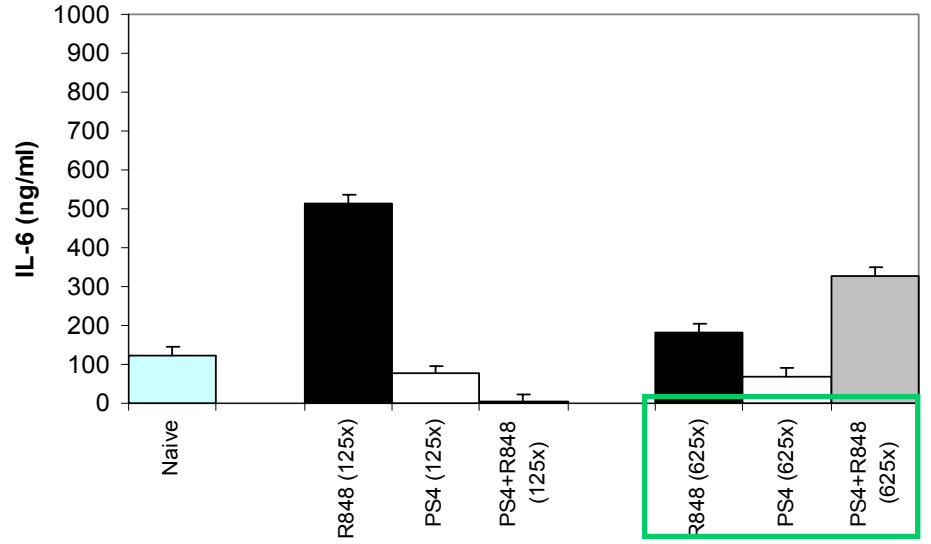


Figure 4.22. IL-6 ELISA of spleen cells when stimulated with PS-4 only, TLR ligands only and PS-4 complexed TLR ligands after 42h. *A)* Control ODN *B)* CpG ODN (ODN 2006) *C)* pI:C (2.0 µg/ml) *D)* R848. $p < 0{,}04$ (compared to ligand only and PS only levels at 1X, 5X, 125X and 625X dilution). ***(green boxes; synergism).***

4.3.3. TLR Expression Pattern of Spleen Cells

We checked the TLR gene expression of spleen cells after incubation with pI:C, and R848 alone or complexed with PS4, at 125x dilution for 4 hrs (Fig. 4.24). Despite to the RAW TLR panel (Fig. 4.16) in spleen cells *tlr3*, *tlr4*, *tlr6* and *tlr7* messages were strongly upregulated after the stimulation with PS4 alone. *Tlr1, tlr2, tlr6* were up-regulated with PS4-pI:C complex, as expected. In stimulation of spleen cells with i) PS4 only, ii) R848 only and iii) PS4-R848 complex we couldn't see any enhancement in any TLR gene messages. This is not surprising since R848 is so potent in inducing gene upregulation at 125x dilution there is no additive effect imposed by PS4.

