IMMUNOTHERAPEUTIC APPLICATIONS OF NUCLEIC ACID BASED TLR AGONISTS AND ANTAGONISTS

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BY
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JANUARY, 2014

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

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ABSTRACT

IMMUNOTHERAPEUTIC APPLICATIONS OF NUCLEIC ACID BASED TLR AGONISTS AND ANTAGONISTS

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Recent evidence suggests that genetic material is not just the blueprint of life. Depending on the type of the source that genomic material is extracted or the type of motif that DNA harbors DNA could be immunostimulatory or immunosuppressive to innate immune system cells. Unmethylated CpG motifs, from bacterial genome is recognized by TLR9 expressing cells as "non-self" and initiates an orchestrated immune activation characteristics of Th1-biased immunity. Conversely, mammalian genome rich in G-runs motifs, such as telomeric ends expressing TTAGGG repeats downregulate Th1-biased immune responses and contributes to restore over exuberant immune response. Several TLR agonists/antagonists candidates are currently under preclinical/clinical trials to prevent or treat cancer, infectious disease, allergic disorders and autoimmune or autoinflammatory diseases. In this thesis, we attempted to broaden the application of nucleic acid based TLR therapy by investigating their beneficial effects either as vaccine adjuvants against Foot and Mouse Disease or to test as immunosuppressive agents that may control autoimmune uveitis. We showed that an immunosuppressive ODN namely A151 downregulated severity of an established animal model of uveitis, endotoxin induced uveitis (EIU), up to more than 60% histologically, or more than 80% based on cytokine production such as MIP3α, IL6, IFNγ from uveitic animals. In order to compare effects of this biological immunosuppressive agent, next we studied effects of a broad-spectrum immunosuppressive drug, namely cyclosporine A in an emulsion form, commercially known as Restasis™. Our results indicated that Restasis™ had significantly lower capacity to reduce disease severity and downregulate *in vivo* chemokine or cytokine levels compared to A151. The second theme of this thesis was to demonstrate effective utilization of CpG ODN as an immunostimulatory agent. The adjuvant effect of CpG ODN 1555-PS in Foot and Mouth Disease (FMD) vaccine formulations were tested. In this study, CpG ODNs were formulated either with commercially used monovalent vaccine or mixed with free Serotype-O Ag. Data suggested that, in mice, inclusion of CpG ODN as an adjuvant, spared the Ag by 6 fold and the vaccine dependent virus neutralization titers were not only higher but also long lasting compared to commercial monovalent vaccine. CpG inclusion in the FMD vaccine helped to generate 1.5-2 fold more cell mediated immunity 24 h after booster injection. Implicating that virus infection could be more effectively controlled by the novel approach. In the last part of this thesis, effects of CpG ODNs as prophylactic agents for newborn broiler chicken were tested *in vivo*. Our results suggested that CpG ODN pretreatment not only significantly reduced mortality rates (> 2.0 %) but also contributed to growth performance of these industrially important animals. We demonstrated that nucleic acid based agonists and antagonists might be

of great potential to be developed as therapeutic agents either in the clinic or for controlling health of industrially important animals.

Keywords: Innate immunity, TLR, suppressive ODN A151, Cyclosporine A EIU, FMD, vaccine, adjuvant, chicken, prophylactic agent.

ÖZET

NÜKLEİK ASİT TEMELLİ TLR AGONİST VE ANTAGONİSTLERİNİN İMMÜNÖTERAPÖTİK UYGULAMALARI

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Yapılan son çalışmalar genetik materyalin yaşamın sadece mavi baskısı olmadığını göstermektedir. Genetik materyal, elde edildiği kaynağa ve barındırdığı DNA örgesine göre immün-stimülan ya da immün baskılayıcı özellik göstermektedir. Bakteri genomu kökenli metillenmemiş CpG örgeleri, TLR9 eksprese eden hücreler tarafından yabancı madde olarak algılanıp Th1 tipte bağışıklık yanıtına neden olmaktadır. Memeli genomu kökenli G-zengini sekanslar, telomerik TTAGGG örgesi gibi, ise aksine Th1 tip immün yanıtı baskılamakta ve immün homeostazın sağlanmasını desteklemektedir. Çeşitli TLR ulağı agonist/antagonistleri preklinik/klinik test fazlarında kanserin, bulaşıcı hastalıkların, alerjik hastalıkların ve otoimmün ya da otoenflammatuvar hastalıkların tedavisi için aday ajanlar olarak denenmektedir. Bu tezde, nükleik asit bazlı TLR terapisinin kullanım alanları ŞAP hastalığına karşın aşı formülasyonlarında adjuvant olarak kullanımları ve otoimmün üveit hastalığının kontrolünde immün baskılayıcı ajan olarak kullanımları test edilerek genişletilmeye çalışılmıştır. Yaptığımız çalışmalarda A151 isimli immün baskılayıcı özellikteki nükleik asidin endotoksin kaynaklı üveiti histolojik olarak %60 gerilettiğini, aynı zamanda üveitli hayvanlardaki MIP3α, IL6 ve IFNγ gibi pro-enflammatuvar sitokinleri de %80 oranında baskıladığını gösterdik. Bu immün baskılayıcı ajanın biyolojik etkilerini geniş spektrumlu immün baskılayıcı ilaç olan Restasis™ (emülsiyon formda siklosporin A) ile karşılaştırdık. Yaptığımız bu çalışmada A151'e göre Restasis™ hem hastalık şiddetini gidermede hem de in vivo sitokin ve kemokin düzeylerini dengelemekte daha düşük etkinlik göstermiştir. Tezin ikinci bölümünde ise metillenmemiş CpG DNA örgülerinin (CpG ODN) immün stimülan ajan olarak kullanılması yer almaktadır. Yapılan çalışmada 1555-PS tipi CpG ODN ŞAP aşısı formülasyonlarında adjuvant olarak kullanılmıştır. Bu çalışmada CpG ODN'ler ticari monovalent ŞAP aşısına katılarak ya da serbest Serotip-O antijeni ile formüle edilerek test edilmiştir. CpG ODN katılımının aşı formülasyonlarında kullanılan antijen ihtiyacını 6 kat azalttığı ve virüs nötralizasyon sonuçlarına göre hem daha güçlü hem de daha uzun süreli koruma sağladığı fare çalışmalarında görülmüştür. ŞAP aşı formülasyonlarına CpG ODN katılımı güçlendirici (ikincil) enjeksiyondan 24 saat sonra hücresel bağışık yanıtı 1,5-2 kat arttırmıştır. Sonuçlarımız, bu özgün yaklaşımın virüs enfeksiyonunun çok daha efektif bir şekilde kontrol altına alınabileceğini göstermektedir. Bu tezin son bölümünde CpG ODN'lerin yeni doğan besi piliçlerinde profilaktik ajan olarak kullanımları *in vivo* etkileri test edilmiştir. Sonuçlarımız CpG ODN kullanımının sadece mortalite oranlarını düşürmekle kalmadığını (> 2.0 %) aynı zamanda endüstriyel açıdan önemli bu hayvanların büyüme performanslarını da olumlu yönde etkilediğini göstermiştir. Bu tezde sunulan çalışmalar, nükleik asit bazlı TLR ulağı agonist/antagonistlerinin gerek klinik açıdan önemli hastalıkların kontrolünde

gerekse endüstriyel açıdan önemli hayvanların korunmasında terapötik amaçlı ajan olarak geliştirilmesi için yüksek potansiyele sahip olduğunu göstermektedir.

Anahtar kelimeler: Doğal bağışıklık, polisakkarit, nanokompleks, ilaç salım sistemi, TLR, CpG ODN, kanser immünöterapi, aşı, adjuvan.

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Abbreviations

Ab	Antibody
AIM2	Absent in melanoma 2
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein
BAL	Bronchoalveolar lavage
BHK-21	Baby Hamster Kidney -21
BCR	B-cell receptor
BEI	Binary ethyleneimine
bp	Base pairs
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CIA	Collagen-induced arthritis
ck	Chicken
CLR	C-type lectin receptors
CMV	Cytomegalovirus
CpG	Unmethylated cytosine-guaniosine motifs
CRP	C-reactive protein
CsA	Cyclosporin A
CXCL	CXC-chemokine ligand
DC	Dendritic cell
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental Autoimmune Uveitis
EDTA	Ethylenediaminetetraacetic acid
EIU	Endotoxin induced Uveitis
ELISA	Enzyme Linked-Immunosorbent Assay
EPEF	European Efficiency of Productivity Factor
ER	Endoplasmic reticulum
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FASL	Fas ligand
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FCR	Feed Conversion Ratio
Flt3	Fms-like tyrosine kinase 3
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GPI	Glycophosphatidylinositol
GVHD	Graft versus Host Disease
HBV	Hepatitis-B Virus
HEK	Human embryonic kidney
HSV	Herpes Simplex Virus
hu	Human
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
ip	Intraperitonea

IP-10	Interferon gamma-induced protein 10
IRAK	IL-1 receptor-associated kinase
IRBP	interphotoreceptor retinoid-binding protein
IRF3	Interferon-regulatory factor 3
IVA	Influenza A Virus
KO	Knock out
KC	Kerationcyte chemoattractant
LAL	Limulus amebocyte lysate
ICAM-1	Intercellular Adhesion Molecule 1
IFI16	Gamma-interferon-inducible protein
IκK	Inhibitor kappa B kinase
INH ODN	Inhibitory oligonucleotides
IRS	Immunoregulatory sequences
LBP	LPS-binding protein
LITAF	Lipopolysaccharide-induced tumor necrosis factor-alpha factor
LGP	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
mAb	Monoclonal antibody
MALP	Mycoplasmal lipopeptide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCMV	Mouse cytomegalovirus
MCP	Monocyte Chemoattractant Protein
MD	Myeloid differentiation protein
MDA	3,4-methylenedioxyamphetamine
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MS ODN	Microsatellite DNA mimicking ODNs

mu	Murine
MyD-88	Myeloid differentiation primary response gene (88)
NALP	NAcht leucine-rich-repeat protein 1
NFAT	Nuclear factor of activated T-cells
NZB/W	New Zealand Black/White
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NKG2D	Natural-killer group 2, member D receptor
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerization Domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pIC (polyIC)	Polyriboinosinic polyribocytidylic acid
PNPP	Para-nitrophenyl phosphate
PRAT4	Protein Associated with TLR4
PRR	Pattern Recognition Receptors
PS CpG ODN	Phosphorothioate backbone modified CpG ODN
PS	Phosphorothioate
RA	Rheumatoid Arthritis
RANTES	Regulated upon activation, normal T-cell expressed, and secreted

RIG	Retinoic acid-inducible gene
RLR	Retinoic acid-inducible gene-I-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse transcriptase PCR
RT	Room temperature
SA-AKP	Streptavidin Alkaline-phosphatase
SAP	Serum amyloid protein
SAT	Southern African Territories
SLE	Systemic Lupus Erythematosus
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
ssDNA	Single stranded DNA
ssRNA	Single-stranded RNA
TAK1	TGF- β -activated kinase 1
TBK	TANK-binding kinase
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
TPA	7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-l3-acetate
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain containing adaptor inducing IFNβ
UNC93B1	Unc-93 homolog B1

UV	Ultraviolet
VKH	Vogt-Koyanagi-Harada syndrome
WNV	West Nile Viruses

Chapter 1

Introduction

1.1 Immune System

All living organisms from bacteria to human have evolved several strategies to combat invading pathogens. In higher organisms the mechanisms involved in providing protection collectively constitute the immune system [1]

In mammals, the epithelial surfaces of the body establish the first lines of defense against invading pathogens. Epithelial cells of the skin, gastrointestinal, respiratory and urogenital tracts are joined by tight junctions and provide a physical barrier between the internal milieu and the external world. Moreover, movement of mucus by cilia and longitudinal flow of air and fluids through internal body surfaces prevents adhesion of pathogens to the epithelium. Other then these physical protection mechanisms, several chemical and enzymatic factors such as antimicrobial peptides, salivary enzymes and low pH of the stomach also contribute to protection from infections. In addition to these defenses, most epithelial surfaces are in contact

with microbial flora which consists of non-pathogenic bacteria, fungi and viruses that compete with the pathogenic ones.[2, 3]

Mammalian immune system consists of two interrelated arms: the evolutionarily conserved, immediate, but non-specific innate immunity and the highly specific but comparably late-acting adaptive immune system. These two systems are interlinked and work together to enable the recognition and elimination of invading pathogens. The innate and the adaptive immune system use different strategies to recognize and eliminate infection. While the innate immune system uses several germ-line encoded receptors to detect invading pathogens, the adaptive immune system uses randomly generated and highly specific receptors for detection of infection. [1]

One unifying feature of both the innate and the adaptive immune system is their ability to discriminate *self* from *non-self*. In immunology, "self-molecules" are defined as the host's own molecules that can be distinguished from other foreign substances. In contrast, "non-self molecules" are those that are recognized as "foreign" by the immune system. By means of this ability to discriminate "self" from "non-self", the immune system can detect various pathogens such as viruses, bacteria and parasitic worms and distinguish them from normal cells and tissues of the host [4].

Table 1.1. Components of the Immune System *(Adapted from[5])*

Attribute	**Innate Immunity**	**Adaptive Immunity**
Response time	Minutes/hours	Highly specific; discriminates even minor differences in molecular structure; details of microbial or nonmicrobial structure recognized with high specificity
Diversity	A limited number of germ line-encoded receptors	Highly diverse; a very large number of receptors arising from genetic recombination of receptor genes
Memory responses	None	Persistent memory, with faster response of greater magnitude on subsequent infection
Self/nonself discrimination	Perfect; no microbe-specific pattern in host	Very good; occasional failures of self/nonself discrimination result in autoimmune disease
Soluble components of blood or tissue fluids	Many antimicrobial peptides and proteins	Antibodies
Major cell types	Phagocytes (monocytes, macrophages, neutrophils), natural killer (NK) cells, dendritic cells	T cells, B cells, antigen-presenting cells

Recognition of microbial pathogens by innate immune cells is mediated by germline-encoded pattern recognition receptors (PRRs). PRRs recognize molecular structures that are expressed by a diverse group of pathogens, collectively known as pathogen associated molecular patterns (PAMPs). Following PAMP recognition, PRRs initiate a series of signaling cascades that generate an initial response against the pathogen [6]. PAMP recognition leads to the maturation and activation of antigen presenting cells (APCs) and allows effective presentation of microbial antigens to cells of the adaptive immune system..[7]

Adaptive Immunity can be divided into two subsections: humoral immunity and cell mediated immunity. Humoral immunity involves the production of antibodies by B cells that can opsonize or neutralize pathogens in blood or other body fluids. On the other hand, cell mediated immunity is responsible for eradication of cancer cells or cells that are infected with intracellular pathogens via cytotoxic T cells. Another class of T cells called T helper cells do not have cytotoxic or phagocytic activity rather they activate other immune cells such as B cells, cytotoxic T cells, macrophages and decide the type of immune response either Th1 or Th2, Th17 or Th9 biased [8, 9] B cells and T cells express B cell receptors (BCR) and T cell receptors (TCR), respectively. These receptors are generated via DNA recombination during the development of these cells and are able to recognize huge numbers of pathogens. Each TCR and BCR consist of a constant and a variable region. Variable region of these receptors are encoded via different gene segments. By random assembly of these gene segments during creation of each receptor, a huge receptor repertoire can be produced. If a pathogen invades the body, T and B cells with corresponding receptors become activated and induce humoral and cellular immune responses. In contrast to the innate immune system, the adaptive immune system has memory When the same pathogen re-invades the body, memory B and T cells expand monoclonally and induce a more rapid and robust immune response [10].

1.2 Innate Immune System

Our bodies are constantly exposed to infectious microorganisms present in our environment. In spite of this frequent exposure, disease occurance is quite low. Most of the microorganisms that can cross the protective epithelial surfaces are detected and eliminated within minutes or hours by defence mechanisms that do not require a prolonged period of induction since they do not rely on the clonal expansion of antigen specific lymphocytes. [2, 11]

Innate immune system is an evolutionarily ancient part of the immune system. Since plants and animals share the same modules, it seems that it arose before the split of these two kingdoms. Defects in innate immunity are almost always lethal, further supporting the evidence that this system is evolutionarily conserved [2, 12].

Innate immune system is composed of many types of cells such as Mast cells, natural killer cells, eosinophils, basophils; and the phagocytic cells including macrophages, neutrophils and dendritic cells (DCs) [13, 14].

If a pathogen can breach the epithelial layer and start to replicate in tissues, it would be recognized by the phagocytes such as DCs, In the classical view of the immune system, DCs engulf invading pathogens, digest their proteins into small peptides and then present these digested peptides on their surface in association with major histocompatibility complex (MHC) proteins. DCs then migrate to the regional lymph nodes and present the antigens to naïve T cells. Once a T cells with a corresponding T-cell receptor recognizes the MHC:peptide complex displayed on the surface of the DC, it becomes activated and starts proliferating (Figure 1.1)

This view is an incomplete one as recent evidence suggests that a DC can only prime a T cell if the DC itself is activated in the first place. Such a prerequisite for DC activation explains why DCs that capture self-antigens from dead or dying cells do

not activate T cells under steady-state conditions (i.e, when there is no infection). For efficient T cell activation to occur, the DCs must first be activated following recognition of PAMPS via the PRRs they express. This recognition leads to a series of signaling events that culminates in induction of co-stimulatory molecule expression on the surface of the DC and provides the "second signal" necessary to fully activate the naive T cell [10].

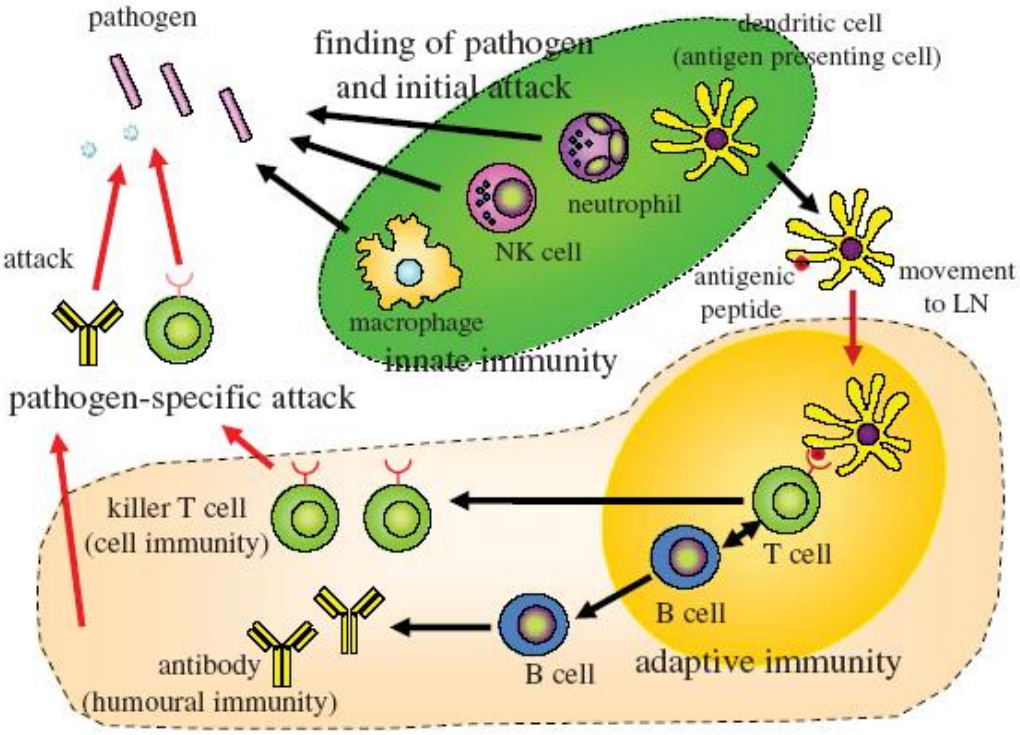


Figure 1.1. Interactions of Innate and Adaptive Immunity *(Adapted from:[10])*

1.3 Pattern Recognition Receptors

Innate immune cells express various PRRs that enable them to recognize PAMPs from different origins. Several classes of PRRs such as Toll-like Receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) have been identified in mammals, each specialized to sense the presence of pathogen at a unique subcellular compartment such as the plasma membrane, endolysosomes or the cytoplasm [15]. PAMP recognition triggers the activation and proliferation of B cells, natural killer

(NK) cells and professional antigen presenting cells (APCs) and induces release of several inflammatory and proinflammatory cytokines and chemokines such as TNFα, IL1α/β, IP10, MIP1α, MIP3α, MCP Regulated upon activation, normal T-cell expressed, (RANTES), IL6, IL12, IL15, IL18, IFNγ and type I interferons (IFNα and IFNβ; important for anti-viral response) that are pivotal for host defense. [2, 11, 14-17]

Toll-like Receptors (TLRs) are the first identified and most well characterized class of PRRs that can recognize a wide range of PAMPs [6, 13, 14, 18]. TLRs are type I transmembrane proteins containing 3 domains. Ectodomains of TLRs consists of leucine-rich repeats (LRRs) which mediates the recognition of PAMPs. Transmembrane and intracellular Toll interleukin 1 (IL-1) receptor (TIR) domains are required for downstream signal transduction. To date 12 different functional TLRs in mice and 10 different functional TLRs in human are identified [19]. Each TLR detects distinct PAPMs originating from viruses, mycoplasma, bacteria, fungi and parasites such as lipoproteins (TLR1,TLR2,TLR6), double stranded (ds) RNA (TLR3), lipopolysaccaride (LPS) (TLR4), flagellin (TLR5), single stranded (ss) RNA (TLR7, TLR8) and DNA (TLR9) [6, 13, 19].

TLRs are expressed on various cell types including immune and non-immune cells such as B cells, macrophages, NK cells, DCs, epithelial cells, endothelial cells and fibroblasts. However, cellular localization of TLRs differs according to the specific ligands they recognize. While TLR1, TLR2, TLR4, TLR5, TLR6 are localized on the plasma membrane, nucleic acid sensing TLRs such as TLR3, TLR7, TLR8 and TLR9 are localized in endosomes [6, 10, 13, 14].

PAMP recognition with corresponding TLRs initiates several downstream signaling pathways that lead to the production of inflammatory cytokines, type I IFNs, chemokines and antimicrobial peptides. These modulators cause recruitment of neutrophils, induction of IFN stimulated genes and direct killing of the pathogen.

Furthermore, activation of TLR signaling leads to the activation of DCs, thereby contributing to the induction of adaptive immune responses [6, 19]

Although TLRs can recognize several PAMPs that are extracellular or present in endosomes, other non-TLR PRRs such as membrane-bound C-type lectin receptors (CLRs)[20], cytosolic proteins such as NOD-like receptors (NLRs) [21] and RIG-I-like receptors (RLRs) [22], also contribute to the recognition of PAMPs. CLRs are membrane bound proteins that can induce inflammatory responses upon recognition of PAMPs from different origins such as bacteria and fungus. NLRs are a large group of cytosolic proteins that contains more than 20 members. NLRs respond to various types of PAMPs to trigger an innate immune response. NLRs such as NALP1 and NALP3 form inflammasomes with the contribution of ASC and Caspase-1 and mediate the processing of pro- IL1β to mature IL1β [21, 23]. RLRs are RNA helicases that can recognize RNA species in various types of cells. RLRs such as RIG-1, MDA5 and LGP2 recognize RNA in the cytoplasm and coordinate antiviral responses via type I IFN induction [24]

In case of an infection with an intact pathogen, several classes of PRRs recognize several molecular structures expressed on that pathogen which leads to multiple activation of PRRs due to multiple PAMP exposure. Moreover, different PRRs can recognize the same PAMP. The nature and the strength of the generated innate immune response depends on the coordination between TLRs and other non-TLR PRRs. In conclusion, TLRs in concert with other PRRs orchestrates innate immune responses against various pathogens such as bacteria, fungi, viruses and other protozoan parasites [6]. Table 1.2 shows PAMPs originating from different pathogens and their relevant PRRs expressed by immune cells.

Table 1.2. PAMP Detection by TLRs and other PRRs *(Adapted from [6])*

Species	PAMPs	TLR Usage	PRRs Involved in Recognition
Bacteria, mycobacteria	LPS	TLR4	
	Lipoproteins, LTA PGN	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-1, MDA5, NALP3
	structural protein	TLR2, TLR4	
Fungus	zymosan, β glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	tGTPI-mutin (Trypanosoma)	TLR2	
	glycoinositolphospholipids, (Trypanosoma)	TLR4	
	DNA	TLR9	
	hemozoin (Plasmodium)	TLR9	NALP3
	Profiling-like molecule (Toxoplasma gondii)	TLR11	

1.4 Toll-Like Receptors

Toll protein was first identified in fruitflies (Drosophila) as a protein that is involved in defining dorsoventral polarity during embryogenic development.[25] Following this, its role in mounting an effective immune response against the fungus *Aspergillus fumigatus* was also described, causing researches to focus on the identification of its homologues in mammals. In 1997, Janeway and his colleagues reported that “A human homologue of the Drosophila Toll protein signals activation

of adaptive immunity." and named the homologue as Toll-like Receptors [26]. As mentioned in the previous section, to date several TLRs have been identified in mouse and human and each recognize a distinct PAMP. Table 1.3 shows the chromosomal locations of the genes encoding TLRs [6, 13, 19] (Table 1.2 and Table 1.3).

Table 1.3.Chromosomal localizations of TLRs.

	Chromosome	
TLR	**Mouse**	**Human**
TLR1	5 37.0 cM	4p14
TLR2	3 E3	4q32
TLR3	8 B2	4q35
TLR4	4 33.0 cM	9q32-q33
TLR5	1 98.0 cM	1q41-q42
TLR6	5 37.0 cM	4 4p14
TLR7	X F5	Xp22.3
TLR8	X F5	Xp22
TLR9	9 F1	3p21.3
TLR10	N/A	4p14
TLR11	14 C1	N/A
TLR12	4 D2.2	N/A
TLR 13	X D	N/A

TLRs can be devided into two groups with regard to their cellular localizations: One group is expressed on the cell surface and include TLR1, TLR2, TLR4, TLR5, TLR6, TLR11 responsible for recognition of mainly microbial membrane components such as proteins, lipids and lipoproteins [19] (Figure 1.3). The other group consists of TLR3, TLR7, TLR8 and TLR9 which are expressed in the intracellular compartments and recognize nucleic acid ligands [19] (Figure 1.3).

1.4.1 Cell Surface Toll-Like Receptors

1.4.1.1 TLR1, TLR2 and TLR6

TLR2 recognizes a wide range of PAMPs form different origins such as bacteria, fungi, parasites and viruses. This promiscuous ligand recognition property of TLR2

can be explained by its ability to form heterodimers with TLR1, TLR6 or with non-TLR molecules such as CD36, CD14 and dectin-1. TLR2-TLR1 heterodimers and TLR2-TLR6 heterodimers recognize triacyl-lipopeptide from Gram negative bacteria and diacyl-lipopeptide from mycoplasma, respectively. [13, 27, 28] Thus, TLR1 and TLR6 functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides [29]. TLR2 also recognizes zymosan with association with dectin-1, a C-type Lectin Receptor. Furthermore, a type of class II scavenger receptor called CD36 functions as a co-receptor in sensing LTA and MALP-2 molecules of *Staph. Aureus* by the TLR2-TLR6 heterodimer [30].

1.4.1.2 TLR4

TLR4 is the receptor that recognizes lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. LPS is a potent immunostimulatory molecule and causes septic shock [31]. The recognition of LPS by TLR4 is dependent on the complex formation of TLR4 with a recognition subunit, MD2 (myeloid differentiation protein-2) and the membrane bound GPI (glycosylphosphatidylinositol) anchored CD14 [32].Additionaly, LPS binding protein (LBP) is another important protein that plays a role in LPS recognition [33]. LBP acts as a soluble or plasma membrane protein that binds LPS. CD14 binds LBP and delivers LPS-LBP complex to the TLR4-MD2 complex. MD2 binds to lipid A (active component of LPS) and this binding activates the dimerization of two TLR4 receptors and TLR4 signalling pathway [24]. In addition to LPS, TLR4 also recognizes glycoinositolphospholipids from *Trypanosoma* [34] and the fusion protein from RSV (respiratory syncytial virus) [13, 35].

1.4.1.3 TLR5

TLR5 recognizes flagellin, which is the monomer of an important structural protein of pathogenic and non-pathogenic bacteria called the flagella [36]. Flagellin induces mucosal immune responses by acting on TLR5 expressed on epithelial cells or

macrophages. The amino acid sequence of flagellin is highly conserved and hence is a target of innate immunity. TLR 5 is expressed in the digestive tract, the urinary tract and the respiratory tract, all constituting surfaces that are prone to infection by flagellated bacteria. In intestinal eptihelia, TLR 5 is expressed only in the basolateral surface and not on the apical surface of the cells. This polarized expression pattern, enables TLR5 to recognize only invading bacteria that have breached the epithelial barrier but not the non-invading commensals. TLR5 activation induces chemokine expression from epithelial cells and thereby contributes to inflammatory responses and leukocyte accumulation at the site of infection [10].

1.4.1.5 TLR11

TLR11 recognizes profilin-like molecules of *Toxoplasma Gondii*, an intracellular protozoan parasite [37]. The active component of this profilin-like molecule has a role in parasite motility and invasion [38]. In addition to its role in recognition of profilin-like molecules originating from *Toxoplasma Gondii*, TLR11 also contributes to protection from uropathogenic bacteria as mice deficient for TLR11 were shown to be highly susceptible to infection with such bacteria [39]. Moreover, TLR11 expression in the kidney and bladder is higher than that of TLR5, suggesting that this receptor is likely to sense uropathogenic bacteria-derived products, although no such "specific ligand" has been identified thus far [24, 39]. Interestingly, TLR11 is expressed only in mice but not in humans which is attributed to the presence of at least one premature stop codon [24].

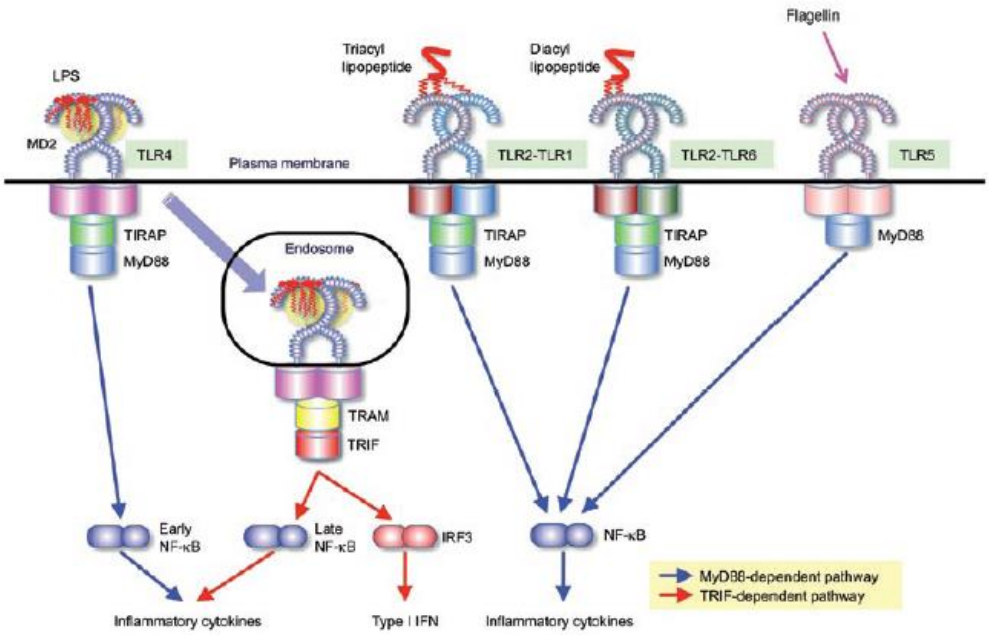


Figure 1.2. PAMP recognition by cell-surface TLRs *(Adapted from [19])*

1.4.2 Intracellular Toll-Like Receptors

TLR3, TLR7, TLR8, TLR9 are the receptors specialized for sensing foreign nucleic acids. These receptors are expressed within the intracellular compartments such as endosomes, lysosomes and the endoplasmic reticulum (ER) [40, 41]. Upon ligand recognition, these receptors initiate an anti-viral innate immune response characterized by production of type I IFNs and inflammatory cytokines [24] (Figure 1.2).

1.4.2.1 TLR3

TLR3 recognizes viral ds RNA from etiher ds RNA viruses or ssRNA viruses that are producing dsRNA during their replication [42-44]. TLR3 also recognizes a synthetic analogue of ds RNA known as polyI:C. TLR3 is expressed both in immune cells such as conventional DCs, macrophages, B cells, NK cells and non-immune cells such as uterine, airway, vaginal, cervical, corneal, biliary, and intestinal epithelial cells, which function as efficient barriers to infection [13]. Interestingly,

TLR3 is not expressed in plasmocytoid DCs (pDCs) which are potent type I IFN producers. In addition, TLR3 is highly expressed in the brain [13, 45]. Viral RNA recognition via TLR3 triggers production of type I IFNs and inflammatory cytokines. Nevertheless, despite such a response, TLR3's role in protection against viral infections is controversial. Some reports indicate that TLR3 deficient mice are more susceptible to infections with mouse cytomegalovirus (MCMV) and West Nile Viruses (WNV) [46, 47]. In contrast, other reports indicate that TLR3 plays a role in pathogenesis rather that protection. For example, TLR3 deficient mice were shown to have increased survival rates following infection with WNV or influenza A virus (IVA) [43, 48]. Moreover, it has been shown that TLR3 deficiency does not affect anti-viral responses of CD4+ and CD8+ T cells following infection with MCMV, reoviruses and lymphocytic choriomeningitis virus [49].

1.4.2.2 TLR7 and TLR8

TLR7 and TLR8 recognizes ssRNA, synthetic antiviral imidazoquinoline compounds (R848, Imiquimod, etc.) and some guanine nucleotide analogs (loxoribine etc.) [6, 50]. TLR7 and TLR 8 genes show high homology to each other and both are expressed on X chromosome. Although both genes are expressed in mouse, mouse TLR8 seems to be non-functional [13]. TLR7 and TLR8 are located in endosomal compartments .In general, viruses gain entry into cells via receptor mediated endocytosis and reach the phagolysosomes. Phagolysosomes have a highly acidic environment and contain degradative enzymes that hydrolyse virus coat proteins, leading to the exposure of viral RNA to TLRs. In contrast, host's ssRNA cannot enter into these compartments due to the activity of RNases in plasma [51]. TLR 7 is highly expressed in pDCs and triggers the secretion of huge amounts of type I IFNs from these cells in response to viral infection [24, 52].

1.4.2.3 TLR9

TLR9 recognizes bacterial and viral genomic DNA [53, 54] such as those originating from MCMV, and herpes simplex viruses 1 and 2 [55, 56]. This recognition induces

production of several inflammatory cytokines and type I IFNs from DCs, B cells and macrophages [19]. Synthetic CpG dinucleotide motif containing ssDNAs can mimic the immune stimulatory activity of bacterial or viral dsDNA [51]. Several different factors seem to contribute to the recognition of DNA by TLR9: Most reports indicate that recognition is due to the presence of unmethylated CpG motifs expressed 20 times more abundantly in bacterial and viral genomes than the mammalian genome. Furthermore, in mammals, CpG motifs are highly methylated and do not activate the mammalian immune cells [13, 51, 57-59]. This theory is supported by publications indicating that bacterial DNA must be delivered to endosomal compartments with acidic environments and degraded into multiple CpG containing ssDNAs that can interact with TLR9 [40, 60]. In contrast, some reports correlate the recognition of DNA by TLR9 to the presence of 2' deoxyribose-phosphate backbone [61, 62] specifically in endosomes. It has been shown that a chimeric form of TLR9 localized on the cell surface has been shown that can respond to self DNA [63]. It is speculated that CpG motifs may play a role in the stability, aggregation and uptake of the DNA by cells but is not necessary for TLR9 recognition [50] A more recent publication challenged the role of CpG motifs claiming that the 2' deoxyribose-phosphate backbone is important in recognition of phosphodiester backbone DNA irrespective of its CpG content while CpG motif is indispensible in recognition of phosphorothioate backbone modified DNA [61] Whether TLR9 recognition of DNA depends strictly on CpG recognition or on the presence of 2' deoxyribose-phosphate backbone in endosomes, it seems that endosomal co-localization is essential for this recognition [50]. For instance, even though malarial DNA itself is not highly stimulatory, when it is delivered into the endosomal compartments via transfection or bound to malarial hemozoin, it induces a strong immune responses [64]. In another study it has been shown that LL37, a human antimicrobial peptide released from keratinocytes during skin injury binds to self-DNA and creates aggregates that can enter endosomes, contributing to immune activation in an autoimmune disease called psoriasis [65].

1.4.2.4 Trafficking of intracellular TLRs

As mentioned above, nucleic acid sensing TLRs such as TLR3, TLR7, TLR8 and TLR9 are expressed in various intracellular compartments such as endosomes, lysosomes and the endoplasmic reticulum (ER) [19, 50]. This intracellular localization is important in terms of two situations. Firstly, viral nucleic acids are delivered via receptor mediated endocytosis and they are recognized in these endosomes that express TLRs. Secondly, this localization is also important in terms of discriminating *self* from *non-self* [24]. It has been shown that macrophages express TLR9 on the cell surface responds DNA originating from *self* [63]. These intracellular TLRs are expressed in the ER in resting cells and upon PAMP stimulation they traffic to the endosomal compartments [24]. It has been shown that activation occurs only in the acidified endosomes since usage of agents that prevents endosomal acidification abrogated TLR3, TLR7, TLR8 and TLR9 responses [66].

Trafficking of intracellular TLR3, TLR7 and TLR9 are mainly regulated by a12 membrane spanning ER protein called UNC93B1. UNC93B1 interacts with transmembrane domains of these receptors and assists in delivery from ER to the endolysosome [67, 68] (Figure 1.2). It has been shown that only a single missense mutation on UNC93B1 causes defects in cytokine production and co-stimulatory molecule up-regulation in response to TLR3, TLR7, TLR9 ligands [69]. In addition, it has been also reported that UNC93B1 deficiency is responsible for HSV-1 encephalitis in human [70].

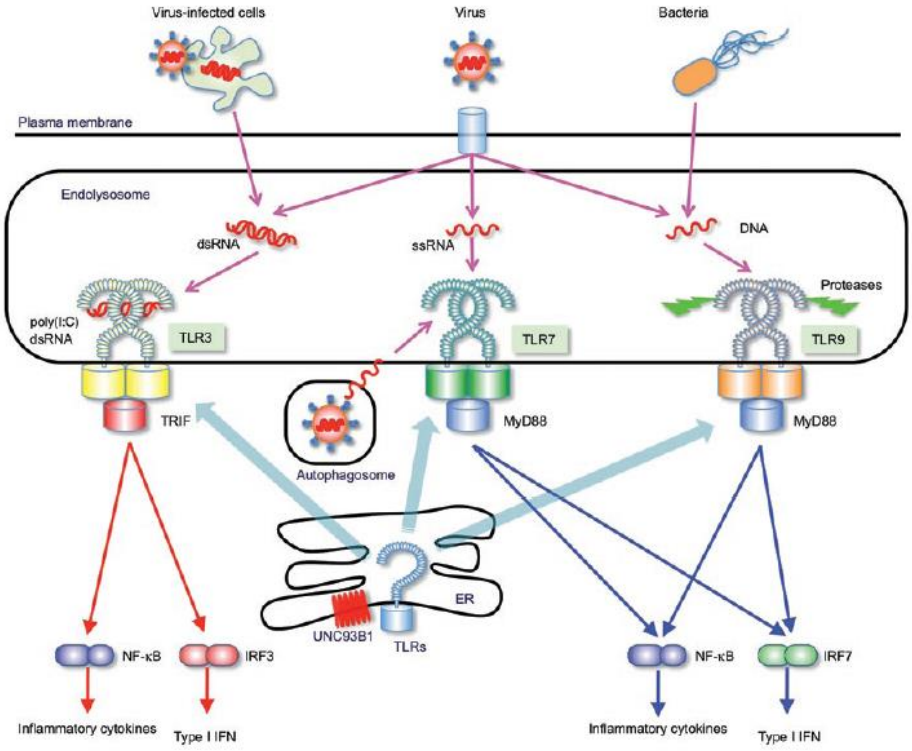


Figure 1.3. PAMP recognition by intracellular TLRs and the role of UNC93B1 on their trafficking from ER to endolysosome *(Adapted from: [19])*

Two other ER proteins are also involved in TLR trafficking PRAT4A and gp96. PRAT4A plays role in the trafficking of TLR1, TLR2, TLR4, TLR7 and TLR9 from ER to plasma membrane or endolysosome [71]. However it does not affect TLR3 trafficking indicating that TLR3 trafficking is regulated in a different way than TLR7 and TLR9 [72]. On the other hand, gp96, another ER protein, functions as a general chaperone for most of the TLRs including cell surface expressed ones such as TLR1, TLR2, TLR4,TLR5 and intracellular ones such as TLR7 and TLR9 [73].

1.4.2.5 Endosomal Cleavage of Intracellular TLRs

As mentioned in previous sections endosomal acidification is required for the activation of intracellular TLRs. This acidification is important from two aspects. Firstly, acidic environment and enzymes in endosomes facilitates the disassembly of microbes in order to release their contents such as CpG DNA [40, 60]. Secondly, endosome maturation is involved in essential processing of TLRs themselves. For

instance, ectodomain of TLR9 is proteolytically cleaved by proteases upon its arrival to the endosomal compartment which generates a functional receptor that can recognize ligand and initiate signal transduction [74, 75]. Proteases involved in the cleavage of TLR9 involves cathepsin B, cathepsin S, cathepsin L, cathepsin H and cathepsin K, and asparaginyl endopeptidase [74-78]. Similar cleavage process was also shown for TLR7 but not TLR3 [74] indicating that cleaved forms of TLR7 and TLR9 may display a similar ligand binding structure to full-length TLR3 [50]

In conclusion, in addition to other pre and post translational regulatory mechanisms, cleavage appears to be an additional step in the maturation of TLRs [50].

1.5 TLR Signalling

Upon PAMP recognition, TLRs trigger specific immune responses. For instance, while TLR3 and TLR4 initiates both type I IFNs and inflammatory cytokine responses, cell surface TLRs such as TLR1-TLR2, TLR2-TLR6 and TLR5 induces mainly inflammatory cytokines. This specificity depends on the recruitment of a single or a specific combination of The Toll/Interleukin-1 receptor (TIR) domain containing adaptor protein such as Myeloid differentiation primary response gene 88 (MyD88), TIR domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF related adaptor molecule (TRAM) [6]. TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11 recruit MyD88 to their TIR domains upon ligand recognition whereas TLR3 and TLR4 recruits TRIF. TLR1, TLR2, TLR4 and TLR6 use TIRAP, which serves as a linker between TIR domain of these TLRs and MyD88 whereas TLR5 and TLR11 binds directly to MyD88. Similarly, TLR4 uses TRAM in order to bind TRIF. These recruitments of adaptor molecules to specific TLRs initiates the signaling cascade and activation of transcription factors such as nuclear factor kappa B (NFκB) and interferon regulatory factor (IRFs) [15] (Figure 1.4)

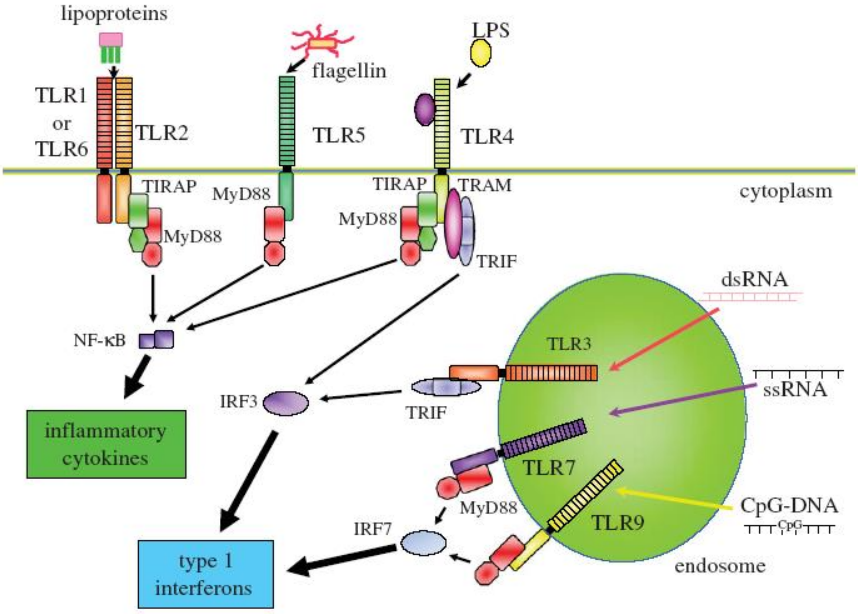


Figure 1.4. TIR domain containing adaptors in TLR signaling *(Adapted from [10])*

TLR signaling can be divided into two categories, MyD88-dependent and TRIF-dependent pathways. TLR4 is the only TLR that uses all four adaptors and induces both pathways [19].

