SUPPRESSIVE OLIGODEOXYNUCLEOTIDES AS A TLR ANTAGONIST: EFFORTS TO TREAT AUTOIMMUNE DISEASES

A THESIS SUBMITTED TO

THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF

BILKENT UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

BY

FUAT CEM YAĞCI

SEPTEMBER 2007

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

Assist. Prof. Dr. İhsan Gürsel

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

Assist. Prof. Dr. K. Can Akçalı

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

Assist. Prof. Dr. İsmail Şimşek

Approved for the Institute of Engineering and Science

Director of Institute of Engineering and Science
Prof. Mehmet Baray

ABSTRACT

SUPPRESSIVE OLIGODEOXYNUCLEOTIDES AS A TLR ANTAGONIST: EFFORTS TO TREAT AUTOIMMUNE DISEASES

FUAT CEM YAĞCI
M. Sc. in Molecular Biology and Genetics
Supervisor: Assist. Prof. Ihsan Gürsel
September 2007, 61 Pages

Synthetic oligodeoxynucleotides (ODN) expressing suppressive TTAGGG motifs effectively down-regulate the production of proinflammatory and Th1 cytokines elicited by a variety of Toll-Like Receptor (TLR) dependent or independent immune stimuli. Although initially identified by their ability to block CpG-induced immune activation, this class of suppressive ODN (typified by ODN A151) was subsequently shown to block multiple forms of immune stimulation and to be effective in the prevention and treatment of pathologic autoimmune diseases. Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation. It is induced by either systemic or intravitreal administration of lipopolysaccharide (LPS). FMF is an autosomal recessive periodic fever disease characterized by recurrent, self-limiting, febrile, inflammatory attacks of the serosal membranes such as peritoneum, pleura, and synovia. FMF patients in clinical remission are reported to have increased baseline inflammation. Present study aims to demonstrate that the downregulatory effect of the suppressive DNA could prove benefit to alleviate the symptoms associated with i) LPS induced EIU in rabbit or murine models as model for local autoimmune disease and ii) Familial Mediterranean Fever a model for systemic autoinflammatory disease. Results from this research strongly implicated that A151 treated EIU induced animals downregulated IL6 and IL1b cytokine secretion or expression as well as chemokines such as or MIP3a, or iNOS levels. Our data suggest that FMF patient PBMCs to that of healthy donor`s blood were more responsive to TLR ligand stimulation and A151 incubation strongly reversed this activation and suppressed certain key cytokine/chemokine levels.

Keywords*:* Suppressive DNA, autoimmunity, immunoregulatory effect, TLR, antagonizm.

ÖZET

TLR ANTAGONİSTİ OLARAK BASKILAYICI OLİGODEOKSİNÜKLEOTİDLER: OTOİMMÜN HASTALIKLARI TEDAVİ ÇABALARI

Fuat Cem Yağcı
Moleküler Biyoloji ve Genetik Yüksek Lisans
Tez Yöneticisi: Yar. Doç. Dr. İhsan Gürsel
Eylül 2007,61 Sayfa

Baskılayıcı TTAGGG motiflerini içeren sentetik oligodeoksinükleotidler (ODN) Toll-Benzeri Almaçlar (TLR) aracılığıyla ya da başka yollarla oluşan proenflamatuar ve Th1 sitokinlerin üretimini etkin bir şekilde azaltabilmektedirler. CpG ile indüklenen immün aktivasyonu bloke edebilmelerine ek olarak bu baskılayıcı ODN sınıfının (ODN A151 tipik bir örneğidir) değişik tipteki immün aktivasyonu bloke edebildikleri ve patolojik otoimmün hastalıklardan korunmada ve bu hastalıkların tedavisinde etkili oldukları gösterilmiştir. Endotoksin ile indüklenen üveyit (EIU) akut oküler enflamasyon için kullanılabilen bir hayvan modelidir. Bu hastalık gram-negatif bakterilerin tipik yan ürünü olan lipopolisakkaritleri (LPS) sistemik veya göz içine uygulayarak oluşturulur. Ailesel Akdeniz Ateşi (FMF), ateş, peritonit, sinovya, plörit ve nadiren perikardit ve menenjiti de içeren, serosit ve tekrarlayan kısa enflamatuvar ataklar ile karakterize otozomal resesif bir hastalıktır. Bu çalışmada i) lokal otoimmün bir hastalık olan, tavsan ve fare hayvan modellerinde LPS ile indüklenerek oluşturulan üveite ve ii) sistemik otoenflamatuvar bir hastalık olan Ailesel Akdeniz Ateşine bağlı oluşan semptomların ortadan kaldırılmasında baskılayıcı DNA'ların etkili olabileceğinin gösterilmesi amaçlanmıştır. Bu araştırmanın sonuçları, EIU oluşturulmuş hayvanlara, A151 uygulanmasının, IL6 ve IL1b salımının yanında, MIP3a, ve iNOS seviyelerinin baskılanmasına etkili olduğunu kuvvetle işaret etmektedir. Verilerimiz, FMF hastalarının kan hücrelerinin, sağlıklı gönüllüler ile karşılaştırıldığında TLR ulaklarının oluşturduğu uyarıya daha yüksek seviyede tepki gösterdiğini ve A151 ile inkübasyon sonrası bu aktivasyonun bazı önemli sitokin ve kemokinlerin salım ve gen ifadelerinin bastırılabildiğini göstermiştir.

Anahtar Kelimerler: Baskılayıcı DNA, otoimmünite, immün düzenleyici etki, TLR, antagonizm.

TO MY FAMILY

FOR BEING MY SHOULDER TO CRY AND LAUGH

AND ALWAYS BEING THERE FOR ME

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Assoc. Prof. İhsan Gürsel who gave me invaluable support, guidance and motivation in completing this thesis. His personal and academic advices besides his guidance and patience, was very valuable for me. I am indebted to him for showing me the beauty of molecular biology and immunology and for teaching me how to be a good scientist.

I would also like to thank Assoc. Prof. Mayda Gürsel for teaching me invaluable concepts and sharing her scientific experiences.

I would like to thank to my undergraduate advisor Assoc. Prof. Neşe Akış for giving me the "first" chance to be a part of a scientific study.

Thanks to Assist. Prof. Can Akçalı and his graduate students Zeynep and Fatma for scientific discussions on our journal club. I would also like to thank Assist. Prof. İsmail Şimşek for providing blood samples whenever I needed and sharing his scientific experiences.

Very special thanks to my group friends Gizem, Resad and Hande for their unconditional support and friendship. I would also thank to senior students Kutay, Seda and Erdem for their friendship and help in my studies. Thanks to Veli, Yetiş, Burcu and Gökhan for their helps during their internships. Especially I would like to thank another summer intern Volkan Yazar for working with me and helping me whenever I needed.

I would also like to thank Emre Onat, Elif Yaman, Melda Kantar and Pelin Telkoparan for their unconditional support and friendship. Life would not be so colorful without them.

Thanks to Bala Gür Dedeoğlu, Esen Oktay, Guvanchmurad Ovezmuradov, Hani AlOtaibi, Haluk Yüzügüllü, Elif Uz, Sevgi Bağışlar and Tolga Acun for being there with all their knowledge and patience whenever I had trouble with my studies and experiments.

In addition, I would like to thank MBG family members for their support, friendship, and help.

My very special thanks go to Gökçen for her great love and unlimited support. You make me feel the luckiest man by being mine.

Last but not least, I would like to thank my family for being there whenever I needed them and supporting me in every decision I gave. Without them and their unconditional love, nothing would be possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

APC	Antigen presenting cell
BCR	B-cell receptor
Bp	Base pairs
CARD	Caspase-recruiting domain
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freud's Adjuvant
CpG	Unmethylated cytosine-guaniosine motifs
CREB	cAMP-responsive element binding protein
CXCL	CXC-chemokine ligand
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EAE	Experimental autoimmune encephalomyelitis
EIU	Endotoxin Induced Uveitis
ELISA	Enzyme Linked-Immunosorbent Assay
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
FMF	Familial Mediterranean Fever
HBV	Hepatitis-B Virus
HEK	Human embryonic kidney
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPF	Hereditary Periodic Fever
HPV	Human papillomavirus
HSP	Heat-shock Protein
HSV	Herpes Simplex Virus
HZ	Hemozoin
ICAM	Intercellular Adhesion Molecule

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP	Interferon gamma Inducible Protein
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
IκK	Inhibitor kappa B kinase
LBP	LPS-binding protein
LFA	Lymphocyte Function Associated Antigen
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
MAP	Mitogen-activated protein
MCP	Monocyte Chemoattractant Protein
mDC	Myeloid dendritic cells
MEFV	Mediterranean Fever
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MyD-88	Myeloid Differentiation Primary Response gene (88)
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
pI:C	Polyriboinosinic polyribocytidylic acid

PMN	Polymorphonuclear Cell
PNPP	Para-nitrophenyl phosphate
PRR	Pattern recognition receptors
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RIG	Retinoic acid-inducible protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA-AKP	Streptavidin Alkaline-phosphatase
ssRNA	Single-stranded RNA
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
TNFR	TNF Receptor
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain containing adaptor inducing IFN-β
UV	Ultraviolet
WBC	White Blood Cell

INTRODUCTION

1.1 The Immune System

Immune system is a set of mechanisms that protects the host against infection by identifying and killing pathogens and tumor cells. The immune system detects various pathogens, such as viruses and parasitic worms and distinguishes them from the organism's normal cells and tissues (Beck et al., 1996). The immune systems of humans interact in a detailed and dynamic network which is consist of many types of proteins, cells, organs, and tissues. The vertebrate system adapts over time to recognize particular pathogens more efficiently as part of this more complex immune response. The adaptation process creates immunological memories and allows even more effective protection during future encounters with these pathogens (Litman et al., 2005).

The immune system has layered defenses of increasing specificity which protects organisms from infection. Most simply, physical barriers prevent pathogens such as bacteria and viruses from entering the body. The innate immune system provides an immediate, but non-specific response if a pathogen breaches these barriers (Litman et al., 2005). The adaptive immune system, third layer of protection, takes place if pathogens successfully evade the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered (Mayer., 2006). Components of the innate and adaptive immune response is summarized in Table 1.1.

Table1.1: Components of the immune system

(Adapted from: Albertz et al., 2002)

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

Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules. In immunology, *self* molecules are those components of an organism's body that can be distinguished from foreign substances by the immune system (Smith., 1997). Conversely, *non-self* molecules are those recognized as foreign molecules. (Alberts et al., 2002). The innate immune system is activated by exposure to this *non-self* molecules called pathogen associated molecular patterns (PAMPs) that are expressed by a diverse group of infectious organisms. (Metzhitov et al., 1998)

1.2 Innate Immunity

The innate immune system is composed of the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. This means that the cells of the innate immune system recognize, and respond to, pathogens in a generic way, but unlike the adaptive immune system, it does not accommodate long-lasting or protective immunity to the host (Alberts et al., 2002).

The major functions of the vertebrate innate immune system include:

- Recruiting immune cells to sites of infection and inflammation, through the production of chemical factors, including specialized chemical mediators, called cytokines.
- Activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes.
- The identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.
- Activation of the adaptive immune system through a process known as antigen presentation (Janeway et al., 2001)

In contrast to the adaptive system, the innate immune system was relatively neglected for many decades until recent discoveries provided a remarkable new understanding of how it accomplishes its crucial mission. In order to protect the host from infections the innate immune system must accomplish four fundamental tasks.

- Detection of any infectious agent regardless of it is a virus, bacteria, fungus or parasite.
- Categorizing the type of invading infectious agent whether it is located intracellularly or extracellularly.
- Appropriating to the pathogen class activated to either eradicate or at least temporarily contain the infection.
- Inducing the appropriate type of adaptive immune response to eliminate the infection and prevent its recurrence. (Krieg., 2006)

Stimulation of the innate immune response limits the early proliferation and spread of infectious organisms via production of immunoprotective cytokines, chemokines and polyreactive antibodies (Metzhitov et al., 1998). The main cytokines/chemokines appears during the onset of innate immune activation are; TNF-α, IL-1α/β, IP-10, MIP-1α, MIP-3α, MCP and Regulated upon activation, normal T-cell expressed, and secreted (RANTES).

These mediators can induce fever, apoptosis, neutrophil activation, recruitment of T and B cells and induction of inflammation as well as regulating the trafficking of immune effector cells to the site of infection.

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

1.2.1 Cells of Innate Immune Response

All white blood cells (WBC) are called leukocytes. Leukocytes are different from other cells of the body in that they are not tightly associated with a particular organ or tissue; thus, they function similar to independent, single-celled organisms. Leukocytes are able to move freely and interact and capture cellular debris, foreign particles, or invading microorganisms. Unlike many other cells in the body, most innate immune leukocytes cannot divide or reproduce on their own, but are the products of pluripotential hemopoietic stem cells present in the bone marrow (Alberts et al., 2002).

The innate leukocytes include: Mast cells, natural killer cells, eosinophils, basophils; and the phagocytic cells including macrophages, neutrophils and dendritic cells, and function within the immune system by identifying and eliminating pathogens that might cause infection. (Janeway et al., 2001)

Mast Cells

Mast cells are a type of innate immune cell that resides in the connective tissue and in the mucous membranes, and are intimately associated with defense against pathogens, wound healing, but are also often associated with allergy and anaphylaxis. When activated, mast cells rapidly release characteristic granules, rich in histamine and heparin, along with various hormonal mediators, and chemokines, or chemotactic cytokines into the environment. Histamine dilates blood vessels, causing the characteristic signs of inflammation, and recruits neutrophils and macrophages (Viera et al., 1995).

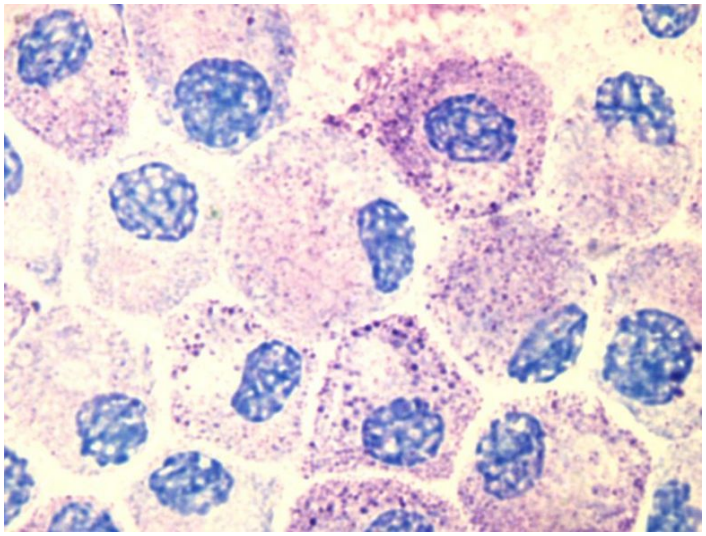


Fig 1.1 Mast Cells (Adapted from: Albertz et al., 2002)

Natural Killer Cells

Natural killer cells, or NK cells, are a component of the innate immune system. NK cells attack host cells that have been infected by microbes, but do not directly attack invading microbes. For example, NK cells attack and destroy tumor cells, and virally infected cells, through a process known as "missing-self". This term describes cells with low levels of a cell-surface marker called MHC I (major histocompatibility complex)—a situation which can arise in viral infections of host cells. They were named "natural killer" because of the initial notion that they do not require activation in order to kill cells that are "missing self." (Janeway et al., 2005).

Basophils and Eosinophils

Basophils and Eosinophils are cells related to the neutrophil . When activated by a pathogen encounter, basophils releasing histamine are important in defense against parasites, and play a role in allergic reactions (such as asthma) (Janeway et al., 2001). Upon activation, eosinophils secrete a range of highly toxic proteins and free radicals that are highly effective in killing bacteria and parasites, but are also responsible for tissue damage occurring during allergic reactions. Activation and toxin release by eosinophils is therefore tightly regulated to prevent any inappropriate tissue destruction (Viera et al., 1995).

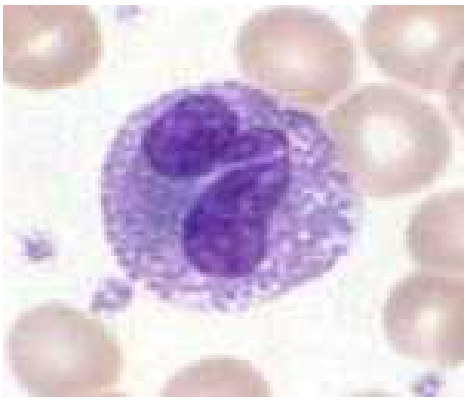


Fig 1.2 An Eosinophil (Adapted from: Albertz et al., 2002)

Phagocytes

The word 'phagocyte' literally means 'eating cell'. These are immune cells that engulf, i.e. phagocytose, pathogens or particles. To engulf a particle or pathogen, a phagocyte extends portions of its plasma membrane, wrapping the membrane around the particle until it is enveloped (i.e. the particle is now inside the cell). Once inside the cell, the invading pathogen is contained inside an endosome which merges with a lysosome (Janeway et al., 2001). The lysosome contains enzymes and acids that kill and digest the particle or organism. Phagocytes generally search the body for pathogens, but are also able to react to a group of highly specialized molecular signals produced by other cells, called cytokines. The phagocytic cells of the immune system include macrophages, neutrophils, and dendritic cells (Janeway et al., 2005).

Phagocytosis of the hosts' own cells is common as part of regular tissue development and maintenance. When host cells die, either internally induced by processes involving programmed cell death (also called apoptosis), or caused by cell injury due to a bacterial or viral infection, phagocytic cells are responsible for their removal from the affected site. By helping to remove dead cells preceding growth and development of new healthy cells, phagocytosis is an important part of the healing process following tissue injury (Alberts et al., 2002).