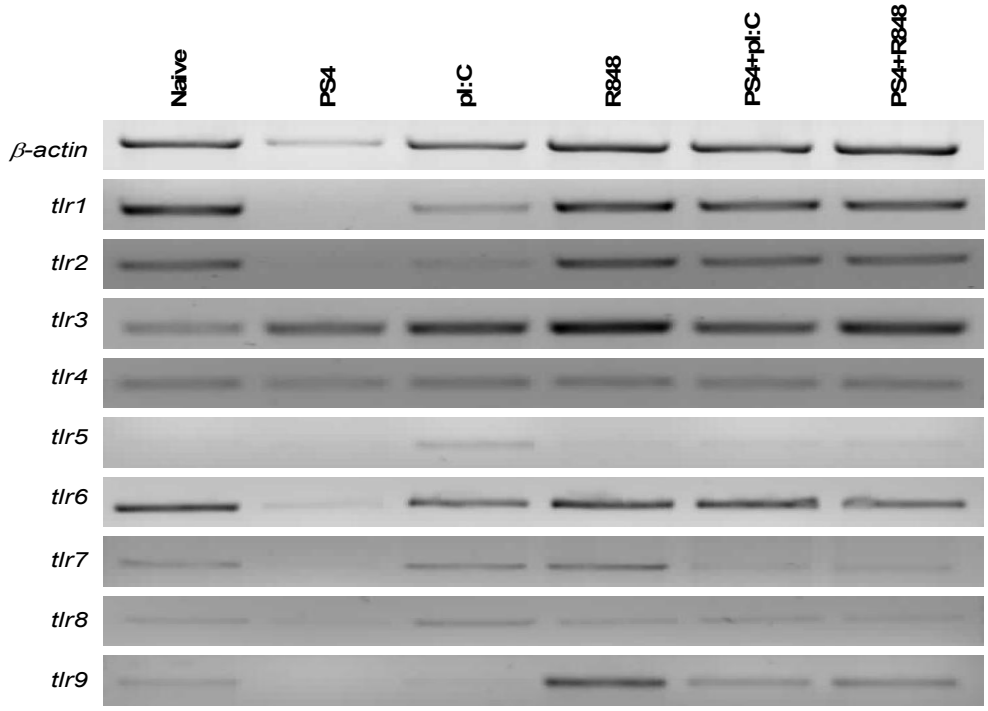


Figure 4.23. TLR mRNA expression of PS4 complexed pI:C or R848. After stimulation for 4h, spleen cells' total RNA were subjected to RT-PCR and amplified with *tlr-1* to *tlr-9* specific primers.

4.3.4. Cytokine and Chemokine Expression Pattern of Spleen Cells

In addition to TLR panel we tested the specific cytokines and chemokine gene level differentiation of these two mixtures despite to their alone stimulation affects.

(Fig 4.24). When PS4-pI:C or R848 complexes were prepared the messages of all the tested cytokines and chemokines were upregulated for PS4-pI:C set (compared to either PS4 or pI:C alone counterparts). The only exception is that *il-15*, *mcp-1* and *il-6* message expression intensities were slightly lower compared to the rest of the cytokines/chemokines. This is in general true for PS4-R848 set (with two exceptions). While *il-18* expression level decreased, *il-6* message remained unchanged for PS4-R848 set (compared to either PS4 or R848 alone counterparts).