1.5.1 MyD88-dependent pathway

All TLRs (except TLR3) use MyD88-dependent pathway. Upon ligand recognition TLRs recruit MyD88 molecules to their TIR domains and MyD88 recruits IL1 receptor associated kinase (IRAK) family of proteins and TNF receptor associated factor (TRAF) 6. TRAF6 activates TAK1 and TAK1 activates the IκB kinase (IKK)complex in order to activate NFκB and also activates the mitogen-activated protein kinase (MAPK) pathway [15] (Figure 1.5)

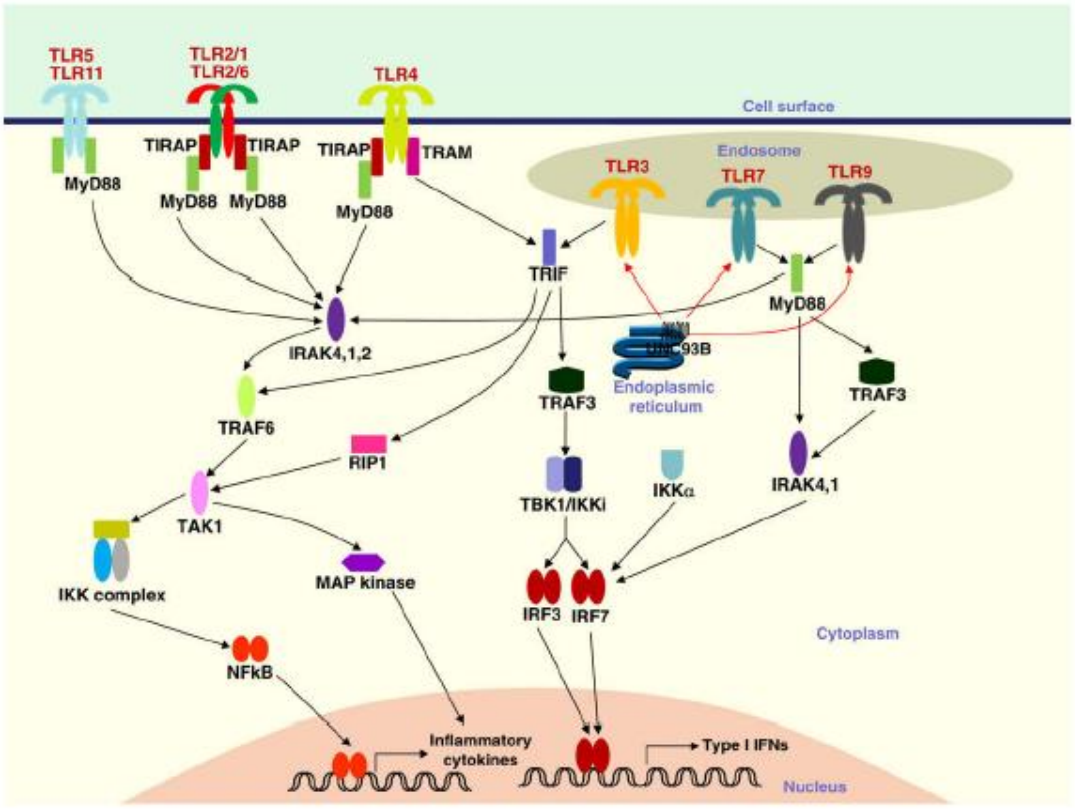


Figure 1.5. MyD88 and TRIF dependent pathways *(Adapted from [15])*

1.5.2 TRIF-dependent pathway

TLR3 and TLR4 use TRIF-dependent pathway upon ligand recognition and initiate the production of type I IFNs and inflammatory cytokines from DCs and macrophages. TRIF activates the transcription factor IRF3 via activating the kinase protein that phosphorylates IRF3 called TBK1. After phosphorylation IRF3 translocates from cytoplasm to nucleus and triggers the production of type I IFNs. Since inducing type I IFNs, TRIF-dependent pathway is involved in anti-viral responses. TLR7 and TLR9 recognizes viral nucleic acids however molecular mechanism underlying the production of type I IFNs upon TLR7 and TLR9 activation is different. In pDCs MyD88 directly interacts with IRF7 and induces type I IFN production [10] (Figure 1.5)

1.6 Effects of DNA on Immune System

DNA and RNA are the essential components of all living organisms. Accumulated evidence strongly suggests that these nucleic acids have multiple and complex effects on the immune system and are more than a blueprint of life [79-81]. DNA is normally isolated from the extracellular world via nuclear or mitochondrial membrane in eukaryotes, the cell wall in bacteria, or the envelope in viruses. Following microbial infection or tissue damage DNA can be released from microbes or damaged host cells. Such DNA is detected by the immune system [13, 82] On one hand, due to their high unmethylated CpG motif frequency, bacterial DNAs are recognized as “non-self” via TLR9 and trigger an innate immune response characterized by the proliferation and maturation of B cells, natural killer cells, and pDCs and the secretion of Th1 type cytokines, chemokines, and/or multivalent immunoglobulins [83-88]

On the other hand, telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that can inhibit several TLR-dependent and TLR-independent Th1-mediated immune responses. Of note, these motifs are underrepresented in the prokaryotic genome [31, 89, 90]. Novel therapeutics including nucleic acid based agonists/antagonists are currently under preclinical/clinical trials to prevent or treat cancer, infectious disease, allergic disorders and autoimmunity [91-93].

1.6.1 Immunostimulatory CpG ODN

CpG ODN story started with the observation that synthetic nucleic acid fractions originally found in bacteria has antitumor effects. It was first hypothesed palindromic sequences within these sequences were the cause of this immune stimulation [94] but it was shown by Krieg and Klinmann in 1995 that specific sequence motifs consisting of unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines were responsible for the innate immune response triggered by such DNAs [84, 95]. Mammalian DNA has approximately 20 times less frequent CpG motifs and due to methylation status they are not immune stimulatory like microbial DNA (Table 1.4)

Table 1.4. Immunostimulatory effect of CpG ODN *(Adapted from [96])*

	Fold Increase in Cytokine secreting cell number			
	IL6	**IL12**	**IFNγ**	**IgM**
Bacterial DNA	3.2±0.2	3.8±0.4	4.7±2.3	3.9±1.1
Mammalian DNA	0.8±0.2	1.1±0.2	0.8±0.3	0.7±0.2
CpG ODN	5.5±1.1	8.3±1.7	4.7±1.1	4.2±1.6
CpG ODN (methylated)	0.9±0.2	1.2±0.3	0.8±0.2	1.1±0.2
CpG ODN (DNase Rx'd)	1.3±0.2	0.8±0.2	1.1±0.2	0.9±0.2
GpC ODN	1.2±0.3	1.3±0.3	1.2±0.3	1.3±0.3

Bacterial DNA or synthetic ODNs expressing these CpG motifs induce immunostimulation characterized by a proinflammatory (IL1, IL6, IL18, TNFα) and T helper 1 (Th1) biased (IFNγ and IL12) immune milieu [96]

Five years after the discovery of CpG motifs, in 2000 Akira et al., indicated that the receptor responsible for CpG ODN recognition is TLR9 [53] As mentioned in previous section CpG ODNs are internalized into endosomal compartments where they are recognized by TLR9 and induce a robust innate immune response [51].

There are three known types of CpG ODN: D-type (also known as A-class), K-type (also known as B-class) and the recently described C-class, all of which possess unmethylated CpG dinucleotides and require TLR9 to activate the immune system [92, 97]. These 3 types of ODN possess CpG dinucleotides, but their flanking sequences and compositions are different. For example, K-type ODN contain multiple CpG motifs, whereas D-type ODN have one CpG with palindromic flanking sequences. D-, but not K- nor C-type ODN have a poly-G (5-6 bases) tail at the 3'-end, which may account for their distinct activity. K- and C- but not D type ODN have phosphorothioate linkage between all nucleotides. D-type ODN stimulate plasmacytoid DC (pDC) to secrete large amounts of IFNα, whereas K-type ODN strongly stimulate B cells to proliferate and to secrete IL6 and IgM. C-type ODN show a combined activity of K- and D-type ODN, but to a lesser extent [16, 80].

Several reports indicated that the IFNα induction from pDCs by D type ODNs were due to the ability to spontaneously form higher order structures because of their palindromic sequences and poly (G) since such higher order structures are not observed in K types [98, 99]

In 2006, Gursel et al., discovered the co-receptor CXCL16 expressed on pDC which can contribute to discribe the dichotomy of response between D and K types. In this work, her group demonstrated for the first time that a type of surface expressed scavenger receptor is required for the D-ODN activation of pDC to secrete robust IFNα [17].

1.6.2 CpG DNA as adjuvants for vaccines

CpG ODN which acts as ligand for innate immune receptors also supports the development of adaptive immune responses via improving the functional activity of professional APCs and triggering the production of cytokine and chemokines that are critical in the development of adaptive immune responses [84, 88, 100, 101].

CpG ODNs activate innate immunity by binding their receptor TLR9 located in endosomal compartments of pDCs, B-cells, and multiple cells of myeloid lineage in mice [102]. CpG ODNs have been shown to activate B cells via upregulating the secretion of IgM, IL6 and IL10, inducing the expressions of Fc receptor (FcR) and costimulatory molecules such as CD40, CD80, CD86 and major histocompatibility complex II (MHCII), inhibiting the apoptosis and enhancing the antigen presentation function [95, 102-104]. Effects of CpG ODNs are not limited on B cells. CpG ODNs have also been shown to induce the maturation and differentiation of other antigen-presenting cells such as plasmacytoid dendritic cells (pDCs) and macrophages. pDCs and macrophages show increased expression MHCII and costimulatory molecules (CD40, CD80, CD86) and cytokines such as IL6, IL12, IL1β upon CpG stimulation [102, 105].

Several studies indicated that use of CpG ODN as an adjuvant increased protein immunogenicity, cytokine production and IgG2a:IgG1 ratio [102, 103, 106, 107]. Table 1.5 summarizes the type and magnitude of immune response induced with the use of CpG ODN with the conventional protein or vaccine based antigens

Table 1.5. Use of CpG ODN as an adjuvant with various peptides and vaccines *(Adapted from [88].)*

Antigen	**Fold Ab Titer**	**Ig Profile**	**Cytokine Profile**	**References**
Ovalbumin	>7fold (3wk)	IgG2a>IgG1	IFNγ	[106]
Hen eggwhite lysozyme	>10fold (3wk)	IgG2a>IgG1	IFNγ, IL5	[108]
Hepatitis B Surface Antigen	$>10^4$fold (4wk)			[109]
Influenza virus	>10 fold (4wk)	Intranasal IgA	IFNγ	[110]
Measles virus	>20 fold (4wk)	IgG2a>IgG1	IFNγ, IL5	[111]
Tetanus Toxoid	> 3fold (6wk)			[112]
Brucella	>100 fold (3wk)	IgG2a>IgG1	IFNγ	[113]
rPA (anthrax)	>8 fold (3wk)	IgG2a>IgG1	IFNγ	[114, 115]
L1 and A33 proteins (smallpox)	>10 fold (3wk)	IgG2a>IgG1		[116]

1.6.3 Immunosuppressive ODN

During infection or tissue damage, inflammation must be waned and terminated with tissue remodeling and healing [80]. CpG-driven immune activation can exacerbate inflammatory tissue damage, or increasing sensitivity to autoimmune diseases or toxic shock. Similarly, other immune responses designed to protect the host can have deleterious consequences if not adequately regulated [117]. Recent evidence suggested that host DNA contained some antagonistic elements to the immunostimulatory effect in their DNA or against pathogen derived CpG rich DNA,

possibly suppressing DNA-driven immunostimulation [118]. Neutralizing or suppressive motifs can selectively block CpG-mediated immune stimulation [119]. Suppressive motifs are rich in poly-G or GC sequences, and optimal motifs are surprisingly identical to telomere sequences (with a repeat of TTAGGG), which are present in DNA of mammals, but not in bacteria [79]. Gursel and his colleagues demonstrated for the first time that telomeric DNA consisting of TTAGGG repeats can significantly suppress immune activation while non-telomeric mammalian DNA cannot. Furthermore they also showed that DNA isolated from telomerase deficient KO mice was not capable to suppress immune responses compared to normal DNA [79].

In vitro, it has been shown by many groups that mammalian telomeric suppressive ODN (A151) inhibits the production of several proinflammatory cytokines and chemokines induced by bacteria such as IL6, IL12, IFNγ, TNFα and MIP2α [79, 120-122]. As summarized in Table 1.6and Table 1.7, Suppressive ODN can significantly reduce cytokine/chemokine production and immune cell proliferation induced by CpG ODN or several other different immune activators [122]

Table 1.6. Effect of Suppressive ODN in CpG induced immune activation (Adapted from [122]).

Treatment	**% Activation**
CpG ODN alone	100±2
+Mammalian DNA	27±4
+Telomeric DNA	13±3
+Non-telomeric DNA	87±5
+Control ODN	97±3
+Suppressive ODN (A151)	8±2

Table 1.7. Effect of Suppressive ODN on IFNγ production induced by several immune activators (Adapted from [122]).

Stimulus	None	Control ODN	Suppressive ODN (A151)
CpG DNA	27±5	24±6	6±2
ds RNA	8±2	10±1	2±1
Peptidoglycan	28±5	31±4	5±2
LPS	24±7	21±3	6±2

Effects of Suppressive ODN (A151) in the treatment of different autoimmune and inflammatory diseases in several animal models have been studied by us and others. In 2005, Shirota and Gursel showed that suppressive ODN treatment immediately prior to LPS challenge significantly improved survival rates in BALB/c mice injected with lethal dose of LPS [31].

In another study, effect of Suppressive ODN (A151) on the onset of systemic lupus erythematosus in NBZ/W mice has been studied. In this study, investigators injected mice twice per month starting at age 6 weeks with Suppressive ODN or Control ODN and several characteristics of the disease such as proteinuria, kidney inflammation and also survival has been followed. According to their results Suppressive ODN significantly delayed the onset of proteinuria and inflammation. Most importantly survival rates of the animals have been prolonged [123].

Rheumatoid Arthritis (RA) is the most common rheumatic disease which is characterized by destruction, disability and deformity of the joints. Effect of Suppressive ODN (A151) on RA is studied in an animal model called collagen-induced arthritis (CIA). In this study arthritis was induced by collagen administration. Authors indicated that Suppressive ODN (A151) treatment significantly reduced the incidence and severity of the arthritis and also decreased serum titers of pathogenic IgG autoantibody titers and IFNγ production by collagen reactive T cells [124]

Furthermore, effects of Suppressive ODN (A151) in Silica induced Pulmonary Inflammation have been studied. Silicosis was induced via instilling silica into the lungs of normal BALB/c mice. Silica instilling increased bronchoalveolar lavage (BAL) cellularity and production of IL12 and kerationcyte chemoattractant (KC) proteins. These effects were significantly reduced in Suppressive ODN treated groups [125].

Uveitis is an ophthalmic disorder that causes vision loss in developed countries [126, 127] and is characterized by acute, recurrent, or persistent ocular inflammation, the breakdown of the blood-ocular barrier, and infiltration of leukocytes [128]. Fujimoto et al. [129] reported that suppressive A151 ODN can inhibit ocular inflammation in two murine models, IRBP (interphotoreceptor retinoid-binding protein)-induced experimental autoimmune uveitis and adoptively transferred ocular inflammation. In 2010, we published that Suppressive ODN can reduce the symptoms associated with a very aggressive form of experimental uveitis initiated via endotoxin administration [81]. Compared to previous studies our animal model was a much more aggressive form of the disease and Suppressive ODN was still able to alleviate several disease induced parameters as described in Results section of this thesis.

Over the last few years, remarkable improvements have been achieved by researchers in Suppressive ODN field. In 2011, Klinman and colleagues showed the effects of Suppressive ODN (A151) on a type of cancer that inflammation plays a critical role called papilloma. In this study, authors used 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-l3-acetate (TPA) in order to induce skin carcinogenesis. Their results indicated for the first time that Suppressive ODN blocked TPA-dependent skin hyperplasia, edema, and leukocytic infiltration. Most importantly, their results suggested that Suppressive ODN reduced papilloma formation in a dose and sequence dependent manner. Up to date, this was the first and only study suggesting that Suppressive ODN can be used in inflammation associated oncogenesis [130]

Their observations were supported by other researchers indicating that Suppressive ODNs can improve the antiproliferative effects of anticancer drugs [131]. Most recently, again Klinman and colleagues demonstrated that Suppressive ODN A151 is capable of reducing lung cancer susceptibility of mice with silicosis [132]. As mentioned before in this section of this thesis, Klinman and colleagues have already established the inhibitory role of Suppressive ODN on silicosis. In addition to their previous observations, in this study researchers investigated the role of potent carcinogens present in cigarette smoke (NNK) in development of lung cancer in mice with silicosis and protective effects on Suppressive ODNs in pathologic inflammation and cancer. In conclusion, authors reported that Suppressive ODN can inhibit pulmonary fibrosis and other inflammatory manifestations of chronic silicosis and so as lung cancer [132].

As mentioned in the beginning of this section, Suppressive ODN A151 is a sequence that contains poly G runs. Guanosines are tend to make interchain and intrachain Hoogstein Bonds and these interactions led the sequence to form higher order structures called G-tetrads. It has been shown by Gursel et al., that the suppressive activity of ODN A151 is correlated with its ability to form G-tetrads. They showed that nucleotide substitutions that inhibit G-tetrad formation abrogated the suppressive activity of these sequences [79].

Suppressive ODN (A151) is not the only sequence motif with suppressive properties. Several reports indicate that sequences other than A151 can also reduce immune responses. Sequences showing suppressive activity, used disease models, potential modes of actions and TLR dependence are given in Table 1.8

Table 1.8. Different types of immunosuppressive ODNs used for several immunotherapeutic applications

ODN Name	Sequence	Disease	Potential Mode of Action	TLR dependence	Reference
A151 ODN	$(TTAGGG)_4$	Endotoxic Shock	Prevent phosphprylation of STAT1 and STAT4	TLR4	[31]
		Collagen induced Arthritis	N/A	TLR-independent	[124]
		Systemic Lupus Erythematosus (SLE)	N/A	TLR-independent	[123]
		Silicosis	N/A	TLR-independent	[125]
		CpG induced Lung Inflammation	N/A	TLR9	[133]
		Endotoxin induced Uveitis (EIU)	N/A	TLR4	[81]
		Experimental Autoimmune Uveitis (EAU)	N/A	TLR-independent	[129].
		Inflammation induced Papillomas	N/A	TLR-independent	[130]
		Herpes Simplex Virus Infection	N/A	TLR9	[134]
		Candida Albicans Infection	Inhibits SOCS3 and promotes STAT3	TLR independent	[102]
		Mouse CMV infection	Inhibits AIM2 inflammasome activation	TLR independent	[135]
		Small cell lung cancer	Modifying Akt and the extracellular signal-regulated kinase 1/2 pathway	TLR independent	[131]
		Lung Cancer Susceptibility in Mice with Silicosis	N/A	TLR independent	[132]
H154 ODN	CCTCAAGCTTGAGGGG	CpG-induced Arthritis	N/A	TLR9	[136, 137]
		Experimental Autoimmune Neuritis	N/A	TLR9	[138]
		CpG-induced myocardial dysfunction	N/A	TLR9	[139]

GpG ODN	TGACTGTGAAGGTTAGAGATGA	Experimental autoimmune encephalomyelitis (EAE)	Inhibits phosphorylation of IκB-α at Ser32	TLR9	[140, 141]
		Lupus Nephritis	N/A	TLR3, TLR7, TLR8, TLR9	[142]
Class-R INH ODN1 and ODN2008	CCTGGATGGGAATTCCCATCCAGG	Systemic lupus erythematosus (SLE)	N/A	TLR7 and TLR9	[143, 144]
Multiple IRS ODNs	Various	Systemic lupus erythematosus (SLE)	N/A	TLR7 and TLR9	[145]
IRS 869	TCCTGG AGGGGT TGT	Systemic Inflammation	N/A	TLR9	[146]
IRS 954	TGCTCCTGGAGGGGTTGT	Skin Inflammation	N/A	TLR7 and TLR9	[147]
		SLE	N/A	TLR7 and TLR9	[148, 149]
G-Rich ODN	CCTGGAGGGGAAGT	(SLE)	N/A	TLR9	[150].???
SAT05f	$(CCT)_8$	GVHD	N/A	TLR7 and TLR9	[151-153]
MS19	$(AAAG)_6$	Acute lung inflammation	N/A	TLR9	[154]

As seen on the Table 1.8 most of the sequences given in the table are G rich sequences and their suppressive activity seems to be their ability to form G-tetrad structures.

ODN H154 is another G-rich suppressive sequence that has beeen shown to alleviate symptoms associated with reactive arthritis and experimental autoimmune neuritis (EAN). Reactive arthritis is an inflammatory condition that develops several weeks after infection of gastrointestinal or genitourinary tracts with bacteria. Zeuner and colleagues injected CpG ODNs directly into the knee joints of BALB/c mice and induced a similar form of arthritis in their study. They showed that co-administration of equal amount of ODN H154 with CpG ODN significantly reduced both

inflammation and swelling on the injected joint. Since arthritis can affect multiple joints, systemic effect of the ODN has also been studied. Their results suggested that administration of ODN H154 3 days before CpG challenge significantly reduced the development of inflammatory arthritis [136, 137].

Guillain-Barre Syndrome is a Th1 type autoimmune disease characterized by demyelination and inflammation in peripheral nervous system. In 2012, Wang et al., reported beneficial effects of ODN H154 on EAN which serves as an established animal model of GBS in humans. In this study authors induced EAN by injecting P2 peptide and complete Freund's adjuvant into both hindpads of Lewis rats. Following immunization at days 3 and 6 authors injected different types of ODNs (such as CpG ODN, control ODN and ODN H154) in shoulders of rats. Their results indicated that EAN rats inoculated with ODN H154 had significantly better clinical scores and reduced TLR9 activation [138]

Most recently, Boehm et al., indicated that ODN H154 can inhibit CpG induced myocardial dysfunction. In this study, researchers induced sepsis via i.p. application of the TLR9 agonist 1668-thioate in C57BL/6 wild type (WT) and TLR9-deficient (TLR9-D) mice. Their results indicated that inhibition of TLR9 by the suppressive ODN H154-thioate significantly ameliorated cardiac inflammation, preserved cardiac function, and improved survival [139]

Effects of another G-rich immunosuppressive sequence called GpG ODN have been studied. Ho and colleagues showed that GpG ODN can effectively enhance the efficacy of myelin cocktail/IL4 tolerizing DNA vaccination in experimental autoimmune encephalomyelitis (EAE) which is a prototypical animal model of multiple sclerosis in humans. They showed that combining GpG ODN with myelin cocktail/IL4 tolerizing DNA can reduce mean disease severity via inducing a shift in the autoreactive B and T-cell responses into a protective IgG1 isotype and Th2 type cytokine pattern respectively [140, 141].

Like Suppressive ODN (A151), effects of GpG ODN on an animal model of lupus nephritis have also been studied. Authors indicated that GpG ODN treatment promoted Th2 type T and B cell responses and delayed the onset of proteinuria in NZB/W mice [142].

Another type of G-rich suppressive sequences called class R inhibitory oligonucleotides (INH-ODNs) were developed by Lenert and colleagues. They indicated that INH-ODNs have high potency in suppressing autoreactive B cell and DC responses *in vitro* and are effective in reducing autoantibody production in lupus prone MRL-Fas$^{lpr/lpr}$ mice *in vivo* [143]

Immunoregulatory sequences (IRS) developed by Dynavax Technologies Company should be also considered as other types of immunosuppressive sequences. Publications of Barrat and colleagues indicated that some of these IRS can selectively block TLR7 and TLR9 activation therefore play beneficial roles in the treatment of animal models of SLE and skin inflammation[145-149].

In addition to the G-rich sequences displaying immunosuppressive properties mentioned above, microsatellite DNA mimicking ODNs (MS ODNs and Sat05f) have been shown to have similar effects on the immune system while their sequences lack G residues. Wang and colleagues reported that MS ODNs and Sat05f can inhibit TLR7 and TLR9 mediated innate immune responses, protect mice from D-GalN/CpG ODN induced lethal shock, alleviate symptoms associated with influenza virus induced acute lung injury, and reduce anti-ssDNA antibody level in the lupus-prone mice induced by chronic graft versus host disease (cGVHD) [151-153].

Studies indicated that Suppressive ODN (A151) did not interfere with binding or uptake of CpG ODN [87]. Rather, they blocked either TLR9 binding or assembling of CpG DNA or the signaling cascade initiated by CpG DNA upstream of NF-κB translocation to the nucleus [79, 87].

The effect of Suppressive ODN (A151) on other inflammatory events that are TLR9 independent has been explored. Suppressive ODN were shown to bind STAT1 and STAT4, thereby inhibiting their downstream signaling cascade that is independent of TLR9 signaling, resulting in reduced incidence of LPS-induced endotoxic shock and Th2 biased adaptive immune responses [31, 121].Most recently, studies conducted by Klinman and colleagues revealed new insights on the mechanism of action of suppressive ODNs. In their study published in Plos One journal in 2013, authors indicated for the first time that Suppressive ODNs promote development of naïve CD4+Tcells into Th17 effector cells. It was quite interesting because reports published until that study were indicating that Th17cells play role in the development of autoimmune and autoinflammatory diseases and reduction on the severity of these diseases via Suppressive ODN administration were thought to be the result of inhibitory effects on Th17 cells. Contrary to expectations, their results indicated that Suppressive ODN administration significantly promoted the maturation of naïve CD4T cells into Th17 cells via inhibiting the expression of suppressor of cytokine signaling 3 (SOCS3) and promoting activation of STAT3. These observations were also supported with the protection of mice injected with Suppressive ODN against *Candida albicans* infection where Th17 cells play critical roles [102]

In another study of the same group, it was demonstrated for the first time that Suppressive ODNs can inhibit AIM2 inflammasome activation. In this study, authors revealed that in addition to blocking TLR9 dependent inflammatory pathways, Suppressive ODN A151 can also abrogate activation of cytosolic nucleic acid sensing pathways via competing with immunostimulatory ODN to bind cytosolic nucleic acid sensors, AIM2 and IFI16 [135]

It is quite interesting that suppressive sequences in self-DNA may play a role in neutralizing exacerbating inflammation or modulating both innate and adaptive immune responses in a TLR9 independent manner, thereby providing potential therapeutic uses as natural anti-inflammatory agents or Th2 inducing adjuvants.

1.7 Endotoxin Induced Uveitis

The eye is an immune privileged organ that allows its protection from the potentially damaging effects of an inflammatory immune response. Several complex mechanisms contribute to the persistence of immune privilege in the eye [155]. The presence of a physical barrier between the blood circulation and the retina (the blood-retinal barrier (BRB)), constitutes one such mechanism where the non-fenestrated capillaries of the retinal circulation and tight-junctions between retinal epithelial cells prevent entry and exit of large molecules such as proteins into or from the eye [156].

Furthermore, certain soluble and cell-bound ocular immunosuppressive factors inhibit the activity of immune cells that may have gained entry, while other proteins that are released from ocular cells following a damage, elicit a deviant systemic immunity via limiting the generation of proinflammatory effector cells [157, 158] . All of these elements collectively create an environment that protects the visual axis from damage by inflammatory processes. However, in spite of the presence of such protective mechanisms, the integrity of the BRB can be breached under certain circumstances such as the case with uveitis or retinopathy [156].

Uveitis is an ophthalmic disorder that causes vision loss in developed countries [126, 127] and is characterized by acute, recurrent, or persistent ocular inflammation, the breakdown of the blood-ocular barrier, and infiltration of leukocytes [128]. The underlying causes of uveitis can vary. For example, acute anterior uveitis is often associated with (i) Behcet disease, (ii) Reiter syndrome, and (iii) ankylosing spondylitis, as well as other systemic inflammatory diseases [159].

Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation. It is triggered by the administration of LPS, which is a component of the Gram-negative bacterial outer membrane [160]. A ligand for TLR4, LPS enhances the expression of various proinflammatory cytokines and chemokines such as IL6 [161, 162], TNFα [163], and MCP1 (monocyte chemoattractant protein 1)

[164] and the production of nitric oxide. All of these mediators contribute to the breakdown of the blood-ocular barrier and infiltration of leukocytes, resulting in the development of EIU [160].

It has been shown that suppressing proinflammatory cytokines, including IL6, TNFα, MCP1, and inducible nitric-oxide synthase (iNOS), retards if not prevents the development of EIU [165]. Conventional drugs used to control these concerted inflammatory activation are mainly immunosuppressive in character and are associated with undesirable systemic side effects [128]. It is of the utmost importance to develop effective, less toxic agents that selec tively block proinflammatory immune activation while eliminating the unwanted systemic side effects.

1.8 Cyclosporin A

Cyclosporin A is a fungal antimetabolite that has been widely used for solid organ transplantation. Although full mechanism of action is currently unknown, it has been shown that CsA can reversibly inhibit T-cell mediated autoimmune responses [166, 167]. It is speculated that CsA binds and inhibits calcineurin and prevents the nuclear translocation of nuclear factor of activated T-cells (NFAT). This inhibition blocks the signaling cascade starting from T cell receptor to genes encoding multiple cytokines and chemokines such as IL2 which are necessary for the activation of resting T-cells [168].

Clinical use of CsA in ophthalmology has been studied by many groups. Palestine et al., were the first group demonstrated that use of CsA in patients with intractable uveitis in Behcet disease, Vogt-Koyanagi-Harada syndrome (VKH), sarcoidosis, and sympathetic ophthalmia has beneficial effects [169]. Furthermore, the same group also indicated that CsA monotheraphy was able to decrease inflammation in 15 out of 16 patients showing resistance to steroid theraphy [170]. In another study efficacy of colchicine and CsA treatment in Behcet disease has been compared and superiority of CsA treatment was reported [171]. Along with those studies data

demonstrated that CsA reduced the production of the proinflammatory cytokines IL1β, TNFα, IL6 and IL8 from U937 human monocyte-like cells in vitro [172]. Recently, Chi et al., reported that CsA suppressed IL17 production from Behcet`s patients` PBMC [173]. Moreover, several other studies report that systemic cyclosporine treatment can reduce the severity of uveitis and can downregulate the expression of proinflammatory cytokines such as IL12, IL18 and TNFα [174-176].

Topical use of CsA has been shown to be effective in many disease conditions such as vernal conjunctivitis, ligneous conjunctivitis, Sjogren syndrome, and atopic keratoconjunctivitis[177]. Of interest, a recent study showed that topical treatment with 1% cyclosporine A reduced the severity of subepithelial corneal infiltrates [178].

1.9 Foot and Mouth Disease

Foot and Mouth Disease (FMD) is one of the major contagious and devastating viral disease of the cloven hoofed animals [179]. FMD outbreaks all around the world caused serious economic losses during the last century. The last FMD outbreak in United Kingdom (UK) in 2001 resulted with the loss of 11.7-18.4 billion US dollars due to slaughter of nearly 7 million animals. Although USA has been FMD-free since 1929, it is predicted that, if there were an FMD epidemic in USA (similar to the 2001 UK FMD outbreak) the cost would be around 7-21 billion US dollars due to reduced farm incomes [180]. In the past decade FMD arouse global concerns by not only reducing animals and their products commercial value but also its potential use as a bio-terrorist threat in the context of several new outbreaks in previously FMD-free countries [181].

The causative agent of the disease FMD virus (FMDV) belongs to the Picornaviridae family and is a member of the genus Aphthovirus. Infection with FMDV occurs commonly with the respiratory tract following contact or inhalation of airborne virus [182]. The disease is characterized by fever, lameness and vesicular lesions on the

feet, tongue, snout and teats, with high morbidity but low mortality [183]. To date seven serotypes (A, O, C, Asia1, Southern African Territories [SAT] 1, SAT2 and SAT3) of the virus have been identified which differ in distribution across the world. The widest distributed one of them is serotype O including Middle East, Asia and Southern America [184].

Vaccines against FMD have been available since the early 1900s. The current vaccines against FMD are produced by infecting BHK-21 cells with growing live velogenic foot and mouth disease virus (FMDV) under bio-secure conditions and inactivating it by using a chemical such as binary ethyleneimine. Inactivated FMD vaccines have proven to be an important component of control and eradication of the disease so far. However due to difficulties to grow certain serotypes and subtypes in cell culture to get sufficient amount of Ag for vaccine production and the lack of longevity and rapidity features of currently used vaccines, more rapid and potent vaccination strategies are urgently needed [179, 185-188].

1.10 Immunostimulatory Effects of CpG ODNs in Chicken

CpG ODN stimulates innate immune responses of human and a variety of species including mice, sheep, cats, dogs, fish, horses and chicken [189]. These responses can vary from species to species. It has been shown that mice can respond differently and generally stronger than human to CpG ODN [190]. This phenomenon can be associated with the differences in TLR9 expression in different cell types between these two species. In human TLR9 is expressed only in pDCs and B cells however in mice TLR9 is expressed in all DC subsets (conventional and plasmacytoid), macrophages and B cells [191, 192]. Although cellular TLR9 expression is one of the criteria in responding to CpG ODN, sequences of CpG ODN also differs the level of immune responses in different animal species [16, 193].

Following *in vivo* administration immune effects of CpG ODN has been studied in different species. For example it has been shown that CpG ODN administration leads

to the protection against many pathogens including viruses, bacteria and protozoa in mice [194-196]. When used with an antigen CpG ODNs can be used as an effective adjuvant in many species including human, mice, cattle, turkey and horses [197-200]

CpG ODN can protect chicken against different bacterial infections such as *S. typhimurium, S. enteritidis* and *E. coli.* [201-204]. Surprisingly, chicken do not express TLR9 which leaded to the questions to identify the receptor that recognizes CpG ODN and mediates immune responses against CpG ODN in chicken[192]. In 2009, Brownlie et al., showed that TLR21 is the functional homologue of mammalian TLR9 that is responsible for CpG recognition [205].

High mortality rates of newborn broiler chickens cause serious economic losses for the industry in Turkey and abroad. Administration of antibiotics *in ovo* or drinking water in the first week of age in order to protect chicken from bacterial infections is becoming unacceptable. Therefore, developing prophylactic agents that can decrease newborn mortality rates of broiler chicken is extremely important [192, 202].

1.11 Aim and Strategy

This thesis is designed to broaden the spectrum of ODNs with known immunosuppressive and immunostimulatory properties.

The first part of the thesis is devoted to demonstrate the beneficial role of suppressive ODN A151 on endotoxin induced uveitis. For this, we have selected to work with two different animal models. Upon parenteral or local LPS administration, EIU was established either in rabbit or in murine models respectively, as a local autoimmune disease. To date, the inhibitory effect of suppressive ODN on LPS mediated EIU at both the local and systemic levels has not been studied by others. In this study, a very aggressive form of experimental uveitis was initiated via endotoxin administration. We investigated whether the suppressive ODN “A151” can inhibit the induction and development of ocular inflammation (before or at the time of LPS

insult or even 2 h after LPS treatment) and help to reduce the symptoms of EIU in rabbits and mice. Our results revealed, for the first time, that A151 is capable of downregulating the mRNA expression and protein levels of several potentially pathologic chemokines and cytokines at both the local and systemic levels. Consequently, suppressive ODN mimicking telomeric DNA offers a novel nucleic acid-based immunotherapeutic agent to control overexuberant undesirable immune responses such as seen in autoimmune and autoinflammatory diseases.

In the second part of this thesis the immunosuppressive potential of ocularly administered Restasis (an ophthalmic emulsion consisting of 0.05% cyclosporine A) was tested in rabbits and mice following local or intraperitoneal LPS injection, respectively, to induce EIU. To our knowledge, the present study demonstrates for the first time that Restasis suppresses the expression and protein levels of several Th1- biased proinflammatory cytokines and chemokines that were upregulated as a result of EIU.

In the third part of this thesis adjuvant effect of CpG ODNs were tested either by formulating them in a lipidic nanoemulsion together with Serotype -O Ag containing Monovalent vaccine or with their Ag alone mixture. Data suggested that, in mice, inclusion of CpG ODN as an adjuvant, significantly improved FMD vaccine formulation that conferred rapid, long lasting humoral and cell mediated immunity as well as persisting virus neutralization titers. These new vaccine candidates may prove to be more protective against FMD epidemics in the field trials.

Finally, in an auxiliary project the effects of immunostimulatory CpG ODNs as prophylactic agents for newborn chickens were tested *in vivo.* Several studies in the literature were strongly indicating the potential of these sequences as prophylactic agents for different species. High mortality rates of newborn broiler chickens cause serious economic losses for the industry in Turkey and abroad. Therefore we aimed to exhibit the prophylactic effects of these sequences in chickens. 3 doses of two different CpG ODNs were injected to 1 day old broiler chickens and percent of mortality, (Feed Conversion Ratio) FCR ratios, live body weights and (European

Efficiency of Productivity Factor) EPEF values of animals were investigated Our results suggested that CpG ODN pretreatment not only significantly reduced mortality rates and FCR ratios but also increased live body weights and EPEF values for a duration of 41 days.

Chapter 2

Materials and Methods

2.1. Materials and Methods Used in EIU Experiments

2.1.1. The Maintenance of the Animals

Adult female BALB/C mice and New Zealand rabbits 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 conditions at 22^{o} C 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 No: 06/027).

2.1.2. Induction of Endotoxin Induced Uveitis

Specific pathogen-free ten weeks old female BALB/c mice were obtained from the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University. In A151 experiments mice grouped as 1) Untreated, 2) LPS Only,

3) A151 Only, 4) Cont. ODN Only, 5) LPS+Cont.ODN, 6) LPS+A151, 7) Cont. ODN then LPS, 8) A151 then LPS, 9) LPS then Cont. ODN, 10) LPS then A151. (5 mice/group) In Table 2.1 injection and treatment protocols for these experiments are presented. The mice were injected i.p with 25, 50, 100 or 200 μg LPS in 200μl of PBS and/or 100-250 μg suppressive ODN (2h before and at the time of LPS injection and 2h after LPS treatment. In Restasis experiments mice grouped as ; 1: Untreated, 2: LPS only, 3: Restasis only, 4: Restasis then LPS, and 5: LPS then Restasis (4 mice/group). In order to induce EIU some groups were injected i.p with 20 or 50 μg LPS in 200 μl of PBS. Restasis was topically administered ocularly either before or after the establishment of EIU with 1 h intervals for 6 times (1.0 μg CsA emulsion/administration). Mice were killed 24 hrs following the LPS injection. In Table 2.2, injection and treatment protocols for mouse studies are presented. Clinical assessment of EIU explained in detail in Clinical Evaluation and Histopathological Investigation Section. Mice were sacrificed at the end of clinical evaluation. Both eyes were enucleated and used for cytokine expression assays. Spleens were removed and split into two, and splenocytes were incubated on tissue culture plates for 6, 12 and 24 h and supernatants were collected for cytokine determination by ELISA. IL6 was measured as an indicator of EIU response. The other half of the spleen was used to extract total RNA for further cytokine/chemokine gene transcript expression analysis by RT-PCR. In rabbit experiments for A151 treatment (3-4 animal/groups, 1500 g each housed in Ankara Hospital animal facility, Cebeci, Ankara) animals were separated into six different treatment groups (1-Untreated, 2-LPS Only, 3-LPS+A151, 4-A151 then LPS, 5-LPS then Cont. ODN, 6-LPS then A151) and EIU was initiated via intraocular LPS injection (100 μg) with or without suppressive ODN treatment. In Table 2.3 injection and treatment protocols for rabbit A151 studies are presented. In rabbit experiments for Restasis treatment (3-4 animal/groups, 1500 g each housed in Ankara Hospital animal facility, Cebeci, Ankara) animals were separated into three different treatment groups (1-Untreated, 2-LPS Only, 3-LPS then Restasis) and EIU was initiated via intraocular LPS injection (100 μg) with or without Restasis treatment. In Table 2.4 injection and treatment protocols for rabbit Restasis studies are presented. Rabbit eyes were removed and gene expression analyses that were described for mice were conducted.

Table 2.1. EIU induction and A151 treatment schedule of mouse experiments

Group ID	Time (h)													
	0	1	2	3	4	5	6	7	8	20	21	22	23	24
Untreated							P							X
LPS Only							L							X
A151 Only							A							X
Cont. ODN Only							C							X
LPS+Cont. ODN							L+C							X
LPS+A151							L+A							X
Cont.ODN then LPS					C		L							X
A151 then LPS					A		L							X
LPS then Cont.ODN							L		C					X
LPS then A151							L		A					X

P: PBS Injection, L: LPS Injection, A: A151 Treatment, C: Cont. ODN Treatment, X: Sacrifice Animals

Table 2.2: EIU induction and Restasis treatment schedule of mouse experiments

Group ID	Time (h)													
	0	1	2	3	4	5	6	18	19	20	21	22	23	24
Untreated	P													X
LPS Only							L							X
Restasis Only	R	R	R	R	R	R								X
Rest. then LPS	R	R	R	R	R	R	L							X
LPS then Rest.							L	R	R	R	R	R	R	X

P: PBS Injection, L: LPS Injection, R: Restasis Treatment, X: Sacrifice Animals

Table 2.3. EIU induction and A151 treatment schedule of rabbit experiments

Group ID	Time (h)													
	0	1	2	3	4	5	6	7	8	20	21	22	23	24
Untreated							P							X
LPS Only							L							X
LPS+A151							L+A							X
A151 then LPS					A		L							X
LPS then Cont.ODN							L		C					X
LPS then A151							L		A					X

P: PBS Injection, L: LPS Injection, A: A151 Treatment, C: Cont. ODN Treatment, X: Sacrifice Animals

Table 2.4. EIU induction and Restasis treatment schedule of rabbit experiments

Group ID	Time (h)													
	0	1	2	3	4	5	6	18	19	20	21	22	23	24
Untreated	P													X
LPS Only	L													X
LPS then Rest.	L							R	R	R	R	R	R	X

P: PBS Injection, L: LPS Injection, R: Restasis Treatment, X: Sacrifice Animals

2.1.3. Clinical Evaluation and Histopathological Investigation

Animals were subjected to blind investigation by an ophthalmologist under a dissection microscope 18–24 h after injection, corresponding to the time of maximal severity of EIU. Clinical ocular inflammation was graded on a scale from 0 to 4 for each animal described previously: no sign of inflammation = 0; discrete inflammation in iris and conjunctiva = 1; dilatation of iris and conjunctiva vessels = 2; hyperemia in iris associated with Tyndall effect in anterior chamber = 3; in addition to the signs in scale 3, synechia or fibrin is formed = 4 ([206]). For histopathological investigations, enucleated eyes were fixed in 10% formalin for 24 h, washed with running tap water for 1 h, and placed in 60% ethyl alcohol for an extra 3 h. Eyes were embedded in paraffin, which was sectioned and stained with hematoxylin and eosin. Sections were examined blindly by a histopathologist, using score systems of severity ranging from 0 to 4. Focal non-granulomatous monocytic infiltration in the choroid, the ciliary body and retina were scored as 0.5. Retinal perivascular infiltration and monocytic infiltration in the vitreous were scored as 1.

Granuloma formation in the uvea and retina and the presence of occluded retinal vasculitis along with photoreceptor folds, serous detachment, and loss of photoreceptor were scored as 2. In addition, the formation of Dalen-Fuchs nodules (granuloma at the level of the retinal pigmented epithelium) and the development of subretinal neovascularization were scored as 3 and 4 according to the number and size of the lesions ([207]).

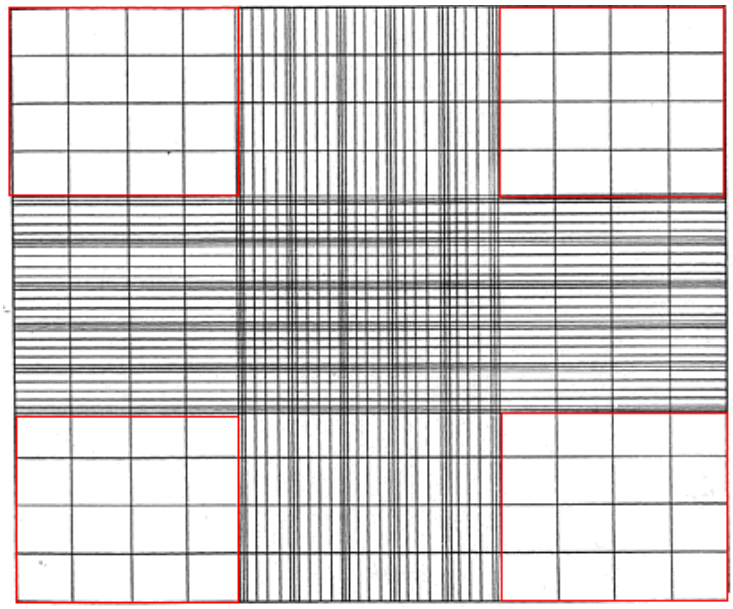
2.1.4. Spleen and Ocular Cell Preperation

Spleens and eyes were removed from the BALB/C female mice after cervical dislocation. Single cell suspensions were obtained by smashing of spleens and eyes with the back of the sterile syringes by circular movements in the 2% FBS supplemented regular RPMI. 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 cell suspensions were counted and adjusted to 2-4x10^6/ml unless otherwise stated.

2.1.5. Cell Number Detection with Cell Count

After the spleen cells were smashed, washed and precipitated, they were suspended in 10 ml of 5% Regular RPMI-1640 media. Cells were diluted 10 fold and micropipetted into 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:

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

2.1.6. Cytokine and IgM ELISAs and NO Assays

Immulon 2 HB microtiter plates (Thermo Scientific) were coated with anti-cytokine or anti-IgM antibodies (BD Pharmingen) and then blocked with PBS and 1% BSA ([79],[107]). Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine antibody followed by phosphatase-streptavidin (Perbio), whereas bound IgM was detected using phosphatase-conjugated anti-IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) as described ([79]). Nitric oxide detection by the Griess method was conducted on murine peritoneal exudate cells (10^6/ml) after 12–36 h of ex vivo incubation as described by the supplier (Promega).

2.1.7. Analysis of Cell-surface Molecule Expression by FACS

2×10^6spleen cells/ml were isolated from 24-h post-treated mice. Cells were washed, fixed, and co-stained with one of the phycoerythrin-labeled anti-CD40, anti-CD86, and anti-ICAM-1 and FITC-labeled cell-specific antibodies (i.e.CD11c for dendritic cells, CD11b for macrophages, and B220 for B cells (BD Pharmingen) for 30 min at room temperature. Following washing, they were studied using a FACSCalibur (BD Biosciences) and analyzed with CellQuest Pro software.

