

**IMMUNOSTIMULATORY MECHANISMS AND
IMMUNOTHERAPEUTIC APPLICATIONS OF
POLYSACCHARIDE NANOCARRIER COMPLEXED
WITH NUCLEIC ACID BASED TLR LIGANDS**

A THESIS
SUBMITTED TO THE DEPARTMENT
OF MOLECULAR BIOLOGY AND GENETICS
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

By
Gizem Tincer König
January, 2013

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

Assoc. Prof. Dr. İhsan Gürsel (Advisor)

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

Prof. Dr. Gülay Özcengiz

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

Prof. Dr. Tamer Yağcı

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

Assist. Prof. Dr. Kamil 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 Doctor of Philosophy.

Assist. Prof. Dr. Ali Osmay Güre

Approved for the Graduate School of Engineering and Science

Prof. Dr. Levent Onural
Director of the Graduate School

ABSTRACT

IMMUNOSTIMULATORY MECHANISMS AND IMMUNOTHERAPEUTIC APPLICATIONS OF POLYSACCHARIDE NANOCARRIER COMPLEXED WITH NUCLEIC ACID BASED TLR LIGANDS

Gizem Tincer König

Ph.D. in Molecular Biology and Genetics

Supervisor: Assoc. Prof. Dr. İhsan Gürsel

January, 2013

Toll-like receptors (TLR) contribute to the establishment of innate and adaptive immune response by inducing proinflammatory signaling cascades leading to a Th1 dominant cytokine/chemokine production from antigen presenting cells. This lead to an enhanced T-helper and cytotoxic T-cell effector functions as well as better control of infection/inflammation and cancer. However, when these ligands are administered *in vivo*, endonuclease digestion and adsorption onto serum proteins hampers their human phase clinical trial performances. Modifications or formulations harboring nucleic acid ligands significantly improve immunotherapeutic potential. Less expensive and less tedious formulation strategies are preferable approaches throughout the FDA approval phases. In this thesis, we first explored the mechanism of action of a group of mushroom derived polysaccharides (PS) and both *in vitro* and *in vivo*, we demonstrated that either PS directly interact with TLR2 and initiates MyD88-mediated signaling cascade or when it reaches cytosol it induces inflammasome activation leading to IL-1 β production from immune cells. This PS recognition was not through either Dectin-1, or TLR4, nor through cytosolic sensors such as NOD1 and NOD2. Next, the most promising PS, was nanocomplexed with TLR3, TLR7 and TLR9 ligands in a single, double or triple combinations. These nanocomplex formulations were tested immunization studies against i) a model antigen ovalbumin or ii) influenza A virus (split vaccine, SV) in mice. PS/TLR ligand nanocomplexes boosted Th1-biased antibody production. Furthermore, PS nanocomplex treatment of established EG.7-mediated T-cell thymoma model in C57BL/6 mice was sufficient to induce significant tumor growth regression as a standalone therapeutic agent. In the second part of this thesis, self-nanoparticle forming CpG ODNs were used to control HuH-7-dependent hepatocellular carcinoma xenograft in athymic mice. Our data implicated that a significant HuH-7 killing was carried out by innate phagocytic/cytotoxic cells, which *in vivo* led to tumor regression when a nanoparticle ODN was used as the sole therapeutic choice. In the last part, we aimed to use CpG ODN motif(s) that could control infections in chicken. Different CpG ODNs, were synthesized/characterized and optimized for chicken immune system and proposed for prophylactic use to reduce uncontrolled deaths in broiler industry.

Keywords: Innate immunity, polysaccharide, nanocomplex, drug delivery system, TLR, CpG ODN, cancer immunotherapy, vaccine, adjuvant.

ÖZET

NÜKLEİK ASİT TEMELLİ TLR ULAKLARIYLA KOMPLEKS OLUŞTURAN POLİSAKKARİT NANOTAŞIYICILARIN İMMÜNSTİMULAN MEKANİZMALARI VE İMMÜNÖTERAPÖTİK UYGULAMALARI

Gizem Tincer König

Moleküler Biyoloji ve Genetik, Doktora Tezi

Tez Yöneticisi: Doç. Dr. İhsan Gürsel

Ocak, 2013

Toll benzeri algaçlar (TLR), proinflatuvar sinyal yollarını uyararak, antijen sunan hücrelerin Th1 yönlü sitokin ve kemokin üretimini sağlayarak, doğal ve edinilmiş bağışıklık cevabı oluşturmaya katkıda bulunmaktadır. Bu etki, daha iyi enfeksiyon/yanğı ve kanser kontrolü sağlayan efektör işlevleri arttırılmış yardımcı ve sitotoksik T hücrelerinin aktifleşmesini sağlar. Ancak nükleik asit ligandlarının *in vivo* ortamda endonükleaz enzimleriyle parçalanmaları ve serumdaki proteinler tarafından emilmeleri insan klinik deneme çalışmalarının başarısını engellemektedir. Nükleik asit ligandlarının modifikasyonları veya ilaç formülasyonlarına dahil edilmeleri, bu ajanların immünoterapötik potansiyellerini arttırmaktadır. Az masraflı ve kolay formülasyonlar, FDA kabul aşamasında daha çok tercih edilen uygulamalardır. Bu tezde, mantarlardan izole edilmiş polisakkaritlerin (PS) mekanizması *in vitro* ve *in vivo* koşullarda araştırılmıştır ve PS'in, TLR2 reseptörüyle direkt olarak etkileşime girdiğı ve MyD88 aracılığıyla sinyal yollarını başlattığı ayrıca sitosola ulaştığında bağışıklık hücrelerinden IL-1 β üretimine neden olarak inflamazom aktivasyonunu başlattığı gösterilmiştir. PS, Dektin-1, TLR4 ya da NOD1 ve NOD2 gibi sitosolik sensörler tarafından tanınmamaktadır. Ardından immüstimulan etkisi en güçlü PS, TLR3, TLR7 ve TLR9 ligandlarıyla, tek, çift, ve üçlü kombinasyonlarla nanokompleks haline getirilmiştir. Bu nanokompleks formülasyonları aşılama çalışmalarında i) model antijen ovalbumin veya ii) influenza A virüsüyle (Split virüs aşısı, SV) farelerde test edilmiştir. PS/TLR ligand nanokompleksleri Th1 yanlı antikor üretimini arttırmaktadır. Ayrıca, C57BL/6 farelerde EG.7 timoma modeline PS nanokompleks uygulaması, tek başına terapötik ajan olarak tümör büyümesini etkin bir biçimde geriletmiştir. Tezin ikinci kısmında, kendiliğinden nano parçacık oluşturabilen CpG ODN, atimik farelerde HuH-7 hepatoselüler karsinoma ksenogref modelinde denenmiştir. Sonuçlarımız, nanopartikül ODN tek başına terapötik olarak kullanıldığında, tümör gerilemesine neden olan fagositik/sitotoksik doğal bağışıklık hücrelerinin yardımıyla belirgin bir HuH-7 ölümüne neden olduğunu göstermiştir. Son kısımda, tavuklardaki bulaşıcı hastalıkları kontrol edebilmek için CpG ODN motif(ler)ini kullanmayı amaçladık. Farklı CpG ODNler, tavuk bağışıklık sistemi için sentezlenmiş, karakterize ve optimize edilmiştir ve etlik piliç endüstrisinde kontrol edilemeyen ölümleri düşürmek için hastalıktan koruyucu ajan olarak önerilmiştir.

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

To my precious family...

Acknowledgements

Most foremost, from my heart, I would like to express my gratitude to my supervisor Assoc. Prof. İhsan Gürsel for giving me the opportunity to work in his lab and providing his continuous support, knowledge, encouragement and patience during my PhD thesis. Working with him was the biggest honor of my lifetime.

Moreover, I'd like to dedicate my heartfelt thanks to my advisors in Osaka University Immunology Frontier Research Center, Assoc. Prof. Dr. Cevayir Coban and Prof. Dr. Ken J. Ishii for entitling to work in their labs and sharing all of their invaluable knowledge during my stay in Japan. They were not only my advisors but also like my family in Japan with all of their support. I would like to convey my sincere thanks to Dr. Kouji Kobiyama, Dr. Taiki Aoshi, Masayuki Hayashi, Kosaku Murase, Yasunari Haseda, Dr. Zhao Hong, Keiichi Ohata and other Ishii and Coban lab members for their assistance with the collection of my data in Japan.

I am really grateful to the past and present members of Gürsel Group, Fuat, Tamer, Gözde, Banu, Arda, İhsan, Mehmet, Begüm, Kutay, Erdem, Reshad, Hande and Fırat for their tremendous help and understanding during my research. I would also like to thank to all the senior and intern students who took part in this project with me.

My heartfelt thanks to Assoc. Prof Mayda Gürsel, for her enthusiastic and resourceful scientific assistance during my thesis project. I would like to offer my special thanks to Mayda Gürsel Group members, Bilgi, Esin and Soner for helping me in my research.

Prof. Dr. Oktay Erbatur and his group, from ukurova University Adana, are sincerely acknowledged for providing the polysaccharides for my studies.

I am truly indebted to all of my dearest friends in MBG family, especially Zeynep, iğdem, Işıl, Merve, Ece, Emre O., Tolga, Bala, Sevgi for giving me full support and morale during this long and rough journey.

Without my family, none of the outstanding things in my life would have been achievable. I would like to express my deepest love and thankfulness to my mother Sevtap, my father Teoman and my best friend, little sister Ezgi for their invaluable and everlasting support.

Once for all, I would like to thank to my dearest husband Teoman for cheering me up whenever I need, and supporting throughout this period with his eternal love and patience.

This work is supported by TÜBİTAK (SBAG 106S102, SBAG 108S316) and SANTEZ Grant (#STZ-0448-2009-2)

My stay in Japan was supported by Kishimoto Foundation as a research fellow in IFRc.

Thanks to all people for being always ready to offer, advise and encouragement.

Contents

1	Introduction	1
1.1	The Immune System.....	1
1.2	Innate Immune System.....	3
1.2.1	Pattern Recognition Receptors.....	5
1.2.2	Toll-like Receptors	9
1.2.2.1	Cell Surface Toll-like Receptors	10
1.2.2.2	Intracellular Toll-like Receptors.....	13
1.2.2.3	TLR Signaling	17
1.2.3	C-type Lectin Receptors	20
1.2.3.1	Dectin-1	20
1.2.3.2	Dectin-2	22
1.2.4	NOD-like Receptors	22
1.2.4.1	NOD1 and NOD2	23
1.2.4.2	Inflammasomes.....	24
1.3	Cooperation between Pattern Recognition Receptors.....	28
1.3.1	Collaboration between TLRs	29
1.3.2	Collaboration between TLRs and other PRRs.....	32
1.4	Immunostimulatory Synthetic CpG ODN Sequences.....	34
1.4.1	An Insight to TLR9 Activation and Signaling.....	35
1.4.2	Types of CpG ODNs.....	35

1.4.2.1	A/D–type CpG ODN	36
1.4.2.2	B/K–type CpG ODN.....	38
1.4.2.3	C-type CpG ODN	39
1.4.2.4	P-type CpG ODN.....	40
1.4.2.5	Y-shaped CpG ODN.....	40
1.4.2.6	X-shaped CpG ODN.....	41
1.4.2.7	Dendrimeric 70-mer ODN.....	42
1.5	Immune System and Cancer.....	43
1.6	Drug Delivery with Nanoparticles	46
1.7	Immunotherapeutic Applications of PRR Ligands	51
1.7.1	Anticancer Applications	53
1.7.2	Antibacterial and Antiviral Applications.....	55
1.8	Immune System in Chickens.....	62
1.8.1	Immunotherapeutic Effects of CpG ODNs in Chicken	64
1.9	Subject and outline of the Thesis	67
2	Materials and Methods	69
2.1	Materials.....	69
2.1.1	Reagents for ELISA.....	69
2.1.2	Pathogen Recognition Receptor Ligands.....	71
2.1.3	Oligodeoxynucleotides	71
2.1.4	Adjuvants, Antigens and Peptides	73
2.1.5	Polysaccharides.....	74
2.1.6	Standard Solutions, Buffers, Cell Culture Media Components	75
2.2	Methods.....	76

2.2.1	Model Animals	76
2.2.2	Cell Culture.....	76
2.2.2.1	Spleen Cell Suspension Preparation	76
2.2.2.2	Peritoneal Exudate Cell Preparation.....	77
2.2.2.3	Bone Marrow Derived Dendritic Cell Generation.....	77
2.2.2.4	Cell Lines.....	78
2.2.2.5	Cell Counting.....	78
2.2.2.6	Adjusted Cell Densities for Different Experiments.....	80
2.2.2.7	Ligands and Polysaccharides used During Cell Stimulations	80
2.2.2.8	Delivery of TLR Ligands with Polysaccharide Nanocomplexes	81
2.2.3	Enzyme Linked-Immunosorbent Assay (ELISA).....	81
2.2.3.1	Cytokine ELISA	81
2.2.3.2	IgG and IgA ELISA.....	82
2.2.4	Transfection of Cells.....	83
2.2.5	Luciferase Assay.....	83
2.2.6	In vitro Cytotoxicity Experiment.....	84
2.2.7	Fluorescence Activating Cell Sorting (FACS)	85
2.2.7.1	Cell Surface Marker Staining	85
2.2.8	Determining the Binding of PS to TLR2.....	85
2.2.9	Determination of the Gene Expression.....	86
2.2.9.1	Total RNA Isolation	86
2.2.9.2	cDNA Synthesis.....	87
2.2.9.3	Semi-quantitative RT-PCR.....	87

2.2.9.4	Agarose Gel Electrophoresis	90
2.2.10	DNase Treatment of the CpG ODNs and PS(CpG ODN) nanocomplexes	90
2.2.11	In vivo Experiments.....	91
2.2.11.1	Injection of animals with polysaccharides in various concentrations.....	91
2.2.11.2	Immunization against OVA either with TLR ligands or PS nanocomplexes	91
2.2.11.3	Immunization with TLRL-PS nanocomplexes against SV.....	92
2.2.11.4	Tumor Xenograft Model.....	92
2.2.12	Size measurement studies of the nanocomplexes	93
3	Results	94
3.1	Mechanism of action of mushroom derived polysaccharides	95
3.1.1	PS4 isolated from <i>Ganoderma lucidum</i> directly binds to TLR2	95
3.1.2	PS4 acts through TLR2 and requires MyD88 adapter protein.....	96
3.1.3	Immune cell activation by polysaccharides are Dectin-1 receptor independent	99
3.1.4	Inflammasome-dependent mechanisms were involved in PS mediated immune response	105
3.1.5	Polysachharides induce immunomodulatory IL-10 cytokine from murine spleen cells	107
3.2	In vivo activity of PSs in mice	108
3.3	Generation and physical characterization of PS nanocomplexes.....	110
3.4	Double or triple nanocomplexes with PS4 enhances IL-6 production from mouse splenocytes.....	112

3.5	Ability of PS to protect CpG ODN in nanocomplexes after DNase treatment	114
3.6	In vivo immune activation of PS4-TLR ligand nanocomplexes	116
3.7	PS nanoparticles as anti-tumor agents.....	119
3.8	Adjuvant effect of PS/TLRL nanocomplexes	123
3.8.1	Immunization with PS-nanocomplexes against model antigen ovalbumin	123
3.8.2	Adjuvant effect of PS-TLRL nanocomplexes against influenza virus ..	126
3.8.2.1	Intranasal immunization	126
3.8.2.2	Intradermal immunization	130
3.9	New generation self assembled nanoparticle forming CpG ODN as effective anti cancer agent.....	135
3.9.1	ODN420 treated PEC cells were cytotoxic against Huh7/luc cells	137
3.9.2	Established tumor regression by nanoparticle forming ODNs	139
3.10	Efforts to establish the optimum sequence in chicken	141
3.10.1	Testing the immunopotency of various CpG ODN types.....	141
3.10.2	Immunostimulatory effect of CpG ODN sequences on chicken immune cells	142
4	Discussion.....	147
5	Future Perspectives	156
6	References	158
7	Appendices	188
	Appendix A.....	189

	Appendix B	192
	Appendix C	194
	Appendix D	196
8	Curriculum vitae and Publications.....	197
9	Permissions to the Copyrighted Materials.....	227

List of Figures

Figure 1.1. PAMP recognition by surface Toll-like receptors	12
Figure 1.2. Ligand recognition by intracellular TLRs	16
Figure 1.3. MyD88 dependent and TRIF dependent TLR signaling pathways in immune cells.	18
Figure 1.4. Dectin-1, Dectin-2 and Mincle induce innate immune response via NF- κ B signaling cascade by recruiting Syk adapter protein.	21
Figure 1.5. Intracytosolic signaling cascade mediated by NODs	24
Figure 1.6. Multimeric inflammasome protein complexes regulate both homeostasis and inflammation upon PAMP and DAMP exposure.	25
Figure 1.7. Alternative entry/activation steps of inflammasome pathway leading to IL-1 β production..	27
Figure 1.8. The structures and the signaling pathways of the various pattern recognition receptors	33
Figure 1.9. Spontaneous higher-order G-quadruplex formation of D-type CpG ODNs..	38
Figure 1.10. Duplex formation via palindromic sequences by two phosphorothioate backbone C ODNs.....	40
Figure 1.11. Representative structure of Y-shaped CpG ODN.....	41
Figure 1.12. Structure of X-shaped CpG ODN.....	41
Figure 1.13. Generation and regulation of antitumoral immune response.....	46
Figure 1.14. Schematic representation of an engineered nanoparticle.....	49
Figure 2.1. Schematic representation of the ODN420 formation via the use of two bifunctional linkers at the 3'-end of ODN 1466 sequences.	73

Figure 2.2. ODN421: Schematic representation of the ODN421 formation via the use of two bifunctional linkers at the 3'-end of ODN 1471 sequences.	73
Figure 2.3. The schedule of <i>in vitro</i> cytotoxicity experiment.	84
Figure 2.4. Immunization schedule for <i>in vivo</i> vaccination against ovalbumin.....	92
Figure 3.1. Detection of direct PS4-TLR2 interaction by binding ELISA.	96
Figure 3.2. TLR2 and MyD88-dependent immune response of PSs from peritoneal macrophages.....	97
Figure 3.3. Cytokine production from wild type, TLR2 ^{-/-} , TLR4 ^{-/-} , and MyD88 ^{-/-} mice splenocytes.	99
Figure 3.4. Polysaccharides act upon Dectin-1 independent immune response..	100
Figure 3.5. Dectin-1 independent production of IL-6 and TNF- α and IL-12/IL-23p40 from w/t and Dectin-1 ^{-/-} PECs	101
Figure 3.6. Dectin-1 was not required for the immune activation upon PSs by GMDC..	102
Figure 3.7. Dectin-1 was not required for the immune activation upon PSs by FLDC.....	103
Figure 3.8. Inflammasome activation by PSs governs IL-1 β production from mouse PECs.....	106
Figure 3.9. IL-10 production from splenocytes after dose dependent PS treatment.....	107
Figure 3.10. Immunostimulatory properties of polysaccharides <i>in vivo</i>	109
Figure 3.11. Size distribution histograms of PS and its nanocomplexes.	111
Figure 3.12. Nanocomplexes of double and triple combinations of nucleic acid TLR ligands have an additive effect on IL-6.....	114
Figure 3.13. PS(CpG ODN) nanocomplexes were resistant to DNase I treatment.....	115
Figure 3.14. The agarose gel picture of CpG ODN, PS(CpG ODN) and their DNase treated samples.	116
Figure 3.15. Improved <i>in vivo</i> immunostimulatory activity of PS4-TLR ligand nanocomplexes.....	118

Figure 3.16. PCR results that were performed from total RNA of splenocytes from BALB/C mice treated with of PS and its TLRL nanocomplexes.	118
Figure 3.17. The effect of double and triple PS nanocomplexes as potent immunotherapeutic anti-cancer agents against EG.7 mouse thymoma cells.	121
Figure 3.18. Representative pictures showing tumor mass of BL/6 mice post-treatment	122
Figure 3.19. Double and triple TLR NA agonist formulated PS nanocomplexes, significantly induced tumor regression of EG7 thymoma generated C57BL/6 mice.. ..	123
Figure 3.20. Single or double TLRL and OVA containing nanocomplex formulations boosted anti-OVA immunity.....	125
Figure 3.22. IgG2c/IgG1 OD ratios of 2700x titrated mice sera against anti-SV.....	130
Figure 3.23. Immunogenicity of the SV (H1N1), after primary and secondary intradermal vaccination.....	133
Figure 3.24. IgG2c/IgG1 OD ratios of 2700x titrated mice sera against anti-SV.....	133
Figure 3.25. Influenza virus specific IgA production from nasal wash and blood.	134
Figure 3.26. IL-6 secretion.from BALB/C or C57BL/6 mice BMDC treated with different doses of ODN420	136
Figure 3.27. In vitro cytotoxicity assay showed that ODN420 was the most promising anti-cancer agent..	138
Figure 3.28. ODN420 was the most prominent anti-tumor agent in HCC model.	140
Figure 3.29. Immunostimulatory effects of different CpG ODNs on BALB/C splenocytes.	142
Figure 3.30. Percentage of CD80 expressing chicken splenocytes upon CpG ODN stimulation.....	143
Figure 3.31. TLR Message transcript changes upon CpG ODN treatment in chicken B cell line DT40 or chicken splenocytes	144
Figure 3.32. RT-PCR products of cytokine-related gene transcripts in DT40 or chicken splenocytes.	145

Figure 4.1. PS to induce immune response requires both extracellular and cytosolic signaling mechanism as depicted on a hypothetical immune cell.....	150
Figure 4.2. Schematic representation of the signaling pathways of PS(TLRL) nanocomplexes on immune cells.	153
Figure B.1 TNF- α and IL-6 cytokine production of splenocytes, PEC macrophages , GMDC and FLDCs from w/t and Dectin ^{-/-} C57BL/6 mice after stimulation with, PS, and PS/TLRL nanocomplexes.....	192
Figure C.1 Serum anti SV titers of IgG2b of primary, secondary bleedings of intradermal and secondary bleeding of intranasal immunized mice.....	194
Figure D.1 T cell dependent immune response from intranasal immunized mice splenocytes against H1N1 influenza virus.	196

List of Tables

Table 1.1. Major differences of innate and adaptive immunity	2
Table 1.2. Influence of inflammatory cytokines on the fate of immune cells.	4
Table 1.3. Chemokines recruit immune cells to the inflammation site.....	5
Table 1.4. Pattern recognition receptors and their ligands.....	8
Table 1.5. Comparison of A/D, B/K, C and P-type CpG oligodeoxynucleotides.....	36
Table 1.6. Immune response outcomes and components of approved and under clinical trial adjuvants	52
Table 1.7. CpG ODNs as vaccine adjuvants.	58
Table 1.8. Prophylactic and therapeutic targeting of TLRs in clinical trials of infectious diseases.....	60
Table 1.9. Similarities of the pattern recognition receptor between human and chicken cells	64
Table 1.10. CpG ODN administrations against chicken pathogens.....	66
Table 2.1. Antibodies and reagents used in cytokine ELISA experiments.	70
Table 2.2. Influenza peptide sequences, brands, and commercial names.	74
Table 2.3. Properties of the polysaccharide extracts.....	75
Table 2.4. Designed mouse and chicken primer sequences and product sizes.	88
Table 2.5. RT-PCR reaction reagents.	89
Table 2.6. RT-PCR conditions of mice primers.....	89
Table 2.7. RT-PCR conditions of chicken β -actin & TLR primers.	89
Table 2.8. RT-PCR conditions of chicken cytokine primers.	90
Table 3.1. Immunological features of different polysaccharides in splenocytes, PECs, GMDCs and FLDCs from wild-type and K/O mice.	104
Table 3.2. Size distribution analyses of PS4 containing nanoparticles after 0, 7 and 14 days.	112

Abbreviations

Ab	Antibody
ACK	Ammonium-Chloride-Potassium
AIM	Absent in melanoma
AP-1	Activator protein 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine triphosphate
AVA	Anthrax vaccine adsorbed
BCG	Bacille Calmette Guerin of Mycobacterium bovis
Bcl	B-cell lymphoma protein
BCR	B-cell receptor
bp	Base pairs
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freud's Adjuvant
ck	Chicken
CRP	C-reactive protein
CLR	C-type lectin receptors
CMV	Cytomegalovirus
CpG	Unmethylated cytosine-guanosine motifs
CXCL	CXC-chemokine ligand
DAMP	Danger-associated molecular pattern molecules

DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion
ddH ₂ O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked-Immunosorbent Assay
EPR	Enhanced permeability and retention
ER	Endoplasmic reticulum
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FASL	Fas ligand
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
Flt3	Fms-like tyrosine kinase 3
FLDC	Flt3L treated dendritic cells
GMDC	GM-CSF treated dendritic cells
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycophosphatidylinositol
HBV	Hepatitis-B Virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HIV	Human Immunodeficiency Virus

HMGB1	High-mobility group protein B1
HPSEC-MALLS	High Pressure Size Exclusion–The Multiple Angle Laser Light Scattering Chromatography
HPV	Human papillomavirus
HSV	Herpes simplex virus
hu	Human
id	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
in	Intranasal
iNOS	Inducible Nitric Oxide Synthase
ip	Intraperitonea
IP-10	Interferon gamma-induced protein 10
IRAK	IL-1 receptor-associated kinase
IRF3	Interferon-regulatory factor 3
LAL	Limulus amebocyte lysate
IκK	Inhibitor kappa B kinase
LBP	LPS-binding protein
LGP	Lipophosphoglycan
LITAF	Lipopolysaccharide-induced tumor necrosis factor-alpha factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic Acid
mAb	Monoclonal antibody
MALP	Mycoplasmal lipopeptide
MALT	Mucosa Associated Lymphoid Tissue

MAP	Mitogen-activated protein
MARCO	Macrophage receptor with a collagenous structure
MBL	Mannose-binding lectin
MCA	Methylcholanthrene
MCMV	Mouse cytomegalovirus
MCP	Monocyte Chemoattractant Protein
MD-2	Myeloid differentiation protein-2
MDA	3,4-methylenedioxyamphetamine
MDP	Muramyl dipeptide
MHC	Major Histocompatibility Complex
MINCLE	The macrophage-inducible C-type lectin
MIP	Macrophage Inflammatory Protein
moDC	Myeloid dendritic cells
	molecule-3-grabbing non-integrin
MPLA	Monophosphoryllipid A
MSR	Macrophage scavenger receptor
mu	Murine
MyD-88	Myeloid differentiation primary response gene (88)
NEMO	NF- κ B essential modulator
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NKG2D	Natural-killer group 2, member D receptor
NLR	Nucleotide-binding oligomerization domain like proteins or receptors
NLRC	CARD domain containing NOD
NLRP	NOD-like receptor protein
NO	Nitric oxide

NOD	Nucleotide-binding Oligomerization Domain
NP	Nanoparticle
NP-ODN	Nanoparticle forming CpG ODNs
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PA	Protective-antigen
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PEC	Peritoneal exudates cells
PEG	Polyethylene glycol
PGN	Peptidoglycan
pIC (polyIC)	Polyriboinosinic polyribocytidylic acid
PLG	Poly(lactide-co-glycolide)
PLGA	Poly(lactic-co-glycolic acid)
PNPP	Para-nitrophenyl phosphate
PO	Phosphodiester backbone
PRAT4	Protein Associated with TLR4
PRR	Pattern Recognition Receptors
PS CpG ODN	Phosphorothioate backbone modified CpG ODN
PS	Polysaccharide
PYR	Pyrimidine binding domain
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RIG	Retinoic acid-inducible gene

RIH	Retinoic acid-inducible gene I (RIG)-like helicases
RIP	Receptor-interacting protein
RLR	RIG-I-Like Receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse transcriptase PCR
RT	Room temperature
SA-AKP	Streptavidin Alkaline-phosphatase
SA-HRP	Streptavidin horseradish peroxidase
SAP	Serum amyloid protein
sc	Subcutaneous
SDS	Sudden death syndrome
SLE	Systemic Lupus Erythematosus
siRNA	Small interfering RNA
SLN	Solid-lipid nanoparticles
SPG	β -(1 $\rightarrow$ 3)-D-glucan schizophyllan
SSCL	Sterically stabilized cationic liposomes
ssDNA	Single stranded DNA
ssRNA	Single-stranded RNA
STF	Soluble tuberculosis factor
SUV	Small unilamellar vesicle
SV	Split vaccine
TBK	TANK-binding kinase
TCR	T-cell receptor
T _H	T-helper
TGF- β	Transforming growth factor beta
TIR	Toll/IL-1 receptor

TIRAP	Toll/IL1 receptor-associated protein
TLR	Toll-like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecules
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIF	TIR domain containing adaptor inducing IFN- β
UNC93B1	Unc-93 homolog B1
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 The Immune System

The mammalian immune system relies on one simple rule while combating against intruders and that is proper and timely differentiation of self from non-self (Murphy *et al.*, 2011). In addition to physical barriers surrounding the body, such as skin and epithelial cells present on, gastrointestinal, respiratory and urogenital surfaces, (Janeway and Medzhitov, 2002), there are chemical and cellular components act during protection and neutralization of insulting organisms (Murphy *et al.*, 2011).

The immune system is divided into two major arms, i) innate and ii) adaptive (or acquired) immune system. Their crosstalk and collaborative action confers lifelong protection against microbial world such as viruses, bacteria, parasites, fungi (Kabelitz and Medzhitov, 2007). Innate immune system uses various germ-line encoded receptors to detect antigens does not generate memory, but adaptive immune system uses highly sophisticated ways such as antigen-specific receptors for detecting infections (Iwasaki

and Medzhitov, 2010). The major differences of two systems are summarized in Table 1.1.

Table 1.1. Major differences of innate and adaptive immunity (*Adapted from Murphy et al., 2011*).

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, LTA, mannans, glycans)	Details of molecular structure (proteins, peptides, carbohydrates)
Self-Nonself discrimination	Selected over evolutionary time	Selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokines: IL-1 β , IL-6 Chemokines: IL-8	Clonal expansion or anergy Effector cytokines: IL-2, IL-4, IFN- γ

Evolutionarily conserved germline encoded pattern recognition receptors (PRRs) which are expressed mainly on innate immune cells are capable of recognizing diverse group of microbial byproducts that are only expressed on them but not on mammalian cells, these molecular structures are known as pathogen associated molecular patterns (PAMPs). Activation of APCs by PRR induced triggering, following pathogen internalization, lead to effective processing and presentation of antigenic epitopes to T and B-cells of the adaptive immune system (Akira *et al.*, 2006).

Adaptive immunity is further sub-divided into humoral and cell mediated immunity. While major player of the humoral response is B-cells, T cells make up the cell-mediated immunity (Boehm, 2011). The antibodies by B cells which present in the blood plasma help to neutralize or opsonize foreign particles, whereas eliminating infected cells, or fight with our own cancer cells are controlled by cytotoxic T cells (Hoebe *et al.*, 2004).

1.2 Innate Immune System

Pathogens that could pass across the hosts epithelial barriers are detected and eliminated within minutes or hours by defense mechanisms of innate immune cells. These mainly include but not limited to macrophages, DCs, NK cells, and neutrophils, or granulocytes (Kumar *et al.*, 2009a).

The first line of host defense is mainly composed of various components such as, epithelial surfaces, complement system, inflammasomes, phagocytic cells, cytokines (type-I IFNs), natural killer cells, mast cells, eosinophils and basophils. Major functions of the vertebrate innate immune system include; i) recruitment of immune cells to the sites of inflammation via cytokines/chemokines, ii) activation of the complement system to identify and iii) eliminate bacteria, iv) activate other immune cells for the clearance of dead cells or antibody complexes, v) identification and removal of foreign substances in organs, tissues, blood and lymph by specialized lymphocytes, and vi) induction of the adaptive immune system through antigen presentation (Akira *et al.*, 2006). Furthermore, mucosal epithelia and keratinocytes found in the skin produce antimicrobial proteins which prevent pathogens to invade to the host (Pasparakis, 2012). One of the main cell types of the innate immune system natural killer cells (NK), demolish the virus infected or tumor cells which are tagged as “missing self”. The downregulation or absence of “self” MHC I molecule on infected cell activates NK cells for killing (Shereck *et al.*, 2007). Other group of innate immune cells, which are mast cells, eosinophils and basophils release histamine, heparin, also various chemokines and toxic proteins. Histamine protein helps the dilation of blood vessels, and recruits other immune cells to sites of infection. Chemokines and other secretory products effectively kill pathogens but the excess production could cause tissue damage throughout the allergic responses (Akuthota *et al.*, 2008; Stone *et al.*, 2010).

Neutrophils, macrophages and DCs are the phagocytic cells of the innate immune system. Initially neutrophils arrive to the site of infection. With the release of toxin containing granules or reactive oxygen species (such as H₂O₂) they destroy or inhibit the growth of pathogens (Nauseef, 2007). The most efficient phagocytes, macrophages, which are derived from the blood circulating monocytes could phagocytose large numbers of microbes. Dendritic cells are the major cell type responsible for the antigen presentation, which are the transformed from hematopoietic bone marrow progenitor cells (Soehnlein and Lindbom, 2010). Activated and matured DCs migrate to the lymph nodes through lymphatic vessels and interact with the T cells to present the antigens already loaded onto either MHC class I or MHC class II molecules. The clonal expansion of T cells leads to the effective and prolonged response against pathogen as well as providing immunological memory (Hansen and Bouvier, 2009; Watts *et al.*, 2010). The properties and types of main cytokines and chemokines are summarized in Table 1.2 and Table 1.3.

Table 1.2. Influence of inflammatory cytokines on the fate of immune cells (*Adapted from Akdis et al., 2011*).

Cytokines	Produced by	Function
TNF- α	MØ, DC, NK and T cells	Local inflammation, induce apoptotic cell death, inhibit tumorigenesis and viral replication.
IL-1 β	MØ, keratinocytes	Induction of proinflammatory proteins, T cell and MØ activation, hematopoiesis.
IL-2	T cells	T and B cell growth, differentiation and survival, NK cell proliferation.
IL-6	MØ, DC	T and B cell growth and differentiation,
IL-10	MØ, DC, T and B cells	Suppression of T cell and MØ responses, B cell survival, proliferation and differentiation.
IL-12 (p35/p40)	MØ, DC, neutrophils, B cells	Activation of NK cells, induction of T _H 1 differentiation and cytotoxicity.
IL-15	Monocytes, keratinocytes	CD8 memory T cell homeostasis, stimulation and proliferation of NK and NK T cells.
IL-18	Activated MØ	Induction of IFN γ secretion via NK and T cells, promotes T _H 1 immunity.
IFN- α	DC	Antiviral immunity, induction of MHC I expression.
IFN- γ	T cells, NK cells, MØ	Promotes cytotoxic activity, MØ activation, increased expression of MHC class I and II, antiviral properties.

MØ: Macrophages, DC: Dendritic cells, NK: Natural killer cells

Table 1.3. Chemokines recruit immune cells to the inflammation site (*Adapted from Bendall, 2005; Laing and Secombes, 2004*).

Chemokines	Produced by	Attracted cells	Major effect
CCL2 (MCP-1)	Monocytes, MØ, fibroblasts, keratinocytes	Monocytes, NK, memory T, DC	Promotes T _H 2 immunity, activates MØ, basophile histamine release
CCL3 (MIP-1 α)	Monocytes, T, Mast cells, fibroblasts	Monocytes, NK, T, basophiles, DC	Promotes T _H 1 and antiviral response, competes with HIV-1
CCL4 (MIP-1 β)	Monocytes, MØ, Neutrophils, endothelium	Monocyte, NK, T, DC	T cell, DC and NK cell recruitment, HIV coreceptor
CXCL8 (IL-8)	Monocytes, MØ, DC	Neutrophils, naïve T cells	Mobilization, activation and degranulation of neutrophils
CXCL10 (IP-10)	T, fibroblasts, endothelial cells, monocytes, keratinocytes	Resting T cells, NK, monocytes	Induces T _H 1 immunity, antiangiogenic, immunostimulant

MØ: Macrophages, DC: Dendritic cells, NK: Natural killer cells, T: T cells

1.2.1 Pattern Recognition Receptors

Pattern recognition receptors (PRR) which are highly expressed on innate immune cells recognize the repeating patterns of microorganisms including their unique motifs of nucleic acids. These are collectively referred to as Pathogen/Microbial Associated Molecular Patterns (P/MAMPs) (Gordon, 2002).

Lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acids and cell-wall lipoproteins, fungal cell wall product β -glucan, unmethylated cytosine-phosphate-guanine (CpG) motif rich bacterial genomic DNA, viral single and double stranded RNAs (ssRNA and dsRNA), are well known members of PAMPs (Takeuchi and Akira, 2010).

PRRs are expressed on the cell surface, in intracellular compartments (i.e. endosomal organelles or ER) or secreted into bloodstream and tissue fluids. They share common properties such as, i) PRRs are expressed ubiquitously in the host and detect the

pathogens regardless of their life-cycle stage. ii) PRRs are germline encoded, nonclonal and expressed on all members of specific cell types iii) the fundamental components of innate immune system and are highly conserved among various species, from plants, fruit flies to mammals (Kawai and Akira, 2009).

Various types of PRRs are well studied. Secreted PRRs known as, Mannan-binding lectin (MBL), C-reactive protein (CRP), and serum amyloid protein (SAP) are synthesized by liver cells in response to the cytokines secreted by macrophages and adipocytes. CRP and SAP belong to pentraxin family, but MBL is a member of the collectin family. CRP and SAP recognize the specific phospholipids on bacterial surfaces and opsonize microorganisms initiating their phagocytosis by macrophages (Degn *et al.*, 2007; O'Brien and Chait, 2006; Paraskevas and Mikhailidis, 2008). MBL binds specifically to terminal carbohydrate mannose residues, which is found on the surface of many pathogenic microorganisms, and initiates the complement system by cleaving C2 and C4 proteins (Degn *et al.*, 2007).

Other group of receptors which are present in the cytoplasm of the immune cells are mainly divided into two families, nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG)-like helicases (RLRs). NLRs mainly recognize viral and bacterial components, while members RLRs such as RIG-I and MDA5 specifically recognize viral nucleic acids (Inohara *et al.*, 2005).

C-type lectin receptors (CLR) and Toll-like receptors (TLR) are either expressed on cell surface or endosomal membranes. Mannose receptor, DC-SIGN, Dectin-1 Dectin-2, CARD9 are the main components of CLRs that involved in the recognition of PAMPs from different origins such as bacteria and fungi (Geijtenbeek and Gringhuis, 2009). The best-characterized and highly conserved PRR family TLRs are mainly consist of 11 members. Homo or heterodimer forms of these receptors are able to recognize different microbial components from viral dsRNA to bacterial flagellin protein (Beutler, 2009).

Scavenger receptors are also located in the cell membrane of the macrophages. Macrophage scavenger receptor (MSR) has wide specificity for many ligands, dsRNA, LPS, and lipoteichoic acid (LTA). Another scavenger receptor expressed on macrophages is class A scavenger receptor family member MARCO, accomplish an important role in antibacterial host defense with the recognition of bacterial cell walls and LPS, also mediate the phagocytosis of both Gram-positive and Gram-negative bacteria (Bowdish and Gordon, 2009; Murphy *et al.*, 2005). Summary of PRRs and their ligands are mentioned in Table 1.4.

Table 1.4. Pattern recognition receptors and their ligands (*Adapted from Hornung et al., 2009; Jacobs and Damania, 2012; Takeuchi and Akira, 2010*).

	Microbial Signature	TLRs	RLRs	NLRs	CLRs
Viruses	Structural proteins (capsid, envelope proteins)	TLR2, TLR4			
	DNA	TLR9		AIM2 (dsDNA)	FcγR
	RNA	TLR3 (dsRNA), TLR7, TLR8 (ssRNA)	RIG-I, MDA5, LGP2	NALP3	FcγR
Bacteria mycobacteria	Cell wall components, LPS, PGN, lipoteichoic acid, lipoproteins, uropathogenic <i>E. coli</i>	TLR2/1, TLR2/6, TLR4, TLR11		NOD1, NOD2, NALP1, NALP3	Collectins (MBL)
	Flagellin	TLR5		IPAF, NAIP5, NLRC4	
	Perotoxins			NALP3	
	DNA	TLR9		AIM2	
	RNA			NALP3	
Protozoan parasites	GPIs	TLR2, TLR4			
	Malaria hemozoin	TLR9		NALP3	
	Proteins (<i>T. cruzi</i> Tc52, <i>T. gondii</i> profilin)	TLR2, TLR11			
	DNA	TLR9			
Helminths	Lipids	TLR2			
	RNA	TLR3			
Fungi	Cell wall components (zymosan, mannan, β-glucan)	TLR2, TLR4, TLR6		NALP3	Mannose receptor, DC-SIGN, Dectin-1, Dectin-2, CARD9
	DNA	TLR9			

1.2.2 Toll-like Receptors

Toll receptors are initially identified in *Drosophila*, and the homologous form is named as Toll-like receptor in mammalian cells. These receptors are the members of IL-1R superfamily, which contain an ectodomain leucine-rich repeats (LRR) that mediate the recognition of PAMPs, a transmembrane region, and intracellular Toll-IL-1 receptor (TIR) domains that mediate downstream signaling pathways. They are found on the cell surface or in intracellular compartments as they tend to recognize microbial membrane compounds as well as genomic structures of pathogenic factors (Uematsu and Akira, 2008).

TLRs direct the course and type of the innate immune response against foreign antigen. It has been previously shown that TLRs can promote proinflammatory and antiviral responses, induce the expression of costimulatory molecules on APCs, initiate the release of various immune response related chemokines to recruit the related cells to the site of inflammation and cross-priming of T cells by mature and active DCs (Kumar *et al.*, 2009b). Major signals for the activation of adaptive immunity such as upregulation of costimulatory molecules CD80/86 which are essential for the activation of T cells and production of proinflammatory cytokines which are, orchestrated by the TLR-dependent recognition of PAMPs by DCs (Reis e Sousa, 2004; Ueno *et al.*, 2011). TLRs also contribute to the initiation of the immune responses of adjuvant activity in vaccination. Some pathogen-derived adjuvants such as complete freund's adjuvant, (CFA) bacilli calmette Guerin of mycobacterium bovis (BCG) are recognized by TLR2 and TLR4, TLR9 respectively (McKee *et al.*, 2007). Activation of different TLRs on DCs can lead to either Th1 or Th2 type cell differentiation depending on the variety of the exposed PAMP.

The initially characterized TLR, TLR4 in mammalian cells, which is expressed in many types of cells including mainly DCs and macrophages. Toll-like receptors in mammals

subcategorized into two groups corresponding to their cellular localization. TLR1, 2, 4, 5, 6 and 11 are expressed on cell surfaces, that recognize microbial membrane compounds like, proteins, lipopolysaccharides and lipoproteins (Figure 1.1). TLR 3,7,8 and 9 are localized in the endosomal or ER compartments and detect viral and bacterial nucleic acids to start the downstream signaling events that anticipates to the production of inflammatory cytokines, chemokines and antimicrobial peptides (Figure 1.2).

1.2.2.1 Cell Surface Toll-like Receptors

1.2.2.1.1 TLR1, TLR2 and TLR6

TLR2 forms heterodimers with TLR1, TLR6 and non-TLR molecules, CD36, CD14 and Dectin-1, therefore, detect very wide spectrum of PAMPs from different origins. These include peptidoglycan and lipoteichoic acid of Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from *Saccharomyces cerevisiae*, viral proteins of herpes simplex virus (HSV) and hepatitis C virus (HCV), hemagglutinin protein from measles virus, glycosylphosphatidylinositol anchors from *Trypanosoma cruzii*, as well as whole pathogens. Heterodimers of TLR2-TLR1 and TLR2-TLR6 specifically detects triacyl-lipopeptides (synthetic bacterial lipopeptide, Pam3Cys) from Gram negative bacteria and diacyl-lipopeptides from mycoplasma respectively. Zymosan is recognized by C-type lectin receptor Dectin-1 and in corporation with TLR2. TLR2-TLR6 heterodimer also sense LTA and macrophage-activating lipopeptide (MALP)-2 of *Staphylococcus aureus* with the help of co-receptor CD36. TLR2 agonists stimulate the production of inflammatory cytokines, but, also induce induced the production of Type I IFN in response to vaccinia virus (Barbalat *et al.*, 2009; Hoshino *et al.*, 1999; Jin *et al.*, 2007a; Takeuchi *et al.*, 2001).

1.2.2.1.2 TLR4

The first identified TLR is endotoxin sensor TLR4 (Hoshino *et al.*, 1999). This recognition is performed with the help of accessory molecules such as myeloid differentiation protein-2 (MD-2), LPS-binding protein (LBP), which transfers LPS monomers into GPI-anchored CD14, to transport LPS to the TLR4–MD-2 complex (Jiang *et al.*, 2000; Park *et al.*, 2009). In addition to LPS, reports suggest that TLR4 also recognizes glycoinositolphospholipids from *Trypanosoma*, fusion viral protein of respiratory syncytial virus (RSV), envelope protein from mouse mammary tumor virus and virulent factor pneumolysin from gram positive *S. pneumoniae* bacteria (Marr and Turvey, 2012; Park *et al.*, 2009).

1.2.2.1.3 TLR5

TLR5 recognizes flagellin protein of flagella (Gewirtz *et al.*, 2001). Flagella, are indispensable for viability, a mutation that affects flagellin function would have lethal consequences for the bacteria, therefore the amino acid structure of this protein is highly conserved (Eaves-Pyles *et al.*, 2001). TLR5 is expressed only in the basolateral surface not on the apical surfaces of the cells that reside in the mucosal sites of the body, therefore TLR5 only detects invading bacteria flagellin, not the non-invading ones. Upon flagellin recognition by TLR5 mucosal immune responses (i.e. induction of chemokine expression) by epithelial cells and macrophages are initiated (Hayashi *et al.*, 2001).

1.2.2.1.4 TLR11

Profilin-like molecules of *Toxoplasma gondii* and uropathogenic *E.coli*. are the major ligands for TLR11, which have a role in parasite motility and invasion. TLR11, is highly expressed kidney and bladder in contrast to TLR5. This receptor shows close homology to TLR5 and present in mice but not in humans because of having at least one stop codon containing mRNA (Plattner *et al.*, 2008; Yarovinsky *et al.*, 2005).

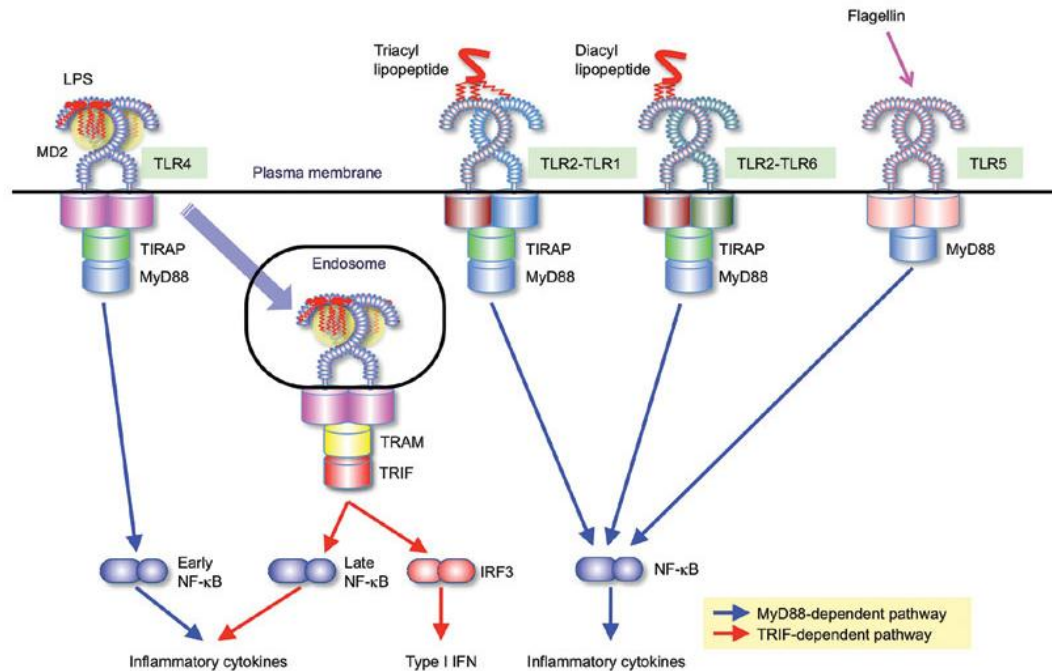


Figure 1.1. PAMP recognition by surface Toll-like receptors. (Adapted from Kawai and Akira, 2010)

1.2.2.2 Intracellular Toll-like Receptors

Nucleic acids are the ligands of intracellular TLRs. These are TLR3, TLR7/8 and TLR9. They are expressed on the intracellular compartments, such as endosomes, lysosomes and even Golgi apparatus and endoplasmic reticulum (ER). The receptor-ligand interaction leads MyD88-dependent or independent induction of signaling cascade leading production of chemokines, inflammatory cytokines and type I IFNs (Latz *et al.*, 2004; Nishiya *et al.*, 2005).

1.2.2.2.1 TLR3

Viral ssRNA and dsRNA are mainly recognized by intracellular TLR3. Synthetic analog of dsRNA, polyinosinic-polycytidylic acid (polyIC) that mimics viral infection therefore promoting antiviral interferons and inflammatory cytokines is defined as the ligand of TLR3 (Alexopoulou *et al.*, 2001; Wang *et al.*, 2004). The large horseshoe-like shape ectodomain of TLR3 has a role to increase its surface area and assist dsRNA detection. On the lateral side of the surface of the TLR3 ectodomain dsDNA binds two N and C terminal regions therefore increasing the stability of receptor-ligand interaction (Luo *et al.*, 2012; Sarkar *et al.*, 2003). Wide range of cells including, myeloid DCs (not plasmacytoid DCs), macrophages, B cells, NK cells, vaginal, cervical, biliary, corneal and intestinal epithelial cells and brain cells express TLR3. Mice deficient in TLR3 gene expression didn't have altered anti-viral responses of T cells after exposure to mouse cytomegalovirus (MCMV), lymphocytic choriomeningitis virus, reoviruses, influenza virus or to herpes simplex virus type 1 (HSV-1) infection but became more susceptible to these diseases (Daffis *et al.*, 2008; Edelmann *et al.*, 2004; Tabeta *et al.*, 2004).

1.2.2.2.2 TLR7 and TLR8

Both of these TLRs recognize single stranded RNA derived from vesicular stomatitis virus, influenza A virus and human immunodeficiency virus (HIV) and also synthetic antiviral chemicals such as imidazoquinoline derivatives, imiquimod and resiquimod (known as R848) and guanine analog, loxoribine (Crozat and Beutler, 2004; Triantafilou *et al.*, 2005). The genes of these receptors show close homology and expressed on the same chromosome X, but in contrast to TLR7, TLR8 is non-functional in the mouse. TLR7 is highly expressed on pDCs, resulting large amounts of type I interferon production (Wei *et al.*, 2011). RNA viruses recognized by pDC via replication-independent manner, by the internalization of the virus through endocytosis and therefore recruiting virus to the endolysosomes where TLR7-ssRNA recognition initiated and antiviral responses are generated (Gilliet *et al.*, 2008). Also, TLR7 senses replicating vesicular stomatitis virus that invades into cytoplasm via autophagy, that causes the lysosomal degradation of viral coat proteins that involves the formation of double-membrane vesicles called autophagosomes (Green *et al.*, 2008). Human TLR8 mainly expressed in monocytes in high-levels recognize R848, viral ssRNA and upregulated upon bacterial infection (Wei *et al.*, 2009).

1.2.2.2.3 TLR9

The most well studied TLR, TLR9 recognizes unmethylated CpG dinucleotides found in both bacterial and viral genome (Hemmi *et al.*, 2000; Lund *et al.*, 2003). The recognition of dsDNA of herpes simplex virus 1 and 2, MCMV as well as synthetic CpG motif containing oligodeoxynucleotides induce the production of type I IFN cytokines from B cells, macrophages and DCs (Krug *et al.*, 2001a). Recognition of bacterial and viral DNA by TLR9 are due to the high abundance of unmethylated CpG motifs in these

organisms compared to the methylated CpG motif containing mammalian genome. Bacterial lysis and the acidic environment in endosomal compartments leads to the degradation of dsDNA into many CpG motif containing ssDNA sequences therefore helps the TLR9-ligand interaction in endosomes or lysosomes by inducing inflammatory cytokines such as IL-6, IL-12 and TNF- α and up-regulation of CD80, CD86 and MHC class II co-stimulatory molecules in pDCs and in B-cells (Klinman *et al.*, 2000; Krug *et al.*, 2001a). It has been reported as 2' deoxyribose-phosphate backbone is important in recognition of phosphodiester backbone DNA while CpG motif is indispensable in recognition of phosphorothionate modified backbone and also it seems that co-localization in the endosomal compartments is essential for recognition (Haas *et al.*, 2008; Yasuda *et al.*, 2006). As an example, when non stimulatory malaria DNA significantly boosted immune response when delivered into the endosomal compartments via transfection or bound to the malarial hemozoin protein (Coban *et al.*, 2010; Coban *et al.*, 2005).

In addition to these, mammalian DNA can be detected by TLR9 if its delivered via transfection reagent. Also human antimicrobial peptide such as LL37 which is released from keratiocytes interacts with self DNA and lead to an autoimmune disease called psoriasis (Coch *et al.*, 2009; Lande *et al.*, 2007). TLR9 signaling antagonist, chloroquine is used for the autoimmune disease treatments such as, rheumatoid arthritis and systemic lupus erythematosus (Sun *et al.*, 2007). Properties and therapeutic potentials of various types synthetic CpG ODNs will be discussed in detail on the following sections.

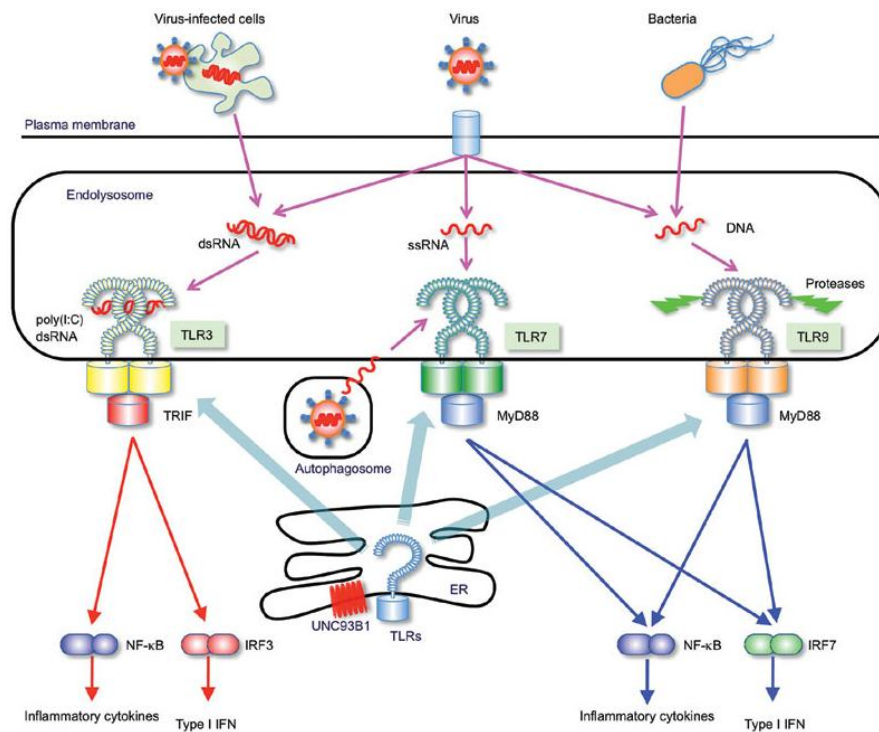


Figure 1.2. Ligand recognition by intracellular TLRs (*Adapted from Kawai and Akira, 2010*).

TLR10, 12 and 13 are the novel members of the TLR family. Their exact role in mammalian immune system yet to be discovered. A gene coding for a protein analogous to TLR10 in humans is present in mice, but appears to be nonfunctional because of the retrovirus insertion. Also mice express TLRs 12 and 13 but none of these are present in humans (Wu *et al.*, 2008). Human TLR10 which are related to TLR1, 2 and 6, highly expressed in spleen, lymph node, thymus, and tonsils. Protein-ligand docking approaches showed that similar to TLR2 ligand family members a diacylated lipoprotein Pam(3)CSK(4) can be the potential ligand for TLR10 (Chuang and Ulevitch, 2001; Govindaraj *et al.*, 2010). TLR12 is localized in chromosome 4, and involved in the immune system against bacteria in the kidney, but its potential ligands and mechanism of action are not known up to date (Hageman *et al.*, 2011). Intracellularly localized, TLR13 is expressed in dendritic cells and macrophages and activates MyD88-dependent antiviral responses upon recognition of vesicular stomatitis virus (Shi *et al.*, 2011). Moreover, it has been shown that TLRs 11-13 are constitutively expressed in mice brain

and upon infection with parasite *Taenia solium*, gene expressions and protein levels of all three TLRs are highly upregulated (Mishra *et al.*, 2008).

1.2.2.3 TLR Signaling

TLR-ligand interaction leads to the activation of various signaling pathways that induce the upregulation of the transcription factors such as nuclear factor kappa B (NF- κ B), activator protein (AP-1) and interferon regulatory factors (IRFs) in the production of Type 1 IFNs, proinflammatory cytokines and expression of IFN-inducible genes (Kawai and Akira, 2007). The TLR signaling cascade is promoted with the interaction of Toll/Interleukin-1 receptor (TIR)-domain-containing adaptors to the TIR domain of TLRs. TLR5, 7, 9 and TLR11 use Myeloid differentiation primary response gene 88 (MyD88). In addition to MyD88, TLR1, 2, 4 and 6 use TIR domain containing adaptor protein (TIRAP), which links TLR TIR domain to MyD88. TLR3 uses solely TIR-domain-containing adapter inducing interferon β (TRIF). TLR4 uses both TRIF and TRIF related adaptor molecule (TRAM), and TRAM binds TLR4 with TRIF (Janssens and Beyaert, 2002; Takeda and Akira, 2005). These protein complexes initiate the signaling cascade and the activation of transcription factors described above.

Generally TLR signaling subdivided into two groups, MyD88-dependent and MyD88-independent/TRIF dependent signaling. Both of the pathways can be activated by only TLR4. Figure 1.3 summarizes the TLR dependent signaling.

1.2.2.3.1 MyD88-Dependent Pathway

The engagement of TLRs (except TLR3) by their appropriate PAMPs, followed by the recruitment of the IL-1 receptor–associated kinases IRAK4, IRAK1, IRAK2 and IRAK-M by MyD88 respectively (Janssens and Beyaert, 2003).

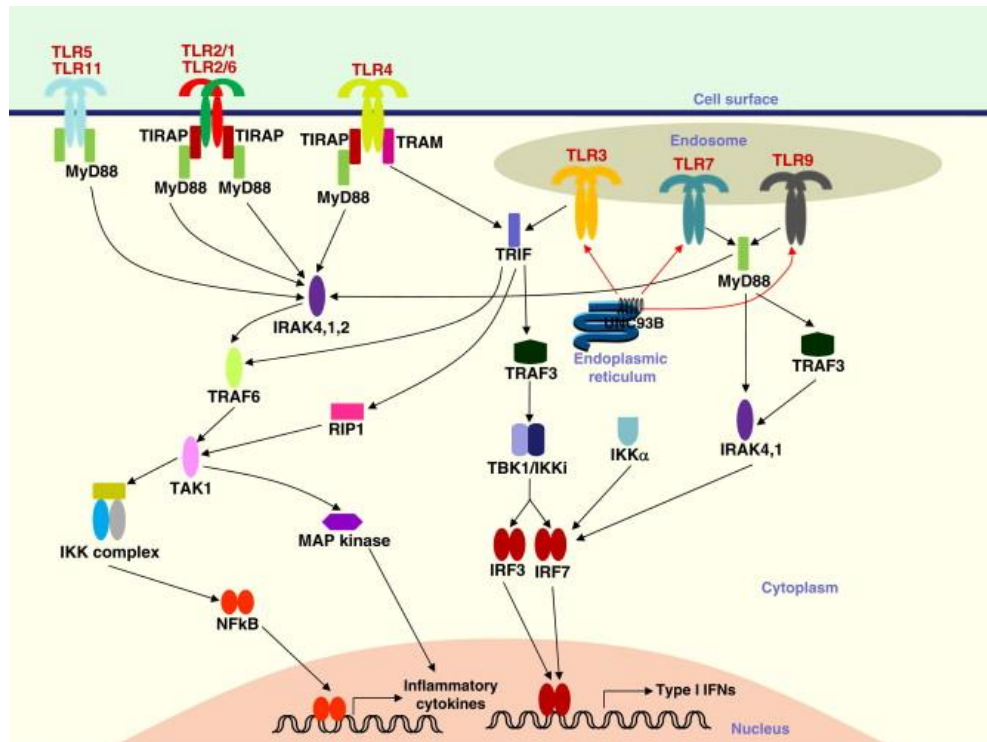


Figure 1.3. MyD88 dependent and TRIF dependent TLR signaling pathways in immune cells (Adapted from Kumar *et al.*, 2009b).

Phosphorylation of IRAK1 results in an interaction with TRAF6, which is an ubiquitin ligase that polyubiquitinates itself and NF-κB essential modifier (NEMO) through Lys63 (Deng *et al.*, 2000; Wang *et al.*, 2001). The activated TRAF6 triggers the protein kinase TAK (transforming growth factor-β-activated kinase 1)/TABs (TAK1-binding proteins) complexes and subsequently enhances the nuclear translocation of transcription factors such as NF-κB and AP-1 through the inhibitor kappa B kinase (IkK) complex and the MAPK [ERK (extracellular-signal-regulated kinase), JNK (c-jun N-terminal kinase) and

p38] pathways respectively for the production of inflammatory cytokines (Gohda *et al.*, 2004). TLR7 and TLR9 are highly expressed in pDCs, therefore able to produce large amounts of type I IFNs, especially IFN- α , upon virus infection. After activation, MyD88 forms a multimeric complex with IRF7 (highly expressed in pDCs) and TRAF6 to induce the production of type I IFNs (Honda *et al.*, 2005).

MyD88 knock-out mice do not produce inflammatory cytokines such as TNF- α and IL-12p40 when treated with TLR agonists. MyD88 gene deficient macrophages, show impaired inflammatory cytokine production in response to TLR2 and TLR4 ligands contrary to TLR3, TLR5, TLR7 and TLR9 ligands (Kawai *et al.*, 1999).

1.2.2.3.2 MyD88-Independent/TRIF Dependent Pathway

Exposure to the TLR3 and TLR4 ligands activate TRIF-dependent signaling pathway and trigger the inflammatory cytokines besides type I IFNs and IFN-inducible genes (such as IP-10, MCP-1, MCP-2) particularly in DCs and macrophages (Oshiumi *et al.*, 2003; Zhai *et al.*, 2004). Interferon regulatory factors (IRF), IRF3 and IRF7 were activated by the recruitment of TRAF3 and the activation of the TANK-binding kinase 1 (TBK1) and I κ Ki. TBK1 and I κ Ki interacts with TANK which then phosphorylates IRF3 and IRF7. Phosphorylated IRF3 and IRF7 form a homodimer, then translocated into the nucleus and induce type I IFNs and related gene products (Gohda *et al.*, 2004). In TRAF3-deficient cells IFN- β production is abrogated suggesting the TRAF3 is the linker between TRIF to TBK1. TRIF knock-out mice showed weakened expression of IFN- β and IFN-inducible genes when subjected to TLR3 and TLR4 ligands (Mao *et al.*, 2010; Song *et al.*, 2008).

Entire MyD88 and TRIF expression is essential for the efficient collaboration, resulting in the production of IL-6, IL-12, IL-23 cytokines but not of IP-10 and TNF- α upon MyD88- and TRIF-dependent TLR stimulation. The synergism of transcription factors

for IL-6, IL-12, and IL-23 gene expressions are highly related to the downstream of MyD88, TRIF and IRF5 cooperation (Murphy *et al.*, 2011).

1.2.3 C-type Lectin Receptors

The C-type lectin family of receptors (CLR), made up of an extracellular C-terminal C-type lectin domain, which are expressed on the cell surfaces of DCs, neutrophils and macrophages. CLRs detect carbohydrates on bacteria, viruses and fungi and therefore induce the production of proinflammatory cytokines that determine T cell polarization fates or could inhibit TLR-mediated immune complexes (Geijtenbeek and Gringhuis, 2009). Main ligands for CLRs are β -glucans found in fungi cell wall which activate DCs by Dectin-1 or Dectin-2 therefore to initiate T cells to create immune response against *Candida albicans* (Robinson *et al.*, 2009). Another C-type lectin MINCLE expressed in macrophages (also known as Clec4e and Clecsf9) recognizes fungi such as *Malassezia* and *Candida* (Yamasaki *et al.*, 2009). Different from pathogen recognizing CLRs, endogenous protein, spliceosome-associated protein 130 (SAP130) and CLEC9A which is expressed on DCs could detect necrotic cells.