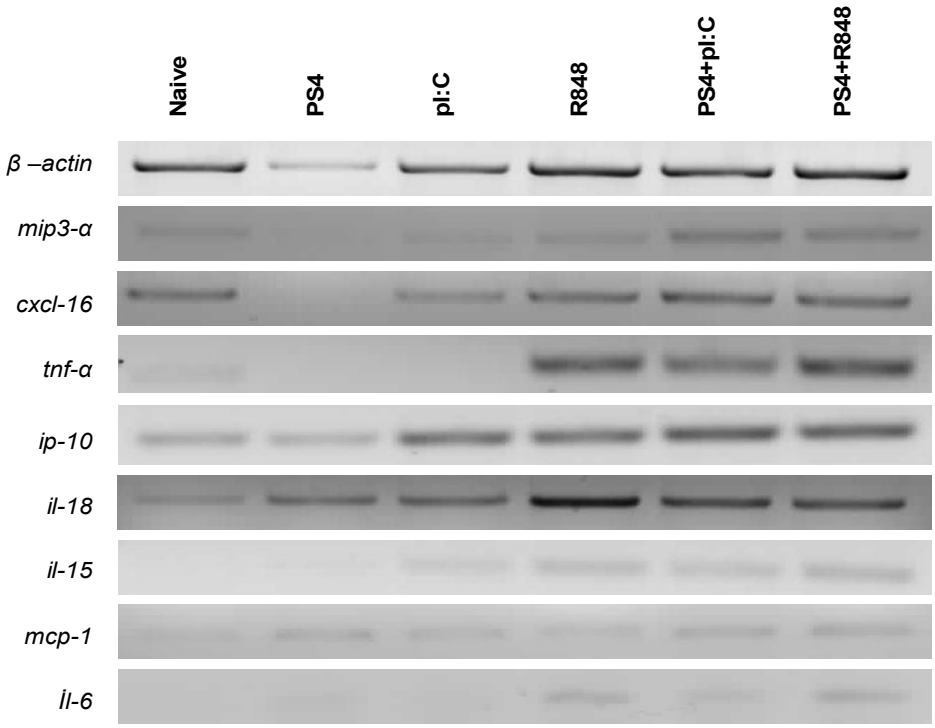


Figure 4.24. RT-PCR analysis for various chemokines and cytokines after incubation for 4h spleen cells with with PS-4 only, R848 only, pI:C only and PS-4 complexed R848 or pI:C in 125x diluted concentrations.

4.3.5. Particle Size Determination of PS4-complexed TLR ligand

In the literature, there is building evidence that some of the TLR ligands induce their effects more effectively when they are in nanoparticle structures. To establish whether PS4-TLR ligand complexes can form nanoparticles we have investigated the sizes of PS4, pI:C and their complexed form. pI:C showed relatively homogenous size distribution (20-70 nm, Fig. 4.25*C*). The images were taken with AFM. Sonicated PS4 (Fig 4.25*A*) showed more homogenous particle size distribution

compared to unsonicated PS4 (Fig 4.25*B)*. The size of the sonicated PS4 (30 – 100 nm) as expected was much smaller than unsonicated one (ca. 150 – 250 nm). Indeed, in our preliminary experiments, we have observed that sonicated PS4 gave significantly higher immune activation than that of unsonicated counterpart (data not shown). When we checked PS4-pI:C complex size were in between 100–300 nm, indicating that dsRNA complexation increased the nanoparticle diameter (Fig 4.25*D*)