2.1.8. Determination of the Gene Expression

2.1.8.1. Total RNA Isolation from the Cells

Cells extensively mixed and homogenated by a mono-phasic solution of phenol and guanidinium thiocyanate: TRItidy G. 200 µl of chloroform for every 1ml of TRItidy G was used and tubes were vigorously shaken for 15 seconds and incubated at room temperature for 2-3 mins followed by a centrifugation for 15 mins at 13.900 rpm at 4°C. The 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, 75% EtOH for every 1 ml of TRItidy G used. Tubes were vortexed and centrifuged at 8000 rpm for 7 mins 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 discussed. After centrifugation, the alcohol was removed and pellet was air-dried under laminar flow hood, and dissolved with 20-30 µl RNase/DNase free ddH_2O. The OD measurements were taken at 260/280 nm wavelengths using a spectrophotometer (NanoDrop® ND-1000). 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 is between 1.8-2.0 OD. The isolated RNA was stored at -80°C.

2.1.8.2. cDNA Synthesis

The cDNAs were synthesized from the total RNA samples with the cDNA synthesis kit according to the manufacturers' protocol. 2µg RNA was mixed with 1µl of Oligo(dT) primer and completed to a total volume of 12 µl with RNase/DNase free ddH_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.

2.1.8.3 Primers

Primers such as; *il-18*, *cxcl-16*, *mip 3-α* 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 different articles and sources (Table 2.5).

Table 2.5. Oligonucleotide PCR primers used in mouse and rabbit experiments

Primer		Sequence	Product Size
m β-actin**	Forward Reverse	5'-GTATGCCTCGGTCGTACCA-3' 5-'CTTCTGCATCCTGTCAGCAA-3'	450bp
m GAPDH*	Forward Reverse	5'-TCCTGCACCACCAACTGCTTAGCC-3' 5'-GTTCAGCTCTGGGATGACCTTGCC-3'	510bp
m TLR2*	Forward Reverse	5'-TCTCTGGGCAGTCTTGAACATTTG-3' 5'-CGCGCATCGACTTTAGACTTTG-3'	320bp
m TLR4*	Forward Reverse	5'-TGCCGTTTCTTGTTCTTCCTCT-3' 5'-CTGGCATCATCTTCATTGTCCTT-3'	240bp
m TLR5*	Forward Reverse	5'-TGGGGCAGCAGGAAGACG-3' 5'-AGCGGCTGTGCGGATAAA-3'	380bp
m TLR6*	Forward Reverse	5'-GCCCGCAGCTTGTGGTATC-3' 5'-GGGCTGGCCTGACTCTTA-3'	650bp
m TLR7*	Forward Reverse	5'-TTAACCCACCAGACAAACCACAC-3' 5'-TAACAGCCACTATTTTCAAGCAGA-3'	700bp
m TLR9*	Forward Reverse	5'-GATGCCCACCGCTCCCGCTATGT-3' 5'-TGGGGTGGAGGGGCAGAGAATGAA-3'	430bp
m IP10**	Forward Reverse	5'-GCCGTCATTTTCTGCCTCAT-3' 5'-GCTTCCCTATGGCCCTCATT-3'	127bp
m iNOS**	Forward Reverse	5'-CAGCTGGGCTGTACAAACCTT-3' 5'-CATTGGAAGTGAAGCGTTTCG-3'	95bp
mMIP1α*	Forward Reverse	5'-ACCATGACACTCTGCAACCA-3' 5'-AGGCATTCAGTTCCAGGTCA-3'	238bp
mIL5**	Forward Reverse	5'-AGCACAGTGGTGAAAGAGACCTT-3' 5'-TCCAATGCATAGCTGGTGATTT-3'	117bp
mIL15**	Forward Reverse	5'-CATCCATCTCGTGCTACTTGTGTT-3' 5'-CATCTATCCAGTTGGCCTCTGT-3'	126bp
mIL18*	Forward Reverse	5'-GATCAAAGTGCCAGTGAACC-3' 5'-ACAAACCCTCCCCACCTAAC-3'	384bp
mMCP1*	Forward Reverse	5'-AGGTCCCTGTCATGCTTCTG-3' 5'-TCTGGACCCATTCCTTCTTG-3'	249bp
mMIP3α*	Forward Reverse	5'-CGTCTGCTCTTCCTTGCTTT-3' 5'-CCTTTTCACCCAGTTCTGCT-3'	250bp
mCXCL16*	Forward Reverse	5'-CCTTGTCTCTTGCGTTCTTC-3' 5'-GGTTGGGTGTGCTCTTTGTT-3'	384bp
mMIP1β*	Forward Reverse	5'-CCAGCTCTGTGCAAACCTAA-3' 5'-CTGTCTGCCTCTTTTGGTCA-3'	250bp
Rb GAPDHω	Forward Reverse	5'-TCACCATCTTCCAGGAGCGA-3' 5'-CACAATGCCGAAGTGGTCGT-3'	319bp
Rb IL6ω	Forward Reverse	5'-GCTCCTGGTGGTGGCTAC-3' 5'-GGGTGGCTTCTTCATTCAAA-3'	450bp
Rb IL1βω	Forward Reverse	5'-GCCGATGGTCCAATTACAT-3' 5'-ACAAGACCTGCCGGAAGCT-3'	121bp

*m: mouse, rb: rabbit, * in house designed primers, **taken from Ref. [208], ωtaken from Ref [209]*

2.1.8.4. 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 with the quantitated values for the *β- actin* for each sample separately.

2.1.8.5. Agarose Gel Electrophoresis

2% agarose gel was prepared with 1X TAE Buffer and 1 mg/ml ethidium bromide solution. Samples were prepared by mixing of 5µl Agarose Gel Loading Dye to 10µl of cDNA sample and loaded to the agarose gel. The gel was run at 80V for 60 minutes and visualized under transilluminator (Gel-Doc BIO-RAD, USA and Vilber Lourmat, France) softwares were used to take photographs of the gels and compare the cDNA band intensities for the analysis. The, Low Range DNA Ladder (Jena Biosciences) and 100 bp DNA ladder (Jena Biosciences) were used as a marker and 3µl was loaded to every gel.

2.1.6. Statistical Analysis

Assays were performed in triplicate on at least three to five different cell preparations. Statistical significance between untreated (or control) and treated groups was evaluated using Student's t-test.

2.2. Materials and Methods Used in Foot and Mouth Disease Experiments

2.2.1. Mice

Adult female BALB/c mice were used for the experiments. The animals were kept in the animal holding facilities of the Department of Molecular Biology and Genetics at Bilkent University under controlled conditions at 22°C with 12 h light and 12 h dark cycles. They were provided with unlimited access to food and water. Animal procedures were approved by the animal ethical committee of Bilkent University.

2.2.2. CpG Oligodeoxynucleotiedes

The ODNs were either synthesized by (Alpha DNA, Canada) or synthesized in house on a Mermade 6 automated ODN synthesizer (BioAutomation, USA). Sequences and properties of the sequences were provided in Table 2.6. All ODNs were tested for endotoxin by LAL assay, or TLR4-stably transfected HEK293 cell based promoter assay and was found to contain no detectable LPS.

Table 2.6. List of CpG ODNs used in FMD study

ODN Name	Sequence	Length	Size	Backbone
1466_Acore_PO	TCAACGTTGATTCAAA	16	4864	PO
1466_Acore_PS	tcaacgttgattcaaa	16	4864	PS
1466_Acore_MB	tcAACGTTGATTCAaa	16	4864	MB
1555_PO	GCTAGACGTTAGCGT	15	4608	PO
1555_PS	gctagacgttagcgt	15	4608	PS
1555_MB	gcTAGACGTTAGCgt	15	4608	MB
D35_3CG_PO	GGTCGATCGATCGAGGGGGG	20	6279	PO
D35_3CG_PS	ggtcgatcgatcgaggggggg	20	6279	PS
D35_3CG_MB	ggTCGATCGATCGAGGGGgg	20	6279	MB
1466_pG6_PO	TCAACGTTGAGGGGGG	16	5002	PO
1466_pG6_PS	tcaacgttgaggggggg	16	5002	PS
1466_pG6_MB	tcAACGTTGAGGGGgg	16	5002	MB
ODN5547_PO	TCGACGTTCGACGTTGGG	18	5547	PO
ODN5547_PS	tcgacgttcgacgttggg	18	6782	PS
ODN5547_MB	TcGACGTTCGACGTTGGg	18	5547	MB
ODN7440_PO	GTCGATCGATTTTCGATCGAGGGG	24	7440	PO
ODN7440_PS	gtcgatcgattttcgatcgagggg	24	7440	PS
ODN7440_MB	gtCGATCGATTTTCGATCGAGGgg	24	7440	MB
1466_PS	tcaacgttga	10	3027	PS
K3_PS	atcgactctcgagcgttctc	20	6044	PS
K23_PS	tcgagcgttctc	12	3612	PS
1826_PS	tccatgacgttcctgacgtt	20	6059	PS
2006_PS	tcgtcgttttgtcgttttgtcgtt	24	7329	PS
2007_PS	tcgtcgttgtcgttttgtcgtt	22	6720	PS

Upper case: phosphodiester (PO), Lower case: phosphorothioate (PS), Mixed Backbone (MB)

2.2.3 Preparation of vaccines

FMDV strain O/TUR/07 was grown in BHK-21(Baby Hamster Kidney) suspension cultures. The virus was harvested after propagation and clarified by centrifugation at 3000 x g for 30 minutes. After inactivation with binary ethyleneimine (BEI), 10% (w/v) polyethyleneglycol 6000 was added to the supernatant and the mixture was stirred overnight at 4°C. The antigens were precipitated by centrifugation at 600g and resuspended in 1/10 volume of PBS. Aliquots were frozen at liquid nitrogen and stored until use. 146S antigen amount was measured by Sucrose Density Gradient method. In order to get the required final concentration, the concentrated antigen was diluted in PBS (pH 7.6) to yield 22ug Ag/ml.

Monovalent vaccine containing FMDV O/TUR/07 inactivated antigen in double oil emulsion with montanide ISA 206 (Seppic, France) were prepared. Equal ratios of antigen and oil volumes were used and antigen and oil slowly blended to ensure water-in-oil-in-water emulsion.

2.2.4. Immunization

4-6 weeks old female BALB/c mice (8/group) were injected twice (ip, at day=0 and 15) either monovalent vaccine (labeled as "Mono") or free FMDV serotype-O antigen (labeled as "Ag") or their combinations with CpG ODN mixture (10µg/animal). Optimum Dose antigen (ODAg) indicates a 3µg serotype O antigen per mouse, Low Dose antigen (LDAg) indicates 0.5µg serotype O antigen per mouse. Either serotype-O antigen(Ag) or monovalent vaccine containing formulations then mixed with the CpG ODN to yield 3µg or 0.5ug Ag or Monovalent Vaccine + 10µg CpG ODN /300µl injections.
Treatment Groups and formulation ingredients used throughout immunization study was given in Table 2.7.

Table 2.7. Injection groups and formulation ingredients used throughout immunization studies

Treatment Groups	**Formulation Ingredients**
Untreated	PBS
OD Ag	3µg Ser-O Ag
LD Ag	0.5µg Ser-O Ag
OD Ag+CpG	3µg Ser-O Ag+10µg CpG ODN
LD Ag+ CpG ODN	0.5µg Ser-O Ag+10µg CpG ODN
OD Mono	3µg Monovalent Vaccine
LD Mono	0.5µg Monovalent Vaccine
OD Mono+CpG	3µg Monovalent Vaccine+10µg CpG ODN
LD Mono+CpG	0.5µg Monovalent Vaccine+10µg CpG ODN

Mouse was bled via tail vein and sera were collected with 2-4 weeks intervals for a duration of 5 months from the time of post-booster injection. Detailed information about injection/bleeding schedule is given in Figure 2.1. Sera were prepared by centrifugation at 8000 rpm for 5 min followed by incubation of blood at 37ºC for one hour and were stored at −20ºC until for further use.

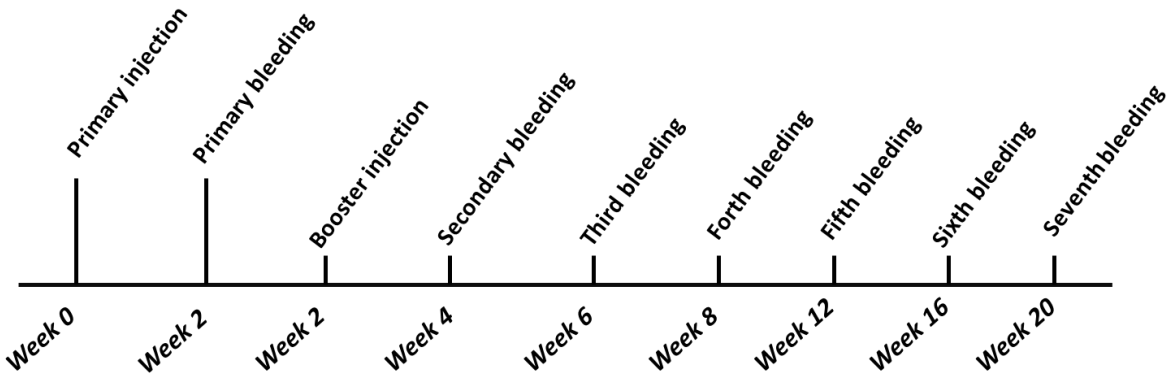


Figure 2.1. Injection and bleeding schedule of mice used in immunization studies.

2.2.5. Ig ELISA

Serum Total IgG, IgG1 and IgG2a antibody in mice was determined by ELISA. Briefly, wells (96 wells, Immulon 1B, Thermo Scientific, USA) were coated with appropriately diluted of Rb-α mouse SerO Ab in PBS (pH 7.2) and incubated overnight at 4ºC and blocked by using 200µl of PBS- BSA (5%) for 3h at RT. After 5X wash with PBST, 1/20 diluted supernatants of the cell lysates of FMDV-infected BHK added to the plates in PBS and incubated overnight at 4ºC. After 5X wash with PBST, 80X diluted mouse sera added to the first wells and subjected to 2X serial dilutions with PBS and incubated overnight at 4ºC. After 5X wash with PBST, appropriately diluted alkaline phosphatase conjugated goat anti- mouse IgG-AP, IgG_1-AP and IgG_{2a}-AP (Southern Biotech) were added to the plates and incubated at RT for 2h. After removal of unbound conjugated antibody, positive binding was detected by adding PNPP substrate (Perbio, Pierce, USA) and the absorbance was read at 405nm.

2.2.6. IFNγ ELISA

Serum IFNγ levels in mice was determined by ELISA. Briefly microtiter plates (Immulon 2 HB, Thermo Scientific, USA) were coated with mouse anti-IFNγ antibodies (MABTECH, Sweden) in PBS (pH 7.2) and incubated overnight at 4ºC and then blocked using 200µl of PBS- BSA (5%) at RT for 3 h [79, 81] After 5X wash with PBST, serially diluted standards and mouse sera were added to these plates and incubated overnight at 4ºC. After 5X wash with PBST, appropriately diluted biotinylated mouse anti IFNγ Ab (MABTECH, Sweden) were added to the plates and incubated at RT for 2 h. After removal of unbound conjugated antibody, positive binding was detected by adding substrate PNPP and the absorbance was read at 405 nm.

2.2.7. Virus Neutralization Assay

Neutralizing antibody titers to FMDV from mice sera were measured by micro-neutralization assay described by OIE Terrestial Manual for Foot and Mouth Disease (2009). Briefly, all sera samples and positive and negative controls were heat inactivated (56°C, 30 min) and used for microneutralization assay on BHK-21 cells. Serial two fold dilutions of serum were made in 5% FCS supplemented Glasgow MEM and incubated with a virus doses of 100 TCID50 of FMDV O/TUR/07 for 1h at 37°C. The BHK-21 cells ($6x10^5$ cells/ml) were added to the wells and incubated further for 48–72 h at 37°C. The cells were monitored every day for Cytopathological Effects (CPE) under cell culture microscope. Wells then were stained with crystal violet-formalin. End-point titers were calculated as the reciprocal of the last serum dilution to neutralize 100 TCID50 of homolog virus CPE in 50% of the wells.

2.2.8. Statistical Analysis

Statistical significance between CpG formulations vs non-CpG formulations were evaluated using Student`s T- test. $p<0.05$ was considered as significant over control group.

2.3. Materials and Methods Used in Chicken Experiments

2.3.1 Chicken Housing

Chickens were kept in 60 separate sections (200 chickens/section) randomly distributed within Research and Development coop facility of the Beypilic. They were provided with unlimited access of food and water. Survival rates, live body weights, water and food consumption rates were routinely monitored by a computerized system within the integrated facility.

2.3.2 Immunization

All procedures involving chicken were done with one day old animals provided by Beypilic hatchery in Bolu, Turkey.
Groups of chickens (1000/group) were subcutaneously injected once either with 3 different doses (0.8µg, 4µg, 20µg) of 1466 Acore PO or D35 3CG MB CpG ODNs in 200 µl Hanks Buffer or Hanks Buffer alone (6000 chicken) as control.

2.3.3 FCR and EPEF Calculation

FCR (Feed conversion ratio) and EPEF (European Poultry Efficiency Factor) were calculated with following formulations.

FCR= Total Feed (kg)/Total Weight (kg)
EPEF= [(Live weight, kg X Viability %) / (FCR X Age, days)]X100

Chapter 3

Results

3.1 Immunotherapeutic Use of Suppressive ODN in Endotoxin Induced Uveitis

EIU is an established animal model of acute ocular inflammation. It is induced by either systemic or intravitreal administration of LPS, the major component of Gram-negative bacteria. LPS acts through the TLR4-triggering proinflammatory signaling cascade. The expression of Th1 cytokines and chemokines, including IL6, IL1β, and MIP3α (macrophage inflammatory protein 3α), contributes to the development of EIU.

Initial experiments were conducted to optimize the induction of EIU (Figure 3.1). For the mouse experiments, systemic administration of LPS doses between 20 and 100 μg/mouse were sufficient to induce uveitic eyes within 24 h as judged by clinical and histopathological investigations (Figure 3.1 and Figure 3.3). For the rabbit experiments, intraocular 100 μg LPS injection was found to be optimal to induce EIU. Following local or intraperitoneal LPS and/or suppressive ODN administration,

rabbit and mouse eyes were removed, and RNAs from the irises, vitreous, and corneas of the rabbit eyes were obtained. PCR was run with the cDNA from each sample, and then RNA levels of IL6, IL15, IP10 (interferon-γ-inducible protein 10), iNOS, MIP1α, IL-18, MIP3α, CXCL16 (CXC chemokine ligand 16), MIP1β, and IL1β were monitored. In addition, 24 h post-LPS and/or A151 treatment, splenocyte suspensions were incubated ex vivo for 6 –24 h, and IgM, IL6, IL10, IL12, and IFN γ levels from the supernatants were determined by ELISA. FACS analyses were conducted on spleen cells to monitor co-stimulatory/surface marker molecule expressions.

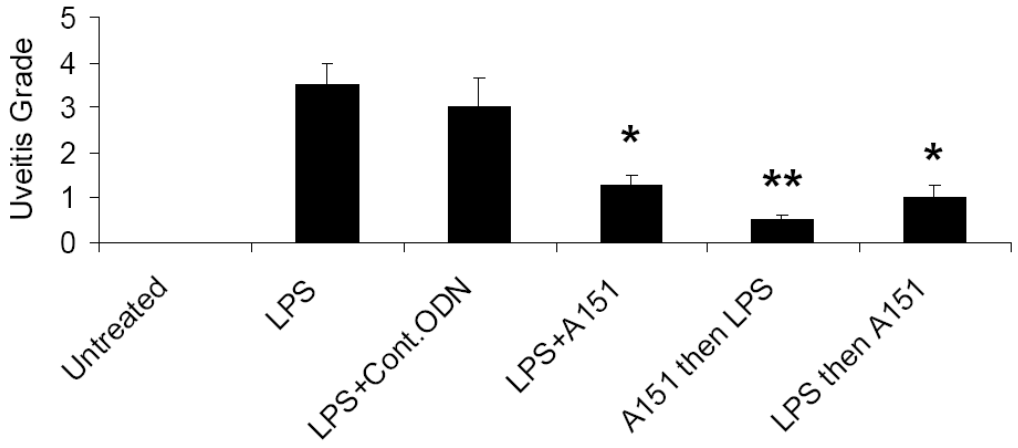


Figure 3.1. Effect of A151 treatment on clinical EIU. Following ip LPS injection animals were treated with A151. Before animals were sacrificed clinical EIU evaluation was performed on both eyes and scored as described in methods section 2.1.3. * $p<0.05$, ** $p<0.01$ between LPS treated and LPS+A151 ODN co-administered groups.

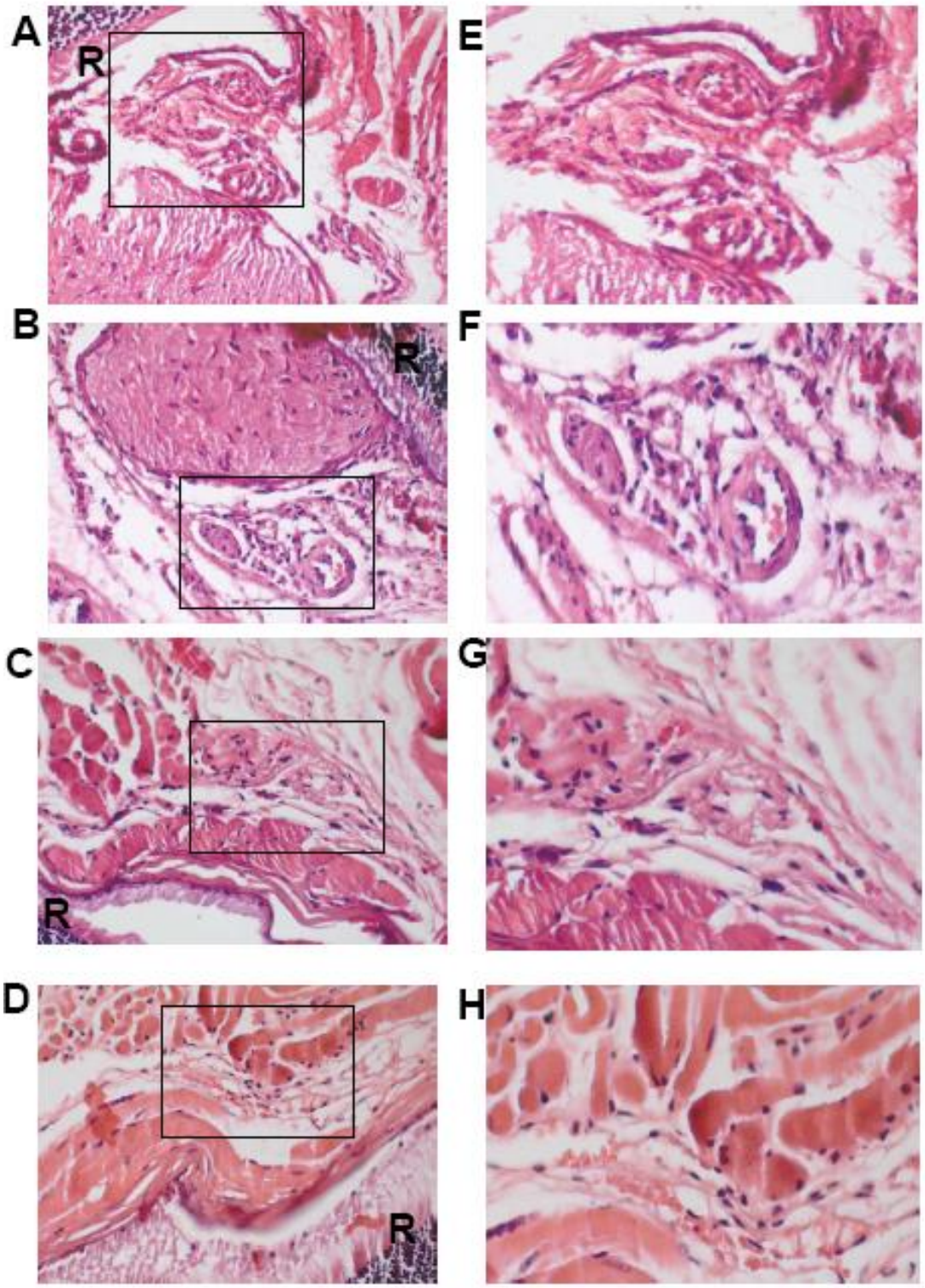


Figure 3.2. Histopathological changes showing retinal perivascular infiltration in eyes of mice following LPS and/or suppressive ODN treatment. A) retinal vessels of untreated eye. B) retinal perivascular infiltration of mononuclear cells (MNC) and neutrophils in LPS and Cont.ODN injected eye. C) decreased MNCs post-A151 treatment before and D) simultaneous with LPS challenge. E-H) represent inlets of A-D respectively (Mag:40X)

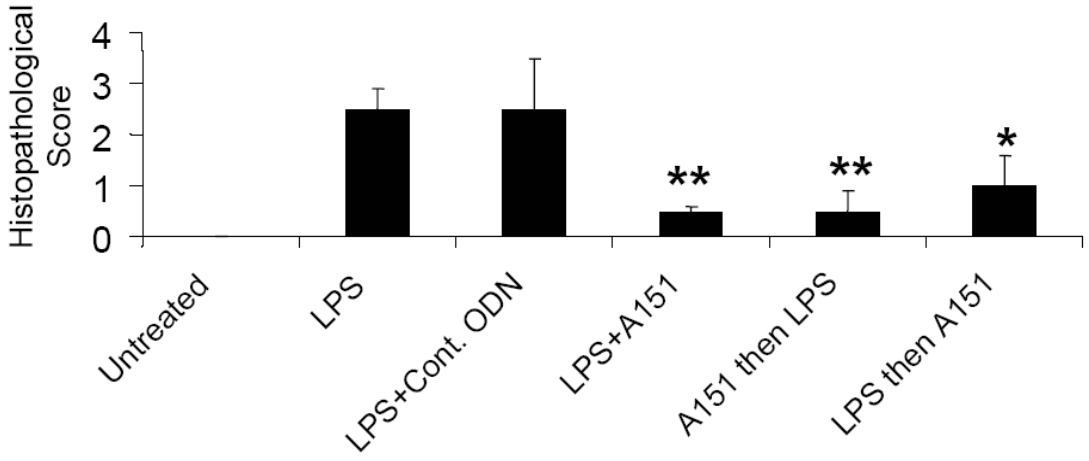


Figure 3.3. A151 mediated changes in histopathological scoring following EIU (for details see Figure 3.2). * $p<0.05$, ** $p<0.01$ between LPS vs LPS & A151 treated groups.

Our results revealed that suppressive ODN administration significantly diminished disease severity proven by reduction on clinical (Figure 3.1) and histopathological (Figure 3.2 and Figure 3.3) scores of groups injected with A151 ODN.

The results indicated that in rabbits, suppressive ODN administered before or after 100 μg LPS treatment or co-injected with LPS significantly down-regulated the expression of IL1β message from the iris (Figure 3.4). In cornea, IL6 was downregulated when suppressive ODN was given before or simultaneously with LPS administration (Figure 3.5). There was no significant inhibitory effect when A151 was given post-LPS treatment. In all these experiments, the mRNA message reduction was suppressive ODN-dependent because control ODN administration did not show any benefit for alleviation of LPS reactogenicity.

IL1β

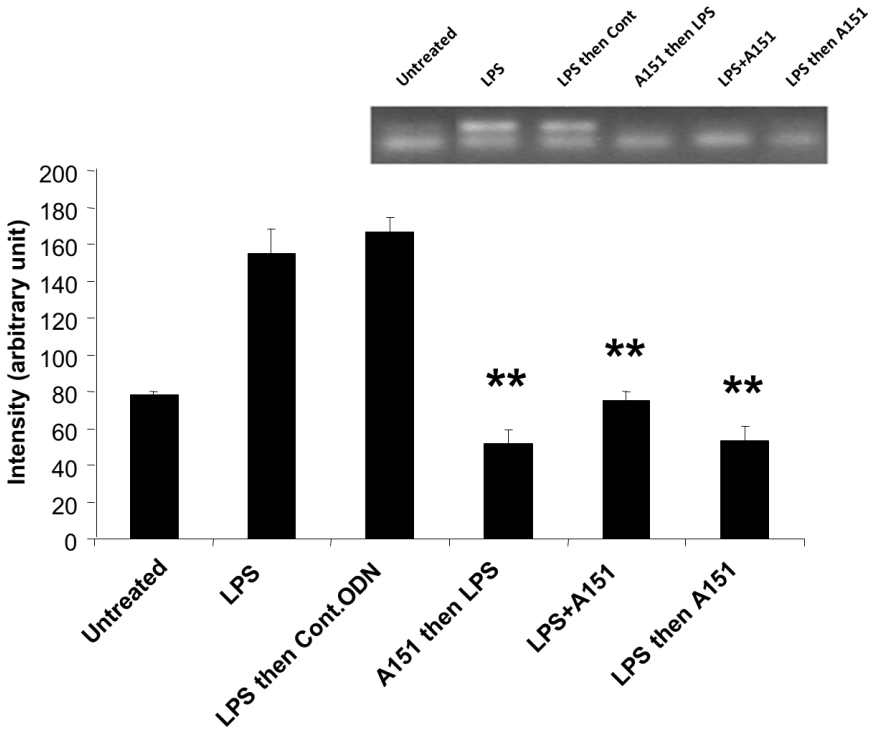


Figure 3.4 Suppressive ODN A151 administration after LPS challenge downregulated IL1β expressions in iris. Rabbits were injected intraocularly with 100 µg LPS and 250 µg suppressive ODN. Average of four animals' densitometric measurements for IL1β mRNA from iris is shown. Inlets are the representative gel image of each group ** $p<0.01$ between LPS vs LPS & A151 ODN treated groups.

IL6

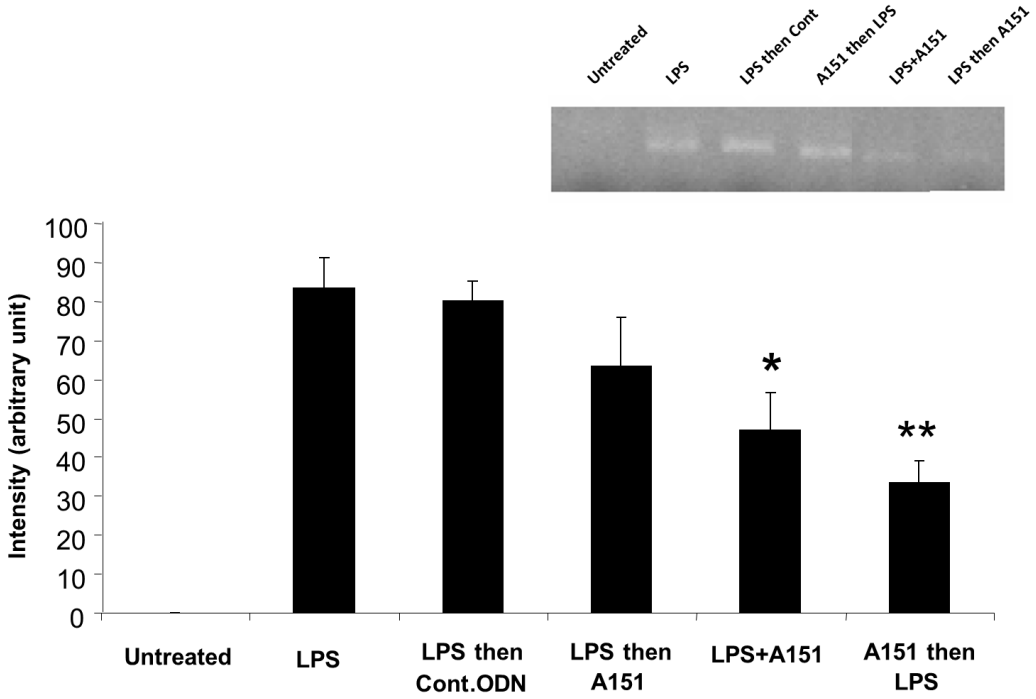
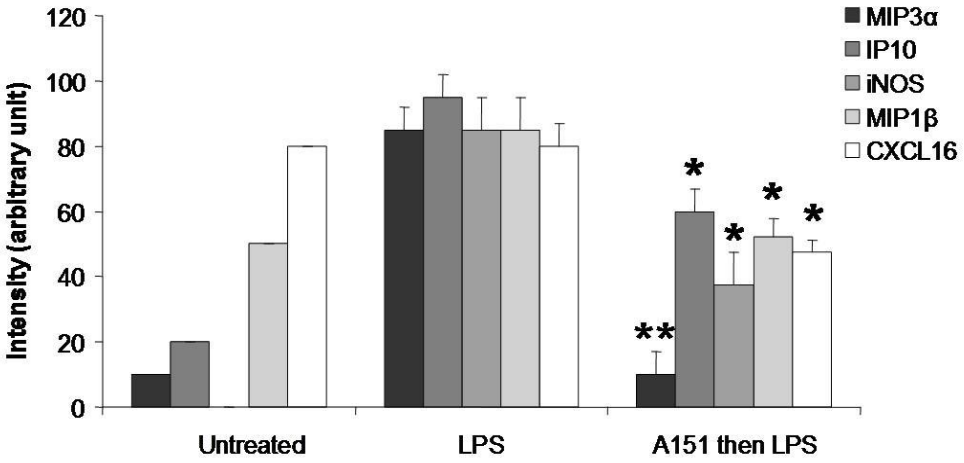


Figure 3.5. Suppressive ODN A151 administration after LPS challenge downregulated IL6 expressions in cornea. Rabbits were injected intraocularly with 100 µg LPS and 250 µg suppressive ODN. Average of four animals' densitometric measurements for IL6 mRNA from cornea is shown. Inlets are the representative gel image of each group * $p<0.05$, ** $p<0.01$ between LPS vs LPS & A151 ODN treated groups.

On the murine EIU model, mouse tolerated up to 100 µg LPS i.p. treatment. Doses over 150 µg caused animals to succumb to endotoxin treatment. The mice experiments were conducted with three doses of LPS; 25, 50, and 100 µg. Suppressive A151 ODN and Control ODN (2h before, at the time of LPS injection and 2 h after LPS treatment) were used in the range of 100-250 µg. While in rabbits the injection of ODNs and endotoxin was intraocular, in mice injections were via intraperitoneal route in 200 µl PBS.

(A)



(B)

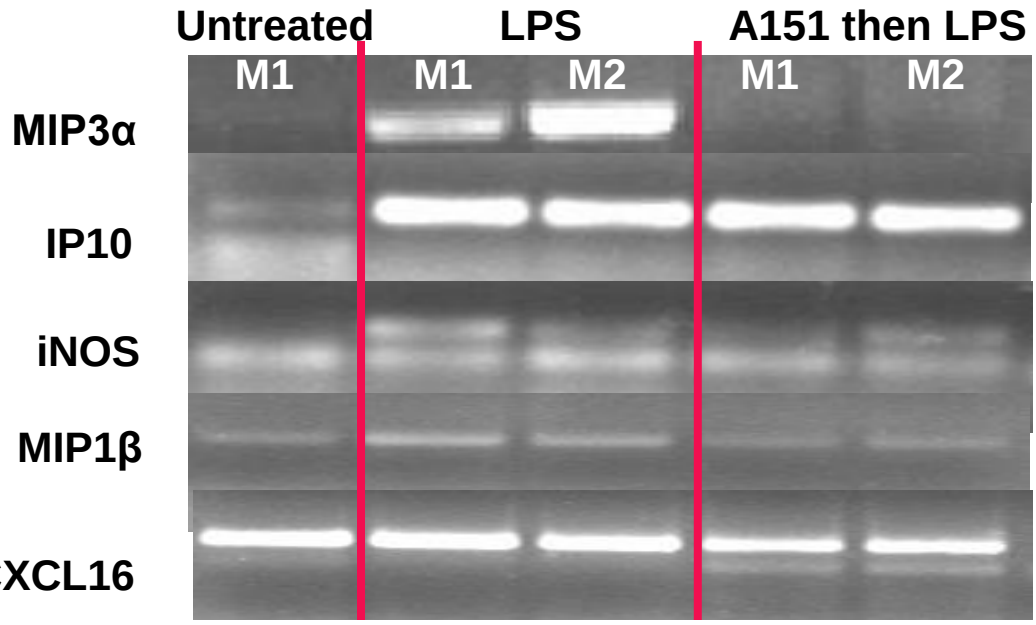


Figure 3.6. A) Suppressive ODN A151 treatment after 100 µg LPS challenge significantly downregulates MIP3α, IP10, iNOS, MIP1β and CXCL16 mRNA expression levels on the eyes of mice. Mice were injected intraperitoneally with 100 µg LPS and 250 µg suppressive ODN and were killed at 18 h after injection. * $p<0.05$, ** $p<0.01$ between LPS treated and LPS+A151 ODN co-administered groups. B) Representative agarose gel picture (M1 indicates mouse #1, M2 indicates mouse #2).

Results showed that when 250 μg suppressive ODN was administered before LPS injection (2h) it significantly downregulated the expression of MIP3α, IP10, iNOS, MIP1β and CXCL16 in 100 μg LPS injected mEIU model (Figure 3.6). The other cytokines like MIP1α and IL18 also showed substantial but insignificant down regulation at these doses (please see Appendix Bi).

In order to understand the systemic effect of suppressive ODN A151, IL6 secreted from murine splenocytes after ex-vivo incubation for up to 24h in culture were monitored by ELISA (Figure 3.7). Our results revealed that suppressive ODN was able to reduce more than 65 % of the secreted IL6 (430±70 vs 135±55 ng/mL, for LPS and A151+LPS groups, respectively). When suppressive ODN were co-administered with LPS, cytokine mRNA levels failed to rise in vivo, and in ex-vivo spleen cells failed to increase their cytokine production (*$p<0.05$, and **$p<0.01$ Figure 3.6 & Figure 3.7). These effects were attributable to the activity of suppressive motifs, since control ODN did not reduce the cytokine production elicited by co-administered LPS (Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7).

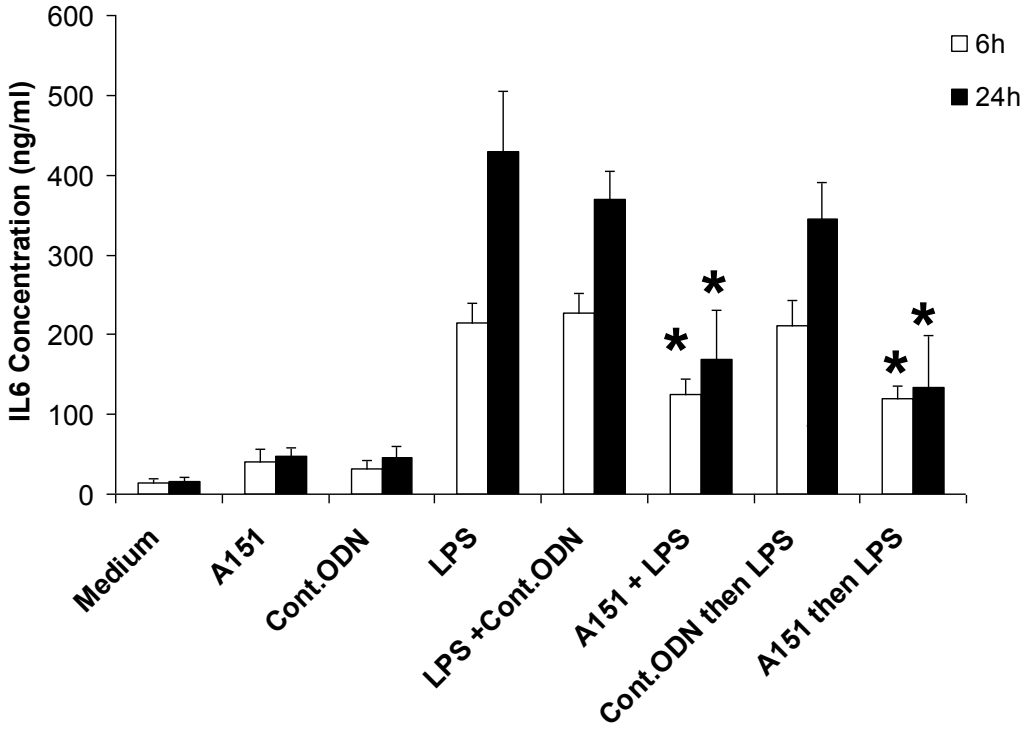


Figure 3.7. Suppressive ODN A151 administration significantly suppressed IL6 release from murine splenocytes. Mice were injected ip with 100 μg LPS and 250 μg suppressive or control ODN as indicated. Spleen cells were removed (post 24h Rx) and incubated for further 6-24 h and supernatants were collected for cytokine ELISA. IL6 was measured as an indicator of EIU response (* $p<0.05$, LPS treated vs LPS & A151 groups)

The $(TTAGGG)_4$ multimers inhibited LPS-dependent up-regulation of co-stimulatory and surface marker molecules on antigen presenting cells (CD40, CD86 and ICAM-1), IgM production by B cells, and NO release from peritoneal macrophages ($p< 0.01$, Figure 3.8). Furthermore co-administration of LPS (50 μg) with A151 ODN (250 μg) inhibited >65% of several immunoregulatory and inflammatory cytokines (i.e. IL6, IL10, and IL12, $p<0.001$, Figure 3.8). This reduction reached over 85% for IFNγ (176±29 vs 26±15 ng/mL, for LPS and A151+LPS groups, respectively).

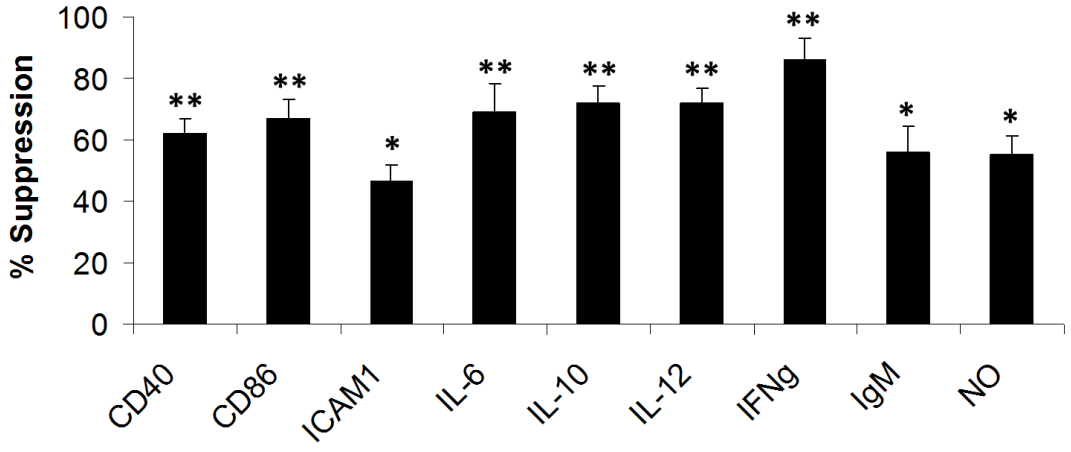


Figure 3.8. Inhibitory effect of suppressive ODN on LPS mediated immune activation. Levels of CD40, CD86, and ICAM-1 expression (MFI) were determined by FACS after 24 hr of in vivo LPS or LPS plus A151 (50 µg LPS and 250 µg ODN, respectively) injection. IL6, IL10, IL12, IFNγ and IgM levels in culture supernatants were determined by ELISA following 36h of ex vivo incubation. The Griess method was used to detect nitric oxide from isolated PEC cell supernatants after 24 hr post Rx.* $p<0.01$, **$p<0.001$

3.2 Immunosuppressive Effects of Restasis in EIU

Cyclosporin A (CsA) is an effective orally-administered immune suppressor, widely used during organ transplantation or for the control of several systemic and ocular autoimmune diseases [210, 211]. A CsA formulation suitable for topical application is the ophthalmic emulsion under the trade name of "Restasis", consisting of 0.05% cyclosporine. This drug was shown to increase the production of tear in patients where this process was presumed to be suppressed due to ocular inflammation associated with keratoconjunctivitis sicca (Product data sheet, Allergan, USA). In order to compare immunosuppressive effects of ODN A151 originated from mammalian telomeric DNA with a commercially used immunosuppressive drug with a non-biological origin Restasis™ was used in further experiments. Of note, initial optimization experiments with Restasis revealed that when EIU was established with 100µg LPS, there was no effect of topical Restasis administration (please see Appendix Bii). These studies, therefore, was conducted with 50µg or 20µg of LPS administration.

Administration of Restasis (detailed in Methods Section 2.2.2) prior to or following LPS injection reduced the severity of disease since the clinical scores of these animals ranged between 1.7 and 2.2 (Figure 3.9)

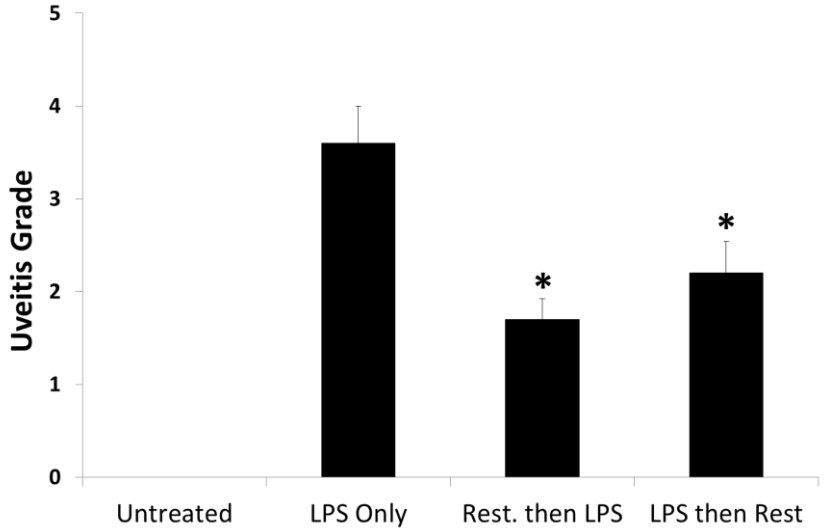


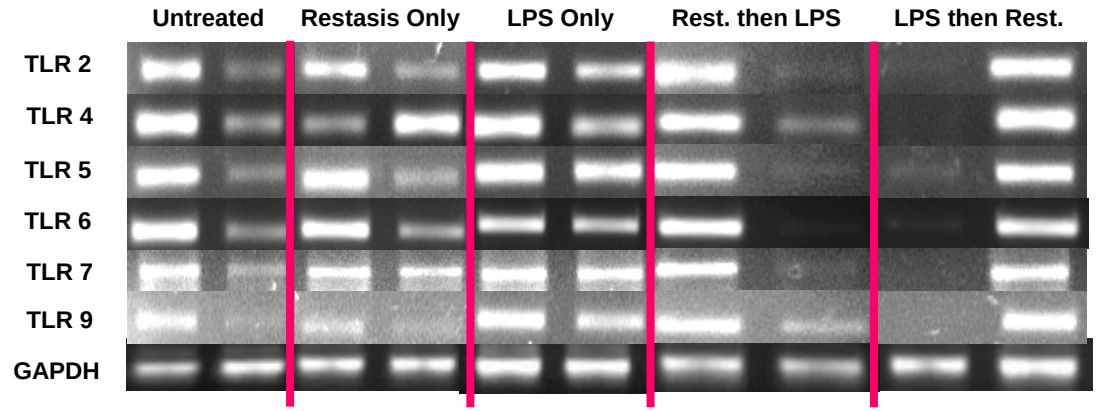
Figure 3.9. Effect of Restasis treatment on clinical EIU. Following ip LPS injection animals were treated with Restasis. Before animals were sacrificed clinical EIU evaluation was performed on both eyes and scored as described in methods section 2.1.3. * $p<0.05$ between LPS treated vs LPS & Restasis treated groups.