Macrophages

Macrophages meaning "large eating cell", are large phagocytic leukocytes, which are able to move outside of the vascular system by moving across the cell membrane of capillary vessels and entering the areas between cells in pursuit of invading pathogens. In tissues, organ-specific macrophages are differentiated from phagocytic cells present in the blood called monocytes. Macrophages are the most efficient phagocytes, and can phagocytose substantial numbers of bacteria or other cells or microbes. The binding of bacterial molecules to receptors on the surface of a macrophage triggers it to engulf and destroy the bacteria through the generation of a “respiratory burst”, causing the release of reactive oxygen species such as H_2O_2 or NO. Pathogens also stimulate the macrophage to produce cytokines and chemokines, which summons other cells to the site of infection (Janeway et al., 2001)

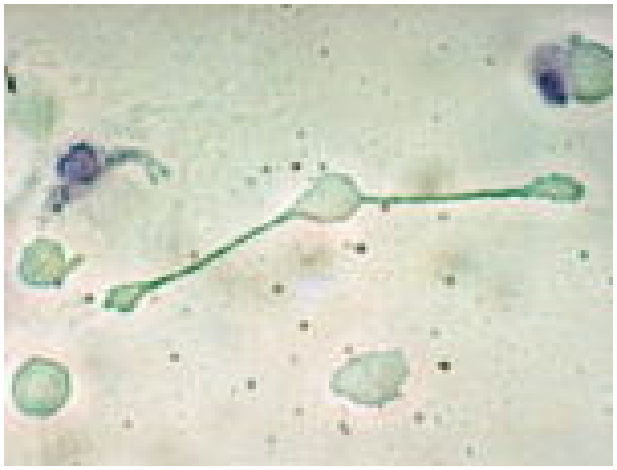


Fig 1.3: A Macrophage (Adapted from: Albertz et al., 2002)

Neutrophils

Neutrophils, along with two other cell types; eosinophils and basophils, are known as granulocytes due to the presence of granules in their cytoplasm, or as polymorphonuclear cells (PMNs) due to their distinctive lobed nuclei. Neutrophil granules contain a variety of toxic substances that kill or inhibit growth of bacteria and fungi. Similar to macrophages, neutrophils attack pathogens by activating a "respiratory burst". The main products of the neutrophil respiratory burst are strong oxidizing agents including hydrogen peroxide, free oxygen radicals and hypochlorite. Neutrophils are the most abundant type of phagocyte, normally representing 50 to 60% of the total circulating leukocytes, and are usually the first cells to arrive at the site of an infection (Viera et al., 1995).

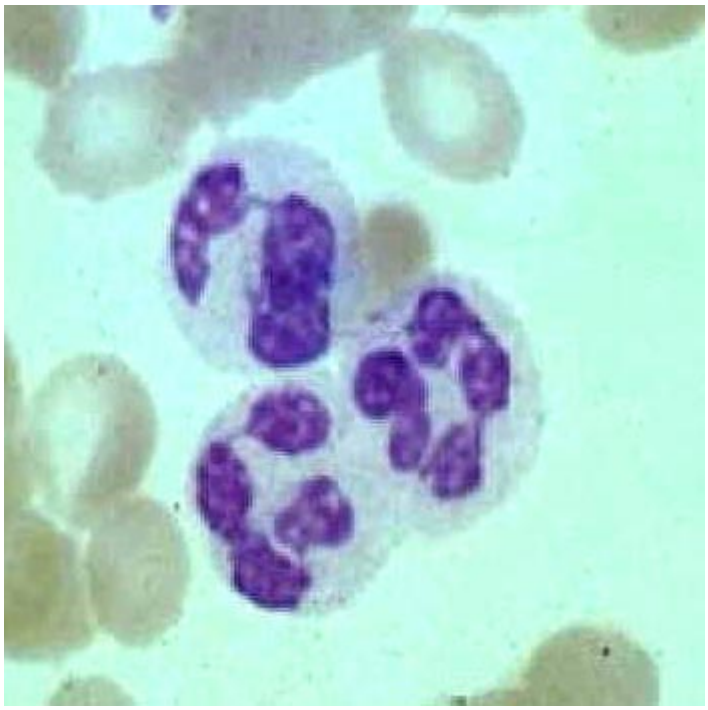


Fig 1.4: Neutrophils (Adapted from: Albertz et al., 2002)

Dendritic Cells

Dendritic cells (DC) are phagocytic cells present in tissues that are in contact with the external environment, mainly the skin (where they are often called Langerhans cells), and the inner mucosal lining of the nose, lungs, stomach and intestines. They are named for their resemblance to neuronal dendrites, but dendritic cells are not connected to the nervous system. Dendritic cells are very important in the process of antigen presentation, and serve as a link between the innate and adaptive immune systems (Alberts et al., 2002).

Dendritic cells are derived from hemopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature dendritic cells. These cells are characterized by high endocytic activity and low T-cell activation potential. Immature dendritic cells constantly sample the surrounding environment for pathogens such as viruses and bacteria. This is done through pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs). TLRs recognize specific chemical signatures found on subsets of pathogens. Once they have come into contact with such a pathogen, they become activated into mature dendritic cells. Immature dendritic cells phagocytose pathogens and degrade its proteins into small pieces and upon maturation present those fragments at their cell surface using MHC molecules. Simultaneously, they upregulate cell-surface receptors that act as co-receptors in

T-cell activation such as CD80, CD86, and CD40 greatly enhancing their ability to activate T-cells. They also upregulate CCR7, a chemotactic receptor that induces the dendritic cell to travel through the blood stream to the spleen or through the lymphatic system to a lymph node. Here they act as antigen-presenting cells: they activate helper T-cells and killer T-cells as well as B-cells by presenting them with antigens derived from the pathogen, alongside non-antigen specific costimulatory signals (Mc Kenna et al., 2005)

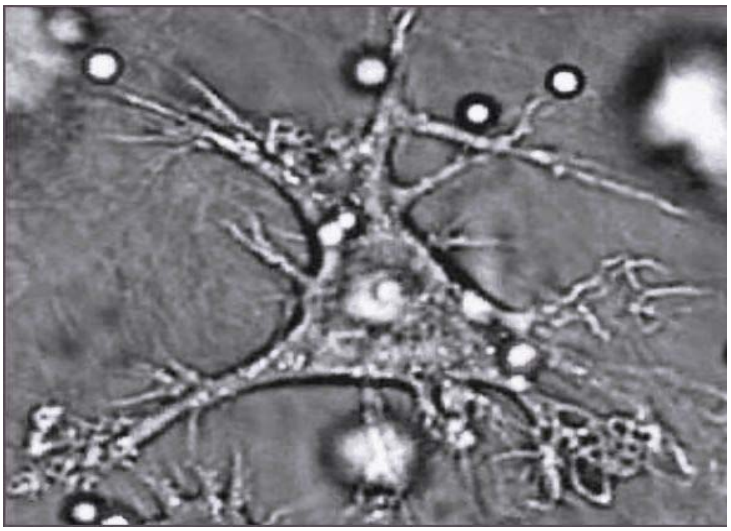


Fig 1.5: A Dendritic Cell (Adapted from: Albertz et al., 2002)

1.2.2 Pattern Recognition Receptors

Multi-cellular organisms are constantly exposed to various pathogenic microbes during their lifetime. To combat these pathogens effectively, most, if not all, multi-cellular organisms have developed some forms of innate immune defense mechanisms such as antimicrobial peptide production and phagocytosis, which rely on detection of the pathogens by a set of germline-encoded pattern-recognition receptors (PRRs) (Janeway et al., 2002) To initiate immune responses to pathogens, PRRs recognize highly conserved microbial structures, so-called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), a major Gram-negative bacterial cell-wall component . The proteins of PRR families such as complement, pentraxin, and collectin present in extracellular space, play a main role in pathogen opsonization for phagocytic clearance and in activation of complement pathways. (Garlanda et al., 2005; Gasque, 2004). Table 1.3 summarizes PRR types and their members with their corresponding ligands.

Table 1.3: Types of PRRs and their associated members or ligands (Adapted from: Lee et al., 2007)

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

PRRs on the cell membrane have two major functions: the promotion of microbial phagocytosis and the initiation of intracellular signaling pathways (Brown, 2006; Underhill and Ozinsky, 2002). Cytoplasmic PRRs can be grouped into three families: (i) interferon (IFN)-inducible proteins, (ii) caspase-recruiting domain (CARD) helicases, and (iii) nucleotide- binding oligomerization domain (NOD)-like receptors (NLRs). IFN-inducible proteins such as double-stranded RNA (dsRNA)-activated protein kinase (PKR) (Stark et al.,1998), and CARD helicases such as retinoic acid-inducible protein I (RIG-I), mediate antiviral defense, whereas NLRs primarily mediate antibacterial defense.

1.2.3 Toll-Like Receptor Family

The best understood family of PRRs are the Toll-like receptors (TLRs). (Krieg., 2006). Evolutionarily conserved TLR molecules were originally identified in vertebrates on the basis of their homology with a molecule that stimulates the production of antimicrobial proteins in *Drosophila melanogaster* called Toll. (Trinchieri et al., 2007)

To date, 11 members of the TLR family have been identified in mammals (Krieg., 2006). TLR family members recognize and respond to diverse molecules containing PAMP including lipids, proteins and nucleic acids. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR5 recognizes flagellin. TLR11 recognizes profilin-like protein from a parasite. TLR3, 7(8) or 9 were found to recognize nucleic acids such as double stranded (ds), single stranded (ss) RNA or ss/ds DNA, respectively. (Ishii et al., 2005)

TLRs are composed of an ectodomain of leucine-rich repeats (LRRs), which are involved directly or through accessory molecules in ligand binding, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain that interacts with TIR-domain-containing adaptor molecules (Takeda et al., 2003). Table 1.4 presents TLRs and their ligands.

Table 1.4: Toll-like receptors (TLRs) and some of their important ligands. (Adapted from: Akira et al., 2004)

TLR	Typical Ligands
TLR1	Bacterial lipoproteins
TLR2	lipoteichoic acid (LTA), peptidoglycan (PGN), bacterial lipoproteins, zymosan
TLR3	Double-stranded RNA
TLR4	Lipopolysaccharide (LPS), viral envelope protein MMTV, RSV F protein
TLR5	Flagellin
TLR6	LTA, diacyl lipoproteins, zymosan
TLR7	ssRNA, imidazoquinolines
TLR8	ssRNA, imidazoquinolines
TLR9	Unmethylated CpG DNA
TLR10	Undetermined
TLR11*	Uropathogenic bacteria, profilin-like protein

** a functional gene for TLR11 has only been found in mice.*

Table 1.5: Examples of pathogens expressing ligands for multiple TLRs. (Adapted from: Trinchieri et al., 2007).

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

Table 1.6: Chromosomal localization of TLRs.

(Adapted from: http://www.ncbi.nlm.nih.gov/sites/entrez)

	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

TLR 1, TLR 2 and TLR 6

TLR2 recognizes a variety of microbial components. These include lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from gram-positive bacteria. (Takeda et al., 2003) There are two aspects proposed for mechanisms that could explain why TLR2 recognizes a wide spectrum of microbial components. The first explanation is that TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2 (Takeuchi et al., 2001) Thus, TLR1 and TLR6 functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides. (Alexopoulou et al., 2002) The second explanation involves recognition of fungal-derived components by TLR2 (15). In this model, TLR2 has been shown to functionally collaborate with distinct types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component b-glucan. Thus, TLR2 recognizes a wide range of microbial products through functional cooperation with several proteins that are either structurally related or unrelated (Gantner et al., 2003).

TLR 3

TLR3-deficient mice are impaired in their response to dsRNA (Alexopoulou., 2001) dsRNA is produced by most viruses during their replication and induces the synthesis of type I interferons (IFN-a/b), which exert anti-viral and immunostimulatory activities (Takeda et al.,2005). This activation is MyD88 independent and TRIF dependent (Jiang et al., 2004, Oshiumi et al., 2003). NK cells are the major players in the antiviral immune response and express TLR3 and are activated directly in response to synthetic dsRNA, polyriboinosinic polyribocytidylic acid (poly I:C) (Schimdt, *2004*). Thus, TLR3 is implicated in the recognition of dsRNA and viruses.

TLR 4

TLR4 is an essential receptor for LPS recognition. (Poltorak et al., 1998, Hoshino et al., 1999) Toll-like receptor 4 was identified as the first human homologue of the Drosophila Toll. This extracellular TLR is expressed in variety of cell types, most predominantly in macrophages and DCs (Medzhitov et al., 1997). The extracellular domain of TLR4 that contain over 600 amino acids is highly polymorphic compared with the transmembrane and intracellular domain of the protein (Smirnova et al., 2000) This TLR4 polymorphism contributes to species-specific differences in recognition of LPS, the prototypic TLR4 ligand (Hajjar et al., 2002). The intracellular TIR domain, which is composed of three highly

conserved regions, contains 150 amino acids. The TIR domain modulates protein–protein interactions between the TLRs and signal transduction elements (O'Neill et al., 2000). Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS is first bound to a serum protein, LPS-binding protein (LBP), which functions by transferring LPS monomers to CD14. MD-2 act as an accessory protein and is required for LPS mediated signaling through TLR(Wright, 1999). In addition to LPS, TLR4 recognizes several other ligands, such as lipoteichoic acid, heatshock proteins (HSP), and EDA in fibronectin (Li et al., 2007). Similar to TLR3 MyD88 independent TLR4 activation is also lead to Type I IFN production this is known as the TRIF pathway (Uematsu et al., 2007).

TLR 5

It has been demonstrated that flagellin was the component of *Listeria* culture supernatants that activated TLR5, and subsequent work in many laboratories confirmed this finding for flagellins from various organisms (Hayashi et al., 2001). To date, flagellin is the only known activator of TLR5 (an extracellular member of the TLR family) and until recently flagellin- induced inflammation was believed to be fully dependent on TLR5 expression. It has been shown in various studies that TLR5 is responsible for flagellin-induced responses in epithelial cells, endothelial cells, macrophages, dendritic cells (DCs), and T cells (Steiner., 2007)

TLR 7 and TLR 8

TLR7 and TLR8 are structurally highly conserved proteins (Akira et al., 2006). The synthetic imidazoquinoline-like molecules imiquimod (R837) and resiquimod (R848) have potent antiviral activities and are used clinically for the treatment of viral infections. Murine TLR7 and human TLR7 and TLR8 recognize imidazoquinoline compounds (Hemmi et al., 2002, Ito et al., 2002). Furthermore, murine TLR7 has been shown to recognize guanosine analogs such as loxoribine, which has antiviral and anti-tumor activities (Akira et al., 2006). Recently, TLR7 and human TLR8 have been shown to recognize guanosine- or uridine-rich ssRNA from viruses such as human immunodeficiency virus, vesicular stomatitis virus, and influenza virus (Diebold et al., 2004, Heil et al., 2004). Similar to TLR3, TLR9, TLR7/8 constitutes the members of the nucleic acid sensing endozome- associated receptors.

TLR 9

Bacterial DNA, which contains unmethylated CpG motifs, is a strong activator of host immunity. In vertebrates, the frequency of CpG motifs is remarkably reduced, and the cystosine of CpG motifs are highly methylated, in addition to CpG supression leading to abrogation of immunostimulatory activity. TLR9 mediates the recognition of CpG DNA (Hemmi et al., 2000). CpG DNA motifs are also found in the genomes of DNA viruses. Mouse pDCs produce IFN-α_by recognizing the CpG containing DNA of herpes simplex virus type 2 (HSV-2) via TLR9 (Lund et al., 2003). TLR9-deficient mice were also shown to be susceptible to mouse cytomegalovirus infection, suggesting that TLR9 induces antiviral responses by sensing the CpG containing DNA of DNA viruses (Tabeta et al., 2004, Krug et al., 2004).

TLRs specific for signature molecules of extracellular pathogens such as lipopolysaccharide, lipopeptides, peptidoglycan, flagellin and zymosan are expressed at the cell surface, whereas TLRs that recognize intracellular pathogens are expressed within the subcellular compartments of innate immune cells and these TLRs are specific for nucleic acids (Krieg., 2006). One controversial exception is the protein hemozoin (HZ) extracted from plasmodium (Coban et al., 2005). On the contrary, Parroche et al reported that "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9" (Parroche et al., 2007). The endosomal localization of TLR9 allows efficient detection of invading viral nucleic acids, while preventing 'accidental' stimulation by CpG motifs within self DNA. (Barton et al., 2005).

Cellular activation of innate immunity by most of the members of TLR family involves a signaling cascade that proceeds through myeloid differentiation primary response gene 88 (MyD88), interleukin-1 (IL1) receptor-activated kinase (IRAK) and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), and culminates in the activation of several transcription factors , including nuclear factor –κB (NF- κB) , activating protein 1 (AP1), CCAAT/enhancer binding protein (CEBP) and cAMP-responsive element binding protein (CREB) (Takeshita et al., 2000, Hacker et al., 2000) As a result of TLR stimulations by cognate ligands, pro-inflammatory response genes including cytokines such as TNFα, IL-6, IL-12 and co-stimulatory molecules are induced via activation of NF-κB and MAP kinases, whereas Type-1 IFN and their inducible genes are induced via interferon regulatory factors (IRF) 3 or 7 (Ishii et al., 2005). Figure 1.6 summarizes the major key players in the MyD88 dependent and independent signaling cascade mediated by endosomal/extracellular TLRs.