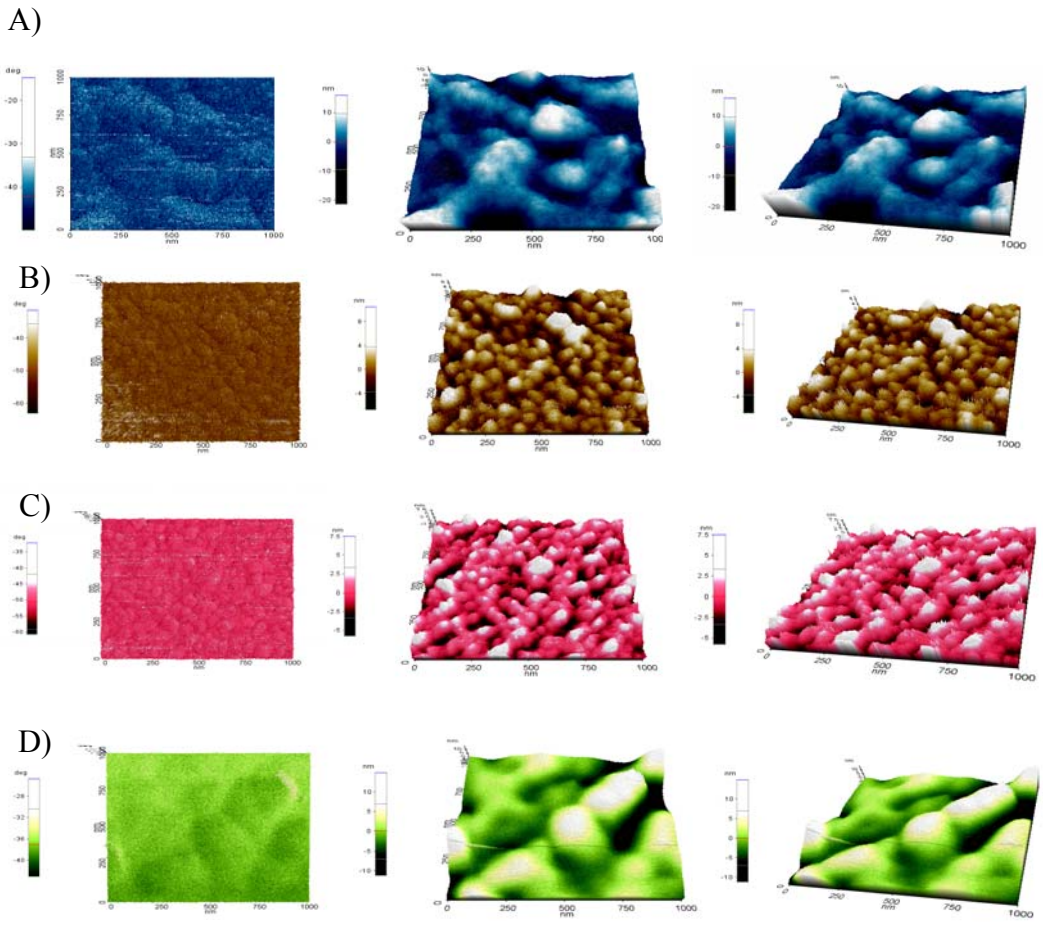


Figure 4.25. AFM phase, 2D topography, 3D topography images of *A)* PS-4 unsonicated *B)* PS-4 sonicated *C)* pI:C *D)* PS-4 + pI:C at 1 µm x 1 µm areas.

5. DISCUSSION

One of the main innate immune activation system against microbes in mammals are controlled by Toll-like receptors; which is first characterized in 1997 by Medzhitov et. al. Since then, TLR mediated immunity become one of the most popular subject in immunobiology. It has been reported that several TLR ligands are the future candidates for many therapeutic applications. and can be the cure for many infectious diseases such as Anthrax, AIDS, Hepatitis, (Klinman DM, *2004*), Listeriosis, (Gursel, *2001*) and cancer (Krieg, *2007*).

To reach our aim we took the following approach; we incubated the spleen cells with 15 different combinations at two different ligand concentrations of bacterial and viral origin TLR ligands. We found out the most potent TLR ligand combination from these groups (Tables 4.2, 4.3 and 4.4. summarize these findings). Independent from these experiments we determined the most effective PS (out of 4 PS candidates) using mouse macrophage cell line RAW 264.7. After establishing the potency of the most promising PS, we have initiated the complexation studies with nucleic acid TLR ligands (pI:C, R848, Control ODN and CpG DNA).

Six different TLR ligands were used for the cooperation studies. Cytokines such as IFN-γ, IL-6 and TNF-α were checked to demonstrate the changes in Th1 biased immunity. Mainly TLR7/8 ligand, R848 plus other TLR ligands, which are bacterial and viral synthetic products, induced pro-inflammatory cytokines. The R848 mainly acted in synergistically with several other ligands and was the most potent inducer of innate immunity.

mRNA expression analysis of TLR genes resulted as *tlr2*, *tlr4, tlr7* and *tlr9* in the combination of R848 plus other TLR ligands, in accordance with ELISA results means that both extracellular and endosomal TLRs act in concert. It was an interesting result that Control ODN, when combined with LPS or R848 increased *tlr7* expression, but decreased the *tlr9* gene expression. Further studies should be done to explain this unexpected result. Combination of two intracellular TLR ligands; TLR3 and TLR9 enhanced the extracellular *tlr2*, *tlr4, and tlr5* expression. This finding is interesting,

indication of this could mean that immune cells may want to make sure if there is an extracellular or intracellular infection is going on pathogen insult. Upon this extracellular TLR message upregulation, may be it differentiates the type of infection and becomes committed to upregulate only endosome associated TLRs and prepares itself for combat against an intracellular pathogen.