1.2.3.1 Dectin-1

Dendritic cell-associated C-type lectin-1 (Dectin-1) also known as CLEC7A is a type-II transmembrane protein that possesses an extracellular C-type lectin-like domain (CTLD) which is connected by a immunoreceptor tyrosine based activation (ITAM)-like motif containing cytoplasmic tail. Dectin-1 is mainly expressed by myeloid cells in many tissues, with highest levels of expression on inflammatory cells such as macrophages, monocytes, subsets of DCs, neutrophils and subpopulations of T cells (Tsoni and Brown, 2008). Moreover this receptor is also expressed in human eosinophils, B cells and mast cells. Dectin-1 is the primary receptor for the recognition of β -1,3 or β -1,6 linked glucans found in bacteria, fungi and plant cell walls (Werner *et al.*, 2009). How Dectin-1

is actually able to recognize β -glucans yet to be answered. Zymosan, a cell wall fraction derived from *Saccharomyces cerevisiae*, its modified form hot alkali treated zymosan, depleted zymosan and unknown molecule on mycobacteria, which didn't include β -glucans also induce immune response through Dectin-1. Zymosan is also composed of mannan, chitin and other components that result in immune activation through cooperative induction of MyD88-dependent TLRs by inducing IL-2, IL-6, IL-10, IL-12, IL-23, TNF- α and some chemokines (Dillon *et al.*, 2006; Goodridge *et al.*, 2007). This is a novel example of collaborative induction of immune responses between TLRs and non-TLR receptors. As seen in figure 1.4 after the activation of Dectin-1 by its ligands, ITAM sequences are phosphorylated by Src family kinase spleen tyrosine kinase (Syk) with the adaptor CARD9 leads to the initiation of downstream signaling events resulting with the activation of nuclear factor of activated T cells (NFAT) and NF- κ B (Figure 1.4) (Saijo and Iwakura, 2011).

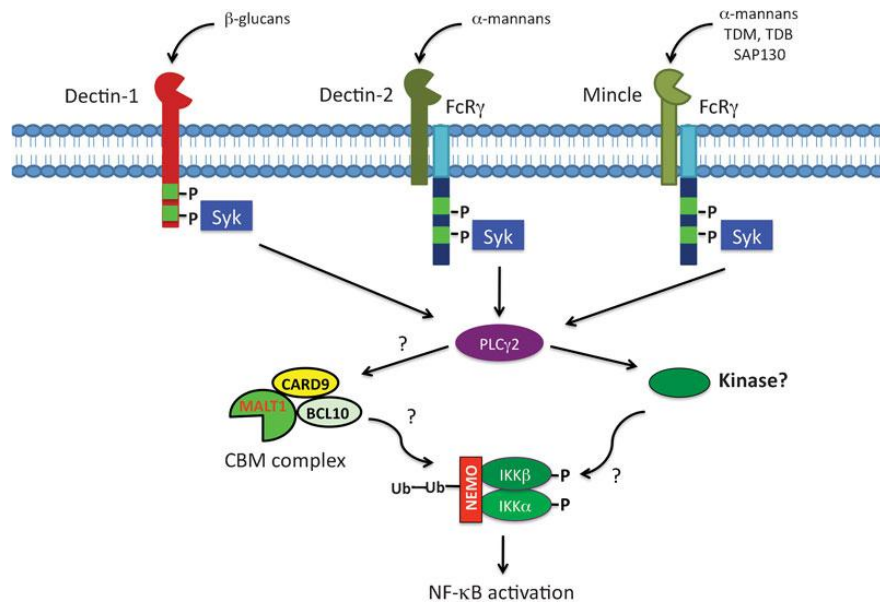


Figure 1.4. Dectin-1, Dectin-2 and Mincle induce innate immune response via NF- κ B signaling cascade by recruiting Syk adaptor protein (Adapted from Kingeter and Lin, 2012).

1.2.3.2 Dectin-2

Dectin-2 receptor belongs to the Dectin-2 family of C-type lectin family which is mainly expressed in pluripotent myeloid precursors, macrophages, neutrophils, Langerhans cells and human plasmacytoid dendritic cells (Graham and Brown, 2009). It is the best characterized receptor in this family among blood dendritic cell antigen 2 (BDCA-2), dendritic cell immunoactivating receptor (DCAR), dendritic cell immunoreceptor (DCIR), Clecsf8 and MINCLE. This receptor could bind to the zymosan and high-mannose structures found in the various pathogens including *Saccharomyces cerevisiae*, *Candida albicans*, and *Mycobacterium tuberculosis* (Willment and Brown, 2008). Lately it has been showed that Dectin-2 on bone-marrow-derived DCs (BMDCs) is able to recognize allergen extracts from different house dust mites and therefore resulting the stimulation of mast cells as an allergic response (Barrett *et al.*, 2009).

1.2.4 NOD-like Receptors

Evolutionarily conserved NLR family of the immune system detect both host-derived danger signals (danger associated molecular patterns, DAMPs) and PAMPs. The members of these receptors are defined by the existence of a central nucleotide-binding and oligomerization (NACHT) domain which is usually followed by C-terminal leucine-rich repeats (LRR) and N-terminal caspase recruitment (CARD) or pyrin (PYR) domains. NACHT domain mediates NLR oligomerization, LRRs function in ligand sensing, while CARD and PYD domains regulate the protein-protein interaction for downstream signaling (Inohara *et al.*, 2005) (Kersse *et al.*, 2011). NLR family is mainly classified into four subfamilies which are composed of 23 members in humans, and at least 34 NLRs are determined in mouse genome. The NLRA subfamily contains one member, class II, major histocompatibility complex, transactivator (CIITA), that acts in the transcriptional regulation of MHC Class II genes. Mutations in this protein cause various lymphocyte related immunological disorders. NRLB subfamily also has only

one member, nucleotide oligomerization domain-like receptor family apoptosis inhibitory protein, NAIP (also known as BIRC3) shows homology to two baculovirus inhibitor of apoptosis proteins, and therefore capable of suppressing apoptosis by inhibiting caspase-3, -7, and -9 (Katagiri *et al.*, 2012). But one functional NAIP member in human genome, Naip5 induces pyroptosis by activating caspase-1 pathway in macrophages which were infected with *Legionella pneumophila* gram-negative bacteria (Kofoed and Vance, 2012). NLRC subfamily members consist of, Nucleotide-binding oligomerization domain-containing protein 1 (NOD1), NOD2, NLRC3, NLRC4 (IPAF), NLRC5 and NLRX1. Finally, NLRP1 to NLRP14 generate the NLRP subfamily. Well known and studied members of the last two subfamilies will be mentioned below in detail.

1.2.4.1 NOD1 and NOD2

NOD1 and NOD2 receptors which are located in the cytoplasm of macrophages, monocytes also known as CARD4 and CARD15 because their N-terminals include one or two CARDs respectively (Kanneganti *et al.*, 2007). NOD1, binds to the bacterial cell wall product *meso*-diaminopimelic acid (*meso*-DAP), while NOD2 recognizes a conserved structure within peptidoglycan called muramyl dipeptide (MDP). *E. coli*, *Helicobacter pylori* and *S. pneumonia*, *M. tuberculosis* are also sensed by NOD1 and NOD2 respectively (Figure 1.5) (Kersse *et al.*, 2011). Both of them could detect the intracellular localized Gram-negative bacterium *Shigella flexneri* (Suzuki and Nunez, 2008). Upon recognition of various PAMPs, CARD domain containing receptors oligomerize leading to the recruitment of another CARD-containing adapter protein receptor interacting protein-2 (RIP-2) (Fairhead *et al.*, 2008). Afterwards RIP2 directly interacts with NEMO, resulting NF- κ B activation, and translocation to the nucleus for the upregulation of proinflammatory cytokine, chemokine genes (Strober *et al.*, 2006). Response to *H. pylori* by NOD1 results the activation of IRF7, therefore induce Type I IFN production (Ferrero, 2005). Also NOD2, detects viral ssRNA of RSV, therefore

activates IRF3 and result as induction of Type I IFN cytokines. Sensing of *S. pneumonia* by NOD2 resulting the stimulation of CCL2 chemokine by macrophages (Kapetanovic *et al.*, 2010). A frameshift mutation of NOD2 causes an inflammatory bowel disease, Crohn's disease in humans which is defined as a type of immune deficiency disease (Ogura *et al.*, 2001).

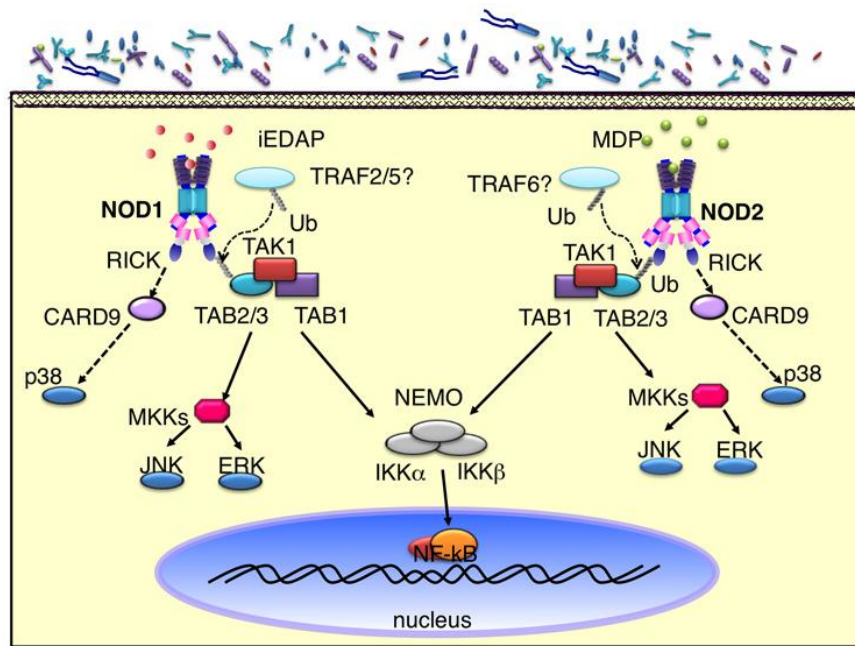


Figure 1.5. Intracytosolic signaling cascade mediated by NODs (Adapted from Shaw *et al.*, 2008).

1.2.4.2 Inflammasomes

NOD like receptors are the main initiators of inflammasomes, the cytosolic multiprotein oligomeric complexes of the innate immune system that include NALP, apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC; also known as PYCARD), caspase-1 and/or caspase-5 (Henao-Mejia *et al.*, 2012). The NLR members NLRP1 (NALP1), NLRP3 and NLRC4 (IPAF), and the non-NLR protein AIM2 have been able to form inflammasomes which could recognize both PAMPs and DAMPs (Figure 1.6).

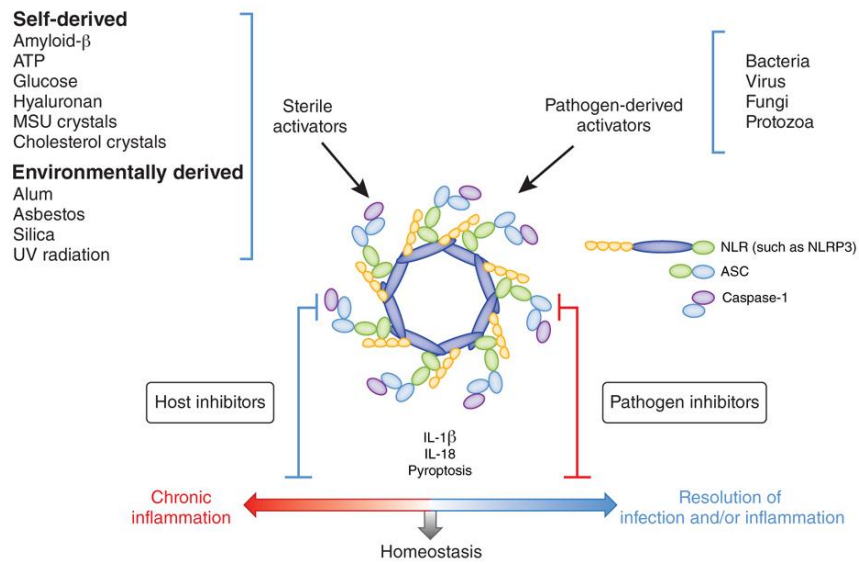


Figure 1.6. Multimeric inflammasome protein complexes regulate both homeostasis and inflammation upon PAMP and DAMP exposure (*Adapted from Henao-Mejia et al., 2012*).

1.2.4.2.1 NLRP1

The NLRP1 (NALP1) inflammasome consists of caspase-1, caspase-5, ASC and triphosphate ribonucleotide. It is expressed on thymus, spleen, heart, liver, lung, macrophages and peripheral blood lymphocytes (Tschopp *et al.*, 2003). By the recognition of bacterial cell wall component MDP and *Bacillus anthracis* lethal toxin N-terminal PYD of NALP1 and ASC assemble to form the inflammasome to recruit and activate procaspase-1, while the C-terminal CARD of NALP1 activates caspase-5 (Boyden and Dietrich, 2006). Caspase-1 is necessary for the cleavage of inactive precursors of proinflammatory cytokines such as $IL-1\beta$ and $IL-18$, which contribute to the host responses upon infection and injury (Faustin *et al.*, 2007).

Moreover, the anti-apoptotic proteins Bcl-2 and Bcl-X(L) bind to NLRP1, therefore resulting the suppression of caspase-1 activation and $IL-1\beta$ production. Increased risks of an autoimmune skin disease vitiligo, a neurodegenerative disease Alzheimer's, are related with the genetic variations on NLRP1 (Jin *et al.*, 2007b; Pontillo *et al.*, 2011).

1.2.4.2.2 NLRP3

One of the well-studied NLRP family member NLRP3, also named as NALP3 or cryopyrin is ubiquitously and highly expressed in neutrophils, monocytes, macrophages and myeloid DCs (Schroder *et al.*, 2010). NLRP3 could recognize diverse ligands both DAMPs and PAMPs. The wide spectrum of stimuli include: i) crystalline particulates such as silica, asbestos and uric acid crystals (or monosodium urate, MSU) which are the causative agents of silicosis, asbestosis and gout, ii) widely used adjuvant, aluminum hydroxide (Alum), iii) an acute-phase protein, serum amyloid A (SAA) in amyloid A-type amyloidosis, iv) fibrillar amyloid- β proteins which are involved in the Alzheimer's disease, v) mitochondrial reactive oxygen species, vi) cholesterol crystals as atherosclerosis indicators, vii) zymosan, mannan and β -(1,3)-glucans combined with ATP from *Candida albicans* and *Aspergillus fumigatus* species, viii) lipooligosaccharide of *Neisseria gonorrhoeae*, ix) alpha-hemolysin from *Staphylococcus aureus* and hemolysins, MDP, and toxins derived from *Vibrio vulnificus* and *Vibrio cholerae* x) Influenza A virus, Sendai virus, RSV, Hepatitis C, HIV, vaccinia virus and encephalomyocarditis viruses double stranded and viral RNA (Burdette *et al.*, 2012; Duewell *et al.*, 2010; Duncan *et al.*, 2009; Iyer *et al.*, 2009; Leemans *et al.*, 2011; Pontillo *et al.*, 2012; Thomas *et al.*, 2009) (Figure 1.7). Also expression of NALP3 induced by various TLR agonists and inflammatory cytokines, including LPS, CpG ODNs, Pam(3)Cys(4), R848 and TNF- α (Franchi *et al.*, 2009; Nixon Tangi *et al.*, 2012).

After the stimulation of NLRP3, proteins engage and interact with the ASC adaptor, that promotes the recruitment of pro-caspase-1 with CARD–CARD associations, resulting the activation of IL-1 family cytokines such as IL-1 β and IL-18. Extracellular ATP and bacterial toxins, nigericin and maitotoxin are the activators of purinergic ATP-gated P2X7 receptor (P2X7R) that induce cellular K⁺ release from cells therefore triggering

both NLRP1 and NLRP3 inflammasome complexes (Martinon *et al.*, 2002; Martinon *et al.*, 2007).

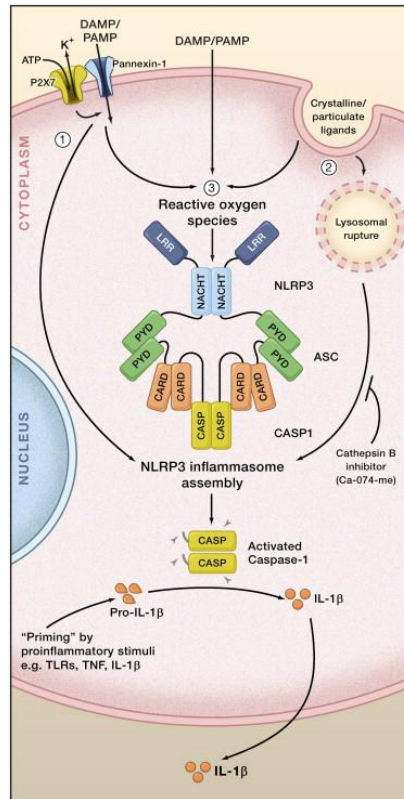


Figure 1.7. Alternative entry/activation steps of inflammasome pathway leading to IL-1 β production (Adapted from Schroder and Tschopp, 2010).

1.2.4.2.3 AIM2

AIM2 is also known as ‘Interferon-inducible protein’ or ‘absent in melanoma 2’. This type of inflammasome is defined as a receptor for the cytosolic double stranded DNA, *Francisella tularensis*, *Listeria monocytogenes* as well as vaccinia virus and mouse cytomegalovirus genetic materials and has partial role in sensing the bacteria (Fernandes-Alnemri *et al.*, 2010; Rathinam *et al.*, 2010). Being constitutively expressed in mouse macrophages and splenocytes, AIM2 induces IL-1 family cytokine (IL1- β and IL-18) production with ASC protein, via caspase-1 pathway, NF- κ B pathway and natural

killer cell–dependent production of interferon- γ against virus replication (Hornung *et al.*, 2009; Rathinam *et al.*, 2010). Endoplasmic reticulum-associated molecule stimulator of IFN gene (STING) regulates an antiviral effect via IRF3-dependent production of type I IFNs (especially IFN- β) after recognizing the cytoplasmic viral DNA (Ishikawa *et al.*, 2009). Recently it is shown that this intracellular inflammasome mutliprotein could recognize both *Streptococcus pneumoniae* and cyclic diguanylate monophosphate (cyclic di-GMP) (Koppe *et al.*, 2012; Shang *et al.*, 2012).

1.3 Cooperation between Pattern Recognition Receptors

Host invading pathogens present a wide variety of surface antigens and released products which are detected by the innate immune system components as well as multiple PRRs. The crosstalk between various extracellular and intracellular receptors leads to the activation of signaling pathways and immune response by innate immune cells. The proinflammatory transcription factor activation (i.e NF- κ B, AP-1, NFAT) trigger phagocytosis and eventually shape the adaptive immune response (Underhill, 2007).

The receptor collaboration is executed by, i) the interaction of two or more receptors with each other in the same APC to identify the transcriptional and cellular response and ii) the activation of the set of receptors that recognize a particular microbe by many types of phagocytes that direct the adaptive immunity (Kawai and Akira, 2011).

The interaction of receptors and pathways have various advantages for the organisms. Firstly, the immune system robust against infection with the inclusion of numerous receptors for the recognition of pathogens. Secondly, the genetic variety in innate immune system of humans for having multiple pathogen recognition systems, compensating the genetic weakness of individuals. Finally, multiple receptor

involvement in the recognition of a microbe could result an appropriate and specific immune response (Trinchieri and Sher, 2007).

1.3.1 Collaboration between TLRs

TLRs sense various molecules carbohydrates, proteins, peptides, lipids and nucleic acids which are expressed by bacteria, virus, fungi and parasites. Revealing the relationship between TLR family members against pathogens will help us to formulate more effective vaccines for strong and long-lasting protection.

The synergism between TLR2 and TLR4 firstly reported *in vitro* studies showing improved TNF- α , IL-6 and MIP-1 α production upon incubation of macrophages by Mycoplasma lipopeptide (MALP-2) or MDP and LPS (Bagchi *et al.*, 2007; Paul-Clark *et al.*, 2006). The engagement of these two receptors also results in the recruitment of mitochondria to macrophage phagosomes and enhanced production of bactericidal mitochondrial reactive oxygen species (ROS) and nitric oxide (NO) (Sato *et al.*, 2000). When TLR4-deficient mice were challenged with *Salmonella typhimurium* they were more susceptible to the infection as evidenced by increased bacterial accumulation in the lymph nodes (Gerold *et al.*, 2007; Weiss *et al.*, 2004). These results infer the sequential involvement of TLR2 and TLR4 in bacterial infection. *Mycobacterium tuberculosis* infection is also disposed with TLR2, TLR4 and TLR9 collaboration by the induction of IL-1 β , IL-6, IL-12 and TNF- α . MyD88 knock-out mice were highly susceptible to the *M. tuberculosis* infection proving the TLR involvement in immune response (Gerold *et al.*, 2007; Saiga *et al.*, 2011). TLR2- and TLR9- deficient mice were highly susceptible to the high dose infection and demonstrated enhanced bacterial stress but not as much as the TLR4- deficient mice (Saiga *et al.*, 2011). Interestingly TLR2, 4 and 9- triple knock-out mice showed same immune response against *M. tuberculosis* infection as wild type mice with enhanced pro-inflammatory cytokines production and expanded IFN- γ -producing antigen-specific T cells (Holscher *et al.*, 2008). These results interpreted as,

other PRRs rather than TLRs are involved in the induction of adaptive immunity against these bacteria.

Viral infections are sensed by TLR2, TLR3, TLR4 and TLR13. TLR2 senses the measles virus hemagglutinin protein, human CMV and HSV-1 therefore stimulate the type I IFN secretion by monocytes (Barbalat *et al.*, 2009). TLR3 knock-out mice were susceptible to the MCMV, coxsackievirus, WNV because of the impaired type I IFN, IFN- γ and IL-12p40 cytokine production and NK cell activation. Fusion protein of RSV, envelope protein of mouse mammary tumor virus (MMTV) induces the inflammatory cytokine production by TLR4. TLR4- deficient mice showed impaired viral clearance along with reduced IL-12 production and infiltrating immune cells (Cyr *et al.*, 2009; Datta *et al.*, 2006). Intracellular TLR13 detects the vesicular stomatitis virus (VSV) and induces type I IFN production (Shi *et al.*, 2011).

Fungus, *Candida albicans* is sensed by at least five TLRs, TLR2, 4, 6, 7 and 9 via multiple patterns such as β -glucan, chitin, mannan, proteins and nucleic acids (Netea and Marodi, 2010; Romani, 2011). Controversial findings indicate that TLR2-deficient mice is either more resistant to candidiasis infection because of the decreased IL-10 and increased IFN- γ production or more susceptible to the exposure because of the low levels of TNF- α and chemokines (Netea *et al.*, 2004; Suttmüller *et al.*, 2006). Almost the same contrast results were obtained from the TLR4- deficient mice studies upon *C. albicans* infection (Netea and Marodi, 2010). Fungal nucleic acid containing vesicles were recognized by TLR7 and TLR9 in DCs leading IFN- γ production. T cells from TLR9- defective mice show higher IL-4 levels, proving that TLR9 acts toward Th1 cell polarization upon fungal infections (Bellocchio *et al.*, 2004).

Parasites such as, *Plasmodium falciparum*, *Leishmania major*, *Trypanosoma cruzi* and *Toxoplasma gondii* are recognized by TLRs (Gazzinelli and Denkers, 2006). Cell membrane glycolipid, glycosylphosphatidylinositol (GPI) anchors detected by TLR2 and

TLR4. GPI induces chemokine and cytokine production from macrophages (Debierre-Grockiego *et al.*, 2007). Profilin-like molecule of *Toxoplasma gondii* is the ligand for mouse TLR11, as proven by higher susceptibility of TLR11-deficient mice to the infection with reduced IFN- γ and IL-12 production (Yarovinsky *et al.*, 2005). Hemozoin and heat labile non-DNA fractions of *P. falciparum* binds to the TLR9, induces both type I IFN production from DCs and NALP3 inflammasome (Coban *et al.*, 2005; Kumar *et al.*, 2009c). TLR2- and TLR9- double knock-out mice were highly sensitive to the *T. cruzi* infection compared to the wild type mice (Bafica *et al.*, 2006).

Various *in vitro* studies showed that, stimulation of DCs with LPS and R848 lead to the upregulation of gene expressions of IL-6, IL-10, IL-12, IL-23 and TNF- α even after 8hrs. Also both in human and mouse DCs, TLR3 and TLR7/8 and TLR9 acted in synergy and enhanced the expression of set of genes to prime Th1 responses (Napolitani *et al.*, 2005). The underlying mechanism is not really understood but implicated as the two TLR receptors stimulate the production of IFN- β via IRF3 and IRF7 activation maintains higher IL-12 secretion from DCs (Goriely *et al.*, 2006).

Stimulation of both MyD88 and TRIF dependent pathways could not necessarily lead to the synergism. TLR2/6 ligand and TLR3 ligand or TLR9 ligand stimulation didn't favor the IL-12 production from DCs (Zhu *et al.*, 2008). No synergy was observed in macrophages when incubated with double combinations of TLR2 and TLR5, TLR2 and TLR9, or TLR5 and TLR9 ligands (Makela *et al.*, 2009). Adeno-associated virus vector cancer vaccine in combination with TLR7 and TLR9 agonists showed decline in Th1 biased immune response and DC activation (Triozi *et al.*, 2010).

The main factors for the synergism/antagonism between TLRs could be attributed to the difference in cellular localization of these receptors, the interaction of TLRs with other PRRs and accessory molecules that could connect TLR to distinct signaling pathways and regulatory mechanisms.

1.3.2 Collaboration between TLRs and other PRRs

Different PRRs recognize same PAMPs, therefore TLRs collaborate with other PRRs such as Dectin-1, NOD1, NOD2, RLRs and inflammasomes to coordinate both innate and adaptive immune responses against pathogens (Figure 1.8).

Both NLRs and CLRs act in concert with TLRs against bacterial infection. NOD2 acts in synergy with TLR2 after *M. tuberculosis* infection (Gandotra *et al.*, 2007). IL-1R deficient mice showed high mortality rate upon *M. tuberculosis* similar to MyD88-deficient mice (Fremond *et al.*, 2007). Also caspase-1, NALP3 and ASC are partially involved in the recognition of this infection shown by IL-1 β production (Mayer-Barber *et al.*, 2010; McElvania Tekippe *et al.*, 2010). Moreover TLR2, TLR3 and TLR4 agonists and NOD2 ligand MDP synergize to produce IL-1 β , IL-10, TNF- α and IL-6 from monocytes and macrophages. The underlying mechanism of this synergism could be attributed to the activation common pathways such as, NF- κ B and MAPKs (Underhill, 2007).

RLRs and TLR7 act together in antiviral responses. TLR7 expressed in pDCs and RIG-I in cDCs, macrophages and fibroblasts recognizes the RNAs of IAV, VSV and Newcastle disease virus to induce type I IFN production. Since viruses could be able to suppress RLR signaling, TLR7 acts as a support system against viral infections (Kumagai *et al.*, 2007). Hepatitis C virus is sensed by both TLR3 and RIG-I in hepatocytes and upon cell-to-cell contact with pDCs that triggers the type I IFN secretion (Horner and Gale, 2009; Lemon, 2010). Poly IC is recognized by both TLR3 and MDA5 by DCs, monocytes, therefore boosting adaptive immune response (Longhi *et al.*, 2009).

Cooperation of TLRs with CLRs boosts anti-fungal immune response. TLR2 or TLR4 and Dectin-1 collaborate upon recognition of zymosan and β -glucan resulting the induction of phagocytosis, ROS and chemokine, cytokine production in immune cells (Goodridge and Underhill, 2008; Netea and Marodi, 2010). IL-1 β production by fungal β -glucan involving both Dectin-1 dependent induction of pro-IL-1 β and NALP3-mediated processing to mature IL-1 β (Gross *et al.*, 2009; Lamkanfi *et al.*, 2009a).

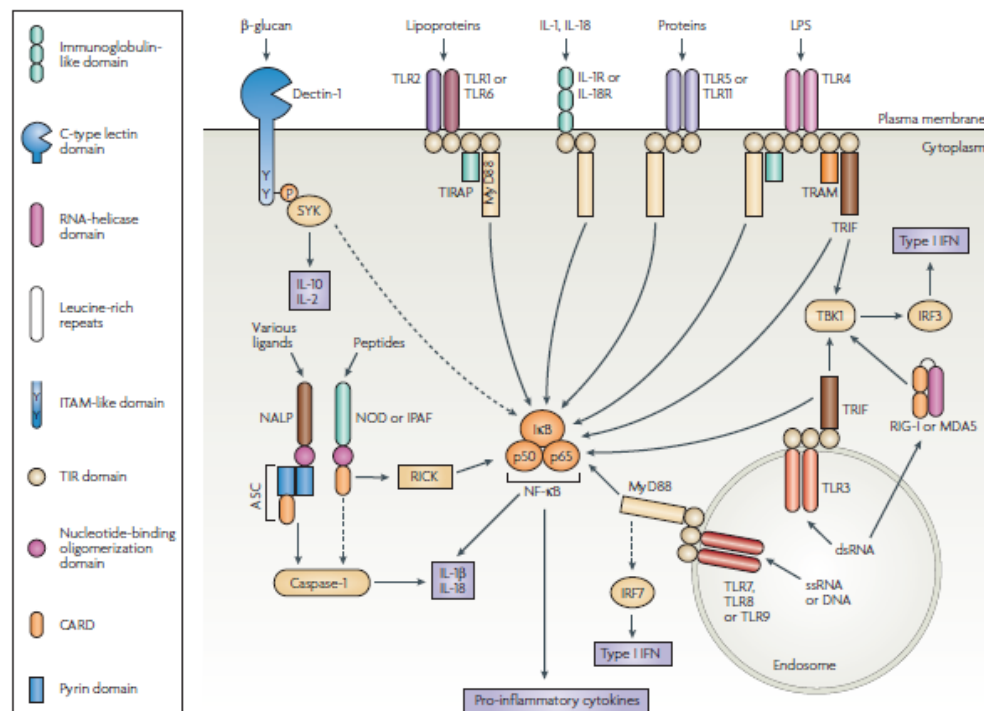


Figure 1.8. The structures and the signaling pathways of the various pattern recognition receptors (Adapted from Trinchieri, 2007).

1.4 Immunostimulatory Synthetic CpG ODN Sequences

The immunostimulatory CpG motif was initially discovered when a DNA fraction from *Mycobacterium bovis* strain (BCG) was found to produce type I IFN. This secretion led to NK cell activation, directing effective antitumor effect (Tokunaga *et al.*, 1984). It was initially thought that the palindromic sequences in the bacterial DNA were the main cause for this immune response, however, later it was proven that not only the palindromic sequences but also unmethylated CpG dinucleotides, accompanied by two 5' purines and two 3' pyrimidines were in charge for the activation of mammalian immune system (Klinman *et al.*, 1999; Krieg *et al.*, 1995). Compared to mammalian DNA having at least 20 times more frequent unmethylated CpG motifs, bacterial DNA or synthetic antisense oligodeoxynucleotide sequences were designed as an analogue of BCG DNA act as an antigen to induce T helper 1 biased immune response, which was characterized by the production of TNF- α , IFN- γ , type I IFN and IL-1 family members, IL-6, IL-12 cytokines (von Meyenn *et al.*, 2006). In 2000 Akira *et al.* showed that CpG ODN is recognized by the TLR9, as evidenced by unresponsiveness to the CpG motif containing DNA in TLR9-deficient mice cells including the proliferation of splenocytes, macrophages, maturation of dendritic cells, inflammatory cytokine production and also being resistant to the side effects of CpG DNA (Hemmi *et al.*, 2000). Complexation with DOTAP or PolyG enrichment enhanced the immunostimulatory effects of PO backbone CpG ODNs by targeting them into the endosomes (Yasuda *et al.*, 2006). In contrast to PO backbone, PS modified sugar backbones could bind both TLR9 and TLR7 with a higher affinity.

1.4.1 An Insight to TLR9 Activation and Signaling

TLR9 resides in the endoplasmic reticulum, which transported into the early endosomes, later to the lysosomes after CpG ODN stimulation. The intracellular localization is essential in two main reasons, i) viral nucleic acids are delivered with receptor mediated endocytosis, and recognized by TLR expressing endosomes and ii) *self* from *non-self* molecules are distinguished, therefore host could be protected from autoimmune response (Blasius and Beutler, 2010). Upon stimulation, the trafficking of TLR9 (also TLR 3, 7 and 8) is mainly regulated by an indispensable a multitransmembrane ER resident protein, UNC93B1, which interacts with the transmembrane domain of the receptor (Kim *et al.*, 2008). PRAT4 and gp96 are also involved in the trafficking of TLR9 from ER to endolysosomes. These proteins are also included in the transportation of TLR 1, 2, 4, 5 and 7 to the plasma membrane or endosome (Saitoh and Miyake, 2009). Secretory (including Golgi) or non-secretory pathways could mediate both the translocation from ER to endosomes. Next, endosomal acidification is required for the activation of TLR9 which contributes to the release of microbial components, such as nucleic acids and also supply the crucial cleavage of the TLRs themselves for more effective binding to CpG DNA (Barton *et al.*, 2006). The cleavage of carboxy terminal fragment of TLR9 is achieved by proteases for instance cathepsins B, S, L, H and K in order to form a functional receptor for the recognition of the specific antigen and leading to the activation of signal cascade by the recruitment of adapter protein MyD88 (Latz *et al.*, 2004; Muller *et al.*, 2008).

1.4.2 Types of CpG ODNs

Synthetic CpG oligodeoxynucleotides designed to date are classified as, well studied i) class A/D CpG ODN ii) class B/K-type ODN iii) class C-type ODN, less well characterized sequences iv) class P CpG ODN v) Y-shaped ODN vi) X-shaped ODN and vii) the novel dendrimeric 70-mer ODN developed in our Biotherapeutic ODN Res.

Lab. Control sequences of CpG ODNs, which contained either methylated cytosine, or CpG flipped into GpC, didn't induce immune response (Gursel *et al.*, 2002; Hanagata, 2012; Karatepe, 2009; Mammadov, 2009). The structural and immunologic properties of four well studied CpG ODNs were summarized in Table 1.5.

Table 1.5. Comparison of A/D, B/K, C and P-type CpG oligodeoxynucleotides (*Adapted from Bode et al., 2011; Klinman et al., 2009*).

ODN type	Representative sequence and examples	Structural characteristics	Immune effects
A/D type ODN	GGTGCAT <u>CG</u> ATGCAGGGGGG ODN 2216 & ODN 2336 (for human) ODN 1585 (for mouse)	• Mixed PO/PS backbone • Single CpG motif • CpG flanking region forms a palindrome • Poly G tail at 3' end	• Induces strong pDC, IFN-α secretion • APC maturation
B/K type ODN	TCCATGGAC <u>GTTC</u> CTGAG <u>CG</u> TT ODN2006 (or PF-3512676, CpG7909) (for human) ODN 1668 & ODN 1826 (for mouse)	• PS backbone • Multiple CpG motifs • 5' motif most stimulatory	• Induces strong B cell activation • pDC maturation • Preferentially enhance production of TNF-α and IL-6
C type	TCGTC <u>GTT</u> CGA <u>ACG</u> AC <u>GTT</u> GAT ODN 2395 & ODN M362 (for human and mouse)	• PS backbone • Multiple CpG motifs • TCG dimer at 5' end • CpG motif imbedded in a central palindrome	• Induces B-cell and pDC proliferation and differentiation • Induces production of IL-6 and IFN-α
P type	<u>TCGTCGACGATCGGCGCGCGCCG</u> ODN 21798 (for human and mouse)	• PS backbone • Two palindromes • Multiple CpG motifs	• Stimulates pDC and B cells • Strong IFN-α secretion

Bold letters in ODN sequences indicate self-complementary palindromes; CpG motifs were underlined. PO: phosphodiester backbone; PS: phosphorothioate backbone

1.4.2.1 A/D-type CpG ODN

Class A, also known as type D CpG ODN is made up of mainly phosphodiester backbone, palindromic CpG motifs at the center and poly(G) sequences on phosphorothioate backbone at the 3' and 5' ends (Krug *et al.*, 2001a). D-ODNs can spontaneously form, duplex or G-quadruplex globular structures because of the palindromic sequences in the center and poly(G) sequences at the ends respectively, that

would efficiently protect these sequences from the endonucleases (Figure 1.9). The interaction between guanosines to form quadruplex is known as “Hoogsteen Base Pairing”. The minimum length of an active D-ODN is 18 bp and size of the higher order structures of D-ODNs varies from 50 nm to 100 nm (Klein *et al.*, 2010).

Mixed backbone structure of D-ODNs mediate the aggregation of these sequences, resulting an extended retention time in early endosome, which leads to the prolonged activation of MyD88-IRF7 complexes and finally promoting strong IFN- α and β production from pDCs. A scavenger receptor, CXCL-16 which is highly expressed on pDCs –but not on B cells, essential for the D-ODN activation to produce strong IFN- α . D-type ODNs activate indirectly IFN- γ and IP-10 production from PBMCs and NK cells as well (Gursel *et al.*, 2006; Kerkmann *et al.*, 2005). They also upregulate the costimulatory molecules such as, CD80, CD86 and HLA-DR on pDCs and B cells. Monocytes are stimulated and matured into CD83⁺/CD86⁺ dendritic cells upon D-ODN encounter (Gursel *et al.*, 2002). NK cell-mediated cytolytic activity, stimulation of splenocytes to produce IL-6, IL-12 and IFN- γ are also strongly initiated by this type of CpG ODN (Krug *et al.*, 2001a). However, having batch-to-batch variations and because of the formation of secondary structures leading to the non-specific binding, effect the therapeutic potential of this type of CpG ODN negatively. But recent studies showed that using D-ODNs in virus-like particles as an adjuvant, have demonstrated strong immunotherapeutic effects against diverse types of diseases such as allergic rhinoconjunctivitis or melanoma patients (Klimek *et al.*, 2011; Speiser *et al.*, 2010; Wang *et al.*, 2009a).

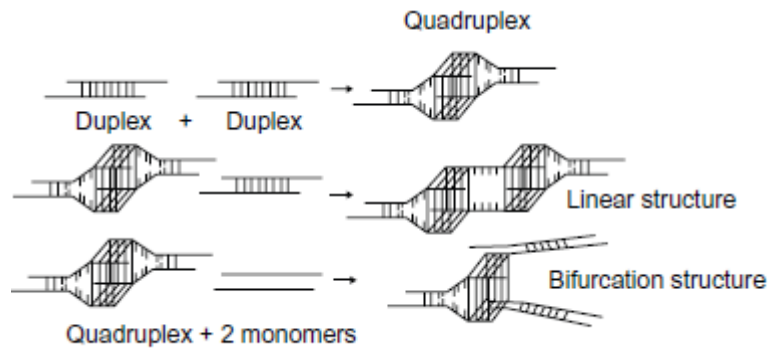


Figure 1.9. Spontaneous higher-order G-quadruplex formation of D-type CpG ODNs (*Adapted from Klein et al., 2010*).

1.4.2.2 B/K-type CpG ODN

B CpG ODN also known as K-ODN contains at least one CpG motif on the full phosphorothioate backbone, two-four CpG motifs and at least 12 bases makes the sequence optimal. Depending on the location of the CpG motif the immune activity of this ODN differs in both mice and humans. The sequence (i.e. ODN 2006) is highly active when CpG motif is located at least one more base upstream or closer to the 5' end of the K-ODN, and having thymidine at the end of the 5' as well in humans (Verthelyi *et al.*, 2001). In mice the closeness of CpG motifs into the 5' didn't affect the activity of K-ODN (ODN 1555, ODN 1826). Unlike D-ODNs, K-ODN didn't form any globular structures and remained single stranded when given *in vivo* therefore weakly induce IFN- α production but robust IL-6 and IgM secretion from B-cells, by inducing these cells proliferation (Krug *et al.*, 2001b). K-ODNs, *i*) activate CD19⁺ B-cells by inducing CD69 and CD25 (early and late activation markers) gene expression, also *ii*) highly effective for the pDC maturation by synergizing with GM-CSF, MHC II, CD40 and CD83. Splenocytes and PBMCs are highly responsive to K-ODNs as evidenced by the production of IP-10, IFN- γ , IL-10 and elevated amounts of IL-6, IL-12 (Gray *et al.*, 2007; Verthelyi *et al.*, 2001); (Hartmann and Krieg, 2000).

K-ODN have been studied as a very effective vaccine adjuvant in the formulations including live or attenuated viruses, peptide/protein antigens, dendritic cell vaccines and polysaccharide conjugates from various routes of administration, such as injection, inhalation and oral administration (Klinman *et al.*, 2004). K-ODN in combination with alum, against hepatitis B virus showed improved vaccine responses. Also mixing K-ODN with a fraction of ragweed allergen proved to be an effective immunotherapeutic agent (Fonseca and Kline, 2009).

1.4.2.3 C-type CpG ODN

C-ODNs are the mixture of D and K-ODNs by being composed of full phosphorothioate backbone, but having 12 to 16 bp palindromic CpG motifs at 3'-end. The activity of this ODN is highly dependent on the ODN length (optimal length 22 bp), the base content, having poly-T at the 3' end and TCG at 5'end (Marshall *et al.*, 2003). The IFN- α production capability is decreased when TCG motif moved towards 3'-end. Having palindromic sequences, that allow the duplex formation in endosomes are also effective for the immunostimulatory property of C type ODN in the context of type I IFN production (Fig. 1.10) (Hartmann *et al.*, 2003).

As indicated above C-ODN combined the immunostimulatory activities of two previously mentioned CpG ODN types. They could directly stimulate human B cells, lead to the production of IFN- α from pDCs –lower than D-ODNs but higher than K-ODNs, induce high levels of IP-10, IFN- γ from PBMC and upregulate NK cell activation markers more efficiently than K-ODNs (Poeck *et al.*, 2004). Basically they are the intermediate CpG ODN sequences by means of their structure and immunological properties (Libri *et al.*, 2009).



Figure 1.10. Duplex formation via palindromic sequences by two phosphorothioate backbone C ODNs (*Adapted from Krieg, 2006*).

1.4.2.4 P-type CpG ODN

Class P CpG ODNs consist of two palindromic motifs on phosphorothioate backbone which enables them to form multimeric units, where each molecule is bound via Watson–Crick basepairing. P-ODNs are less effective on B cell activation but improved pDC stimulation therefore induce greater IFN- α production when compared with C-ODN. They induce greater IL-6 and IP-10 production when given *in vivo*. Even in monomeric or dimeric forms, they retain the strong and effective immune activities (Samulowitz *et al.*, 2010).

1.4.2.5 Y-shaped CpG ODN

Dendrimer-like CpG ODN is designed with phosphodiester backbone, having three linear ODNs with the halves of each ODN being complementary to the half of the other two sequences. The special structure of 30 bp Y-shaped ODN increases the efficiency of uptake into the cells, but less stable in serum. Incubation of mouse macrophage cell line RAW 264.7 cells with Y-shaped ODN resulted in a significant production of TNF- α and IL-6 compared to conventional ssDNA and dsDNA sequences (Fig 1.11) (Nishikawa *et al.*, 2008).

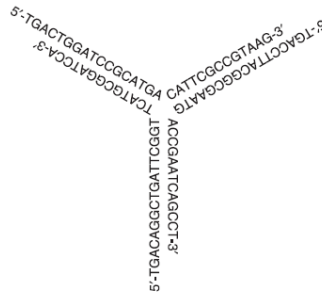


Figure 1.11.. Representative structure of Y-shaped CpG ODN (*Adapted from Nishikawa et al., 2008*).

1.4.2.6 X-shaped CpG ODN

X-shaped ODNs are prepared from four ODN sequences, each one of them containing six potent CpG motifs (Figure 1.12). This type of novel CpG DNA was more effective than its components by inducing TNF- α production from RAW 264.7 cells and leading to the maturation of murine DCs as well. At least 8 hrs serum stable X-shaped ODN also possess antitumoral properties by inhibiting the proliferation of colon26/Luc cells when injected into the mice, in hydrogels (Nishikawa *et al.*, 2011).



Figure 1.12. Structure of X-shaped CpG ODN (*Adapted from Nishikawa et al., 2011*).

1.4.2.7 Dendrimeric 70-mer ODN

Our lab identified a novel phosphodiester and phosphorothioate backbone modified 70-mer dendrimeric ODN, having 7 times repeated 1466-ODN sequence which are linked via two symmetric bifunctional linkers, known as ODN420. GpC containing control ODN named as ODN421. Unlike Linear 7x-1466 CpG ODN, ODN420 are uniform-sized nanoparticles with an average size of 35 ± 10 nm. The nanoparticulate form of CpG ODN, both induce its resistance to endonucleases, therefore resulting an effective immune response as evidenced by works performed by Mammadov and Karatepe.

Novel dendrimeric structure results to a higher immunostimulatory potential compared to conventional ODNs. *In vivo* ODN administration into mice indicated that nanoparticle-forming CpG ODNs (NP-ODN) initiated substantially high by IFN- γ and IL-12 production from splenocytes and PECs. At least one type of scavenger receptor is important for the internalization of NP-ODN. In addition, it combines advanced properties of conventional classes of D and K-ODNs such as activating mice macrophages, inducing DC maturation, B cell proliferation as well as activating human PBMCs. Type I interferon, TNF- α , IFN- γ , IL-6 and various chemokines were up regulated as evidenced by ELISA, flow cytometer and PCR studies. Our group also studied the adjuvant potential of ODN420 against model antigen ovalbumin. Results demonstrated that ODN420 induced higher Th1 biased response compared to conventional CpG ODN sequences (Karatepe, 2009; Mammadov, 2009).

1.5 Immune System and Cancer

Acute inflammation contribute to the development of protective adaptive immune response to microbes and cancer (Grivennikov *et al.*, 2010). In contrast, chronic inflammations contribute to the different stages of tumorigenesis including intitation, growth and metastasis. Hepatitis B or C virus infections enhance the risk of hepatocellular carcinoma, *Schistosoma* flatworm or *Bacteroides* bacteria infections are associated with bladder and colon cancer, continuous *Helicobacter pylori* infection was associated with gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma (Karin, 2006; Nair *et al.*, 2011; Shibata *et al.*, 2010; Wong *et al.*, 2009). Carcinogens and particulate materials from tobacco smoke participate to the chronic pulmonary diseases which might lead to the higher lung cancer risk (Takahashi *et al.*, 2010).

Solid masses induce inflammatory responses that create the tumorigenic microenvironment. Both cell proliferation and expression of certain oncogenes from RAS and MYC families lead to the modeling of tumor microenvironment via leukocytes that produce tumor-promoting chemokines and cytokines. But in some point, oxygen and nutrient deprived solid malignancies, resulting the release of the DAMPs such as, high mobility group B1 (HMGB1), adenosine triphosphate (ATP), as well as proinflammatory cytokines IL-1 β and IL-17A (Kortylewski *et al.*, 2009; Vakkila and Lotze, 2004).

DAMP molecule, non-histone protein HMGB1 is secreted passively and actively by necrotic and immune cells respectively. It is highly expressed in melanoma, prostate, breast and colon cancers. HMGB1, i) stimulates neutrophils or monocytes to produces proinflammatory cytokines and chemokines ii) also activates endothelial cells inducing the angiogenesis in the tumor environment iii) provokes IFN- γ producing tumor specific

T cells by cross-presentation of tumor antigens. This protein is recognized by TLR2 and 4, also by receptor for advanced glycation end products (RAGE) receptors. Also could form complex with DNA and bind to the TLR9 (Dumitriu *et al.*, 2005; Fiuza *et al.*, 2003; Maeda *et al.*, 2007; Mitola *et al.*, 2006).

ATP is released upon dying cells after chemotherapeutic application and sensed as danger signal by DCs that activates P2RX7 receptors on these cells. ATP activates Nalp3/ASC/caspase-1 multimeric inflammasome complexes, causing the secretion of IL-1 β which leads to the IFN- γ producing CD8⁺ divergence (Stagg and Smyth, 2010).

IL-17A is predominantly produced by Th17 cells, NK T cells and $\gamma\delta$ T cells, which contribute to the induction of TNF- α , IL-1 β , IL-6, chemokines and granulocyte colony stimulating factors also to the activation of neutrophils (Xu and Cao, 2010). Th17 cells were shown to be more successful than Th1 cells in the retention of tumor growth by increasing the proliferation of CD8⁺ T cells. IL-17^{-/-} mice were more susceptible to lung metastasis and colon adenocarcinoma in contrast to wild-type mice (Kryczek *et al.*, 2009; Muranski *et al.*, 2008). Having all negative effects on tumor growth, IL-17 could promote tumor growth by inducing angiogenic mediators such as VEGF, IL-6 and IL-8 (Numasaki *et al.*, 2003).

Like IL-17A, IL-1 β is required for the polarization of IFN- γ producing CD8⁺ T cells, as well as inducing IL-17 production and the generation of anti-tumor $\gamma\delta$ T cells. However, enormous IL-1 β production has been involved in chronic inflammatory diseases and malignancies such as, various lung and skin cancers, fibrocarcinomas and melanoma tumors (Carmi *et al.*, 2011; Dinarello, 2009).

The tumor microenvironment consists of mast cells, neutrophils, natural killer cells, macrophages, DCs, myeloid-derived suppressor cells, T and B cells certainly with cancer cells and stromal cells, such as fibroblasts, endothelial cells and mesenchymal

cells (de Visser *et al.*, 2006) (Figure 1.13). The interaction between these cells in accordance with cytokine and chemokines that act both as an autocrine and paracrine manners, coordinate the tumor environment. Tumor-associated macrophages (TAMs) and CD8⁺, IFN- γ producing, Th2 and Th17 T cells were the most commonly occurred immune cells within the tumor microenvironment. TAMs, promote tumor growth by angiogenesis, invasion and metastasis however T cells could instruct both tumor-promoting and suppressive responses (Murdoch *et al.*, 2008). Divided into two main subtypes M1 and M2 TAMs were analogous to Th1 and Th2 T cells. M1 macrophages are activated by IFN- γ and produce robust proinflammatory cytokines such as, TNF- α , IL-1 β , IL-6, IL-12 and IL-23 and MHC molecules and inducible nitric oxide synthase (iNOS) therefore able to dismiss pathogens and initiating antitumor immune response (Sica *et al.*, 2008). But, M2 TAMs, which are stimulated with Th2 cytokines (IL-4, IL-10 and IL-13) down-regulate MHC class II and IL-12 expression and induce anti-inflammatory IL-10 cytokine (Allavena *et al.*, 2008). Neutrophils also exert both tumor-promoting and tumoricidal functions depending on their differentiation and the existence of TGF- β (Fridlender *et al.*, 2009). DCs are the key components for the tumor specific antigen presentation, responding to the stress and danger signals, production of immunostimulatory and cytotoxic molecules such as, type I IFNs, IFN- γ , GM-CSF, IL-12, FASL and the activation of adaptive anti-tumor immune response and in contrast, suppression of immune response in conventional tumors (Palucka *et al.*, 2007).

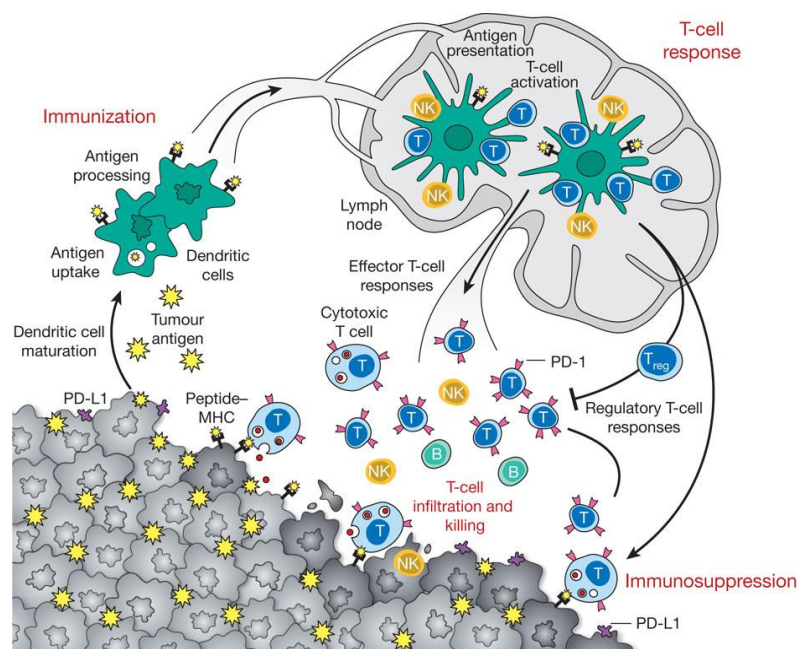


Figure 1.13. Generation and regulation of antitumoral immune response (*Adapted from Mellman et al., 2011*).

1.6 Drug Delivery with Nanoparticles

Delivering therapeutic agents in most optimized platforms have becoming more important and popular each day. Having safe and targeted drug delivery could improve the performance of the medicines in the market and contribute to the development and success of novel, effective therapeutic strategies for the delivery of antigens, adjuvants, anticancer drugs and gene therapy (Orive *et al.*, 2003).

Majority of the drugs are limited by their, high toxicity, poor solubility, aggregation, high toxicity, nonspecific targeting and delivery, *in vivo* instability and having a short half-lives, therefore current advances in biotechnology field are assisting the discovery, design and the improvement of nanoscale drug delivery systems (Sahoo *et al.*, 2007). Nanoparticles (NPs), nanocapsules, dendrimers, micellar systems, liposomes and other

carrier systems shape the kinetics, distribution and drug release of an associated drug (Okada, 2011).

One of the main advantage that nanotechnology offer is targeted drug delivery to the site of inflammation, which could be performed either via passive targeting of drugs to the site of interest or by active targeting of the drug. Targeted drug-delivery systems transmit effective molecules more efficiently and easily resulting the increase in patient fulfillment, lengthen product life cycle and decrease healthcare costs (Moghimi *et al.*, 2005; Torchilin, 2000).

Passive targeting takes advantage of the molecular differences between normal and transformed tissues to deliver the appropriate drugs to the necessary site. Enhanced permeability and retention (EPR) is the main physiological alteration achieved by this type of targeting (Maeda *et al.*, 2009). Sterically stabilized PEGylated liposome encapsulating doxorubicin, Doxil™, was approved for the treatment of metastatic breast cancer, ovarian cancer, and Kaposi's sarcoma. Cisplatin-loaded glycol chitosan nanoparticles are also effective against tumor growth, by having prolonged circulation and robust EPR effect in tumor-xenografted mice (Gullotti and Yeo, 2009). NP ODNs could also be delivered into the local cancers, such as prostate, head and neck cancers by injection. Transferrin conjugated paclitaxel-loaded biodegradable NPs were successful by having substantial antiproliferative effect in addition to the complete tumor regression in mice because of their greater cellular uptake and persistent intracellular retention abilities (Sahoo *et al.*, 2004) (Sahoo and Labhasetwar, 2005).

Active targeting is described as the combination of receptor specific ligands which could lead the NPs directly to the specific site. To be successful at this type of drug delivery and targeting system, the affinity, specificity of the targeted moiety and the chemical modification should be well studied. Cancer cells were targeted with poly(lactic-co-glycolic acid) (PLGA) NPs which contain surface-modified with specific monoclonal

antibodies, therefore lead to the enhanced binding of NPs to the cancer cells and resulting more pronounced antitumoral effect (Kocbek *et al.*, 2007). PEGylated gold NPs, were modified with human transferrin (Tf) strongly induce active targeting by having greater intracellular delivery of therapeutic agents into cancer cells in contrast to their nonmodified analogs (Choi *et al.*, 2010).

10-1000 nm sized nanoparticles are formulated from very wide range of biodegradable materials with very novel chemical structures in order to provide the most effective depot system against the related disease. Loading of the drugs could be performed by the encapsulation, surface attachment or entrapment. Because of their small sizes NPs efficiently pass through the barriers into target site with the accurate drug accumulation. This property, reduces the toxicity and unwanted side effects of the therapeutic agent and furthermore, improved its therapeutic effect (Parveen *et al.*, 2012).

Both biodegradable and biocompatible polymeric NPs are one of the most optimized drug delivery systems having high potential for surface and ligand modifications (Lu *et al.*, 2011). NPs are divided into two depending on their preparation process as, nanospheres and nanocapsules. Nanospheres are made up of matrix-like structure allowing the convenient entrapment or binding of target molecules, whereas nanocapsules contain polymeric outer and inner core (Figure 1.14) (Vrignaud *et al.*, 2011). As mentioned above PLGA and polylactide (PLA) have been studied for the persistent drug delivery. PLGA nanoparticles are effectively capable of delivering both chemotherapeutic agents and chemosensitizers, such as verapamil for the effective treatment of drug-resistant cancers *in vivo* (Song *et al.*, 2009). Encapsulation of PLGA with TLR agonists, TLR3L: pIC, TLR4L: LPS, TLR9L: CpG ODN and bacterial or viral antigens also lead to the more pronounced innate and adaptive immune responses as evidenced by the DC maturation, induced proliferation of CD8⁺ and CD4⁺ T cells, and production of cytokines, such as IFN- γ (Hamdy *et al.*, 2008; Lee *et al.*, 2010; Wischke *et al.*, 2009). Modification of PLGA with trimethylated chitosan direct this NP as drug

carrier for brain delivery. Adding polyethylamine to the surface of PLGA aid siRNA delivery (Wang *et al.*, 2010b).

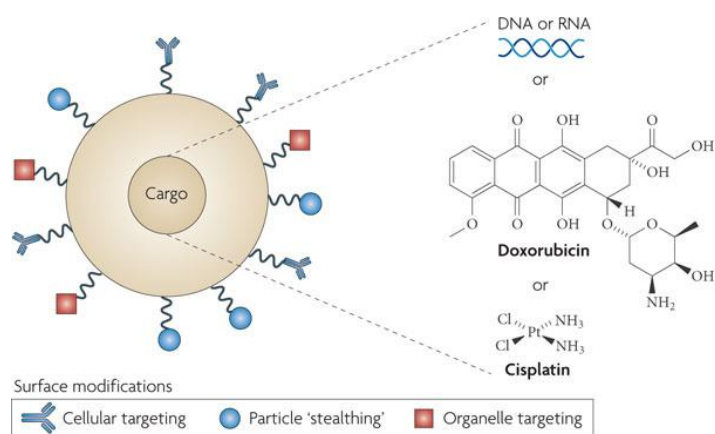


Figure 1.14. Schematic representation of an engineered nanoparticle (*Adapted from Petros et al. 2010*).

Solid-lipid nanoparticles (SLN) are designed from solid lipids including mono– di– tri– glycerides, fatty acids and cholesterol (Wissing *et al.*, 2004). The advantages of SLNs over polymeric NPs are, they could easily incorporate with lipophilic and hydrophilic drugs, biodegradable, nontoxic, easily dissolved in the water, large scale production. However they are not as stable as the polymeric nanoparticle systems *in vivo* (Pathak *et al.*, 2011). PEGylated stealth SLN nanoparticles enhance the tumor accumulation as well as promote antibacterial activity of antiparasitic and antifungal drugs (Uner *et al.*, 2004).

Well studied liposome nanoparticles are composed of both hydrophilic and hydrophobic lipid bilayers therefore spontaneously form closed vesicles in water. Types of liposomes are classified depending on their size (100 nm–5µm) and lipid bilayers as, i) large multilamellar vesicles (LMV), ii) small unilamellar vesicles (SUV), and iii) the large unilamellar vesicles (LUV). In order to increase the stability of liposomes in circulation, surface modification with PEG was tested (Torchilin, 2005). Liposomes carrying antibodies or antibody fragments are called, ‘immunoliposomes’ and able to migrate to

the area in the body, where immunoliposome recognizes its appropriate antigen in. This type of liposome is effectively used in cancer immunotherapy. Using liposomes are advantageous in many ways including, they do not accumulate in the tissues, therefore lowering the side effects of encapsulated drugs, could be easily modified, could entrap water-soluble molecules. Disadvantages will be listed as, having short shelf life (not more than 2 years), being not so much stable, organic compounds such as chloroform and ethanol were used during the preparation of liposomes (Mozafari, 2005). Encapsulation of CpG ODN with various antigens for instance OVA or AVA into sterically stabilized cationic and polylactide-co-glycolide cationic liposomes respectively, have been shown to improve the Ag specific IFN- γ production as well as anti-IgG2a antibody production against specific Ag (Erikci *et al.*, 2011). Another TLR ligand pIC is also enclosed with cationic liposomes leading to robust type I IFN, IFN- γ production and highly effective on CD4⁺ and CD8⁺ T cell responses *in vivo*. In addition, recently it has been showed that DOTAP cationic liposome nanocomplexed with pIC boosted antitumor immunity by enhancing IRF3 dependent signaling and type I interferons in dendritic cells (Wang *et al.*, 2012).

Natural polymers, polysaccharides are amphiphilic, highly stable, safe, non toxic, low-cost, biodegradable and found in abundant resources in nature. They could be extracted from algae (alginate), plants (starch), microbes (dextran), fungi (β -glucan) and animals (chitosan). Polysaccharides are divided into various groups depending on their chemical structures. Widely used chitosan-based nanoparticles offer many advantages due to simple preparation, low toxicity, high stability, ensuring various routes of administration from intravenous to orally. Chitosan-DNA nanoparticles could modulate anti-allergic responses as shown by preventing anaphylactic shock, atopic dermatitis and asthma (Fujii *et al.*, 2011; Wang *et al.*, 2009b). They are also used as siRNA delivery agents, having high transfection efficiency as lipofectamine. Uptake of chitosan, activate macrophages therefore it could be effectively used as vaccine carrier. Cationic chitosan NP-based DNA flu vaccine showed elevated IgA antibody level in mice when given via

intranasal route (Illum *et al.*, 2001). Chemically modified chitosan NPs which are separately encapsulated with TLR2, 7 and 9 agonists, activate macrophages, induce higher IgG2a, secretory IgA levels and enhance CD8⁺ T cell dependent IFN- γ production (Arias *et al.*, 2012).

β -glucans are used as delivery systems as well. They have some pharmacological effects against infection, cancer and also contribute to the wound healing and cholesterol reduction (Tsoni and Brown, 2008).

β -(1 $\rightarrow$ 3)-D-glucan schizophyllan (SPG) polysaccharide extracted from a fungi, *Schizophyllum commune*. SPG was modified with peptides and cholesterol, complexed with CpG ODN boosted production of IL-6 and IL-12 from macrophages. Furthermore, antisense ODN-SPG complexation was recently shown to have immunotherapeutic role against inflammatory bowel disease (Shimada *et al.*, 2006; Takedatsu *et al.*, 2012; Wang *et al.*, 2009b). In 2011, Tincer *et al.* showed β -glucan polysaccharide extracted from *Ganoderma lucidum* stably nanocomplexed with TLR3L pIC, leading to a production of higher amounts of TNF- α , IL-6, IFN- γ and bacteriocidal nitric oxide (NO). Nanocomplexes also induced anti-OVA IgG antibody response from C57BL/6 mice compared to pIC and polysaccharide alone groups. This type of natural occurring PS forms also complexes with TLR7/8 and TLR9 agonists to improve immune response (Tincer *et al.*, 2011).

1.7 Immunotherapeutic Applications of PRR Ligands

Development of new vaccines against cancer, viral, bacterial infections and autoimmune diseases are shifting from traditional vaccines into more specialized, defined and safer ones. Generation of novel adjuvants aim to target innate immune system components such as PRRs on APCs, improve immunogenicity of co-administered antigens therefore strengthen up the regulation of humoral and cellular responses (Lam, 2007). Using

PAMPS to trigger, TLRs, NLRs, and other pattern recognition receptors in the formulations of, vaccines and antitumor agents, strongly induce the production of pro-inflammatory cytokines/chemokines and type I IFNs that boost the host's ability to abolish the pathogen (Olive, 2012). Vaccine adjuvants are classified into another group including, microparticles, liposomes, emulsions and saponins particulate materials, which are shown to regulate antigen persistency and antigen uptake by DCs, therefore also defined as delivery systems (Bachmann and Jennings, 2010). Combining TLR ligands, with particulate adjuvants are proven to induce the immunogenic properties of the proper vaccine (Steinhagen *et al.*, 2011). Table 1.6 shows components and results of immune response of various vaccine adjuvants.

Table 1.6. Immune response outcomes and components of approved and under clinical trial adjuvants (*Adapted from Olive, 2012*).