In order to evaluate the levels of expression of inflammatory mediators in LPS treated animals, rabbit and mouse eyes were removed 18 h post treatment and RNA was isolated from iris, vitreous and cornea for the rabbits and from the whole eye for mice. Following PCR amplification, changes in transcript levels were monitored from gel pictures.

EIU experiments in mice revealed that LPS treatment alone led to a significant upregulation of TLR2, 4,5,6,7 and 9 gene products (Figure 3.10) when compared to expression levels in untreated animals, suggesting the presence of an inflammatory response.

Although Restasis alone had no effect on TLR expression in the absence of LPS challenge, administration of Restasis prior to or post LPS treatment inhibited the LPS-induced TLR gene upregulation (Figure 3.10).

(A)



(B)

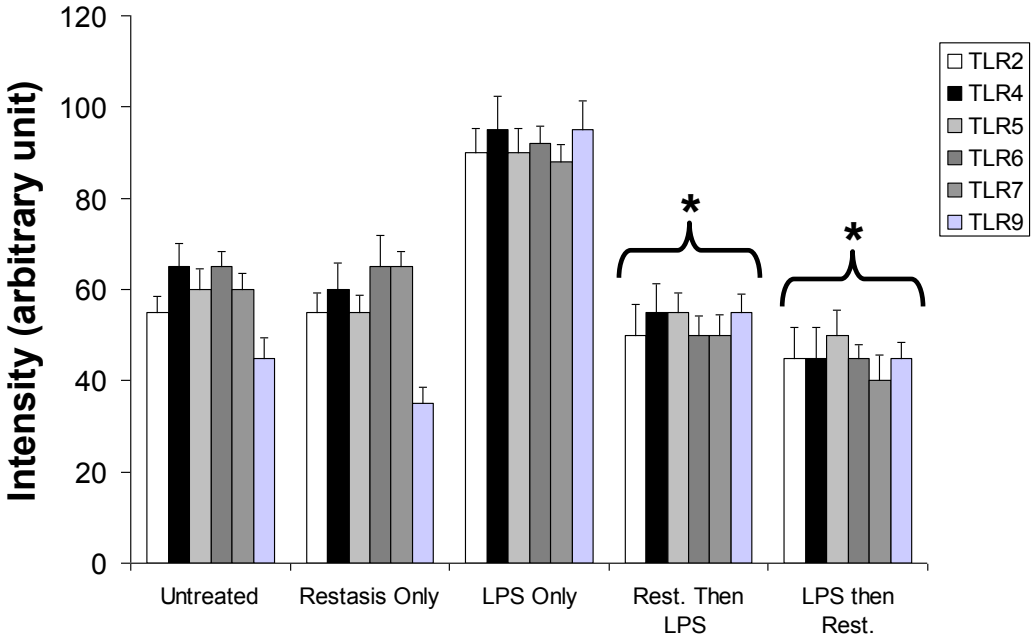
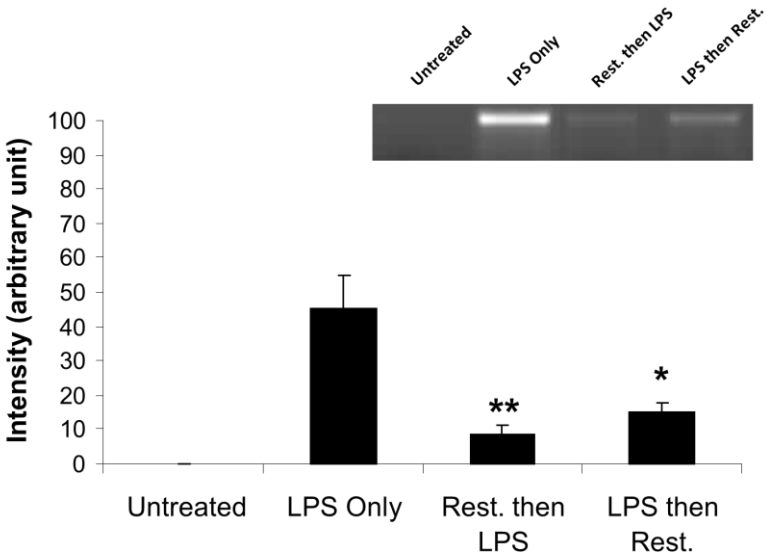


Figure 3.10. Restasis treatment pre- or post 20 µg LPS challenge (4 mouse/group) significantly downregulated several TLR mRNA expression levels from mice eyes 18 h after LPS challange (* =p<0.05, ** =p<0.01 between LPS Only and LPS & Restasis treated groups). A) Representative gel image of the TLR panel (2 mouse/group). B) Densitometric analyses of several TLR gene transcripts.

This suppression encouraged us to analyze the expression levels of other key proinflammatory chemokines. Similarly, administration of Restasis prior to or following LPS injection suppressed the LPS-induced MCP1 and IP10 production in mice (Figure 3.11).

A)MCP1



(B) IP10

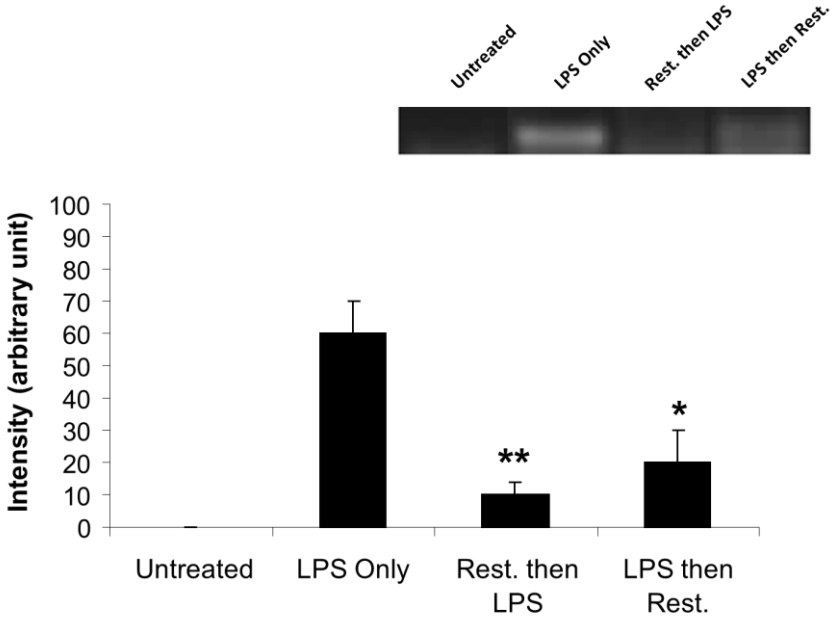


Figure 3.11. Restasis treatment pre- or post 20 µg LPS challenge (4 mouse/group) significantly downregulated A) MCP1, B) IP10 expression levels of mice eyes 18 h after LPS injection (* =p<0.05, ** =p<0.01 between LPS Only and Restasis treated groups)

Of note, treatment with Restasis was more effective when administered prior to LPS (85% and 75% inhibition for MCP1 and IP10, respectively) in contrast to post-treatment (70% and 65% inhibition for MCP1 and IP10, respectively).

Next, in order to investigate whether locally administered Restasis induced a systemic effect, IL6 secretion from murine spleen cells was studied following a 24 h ex-vivo incubation of splenocytes (Figure 3.12). As expected, LPS injection resulted in secretion of high levels of IL6 from mouse splenocytes. Restasis suppressed the LPS-induced IL6 production by 50% when administered prior to the endotoxin. Surprisingly, Restasis had a strong systemic effect and inhibited the IL6 production in the spleen (>90%) when administered after LPS (Figure 3.12). It is possible that the concentration of the drug in the circulation is still high in the post-treatment group whereas in the pre-treated animals some of it is cleared from the circulation as more time has elapsed since exposure.

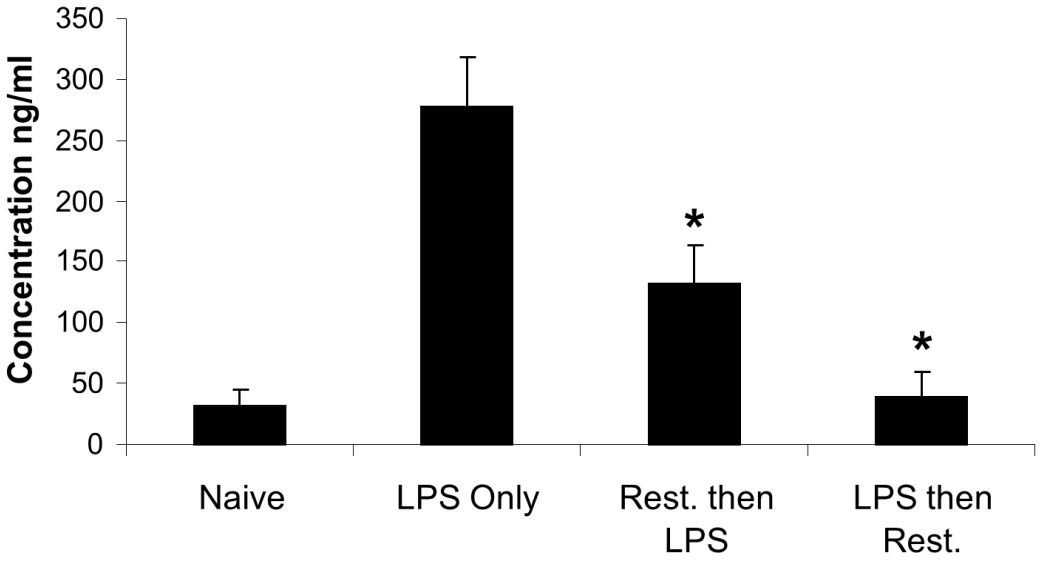
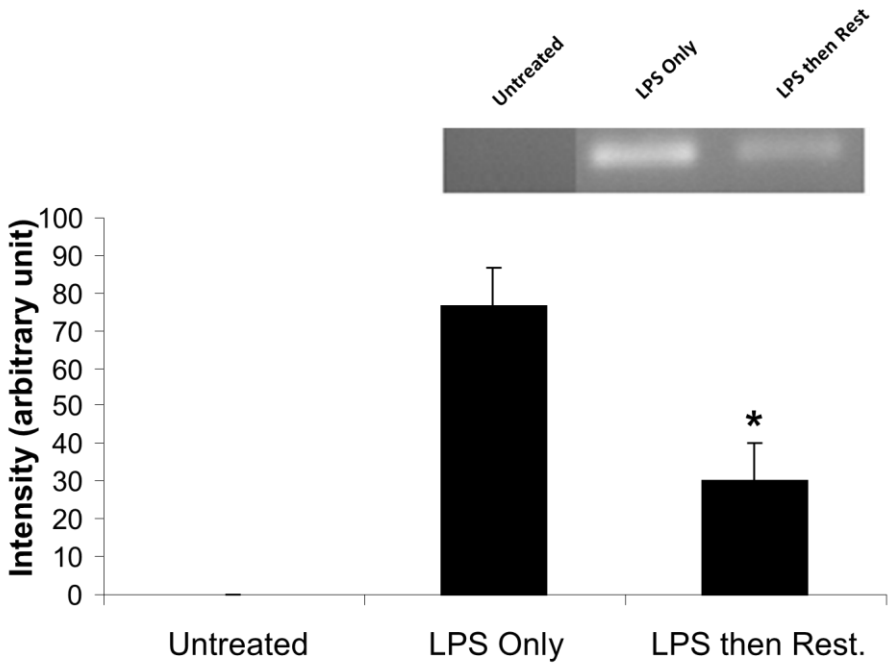


Figure 3.12. Restasis treatment pre- or post-20 μg LPS challenge (4 mouse/group) significantly diminished IL6 levels from murine splenocytes 24 h after LPS challenge (* =p<0.05 between LPS Only and Restasis treated groups).

In an attempt to simulate the clinical scenario and assess the suppressive potential of Restasis, another set of animals were treated with higher doses of LPS (50 and 100 μg/mouse). Similar responses were checked to assess the inhibitory effect of the drug. Since patients are admitted to hospital with uveitis application of Restasis before LPS challenge was abandoned at this stage. Induction of EIU by 100 μg LPS and subsequent treatment of animal eyes with 6x Restasis demonstrated no beneficial therapeutic value (please see Appendix Bii). However, when a 50 μg LPS dose was used to initiate EIU Restasis suppressed the MCP1 (Figure 3.13A, 55%) and IP10 (Figure 3.13B; 40%) expressions in the eyes.

(A) MCP1



(B) IP10

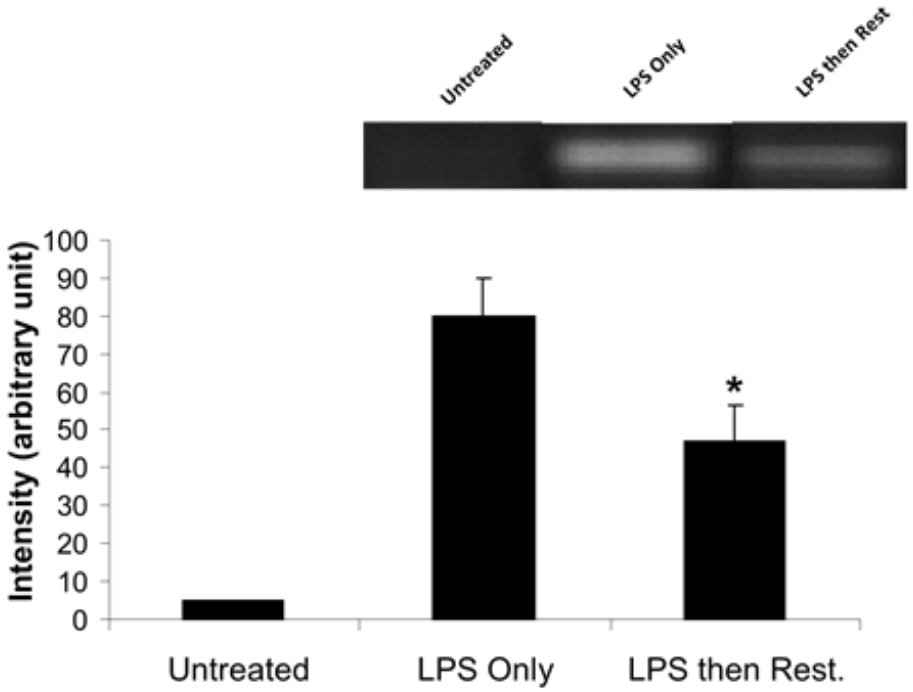
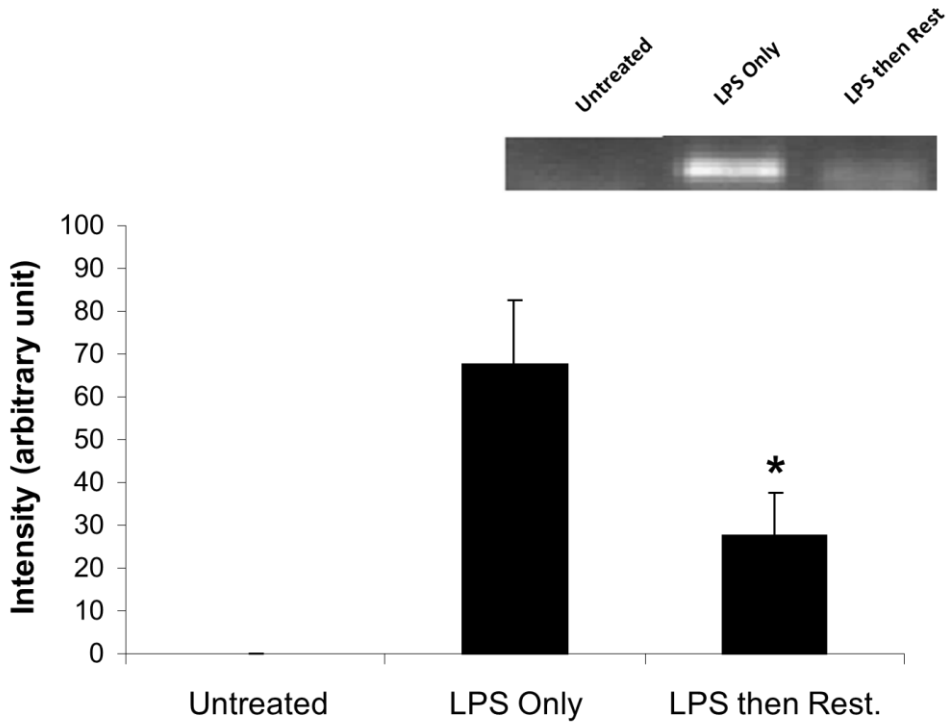


Figure 3.13. Restasis treatment post 50 µg (high dose) LPS challenge (4 mouse/group) significantly downregulated A) MCP1, B) IP10 expression levels of mice eyes (* =p<0.05)

Restasis showed no inhibitory effect on LPS-induced upregulation of IL-15, CXCL16, MIP1α, IL18, MIP3α, and MIP1β in murine eyes (please see Appendix Biii).

The EIU experiments in mice were limited with gene expression analyses from the whole eyes owing to the difficulty in sub-sectioning of the organ from this species. Therefore, in order to analyze whether Restasis was effective in controlling the inflammation occurring at separate anatomical locations in the eye, a rabbit EIU model was used where the uveitis was induced following intravitreal injection of 100 μg LPS. At the end of Restasis treatment rabbit eyes were removed and iris, cornea and vitreous sections were recovered for further analyses. The rabbit model of EIU revealed that Restasis administration downregulated IL1β and IL6 expressions in cornea and vitreous, respectively (Figure 3.14). PCR analyses of the iris section did not provide any significant downregulation of these genes (please see Appendix Biv).

(A) IL1β



(B) IL6

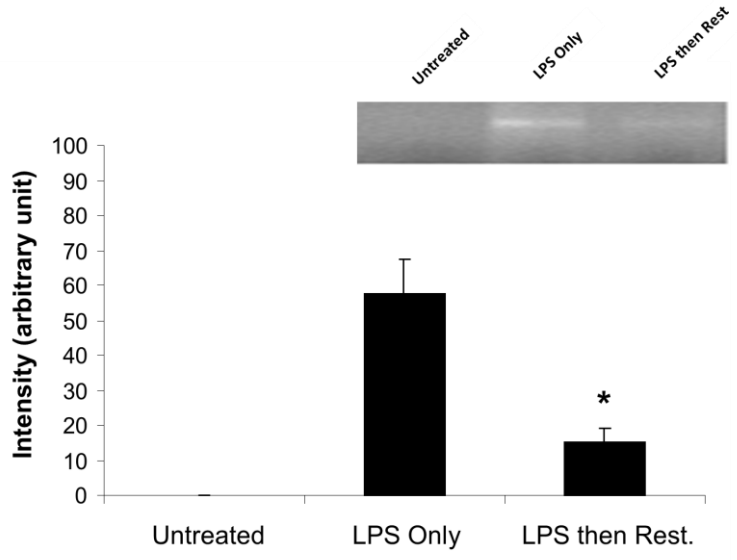


Figure 3.14. Restasis administration after LPS treatment downregulated IL1β and IL6 expressions of rabbit (5 rabbit/group) cornea and vitreous respectively. A) IL1β and B) IL6 mRNA levels. Representative agarose gel images. (* =p<0.05)

As mentioned in the beginning of this part, along with exploring immunosuppressive effects of Restasis in EIU, another aim of this study was to compare immunosuppressive properties of ODN A151 originated from mammalian telomeric DNA with a commercially used immunosuppressive drug from a non-biological origin. As summarized in Table 3.1, although Restasis showed some immunosuppressive properties, its effect in EIU was limited compared to A151 ODN.

Table 3.1. Comparative effects of biological and non-biological immunosuppressive agents to control established LPS induced EIU.

Name of Immunosuppressive Agent	**Origin**	**Administration Route**	**Clinical Score Reduction**	**Suppressed Immunemediators**
A151 ODN	Biological	Intraperitoneal inj. Intraocular inj.	~%80	IL1β, IL6, MIP3α,MIP1β, IP10, iNOS, CXCL16, CD40, CD86, ICAM1, IL10, IL12, IFNγ, IgM, NO
Restasis™	Non-biological	Dropped into eye	~%47	TLR2, TLR4, TLR5, TLR6, TLR7, TLR9, MCP1, IP10, IL6, IL1 β

A151 ODN Study: LPS 100μg/mouse
Restasis Study: LPS 20 μg/mouse

3.3 Use of CpG ODN as a Vaccine Adjuvant Against Foot And Mouth Disease

To determine the most potent CpG ODN sequence(s) as a potential adjuvant for the intended vaccine formulation, we synthesized more than 50 different CpG/Non CpG ODN sequences (i.e with their proper CpG flip controls) and tested them in vitro on spleen cells for their ability to induce pro-inflammatory and inflammatory cytokine secretions. Of note, three different backbone of these sequences were synthesized (PO/PS/MB) and tested on spleen cells Part of these CpG ODN sequences were either newly synthesized or they were previously reported as an effective adjuvant against various infections [16, 17, 87, 212-218] Table 2.6, summarizes these sequences and their general properties. Next, splenocytes from BALB/C mice were incubated with different concentrations (0.1-3.0 μM) of CpG ODNs and supernatants from 12-72 hours culture were collected to assess IL6, IL12p40, and IFNγ productions by ELISA. In Figure 3.15, data obtained only from 1μM ODN were presented (@ t=42h stimulation). When secretion profiles of the three cytokines were considered, ODN Seq 1555-PS ODN were determined to be the most potent inducers. Corresponding control ODNs did not result any significant cytokine production (please see Appendix Bv) indicating that activation of spleen cells were strictly CpG motif dependent. Immunization studies were conducted by using 1555-PS ODN.

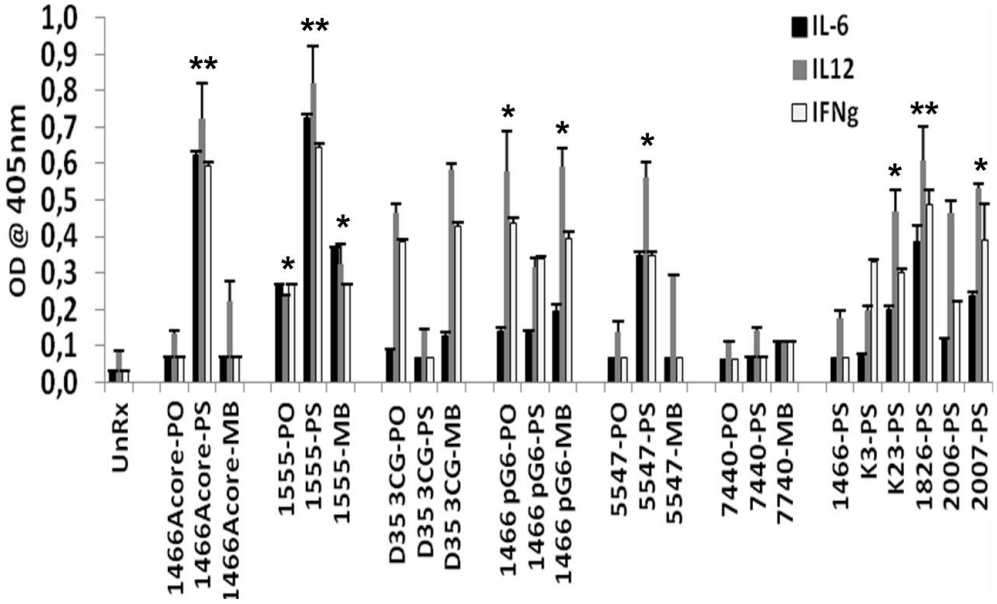
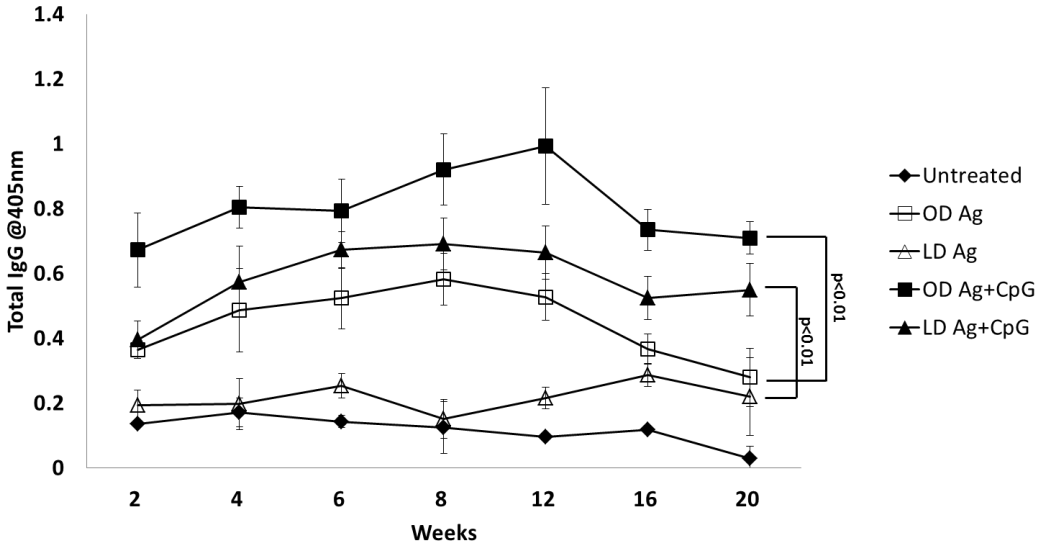


Figure 3.15. Immunostimulatory effects of different CpG ODNs. Balb/C splenocytes ($4X10^6$) were stimulated with phosphorothioate (PS), phosphodiester (PO), and mixed backbone (MB) modified K and D type CpG ODNs for 42hrs. IL6, IL12p40 and IFNγ production were assessed by ELISA. Data represents average ±SEM of three independent experiments carried out in triplicate repeats for each treatment groups (** =p<0.01 between CpG treated groups vs UnRx for all cytokines).

Here, in order to document the benefit of CpG ODN involvement in the formulations, 2 different doses of either antigen or monovalent vaccine was selected. As explained in detail in Materials and Methods section 2.3.4 animals injected with Optimum Dose (OD-3µg/animal) or Low Dose (LD-0.5 µg/animal) with Serotype-O Ag/Monovalent Vaccine or their CpG ODN including counterparts. After booster injection, mice were bled regularly for the next 5 months and anti Serotype-O Ag responses were studied. Formulations containing CpG ODN induced significantly higher levels of anti Serotype-O total IgG (Figure 3.16)

A)



B)

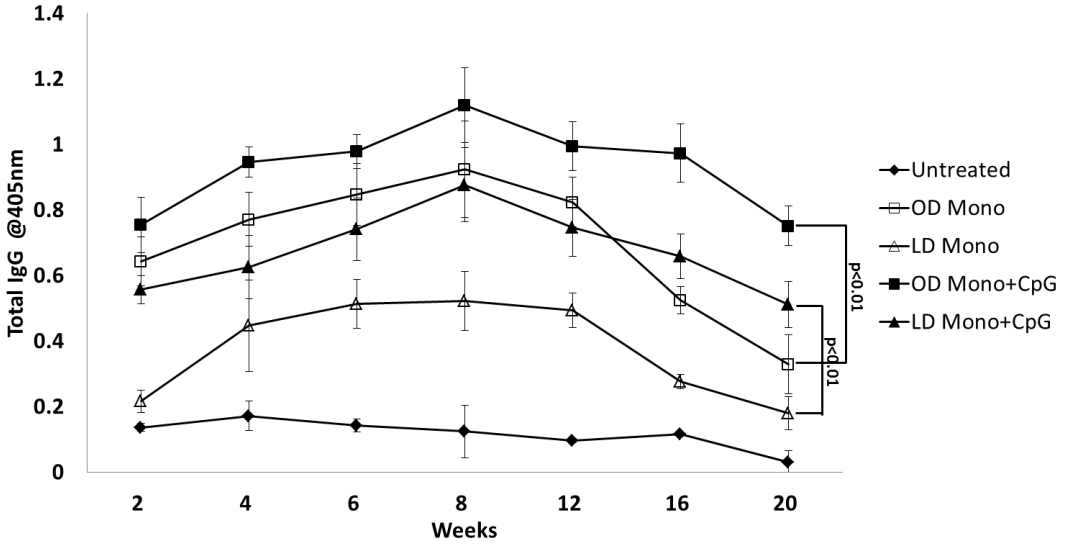
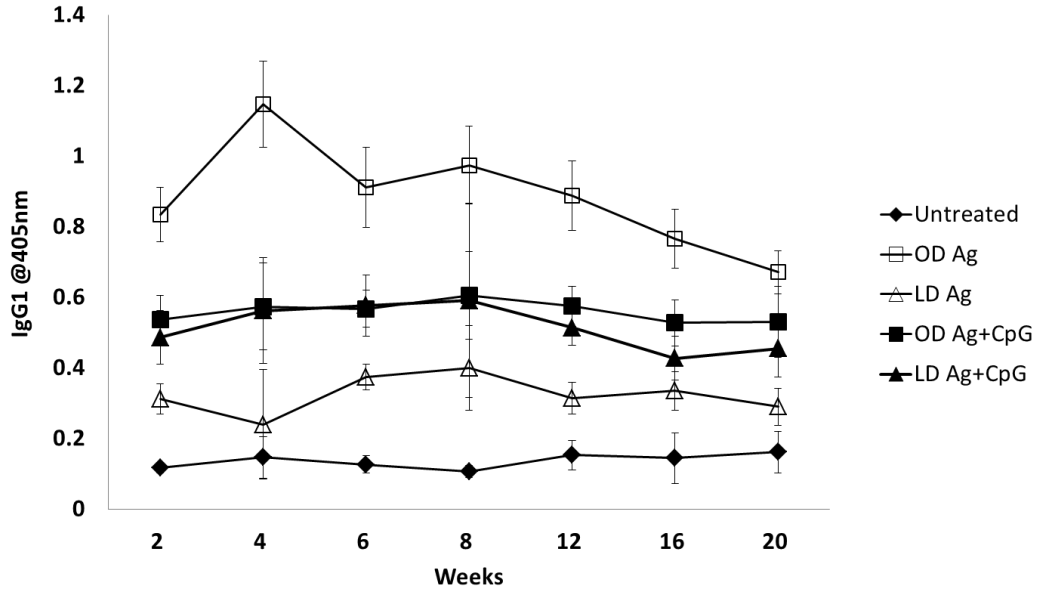


Figure 3.16. Anti Serotype-O total IgG responses of mice immunized twice either with A) Ser-O Ag or B) Monovalent Vaccine or their CpG ODN containing groups and followed for a period of 20 weeks. Anti serotype-O total IgG responses significantly enhanced with inclusion of CpG ODN within formulations. Serum total IgG levels were determined by ELISA. Plots are the OD readings of anti-FMDV total IgG responses at 900 titer (8 mice/group)

In order to evaluate the type of Th response, different IgG isotypes (IgG1 and IgG2a) were investigated. In BALB/c mice predominance of IgG1 or IgG2a indicates the

character of immune response either Th2 or Th1 biased respectively. Our results suggested that CpG ODN including formulations induced significantly higher levels of IgG2a (Figure 3.18) but not IgG1 (Figure 3.17).

A)



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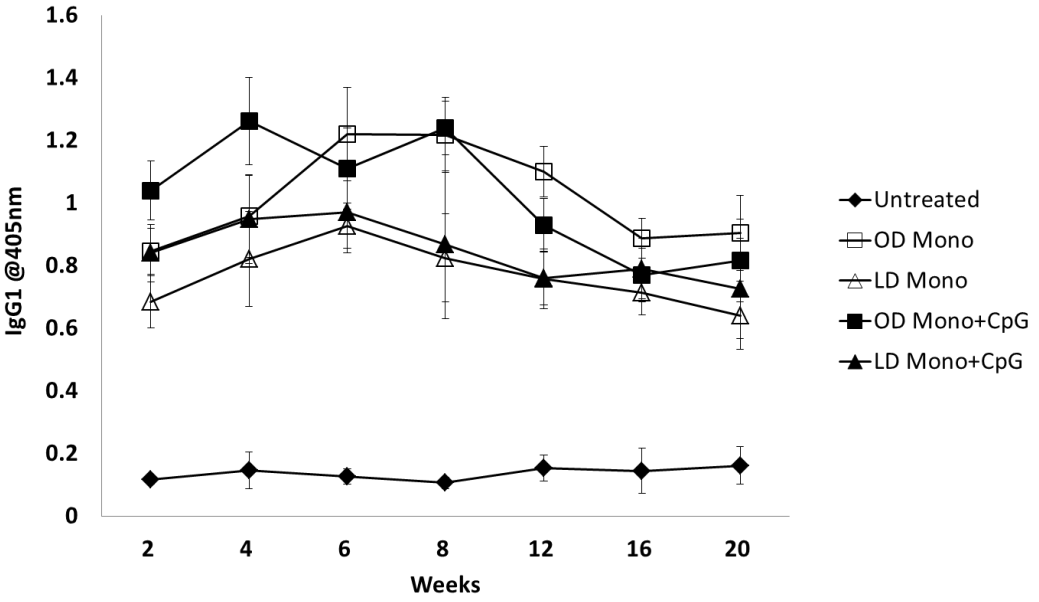

Figure 3.17. Anti Serotype-O IgG1 responses of immunized mice (for details please see Figure 3.16 legend). LD: Low Dose, OD: Optimum Dose.

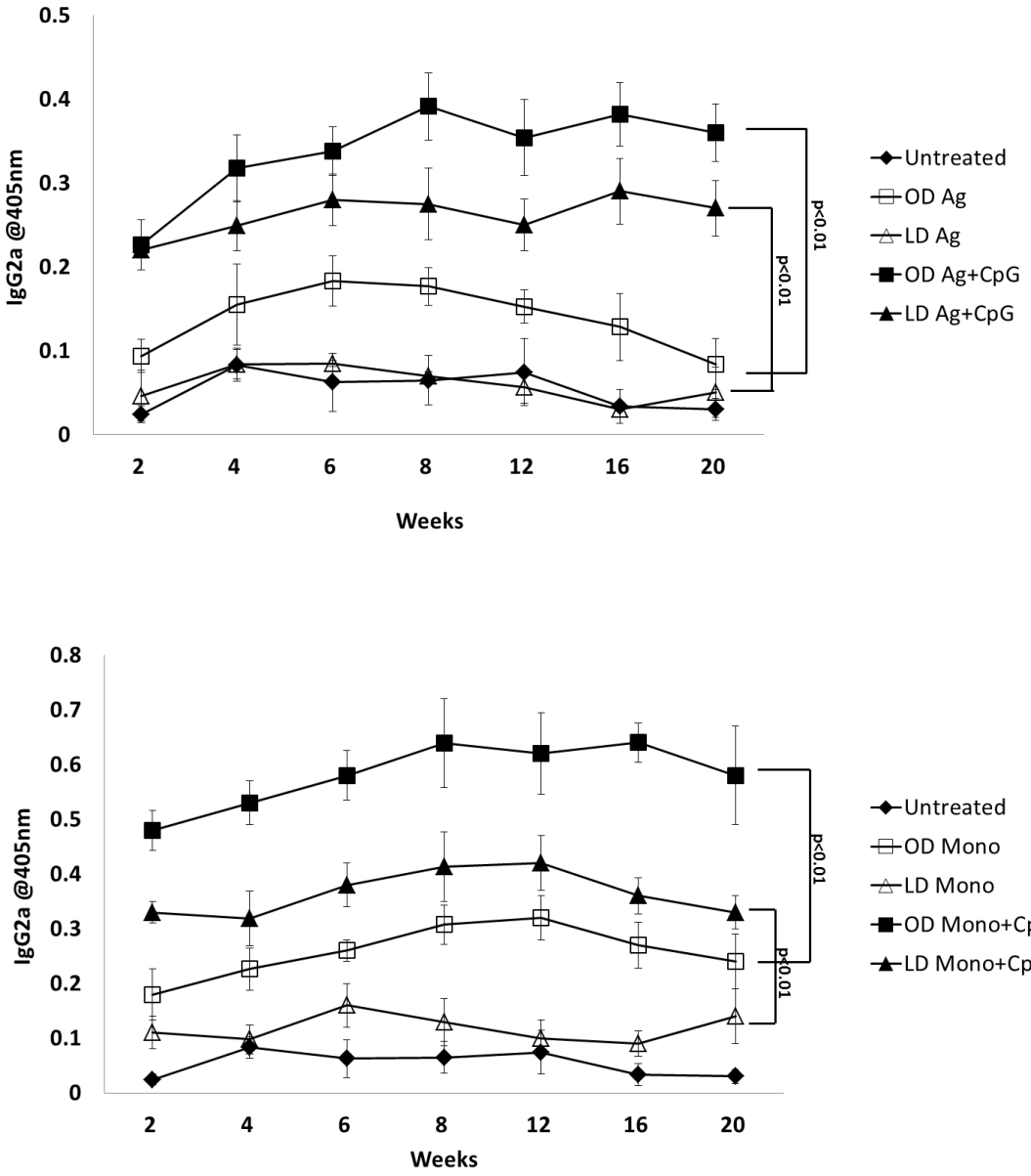

Figure 3.18. Anti Serotype-O IgG2a responses of immunized mice (for details please see Figure 3.16 legend). LD: Low Dose, OD: Optimum Dose.

When IgG2a/IgG1 ratios were compared among treatment groups, our results revealed that anti Serotype-O immunity induced via CpG ODN adjuvanted groups showed more Th1 biased immunity compared to non-CpG formulations (please see Appendix Bvi).

ELISA experiments indicated that anti Serotype-O antibody levels are higher in CpG including formulations, however it does not give any information that whether these antibodies are capable to neutralize viruses or not. For this reason, virus neutralization tests were conducted and neutralization capabilities of immune sera collected from different treatment groups studied.

As seen in Figure 3.19A, neither ODAg nor LDAg injected mice gave any detectable neutralization titers at 2 weeks. CpG ODN addition brought these neutralization titers to very high levels (480±450, 150±124, $p<0.05$). Similar responses were observed in monovalent vaccine treated groups. As seen in Figure 3.19A, while ODMono and LDMono injected mice gave average neutralization titers of 80±113 and 15±30 respectively these titers enhanced to 1040±872 and 210±234 with CpG ODN inclusion within formulations at 2 weeks post booster injection. . Similar trend were observed over the course of 5 months (Figure 3.19, Figure 3.20, Figure 3.21 and please see Appendix Bvii).

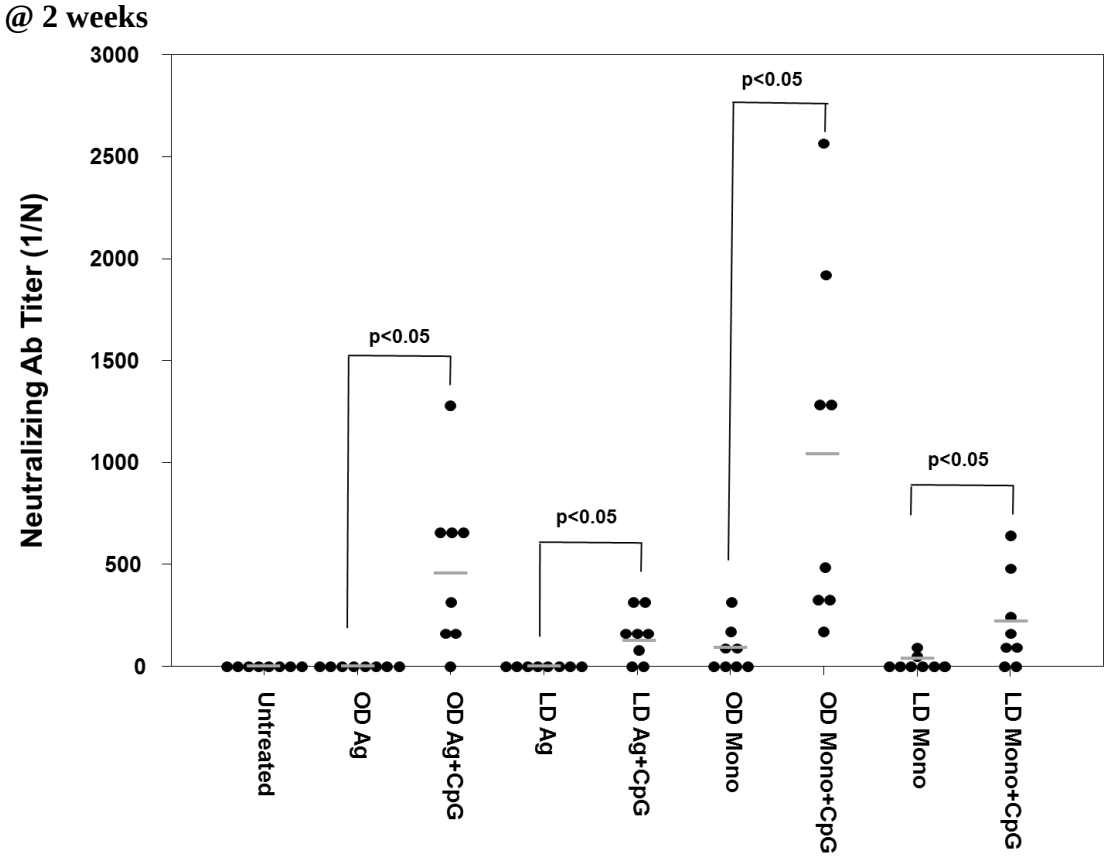

Figure 3.19. FMDV neutralizing Ab Titers of mice injected either with Optimum Dose (OD) and Low Dose (LD) Serotype O Ag/Monovalent Vaccine or their CpG ODN containing counterparts at 2 weeks post booster injection was assesed by Virus Neutralization Assay. Each dot represents the neutralizing Ab levels of one mouse ($p<0.01$, $p<0.05$)

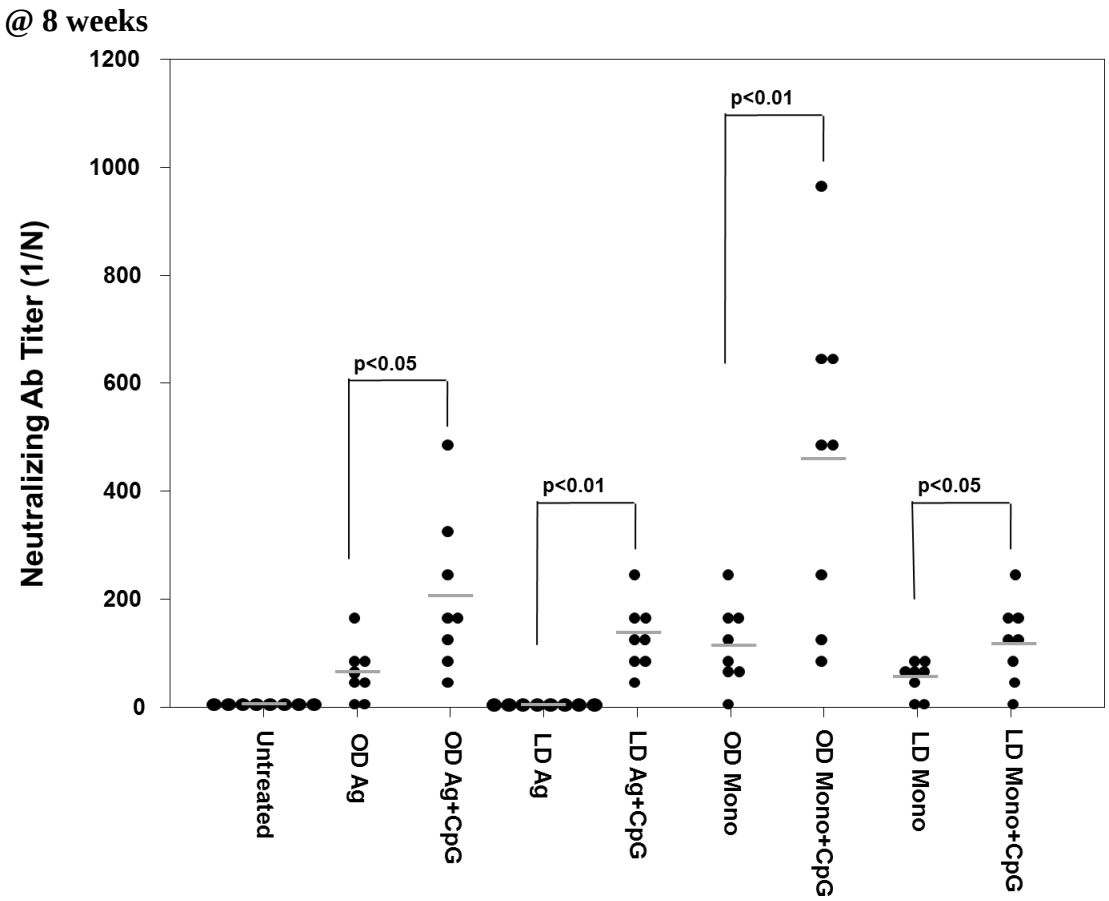

Figure 3.20. FMDV neutralizing Ab titers of immunized mice at post booster 8 weeks (for details please see Figure 3.19 legend). LD: Low Dose, OD: Optimum Dose.