Ganoderma and *Shiitake* mushrooms have been used for medicinal purposes. The anti-tumor and immuno-modulating acitivites of these mushrooms have been reported as early as in 1957. Extracts from *G.lucidum* have been shown to activate the host-mediated immune responses by the production of cytokines (Lieu, *1992*).

One aproach to get an improved vaccine immunity, is to design formulations that provide simultaneous presentation of an antigen and an adjuvant to immunologically relevant cell in a carrier depot system. Only in these cases Ag specific immune response reach to a level where appreciable protection from pathogen is created. Inclusion of one or more TLR ligand with a specific carrier that can act as a co-adjuvant would activate innate immunity, and lead to an increased and extended cytokine production. It has been previously reported that TLR2 and TLR4 synergistically act on macrophages resulted in enhancement of TNF production (Beutler, *2001*). Costimulation with TLR4 and TLR2 or TLR9 induces synergistic release of Th1 cytokines (Equils, *2003*). A subsequent study showed that stimulation of mouse macrophages with both TLR3 ligand, pI:C and TLR9 ligand; CpG DNA induced more-than-additive levels of TNF, IL-6 and IL-12p40 which again confirmed that cooperation between certain TLRs exist (Whitmore, *2004*). A study about TLR7/8 described that the effects of coupling a TLR ligand to antigen induced pronounced vaccine mediated antigen-specific response in a primate model (Wille-Reece, *2005*). Another primate conducted study showed that licensed anthrax vaccine in the combination of CpG ODN improved the protective immune response (Klinman, *2006*).

It has been showed that a type of polysaccharide from *Spirulina* bacteria was a potent activator of TLR2 through NF-κB pathway which induces IL-1β and TNF-α in human monocytes (Balachandran, *2006*). The relation between *G.lucidum*, *Shiitake*

mushrooms and TLRs are coming to light these days. There are limited studies concerning TLR and PS extracts from mushrooms. Recent study showed that *G. lucidum* polysaccharides enhanced CD14 mediated endocytosis of LPS and promote TLR2/4 signal transduction of cytokine expression (Hua, *2007*).

We hypothesized that by achieving PS/TLR ligand complexes we have two fold benefits, i) while constructing a depot delivery system, ii) we can have an effective immunoadjuvant action that could be used for generating novel vaccine formulations.

Polysaccharides mainly act on innate immune system through TLR2/6 (similar to peptidoglycan, and zymosan) and TLR4 (lipopolysaccharide). So, we did check the pro-inflammatory cytokines TNF-α and IL-6 which were primarly secreted from immune cells, after the exposure of polysaccharides. IL-6; secreted from macrophages in response to pathogen associated molecular patterns (PAMPs) seemed to be released at high levels with the incubation of PS2 and PS4 in two different doses for 24 and 42 hours. An interesting result from the IL-6 ELISA, is that at 42 hour stimulation in low doses (1,6 μg/ml) with PS2 and PS4 secreted more IL-6 then in high doses (16 μg/ml). The dose and time-dependent stimulation assays we found out that incubation of PS2 and PS4, with one of the main APC macrophages, at least 2μg/ml (0,2μg/ml seems the optimum dose) dose was enough to induce TNF-α level. In time-dependent experiment we saw that PS2 and PS4 seemed to release more TNF-α from macrophages after 12-24 hours than PS1 and PS3.

We further checked for the nitric oxide levels after the stimulation with these PSs. Since NO is an important regulator and mediator of macrophage-mediated cytotoxicity for microbes and tumor cells (Nathan, *1993*), the results from NO Assay gave us the direct evidence for the activation of RAW 264.7 in culture via PS. As similar with ELISA it was turned out that both PS2 and PS4 were the most potent PSs. But time-dependent NO assay didn't result like TNF-α ELISA. After 24h stimulation the NO levels reached saturation with PS2 and PS4 in parallel to the one of the control ODN group plus LPS. It seemed that PS1 and PS3 were ineffective until 24h and secreted nitric oxide almost 2-3 fold lower than the other two PS extracts.