Adjuvant	Components	Immune response outcomes
Licensed vaccine adjuvants		
Alum	Aluminum salts	Ab, Th2
MF59	Oil-in-water emulsion	Ab, Th1/Th2, long-lived memory
AS03	Oil-in-water emulsion	Ab, B cell memory, Th
AS04	Alum-absorbed MPL	Ab, Th1
Virosomes	Liposome, neuraminidase and hemagglutinin	Ab, Th1
Vaccine adjuvants in clinical development		
Poly(I:C)	Synthetic dsRNA, TLR3 agonist	Ab, Th1, CD8+ T cells
Flagellin	FL-antigen fusion proteins, TLR5 agonist	Ab Th1/Th2, CD8+ T cells
Imidazoquinolines	Small molecules, TLR7 and TLR8 agonists	Ab, Th1, CD8+ T cells
CpG 7909, CpG 1018	CpG ODNs alone or combined with alum/emulsion, TLR9 agonists	Ab, Th1
AS01	Liposome, MPL and saponin (QS-21)	Ab, Th1
AS02	Oil-in-water emulsion, MPL and saponin	Ab, Th1
IC31	Synthetic peptide KLK and ODN	Ab, Th1, long-lived memory
CAFO1	Cationic liposomes (DDA and TDB)	Ab, Th1
ISCOMS/ISCOMATRIX	Saponin-based particulate	Ab, broad Th, CD8+ T cells

1.7.1 Anticancer Applications

Synthetic TLR3 agonist, pIC, directly triggers cancer cell apoptosis through the activation of intrinsic and mitochondrial apoptotic pathways with the cooperation of other two RNA recognizing PRRs, RIG-I and MDA5 (Basith *et al.*, 2011). TLR3, is expressed in melanoma cells therefore effectively induce proapoptotic and antiproliferative signaling and DC maturation upon pIC treatment. Complexation of pIC with liposome decreased the occurrence of metastatic liver cancer in mice. Peptide from the HPV16 E7 protein plus pIC boosted specific peptide cytotoxic T cell responses that resulted considerable regression of cervical cancer tumors pre-established in mice. Upon pIC exposure, proliferation of breast cancer cells, urinary tract tumors, prostate cancer and hematopoietic tumors were effectively diminished by the activation of apoptotic pathways, antitumoral T cell and NK cell activation (Cheng and Xu, 2011; Peng *et al.*, 2009; Salaun *et al.*, 2007; Wu and Lanier, 2003).

Clinically first and only approved TLR7/8 agonist, imiquimod was used for topical administration to treat human papilloma virus (HPV) derived warts, basal cell carcinoma and solar keratosis where it improved Ag-specific Ab and CD4⁺ T cell responses in patients (Hagman *et al.*, 2003; Terlou *et al.*, 2010). Imiquimod in combination with various melanoma peptides and Flt3 ligand enhanced peptide-specific CD8⁺ T cell responses in treated patients. TLR7/8 ligand was more effective adjuvants than GM-CSF and multi-peptide vaccine against prostate cancer as proven with the phase I/II clinical trials (Bong *et al.*, 2002; Kreiter *et al.*, 2011; Morse *et al.*, 2011).

Immunotherapeutic applications of TLR9 ligand, CpG ODNs were generally tested in highly immunogenic melanoma and other skin cancers. K-type CpG ODN, CPG 7909 was applied in intradermal and subcutaneous routes into melanoma patients, resulting release of inflammatory cytokines, induced tumor-specific CD8⁺ T cells, pDCs, NK

cells and declined regulatory T cells of these patients (Bode *et al.*, 2011). Phase I/II trials of this CpG ODN were performed on renal cell carcinoma, glioblastoma, non-Hodgkin's lymphoma patients concluding promising optimistic results. Non-Hodgkin's lymphoma patients were administered with CpG-K 1018 in combination of chemotherapeutic agent rituximab, showed relapsed/refractory follicular lymphoma or at least indication of immune response, such as infiltration of CTL and macrophages into tumor area (Leonard *et al.*, 2007; Link *et al.*, 2006). Phase I/II clinical trials on breast cancer, ovarian cancer, sarcoma and non-small-cell lung cancer patients of TLR9 agonists in combination with specific chemotherapeutic agents were still undergoing by various companies and sponsors. Diverse studies with nanoparticle encapsulated CpG ODNs performed against cancer cells. Combining, PS backbone modified K-type CpG ODN 1668 with liposomes and C-dextran lead to the enhancement of CD8⁺, CD4⁺ T cell infiltration, NK cell activation, TAM specific anti-tumor activities as well as suppressed the pro-tumor functions in RIP1-Tag5-transgenic mice and hepatoma murine model respectively (Hamzah *et al.*, 2009; Huang *et al.*, 2012). Cationized gelatin based nanoparticle-delivered CpG ODN 1826, initiated immune stimulation in the lymph nodes by APC and T cell activation in thymoma model in mice (Bourquin *et al.*, 2008).

Alum and TLR4 ligand monophosphoryl lipid A (MPLA), combined with other peptides, proteins were tested in the melanoma and non-small cell lung cancer patients. That formulation resulted enhanced survival rates and boosted immune response. MPLA also used in human papilloma virus (HPV) vaccine Cervarix™ which targets HPV types 16 and 18, which were the main cause of 70% of the cervical cancer (Garcon *et al.*, 2007; Giannini *et al.*, 2006; Romanowski *et al.*, 2009).

β-glucan purified from mutated *S. cerevisiae* inhibited metastasis of colon carcinoma which was related with the tumoricidal activity of peritoneal macrophages, NK cell cytotoxicity and prolonged the survival time of tumor-bearing mice (Yoon *et al.*, 2008). When administered with chemotherapy and radiotherapy, lentinan β-glucan improved

the survival rate of hepatocellular carcinoma patients also lowered tumor recurrence rates (Yang *et al.*, 2008).

1.7.2 Antibacterial and Antiviral Applications

TLR2 agonist Pam3Cys linked to outer surface protein A (OspA) of *B. burgdorferi* and approved as vaccine against tick-borne lyme disease by FDA (Nigrovic and Thompson, 2007; Steere *et al.*, 1998). Pam3Cys in combination with peptide vaccine containing *Plasmodium falciparum* circumsporozoite protein, also tested as an adjuvant against malaria and resulted enhanced IgG Abs in subjected volunteers (Nardin *et al.*, 2000). Another agonist palmitic acid was combined with peptides and proteins to target hepatitis B virus (HBV) and human immunodeficiency virus (HIV) in phase I/II trials showed induced and persistent CTL response against the virus (Durier *et al.*, 2006; Livingston *et al.*, 1999).

Intranasal administration of pIC plus haemagglutinin-modified recombinant split-H5N1 vaccine boosted antigen-specific mucosal IgA production and systemic humoral responses in an influenza infection mouse model. Following challenge resulted high number of protection from lethal H5N1 infection (Asahi-Ozaki *et al.*, 2006; Ichinohe *et al.*, 2007). Mismatched poly I:poly C dsRNA (polyI-polyC12U) was in Phase II clinical trials for the HIV treatment and severe acute respiratory syndrome (Stevceva, 2011). These adjuvants were studied as an adjuvant against *Neisseria meningitides*, *Mycobacterium tuberculosis* and *Plasmodium falciparum* in mouse models. Having similar safety profile as Alum, inducing neither renal nor hepatic toxicity AS04 (MPLA and Alum combination) with hepatitis B vaccine (FENDrix[®]) was tested on immunocompromised patients and stimulated higher seroprotection rates and antibody titers than the licensed HBV vaccine (Boland *et al.*, 2004; Kundi, 2007).

Intramuscular and subcutaneous delivery of TLR7/8 agonist imidazoquinolines could improve both quality and the amount of antigen specific T cell and antibody responses. Shown in non-human primates HIV-1 gag protein combined with TLR7/8 agonists induced anti-HIV IgG levels and CD8⁺ T cell responses (Wille-Reece *et al.*, 2006; Wille-Reece *et al.*, 2005). Herpes simplex virus-2 glycoprotein vaccine plus imiquimod increased antibody production up to 10-fold and attenuated lesion recurrences in guinea pig model (Harrison *et al.*, 2001).

Synthetic TLR9 ligand can provide temporary protection against a wide range of viral, bacterial and parasitic pathogens, such as *Bacillus anthracis*, *Listeria monocytogenes*, vaccinia virus, *Francisella tularensis*, Hepatitis B. Administration of K-type CpG ODN through multiple routes such as oral administration, injection and inhalation showed protection against pathogens (Krieg, 2012; Vollmer and Krieg, 2009). Phase I clinical trial of, C-class CpG ODN 10101 plus pegylated IFN and ribavirin against hepatitis C virus improved diverse Th1 and CTL response against the virus and the infection was cleared ≈20% of the patients (Houghton, 2010). Like anticancer applications, B/K type CpG ODN have been shown the most studied vaccine adjuvant for improving Th1 biased immune response when combined with antigens and formulations including live or killed viruses, peptide or protein antigens, autologous cellular vaccines, dendritic cell vaccines and polysaccharide conjugates. A/D type CpG ODNs with virus like particles were used as adjuvants as well as C type CpG ODNs in murine and primates (Jennings and Bachmann, 2008).

CpG 7909-adjuvanted hepatitis B surface antigen (HBsAg) containing Hepsilav, increased antibody response up to 10-fold in concordance with an earlier and higher avidity antibody responses in phase III trials, hopefully could become the first approved CpG-including vaccine (Halperin *et al.*, 2006; Siegrist *et al.*, 2004). Phase I trial of CpG 7909, accelerated detectable robust IgG antibody production against *Bacillus anthracis* was observed. In mice and rhesus macaque pre-clinical trials, administration of

PLG/CpG 7909 nanoparticles enhanced IgG anti-A responses up to 100 fold compared to control groups (Xie *et al.*, 2005).

Formulations including recombinant malarial protein vaccine or merozoite surface protein 1 in addition with CpG 7909 and Alhydrogel, induced the functional capacity of the antibody, generation of malaria-specific memory B cells as shown with both *in vitro* parasite growth inhibition assays and Phase I trial studies (Ellis *et al.*, 2010; Mullen *et al.*, 2008). Additional research of these and other malaria vaccine should be performed for the optimal vaccination formulations. BALB/C mice subjected with trimeric recombinant HA protein from H5N1 viruses coupled with non-emulsion adjuvant PELC, K3 CpG ODN resulted highest neutralizing antibody levels and boosted antibody responses (Lin *et al.*, 2011). Another study showed immunization of mice intranasally with, CpG ODN plus formalin-inactivated influenza virus enhanced flu-specific antibody levels in the serum and saliva (Moldoveanu *et al.*, 1998). K type CpG ODN 7909 with 1/10th of the optimum dose of commercial influenza vaccine was tested in randomized, double-blind Phase I clinical trial and refined the influenza specific IFN- γ production in PBMC, compared to full-dose vaccine. These results were promising but, should be reproduced in future studies (Cooper *et al.*, 2004).

Table 1.7 provides the clinical trials of TLR9 agonists against inflammatory diseases and cancer.

Table 1.7. CpG ODNs as vaccine adjuvants (*Adapted from Bode et al., 2011*).

Treatment	Disease	Study phase	Outcome
Infectious diseases			
CpG 7909 + Engerix B®	Hepatitis B	Phase I/II	Seroprotection achieved faster and anti-HBs Ab titers 45-fold higher after secondary immunization vs Engerix B alone. 100% seroconversion after 6 weeks (vs 63% in controls) in HIV-infected adults, seroprotection maintained 5 years
CpG 7909 + AMA1	Malaria	Phase I	5.5-fold increase in anti-AMA1 Ab titer, enhanced Ab after 236 days post immunization and decreasing the Ag dose by a quarter compared to AMA1 alone. No enhancement in the acquisition of memory B cells in semi-immune adults
CpG 7909 + PCV-7/ PPV-23	Pneumonia	Phase I/II	Twofold increase in Ag-specific IgG levels at 3, 4 and 10 months in HIV-infected adults
Cancer			
CpG 7909 + Melan A MART 1	Melanoma	Phase I	Ten fold increase in Melan A-specific CD8+ T cells (>3%)
CpG 7909 + NY-ESO-1	Melanoma, breast cancer, sarcoma, ovarian cancer	Phase I	Increased CD8+ T-cell response and prolonged survival
CpG 1018 + GM-CSF + Htert peptide	Sarcoma, glioblastoma	Phase I	CD8+ T-cell response in only one subject
CpG 7909 + low-dose radiotherapy	Lymphoma	Phase I/II	Four of 15 patients with complete or partial clinical response, induction of tumor reactive memory CD8+ T cells

Ab: Antibody; Ag: Antigen; AMA1: Apical membrane antigen 1; Engerix B: Licensed hepatitis B vaccine; GM-CSF: Granulocyte-macrophage colony-stimulating factor; hTERT: Human telomerase reverse transcriptase; MART: Melanoma antigen recognized by T cells; NY-ESO-1: New York-esophageal cancer-1; PCV-7: 7-valent pneumococcal conjugate vaccine; PPV-23: 23-valent pneumococcal polysaccharide vaccine.

β -glucan from different sources enhanced resistance and protective response to *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* in mice (Hetland *et al.*, 2000; Markova *et al.*, 2003). Sulfated β -(1-3) glucan showed potent activity against the HIV isolate in human PBMCs. *Lentinan* β -glucan also had a potent activity against HIV, with the combination of didanosine as evidenced by enhanced CD4⁺ T cell numbers in

HIV positive patients (Gordon *et al.*, 1995). SPG β -1,3-glucan and an antisense-ODN sequence complexation significantly decreased LPS-induced TNF- α secretion both in macrophages *in vitro* and in mice sera.

Aluminum hydroxide and aluminum phosphate (Alum) were used as adjuvants for human vaccines such as Hepatitis A and B, diphtheria and tetanus. Alum acts as a depot and delivery system for the antigen *in vitro* which targets DCs. Also it induces local proinflammatory reaction at the site of infection by synergizing TLR agonists to stimulate IL-1 β production. NLRP3 inflammasome complex is required for the alum adjuvant impact using ovalbumin and diphtheria toxin for IgE responses but dispensable for IgG1 and IgG2c (Eisenbarth *et al.*, 2008).

Applications of TLR agonists as immunotherapeutic agents were summarized above in Table 1.8.

Table 1.8. Prophylactic and therapeutic targeting of TLRs in clinical trials of infectious diseases. (Adapted from Hedayat et. al. 2011).

	Ligand	Implication	Compound
Hepatitis B virus			
TLR4	MPL	Vaccine adjuvant	AS04 adjuvant system + hepatitis B antigen (Fendrix; GlaxoSmithKline, London, UK)*; AS02 adjuvant system + hepatitis B antigen; RC-529 + hepatitis B antigen (Supervax; Dynavax Technologies, Berkeley, CA, USA)*
TLR9	Class B CpG	Vaccine adjuvant	1018 ISS + hepatitis B antigen (Heplisav; Dynavax Technologies); CPG 7909 + Engerix-B (GlaxoSmithKline) vaccine
Hepatitis C virus			
TLR7	Isatoribine	Antimicrobial treatment	...
TLR7	ANA975	Antimicrobial treatment	Oral prodrug of isatoribine
TLR9	Class C CpG	Antimicrobial treatment	CPG 10101 (Actilon; Coley Pharmaceutical Group, Wellesley, MA, USA)
TLR9	IMO-2125	Antimicrobial treatment	...
HPV			
TLR4	MPL	Vaccine adjuvant	AS04 adjuvant system + HPV16 and HPV18 L1 protein (Cervarix; GlaxoSmithKline)*
TLR7	Imiquimod	Antimicrobial treatment	Imiquimod 5% cream (Aldara; 3M Company, Maplewood, MN, USA)*
TLR7/8	Gardiquimod	Antimicrobial treatment	...
HIV			
TLR3	PolyI:polyC ₁₂ U	Antimicrobial treatment	Ampligen (Hemispherx Biopharma, Philadelphia, PA, USA)
TLR4	MPL	Vaccine adjuvant	3D-MPL + QS21 ± SB62 + rgp120; RC-529-SE + HIV cytotoxic T lymphocyte multiepitope vaccine
HSV			
TLR4	MPL	Vaccine adjuvant	AS04 adjuvant system + HSV2 gD subunit vaccine
TLR7/8	Resiquimod	Antimicrobial treatment	...
EBV			
TLR4	MPL	Vaccine adjuvant	AS04 adjuvant system + EBV subunit gp350
Influenza virus			
TLR5	Flagellin	Vaccine adjuvant	SFT2.HA1(SI) infl uenza vaccine (VAX125; VaxInnate, Cranbury, NJ, USA)
TLR9	Class B CpG	Vaccine adjuvant	CPG 7909 + Fluarix (GlaxoSmithKline) vaccine

<i>Molluscum contagiosum</i>			
TLR7	Imiquimod	Antimicrobial treatment	Imiquimod 5% cream (Aldara; 3M Company)
Norovirus			
TLR4	MPL	Vaccine adjuvant	AS04 adjuvant system + norovirus GI.1/GII.4 bivalent VLP vaccine
<i>Plasmodium falciparum</i>			
TLR4	MPL	Vaccine adjuvant	AS02 adjuvant system + RTS,S antigen; AS02 adjuvant system + FMP2.1 antigen; AS01 adjuvant system + RTS,S antigen
TLR9	Class B CpG	Vaccine adjuvant	CPG 7909 + AMA1-C1/Alhydrogel or MSP1(42)-C1/Alhydrogel (Brenntag Biosector, Frederikssund, Denmark)
<i>Leishmania spp.</i>			
TLR4	MPL	Vaccine adjuvant	MPL-SE + LEISH-(111F or F2)
TLR7	Imiquimod	Antimicrobial treatment	Imiquimod 5% cream (Aldara; 3M Company)
<i>Streptococcus pneumoniae</i>			
TLR9	Class B CpG	Vaccine adjuvant	CPG 7909 + seven-valent pneumococcal conjugate vaccine
<i>Bacillus anthracis</i>			
TLR9	Class B CpG	Vaccine adjuvant	CPG 7909 + BioThrax (Emergent BioSolutions, Rockville, MD, USA) vaccine (VaxImmune, Coley Pharmaceutical Group)
Severe sepsis			
TLR4	E5564 TAK-242	Antimicrobial treatment Antimicrobial treatment	Eritoran (Eisai, Tokyo, Japan) ...

1.8 Immune System in Chickens

The central role and specificity of innate immune response in controlling the adaptive immune are still being investigated in avian species as well as in chicken. The roles of T cell subtypes in the regulation of adaptive immune were being characterized. Chicken demonstrated Th1/Th2 paradigm also having CD4 T-cell subsets. Th1 cytokine responses, IFN- γ , IL-12 and IL-18 act against intracellular pathogens. Th2 cytokine responses, IL-4, IL-13 and IL-19 predominate in response to extracellular pathogens (Kaiser, 2010).

Chicken express duplicated TLR2 genes, TLR2 type 1 and type 2, that could form heterodimers with two chTLR1A and chTLR1B molecules. MALP-2 and Pam3Cys stimulations lead NF- κ B activation in HEK293 cells that express chTLR1 and chTLR2, therefore covering the functions of mammalian TLR1, 2 and 6. Orthologues of cell surface TLR4 and TLR5 were found in chickens. Chicken CD14, chMD-2 and chTLR4 co-expressions are shown to allow LPS-mediated NF- κ B activation (Cormican *et al.*, 2009; Kestra and van Putten, 2008). The chTLR5 has been shown to mediate NF- κ B activation in heterophils, upon live *Salmonella enteric* or purified flagellin incubation resulting IL-6 and the chemokine CXCLi2 production (Kogut *et al.*, 2008).

Chickens also have orthologues of intracellular TLR3 and TLR7 as well which are expressed in splenocytes, PBMCs, tonsils. Upon incubation with pIC, IFN- α , IFN- β cytokine expressions, R848 and imiquimod up-regulated chicken IL-1 β , and chicken IL-8 mRNAs in immune cells (Iqbal *et al.*, 2005; Philbin *et al.*, 2005). Chicken cells respond to synthetic CpG ODN sequences, via mammalian TLR9 homologue TLR21 (Roach *et al.*, 2005). Similar to the mammalian TLR9, chTLR21 is localized to the ER and its activation is blocked with the endosomal acidification blocking agent, bafilomycin A. Once showing the homology to the functional mammalian TLR9 (Rankin *et al.*, 2001). Novel chTLR15, expressed in chicken spleen, bursa, and bone

marrow, up regulated upon *Salmonella enterica* infection (Higgs *et al.*, 2006). Briefly, chicken immune cells are able recognize almost similar PAMPs as mammals yet using different TLRs (Table 1.9).

Other PRR family members such as NOD1, NOD2 and MDA5 are expressed in chicken cells but inflammasome multiprotein complexes were absent. Anti-microbial peptides β -defensins which could bind to the microbial cell walls, to cause cell death and act as a chemo attractant to immune cells were expressed in chicken leukocytes, epithelial cells and heterophils (Lynn *et al.*, 2007). Mammalian equivalent of neutrophils, heterophils are assigned to eliminate pathogens by the release of cathepsins, defensins and cathelocidins into phagosomes. They also release net of fibers to entrap and destroy microbes extracellularly, which act as physical barriers (Lynn *et al.*, 2007). Like other non-mammalian species chickens lack lymph nodes, therefore antigen presentation by DCs to adaptive immune system cells were poorly understood. Chicken DCs are subdivided as follicular and epidermal DCs, depending on the expression patterns as CD83⁺, ATPase⁺, MHC Class II⁺, CD45⁺ which reside mainly in the spleen and caecal tonsil tissues (Del Cacho *et al.*, 2009; Del Cacho *et al.*, 2008). The potential relations of DCs with other cells, such as NK cells, NK T cells and $\gamma\delta$ T cells were still under investigation. Various pro and anti inflammatory cytokines were identified in chickens, including 23 types of interleukins, IFN- γ , eight type I IFNs, GM-CSF, 24 different chemokines (XCL, predominantly CCL and CXCL subtypes) and 10 TNF superfamily members (including, lipopolysaccharide-induced TNF- α factor, LITAF) also, IL-1 family members, IL-11 and IL-23, IL-17 family member IL-34 and M-CSF (Garceau *et al.*, 2010; Jeurissen *et al.*, 2000; Kaiser *et al.*, 2009; Klasing, 1994).

Table 1.9. Similarities of the pattern recognition receptor between human and chicken cells (Adapted from Kaiser, 2010).

Pattern recognition receptors	Human	Chicken
Membrane-bound TLRs (signalling) recognizing cell surface PAMPs	TLR1/6/10	TLR1LA and TLR1LB
	TLR2	TLR2A and TLR2B
	TLR4	Present
	TLR5	Present
	TLR11	Absent
		TLR15 (predicted ^y)
TLRs (signalling) recognizing pathogen nucleic acid	TLR3	Present
	TLR7	Present
	TLR8	Pseudogene
	TLR9	TLR9 absent; CpG recognized by TLR21
Endocytic	Mannose receptor	Present
	Glucan receptors	Present
	Scavenger receptors	Present
Cytoplasmic		
NLRs		
NODs	NOD1	Present
	NOD2	Absent
NALPs	14 genes	All absent
RNA helicases	RIG-I	Absent, but present in duck
	MDA5	Present
	LPG2	Absent

1.8.1 Immunotherapeutic Effects of CpG ODNs in Chicken

Sudden death syndrome (SDS) and infectious mortalities during the first week of life in broiler chickens are serious problems for the broiler industry with an incidence of 1.5–2% of the population (Hopkinson *et al.*, 1984; Nain *et al.*, 2007; Steele and Edgar, 1982).

Treatment with antibiotics *in ovo* or including in the drinking water, physical feed restriction and/or the use of low nutrient dense diet starting as early as 2 days of age to

avoid SDS are becoming increasingly unfavorable (Rotter *et al.*, 1985; Steele *et al.*, 1982).

In vitro studies revealed promising results for the usage of CpG ODN as a protective agent. Avian macrophage cell line HD11 when incubated with K-type CpG ODN 2006 showed robust IL-6 and nitric oxide production as well as increased intracellular killing of *S. enteritidis* (He *et al.*, 2003).

Pretreatment of 2-day old chicks with 10µg and 50µg CpG ODN via subcutaneous route following challenge with *E.coli* resulted considerable reduction in mortality, in contrast to 90% death rate in control group within 7 days after infection (Gomis *et al.*, 2004).

The route of administration of CpG ODN should be adapted to the industry since vaccination of large number of animals individually is difficult to achieve and ineffective. *In ovo* route of delivery is the most preferred type of injection in animal health industry, since chickens were vaccinated before they were hatched from their eggs in embryonic stage through the allantoic sac (waste compartment), the amniotic fluid, air cell, the body of the embryo and the yolk sac (Dar *et al.*, 2009a).

Because of this reason, chickens were vaccinated with CpG ODN *in ovo* route before performing systematic infection with *E.coli*. Results showed reduced infection levels even after the 19 days of CpG ODN treatment (Gomis *et al.*, 2004).

TLR9 is also affective in the protection against *Salmonella* infection when given 50µg intraperitoneally into newly hatched chicks. 4h after the vaccination, they were challenged with *Salmonella enteritidis* outcome of reduced mortality rates as well as decreased colonization of the bacteria into the liver and spleen (He *et al.*, 2007; He *et al.*, 2005). In addition to that, doses of 5 µg and 10 µg *in ovo* injections of CpG ODN 2007, protected and reduced the mortality rates of chickens against *S. typhimurium*

challenge (Taghavi *et al.*, 2008). Other than K-type CpG ODNs, C-type CpG ODNs were tested for their potent protective properties against Salmonella infection, but didn't show any improved response in chickens in contrast to mice (Booth *et al.*, 2007).

Antiviral effects of CpG ODNs were also tested on chickens after *in ovo* injection. Chicken embryos (18 days old) were injected with CpG ODN 2007, and 1 day after they were challenged with infectious bronchitis virus (IBV). The proliferation of IBV in embryos were significantly diminished compared to controls treatments (Dar *et al.*, 2009b). In addition the mRNA expressions of IFN- γ , IL-1 β , IL-6, and IL-8 cytokines.

The adjuvant potency of CpG ODN in chickens was studied with the model antigen bovine serum albumin (BSA). After one injection of BSA combined with 20 μ g CpG ODN resulted enhanced IgG and IgM antibody levels compared to Freund's adjuvant treated group (Vleugels *et al.*, 2002). Up to date, CpG ODNs didn't show any side effects on the health of chickens and could be further used as a protective agent against chicken infections in industry. Table 1.10 summarizes the CpG ODN trials against various bacterial infections in chicken.

Table 1.10. CpG ODN administrations against chicken pathogens (*Adapted from Dar et al., 2009a*).

CpG treatment				Infection	
Age	Route	Dose	Time prior to challenge	Pathogen	Route
4 weeks	SC, IM	100, 50, 31.6, 10, 3, 16 μ g	3 days	<i>E. coli</i>	Scratch
18 day embryo	<i>in ovo</i>	10 μ g, 50 μ g	3 days	<i>E. coli</i>	SC
18 day embryo	<i>in ovo</i>	50, 10, 5, 1 μ g	3 days	<i>S. typhimurium</i>	SC
1 day	IP	25 or 50 μ g	4 hours	<i>S. enteritidis</i>	IP, Oral
1 day	IV oral 30 μ g, Subcutaneous 75 or 100 μ g	30 or 40 μ g	Oral 1 day of age IV day of age or weeks of age Sc weeks of age	<i>Eimeria acervulina</i>	Oral 1 day after CpG

SC Subcutaneous, IM Intramuscular, IP Intraperitoneal.

1.9 Subject and outline of the Thesis

Microbial motifs, known as pathogen-associated molecular patterns are recognized by germline encoded pattern recognition receptors (PRRs). The crosstalk between TLRs, NLRs, RLRs, and CLRs, generate a complex signaling network, leading to pronounced secretion of an array of cytokines, chemokines and type I interferon from different immune cells such as macrophages, dendritic cells, natural killer cells, B cells and even epithelial cells. Lately, strategies that help to improve host's immune response against viral, bacterial, allergic diseases and cancer gained significant importance, especially if the goal was to harness effective, safer and targetable formulations involving PAMPs and their ligands.

One of the main purposes of this study was dedicated to reveal the molecular mechanism of action of a newly identified mushroom derived immunostimulatory polysaccharides. In order to address these questions, we studied four different polysaccharides in dose dependent manner with mouse splenocytes, PECs and DCs derived from MyD88-, TLR2-, TLR4-, Dectin-1-, ASC-, Caspase-1-, NALP3-deficient and wild-type mice.

Next, we aimed to nanocomplex this amphiphilic PS with labile, negatively charged nucleic acid based TLR ligands, TLR3L: pIC, TLR7/8L: R848 and TLR9L: CpG ODN. Single, double (i.e PS(pIC+R848)) or triple combinations were formulated with PS4. Their physical characteristics (size, stability) were studied by dynamic light scattering method. Immune potentiating activities of these nanocomplexes were tested in splenocytes, macrophages *in vitro* and *ex vivo*.

The adjuvant potency of these PS nanocomplexes were studied against model antigen ovalbumin (OVA) and influenza A virus (split vaccine, SV). Wild type BALB/C mice were intraperitoneally immunized with PS(TLRL) nanocomplexes intraperitoneally, and anti-OVA immunoglobulin production was determined by ELISA. Wild type C57BL/6

mice were either intranasally (i.n.) or intradermally (i.d.) immunized with alone and single nanocomplex (PS(pIC/R848/CpG ODN)) formulations including SV. IgG, IgG subtype and IgA antibody responses against H1N1 Ag from sera were identified. Cellular immune response from i.n. immunized mouse splenocytes *ex vivo* via IL-2 and IFN- γ ELISA were also studied.

Anti-cancer studies were performed by using two different forms of nucleic acid based nanoparticles: i) either as a nucleic acid-polysaccharide nanocomplexes or ii) self-nanoparticle forming ODN forms to control establish tumor growth in C57BL/6 (mouse thymoma) and nude mice (human hepatocellular transplanted) respectively. Tumor sizes were followed daily for 3-5 weeks and volumes were calculated as mm³.

Finally, in an auxiliary project the immunomodulatory activities of CpG ODN sequences on chicken immune cells (B cells, splenocytes) were tested. The most potent sequence candidates were determined for chicken. These *in vitro* data was channelled to *in vivo* protection studies in a subsequent study in broiler chicken in order to improve immune response against uncontrolled infections.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents for ELISA

Cytokine pairs and recombinant proteins for ELISA were from Endogen (USA), Biolegend (USA) and R&D (USA). IgG and IgA ELISA antibodies were supplied from Southern Biotech (USA). The specific information of types, clones, brands and working concentrations of ELISA antibodies and reagents were mentioned in table 2.1.

Table 2.1. Antibodies and reagents used in cytokine ELISA experiments.

Product Name	Brand	Clone	Working concentration
Anti-Mouse IL-6 mAb	Pierce	32C11	10 µg/ml
Anti-Mouse TNF- α mAb	Pierce	XT3	5 µg/ml
Mouse IFN- γ mAb	Pierce	R4-6A2	10 µg/ml
Mouse IL-6 Biotinylated mAb	Pierce	2OF3	1:1000 dilution
Mouse TNF- α Biotinylated mAb	Pierce	XT22	1:1000 dilution
Mouse IFN- γ Biotinylated mAb	Pierce	XMG1.2	1:1000 dilution
Mouse recombinant IL-6	Pierce	N/A	2000 ng/ml
Mouse recombinant TNF- α	Pierce	N/A	2000 ng/ml
Mouse recombinant IFN- γ	Pierce	N/A	1000 ng/ml
Mouse IL-1 β /IL-1F2 DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 1000 pg/ml Detection Ab : 600 ng/ml
Mouse IL-4 DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 1000 pg/ml Detection Ab : 600 ng/ml
Mouse IL-6 DuoSet	R&D	N/A	Capture Ab : 2 µg/ml Recombinant Ab : 1000 pg/ml Detection Ab : 400 ng/ml
Mouse IL-10 DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 2000 pg/ml Detection Ab : 400 ng/ml
Mouse IL-12/IL-23 p40 DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 400 pg/ml Detection Ab : 2000 ng/ml
Mouse IL-13 DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 4000 pg/ml Detection Ab : 200 ng/ml
Mouse TNF- α DuoSet	R&D	N/A	Capture Ab : 0.8 µg/ml Recombinant Ab : 400 pg/ml Detection Ab : 2000 ng/ml
Mouse IFN- γ DuoSet	R&D	N/A	Capture Ab : 4 µg/ml Recombinant Ab : 100 pg/ml Detection Ab : 2000 ng/ml
Immunopure streptavidin alkaline phosphatase	Pierce	N/A	1:3000 dilution

Streptavidin conjugated horseradish peroxidase	R&D	N/A	1:200 dilution
PNPP (p-Nitrophenyl Phosphate, Disodium Salt) substrate	Pierce	N/A	Dissolve one, 5 mg PNPP tablet into 4 ml ddH ₂ O, then mix 1ml Diethanolamine Buffer.
TMB Microwell Peroxidase Substrate	KPL	N/A	Mix equal volumes of TMB Peroxidase Substrate with Peroxidase Substrate Solution B.

N/A: non-available

2.1.2 Pathogen Recognition Receptor Ligands

TLR and Dectin-1 ligands were purchased from several vendors as outlined below: i) peptidoglycan (PGN) (isolated from *B.subtilis*, Fluka, Switzerland), ii) pIC (Amersham, UK), iii) lipopolysaccharide (LPS) (isolated from *E.coli*, Sigma, USA), iv) Zymosan (isolated from *S.cerevisiae*, Invivogen, USA), v) R848 or vi) gardiquimod (Invivogen, USA), vii) depleted zymosan (isolated from *S.cerevisiae*, Invivogen, USA), viii) curdlan (isolated from *A.faecalis*, Sigma, USA).

2.1.3 Oligodeoxynucleotides

Stimulatory synthetic phosphodiester (PO, indicated with lower cases), phosphorothioate (PS, indicated with higher cases) or mixed backbone (MB) oligodeoxynucleotides (ODN hereafter) sequences given below were purchased from either Alpha DNA, (Canada) or NIH or USFDA CBER Core Facility (USA), or synthesized in the Biotherapeutic ODN Research Lab. Facility on a MerMade6 Oligonucleotide synthesizer machine:

D35 3CG PO (20mer) : 5'-ggtcgatcgatcgagggggg-3'

D35 3CG PS : 5'-GGTCGATCGATCGAGGGGGG-3'

D35 3CG MB : 5'-GGtcgatcgatcgaggggGG-3'

1466 Acore PO (16mer) : 5'-tcaacgttgattcaaa-3'

1466 Acore PS : 5'-TCAACGTTGATTCAAA-3'
 1466 Acore MB : 5'-TCaacgttgattcaAA-3'
 1466 pG6 PO (16mer) : 5'-tcaacgttgagggggg-3'
 1466 pG6 PS : 5'-TCAACGTTGAGGGGGG-3'
 1466 pG6 MB : 5'-TCaacgttgaggggGG-3'
 5547 PO (22mer) : 5'-tcgatcgacgttcgacgttggg-3'
 5547 PS : 5'-TCGATCGACGTTTCGACGTTGGG-3'
 5547 MB : 5'-tCgatcgacgttcgacgttggG-3'
 1466 (10mer) : 5'-TCAACGTTGA-3'
 1826 (20mer) : 5'-TCCATGACGTTCTGACGTT-3'
 1555 (15mer) : 5'-GCTAGACGTTAGCGT-3'
 2006 (24mer) : 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'
 2007 (22mer) : 5'-TCGTCGTTGTCGTTTTGTCGTT-3'
 7440 PO (24mer) : 5'-gtcgatcgattttcgatcgagggg-3'
 7440 PS (24mer) : 5'-GTCGATCGATTTTCGATCGAGGGG-3'
 K3 (20mer) : 5'-ATCGACTCTCGAGCGTTCTC-3'
 K23 (12mer) : 5'-TCGAGCGTTCTC-3'
 K3 Flip (20mer) : 5'-ATGCACTCTGCAGGCTTCTC-3'
 Linear-7x-1466 (70-mer) : 7 x 1466 (linear sequence of 5'-TCAACGTTGA-3')
 Linear-7x-1471 (70-mer) : 7 x 1471 (linear sequence of 5'-TCAAGCTTGA-3')
 ODN420 (70-mer) : 7 x 1466 (dendrimeric sequence linked via two symmetric bifunctional linkers, please see Figure 2.1.
 ODN421 (70-mer) : 7 x 1471 (dendrimeric sequence linked via two symmetric bifunctional linkers, please see Figure 2.2.)

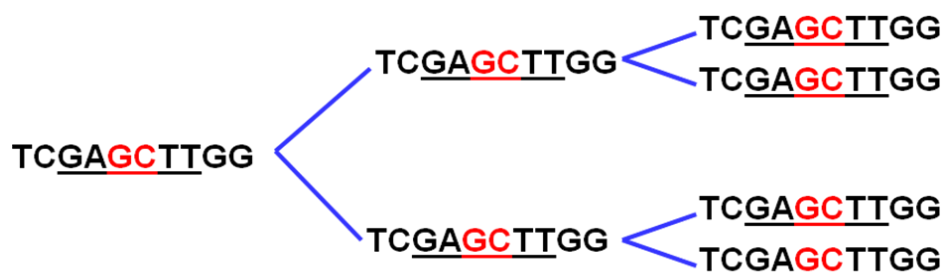


Figure 2.1. Schematic representation of the ODN420 formation via the use of two bifunctional linkers at the 3'-end of ODN 1466 sequences.

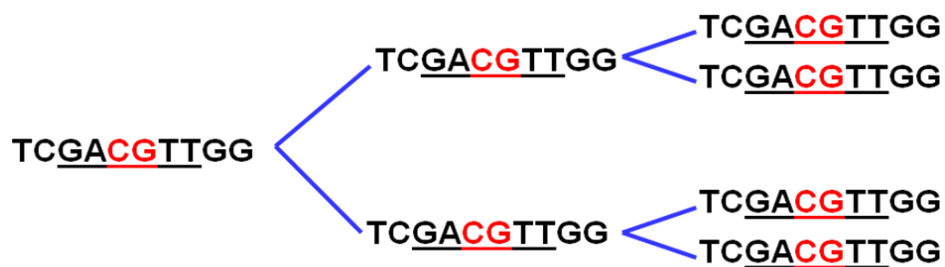


Figure 2.2. ODN421: Schematic representation of the ODN421 formation via the use of two bifunctional linkers at the 3'-end of ODN 1471 sequences.

2.1.4 Adjuvants, Antigens and Peptides

Imject Alum adjuvant and Imject ovalbumin purchased from Thermo (USA). Split vaccine (SV) of H1N1 (New Caledonia strain) was prepared by mixing virus with ether, incubation for 30 mins (at room temperature) then centrifugation (3000 rpm, 15 mins), and collection of aqueous phase followed by evaporation. Whole virus were prepared by the purified from allantoic fluid by filtration (0.45 µm) followed by sedimentation through a linear sucrose gradient (Koyama *et al.*, 2010). MHC class I and II type influenza peptides were from various manufacturers. Detailed information about these peptides were mentioned in table 2.2. Recombinant murine GM-CSF and recombinant human Flt3 ligands were obtained from Peprotech (USA). All reagents used in these experiments are endotoxin free.

Table 2.2. Influenza peptide sequences, brands, and commercial names.

Name	Sequence	Brand	Epitope
M-502-P	ASNENMDTM	MBL	MHC I
M508-P	ASNENMETM	Operon	MHC I
PA 224-223	SSLENFRAYV	Operon	MHC I
NP260-283/I-1006061N-1	ARSALILRGSVAHKSCLPACVYGP	Operon	MHC II

2.1.5 Polysaccharides

Four different polysaccharide (PS) extracts (which abbreviated as PS1 to PS4) were kindly provided by Prof. Oktay Erbatur (Cukurova University, Chem. Dept., Adana, Turkey). Polysaccharides belong to the Basidiomycota phylum and Agaricomycetes class. PSs were isolated from *Shiitake* and the *Ganoderma lucidum* (Balcalı and Alata strains), under subcritical water conditions at 100°C and 150°C which were endotoxin free as determined by LAL assay (<0.01 EU/ml). PS were resuspended in DNase/RNase free water (Hyclone), vortexed and stored at +4°C. PS nanocomplexes with pIC, R848 and CpG ODNs (1:1 weight/weight, PS to ligand ratio) were mixed and incubated overnight at +4°C. Unbound nucleic acids were filtered and nucleic acid complexation yield determination was done on Nanodrop™ from the eluents. In all preparations the nanocomplex formation yields were ≥90%. For the coding and physicochemical properties of four different polysaccharides used throughout this study, please see table 2.3. Molecular weight determination was carried out by high performance size exclusion chromatography (on a HPSEC-MALLS system).

Table 2.3. Properties of the polysaccharide extracts

Name	Code	Our Code	Molecular Weight	pKa
<i>Ganoderma lucidum</i> (Alata strain)	AR150	PS1	2.9x10 ⁶ Da	6.64
<i>Ganoderma lucidum</i> (Balcali strain)	BROsm	PS2	3.88x10 ⁶ Da	6.99
<i>Shiitake</i>	SR150	PS3	1.6x10 ⁶ Da	6.67
<i>Ganoderma lucidum</i> (Alata strain)	AROsm	PS4	5.2x10 ⁶ Da	6.69

2.1.6 Standard Solutions, Buffers, Cell Culture Media Components

Cell culture media components were from Hyclone (USA), Lonza (Switzerland) and Nacalai Tesque (Japan). Preparation of `Oligo` and `Regular` RPMI1640 or DMEM media was described in Appendix 1. BSA was from Roche (Germany). Thioglycollate broth was from Sigma (USA), (please see Appendix A for details). For RNA isolation and for cDNA synthesis, TRIzol[®] (Invitrogen, USA) and DyNAmo[™] cDNA Synthesis kit (Finnzymes, Finland) were used respectively according to the manufacturer's protocol.

2.2 Methods

2.2.1 Model Animals

Wild type and specific gene deficient mice (either at C57BL/6 or BALB/C background) and T-cell deficient athymic nude mice (female, 6-8 weeks old) were housed in Bilkent University, Department of Molecular Biology and Genetics, Osaka University, Immunology Frontier Research Center (IFReC) and Japan National Institute of Biomedical Innovation facilities. The generation of MyD88, TLR2 and TLR4 deficient mice, on C57BL/6 background, were established in Shizuo Akira's laboratory at IFReC, Osaka University (Adachi *et al.*, 1998; Hoshino *et al.*, 1999; Takeuchi *et al.*, 1999). ASC^{-/-}, Nalp3^{-/-} and Casp1^{-/-} mice (C57BL/6 background) were obtained from Vishva M. Dixit at UCSD, USA (Lamkanfi *et al.*, 2009b; Mariathasan *et al.*, 2006). The generation of Dectin-1 deficient mice was generated by homologous recombination as described by Saijo *et al.* (Saijo *et al.*, 2007). 1 week old female broiler chickens were housed in Beypi Research and Development Facility in Bolu. They were supplied overnight shipments and they were sacrificed and used as they arrived to Bilkent, MBG Animal Facility.

2.2.2 Cell Culture

2.2.2.1 Spleen Cell Suspension Preparation

Spleens were surgically removed from female mice (C57BL/6 or BALB/C strains) or 1 week old broiler chickens after cervical dislocation. Single cell suspensions were prepared by smashing spleen by circular movements suspended in the 2% FBS supplemented regular RPMI1640 media. Cells were washed at least 2x following centrifugation at 1500 rpm for 5 mins. Cell pellet was gently disrupted with fresh media,

the tissue debris was removed and finally the splenocyte suspension was counted and adjusted to $4-10 \times 10^6/\text{ml}$ depending on the type of intended experiments.

2.2.2.2 Peritoneal Exudate Cell Preparation

Peritoneal macrophages from mice collected into cold PBS, 4-6 days after intraperitoneal (i.p.) injection of 2.5 ml, sterile 4% thioglycollate broth. Cells were washed at 1500 rpm for 5 mins in regular RPMI1640 media supplemented with 2% FBS. Pellet was resuspended in 1 ml ACK lysis buffer (Please see appendix A for the preparation conditions) and incubated for 5 mins, volume was completed into 10 ml with FBS containing media and then centrifuged. Cells were resuspended at 10% FBS regular RPMI1640, counted and seeded into plates as $1.25 \times 10^6/\text{ml}$.

2.2.2.3 Bone Marrow Derived Dendritic Cell Generation

After removal of both back legs from executed mice, skin and muscles of mice were dissected to expose the femur and tibia bones. Bones were washed twice with 70% EtOH for 5 mins afterwards with 1xPBS for 5 mins. and finally transferred into complete medium. Both ends of the bones were cut with scissors and 5 ml syringe, 25G needle were used to flush out the marrow using complete medium. Clusters in the suspension were disintegrated with the help of the back of the syringe with circular movements. Cells were washed at 1500 rpm for 5 mins in 2% FBS regular RPMI1640. Pellet was resuspended in 1 ml ACK lysis buffer and incubated for 5 mins, volume was completed into 10 ml with FBS containing media and then centrifuged. Bone marrow cells were resuspended at 10% FBS regular RPMI1640, counted and were seeded as $4 \times 10^6/\text{ml}$ with 10 ng/ml muGM-CSF and as $4 \times 10^6/\text{ml}$ with 100ng/ml huFlt3L into 60 mm and/or 100 mm petri dishes at day 0. After 7 days, cells were detached from petri dishes with 50mM sterile EDTA and distributed into 96 well plates ($5 \times 10^5/\text{ml}$) for stimulation.

2.2.2.4 Cell Lines

2.2.2.4.1 E.G7

EG.7 cells (ATCC, CRL-2113™) were derived from EL4, a mouse lymphoma cell line. EG.7 was stably transfected with pAc-neo-OVA that carries a complete copy of chicken ovalbumin (OVA) mRNA and the neomycin (G-418) resistance gene. Cells were sustained in a RPMI1640 media with 5% or 10% regular FBS supplemented with 1 mg/ml G-418 (Hyclone). Culture maintenance was performed by refreshment of media in every 2-3 days intervals as the cells reaches ~90% confluency.

2.2.2.4.2 HuH-7

Human hepatocellular carcinoma cell line HuH-7 (ATCC) was cultured with 10% FBS supplemented High-glucose DMEM. These adherent cells were sub-cultured when they reached 90% confluency with fresh media following trypsinization and washing with 1x PBS.

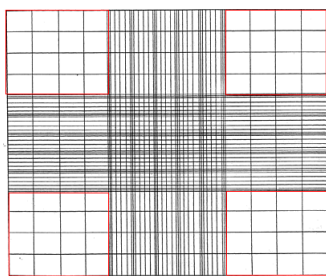
2.2.2.4.3 DT40

The chicken B cell line DT40 (ATCC, CRL-2111™) was cultured in High-glucose DMEM media with 10% regular FBS. Chicken lymphocyte suspensions were cultured within 2-3 days by addition or replacement of fresh medium.

2.2.2.5 Cell Counting

After the cell lines and primary cells were pooled, washed and precipitated, they were suspended in 10 ml of 5% regular RPMI1640 media. Cells were diluted 10 fold and

micropipetted on a hemocytometer (10 μ l from cell suspensions into 90 μ l medium to reduce the cell number). The number of cells in the tahoma chamber was determined by counting under the light microscope from these red areas made up of big gridlines, which are 0.1 mm³ by volume:



The cell number was calculated according to the following formula:

$$(\text{Counted cell number} / 4) \times 10 (\text{dilution factor}) \times 10^4 = \text{Total cell number per ml}$$

Cells were also counted with Z1 coulter counter (Beckman Coulter, USA) machine as follows, cell suspensions were diluted for 500 times, by mixing 20 μ l cell with 10 ml Isoton II Diluent buffer into Z1 cup, adding 2-3 drops of ZAP-OGLOBIN II Lytic Reagent (red blood cell lytic agent). Following gentle tilting of the vial up and down for 5-6 times, cells were then counted on the instrument and were recorded as the number of the cells per ml.

Accuri™ C6 flow cytometer also used for the cell counting. Cells were dissolved at 1 ml media. Diluted, by adding 20 μ l cell into 10 ml Isoton II Diluent buffer in Z1 cuvette (dilution #1). Cuvette was directly located into the SIP of Accuri C6, 10 μ l from the total volume was counted (dilution #2). Live cells were gated, apoptotic cells or cell clusters were ignored.

The cell number in the falcon was calculated according to the following formula:

$$\text{Counted cell number} \times 500 (\text{dilution\#1}) \times 100 (\text{dilution\#2}) = \text{Total cell number in the falcon tube.}$$

2.2.2.6 Adjusted Cell Densities for Different Experiments

For cytokine ELISA, $2\text{--}5 \times 10^6/\text{ml}$ spleen cells, $1.25 \times 10^6/\text{ml}$ PEC and $5 \times 10^5/\text{ml}$ bone marrow derived dendritic cells were distributed into 96 well plates with a final volume of 200 μl or 250 μl media per well. Stimulations were ran in either duplicate or triplicate wells for each treatment and was repeated at least twice on different time. After 24 to 42 hrs stimulation, supernatants were collected from the plates and stored at -20°C . Supernatants were layered on pre-coated 96 well ELISA plates for specific determination of cytokine production for that assay.

For RNA isolation, after stimulation with PS and TLR ligands for 2-4 hrs, $8\text{--}10 \times 10^6$ splenocytes, or $2\text{--}4 \times 10^6$ PECs were transferred into 15 ml falcons with a final volume of 2 ml.

2.2.2.7 Ligands and Polysaccharides used During Cell Stimulations

For the stimulation assays, spleen cells, DCs and PEC were incubated with i) four different PSs (PS1-PS4) at concentration ranges between 0.02 $\mu\text{g}/\text{ml}$ to 20 $\mu\text{g}/\text{ml}$ ii) PGN, 0.02–20 $\mu\text{g}/\text{ml}$, iii) zymosan 0.02–20 $\mu\text{g}/\text{ml}$, iv) depleted zymosan 1–100 $\mu\text{g}/\text{ml}$, v) pIC 0.02–100 $\mu\text{g}/\text{ml}$, vi) R848 (or in some cases with gardiquimod) 0.02–5 $\mu\text{g}/\text{ml}$, vii) curdlan 0.01–100 $\mu\text{g}/\text{ml}$, viii) Alum 50–500 $\mu\text{g}/\text{ml}$, ix) LPS 0.5–5 $\mu\text{g}/\text{ml}$, and x) CpG ODNs and their proper non-CpG control ODNs at 0.1–10 $\mu\text{g}/\text{ml}$ or 1 μM .

Cells were incubated with 5% FBS, oligo RPMI1640 or 5% FBS oligo DMEM when stimulated with ODNs and cultured with 5% regular RPMI1640 or 5% FBS regular supplemented DMEM as they stimulated with other TLR Ligands and polysaccharides.

2.2.2.8 Delivery of TLR Ligands with Polysaccharide Nanocomplexes

Polysaccharides were mixed as 1:1, PS:ligand ratio (w/w), i) K3 CpG ODN, ii) K23 CpG ODN, iii) 1555 CpG ODN, iv) K3 Flip ODN, v) pIC, and vi) R848 separately in double or in triple combinations. In order to induce nanocomplex formation the ligand(s) and PS were incubated overnight at 4°C prior to treat immune cells. The starting concentrations of these nanocomplexes for cytokine ELISA were adjusted to 1 µM for CpG or control ODNs, 20 µg/ml or 6 µg/ml for pIC, and 2–6 µg/ml for R848 (TLR9, 3, and TLR7 ligands, respectively). In later stages of the study, these stimulants were further diluted and used for cell stimulations.

2.2.3 Enzyme Linked-Immunosorbent Assay (ELISA)

2.2.3.1 Cytokine ELISA

After the incubation of cells with stimulants for various time periods as indicated previously, 96 well plates were spun at 1500 rpm for 5min. Supernatants (170 µl-220 µl) were transferred into new plates and stored at –20°C until further use. Properties, brands, clones and concentrations of antibodies and chemicals used in different cytokine ELISA were indicated in the table 2.1. The ELISA protocol followed for all cytokine antibodies were taken from Gursel et al. with slight modifications (Gursel *et al.*, 2003). Briefly, high binding polysorp (Nunc, Denmark) or Immulon 2HB plates (Thermo Scientific, USA) were coated with anti-cytokine monoclonal antibodies (50 µl/well) for 4-5 hrs at RT or overnight at +4°C. Then the wells were blocked with 200 µl blocking buffer for 2 hrs at RT or at +4°C for overnight and washed with wash buffer for 5 times for 5 mins intervals and rinsed with ddH₂O. Cell supernatants and 11x, 2-fold serially diluted recombinant proteins (diluted in 1xPBS) were added 50 µl/well and incubated for 2 hrs at RT or overnight at +4°C. Plates were washed as previously mentioned. Afterwards, biotinylated anti-cytokine antibodies (50 µl/well) which were prepared in a T-cell buffer,

either 1:200 or 1:1000 dilutions, were added to the wells and incubated for further 2 hrs at RT or overnight at +4°C for the detection of cytokine levels. Again followed by washing as described earlier. 1:3000 diluted Streptavidin alkaline-phosphatase or 1:200 diluted streptavidin horseradish peroxidase (SA-AKP or SA-HRP should be prepared in T-cell buffer at least 1h prior to use and must be kept at RT, if prepared overnight then at +4°C) were added to the plates as 50 µl/well and incubation was performed for 60 mins or 2 hour at RT (or o/n in fridge). After washing the plates, PNPP (for SA-AKP detection) or TMB (for HRP detection) substrates were prepared according to manufacturers protocol and 50 µl/well aliquots were pipetted to each wells. Yellow color development (or Blue for TMB substrate) were followed by multiple optical density readings at 405 nm for PNPP (or @450 nm for TMB) until recombinant cytokine standards reach a saturation. Cytokine levels for every sample were calculated based on the selected standard curve and was kept constant for that specific experiment. 15-20 mins after TMB addition, (could be interpreted with the dark blue color formation by recombinant proteins) 50 µl/well stop solution (2N sulfuric acid) was added and absorbance reading was taken at OD 450 nm. Data were recorded on an ELISA plate reader (BioTek, µQuant).

2.2.3.2 IgG and IgA ELISA

In Immunoglobulin ELISA, Immulon 1B plates (Thermo, USA) were used. Each well was coated with 50 µl of OVA or SV (Pierce, USA) (10 µg/ml OVA or 1 µg/ml SV, diluted in 1x PBS) diluted in sodium carbonate buffer. Blocking and washing steps were same as cytokine ELISA as mentioned in Section 2.2.3.1. Instead of culture supernatants, here immune mouse sera from each animal was pipetted on the first row in 1/7 titration (10 µl serum was diluted with 60 µl 1x PBS) for OVA and 1/100 titration for SV. All wells (excluding 1st row) were filled with 52.5 µl 1x PBS and 4-fold serial dilution was performed on vertical axis by transferring 17.5 µl from the previous well to the next well (total of 7 dilutions were carried out) for anti-OVA IgG ELISAs. For anti-

SV IgG and IgA ELISA mice sera were diluted 3-fold for 7 times. 33 μ l diluted sera from previous well was mixed with the 66 μ l 1xPBS in the following well. The dilution was adjusted as 2-fold for IgA ELISA from nasal wash. Antibodies against mouse IgG, IgG1, IgG2a, IgG2b, IgG2c and IgA (Southern Biotech, USA) were either directly linked with AP or HRP. AP or HRP-linked IgG antibodies (1 mg/ml) were 1:4000 diluted in T-cell buffer and again prepared one day prior to their use. After the addition of PNPP, yellow color formation was read at OD405 nm was on an ELISA reader. TMB substrate was added into the wells for HRP-conjugated IgG and IgA antibodies, blue color formation was observed and after the addition of stop solution the color changed into yellow with a maximum absorbance at OD450 nm.

2.2.4 Transfection of Cells

HuH-7 cells (1×10^4 /well) were transfected with FuGENE6 according to manufacturer's protocol in 96 well round bottom plates. Briefly, 6 μ l FuGENE6 (Roche, Switzerland) was added to 94 μ l (serum and penicillin/streptomycin free media) in an eppendorf. Mixture was vortexed for 3-5 secs, then incubated at RT for 5 mins. 0.3 μ g pMIR-REPORT Luciferase miRNA expression reporter plasmid (Applied Biosystems, USA) was added to the mixture and further incubated for at least 15 mins. The transfection mixture was distributed 15 μ l/well in a dropwise manner. Wells were swirled to ensure distribution over the entire plate surface. Cells were incubated for 24 hrs prior to co-culturing with mouse peritoneal exudates cells in round bottom microtiter plates.

2.2.5 Luciferase Assay

After 20 hrs of co-culturing HuH-7 cells with PECs or splenocytes from ODN treated mice, media was removed, each well was washed smoothly with warm 1xPBS. 100 μ l/well Luciferase substrate reagent was added for cell lysis. Plates left on a rocking shaker for 15 mins at room temperature. Supernatants were transferred into a new white

96 well plate and relative light unit from cell extracts were recorded on a Reporter Machine™ (Turner Biosystems, USA).

2.2.6 In vitro Cytotoxicity Experiment

Athymic nude mice were i.p. injected with 100 µg ODN420, ODN421, Linear-7x-1466, Linear-7x-1471 and 1xPBS for three times with 2 days intervals. One day after the last treatment Huh7 cells were transfected with pMIR plasmid as mentioned in Section 2.2.2.10. 24 hrs after transfection, mice were euthanized by cervical dislocation, spleen and PECs were collected from animals. Splenocytes and PECs were co-cultured with pMIR transfected human hepatocellular carcinoma cells with the ratios of 1:0 (i.e 1x10⁴ HuH-7 cells: no PEC or splenocytes), 1:1 and 1:5 in a round bottom 96 well plate at 200 µl final volume. 24 hrs after incubation of HuH-7 and CpG ODN treated PECs and spleen cells, luciferase assay was performed as previously explained (Figure 2.3).

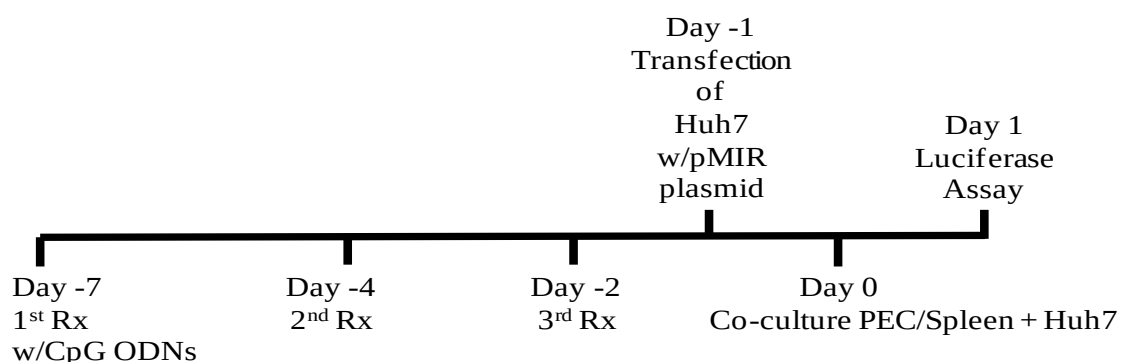


Figure 2.3. The schedule of in vitro cytotoxicity experiment.

2.2.7 Fluorescence Activating Cell Sorting (FACS)

2.2.7.1 Cell Surface Marker Staining

Chicken splenocytes were centrifuged at 1500 rpm for 7 mins at the end of the 24 hrs incubation period. Supernatants were discarded. Pellets were disrupted by using a pin rack holder. 1 ml PBS-BSA-Na-Azide added into each tube to wash cells. Cells were spun at 2000 rpm for 5 mins. Supernatant was discarded and the washing step was repeated. At the end of the second washing step, PBS-BSA-Na-Azide was discarded. Indirect staining was performed as, the cells were incubated in 50 μ l PBS-BSA-Na-Azide containing 1.5 μ g/tube biotin CD80 (AbD Serotec) for 15 mins at dark. Samples washed as described previously. Then stained with 50 μ l, 1:250 diluted SA-PE for 15 mins in dark. Cells were washed twice, resuspended in 500 μ l PBS-BSA-Na-Azide, transferred to FACS tubes and analyzed in Accuri C6 (BD, USA).

2.2.8 Determining the Binding of PS to TLR2

Recombinant fusion protein consisting of the extracellular domains of the human TLR2 fused to mouse IgG2b-Fc was prepared. Protein was constructed by amplifying the corresponding extracellular domain and ligating the fragment in frame into a pCIneo vector containing the coding sequence for mouse IgG2b-Fc and purified from cell lysates by protein G affinity chromatography (Coban *et al.*, 2010). PGN, PS, depleted zymosan and zymosan (10 μ g/ml), were coated on high binding polysorp 96-well plates in 1xPBS overnight at +4°C and then blocked with blocking buffer. The binding of TLR2 (0.25–4 μ g/ml) fusion protein was detected with HRP-labeled mouse anti-IgG.

2.2.9 Determination of the Gene Expression

2.2.9.1 Total RNA Isolation

Cells ($4\text{--}10 \times 10^6/\text{ml}$) stimulated with various ligands and PSs were centrifuged at 1500 rpm for 10 mins at $+4^\circ\text{C}$. Supernatants were gently discarded without disrupting the pellet and cells were extensively mixed and lysed with 1 ml TRIzol (a phenol, guanidine isothiocyanate solution). Cell lysates were transferred into eppendorf tubes and could be stored at -80°C for a week. All steps must be performed on ice and centrifugation must be carried at $+4^\circ\text{C}$. 200 μl chloroform was added into each 1ml TRIzol containing tubes and vigorously shaken at least for 40 secs and incubated at RT for 3 mins. Samples were centrifuged at 13.900 rpm for 17 mins at $+4^\circ\text{C}$. Subsequently, $\sim 550\text{--}600$ μl of the clear upper aqueous portion was transferred into a new eppendorf tube and total RNA was precipitated by adding 500 μl of 2-propanol, gently inverted up-down for proper mixing, incubated at RT for 10 mins and centrifuged for 10 mins at 13.900 rpm. Supernatant was removed, and the pellet was washed with 1 ml of 75% EtOH, vortexed for 10 secs, again centrifuged at 8000 rpm for 7 mins. After centrifugation ethanol was removed and pellets were washed again with 1ml $>99,8\%$ EtOH followed by centrifugation at 8000 rpm for 7 mins. After the last centrifugation, the pellet was left under laminar flow hood to air-dry. Purified total RNA then dissolved with 20-30 μl RNase/DNase free H_2O . The OD measurements of the samples were carried out at 260 nm and 280 nm with the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNA samples were expected to give 1.8-2.0 ratio at A260/280 which was the indicator of minimal DNA, protein or chemical organic solvent contaminations. RNA samples were stored at -80°C for further use.

2.2.9.2 cDNA Synthesis

Extracted total RNA samples were transcribed into cDNA with cDNA synthesis kit (Finnzymes, USA) according to the manufacturers' protocol. Briefly, 2 µg total RNA was mixed with 1.5 µl of Oligo (dT) 15-mer (100 ng) primer and total volume was completed to 14 µl with RNase DNase free H₂O PCR tubes. Tubes were pre-denatured at 65°C for 5 mins in MJ Mini thermocycler (BIO-RAD, USA) and then spun down afterwards 10 µl RT Buffer (includes dNTP mix and 10 mM MgCl₂) and 2 µl M-MuLV RNase H⁺ reverse transcriptase (contains RNase inhibitor) were added to the mixture. Samples were incubated at i) 25°C for 10 mins, ii) 40°C for 45 mins and iii) 85°C for 5 mins. cDNA samples were preserved either at -20°C or -80°C for further use.

2.2.9.3 Semi-quantitative RT-PCR

Primers were designed with Primer3 Input v.0.4.0 program (<http://frodo.wi.mit.edu/primer3/input.htm>) and PerlPrimer (<http://perlprimer.sourceforge.net/>) using the cDNA sequences of mouse and chicken genes available at the EnsemblTM database. Each primer pair was blasted (<http://www.ncbi.nlm.nih.gov/BLAST/> or <http://genome.ucsc.edu/>) against the mouse or chicken genome. For each gene, a specific primer pairs (Table 2.4) optimal PCR conditions were set and following mixing the reagents, reverse-transcriptase PCR was performed on MJ Mini thermocycler (Bio-Rad, USA). PCR mixtures were prepared according to the protocol in Table 2.5. PCR conditions for mice and chicken genes were summarized in Table 2.6, Table 2.7, and Table 2.8. PCR products kept at 4°C until they were loaded on 1.5% agarose gel. Mouse *IP-10* primer pairs was obtained from the literature (Giulietti *et al.*, 2001).

Table 2.4. Designed mouse and chicken primer sequences and product sizes.