@ 20 weeks

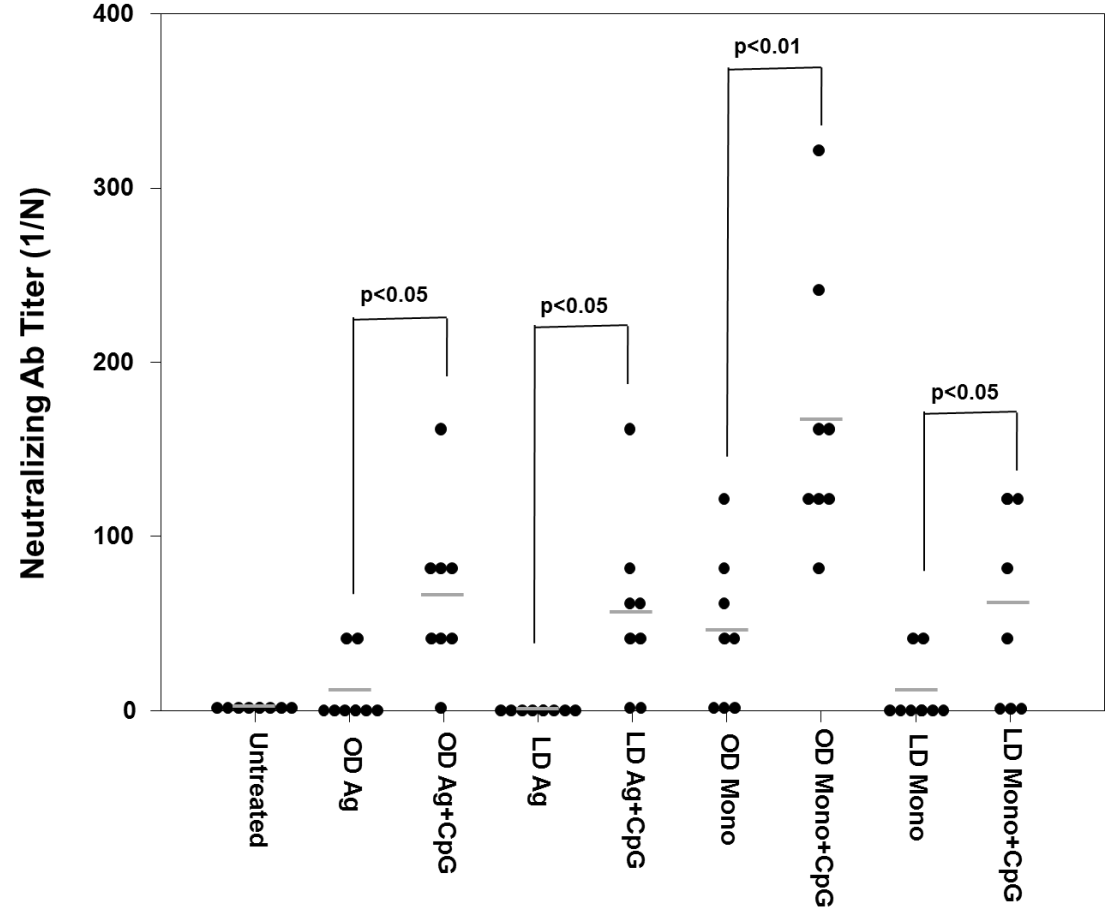

Figure 3.21. FMDV neutralizing Ab titers of immunized mice at post booster 20 weeks (for details please see Figure 3.19 legend). LD: Low Dose, OD: Optimum Dose.

The most fascinating outcome of virus neutralization assay can be observed when number of non-responsive and responsive animals were compared. As seen in Table 3.2 number of responsive mice according to the virus neutralization titers are superior in CpG ODN including formulations compared to Ag alone or Monovalent vaccine formulations.

Table 3.2. Number of immunized mice developed detectable virus neutralization titers for a duration of 20 weeks.

		Serotype-O Antigen				**Monovalent Vaccine**			
Weeks	Untreated	ODAg	**ODAg+CpG**	LDAg	**LDAg+CpG**	ODMono	**ODMono+CpG**	LDMono	**LDMono+CpG**
2 weeks	0/8	0/8	**7/8**	0/8	**7/8**	4/8	**8/8**	2/8	**6/8**
4 weeks	0/8	3/8	**8/8**	0/8	**8/8**	8/8	**8/8**	4/8	**8/8**
6 weeks	0/8	5/8	**8/8**	0/8	**8/8**	8/8	**8/8**	4/8	**8/8**
8 weeks	0/8	6/8	**8/8**	0/8	**8/8**	7/8	**8/8**	6/8	**7/8**
12 weeks	0/8	6/8	**8/8**	0/8	**7/8**	6/8	**8/8**	4/8	**7/8**
16 weeks	0/8	2/8	**8/8**	0/8	**7/8**	5/8	**8/8**	3/8	**6/8**
20 weeks	0/8	2/8	**7/8**	0/8	**6/8**	5/8	**8/8**	2/8	**5/8**

In conclusion, CpG ODN addition brought neutralization titers and percent of responder animals to a higher extent even in 6 fold less Serotype-o Ag or Monovalent vaccine injected animals.

Rapidity of the immune response generated by vaccine is another important parameter in vaccine development. In addition to the long lasting humoral immune responses, a good vaccine formulation should also confer rapid immune responses as well. It has been shown that IFNγ induced in vaccinated cattle is correlated with the animal`s ability to control the replication of FMD virus before humoral immune responses established [219]. In order to understand whether our vaccine formulation can induce a rapid cell mediated immunity sera collected from immunized animals only 24h after booster injection and IFNγ levels determined by ELISA. Our results revealed that mice injected with CpG ODN including formulations induced much more IFNγ ($p<0.05$) responses compared to non-CpG formulations either formulated with Serotype-O Ag or Monovalent vaccine (Figure 3.22).

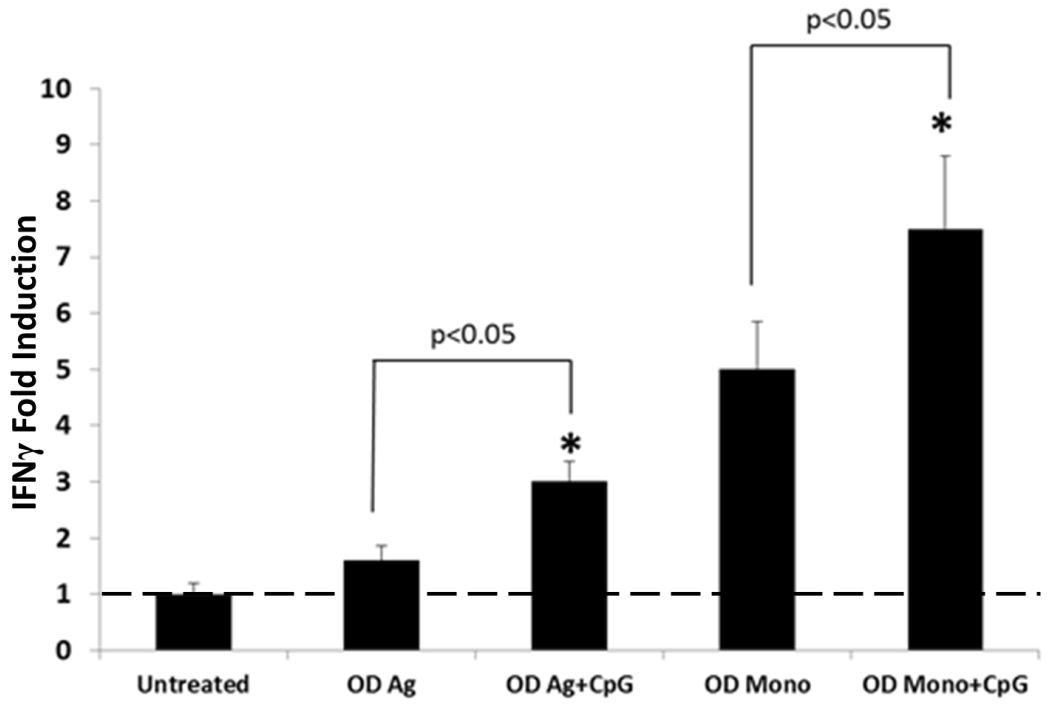

Figure 3.22. Rapid serum IFNγ response of mice immunized with indicated formulations mice 24h after booster injection IFNγ levels were determined by ELISA (Untreated mice gave 423±38 pg/ml).

3.4 Prophylactic Use of CpG ODNs in Broiler Chickens

As mentioned in Introduction section high mortality rates of newborn chickens cause serious economic losses for the industry in Turkey and abroad. In this part of this thesis, different classes of CpG ODNs were tested in order to develop effective prophylactic agents that can decrease newborn mortality rates of broiler chicken. For this purpose, effects of different classes and types of CpG ODNs were tested both in mice (Figure 3.15) and chicken[220]. As indicated by Tincer et al., especially D35 3CG MB and 1466 Acore PO sequences induce stronger immune stimulation and could mediate Th-1 biased immune response on both B cells (DT40 cell line) and chicken splenocytes.

After determination of most potent CpG ODN types and sequences these ODNs were tested in broiler chickens *in-vivo*. Briefly, 1 day old broiler chickens (1000

chicken/group) were subcutaneously injected with 3 doses of D35 3CG MB and 1466 Acore PO in 200µl Hanks Buffer or Hanks Buffer alone (detailed in Methods Section) and mortality rates, live body weights, feed conversion ratios (FCR) and European Efficiency of Productivity Factor (EPEF) values were controlled.

Our results indicated that chicken injected with both 1466 Acore PO ODN or D35 3CG MB ODN exhibited lower mortality percentages at the end of 41 days (Figure 3.23). As seen on Figure 3.23, while all 3 doses of 1466 Acore PO ODN were able to reduce mortality, only 4 µg dose of D35 3CG MB was able to reduce mortality significantly.

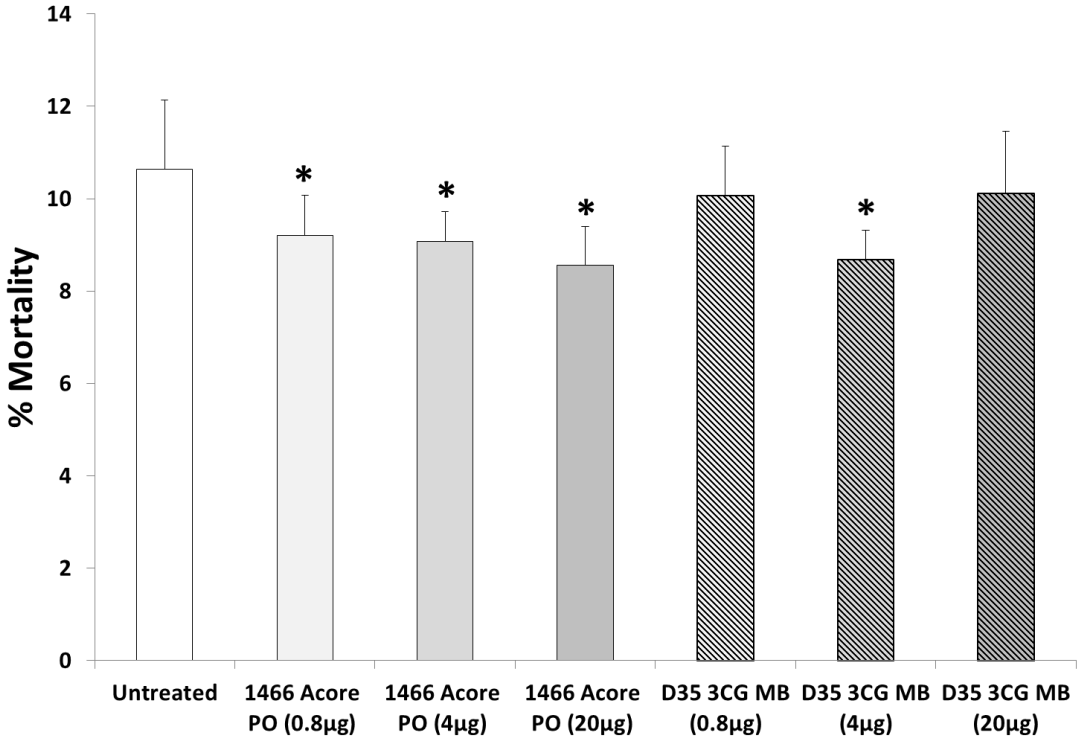

Figure 3.23. Mortality percentages of chickens at the end of 41 days. 1 day old chicken injected s.c with 3 doses (0.8µg, 4µg, 20µg) of 1466 Acore PO ODN or D35 3CG MB ODN in 200 µl Hanks buffer or Hanks buffer alone (Untreated). ($p<0.05$) (N=1000 chicken/group, Untreated: 6000/group).

In broiler chicken industry, animals are sent to slaughter house at the end of 41 days. During this period, mass gained by animals and ability of the animal to convert feed mass to body mass are important economical parameters. For this reason, effect of CpG ODN injection on live body weights, FCR and EPEF values were studied.

Our results revealed that chickens injected with D35 3CG MB ODN (4 μg and 20 μg doses) significantly gained more mass in 41 days compared to untreated chickens (Figure 3.24).

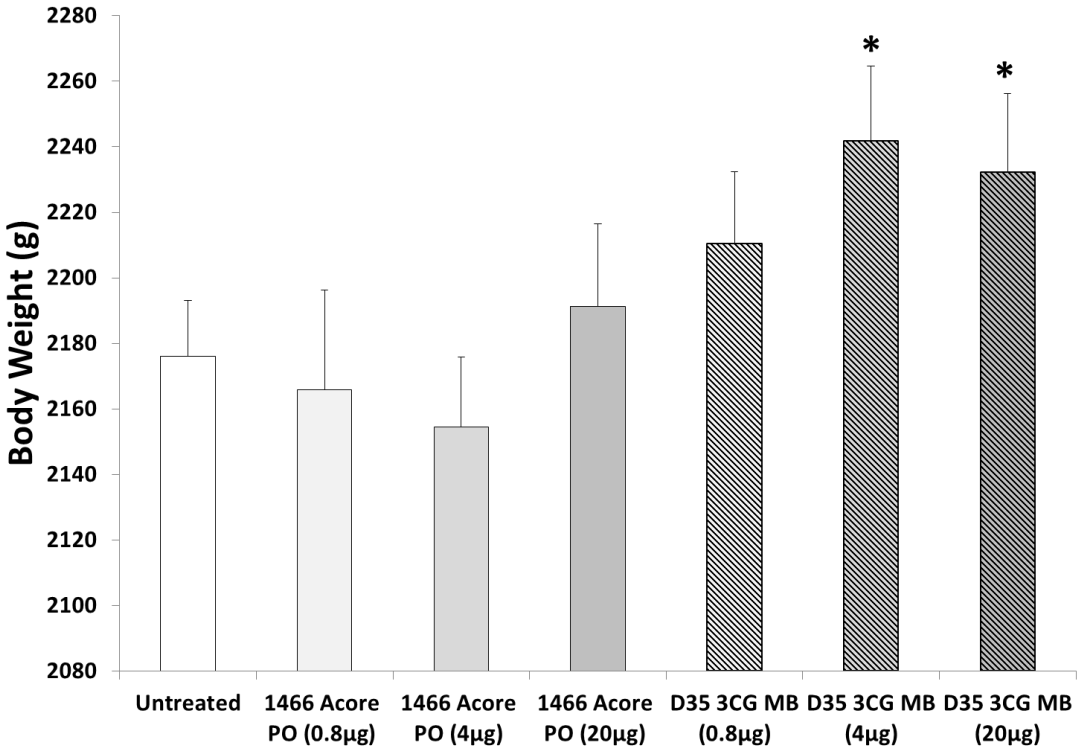

Figure 3.24 Body weights of chickens at the end of 41 days (for details please see Figure 3.23).

Since gained live body weight within a specific time period is directly related to the amount of feed mass, FCR values were also calculated in order to understand the effect of CpG ODNs in nutrition. Briefly, FCR is a measure of animals efficiency to convert feed mass to body mass. Animals that have lower FCR are considered as efficient users of the feed.

As seen in Figure 3.25, chicken injected with 0.8µg and 20µg 1466 Acore PO ODN exhibited significantly lower FCR values.

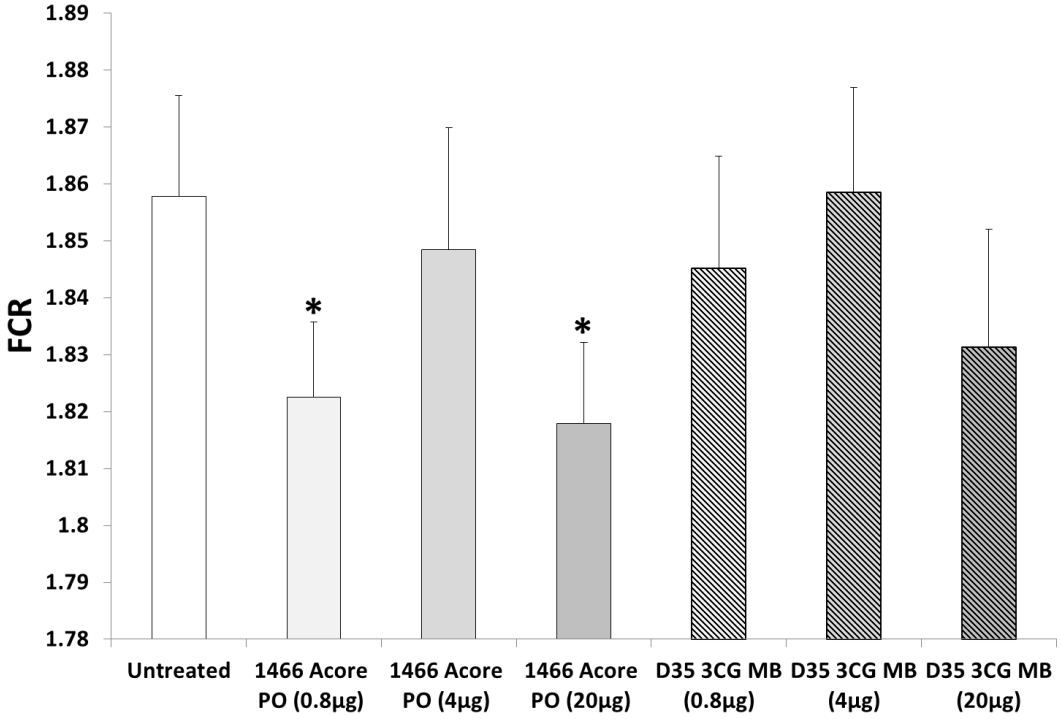

Figure 3.25. FCRs (Feed conversion ratio) of chickens at the end of 41 days. (for details please see Figure 3.23).

Another parameter that indicates growth performance is European Efficiency of Productivity Factor (EPEF) value. As seen in Figure 3.26, 20µg 1466 Acore PO ODN and 4 µg and 20 µg D35 3CG MB ODN injection significantly enhanced EPEF values.

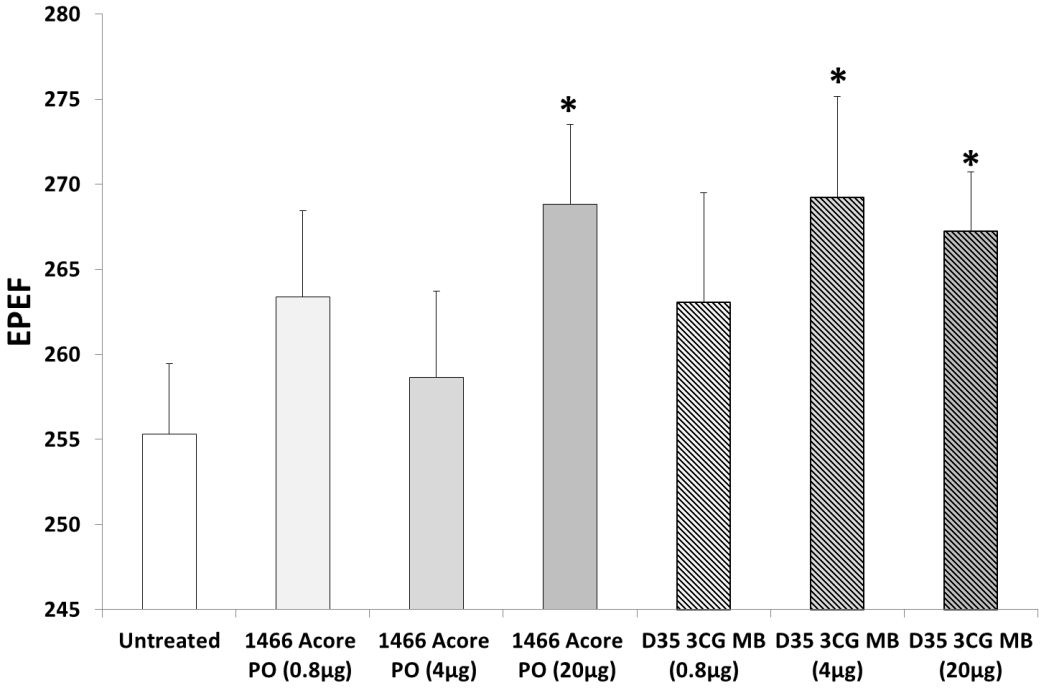

Figure 3.26. EPEF (European Efficiency of Productivity Factor) of chickens at the end of 41 days. (for details please see Figure 3.23).

Although mortality, body weight, FCR and EPEF values are not perfectly correlated our results collectively representing the benefits of CpG ODNs on growth performance.

Chapter 4

Discussion

4.1 Immunotherapeutic Use of Suppressive ODN in Endotoxin Induced Uveitis

In this study, we examined the effect of synthetic telomeric repeat units (suppressive A151 ODN) localized at the end of mammalian chromosomes on EIU, which is an established animal model of acute ocular inflammation in both mouse and rabbit models. The results indicated that suppressive ODN was able to down-regulate the expression and protein levels of several proinflammatory and immunoregulatory cytokines/chemokines at local and systemic levels when administrated (i) prior to, (ii) simultaneously with, or (iii) even after LPS challenge (Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8).

Compared with local injection of (intraocular) LPS and/or LPS- and control ODN-treated rabbits, suppressive ODN-administrated animals exhibited reduced levels of IL1β and IL6 expression in the iris and cornea, respectively. In the mouse model, the results revealed that pretreatment with 250 μg of suppressive ODN reduced the expression of IP10, iNOS, MIP1α, IL-18, MIP3 α, CXCL16, and MIP1β in 100-μg

LPS injected mice. The suppressive action of this class of ODN was not only on the mRNA levels of several Th1-type cytokines and chemokines but also on the secreted protein level. ELISA experiments showed that suppressive ODN pre- and post-treatments significantly diminished IL6 secretion at 6 and 24 h; simultaneous administration of suppressive ODN also reduced IL6 production. Several studies indicated that suppressing proinflammatory cytokines, including IL6, TNFα, MCP1, and iNOS, prevents the development of EIU [160, 163-165]. Here, we have shown that either local (intraocular) or systemic (intraperitoneal) administration of suppressive A151 ODN can significantly reduce several proinflammatory cytokines and chemokines even 2 h after in vivo LPS challenge.

Corticosteroids and chemotherapeutic agents are currently in use in uveitis therapy.[221] However, long-term treatment with these drugs may have grave side effects such as increased intraocular pressure [222] and cytotoxicity [223] and thus limit their use [159] [207, 221]. Therefore, a new therapeutic strategy is urgently needed [224, 225]. The mechanism of action of this novel ODN-based immunosuppressive drug candidate is currently unknown. Previous studies revealed that suppressive ODN can inhibit immune response by blocking the stimulatory effects of CpG motifs [79, 136]. It also has been shown by Shirota et al. [31] that suppressive A151 ODN can also protect mice from lethal endotoxic shock that is induced by LPS. It has been shown that suppressive ODN can also inhibit several signal transduction cascades related to the production of Th1 cytokines such asIFNγ and IL12 by binding and inhibiting the phosphorylation of STAT1 (signal transducer and activator of transcription 1) and STAT4 proteins [31, 121]. Our study has demonstrated that suppressive ODN can block immune responses mediated by endotoxin in the eye (an immune privileged site), an established animal model of acute ocular inflammation. Recently, Fujimoto et al. [129] reported that suppressive A151 ODN can inhibit ocular inflammation in two murine models, IRBP (interphotoreceptor retinoid-binding protein)-induced experimental autoimmune uveitis and adoptively transferred ocular inflammation. These forms are antigen-driven and, compared with LPS, are significantly less aggressive forms of experimental uveitis models. The control of LPS-mediated EIU at both the local and

systemic levels has not been studied by others and increases the breadth of the suppressive ODN-mediated therapy for the eye.

Collectively, these observations support the provocative possibility that the evolutionary expansion of TTAGGG repeats in telomeres, in addition to known properties such as, protecting genomic DNA from degradation, and chromosome capping [226, 227] may also be linked to their ability to down-regulate sustained/pathologic microbe-associated molecular pattern-induced immunity. In conclusion, we have provided evidence that suppressive A151 ODN is able to significantly reduce the ocular inflammatory responses in both rabbit and murine EIU models.

4.2 Immunosuppressive Effects of Restasis in EIU

To our knowledge, the present study demonstrates for the first time that Restasis suppresses the expression and protein levels of several Th1- biased proinflammatory cytokines and chemokines that were upregulated as a result of EIU. Moreover, the topically administered drug was effective both locally and systemically. Compared to rabbits and mice that received LPS only, animals that were treated with Restasis exhibited reduced expression levels of IP10, MCP1, IL6 and IL1β in their eyes (Figure 3.11, Figure 3.12, Figure 3.13, Figure 3.14). This reduction in proinflammatory cytokine and chemokine expression could stem from the downregulatory effect of Restasis on TLRs message. Our results indicated that either pre or post- treatment with Restasis was able to downregulate the expressions of several TLRs such as TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 in the mouse eye (Figure 3.10). Therefore, the decrease in IL6 levels in Restasis treated animals (Figure 3.12) could be due to the downregulation of TLR4, the receptor required for LPS signaling. In this respect, our results are consistent with the literature where oral administration of cyclosporines were proven to be beneficial in the treatment of several systemic and ocular autoimmune diseases as well as the prevention of transplant rejection [210, 211]. Along with those studies data demonstrated that CsA

reduced the production of the proinflammatory cytokines IL1β, TNFα, IL6 and IL8 from U937 human monocyte-like cells in vitro [172]. Recently, Chi et al., reported that CsA suppressed IL17 production from Behcet`s patients` PBMC [173]. Moreover, several other studies report that systemic cyclosporine treatment can reduce the severity of uveitis and can downregulate the expression of proinflammatory cytokines such as IL12, IL18 and TNFα [174-176]. Of interest, a recent study showed that topical treatment with 1% cyclosporine A reduced the severity of subepithelial corneal infiltrates [228].

The present study established that topical Restasis treatment downregulated both the systemic and the local proinflammatory response. The finding that topically administered Restasis had a systemic effect (Figure 3.12) was an unexpected one and can be explained by an enhanced diffusion of the drug into circulation due to the breached BRB. Several studies indicated that suppressing proinflammatory cytokines including IL6, TNFα, MCP1 and inducible nitric oxide synthase (iNOS) prevents the development of EIU [160, 163, 164, 229]. Current practice in treatment of uveitis involves systemic administration of corticosteroids and chemotherapeutic agents [221, 230]. However, long-term treatment with these agents may have serious side effects such as increased intraocular pressure [222] or cytotoxicity [223] , thereby limiting their use [159, 221, 222, 230]. The study on patients with adenoviral keratoconjunctivitis demonstrates that topically administered CsA has fewer side effects and offers a safe and effective alternative [228]. While our work supports this observation, it extends the potential of topical CsA administration to treatment of intraocular diseases such as uveitis.

In conclusion, present work extended the breadth of the immunoregulatory potential of Restasis and may offer a safe alternative in the treatment of ocular inflammatory diseases in the clinic. Collectively, the present findings suggest that Restasis might constitute a relevant therapeutic alternative for the treatment of uveitis.

4.3 Use of CpG ODN as a Vaccine Adjuvant Against Foot And Mouth Disease

Major problem associated with FMDV vaccine is that, there is very limited amount of antigen for the production of sufficient amounts of commercial vaccine doses. Moreover, due to inability of providing long lasting immunity and the lack of inducing rapid immune responses in case of a rapid virus spread, frequent re-vaccinations are required [179, 185-188].

Protection against FMDV correlates with the high levels of neutralizing antibodies in the serum. Viruses are neutralized via neutralizing antibodies in serum which is proceeded by the clearance of the opsonized virus complexes by phagocytic cells [182]. Although it is established that FMD infection elicits an appreciable amount of humoral response some studies indicated that despite antibody levels, early protection could also be maintained by initiating sufficient amount of innate immune activity [185]. In this respect DCs play the most important role, since they express a panel of TLRs designed to recognize pathogenic components. Upon recognition of pathogenic molecular patterns a number of co-stimulatory molecules, proinflammatory cytokines and chemokines are secreted as well as the enhancement of antigen presentation function of these cells. All these processes licences DCs to maturate and help to elicit FMDV specific adaptive immunity [185, 231, 232]. Therefore, aiming to induce innate immune responses is a desirable approach to overcome undesirable problems associated with the conventional FMDV specific vaccine since innate immune system induces a rapid immune response against invaders and alerts adaptive immunity.

To date several attempts were made in the context of inducing innate immunity to develop more potent FMDV vaccines. For instance, Su et al., indicated that use of IL6 and TNFα as molecular adjuvants can enhance both innate and adaptive immune responses against FMDV [185]. In another study, Barnett et al., denoted that protection against FMDV in pigs is correlated with high serum IL12 levels [233].

Ren et al., demonstrated that CpG ODN RW03 in combination with montanide ISA 206 can enhance immune responses against a recombinant FMDV vaccine named A7 [186]. Most recently, Guo et al., reported that use of CpG rich plasmids can enhance the efficacy of traditional FMD vaccine [234].

In this study, we investigated the effects of 1555 PS CpG ODN as an adjuvant on both Ag alone or a commercially relevant form of FMD vaccine which includes nanoemulsion of montanide ISA 206 comlexed serotype-O-Ag. Previously, it has been shown that combination of CpG ODN with montanide ISA 206 enhanced protective efficacy of vaccine against malaria.[216] Since challenge studies in mice are not well established, the potency of FMDV vaccine is tested in vaccinated animals indirectly according to the standard protocol of OIE. The well established test for assessing antibodies to FMD vaccines is virus neutralization test (VNT). A good correlation was reported between VNT and protection [235, 236]. Ab tests revealed that formulations with CpG ODN induced significantly higher anti-FMDV Ab titers compared to non-CpG formulations (Figure 3.16 and Figure 3.18). Our results implied that reducing monovalent vaccine (or serotype O-Ag alone) amount by 6 fold and including a potent CpG ODN sequences on to these formulations was much effective than high Monovalent vaccine or Ag alone doses (Figure 3.16 and Figure 3.18).

Several studies demonstrated that CpG ODN mediate a Th1 biased immune response. In this study, we planned to study the induction levels of IgG subtypes. As expected, our results showed that formulations with CpG ODNs induced a strong Th1 type immune response evidenced by increased IgG2a/IgG1 ratios compared to non-CpG ones (Please see appendix Bvi). IFNγ is one of the major Th1 type cytokine which plays critical roles in the improvement of CD8+ T cell responses. It has been reported that IFNγ induced in vaccinated cattle is correlated with the animals ability to control the replication of FMD virus [219]. Since mice injected with CpG ODNs induced such high IgG2a/IgG1 ratios, we investigated whether CpG ODN including formulations can also induce cell-mediated immunity. As an indicator of cellular immune responses serum IFNγ levels of mice have been measured by ELISA. Our

results revealed that 24h after injection CpG containing formulations induced 1,5 to 2 fold more IFNγ in serum which indicates the contribution of cell-mediated immune response (Figure 3.22). This type of rapid immune response also shows the emergency vaccine character of CpG including formulations.

As a hallmark of FMD vaccine's efficacy, virus neutralizing Ab titers of the vaccinated mice was studied. Consistent with the Ab results, virus neutralization assays suggested that mice injected with CpG including formulations have more and persisting titers in their sera (Figure 3.19 and please see appendix Bvii).

Attaining high anti-FMDV immunity and maintaining this response for longer periods is another critical feature of an ideal FMD vaccine. Current practice in the field is to protect animals from FMDV infections via frequent re-vaccinations that would provide protective Ab levels[187]. Our mice study results not only revealed that CpG inclusion in the formulations induced higher anti-FMDV total IgG and IgG2a responses even 5 months after booster injection (Figure 3.16 and Figure 3.18). but also virus neutralization studies supported that higher levels of circulating neutralizing Abs were maintained in their sera after 5 months (Figure 3.19 and please see appendix Bvii).

In summary, data presented in this study demonstrated that CpG ODNs can be used either in nanoemulsion form together with montanide ISA 206 or even as a standalone mixture for enhancing both humoral and cell mediated immune responses in mice. In the light of these findings, studies in cloven-hoofed animals are underway to examine the vaccine efficacy, protection performance as well as anti-FMD-Ab persistence of the candidate vaccine.

4.4 Prophylactic Use of CpG ODNs in Broiler Chickens

Mortality during the first week of life in broiler chickens is one of the most important problems for broiler industry. Use of antibiotics via *in ovo* injection or adding into drinking water or food is becoming unacceptable due to several safety and human health issues. For instance, antibiotic use can been linked to the emergence of drug resistant bacteria strains and also possible drug residues in the meat products. Therefore, animal industry is seeking for novel therapeutics or prophylactic agents that can be used instead of antibiotics.

As detailed in Introduction section, it is known that CpG ODNs activate innate immune responses to protect host against a variety of pathogens. It has been shown in many scientific publications that CpG can protect mice against bacterial, viral and protozoal infections (Krieg 2006). In most of these articles it is indicated that pre-treatment with CpG ODNs is much more effective than post-treatment in case of protection (Krieg 2006). Protection time with CpG ODNs varies between a few days to two weeks, but that can be extended to months by repeated treatments (Klinman 1999).

There are several studies indicating the role and ability of CpG ODNs to stimulate innate immune responses and protection against infections in chicken. It has been shown that CpG pre-treatment improved survival rates of chickens upon challenge with E.coli (Gomis 2004). In this study 2 day old birds were subcutaneously or intramuscularly injected with 10-50 μg of CpG ODN and challenged with E.coli with the same route 3 days after CpG injection. Results were encouraging because nearly 90% of the animals in the control group were died. In contrast, CpG pre-treated groups had significantly higher survival rates (Gomis 2004). In addition, it has been reported that CpG ODNs are effective in prevention of Salmonella infections in chicken. He et al., demonstrated that intraperitoneal 50 μg pre-CpG injection protected chicken from S.enteriditis challenge (He 2005). In another study, same group showed that CpG injection 24h prior to oral S.enteridis challenge prevented

the colonization of bacteria in liver and spleen (He 2007). Furthermore, effect of CpG ODNs on S.typhimurium infection has been studied. Taghavi et al., reported that 50 µg intramuscular CpG ODN injection in newborn chickens was able to reduce mortality and clinical symptoms associated with S.typhimurium infection upon bacterial challenge. (Taghavi 2008).

While there are a plethora of studies indicating the effect of CpG ODNs in viral infection in mice, studies demonstrating the antiviral effects in chicken are only a few. One of those studies was conducted against infectious bronchitis virus (IBV) infection. In this study, eighteen day old chicken embryos were *in-ovo* injected with CpG ODN and 24h later challenged with IBV virus. Results indicated that chickens injected with CpG ODN were significantly inhibited viral replication compared to controls (Dar 2009). With respect to results obtained in IBV study, same group reported that CpG ODNs were effective in avian influenza virus (AIV) infection. In this *in-vitro* study, chicken embryo fibroblasts were treated with CpG ODN and 24h later infected with AIV virus and viral replication was assessed by real time PCR. Similar with IBV study results indicated that CpG ODNs were able to inhibit viral replication (Dar 2009). These two independent studies suggest that CpG ODNs may have potential in controlling viral infections in chicken.

Parasitic diseases like avian coccidiosis are also very important for poultry industry. It is reported that poultry industry loses 800 million dollars every year due to avian coccidiosis. Currently, some expensive prophylactic agents are in use in order to control parasitic infections but their effectiveness are limited. Therefore new therapeutic applications are needed. In this context, effect of CpG ODNs against avian coccidiosis infection was studied by Dalloul et al. They indicated that use of CpG ODNs enhanced resistance against parasite in a susceptible chicken strain (Dalloul 2004).

In the light of these studies, we decided to check the effects of CpG ODNs as a prophylactic agent in newborn chicken in order to reduce high mortality rates and provide a better quality and profit for poultry industry. For this purpose, we

synthesized more than 50 different CpG/Non CpG ODN sequences (i.e with their proper CpG flip controls) and tested them in vitro on spleen cells of both mice and chicken for their ability to induce pro-inflammatory and inflammatory cytokine secretions (Figure 3.15 and G. Tincer PhD Thesis, Bilkent Univ., 2013). Of those two different types of CpG ODN sequences were selected and tested *in-vivo* in 1 day old broiler chicken. Most of the studies conducted so far with newborn chicken have been carried out with high doses of CpG ODNs which may not be economical to use in poultry industry. In our study, we evaluated minimal amount of CpG ODN required for protection against pathogens. As reported in detail in Results section, even 0.8 µg CpG ODN per animal was sufficient to reduce mortality rates (Figure 3.23). Although mortality percent reduction upon CpG administration is only about 2%; this reduction, when considered on industrial scale (1.2 billion chicken reproduction in Turkey/year), corresponds to survival of extra 24 million chicken per year in Turkey.

Effect of such prophylactic agents on growth performance is another important parameter that should be taken into consideration. For this reason live body weights (Figure 3.24), FCR (Figure 3.25), and EPEF (Figure 3.26) values were also determined. Although, results obtained from these studies are not perfectly correlated, it is obvious that use of CpG ODNs had significant beneficial effects on growth performance. Collectively, our results represented that CpG ODNs can be used as prophylactic agents in order to prevent bacterial, viral and parasitic infections and also have beneficial effects on growth performance in newborn chicken.

Chapter 5

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Chapter 6

Appendices

Appendix A

Standard Solutions, Buffers, Media

Sodium Carbonate Buffer (0.1M) (ELISA)

- 1.696 grams Na_2CO_3
- 2,856 grams $NaHCO_3$

into 500 ml ddH_2O. pH should be adjusted to 9.6. The buffer should be stored at +4°C.

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 stirrer for 20
30 min. The buffer should be stored at -20°C.

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

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.

HANKS BUFFER [10x]

- 74.25 grams Nacl
- 26.03 grams HEPES (sodium salt)
- 23.83 grams HEPES (free acid)
- 4 grams KCL
- 0.47 grams Na_2HPO_4
- 0.6 grams KH_2PO_4

Into 1 lt dd H2O

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

High Glucose DMEM and RPMI-1640

- 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)

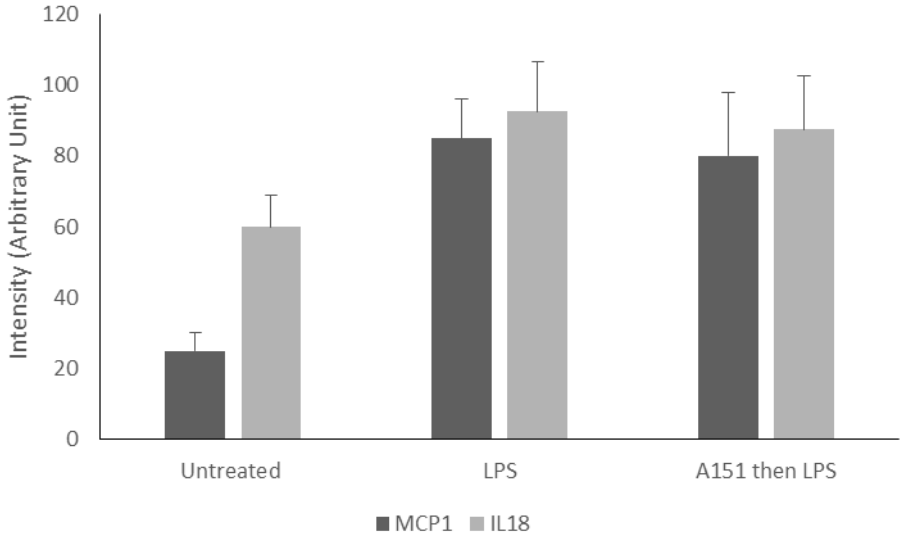
- 5 ml Na Pyruvate, (0.11 mg/ml final concentration from 100mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

In 500 ml media

Appendix B

Figure B i: A) Suppressive ODN A151 treatment after 100 µg LPS challenge induced substantial but insignificant down regulation of MCP1 and IL18 mRNA expression levels on the eyes of mice. Mice were injected intraperitoneally with 100 µg LPS and 250 µg suppressive ODN and were killed at 18 h after injection. B) Representative agarose gel picture (M1 indicates mouse #1, M2 indicates mouse #2).

A)



B)

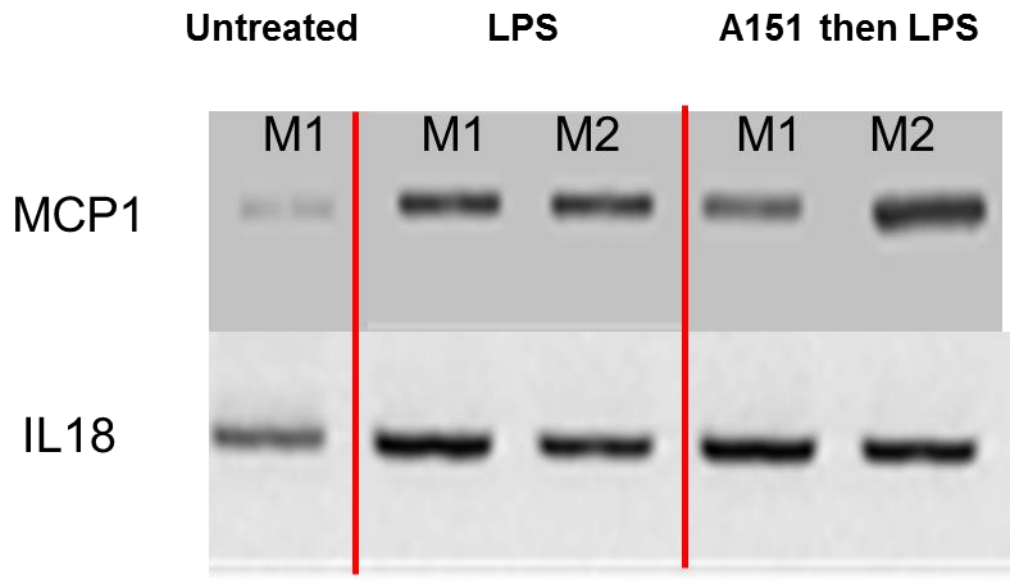


Figure B ii: Topical Restasis treatment did not reduce disease severity in 100µg LPS induced EIU. Animals treated 6 times with Restasis either 2h before or 2h after LPS challenge. Clinical EIU evaluation was performed on both eyes and scored as described in methods section 2.1.3.

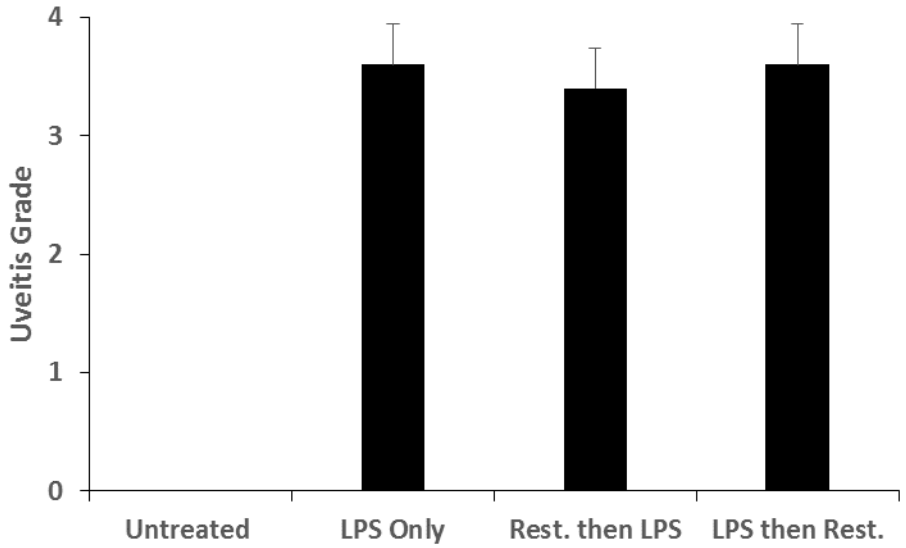


Figure B iii: Restasis treatment post 50 µg (high dose) LPS challenge (4 mouse/group) did not downregulated IL15, CXCL16, MIP1α, IL18, MIP3α and MIP1β expression levels of mice eyes.