The gene expression analysis of these polysaccharide extracts (PS1 to PS4) for entire mouse TLR panel, showed us that within 4 hours of stimulation with PS2 or PS4 *tlr-1, 2, 3, 4, 6* and *7* upregulated. In spite of that, after the first 2 hours the gene expression of *tlr-1, 2, 3, 4, 6* and *7* were down-regulated with the incubation of PS1 and PS3. Once again these results showed us that, all four polysaccharides change the gene expression pattern of TLRs and PS2 with PS4 were the most potent immune-activators when compared with unstimulated group.

To prove if these PS extracts acts on immune-system throughout the TLR-dependent pathway, we transformed hTLR2/6 HEK cells with NF-κB plasmid. Incubation with PS1, 2, 3, and 4 with positive controls, PGN or Zymosan, and LPS as a negative control for 24 hours, showed that PS2 and PS4 were triggering through TLR2/6 pathway and are the strongest inducers (compared to PS1 andPS3) of this TLR2/6 induced NF-κB mediated signaling pathway.

Finally we formulated the most potent PS extract (PS4) with three nucleic acid TLR ligands and incubated them with murine macrophages (and splenocytes) to investigate whether there is an added benefit to create PS/Nucleic acid complexations in the augmented activation of the innate immunity. PS4-R848 or PS4-pI:C complexes even at very low doses, showed highly significant IL-6 production compared to their alone treatments at the same dose. These findings collectively, strongly indicated that these complexes indeed impose a synergism when they are brought together and simultaneously presented to the relevant immune cells. Like in the first TLR cooperation experiments which were done to determine the most effective TLR ligand combination, when combined with PS4, TLR7/8 ligand R848 seemed to be the most active stimulant.

In conclusion, the combination of certain TLR ligands induces immune response (both cytokine and mRNA expression level) in a TLR-dependent manner. To conceive an effective vaccine against infectious agents, inclusion of more than one TLR ligand in the final formulation will be of great benefit.

Polysaccharides which were extracted from edible mushrooms, when given as such could stimulate the immune system via TLR2/6 dependent fashion but can also serve as a depot delivery vehicle. This carrier may be used to deliver certain nucleic acid based vaccine adjuvants to enhance cytokine secretion from splenocytes (Mizu, *2003*). These PS extracts could be a potent carrier. Ultimately, the whole knowledge I this thesis will guide us to develop more effective/specialized vaccine formulations against infectious agents.

6. FUTURE STUDIES

In future, we are planning to work on the effect of these combinations on specific cell types which are important in innate-immunity and regulate Toll-like receptor induction from plasmacytoid dendritic cells (pDC that expresses TLR7 and TLR9) which are the main producers of IFN-α cytokine that is highly efficient in inducing cross-priming $CD8^+$ T cells against exogenous viral antigens (Lapenta, *2006*) and also isolate myeloid dendritic cells (mDC, express TLR2/6 TLR3) that induces IL-12 production by stimulating T-cells. One main reason for choosing these types of cells to work with is that the *ex vivo* manipulation of the DCs. Accumulating data point out that these manipulated cells becomes potent antigen presenting cell and can be harnessed in clinics to induce effective anti-tumor activity *in vivo* (Gilboa, *2007*).

Specific cluster of differentiation (CD) markers such as; CD36; a monocyte marker, CD80 and CD86; provides a co-stimulatory signal necessary for T cell activation and survival, CD11b; a macrophage marker, BDCA; pDC marker, CD4; expressed on the surface of T helper cells, regulatory T cells (together with CD25), monocytes, macrophages, and dendritic cells, CD69; a T-cell activator molecule will be used as a marker to determine the differentiation/activation status and effect on a collection of diverse group of immune cells from spleen, peritoneal exudate and lymph nodes will be studied. In addition, we are expecting establish intracellular cytokine staining of specific immune cells in order to display differential activity of our PS formulations and the production/secretion characterization from different cell populations.