Primer		Sequence	Product Size
<i>mβ-actin</i>	Forward Reverse	5'-GTATGCCTCGGTCGTACCA-3' 5'-CTTCTGCATCCTGTCAGCAA-3'	450 bp
<i>mIP10</i> [§]	Forward Reverse	5'-GCCGTCATTTTCTGCCTCAT-3' 5'-GCTTCCCTATGGCCCTCATT-3'	127 bp
<i>mTNF-α</i>	Forward Reverse	5'-CCACCACGCTCTTCTGTCTAC-3' 5'-ACTCCAGCTGCTCCTCCACT-3'	189 bp
<i>ckβ-actin</i>	Forward Reverse	5'-CATCTTCCAACCTTCCTTCC-3' 5'-GCCAGACTCATCATACTCCT-3'	325 bp
<i>ckTLR1</i>	Forward Reverse	5'-AAGGTTGGACTTCTTATTGAGG-3' 5'-AAGGCACTAAAGTCAAGCTC-3'	366 bp
<i>ckTLR3</i>	Forward Reverse	5'-GAGTGTTTGATTATGACGCCT-3' 5'-ATGATGCACCTTAAACTTCCTG-3'	250 bp
<i>ckTLR4</i>	Forward Reverse	5'-GCAGTTTCTGGATCTTTCAAGG-3' 5'-CAGGGTATTCAAGTGTCCGA-3'	216 bp
<i>ckTLR5</i>	Forward Reverse	5'-ATCTTGAAAGCTGTGAACCTG-3' 5'-ATCCTGAGCCCATTGTATGAG-3'	294 bp
<i>ckTLR21</i>	Forward Reverse	5'-AGAAGAAGGTTTGGGTGGAG-3' 5'-CTGGGTATTTGGAATGAGAAAGG-3'	541 bp
<i>ckIFN-α</i>	Forward Reverse	5'-CCTCCAAGACAACGATTACAG-3' 5'-ATAAATAGGACAAGGGTGGGA-3'	139 bp
<i>ckIFN-β</i>	Forward Reverse	5'-CAAGGACAAGAAGCAAGCAG-3' 5'-CTGTAGGAAGTTGTGGATGGA-3'	252 bp
<i>ckLITAF</i>	Forward Reverse	5'-CCTATGCCAACAAAGTACATCTG-3' 5'-AAGTGTTCCACATCTTTCAGAG-3'	229 bp
<i>ckIL-1β</i>	Forward Reverse	5'-CGACATCTTCGACATCAACC-3' 5'-CGACATGTAGAGCTTGTAGCC-3'	201 bp
<i>ckIL-4</i>	Forward Reverse	5'-CTACCAGTCCCTTAGACCAG-3' 5'-AGACAGAGCCTTACCTTGAG-3'	278 bp
<i>ckIL-6</i>	Forward Reverse	5'-GGTGATAAATCCCGATGAAGTG-3' 5'-GGCAGATTGGTAACAGAGGA-3'	232 bp
<i>ckIL-8</i>	Forward Reverse	5'-CACTCATTCTAAGTTCATCCACC-3' 5'-CCCAGTAGAAATCAGGAGCA-3'	252bp
<i>ckIL-15</i>	Forward Reverse	5'-ACCATCTTCTTCTATGTGCT-3' 5'-GTTCCGTACATCATGCTTCC-3'	246 bp
<i>ckIL-18</i>	Forward Reverse	5'-TGAAGATGTAACAGATCAGGAGG-3' 5'-TCAAAGGCCAAGAACATTCC-3'	308 bp

[§]Adapted from Giulietti et al., 2001

Table 2.5. RT-PCR reaction reagents.

Reaction Ingredients	Volume
2x DyNAzyme™ II PCR Master Mix*	12.5 µl
DNase/RNase free H ₂ O	9 µl
Forward primer	1 µl (10pmol)
Reverse primer	1 µl (10pmol)
cDNA	1.5 µl
Total	25 µl

Table 2.6. RT-PCR conditions of mice primers.

PCR Step	mβ-actin, mIP10	mTNF-α
Initial Denaturation	—	—
Denaturation	94°C, 30''	94°C, 30''
Annealing	55°C, 30''	60°C, 30''
Extension	72°C, 30''	72°C, 30''
Number of cycles	35	40
Final Extension	72°C, 5'	72°C, 5'

' denotes mins; '' denotes secs

Table 2.7. RT-PCR conditions of chicken β-actin & TLR primers.

PCR Step	ckβ-actin	ckTLR1	ckTLR2, ck TLR4	ckTLR5	ckTLR21
Initial Denaturation	95°C, 7'	95°C, 7'	95°C, 7'	95°C, 7'	95°C, 7'
Denaturation	95°C, 30''	95°C, 30''	95°C, 30''	95°C, 30''	95°C, 30''
Annealing	61°C, 30''	63.5°C, 30''	63.5°C, 30''	57.7°C, 30''	64°C, 30''
Extension	72°C, 30''	72°C, 30''	72°C, 30''	72°C, 30''	72°C, 30''
Number of cycles	35	35	50	40	50
Final Extension	72°C, 3'	72°C, 3'	72°C, 3'	72°C, 3'	72°C, 3'

' denotes mins; '' denotes secs

Table 2.8. RT-PCR conditions of chicken cytokine primers.

PCR Step	ckIFN- α , ckLITAF, ckIL-8	ckIFN- β , ckIL-4, ckIL-6, ckIL-15, ckIL-18	ckIL1- β
Initial Denaturation	95°C, 7'	95°C, 7'	95°C, 7'
Denaturation	95°C, 30''	95°C, 30''	95°C, 30''
Annealing	62.4°C, 30''	62.4°C, 30''	61.4°C, 30''
Extension	72°C, 30''	72°C, 30''	72°C, 30''
Number of cycles	35	50	50
Final Extension	72°C, 3'	72°C, 3'	72°C, 3'

' denotes mins; '' denotes secs

2.2.9.4 Agarose Gel Electrophoresis

1.5% agarose gel was prepared with 1x TAE buffer and 1 mg/ml ethidium bromide solution. Gel lanes were loaded with 10 μ l of PCR product and 2 μ l loading dye mixtures. 5 μ l of the low range 50-500 bp and 100-1000 bp DNA ladders (Jena Biosciences) were used as markers and the gels were ran for 40-60 mins at 80V or 100V power. Visualization of the gels were performed under UV transilluminator (Vilber Lourmat, France) with BIO-PROFILE Bio-1D V 11.9 software The gel exposure time was kept fixed at 0.1 second for each run. In some experiments, blanked intensities were calculated by subtracting background intensity for each product and were divided by blanked house-keeping gene (β -actin) values to normalize semi-quantitative data.

2.2.10 DNase Treatment of the CpG ODNs and PS(CpG ODN) nanocomplexes

CpG ODN and its nanocomplex with PS4 were treated with DNase I (1U enzyme for 1 μ g DNA) according to manufacturer's protocol (Ambion). Each reaction tube, contained 5 μ l DNase I (10U), 10 μ l CpG ODN (10 μ g), 5 μ l enzyme buffer and 30 ml ddH₂O. Incubations at 37°C were continued either for 10 or 20 mins. At the end of digestion, tubes were incubated at 75°C for 10 minutes to inactivate the DNase I. Remaining ODN activity was tested from splenocyte supernatants by ELISA. ODN integrity was assessed

by agarose gel electrophoresis technique (2µg ODN was loaded into each lane of 4% agarose).

2.2.11 In vivo Experiments

2.2.11.1 Injection of animals with polysaccharides in various concentrations

C57BL/6 mice were injected intraperitoneally with 0.2 µg, 2 µg and 20 µg PS1-4. Six hours later mice were sacrificed. The peritoneal exudate cells and spleens were collected. 1×10^5 PECs/well were incubated for 24 hrs and 48 hrs in 96 well plates. To evaluate the level of *ex vivo* stimulation, cell supernatants were subjected to detect IL-6 by ELISA. For RNA isolation, 5×10^6 /ml splenocytes from each treated animal were incubated in 15 ml falcon tubes in CO₂ incubator for 4 hrs and mouse *ip-10*, *tnf-α* gene expressions were assessed by RT-PCR.

2.2.11.2 Immunization against OVA either with TLR ligands or PS nanocomplexes

Adult female C57BL/6 mice (5/group) were injected intraperitoneally with PS, pIC, R848, CpG ODN (15 µg each) mixed with 7.5 µg of ovalbumin. The treatments were either carried with alone ligands or their double or triple combinations. Similarly, PS nanocomplexes were prepared as listed below: i) PS-pIC ii) PS-R848 iii) PS-CpG ODN iv) PS-pIC+CpGODN v) PS-pIC+R848 vi) PS-R848+CpG ODN vii) PS-pIC+R848+CpG ODN. All complexes contained 7.5 µg of OVA antigen. Immunization protocol was given in Figure 2.4. Animals were tail bled one day before (primary response) and 14 days after the booster injection (secondary response). In order to obtain sera, blood was collected in glass tubes and incubated at 37°C for 1 hour. The top liquid part was collected and was spun at 8000 rpm for 5 mins. The mice sera (>50 µl), were collected in microtiter plates and stored at -20°C until for further use.

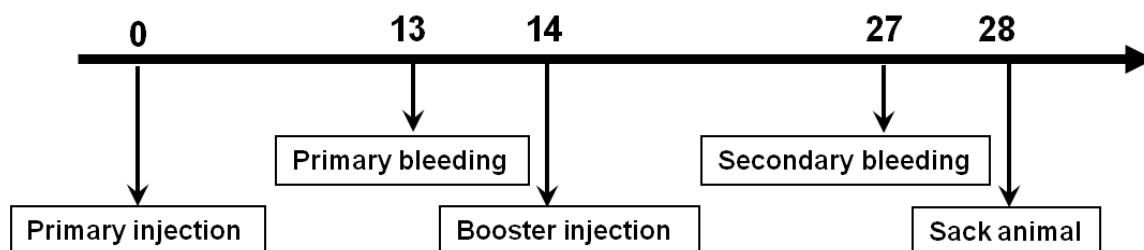


Figure 2.4. Immunization schedule for *in vivo* vaccination against ovalbumin.

2.2.11.3 Immunization with TLRL-PS nanocomplexes against SV

Adult female C57BL/6 mice (5/group) were either injected intradermally or intranasally under anesthesia with PS, pIC, R848, CpG ODN (15 µg each) alone or PS-pIC/R848/CpG ODN nanocomplexes (15 µg:15 µg) in combination with 0.1 µg and 1 µg of SV antigen, respectively. The immunization protocol kept identical as described above with few modifications. Secondary injections were performed at day=10 post-primary injections (instead of d=14). Mouse sera and nasal wash collections were done @ day:7 after booster injection (instead of d=28). Mice was bled and sera was collected in to 96 well plates as indicated above. Nasal wash samples were collected directly into eppendorf tubes by back-flushing 1 ml 1xPBS from the trachea into the nose with 22G cathethers. The mice sera and nasal wash were stored at -20°C for further use.

2.2.11.4 Tumor Xenograft Model

5×10^6 EG.7 cells or HuH-7 cells in 100 µl sterile 1x PBS were injected subcutaneously to adult C57BL/6 (5-10 mice/group) and T-cell deficient nude mice (5 mice/group) respectively. Animals checked daily for their tumor development. The day when a ~50 mm³ palpable tumor was observed was considered as the "day 0". Three doses of 50 µg of pIC, R848, CpG ODN and their single, double or triple nanocomplexes with PS4 (1:1, weight ratio) were injected peritumorally with one day-on and one day-off treatment starting from the day of palpable tumor formation. Same injection was performed for

HuH-7 xenografted nude mice, with 100 µg of ODN420, 1466 CpG ODN. Tumor volumes were recorded daily as width, height and length by a caliper and reported as mm³.

2.2.12 Size measurement studies of the nanocomplexes

PS4 and their nanocomplexes were diluted in 1x PBS and particle size analyses of the generated nanocomplexes were measured using dynamic light scattering method on a Zetasizer (Model: Nano ZS, Malvern, UK).

Chapter 3

Results

The present thesis is an extension of previously established observations with regard to immunostimulatory properties of mushroom derived β -glucan based different polysaccharides (Tincer, 2007). In that study, four different polysaccharides (extracted and purified) from different mushrooms were used as standalone immunostimulatory nanocomplexes either alone or in combination with different TLR ligands of nucleic acids (namely, TLR3, 7, and 9 for dsRNA, ssRNA, and ss/dsDNA, respectively). However, at the end of that study, we have not determined the potential mechanism of action of these PSs, as well as their potential binding site/location that may facilitate their intracellular entry or mediate initiation of signaling cascade of responsive cells. Furthermore, the broad immunotherapeutic spectra of these novel nanocomplexes were not established up to their full potential at the end of previous work.

Therefore, substantial amount of work in this thesis was planned to be dedicated to unearth these issues and establish the breadth of action of these PS molecules for immunotherapeutic applications.

3.1 Mechanism of action of mushroom derived polysaccharides

3.1.1 PS4 isolated from *Ganoderma lucidum* directly binds to TLR2

In our previous studies we showed that natural polysaccharides purified from mushrooms induces NF- κ B mediated immune activity mainly via TLR2 in a model cell line that expresses TLR2 but not TLR4 or any other TLRs (Tincer, 2007; Tincer *et al.*, 2011). However, in the literature, there were contradicting reports describing that different β -Glucan based PSs may signal through either via different extracellular or intracellular receptors. These includes different TLRs (TLR2, 4, or even 6), as well as other cell surface receptors such as Dectins or even cytosolic receptors such as NODs (Batbayar *et al.*, 2011; Kikkert *et al.*, 2007; Yadav and Schorey, 2006).

In order to delineate which receptor(s) bind to PSs, we embarked on a systematic investigation that involves candidate immune related gene knockout mice and attempted to study the alterations in immune activity mediated by different β -Glucan polymers that were isolated from mushrooms.

To further confirm our previous observation on TLR2, polysaccharides, zymosan and PGN were immobilized on a solid plastic surface, and binding affinity of rTLR2 was analyzed. As seen in Figure 3.1, direct interaction between TLR2 and PSs was obtained following an ELISA-based binding assay with recombinant TLR2 (rTLR) protein. This data strongly confirmed our previous finding (Tincer *et al.*, 2011). In contrast to PS1, PS2 and PS3, only PS4 (10 μ g/ml) displayed a very strong and significant binding with rTLR2. This interaction is stronger than the classic TLR2 agonists that were used as positive control during the assay (i.e. zymosan and PGN).

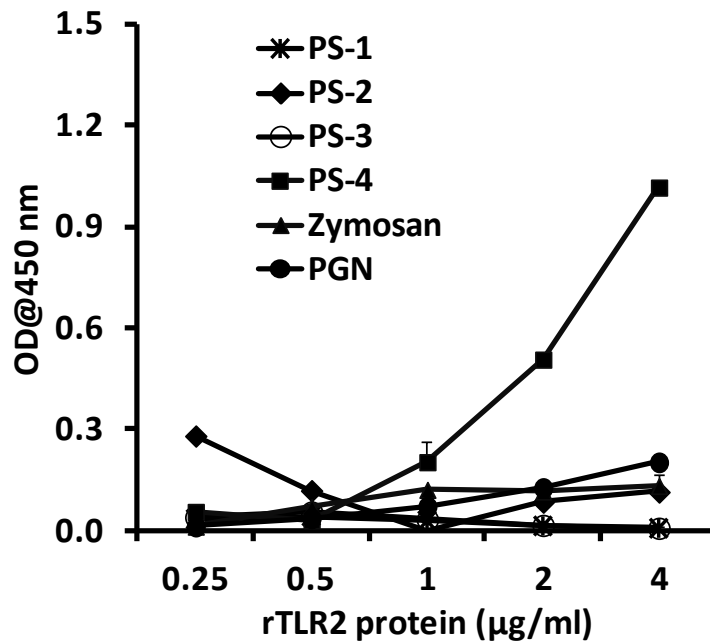


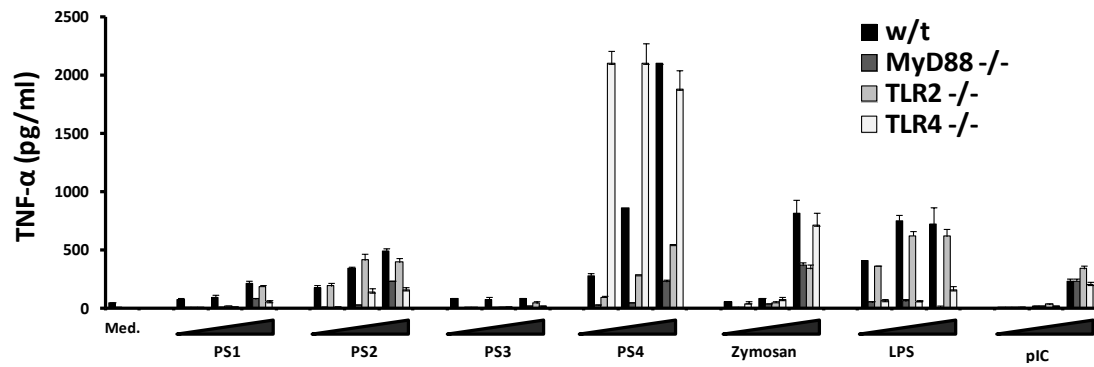
Figure 3.1. Detection of direct PS4-TLR2 interaction by binding ELISA. PS1–PS4, zymosan and PGN were coated (10 μg/ml) on ELISA plates and specific binding of recombinant TLR2 protein was detected at 450 nm. Results of two independent experiments were shown. PS4 vs. other groups' comparison gave $p < 0.005$ at 2 μg/ml, 4 μg/ml rTLR.

3.1.2 PS4 acts through TLR2 and requires MyD88 adapter protein

Although there are exemptions to this, mostly TLR-mediated signaling depend on MyD88 adapter molecule, furthermore, several PSs containing β-glucan chains are implicated to use TLR4 for signaling to initiate. In order to uncover these, another set of experiment was designed where wild-type (black bars), MyD88-deficient (dark grey bars), TLR2-deficient (light grey bars), and TLR4-deficient (white bars) C57/BL6 mice were used side by side to differentiate PS-dependent activities. PEC macrophages isolated from 4 days post-thioglycollate treated mice were either incubated with different doses of PSs (10, 1 and 0.1 μg) or zymosan (a conventional TLR2/MyD88-dependent agonists) and LPS (a TLR4 ligand) showed significantly decreased or almost complete regression of TNF-α or IL-6 cytokine production after 24h incubation in contrast to MyD88-independent TLR3 ligand pIC treated groups (Fig. 3.2). Neither PS2 nor PS4 were able to produce appreciable amounts of IL-6 and TNF-α from TLR2 and MyD88-

deficient PECs (Fig. 3.2). Importantly, these PSs when incubated with TLR4^{-/-} macrophages, they induced strong TNF- α (Fig. 3.2A) and IL-6 (Fig. 3.2B) production. Interestingly, compared to w/t mice, at 0.01 and 1.0 $\mu\text{g/ml}$ PS4 doses. Unexpectedly these cytokine responses were significantly higher in TLR4-deficient mice upon PS4 stimulation than w/t mice. These data, suggests that TLR4 is not involved in the initiation of immune cascade but PS4 mediated activation is strictly dependent on TLR2/MyD88 axis.

A)



B)

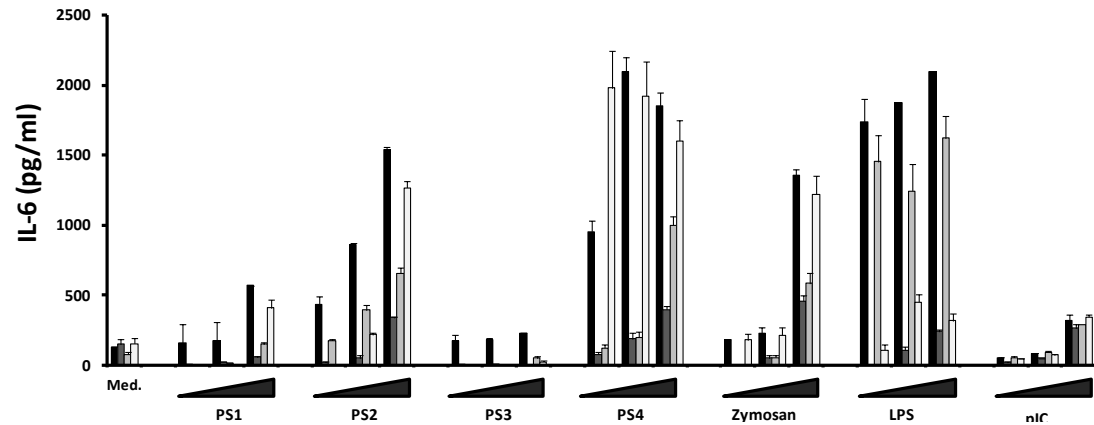
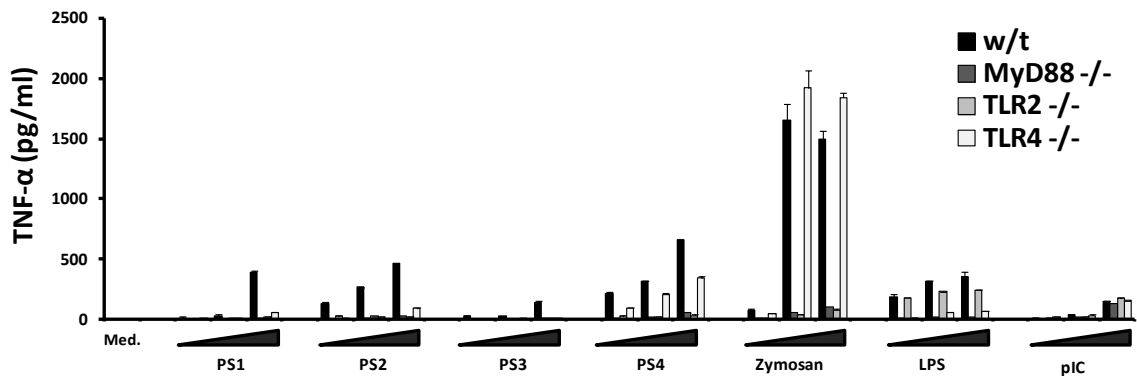


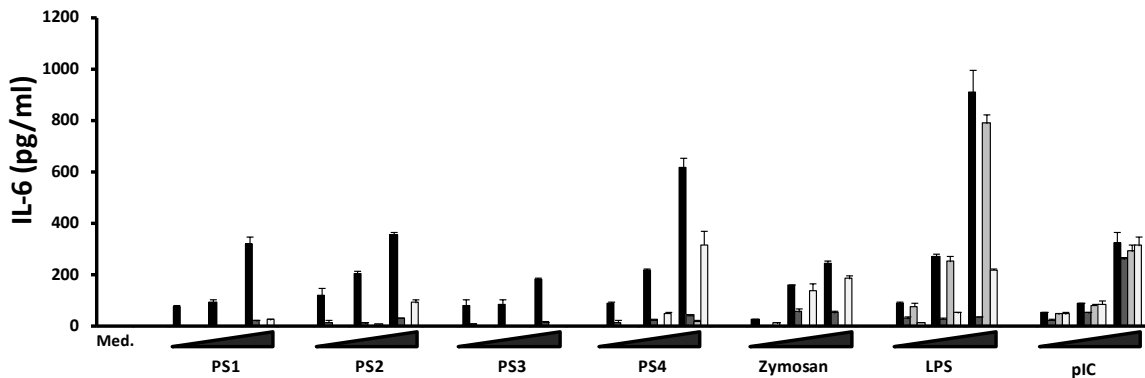
Figure 3.2. TLR2 and MyD88-dependent immune response of PSs from peritoneal macrophages. ELISA result of (A) TNF- α and (B) IL-6 production from w/t, MyD88^{-/-}, TLR2^{-/-} and TLR4^{-/-} macrophages ($1 \times 10^6/\text{ml}$) after 24h induction with PSs (0.1-10 $\mu\text{g/ml}$, 10x dil.), zymosan (0.1-10 $\mu\text{g/ml}$, 10x dil.), LPS (0.01-1 $\mu\text{g/ml}$, 10x dil.) and pIC (1-100 $\mu\text{g/ml}$, 10x dil.). Data represents combination of at least three independent experiments (mean $\pm$ SEM) of triplicate samples treated with indicated stimulants. $p < 0.005$, w/t versus TLR2^{-/-} or MyD88^{-/-} mice upon PS4 treatment.

After stimulation of splenocytes with PSs in dose-dependent manner, similar to what was seen for PECs, production of TNF- α (Fig. 3.3A), IL-6 (Fig. 3.3B) and IFN- γ (Fig. 3.3C) were defective in TLR2^{-/-} and MyD88^{-/-} mice, but did not significantly differ in TLR4^{-/-} mice. As expected, positive controls zymosan (TLR2/6 agonist) and LPS (TLR4 agonist) induced low or undetectable amounts of cytokines in TLR2-deficient and TLR4-deficient splenocytes, respectively.

A)



B)



C)

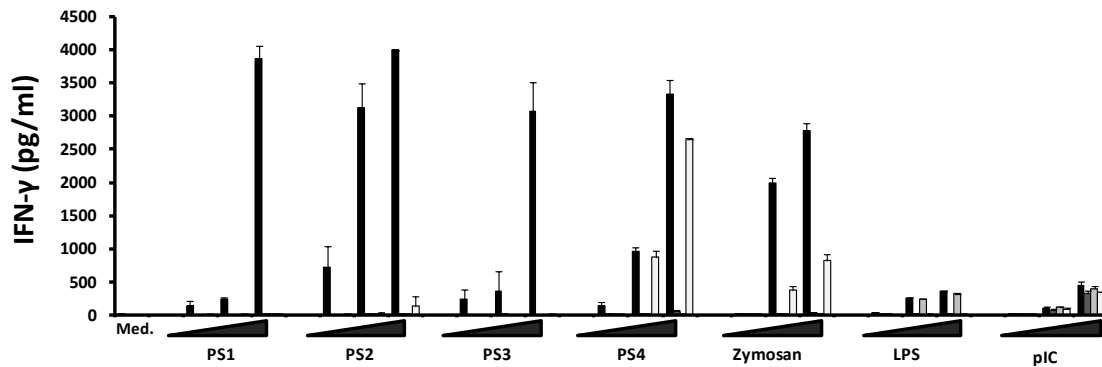


Figure 3.3. Cytokine production from wild type, TLR2^{-/-}, TLR4^{-/-}, and MyD88^{-/-} mice splenocytes. (A) TNF- α , (B) IL-6 and (C) IFN- γ induction from w/t (black bars), MyD88^{-/-} (dark grey bars), TLR2^{-/-}, (light grey bars), and TLR4^{-/-} (white bars), spleen cells stimulated for 42h (4×10^6 /ml) with PS1–PS4, zymosan, LPS, and pIC (amounts were given in Fig 3.2) were shown. Results of two independent experiments gave similar trends and is expressed as Ave $\pm$ SEM. $p < 0.001$ at all concentrations of PS1–PS4 when w/t vs. either TLR2 or TLR4 deficient splenocyte comparisons were analyzed.

3.1.3 Immune cell activation by polysaccharides are Dectin-1 receptor independent

Recent findings suggested that β -glucan based PS biopolymers may activate immune cells via their Dectin-1 or 2 receptors (C type-lectins) as they are expressed by most antigen presenting cells. To test, whether polysaccharides extracted from mushrooms also triggers through Dectin-1 we isolated i) splenocytes, ii) PECs, iii) GM-CSF derived conventional myeloid DCs (mDCs) and iv) FLT3 ligand induced plasmacytoid DCs (pDCs) from w/t and Dectin-1^{-/-} mice and incubated with our four PSs. Of note, two conventional Dectin-1 ligands, i) curdlan (a β -1,3-glucan) or ii) depleted zymosan (hot alkali treated zymosan) were included in these assays as positive controls and PGN a Dectin-1 independent ligand was used as a negative control. As seen in Figure 3.4, PS2 and PS4, in a dose dependent manner (0.1, 1, 10 μ g/ml) induced TNF- α production both in wild type and Dectin-1 deficient mice splenocytes. In contrast, depleted zymosan did not induce any detectable TNF- α from k/o mice splenocytes even at the highest dose, as expected. A Dectin-1 independent ligand PGN, led to similar TNF- α from both Dectin-1

k/o and wt mice spleen cells. TNF- α , IL-6 and IL-12 productions of Dectin-1 deficient PECs which were stimulated with PSs slightly increased or did not change cytokine profiles to that of curdlan responses (Fig. 3.5 A,B,C). Notably, in those results PS4 was the most active stimulant at doses greater or equal to 0.1 μ g/ml. Next, GM-CSF treated bone marrow derived DCs (i.e. GMDCs) at day 7 were stimulated with PSs, zymosan and curdlan. Consistent with previous results seen from splenocytes and PECs no decrease in the levels of IL-6 and IL-12 were seen in Dectin-1 deficient mice GMDCs upon PS treatments. Again, PS4 was the most effective PS among all tested PSs (Figure 3.6). PS2 and PS4 were not effective on Dectin-1^{-/-} FLDCs since these PSs did not yield any IL-6 and IL-12 production (Figure 3.7). In conclusion, data from these set of experiments implicated that PS4 is not a Dectin-1 ligand and does not contribute to immune activation via this receptor.

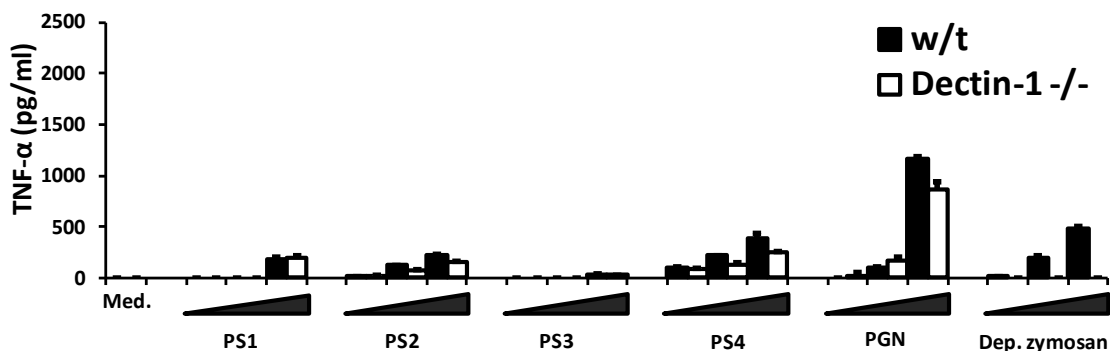
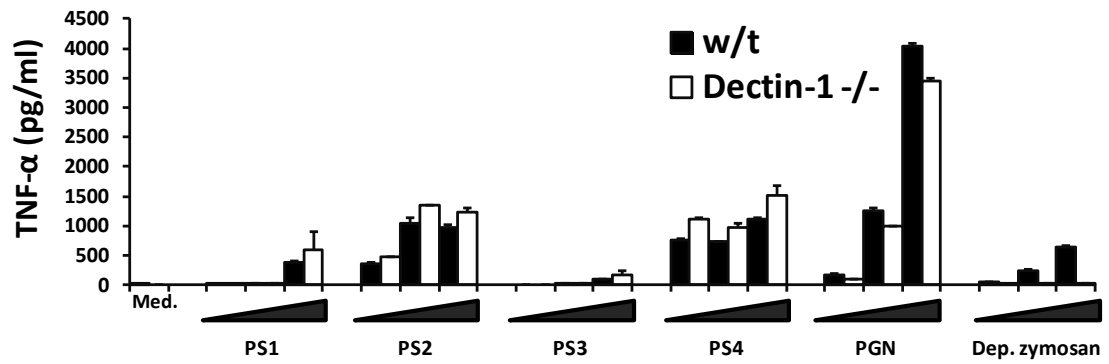
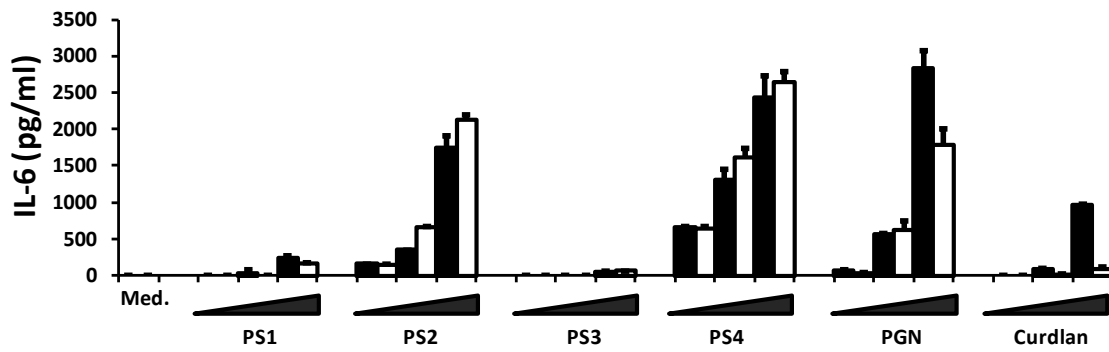


Figure 3.4. Polysaccharides act upon Dectin-1 independent immune response. TNF- α production from w/t or Dectin-1 deficient mice splenocytes (1×10^6 /well) were incubated with PS1-4, PGN (0.1–10 μ g/ml) and depleted zymosan (1–100 μ g/ml) in dose dependent manner for 36 hours. Result represents combination of at least three independent experiments (mean $\pm$ SEM) of triplicate samples. $p < 0.004$ PS1–PS4 and PGN at high doses compared to depleted zymosan.

A)



B)



C)

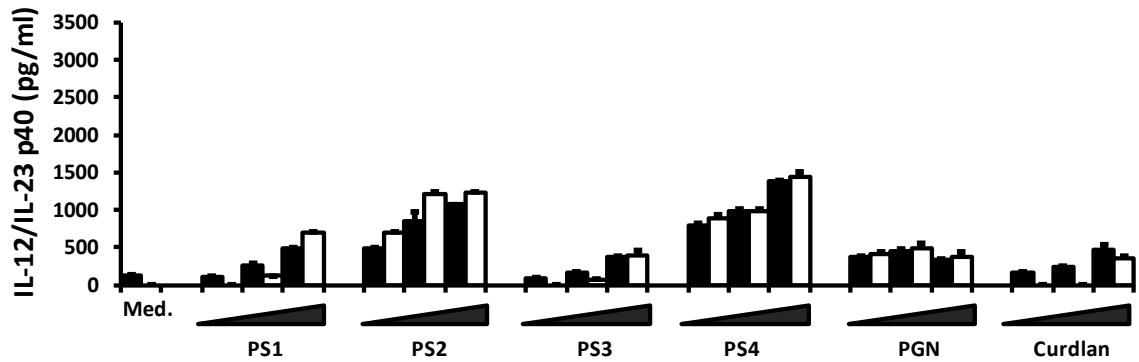


Figure 3.5. Dectin-1 independent production of IL-6 (A) and TNF- α (B) and IL-12/IL-23p40 (C) from w/t and Dectin-1^{-/-} PECs (1.25×10^6 /ml) after 24h stimulation with polysaccharides, PGN (0.1–10 μ g/ml, 10x dil.) and curdlan (1–100 μ g/ml, 10x dil.) detected via cytokine ELISA. Result represents combination of at least three independent experiments (mean $\pm$ SEM) of triplicate samples. PS4 vs curdlan gave $p < 0.001$ at TNF- α , $p < 0.002$ for IL-6 and IL-12 (at 0.1 and 1 μ g/ml doses) in Dectin1^{-/-} cells.

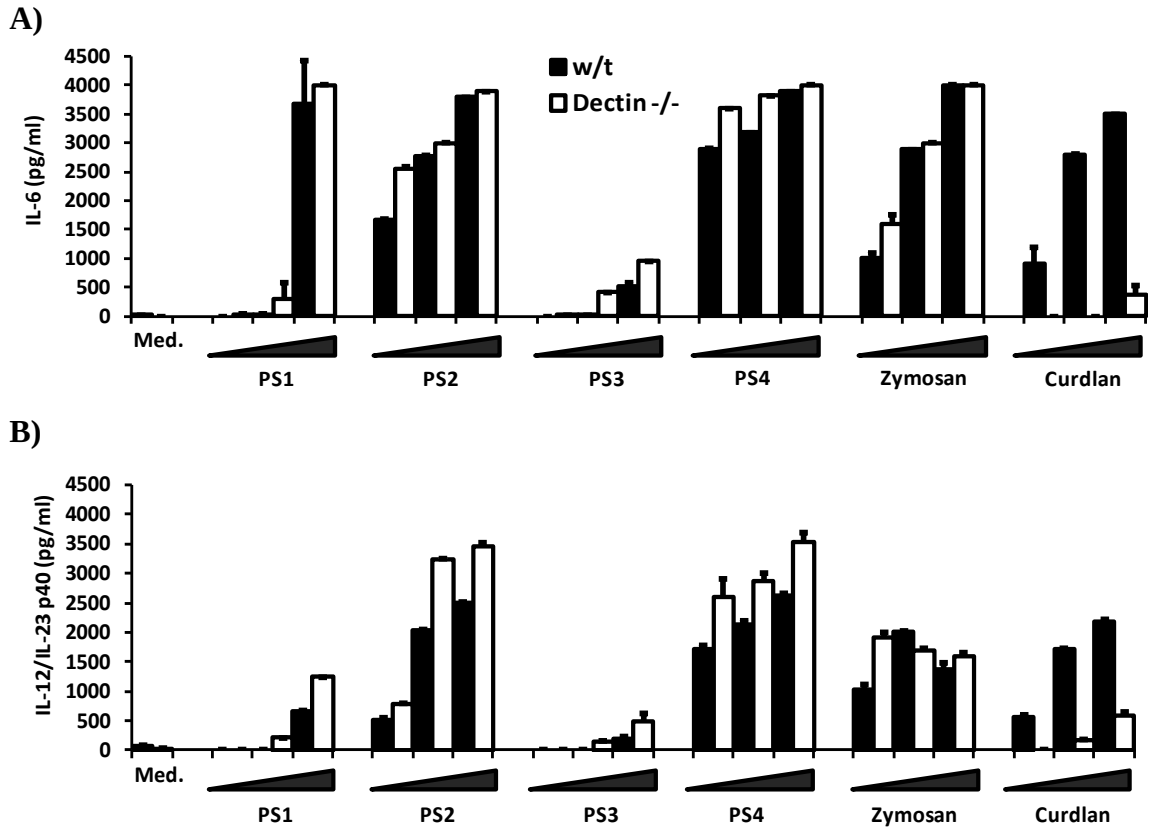
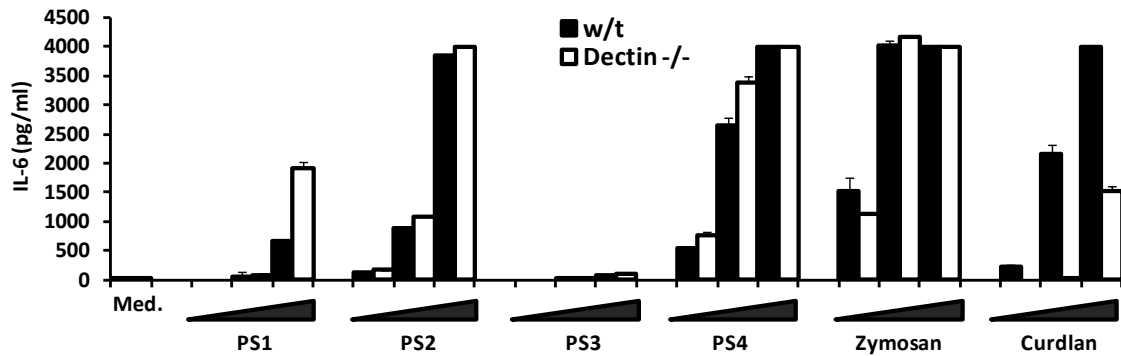


Figure 3.6. Dectin-1 was not required for the immune activation upon PSs by GMDC. GMCSF (5×10^5 /ml) treated bone marrow derived DCs were left untreated or either treated with PS1–4 (0.1–10 μ g/ml, 10x dil.), zymosan (0.1–10 μ g/ml, 10x dil.) and curdlan (1–100 μ g/ml, 10x dil.) for 24h. IL-6 (A) and IL-12/IL-23 p40 (B) cytokine production was assessed by ELISA. Data shows the mean $\pm$ SEM of triplicate wells which were the representative of three independent experiments. PS4 vs curdlan gave $p < 0.002$ for IL-6 and $p < 0.003$ IL-12 (at all doses) in Dectin1^{-/-} cells.

A)



B)

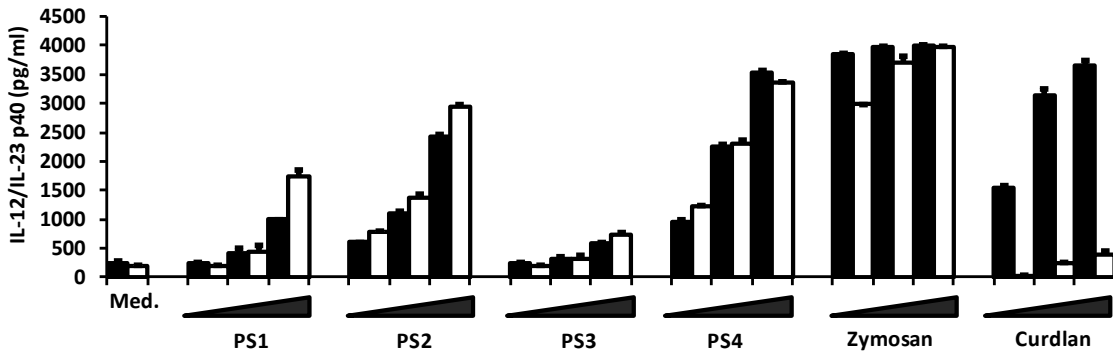


Figure 3.7. FLDCs derived from bone marrow of Dectin-1^{-/-} mice didn't show any difference in (A) IL-6 and (B) IL-12/IL-23p40 cytokine induction when stimulated with PSs and zymosan (0.1-10 μ g/ml, 10x dil.), in contrast to curdlan (1-100 μ g/ml, 10x dil.) for 24 hrs (1×10^5 /ml FLDCs in 96 well plates). Data were representative of combination of at least three separate experiments (mean $\pm$ SEM) of triplicate samples. PS4 vs curdlan gave $p < 0.006$ for IL-6 and $p < 0.002$ IL-12 (at all doses) in Dectin1^{-/-} cells.

Table 3.1 summarizes the PS mediated immune activation parameters from different immune cells of wild-type and k/o mice.

Table 3.1. Immunological features of different polysaccharides in splenocytes, PECs, GMDCs and FLDCs from wild-type and k/o mice.

Cell Types Stimulants	DECTIN-1 DEPENDENCY				TLR-2 DEPENDENCY		TLR-4 DEPENDENCY	
	Splenocytes	PEC	GMDC	FLDC	Splenocytes	PEC	Splenocytes	PEC
PS1	—	—	—	—	+	+ ^a	+	+ ^b
PS2	—	—	—	—	+	+ ^a	+	+ ^b
PS3	—	—	—	—	+	+	+	+
PS4	—	—	—	—	+	+	—	—
PGN	—	—	NT	NT	NT	NT	NT	NT
Zymosan	NT	NT	—	—	+	+	—	—
Depleted zymosan	+	+	+	NT	NT	NT	NT	NT
Curdian	NT	+	NT	+	NT	NT	NT	NT
LPS	NT	NT	NT	NT	—	—	+	+

“—”, for all tested cytokines, “+”, for all tested cytokines, +^a, positive only for IL-6, +^b, positive only for TNF- α , NT, Not-tested

As discussed in preceding sections, in this study, the aim was to develop a PS4/TLR ligand nanocomplexes and characterize synergistic effects between PS and TLRL. In this context, we tested the Dectin-1 dependency for the recognition or signaling initiation of immune cells upon PS-TLRL nanocomplex stimulation. These results were presented in Appendix C Section and were covered here. As before, splenocytes, peritoneal exudates cells, GMDCs and FLDCs from wild type and Dectin-1 deficient BL/6 mice were harvested, incubated with varying doses of PS4/TLRL nanocomplexes and assessed for TNF- α and IL-6 induction capacities. Upon dose dependent stimulation of i) PS4-pIC, ii) PS4-R848, and iii) PS4-CpG ODN nanocomplexes, a synergistic dose dependent IL-6 production upon PS-CpG ODN nanocomplex treatment was observed from spleen cells (please see Appendix Bi). TNF- α secretion was slightly increased in PS-R848 and PS4-CpG ODN groups compared to PS4 alone treatment (please see Appendix Bii). In PEC studies, however, PS-R848 and PS-CpG ODN were the most effective formulations since they led to a significant boost in TNF- α and IL-6 secretion

(even at the lowest dose 0.24 $\mu\text{g/ml}$). PS-pIC nanocomplex induced IL-6 secretion from w/t macrophages especially at 1.2 $\mu\text{g/ml}$ and its 5x diluted concentration, but there was no TNF- α production by this nanocomplex (please see Appendix Biii and Biv). In GMDCs and FLDCs studies, data revealed that PS-R848 nanocomplexes induced very high IL-6 levels at all doses. PS-CpG ODN group show synergistic effect for IL-6 production at higher concentrations on FL-dendritic cells (please see Appendix Bv, and Bvi). Interestingly, upon PS-pIC nanocomplex stimulation on all immune cell types there was no significant improvement in TNF- α production. Of note, in all these studies, there were no differences in the cytokine productions between w/t vs Dectin-1 knockout mice cells for all tested nanocomplex groups. Once again these results suggests that Dectin-1 is not necessary for the PS4-derived nanocomplex recognition/signaling by immune cells.

3.1.4 Inflammasome-dependent mechanisms were involved in PS mediated immune response

Next we analyzed IL-1 β production from macrophages when incubated with β -glucan polymers and delineated the involvement of inflammasome related mechanisms. Peritoneal macrophages collected from w/t and i) ASC, ii) caspase-1, and iii) Nalp3-deficient mice were either primed with LPS or left untreated and then stimulated with PSs and Alum (as positive control) for total 24 hours. The supernatants were assayed for IL-1 β production. To our surprise, PS2 and PS4 (at 1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ doses) induced significantly higher IL-1 β production than PS1 and PS3 in macrophages from wild-type mice. But when ASC- and caspase-1- deficient macrophages were stimulated with PS and Alum, IL-1 β induction was completely abolished. Macrophages from Nalp3-deficient mice showed partial reduction of IL-1 β release in the presence of PS2 and PS4 (Fig. 3.8A). Interestingly, LPS priming led to a decreased IL-1 β release from normal mice in response to PS and complete regression in Nalp 3^{-/-} mice macrophages (Fig. 3.8B). IL-1 β production especially by PS was ASC and caspase-1 dependent but

partially Nalp-3 dependent, which could be concluded that other inflammasome multiprotein complexes rather than NLRP-3 could also be responsible from PS-mediated inflammasome activation.

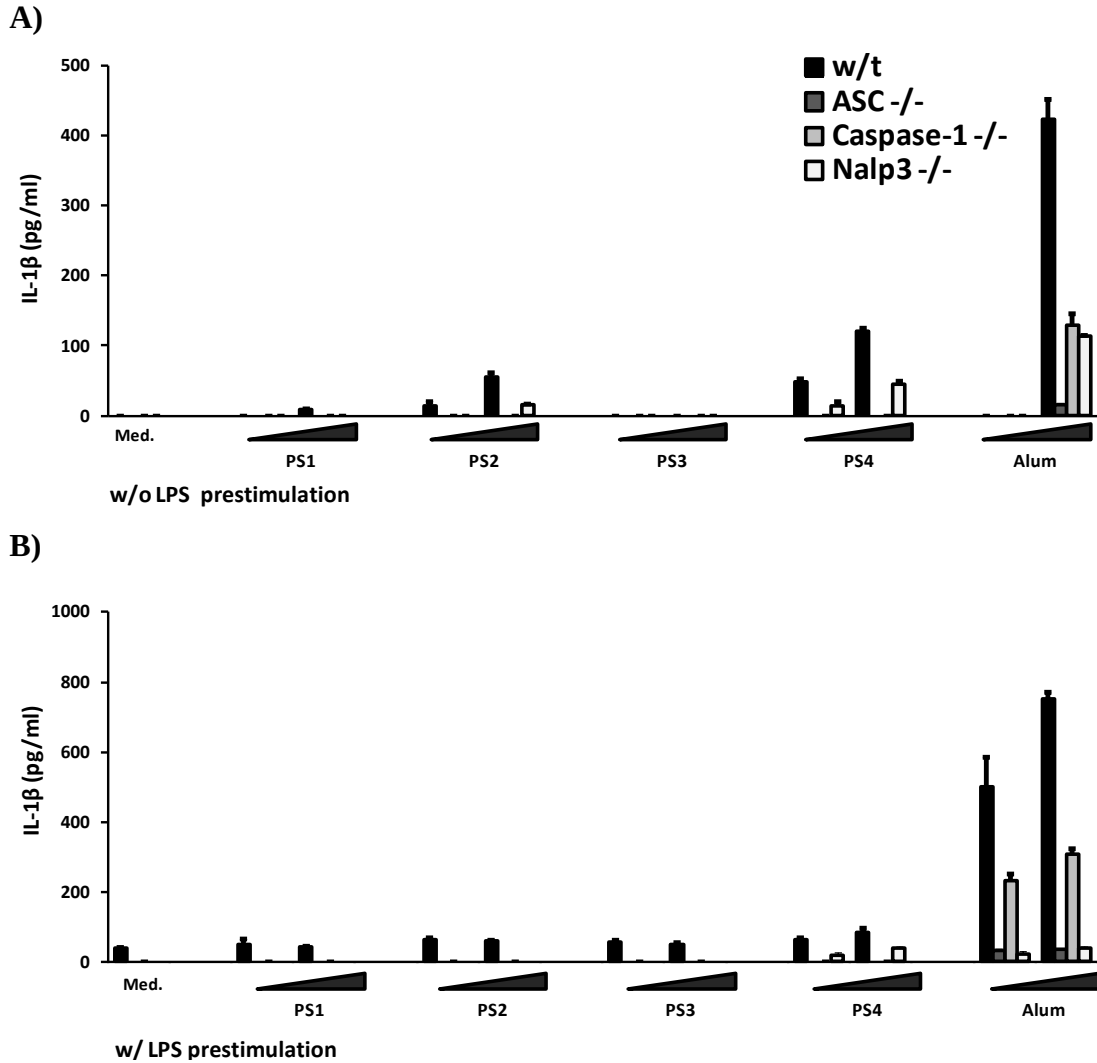


Figure 3.8. Inflammasome activation by PSs governs IL-1 β production from mouse PECs. Quantification of IL-1 β production (A) without LPS pre-stimulation and (B) with 6h LPS (50 ng/ml)-priming prior to stimulation of PECs with PSs (1 μ g/ml and 10 μ g/ml) and as positive control with Alum (50 μ g/ml and 500 μ g/ml) for 24h. Representative plots of three independent experiments are shown. $p < 0.001$ between w/t and knock-out mice macrophages except Nalp-3^{-/-} in PS1–4 groups.

3.1.5 Polysaccharides induce immunomodulatory IL-10 cytokine from murine spleen cells

In order to evaluate whether mushroom derived polysaccharides induces any Th2 biased or immunomodulatory cytokines such as IL-4, IL-5, IL-13 or IL-10 from BL/6 splenocytes, PSs were incubated for 48h and supernatants were assayed by ELISA. There were no remarkable IL-4, IL-5 or IL-13 productions from spleen cells upon PS1–4 stimulation even at 10 μ g/ml doses (data not shown). In brief, PS1 and PS3 did not induce any IL-10 production, but PS2 and more potently, PS4 led to a dose-dependent IL-10 secretion from spleen cells (Figure 3.9). Besides with its anti-inflammatory properties such as, the inhibition of the expression of proinflammatory cytokines, chemokines, and chemokine receptors, downregulating the expression of MHC class II and costimulatory molecules, IL-10 also induces the survival, proliferation, and differentiation of B cells. It is also reported elsewhere that it regulates establishment of Treg effector population (Akdis *et al.*, 2011). Therefore, surprisingly, our data pointed that PS4 could modulate establishment of a later phase of the immune homeostasis by inducing IL-10 production, which may then contribute to the down-regulation of the existed inflammatory response.

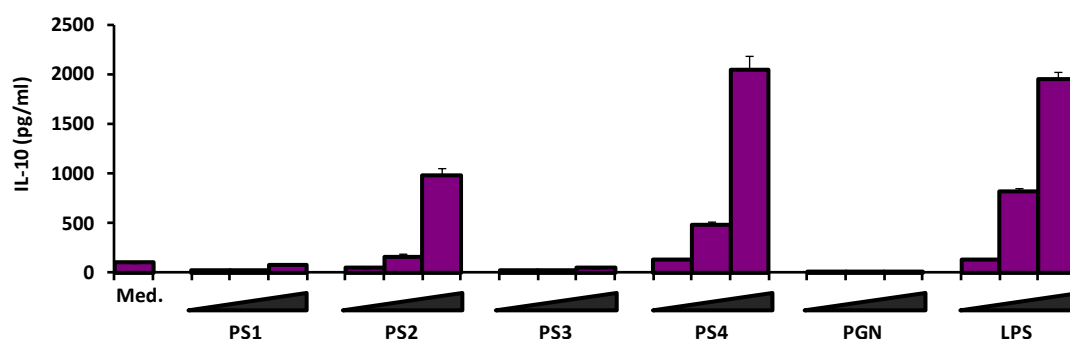
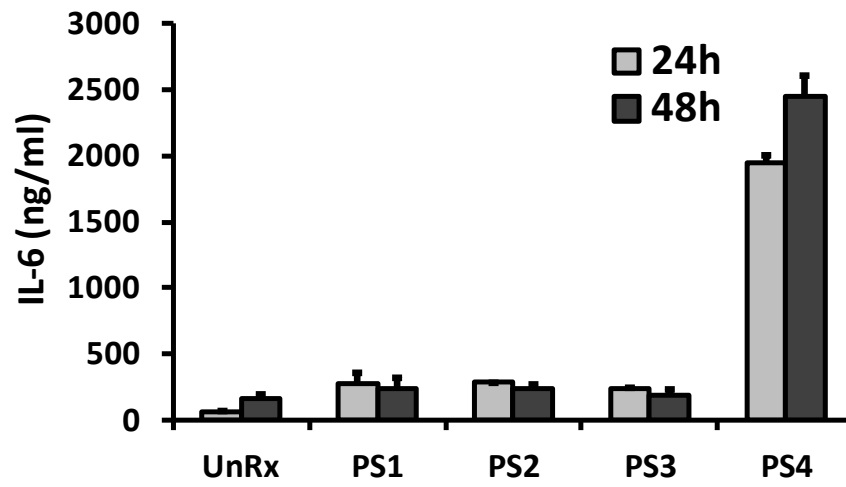


Figure 3.9. IL-10 production from splenocytes after dose dependent PS treatment. Sandwich ELISA was studied from w/t C57BL/6 spleen cells (5×10^6 /ml) supernatants after PS1–4 and PGN (0.1-10 μ g/ml, 10x dil.), or LPS (0.01-1 μ g/ml, 10x dil.) stimulation for 48 hrs. Data shown were from triplicate samples of at least two independent experiments. PS2 and PS4 vs medium alone gave $p < 0.003$ at high dose.

3.2 In vivo activity of PSs in mice

Although improved *in vitro* activity is important, we felt that this superior behavior of the PS4 nanocomplexes must be reproduced in *in vivo*, before using any of them for therapeutic applications in animal disease models. For that, mice were injected with PS1–4 in 0.2, 2, 20 µg/mice and 6h later peritoneal exudate cells and spleen cells were collected and incubated *ex vivo* without further stimulation for the evaluation of cytokine or chemokine production and transcriptional regulation by ELISA and RT-PCR, respectively. Similar to our previous *in vitro* data, PS4 was the most active stimulant among other studied PSs. It yielded the highest IL-6 production from PECs after 24h and 48h *ex vivo* incubation (Fig. 3.10A) at 2µg dose. The spleen cells` TNF- α and IP-10 transcript levels were significantly upregulated when PS1, 2, and 3 versus PS4 band intensities were compared (Fig. 3.10B), in various concentrations. PS4 was also upregulated the cytokine and chemokine gene expression profile even at the lowest dose 0.2 µg when injected *in vivo*. In our previous results, PS2 activity was comparable with PS4, however it failed to reproduce this trend when administered *in vivo*. When accumulated *in vitro* and *in vivo* data were taken together, of these four different PS candidates, PS4 repeatedly displayed the highest immunostimulatory performance, thus it was selected to PS4-TLR ligand nanocomplex development.

A)



B)

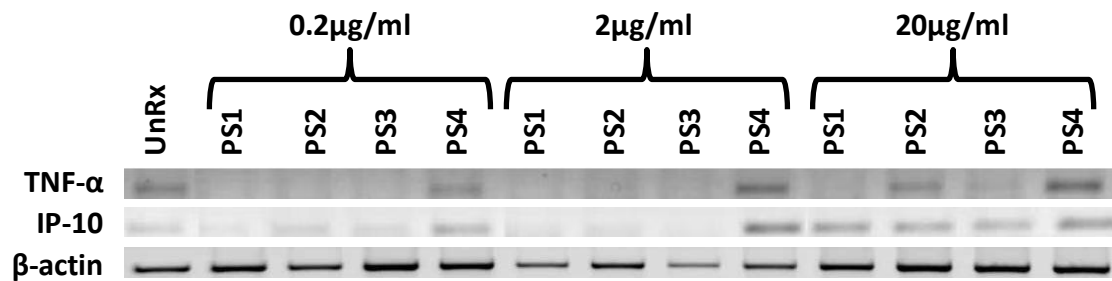


Figure 3.10. Immunostimulatory properties of polysaccharides *in vivo*. (A) Six hours after i.p. PS injection (2 μg/ml), PECs were isolated (3 mice/group). Cells (4×10^6 /ml) were extra incubated ex vivo for 24h and 48h. IL-6 production were assessed by cytokine ELISA. (B) mRNA levels of TNF-α and IP10 were assessed by RT-PCR from spleen cells. PS4 vs unrx gave $p < 0.002$ after 24h and 48h ex vivo incubation.

3.3 Generation and physical characterization of PS nanocomplexes

When nucleic acids were injected *in vivo*, they were either subjected to premature digestion by nucleases or adsorbed onto serum proteins, hampering their bioavailability significantly. Moreover, previous studies indicated that human clinical trials of these ligands were limited due to their labile nature. Therefore, strategies aiming to improve their stability as well as attempts to avoid reducing their premature clearance and improve their internalization via target cells was of great importance to pursue these ligands into clinic.

Initial analyses were designed to investigate whether these complexes formed stable nanoparticles. For this, dynamic light scattering technique (DLS, Zetasizer Nano, ZS) was employed. Zetasizer results revealed that PS (as of PS4) appeared as individual nanoparticles around 40 ± 20 nm in diameter (Ave $\pm$ SEM) (Fig. 3.11A). PS nanocomplexed with pIC led to an increase in the nanoparticle size from 40 nm to 165 nm (Fig. 3.11B). PS(CpG ODN) nanocomplex was also studied by DLS. When a 20mer CpG ODN formed nanocomplex with PS, it was found to be around 190 ± 10 nm in diameter (Fig. 3.11C). The stability in solution was also studied and found that no significant change in the sizes of the generated nanocomplexes over a period of two weeks. These results indicated that there was no spontaneous fusion (or disintegration) of the individual particles when suspended either in PBS or in FBS supplemented cell culture media (Table 3.2).

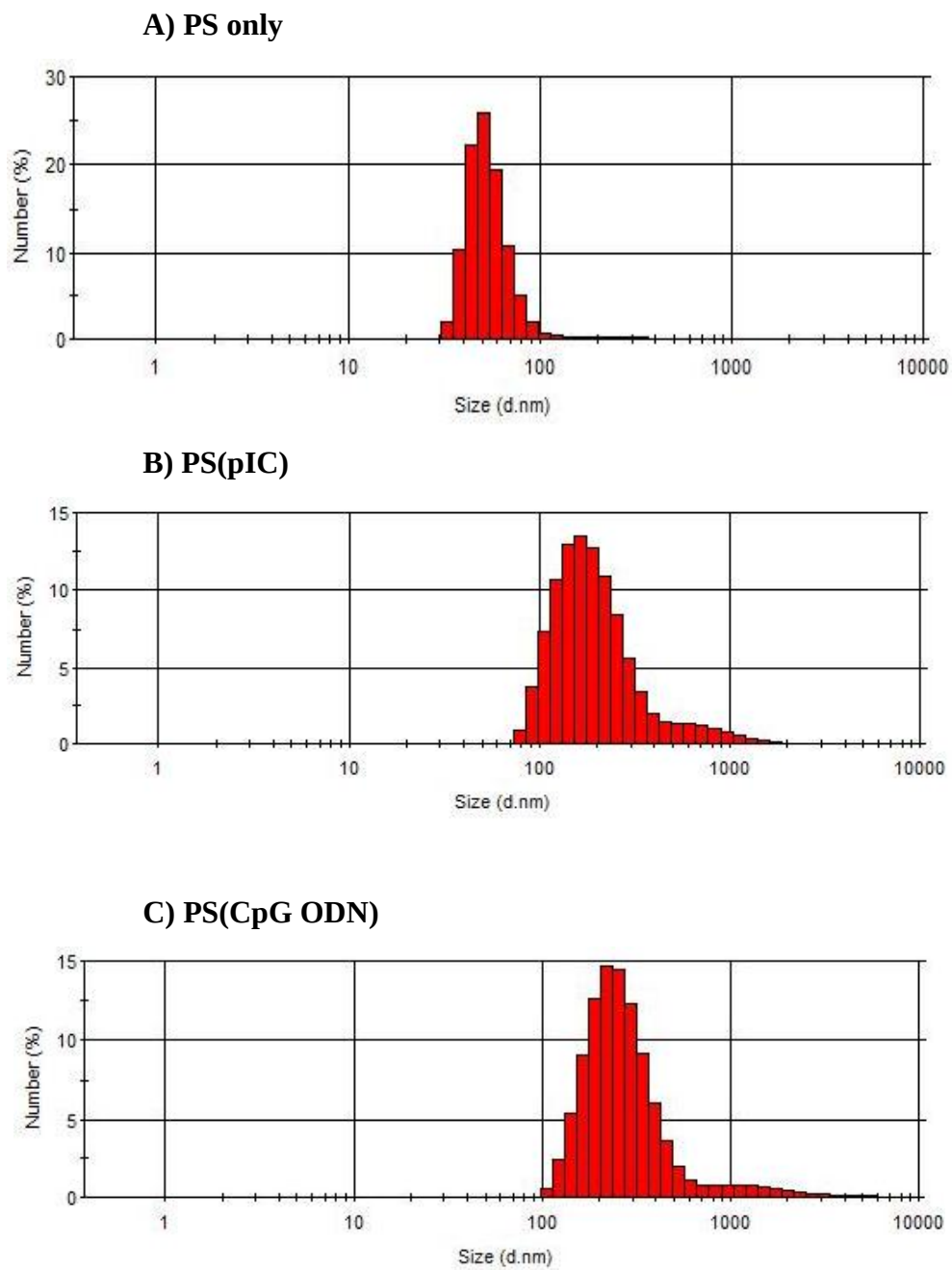


Figure 3.11. Size distribution histograms of (A) PS, (B) PS(pIC), and (C) PS(CpG ODN) nanocomplexes.

Table 3.2. Size distribution analyses of PS4 containing nanoparticles after 0, 7 and 14 days.

Type	Size (nm), Ave $\pm$ SEM (days after complexes were formed)		
	Day 0	Day 7	Day 14
PS	40 $\pm$ 20	50 $\pm$ 15	55 $\pm$ 20
PS(pIC)	165 $\pm$ 30	170 $\pm$ 35	185 $\pm$ 35
PS(CpG ODN)	190 $\pm$ 10	195 $\pm$ 15	200 $\pm$ 20

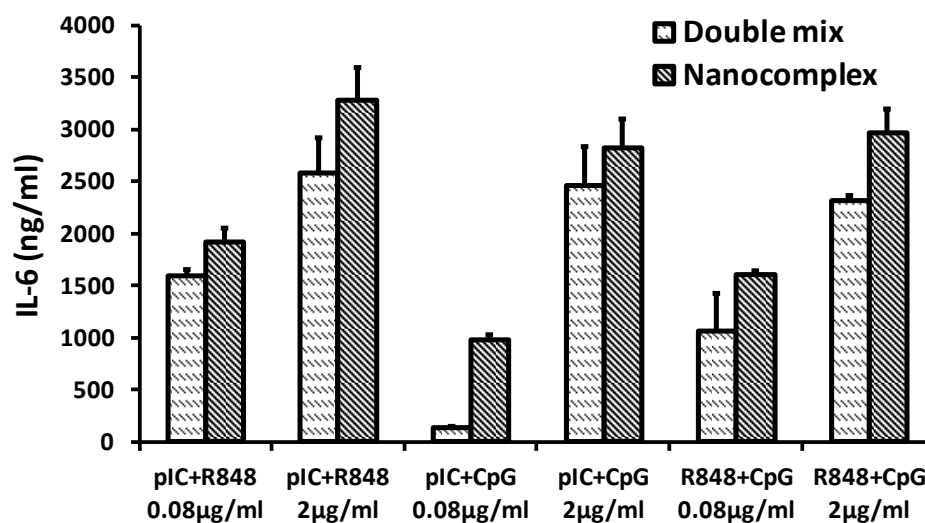
3.4 Double or triple nanocomplexes with PS4 enhances IL-6 production from mouse splenocytes

One of the major goals of this study was to determine the most potent PS type and apply it as a nucleic acid carrier preferably displaying synergistic immunological activities suitable for immunotherapeutic applications. Previously, we have demonstrated that PS4 is inducing synergistic effects when nanocomplexed with one of the TLR3, 7, and 9 ligands. However, in that study, we did not prepared double or triple TLRL combinations and their nanocomplexes with PS4. Here, we tested their immunological performances on immune cells and aimed to improve benefit of nanocomplexation with PS4 in either double or triple combinations (complexed at 1:1 weight ratios with pIC (TLR3L), R848 (TLR7L), and CpG ODN (TLR9L).