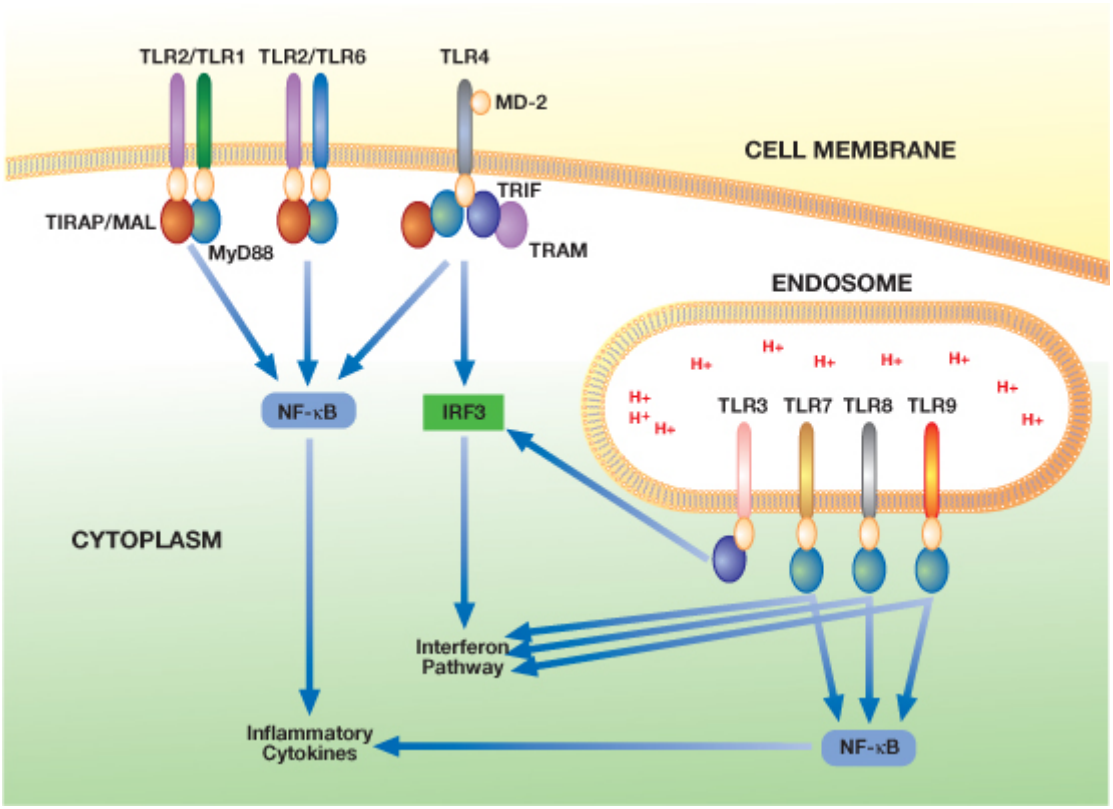


Fig 1.6: Summary of the MyD88 dependent/independent signaling pathway initiated by TLRs (Adapted from: Akira et al., 2004)

Microbial TLR ligands can activate dendritic cells (DCs), macrophages, and other antigen-presenting cells (APCs) and allow the effective presentation of microbial antigens to cells of the adaptive immune system (Rothstein., 2006). Activated DCs produce cytokines and chemokines that will be toxic to pathogen and instruct other immune cells about the nature of antigens. Afterwards, they will present an antigen with their optimally loaded major histocompatibility complex (MHC) class I and MHC class II molecules to T and B cells (Lee, 2007). Activated T and B cells expressing T cell receptor (TCR) and B cell receptor (BCR) will migrate to the infected area of the body on account of the production of chemokines (Luster, *2002*). These cells rapidly differentiate into effector cells whose main role is to get rid of the infection. This, they mainly succeed without recourse to adaptive immunity.

1.3 The effects of DNA on Immune System

Nucleic acids such as DNA and RNA are essential components of all living organisms (Ishii et al., 2005) DNA is normally tightly sequestered within the nuclear or mitochondrial membrane in eukaryotes, the cell wall in bacteria, or the envelope in viruses. However, following microbial infection or failure of host DNA clearance, DNA can be released from microbes or damaged host cells. Such DNA is detected by, and modulates, the innate immune system (Akira et al., 2006, Nagata et al., 2005). Such phenomenon had often been ignored, but are now in the limelight after the recent discovery of Toll-like receptors (TLR) (Medzhitov et al., 2002, Akira et al., 2004) Structure- or sequence-dependent immune recognition of nucleic acids by TLR were shown to play an important role in both innate and adaptive immune responses to infectious organisms including bacteria, virus and parasites (Wagner., 2004, Iwasaki et al., 2004) Novel therapeutics including nucleic acid-based agonists/antagonists via TLR-mediated immunomodulation are being developed for multiple therapeutic applications to prevent or treat infectious diseases, allergic disorders and cancer (Krieg., 2002, Klinman., 2004)

Immunostimulatory CpG ODN

The immune system is effected by DNA in multiple and complex manner. Bacterial DNA contains unmethylated immunostimulatory CpG motifs that trigger a protective innate immune response via TLR9 that contains the proliferation and maturation of B cells, NK cells and dendritic cells and secretion of various cytokines, chemokines and/or polyreactive Ig (Klinmann et al., 2003). CpG DNA binds to, and is taken up by immune cells through endocytic pathways, then co-accumulates with TLR9 in phagosome-like vesicles, a process controlled by PI3 kinase (Klinman., 2004).

Interaction of CpG DNA with TLR9 triggers the recruitment of the MyD88 adaptor molecule, followed by activation of IRAK1/4, TRAF6 and subsequently the MAP kinase signaling cascade, culminating with nuclear translocation of NF-κB. CpG DNA-mediated activation of the innate immune system is characterized by B cell proliferation, dendritic (DC) maturation, NK cell activation and production of pro-inflammatory cytokines (such as IL-6, 12 and Type-I and Type-II IFN), chemokines (such as MCP-1, IP-10, MIP-1α,β,) and immunoglobulins. Single stranded oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG ODN) mimic immunostimulatory activity of bacterial DNA. Impressive immunostimulatory activity of CpG ODN is being recorded for future use in a variety of therapeutic purposes (Klinman., 2004).

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. (Klinman., 2004, Krieg., 2006) 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 IFNa, whereas K-type ODN strongly stimulate B cells to proliferate and to secrete IL-6 and IgM. C-type ODN show a combined activity of K- and D-type ODN, but to a lesser extent (Ishii et al., 2005).

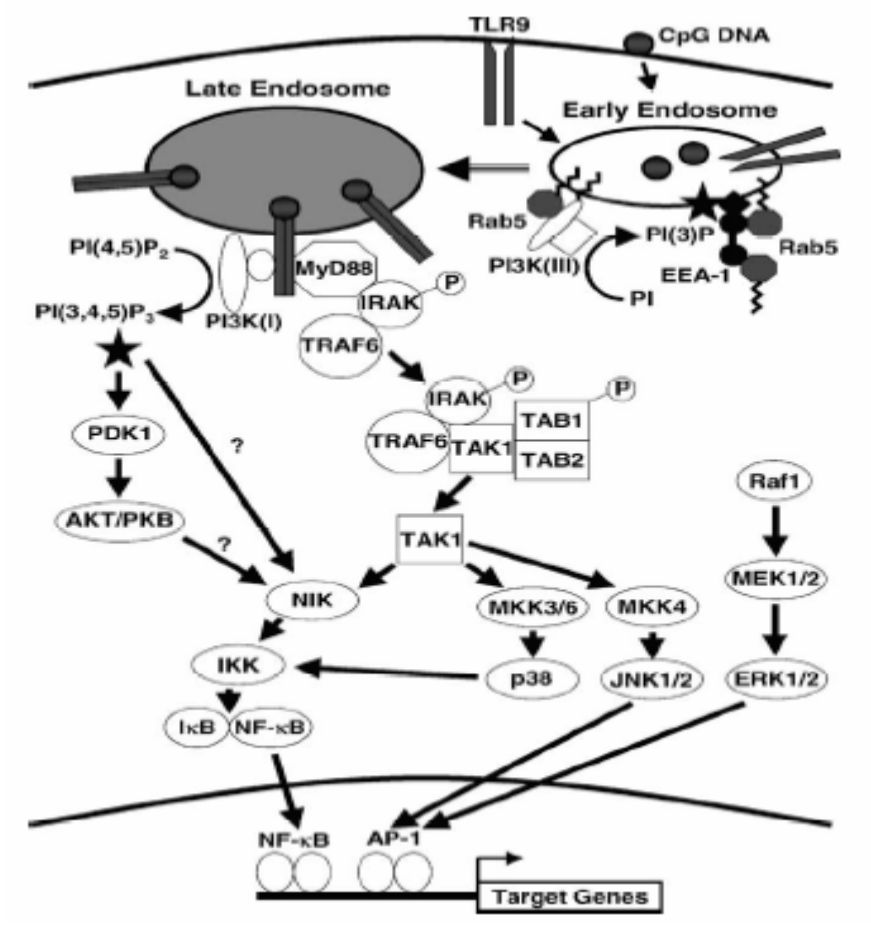


Fig 1.7: TLR9 dependent signaling pathway (Adapted from: Klinman et al., 2004)

Class III PI3K (PI3K (III)), EEA1, and Rab5 mediate the trafficking and maturation of endosomes containing CpG DNA and TLR9, by which TLR9 transduces intracytoplasmic signal. The signal initiates with the recruitment of MyD88 to the TIR, which then activates IRAK-TRAF6-TAK1 complex. This leads to the activation of both MAPKs (JNK1/2 and P38) and IKK complex, culminating upregulation of transcription factors including NF-kB and AP-1. Raf1-MEK1/2-ERK1/2-AP-1 pathway is involved in CpG DNA-induced IL-10 production in macrophages. The alternative pathway mediated by class I PI3K (PI3K (I))-PDK1-AKT/PKB is also suggested to be involved in TLR9-mediated cellular activation.

Recently, 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 fist time that a type of surface expressed scavenger receptor is required for the D-ODN activation of pDC to secrete robust IFNα (Gursel et al., 2006).

Immunosuppressive ODN

During infection or tissue damage, inflammation must be waned and terminated with tissue remodeling and healing. In this case, a negative feedback system of innate immune activation occurs via several inhibitory signals (Ishii et al., 2005). 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. (Klinman et al., 2005)

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 (Ishii et al., 2004). Neutralizing or suppressive motifs can selectively block CpG-mediated immune stimulation (Krieg et al., 1998) 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 (Gursel et al., 2003) Suppressive activity of ODN also correlates with their ability to form higher structures such as G-tetrads (Gursel et al., 2003). Recent studies indicated that suppressive ODN did not interfere with binding or uptake of CpG ODN (Yamada et al., 2002) 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 (Gursel et al., 2003, Yamada et al., 2002). Previous research established that suppressive ODN can down-regulate inflammatory responses that are deleterious to the host (Zeuner et al., 2002, Dong et al., 2004). Suppressive ODN block the production of Th1 and proinflammatory cytokines induced by bacteria in vitro. In vivo, they inhibit the development

of organ-specific autoimmune diseases, such as arthritis and experimental autoimmune encephalomyelitis (EAE) or lung inflammation. (Zeuner et al., 2002, Dong et al., 2004, Ho et al., 2003, Yamada et al., 2004)

The effect of suppressive ODN 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 (Shirota et al., 2005, Shirota et al., 2004). 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.

1.4 Endotoxin Induced Uveitis

It has been known for a long time that the eye has a special relationship with the immune system, known as immune privilege (Simson., 2006). The immune privilege of the eye is a complex phenomenon, involving many layers and mechanisms: (i) physical barriers prevent entry and exit of larger molecules such as proteins from the eye; (ii) cell-bound and soluble immunosuppressive factors within the eye inhibit the activity of immune-competent cells that may gain entry; and (iii) protein antigens released from a damaged eye elicit deviant systemic immunity that limits the generation of proinflammatory effector cells (Streilein., 2003). Acting in concert, these elements serve to create a milieu designed to protect the delicate visual axis from damage by inflammatory processes that in any other organ would not carry adverse functional consequences. Based on accumulated evidence from rodent studies, it is widely accepted that breakdown of immune privilege contributes to bystander damage from infection, to rejection of corneal grafts, and to development of uveitis. However, the ease with which it is possible to elicit autoimmunity in experimental animals to antigens originating from the retina puts in question the role of immune privilege as an effective barrier against ocular autoimmunity (Niederkorn., 2006, Streilein., 2003).

Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation induced by the administration of lipopolysaccharide (LPS), a component of Gram-negative bacterial outer membranes. Because uveitis frequently leads to severe vision loss and blindness with retinal vasculitis, retinal detachment, and glaucoma, it is important to elucidate

further the mechanisms in the development of ocular inflammation (Rosenbaum et al., 1980, Hoekzema et al., 1992) Uveitis can have a variety of underlying causes. For instance, acute anterior uveitis is often associated with Behcet's disease, ankylosing spondylitis, Reiter's syndrome, and human leukocyte antigen (HLA) B27-associated uveitis as well as other systemic inflammatory diseases (Chang et al., 2005)

LPS enhances the expression of various inflammatory mediators, such as IL-6, (Hoekzema et al., 1992, Ohta et al., 2005) TNF-α, (Koizimi et al., 2003) and MCP- 1, (Mo et al., 1999) as well as the production of nitric oxide (Bellot et al., 1996) all of which contribute to the development of EIU, resulting in the breakdown of the blood–ocular barrier and in the infiltration of leukocytes. For the first phase of leukocyte infiltration, cell adhesion to vascular endothelium is essential, in which adhesion molecules play major roles (Springer et al., 1993). Among various adhesion molecules, intercellular adhesion molecule (ICAM)-1 and its receptor, lymphocyte function-associated antigen (LFA)-1, are necessary for the development of EIU (Springer et al., 1993, Whitcup et al., 1993). Although EIU was originally used as a model of anterior uveitis, increasing evidence shows that it also involves inflammation in the posterior segment of the eye with recruitment of leukocytes that adhere to the retinal vasculature and infiltrate the vitreous cavity (Miyamoto et al., 1996, Yamashito et al., 2003).

Current therapies for uveitis include corticosteroids and chemotherapeutic agents to reduce inflammation (Dunn., 2004). However, the grave side effects of these drugs, such as increased intraocular pressure (Moorthy et al.,1997) or cytotoxicity (Lightman., 1997), limit their use (Dunn., 2004, Moorthy et al.,1997). Therefore, a new therapeutic strategy is urgently needed (Adamus et al., 2006, Avunduk et al., 2004).

1.5. Familial Mediterranean Fever

Autoinflammatory diseases are a group of disorders characterised by seemingly unprovoked inflammation in the absence of high-titre autoantibodies or antigen specific T cells (Stojanov et al., 2005). They include the hereditary periodic fever syndromes (HPF) and are thought to be caused by disturbances in the regulation of innate immunity (Kastner., 2005).

Familial Mediterranean Fever (FMF) is the most well known and best characterized of the HPFs. The mutated gene, Mediterranean Fever (MEFV), encoding pyrin/marenostrin protein, was identified in 1997 and found to be predominantly expressed in neutrophils, monocytes and eosinophils but not in lymphocytes (Aksentijevich et al., 1997, Bernot et

al.,1997) , suggesting a potential functional role in the regulation of inflammation. The vast majority of FMF-associated mutations are located in the B30.2 serine-proline-arginine-tyrosine (SPRY) domain, at the carboxy terminus of the protein (Gumucio et al., 2002). This domain is thought to function as a ligand binding or signal transduction domain, and therefore, B30.2 mutations may cause delayed apoptosis and inflammation by the reduced ability of pyrin to moderate IL-1β activation [Gumucio et al., 2002, Stojanov et al.,2005). There are over 100 variants in the MEFV gene recorded to date (de Menthiere et al., 2003), and the majority are disease associated mutations. The three most commonly reported mutations are M694V, M680I and V726A (Eisenberg et al.,1998).

Levels of blood cytokines and acute phase reactants have been measured in FMF patients and the results provide additional enticing clues. Typical laboratory findings during an attack include leukocytosis, an elevated erythrocyte sedimentation rate and increased acute phase reactants (e.g. serum amyloid A, fibrinogen, C-reactive protein) (Sohar et al., 1967, Baykal et al., 2003, Gang et al., 1999). Several studies have now shown that these components are also elevated between attacks in FMF patients [Baykal et al., 2003, Korkmaz et al., 2002, Poland et al., 2001 , Duzova et al., 2003).

A striking finding in FMF patients is the apparent absence of a C5a/interleukin-8 (IL-8) inhibitor activity in the serosal fluids of FMF patients (Matzner et al., 1984). The activity of this inhibitor is also defective in primary fibroblast cultures derived from the serosa of FMF patients (Matzner et al., 2000). The anaphylotoxin C5a is a powerful chemoattractant for many leukocytes (e.g. neutrophils, monocytes) and acts as a pro-inflammatory mediator in a during infection (e.g. it stimulates increased vascular permeability and monocyte/granulocyte oxidative burst responses) (Kohl., 2001). The chemokine IL-8 is also a potent chemoattractant, primarily for neutrophils, although it is also implicated in monocyte adhesion (Ohlson et al., 2002). The C5a/IL-8 inhibitor identified by Matzner and colleagues is a serine protease that inactivates both C5a and IL-8 *via* direct proteolysis. The hypothesis in regards to FMF, therefore, is that even with a normally insignificant inflammatory insult (e.g. minor trauma secondary to running) the absence of the C5a/IL-8 inhibitor allows IL-8 and C5a to accumulate, inducing a massive neutrophil chemotaxis that results in an inflammatory crisis.

FMF patients exhibit increased levels of serum IL-6, IL-8, soluble ICAM-1 and soluble TNF receptors p55 and p75 relative to controls (Kiraz et al., 1998, Baykal et al., 2003). However, the findings seem to vary depending on the timing of cytokine measurement. It has been suggested that later stages of the attack may be characterized by depleted stores of

TNF-α due to a previous massive release of this cytokine from monocytes at the onset of the attacks (Schattner et al., 1991, Schattner et al., 1996). Gang *et al.* (1999) measured IL-1β and IL-1 receptor antagonist levels and suggested that these components are unaltered during attacks (Gang et al., 1999). But in another study, mRNA levels for TNF-α, IL-1β, IL-6 and IL-8 were all increased relative to controls in circulating leukocytes of attack-free FMF patients (Notarnicola et al., 2002). Finally, Aypar *et al.* (2003) reported high levels of IFNγ production in FMF patients. Interestingly, the percentage of IFNγ positive T cells was also increased in FMF patients both during and between attacks (Aypar et al., 2003). Since the percentage of IL-4 positive T cells (Th2 cells) was not increased, these authors concluded that inflammation in FMF shows a Th1 polarization.

Colchicine is one of the most important drug used in the treatment of FMF. The remarkable therapeutic response of FMF to colchicine was identified by Goldfing in 1972 (Goldfing., 1972). It greatly reduces the frequency and intensity of clinical attacks, and by effectively suppressing inflammation generally in this particular disease, very largely prevents the development of amyloidosis (Zemer et al., 1996). However, despite the efficacy of colchicine, amyloidosis remains an important cause of morbidity and mortality in FMF, most likely relating to lack of such treatment, insufficient dosing, poor compliance and, in a small proportion of cases, perhaps approximately 5%, a genuine lack of response (Ben-Chetrit et al., 1998). No other therapy is of proven long-term efficacy in FMF. Therefore, a new therapeutic strategy is needed.