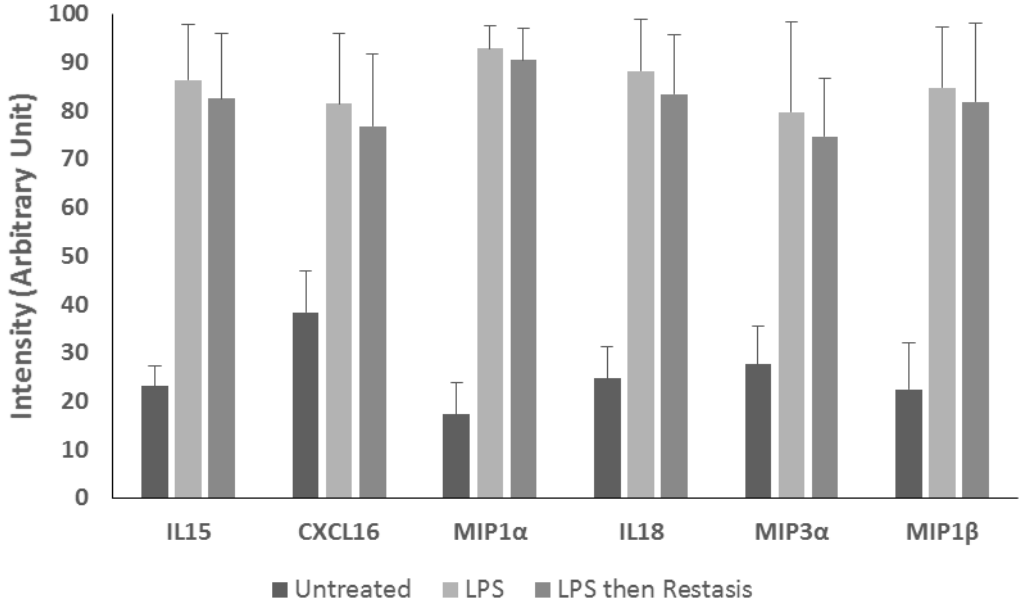
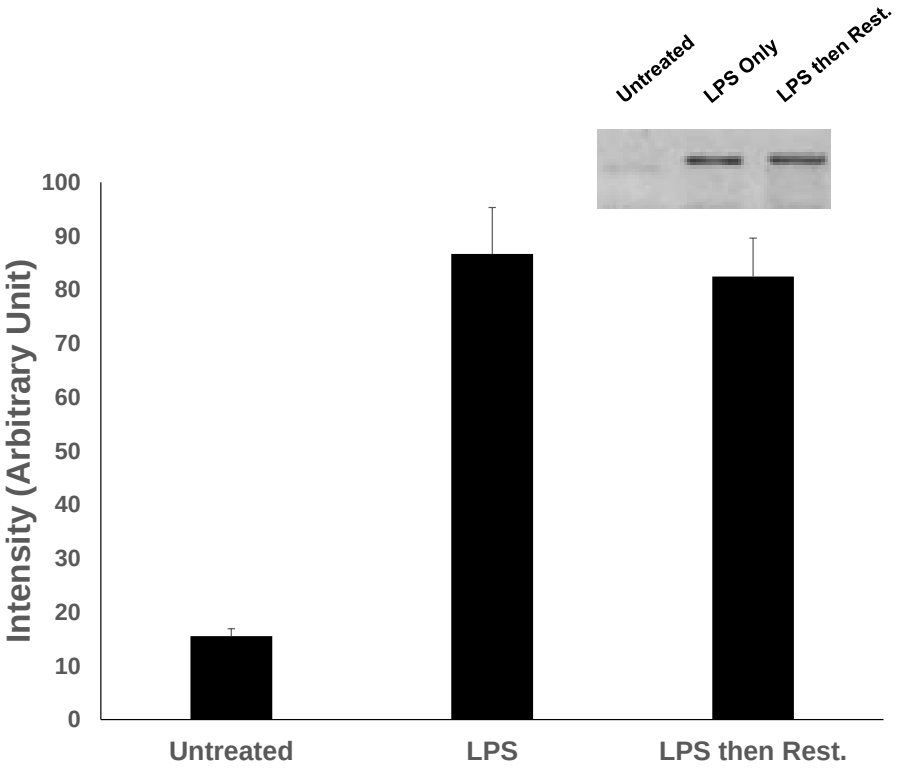


Figure B iv: Restasis administration after LPS treatment did not downregulated IL1β and IL6 expressions in rabbit iris tissues (5 rabbit/group) A) IL1β and B) IL6 mRNA levels. Representative agarose gel images.

A) IL1β



B) IL6

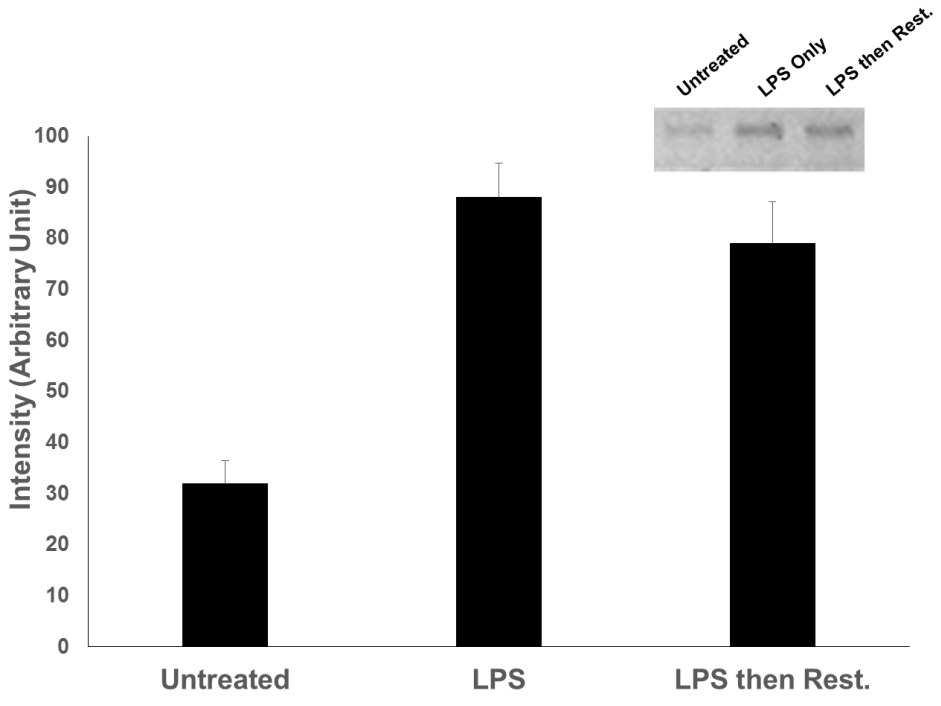


Figure B v: Non-CpG Control ODNs did not induce any cytokine production. BALB/c splenocytes ($4X10^6$) were stimulated with Control ODNs for 42hrs. IL6, IL12p40 and IFNγ production were assessed by ELISA. Data represents average ±SEM of three independent experiments carried out in triplicate repeats for each treatment groups.

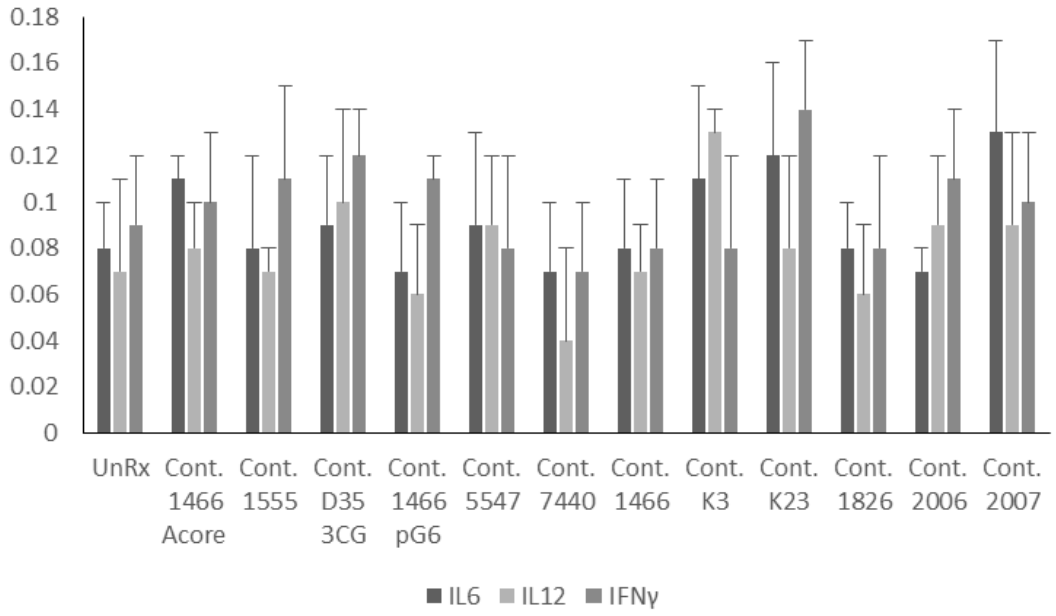
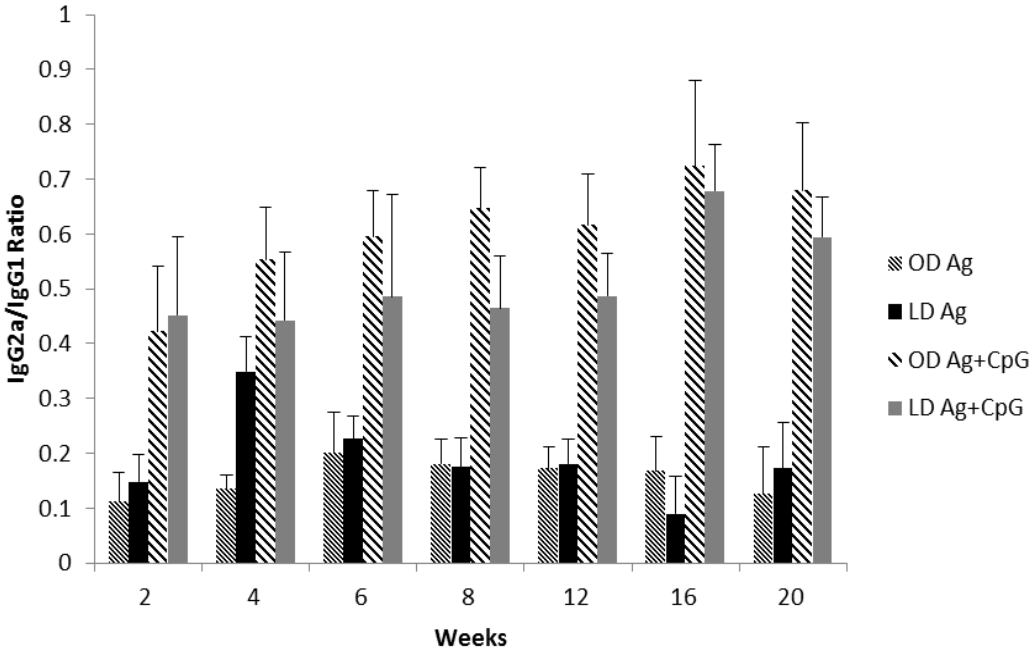


Figure B vi: Graphical representation of IgG2a/IgG1 ratios of mice immunized with either with Ser-O Ag or Monovalent Vaccine or their CpG ODN containing counterparts for a period of 20 weeks. Inclusion of CpG ODN within vaccine formulations significantly effected IgG2a/IgG1 ratios. Serum IgG2a and IgG1 levels were determined by ELISA. Graphics shows the ratios of the OD readings of anti-FMDV IgG2a and IgG1 responses at 900 titer (8 mice/group).



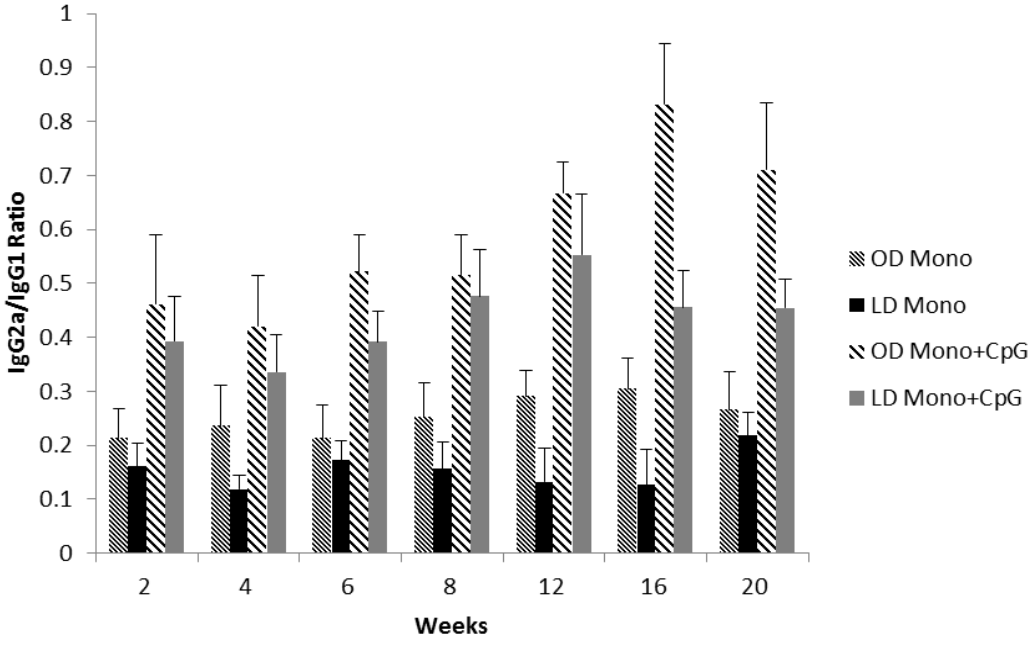
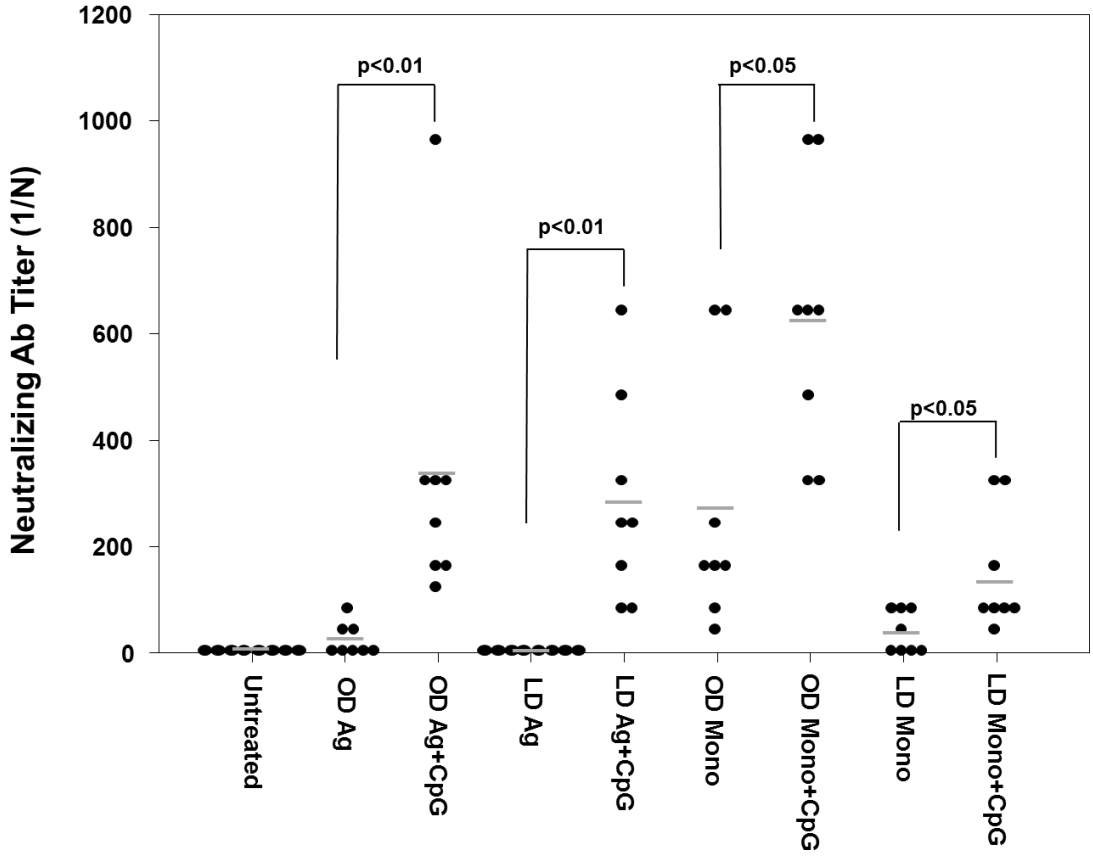
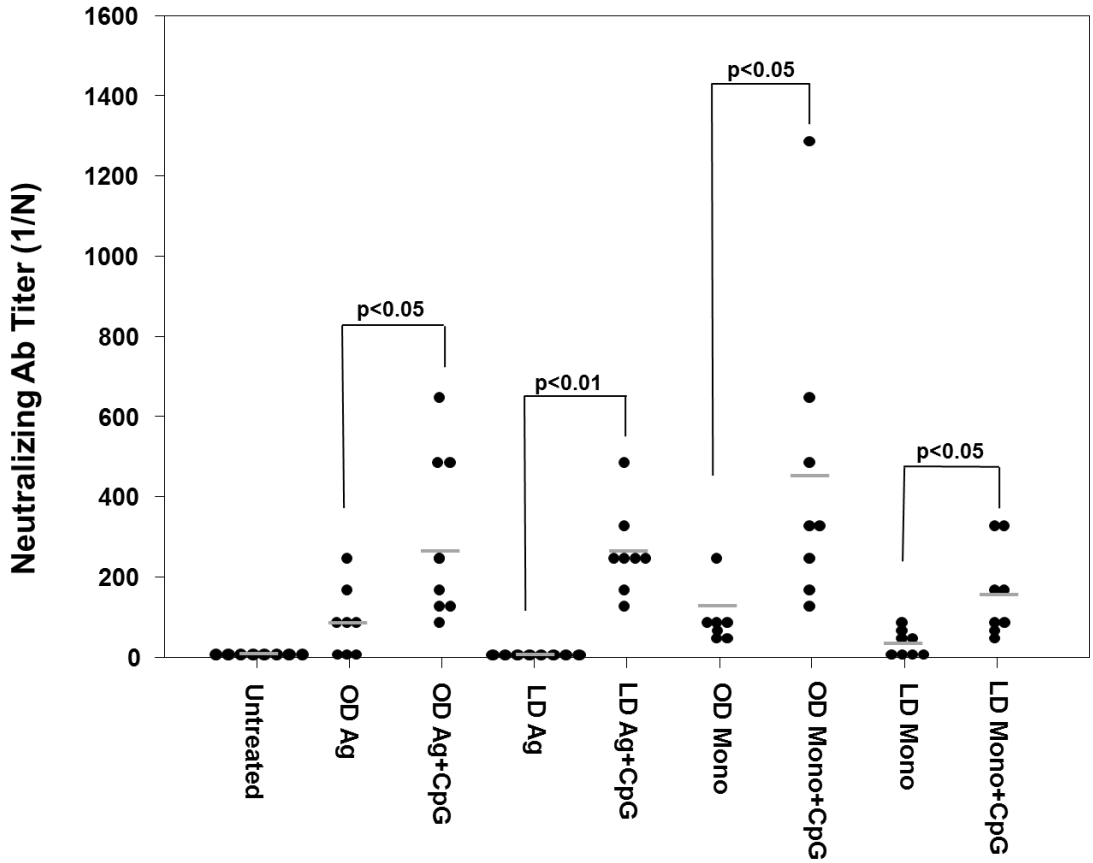


Figure B.vii: FMDV neutralizing Ab Titers of mice injected either with Optimum Dose (OD) and Low Dose (LD) Serotype O Ag/Monovalent Vaccine or their CpG ODN containing counterparts at A) 4weeks B) 6 weeks C) 12 weeks D) 16 weeks post booster injection was assesed by Virus Neutralization Assay. Each dot represents the neutralizing Ab levels of one mouse ($p<0.01$, $p<0.05$)

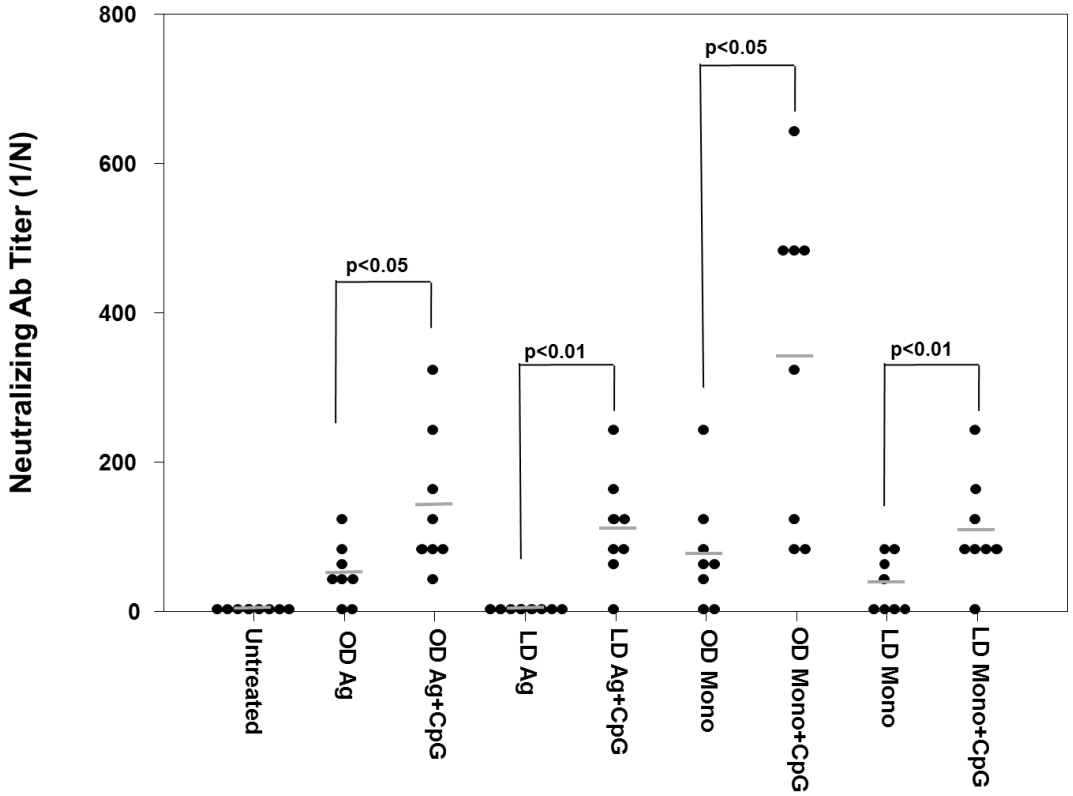
A) Virus Neutralization titers @ 4 weeks post booster injection



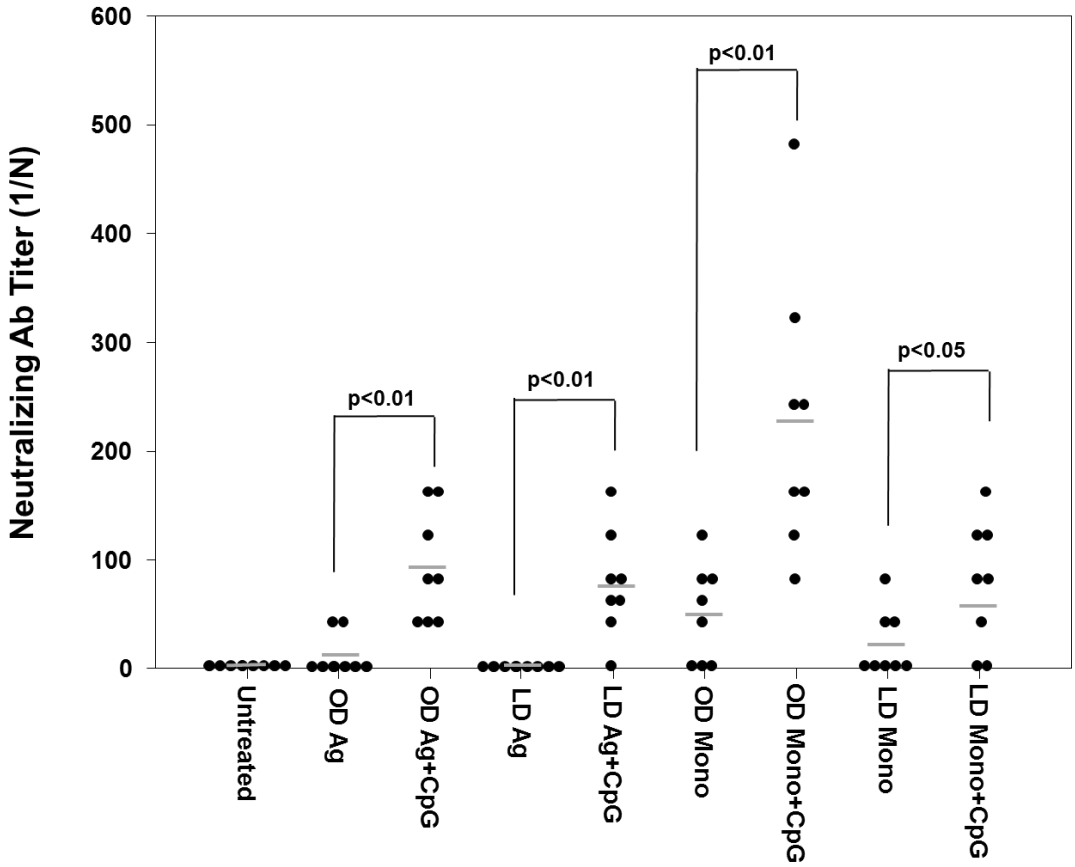
B) Virus Neutralization titers @ 6 weeks post booster injection



C) Virus Neutralization titers @ 12 weeks post booster injection



D) Virus Neutralization titers @ 16 weeks post booster injection



Chapter 7

Curriculum Vitae and Publications

Fuat Cem Yağcı
(MSc/PhD Candidate)

Personal Information

Birth Date: 01.08.1983
Nationality: Turkish
Address: Bilkent University, Faculty of Science, Department of Molecular Biology and Genetics, Biotherapeutic ODN Research Laboratory, Ankara, Turkey
Cellular Phone: +90 533 625 34 40
e-mail: fuatcemyagci@gmail.com

ACADEMIC DEGREES

2007-…..	**Bilkent University,** Department of Molecular Biology and Genetics, Ankara, Turkey (Ph.D. Degree)
2005-2007	**Bilkent University,** Department of Molecular Biology and Genetics, Ankara, Turkey (M.Sc. Degree)
2001-2005	**Halic University,** Department of Molecular Biology and Genetics, Istanbul, Turkey (B.Sc. Degree)
1998-2001	**Antalya Anadolu High School**, Antalya, Turkey

THESIS DEFENDED

2005 – 2007	**Bilkent University, Department of Molecular Biology and Genetics, M.Sc. Degree** "Suppressive Oligodeoxynucleotides as a TLR Antagonist: Efforts to Treat Autoimmune Diseases". Supervisor: Assoc. Prof. Dr. Ihsan Gursel,

PUBLICATIONS

1. Yagci FC, Cokcaliskan C, Gungor B, Alkan M, Gursel M, Gursel I. "Immunization of mice with CpG ODN/lipidic nanoemulsion spares antigen and generates a rapid, persistent humoral and cell mediated immunity against FMDV." Vaccine (In preperation), January 2014.

2. Yagci FC, Aslan O, , Tincer G, Kahraman T, Akcali CK, Bozdogan O, Gursel M, Gursel I. "Immunosuppressive Effect of Topical Cyclosporin A Application on the Endotoxin Induced Uveitis**".** Clin Exp Opthalmol (In preperation), January 2014.

3. Gungor B, Yagci FC, Tincer G, Bayyurt B, Alpdundar E, Yildiz S, Ozcan M, Gursel I, Gursel M. "Production from Plasmocytoid Dendritic Cells and Demonstrate Potent Vaccine Adjuvant Activity". Science Translational Medicine (Under Review), January 2014.

4. Osmanagaoglu O, Kıran F, Yagci FC, Gursel I. "Immunomodulatory function and in vivo properties of Pediococcus pentosaceus OZF, a promising probiotic strain." Ann Microbiol DOI 10.1007/s13213-012-0590-9, December 2012.

5. Tincer G, Yerlikaya S, Yagci FC, Kahraman T, Atanur OM, Erbatur O, Gursel I. "Immunostimulatory Activity of Polysaccharide-Poly(I:C) Nanoparticles." Biomaterials. 2011 Jun;32(18):4275-82, June 2011.

6. Gursel I, Yagci FC, Tincer G, Kahraman T, Gursel M. "DNA Nanoparçacıklarının Nanotıp ve Nanobiyoteknolojideki Yeni Kullanım Alanları." (Novel applications of DNA nanoparticles in nanomedicine and nanobiotechnology) Bilim ve Teknik Dergisi. (Science and Technology Journal), 44-47, March 2011. (Article in Turkish)

7. Yagci FC, Aslan O, Gursel M, Tincer G, Ozdamar Y, Karatepe K, Akcali CK, Gursel I. "Mammalian Telomeric DNA Suppresses Endotoxin Induced Uveitis". J Biol Chem. 10;285(37):28806-11, September 2010.

RESEARCH EXPERIENCE

2013- …..	**Project PI: Ministery of Science and Technology (TEKNOGİRİSİM Project# 1232.TGSD.2013)** " "Use of several TLR agonists to enhance the efficiency of Foot and Mouth Disease (FMD) vaccines as adjuvants"
2009-2011	**Research Assistant: Ministery of Science and Technology (*SANTEZ, Project#00448.STZ.2009-2*), Bilkent University, Department of Molecular Biology and Genetics** "Improving broiler chicken immune resistance by providing protection and reducing losses from stress and bacterial diseases with CpG DNA". Supervisor: Assoc. Prof. Dr. Ihsan Gursel
2009 - 2010	**Research Assistant: TUBITAK, *SBAG-108S316* , Bilkent University, Department of Molecular Biology and Genetics** "Biomedical Applications of DNA Nanoparticles" Supervisor: Assoc. Prof. Dr. Ihsan Gursel
2006-2009	**Research Assistant: TUBITAK, *SBAG-106S102*, Bilkent University, Department of Molecular Biology and Genetics** "Development of Liposomal CpG Oligodeoxynucleotide Carrying Vaccines Against Pathogens" Supervisor: Assoc. Prof. Dr. Ihsan Gursel
2004	**Visiting Scholar: University of Pennsylvania (UPENN), Department of Renal Electrolyte and Hypertension,** "Molecular Analysis of Human Anti-GBM Antibodies" Supervisor: Prof. Dr. Michael P. Madaio

2004 – 2005	**Research Assistant: Halic University, Department of Molecular Biology and Genetics,** "Endhotelial Cell Protective Gene Expression in Response to High Concentrarion of D-Glucose and Insulin in the presence and absence Bradykinin Stimulation"

AWARDS AND SCHOLARSHIPS

2013	**Travel Award** provided by European Federation of Immunological Societies to participate **15 thInternational Congress of Immunology**, Milan, Italy.
2012	**Registration and Travel Awards** provided by Association for Cancer Immunotherapy. **10th CIMT Annual Meeting**. Mainz, Germany, 23–25 May 2012.
2012	**Registration and Accomodation Awards** provided by Turkish Society of Immunology to participate **Molecular Immunology & Immunogenetics Congress (MIMIC2012)**, Antalya, Turkey, April 2012.
2011	**Full Scholarship** provided by Turkish Society of Immunology to participate **21thNational Congress of Immunology**, Marmaris, Turkey.
2010	**Travel Award** provided by European Federation of Immunological Societies to participate **14th International Congress of Immunology**, Kobe, Japan.
2009	**Full Scholarship** provided by Turkish Neurology Society to participate **9th European School of NeuroImmunology**, Istanbul, Turkey.
2009-2011	**Ministery of Science and Technology (SANTEZ, Project#00448.STZ.2009-2) Project Bursary.** "Improving broiler chicken immune resistance by providing protection and reducing losses from stress and bacterial diseases with CpG DNA".
2009 - 2010	**TUBITAK, *SBAG-108S316* Project Bursary** "Biomedical Applications of DNA Nanoparticles"
2006-2009	**TUBITAK, *SBAG-106S102* Project Bursary** "Development of Liposomal CpG Oligodeoxynucleotide Carrying Vaccines Against Pathogens"
2005 -	**PhD Programme Bursary** Institute of Science and Engineering, Bilkent University, Ankara, Turkey.

2005 – 2007	**M.Sc. Programme Bursary** Institute of Science and Engineering, Bilkent University, Ankara, Turkey.
2004	**"Goldie Simon" Preceptorship Award** LUPUS Foundation, Wyncote, PA, USA
2001 - 2005	**B.Sc. Programme Bursary** Higher Education Council, Ankara, Turkey

ORAL PRESENTATIONS

1. Yagci FC, Cokcaliskan C, M, Ozturk N, Gungor B, Gursel M, Gursel I. "Use of CpG Oligodeoxynucleotides enhances rapidity, longevity and potency of FMDV vaccines in mice." Molecular Immunology & Immunogenetics Congress (MIMIC), Antalya, Turkey, April 2012.

2. Yagci FC, Cokcaliskan C, Alkan M, Ozturk N, Bugdayci E, Gungor B, Gursel M, Gursel I. "Use of CpG Oligodeoxynucleotides enhances level and duration of vaccine potency against FMDV in mice" 17. Biomedical Science and Technology Symposium (BIOMED), Ankara, Turkey, November 2011.

3. Yagci FC, Aslan O, Gursel M, Simsek I, Bayyurt B, Gursel I. "İmmün Baskılayıcı DNA'nın Otoimmün ve Otoenflammatuvar Hastalıkların Tedavisine Yönelik Uygulamaları" (Applications of Immunosuppressive DNA in Autoimmune and Autoinflammatory Diseases) 21thNational Congress of Immunology, Marmaris, Turkey, April 2011.

4. Gursel I, Yagci FC Tincer G, Kahraman T, Bayyurt B, Gucluler G, Erikci E, Karatepe K. "Nanobilimin Uygulamalı İmmünolojiye Katkıları: TLR Ulaklarının İmmünterapide Daha Etkin Kullanımının Geliştirilmesi"(Contribution of Nanoscience to Applied Immunology: Improving the use of TLR ligands in Immunotherapy) 21thNational Congress of Immunology, Marmaris, Turkey, April 2011.

5. Yagci FC, Aslan O, Songur MS, Simsek I, Gursel I "Applications of Immunosuppressive Nanoparticle Forming DNA to Treat Autoimmune and Autoinflammatory Diseases". Workshop on Current Trends in Molecular Nanobiosciences, Bilkent University, Ankara, Turkey, January 2010.

6. Yagci FC, Aslan O, Songur MS, Gursel I. "Immunosuppressive DNA Nanoparticles: A Potential Nanodrug to Treat Endotoxin Induced Uveitis in Mice". 5. National Nanoscience and Nanotechnology Conference, Anadolu University, Eskisehir, Turkey, June 2009.

SELECTED CONGRESS COMMUNICATIONS

1. Kahraman T, Yagci FC, Gursel I. "Plasma microparticles and LL37 levels regulate Behcet's disease severity." 15th International Congress of Immunology, Milan, Italy, August 2013. (Poster Presentation)

2.Yagci FC, Kiran F, Almacioglu K, Osmanagaoglu O, Gursel I. "DNA Bakteriyosin komplekslerinin immune düzenleyici etkileri." (Immunomodulatory effects of DNA-Bacteriocin Complexes) 22 th National Immunology Congress, Cesme, Izmir, April 2013 (Poster Presentation in Turkish)

3. Yagci FC, Cokcaliskan C, Alkan M, Gungor B, Gursel M, Gursel I. "CpG Oligodeoxynucleotides enhances both humoral and cellular immune responses against FMDV in mice." ." 10th Cancer Immunotherapy Annual Meeting (CIMT), Mainz, Germany, May 2012. (Poster Presentation)

4. Yagci FC, Gucluler G, Kiran F, Gursel I. "Bacteriocin DNA nanocomplexes as immunotherapeutic carriers." 10th Cancer Immunotherapy Annual Meeting (CIMT), Mainz, Germany, May 2012. (Poster Presentation)

5. Gungor B, Yagci FC, Gursel I, Gursel M. "Potentiating the immunostimulatory properties of CpG oligodeoxynucleotides:aiming to develop a better vaccine adjuvant." 10th Cancer Immunotherapy Annual Meeting (CIMT), Mainz, Germany, May 2012. (Poster Presentation)

6. Gungor B, Cokcaliskan C, Alkan M, Yagci FC, Gursel I, Gursel M. "CpG Oligodexynucleotide/Tat Peptide Complexes Enhance the Potency of Foot and Mouth Disease Vaccine in Mice." Molecular Immunology & Immunogenetics Congress (MIMIC), Antalya, Turkey, April 2012. (Poster Presentation)

7. **Kiran F, Yagci FC, Almacioglu K, Osmanagaoglu O, Gursel I.** "Enhanced Immunostimulatory activity of bacteriocin CpG ODN nanocomplexes" 17. Biomedical Science and Technology Symposium (BIOMED), Ankara, Turkey, November 2011. (Poster Presentation)

8. **Gungor B, Yagci FC, Tincer G, Alpdundar E, Yildiz S, Gursel I, Gursel M.**"Determination of Immune Stimulatory Properties of Synthetic CpG ODN/ Cationic Peptide Complexes". Biomedical Science and Technology Symposium (BIOMED), Ankara, Turkey. November 2011. (Invited speaker, Dr. Mayda Gürsel)

9. **Gungor B, Yagci FC, Tincer G, Alpdundar E, Yildiz S, Gursel I, Gursel M.** "Immunostimulatory Properties of CpG ODN Based Nanoparticles". ". Biomedical Science and Technology Symposium (BIOMED), Ankara, Turkey, November 2011. (Poster Presentation)

10. Yagci FC, Aslan O, Songur MS, Gursel I. "Mammalian Telomeric DNA Sequences Downregulate Symptoms of Autoimmune and Autoinflammatory Diseases". BRIDGE Symposium, Bogazici University, Istanbul, Turkey, April 2011. (Poster Presentation)

11. Yagci FC, Aslan O, Simsek I, Gursel M, Ozdamar Y, Tincer G, Karatepe K, Gursel I. "Immunosupressive Oligodeoxynucleotides: A Potential Nanodrug to Treat Autoimmune and Autoinflammatory Diseases". 14th International Congress of Immunology, Day 1 Abstract Book, P21, Kobe, Japan, August 2010. (Poster Presentation)

12. Yagci FC, Aslan O, Songur MS, Gursel I. "Mammalian Telomeric DNA Sequences Are Immunosuppressive and Downregulate Symptoms of Endotoxin Induced Uveitis in Mice". 15. Biomedical Science and Technology Symposium, P125, Guzelyurt, Turkish Republic of Northern Cyprus, August 2009. (Poster Presentation)

13. Gursel I, Tincer G, Yagci FC, Mammadov R, Kocak H, Kahraman T, Karatepe K, Erikci E, Akcali C, Gursel M. "Nanobiotechnological Applications of Self-Assembled Immunostimulatory DNA Nanoparticles". 15th International Biomedical Science and Technology Symposium, Guzelyurt, Turkish Republic of Northern Cyprus, August 2009. (Invited speaker, Dr. Ihsan Gürsel)

14. Gursel I, Tincer G, Yagci FC, Kahraman T, Mammadov R, Erikci E, Karatepe K, Akcalı C, Gürsel M. "Potential Nanomedical Applications of Self Assembled Immunomodulatory DNA Nanoparticles". 5th National Nanoscience and Nanotechnology Conference, Eskisehir, Turkey, June 2009. (Invited speaker, Dr. Ihsan Gürsel)

15. Gursel I, Tincer G, Yagci FC, Mammadov R, Kocak H, Kahraman T, Karatepe K, Erikci E, Gursel M. "Immunotherapeutic applications of DNA nanoparticles to cancer, infectious and autoimmune diseases". 13th National Biomedical Engineering Conference, Ankara, Turkey, May 2008 . (Invited speaker, Dr.Ihsan Gürsel)

16. Gursel I, Tincer G, Yagci FC, Mammadov R, Kocak H, Kahraman T, Karatepe K, Erikci E, Gursel M. "Immune activation by CpG oligodeoxynucleotides". XX. National Biochemistry Congress, Nevsehir, Turkey, November 2008. (Invited speaker, Dr.Ihsan Gürsel)

17.Gursel I, Yagci FC, Tincer G, Gursel M. "DNA Nanoparticles: Novel Immunomodulatory Adjuvants for Immunotherapy". World Immune Regulation Meeting-II, Davos, Switzerland, March 2008. (Poster Presentation)

18. Yagci FC, Biterge B, Yazar V, Bahcecioglu G, Gursel I. "Nanoparticle Forming TLR Antagonist as Potential Nanodrugs to Treat Autoimmune Diseases". 13. Biomedical Science and Technology Symposium, P90, Istanbul, Turkey, June 2007. (Poster Presentation)

19. Akis N, Aydin Z, Tezil T, Yagci FC, Kahraman S, Endothelial Cell Protective Gene Expression. *2nd* Modern Drug Discovery & Development Summit Abstract Book, 3, Philadelphia, USA, 2006. (Poster Presentation)

PARTICIPATED CONGRESSES

Certificate of Participation "**Winter School on Frontiers in Nanomedicine and Nanobiotechnology**", Bilkent University, Ankara, Turkey, January, 2010.

Certificate of Participation. "**9th European School of NeuroImmunology**", Istanbul, Turkey, September, 2009.

Certificate of Participation. "**1st Congress of the Society of Innate Immunity**", Ankara, Turkey, June 2007.

Certificate of Participation. "**31st FEBS Congress, Molecules in Health and Disease**", İstanbul, Turkey, 24-29 June 2006.

Certificate of Participation. "**First Molecular Medicine Congress**". Hilton Convention Center, Istanbul, Turkey, April 2005.

Certificate of Participation. "**XVI. National Cancer Congress**". Pine Beach City, Antalya, Turkey, April 2005.

Certificate of Participation. "**9. National Biology Students Congress**", Abant Izzet Baysal University, Bolu, Turkey, November 2002.

SOCIETY MEMBERSHIPS

American Association of Immunologists- Student Member
Turkish Society of Immunology
Turkish Neurology Society
Biomaterial and Tissue Engineering Society

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 37, pp. 28806–28811, September 10, 2010

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis*[S]

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Telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that inhibit several proinflammatory and Th1-biased immune responses. Synthetic oligodeoxynucleotides (ODN) expressing suppressive motifs can reproduce the down-regulatory activity of mammalian telomeric repeats and have proven effective in the prevention and treatment of several autoimmune and autoinflammatory diseases. Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation induced by LPS administration. Augmented expression of proinflammatory cytokines/chemokines such as TNFα, IL-6, and MCP1 and bactericidal nitric oxide production mediated by LPS contribute to the development of EIU. Suppressing these mediators using agents that are devoid of undesirable systemic side effects may help prevent the development of EIU. This study demonstrates the selective down-regulatory role of suppressive ODN after (i) local or (ii) systemic treatment in EIU-induced rabbits and mice. Our results indicate that suppressive ODN down-regulate at both the transcript and protein levels of several proinflammatory cytokines and chemokines as well as nitric oxide and co-stimulatory surface marker molecules when administrated prior to, simultaneously with, or even after LPS challenge, thereby significantly reducing ocular inflammation in both rabbit and mouse eyes. These findings strongly suggest that suppressive ODN is a potent candidate for the prevention of uveitis and could be applied as a novel DNA-based immunoregulatory agent to control other autoimmune or autoinflammatory diseases.

DNA and RNA are the essential components of all living organisms. Accumulated evidence strongly suggests that these nucleic acids have multiple and complex effects on the immune system and are more than a blueprint of life (1, 2). On one hand, due to their high unmethylated CpG motif frequency, bacterial DNAs are recognized as "non-self" via TLR9 (Toll-like receptor 9) and trigger an innate immune response characterized by the proliferation and maturation of B cells, natural killer cells, and plasmacytoid dendritic cells and the secretion of T-helper 1-type cytokines, chemokines, and/or multivalent immunoglobulins (3–8). On the other hand, telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that can inhibit several TLR-dependent and TLR-independent Th1-mediated immune responses. Of note, these motifs are underrepresented in the prokaryotic genome. Synthetic single-stranded oligodeoxynucleotides (ODN)[3] containing repetitive TTAGGG motifs mimic this effect (1, 9–11). Previous studies revealed that deleterious inflammatory responses to a host can be down-regulated by suppressive ODN. *In vitro*, suppressive ODN inhibits the production of several proinflammatory cytokines and chemokines induced by bacteria (1, 12–14). Furthermore, *in vivo* suppressive ODN administration reduces the frequency and severity of several autoimmune and inflammatory diseases such as arthritis, systemic lupus erythematosus, pulmonary inflammation, toxic shock, silicosis, and experimental autoimmune encephalomyelitis (10, 15–21).

Uveitis is an ophthalmic disorder that causes vision loss in developed countries (22, 23) and is characterized by acute, recurrent, or persistent ocular inflammation, the breakdown of the blood-ocular barrier, and infiltration of leukocytes (24). The underlying causes of uveitis can vary. For example, acute anterior uveitis is often associated with (i) Behcet disease, (ii) Reiter syndrome, and (iii) ankylosing spondylitis, as well as other systemic inflammatory diseases (25).

Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation. It is triggered by the administration of LPS, which is a component of the Gram-negative bacterial outer membrane (26). A ligand for TLR4, LPS enhances the expression of various proinflammatory cytokines and chemokines such as IL-6 (27, 28), TNFα (29), and MCP1 (monocyte chemoattractant protein 1) (30) and the production of nitric oxide. All of these mediators contribute to the breakdown of the blood-ocular barrier and infiltration of leukocytes, resulting in the development of EIU (26). It has been shown that suppressing proinflammatory cytokines, including IL-6, TNFα, MCP1, and inducible nitric-oxide synthase (iNOS), retards if not prevents the development of EIU (31). Conventional drugs used to control these concerted inflammatory activation are mainly immunosuppressive in character and are associated with undesirable systemic side effects (24). It is of the utmost importance to develop effective, less toxic agents that selec-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed. Tel.: 90-312-290-2408; Fax: 90-312-266-5097; E-mail: ihsangursel@bilkent.edu.tr.