Mouse spleen cells were incubated either with PS4 alone or nano-complexed combinations for 48 hours and IL-6 induction was assayed by ELISA and was found to be significantly increased in nanocomplex formulations in a dose dependent manner (Fig. 3.12). When pIC+CpG ODN was nanocomplexed with PS4 at low dose (0.08 μ g/ml each ligand) a robust synergistic IL-6 induction was observed (almost >9 fold increase compared to mixture of pIC+CpG ODN counterpart (Fig. 3.12A). Interestingly, in other combinations where R848 was used either as a mixture or as a nanocomplex, this trend was not that apparent although nanocomplexes gave much higher IL-6

production. When triple nanocomplex formulation (pIC/R848/CpG ODN; each @ 0.67 μ g) prepared with 2 μ g PS4 and tested for synergistic IL-6 enhancement, only at the lowest dose (equivalent to 0.027 μ g/ml of each ligand) a significant IL-6 production was measured, compared to triple mixture counterpart (Fig. 3.12B). At least 6x more IL-6 was produced by spleen cells at this concentration compared to triple ligands mixture. These results, indicated that the *in vivo* potency of the nanocomplexes of double or triple TLR ligands would be very much stronger than their mixtures. In these assays, it is important to note that PS4 is not only protecting the labile ligands from premature digestion/degradation, but also delivers these ligands much effectively than their free forms to target cell sites. Furthermore, PS4 itself contributes to the overall immune activation process since itself can trigger TLR2 mediated signaling cascade.

A)



B)

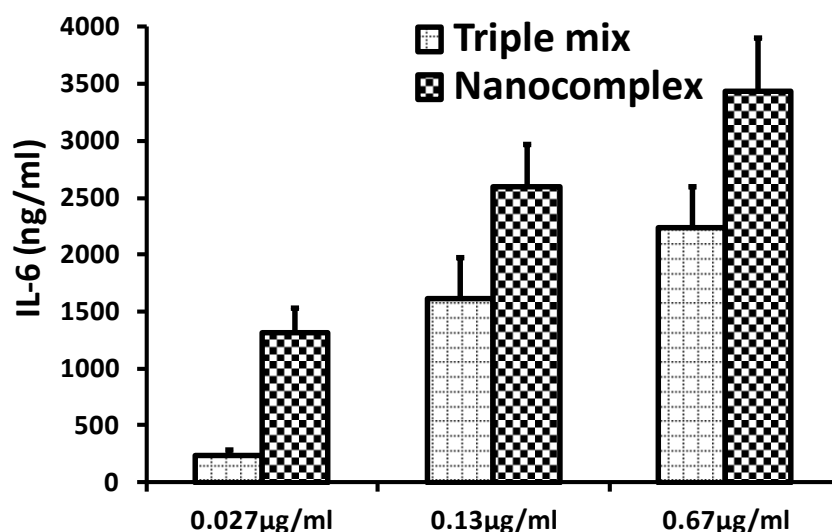


Figure 3.12. Nanocomplexes of double and triple combinations of nucleic acid TLR ligands have an additive effect on IL-6. Mouse splenocytes were incubated 1:1 (w/w) nanocomplexes of (A) double (i.e. 4µg PS + 2µg pIC + 2µg R848) (B) triple pIC, R848 and CpG ODN in dose-dependent manner for 48h. Experiments were ran on either double or triplicate repeats. $p < 0.003$ mix versus nanocomplex of pIC+CpG ODN (at 0.08 µg/ml). $p < 0.006$ mix versus nanocomplex of pIC+R848+CpG ODN (at 0.027 µg/ml).

3.5 Ability of PS to protect CpG ODN in nanocomplexes after DNase treatment

Improved immunoactivity of nanocomplexes could be mainly due to two reasons, i) improved internalization, or ii) improved ODN resistance against DNase attack. Since we did not have any labeled PS at hand, it was not possible to study internalization. However, DNase stability assays were performed either with free ODN or with PS nanocomplexed forms. CpG ODN and PS(CpG ODN) nanocomplexes (1:1, w:w ratio) were subjected to DNase I treatment for up to 20 mins. Next, murine splenocytes were stimulated with DNase I-treated CpG ODNs. IL-6 and IL-12 cytokine productions were checked and determined to be 100% protected (Figures 3.13A, B). with DNase I subjected PS(CpG ODN) (Fig 3.13C). Effect of DNase on the ODN digestion was

further analyzed by gel electrophoresis. As seen in figure 3.14, while free CpG ODN was completely digested after 20 mins DNase treatment, PS(CpG ODN) forms were found to be unaffected. This piece of data suggested that PS nanocomplexation protects labile ODN from DNase digestion and led to an improved immune activation.

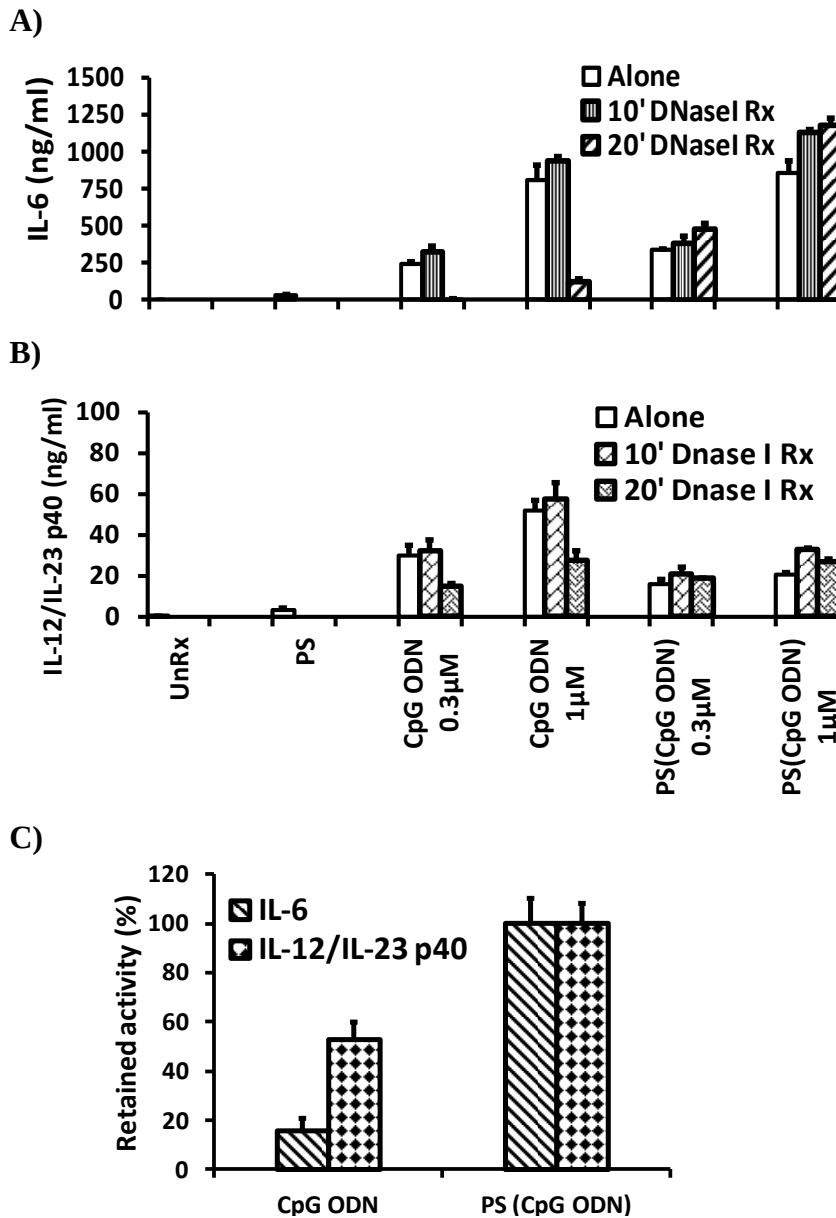


Figure 3.13. PS(CpG ODN) nanocomplexes were resistant to DNase I treatment. CpG ODN and PS(CpG ODN) nanocomplexes were treated with DNase I for 10min and 20min. Samples

(0.3 μ M and 1 μ M of CpG ODN) were used to stimulate splenocytes (4×10^6 /ml) for 24h. (A) IL-6 and (B) IL-12/IL23 p40 productions were checked with cytokine ELISA. (C) Percent retained activity of DNase digested (20min) 1 μ M of CpG ODN vs PS(CpG ODN) nanocomplex plot. $p < 0.0006$ for IL-6 level between DNase treated CpG ODN vs PS(CpG ODN) nanocomplexes (20 min).

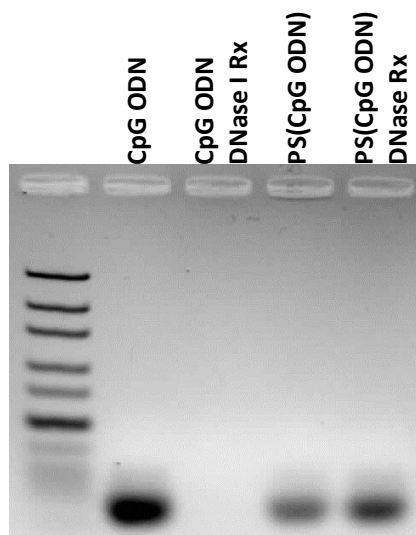


Figure 3.14. The agarose gel picture of CpG ODN, PS(CpG ODN) and their DNase treated samples. 2 μ g of CpG ODN for each lane was loaded into 4% agarose gel.

These results prompted us to test these nanocomplexes in different *in vivo* therapeutic settings.

3.6 In vivo immune activation of PS4-TLR ligand nanocomplexes

In earlier sections of this study, we showed that PS-nucleic acid TLR ligand (single, double and triple combinations) nanocomplexes induced synergistic immune activation. (Tincer, 2007; Tincer *et al.*, 2011). Next, *in vivo* effectiveness of these formulations were analyzed. Single and double combinations of PS4 and TLR ligands (pIC/R848/CpG ODN @ 1:1 w/w ratio, 15 μ g each) were i.p. injected and after 18h BALB/C splenocytes and PEC were harvested and incubated in the culture without any further stimulation to assess i) the cytokine production and ii) change in the chemokine/cytokine message transcript levels. PS4(R848+CpG ODN) nanocomplex led to almost ≥ 3 fold induction compared to PS4 alone and R848+CpG ODN alone

counterparts in IL-6 production (Figure 3.15A). Single nanocomplexes didn't show any enhancement in IL-6 secretion when compared with free forms of PS4 and pIC, R848 and CpG ODN. Although mixture of pIC+R848 or pIC+CpG ODN double combinations as evidenced by almost ≥ 10 fold increase in IL-6 cytokine level boosted response, this result was not reproduced in the nanocomplex formulations from PEC cells. IFN- γ production was parallel to these observations, again PS4(R848+CpG ODN) treated mice gave the best response over others. Contrary with the cytokine IL-6 ELISA result, IFN- γ production from both PS4(R848) and PS4(CpG ODN) PEC cells after 42hrs *ex vivo* incubation showed strong synergistic effect compared to alone group treatments (Figure 3.15B). Compared to PS4, pIC and R848 alone groups, spleen cell's transcript levels of IP-10 and IFN- γ were up-regulated in PS4(R848) and PS4(pIC) groups, respectively (Figure 3.16).

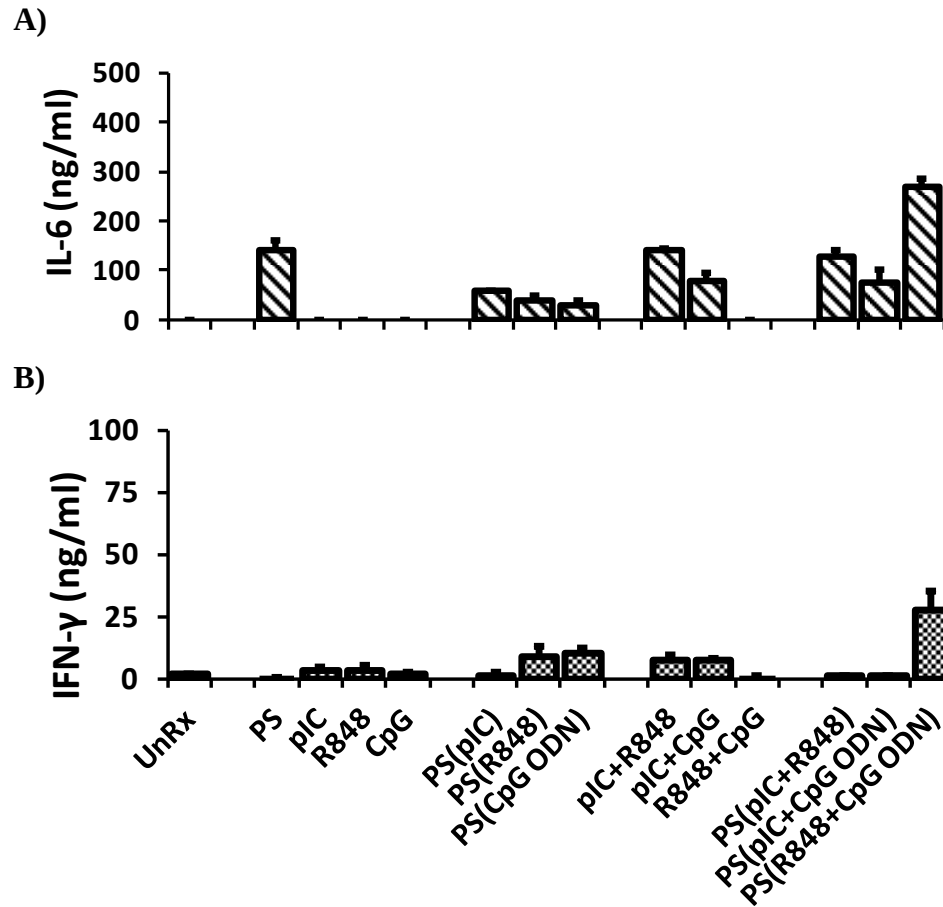


Figure 3.15. Improved *in vivo* immunostimulatory activity of PS4-TLR ligand nanocomplexes. 18 hrs after i.p. PS4(pIC/R848/CpG ODN; 15 μ g:15 μ g) single or double nanocomplex injections, PECs were isolated (3 BALB/C/group) and cells (1.25x10⁶/ml) were incubated *ex vivo* for further 42h, (A) IL-6 and (B) IFN- γ levels from culture supernatants were analyzed by ELISA. Result represents mean $\pm$ SEM of triplicate samples repeated at least twice. pIC+R848/CpG ODN vs. alone stimulations $p < 0.005$, PS4(R848+CpG ODN) vs. PS4 and R848+CpG ODN $p < 0.002$ for IL-6. $p < 0.005$ for PS(CpG ODN) and PS4(R848+CpG ODN) vs alone stimulations for IFN- γ .

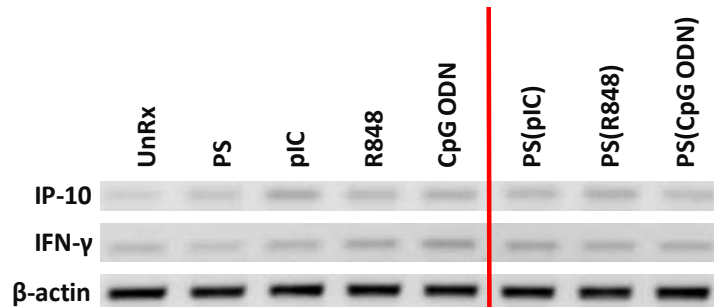


Figure 3.16. PCR results that were performed from total RNA of splenocytes from BALB/C mice 18 hours after i.p. injection of PS, pIC, R848, CpG ODN alone and their nanocomplexes with PS (15 μ g:15 μ g).

3.7 PS nanoparticles as anti-tumor agents

In recent years, cancer immunotherapy has gained a critical importance in the context of combating with established tumors. The interplay between cancer micro-niche and localization of relevant immune system cells at the site are recognized as a valuable target to induce effective control over the spread of tumor mass. Nucleic acid based TLR ligands (NA TLR ligands), such as, pIC, R848 and CpG ODNs are known to boost anti-tumor immunity. Among many, induction of cytotoxic T-cells, improving antigen presenting cell function (i.e. macrophages and dendritic cells) as well as subversion of immunosuppressive microenvironment generated by tumors are some of these mechanisms mediated by these ligands. In addition to these, polysaccharides from mushrooms were reported to contribute to anti-tumoral activity.

Here, in order to check the anticancer effects of PS nanocomplexes harboring TLRL, an EG.7 thymoma model was developed in C57BL/6 mice (5-10/group). After palpable tumor formation i) PS alone, ii) PS(singleTLRL) or iii) PS(doubleTLRL) or iv)PS (tripleTLRL) nanocomplexes were intra and/or peritumorally administered (i.e. three injections on alternating days) and tumor dimensions were recorded daily (for a duration of 21 days and volumes were calculated and reported as mm³ as seen in Fig 3.17 and 3.18.

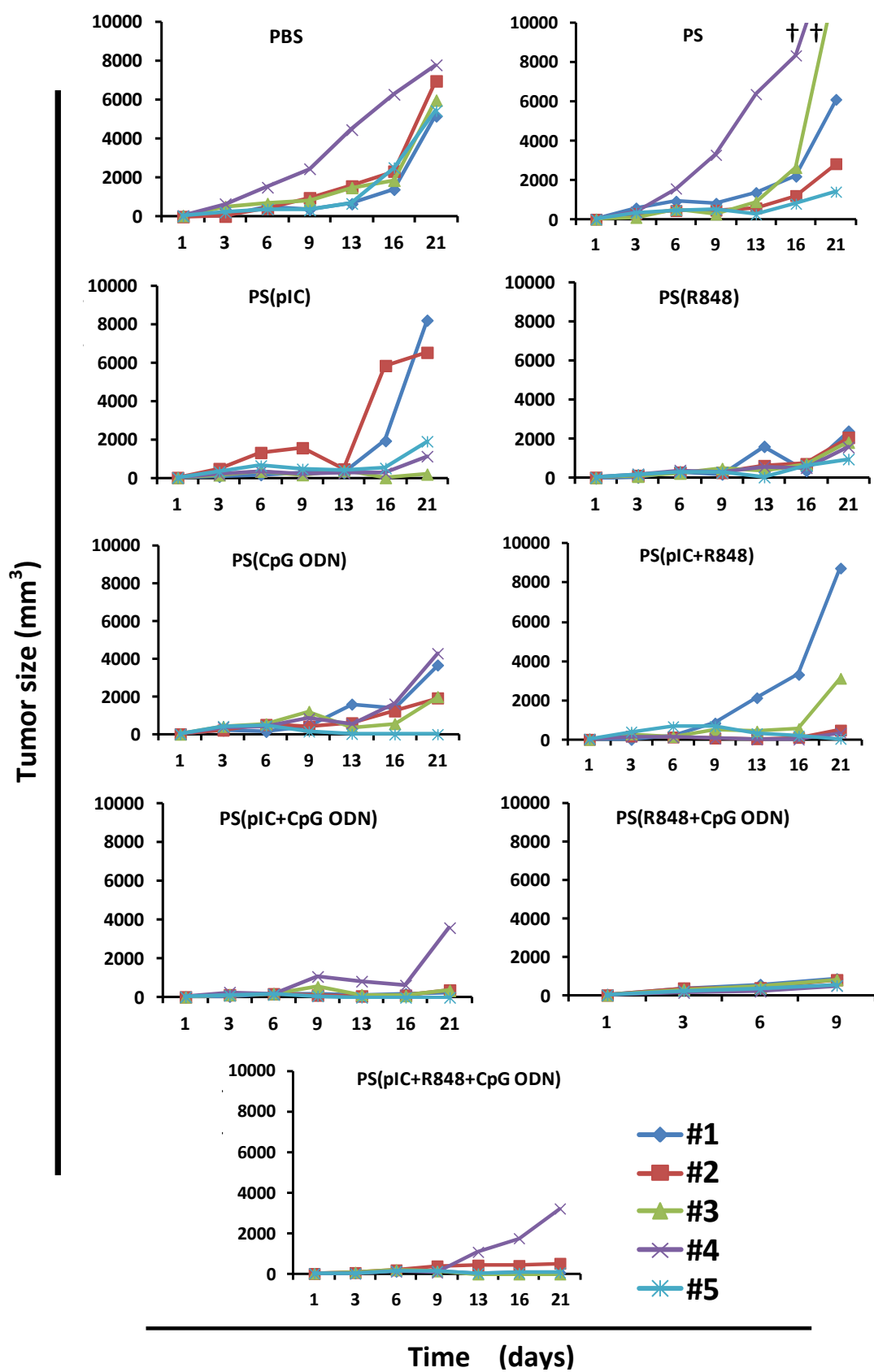


Figure 3.17. The effect of double and triple PS nanocomplexes as potent immunotherapeutic anti-cancer agents against EG.7 mouse thymoma cells. Tumor volumes of untreated mice, mice treated with PS only, PS single, double and triple nanocomplexes (50µg:50µg, w:w) were given in individual (5 mice/group) following 20 days after the palpable tumor formation.

At day twenty one tumor sizes were 6200 mm³ on average in untreated mice. At the same day, tumor volume was 7415 mm³ in PS only treated mice. Single TLR ligand nanocomplex treated mice tumor sizes varied between; 1800-4000 mm³ on average but 1 mice out of 5 mice in the groups treated with PS(pIC) and PS(CpG ODN) showed delay in tumor growth. The partial remission rate was higher on PS(pIC+R848) and PS(pIC+CpG ODN) treated groups, which reached up to 70% on average compared to untreated group two weeks after the first treatment (Fig. 3.19). Starting from one week after the cessation of Rx with nanocomplexes, tumor sizes were significantly reduced and/or complete remission was observed in 75% of animal that had three injections of triple combination nanocomplex (i.e. PS(pIC+R848+CpG ODN) (Fig. 3.17). This formulation implicated that a synergistic activation of immune cells led to an effective tumor cell killing than other combinations tested in established thymoma model. The mechanism responsible from this effect could be attributed to a more effective antigen presenting cells and cytotoxic T-cell activation along with improved NK cell killing. We have yet to demonstrate the possible contributions of these cell types in this model either reproducing these models in knock-out animal models or in models where either T or NK cells were neutralized by the injection of mAbs (following anti-CD54, anti-CD3, or anti-CD4 or anti-CD8 injections).

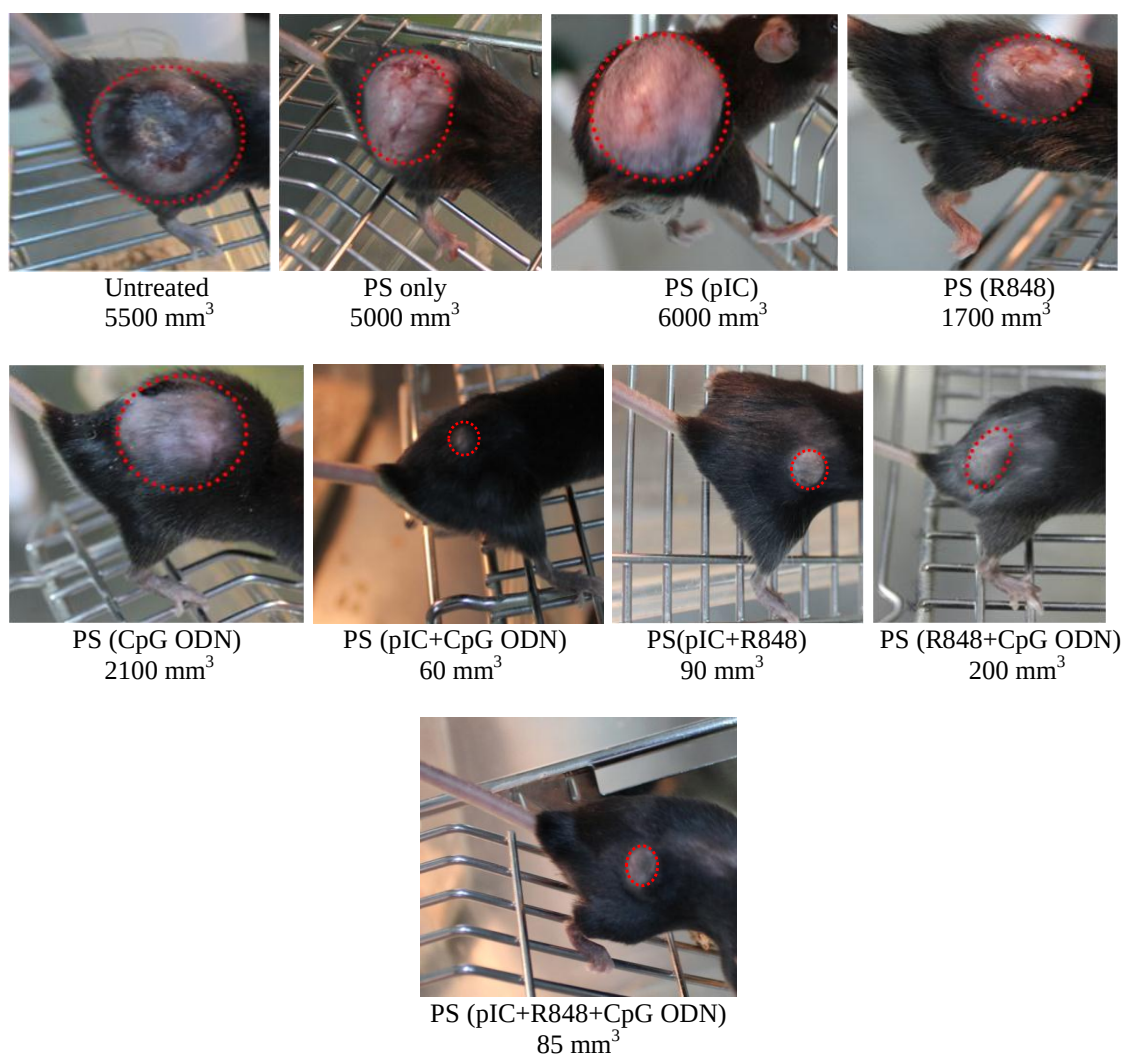


Figure 3.18. Representative pictures showing tumor mass of BL/6 mice post-treatment with indicated treatments at day 20. Picture of PS (R848+CpG ODN) group was taken on day 8.

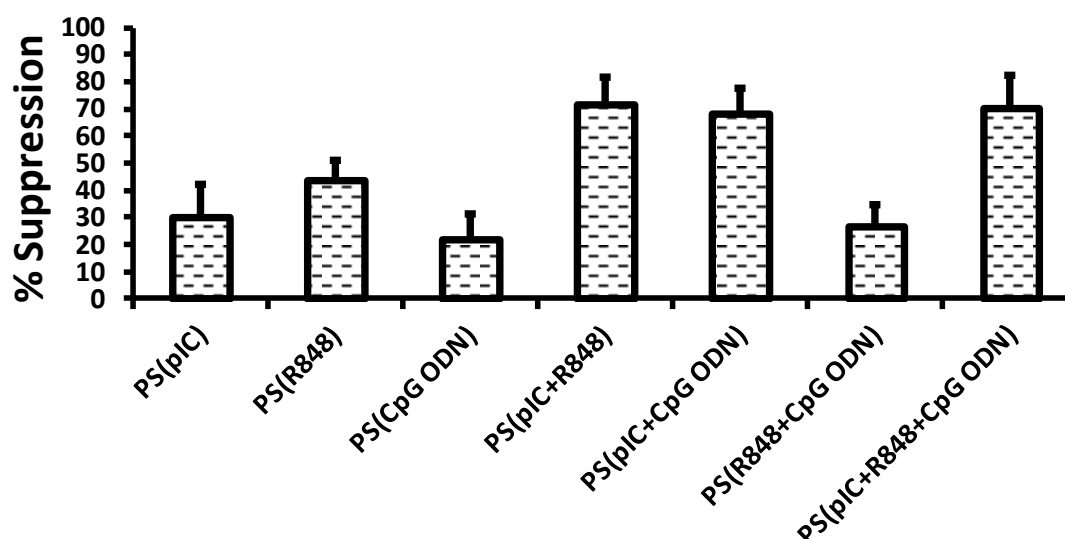


Figure 3.19. Double and triple TLR NA agonist formulated PS nanocomplexes, significantly induced tumor regression of EG7 thymoma generated C57BL/6 mice. Data represent % tumor suppression compared to untreated mice at d=18.

3.8 Adjuvant effect of PS/TLRL nanocomplexes

3.8.1 Immunization with PS-nanocomplexes against model antigen ovalbumin

To assess whether PS-TLR ligand nanocomplexes could be used as an effective immune adjuvant and vaccine delivery system, female C57BL/6 (3 mice/group) animals were immunized with the model antigen ovalbumin (OVA hereafter). In this study, animals were injected 15 μ g TLR ligand(s) plus 7.5 μ g OVA intraperitoneally (i.e. in case of triple combination: pIC+R848+CpG ODN; 5 μ g:5 μ g:5 μ g each) with or without PS. Following serial dilutions of immune sera on ELISA plates against i) total IgG, ii) IgG1 and iii) IgG2a primary and secondary immune response profiles were plotted (primary response: 2 wks after 1st injection and booster response: two weeks after 2nd injection, @d=28). As seen in Fig. 3.20A Anti-OVA IgG and IgG subtypes were significantly boosted in PS-nanocomplex treated groups even after primary injection (shown at 1/2000 titer) compared to TLR combination or PS(OVA) alone treated groups. In

contrast, PS(pIC+R848+CpGODN+OVA) group primary responses showed no benefit in this setting (Fig. 3.20A).

After booster injection, anti-OVA total IgG and IgG1 subtypes (here, compared @ titer 1/8000) were improved at least 2 fold and up, when mice were immunized with i) PS(pIC), ii) PS(R848), iii) PS(pIC+R848), and iv) PS(pIC+CpG ODN) nanocomplexes, but PS(pIC+R848) and triple complex combination showed decrease or no change, respectively (Fig. 3.20B). Greater the ratio of IgG2a/IgG1 means more pronounced Th1 immune response. That ratio was improved after booster injection although it is less than 1.

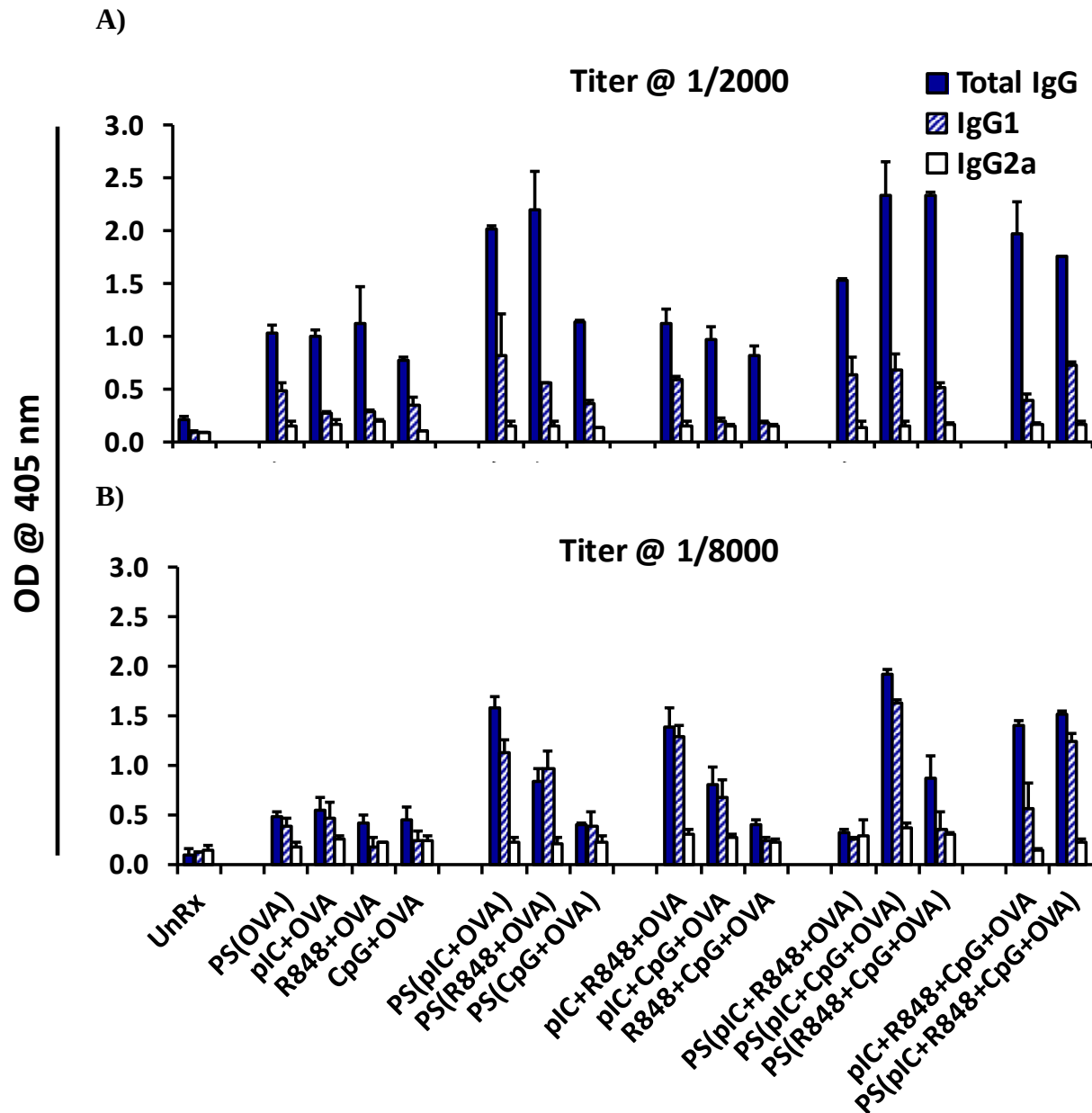


Figure 3.20. Single or double TLR and OVA containing nanocomplex formulations boosted anti-OVA immunity. Sera from (A) primary and (B) secondary bleeding were assessed for total IgG, IgG1, and IgG2a by ELISA. OD values at titer 1/2000 for primary and at 1/8000 for secondary responses were plotted against treatment groups. Data represents the average $\pm$ SD serum IgG anti-OVA response (N=5 mice/group).

3.8.2 Adjuvant effect of PS-TLRL nanocomplexes against influenza virus

3.8.2.1 Intranasal immunization

After establishing that PS nanoparticles were promising vaccine carriers candidates as well as an effective co-adjuvants capable of boosting antigen-specific immunity in an ovalbumin antigen model in mice, we extended these observations to check whether in influenza split vaccine harboring PS nanoparticles offer any advantage against anti-influenza response. To date, no successful protective and/or therapeutic vaccine formulation against flu was developed. Of note, split vaccine of H1N1 virus (SV) is the most promising and relevant antigen under investigation for human use.

Wild-type C57BL/6 mice were either intradermally (i.d.) or intranasally (i.n.) immunized with eight different formulations containing SV. These 8 group of mice had mixtures of TLRL+SV, and their PS nanocomplexed counterparts as already mentioned in Section 2.2.11.3. In order to control the influenza infection one must develop effective and robust mucosal as well as serum antibody responses against H1N1 Ag along with very efficient cellular Th1-type response. To achieve these goals i.n. and i.d. routes were the most promising way of delivering our vaccine candidates. In that respect, sera collected one week after primary and one week after the booster injections were analyzed for the presence of anti-H1N1 response (i.e. total IgG, IgG1 and IgG2b and IgG2c). This strain of mice does not develop IgG2a (an important Ig subtype indicative of T-cell dependent cellular immunity), but instead it induces IgG2c class switch representative of cellular responses. The comparison of IgG2c to that of IgG1 levels provide a valuable insight regarding vaccine`s cellular and humoral immune potency.

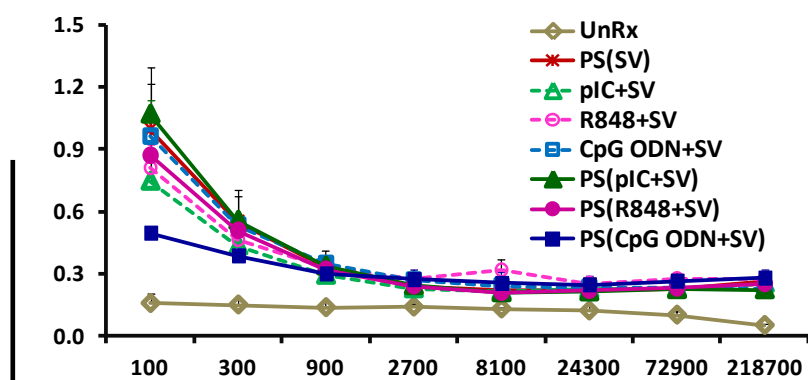
Different from i.d. immunization (please see below section for details), results of secondary bleeding sera after intranasal (i.n.) injection of SV+PS nanocomplex combinations, were the highest among other groups for total IgG, IgG2b and IgG2c. All

nanocomplex formulations of PSs were significantly induced the total IgG production compared to alone formulations at 1/8100 antibody titre (Fig. 3.21) after secondary injection.

Regarding primary response there was no significant difference between sera of all groups when tested 10 days after primary injection with SV antigen. As shown in Figure 3.22, i.n. immunized mice showed more Th1-dependent antibody response as evidenced by higher IgG2c and lower IgG1 levels almost in all groups both after primary (@d10) and booster (@d17) injections. But more strong significant anti-SV response was developed after booster injection. Anti-SV IgG2b response was studied from booster sera, of 8 groups only three of them gave an IgG2b/IgG1 response ≥ 1 , these were, i) PS(SV), ii) pIC+SV and iii) PS(pIC+SV) injected groups (please see Appendix C *i-iii* for more details).

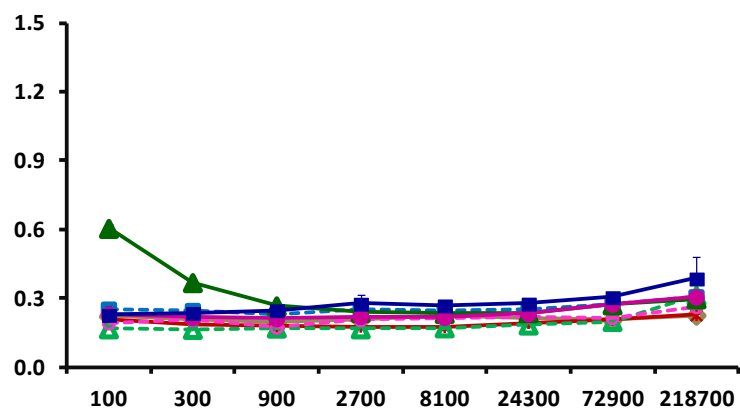
PRIMARY BLEEDING

Total IgG

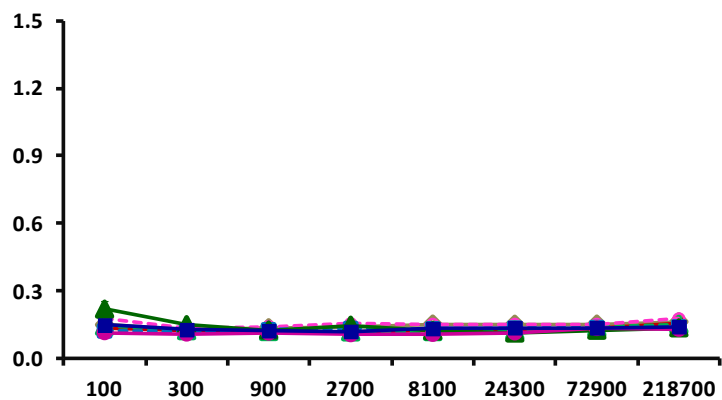


IgG1

OD @ 450 nm



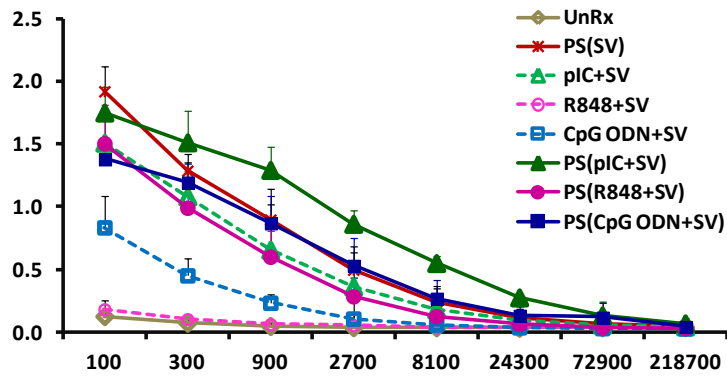
IgG2c



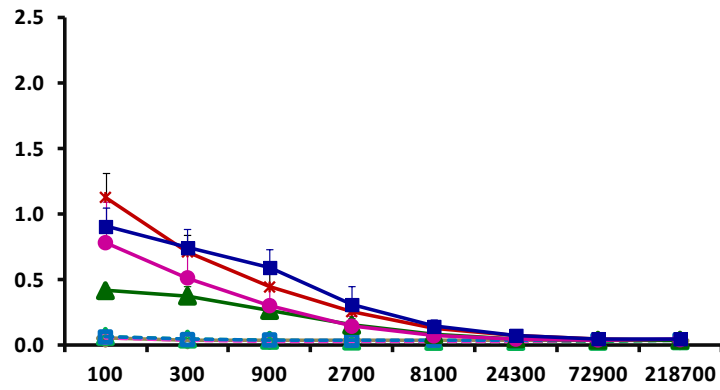
Titer (1/N)

SECONDARY BLEEDING

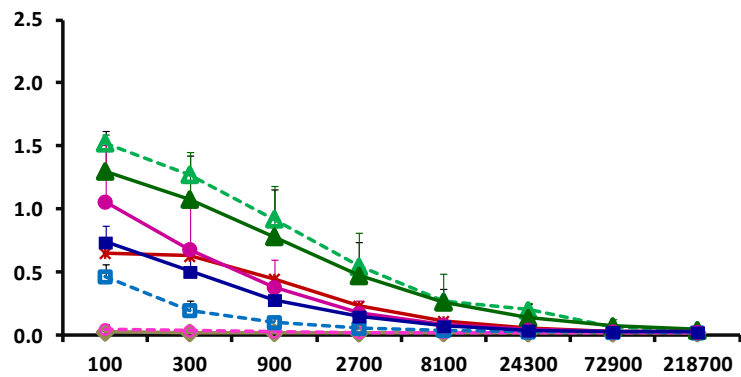
Total IgG



IgG1



IgG2c



Titer (1/N)

OD @ 450 nm

Figure 3.21. Anti-SV Ig responses of i.n. immunized mice. Animals were injected i.n with 15 μ g of PS+TLRL mixtures or PS-TLRL nanoparticles incorporating 1 μ g SV. Serum anti-SV titers at days 10 (primary bleeding, upper panel) and 17 (secondary bleeding, lower panel) were determined by total IgG, IgG1 and IgG2c ELISA. Data represents the average $\pm$ SD serum IgG anti-SV response (5 mice per group).

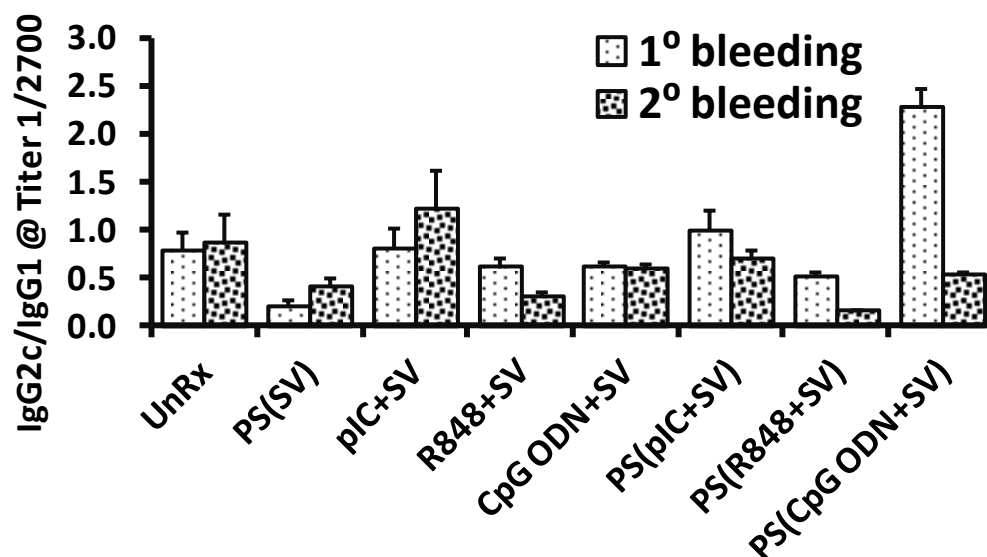


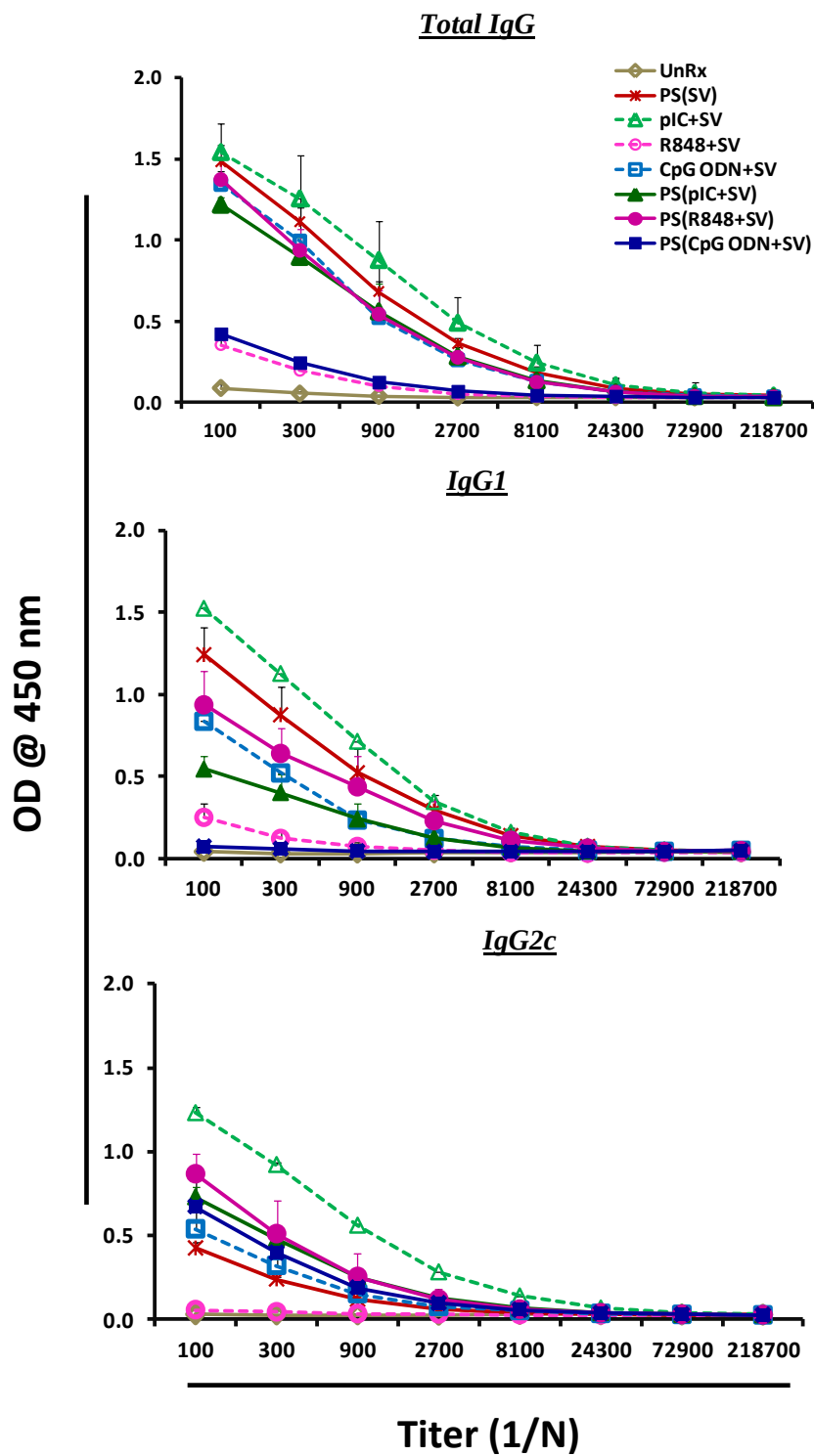
Figure 3.22. IgG2c/IgG1 OD ratios of 2700x titrated mice sera (ave. of 5 mice/group) against anti-SV. Sera collected 1 week after primary and booster intranasal injections.

3.8.2.2 Intradermal immunization

Intradermally immunized mice sera obtained from primary and secondary bleedings revealed that IgG production was much higher for the booster response as opposed to the primary responses (Fig. 3.23). Compared to the groups that had combinations of TLRL+SV, all PS/nanocomplexes gave significantly higher amounts of anti-SV IgG1 levels (Fig. 3.23). The IgG2c levels at all investigated titers after booster injection were not higher than the levels of IgG1, indicating that a weaker Th1 biased immunity was generated after id immunization of SV vaccine formulations. Of note, the higher the IgG2c subtype and the lower the IgG1, represents generation of effective Th1 biased response. When taken together it was observed that PS/nanocomplex-treated groups gave ≤ 1 ratio of IgG2c/IgG1 indicating Th2 biased anti-SV response (Fig. 3.24). Th2 dependent immune response was also supported by the ratio of IgG2b/IgG1 > 1 . IgG2b

levels were higher after secondary bleeding as expected but the IgG2b/IgG1 ratio was compared with the primary bleeding at all treatments (please see Appendix C iv, v).

PRIMARY BLEEDING



SECONDARY BLEEDING

Total IgG

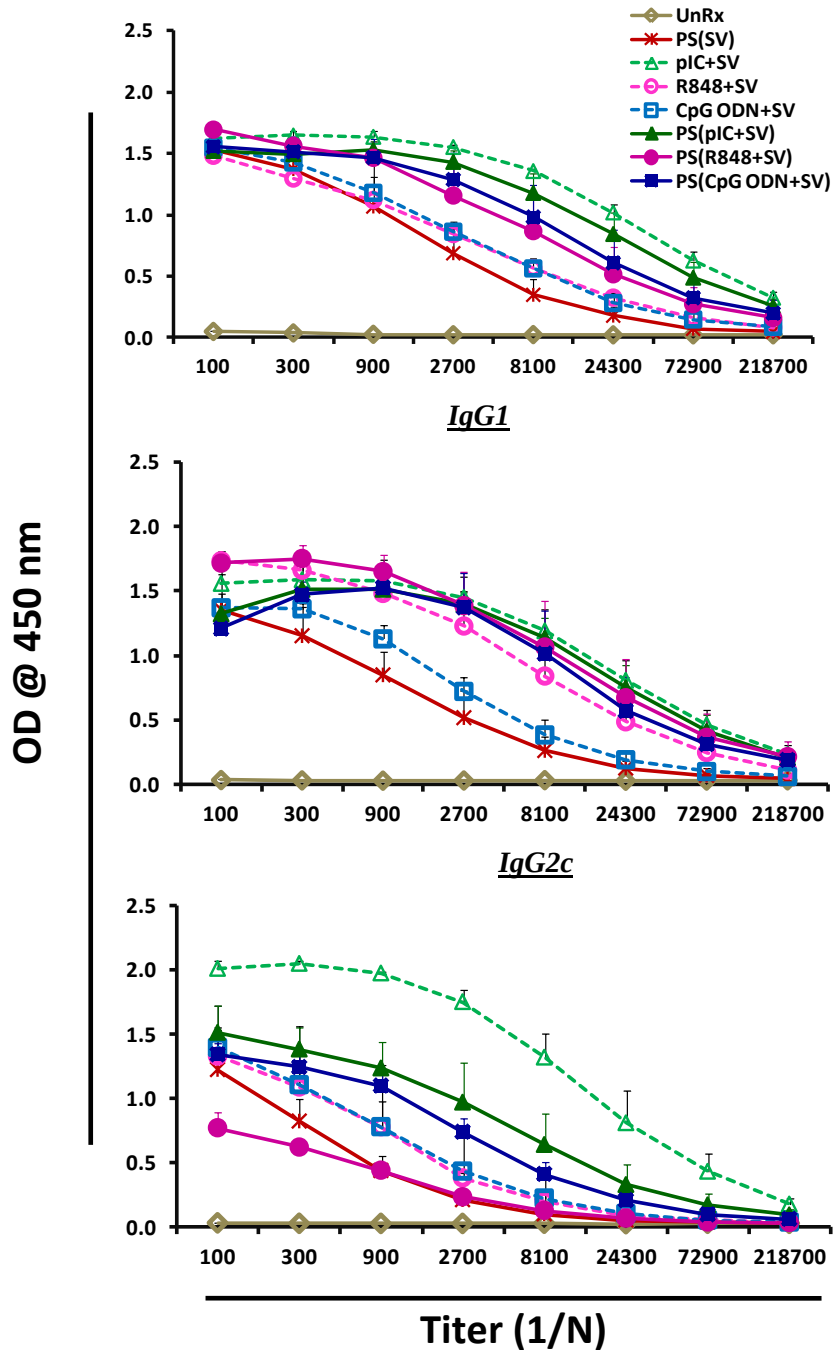


Figure 3.23. Immunogenicity of the SV (H1N1), after primary and secondary intradermal vaccination. Animals were administered intradermally with 15 μ g of PS+pIC/R848/CpG ODN mixtures or PS-pIC/R848/CpG nanoparticles incorporating 0.1 μ g H1N1 influenza split virus (SV). OD (450 nm) versus serum anti-SV titers of Total IgG, IgG1, IgG2c one week after primary (upper panel) and booster injections (lower panel) were plotted. Data represents average $\pm$ SD anti-SV serum IgG response (5 mice/group).

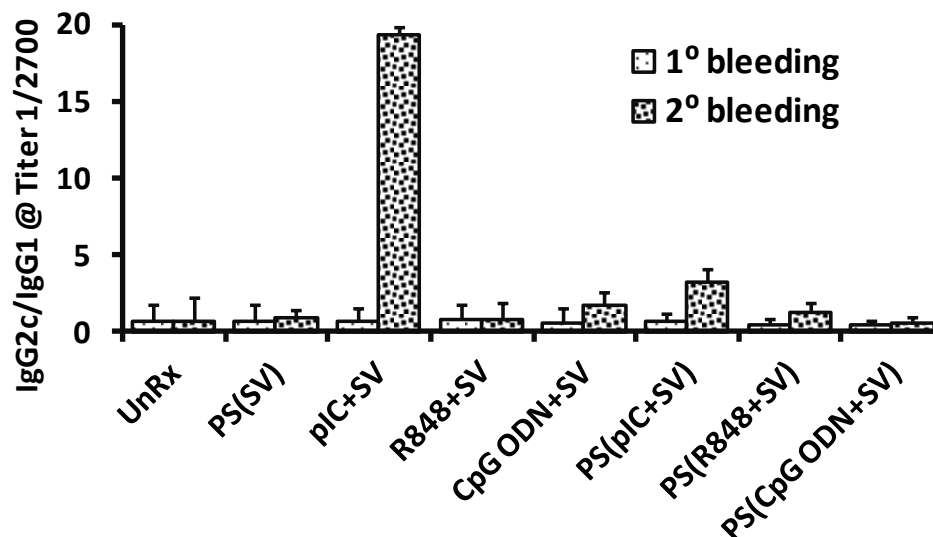


Figure 3.24. IgG2c/IgG1 OD ratios of 2700x titrated mice sera (ave. of 5 mice/group) against anti-SV. Sera collected 1 week after primary and booster intradermal injections.

In addition to Ig subclass analyses, one week after booster injection, an attempt was made to identify influenza virus specific IgA antibody either from nasal wash or from blood sera of i.n. immunized animals (Fig. 3.25). Surprisingly, nasal wash vs blood sera responses varied among groups. Higher IgA titers were detected from PS(SV), PS(pIC+SV), and PS(CpG+SV) from the nasal wash (Fig. 3.25A). Only PS(pIC+SV) group gave higher secreted IgA from the blood sera analyses (Fig. 3.25B). These results collectively implicated that effective mucosal anti-H1N1 immune response could be generated when pIC or CpG ODN(TLR3L, TLR9L, respectively) adjuvants were included in the vaccine formulation.

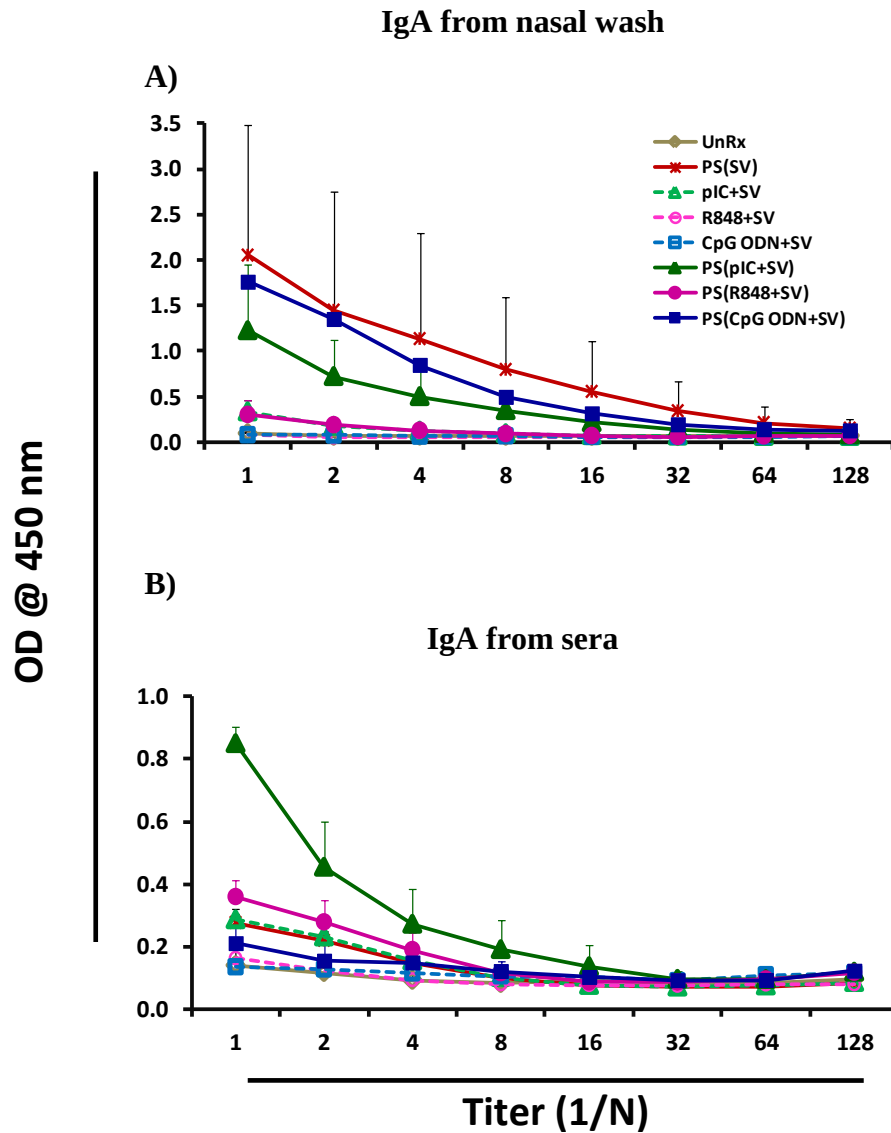


Figure 3.25. Influenza virus specific IgA production from nasal wash and blood. Mice sera of (A) nasal wash and (B) secondary bleeding (@d17) which were i.n. immunized with PS-nucleic acid based TLR ligand complexes. Data represents the average SD serum IgA anti-SV response (three/five mice per group).

The T cell response of i.n. immunized mice was assessed by incubating splenocytes with various influenza-specific MHC Class I and class II peptides, and checked for IL-2 and IFN- γ production by ELISA (please see Appendix D for more details). Splenocytes from PS(pIC+SV) injected mice also effectively responded to the only MHC Class II peptide; NP₂₆₀₋₂₃₃, like pIC+SV and CpG ODN+SV groups in a IL-2 dependent manner. Two

different CD8⁺ dependent peptides; M502-P and PA₂₂₄₋₂₃₃ induced IL-2 production in splenocytes from R848 and CpG ODN nanocomplexes (Appendix Di). No other induction in IL-2 production was seen in splenocytes of TLR-PS nanocomplex groups on either SV or WV treatments compared to PS/pIC/R848/CpG ODN+SV treatments. IFN- γ secretion were the highest in both pIC+SV and PS(pIC+SV) treated mice against WV (Appendix Dii). CpG ODN+SV but not its PS/nanocomplex formulation also responded to both WV and MHC Class I type peptide M502-P as evidenced by both IFN- γ and IL-2 production compared with untreated mice splenocytes. PS(SV) group enhanced both cytokine production when incubated with WV and SV but not with any of the influenza peptides.

3.9 New generation self assembled nanoparticle forming CpG ODN as effective anti cancer agent.

Recently, a novel self-nanoparticle forming ODN class was designed in the Lab and their immunostimulatory potential was characterized by two other M.Sc. students (Karatepe, 2009; Mammadov, 2009). This ODN class was named as a nanoparticle forming CpG ODN (in short ODN420). Their findings suggested that ODN420 captured the superior features of both D and K class CpG ODN. Since then, no one has attempted to utilize these agents as a standalone anticancer agent.

As seen in figure 3.26 dendritic cells generated from mouse bone marrow (BMDC) were treated with ODN420 either in phosphodiester form (PO) or phosphorothioate form (PS). Cell supernatants were assessed for cytokine production either from BALB/C or BL/6 derived spleen cells as opposed to control ODN composed of similar CpG motifs but synthesized as linear 1466ODN (Fig 3.26 A and B).

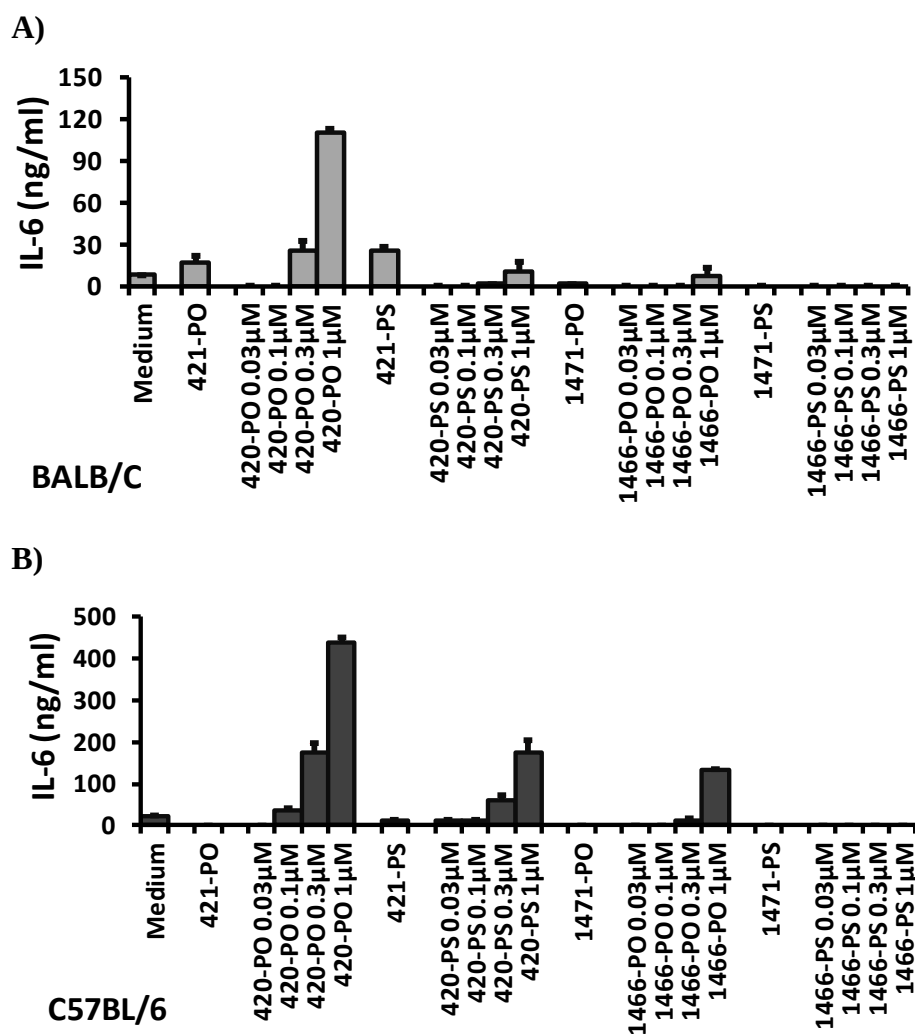


Figure 3.26. BMDC ($5 \times 10^5/\text{ml}$) from (A) BALB/C or (B) C57BL/6 mouse were treated with different doses of ODN420, or other CpG ODNs for 42h. Culture supernatants were collected and assessed for IL-6 secretion.

IL-6 production profile of BMDCs revealed that ODN420 in PO is much potent than any other type of CpG ODN. There was at least 3-10 fold more cytokine production at each dose tested, and this difference was more significant when the treatment dose was lowered. This finding led us to utilize ODN420 as a potential standalone anticancer agent since T cell instruction via APCs induced by ODN420 would lead to an effective CTL activation against cancer cells.