1.6. Aim and Strategy

The innate immune response triggered by several TLR ligands can improve host survival following pathogen challange. Yet unchecked stimulation of the innate immune system can cause tissue damage, autoimmune disease, and even death. Krieg et al were the first to demonstrate that "neutralizing" ODN containing G-run sequences selectively inhibited CpG-induced immune activation (Krieg et al., 1998).

Suppressive ODN were found to limit the immune activation induced by multiple TLR ligands in vitro (Klinman et al., 2003, Zhu et al., 2002). In vivo, suppressive ODN ameliorated a variety of organ-specific autoimmune diseases, including inflammatory arthritis, rheumatoid arthritis, and EAE (Zeuner et al., 2002, Dong et al., 2004 , Ho et al., 2003).

This thesis is designed to broaden the immunosuppressive spectrum of a candidate TLR ligand antagonist A151 ODN. The first part of the thesis will be devoted to demonstrate the beneficial role of suppressive ODN on experimental 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.In the second part, human Familial Mediterranean Fever was selected as a model for a systemic autoinflammatory disease.

On EIU model, different sites from rabbit and/or mouse will be analyzed and downreglatory effect of A151 on the gene expression or protein secretion levels will be assessed either by PCR or by ELISA respectively. Changes in mRNA message of IL1β, IL6, IL-15, IP10, iNOS, MIP3α will be checked by PCR method. ELISA assay will be performed on one of the key proinflammatory cytokine, namely, IL6.

In the second part of the study, we will isolate peripheral blood mononuclear cells (PBMCs) from FMF patients and healthy controls. Whether there is a background TLR (1-10) expression difference or baseline activity of certain proinflammatory or Th1-based cytokine expression levels (such as IL1β, IL6 and TNFα) between FMF and healthy donors will be determined. Then, these cells will be subjected to i) PGN for TLR2, ii) LPS for TLR4, iii) pI:C for TLR3, iv) R848 for TLR7/8 stimulations either alone or in combination with A151, and suppressive ability to downregulate the innate immune response initiated by these ligands will be documented.

MATERIALS AND METHODS

2.1. MATERIALS

All cell culture media components were from Hyclone (USA) unless otherwise stated. Cytokine pairs for ELISA assays were from Endogen (USA) unless otherwise stated. TLR ligands; phosphorothioate backbone modified K-Type (or Type B) ODN, ODNK23: 5`-TCGAGCGTTCTC-3` and D-type (type A) mixed backbone ODN (phosphorothioate-phosphodiester- phosphorothioate: PS-PO-PS) ODND35 5`-GGtgcatcgatgcaggggGG (lower case letters are PO bases) and suppressive ODN A151 5`-TTAGGGTTAGGGTTAGGGTTAGGG-3` were obtained from Alpha DNA (Montreal, Canada), PGN (isolated from *B.subtilis*; Fluka, Switzerland), pI:C (Amersham, UK), LPS (isolated from *E.coli*; Sigma, USA), , phosphorothioate backbone modified synthetic R848 (Invivogen, USA). TRIdity G (AppliChem, Germany) was used for RNA isolation. cDNAs were synthesized by DyNAmo^{TM} cDNA Syntesis kit (Finnzymes, Finland) according to the manufacturer's protocol.

For the information on standard solutions, buffers, and other cell culture media please refer to Appendix 1 for details.

2.2. METHODS

2.2.1. The Maintenance of the Animals

Adult female BALB/C mice 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).

2.2.2. Endotoxin Induced Uveitis Model

40 Ten week old female BALB/c mice were obtained from the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University. The mice were injected i.p with 250-300 ug of ODN (a dose previously found to prevent the development of

autoimmune disease) and/or LPS (30-100 ug in 200ul of PBS). Clinical assessment of EIU was based on redness and discharge of the eye, cloudy anterior chamber, and lack of papillary reactivity to the light. Control mice were injected with 200ul PBS i.p. Mice were killed at 24 hours after injection. Eyes were enucleated and used for cytokine expression assays. Spleens were removed and incubated on tissue culture plates for 4 hrs 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 message expression analysis by RT-PCR.

In another experiment, Rabbits (3-4 animal/groups, 1500 gm each housed in Ankara Hospital animal facility, Cebeci, Ankara) were separated in to different treatment groups and EIU was initiated via intraocular LPS injection (100 ng) with or without A151 suppressive ODN. Eyes were removed and further analyses was conducted on them.

2.2.3. Cell Culture

2.2.3.1. Spleen and Ocular Cell Preparation

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.

2.2.3.2. Peripheral Blood Mononuclear Cell Preparation

50 ml blood collected from each donor with heparinized syringes and seperated into two 50ml falcon tubes from syringes. 25 ml blood slowly layered on the top of each 15 ml histopaque layer and centrifuged 30 minutes at 1800 rpm at room temperature (RT) setting the break off. The opaque interface containing mononuclear cells slowly aspirated into a new 50 ml falcon tube by using a sterile Pasteur pipette. Tubes were then filled with %2 FBS supplemented regular RPMI-1640 and centrifuged 10 minutes at 1800 rpm at RT. Supernatant removed by using a sterile pipette and pellet resuspended in %2 regular RPMI-1640 and centrifuged 10 minutes at 1800 rpm at RT. This washing process repeated for 3 times and

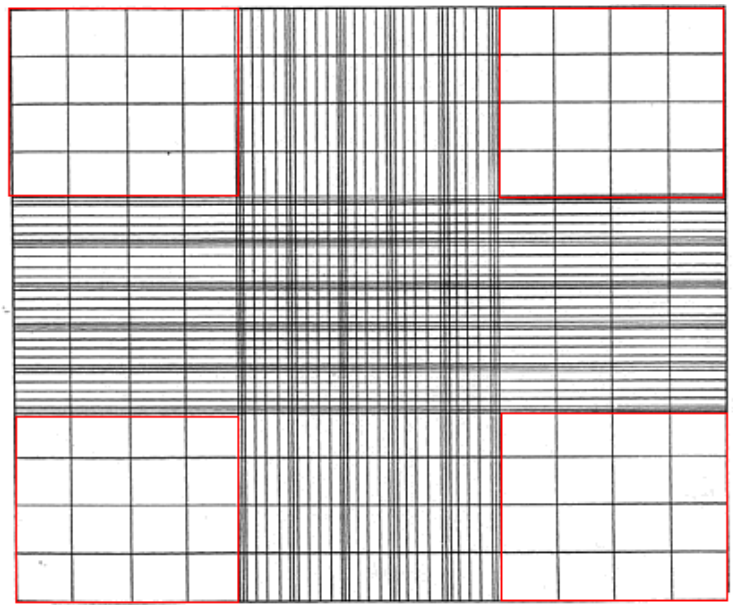
pellet were resuspended in %5 Oligo RPMI-1640 and isolated cells counted by using hemocytometer.

2.2.4. Stimulation Assay

2.2.4.1. Cell Number Detection with Cell Count

After the spleen cells, ocular cells, or peripheral blood mononuclear cells were pooled, 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:

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

2.2.4.2. Cell Distribution

For Cytokine ELISA; 2-4x10^6/ml cells were distributed into 96 well plates with a final volume of 200µl or 250µl media per well. After 6 to 42 hours stimulation supernatants were collected from the plates and stored at -20°C. Supernatants were layered to the plates with or without diluting for two assay as previously mentioned.

Cells were splitted into 6 well plates or 15 ml falcons with a final concentration 2-3 ml, for RNA isolation after stimulation with TLR ligands and/or suppressive ODN for 2 and 4 hours.

2.2.4.3. Stimulation with TLR Ligands and/or Supressive ODN

PBMCs were stimulated with various TLR ligands in optimum doses of: Cont.K23 ODN; 1uM, CpG ODN K23; 1uM, CpG ODN D35; 1uM, PGN; 1ug/ml, LPS; 0.25 ug/ml, pI:C 20ug/ml, R848; 1ug/ml and suppressive ODN A151; 3uM.

Cells were incubated with 5% Oligo RPMI-1640 when stimulated with ODNs and cultured with 5% Regular RPMI-1640 as they stimulated with other TLR Ligands.

2.2.5. Enzyme Linked-ImmunoSorbent Assay

2.2.5.1. Cytokine ELISA

Polysorp (F96 Nunc-Immunoplate, NUNC, Germany) plates were coated with anti-cytokine (Pierce, Endogen) mouse or human IL-6 monoclonal antibody ; 10 µg/ml, 5 µg/ml, respectively for 4-5 hours at room temperature or overnight at +4°C. Plates were blocked with blocking buffer for 2 hours at room temperature and washed with wash buffer for 5 minutes, 5 times and rinsed with ddH_2O. Supernatants and serially diluted recombinant proteins of mouse IL-6 (2000 ng/ml) and human IL-6 (1000ng/ml) were added and incubated for 2 hours at room temperature or overnight at +4°C. Plates were washed as previously described. For the detection of cytokine levels; biotinylated anti-cytokine antibodies (Pierce, Endogen) were prepared in a T-cell buffer, 1:1000 dilution, added to the plates and incubated for 2 hours at room temperature or overnight at +4°C, followed by washing. 1:5000 diluted SA-AKP was prepared in T-cell buffer and added to the plates for 1 hour at room temperature. After washing the plates; PNPP substrate was added and after color formation, in three different intervals optical densities at target wavelengths were measured on an ELISA plate reader (BioTek, µQuant) at 405 nm. The reading was terminated for each plate when an S shaped recombinant cytokine standard curve is obtained.

Concentrations of the cytokines in supernatants were determined by the 4-parameter standard curves generated by using recombinant proteins as mentioned above.

2.2.6. Determination of the Gene Expression

2.2.6.1. Total RNA Isolation from the Cells

After incubating cells (mouse spleen or human PBMC) with various TLR Ligands and/or suppressive ODN A151 for 2 and 4 hours, total RNAs were isolated. The cells were scraped and centrifuged at 2500 rpm for 5 min. in cold media. Then the media was removed and cells were 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.

2.2.7. 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.

2.2.8. PCR

2.2.8.1. 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 2.1 The sequences, the product sizes and the sources of the mouse primers used

Primer	Forward	Reverse	Product Size
m β-actin*	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA	450 bp
m IP10 *	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT	127 bp
m iNOS *	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	95 bp
mMIP1α ¶	ACCATGACACTCTGCAACCA	AGGCATTCAGTTCCAGGTCA	238 bp
mIL-5 *	AGCACAGTGGTGAAAGAGACCTT	TCCAATGCATAGCTGGTGATTT	117 bp
mIL-15 *	CATCCATCTCGTGCTACTTGTGTT	CATCTATCCAGTTGGCCTCTGT	126 bp
mIL-18 ¶	GATCAAAGTGCCAGTGAACC	ACAAACCCTCCCCACCTAAC	384 bp
mMCP-1 ¶	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG	249 bp
mMIP3α ¶	CGTCTGCTCTTCCTTGCTTT	CCTTTTCACCCAGTTCTGCT	250 bp
mCXCL16 ¶	CCTTGTCTCTTGCGTTCTTC	GGTTGGGTGTGCTCTTTGTT	384 bp
mMIP1β ¶	CCAGCTCTGTGCAAACCTAA	CTGTCTGCCTCTTTTGGTCA	250 bp

¶ Designed primer

* An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression, Giulietti et al., 2001, Methods

Table 2.2The sequences, the product sizes and the sources of the human primers used

Primer	Forward	Reverse	Product Size
h GAPDH	ACCACCATGGAGAAGGCTGG	CTCAGTGTAGCCCAGGATGC	532 bp
h TLR 1	CATAACTCTGCTGATCGTCACC	TGCTAGGAATGGAGTACTGCG'	491 bp
h TLR 2	GATGACTCTACCAGATGCCTCC	CAGAAGAATGAGAATGGCAGC	745 bp
h TLR 4	TTACCTGTGTGACTCTCCATCC	CAGAAGAATGAGAATGGCAGC	507 bp
h TLR 5	CCTTGACTATTGACA AGGAGGC	TTGTAGGCAAGGTTCAGAACC'	713 bp
h TLR 6	TCTCATGACGAAGGATATGCC'	CGATCAGCAGAGTTATGTTGC	578 bp
h TLR 7	ACGAACACCACGAACCTCAC	GGCACATGCTGAAGAGAGTTAC	725 bp
h TLR 8	TGGCTTGAATATCACAGACGG	ACCAGGCAGCATTAATCTTCC	584 bp
h TLR 10	GAACTGATGACCAAC TGC TCC'	GAAGTCTTGATTCCATCACGC	557 bp
h IL1 β	AGATGATAAGCCCACTCTACAG	ACATTCAGCACAGGACTCTC	276 bp
h IL 6	ACAGCCACTCACCTCTTCAG	CCATCTTTTTCAGCCATCTTT	168 bp
h TNF α	CCCGAGTGACAAGCCTGTAG	GATGGCAGAGAGGAGGTTGAC	271 bp

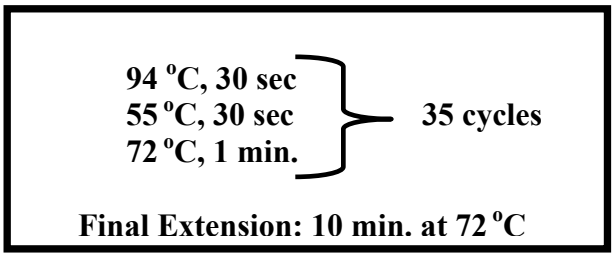
2.2.8.2. Semi-Quantitative RT-PCR

For the comparison of the mRNA expression levels of the samples, semiquantitative reverse-transcriptase PCR (MJ Mini, BIO-RAD, USA) was performed. Quantification of the band intensities was performed using MultiAnalyst and Bio1D softwares. The quantitated values for the samples were normalized by the division with the quantitated values for the *β-actin* for each sample separately. The reaction ingredients used in PCR reactions are shown in Table 2.3 and the condition of PCR reactions are shown in Table 2.4

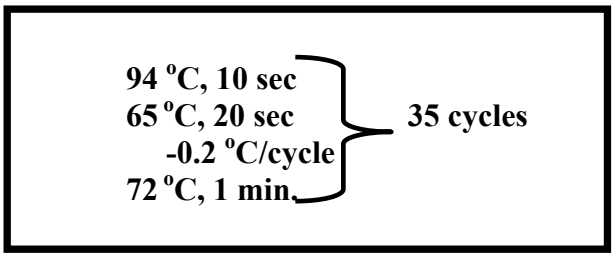
Table 2.3 PCR reaction ingredients

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

Table 2.4: PCR Conditions



PCR conditions used in mouse experiments



PCR conditions used in human experiments

2.2.8.3. 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.

2.2.9. Statistical Analysis

Statistical analysis was done by using SigmaSTAT software significance level between untreated (or control groupd) vs treatments were determined via Student's t- test analysis.

RESULTS

3.1 ENDOTOXIN INDUCED UVEITIS

Endotoxin-induced uveitis (EIU) is an established animal model of acute ocular inflammation. It is induced by either systemic or intravitreal administration of lipopolysaccharide (LPS), the major component of gram-negative bacteria. LPS acts through TLR4 triggering proinflammatory signaling cascade. The expression of Th1 cytokines and chemokines, including interleukin 6 (IL6), IL-1β, and MIP3α contributes to the development of EIU.

Following local or intraperitonial LPS and/or suppressive ODN administration, rabbit and mouse eyes were removed and RNA from iris, vitreous and cornea from rabbit eyes were obtained (for mouse total RNA was obtained from the whole eye(s). PCR was run from cDNA of each sample and mRNA message of IL1β IL6, IL-15, IL18, IP10, iNOS, MIP1α, MIP1β, MIP3α, and CXCL16 levels was monitored. Murine splenocytes were also incubated for 6, 12 and 24 hrs on 96 well plates in triplicates and IL6 secretion level of the culture media was determined by ELISA.

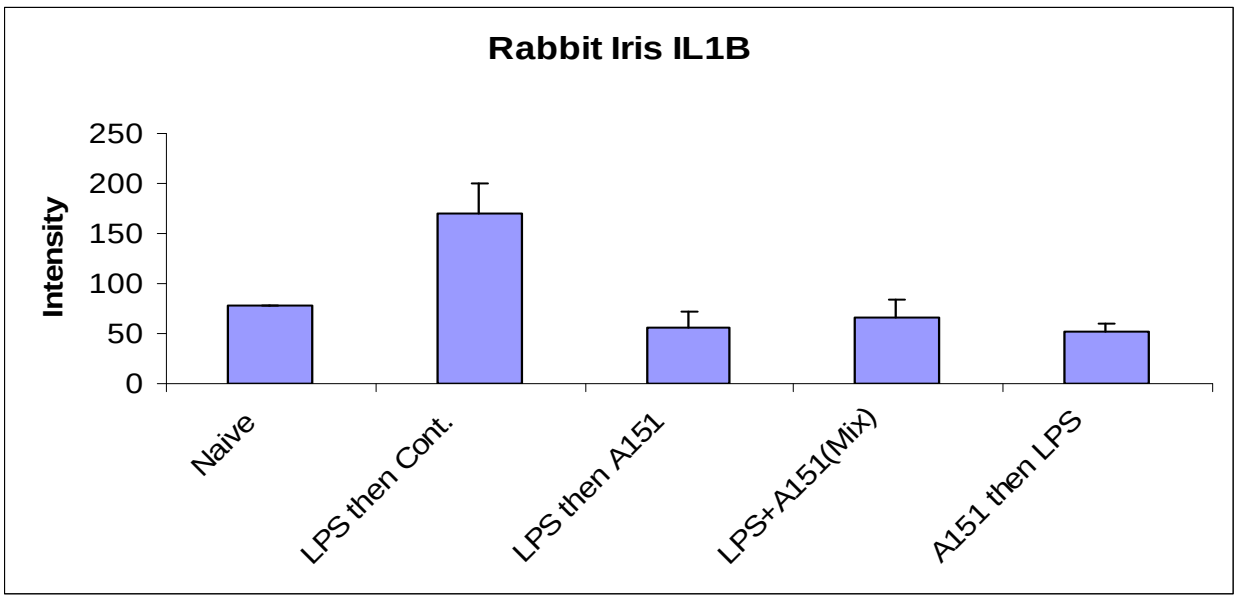


Fig.3.1: IL1β induction (as judged by mRNA level via PCR) from uveitic rabbit iris is suppressed when the eyes are treated with A151.