[3] The abbreviations used are: ODN, oligodeoxynucleotide(s); EIU, endotoxin-induced uveitis; iNOS, inducible nitric-oxide synthase.

tively block proinflammatory immune activation while eliminating the unwanted systemic side effects.

To date, the inhibitory effect of suppressive ODN on LPS-mediated EIU at both the local and systemic levels has not been studied by others. In this study, a very aggressive form of experimental uveitis was initiated via endotoxin administration. We investigated whether the suppressive ODN "A151" can inhibit the induction and development of ocular inflammation (before or at the time of LPS insult or even 2 h after LPS treatment) and help to reduce the symptoms of EIU in rabbits and mice. Our results revealed, for the first time, that A151 is capable of down-regulating the mRNA expression and protein levels of several potentially pathologic chemokines and cytokines at both the local and systemic levels. Consequently, suppressive ODN mimicking telomeric DNA offers a novel nucleic acid-based immunotherapeutic agent to control overexuberant undesirable immune responses such as seen in autoimmune and autoinflammatory diseases.

EXPERIMENTAL PROCEDURES

Materials—All cell culture medium components were from HyClone. Cytokine pairs for ELISAs were from Endogen. LPS (isolated from *Escherichia coli*) was obtained from Sigma. The phosphorothioate-modified suppressive ODN A151 (24-mer, 5′-$(TTAGGG)_4$-3′) and control ODN (24-mer, 5′-$(TTACCC)_4$-3′) were obtained from Alpha DNA (Montreal, Canada). TRIdity G (AppliChem GmbH, Darmstadt, Germany) was used for RNA isolation. cDNAs were synthesized using a DyNAmo™ cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. DyNAzyme™ PCR Master Mix was used for PCRs.

Maintenance of Animals—Adult female BALB/c mice and adult New Zealand rabbits 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 conditions at 22 °C with 12-h light and 12-h dark cycles. They were provided with unlimited access of food and water.

Induction of Endotoxin-induced Uveitis—Specific pathogen-free 10-week-old female BALB/c mice were injected intraperitoneally with 25, 50, 100, or 200 μg of LPS in 200 μl of PBS and/or suppressive ODN. Mice were killed at the end of clinical evaluation. Both eyes were enucleated and used for cytokine expression assays. Spleens were removed and split in two; splenocytes were incubated on tissue culture plates for 6, 12, and 24 h; and supernatants were collected for cytokine determination by ELISA. IL-6 was measured as an indicator of EIU response. The other half of the spleen was used to extract total RNA for further cytokine/chemokine gene transcript expression analysis by RT-PCR. In another experiment, rabbits (three to four animal/group, ~1500 g each; housed in the Ankara Hospital animal facility) were separated into different treatment groups, and EIU was initiated via intraocular LPS injection (100 μg) with or without suppressive ODN treatment. Eyes were removed, and further analyses as described for mice were conducted.

Clinical Evaluation and Histopathological Investigation—Animals were subjected to blind investigation by an ophthalmologist under a dissection microscope 18–24 h after injection, corresponding to the time of maximal severity of EIU. Clinical ocular inflammation was graded on a scale from 0 to 4 for each animal described previously (32): no sign of inflammation = 0; discrete inflammation in iris and conjunctiva = 1; dilatation of iris and conjunctiva vessels = 2; hyperemia in iris associated with Tyndall effect in anterior chamber = 3; in addition to the signs in scale 3, synechia or fibrin is formed = 4 (32). For histopathological investigations, enucleated eyes were fixed in 10% formalin for 24 h, washed with running tap water for 1 h, and placed in 60% ethyl alcohol for an extra 3 h. Eyes were embedded in paraffin, which was sectioned and stained with hematoxylin and eosin. Sections were examined blindly by a histopathologist, using score systems of severity ranging from 0 to 4. Focal non-granulomatous monocytic infiltration in the choroid, the ciliary body and retina were scored as 0.5. Retinal perivascular infiltration and monocytic infiltration in the vitreous were scored as 1. Granuloma formation in the uvea and retina and the presence of occluded retinal vasculitis along with photoreceptor folds, serous detachment, and loss of photoreceptor were scored as 2. In addition, the formation of Dalen-Fuchs nodules (granuloma at the level of the retinal pigmented epithelium) and the development of subretinal neovascularization were scored as 3 and 4 according to the number and size of the lesions (33).

Cytokine and IgM ELISAs and NO Assays—Immulon 2 HB microtiter plates (Thermo Scientific) were coated with anti-cytokine or anti-IgM antibodies (BD Pharmingen) and then blocked with PBS and 1% BSA (1, 34). Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine antibody followed by phosphatase-streptavidin (Perbio), whereas bound IgM was detected using phosphatase-conjugated anti-IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) as described (1). Nitric oxide detection by the Griess method was conducted on murine peritoneal exudate cells (10^6/ml) after 12–36 h of *ex vivo* incubation as described by the supplier (Promega).

Analysis of Cell-surface Molecule Expression by FACS—2×10^6 spleen cells/ml were isolated from 24-h post-treated mice. Cells were washed, fixed, and co-stained with one of the phycoerythrin-labeled anti-CD40, anti-CD86, and anti-ICAM-1 and FITC-labeled cell-specific antibodies (*i.e.* CD11c for dendritic cells, CD11b for macrophages, and B220 for B cells (BD Pharmingen)) for 30 min at room temperature. Following washing, they were studied using a FACSCalibur (BD Biosciences) and analyzed with CellQuest Pro software.

Cytokine and Chemokine RT-PCR—Animals were injected with LPS and/or suppressive ODN. Total RNA was extracted from the eyes or spleens of the mice 4–6 h later (or from the irises or corneas of the rabbits), reverse-transcribed, and amplified to obtain cDNA in a standard PCR for 30 cycles using primers for mouse- or rabbit-specific target genes (Table 1) as described previously (1, 34). PCR-amplified material was separated on 1.5% agarose gels and visualized under UV light after ethidium bromide staining.

TABLE 1
Oligonucleotide PCR primers used in mouse or rabbit experiments
m, mouse; rb, rabbit.

Primer	Forward	Reverse	Product
			bp
mβ-actin[a]	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA	450
mIP10[a]	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT	127
miNOS[a]	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	95
mMIP1α[b]	ACCATGACACTCTGCAACCA	AGGCATTCAGTTCCAGGTCA	238
mIL-5[a]	AGCACAGTGGTGAAAGAGACCTT	TCCAATGCATAGCTGGTGATTT	117
mIL-15[a]	CATCCATCTCGTGCTACTTGTGTT	CATCTATCCAGTTGGCCTCTGT	126
mIL-18[b]	GATCAAAGTGCCAGTGAACC	ACAAACCCTCCCCACCTAAC	384
mMCP1[b]	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG	249
mMIP3α[b]	CGTCTGCTCTTCCTTGCTTT	CCTTTTCACCCAGTTCTGCT	250
mCXCL16[b]	CCTTGTCTCTTGCGTTCTTC	GGTTGGGTGTGCTCTTTGTT	384
mMIP1β[b]	CCAGCTCTGTGCAAACCTAA	CTGTCTGCCTCTTTTGGTCA	250
rbGAPDH[c]	TCACCATCTTCCAGGAGCGA	CACAATGCCGAAGTGGTCGT	319
rbIL-6[c]	GCTCCTGGTGGTGGCTAC	GGGTGGCTTCTTCATTCAAA	450
rbIL-1β[c]	GCCGATGGTCCAATTACAT	ACAAGACCTGCCGGAAGCT	121

[a] Taken from Ref. 43.
[b] In house-designed primers.
[c] Taken from Ref. 44.

Statistical Analysis—Assays were performed in triplicate on at least three to five different cell preparations. Statistical significance between untreated (or control) and treated groups was evaluated using Student's *t* test.

RESULTS

EIU is an established animal model of acute ocular inflammation. It is induced by either systemic or intravitreal administration of LPS, the major component of Gram-negative bacteria. LPS acts through the TLR4-triggering proinflammatory signaling cascade. The expression of Th1 cytokines and chemokines, including IL-6, IL-1β, and MIP3α (macrophage inflammatory protein 3α), contributes to the development of EIU.

This study was performed with 82 mice and 26 rabbits. Initial experiments were conducted to optimize the induction of EIU (supplement Fig. 1). For the mouse experiments, systemic administration of LPS doses between 25 and 100 μg/mouse were sufficient to induce uveitic eyes within 24 h as judged by clinical and histopathological investigations (supplement Figs. 1 and 2). For the rabbit experiments, intraocular 100-μg LPS injection was found to be optimal to induce EIU. Following local or intraperitoneal LPS and or suppressive ODN administration, rabbit and mouse eyes were removed, and RNAs from the irises, vitreous, and corneas of the rabbit eyes were obtained. PCR was run with the cDNA from each sample, and the mRNA levels of IL-6, IL-15, *IP10* (interferon-γ-inducible protein 10), iNOS, *MIP1*α, IL-18, *MIP3*α, *CXCL16* (CXC chemokine ligand 16), *MIP1*β, and IL-1β were monitored. In addition, 24 h post-LPS and/or A151 treatment, splenocyte suspensions were incubated *ex vivo* for 6–24 h, and IgM, IL-6, IL-10, IL-12, and IFNγ levels from the supernatants were determined by ELISA. FACS analyses were conducted on spleen cells to monitor co-stimulatory/surface marker molecule expressions.

The results indicated that in rabbits, suppressive ODN administered before or after 100-μg LPS treatment or co-injected with LPS significantly down-regulated the expression of IL-1β message from the iris (Fig. 1*A*). In cornea, IL-6 was down-regulated when suppressive ODN was given before or simultaneously with LPS administration (Fig. 1*B*). There was no significant inhibitory effect when A151 was given post-LPS treatment. In all these experiments, the mRNA message reduction was suppressive ODN-dependent because control ODN administration did not show any benefit for alleviation of LPS reactogenicity.

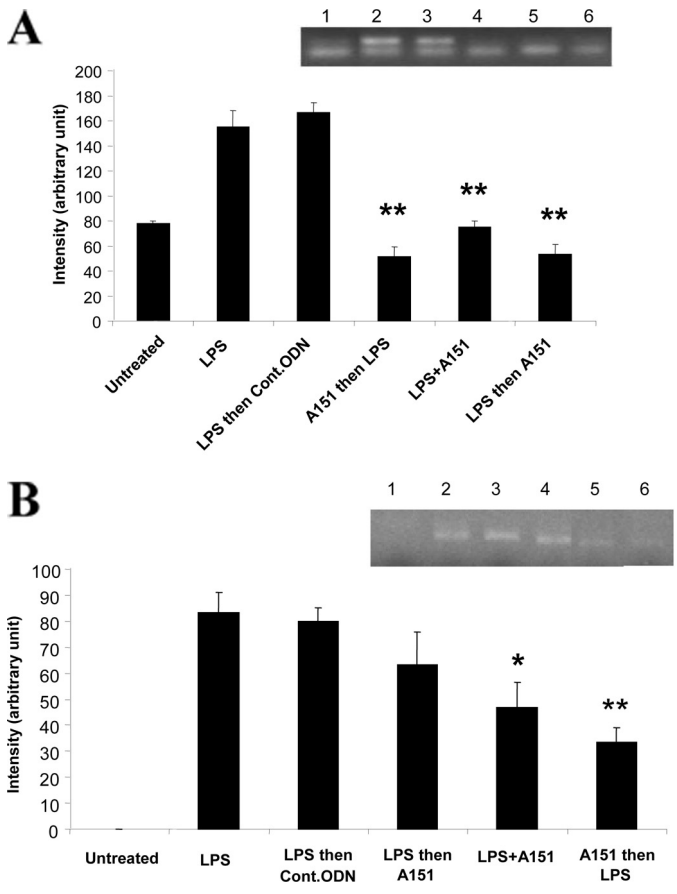

FIGURE 1. **Suppressive A151 ODN administration after LPS challenge significantly down-regulates IL-1β and IL-6 expression in the iris and cornea, respectively.** Rabbits were injected intraocularly with 100 μg of LPS and 250 μg of suppressive ODN. The average of densitometric measurements of four animals for IL-1β mRNA from iris (*A*) and IL-6 mRNA from cornea (*B*) is shown. *Insets* are the representative gel image of each group labeled from untreated to A151 and then LPS as *1* to *6*. *, $p < 0.05$; **, $p < 0.01$ between LPS-treated and A151 ODN-co-administered groups.

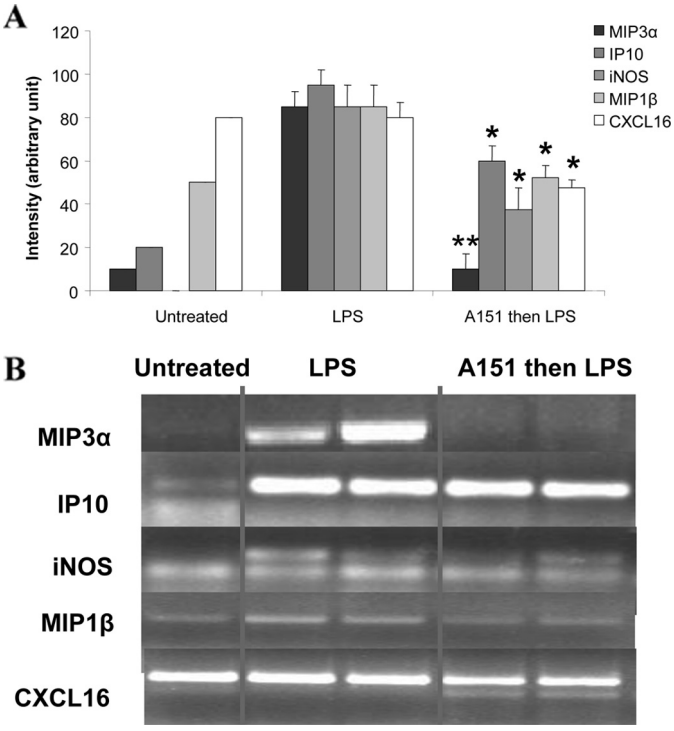

FIGURE 2. *A*, suppressive A151 ODN treatment after 100-μg LPS challenge significantly down-regulates IP10, MIP3α, iNOS, MIP1β, and CXCL16 expression levels in the eyes of mice. Mice were injected intraperitoneally with 100 μg of LPS and 250 μg of suppressive ODN and killed 18 h after injection. *, $p < 0.05$; **, $p < 0.01$ between LPS-treated and LPS + A151 ODN-co-administered groups. *B*, representative gel image.

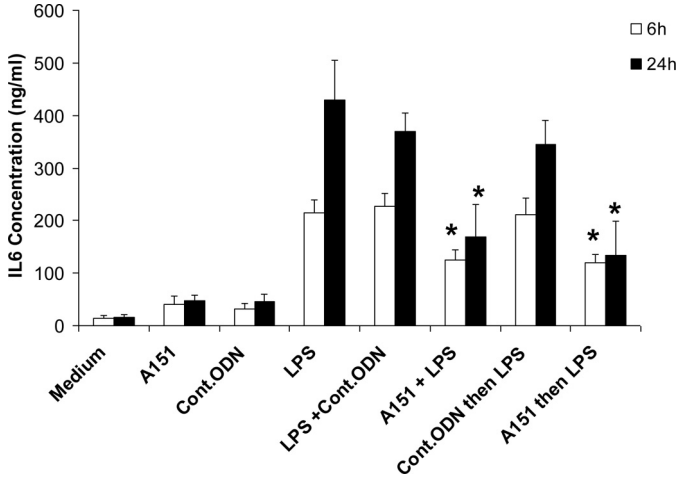

FIGURE 3. **Suppressive A151 ODN administration significantly suppresses IL-6 release from murine splenocytes.** Mice were injected intraperitoneally with 100 μg of LPS and 250 μg of suppressive or control ODN as further indicated. Spleen cells were removed (24 h post injection) and incubated 6–24 h, and supernatants were collected for cytokine ELISA. IL-6 was measured as an indicator of EIU response. *, $p < 0.05$, between LPS and LPS + A151 groups.

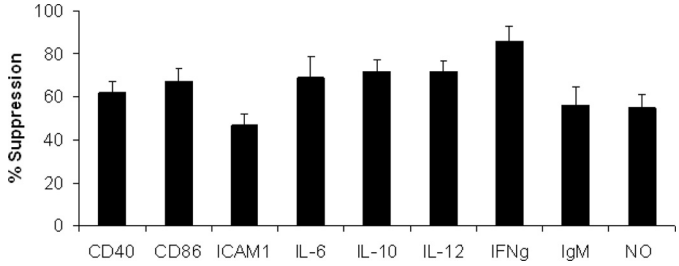

FIGURE 4. **Inhibitory effect of suppressive ODN on LPS-mediated immune activation.** The levels of CD40, CD86, and ICAM-1 expression (mean fluorescence intensity) were determined by FACS 24 h after *in vivo* injection of LPS or LPS plus A151 (50 μg of LPS and 250 μg of ODN). IL-6, IL-10, IL-12, IFNγ, and IgM levels in culture supernatants were determined by ELISA following 36 h of *ex vivo* incubation. The Griess method was used to detect nitric oxide in isolated peritoneal exudate cells supernatants 24 h post-treatment. *% Suppression* was calculated by the following formula: (1 − ((activation by LPS + suppressive ODN) − (background)/(activation by LPS + control ODN) − (background))) × 100.

In the murine EIU model, mice tolerated up to 100-μg LPS intraperitoneal treatment. Doses >150 μg caused animals to succumb to endotoxin treatment. The mouse experiments were conducted with three doses of LPS: 25, 50, and 100 μg. Suppressive A151 ODN and control ODN (2 h before and at the time of LPS injection and 2 h after LPS treatment) were used in the range of 100–250 μg. Although in rabbits, the injection of ODN and endotoxin was intraocular, in mice, injections were given intraperitoneally in 200 μl of PBS.

The results showed that when 250 μg of suppressive ODN was administered before LPS injection (2 h), it significantly down-regulated the expression of IP10, iNOS, MIP3α, CXCL16, and MIP1β in the 100-μg LPS-injected mouse EIU model (Figs. 2, *A* and *B*). Other cytokines such as MIP1α and IL-18 also showed substantial but insignificant down-regulation at these doses (data not shown). To understand the systemic effect of suppressive A151, IL-6 secreted from murine splenocytes after *ex vivo* incubation for up to 24 h in culture was monitored by ELISA (Fig. 3). Our results revealed that suppressive ODN was able to reduce >65% of the secreted IL-6 (430 ± 70 and 135 ± 55 ng/ml for LPS and A151 + LPS groups, respectively). Co-administration of suppressive ODN with LPS significantly decreased cytokine mRNA levels *in vivo* or cytokine production in *ex vivo* spleen cells ($p < 0.01$) (Figs. 2 and 3). These effects were attributable to the activity of suppressive motifs because control ODN did not reduce the cytokine production elicited by co-administered LPS (Figs. 1–3).

The $(TTAGGG)_4$ multimers inhibited LPS-dependent up-regulation of co-stimulatory and surface marker molecules on antigen-presenting cells (CD40, CD86, and ICAM-1), IgM production by B cells, and NO release from peritoneal macrophages ($p < 0.01$) (Fig. 4). Furthermore, co-administration of LPS (50 μg) with A151 ODN (250 μg) inhibited >65% of several immunoregulatory and inflammatory cytokines (*i.e.* IL-6, IL-10, and IL-12; $p < 0.001$) (Fig. 4). This reduction reached >85% for IFNγ (176 ± 29 and 26 ± 15 ng/ml for LPS and A151 + LPS groups, respectively).

DISCUSSION

In this study, we examined the effect of synthetic telomeric repeat units (suppressive A151 ODN) localized at the end of mammalian chromosomes on EIU, which is an established animal model of acute ocular inflammation in both mouse and rabbit models. The results indicated that suppressive ODN was able to down-regulate the expression and protein levels of several proinflammatory and immunoregulatory cytokines/chemokines at local and systemic levels when administrated (i) prior to, (ii) simultaneously with, or (iii) even after LPS challenge (Figs. 2–4).

Compared with local injection of (intraocular) LPS and/or LPS- and control ODN-treated rabbits, suppressive ODN-administrated animals exhibited reduced levels of IL-1β and IL-6 expression in the iris and cornea, respectively. In the mouse model, the results revealed that pretreatment with 250 μg of suppressive ODN reduced the expression of IP10, iNOS, MIP1α, IL-18, MIP3α, CXCL16, and MIP1β in 100-μg LPS-injected mice. In another experiment, with different doses of suppressive ODN and LPS, suppressive ODN also down-regulated the expression of MCP1, which is an important chemokine for monocyte chemoattraction (data not shown). The suppressive action of this class of ODN was not only on the mRNA levels of several Th1-type cytokines and chemokines but also on the secreted protein level. ELISA experiments showed that suppressive ODN pre- and post-treatments significantly diminished IL-6 secretion at 6 and 24 h; simultaneous administration of suppressive ODN also reduced IL-6 production.

Several studies indicated that suppressing proinflammatory cytokines, including IL-6, TNFα, MCP1, and iNOS, prevents the development of EIU (26, 29, 30, 31). Here, we have shown that either local (intraocular) or systemic (intraperitoneal) administration of suppressive A151 ODN can significantly reduce several proinflammatory cytokines and chemokines even 2 h after *in vivo* LPS challenge.

Corticosteroids and chemotherapeutic agents are currently in use in uveitis therapy (35). However, long-term treatment with these drugs may have grave side effects such as increased intraocular pressure (36) and cytotoxicity (37) and thus limit their use (25, 33, 35). Therefore, a new therapeutic strategy is urgently needed (38, 39). The mechanism of action of this novel ODN-based immunosuppressive drug candidate is currently unknown. Previous studies revealed that suppressive ODN can inhibit immune response by blocking the stimulatory effects of CpG motifs (1, 15). It also has been shown by Shirota *et al.* (10) that suppressive A151 ODN can also protect mice from lethal endotoxic shock that is induced by LPS. It has been shown that suppressive ODN can also inhibit several signal transduction cascades related to the production of Th1 cytokines such as IFNγ and IL-12 by binding and inhibiting the phosphorylation of STAT1 (signal transducer and activator of transcription 1) and STAT4 proteins (10, 13). Our study has demonstrated that suppressive ODN can block immune responses mediated by endotoxin in the eye (an immune privileged site), an established animal model of acute ocular inflammation. Recently, Fujimoto *et al.* (40) reported that suppressive A151 ODN can inhibit ocular inflammation in two murine models, IRBP (interphotoreceptor retinoid-binding protein)-induced experimental autoimmune uveitis and adoptively transferred ocular inflammation. These forms are antigen-driven and, compared with LPS, are significantly less aggressive forms of experimental uveitis models. The control of LPS-mediated EIU at both the local and systemic levels has not been studied by others and increases the breadth of the suppressive ODN-mediated therapy for the eye. Collectively, these observations support the provocative possibility that the evolutionary expansion of TTAGGG repeats in telomeres, in addition to known properties such as, protecting genomic DNA from degradation, and chromosome capping (41, 42) may also be linked to their ability to down-regulate sustained/pathologic microbe-associated molecular pattern-induced immunity. In conclusion, we have provided evidence that suppressive A151 ODN is able to significantly reduce the ocular inflammatory responses in both rabbit and murine EIU models.

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Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles

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Immunogenicity

ABSTRACT

Immunostimulatory properties of mushroom derived polysaccharides (PS) as stand-alone agents were tested. Next, PS were nanocomplexed with polyI:C (pIC) to yield stable nanoparticles around 200 nm in size evidenced by atomic force microscopy and dynamic light scattering analyses. PSs were selectively engaged by cells expressing TLR2 and initiated NFκB dependent signaling cascade leading to a Th1-biased cytokine/chemokine secretion in addition to bactericidal nitric oxide (NO) production from macrophages. Moreover, cells treated with nanoparticles led to synergistic IL6, production and upregulation of TNFα, MIP3α, IFNγ and IP10 transcript expression. In mice, PS-Ovalbumin-pIC formulation surpassed anti-OVA IgG responses when compared to either PS-OVA or pIC-OVA mediated immunity. Our results revealed that signal transduction initiated both by TLR2 and TLR3 via co-delivery of pIC by PS in nanoparticle depot delivery system is an effective immunization strategy. The present work implicate that the PS and nucleic acid based nanoparticle approach along with protein antigens can be harnessed to prevent infectious diseases.

1. Introduction

Toll-like receptors (TLRs) are the most extensively studied pathogen recognition receptors (PRRs) that recognizes specific microbial associated molecular patterns (MAMPs) including lipopolysaccharides, peptidoglycans, lipoproteins, flagellin, unmethylated CpG motifs or viral RNA/DNA that triggers innate immune response [1–3]. TLRs are subcategorized as endosomal or intracellular receptors depending on their site of expression. TLR3, 7/8 and 9 are specialized to sense pathogens via their nucleic acids moieties [4]. TLR3, a member of endosome-associated TLR is activated directly in response to dsRNA, or synthetic poly(riboinosinic:polyribocytidylic acid) (pIC) [5] and are harnessed as vaccine adjuvant, anti-cancer or anti-allergic therapeutic agents. When these ligands are given *in vivo*, they are rapidly cleared by nucleases, and could be adsorbed by serum proteins hampering their *in vivo* therapeutic applications [6]. In order to improve their immune stimulatory potential repeated high doses are necessary, however, in many instances this may cause undesirable side effects including septic shock [7]. Nucleic acid backbone modifications such as phosphorothioate linkages are another widely accepted approach but this not only induces undesirable side effects such as granuloma formation, temporary splenomegaly, and lymphadenopathy but also increases the cost of the final product [8–11].

Several strategies were proposed as carriers for labile nucleic acids attempting to increase their *in vivo* performances [12–14]. Among many, liposome encapsulation, biodegradable nano/micro carriers and soluble macromolecules with cationic moieties were widely studied [12–14]. We and others demonstrated that, uptake, duration and *in vivo* immunostimulatory activity of oligodeoxynucleotides enhanced when encapsulated in sterically stabilized cationic liposomes (SSCL) [15–17]. Although co-administration of pIC with liposomes induced anti-viral immunity and activated effective $CD8^+$ T cells *in vivo* [18], still several obstacles prevent these depot delivery systems enter into clinic. Of note, batch to batch variation, limited shelf-life, difficulty in reproducible reconstitution and sterility are the major concerns from FDA stand point. Furthermore, organic solvents used during the production of certain formulations are of major concern [19–21].

In the present study, natural amphiphilic polysaccharides purified from mushrooms were studied to assess their immunostimulatory

Abbreviations: AFM, Atomic Force Microscopy; BMDC, Bone marrow derived dendritic cell; HEK, Human embryonic kidney; NOD, Nucleotide-binding oligomerization domain; ODN, Oligodeoxynucleotide; PEC, Peritoneal exudate cells; PGN, Peptidoglycan; pIC, Poly(riboinosinic:ribocytidylic acid); PS, Polysaccharide.

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potential. Later, a nanoparticle delivery system was formulated to efficiently harbour pIC. The TLR2 mediated PS targeting and subsequent initiation of signal transduction cascade and ability of the nanoparticles to induce synergistic immune activation was evaluated. Furthermore, in an immunization study in mice co-delivering a model protein antigen ovalbumin (OVA), immunogenicity of PS-OVA-pIC nanoparticles was compared to either PS-OVA or pIC-OVA mediated immune response.

2. Materials and methods

2.1. Materials

All cell culture media components were from Hyclone (USA). Cytokine, IgG ELISA were from Endogen and Southern Biotech, respectively (USA). TLR ligands were obtained from several vendors: peptidoglycan (PGN) (Fluka, Switzerland), pIC (Amersham, UK). β-Glucan based polysaccharides were provided by Prof. Oktay Erbatur (Cukurova University, Chem Dept., Adana, Turkey). A high pressure/high temperature stainless steel reactor (Parr 4575 HT/HP) with 500 ml volume was used for extraction of polysaccharide at subcritical water conditions from different mushrooms. The sugar content of the extracts was determined by HPLC (Varian Prostar210, equipped with a RI detector) against monosaccharide standards and protein determination by Lowry assay. Monosaccharide analyses of the PS yielded mainly glucose, in addition to mannose and galactose. The purity was found to be >93%. Following protease digestion to eliminate protein contamination purity reached over 97%. Molecular weight determination was carried out by high performance size exclusion chromatography (on a HPSEC-MALLS system). In this study, polysaccharides were abbreviated as PS1 to PS4. Their physicochemical characteristics were as follow: i) *Ganoderma lucidum* (Alata strain)-PS1 (Ave. MWt: 2.9×10^6 Da and pKa: 6.64), ii) *G. lucidum* (Balcalı strain)-PS2 (Ave. MWt: 3.8×10^6 Da and pKa: 6.99), iii) *Shiitake*-PS3 (Ave. MWt: 1.6×10^6 Da and pKa: 6.67), and iv) *G. lucidum* (Alata strain)-PS4 (Ave. MWt: 5.2×10^6 Da and pKa: 6.69). Endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml). PS nanocomplexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4 °C. Unbound pIC was filtered (Microspin G-25 column) free nucleic acid concentration was measured by Nanodrop™ from the eluent. pIC incorporation was over 90% in all preliminary trials.

2.2. Mice

Adult C57BL/6 and BALB/C mice (female, 6–8 weeks old) were housed in Department of Molecular Biology and Genetics, facility and were provided with unlimited access of food and water. All experimental procedures were approved by the animal ethical committee of Bilkent University (Bil-AEC/Protocol#: 2006/027). RAW 264.7 cells (ATCC) or splenocytes were cultured with RPMI 1640 supplemented with 5% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0,11 mg/ml sodium pyruvate. HEK 293 or stably *hTLR2* transfected cells (Invivogen, USA), were kept in high-glucose DMEM media with 10% FBS,

2.3. Immunization

Adult female C57/BL6 mice (5/group) were injected ip with of PS, pIC (15 μg each) or PS-pIC nanocomplex combined with 7.5 μg of OVA. One day before booster injection ($@d = 13$) animals were bled and next day injected with the same formulations. On day 28 mice were bled. Sera from the primary and secondary bleedings were studied for total IgG, IgG1, and IgG2a by ELISA.

2.4. ELISA and NO assays

Immulon 2 HB microtitre plates (Thermo Scientific, USA) were coated with anti-cytokine or anti-IgG antibodies and then blocked with PBS-BSA 1% [17,35]. Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine Ab (TNFα: XT22 and IL6: 2OF3 clones) followed by phosphatase-streptavidin (Perbio Pierce, USA) whereas bound IgG subclasses were detected using phosphatase-conjugated anti-IgG, IgG1, and IgG2a antibodies as described elsewhere [17]. Nitric oxide detection by Griess method was conducted on RAW 264.7 cells (10^5/ml) after 6–48 h of incubation as described by the suppliers (Promega WI, USA).

2.5. Luciferase assay

hTLR2 expressing HEK cells were transfected using FuGENE6 with p5xlucNFκB luciferase as described in manufacturer's protocol. Following overnight stimulations, cells were further incubated for 24–36 h and were harvested and assayed for luciferase activity (Roche, Germany). Plots were generated from relative light units.

2.6. Cytokine and chemokine RT-PCR

Total RNA was extracted from the cells that were treated either with PS or with pIC nanocomplexes for 2–6 h. They were reverse-transcribed and amplified to obtain cDNA in a standard PCR reaction for 30 cycles using primers for murine specific target genes (Supplementary Table S2) as previously described [35,36]. PCR amplified material was separated on 2% agarose gels and visualized under UV light after ethidium bromide staining.

2.7. Atomic force microscopy (AFM) and size measurement studies of the nanocomplexes

pIC, PS4 and their nanocomplexes were diluted in DNase/RNase free H_2O and were deposited on silicon 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 1×1 μm. Particle size analyses of the generated nanocomplexes were measured using dynamic light scattering method on a zetasizer (Model: Nano ZS, Malvern, UK).

2.8. Statistical analysis

Statistical significant differences between groups were determined using Student's t- test analysis via SigmaSTAT software. P values < 0.05 were considered significant.

3. Results

3.1. TNFα and nitric oxide production by polysaccharides

Initial experiments were designed to understand the immunostimulatory potential of the four PS candidates in dose-titration (20–0.02 μg/ml) assays. A well established positive control peptidoglycan (PGN) was run in parallel to compare the response raised by β-Glucan polymers. Fig. 1 shows that when RAW cells were treated with different PSs for 24 h, a dose dependent TNFα (Fig. 1A) and NO (Fig. 1B) production were induced especially by PS2 and PS4. PS4 was the most active among other tested PSs at doses

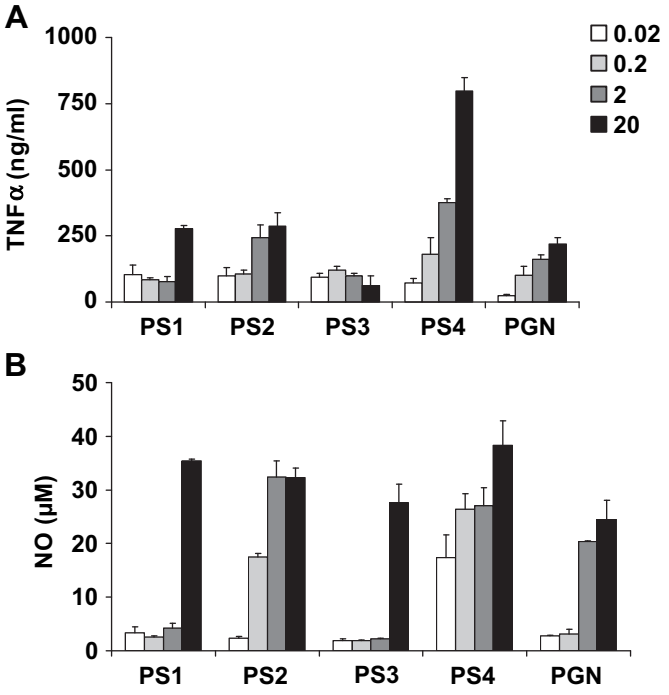

Fig. 1. Dose dependent TNFα, and NO, induction from RAW cells following 24 h post-stimulation with different β-Glucan polymers. A) TNFα and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean ± SEM) of triplicate samples treated with different stimulants (0.02–20 μg/ml). PS4 vs PS2 comparison gave a $p < 0.001$ for TNFα (at all doses) and $p < 0.004$ for NO (at 0.02 μg/ml).

greater or equal to 0.2 μg/ml (Fig. 1A). In the case of NO induction PS4 was superior (at all doses) than the rest of the stimulants including positive control PGN (Fig. 1B).

3.2. PS mediated upregulation of various cytokine/chemokine transcripts

To examine the kinetics of PS driven immune activation RAW cells were incubated with different β-Glucan stimulants. The culture fluids collected at different time intervals (6, 12, 24, 36, and 48 h after stimulation) after 2 μg/ml PS treatments were assayed for TNFα (Fig. 2A) and bactericidal mediator NO (Fig. 2B). As seen in Fig. 2A, as early as 6 h post-stimulation all PS types secreted substantial amount of TNFα. Among the tested groups, only PS4 type continued to increase TNFα level by 48 h. Similarly, nitric oxide secretion profiles of different PS types gave a time-dependent response. Again, consistent with Fig. 1, only PS2 and PS4 (Fig. 2B) displayed time-dependent NO production from macrophages. Also these β-Glucans' effects on mRNA transcript upregulation of TLRs as well as various cytokines were assessed after 4 h incubation. When TLR transcript levels of an untreated mouse splenocytes were compared to that of PS treated cells' transcript levels PS2 and PS4 treatment highly upregulated expression of *tlr1, 2, 5, 7,* and *9* genes (Supplementary Figure S1A). Moreover, compared to untreated vs PS treated cells, mRNA band intensities for *il-15, il-18,* and *TNFα* significantly increased only upon PS4 treatment (Supplementary Figure S1B). These results suggested that PS4 and then PS2 polysaccharides were the most potent stimulants.

3.3. Engagement of PS by TLR2

Several β-Glucan polymers were reported to trigger signaling cascade either using cell surface expressed TLR2 (alone or engaging TLR1 or TLR6), TLR4 or even cytosolic sensors such as NOD1 and NOD2 [22,31]. To differentiate between these alternatives HEK cells stably expressing hTLR2, hTLR4 or TLR2/6 genes were transfected with p5xlucNFκB and in subsequent experiments co-transfected with pcDNA3NOD1 or pcDNA3NOD2 or mock plasmids to check whether these PS polymers either engages only through TLRs or additionally interact with the cytosolic NOD sensors. In Fig. 3, NFκB-mediated luciferase activity studies demonstrated that PS triggers via TLR2 receptor (Fig. 3A) and furthermore regulates IL8 production (Fig. 3B). Expression of TLR6 did not improve TLR2 mediated promoter activation (*data not shown*). Consistent with previous results, PS4 is the most potent inducer. Of note, other TLRs or NODs were not contributing to this immune cascade since subsequent experiments demonstrated that there was no further significant increase (either luciferase activity or IL8 production) mediated by PS treatment of cells expressing TLR4, or NOD1 or NOD2 (*data not shown*).

3.4. PS4 activity upon in vivo administration to mice

These four PSs were given to mice (2 μg/ml) and 6 h later PEC and spleen cells were recovered and incubated in culture without further stimulation to evaluate cytokine production or chemokine message transcript upregulation by ELISA and RT-PCR, respectively. Consistent with previous observations, PS4 was the most active stimulant among the tested polysaccharides. It yielded the highest IL6 production from PECs after 48 h *ex-vivo* incubation (Fig. 4A). The spleen cells *TNFα* and *IP10* transcript levels were significantly upregulated when PS1, 2, and 3 vs PS4 band intensities were

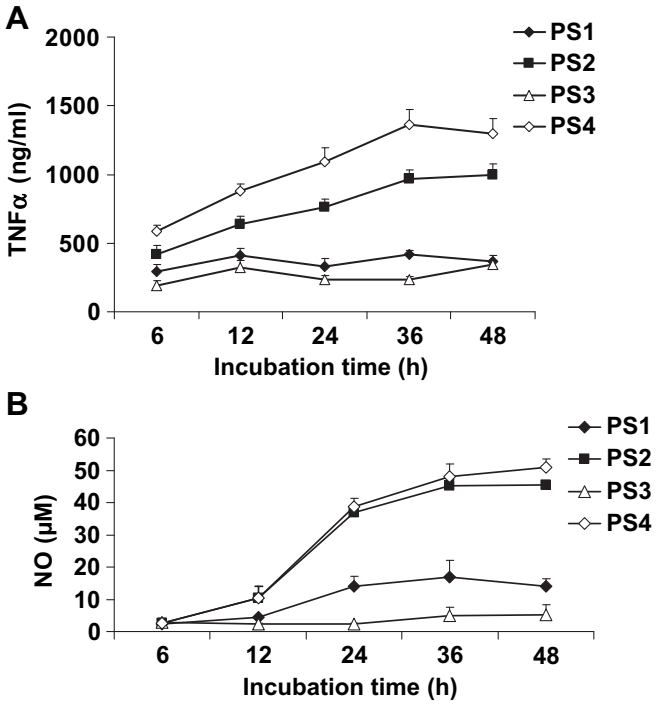

Fig. 2. Time dependent TNFα, and NO release by RAW cells following different PS treatments at 2 μg/ml. A) TNFα and B) NO levels were assessed by ELISA and Griess assay respectively. Result represents combination of at least three independent experiments (mean ± SEM) of triplicate samples treated at different time points (6 h, 12 h, 24 h, 36 h & 48 h). PS4 vs PS1, PS2 (except 12 h) or PS3 comparison gave a $p < 0.01$ for TNFα (at all time points) and for NO, PS4 vs PS2 is NS, and PS4 vs PS1 or PS3 is $p < 0.001$ (at all time points except 6 h).

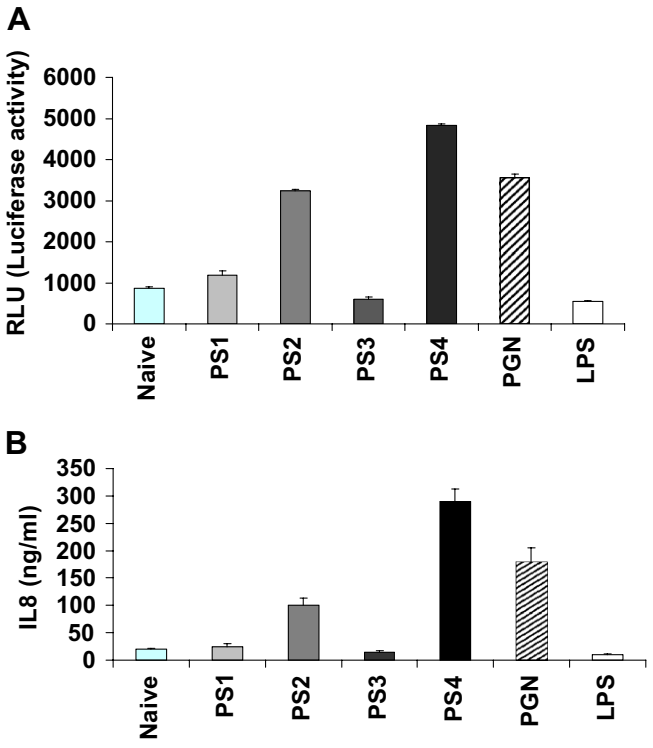

Fig. 3. PS mediated promoter assay and IL8 production by *hTLR2*-HEK cells. A) Relative luciferase activity after PS treatment of stable *hTLR2*-HEK cells. Cells were transfected with p5xlucNFκB plasmid for 24 h and stimulated with 5 μg/ml of each PS for 12 h. B) IL8 production following PS treatment. Stable *hTLR2*-HEK cells were stimulated with 5 μg/ml of each PS for 24 h. IL8 ELISA was studied from the culture supernatants. Data represents combination of at least two independent experiments (mean ± SEM) of triplicate samples treated with different stimulants (PS1-4, PGN and LPS). PS4 vs PS1, 2, 3 and LPS gave a $p < 0.001$.

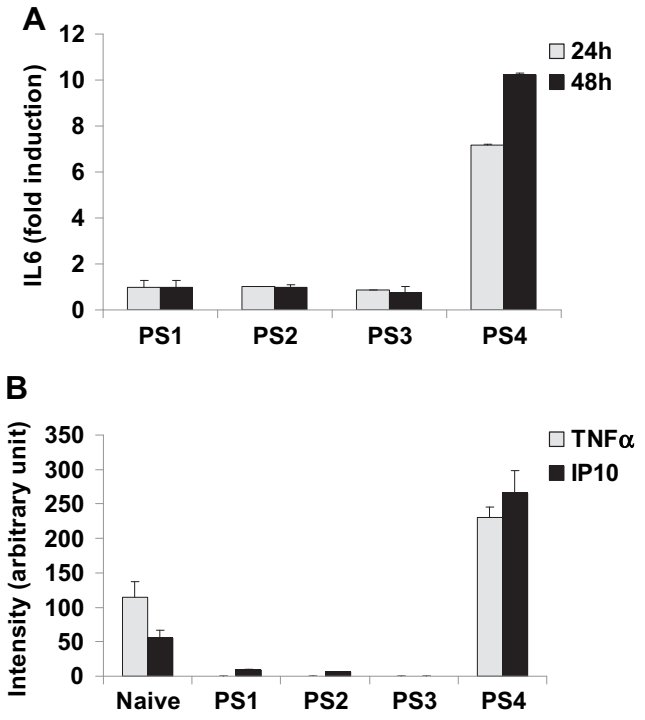

Fig. 4. *In vivo* immunostimulatory activity of polysaccharides. A) Six hours after i.p. PS injection (2 μg/ml), PECs were isolated (3 mice/group). Cells (4×10^6/ml) were incubated *ex-vivo* for further 24 h or 48 h, IL6 levels from supernatants were measured by cytokine ELISA. Fold induction of IL6 production over naive cells was plotted (naive cells induced 56 ± 13 @ 24 h and 164 ± 23 @ 48 h pg/ml IL6). B) mRNA levels of TNFα and IP10 were assessed by RT-PCR from spleen cells. Densitometric measurements (obtained from 3 independent mice) for the gel band intensities were plotted. PS4 vs PS1, 2 and 3 gave a $p < 0.001$.

compared, (Fig. 4B). Although previously PS2 activity in culture was similar to PS4, surprisingly it failed to reproduce this trend when administered *in vivo*. When *in vitro* and *in vivo* data are collectively considered, among four tested PS candidates, PS4 consistently displayed the highest immunostimulatory performance, and was selected to develop PS4-pIC nanoparticles.

3.5. Physical characterization of PS4-pIC nanocomplexes

When nucleic acids are injected *in vivo* they are either subjected to premature digestion by nucleases or adsorbed by serum proteins, therefore, hampering their bioavailability. Accumulating evidence strongly suggested that their *in vivo* applications are limited due to their labile nature. Strategies to improve their stability as well as retaining activity and facilitate their cellular internalization without premature clearance are of great importance to pursue these ligands into clinical use [15,17]. To establish whether PS4-pIC complexes form stable nanoparticles their physical characteristics by i) AFM and by ii) dynamic light scattering techniques (Zetasizer Nano, ZS™) were studied. AFM photomicrographs revealed that PS4 appeared as individual nanoparticles around 50–70 nm in size (Fig. 5). This was further confirmed by Zetasizer and was found to be ca. 40 ± 20 nm (Ave ± SEM) (Supplementary Figure S2). PS4 nanocomplex with pIC led to an increase in the nanoparticle size from 40 nm to 165 nm (Supplementary Figure S2). The stability of these nanocomplexes was also studied by dynamic light scattering method using Zetasizer. There was no significant change in the size of the generated nanocomplexes over a period of two weeks indicating that there was no spontaneous fusion (or disintegration) of the individual particles when suspended either in PBS or in FBS supplemented media (Supplementary Table S1, and data not shown).