3.9.1 ODN420 treated PEC cells were cytotoxic against Huh7/luc cells

To establish whether peritoneal cells and splenocytes from mice treated with the ODN420 and its control 1466ODN were capable of killing human hepatocellular carcinoma cells (HuH-7) in culture, we developed an *in vitro* cytotoxicity assay. In that, a luciferase plasmid transfected HuH-7 cells were co-incubated with PECs and splenocytes from CpG ODN treated athymic nude mice (for details see Section 2.2.6.) Briefly, nude mice were i.p. injected with i) saline, ii) ODN420, iii) 421-Control ODN, iv) Linear-1466ODN or v) Linear-1471Control ODN (100µg/Rx, for three times, on alternating days). Two days after the last injections, splenocytes and PECs were collected and co-cultured with HuH-7/pMIR cells for 24h at various effector-to-target ratios. Next day cells were harvested and luciferase activity was assessed. As shown in figure 3.27A, the lowest luciferase activity was seen when HuH-7 cells were co-incubated with spleen cells extracted from ODN420 pre-treated mice. Up to >65% depletion in luciferase intensity was observed in co-culture of HuH-7 and ODN420 treated spleen cells compared to splenocytes obtained either from ODN1466 or their respective control ODN treated animals. Interestingly, 421-Control ODN treated splenocytes were more cytotoxic than linear 70-mer linear ODN1466 treated cells. When PEC cells were co-cultured with HuH-7/luc cells, at 1:1 and 1:5 effector-to-target ratios, cells from ODN1466 and ODN420 treated mice showed lowest luciferase activity (Fig. 3.27B).

These sets of findings strongly indicated that ODN420 induced a robust antitumor effect. Next, we tested these therapeutic strategies in nude mice model.

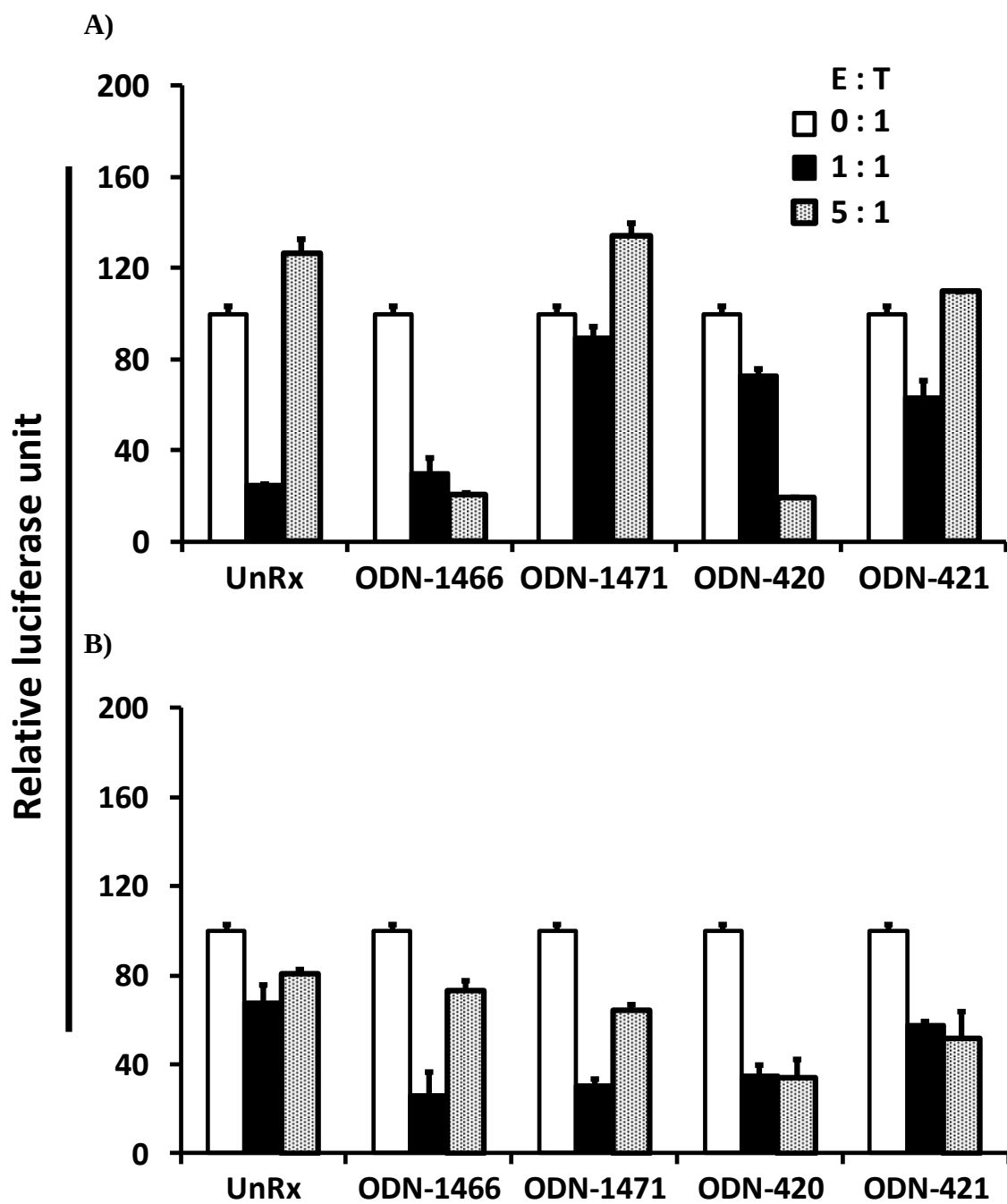


Figure 3.27. In vitro cytotoxicity assay showed that ODN420 was the most promising anti-cancer agent. Luciferase intensity bar graphs of (A) splenocytes and (B) peritoneal exudates cells with the co-culturing of luciferase plasmid transfected HuH-7 cells in 1:1 and 1:5 effector-to-target cells ratios for 24h, one day after the last treatment. Fold induction of luciferase activity over pMIR transfected HuH-7 cells was plotted.

3.9.2 Established tumor regression by nanoparticle forming ODNs

Thymus deficient nude mice were xenografted with human hepatocellular carcinoma cell line HuH-7 as described in the Section 2.2.11.4. Three doses of 100 μ g of ODN420, or ODN1466 (Linear 70mer ODN) were injected 3 times (peritumorally and/or intratumorally) starting from the day of post-palpable tumor formation with one day-on and one day-off regiments. Untreated, ODN1466 and ODN420 treated nude mice developed a tumor volume $\sim 1644 \pm 101$ mm³, 648 ± 56 mm³ and 120 ± 46 mm³, respectively. A reduction >90% of tumor size was reached in animals that was treated with ODN420 (Fig. 3.28A). This mode of tumor immunotherapy proved that self-nanoparticle CpG ODN induce a potent immune milieu that was sufficient to revert a very aggressive form of human tumor in nude mice model. Interestingly, a conventional CpG ODN (i.e. Linear-70-mer-1466 CpG ODN) did not provide any benefit in this *in vivo* setting, although it contains same number of active CpG motifs.

Consequently, a 40% complete remission with NP-ODN420 treatment was observed (Fig. 3.28B). The results indicated that in HuH-7 carcinoma model, 420 CpG ODN significantly improved NK cell activity, and led effective HuH-7 clearance.

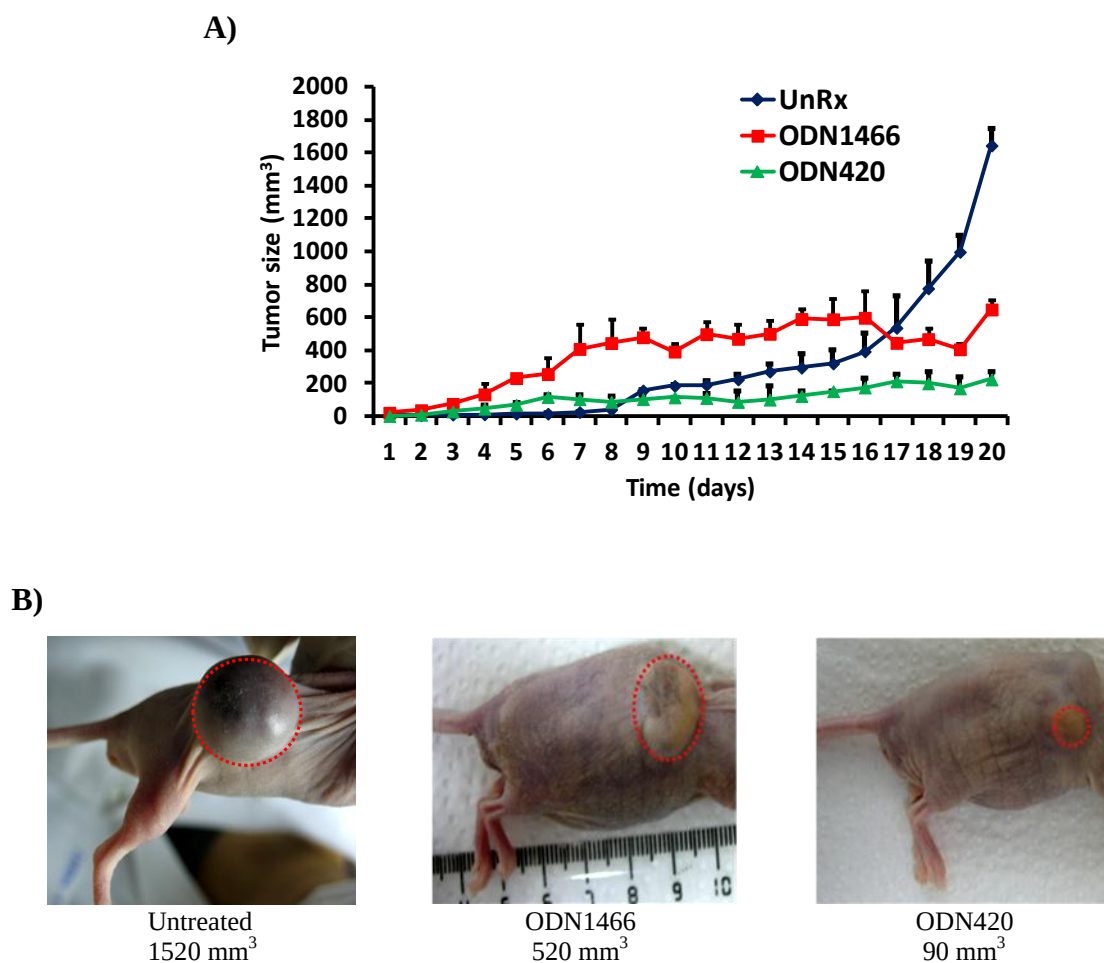


Figure 3.28. ODN420 was the most prominent anti-tumor agent in HCC model. (A) Tumor size measurements of untreated, linear ODN and NP-ODN treated groups for 20 days. Results show mean tumor size from 5 mice/group (B) Tumor size was reduced almost >60% for Lin-ODN and >90% for NP-ODN treated groups compared to untreated nude mice (d=20).

3.10 Efforts to establish the optimum sequence in chicken

This thesis was dedicated to establish immunotherapeutic CpG ODN suitable for several disease conditions. In addition to design more potent sequences and also develop suitable carrier systems that could complement and improve TLR based therapeutics. In that respect we embarked on a project to determine suitable CpG ODN sequences that could control infections in chicken immune system, so that they could be applied as a prophylactic agents in broiler chicken industry thereby reducing uncontrolled deaths.

3.10.1 Testing the immunopotency of various CpG ODN types

To assess the most potent CpG ODN sequence(s) for *in vitro* brewer chicken experiments, we synthesized and/or designed several novel types of CpG ODNs. Sequences and backbone properties were mentioned previously in the Materials and Methods section (please refer to Section 2.1.3). Initially, splenocytes from BALB/C mice were incubated with 1 μ M CpG ODNs. After 42 hours of incubation, supernatants were collected and IL-6 and IL-12 productions were tested by cytokine ELISA. As seen in Figure 3.29 consistent with both cytokine ODs, phosphorothioate (PS) backbone containing 5547-ODN, 1826-ODN, 2007-ODN and 1466 Acore-ODN were the most potent among other PS backbone modified ODNs as evidenced by enhancement in both cytokine levels. Also D35-3CG and 1466-pG6 ODNs which were synthesized as PS, PO and mixed backbones were effective in IL-12 induction but couldn't induce IL-6 cytokine. Control ODNs of all of the studied CpG ODNs did not induced any appreciable amounts of cytokines (data not shown) suggesting that these activities are solely dependent on the expression of CpG motifs. We next, tried to characterize these sequences on chicken immune cells, expecting to identify potent CpG ODNs suitable for immune modulation.

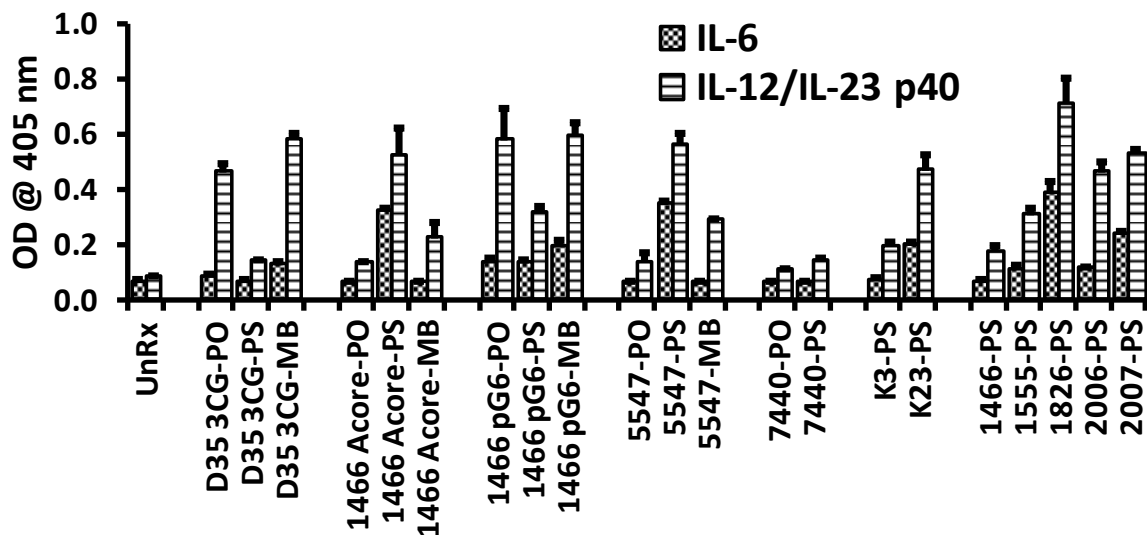


Figure 3.29. Immunostimulatory effects of different CpG ODNs. BALB/C splenocytes ($4 \times 10^6/\text{ml}$) were stimulated with phosphorothioate, phosphodiester and mixed backbone modified K and D type CpG ODNs for 42hrs. IL-6 and IL-12p40 cytokine ELISAs were ran on triplicate. Data represents the average $\pm$ SD in treatment groups.

3.10.2 Immunostimulatory effect of CpG ODN sequences on chicken immune cells

The co-stimulatory molecule, B7-1 (CD80) expression, on chicken APCs is required for T cell activation and survival we next checked whether upregulation of CD80 was improved upon CpG ODN treatment. Chicken splenocytes were incubated with 21 differentially modified CpG ODNs and as a positive control PGN. Treated cells stained with anti-CD80-PE, results indicated that, almost 80% of the splenocyte population which were either stimulated with 1466-Acore-PO, or D35-3CG-MB or 2006 CpG ODNs were positive for CD80. This was equivalent to the positive control peptidoglycan ($\geq 80\%$) (Figure 3.30).

We have also checked TLR message levels from mouse B cell line DT40 and chicken spleen cells after stimulation with ODNs. D35-ODN and 1466-ODNs upregulated TLR3 and TLR21 (functional homologue of mammalian TLR9 in chicken) (Fig 3.31) gene expressions. Expression levels compared to untreated mouse spleen cells demonstrated

that two more TLRs (i.e. TLR1 and TLR5) were also induced by D35. Interestingly, TLR3 was highly overexpressed in splenocytes which were incubated with 7440, D35-3CG-PO, 2006 and 2007 CpG ODN sequences (Fig. 3.31B). However, TLR21 gene expression was down-regulated in all of those groups. There was no change in the gene expression levels of TLR2 and TLR7 before and after CpG treatment (either from splenocytes or DT40 cells, data not shown).

Next, we studied the changes of either anti-virally or anti-bacterially important mediator expression in response to CpG ODN treatment (both from splenocytes and DT40 cells). D35-ODN, 2007 and 7440 stimulated splenocytes and DT40 cells to augment anti-viral cytokines such as type I interferons (IFN- α and IFN- β) by 4 hours of treatment. Chicken lipopolysaccharide-induced TNF- α factor (LITAF) was upregulated in 1466 and D35-ODN treated DT40 cells and 2006-PS, 2007-PS treated splenocytes. In general, 2006 and 2007 stimulated spleen cells showed higher amount of all cytokine transcripts (except IL-4 indicating that CpG-ODN's with phosphorothioate backbone drives more strong Th1 biased immunity in chicken cells Fig. 3.32). Of note, mRNA expressions of proinflammatory cytokines from IL-1 superfamily members, IL-1 β and IL-18 were mainly detected upon 7440, 2007 and 1466-CpG ODN stimulation.

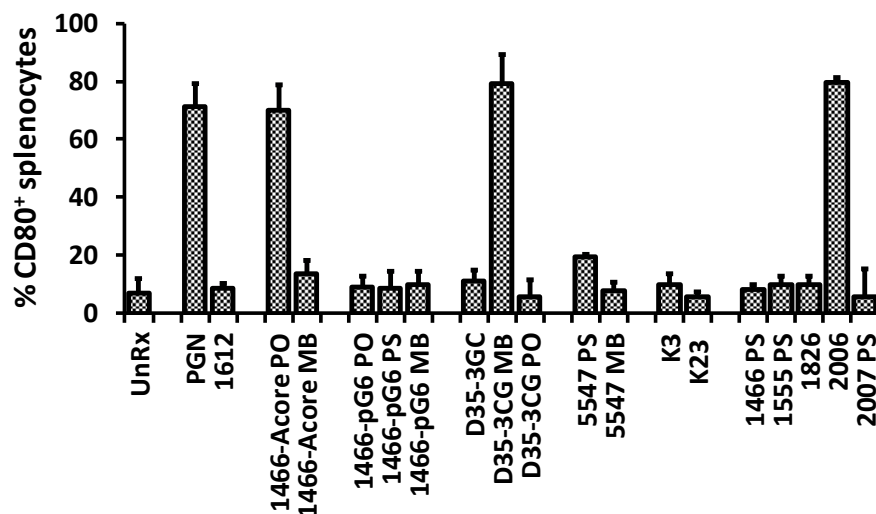


Figure 3.30. Percentage of CD80 expressing chicken splenocytes (4×10^6 /ml) upon ODN (1μ M) and 10μ g/ml PGN stimulation for 24 hours

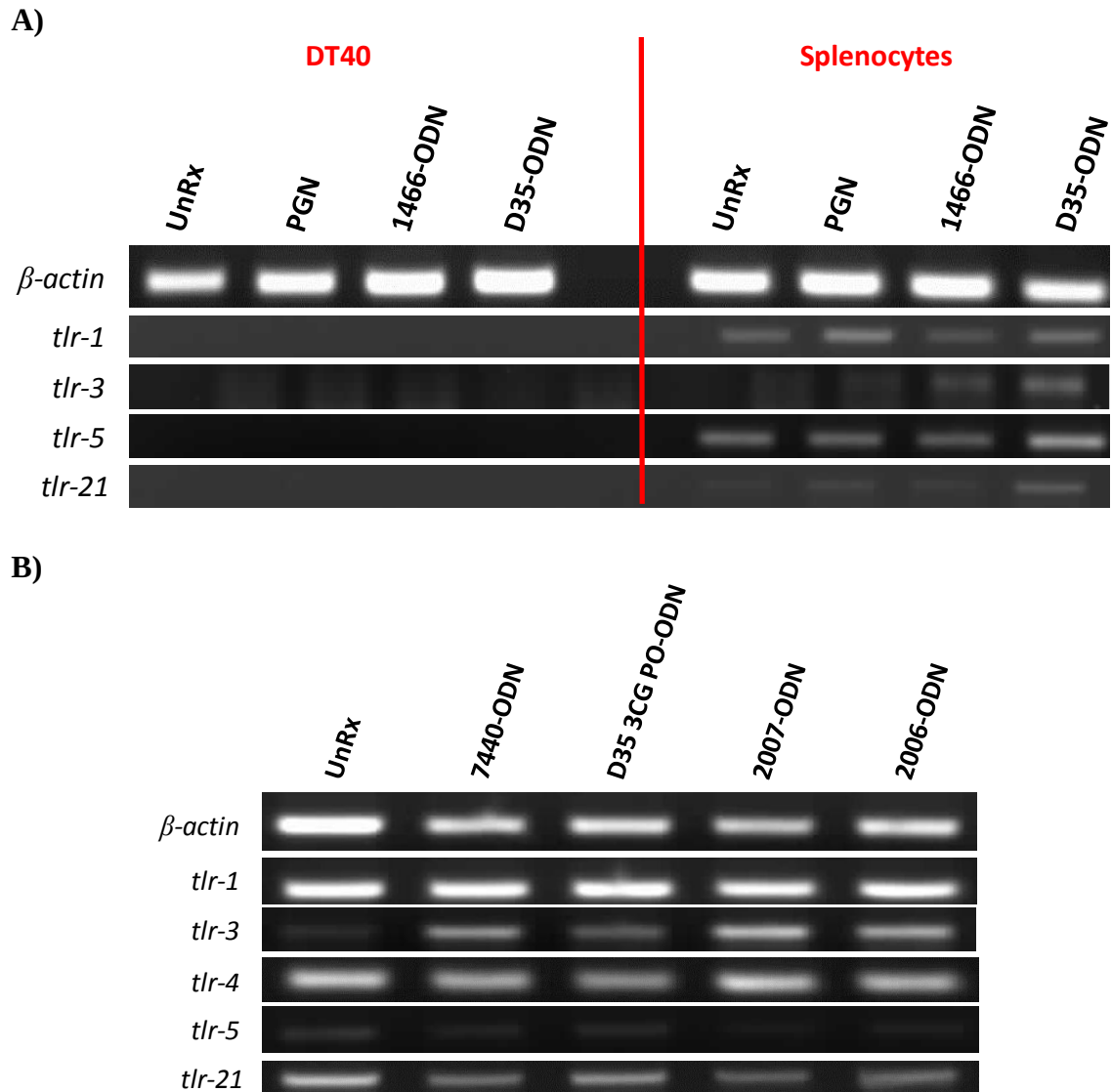
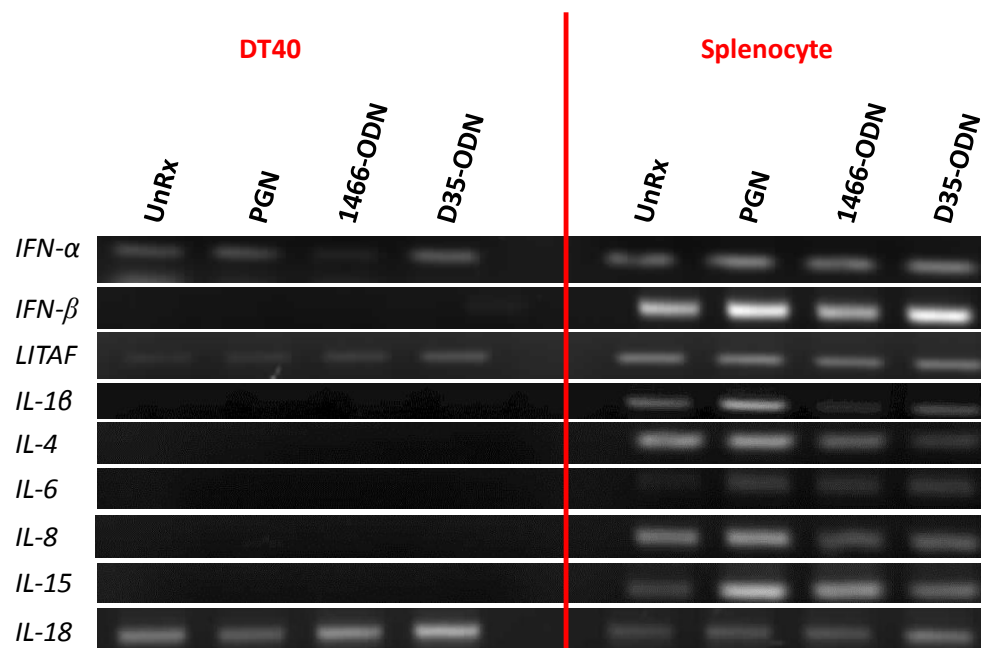


Figure 3.31. TLR Message transcript changes upon CpG ODN treatment. PCR from total RNA of (A) chicken B cell line DT40 ($4 \times 10^6/\text{ml}$) or (B) splenocytes ($4 \times 10^6/\text{ml}$) were studied. Cells were harvested after 4 hrs of incubation with $1 \mu\text{M}$ of indicated CpG ODNs (or PGN ($10 \mu\text{g}/\text{ml}$) as a positive control).

A)



B)

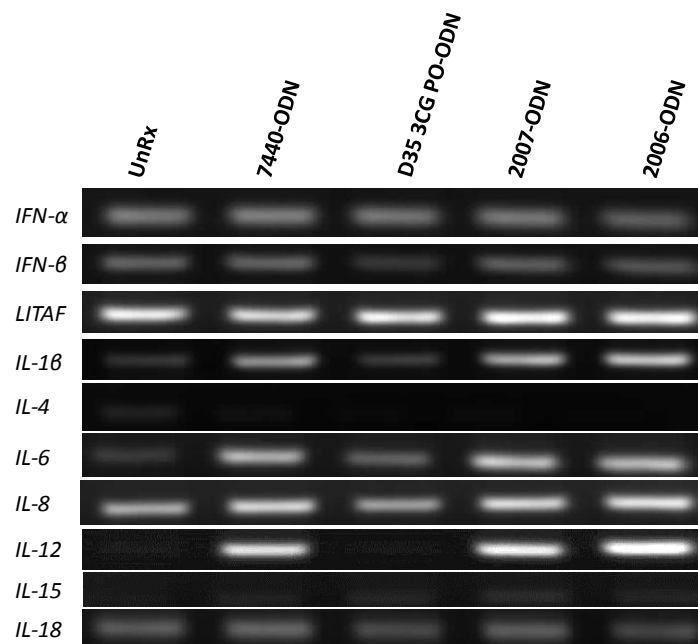


Figure 3.32. RT-PCR products of cytokine-related gene transcripts. (A) DT40 or (B) splenocytes were stimulated with different CpG ODNs (1 μ M) and PGN (10 μ g/ml) for 4 hrs. Gene specific amplicons were run on an agarose gel (1.5%).

Collectively, our findings pointed that D35-3CG and 1466-CpG ODNs induced much stronger and broader immune activation and could mediate Th-1 biased immune response on both B cells (DT40) and chicken splenocytes. This knowledge will pave the way to design novel effective prophylactic agents and/or more effective vaccine adjuvants against viral and bacterial diseases in chickens.

Chapter 4

Discussion

Immune modulatory activities of naturally occurring heterogeneous β -glucan polysaccharides in yeasts, bacteria and fungi have been studied for their potential applications against cancer or infectious diseases. Previous findings suggested that these biomolecules induce various pro-inflammatory (IL-1 β), inflammatory cytokines (TNF- α , IL-6, IL-12) and chemokines (IL-8, IP-10) upon incubating them with macrophages, dendritic cells, natural killer, B- and T-cells (Murphy *et al.*, 2010).

However, still β -glucan recognition is controversial and diverse. Literature findings suggests that different intracellular or cell surface-expressed PRRs such as Dectin-1, TLR2/TLR4 also complement receptor (CR3), NODs, and scavenger receptors triggers β -glucan mediated immune cell activation (Hua *et al.*, 2007; Kumar *et al.*, 2009c; Lin *et al.*, 2005; Tsoni and Brown, 2008). Recently, it has been reported that curdlan (β -1,3 glucan) and zymosan may induce IL-1 β production from human macrophages via NLRP3 inflammasome and Dectin-1 (Goodridge and Underhill, 2008; Kumar *et al.*, 2009c; Tsoni and Brown, 2008). Dectin-1 specifically recognizes β -(1,3)(1,6) glucans

from mushroom, bacteria, yeast and plants (Goodridge and Underhill, 2008; Tsoni and Brown, 2008).

Furthermore, β -glucans polysaccharides from different sources (i.e. yeast, or mushroom) were used as potent adjuvants. They were mixed together or conjugated either with protein antigens or with plasmid DNA against fungal, parasitic and viral diseases (Lehtovaara and Gu, 2011). Another β -glucan, Lentinan, was shown to induce stimulatory effects on virus-specific T cells induced by DNA vaccine against hepatitis B virus core antigen (Lehtovaara and Gu, 2011; Wang *et al.*, 2010a; Zhou *et al.*, 2009).

We previously reported that mushroom derived polysaccharides, namely PS2 and PS4 were recognized through TLR2 (but not through NOD1 or NOD2). These PSs led to significantly higher production of TNF- α , IL-6, IFN- γ , IP-10 and NO from RAW 264.7 cells and mouse splenocytes. We then, optimized stable nanocomplex formation of PS with i) TLR3 ligand pIC (synthetic dsRNA), ii) TLR7/8 ligand, R848 (synthetic ssRNA), and iii) TLR9 ligand, CpG ODN. Nanocomplexes of PS/TLRL were shown to boost immune response in mice even at very low doses where to their standalone counterparts were ineffective (Tincer, 2007; Tincer *et al.*, 2011).

Distinct from previously reported findings, our PS isolates from *Ganoderma lucidum* and *Shiitake* did not recognized by membrane-associated Dectin-1 since macrophages, DCs and splenocytes from Dectin-1^{-/-} led to substantial cytokine production (Figures 3.4–3.7). PS induced TLR dependent signaling is controversial. Previous reports indicated that certain PS types respond to TLR2, TLR2/6, or TLR4. Here, we attempted to differentiate TLR2, TLR4 or MyD88 dependent immune activation specific to our most potent PS type (i.e. PS4). Our data strongly implicated that PS4 directly binds to TLR2, and also initiates promoter activity in stable hTLR2 HEK293 cells in a MyD88-

dependent manner and does not interact with TLR4 (Figures 3.1–3.3, (Tincer *et al.*, 2011)).

While intracellular sensors such as NODs and inflammasome complexes are critical to augment the early inflammatory phase, contrary to previous findings with other PSs, we did not detect any NOD involvement in PS mediated intracellular activation (Tincer, *et.al.*, 2011). However, our β -glucan based PS4, induced strong inflammasome activation that was mainly regulated by ASC, Caspase-1 and partly via NALP3 (Fig. 3.8 and Fig 4.1). In addition to TLR2 recognition, we propose that inflammasome complex, including NLRP1 (also consists of ASC adaptor and caspase-1 proteins) might be involved for the intracellular recognition of PSs.

Lamkanfi *et al.* reported that IL-1 β production by β -glucans could be achieved in two steps: i) Dectin-1 dependent induction of pro-IL-1 β expression and ii) Nalp3-mediated conversion of pro-IL-1 β to active IL-1 β (Lamkanfi *et al.*, 2009a). Our results support this view, PS4 firstly signal for pro-IL-1 β production through extracellular TLR2, then destabilization of PS loaded vesicles leak its cargo into cytosol and initiates the maturation of inflammasome dependent pathways. Figure 4.1 summarizes our findings regarding the molecular mechanisms of action of mushroom derived PS4 on an hypothetical immune cell.

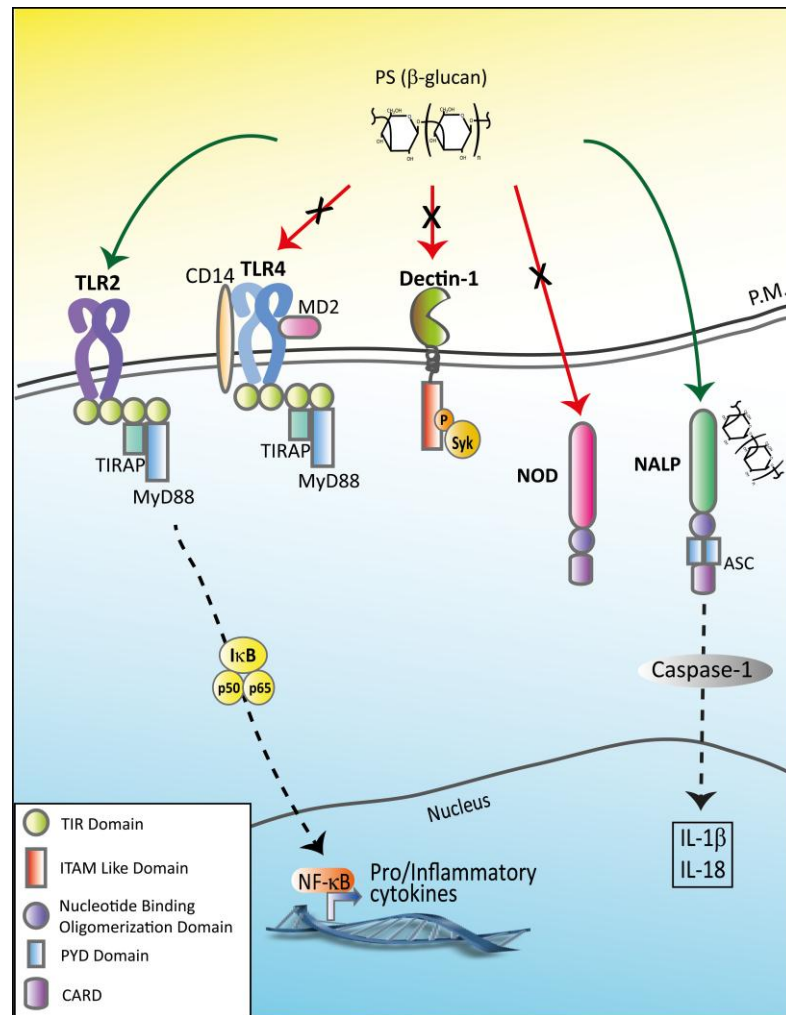


Figure 4.1. PS to induce immune response requires both extracellular and cytosolic signaling mechanism as depicted on a hypothetical immune cell.

The synergism within these receptors could be occurring through the activation common signaling pathways, such as MAPK and NF-κB. NALP3 activation in response to TLR7/8 ligand, double stranded RNA viruses such as Sendai or influenza virus was essential for the induction IL-1 cytokine family members (Kanneganti *et al.*, 2006). Cooperation between cell stress activated inflammasome and TLR signals could trigger both endogenous and exogenous ligands in regulating immune response. Not all TLR agonist combinations were synergistic. Imiquimod and ODN 1826 combination reduced

Th1 response and DC activation in mice in anti-tumor studies (Ghosh *et al.*, 2007). Also IL-10 induction upon TLR2 stimulation inhibited the induction of Th1 biased chemokines/cytokines such as IP-10 and IL-12p35 which were specifically induced by TLR3 or TLR4 in human DCs (Re and Strominger, 2004).

The dire need for development of effective adjuvants, including single/multiple TLR ligand combinations for the bacterial, viral, fungal, parasitic diseases as well as immunotherapeutics for cancer prompted researchers to develop more effective adjuvants, or immunotherapeutic agents harboring multiple components, that ultimately act in synergy to provide better protection against diseases.

To explore the anti-cancer effect single, double and even triple combinations of PS(pIC/R848/CpG ODN) nanocomplexes was studied in established T-cell thymoma model. Almost >70% suppression in tumor size were observed in mice that had PS(pIC+K3/R848) and triple PS nanocomplexes even after 21 days post therapy (Figures 3.24–25). In similar studies, liposomal CpG ODN plus OVA was used to control EG.7 mediated thymoma in mice, and up to 40-50 % reduction of tumor sizes were reported (de Jong *et al.*, 2007; Wakita *et al.*, 2006). In our studies, we have not injected OVA but relied only on the innate immune cell mediated clearance.

In parallel studies PS/TLR agonists nanocomplexes were investigated as a potent vaccine carriers in two separate models; i) OVA antigen (Fig 3.20). and ii) split vaccine against influenza (Figures 3.21–24). In OVA vaccine model, PS(pIC/R848) and pIC+R848/CpG ODN nanocomplexes boosted Total IgG and IgG1 subtypes No up-regulation or suppression was observed in other double or triple nanocomplex combinations.

In the second immunization study, i.n. and i.d. routes were used to mount immune response against influenza A (Figures 3.21–24) in C57BL/6 mice. SV+pIC and its

nanocomplexes induced a Th1 biased antibody response (high IgG2c/IgG1 ratio) upon i.n. immunization. While nearly all nanocomplex formulations boosted total IgG production after secondary injection, detection of IgG2c against SV, was induced in PS(pIC and/or SV) treated mice (Figures 3.21 and 3.22).

As an indicator of mucosal immunity, IgA production was boosted mainly in PS(pIC+SV) and PS(CpG ODN+SV) groups compared to alone groups (PS(SV) or PS(pIC) or PS(CpG ODN)) in nasal wash. Only sera of PS(pIC+SV) treated mice gave higher sIgA, suggesting that TLR3 and TLR9 ligands may help to improve mucosal immunity against H1N1 influenza in mice (Fig. 3.25). T cell activity in splenocytes against SV antigen were studied with either MHC Class I or MHC Class II SV peptides as well as SV and WV. IL-2 production was induced in splenocytes from PS(pIC+SV) when incubated with MHC Class II peptides. but didn't improve in intranasally PS nanocomplex treated mice. Joseph et.al., studied H1N1 vaccine in liposomes containing 25 µg ODN in their formulations (Joseph *et al.*, 2002). We used only 15 µg in the present study. Their IgG titers were nearly 50.000 from 10 animal combined sera and their max OD was ~0.3 whereas ours yielded maximum ~1.5 OD. When we read 0.3 OD our titers were ~73.000.

The PS/TLRL nanocomplexes can act as an anti-cancer agent as well as a potent vaccine carrier. Figure 4.2 is summarizing nanocomplex activity on an immune cell.

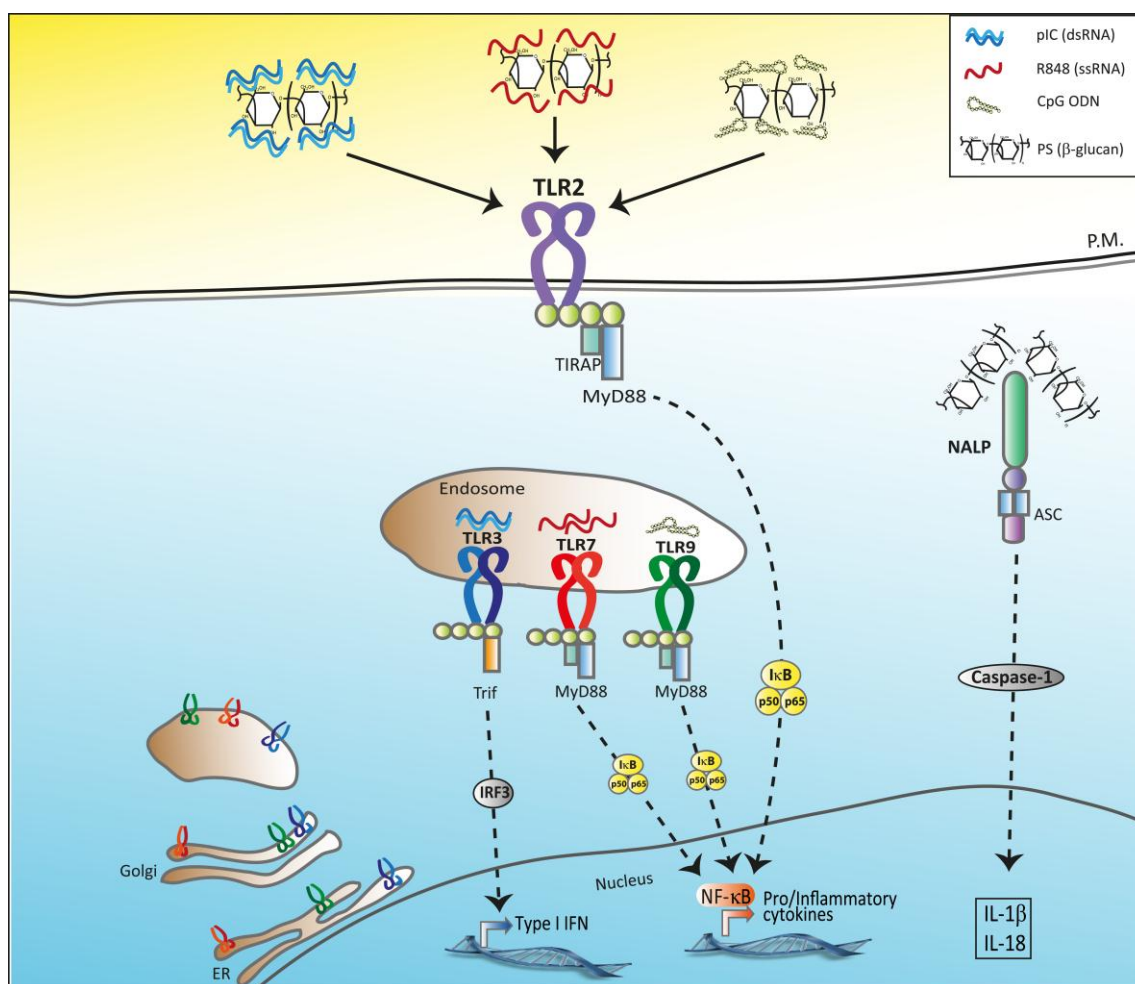


Figure 4.2. Schematic representation of the signaling pathways of PS(TLRL) nanocomplexes on immune cells.

In another study, a next generation CpG ODN molecule was tested for its suitability as a stand-alone effective anti-cancer immunotherapeutic agent. Since their description in mid 90`s, CpG ODNs became one of the most studied TLR agonists against infectious diseases, allergy and cancer. To date, three to four major classes of CpG ODNs were described depending on their backbone and sequence properties (i.e. presence of poly-G tail, flanking sequences), or number of CpG motifs expressed on a single strand and ability of these CpG sequences to adapt concatameric orientation leading to distinct immunological outcomes (Krieg, 2006). Shockingly, D-ODN development towards

clinical use was hampered by the fact that they undergo uncontrolled multimerization tendencies during synthesis. This raises a serious concern from FDA perspective (Vollmer and Krieg, 2009). We have designed a novel 7 CpG motif containing dendrimeric CpG ODN (named as ODN420). It spontaneously forms 30-40 nm sized uniform nanoparticles. ODN420 was more immunostimulatory than the linear 70mer 1466 CpG ODN (Karatepe, 2009; Mammadov, 2009). Moreover, anti-OVA immunization experiments demonstrated that ODN420 act as a potent adjuvant. However, to date, no one has fully investigated the anti-cancer efficacy of ODN420. This thesis fulfilled this task.

In *in vivo* experiments, HuH-7 xenografted nude mice were treated with ODN1466 or ODN420. Almost 50% reduction in tumor sizes were observed in 420ODN compared to linear ODN treated mice (Fig. 3.28). Almost one month after the treatments, half of the NP-ODN treated mice showed prolonged survival compared to linear ODN treated mice.

Both *in vitro* and *in vivo* tests supported the idea that a self-nanoparticle forming 420 CpG ODN is a promising standalone anti-cancer agent. The mechanism responsible from this effect is probably attributed to more pronounced natural killer cell activation. These assays must be performed to describe the validity of these effects. To date self nanoparticle CpG ODN was not described anywhere. In that context, there is no study utilizing this novel class of ODN as an anticancer therapeutic agent. However, in a similar study utilizing conventional ODN sequence plus 5-Fluorouracil (5-FU) as a combination therapy in hepatoma cell line derived tumor model~70% tumor mass reduction in BALB/C mice was reported. In this study, the authors administered four consecutive injections of 50 µg CpG ODN plus 25 µg 5-FU, however, in our case we injected three times 100 µg ODN and observed > 50% reduction in tumor mass (Wang *et al.*, 2005).

The last part of this thesis was dedicated to another economically important species, namely, broiler chickens. Unavoidable death due to acute infections (i.e. avian influenza) or sudden death of 2-3 weeks old broiler chickens due to stress related responses were among the prime problems affecting the industry, for this reason evaluating optimum vaccine types as well as administration routes were gaining importance recently.

Compared to mammals, chickens have different repertoires of immune molecules, antibodies, cytokines, chemokines, TLRs and immune cells. They do not have TLR9 but they express TLR21, therefore like mammals they do respond to CpG motifs but via ckTLR21 signaling cascade (Brownlie and Allan, 2011). *In vivo* studies showed that, *in ovo*, i.p. or s.c. vaccination with K-type CpG ODN prior to bacteria (*E.coli*, *Salmonella*) or virus (avian influenza, infectious bronchitis virus) challenges were highly effective in reducing mortality and increasing protective immune response in broiler chicken (Dar *et al.*, 2009a). In this thesis we aimed to assess the most efficient CpG ODN sequence in chicken rather than mammals, we designed and synthesized PO, PS or mixed backbone as well as D- and K- types of ODNs and tested their immune modulatory effects. Among tested almost 20 types of different CpG ODNs, D35-3CG-PO 2006 and 2007 were the most promising CpG ODNs by suitable for chicken immune system as evidenced by up-regulation of TLR or cytokine/chemokine mRNA message level and APC activation (Fig. 3.29–3.32). The immune activatory properties of these sequences might be important for the development of an immunological approach as well as vaccination strategies for controlling infectious diseases in broiler chicken and avoid uncontrolled death. Thus, *in vivo* potency of these sequences should be further analyzed.

Chapter 5

Future Perspectives

In order to better understand the mode of action of polysaccharides, trials with various knock-out mice would be very informative. For instance, different NALP knock-out mice or TLR2 double knockouts with different NALPs would be very critical as to document the dependence of PS action on these gene deficient mice.

Furthermore, the breadth of PS as a vaccine carrier system could be exploited further by generating novel complexes with other clinically important biomolecules, especially with peptides, proteins, plasmid DNA, siRNA, shRNA, and even miRNA and establish their contribution to better control of infectious diseases.

The entire study is focused on the immunostimulatory potential of PS4. We have however, worked with three other PS molecules. Of these, PS1 and PS3 did not induce any inflammatory response. We have abandoned investigating these PS molecules. A study to explore whether these PS molecules are immunosuppressive or not, would be a

very intriguing one. Further experiments must be planned to investigate the potential of these PS molecules.

In our cancer immunotherapy experiments, we unfortunately could not study the major cell type(s) responsible of tumor mass eradication. Series of experiments where NK cells or CD8⁺ T-cells or macrophages were neutralized during the cancer immunotherapy phase would provide very valuable information regarding the clearance mechanism. For example, mice treated with i) anti-CD4, ii) anti-CD8, iii) anti-CD3 for T cells or iv) anti-CD54, for NK cells or v) clodronate liposomes for macrophages would be very supportive. In addition to depletion experiments, immunohistochemistry analyses in tumor tissues could be performed to reveal the involvement of cell specific regulation of PS nanocomplexes or NP-CpG ODN mediated tumor regression in mice.

Chapter 6

References

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, *et al.* (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143-150.

Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, *et al.* (2011) Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *The Journal of allergy and clinical immunology* 127:701-721 e701-770.

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783-801.

Akuthota P, Wang HB, Spencer LA, Weller PF (2008) Immunoregulatory roles of eosinophils: a new look at a familiar cell. *Clin Exp Allergy* 38:1254-1263.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-738.

Allavena P, Sica A, Solinas G, Porta C, Mantovani A (2008) The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Critical reviews in oncology/hematology* 66:1-9.

Arias MA, Van Roey GA, Tregoning JS, Moutaftsi M, Coler RN, Windish HP, *et al.* (2012) Glucopyranosyl Lipid Adjuvant (GLA), a Synthetic TLR4 Agonist, Promotes Potent Systemic and Mucosal Responses to Intranasal Immunization with HIVgp140. *PloS one* 7:e41144.

Asahi-Ozaki Y, Itamura S, Ichinohe T, Strong P, Tamura S, Takahashi H, *et al.* (2006) Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. *Microbes and infection / Institut Pasteur* 8:2706-2714.

Bachmann MF, Jennings GT (2010) Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 10:787-796.

Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A (2006) Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. *J Immunol* 177:3515-3519.

Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, *et al.* (2007) MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol* 178:1164-1171.

Barbalat R, Lau L, Locksley RM, Barton GM (2009) Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nature immunology* 10:1200-1207.

Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y (2009) Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol* 182:1119-1128.

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. *Nature immunology* 7:49-56.

Basith S, Manavalan B, Lee G, Kim SG, Choi S (2011) Toll-like receptor modulators: a patent review (2006-2010). *Expert opinion on therapeutic patents* 21:927-944.

Batbayar S, Kim MJ, Kim HW (2011) Medicinal mushroom Lingzhi or Reishi, *Ganoderma lucidum* (W.Curt.:Fr.) P. Karst., beta-glucan induces Toll-like receptors and fails to induce inflammatory cytokines in NF-kappaB inhibitor-treated macrophages. *International journal of medicinal mushrooms* 13:213-225.

Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, *et al.* (2004) The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol* 172:3059-3069.

Ben-Baruch A (2006) Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. *Seminars in cancer biology* 16:38-52.

Bendall L (2005) Chemokines and their receptors in disease. *Histology and histopathology* 20:907-926.

- Beutler BA (2009) TLRs and innate immunity. *Blood* 113:1399-1407.
- Bhatia A, Kumar Y (2011) Cancer-immune equilibrium: questions unanswered. *Cancer Microenviron* 4:209-217.
- Blasius AL, Beutler B (2010) Intracellular toll-like receptors. *Immunity* 32:305-315.
- Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM (2011) CpG DNA as a vaccine adjuvant. *Expert review of vaccines* 10:499-511.
- Boehm T (2011) Design principles of adaptive immune systems. *Nat Rev Immunol* 11:307-317.
- Boland G, Beran J, Lievens M, Sasadeusz J, Dentico P, Nothdurft H, *et al.* (2004) Safety and immunogenicity profile of an experimental hepatitis B vaccine adjuvanted with AS04. *Vaccine* 23:316-320.
- Bong AB, Bonnekoh B, Franke I, Schon MP, Ulrich J, Gollnick H (2002) Imiquimod, a topical immune response modifier, in the treatment of cutaneous metastases of malignant melanoma. *Dermatology (Basel, Switzerland)* 205:135-138.
- Booth JS, Nichani AK, Benjamin P, Dar A, Krieg AM, Babiuk LA, *et al.* (2007) Innate immune responses induced by classes of CpG oligodeoxynucleotides in ovine lymph node and blood mononuclear cells. *Veterinary immunology and immunopathology* 115:24-34.
- Bourquin C, Anz D, Zwioerek K, Lanz AL, Fuchs S, Weigel S, *et al.* (2008) Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. *J Immunol* 181:2990-2998.
- Bowdish DM, Gordon S (2009) Conserved domains of the class A scavenger receptors: evolution and function. *Immunological reviews* 227:19-31.
- Boyden ED, Dietrich WF (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nature genetics* 38:240-244.
- Brownlie R, Allan B (2011) Avian toll-like receptors. *Cell and tissue research* 343:121-130.
- Burdette D, Haskett A, Presser L, McRae S, Iqbal J, Waris G (2012) Hepatitis C virus activates interleukin-1beta via caspase-1-inflammasome complex. *The Journal of general virology* 93:235-246.
- Carmi Y, Rinott G, Dotan S, Elkabets M, Rider P, Voronov E, *et al.* (2011) Microenvironment-derived IL-1 and IL-17 interact in the control of lung metastasis. *J Immunol* 186:3462-3471.
- Cheng YS, Xu F (2011) Anticancer function of polyinosinic-polycytidylic acid. *Cancer biology & therapy* 10:1219-1223.

Choi CH, Alabi CA, Webster P, Davis ME (2010) Mechanism of active targeting in solid tumors with transferrin-containing gold nanoparticles. *Proceedings of the National Academy of Sciences of the United States of America* 107:1235-1240.

Chuang T, Ulevitch RJ (2001) Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochimica et biophysica acta* 1518:157-161.

Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T, *et al.* (2010) Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9. *Cell host & microbe* 7:50-61.

Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, *et al.* (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *The Journal of experimental medicine* 201:19-25.

Coch C, Busch N, Wimmenauer V, Hartmann E, Janke M, Abdel-Mottaleb MM, *et al.* (2009) Higher activation of TLR9 in plasmacytoid dendritic cells by microbial DNA compared with self-DNA based on CpG-specific recognition of phosphodiester DNA. *Journal of leukocyte biology* 86:663-670.

Cooper CL, Davis HL, Morris ML, Efler SM, Krieg AM, Li Y, *et al.* (2004) Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine* 22:3136-3143.

Cormican P, Lloyd AT, Downing T, Connell SJ, Bradley D, O'Farrelly C (2009) The avian Toll-Like receptor pathway--subtle differences amidst general conformity. *Developmental and comparative immunology* 33:967-973.

Crozat K, Beutler B (2004) TLR7: A new sensor of viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 101:6835-6836.

Cyr SL, Angers I, Guillot L, Stoica-Popescu I, Lussier M, Qureshi S, *et al.* (2009) TLR4 and MyD88 control protection and pulmonary granulocytic recruitment in a murine intranasal RSV immunization and challenge model. *Vaccine* 27:421-430.

Daffis S, Samuel MA, Suthar MS, Gale M, Jr., Diamond MS (2008) Toll-like receptor 3 has a protective role against West Nile virus infection. *J Virol* 82:10349-10358.

Dar A, Allan B, Gomis S, Potter A, Mutwiri G (2009a) Immunotherapeutic potential of CpG oligonucleotides in chickens. *Journal of Poultry Science* 46:69-80.

Dar A, Potter A, Tikoo S, Gerdtz V, Lai K, Babiuk LA, *et al.* (2009b) CpG oligodeoxynucleotides activate innate immune response that suppresses infectious bronchitis virus replication in chicken embryos. *Avian diseases* 53:261-267.

Datta A, Sinha-Datta U, Dhillon NK, Buch S, Nicot C (2006) The HTLV-I p30 interferes with TLR4 signaling and modulates the release of pro- and anti-inflammatory cytokines from human macrophages. *The Journal of biological chemistry* 281:23414-23424.

de Jong S, Chikh G, Sekirov L, Raney S, Semple S, Klimuk S, *et al.* (2007) Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN. *Cancer Immunol Immunother* 56:1251-1264.

de Visser KE, Eichten A, Coussens LM (2006) Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* 6:24-37.

Debierre-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker U, Resende MG, *et al.* (2007) Activation of TLR2 and TLR4 by glycosylphosphatidylinositols derived from *Toxoplasma gondii*. *J Immunol* 179:1129-1137.

Degn SE, Thiel S, Jensenius JC (2007) New perspectives on mannan-binding lectin-mediated complement activation. *Immunobiology* 212:301-311.

Del Cacho E, Gallego M, Lillehoj HS, Lopez-Bernard F, Sanchez-Acedo C (2009) Avian follicular and interdigitating dendritic cells: isolation and morphologic, phenotypic, and functional analyses. *Veterinary immunology and immunopathology* 129:66-75.

Del Cacho E, Gallego M, Lopez-Bernard F, Sanchez-Acedo C, Lillehoj HS (2008) Isolation of chicken follicular dendritic cells. *Journal of immunological methods* 334:59-69.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, *et al.* (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103:351-361.

Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, *et al.* (2006) Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *The Journal of clinical investigation* 116:916-928.

Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* 27:519-550.

Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, *et al.* (2010) NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature* 464:1357-1361.

Dumitriu IE, Baruah P, Bianchi ME, Manfredi AA, Rovere-Querini P (2005) Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells. *European journal of immunology* 35:2184-2190.

Duncan JA, Gao X, Huang MT, O'Connor BP, Thomas CE, Willingham SB, *et al.* (2009) *Neisseria gonorrhoeae* activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *J Immunol* 182:6460-6469.

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology* 3:991-998.

Durier C, Launay O, Meiffredy V, Saidi Y, Salmon D, Levy Y, *et al.* (2006) Clinical safety of HIV lipopeptides used as vaccines in healthy volunteers and HIV-infected adults. *AIDS (London, England)* 20:1039-1049.

Eaves-Pyles TD, Wong HR, Odoms K, Pyles RB (2001) Salmonella flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *J Immunol* 167:7009-7016.

Edelmann KH, Richardson-Burns S, Alexopoulou L, Tyler KL, Flavell RA, Oldstone MB (2004) Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 322:231-238.

Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453:1122-1126.

Ellis RD, Martin LB, Shaffer D, Long CA, Miura K, Fay MP, *et al.* (2010) Phase 1 trial of the Plasmodium falciparum blood stage vaccine MSP1(42)-C1/Alhydrogel with and without CPG 7909 in malaria naive adults. *PloS one* 5:e8787.

Erikci E, Gursel M, Gursel I (2011) Differential immune activation following encapsulation of immunostimulatory CpG oligodeoxynucleotide in nanoliposomes. *Biomaterials* 32:1715-1723.

Fairhead T, Lian D, McCully ML, Garcia B, Zhong R, Madrenas J (2008) RIP2 is required for NOD signaling but not for Th1 cell differentiation and cellular allograft rejection. *Am J Transplant* 8:1143-1150.

Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, *et al.* (2007) Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Molecular cell* 25:713-724.

Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, *et al.* (2010) The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nature immunology* 11:385-393.

Ferrero RL (2005) Innate immune recognition of the extracellular mucosal pathogen, Helicobacter pylori. *Molecular immunology* 42:879-885.

Fiuza C, Bustin M, Talwar S, Tropea M, Gerstenberger E, Shelhamer JH, *et al.* (2003) Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 101:2652-2660.

Fonseca DE, Kline JN (2009) Use of CpG oligonucleotides in treatment of asthma and allergic disease. *Advanced drug delivery reviews* 61:256-262.

Franchi L, Eigenbrod T, Nunez G (2009) Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol* 183:792-796.

- Fremont CM, Togbe D, Doz E, Rose S, Vasseur V, Maillet I, *et al.* (2007) IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. *J Immunol* 179:1178-1189.
- Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, *et al.* (2009) Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer cell* 16:183-194.
- Fujii M, Shimizu T, Nakamura T, Endo F, Kohno S, Nabe T (2011) Inhibitory effect of chitosan-containing lotion on scratching response of hairless mice with atopic dermatitis-like dry skin. *Biological & pharmaceutical bulletin* 34:1890-1894.
- Gandotra S, Jang S, Murray PJ, Salgame P, Ehrt S (2007) Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with *Mycobacterium tuberculosis*. *Infection and immunity* 75:5127-5134.
- Garceau V, Smith J, Paton IR, Davey M, Fares MA, Sester DP, *et al.* (2010) Pivotal Advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. *Journal of leukocyte biology* 87:753-764.
- Garcon N, Chomez P, Van Mechelen M (2007) GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. *Expert review of vaccines* 6:723-739.
- Gazzinelli RT, Denkers EY (2006) Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. *Nat Rev Immunol* 6:895-906.
- Geijtenbeek TB, Gringhuis SI (2009) Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* 9:465-479.
- Gerold G, Zychlinsky A, de Diego JL (2007) What is the role of Toll-like receptors in bacterial infections? *Seminars in immunology* 19:41-47.
- Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 167:1882-1885.
- Ghosh TK, Mickelson DJ, Solberg JC, Lipson KE, Inglefield JR, Alkan SS (2007) TLR-TLR cross talk in human PBMC resulting in synergistic and antagonistic regulation of type-1 and 2 interferons, IL-12 and TNF-alpha. *International immunopharmacology* 7:1111-1121.
- Giannini SL, Hanon E, Moris P, Van Mechelen M, Morel S, Dessy F, *et al.* (2006) Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine* 24:5937-5949.
- Gilliet M, Cao W, Liu YJ (2008) Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* 8:594-606.

- Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, *et al.* (2001) Regulation of cutaneous malignancy by gammadelta T cells. *Science (New York, NY)* 294:605-609.
- Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods (San Diego, Calif)* 25:386-401.
- Gohda J, Matsumura T, Inoue J (2004) Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. *J Immunol* 173:2913-2917.
- Gomis S, Babiuk L, Allan B, Willson P, Waters E, Ambrose N, *et al.* (2004) Protection of neonatal chicks against a lethal challenge of *Escherichia coli* using DNA containing cytosine-phosphodiester-guanine motifs. *Avian diseases* 48:813-822.
- Goodridge HS, Simmons RM, Underhill DM (2007) Dectin-1 stimulation by *Candida albicans* yeast or zymosan triggers NFAT activation in macrophages and dendritic cells. *J Immunol* 178:3107-3115.
- Goodridge HS, Underhill DM (2008) Fungal Recognition by TLR2 and Dectin-1. *Handbook of experimental pharmacology*:87-109.
- Gordon M, Guralnik M, Kaneko Y, Mimura T, Goodgame J, DeMarzo C, *et al.* (1995) A phase II controlled study of a combination of the immune modulator, lentinan, with didanosine (ddI) in HIV patients with CD4 cells of 200-500/mm³. *Journal of medicine* 26:193-207.
- Gordon S (2002) Pattern recognition receptors: doubling up for the innate immune response. *Cell* 111:927-930.
- Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, Lin R, *et al.* (2006) Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. *Blood* 107:1078-1084.
- Govindaraj RG, Manavalan B, Lee G, Choi S (2010) Molecular modeling-based evaluation of hTLR10 and identification of potential ligands in Toll-like receptor signaling. *PloS one* 5:e12713.
- Graham LM, Brown GD (2009) The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine* 48:148-155.
- Gray RC, Kuchtey J, Harding CV (2007) CpG-B ODNs potently induce low levels of IFN- α and induce IFN- α -dependent MHC-I cross-presentation in DCs as effectively as CpG-A and CpG-C ODNs. *Journal of leukocyte biology* 81:1075-1085.
- Green DS, Lum T, Mullins BT, Green JA (2008) Antibody-mediated enhancement of the rate, magnitude, and responsiveness of vesicular stomatitis virus induced α interferon production. *Journal of medical virology* 80:1675-1683.

- Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. *Cell* 140:883-899.
- Gross O, Poeck H, Bscheider M, Dostert C, Hanneschlager N, Endres S, *et al.* (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459:433-436.
- Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, *et al.* (2008) NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 28:571-580.
- Gullotti E, Yeo Y (2009) Extracellularly activated nanocarriers: a new paradigm of tumor targeted drug delivery. *Molecular pharmaceutics* 6:1041-1051.
- 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* 171:1393-1400.
- Gursel M, Gursel I, Mostowski HS, Klinman DM (2006) CXCL16 influences the nature and specificity of CpG-induced immune activation. *J Immunol* 177:1575-1580.
- Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM (2002) Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *Journal of leukocyte biology* 71:813-820.
- Haas T, Metzger J, Schmitz F, Heit A, Muller T, Latz E, *et al.* (2008) The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. *Immunity* 28:315-323.
- Hageman RS, Leduc MS, Caputo CR, Tsaih SW, Churchill GA, Korstanje R (2011) Uncovering genes and regulatory pathways related to urinary albumin excretion. *J Am Soc Nephrol* 22:73-81.
- Hagman JH, Bianchi L, Marulli GC, Soda R, Chimenti S (2003) Successful treatment of multiple filiform facial warts with imiquimod 5% cream in a patient infected by human immunodeficiency virus. *Clinical and experimental dermatology* 28:260-261.
- Halperin SA, Dobson S, McNeil S, Langley JM, Smith B, McCall-Sani R, *et al.* (2006) Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. *Vaccine* 24:20-26.
- Hamdy S, Molavi O, Ma Z, Haddadi A, Alshamsan A, Gobti Z, *et al.* (2008) Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8⁺ T cell-mediated anti-tumor immunity. *Vaccine* 26:5046-5057.
- Hamzah J, Altin JG, Herringson T, Parish CR, Hammerling GJ, O'Donoghue H, *et al.* (2009) Targeted liposomal delivery of TLR9 ligands activates spontaneous antitumor immunity in an autochthonous cancer model. *J Immunol* 183:1091-1098.

- Hanagata N (2012) Structure-dependent immunostimulatory effect of CpG oligodeoxynucleotides and their delivery system. *International journal of nanomedicine* 7:2181-2195.
- Hansen TH, Bouvier M (2009) MHC class I antigen presentation: learning from viral evasion strategies. *Nat Rev Immunol* 9:503-513.
- Harrison CJ, Miller RL, Bernstein DI (2001) Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine. *Vaccine* 19:1820-1826.
- Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, *et al.* (2003) Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- α induction in plasmacytoid dendritic cells. *European journal of immunology* 33:1633-1641.
- Hartmann G, Krieg AM (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 164:944-953.
- Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, *et al.* (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410:1099-1103.
- He H, Crippen TL, Farnell MB, Kogut MH (2003) Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. *Developmental and comparative immunology* 27:621-627.
- He H, Genovese KJ, Swaggerty CL, Nisbet DJ, Kogut MH (2007) In vivo priming heterophil innate immune functions and increasing resistance to *Salmonella enteritidis* infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides. *Veterinary immunology and immunopathology* 117:275-283.
- He H, Lowry VK, Swaggerty CL, Ferro PJ, Kogut MH (2005) In vitro activation of chicken leukocytes and in vivo protection against *Salmonella enteritidis* organ invasion and peritoneal *S. enteritidis* infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. *FEMS immunology and medical microbiology* 43:81-89.
- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, *et al.* (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740-745.
- Henao-Mejia J, Elinav E, Strowig T, Flavell RA (2012) Inflammasomes: far beyond inflammation. *Nature immunology* 13:321-324.
- Hetland G, Ohno N, Aaberge IS, Lovik M (2000) Protective effect of beta-glucan against systemic *Streptococcus pneumoniae* infection in mice. *FEMS immunology and medical microbiology* 27:111-116.
- Higgs R, Cormican P, Cahalane S, Allan B, Lloyd AT, Meade K, *et al.* (2006) Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. *Infection and immunity* 74:1692-1698.