Results indicate that when suppressive ODN administered before or after LPS treatment or included simultaneously with LPS treatment significantly down regulated the expression of IL1β message from iris (Fig. 3.1). In cornea, however, only IL6 was downregulated when suppressive ODN was given before LPS administration (Fig. 3.2).

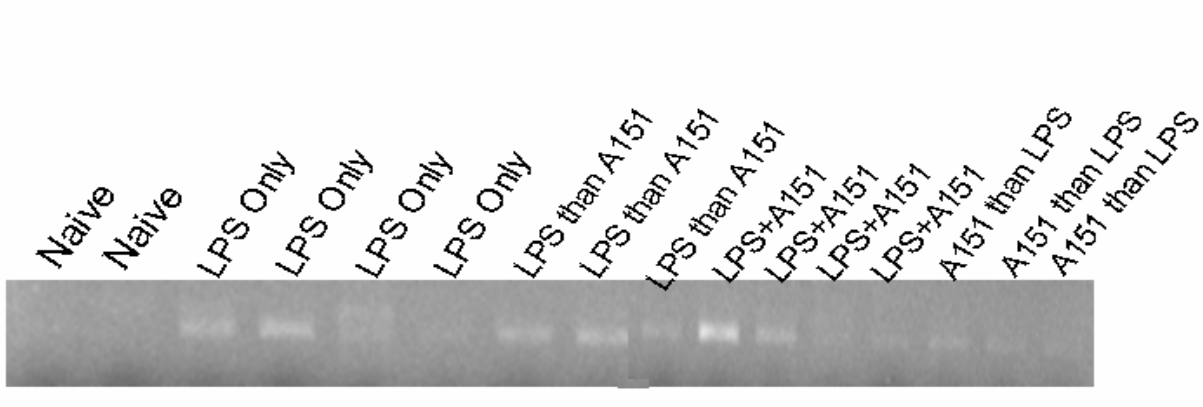


Fig 3.2: IL6 message is strongly suppressed in cornea of uveitic rabbit eye upon A151 treatment.

Eventhough it is pricy to conduct rabbit experiment and logistically problemmatic to house the animals in a different site instead of our animal facility, the information obtained from this set of experiment was critical, because the direct injection of the agent (LPS) to the vitreous part of the eye was possible in rabbit system, thus we could induce local EIU at the site of the A151 therapy. Another problem associated with the rabbit experiment worth mentioning was that, after we have initiated the study we have realized that it was not possible to set the best time to retrieve the eye for further investigations. Since LPS mediated inflammation was initiated at the vitreous site, it was not possible to deduce when to collect the tissues post-LPS treatment. We have decided to sacrifice the animals 24 hrs after the treatment, and check as many sites as possible, (i.e. vitreous, iris, and cornea). Data indicated that we have unfortunately could not detect any significant change on the gene expression levels of several cytokines/chemokines from vitreous region (data not shown). Nevertheless, downregulation of the proinflammatory cytokine IL1β and IL6 could be detected from iris and cornea respectively. Instead of repeating rabbit experiment, it was much more reliable to move to mouse model.

On the murine EIU experiments LPS administrated via i.p. in two different doses; 30 and 100ug. Supressive ODN (or Control ODN) were given at 250ug and 300ug doses.

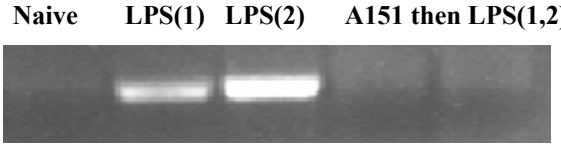


Fig.3.3: MIP3α expression level of mouse eyes either injected i.p. with 100µg LPS alone or following 250 µg A151 supressive ODN Rx (Numbers in paranthesis indicate the number of mice used in the study).

Results showed that when 250ug supressive ODN administered before LPS treatment it significantly down regulated the expression of several important Th1 cytokines and proinflammatory chemokines IL18, IP10, iNOS, MIP1α, MIP1β, MIP3α, and CXCL16 in 100ug LPS injected mEIU model. Of these, MIP3α and iNOS were the most significant down regulated mediators. The other cytokines also showed substantial but non significant down regulation. (data not shown)

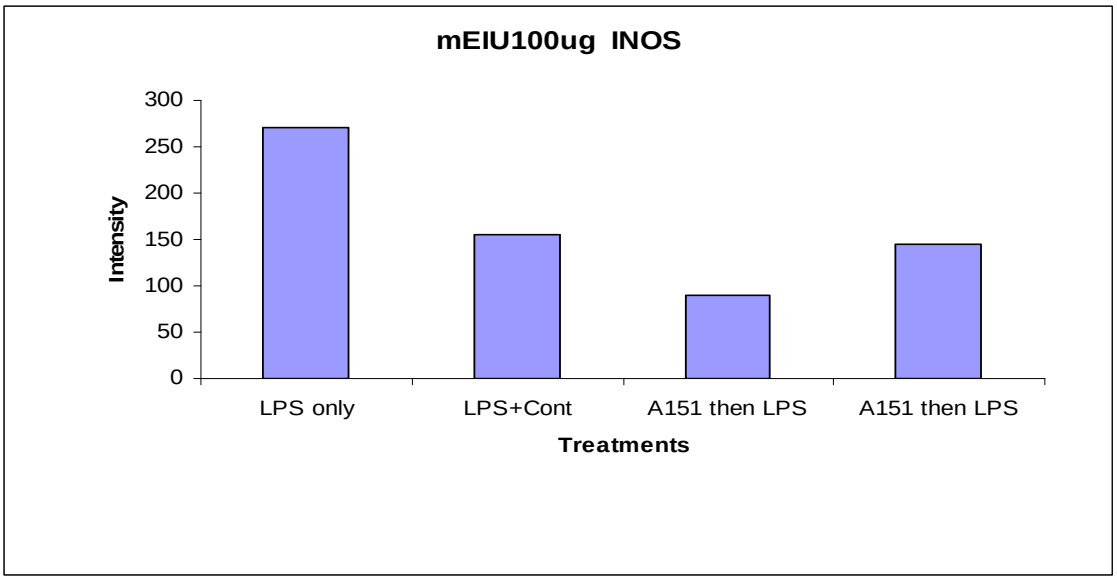


Fig 3.4: Suppression of LPS triggered inducible nitric oxide synthase gene by A151 treatment.

Results demonstrates that when 300ug supressive ODN administered before LPS treatment, it significantly down regulated the expression of IP10 and MCP1 in 30ug LPS injected mEIU model (data not shown). Results show that when supressive ODN administered before or at the same time with LPS treatment it significantly reduced the IL6 level in mouse splenocytes (for 30ug LPS injected experiment).

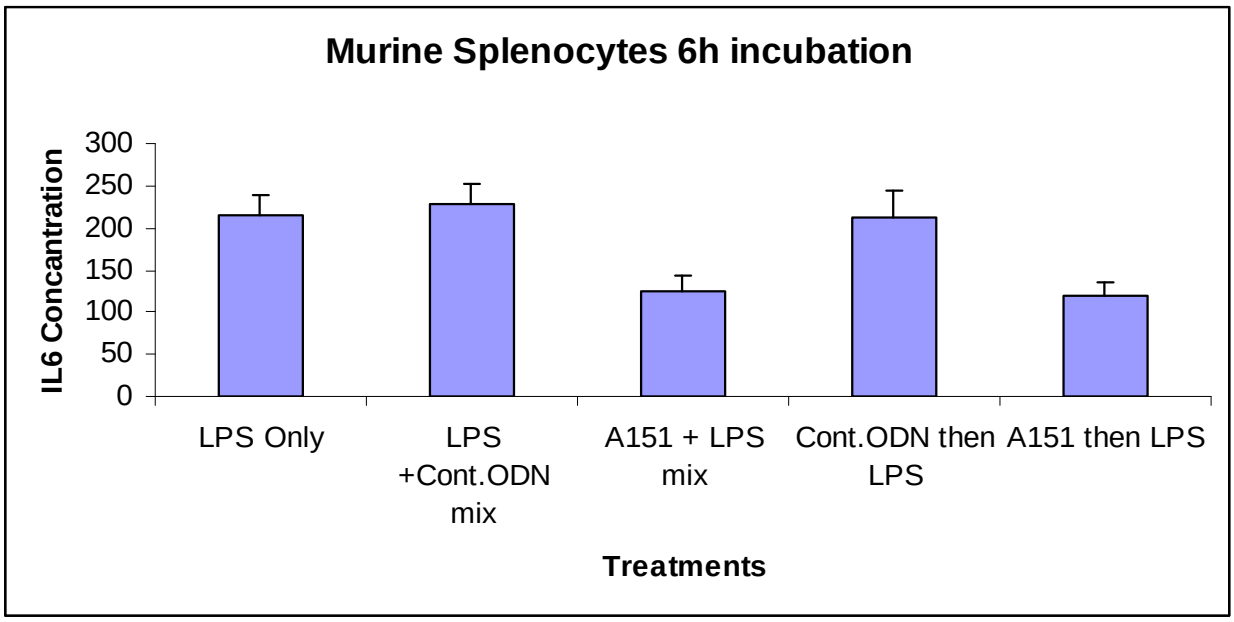


Fig 3.5: Reduction of IL6 production by A151 ODN from murine spleen cells induced by LPS

When taken together, these results indicated that rabbit and mouse EIU can be reproducibly estaliblished by either parenteral or local administration of LPS, and the disease severity and progression at least in part can be controlled by the administration of A151 suppressive ODN either simultanously or ahead of the insult initiation.

3.2. FAMILIAL MEDITERRANEAN FEVER (FMF)

FMF is an autosomal recessive periodic fever disease characterized by recurrent, self-limiting, febrile, inflammatory attacks of the serosal membranes such as peritoneum, pleura, and synovia. FMF patients in clinical remission are reported to have increased baseline inflammation. It has been demonstrated that A151 ODN can either neutralize or down regulate overexuberant ongoing immune response and may control local, tissue specific, and even certain systemic autoimmune diseases (Zeuner et al., 2002, Dong et al., 2004, Ho et al., 2003, Yamada et al., 2004). Our preliminary experiments prompted us to focus on FMF as an autoinflammatory disease, and use suppressive A151 ODN hoping to neutralize this increased baseline inflammation.

The study was performed on 7 healthy donors and 7 FMF patients. Peripheral blood mononuclear cells (PBMC) are isolated from FMF patients and healthy controls. We first wanted to establish whether there is a difference between the background TLR (1-10) expression between FMF and healthy donors, furthermore we wanted to check the baseline

activity of certain proinflammatory or Th1-based cytokine expression levels (such as IL1β, IL6 and TNFα) by RT-PCR. As seen in figures 3.6 and 3.7 baseline IL1B and IL6 message levels were significantly higher in FMF patients compared to healthy donors. This difference is more pronounced for IL6. While there is about 2.5 fold difference in the IL1B message level of FMF patients to that of healthy subjects, this rose to more than 4 fold for IL6.

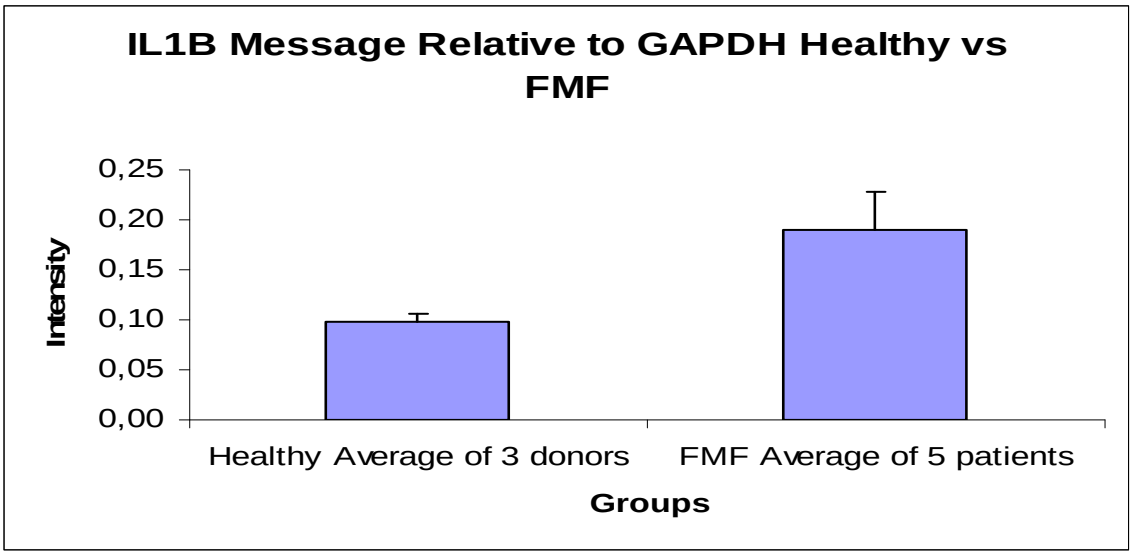


Fig 3.6: The baseline mRNA level of IL1B is significantly elevated in FMF patients ($p<0.01$)

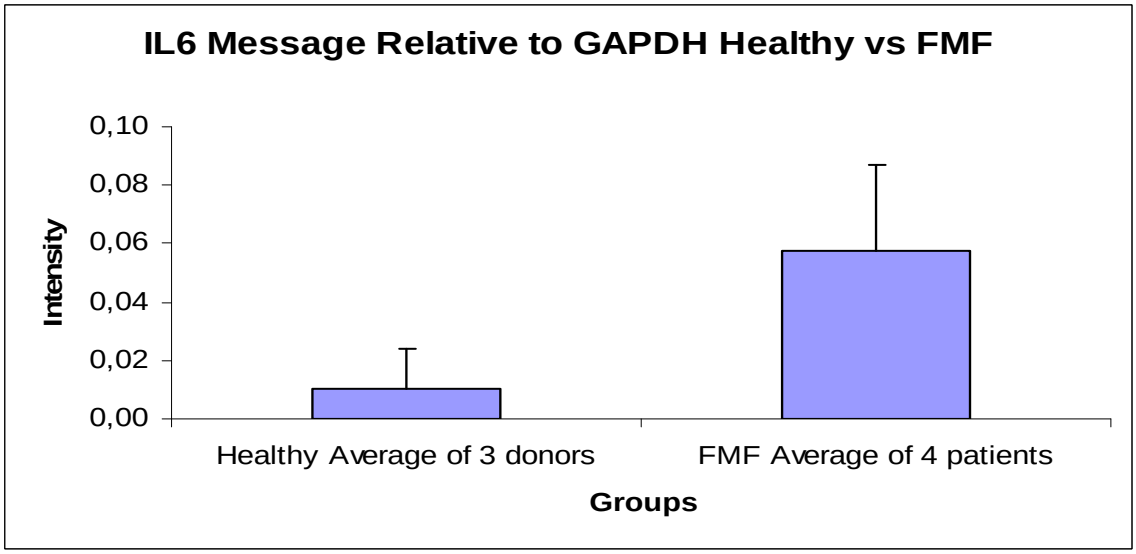
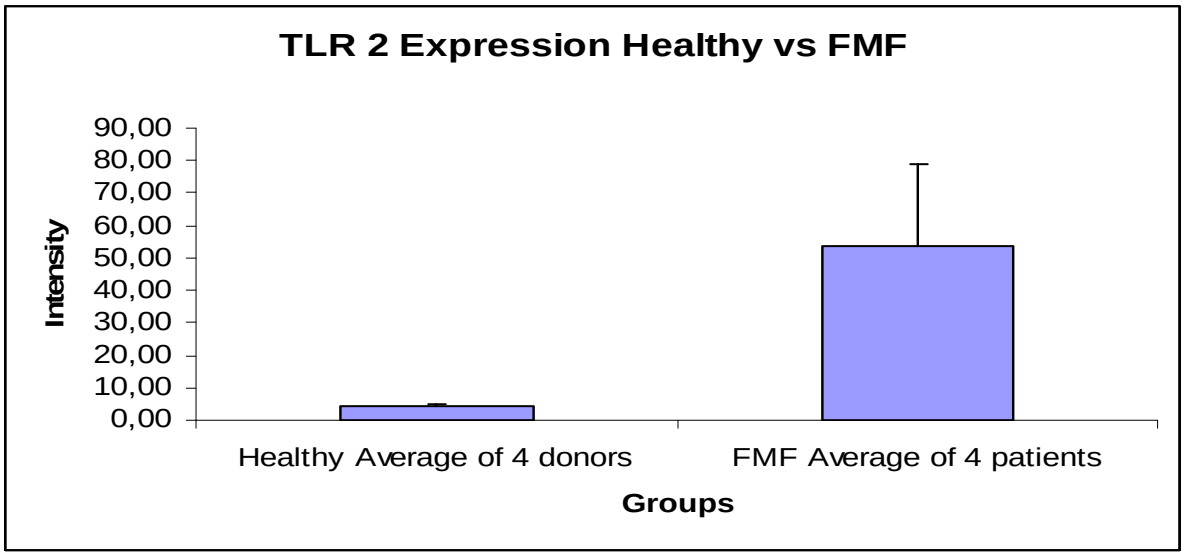


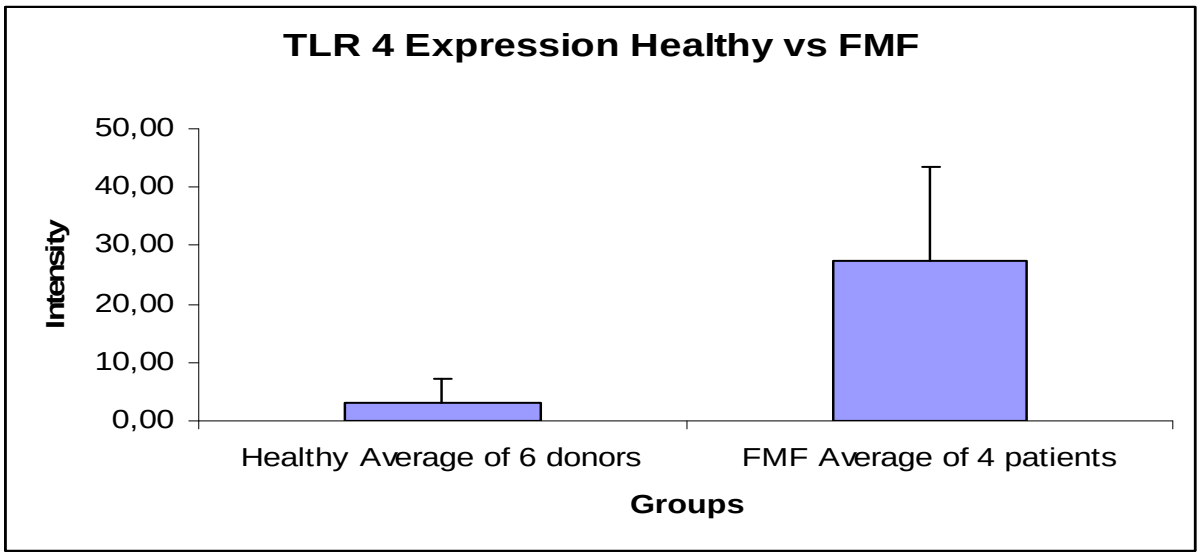
Fig 3.7: The baseline mRNA level of IL6 is significantly elevated in FMF patients ($p<0.01$)

In additon to the cytokine levels, the mRNA from these donors were also checked for the expression level of the TLR panel. As seen in Figs 3.8 (A), (B), and (C) for TLR2, 4 and 7 baseline expression (from unstimulated FMF PBMC) level showed significant mRNA message intensities compared to blood taken from unstimulated healthy donor.