To see in which manner these combinations triggers the immune-system, silencing of specific genes for example TLR genes or the genes of adaptor proteins that are involved in the TLR pathway; Myeloid differentiation primary response gene 88 (MyD88), Toll/IL1 receptor-associated protein (TIRAP), TIR domain-containing adaptor protein inducing IFN-b (TRIF) and TRIF-related adaptor molecule (TRAM) is planned for further studies.

Since TLRs and other pathogen recognition receptor (PRR), such as NOD-like receptors (NLR) NOD-1 and NOD-2 or even Dectin-1 can synergize with several TLR1, 2 and 6 ligands, especially when delivered into cytosol (such as PS molecules) or even endosome associated TLR was reported to cooperate with NOD-2 and regulate the induction of more robust TNF and IL6 and IFNγ production (Uehara, *2005*). We will transfect hTLR2/6 HEK cells with NOD-1 and NOD-2 expressing plasmids, and stimulate these cells with different PS4 nucleic acid complexes to observe if there is an interaction between NLRs and TLRs while inducing this synergism.

It is of great importance to establish whether *in vitro* observations can be translated to *in vivo* setting. These mixtures could be used as an anti-tumorogenic, immunoprotective agents. Also they could be used as vaccine adjuvants by coadministirating with specific vaccine antigens such as; anthrax vaccine adsorbed (AVA), tuberculosis vaccine; Bacillus of Calmette and Guerin (BCG), influenza, and hepatitis B.

Indeed synergism between these TLR ligands and PS extracts is important not only for understanding innate response to microbial infection but also in the context of vaccination and cancer immunotherapy. Thus the additional studies exploring these interactions will lead to an improved understanding of innate immunity to pathogen infection and the utility for TLRs in immune based therapies for diseases.

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9. APPENDICES

9.1 Appendix A

Four different polysaccharide extracts code table:

Name	Code	Our Code	Weight	Solubility in ddH_2O
Ganoderma lucidum (Alata strain)	AR150	PS-1	39,21 mg	70-80 %
Ganoderma lucidum (Balcalı strain)	BROsm	PS-2	24,55 mg	80-90 %
Shiitake	SR150	PS-3	34,18 mg	40 %
Ganoderma lucidum (Alata strain)	AROsm	PS-4	23,22 mg	60 %

9.2 Appendix B

Standard Solutions, Buffers, Media

Blocking Buffer (ELISA)

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

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

Loading Dye (Agarose gel)

- 0,009 grams Bromofenol blue
- 0,009 grams Xylen cyanol
- 2,8 ml ddH_2O
- 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 Na_2HPO_4 . $2H_2O$
- 2 grams KH_2PO_4

into 1 lt ddH_2O

pH= 6,8. For 1xPBS's pH should be ≈ 7,2-7,4. Should be autoclaved prior to use.

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

- 242 grams Tris ($C_4H_{11}NO_3$)
- 37,2 grams Tritiplex 3 (EDTA= $C_{10}H_{14}N_2Na_2O_2$. $2H_2O$)
- 57,1 ml Glacial acetic acid

into 1 lt ddH_2O

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

T-cell Buffer [ELISA]

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

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

- 500 ml 10x PBS
- 2,5 ml Tween20
- 4,5 lt dH_2O

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

- 2 %: 10 ml FBS (Oligo FBS = inactivated at 65°C, Regular FBS = inactivated at 55°C)

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

In 500 ml media

$NaNO_2$ (100µM)

- 34,5 µg $NaNO_2$
- 50 ml Ultrapure H_2O [100x]

Dilute this solution into 1x for 100µM concentration.