- Hoebe K, Janssen E, Beutler B (2004) The interface between innate and adaptive immunity. *Nature immunology* 5:971-974.
- Holscher C, Reiling N, Schaible UE, Holscher A, Bathmann C, Korb D, *et al.* (2008) Containment of aerogenic *Mycobacterium tuberculosis* infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9. *European journal of immunology* 38:680-694.
- Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, *et al.* (2005) Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* 434:1035-1040.
- Hopkinson WI, Williams W, Griffiths GL, Jessop D, Peters SM (1984) Dietary induction of sudden death syndrome in broiler breeders. *Avian diseases* 28:352-357.
- Horner SM, Gale M, Jr. (2009) Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. *J Interferon Cytokine Res* 29:489-498.
- Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, *et al.* (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458:514-518.
- Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, *et al.* (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162:3749-3752.
- Houghton M (2010) Prospects for prophylactic and therapeutic vaccines against the hepatitis C viruses. *Immunological reviews* 239:99-108.
- Hua KF, Hsu HY, Chao LK, Chen ST, Yang WB, Hsu J, *et al.* (2007) *Ganoderma lucidum* polysaccharides enhance CD14 endocytosis of LPS and promote TLR4 signal transduction of cytokine expression. *Journal of cellular physiology* 212:537-550.
- Huang Z, Zhang Z, Jiang Y, Zhang D, Chen J, Dong L, *et al.* (2012) Targeted delivery of oligonucleotides into tumor-associated macrophages for cancer immunotherapy. *J Control Release* 158:286-292.
- Ichinohe T, Kawaguchi A, Tamura S, Takahashi H, Sawa H, Ninomiya A, *et al.* (2007) Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes and infection / Institut Pasteur* 9:1333-1340.
- Illum L, Jabbar-Gill I, Hinchcliffe M, Fisher AN, Davis SS (2001) Chitosan as a novel nasal delivery system for vaccines. *Advanced drug delivery reviews* 51:81-96.
- Inohara, Chamaillard, McDonald C, Nunez G (2005) NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* 74:355-383.

- Iqbal M, Philbin VJ, Smith AL (2005) Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Veterinary immunology and immunopathology* 104:117-127.
- Ishikawa H, Ma Z, Barber GN (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 461:788-792.
- Iwasaki A, Medzhitov R (2010) Regulation of adaptive immunity by the innate immune system. *Science (New York, NY)* 327:291-295.
- Iyer SS, Pulskens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, *et al.* (2009) Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proceedings of the National Academy of Sciences of the United States of America* 106:20388-20393.
- Jacobs SR, Damania B (2012) NLRs, inflammasomes, and viral infection. *Journal of leukocyte biology*.
- Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. *Annual review of immunology* 20:197-216.
- Janssens S, Beyaert R (2002) A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends in biochemical sciences* 27:474-482.
- Janssens S, Beyaert R (2003) Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Molecular cell* 11:293-302.
- Jennings GT, Bachmann MF (2008) The coming of age of virus-like particle vaccines. *Biological chemistry* 389:521-536.
- Jeurissen SH, Boonstra-Blom AG, Al-Garib SO, Hartog L, Koch G (2000) Defence mechanisms against viral infection in poultry: a review. *The Veterinary quarterly* 22:204-208.
- Jiang Q, Akashi S, Miyake K, Petty HR (2000) Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. *J Immunol* 165:3541-3544.
- Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, *et al.* (2007a) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 130:1071-1082.
- Jin Y, Mailloux CM, Gowan K, Riccardi SL, LaBerge G, Bennett DC, *et al.* (2007b) NALP1 in vitiligo-associated multiple autoimmune disease. *The New England journal of medicine* 356:1216-1225.
- Joseph A, Louria-Hayon I, Plis-Finarov A, Zeira E, Zakay-Rones Z, Raz E, *et al.* (2002) Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines. *Vaccine* 20:3342-3354.

Kabelitz D, Medzhitov R (2007) Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Current opinion in immunology* 19:1-3.

Kaiser P (2010) Advances in avian immunology--prospects for disease control: a review. *Avian Pathol* 39:309-324.

Kaiser P, Wu Z, Rothwell L, Fife M, Gibson M, Poh TY, *et al.* (2009) Prospects for understanding immune-endocrine interactions in the chicken. *General and comparative endocrinology* 163:83-91.

Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, *et al.* (2006) Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *The Journal of biological chemistry* 281:36560-36568.

Kanneganti TD, Lamkanfi M, Nunez G (2007) Intracellular NOD-like receptors in host defense and disease. *Immunity* 27:549-559.

Kapetanovic R, Jouvion G, Fitting C, Parlato M, Blanchet C, Huerre M, *et al.* (2010) Contribution of NOD2 to lung inflammation during Staphylococcus aureus-induced pneumonia. *Microbes and infection / Institut Pasteur* 12:759-767.

Karatepe K (2009) Immunoregulatory Activities of Nanoparticle-Forming Oligodeoxynucleotides. *Master of Science Thesis*.

Karin M (2006) Nuclear factor-kappaB in cancer development and progression. *Nature* 441:431-436.

Katagiri N, Shobuike T, Chang B, Kukita A, Miyamoto H (2012) The human apoptosis inhibitor NAIP induces pyroptosis in macrophages infected with Legionella pneumophila. *Microbes and infection / Institut Pasteur*.

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11:115-122.

Kawai T, Akira S (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends in molecular medicine* 13:460-469.

Kawai T, Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology* 21:317-337.

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* 11:373-384.

Kawai T, Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637-650.

- Keestra AM, van Putten JP (2008) Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. *J Immunol* 181:4354-4362.
- Kerkmann M, Costa LT, Richter C, Rothenfusser S, Battiany J, Hornung V, *et al.* (2005) Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon- α induction by CpG-A in plasmacytoid dendritic cells. *The Journal of biological chemistry* 280:8086-8093.
- Kersse K, Bertrand MJ, Lamkanfi M, Vandenabeele P (2011) NOD-like receptors and the innate immune system: coping with danger, damage and death. *Cytokine & growth factor reviews* 22:257-276.
- Kikkert R, Bulder I, de Groot ER, Aarden LA, Finkelman MA (2007) Potentiation of Toll-like receptor-induced cytokine production by (1 $\rightarrow$ 3)- β -D-glucans: implications for the monocyte activation test. *Journal of endotoxin research* 13:140-149.
- Kim YM, Brinkmann MM, Paquet ME, Ploegh HL (2008) UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* 452:234-238.
- Kingeter LM, Lin X (2012) C-type lectin receptor-induced NF- κ B activation in innate immune and inflammatory responses. *Cellular & molecular immunology* 9:105-112.
- Klasing KC (1994) Avian leukocytic cytokines. *Poultry science* 73:1035-1043.
- Klein DC, Latz E, Espevik T, Stokke BT (2010) Higher order structure of short immunostimulatory oligonucleotides studied by atomic force microscopy. *Ultramicroscopy* 110:689-693.
- Klimek L, Willers J, Hammann-Haenni A, Pfaar O, Stocker H, Mueller P, *et al.* (2011) Assessment of clinical efficacy of CYT003-QbG10 in patients with allergic rhinoconjunctivitis: a phase IIb study. *Clin Exp Allergy* 41:1305-1312.
- Klinman DM, Barnhart KM, Conover J (1999) CpG motifs as immune adjuvants. *Vaccine* 17:19-25.
- Klinman DM, Currie D, Gursel I, Verthelyi D (2004) Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunological reviews* 199:201-216.
- Klinman DM, Kamstrup S, Verthelyi D, Gursel I, Ishii KJ, Takeshita F, *et al.* (2000) Activation of the innate immune system by CpG oligodeoxynucleotides: immunoprotective activity and safety. *Springer Semin Immunopathol* 22:173-183.
- Klinman DM, Klaschik S, Sato T, Tross D (2009) CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. *Advanced drug delivery reviews* 61:248-255.
- Kocbek P, Obermajer N, Cegnar M, Kos J, Kristl J (2007) Targeting cancer cells using PLGA nanoparticles surface modified with monoclonal antibody. *J Control Release* 120:18-26.

Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, *et al.* (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450:903-907.

Kofoed EM, Vance RE (2012) NAIPs: Building an innate immune barrier against bacterial pathogens: NAIPs function as sensors that initiate innate immunity by detection of bacterial proteins in the host cell cytosol. *Bioessays* 34:589-598.

Kogut MH, Genovese KJ, He H, Kaiser P (2008) Flagellin and lipopolysaccharide up-regulation of IL-6 and CXCLi2 gene expression in chicken heterophils is mediated by ERK1/2-dependent activation of AP-1 and NF-kappaB signaling pathways. *Innate immunity* 14:213-222.

Koppe U, Hogner K, Doehn JM, Muller HC, Witzernath M, Gutbier B, *et al.* (2012) *Streptococcus pneumoniae* stimulates a STING- and IFN regulatory factor 3-dependent type I IFN production in macrophages, which regulates RANTES production in macrophages, cocultured alveolar epithelial cells, and mouse lungs. *J Immunol* 188:811-817.

Kortylewski M, Xin H, Kujawski M, Lee H, Liu Y, Harris T, *et al.* (2009) Regulation of the IL-23 and IL-12 balance by Stat3 signaling in the tumor microenvironment. *Cancer cell* 15:114-123.

Koyama S, Aoshi T, Tanimoto T, Kumagai Y, Kobiyama K, Tougan T, *et al.* (2010) Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Science translational medicine* 2:25ra24.

Kreiter S, Diken M, Selmi A, Diekmann J, Attig S, Husemann Y, *et al.* (2011) FLT3 ligand enhances the cancer therapeutic potency of naked RNA vaccines. *Cancer research* 71:6132-6142.

Krieg AM (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nature reviews* 5:471-484.

Krieg AM (2012) CpG still rocks! Update on an accidental drug. *Nucleic acid therapeutics* 22:77-89.

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, *et al.* (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374:546-549.

Krug A, Rothenfusser S, Hornung V, Jahrsdorfer B, Blackwell S, Ballas ZK, *et al.* (2001a) Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *European journal of immunology* 31:2154-2163.

Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, *et al.* (2001b) Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *European journal of immunology* 31:3026-3037.

- Kryczek I, Wei S, Szeliga W, Vatan L, Zou W (2009) Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114:357-359.
- Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, *et al.* (2007) Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27:240-252.
- Kumar H, Kawai T, Akira S (2009a) Pathogen recognition in the innate immune response. *Biochem J* 420:1-16.
- Kumar H, Kawai T, Akira S (2009b) Toll-like receptors and innate immunity. *Biochemical and biophysical research communications* 388:621-625.
- Kumar H, Kumagai Y, Tsuchida T, Koenig PA, Satoh T, Guo Z, *et al.* (2009c) Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal beta-glucan. *J Immunol* 183:8061-8067.
- Kundi M (2007) New hepatitis B vaccine formulated with an improved adjuvant system. *Expert review of vaccines* 6:133-140.
- Laing KJ, Secombes CJ (2004) Chemokines. *Developmental and comparative immunology* 28:443-460.
- Lam KS (2007) New aspects of natural products in drug discovery. *Trends in microbiology* 15:279-289.
- Lamkanfi M, Malireddi RK, Kanneganti TD (2009a) Fungal zymosan and mannan activate the cryopyrin inflammasome. *The Journal of biological chemistry* 284:20574-20581.
- Lamkanfi M, Mueller JL, Vitari AC, Misaghi S, Fedorova A, Deshayes K, *et al.* (2009b) Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *The Journal of cell biology* 187:61-70.
- Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, *et al.* (2007) Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 449:564-569.
- Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, *et al.* (2004) TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature immunology* 5:190-198.
- Lee YR, Lee YH, Im SA, Yang IH, Ahn GW, Kim K, *et al.* (2010) Biodegradable nanoparticles containing TLR3 or TLR9 agonists together with antigen enhance MHC-restricted presentation of the antigen. *Archives of pharmacal research* 33:1859-1866.
- Leemans JC, Cassel SL, Sutterwala FS (2011) Sensing damage by the NLRP3 inflammasome. *Immunological reviews* 243:152-162.

Lehtovaara BC, Gu FX (2011) Pharmacological, structural, and drug delivery properties and applications of 1,3-beta-glucans. *Journal of agricultural and food chemistry* 59:6813-6828.

Lemon SM (2010) Induction and evasion of innate antiviral responses by hepatitis C virus. *The Journal of biological chemistry* 285:22741-22747.

Leonard JP, Link BK, Emmanouilides C, Gregory SA, Weisdorf D, Andrey J, *et al.* (2007) Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma. *Clin Cancer Res* 13:6168-6174.

Libri NA, Barker SJ, Rosenberg WM, Semper AE (2009) A class C CpG toll-like receptor 9 agonist successfully induces robust interferon-alpha production by plasmacytoid dendritic cells from patients chronically infected with hepatitis C. *Journal of viral hepatitis* 16:315-324.

Lin SC, Huang MH, Tsou PC, Huang LM, Chong P, Wu SC (2011) Recombinant trimeric HA protein immunogenicity of H5N1 avian influenza viruses and their combined use with inactivated or adenovirus vaccines. *PloS one* 6:e20052.

Lin YL, Liang YC, Lee SS, Chiang BL (2005) Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. *Journal of leukocyte biology* 78:533-543.

Link BK, Ballas ZK, Weisdorf D, Wooldridge JE, Bossler AD, Shannon M, *et al.* (2006) Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma. *J Immunother* 29:558-568.

Livingston BD, Alexander J, Crimi C, Oseroff C, Celis E, Daly K, *et al.* (1999) Altered helper T lymphocyte function associated with chronic hepatitis B virus infection and its role in response to therapeutic vaccination in humans. *J Immunol* 162:3088-3095.

Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, *et al.* (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4⁺ Th1 immunity with poly IC as adjuvant. *The Journal of experimental medicine* 206:1589-1602.

Lu XY, Wu DC, Li ZJ, Chen GQ (2011) Polymer nanoparticles. *Progress in molecular biology and translational science* 104:299-323.

Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A (2003) Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *The Journal of experimental medicine* 198:513-520.

Luo J, Obmolova G, Malia TJ, Wu SJ, Duffy KE, Marion JD, *et al.* (2012) Lateral Clustering of TLR3:dsRNA Signaling Units Revealed by TLR3ecd:3Fabs Quaternary Structure. *Journal of molecular biology* 421:112-124.

Lynn DJ, Higgs R, Lloyd AT, O'Farrelly C, Herve-Grepinet V, Nys Y, *et al.* (2007) Avian beta-defensin nomenclature: a community proposed update. *Immunology letters* 110:86-89.

Maeda H, Bharate GY, Daruwalla J (2009) Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect. *Eur J Pharm Biopharm* 71:409-419.

Maeda S, Hikiba Y, Shibata W, Ohmae T, Yanai A, Ogura K, *et al.* (2007) Essential roles of high-mobility group box 1 in the development of murine colitis and colitis-associated cancer. *Biochemical and biophysical research communications* 360:394-400.

Makela SM, Strengell M, Pietila TE, Osterlund P, Julkunen I (2009) Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *Journal of leukocyte biology* 85:664-672.

Mammadov R (2009) Self-Nanoparticle Forming Immunostimulatory DNA: Structure-Function Relationship Studies. *Master of Science Thesis*:121.

Mao AP, Li S, Zhong B, Li Y, Yan J, Li Q, *et al.* (2010) Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of interferon-beta (IFN-beta) and cellular antiviral response. *The Journal of biological chemistry* 285:9470-9476.

Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, *et al.* (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440:228-232.

Markova N, Kussovski V, Drandarska I, Nikolaeva S, Georgieva N, Radoucheva T (2003) Protective activity of Lentinan in experimental tuberculosis. *International immunopharmacology* 3:1557-1562.

Marr N, Turvey SE (2012) Role of human TLR4 in respiratory syncytial virus-induced NF-kappaB activation, viral entry and replication. *Innate immunity*.

Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, *et al.* (2003) Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *Journal of leukocyte biology* 73:781-792.

Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Molecular cell* 10:417-426.

Martinon F, Gaide O, Petrilli V, Mayor A, Tschopp J (2007) NALP inflammasomes: a central role in innate immunity. *Seminars in immunopathology* 29:213-229.

Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, *et al.* (2010) Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. *J Immunol* 184:3326-3330.

McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, Sandor M, *et al.* (2010) Granuloma formation and host defense in chronic Mycobacterium tuberculosis infection requires PYCARD/ASC but not NLRP3 or caspase-1. *PloS one* 5:e12320.

- McKee AS, Munks MW, Marrack P (2007) How do adjuvants work? Important considerations for new generation adjuvants. *Immunity* 27:687-690.
- Mellman I, Coukos G, Dranoff G (2011) Cancer immunotherapy comes of age. *Nature* 480:480-489.
- Mishra BB, Gundra UM, Teale JM (2008) Expression and distribution of Toll-like receptors 11-13 in the brain during murine neurocysticercosis. *Journal of neuroinflammation* 5:53.
- Mitola S, Belleri M, Urbinati C, Coltrini D, Sparatore B, Pedrazzi M, *et al.* (2006) Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. *J Immunol* 176:12-15.
- Moghimi SM, Hunter AC, Murray JC (2005) Nanomedicine: current status and future prospects. *Faseb J* 19:311-330.
- Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* 16:1216-1224.
- Morse MA, Chapman R, Powderly J, Blackwell K, Keler T, Green J, *et al.* (2011) Phase I study utilizing a novel antigen-presenting cell-targeted vaccine with Toll-like receptor stimulation to induce immunity to self-antigens in cancer patients. *Clin Cancer Res* 17:4844-4853.
- Mozafari MR (2005) Liposomes: an overview of manufacturing techniques. *Cellular & molecular biology letters* 10:711-719.
- Mullen GE, Ellis RD, Miura K, Malkin E, Nolan C, Hay M, *et al.* (2008) Phase 1 trial of AMA1-C1/Alhydrogel plus CPG 7909: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *PloS one* 3:e2940.
- Muller T, Hamm S, Bauer S (2008) TLR9-mediated recognition of DNA. *Handbook of experimental pharmacology*:51-70.
- Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, *et al.* (2008) Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 112:362-373.
- Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 8:618-631.
- Murphy EA, Davis JM, Carmichael MD (2010) Immune modulating effects of beta-glucan. *Current opinion in clinical nutrition and metabolic care* 13:656-661.
- Murphy JE, Tedbury PR, Homer-Vanniasinkam S, Walker JH, Ponnambalam S (2005) Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis* 182:1-15.
- Murphy MK, Travers P, Walport M, Janeway C (2011) Janeway's Immunobiology. *Garland Science* Eighth Edition.

- Nain S, Laarveld B, Wojnarowicz C, Olkowski AA (2007) Excessive dietary vitamin D supplementation as a risk factor for sudden death syndrome in fast growing commercial broilers. *Comparative biochemistry and physiology* 148:828-833.
- Nair SS, Bommana A, Bethony JM, Lyon AJ, Ohshiro K, Pakala SB, *et al.* (2011) The metastasis-associated protein-1 gene encodes a host permissive factor for schistosomiasis, a leading global cause of inflammation and cancer. *Hepatology (Baltimore, Md)* 54:285-295.
- Napolitani G, Rinaldi A, Berton F, Sallusto F, Lanzavecchia A (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nature immunology* 6:769-776.
- Nardin EH, Oliveira GA, Calvo-Calle JM, Castro ZR, Nussenzweig RS, Schmeckpeper B, *et al.* (2000) Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccinees of defined HLA genotypes. *The Journal of infectious diseases* 182:1486-1496.
- Nauseef WM (2007) How human neutrophils kill and degrade microbes: an integrated view. *Immunological reviews* 219:88-102.
- Netea MG, Marodi L (2010) Innate immune mechanisms for recognition and uptake of *Candida* species. *Trends in immunology* 31:346-353.
- Netea MG, Suttmüller R, Hermann C, Van der Graaf CA, Van der Meer JW, van Krieken JH, *et al.* (2004) Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol* 172:3712-3718.
- Nigrovic LE, Thompson KM (2007) The Lyme vaccine: a cautionary tale. *Epidemiology and infection* 135:1-8.
- Nishikawa M, Matono M, Rattanakit S, Matsuoka N, Takakura Y (2008) Enhanced immunostimulatory activity of oligodeoxynucleotides by Y-shape formation. *Immunology* 124:247-255.
- Nishikawa M, Mizuno Y, Mohri K, Matsuoka N, Rattanakit S, Takahashi Y, *et al.* (2011) Biodegradable CpG DNA hydrogels for sustained delivery of doxorubicin and immunostimulatory signals in tumor-bearing mice. *Biomaterials* 32:488-494.
- Nishiya T, Kajita E, Miwa S, Defranco AL (2005) TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *The Journal of biological chemistry* 280:37107-37117.
- Nixon Tangi T, Elmabsout AA, Bengtsson T, Sirsjo A, Fransen K (2012) Role of NLRP3 and CARD8 in the regulation of TNF- α induced IL-1 β release in vascular smooth muscle cells. *International journal of molecular medicine* 30:697-702.
- Numasaki M, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, *et al.* (2003) Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101:2620-2627.

- O'Brien KD, Chait A (2006) Serum amyloid A: the "other" inflammatory protein. *Current atherosclerosis reports* 8:62-68.
- Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411:603-606.
- Okada H (2011) Drug discovery by formulation design and innovative drug delivery systems (DDS). *Yakugaku Zasshi* 131:1271-1287.
- Olive C (2012) Pattern recognition receptors: sentinels in innate immunity and targets of new vaccine adjuvants. *Expert review of vaccines* 11:237-256.
- Orive G, Hernandez RM, Rodriguez Gascon A, Dominguez-Gil A, Pedraz JL (2003) Drug delivery in biotechnology: present and future. *Current opinion in biotechnology* 14:659-664.
- Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nature immunology* 4:161-167.
- Ostrand-Rosenberg S, Sinha P (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182:4499-4506.
- Palucka AK, Ueno H, Fay JW, Banchereau J (2007) Taming cancer by inducing immunity via dendritic cells. *Immunological reviews* 220:129-150.
- Paraskevas KI, Mikhailidis DP (2008) C-reactive protein (CRP): more than just an innocent bystander? *Current medical research and opinion* 24:75-78.
- Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458:1191-1195.
- Parveen S, Misra R, Sahoo SK (2012) Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine* 8:147-166.
- Pasparakis M (2012) Role of NF-kappaB in epithelial biology. *Immunological reviews* 246:346-358.
- Pathak K, Keshri L, Shah M (2011) Lipid nanocarriers: influence of lipids on product development and pharmacokinetics. *Critical reviews in therapeutic drug carrier systems* 28:357-393.
- Paul-Clark MJ, McMaster SK, Belcher E, Sorrentino R, Anandarajah J, Fleet M, *et al.* (2006) Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. *British journal of pharmacology* 148:1067-1075.

Peng S, Geng J, Sun R, Tian Z, Wei H (2009) Polyinosinic-polycytidylic acid liposome induces human hepatoma cells apoptosis which correlates to the up-regulation of RIG-I like receptors. *Cancer science* 100:529-536.

Philbin VJ, Iqbal M, Boyd Y, Goodchild MJ, Beal RK, Bumstead N, *et al.* (2005) Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* 114:507-521.

Plattner F, Yarovinsky F, Romero S, Didry D, Carlier MF, Sher A, *et al.* (2008) Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell host & microbe* 3:77-87.

Poeck H, Wagner M, Battiany J, Rothenfusser S, Wellisch D, Hornung V, *et al.* (2004) Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. *Blood* 103:3058-3064.

Pontillo A, Catamo E, Arosio B, Mari D, Crovella S (2011) NALP1/NLRP1 Genetic Variants are Associated With Alzheimer Disease. *Alzheimer disease and associated disorders*.

Pontillo A, Silva LT, Oshiro TM, Finazzo C, Crovella S, Duarte AJ (2012) HIV-1 induces NALP3-inflammasome expression and interleukin-1 β secretion in dendritic cells from healthy individuals but not from HIV-positive patients. *AIDS (London, England)* 26:11-18.

Rankin R, Pontarollo R, Ioannou X, Krieg AM, Hecker R, Babiuk LA, *et al.* (2001) CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense & nucleic acid drug development* 11:333-340.

Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, *et al.* (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nature immunology* 11:395-402.

Re F, Strominger JL (2004) IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J Immunol* 173:7548-7555.

Reis e Sousa C (2004) Toll-like receptors and dendritic cells: for whom the bug tolls. *Seminars in immunology* 16:27-34.

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, *et al.* (2005) The evolution of vertebrate Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America* 102:9577-9582.

Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, *et al.* (2009) Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *The Journal of experimental medicine* 206:2037-2051.

Romani L (2011) Immunity to fungal infections. *Nat Rev Immunol* 11:275-288.

- Romanowski B, de Borba PC, Naud PS, Roteli-Martins CM, De Carvalho NS, Teixeira JC, *et al.* (2009) Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years. *Lancet* 374:1975-1985.
- Rotter B, Guenter W, Boycott BR (1985) Sudden death syndrome in broilers: dietary fat supplementation and its effect on tissue composition. *Poultry science* 64:1128-1136.
- Sahoo SK, Labhasetwar V (2005) Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. *Molecular pharmaceutics* 2:373-383.
- Sahoo SK, Ma W, Labhasetwar V (2004) Efficacy of transferrin-conjugated paclitaxel-loaded nanoparticles in a murine model of prostate cancer. *International journal of cancer* 112:335-340.
- Sahoo SK, Parveen S, Panda JJ (2007) The present and future of nanotechnology in human health care. *Nanomedicine* 3:20-31.
- Saiga H, Shimada Y, Takeda K (2011) Innate immune effectors in mycobacterial infection. *Clinical & developmental immunology* 2011:347594.
- Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, *et al.* (2007) Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nature immunology* 8:39-46.
- Saijo S, Iwakura Y (2011) Dectin-1 and Dectin-2 in innate immunity against fungi. *International immunology* 23:467-472.
- Saitoh S, Miyake K (2009) Regulatory molecules required for nucleotide-sensing Toll-like receptors. *Immunological reviews* 227:32-43.
- Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P (2007) Toll-like receptor 3 expressed by melanoma cells as a target for therapy? *Clin Cancer Res* 13:4565-4574.
- Samulowicz U, Weber M, Weeratna R, Uhlmann E, Noll B, Krieg AM, *et al.* (2010) A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties. *Oligonucleotides* 20:93-101.
- Sarkar SN, Smith HL, Rowe TM, Sen GC (2003) Double-stranded RNA signaling by Toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. *The Journal of biological chemistry* 278:4393-4396.
- Sato S, Nomura F, Kawai T, Takeuchi O, Muhlradt PF, Takeda K, *et al.* (2000) Synergy and cross-tolerance between toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. *J Immunol* 165:7096-7101.
- Schroder K, Tschopp J (2010) The inflammasomes. *Cell* 140:821-832.

Schroder K, Zhou R, Tschopp J (2010) The NLRP3 inflammasome: a sensor for metabolic danger? *Science (New York, NY)* 327:296-300.

Shang G, Zhu D, Li N, Zhang J, Zhu C, Lu D, *et al.* (2012) Crystal structures of STING protein reveal basis for recognition of cyclic di-GMP. *Nature structural & molecular biology* 19:725-727.

Shaw MH, Reimer T, Kim YG, Nunez G (2008) NOD-like receptors (NLRs): bona fide intracellular microbial sensors. *Current opinion in immunology* 20:377-382.

Shereck E, Satwani P, Morris E, Cairo MS (2007) Human natural killer cells in health and disease. *Pediatric blood & cancer* 49:615-623.

Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, *et al.* (2011) A novel Toll-like receptor that recognizes vesicular stomatitis virus. *The Journal of biological chemistry* 286:4517-4524.

Shibata W, Takaishi S, Muthupalani S, Pritchard DM, Whary MT, Rogers AB, *et al.* (2010) Conditional deletion of IkappaB-kinase-beta accelerates helicobacter-dependent gastric apoptosis, proliferation, and preneoplasia. *Gastroenterology* 138:1022-1034 e1021-1010.

Shimada N, Ishii KJ, Takeda Y, Coban C, Torii Y, Shinkai S, *et al.* (2006) Synthesis and in vitro characterization of antigen-conjugated polysaccharide as a CpG DNA carrier. *Bioconjugate chemistry* 17:1136-1140.

Sica A, Allavena P, Mantovani A (2008) Cancer related inflammation: the macrophage connection. *Cancer letters* 267:204-215.

Siegrist CA, Pihlgren M, Tougne C, Efler SM, Morris ML, AlAdhami MJ, *et al.* (2004) Co-administration of CpG oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response. *Vaccine* 23:615-622.

Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ (2010) HMGB1 and RAGE in inflammation and cancer. *Annual review of immunology* 28:367-388.

Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA (2000) Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *The Journal of experimental medicine* 192:755-760.

Soehnlein O, Lindbom L (2010) Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* 10:427-439.

Song XR, Cai Z, Zheng Y, He G, Cui FY, Gong DQ, *et al.* (2009) Reversion of multidrug resistance by co-encapsulation of vincristine and verapamil in PLGA nanoparticles. *Eur J Pharm Sci* 37:300-305.

Song YJ, Izumi KM, Shinnars NP, Gewurz BE, Kieff E (2008) IRF7 activation by Epstein-Barr virus latent membrane protein 1 requires localization at activation sites and TRAF6, but not

TRAF2 or TRAF3. *Proceedings of the National Academy of Sciences of the United States of America* 105:18448-18453.

Speiser DE, Schwarz K, Baumgaertner P, Manolova V, Devedre E, Sterry W, *et al.* (2010) Memory and effector CD8 T-cell responses after nanoparticle vaccination of melanoma patients. *J Immunother* 33:848-858.

Stagg J, Smyth MJ (2010) Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29:5346-5358.

Steele P, Edgar J (1982) Importance of acute death syndrome in mortalities in broiler chicken flocks. *Australian veterinary journal* 58:63-66.

Steele P, Edgar J, Doncon G (1982) Effect of biotin supplementation on incidence of acute death syndrome in broiler chickens. *Poultry science* 61:909-913.

Steere AC, Sikand VK, Meurice F, Parenti DL, Fikrig E, Schoen RT, *et al.* (1998) Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. *The New England journal of medicine* 339:209-215.

Steinhagen F, Kinjo T, Bode C, Klinman DM (2011) TLR-based immune adjuvants. *Vaccine* 29:3341-3355.

Stevceva L (2011) Toll-like receptor agonists as adjuvants for HIV vaccines. *Current medicinal chemistry* 18:5079-5082.

Stone KD, Prussin C, Metcalfe DD (2010) IgE, mast cells, basophils, and eosinophils. *The Journal of allergy and clinical immunology* 125:S73-80.

Strober W, Murray PJ, Kitani A, Watanabe T (2006) Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* 6:9-20.

Sun S, Rao NL, Venable J, Thurmond R, Karlsson L (2007) TLR7/9 antagonists as therapeutics for immune-mediated inflammatory disorders. *Inflammation & allergy drug targets* 6:223-235.

Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, *et al.* (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *The Journal of clinical investigation* 116:485-494.

Suzuki T, Nunez G (2008) A role for Nod-like receptors in autophagy induced by *Shigella* infection. *Autophagy* 4:73-75.

Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, *et al.* (2004) Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America* 101:3516-3521.

- Taghavi A, Allan B, Mutwiri G, Van Kessel A, Willson P, Babiuk L, *et al.* (2008) Protection of neonatal broiler chicks against *Salmonella Typhimurium* septicemia by DNA containing CpG motifs. *Avian diseases* 52:398-406.
- Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M (2010) Tobacco smoke promotes lung tumorigenesis by triggering IKK β - and JNK1-dependent inflammation. *Cancer cell* 17:89-97.
- Takeda K, Akira S (2005) Toll-like receptors in innate immunity. *International immunology* 17:1-14.
- Takedatsu H, Mitsuyama K, Mochizuki S, Kobayashi T, Sakurai K, Takeda H, *et al.* (2012) A new therapeutic approach using a schizophyllan-based drug delivery system for inflammatory bowel disease. *Mol Ther* 20:1234-1241.
- Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. *Cell* 140:805-820.
- Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11:443-451.
- Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, *et al.* (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International immunology* 13:933-940.
- Terlou A, van Seters M, Kleinjan A, Heijmans-Antonissen C, Santegoets LA, Beckmann I, *et al.* (2010) Imiquimod-induced clearance of HPV is associated with normalization of immune cell counts in usual type vulvar intraepithelial neoplasia. *International journal of cancer* 127:2831-2840.
- Thomas PG, Dash P, Aldridge JR, Jr., Ellebedy AH, Reynolds C, Funk AJ, *et al.* (2009) The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30:566-575.
- Tincer G (2007) Immunomodulatory Effects of TLR Ligands and Polysaccharide Combinations: Strategies to Augment Innate Immune Response. *Master of Science Thesis*.
- Tincer G, Yerlikaya S, Yagci FC, Kahraman T, Atanur OM, Erbatur O, *et al.* (2011) Immunostimulatory activity of polysaccharide-poly(I:C) nanoparticles. *Biomaterials* 32:4275-4282.
- Tokunaga T, Yamamoto H, Shimada S, Abe H, Fukuda T, Fujisawa Y, *et al.* (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *Journal of the National Cancer Institute* 72:955-962.
- Torchilin VP (2000) Drug targeting. *Eur J Pharm Sci* 11 Suppl 2:S81-91.

- Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. *Nature reviews* 4:145-160.
- Triantafilou K, Vakakis E, Orthopoulos G, Ahmed MA, Schumann C, Lepper PM, *et al.* (2005) TLR8 and TLR7 are involved in the host's immune response to human parechovirus 1. *European journal of immunology* 35:2416-2423.
- Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7:179-190.
- Triozi PL, Aldrich W, Ponnazhagan S (2010) Regulation of the activity of an adeno-associated virus vector cancer vaccine administered with synthetic Toll-like receptor agonists. *Vaccine* 28:7837-7843.
- Tschopp J, Martinon F, Burns K (2003) NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 4:95-104.
- Tsoni SV, Brown GD (2008) Beta-glucans and Dectin-1. *Annals of the New York Academy of Sciences* 1143:45-60.
- Uematsu S, Akira S (2008) Toll-Like receptors (TLRs) and their ligands. *Handbook of experimental pharmacology*:1-20.
- Ueno H, Klechevsky E, Schmitt N, Ni L, Flamar AL, Zurawski S, *et al.* (2011) Targeting human dendritic cell subsets for improved vaccines. *Seminars in immunology* 23:21-27.
- Underhill DM (2007) Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. *Immunological reviews* 219:75-87.
- Uner M, Wissing SA, Yener G, Muller RH (2004) Influence of surfactants on the physical stability of solid lipid nanoparticle (SLN) formulations. *Die Pharmazie* 59:331-332.
- Vakkila J, Lotze MT (2004) Inflammation and necrosis promote tumour growth. *Nat Rev Immunol* 4:641-648.
- Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM (2001) Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J Immunol* 166:2372-2377.
- Vleugels B, Ververken C, Goddeeris BM (2002) Stimulatory effect of CpG sequences on humoral response in chickens. *Poultry science* 81:1317-1321.
- Vollmer J, Krieg AM (2009) Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Advanced drug delivery reviews* 61:195-204.
- von Meyenn F, Schaefer M, Weighardt H, Bauer S, Kirschning CJ, Wagner H, *et al.* (2006) Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. *Immunobiology* 211:557-565.

Vrignaud S, Benoit JP, Saulnier P (2011) Strategies for the nanoencapsulation of hydrophilic molecules in polymer-based nanoparticles. *Biomaterials* 32:8593-8604.

Wakita D, Chamoto K, Zhang Y, Narita Y, Noguchi D, Ohnishi H, *et al.* (2006) An indispensable role of type-1 IFNs for inducing CTL-mediated complete eradication of established tumor tissue by CpG-liposome co-encapsulated with model tumor antigen. *International immunology* 18:425-434.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412:346-351.

Wang C, Zhuang Y, Zhang Y, Luo Z, Gao N, Li P, *et al.* (2012) Toll-like receptor 3 agonist complexed with cationic liposome augments vaccine-elicited antitumor immunity by enhancing TLR3-IRF3 signaling and type I interferons in dendritic cells. *Vaccine* 30:4790-4799.

Wang D, Bhagat L, Yu D, Zhu FG, Tang JX, Kandimalla ER, *et al.* (2009a) Oligodeoxyribonucleotide-based antagonists for Toll-like receptors 7 and 9. *Journal of medicinal chemistry* 52:551-558.

Wang J, Dong S, Liu C, Wang W, Sun S, Gu J, *et al.* (2010a) Beta-Glucan oligosaccharide enhances CD8(+) T cells immune response induced by a DNA vaccine encoding hepatitis B virus core antigen. *Journal of biomedicine & biotechnology* 2010:645213.

Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 10:1366-1373.

Wang X, Xu W, Kong X, Chen D, Hellermann G, Ahlert TA, *et al.* (2009b) Modulation of lung inflammation by vessel dilator in a mouse model of allergic asthma. *Respiratory research* 10:66.

Wang XS, Sheng Z, Ruan YB, Guang Y, Yang ML (2005) CpG oligodeoxynucleotides inhibit tumor growth and reverse the immunosuppression caused by the therapy with 5-fluorouracil in murine hepatoma. *World J Gastroenterol* 11:1220-1224.

Wang ZH, Wang ZY, Sun CS, Wang CY, Jiang TY, Wang SL (2010b) Trimethylated chitosan-conjugated PLGA nanoparticles for the delivery of drugs to the brain. *Biomaterials* 31:908-915.

Watts C, West MA, Zaru R (2010) TLR signalling regulated antigen presentation in dendritic cells. *Current opinion in immunology* 22:124-130.

Wei T, Gong J, Jamitzky F, Heckl WM, Stark RW, Rossle SC (2009) Homology modeling of human Toll-like receptors TLR7, 8, and 9 ligand-binding domains. *Protein Sci* 18:1684-1691.

Wei T, Gong J, Rossle SC, Jamitzky F, Heckl WM, Stark RW (2011) A leucine-rich repeat assembly approach for homology modeling of the human TLR5-10 and mouse TLR11-13 ectodomains. *Journal of molecular modeling* 17:27-36.

Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A (2004) Toll-like receptors are temporally involved in host defense. *J Immunol* 172:4463-4469.

- Werner JL, Metz AE, Horn D, Schoeb TR, Hewitt MM, Schwiebert LM, *et al.* (2009) Requisite role for the dectin-1 beta-glucan receptor in pulmonary defense against *Aspergillus fumigatus*. *J Immunol* 182:4938-4946.
- Whiteside TL (2006) Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Seminars in cancer biology* 16:3-15.
- Wille-Reece U, Flynn BJ, Lore K, Koup RA, Miles AP, Saul A, *et al.* (2006) Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *The Journal of experimental medicine* 203:1249-1258.
- Wille-Reece U, Wu CY, Flynn BJ, Kedl RM, Seder RA (2005) Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. *J Immunol* 174:7676-7683.
- Willment JA, Brown GD (2008) C-type lectin receptors in antifungal immunity. *Trends in microbiology* 16:27-32.
- Wischke C, Zimmermann J, Wessinger B, Schendler A, Borchert HH, Peters JH, *et al.* (2009) Poly(I:C) coated PLGA microparticles induce dendritic cell maturation. *International journal of pharmaceutics* 365:61-68.
- Wissing SA, Kayser O, Muller RH (2004) Solid lipid nanoparticles for parenteral drug delivery. *Advanced drug delivery reviews* 56:1257-1272.
- Wong VW, Yu J, Cheng AS, Wong GL, Chan HY, Chu ES, *et al.* (2009) High serum interleukin-6 level predicts future hepatocellular carcinoma development in patients with chronic hepatitis B. *International journal of cancer* 124:2766-2770.
- Wu H, Wang H, Xiong W, Chen S, Tang H, Han D (2008) Expression patterns and functions of toll-like receptors in mouse sertoli cells. *Endocrinology* 149:4402-4412.
- Wu J, Lanier LL (2003) Natural killer cells and cancer. *Advances in cancer research* 90:127-156.
- Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB, *et al.* (2005) CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infection and immunity* 73:828-833.
- Xu S, Cao X (2010) Interleukin-17 and its expanding biological functions. *Cellular & molecular immunology* 7:164-174.
- Yadav M, Schorey JS (2006) The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* 108:3168-3175.

Yamasaki S, Matsumoto M, Takeuchi O, Matsuzawa T, Ishikawa E, Sakuma M, *et al.* (2009) C-type lectin Mincle is an activating receptor for pathogenic fungus, *Malassezia*. *Proceedings of the National Academy of Sciences of the United States of America* 106:1897-1902.

Yang P, Liang M, Zhang Y, Shen B (2008) Clinical application of a combination therapy of lentinan, multi-electrode RFA and TACE in HCC. *Advances in therapy* 25:787-794.

Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, *et al.* (2005) TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science (New York, NY)* 308:1626-1629.

Yasuda K, Rutz M, Schlatter B, Metzger J, Lippa PB, Schmitz F, *et al.* (2006) CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA. *European journal of immunology* 36:431-436.

Yoon TJ, Kim TJ, Lee H, Shin KS, Yun YP, Moon WK, *et al.* (2008) Anti-tumor metastatic activity of beta-glucan purified from mutated *Saccharomyces cerevisiae*. *International immunopharmacology* 8:36-42.

Zhai Y, Shen XD, O'Connell R, Gao F, Lassman C, Busuttil RW, *et al.* (2004) Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IFN regulatory factor 3-dependent MyD88-independent pathway. *J Immunol* 173:7115-7119.

Zhou LD, Zhang QH, Zhang Y, Liu J, Cao YM (2009) The shiitake mushroom-derived immunostimulant lentinan protects against murine malaria blood-stage infection by evoking adaptive immune-responses. *International immunopharmacology* 9:455-462.

Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, Klinman DM, *et al.* (2008) Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. *Proceedings of the National Academy of Sciences of the United States of America* 105:16260-16265.

Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6:295-307.

Chapter 7

Appendices

Appendix A

Standard Solutions, Buffers, Media

Sodium Carbonate Buffer (0.1M) (ELISA)

- 1.696 grams Na_2CO_3
- 2,856 grams NaHCO_3

into 500 ml ddH₂O. pH should be adjusted to 9.6. The buffer should be stored at +4°C.

Blocking Buffer (ELISA)

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

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

T-cell Buffer [ELISA]

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

The buffer should be stored at -20°C.

Wash Buffer [ELISA]

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

PBS (Phosphate Buffered Saline) [10x]

- 80 grams NaCl
- 2 grams KCl
- 8.01 grams Na₂HPO₄ · 2H₂O
- 2 grams KH₂PO₄

into 1 lt ddH₂O

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 (C₄H₁₁NO₃)
- 37.2 grams Tritiplex 3 (EDTA= C₁₀H₁₄N₂Na₂O₂ · 2H₂O)
- 57.1 ml Glacial acetic acid

into 1 lt ddH₂O

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

Thioglycollate Solution (4%)

- 4 grams brewer thioglycollate

into 100 ml ddH₂O

Dissolves with magnetic stirrer for 5 min. Autoclave before using. The buffer should be stored at room temperature.

ACK Lysis Buffer

- 150mM NH₄Cl
- 10mM KHCO₃
- 0.1mM Na₂EDTA

into 1 lt ddH₂O. Autoclave before using. The buffer should be stored at +4°C.

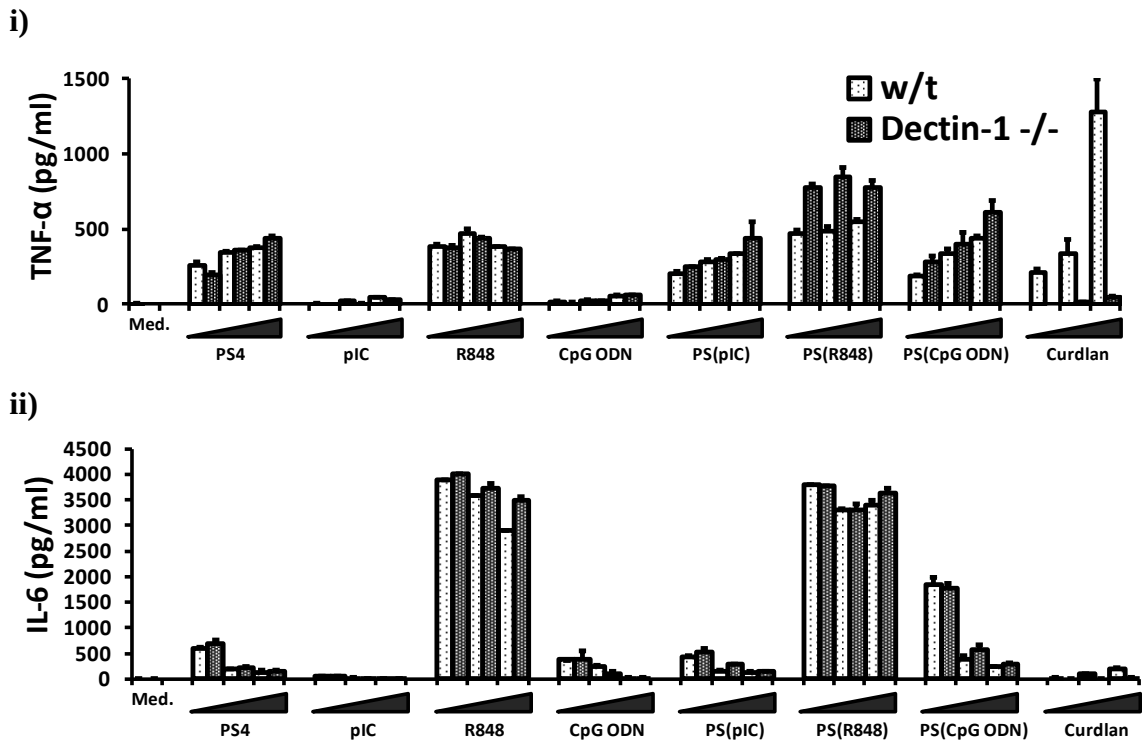
High Glucose DMEM and RPMI-1640

- 2 %: 10 ml FBS (Oligo FBS = inactivated at 65°C, Regular FBS = inactivated at 55°C)
- 5 % : 25 ml FBS
- 10 % : 50 ml FBS
- 5 ml Penicillin/Streptomycin (50 µg/ml final concentration from 10 mg/ml stock)
- 5 ml HEPES (Biological Industries),(10 mM final concentration from 1M stock)
- 5 ml Na Pyruvate, (0.11 mg/ml final concentration from 100mM, 11 mg/ml stock)
- 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock)
- 5 ml L-Glutamine, (2 mM final concentration from 200 mM, 29.2 mg/ml stock)

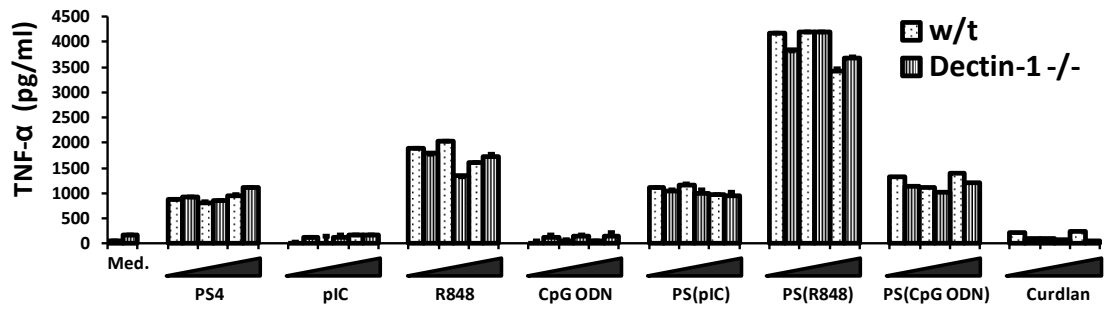
In 500 ml media

Appendix B

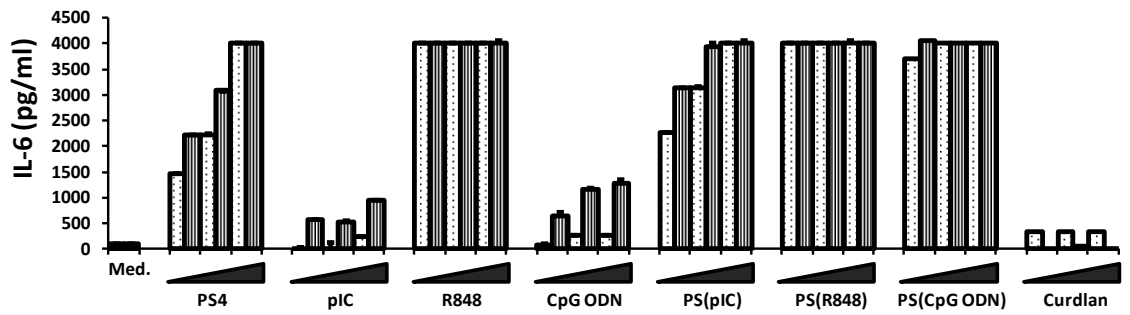
Figure B.1 TNF- α and IL-6 cytokine production of (i, ii) splenocytes ($5 \times 10^6/\text{ml}$), (iii, iv) PEC macrophages ($1.25 \times 10^6/\text{ml}$), (v) GMDC and (vi) FLDCs ($5 \times 10^5/\text{ml}$) from w/t and Dectin-1^{-/-} C57BL/6 mice after 24 hours stimulation with, PS, pIC, R848, CpG ODN, PS/TLRL nanocomplexes (1:1, w:w, $6 \mu\text{g}/\text{ml}$ – $0.24 \mu\text{g}/\text{ml}$, 5x dilutions) and curdlan (1, 10, 100 $\mu\text{g}/\text{ml}$). Cytokine ELISA was performed. Experiments were ran on triplicate.



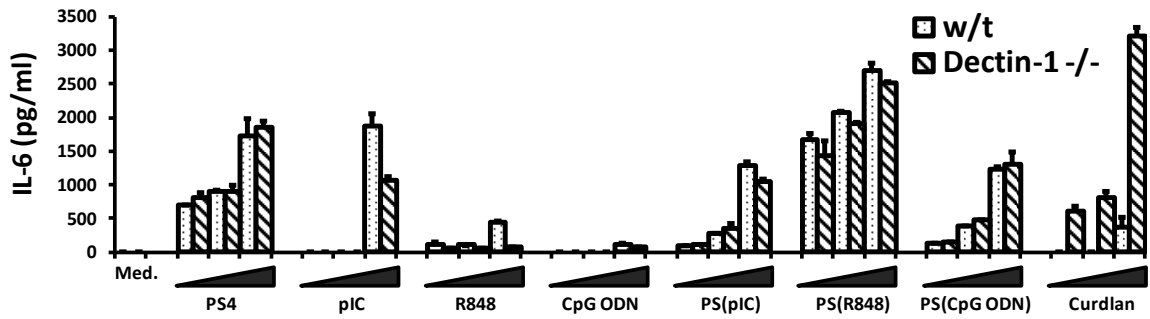
iii)



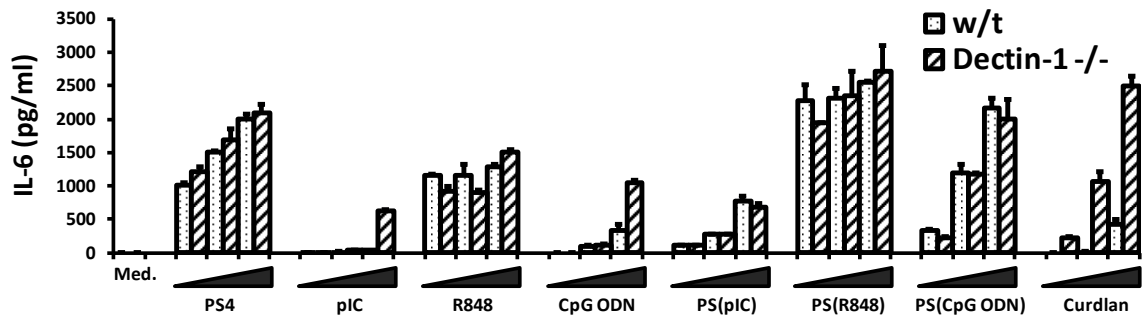
iv)



v)

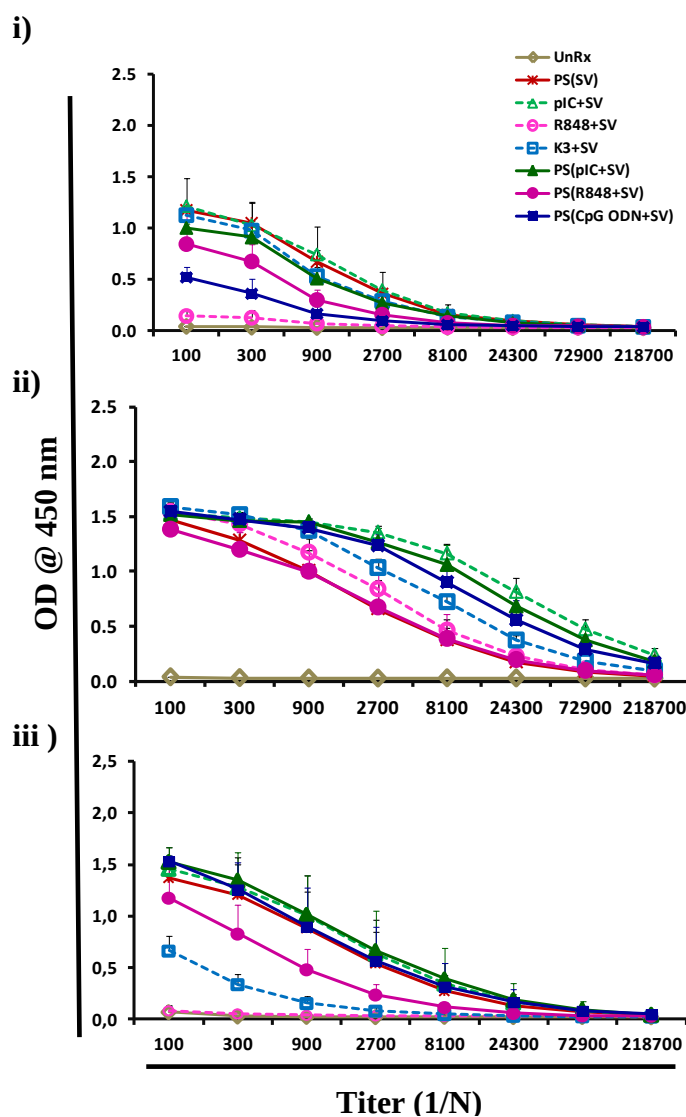


vi)

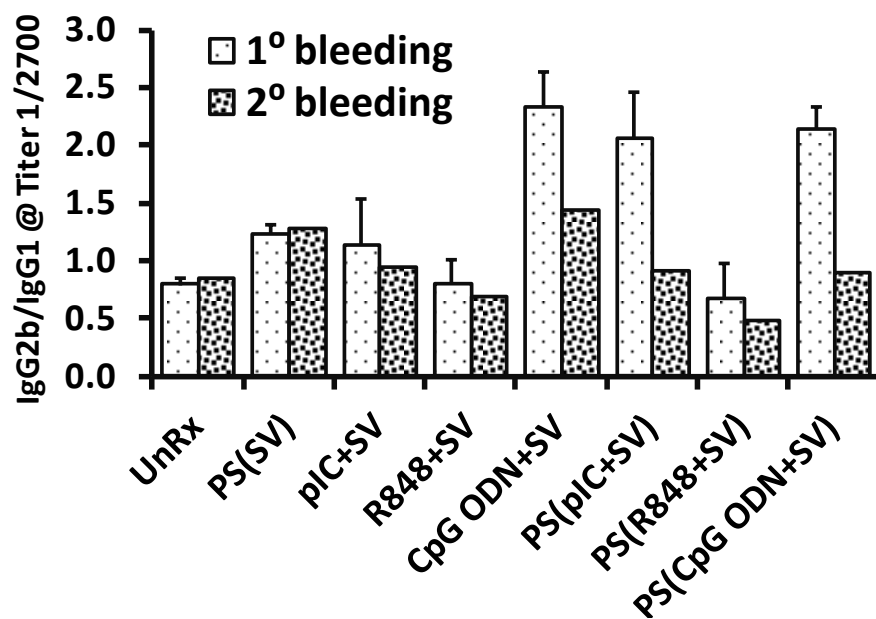


Appendix C

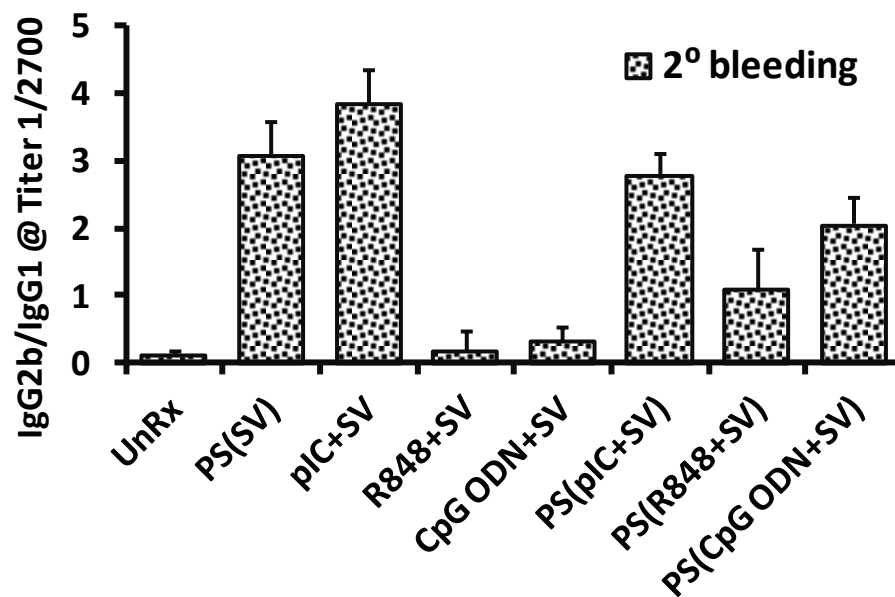
Figure C.1 Serum anti SV titers of IgG2b of (i) primary, (ii) secondary bleedings of intradermal and (iii) secondary bleeding of intranasal immunized mice. IgG2b/IgG1 OD ratios of 2700x titrated mice sera (ave. of 5 mice/group) against anti-SV. Sera collected (iv) 1 week after primary and booster intradermal (v) secondary intranasal injections.



iv)



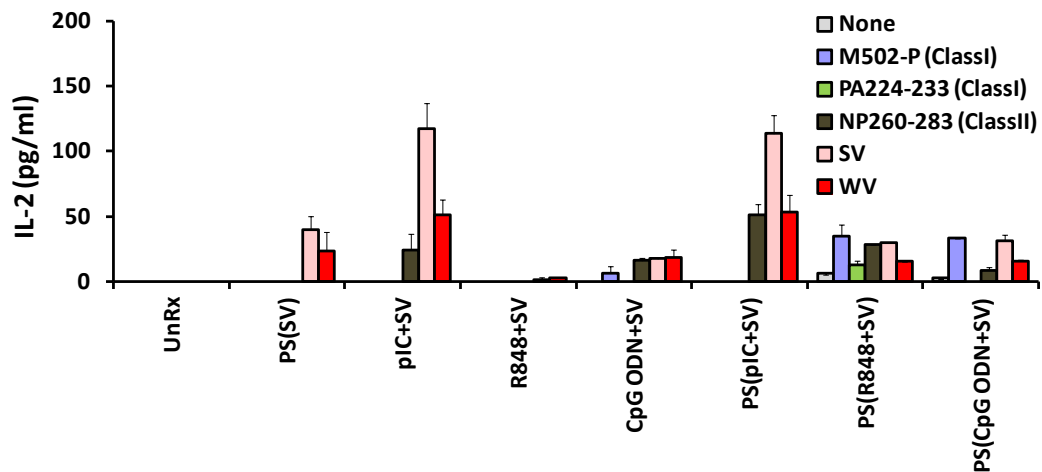
v)



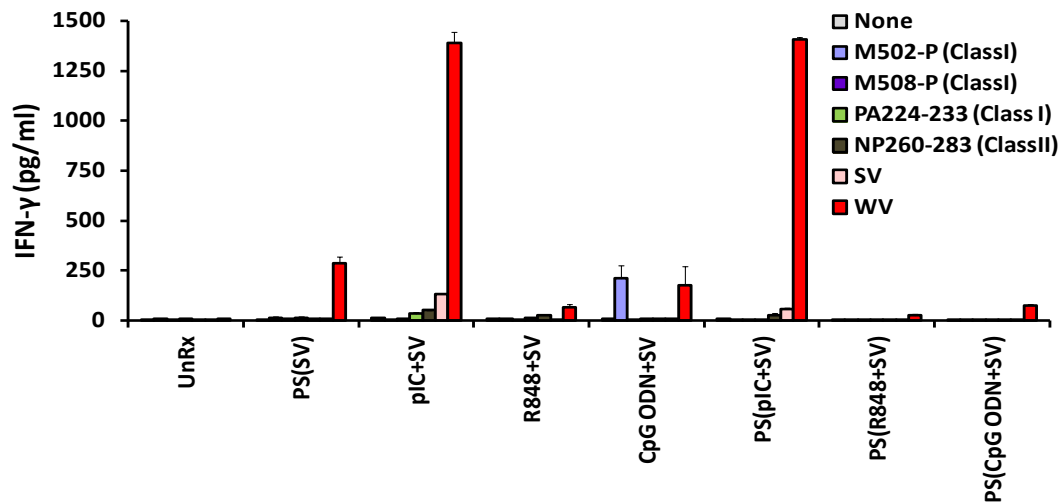
Appendix D

Figure D.1 T cell dependent immune response from intranasal immunized mice splenocytes against H1N1 influenza virus. Splenocytes ($5 \times 10^6/\text{ml}$) were stimulated with class I and class II MHC influenza peptides, SV and WV ($2.5 \mu\text{g}/\text{ml}$), (i) IL-2 and (ii) IFN- γ productions were assessed by cytokine ELISA after 24h. PS(R848+SV) vs PS(SV) or R848+SV comparison gave a $p < 0.003$ for IL-2 (after incubation with both class I peptides).

i)



ii)



Curriculum vitae and Publications

PERSONAL INFORMATION

Name Surname Gizem Tincer König
Date of Birth 27. December. 1983
Nationality Turkish
Languages spoken Turkish (Mother tongue)
English (Fluent)
French (Beginner)
Address Bilkent University Science Faculty
Department of Molecular Biology and Genetics
Ankara/TURKEY
Contact Info Cellular phone: 0090-533-655-1128
Home phone: 0090-312-241-4348
gizemt@fen.bilkent.edu.tr



EDUCATION

2007- ... Bilkent University, Department of Molecular Biology and Genetics,
Ankara, Turkey (PhD Degree)

**September 2010 – March 2011 Osaka University, The WPI Immunology Frontier
Research Center (IFReC), Research fellow.**
Supervisors: Prof. Ken Ishii, Assoc. Prof. Cevayir Coban

2005-2007 Bilkent University, Department of Molecular Biology and Genetics,
Ankara, Turkey (M.Sc. Degree)

2001-2005 Hacettepe University, Department of Biology, Ankara, Turkey (Bs
Degree)

PUBLICATIONS

A) Peer Reviewed Journals

- 1- G. Tincer**, S. Yerlikaya, F.C. Yagci, T. Kahraman, O. M. Atanur, O. Erbatur, I. Gursel., Immunostimulatory Activity of Polysaccharide-Poly(I:C) Nanoparticles, Biomaterials, 32(18):4275-82, Epub 2 Apr 2011. (IF: 8.4)
- 2- E. Birben**, C. Sackesen, S. Kazani, **G. Tincer**, C.Karaaslan, B. Durgunsu, I. Gursel, M.E. Wechsler, E. Israel, O. Kalayci., The effects of an insertion in the 5'UTR of the AMCase on gene expression and pulmonary functions. Respir Med., 105(8):1160-9, Epub 20 Apr 2011. (IF: 2.4)
- 3- F.C. Yagci**, O. Aslan, M. Gursel, **G. Tincer**, Y. Ozdamar , K. Karatepe, K.C. Akcali, I. Gursel., Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis, J Biol Chem., 285(37):28806, Epub 14 Jul 2010. (IF: 5.5)

B) Popular Science Journals

1- I. Gursel, F.C. Yagci, **G. Tincer**, T. Kahraman, M. Gursel., Novel applications of DNA nanoparticles in nanomedicine and nanobiotechnology. Science and Technology Journal (by TÜBİTAK, Turkey), 520:44-48, Mar 2011.

AWARDS & SCHOLARSHIPS

- 1- Registration and travel awards by Association for Cancer Immunotherapy. 10th CIMT Annual Meeting. Mainz, Germany, 23–25 May 2012.
- 2- Registration and accommodation awards by Turkish Society of Immunology. Molecular Immunology & Immunogenetics Congress (MIMIC2012), Antalya, Turkey, April 2012.
- 3- Best poster award, “Polysaccharide-TLR agonist nanocomplexes: Mechanism of action as immune adjuvants.”, 17th International Symposium on Biomedical Science and Technology, Ankara, Turkey, 23–25 November 2011.
- 4- Registration and accommodation awards by Turkish Society of Immunology. 21th National Immunology Congress, Marmaris, Turkey, April 2011.
- 5- Research fellow, IFRc Kishimoto Foundation Fellowship, Osaka University, September 2010 – March 2011
- 6- Travel award by European Federation of Immunological Societies (EFIS) 14th International Congress of Immunology, Kobe, Japan, August 2010.
- 7- Scholarship, Ministry of Industry and Trade (SANTEZ Grant), “Improving broiler chicken immune resistance by providing protection and reducing losses from stress and bacterial diseases with CpG DNA.”, November 2009 (Duration: 24 months)
- 8- Research assistant, TÜBİTAK project (SBAG-106S102). “Development of CpG ODN containing liposomal vaccines against pathogens.”, June 2006-June 2009.
- 9- Travel and accommodation awards by Turkish National Society of Allergy and Clinical Immunology. (World Immune Regulation Meeting-II, Davos, Switzerland, March 2007).
- 10- Full scholarship, Graduate education, Bilkent University, Molecular Biology and Genetics Department, 2005 – ...