(A)


TLR 2 Expression Healthy vs FMF
Intensity
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
Healthy Average of 4 donors
FMF Average of 4 patients
Groups

(B)


TLR 4 Expression Healthy vs FMF
Intensity
50,00
40,00
30,00
20,00
10,00
0,00
Healthy Average of 6 donors
FMF Average of 4 patients
Groups

(C)

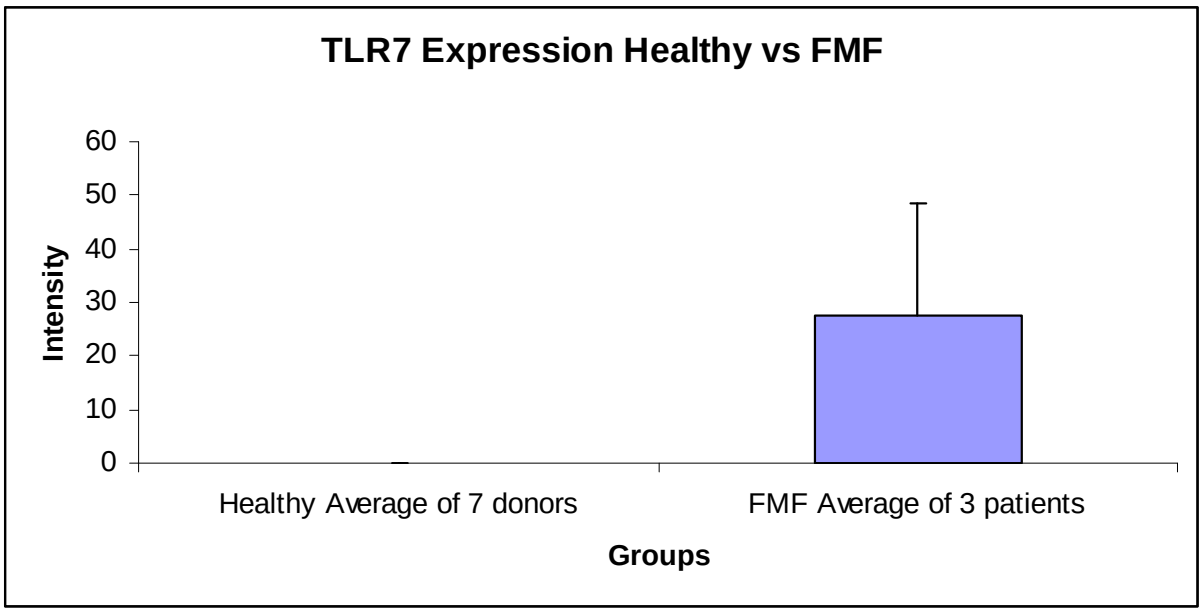


Fig 3.8: (A) TLR2 expression profiles of unstimulated FMF vs healthy subjects, (B) TLR4 expression profiles of unstimulated FMF vs healthy subjects, and (C) TLR7 expression profiles of unstimulated FMF vs healthy subjects. ($p<0.01$, for all TLRs)

Initial characterization results indicated that indeed not only there is significantly increased baseline inflammation, but certain TLRs (both extracellular and endosome associated) have higher TLR2, TLR4, TLR7, levels compared to healthy controls.

These findings encouraged us to stimulate PBMCs with various TLR ligands such as i) PGN for TLR2, ii) LPS for TLR4, iii) pI:C for TLR3, and iv) R848 for TLR7/8 either alone or in combination with supressive ODN (A151). Cells incubated with those ligands and then supernatants were collected 48h after stimulation. Cytokine ELISA was performed to analyse the levels of IL6. As seen in Fig 3.9 , IL6 production in response to TLR2, TLR4, and TLR7/8 ligand stimulation by FMF patients induced substantially higher levels of IL6 (to that of healthy donors` PBMC).

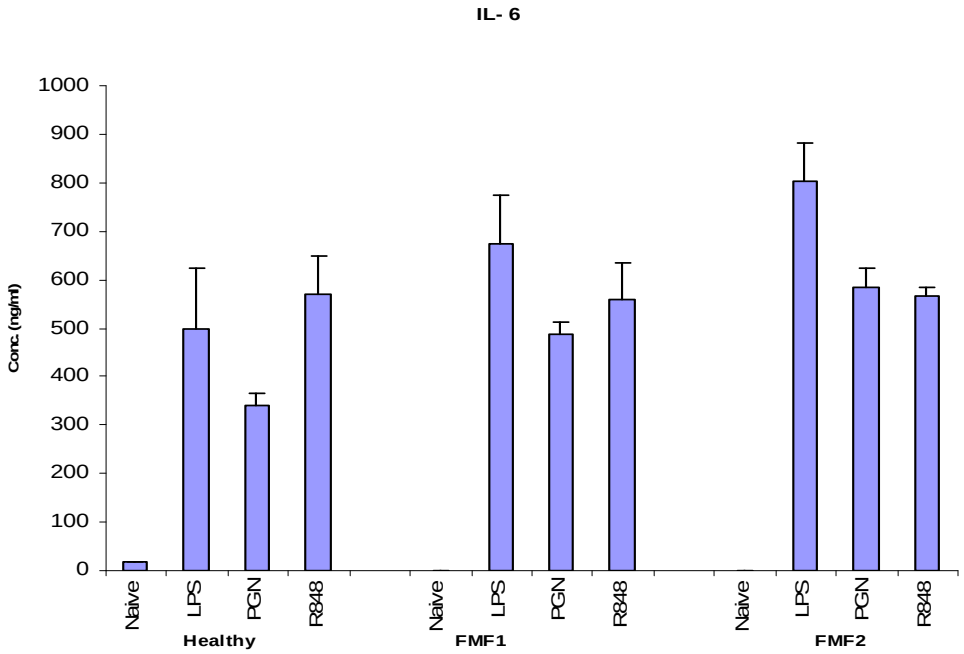


Fig 3.9: IL6 induction level of healthy and FMF PBMCs. ($p<0.01$ for LPS and PGN groups)

Our data suggest that FMF patients are more sensitive than healthy subjents in terms of responding to TLR ligand mediated immune activation. In an attempt to control this overexuberant immune activation of FMF patients, supressive ODN, A151 is used in further experiments in order to determine whether A151 can supress either the cytokine message or cytokine secretion from TLR ligand stimulated healthy and/or FMF patients` PBMCs.

Our data revealed that IL1β expression level of healthy donors significantly suppressed by suppressive A151 ODN. TLR3, and TLR7/8 but not TLR2 and TLR4 ligands mediated IL1β is strongly inhibited by A151 (Fig 3.10). Same level of IL6 suppression was seen for IL6 message (Fig 3.11). One striking feature is that this suppression is suppressive ODN mediated since treatment with control ODN did not show any effect (data not shown).

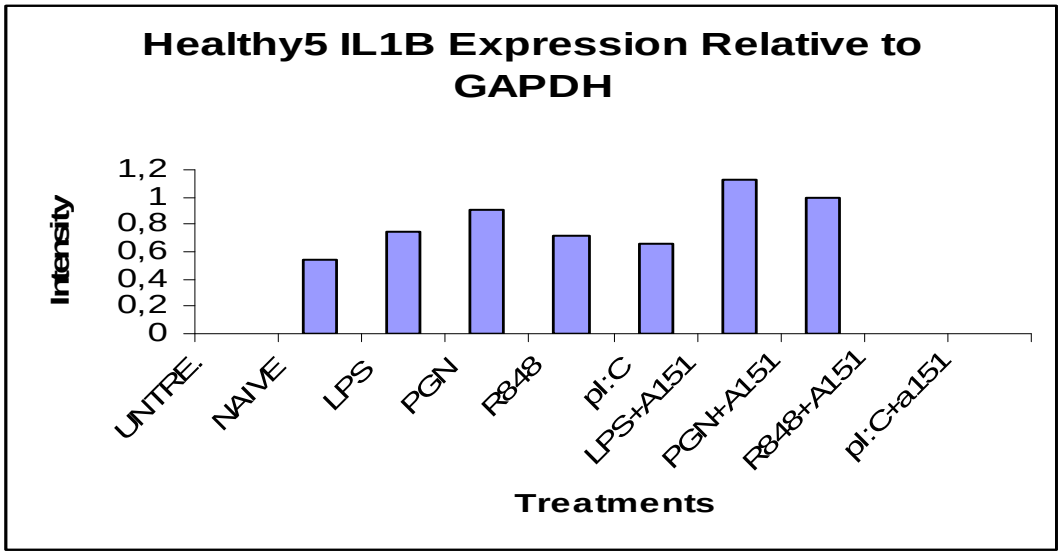


Fig 3.10. TLR3 and TLR7/8 mediated of IL1b message expression is suppressed by A151.

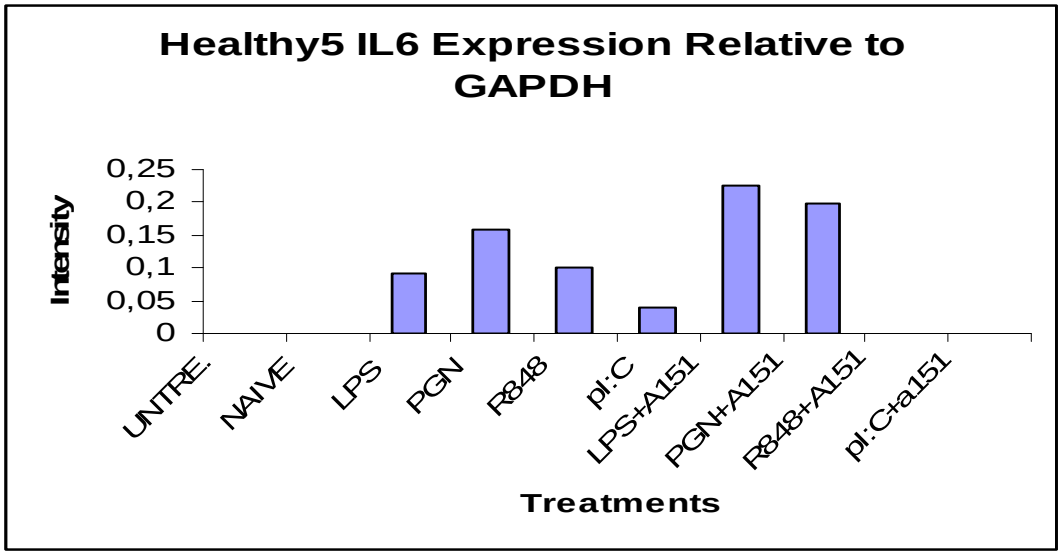


Fig 3.11. TLR mediated IL6 message overexpression is neutralized by treating PBMCs with A151.

The same trend for IL1b and IL6 was repeated for TNFa. Fig 3.12 demonstrate that TNFa expression level upon pI:C and R848 induction increased, and this elevated message is completely abrogated by the addition of suppressive ODN, A151. These results collectively indicate that A151 is strong enough to counteract the proinflammatory activity of TLR3 and 7/8 ligands.

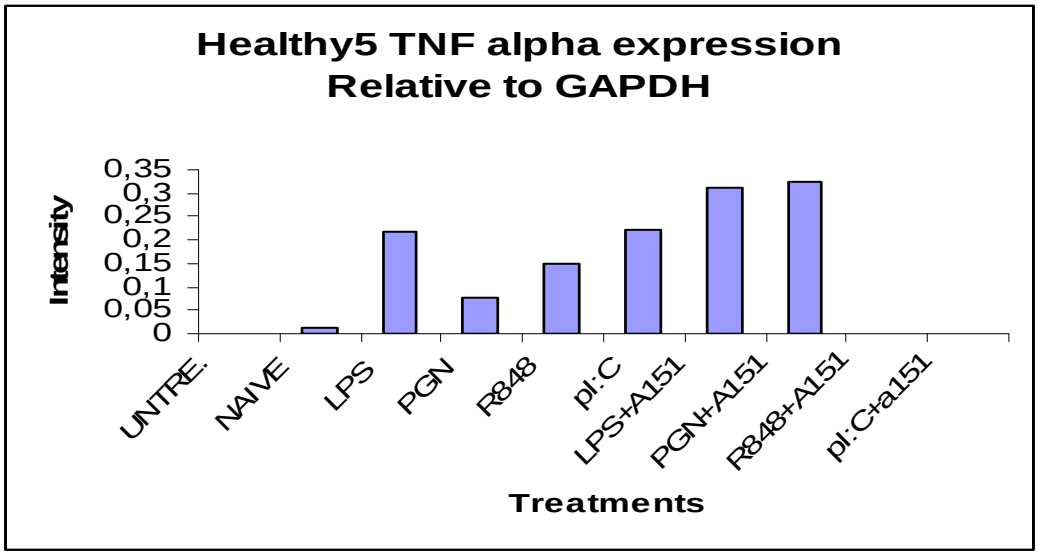


Fig 3.12: TNFa expression is abrogated by A151 when healthy PBMCs were stimulated with either TLR3 or TLR7/8 ligands but not with TLR2 or TLR4 ligands.

Another unexpected observation is that, A151 not only fail to suppress TLR2, and 4 mediated immune activation, but mixing these ODN with the ligands augments the overall immune response (see Figs 3.10, 3.11, and 3.12 for IL1b, IL6 and TNF responses). Fig 3.13 summarizes the RT-PCR band intensities for Healthy subjects.

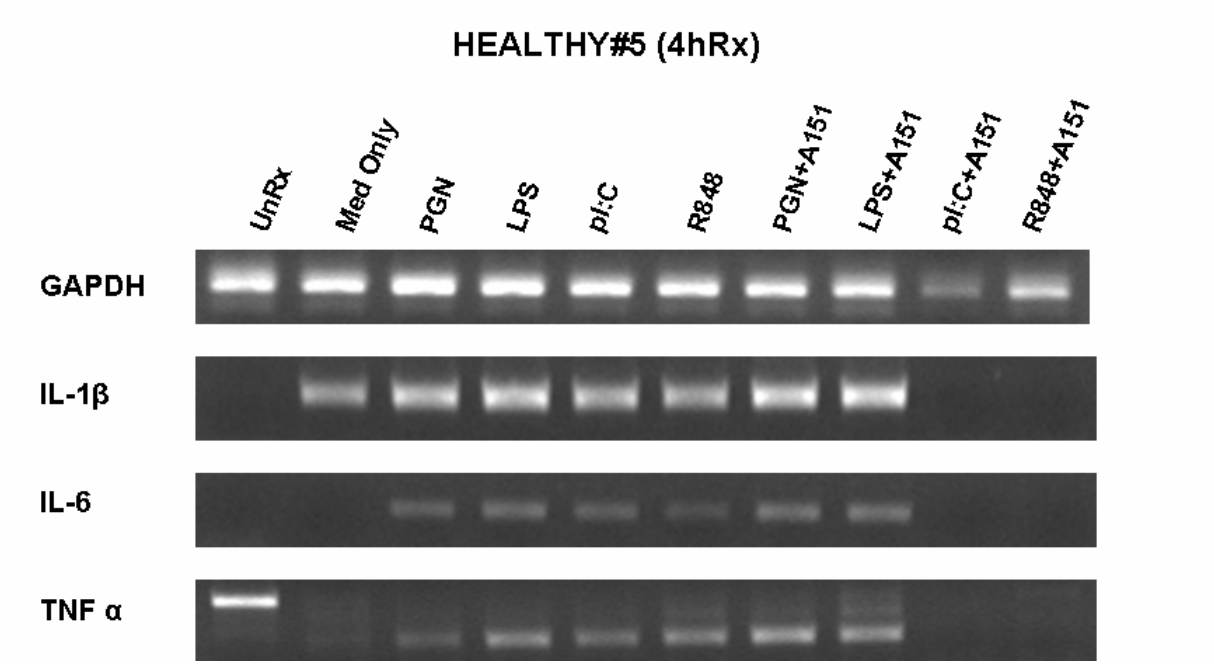


Fig 3.13: PCR band intensities for healthy PBMC subject (representative of 5 repeat experiments).