3.6. Synergistic immune activation by PS4-pIC nanoparticles

Next we analyzed the immunostimulatory effect of PS4 nanocomplexes harbouring one of the labile nucleic acid ligands, pIC. Whether it improves pIC dependent immune activation to that of its free form is of great interest. As presented in Fig. 6, PS4-pIC nanoparticles induced synergistic activation on both RAW 264.7 or mouse spleen cells upon *in vitro* or *in vivo* treatments. PS4–pIC nanocomplexes led to a significantly high IL6 production (Fig. 6 and data not shown). It is important to note that pIC alone, when given at this concentration could not produce any detectable IL6, however, the nanoparticles yielded 1250 ± 57 ng/ml of IL6 secretion. This amount is >6x fold more cytokine production to that of PS4 alone stimulation (Fig. 6A left panel). Furthermore, macrophages stimulated by these nanocomplexes induced very strong bactericidal mediator, NO compared to either of the PS4 alone or pIC alone treatments (Supplementary Figure S3). When spleen cells were incubated with PS4-pIC nanocomplexes, the gene message levels of *tnfa, il15, il18, mip3α, and ip10* were significantly upregulated (compared to either PS4 alone or pIC alone treatments, Fig. 6B). The activity of these nanocomplexes were further analyzed following ip injection of mice (3/group) and checking IFNγ transcript level from recovered spleen cells (Fig. 6C). Consistently, PS4-pIC nanocomplex treated animals displayed the highest *ifnγ* message. These results strongly support the synergistic activation mediated by the nanocomplex.

3.7. Immunization with PS4-pIC nanoparticles

To examine whether the PS4-pIC nanoparticles could act as an effective immune adjuvant/delivery system, ovalbumin (OVA) was loaded as the model antigen and C57BL/6 mice were immunized. Primary and secondary bleeding sera were analyzed to detect the levels of total IgG, IgG1 and IgG2a. As seen in Fig. 7, total IgG and anti-OVA specific IgG subtypes were significantly boosted by the PS4-OVA-pIC nanoparticles when compared to dual combination groups (i.e. pIC + OVA or PS + OVA). This was evident even after primary immunization, since there was 2–3-fold more anti-OVA Ab induction at 1/2000 antibody titre, (Fig. 7A). As expected, significantly higher magnitude of total IgG, IgG1 as well as IgG2a titers were obtained after booster injection. In Fig. 7B, at titre 1/8000, all IgG types were improved at least 2- fold and up when mice were immunized with the nanocomplex. These data implied that generated stable nanoparticles ensured simultaneous internalization of the adjuvant (signal 1) and antigen (signal 2) by antigen presenting cells (APCs), thereby led to stronger anti-OVA immunity.

4. Discussion

Natural polysaccharides extracted from mushrooms was shown to activate host immune system leading to production of various Th1-biased cytokines [23]. One approach to induce an improved immune response is to ensure that these formulations achieve simultaneous presentation of antigen and adjuvant to relevant APCs. Developing such systems that act in synergy with the loaded adjuvant may further improve the success of the therapy and help host defence [14,24–26].

In this study, PS/Nucleic acid ligand nanocomplexes were designed to solve several key problems associated with poor *in vivo* performance. Polysaccharides used in this study are; i) naturally occurring, readily available, easily extracted in bulk ii) cheap iii) does not require sophisticated formulation technology as it is water soluble iv) readily undergoes stable interaction with charged

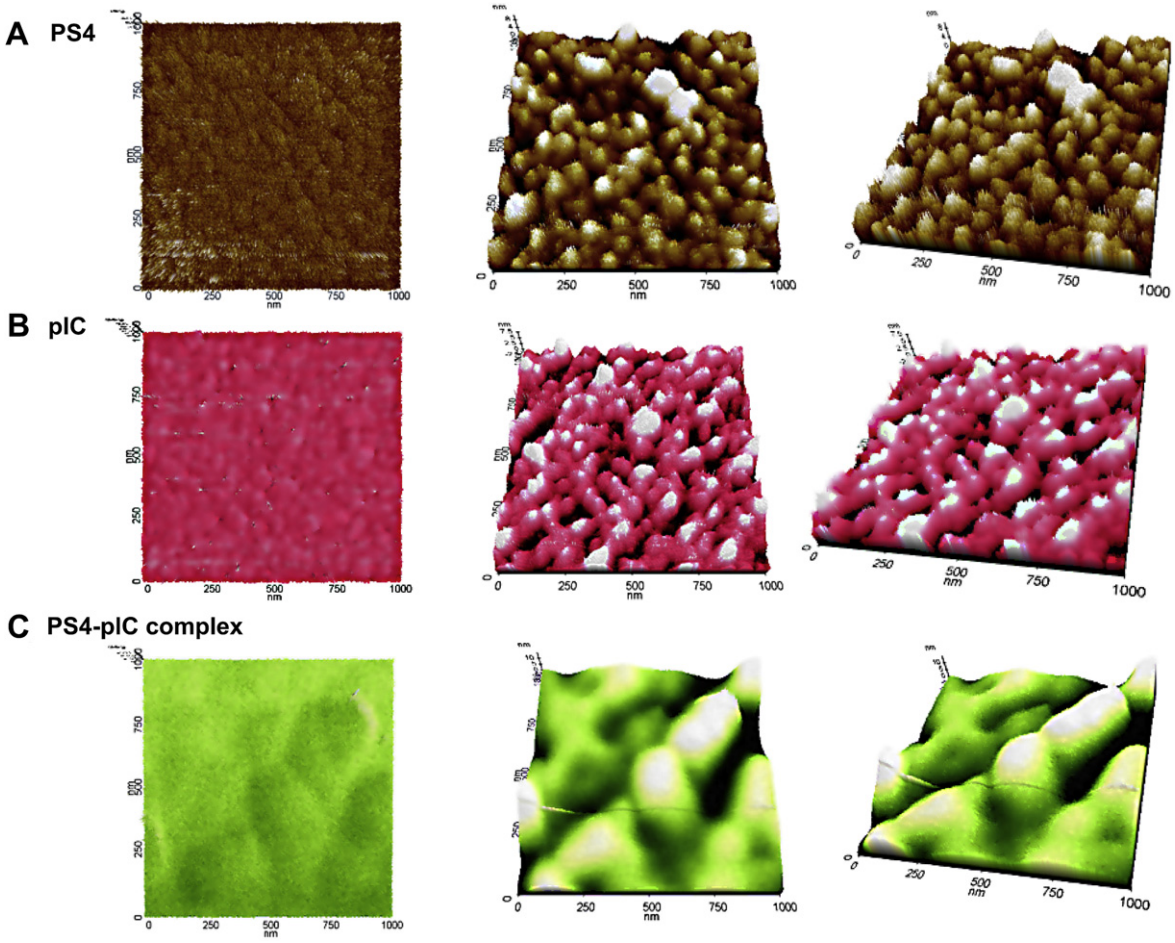

Fig. 5. AFM photomicrographs of phase, 2D phase (left) and 3D topography (middle and right) images. A) PS4, B) pIC and C) PS4-pIC nanoparticles at 1000 nm^2 area. Images were recorded under contact mode.

macromolecules due to their amphiphilic nature v) effectively engage by cells expressing TLR2 on their surface (i.e. natural targeting), vi) the labile cargo is protected from premature degradation/elimination and finally vii) act as a co-adjuvant in vaccine formulations along with the adjuvant to augment Ag-dependent immune activation. Our findings strongly suggest that all these key features are fulfilled by the developed formulation.

Recent studies showed that a natural β-glucan polysaccharide isolated from *Schizophyllan* modified with peptides and cholesterol formed a complex with TLR9 ligand, CpG ODNs and significantly elevated secretion of cytokines (IL12, IL6 and TNFα) from BMDCs, and murine macrophages [27,28]. These compounds were proposed to trigger several immune cells through various receptors including complement receptor (CR3), scavenger receptors, as well as TLR4 or TLR2/6 [29–32]. In other study, *G. lucidum* polysaccharides enhanced CD14 mediated endocytosis of LPS and promoted TLR2/4 signal transduction of cytokine expression [31].

Contrary to previous reports, our findings suggested that mushroom derived PS polymers recognized only via TLR2 but not via TLR4 or TLR2/6. Furthermore, to date, there is no document reporting the collaborative activation of TLR3 ligand with PS obtained from edible mushrooms. Moreover, in the present study plain PS compounds were used with no further modification (i.e cholesterol or peptide). The cytokine secretion panels (dose-dependent and time-dependent findings) presented in Figs. 1 and 2 clearly suggested that PS4 is the most potent stimulant. Moreover, in HEK cell system we established that these PS types only interact with TLR2 (Fig. 3) but not with other cell surface or cytosolic receptors (NOD1 or 2). In the literature, receptor usage of PS is still elusive. This is probably due to the problem associated with the extraction of PS from different sources. Similar compounds were reported to trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells through various receptors including Dectin-1 and scavenger receptors [31,32]. We have attempted to delineate the contribution of PS mediated activation via the scavenger receptor CXCL16. It was found that it does not involve in PS mediated cytokine production, since there was no up or down regulation of the *cxcl16* gene transcript (Supplementary Figure S2). However, Dectin-1 has not been studied here, and must be checked to resolve its involvement.

We further assessed the nitric oxide production mediated by different PS compounds. Since NO is an important regulator and mediator of macrophage directed cytotoxicity against microbes and tumour cells, detecting the most potent NO inducer as well as establishing the breadth of activation pattern of PS4 was very critical [33,34]. The results of *in vitro* findings were reproduced *in vivo* (Figs. 1, 2 and 4). Among all tested PSs, consistently PS4 triggered the highest IL6 production in addition to NO secretion as well as enhanced several key cytokine/chemokine gene expression in PECs and splenocytes. This is the first example to demonstrate that a polysaccharide extracted from mushrooms induced strong NO production from immune cells.

PS4-pIC nanoparticles supported the added effect of formulating them as a carrier depot system. In addition to IL6 production by

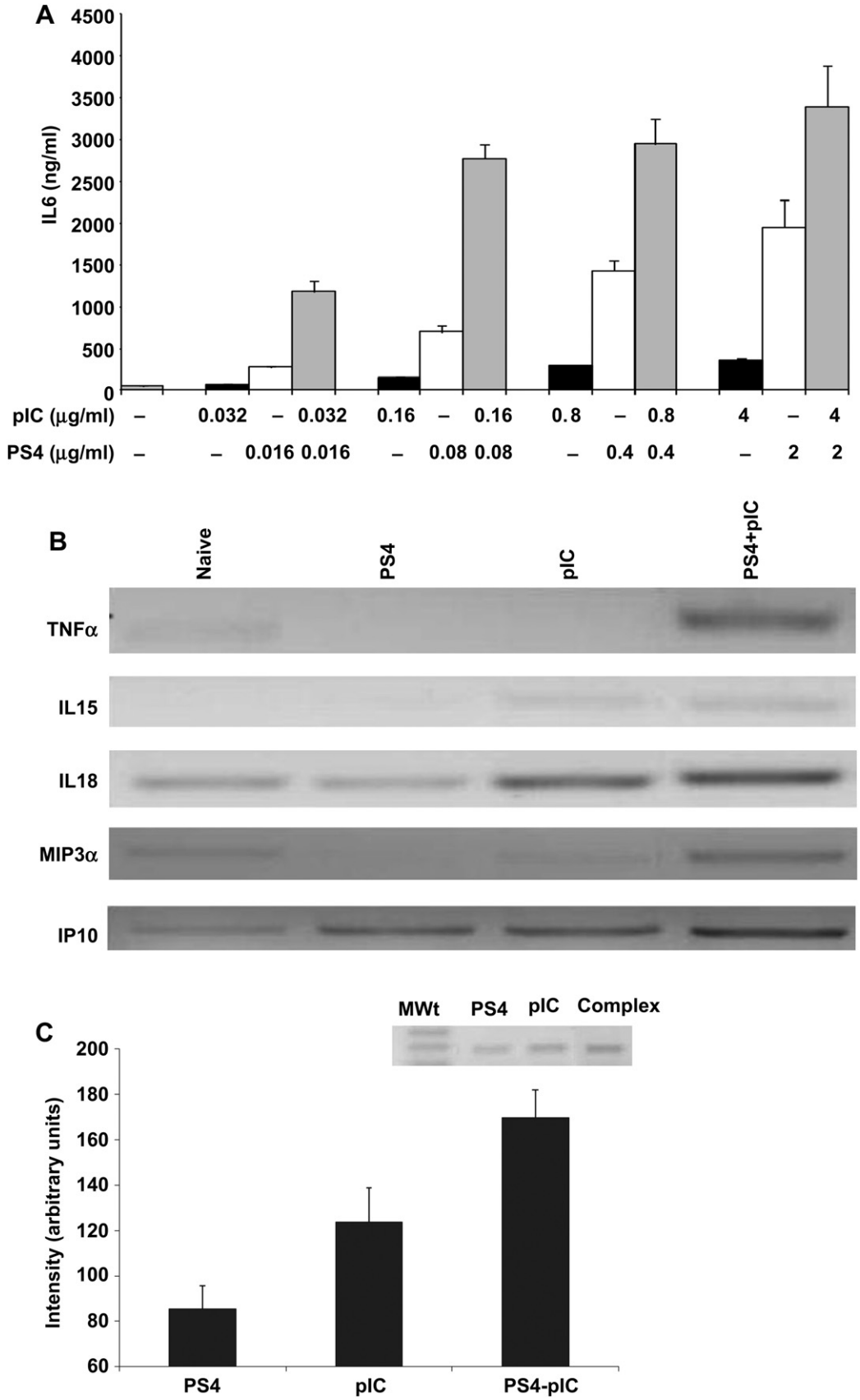

Fig. 6. PS-pIC nanoparticles induced higher IL6 production and strong gene upregulation of various cytokine and chemokines after *in vitro* and *in vivo* stimulations. A) RAW cell supernatants from 36 h culture were analyzed by ELISA. Result represents combination of at least two independent experiments (mean ± SEM) of triplicate samples. $p < 0.002$ nanocomplexes vs nucleic acid alone treated groups. B) After stimulation with PS4 (0.016 μg/ml) or PS4-pI:C (0.16 μg/ml each) for 4 h in culture, total RNA from spleen cells were subjected to RT-PCR and cDNA were amplified against gene-specific primers. C) PS4-pIC nanoparticles (2 μg each) was injected to mice and spleen cells were recovered 18 h post-treatment for IFNγ transcript analyses (inlet; representative band intensities from a single animal). Band intensities were reported as average of 3 mice/group.

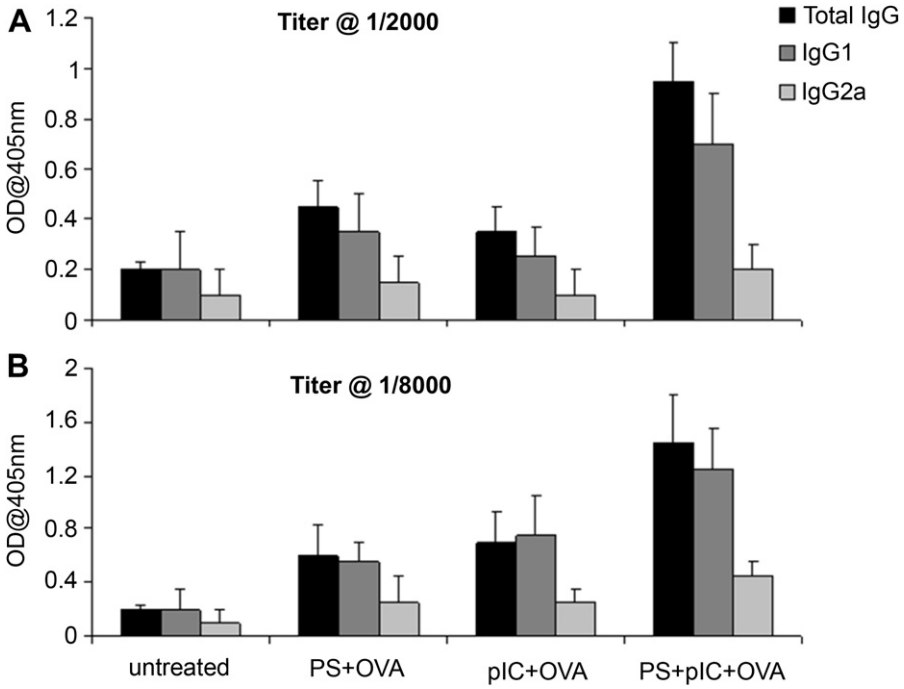

Fig. 7. IgG anti-OVA response of immunized mice. Animals were injected i.p with 15 μg of PS4 + pIC combination or PS4-pIC nanoparticles incorporating 10 μg OVA. Serum anti-OVA titers at day 13 (A) and day 28 (B) were determined by Total IgG, IgG1 and IgG2a ELISA. Data represents the average ± SD serum IgG anti-OVA response (five mice per group).

immune cells compared to their alone treatments (Fig. 6A), data also demonstrated that several cardinal chemokine and cytokine mRNA messages were upregulated (Fig. 6B). The *in vivo* benefit of PS4-pIC nanocomplexes were documented in *ex vivo* study along with model antigen immunization study. Data suggested that these formulations could improve primary and booster immunity against OVA antigen (Fig. 7). This is primarily due to the fact that, the present formulation helped avoiding premature clearance and digestion of its cargo as well as increased stability and efficient delivery of OVA + adjuvant simultaneously to APCs, thereby resulting more pronounced immune response.

5. Conclusions

The present work established that combination of mushroom derived polysaccharide and pIC forms stable nanocomplexes and triggered upregulation of inflammatory cytokines, and bactericidal mediators as evidenced by dose and time-dependent kinetic profiles of, IL6, IL18, TNFα and NO productions. Immune enhancement of PS is dependent on TLR2 signaling, compounded by the pIC mediated TLR3 signaling resulted stronger pro-inflammatory effect of the formulation. When used *in vivo* as a protein vaccine carrier, nanocomplex led to elevated IgG titers against ovalbumin antigen in mice, compared to either PS + OVA or pIC + OVA immunizations. In conclusion, present system can be formulated with other clinically valuable bioactive agents such as siRNA, plasmids, and different nucleic acid based TLR ligands, along with peptides or proteins as vaccine carriers to control infectious pathogenic insults.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biomaterials.2011.01.028.

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ORIGINAL ARTICLE

Immunomodulatory function and in vivo properties of *Pediococcus pentosaceus* OZF, a promising probiotic strain

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Abstract Some of the important properties of probiotics are the ability to survive during gastrointestinal transit and to modulate the immune functions. The objectives of the reported study were to assess in vivo gastrointestinal survival of orally administered *Pediococcus pentosaceus* OZF using an animal model BALB/c mice, and to examine its effects on the immune response. Following oral administration to mice, the ability of *Pediococcus pentosaceus* OZF to pass and survive through the mouse gastrointestinal system was investigated by analyzing the recovery of the strain in fecal samples. Microbiological and polymerase chain reaction (PCR) methods proved that the strain OZF could overcome specific conditions in the gastrointestinal tract of mice and reach the intestine alive after ingestion. To observe the effect of oral administration on immune response, IL-6, IL-12 and IFN-γ were measured by ELISA, and the strain OZF was found to cause increases in IL-6 synthesis in regularly fed mice. However, stimulation was carried out with various concentrations of bacterial ssDNA and heat killed cells of *Pediococcus pentosaceus* OZF. The heat killed cells of the strain OZF were shown to produce IFN-γ independently from IL-12. On the other hand, a significant difference between control and experimental group was noticed when lipopolysaccharide, a TLR4 (toll like receptor) ligand, was used. Overall, *Pediococcus pentosaceus* OZF may be a valuable probiotic strain for therapeutic uses. Nevertheless, further studies on the mechanisms of immunomodulatory effect will allow for better clarification of the immune functions of this strain.

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Keywords Probiotic · Survival · Immunomodulation · In vivo

Introduction

Lactic acid bacteria (LAB) are very important in the production of many fermented foods (cheese, yogurt, etc.). Applications of these organisms are now being extended to the area of health improvement, which is known as a probiotic activity. An expert committee defined the term probiotic, popularized by Roy Fuller in 1989, as "living microorganisms, which upon ingestion in certain numbers exert health benefits to the host animal by improving its intestinal microbial balance" (Guarner and Schaafsma 1998). Several aspects, including general, functional and technological characteristics, have to be taken into consideration in the selection process of probiotic strains. The criteria for selecting a good probiotic strain have been listed comprehensively by several authors (Collins et al. 1998; Salminen et al. 1998; Deshpande et al. 2011). A successful probiotic needs to be able to reach the distal part of the intestine successfully in order to have a beneficial effect (Freter 1992; Havenaar et al. 1992; Lo Curto et al. 2011). In order to survive in and colonize the gastrointestinal tract (GIT), ingested bacteria need to express high tolerance to the enzymes in the oral cavity (e.g., lysozyme), as well as to the digestion process in the stomach (e.g., exposure to low pH) and the intestine (e.g., exposure to bile); to have the ability for adhesion to the intestinal surfaces. However, it is reported that many bacteria cannot tolerate these stresses (Fujiwara et al. 2001; Suskovic et al. 2001; Iannitti and Palmieri 2010).

In recent years, the incorporation of probiotic bacteria into foods has received increasing scientific interest for health promotion and disease prevention such as anti-infection properties (Isolauri et al. 1991), beneficial effects in intestinal inflammation (Peran et al. 2005), immunomodulatory activity

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(Olivares et al. 2006), and efficacy in the prevention of allergic diseases (Furrie 2005). Our immune system is one of the most dynamic body components in determining our state of health or disease. Therefore, the immunoregulatory effects of LAB are of primary interest and nowadays there is growing interest in research of immunobiotic LAB that have an ability to modulate or stimulate the gastrointestinal immune system (Nagao et al. 2000; Sheih et al. 2001; Lomax and Calder 2009). Many researchers actively studied the immunomodulatory function of LAB, reporting selected strains as able to prevent or reduce allergies and to preserve the host from various infectious diseases and cancer (Yasui et al. 1999; Takagi et al. 2001; Shida et al. 2002; Repa et al. 2003; Fujiwara et al. 2004; Kimoto et al. 2004). Several studies in the literature focused on the ability of selected probiotics to modulate in vitro cytokine production by human or murine cells (Niers et al. 2005; Baken et al. 2006; Helwig et al. 2006; Shida et al. 2006; Perez-Cano et al. 2010). These effects range from stimulation to inhibition of several pro-inflammatory and anti-inflammatory cytokines, as well as some chemokines, and these effects are often strain-specific and each strain appears to have its own unique immunomodulatory profile (Niers et al. 2007). The pro-inflammatory cytokines secreted by the epithelium, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-8 and IL-12, are hallmarks of the inflammatory responses in the intestine (Isolauri 1999).

Recently, a new potential probiotic strain, *P. pentosaceus* OZF, has been isolated from human breast milk (Osmanagaoglu et al. 2010). The strain OZF has been shown to tolerate low pH, pepsin, bile acid and pancreatic fluid under in vitro conditions, to bind human Caco-2 cells and to exert inhibitory activity against a wide range of bacteria, including some pathogens by producing pediocin AcH/PA-1 (Osmanagaoglu et al. 2010, 2011). Since the strain was shown to fulfill in vitro probiotic selection criteria, the study was extended one step further, and this article presents results of some in vivo pre-selective studies on promising probiotic *P. pentosaceus* OZF, such as the survival in the GIT of mice and its effects on the immune system modulation.

Materials and methods

In vivo survival of *Pediococcus pentosaceus* OZF during passage through GIT of mice

Maintenance of animals Seven-week old female BALB/c mice weighing 20–22 g were purchased from Gazi University Animal Reproduction and Animal Research Center (GUDAM) and were housed in stainless-steel cages with a 12 h light/dark cycle under specific pathogen-free controlled environmental conditions (temperature 22±1 °C, humidity 55±5 °C). Mice were fed with an unlimited commercial rodent diet (Harlan, Barcelona, Spain) and water ad libitum during the experimental protocol. The protocol was carried out according to the guidelines of the Helsinki declaration and was approved by the Local Ethics Committee on Animal Experiments of the Gazi University, Ankara, Turkey (G.U.E.T., Protocol No: 07/0029).

Preparation of bacterial suspension and administration to mice *Pediococcus pentosaceus* OZF was grown in tryptone-glucose-yeast extract (TGE) broth at 37 °C for 18 h. Cells were removed by centrifugation at 3,000 *g* for 10 min, washed twice with physiological saline solution and resuspended in the same solution to a final concentration of 10^8 CFU per 200 μL. Mice in the experimental group (n=7) received a daily dose of 10^8 CFU of bacterial suspension, while the control group (n=7) received 200 μL of physiological saline solution by intragastric gavage. Sera, spleen cells and peritoneal exudate cells (PECs) obtained from mice fed 30 days with *P. pentosaceus* OZF were used for detection of in vivo immunomodulatory capacity of the strain, while feces of mice fed 5 days with OZF were used in viability (survival ability in GIT) studies.

Recovery of Pediococcus pentosaceus OZF from feces The mice were fed with bacterial suspension regulary for 5 days. The survival ability of *P. pentosaceus* OZF through the GIT was investigated every day for 5 days, by analyzing the recovery of the strain in fecal samples as bacteriocin-producing colonies. At the end of 5 days, the feeding trial was stopped, but fecal samples were collected for 5 additional days to check the colonization ability of the strain, and subjected to the same analysis. For this aim, daily collected fecal samples were pooled, resuspended in physiological saline solution (100 mg mL^{-1}) and mechanically homogenized. Dilutions were plated onto Pediococci Selective Medium (PSM) agar and incubated at 37 °C for 24 h before enumeration (Simpson et al. 2006). To verify existence of *P. pentosaceus* OZF in feces, bacteriocin assay and PCR-based methods were applied before and after administration.

For bacteriocin assay, following pour plating, the plates containing ~50 to 100 colonies were overlaid with TSB (Tyriptic soy broth) soft agar seeded with *Listeria monocytogenes* ATCC 7644 as an indicator. Plates were further incubated overnight at 37 °C and examined for the presence of an inhibition halo against *L. monocytogenes* ATCC 7644 (Biswas et al. 1991). The bacteriocin-producing colonies on PSM medium were counted as CFU mL^{-1}. Approximately 50 colonies passed through GIT were randomly picked from plates, observed microscopically, Gram-stained and then subjected to API kit for identification. Identification was confirmed by 16S rDNA sequencing using specific universal primers (Edwards et al. 1989; Osborne et al. 2005) as previously discussed (Osmanagaoglu et al. 2011). Amplified

PCR fragments were purified by PCR purification kit (Promega, Agarose Gel DNA Extraction Kit) and sequenced by REFGEN Biotechnology (METU Technocity, Ankara, Turkey). Basic local alignment search tool (BLAST) was used to compare the sequences with the one deposited for *P. pentosaceus* OZF (accession number 1337739, 706 bp) in National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov.tr/BLAST).

For *P. pentosaceus* OZF detection from feces by using PCR based methods, multiplex PCR was carried out first. DNA templates for PCR reactions were prepared from bacteriocin producing colonies. One colony was resuspended in 50 μL of sterilized TE (10 mM Tris–HCl, 1 mM EDTA pH 7.5) buffer and boiled for 10 min. Supernatant containing released DNA was directly used as template in PCR amplification. Target specific genes for pediococci, *P. pentosaceus* and pediocin AcH/PA-1 were amplified by using the method proposed by Suwanjinda et al. (2009). To determine whether or not the original OZF strain and the strains obtained in the feces following oral administration were the same strain, the recovered strains were further typed by RAPD profile. DNA was extracted by using Promega DNA isolation kit, and PCR was carried out with using different RAPD primers [OPO-09 (5′ TCCCACGCAA 3′), OPF-14 (5′ TGCTGCAGGT 3′), and OPA-07 (5′ GAAACGGGTG 3′)].

Immunomodulatory function of *Pediococcus pentosaceus* OZF

Culture medium, cytokines, antibodies and reagents All cell culture media components were obtained from Hyclone (USA). Cytokine ELISA assay reagents, recombinant mouse IL-6, IL-12 and IFN-γ, and their monoclonal and biotinylated antibodies were obtained from Thermo, Pierce Endogen and BD Biosiences, respectively. Streptavidin-alkaline phosphatase (SA-AKP) and p-nitrophenyl phosphate disodium salt substrate (PNPP) were purchased from Thermo. Lipopolysaccharide (LPS) and peptidoglycan (PGN), isolated from *Escherichia coli*, was obtained from Sigma. Immunsuppressive CpG oligodeoxynucleotide (ODN) K-type; K23 (12mer) and control ODN were chemically synthesized by Alpha DNA (Montreal, Canada).

Cell culture and stimulation assay BALB/c mice involved in control and experimental groups were used after a 30 day daily feeding period. Mice were euthanized by cervical dislocation. Blood, spleen cells and PECs were aseptically extracted and used for further analysis. Sera were obtained by centrifugation (1,000 *g* for 5 min at room temperature, RT) and stored at −20 °C until ELISA (Enzyme-linked immunosorbent assay) was carried out. Unstimulated spleen cells (4 millions of cells in 200 μL), PECs (200.000 cells in 200 μL) and serum samples of mice were used for in vivo detection of immunomodulatory function in strain OZF. In addition, stimulated forms were used to understand the differentation of immunostimulatory effect between the control and experimental group. For stimulation assay, spleen cells and PECs in 96 well plates were stimulated with three increasing concentrations of ss DNA (0.08, 0.8 and 8 μg in 200 μL) and heat killed cells of *P. pentosaceus* OZF (10^7 to 10^2CFU mL^{-1}). DNA of *P. pentosaceus* OZF was extracted with Promega Wizard DNA purification kit. To avoid stimulatory effects due to contamination of bacterial proteins or LPSs, DNA preparations used in the experiments had purity values higher than 1.8 ($OD_{260/280}$). Single-stranded DNA was prepared by heat denaturing of double-stranded DNA at 95 °C for 5 min, followed by cooling on ice. To obtain heat killed cells, the remaining culture fluid was heated to 121 °C for 15 min, and washed three times with PBS (phosphate buffer saline pH 7.2). For positive controls, LPS (5 μg mL^{-1}), PGN (5 $\mu g mL^{-1}$), CpG-ODN K23 (1 μM) and CONTROL ODN (1 μM) were used. Stimulations were performed in duplicate wells for each indicated treatment. The cells were incubated overnight at 37 °C in a 5 % CO_2 incubator and following incubation, collected supernatants were stored at −20 °C for further use.

Evaluation of cytokines production by ELISA Unstimulated and stimulated serum and cells supernatants were immediately analyzed by ELISA, to measure the IL-6, IL-12 and IFN-γ concentrations as described in Erikci et al. (2011). The optical densities of the enzymatic reaction solutions were read using an automatic ELISA plate reader (Molecular Devices, SoftmaxPro Software V5) at 405_{nm} until recombinant standards (with the starting concentrations of 4,000 ng mL^{-1} for IL-6, 100 ng mL^{-1} for IL-12 and 1,000 ng mL^{-1} for IFN-γ) reached a four parameter saturation. All ELISA assays were performed in duplicate for each group.

Statistical analysis

A student's *t* test was used to evaluate the statistical significances ($p<0.05$) of the differences in cytokine production of mice before and after exposure to tested bacterium. Values of $p<0.05$ were considered significant.

Results and discussion

In vivo survival of *Pediococcus pentosaceus* OZF during passage through GIT of mice

From a safety as well as a functional point of view, it is essential to determine if a strain survives in the GIT. In the present study, following oral administration to mice, the

ability of *P. pentosaceus* OZF to pass and survive through the mouse GIT was investigated by analyzing the recovery of the strain in fecal samples as bacteriocin-producing colonies. Clear inhibition zones characterized as bacteriocin production against *L. monocytogenes* ATCC 7644 were observed in all colonies recovered from fecal samples after administration with the strain OZF, and it has been suggested that ingested bacteria as probiotics cannot affect the intestinal environment unless their population reaches a certain minimum level of between 10^6 and 10^8CFU g^{-1} in intestinal content (Marteau and Rambaud 1993). According to PSM counts, no Pediococcus was recorded in feces of mice belonging to control groups, highlighting the lack of this genus within the intestinal flora of mice (Data not shown). Viable cells of *P. pentosaceus* OZF administered orally to mice decreased time dependently (Fig. 1). Twelve hours after the first administration, the strain OZF appeared at a level of 10^7 CFU g^{-1} in feces of all mice in the treated group and at a level of $10^6$to 10^7CFU g^{-1} after further 24 h . The strain OZF was detected in three of seven mice at gradually reduced levels at 48, 72, 96 and 120 h. This indicates that *P. pentosaceus* OZF administered to mice can survive in the stomach and reach the mouse intestine alive. However, the strain OZF was not detected in any mouse at 144 h (24 h after the administration had ceased). Feeding trials with the strain OZF showed that the promising probiotic strain disappears from the GIT within 1 day after the feeding was discontinued. This indicates that the strain could not get through to colonize in the mouse intestine and although it could reach the intestine alive, is eliminated from the intestine. This is because the normal intestinal microbiota provide an excellent resistance against colonization by introduced bacteria (Wells et al. 1988). On the other hand, pediococci were not detected in the control group. However, other studies showed that the number of probiotic bacteria detected 14 days after the administration of probiotic strains was lower than on the first day after administration (Murphy et al. 1999). This observation is supported by results of a human study completed by Goldin et al. (1992) where it was demonstrated that 60–80 % of individuals consuming *Lactobacillus rhamnosus* GG excreted the bacterium for 3–4 days, but only 33 % of the population excreted the bacterium after 7 days. In a similar study, the probiotic strain could be recovered (with no oral supplementation) from feces up to 3 days after cessation of feeding. Therefore, it appears increasingly likely that daily administration of the preferred strain is necessary for maintenance of high levels of probiotics (Murphy et al. 1999).

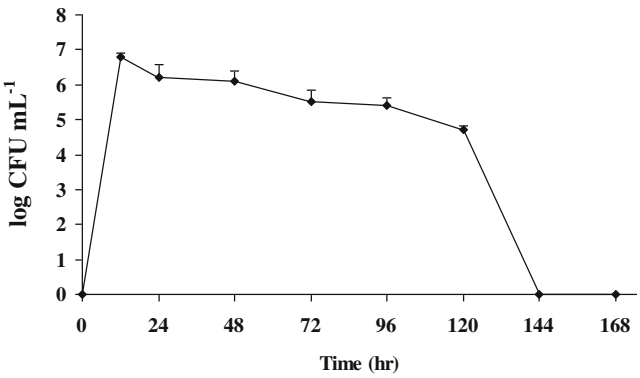

Fig. 1 Survival and time dependent changes in the fecal populations of *P. pentosaceus* OZF after oral administration to mice

By using bacteriocin-producing ability, the survival and persistence of the administered strain could be monitored after transit in the GIT of mice. Almost 50 (ten for each day of administration) randomly selected bacteriocin-producing colonies were found in tetrad morphology, Gram-positive and catalase-negative. Nevertheless, to avoid the risk of mistaking colonies of *Pediococcus* spp. grown on PSM agar medium, multiplex and RAPD PCR were used. PCR was performed on the colonies grown on PSM agar to detect live bacterial cells in feces (Fig. 2). Biochemical profiles of five randomly chosen strains were obtained with API 50CH (bioMerieux) following the manufacturer's guidelines and identified as *P. pentosaceus.* Besides, 16S rRNA sequence analysis allowed us to identify the five strains as *P. pentosaceus*, and the obtained sequences were found to be the same (99 % similarity) that have already been registered for OZF strain in GenBank database system under accession number HM051378 (706 bp). Multiplex PCR profiles of 50 randomly selected colonies from the fecal samples of treated mice group were all found to be identical to the pattern of administered *P. pentosaceus* OZF. Amplification of template DNA obtained from *P. pentosaceus* OZF before and after administration, using specific primer sets, resulted in two well-differentiated PCR

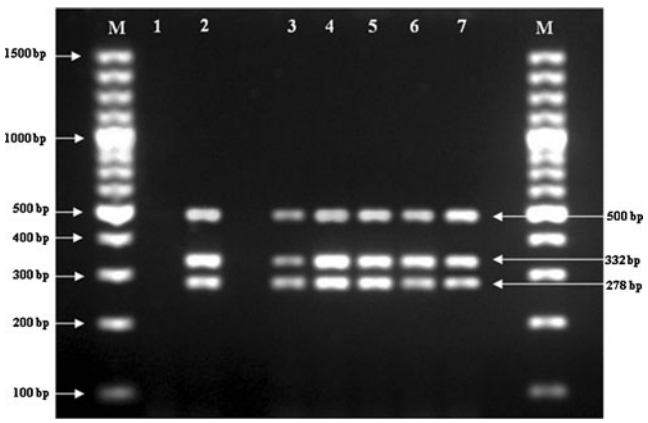

Fig. 2 Molecular identification of *Pediococcus pentosaceus* OZF in mouse feces by multiplex-PCR. Lane M, 100 bp DNA ladder (GeneRulerTM 100 bp DNA Ladder Plus, Fermentas); *Lane 1*, negative PCR control (no template DNA); *Lane 2*, *P. pentosaceus* OZF used for oral administration to mice as positive control; *Lane 3–7*, multiplex-PCR products derived from colonies on PSM agar after oral administration of *P. pentosaceus* OZF at 1, 2, 3, 4 and 5 days respectively

fragments with molecular weights of 500 and 278 bp specific for *P. pentosaceus*, and a 332 bp DNA fragment for pediocin AcH/PA-1 (Fig. 2). RAPD-PCR for the same randomly selected colonies generated reproducible patterns identical to fingerprint obtained for *P. pentosaceus* OZF (Fig. 3). Therefore, the one representing each day was choosen randomly and its band profiles are shown in Fig. 3. Multiplex PCR was found to be an easy, fast, reliable and reproducible method, and can be used as an alternative for verification of bacteriocin-producing pediococci strains. RAPD-PCR was also found as a rapid and reliable molecular technique to generate DNA fingerprints for each strain, and to distinguish the ingested strain from the potentially thousands of other bacterial strains that make up the gastrointestinal ecosystem.

Immunomodulatory function of *Pediococcus pentosaceus* OZF

Some LAB strains may be able to activate the immune system cells (Christensen et al. 2002; Medina et al. 2007; Vizoso Pinto et al. 2007, 2009). Inducing or enhancing the cytokine production could be a major mechanism for probiotic bacteria to exert immunomodulating activities (Marin et al. 1997). This would open up a promising use of *P. pentosaceus* OZF as an immunomodulator. Our study was performed in two stages, in vivo and in vitro. The analysis of the cytokines profiles revealed that the most remarkable effect was an increase in the IL-6 (Fig. 4), an important mediator both in humoral and cellular host defense. IL-6 plays an important role in host immune mechanism by regulating immune response and acute-phase reactions (Morita et al. 2002). In control group, IL-6 was not detected in sera, while a statistically significant increase was detected in mice regularly fed with bacterial suspension (89.15± 7.89 ng mL^{-1}, $p<0.001$). On the other hand, no significant difference was recorded in IL-12 production between groups

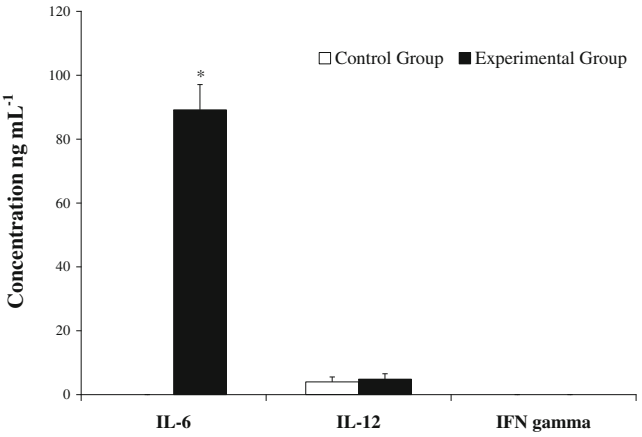

Fig. 4 IL-6, IL-12 and IFN-γ production in serum samples of BALB/c mice involved in control and experimental group after 30 days daily feeding period (*significantly difference: $p<0.001$)

(control: 3.96±1.58 ng mL^{-1}; experimental: 4,82±1.72 ng mL^{-1}). Besides, the resulting amount of IFN gamma level was below the detection level of the method in cell supernatants including serum (Fig. 4). The increase in the production of IL-6, as a result of regular feeding with *P. pentosaceus* OZF, may create an advantage for defense of infections encountered in any way (Van Enckevort et al. 1999). Despite growing evidence for immunomodulatory capability of LAB, especially human milk-derived species, there is still little information regarding their mode of action. Several similar studies have reported the in vitro and in vivo cytokine response patterns of co-culturing cells of the innate immune defense system with different probiotic LAB for understanding their health protection mechanism. Our results are in agreement with the results obtained by Gill (1998); Morita et al. (2002); Kimura et al. (2006); Foligne et al. (2010); Perez-Cano et al. (2010) and Zhu et al. (2011). Intestinal epithelial cells from conventional mice were reported to produce IL-6 in response to the challenge in

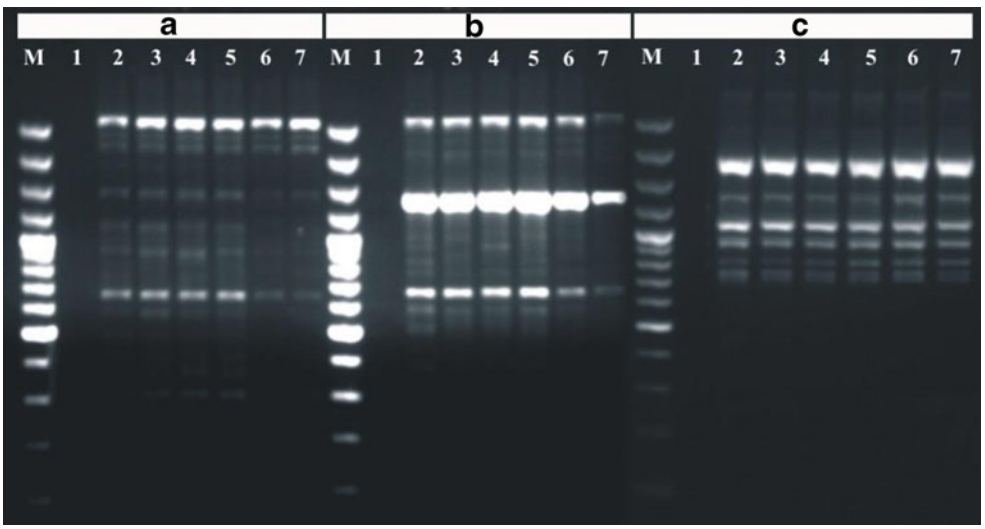

Fig. 3 Molecular typing of *Pediococcus pentosaceus* OZF in mouse feces by RAPD PCR. *Lane M*, 100 bp DNA ladder (GeneRulerTM 100 bp DNA Ladder Plus, Fermentas; Marker bp, ruler was given at Fig. 2); *Lane 1*, negative PCR control (no template DNA); *Lane 2*, *P. pentosaceus* OZF used for oral administration to mice as positive control; *Lane 3–7*, RAPD PCR profiles derived from colonies on PSM agar after oral administration of *P. pentosaceus* OZF at 1, 2, 3, 4 and 5 day respectively. Primers used are OPA-9 (**a**), OPF-14 (**b**) and OPA-7 (**c**)

vitro with certain LAB strains that had also demonstrated in vivo immunomodulating capacity (Vinderola et al. 2005). Two *P. pentosaceus* strains isolated from traditional vegetable pickles were shown to have a high capacity to survive in the GIT, and have in vitro immunomodulatory and allergy inhibition effects (Jonganurakkun et al. 2008). In contrast, another *P. pentosaceus* strain, used as preservative to prevent farmer's lung pneumonitis, was shown to induce an inflammatory response in mice in the study of Duchaine et al. (1996).

In order to find out whether or not *P. pentosaceus* OZF exhibit in vitro immunostimulating capacity, ssDNA and heat killed cells of the strain were used, since it is known that cellular components of LAB such as peptidoglycans, lipoteichoic acids, cell surface protein, exopolysaccharide and DNA CpG motifs, as well as live bacteria and inactivated bacteria, may present a capacity for immune system stimulation (Lebeer et al. 2008; Ng et al. 2009). In both experimental and control groups, stimulation studies were carried out by use of both spleen cells and PECs. Following stimulation, the release of IL-6 and IL-12 was not detected in spleen cells. In PECs, when we look at the results from the perspective of IL-6 production after stimulation, the control group was found to react more than experimental group to high dosage of heat killed cells of the strain OZF ($p<0.01$). However, when the concentration of heat killed cells was decreased (10^4CFU mL^{-1} and lower titrations), IL-6 production was found to be higher in the experimental group than in the control group ($p<0.05$). When the results of heat killed bacteria stimulation are evaluated in terms of IL-12 and IFN gamma secretion, no significant difference was recorded between the groups (data not shown).

Although studies are limited in this regard, available references reported that DNA of LAB have immunostimulatory properties (Lammers et al. 2003; Iliev et al. 2005; Li et al. 2005; Medina et al. 2007; Ghadimi et al. 2008; Satokari et al. 2009; Menard et al. 2010). The present study showed that ssDNA of *P. pentosaceus* OZF induced the secretion of IL-6 and IL-12, but this was not found to be significantly different when compared to each group (data not shown). IFN-γ was not detected after stimulation with ssDNA, which could be related to the use of low dosages of ssDNA.

In conclusion, *P. pentosaceus* OZF strain proved to be able to pass live through the GIT after oral administration to mice and to stimulate an immune response. To the authors' knowledge, this is the first article dealing with the effects of a *Pediococcus* strain, isolated from human breast milk, on cytokines activation. The comparison of the stimulant effect of heat killed cells and ssDNA after co-culture with spleen cells and PECs is also novel. On the other hand, further studies are necessary to better assess the fulfill breadth of the immunomodulatory capability of our strain in the gut environment, and to understand its cellular or humoral immune effect by measuring the inductive activity of IgA, IgG and IgM in case of usage as a oral vaccine carrier.

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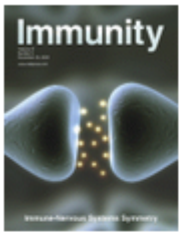

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