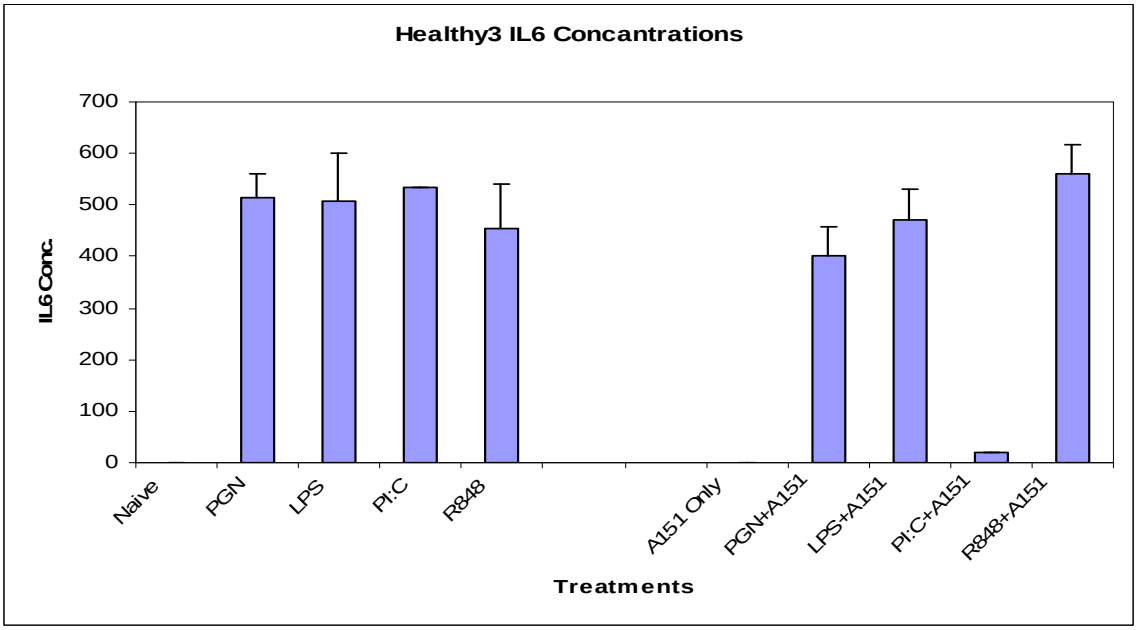


Fig 3.14: IL6 secretion form healthy donors upon stimulated with several TLR ligands.

ELISA results showed that A151 supresses the production of IL6 only in pI:C treated group. These data indicates that A151 down regulates the expression of IL6, IL1β and TNFα at the mRNA message level for R848 and pI:C treated groups and although IL6 message down regulated upon R848 treatment, it could not induce detectable supression on protein level. But, in pI:C treated group IL6 down regulated both the message and the protein secretion.

Later, the supressive effect of A151 tried on FMF patients. PBMCs of the FMF patients stimulated with various TLR ligands and/or supressive ODN checked for IL6 levels determined by ELISA.

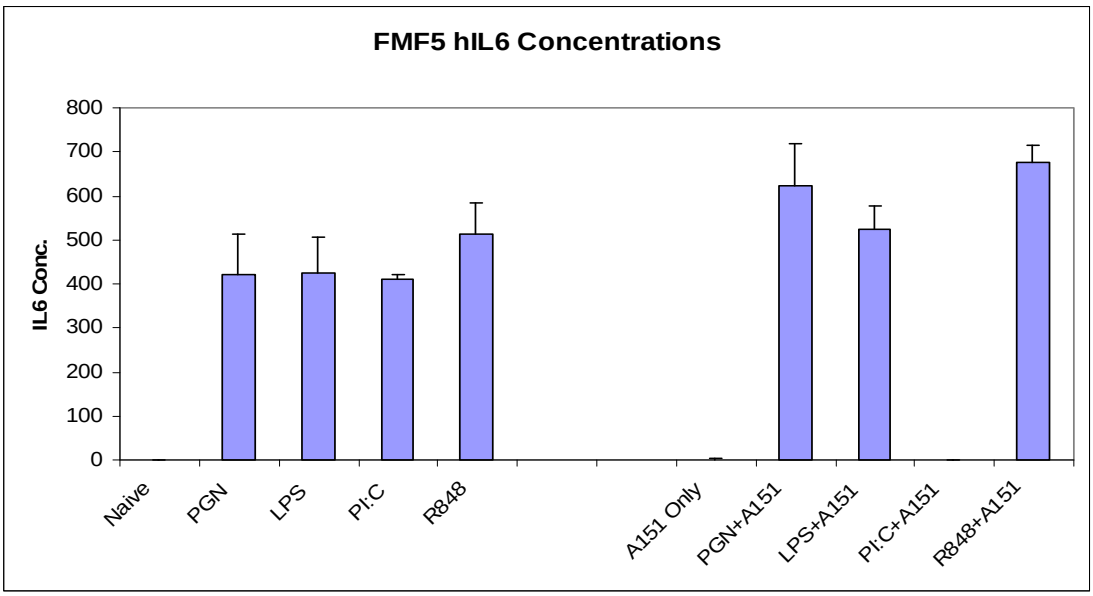


Fig 3.15: Lower A151 dose could only suppress pI:C mediated IL6 secretion, but not the rest of the tested TLR ligands.

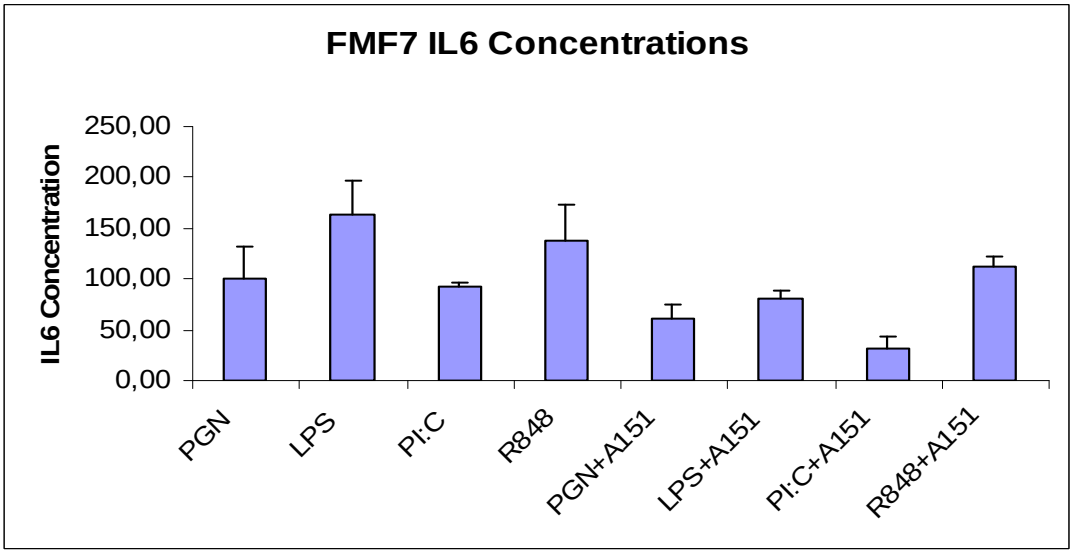


Fig 3.16: Increased A151 treatment dose downregulated IL6 level for pI:C, LPS, PGN but not for R848 ligands. ($p<0.01$ for PGN and LPS alone vs A151plus ligand groups, and $p<0.001$ for pI:C vs A151 plus pI:C group).

As shown in the Figs (3.15 and 3.16), A151 down regulated the production of IL6 in a dose dependent manner. One patients PBMCs when it is stimulated with LPS, pI:C or PGN. On the other hand, in another patient A151 supressed IL6 production only in pI:C stimulated group but not the rest.

It is clear from these data that more patients are needed to enroll in these tests. It is challenging and time consuming to obtain several subjects that can be analyzed to establish the significance level. We are committed to test more patients and gather reliable data to demonstrate the beneficial effect of suppressive DNA on FMF patients.

DISCUSSION

The innate immune response triggered by several TLR ligands can improve host survival following pathogen challange. Yet unchecked stimulation of the innate immune system can cause tissue damage, autoimmune disease, and even death. Krieg et al were the first to demonstrate that "neutralizing" ODN containing GC-rich sequences selectively inhibited CpG-induced immune activation (Krieg et al., 1998).

Other G-rich ODN were also shown to block the interaction of CpG ligands with their Toll-like receptor (TLR9) (Lenert et al., 2001, Zhu et al., 2002). Subsequently, Gursel et al identified a different class of "suppressive" ODN, patterned after the repetitive TTAGGG motifs present at high frequency in mammalian telomeres (but rare in the genomes of bacteria). Studies showed that these ODN had broad immunosuppressive properties and could down-regulate the production of certain Th1 and proinflammatory cytokines (Klinman et al., 2003, Gursel et al., 2003, Yamada et al., 2002). This led scientists to hypothesize that endogenous TTAGGG motifs released by dying host cells might serve to down-regulate pathologic/ overexuberant host immune responses (Klinman et al., 2003, Gursel et al., 2003). Consistent with such a possibility, suppressive ODN were found to limit the immune activation induced by multiple TLR ligands in vitro (Klinman et al., 2003, Zhu et al., 2002). In vivo, suppressive ODN ameliorated a variety of organ-specific autoimmune diseases, including inflammatory arthritis, rheumatoid arthritis, and EAE (Zeuner et al., 2002, Dong et al., 2004, Ho et al., 2003).

This study establishes that suppressive ODN (typified by ODN A151) is capable of suppressing at least in part some features of the Th1 proinflammatory response both in an organ specific autoimmune disease model (endotoxin-induced uveitis) and a systemic autoinflammatory disease Familial Mediterranean Fever.

Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation induced by the administration of lipopolysaccharide (LPS), a component of Gram-negative bacterial outer membranes. Uveitis can have a variety of underlying causes. For instance, acute anterior uveitis is often associated with Behcet's disease, ankylosing spondylitis, Reiter's syndrome, and human leukocyte antigen (HLA) B27-associated uveitis as well as other systemic inflammatory diseases (Chang et al., 2005)

LPS enhances the expression of various inflammatory mediators, such as IL-6, (Hoekzema et al., 1992, Ohta et al., 2005) TNF-α, (Koizimi et al., 2003) and MCP - 1, (Mo et al., 1999) as well as the production of nitric oxide (Bellot et al., 1996) all of which contribute to the development of EIU, resulting in the breakdown of the blood–ocular barrier and in the infiltration of leukocytes.

The current study demonstrates that systemic or intravitreal administration of suppressive ODN down-regulates mRNA message and even production of several proinflammatory cytokines both in the eyes and spleen of mouse and rabbit following local or intraperitoneal LPS administration.

Results from rabbit model indicate that when suppressive ODN administered before or after LPS treatment or included simultaneously with LPS treatment significantly down regulated the expression of IL1β message from iris. In cornea, only IL6 was downregulated when supressive ODN was given before LPS administration.

Results from murine model showed that when supressive ODN administered before LPS treatment it significantly neutralized the expression of IP10, iNOS, MIP1α, IL18, MIP3α, CXCL16 and MIP1β levels. Results also suggest that when supressive ODN administered before or at the same time with LPS treatment, it significantly down regulated IL6 secretion in murine splenocytes.

Autoinflammatory diseases are a group of disorders characterized by seemingly unprovoked inflammation in the absence of high-titer autoantibodies or antigen specific T cells (Stojanov et al., 2005). They include the hereditary periodic fever syndromes (HPF) and are thought to be caused by disturbances in the regulation of innate immunity (Kastner., 2005).

Familial Mediterranean Fever (FMF) is the most well known and best characterized of the hereditary periodic fever syndromes. The mutated gene, MEFV, encoding pyrin/marenostrin protein, was identified in 1997 and found to be predominantly expressed in neutrophils, monocytes and eosinophils but not in lymphocytes (Aksentijevich et al., 1997, Bernot et al.,1997), suggesting a potential functional role in the regulation of inflammation.

Several studies have shown that FMF patients exhibit increased levels of serum IL-6, IL-8, IFNγ, IL12, IL18, TNFα (Kiraz et al., 1998, Baykal et al., 2003, Schattner et al., 1991, Schattner et al., 1996, Aypar et al., 2003). Gang *et al.* (1999) measured IL-1β and IL-1 receptor antagonist levels and suggested that these components are unaltered during attacks (Gang et al., 1999). But in another study, mRNA levels for TNF-α, IL-1β, IL-6 and IL-8 were

all increased relative to controls in circulating leukocytes of attack-free FMF patients (Notarnicola et al., 2002).

Our preliminary FMF study in accordance with the literature pointed that FMF patients have higher IL1β and IL6 expressions, furthermore, current work for the first time demonstrate that baseline TLR2, TLR4, TLR7 levels are significantly elevated compared to healthy controls. FMF patients are producing more IL6 when they are stimulated with certain TLR ligands, (i.e. LPS and PGN), compared to healthy subjects which means FMF patients are more sensitive and responsive to these stimulants compared to healthy individuals. A151 significantly down regulated the expression of IL6, IL1β and TNFα from the PBMCs of healthy donors which are stimulated with TLR7/8 and TLR3 ligands, R848 and pI:C. A151 suppresses pI:C stimulated IL6 production of PBMCs both in FMF patient and healthy donor. We observed that suppression of pI:C was more stronger compared to PGN and LPS. There seems to be a race between the rate of induction/activation via PGN and LPS and A151 down regulatory mechanisms. This observation is supported by the data presented in Figs 3.15 and 3.16. While low dose A151 could not suppress PGN and LPS in Fig 3.15, it was capable of inhibiting LPS and PGN mediated IL6 secretion when A151 dose was raised 2 fold. More work is needed to reveal the mechanism underlying behind this phenomenon.

In conclusion, suppressive ODN A151 inhibits several proinflammatory cytokine messages or cytokine production from immune cells. Our data stongly suggest that mammalian derived suppressive motifs (A151, telomeric sequence) could be used as a therapeutic agent to control either EIU or FMF and might be of use in the treatment of other autoimmune or autoinflammatory diseases.

REFERENCES

1. Adamus G., Burrows G. G., Vandenbark, A. A., and Offner, H. (2006) **Treatment of autoimmune anterior uveitis with recombinant TCR ligands**. Invest. Ophthalmol. Vis. Sci. 47, 2555–2561

2. Akira S, Takeda K. (2004) **Toll-like receptor signalling**. Nat Rev Immunol 004;4:499–511

3. Akira S., Uematsu S., Takeuchi O. (2006) **Pathogen recognition and innate immunity**. Cell124, 783–801

4. Aksentijevich I., Centola M., Deng ZM. (1997) **Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever**. Cell 90, 797

5. Alberts B., Johnson A.,Lewis J., Raff. M.,Roberts K., Walters P. (2002) **Molecular Biology of the Cell; Fourth Edition**. New York and London: Garland Science.

6. Alexopoulou L., Thomas V., Schnare M., Lobet Y., Anguita J., Schoen RT., Medzhitov R., Fikrig E., Flavell RA. (2002). **Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2- deficient mice**. Nat. Medicine 8, 878.

7. Avunduk M. C., Avunduk A. M., Oztekin E., Baltaci A. K., Ozyazgan Y., and Mogolkoc R. (2004) **Etanercept treatment in the endotoxin-induced uveitis of rats**. Exp. Eye Res. 79, 357–365

8. Aypar E., Ozen S., Okur H., Kutluk T., Besbas N., Bakkaloglu A.(2003). **Th1 polarization in familial Mediterranean fever**. J. Rheumatol. 30, 2011.

9. Barton GM., Kagan JC., Medzhitov R. (2006) **Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA**. Nat Immunol.7(1), 49-56.

10. Baykal Y., Saglam K., Yilmaz M.I., Taslipinar A., Akinci, S.B., Inal A. (2003). **Serum sIL-2r, IL-6, IL-10 and TNF-alpha level in familial Mediterranean fever patients** Clin. Rheumatol. 22, 99.

11. Beck G., Gail S., Habicht A., (1996) **Immunity and the Invertebrates**. Scientific American: 60–66.

12. Bellot JL., Palmero M., Garcia-Cabanes C., Espi R., Hariton C., Orts A. (1996) **Additive effect of nitric oxide and prostaglandin-E2 synthesis inhibitors in endotoxin-induced uveitis in the rabbit**. Inflamm Res.45, 203–208.

13. Ben-Chetrit E., Levy M. (1998) **Familial Mediterranean fever**. Lancet 351:659.

14. Bernot A, Clepet C, Dasilva C et al (1997) **A candidate gene for familial Mediterranean fever**. Nat Genet17:25

15. Brown G. D., (2006) **Dectin-1: a signalling non-TLR patternrecognition receptor**. Nat. Rev. Immunol. 6, 33−43.

16. Chang J.H., McCluskey, PJ., and Wakefield, D. (2005) **Acute anterior uveitis and HLA-B27**. Surv. Ophthalmol. 50, 364–388

17. Coban C., Ishii KJ., Kawai T., Hemmi H., Sato S., Uematsu S., Yamamoto M., Takeuchi O., Itagaki S., Kumar N., Horii T., Akira S. (2005) **Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin**. J Exp Med. 3;201(1), 19-25.

18. de Menthiere CS., Terriere S., Pugnere D. (2003) I**NFEVERS: the registry for FMF and hereditary inflammatory disorders mutations.** Nucleic Acids Res 31, 282

19. Diebold S.S., Kaisho T., Hemmi H., Akira S., Reis E., and Sousa C. (2004). **Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.** Science 303, 1529–1531

20. Dong L., Ito S., Ishii KJ., Klinman DM. **Suppressive oligonucleotides protect against the development of collagen-induced arthritis in mice**. (2004) Arthritis Rheum;50, 1686–9

21. Dunn J. P. (2004) **Review of immunosuppressive drug therapy in uveitis**. Curr. Opin. Ophthalmol. 15, 293–298

22. Duzova A., Bakkaloglu A., Besbas N., Topaloglu R., Ozen S., Ozaltin F., Bassoy Y., Yilmaz E. (2003). **Role of A-SAA in monitoring subclinical inflammation and in colchicine dosage in familial Mediterranean fever.** Clin. Exp. Rheumatol. 21, 509.

23. Eisenberg S., Aksentijevich I., Deng ZM. (1998) **Diagnosis of familial Mediterranean fever by a molecular genetics method.** Ann Intern Med 129:539

24. Gang N., Drenth J.P., Langevitz P., Zemer D., Brezniak N., Pras M., van der Meer, J.W., Livneh A. (1999). **Activation of the cytokine network in familial Mediterranean fever**. J. Rheumatol., 26, 890.

25. Gantner B. N., Simmons R. M., Canavera S. J., Akira S. and Underhill D. M. (2003) **Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2**. J. Exp. Med. 197:1107.

26. Garlanda C., Bottazzi B., Bastone A., and Mantovani, A.(2005) **Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility**. Annu. Rev Immunol. 2005;23:337-66.

27. Gasque, P. (2004) **Complement: a unique innate immune sensor for danger signals.** Mol. Immunol. 41, 1089−1098.

28. Goldfing SE (1972) **Colchicine for familial Mediterranean fever.** N Engl J Med 287, 1302

29. Gumucio DL., Diaz A., Schaner P (2002) **Fire and ICE: the role of pyrin domain-containing proteins in inflammation and apoptosis.** Clin Exp Rheumatol 20,S45

30. Gursel I., Gursel M., Yamada H., Ishii KJ., Takeshita F., Klinman DM. (2003). **Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation**. J Immunol;171, 1393–400.

31. Gursel M., Gursel I., Mostowski HS., Klinman DM. (2006). **CXCL16 influences the nature and specificity of CpG-induced immune activation**. J Immunol. 177(3), 1575-80

32. Häcker H., Vabulas RM., Takeuchi O., Hoshino K., Akira S., Wagner H. (2000) **Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF) 6**. J Exp Med.Aug 21;192(4), 595-600.

33. Hajjar AM., Ernst RK., Tsai JH., Wilson CB., Miller SI. (2002) **Human Toll-like receptor 4 recognizes host-specific LPS modifications.** Nat Immunol. 3, 354–9.

34. Heil F., Hemmi H., Hochrein H., Ampenberger F., Kirschning C., Akira S., Lipford G., Wagner H., and Bauer S. (2004). **Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.** Science 303, 1526–1529

35. Hemmi H., Kaisho T., Takeuchi O., Sato S., Sanjo H., Hoshino K., Horiuchi T., Tomizawa H., Takeda K., Akira S. (2002). **Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway**. Nat. Immunol. 3, 196–200.

36. Hemmi H., Takeuchi O., Kawai T., Kaisho T., Sato S., Sanjo H., Matsumoto M., Hoshino K., Wagner H., Takeda K., and Akira, S. (2000). **A Toll-like receptor recognizes bacterial DNA**. Nature 408, 740–745

37. Ho PP., Fontoura P., Ruiz PJ., Steinman L., Garren H. (2003). **An immunomodulatory GpG oligonucleotide for the treatment of autoimmunity via the innate and adaptive immune systems.** J Immunol 171, 4920–6.

38. Hoekzema R, Verhagen C., van Haren M., Kijlstra A. (1992). **Endotoxin induced uveitis in the rat: the significance of intraocular interleukin-6**. Invest Ophthalmol Vis Sci. 33, 532–539.

39. Hoshino K., Takeuchi O., Kawai T., Sanjo H., Ogawa T., Takeda Y., Takeda K. and Akira, S. (1999). **Cutting Edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps hene product**. J. Immunol. 162:749.

40. Ishii KJ., Akira S. (2005). **Innate immune recognition of nucleic acids: beyond toll-like receptors.** Int J Cancer. 20, 117(4):517-23.

41. Ishii KJ., Gursel I., Gursel M., Klinman DM. (2006). **Immunotherapeutic utility of stimulatory and suppressive oligodeoxynucleotides.** Curr Opin Mol Ther 6, 166–74.

42. Ito T., Amakawa R., Kaisho T., Hemmi H., Tajima K., Uehira K., Ozaki Y., Tomizawa H., Akira S., and Fukuhara S. (2002). **Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets.** J. Exp. Med. 195, 1507–1512

43. Iwasaki A., Medzhitov R. (2004). **Toll-like receptor control of the adaptive immune responses.** Nat Immunol.5:987–95.

44. Janeway CA, Jr. (2005). **Immunobiology., 6th ed**., Garland Science.

45. Janeway C. A., Jr. and Medzhitov, R. (2002) **Innate immune recognition.** Annu. Rev. Immunol. 20, 197−216.

46. Janeway Charles; Paul Travers, Mark Walport, and Mark Shlomchik (2001). **Immunobiology; Fifth Edition**. New York and London: Garland Science.

47. Jiang, Z. (2004) **Toll-like receptor 3-mediated activation of NF-kB and IRF3 diverges at Toll–IL-1 receptor domain-containing adapter inducing IFN-b**. Proc. Natl. Acad. Sci. U. S. A. 101, 3533–3538

48. Kastner DL. (2005) **Hereditary periodic fever syndromes**. Hematology (Am Soc Hematol Educ Program); 74–81.

49. Kiraz S., Ertenli I., Arici M., Calguneri M., Haznedaroglu I., Celik, I., Pay S., Kirazli S. (1998) **Effects of colchicine on inflammatory cytokines and selectins in familial Mediterranean fever.** Clin. Exp. Rheumatol., 16, 721.

50. Klinman DM., Gursel I., Klaschik S., Dong L., Currie D., Shirota H. (2005) **Therapeutic potential of oligonucleotides expressing immunosuppressive TTAGGG motifs**. Ann N Y Acad Sci.1058:87-95

51. Klinman DM. (2004) **Immunotherapeutic uses of CpG oligodeoxynucleotides**. Nat Rev Immunol.4(4):249-58

52. Klinman DM., Zeuner R., Yamada H., Gursel M., Currie D., Gursel I. (2003) **Regulation of CpG-induced immune activation by suppressive oligodeoxynucleotides.** Ann N Y Acad Sci.1002:112-23.

53. Koizumi K., Poulaki V., Doehmen S. (2003) **Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis in vivo.** Invest Ophthalmol Vis Sci.44:2184–2191.

54. Korkmaz C., Ozdogan H., Kasapcopur O., Yazici H. (2002) **Acute phase response in familial Mediterranean fever.** Ann. Rheum. Dis., 61, 79.

55. Krieg A.M. (2006) **Therapeutic potential of Toll-like receptor 9 activation**. Nat Rev Drug Discov. 5(6):471-84

56. Krieg AM., Wu T., Weeratna R., Efler SM., Love-Homan L, Yang L. (1998) **Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs**. Proc Natl Acad Sci U S A; 95:12631–6.

57. Krieg AM, Wu T, Weeratna R, Efler SM, Love-Homan L, Yang L, Yi AK, Short D, Davis HL. (1998) **Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs.** Proc Natl Acad Sci USA; 95:12631–6.

58. Krieg AM. (2002). **CpG motifs in bacterial DNA and their immune effects**. Annu Rev Immunol; 20:709–60.

59. Krug, A., French, A. R., Barchet W., Fischer J. A., Dzionek A., Pingel J. T., Orihuela M. M., Akira, S., Yokoyama W. M., and Colonna M. (2004) **TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function.** Immunity 21, 107–119

60. Lee HK, Iwasaki A. (2007). **Innate control of adaptive immunity: dendritic cells and beyond**. Semin Immunol. 19(1):48-55.

61. Lee MS., Kim YJ. (2007) **Pattern-recognition receptor signaling initiated from extracellular, membrane, and cytoplasmic space**. Mol Cells. 28; 23(1):1-10.

62. Lenert P., Stunz L., Yi AK., Krieg AM., Ashman RF.(2003) **CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-κB activation.** Antisense Nucleic Acid Drug Dev.13(3):143-50.

63. Li H., Sun B. (2007). **Toll-like receptor 4 in atherosclerosis**. J. Cell. Mol. Med. 11, 88-95

64. Lightman S. (1997) **New therapeutic options in uveitis**. Eye 11, 222–226

65. Litman G., Cannon J., Dishaw L. (2005). **Reconstructing immune phylogeny: new perspectives**. Nat Rev Immunol 5 (11), 866-79.

66. Lund J., Sato A., Akira S., Medzhitov R., and Iwasaki A. (2003). **Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells**. J. Exp. Med. 198, 513–520

67. Luster AD. (2002). **The role of chemokines in linking innate and adaptive immunity**. Curr. Opin. Immunol 14, 129-35

68. Matzner Y., Abedat S., Shapiro E., Eisenberg S., Bar-Gil-Shitrit A., Stepensky P., Calco S., Azar Y., Urieli-Shoval S. (2000). **Expression of the familial Mediterranean fever gene and activity of the C5a inhibitor in human primary fibroblast cultures**. Blood, 96, 727.

69. Matzner Y., Partridge R.E., Levy M., Babior B.M. (1984). **Diminished activity of a chemotactic inhibitor in synovial fluids from patients with familial Mediterranean fever.** Blood, 63, 629.

70. Mayer G. (2006). **Immunology - Chapter One: Innate (non-specific) Immunity. Microbiology and Immunology On-Line Textbook.** USC School of Medicine.

71. McKenna K., Beignon A., Bhardwaj N (2005). **Plasmacytoid dendritic cells: linking innate and adaptive immunity**. J. Virol. 79 (1), 17-27.

72. Medzhitov R., Janeway CA Jr. (2002). **Decoding the patterns of self and nonself by the innate immune system**. Science; 296, 298–300.

73. Medzhitov R., Preston-Hurlburt P., Janeway CA Jr. (1997). **A human homologue of the Drosophilatoll protein signals activation of adaptive immunity**. Nature. 388: 394–7.

74. Metzhitov R, Janeway C.A. (1998). **Innate Immunity, the virtues of a nonclonal system of recognition**. Cell 91. 295-298

75. Miyamoto K, Ogura Y, Hamada M, Nishiwaki H, Hiroshiba N, Honda Y. (1996). **In vivo quantification of leukocyte behavior in the retina during endotoxin-induced uveitis**. Invest Ophthalmol Vis Sci.37, 2708–2715.

76. Mo JS, Matsukawa A, Ohkawara S, Yoshinaga M. (1999). **Role and regulation of IL-8 and MCP-1 in LPS-induced uveitis in rabbits**. Exp Eye Res. 68, 333–340.

77. Moorthy, R. S., Mermoud, A., Baerveldt, G., Minckler, D. S., Lee, P. P., and Rao, N. A. (1997) **Glaucoma associated with uveitis**. Surv. Ophthalmol. 41, 361–394

78. Nagata, S. (2005) **DNA degradation in development and programmed cell death**. Annu Rev. Immunol. 23, 853–875

79. Niederkorn JY. (2006) **See no evil, hear no evil, do no evil: the lessons of immune privilege**. Nat Immunol. 7(4):354-9

80. Notarnicola, C.; Didelot, M.N.; Seguret, F.; Demaille, J.; Touitou, I. (2002). **Enhanced cytokine mRNA levels in attack-free patients with familial Mediterranean fever.** Genes Immun., 3, 43.

81. O'Neill LA, Dinarello CA. (2000). **The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense**. Immunol Today.; 21: 206–9.

82. Ohta K, Kikuchi T, Miyahara T, Yoshimura N. (2005). **DNA microarray analysis of gene expression in iris and ciliary body of rat eyes with endotoxin-induced uveitis**. Exp Eye Res.80:401–412.

83. Olson, T.S.; Ley, K. (2002) **Chemokines and chemokine receptors in leukocyte trafficking** Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R7.

84. Oshiumi, H. et al. (2003) **TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-b induction**. Nat. Immunol. 4, 161–167

85. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, Halmen KA, Lamphier M, Olivier M, Bartholomeu DC, Gazzinelli RT, Golenbock DT. (2007). **Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9**. Proc Natl Acad Sci U S A. 104(6):1919-24. Epub 2007 Jan 29.

86. Poland, D.C.; Drenth, J.P.; Rabinovitz, E.; Livneh, A.; Bijzet, J.; van het Hof, B.; van Dijk, W. (2001). **Specific glycosylation of alpha(1)-acid glycoprotein characterises patients with familial Mediterranean fever and obligatory carriers of MEFV.** Ann. Rheum. Dis., 60, 777.

87. Poltorak, A. (1998). **Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutation in Tlr4 gene.** Science 282:2085.

88. Rosenbaum JT, McDevitt HO, Guss RB, Egbert PR. (1980). **Endotoxininduced uveitis in rats as a model for human disease**. Nature. 286:611–613.

89. Rothstein A.M. **Toll-like receptors in systemic autoimmune disease**. (2006) Nat Rev Immunol. 6(11):823-35

90. Schmidt, K.N. et al. (2004) **APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA**. J. Immunol. 172, 138–143

91. Shirota H, Gursel I, Gursel M, Klinman DM. (2005) **Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock**. J Immunol 174:4579–83.

92. Shirota H, Gursel M, Klinman DM. (2004) **Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFN-gamma- and IL-12-mediated signaling.** J Immunol 173:5002–7.

93. Simpson E. (2006). **A historical perspective on immunological privilege**. Immunol Rev 213:12–22.

94. Smirnova I, Poltorak A, Chan EK, McBride C, Beutler B. (2000) **Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4)**. Genome Biol. 1: 1–10.

95. Smith A.D. (Ed) (1997). **Oxford dictionary of biochemistry and molecular biology**. Oxford University Press.

96. Sohar, E.; Gafni, J.; Pras, M.; Heller, H. (1967) **Familial Mediterranean fever. A survey of 470 cases and review of the literature** Am. J. Med., 43, 227.

97. Springer TA. (1993) **Signals on endothelium for lymphocyte recirculation and leukocyte emigration: the area code paradigm**. Harvey Lect. 89:53–103.

98. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) **How cells respond to interferons**. Annu. Rev. Biochem. 67, 227−264.

99. Stojanov S, Kastner DL (2005) **Familial autoinflammatory diseases: genetics, pathogenesis and treatment**. Curr Opin Rheumatol 17:586

100. Streilein JW. (2003). **Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation.** J Leukoc Biol 74:179–185.

101. Stvrtinová, Viera; Ján Jakubovský and Ivan Hulín (1995). **Inflammation and Fever from Pathophysiology: Principles of Disease.** Computing Centre, Slovak Academy of Sciences: Academic Electronic Press.

102. Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L., Flavell, R. A., and Beutler, B. (2004) **Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection.** Proc. Natl. Acad. Sci. U. S. A. 101, 3516–3521

103. Takeda, K. and Akira, S. (2005). **Toll-like receptors in innate immunity**. International Immunology, Vol. 17, No. 1, pp. 1–14

104. Takeda, K., Kaisho, T. & Akira, S. (2003). **Toll-like receptors.** Annu. Rev. Immunol. 21, 335–376

105. Takeshita F, Ishii KJ, Ueda A, Ishigatsubo Y, Klinman DM. (2000) **Positive and negative regulatory elements contribute to CpG oligonucleotide-mediated regulation of human IL-6 gene expression.** Eur J Immunol. (1):108-16

106. Takeuchi, O., Kawai, T., Muhlradt, P. F., Radolf, J. D., Zychlinsky, A., Takeda, K. and Akira, S. (2001). **Discrimination of bacterial lipoproteins by Toll-like receptor 6.** Int. Immunol. 13:933.

107. Trinchieri G, Sher A. (2007). **Cooperation of Toll-like receptor signals in innate immune defence.** Nat Rev Immunol. (3):179-90.

108. Uematsu, S. and Akira S. (2007) **Toll Like Receptors and Type I Interferons**. THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 21, pp. 15319–15324, May 25, 2007

109. Underhill D. M. and Ozinsky, A. (2002) **Phagocytosis of microbes: complexity in action.** Annu. Rev. Immunol. 20, 825−852.

110. Wagner H (2004). **The immunobiology of the TLR9 subfamily**. Trends Immunol 25:381–6.

111. Whitcup SM, DeBarge LR, Rosen H, Nussenblatt RB, Chan CC. (1993). **Monoclonal antibody against CD11b/CD18 inhibits endotoxin-induced uveitis.** Invest Ophthalmol Vis Sci. 34:673–681.

112. Wright, S. D., Tobias, P. S., Ulevitch, R. J. & Ramos, R. A. (1989) **Lipopolysaccharide (LPS) binding protein opsonizes LPS bearing particles for recognition by a novel receptor on macrophages.** J Exp Med. 170(4):1231-41.

113. Yamada H, Gursel I, Takeshita F, Conover J, Ishii KJ, Gursel M, Takeshita S, Klinman DM. (2002). **Effect of suppressive DNA on CpG induced immune activation.** J Immunol 2002169:5590–4.

114. Yamada H, Ishii KJ, Klinman DM. (2004). **Suppressive oligodeoxynucleotides inhibit CpG-induced inflammation of the mouse lung**. Crit Care Med 32:2045–9.

115. Yamashiro K, Kiryu J, Tsujikawa A, et al. (2003). **Suppressive effects of selectin inhibitor SKK-60060 on the leucocyte infiltration during endotoxin induced uveitis.** Br J Ophthalmol. 87:476–480.

116. Zemer D, Pras M, Sohar E et al (1986) **Colchicine in the prevention and treatment of the amyloidosis of familial Mediterranean fever**. N Engl J Med 314:1001

117. Zeuner RA, Ishii KJ, Lizak MJ, Gursel I, Yamada H, Klinman DM. (2002). **Reduction of CpG-induced arthritis by suppressive oligodeoxynucleotides.** Arthritis Rheum 46:2219–24.

118. Zhu FG, Reich CF, Pisetsky DS. (2002). **Inhibition of murine dendritic cell activation by synthetic phosphorothioate oligodeoxynucleotides.** J Leukoc Biol 72:1154–63.

APPENDICES

Appendix 1

Standard Solutions, Buffers, Media

Blocking Buffer (ELISA)

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

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

Loading Dye (Agarose gel)

- 0,009 grams Bromofenol blue
- 0,009 grams Xylen cyanol
- 2,8 ml ddH_2O
- 1,2 ml 0,5M EDTA
- 11 ml glycerol

After preparing, just vortex it.

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8,01 grams Na_2HPO_4 . $2H_2O$
- 2 grams KH_2PO_4

into 1 lt ddH_2O

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

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

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

into 1 lt ddH_2O

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

T-cell Buffer [ELISA]

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

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

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

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

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

In 500 ml media

$NaNO_2$ (100µM)

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

Dilute this solution into 1x for 100µM concentration.