CONFERENCE COMMUNICATIONS

- 1- **G. Tincer**, K. Karatepe, C. Coban, K. Ishii, M. Gursel, İ. Gursel. Potential antitumor immunotherapeutic candidates: CpG ODN and PS nanocomplexes. 10th CIMT Annual Meeting, Mainz, Germany, 23–25 May 2012. (Poster presentation).
- 2- **G. Tincer**, K. Karatepe, C. Coban, K. Ishii, M. Gursel, İ. Gursel. CpG ODN nanoparticles and polysaccharide nanocomplexes: Improved antitumor action. Molecular Immunology & Immunogenetics Congress (MIMIC2012), Antalya, Turkey, 27–29 April 2012. (Poster presentation).
- 3- **G. Tincer**, M. Sahin, C. Coban, K. Ishii, M. Gursel, İ. Gursel. Polysaccharide-TLR agonist nanocomplexes: Mechanism of action as immune adjuvants. 17th International Symposium on Biomedical Science and Technology, Ankara, Turkey, 23–25 November 2011. (Poster presentation).

- 4- B. Gungor, F.C. Yagci, **G. Tincer**, E. Alpdundar, S. Yildiz, I. Gursel, M. Gursel. Determination of Immune Stimulatory Properties of Synthetic CpG ODN/Cationic Peptide Complexes. 17th International Symposium on Biomedical Science and Technology, Ankara, Turkey, 23–25 November 2011. (Poster presentation).
- 5- B. Gungor, F.C. Yagci, **G. Tincer**, E. Alpdundar, S. Yildiz, I. Gursel, M. Gursel. Immunostimulatory Properties of CpG ODN Based Nanoparticles. 17th International Symposium on Biomedical Science and Technology, Ankara, Turkey, 23–25 November 2011.(Invited speaker, Dr. M. Gürsel).
- 6- **G. Tincer**, C. Coban, K. Ishii, İ. Gursel. Improving immune response by natural polysaccharide-TLR ligand nanoparticles. Advances in Immunology and Cancer Biology Workshop, Istanbul, Turkey, 15 – 17 April 2011. (Poster presentation).
- 7- **G. Tincer**, C. Coban, K. Ishii, İ. Gursel. Synergistic effects of nucleic acid based TLR ligands complexed with polysaccharide nanoparticles on immune system. 21st National Immunology Congress, Marmaris, Turkey, 6–9 April 2011. (Oral presentation, Turkish).
- 8- **G. Tincer**, C. Coban, K.J. Ishii, I. Gursel. Immunostimulatory Activity of Nucleic Acid Based TLR Ligands Complexed with Polysaccharide Nanoparticles. Towards Comprehensive Understanding of Immune Dynamism (TCUID 2011), Osaka, Japan, 1-2 March 2011. (Poster presentation).
- 9- **G. Tincer**, S. Yerlikaya, I. Gursel. β -Glucans effectively binds TLR3, 7, and 9 ligands and improves innate immune response. 14th International Congress of Immunology, Kobe, Japan, 22-27 August 2010. (Poster presentation).
- 10- I. Gursel, **G. Tincer**, F.C. Yagci, T. Kahraman, R. Mammadov, E. Erikci, K. Karatepe, C. Akcali, M. Gursel. Nanobiotechnological Applications of Self-Assembled Immunostimulatory DNA Nanoparticles. 15th International Biomedical Science and Technology Symposium, Güzelyurt, North Cyprus, 16-19 August 2009. (Invited speaker, Dr. Gürsel).
- 11- **G. Tincer**, O.M. Atanur, T. Balın, O. Erbatur, I. Gursel. Delivery of TLR Ligands with Polysaccharide Nanocomplexes Enhances Immune Activation. 15th International Biomedical Science and Technology Symposium, Güzelyurt, North Cyprus, 16-19 August 2009. (Poster presentation).
- 12- I. Gursel, **G. Tincer**, F.C. Yagci, T. Kahraman, R. Mammadov, E. Erikci, K. Karatepe, C. Akcali, M. Gursel. Potential Nanomedical Applications of Self Assembled Immunomodulatory DNA Nanoparticles. 5th National Nanoscience and Nanotechnology Conference, Eskişehir, Turkey, 8-12 June 2009. (Invited speaker, Dr. Gürsel).
- 13- **G. Tincer**, O.M. Atanur, T. Balın, O. Erbatur, I. Gursel. Delivery of TLR Ligands with Polysaccharide Nanocomplexes Enhances Immune Activation. 5th National Nanoscience and Nanotechnology Conference, Eskişehir, Turkey, 8-12 June 2009. (Oral presentation).
- 14- I. Gursel, **G. Tincer**, F.C. Yagci, R. Mammadov, H. Kocak, T. Kahraman, K. Karatepe, E. Erikci, M. Gursel. Immune activation by CpG oligodeoxynucleotides. XX. National Biochemistry Congress, Nevşehir, Turkey, 29 October-1 November 2008. (Invited speaker, Dr. Gürsel).
- 15- I. Gursel, **G. Tincer**, F.C. Yagci, R. Mammadov, H. Kocak, T. Kahraman, K. Karatepe, E. Erikci, M. Gursel. Immunotherapeutic applications of DNA nanoparticles to cancer, infectious and autoimmune diseases. 13th National Biomedical Engineering Conference, Ankara, Turkey, 29-31 May 2008 . (Invited speaker, Dr. Gürsel).

- 16- I. Gursel, F.C. Yagci, **G. Tincer**, M. Gursel. DNA Nanoparticles: Novel Immunomodulatory Adjuvants for Immunotherapy. World Immune Regulation Meeting-II, Davos, Switzerland, 17–20 March 2008. (Poster presentation).
- 17- **G. Tincer**, S. Yerlikaya, O.M. Atanur, T. Balın, O. Erbatur, I. Gursel. Designing Novel Immunoadjuvants For Immunotherapy. World Immune Regulation Meeting-II, Davos, Switzerland, 17–20 March 2008. (Poster presentation).
- 18- **G. Tincer**, S. Yerlikaya, O.M. Atanur, T. Balın, O. Erbatur, I. Gursel. Immunomodulatory Activity of Polysaccharides Extracted from *Ganoderma* and *Shiitake* Mushrooms. 13th Biomedical Science and Technology Symposium, İstanbul, Turkey, 26-28 August 2007. (Poster presentation).
- 19- **G. Tincer**, I. Gursel. Contrasting and Synergistic Immune Responses Mediated by Nanoparticulate TLR Ligands. 13th Biomedical Science and Technology Symposium, İstanbul, Turkey, 26-28 August 2007. (Poster presentation).
- 20- **G. Tincer**, R. Mammadov, I. Gursel. Efforts to delineate contrasting and synergistic interaction of TLR ligands leading to a robust immune response. 1st Congress of the Society of Innate Immunity, Ankara, Turkey, 13-16 May 2007. (Oral presentation).

CONGRESS & SYMPOSIUM PARTICIPATIONS

- 1- 17th International Symposium on Biomedical Science and Technology, Ankara, Turkey, 23–25 November 2011.
- 2- 21st National Immunology Congress, Marmaris, Turkey, 6–9 April 2011.
- 3- Towards Comprehensive Understanding of Immune Dynamism (TCUID 2011), Osaka, Japan, 1-2 March 2011.
- 4- 14th International Congress of Immunology, Kobe, Japan, 22-27 August 2010.
- 5- 15th International Biomedical Science and Technology Symposium, Güzelyurt, North Cyprus, 16-19 August 2009.
- 6- 5th National Nanoscience and Nanotechnology Conference, Eskişehir, Turkey, 8-12 June 2009.
- 7- World Immune Regulation Meeting-II, Davos, Switzerland, 17-20 March 2008.
- 8- 13th Biomedical Science and Technology Symposium, İstanbul, Turkey, 26-28 August 2007.
- 9- 1st Congress of the Society of Innate Immunity, Ankara, Turkey, 13-16 May 2007.
- 10- 31st FEBS Congress, Molecules in Health and Disease, İstanbul, Turkey, 24-29 June 2006.

WORKSHOPS & COURSES

- **Advances in Immunology and Cancer Biology Workshop**, İstanbul, Turkey, 15–17 April 2011.
- **Workshop on Current Trends in Molecular Nanobiosciences**, Bilkent University, Molecular Biology and Genetics Department, Ankara, Turkey, 13-16 January 2010 (Event asistant).
- **Winter School on Frontiers in Nanomedicine and Nanobiotechnology**, Bilkent University, Molecular Biology and Genetics Department, Ankara, Turkey, 13-16 January 2010 (Event asistant).
- **XIV. Flow Cytometry Workshop**, İstanbul University Institute for Experimental Medicine, İstanbul, Turkey, 2-6 June 2008.

SOCIETY MEMBERSHIPS

Turkish National Society of Allergy and Clinical Immunology

Turkish Society of Immunology

The American Association of Immunologists (2010-2012)

REFEREING FOR JOURNALS

- Biomacromolecules (In Gursel's lab.)
- International Immunology (In Ishii's lab.)
- Journal of Immunology (In Ishii's lab.)
- Vaccine (In Ishii's lab.)
- Microbiology and Immunology (In Ishii's lab.)



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

Gizem Tincer^a, Seda Yerlikaya^{a,1}, Fuat C. Yagci^a, Tamer Kahraman^a, Osman M. Atanur^b, Oktay Erbatur^b, Ihsan Gursel^{a,*}

^a Biotherapeutic ODN Research Laboratory, Molecular Biology and Genetics Department, Faculty of Sciences, Bilkent University, Bilkent, Ankara 06800, Turkey

^b Chemistry Department, Science Faculty, Çukurova University, Balcalı, Adana 01330, Turkey

ARTICLE INFO

Article history:

Received 27 November 2010

Accepted 12 January 2011

Available online 2 April 2011

Keywords:

Polysaccharides

Poly(I:C)

Nanoparticles

Targeted delivery system

TLR

Immunogenicity

ABSTRACT

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

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Toll-like receptors (TLRs) are the most extensively studied pathogen recognition receptors (PRRs) that recognizes specific microbial associated molecular patterns (MAMPs) including lipopolysaccharides, peptidoglycans, lipoproteins, flagellin, unmethylated CpG motifs or viral RNA/DNA that triggers innate immune response [1–3]. TLRs are subcategorized as endosomal or intracellular receptors depending on their site of expression. TLR3, 7/8 and 9 are specialized to sense pathogens via their nucleic acids moieties [4]. TLR3, a member of endosome-associated TLR is activated directly in response to dsRNA, or synthetic poly(riboinosinic:polyribocytidylic acid) (pIC) [5] and are harnessed as vaccine adjuvant, anti-cancer or anti-allergic therapeutic agents. When these ligands are given *in vivo*, they are rapidly cleared by nucleases, and could be adsorbed by serum proteins hampering their *in vivo* therapeutic

applications [6]. In order to improve their immune stimulatory potential repeated high doses are necessary, however, in many instances this may cause undesirable side effects including septic shock [7]. Nucleic acid backbone modifications such as phosphorothioate linkages are another widely accepted approach but this not only induces undesirable side effects such as granuloma formation, temporary splenomegaly, and lymphadenopathy but also increases the cost of the final product [8–11].

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

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

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

* Corresponding author. Tel.: +90 312 290 24 08; fax: +90 312 266 50 97.

E-mail address: ihsangursel@bilkent.edu.tr (I. Gursel).

¹ Present address: Department of Molecular Biology, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland.

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

2. Materials and methods

2.1. Materials

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

2.2. Mice

Adult C57BL/6 and BALB/C mice (female, 6–8 weeks old) were housed in Department of Molecular Biology and Genetics, facility and were provided with unlimited access of food and water. All experimental procedures were approved by the animal ethical committee of Bilkent University (Bil-AEC/Protocol#: 2006/027). RAW 264.7 cells (ATCC) or splenocytes were cultured with RPMI 1640 supplemented with 5% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.11 mg/ml sodium pyruvate. HEK 293 or stably *hTLR2* transfected cells (Invivogen, USA), were kept in high-glucose DMEM media with 10% FBS.

2.3. Immunization

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

2.4. ELISA and NO assays

Immulon 2 HB microtitre plates (Thermo Scientific, USA) were coated with anti-cytokine or anti-IgG antibodies and then blocked with PBS-BSA 1% [17,35]. Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine Ab (TNF α : XT22 and IL6: 20F3 clones) followed by phosphatase-streptavidin (Perbio Pierce, USA) whereas bound IgG subclasses were detected using phosphatase-conjugated anti-IgG, IgG1, and IgG2a antibodies as described elsewhere [17]. Nitric oxide detection by Griess method was conducted on RAW 264.7 cells (10^5 /ml) after 6–48 h of incubation as described by the suppliers (Promega WI, USA).

2.5. Luciferase assay

hTLR2 expressing HEK cells were transfected using FuGENE6 with p5xLucNFkB luciferase as described in manufacturer's protocol. Following overnight stimulations, cells were further incubated for 24–36 h and were harvested and assayed for luciferase activity (Roche, Germany). Plots were generated from relative light units.

2.6. Cytokine and chemokine RT-PCR

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

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

pIC, PS4 and their nanocomplexes were diluted in DNase/RNase free H₂O and were deposited on silicon wafer. Following complete drying images were taken by using non-contact mode XE-100E model AFM (PSIA with XEI 1.6 software incorporated) with a 0.73–0.79 Hz scanning rate. The scanning area sizes were in $1 \times 1 \mu\text{m}$. Particle size analyses of the generated nanocomplexes were measured using dynamic light scattering method on a zetasizer (Model: Nano ZS, Malvern, UK).

2.8. Statistical analysis

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

3. Results

3.1. TNF α and nitric oxide production by polysaccharides

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

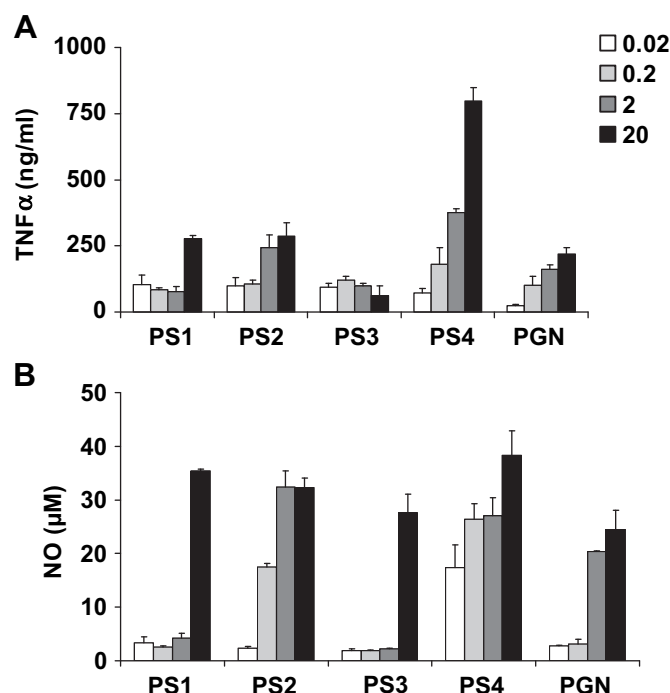


Fig. 1. Dose dependent TNF α and NO induction from RAW cells following 24 h post-stimulation with different β -Glucan polymers. A) TNF α and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean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

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

3.2. PS mediated upregulation of various cytokine/chemokine transcripts

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

3.3. Engagement of PS by TLR2

Several β -Glucan polymers were reported to trigger signaling cascade either using cell surface expressed TLR2 (alone or engaging

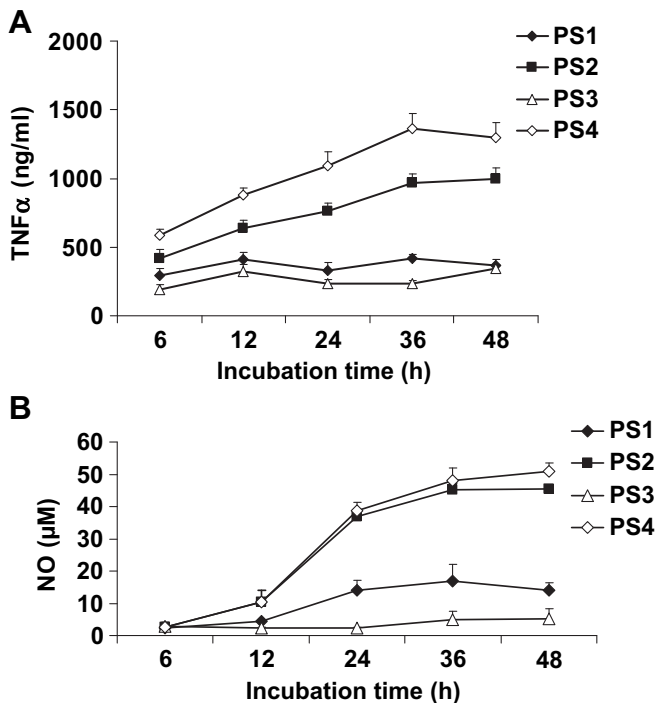


Fig. 2. Time dependent TNF α and NO release by RAW cells following different PS treatments at 2 $\mu\text{g/ml}$. A) TNF α and B) NO levels were assessed by ELISA and Griess assay respectively. Result represents combination of at least three independent experiments (mean $\pm$ SEM) of triplicate samples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

TLR1 or TLR6), TLR4 or even cytosolic sensors such as NOD1 and NOD2 [22,31]. To differentiate between these alternatives HEK cells stably expressing hTLR2, hTLR4 or TLR2/6 genes were transfected with p5xLucNF κ B and in subsequent experiments co-transfected with pcDNA3NOD1 or pcDNA3NOD2 or mock plasmids to check whether these PS polymers either engages only through TLRs or additionally interact with the cytosolic NOD sensors. In Fig. 3, NF κ B-mediated luciferase activity studies demonstrated that PS triggers via TLR2 receptor (Fig. 3A) and furthermore regulates IL8 production (Fig. 3B). Expression of TLR6 did not improve TLR2 mediated promoter activation (*data not shown*). Consistent with previous results, PS4 is the most potent inducer. Of note, other TLRs or NODs were not contributing to this immune cascade since subsequent experiments demonstrated that there was no further significant increase (either luciferase activity or IL8 production) mediated by PS treatment of cells expressing TLR4, or NOD1 or NOD2 (*data not shown*).

3.4. PS4 activity upon in vivo administration to mice

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

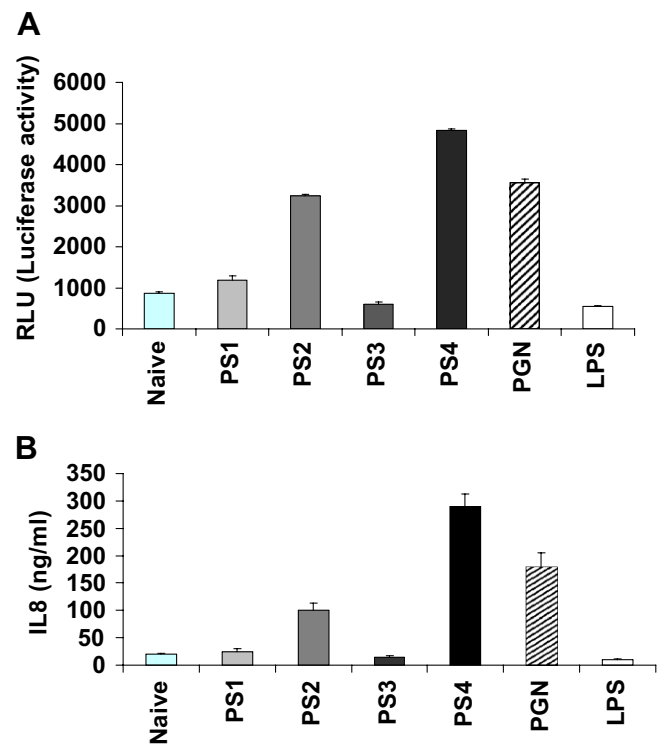


Fig. 3. PS mediated promoter assay and IL8 production by hTLR2-HEK cells. A) Relative luciferase activity after PS treatment of stable hTLR2-HEK cells. Cells were transfected with p5xLucNF κ B plasmid for 24 h and stimulated with 5 $\mu\text{g/ml}$ of each PS for 12 h. B) IL8 production following PS treatment. Stable hTLR2-HEK cells were stimulated with 5 $\mu\text{g/ml}$ of each PS for 24 h. IL8 ELISA was studied from the culture supernatants. Data represents combination of at least two independent experiments (mean $\pm$ SEM) of triplicate samples treated with different stimulants (PS1–4, PGN and LPS). PS4 vs PS1, 2, 3 and LPS gave a $p < 0.001$.

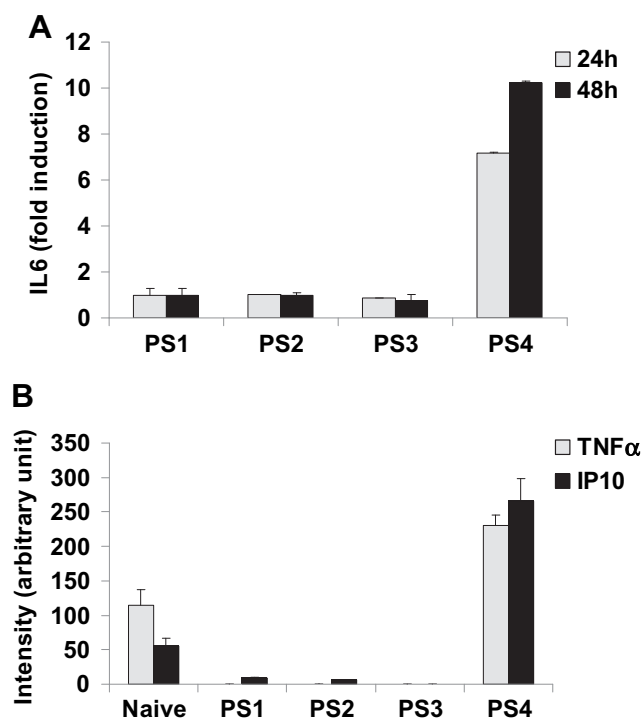


Fig. 4. *In vivo* immunostimulatory activity of polysaccharides. A) Six hours after i.p. PS injection (2 µg/ml), PECs were isolated (3 mice/group). Cells (4×10^6 /ml) were incubated *ex-vivo* for further 24 h or 48 h, IL6 levels from supernatants were measured by cytokine ELISA. Fold induction of IL6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

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

3.5. Physical characterization of PS4-pIC nanocomplexes

When nucleic acids are injected *in vivo* they are either subjected to premature digestion by nucleases or adsorbed by serum proteins, therefore, hampering their bioavailability. Accumulating evidence strongly suggested that their *in vivo* applications are limited due to their labile nature. Strategies to improve their stability as well as retaining activity and facilitate their cellular internalization without premature clearance are of great importance to pursue these ligands into clinical use [15,17]. To establish whether PS4-pIC complexes form stable nanoparticles their physical characteristics by i) AFM and by ii) dynamic light scattering techniques (Zetasizer Nano, ZS™) were studied. AFM photomicrographs revealed that PS4 appeared as individual nanoparticles around 50–70 nm in size (Fig. 5). This was further confirmed by Zetasizer and was found to be ca. 40 ± 20 nm (Ave $\pm$ SEM) (Supplementary Figure S2). PS4 nanocomplex with pIC led to an increase in the nanoparticle size from 40 nm to 165 nm (Supplementary Figure S2). The stability of these nanocomplexes was also studied by dynamic light scattering method using Zetasizer. There was no significant change in the size of the generated nanocomplexes over a period of two weeks indicating that there was no spontaneous fusion (or disintegration) of the individual particles when suspended either in PBS or in FBS

supplemented media (Supplementary Table S1, and data not shown).

3.6. Synergistic immune activation by PS4-pIC nanoparticles

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

3.7. Immunization with PS4-pIC nanoparticles

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

4. Discussion

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

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

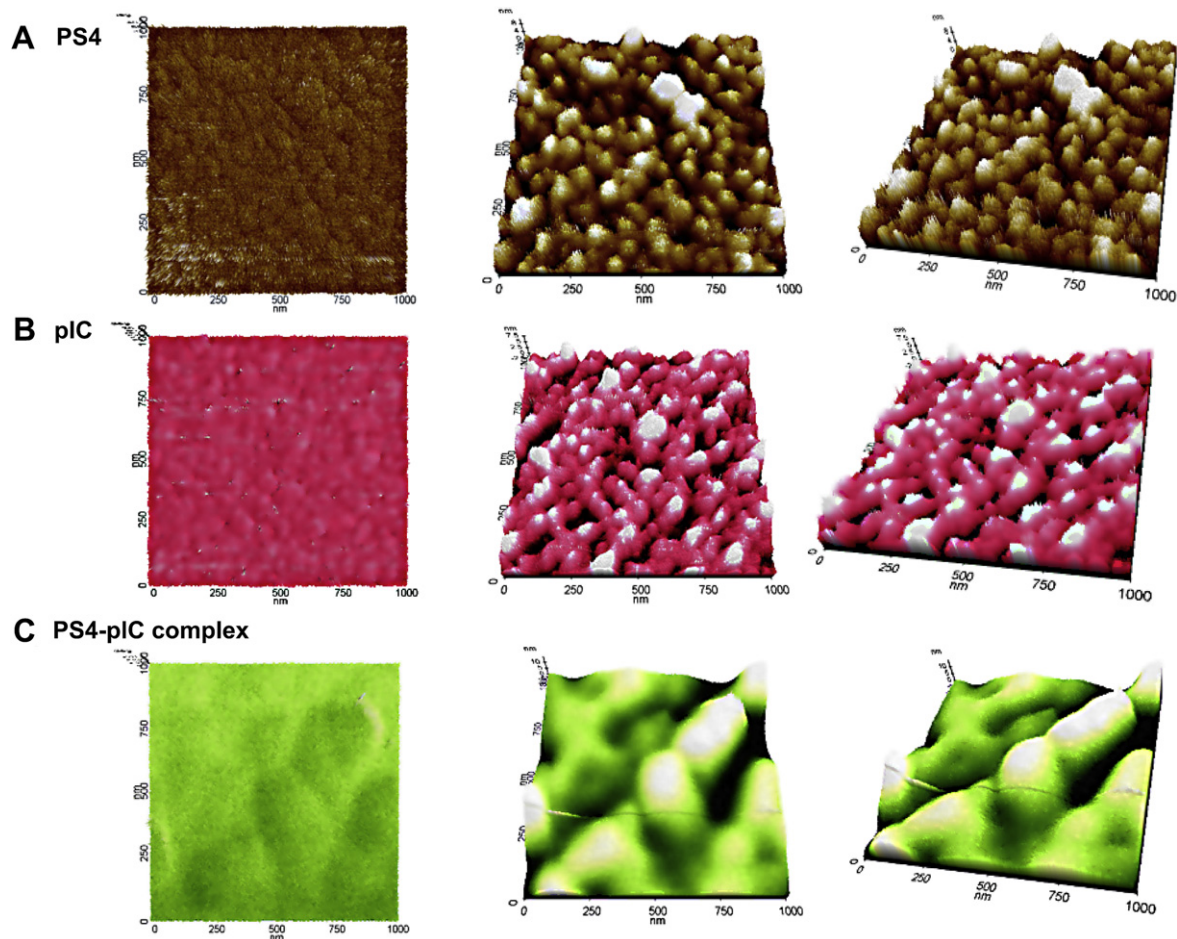


Fig. 5. AFM photomicrographs of phase, 2D phase (left) and 3D topography (middle and right) images. A) PS4, B) pIC and C) PS4-pIC nanoparticles at 1000 nm² area. Images were recorded under contact mode.

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

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

Contrary to previous reports, our findings suggested that mushroom derived PS polymers recognized only via TLR2 but not via TLR4 or TLR2/6. Furthermore, to date, there is no document reporting the collaborative activation of TLR3 ligand with PS obtained from edible mushrooms. Moreover, in the present study plain PS compounds were used with no further modification (i.e. cholesterol or peptide). The cytokine secretion panels (dose-dependent and time-dependent findings) presented in Figs. 1 and 2 clearly suggested that PS4 is the most potent stimulant. Moreover, in HEK cell system we established

that these PS types only interact with TLR2 (Fig. 3) but not with other cell surface or cytosolic receptors (NOD1 or 2). In the literature, receptor usage of PS is still elusive. This is probably due to the problem associated with the extraction of PS from different sources. Similar compounds were reported to trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells through various receptors including Dectin-1 and scavenger receptors [31,32]. We have attempted to delineate the contribution of PS mediated activation via the scavenger receptor CXCL16. It was found that it does not involve in PS mediated cytokine production, since there was no up or down regulation of the *cxcl16* gene transcript (Supplementary Figure S2). However, Dectin-1 has not been studied here, and must be checked to resolve its involvement.

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

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

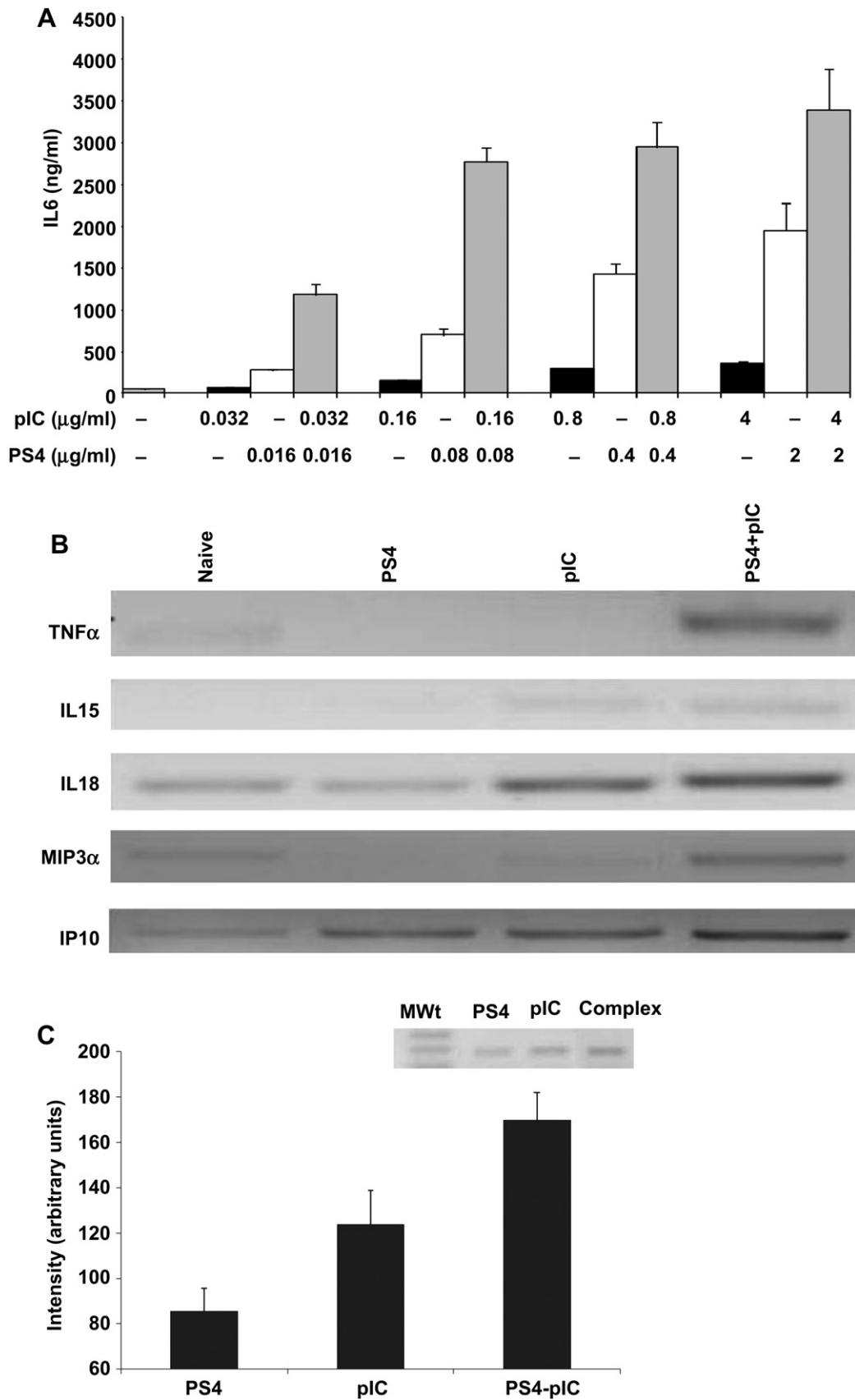


Fig. 6. PS-pIC nanoparticles induced higher IL6 production and strong gene upregulation of various cytokine and chemokines after *in vitro* and *in vivo* stimulations. A) RAW cell supernatants from 36 h culture were analyzed by ELISA. Result represents combination of at least two independent experiments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

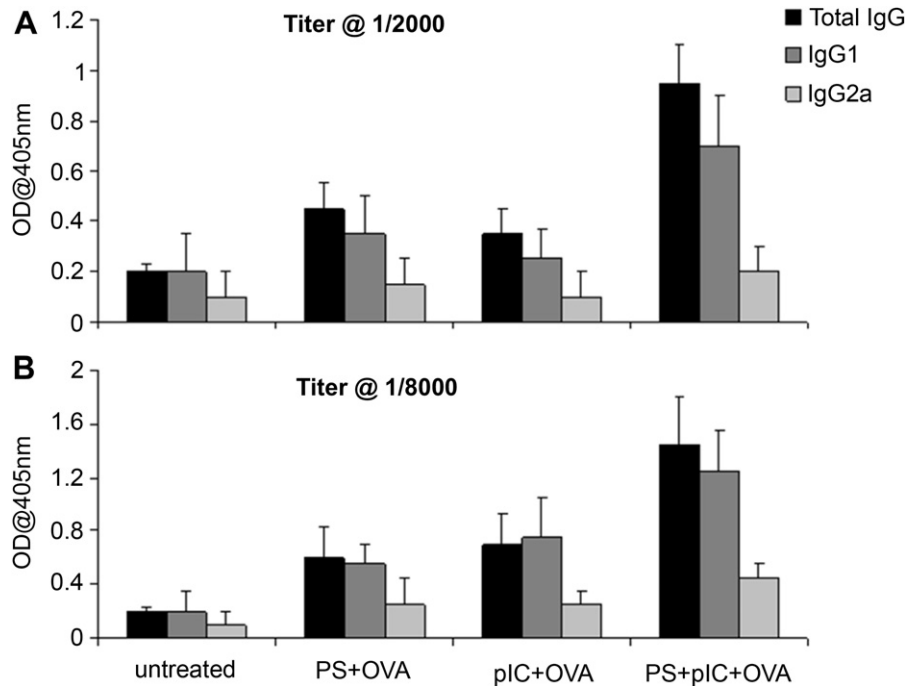


Fig. 7. IgG anti-OVA response of immunized mice. Animals were injected i.p with 15 μ g of PS4 + pIC combination or PS4-pIC nanoparticles incorporating 10 μ g OVA. Serum anti-OVA titers at day 13 (A) and day 28 (B) were determined by Total IgG, IgG1 and IgG2a ELISA. Data represents the average $\pm$ SD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

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

5. Conclusions

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

Acknowledgements

This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant No: 108S316). IG received grant support from EU/FP6/MC IRG (Grant # 036615) and EU 7th Framework Project UNAM-Regpot (Grant # 203953). GT and FCY received scholarship grants from SANTEZ (0448-STZ-2009-2)

and TÜBİTAK. We thank to Dr. Aykutlu Dana and his group members for their invaluable support during AFM studies. Drs. Can K. Akcali and Mayda Gursel are sincerely acknowledged for their critical reading of the manuscript.

Appendix. Supplementary data

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

References

- [1] Janeway Jr CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216.
- [2] Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol* 2001;1:135–45.
- [3] Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;17:1–14.
- [4] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe* 2008;3:352–63.
- [5] Schmidt KN, Leung B, Kwong M, Zarembka KA, Satyal S, Navas TA, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J Immunol* 2004;172:138–43.
- [6] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther* 1999;10:2461–80.
- [7] Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, et al. Bacterial DNA causes septic shock. *Nature* 1997;386:336–7.
- [8] Krieg AM, Efler SM, Wittpoth M, Adhami AI, M.J., Davis HL. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J Immunother* 2004;27:460–71.
- [9] Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF, et al. Immune stimulation—a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des* 1997;12:421–32.
- [10] Sparwasser T, Hultner L, Koch ES, Luz A, Lipford GB, Wagner H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol* 1999;162:2368–74.
- [11] Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J Immunol* 2000;165:1228–35.

- [12] Borges O, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. *Int Immunopharmacol* 2008;8:1773–80.
- [13] Fischer S, Schlosser E, Mueller M, Csaba N, Merkle HP, Groettrup M, et al. Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. *J Drug Target* 2009;17:652–61.
- [14] Schlosser E, Mueller M, Fischer S, Basta S, Busch DH, Gander B, et al. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 2008;26:1626–37.
- [15] Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J Immunol* 2001;167:3324–8.
- [16] Suzuki Y, Wakita D, Chamoto K, Narita Y, Tsuji T, Takeshima T, et al. Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. *Cancer Res* 2004;64:8754–60.
- [17] Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB, et al. CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infect Immun* 2005;73:828–33.
- [18] Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J Immunol* 2006;176:7335–45.
- [19] Mozafari MR. Liposomes: an overview of manufacturing techniques. *Cell Mol Biol Lett* 2005;10:711–9.
- [20] Crommelin DJ, Storm G. Liposomes: from the bench to the bed. *J Liposome Res* 2003;13:33–6.
- [21] Chirila TV, Rakoczy PE, Garrett KL, Lou X, Constable IJ. The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides. *Biomaterials* 2002;23:321–42.
- [22] Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Nunez G. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* 2008;28:246–57.
- [23] Lin ZB, Zhang HN. Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. *Acta Pharmacol Sin* 2004;25:1387–95.
- [24] Bourquin C, Anz D, Zwirok K, Lanz AL, Fuchs S, Weigel S, et al. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. *J Immunol* 2008;181:2990–8.
- [25] Wilson KD, de Jong SD, Tam YK. Lipid-based delivery of CpG oligonucleotides enhances immunotherapeutic efficacy. *Adv Drug Deliv Rev* 2009;61:233–42.
- [26] Mizu M, Koumoto K, Anada T, Matsumoto T, Numata M, Shinkai S, et al. A polysaccharide carrier for immunostimulatory CpG DNAs to enhance cytokine secretion. *J Am Chem Soc* 2004;126:8372–3.
- [27] Shimada N, Coban C, Takeda Y, Mizu M, Minari J, Anada T, et al. A polysaccharide carrier to effectively deliver native phosphodiester CpG DNA to antigen-presenting cells. *Bioconjug Chem* 2007;18:1280–6.
- [28] Mizu M, Koumoto K, Kimura T, Sakurai K, Shinkai S. Protection of polynucleotides against nuclease-mediated hydrolysis by complexation with schizophyllan. *Biomaterials* 2004;25:3109–16.
- [29] Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. *J Hematol Oncol* 2009;2:25.
- [30] Zhu XL, Lin ZB. Effects of *Ganoderma lucidum* polysaccharides on proliferation and cytotoxicity of cytokine-induced killer cells. *Acta Pharmacol Sin* 2005;26:1130–7.
- [31] Hua KF, Hsu HY, Chao LK, Chen ST, Yang WB, Hsu J, et al. *Ganoderma lucidum* polysaccharides enhance CD14 endocytosis of LPS and promote TLR4 signal transduction of cytokine expression. *J Cell Physiol* 2007;212:537–50.
- [32] Lin YL, Liang YC, Lee SS, Chiang BL. Polysaccharide purified from *Ganoderma lucidum* induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. *J Leukoc Biol* 2005;78:533–43.
- [33] Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992;6:3051–64.
- [34] Chin, M. P., Schauer, D. B. and Deen, W. M., Nitric oxide, oxygen, and superoxide formation and consumption in macrophages and colonic epithelial cells. *Chem Res Toxicol*.
- [35] Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol* 2003;171:1393–400.
- [36] Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;25:386–401.



The effects of an insertion in the 5'UTR of the AMCase on gene expression and pulmonary functions

Esra Birben^a, Cansın Sackesen^a, Shamsah Kazani^b, Gizem Tincer^c,
Cagatay Karaaslan^d, Berna Durgunsu^d, Ihsan Gürsel^c, Michael E. Wechsler^b,
Elliot Israel^b, Ömer Kalayci^{a,*}

^a Hacettepe University School of Medicine, Pediatric Allergy and Asthma Unit, Hacettepe, 06100 Ankara, Turkey

^b Pulmonary and Critical Care Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

^c Bilkent University, Faculty of Science, Department of Molecular Biology and Genetics, Ankara, Turkey

^d Hacettepe University, Faculty of Sciences, Department of Molecular Biology, Ankara, Turkey

Received 19 January 2011; accepted 21 March 2011

Available online 20 April 2011

KEYWORDS

Asthma;
Chitinase;
Genetics;
Polymorphism;
Pulmonary function;
Severity

Summary

Background: Studies regarding the physiological role of acidic mammalian chitinase (AMCase) and the effects of its genetic variants on asthma have produced conflicting results.

Objectives: We aimed to determine the genetic variants in the AMCase gene that could regulate the gene expression and thus influence disease severity.

Methods: Genetic variants of the AMCase gene were determined by sequencing of asthmatics and healthy controls in up to –1 kb in the promoter region and exon 1 and 2. In an association study, a population of asthmatic ($n = 504$) and healthy Turkish children ($n = 188$) were genotyped for the observed SNPs. A replication study was performed in a North American adult population of patients with mild ($n = 317$) and severe ($n = 145$) asthma. The functional properties of the insertion were determined by promoter reporter assay, electromobility shift assay and transcription factor ELISA experiments.

Results: Of the identified SNPs, only a ten base pair insertion (CAATCTAGGC) in the 5'UTR region of exon 2 was significantly associated with lower FEV₁ ($\beta = -14.63$ SE = 6.241, $P = 0.019$) in Turkish children with asthma. However, in the adult population, the same insertion showed a trend toward higher FEV₁. The insertion was shown to have enhancer activity and the mutant probe possessing the insertion had higher binding affinity for the nuclear extracts.

* Corresponding author. Tel.: +90 533 727 6459; fax: +90 312 441 9555.

E-mail address: okalayci@hacettepe.edu.tr (Ö. Kalayci).

Conclusion: Our study shows that a ten base pair insertion in the 5'UTR region of AMCase gene may modify gene expression and thus may affect the severity of asthma. However, its effects appear to be different in different populations.

© 2011 Elsevier Ltd. All rights reserved.

Introduction

Chitin is the second most abundant polysaccharide in nature after cellulose. It is found as a structural component of many species such as exoskeleton of crustaceans, parasitic nematodes and walls of fungi. It protects these organisms from harsh conditions.^{1–4} Chitinases are enzymes that hydrolyze chitin and are part of the anti-parasitic response against chitin containing organisms in lower life forms. Although there is no chitin in humans, recent studies have identified functional mammalian chitinase genes such as chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase, CHIA) and several chitinase-like genes such as YKL-40 (CHI3L1).^{5–9}

AMCase is one of the enzymes with true chitinase activity.¹⁰ The gene is located on chromosome 1q13.1–21.3 and contains 12 exons which transcribes into a 50 kD protein. The gene also encodes various splice forms. The enzyme is acid stable and mostly expressed in the gastrointestinal tract and in the asthmatic lung.¹⁰ In their pioneering study, Zhu et al. showed that AMCase is expressed in the epithelial cells and alveolar macrophages in patients with asthma.¹¹ In a murine model of asthma, they showed that AMCase acts downstream of IL-13 driven cytokine response. In support of this observation, Chupp et al. have shown that serum levels of a chitinase-like protein, YKL-40, were higher in asthmatics than control subjects and that they correlated positively with the severity of asthma and inversely with FEV₁%.¹² The same group also showed that a SNP in the promoter region of the gene encoding YKL-40 was associated with elevated serum YKL-40 levels, asthma, bronchial hyper-responsiveness, and measures of pulmonary functions.¹³

Even though many studies have suggested that AMCase is a positive mediator of Th2 response,¹⁴ more recent studies have challenged these findings. For example, Reese et al. showed that in a mouse model, AMCase inhibits chitin induced eosinophil and basophil recruitment to the lungs suggesting that AMCase can prevent allergic response induced by chitin.¹⁵ Similarly, in a human study, Seibold et al. measured chitinase activity in bronchoalveolar lavage (BAL) samples of asthmatics, habitual smokers and healthy controls and found that chitinase activity in human BAL samples was mostly related to chitotriosidase (CHIT1) but not to AMCase.¹⁶ Furthermore, they showed that chitinase activity was not higher, but actually lower in BAL fluids of asthmatics than control subjects and was increased in habitual smokers.^{gr1}

Similar to the conflicting findings regarding its physiological role in asthma, the studies on the effect of genetic variants on disease and disease phenotypes have produced some contradictory findings, as well. Bierbaum et al. identified six SNPs in the promoter and three SNPs in the exonic region of AMCase and reported that a non-synonymous SNP,

Lys17Arg, and possibly a promoter SNP (rs12033184), are associated with pediatric asthma in a German population.¹⁷ In contrast, Seibold et al. could not detect the Lys17Arg SNP in a population composed of adult African American, Puerto Rican, and Mexicans.¹⁸ They genotyped this population for 8 SNPs in the coding region and reported that some haplotypes composed of these SNPs are protective against asthma and are in fact associated with increased levels of chitinase activity *in-vitro*. Chatterjee et al. repeated Bierbaum's study in an Indian population by including a wider region of promoter and 3'UTR region.¹⁹ They reported quite a different set of SNPs with only four being common in both reports. In this report one promoter SNP, rs3806448, was associated with both asthma and IgE; and another one, rs10494132, was associated with IgE only. In a more recent study, however, neither of these SNPs nor any of the 24 SNPs analyzed was found to be associated with asthma or asthma related phenotypes.²⁰

In summary, similar to studies assessing associations with physiological outcomes, the results of studies evaluating genotypic associations of AMCase gene with asthma have produced conflicting results. We aimed to search for genetic variants in the AMCase gene that could regulate the gene expression and thus may influence disease expression in a case-control study involving Turkish children. For this purpose, we sequenced the promoter region up to –1 kD and also exon 1 and exon 2 as several different transcripts of the gene have been shown to include different exons. Here, we report our findings on the association of several SNPs and a 10 base pair insertion in the 5'UTR region of exon 2. We also attempted to replicate our findings with the 10 base pair insertion in a population composed of adult asthmatics from the United States; and also determine the functional properties of this insertion.

Materials and methods

Study population

Children with asthma: The patients in the asthma group have been detailed previously.^{21–23} Briefly, we enrolled children aged 6–18 years who were diagnosed with asthma at the Pediatric Allergy and Asthma Unit of Hacettepe University, School of Medicine, Ankara, Turkey between 2002 and 2009. Asthma diagnosis was confirmed by the examining pediatric allergist according to GINA guidelines.²⁴ All children had evidence of reversible airway obstruction as defined by at least a 12% improvement in FEV₁% following bronchodilator administration, therapeutic response to anti-asthma treatment, or an abnormal result in methacholine bronchoprovocation test (PC20 < 8 mg/dl). Spirometric measurements, total IgE, and eosinophil counts were obtained. Skin testing was performed as detailed

previously.²³ The study was approved by the Ethics committee of Hacettepe University and the parents have provided written informed consent for the study.

Healthy Children: The patients in the control group have been detailed previously.²³ Briefly, the control group was composed of Turkish school children who presented between 2005 and 2006 to the outpatient department of the same hospital. They presented for reasons such as minor trauma or for their regular follow-up. They responded negatively to an established and validated asthma questionnaire (ISAAC),²⁵ never had any diagnosis of asthma or allergic bronchitis by a physician, and never had any history of wheezing. They all had normal pulmonary function tests. All children underwent skin prick testing and had their total IgE measured in serum.

Adults with asthma: To test whether the findings observed in children with asthma could be replicated in an adult asthma population, we genotyped adult asthmatics from the USA composed of patients with mild and severe asthma. 462 subjects classified as either mild or severe asthmatics by the NAEPP guidelines were identified from the Brigham and Women's Hospital's (BWH) Asthma Research Center's database. These patients were recruited between 2001 and 2008, and each had signed a written informed consent agreeing to exploratory genetic analysis related to asthma, which was approved by the BWH institutional review board. Baseline demographic information was available for each subject and baseline spirometry was performed on the day of the blood draw. All the patients in the adult population were non-smokers, defined as less than or equal to 10 pack year smoking history and none in the last 12 months. DNA was stored at the Channing Laboratory where genotyping was performed for the 10 base pair insertion (CAATCTAGGC) identified in the 5'UTR region of exon 2, as described below.

Identification of genetic variants

DNA was extracted from whole blood by standard techniques. To determine the polymorphisms in the promoter region and 5'UTR region of the transcript including exon 1 and exon 2 of AMCase gene, DNA samples from 20 children with asthma and 20 healthy children were sequenced with Big Dye Terminator cycle sequencing kit (3.2 version) from ABI Prism (Foster City, CA, USA). Sequencing of the promoter and the first 2 exons revealed the presence of 10 SNPs, 8 in the promoter, 2 in exon 1 and a ten base pair insertion in the 5'UTR of Exon 2. According to the initial analysis, 8 of the 10 SNPs, six SNPs in the promoter region and two SNPs in Exon 1, seemed to be in linkage disequilibrium. To prove this, 100 individuals were further sequenced in the promoter region.

Genotyping of the promoter region

Sequencing of 100 samples showed that eight of the promoters' SNPs were in perfect linkage disequilibrium. Therefore, we chose one of them, rs4442363 SNP, to determine the genotype by restriction fragment length polymorphism (RFLP) analysis. A 613 base pair of the promoter region was amplified using the following primer pairs: forward 5'-CGGACACTGGACTTAAGTTGT-3' and reverse 5'-GAAGCTTTGGCACCCTCT-3'. The amplicon was digested with *Hinf I* (New England Biolabs, MA, USA) and

the products were fractionated on a 2% agarose gel. Digestion occurs only in the presence of the T allele and produces a 214 and 399 base pair products; whereas the undigested C allele is visualized as a single 613 base pair band. 10% of samples randomly selected but including from all genotypes were re-genotyped for quality control.

Genotyping of the insertion at 5'UTR region of exon 2

The genotyping for the 10 base pair insertion (CAATCTAGGC) was again done by PCR-RFLP analysis. A 451 base pair segment in the 5'UTR region of exon 2 was amplified using the following primers: Forward 5'-CTGACCACAGTATCTAAACAG-3', reverse 5'-GGGATGTAGTATGAAGACCACT-3'. The amplicon was digested with *Bfal* (New England Biolabs) and the product was fractionated on an agarose gel which resulted in three fragments 308, 94 and 59 base pairs in the presence of insertion; while two fragments of 392 and 59 base pairs were obtained in the absence of the insertion. 10% of samples randomly selected but including from all genotypes were re-genotyped for quality control.

Transient transfection analysis with reporter constructs

Genomic DNA from asthmatic individuals with and without the 10 base pair insertion was amplified to include 200 base pairs upstream from the start codon of exon 2. Purified PCR products were cloned into two different types of vectors: *i.* a pGL3 basic vector without a promoter to check for the promoter activity of the inserted sequence *ii.* a pGL3 control vector possessing a SV40 promoter region (Promega, Madison, WI, USA) at *Kpn I* and *Xho I* to check for the enhancer activity of inserted sequence.

A549 bronchial type II alveolar cells (DSMZ, Braunschweig, Germany) were used for the transfection assays. A549 cells were grown to 90% confluence in 24-well plates, transiently transfected with either promoter reporter constructs or empty vectors and co-transfected with a pRL-TK vector ($n = 4$) (Promega, Madison, WI, USA) containing Renilla Luciferase gene using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. 48 h after the transfection, cells were lysed with reporter lysis buffer and luciferase activity of each lysate was measured using Dual Luciferase Assay kit (Promega). Firefly luciferase activity was normalized to renilla luciferase activity to control for differences in transfection efficiency.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from A549 cells either without any stimulation or after stimulation with IL-4 (25 ng/ml for 24 h) (Biosource, Camarillo, CA, USA) and/or IL-13 (25 ng/ml for 24 h) (PeproTech Inc., Rocky Hill, NJ, USA) using NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations of nuclear extracts were measured using Bradford Assay (Bio-Rad, Munich, Germany). Double-stranded, biotin labeled oligonucleotides (IDT, Coralville, IA, USA) with and without the insertion were synthesized: without the insertion 5'-AGAACATATAAAAAGCTCTGCGGGACTGGT-3'; with the insertion 5'-TATAAAAAGCCAATCTAGGCTCTGCGGG-AC-3'. Protein-DNA binding reactions were performed with chemiluminescent LightShift EMSA kit (Pierce Biotechnology). Briefly, 2 µg of nuclear protein and 2 pmol of labeled

probes were incubated in binding mix containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 50 ng/μl Poly(dI.dC), 0.05% NP-40 at room temperature for 20 min. Protein–DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel in a Tris–borate–EDTA buffer in a cold room. Complexes were transferred onto a Biotinylated Nylon membrane and cross-linked with UV. Biotin labeled free oligos and oligo–protein complexes were detected using streptavidin–horseradish peroxidase system. Gel bands were visualized using the CDD camera system (Vilber Lourmat, Marne-la-Vallée, France).

Intensity of binding bands was measured by image analyzer (Syngene Gene Genius, Cambridge, UK) by using Gene Tools Analysis Software 3.02.00 (SynGene software). Intensity of band which represents the binding of wild type oligos to unstimulated extract was defined as 1 and intensity of other conditions calculated relatively according to this one's.

Transcription factor ELISA

Since the insertion sequence contained a CAAT motif for C/EBP, a custom designed transcription factor ELISA (Active Motif, Carlsbad, CA, USA) was performed to show the binding of insertion to C/EBP protein. In this method, the plates were initially coated with biotin labeled double stranded oligonucleotides with and without the insertion; followed by incubation with 5 μg of nuclear extract for 1 h. The wells were then washed and incubated with a C/EBP alpha or beta antibody. The reaction was developed with an anti-IgG HRP conjugate and the colorimetric reaction was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The experiment was performed in quadruplicate for each condition.

Statistical analyses

Statistical analyses were done with SPSS 15 for Windows (Chicago, IL, USA), Prism 5 for Windows (GraphPad Software, Inc. CA, USA) and SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Comparison of quantitative variables such as age, eosinophil counts, IgE levels and FEV₁% were done with parametric or non-parametric tests depending on the normalcy of distribution. In case of non-normal distribution, non-parametric tests were used only if various transformation methods failed to normalize the data. Chi square or Fisher's exact test was used for comparison of categorical variables as appropriate. As expected, there was a significant difference in the gender distribution between asthmatic children and healthy controls.²⁶ Therefore, all data are stratified to account for this gender difference. For all analyses, a p value <0.05 was considered significant.

We performed linear regression analysis to establish the factors that were associated with FEV₁% and eosinophil counts. With this regression model, we calculated the residuals which are the difference between observed values minus values predicted in the regression. Then, we constructed a box plot as well as a histogram to see whether residual distributions are skewed. Both graphs showed that the distribution of the residuals was not skewed. In this regression model, our primary endpoint was the effect of genotype on FEV₁% levels or eosinophil counts.

We considered the following as covariates in the regression model: age, gender, age of onset, skin test positivity and plasma total IgE levels. The model was constructed using backward elimination. In order to ensure that the assumptions for linear regression analysis are met, we checked for the normality of residuals by Q–Q plots and Shapiro–Wilk test and in addition we constructed a scatter plot of residuals versus predicted values.

Results

Five hundred and four children with asthma and 188 healthy controls were included in the study. Characteristics of study population are summarized in Table 1. Asthma and atopy-related findings including IgE, skin prick tests, FEV₁%, eosinophil counts and family history of allergic diseases were significantly different between the 2 groups.

Sequencing results

We sequenced 20 asthmatic and 20 healthy controls up to –1 kb in the promoter region and Exon 1 and 2. We identified 10 SNPs in the promoter and 5'UTR region and a 10 base pair insertion (CAATCTAGGC) in the 5'UTR region of exon 2.

Of the 10 SNPs, eight (rs12033184, rs35042265, rs4546919, rs4554721, rs4442363, rs11102235, rs34698010, rs12026825) were in complete linkage disequilibrium as shown by sequencing of 100 DNA samples from each of asthmatic children and healthy controls. The study population was genotyped for the presence of only one of these SNPs, rs4442363 polymorphism, in order to investigate the association between this block of SNPs and asthma and asthma phenotypes.

Of the remaining two SNPs in the promoter region, sequencing studies of 63 healthy and 80 asthmatic children showed that there was no genotypic variance in rs11102234; and that the genotypic distribution of the rs12023459 was very similar between children with asthma and healthy controls and failed to show any association with asthma and atopic phenotypes. Therefore, these two SNPs were not pursued any further.

Association studies between the promoter SNPs, asthma diagnosis and asthma phenotypes

Promoter SNPs were analyzed by PCR-RFLP in a subpopulation of the whole cohort involving 352 children with asthma and 172 healthy controls. There was no difference in the genotype distribution of the promoter SNPs between children with asthma and healthy controls (Table 2). There was no association between the reported SNPs and asthma phenotypes such as atopy, FEV₁%, eosinophil numbers, either.

Association studies between the 5'UTR insertion (CAATCTAGGC), asthma diagnosis and asthma phenotypes

504 children with asthma and 188 healthy controls were genotyped for the insertion at 5'UTR of exon 2 by PCR-RFLP

Table 1 Characteristic of study population.

	Healthy controls <i>n</i> = 188	Children with asthma <i>n</i> = 504	<i>p</i>
Age (years) ^a	10.8 (8.0–13.0)	10.0 (7.8–12.7)	>0.05
Gender			
Male (%)	90 (47.6)	324 (64.5)	<0.001
Female (%)	99 (52.4)	179 (35.5)	
FEV ₁ (% predicted) ^a	101 (91–108)	92 (79–102)	<0.001
Eosinophil count/mm ^{3a}	150 (98.9–280.8)	300 (170–500)	<0.001
Skin test positivity (%)	29 (15.4)	295 (59.4)	<0.001
IgE (kU/L) ^a	35 (17–72)	178 (55–454)	<0.001
Family history of atopic diseases (%)	22 (11.8)	162 (32.3)	<0.001
Pet ownership (%)	25 (13.4)	34 (6.8)	>0.05
Smoke exposure	106 (57.0)	202 (40.2)	>0.05

^a Median (Interquartile range).

analysis. There was no difference in the genotype frequencies between children with asthma and healthy controls (Table 3) showing that this insertion is not associated with asthma.

Within the asthmatic population, in a model where the presence of the insertion behaves as the recessive allele [i.e., wild type + heterozygotes vs. mutant (insertion present in both alleles)] the insertion was significantly associated with lower FEV₁% and lower eosinophil counts. Multiple linear regression analysis showed that the homozygous presence of the insertion was significantly and independently associated with lower FEV₁% values ($\beta = -14.63$ SE = 6.241, $P = 0.019$). In this model, eosinophil counts had a small but significant effect, as well ($\beta = -0.008$ SE = 0.003, $P = 0.003$).

The effect of the 10 base pair insertion in the 5'UTR of exon 2 on the expression of AMCase gene

Due to the observed association between insertion and FEV₁%, we attempted to determine the function of the mutation and tried to establish whether it affects the expression of AMCase gene using A549 alveolar cell line. In order to see whether this insertion independently increases the promoter activity or whether it behaves as an enhancer, we designed two types of reporter constructs: promoter type and enhancer type. Cloning of the 200 bp region of 5'UTR into a basic vector from both the wild and mutant genotypes failed to show any difference between the genotypes ($p > 0.05$; data not shown). However, cloning of the same region into a control vector just in front of the SV40 promoter as an enhancer element showed that the sequence with the insertion displayed significantly higher luciferase activity compared to the wild type ($p < 0.05$)

(Fig. 1). Stimulation of A549 cells with IL-4, IL-13 or both further increased the enhancer activity conferred by the presence of the insertion (Fig. 1). The results of this experiment suggested that the studied insertion behaves as an enhancer element for the AMCase gene and that this enhancer activity increases in the presence of IL-4.

The effect of the 10 base pair insertion in the 5'UTR on nuclear protein avidity and specific binding of C/EBP

Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts obtained from A549 cells with and without stimulation with IL-4 and IL-13. The EMSA assay showed more intensive bands with the mutant probe possessing the insertion suggesting stronger binding affinity of the mutant sequence with the insertion to the nuclear extracts (Fig. 2).

In order to identify the transcription factor that is responsible for higher binding avidity of the mutant probe, we performed supershift experiments with antibodies to various transcription factors. Our main candidate was the transcription factor C/EBP (CAAT enhancer binding protein) as the inserted sequence contains a CAAT motif for C/EBP binding. However, antibodies to C/EBP failed to induce any supershift (Fig. 2). We were unable to induce any supershift with antibodies to other transcription factors such as Oct-1 and GATA-3 (data not shown).

Since EMSA assay failed to show whether C/EBP is the transcription factor responsible for the specific binding, a more sensitive TransAM transcription factor ELISA was performed. Plates were covered with biotin labeled double stranded wild type and mutant type oligonucleotides; incubated with nuclear extracts and C/EBP α or β antibodies were

Table 2 Genotype frequencies of the promoter polymorphism rs4442363 in the AMCase gene.

Genotype	Healthy Controls <i>n</i> = 172 <i>n</i> (%)	Children with asthma <i>n</i> = 352 <i>n</i> (%)	<i>P</i>
CC	59 (34.3)	97 (27.6)	
CT	78 (45.3)	190 (54.0)	
TT	35 (20.3)	65 (18.5)	>0.05

Table 3 Genotype frequencies of 10 the base pair insertion in the 5'UTR region of exon 2

Genotype	Healthy controls <i>n</i> = 188 <i>n</i> (%)	Children with asthma <i>n</i> = 504 <i>n</i> (%)	<i>p</i>
Wild type (no insertion)	153 (84.1)	409 (81.2)	
Heterozygote	33 (17.6)	87 (17.3)	
Mutant (insertion present in both alleles)	2 (1.1)	8 (1.6)	>0.05

used as a primary antibody. Data revealed that antibodies to C/EBP β but not to α showed specific binding. Fig. 3 shows that stimulation with IL-4 significantly reduced binding of C/EBP β to nuclear extracts ($p = 0.002$) but there was no difference in the binding avidity between the oligonucleotides with or without the insertion. Further experiments with GATA family transcription factors including GATA-1, 2 and 3 failed to show any difference between the two genotypes.

Genotyping of the adult population

In order to confirm our findings in another population, we genotyped 317 patients with mild and 145 patients with severe asthma for the presence of the insertion (Table 4). The severe asthmatics were older and had lower lung function than the mild asthmatics. There was no difference in the frequency of the insertion between the two groups. In contrast to our observation in the pediatric population, adults with the homozygous form of insertion had showed a trend toward higher FEV₁% values that approached significance [$90.4 \pm 4.6\%$ in mutant (insertion present in both alleles) vs 81.2 ± 0.9 in wild type + heterozygotes, $p = 0.06$]. Because of our adult population composed of

different ethnic backgrounds population stratification was assessed, but not significant differences was found between groups.

Discussion

There is a great deal of controversy surrounding the physiological effects of chitinase proteins as well as the effects of the variants of the encoding genes in asthma. In this report, we show that a genetic variant in the second exon of the AMCase gene may modify the function of the gene and the same variant may have different and even opposite effects in two different populations.

In their initial report, Zhu et al. have shown that AMCase is expressed in the asthmatic lung and is found in the BAL fluid of asthmatics.¹¹ They have also shown that it acts downstream of IL-13 in the inflammatory pathway. These results were challenged in a mouse model where AMCase was shown to inhibit chitin induced eosinophil and basophil recruitment to the lungs suggesting that AMCase can prevent allergic response induced by chitin. In a human study Seibold et al.¹⁶ showed that chitinase activity in BAL samples was mostly related to chitotriosidase (CHIT1) but not to AMCase. More importantly, they showed that chitinase activity was not higher, but actually lower in BAL fluids of asthmatics than control subjects and was increased in habitual smokers. Similar to the controversies regarding the function of chitinase, there have been conflicting reports on the effect of AMCase genotypes on asthma and asthma related phenotypes, as well.

We attempted to search for the genetic variants of the AMCase gene that may modify the disease expression and thus may affect the severity of asthma. We found that an insertion containing a CAAT motif (CAATCTAGGC) in the 5'UTR region of exon 2 was associated with lower FEV₁% in children with asthma. In a reporter gene system, we also showed that this variant enhances the promoter activity of the gene especially in the presence of IL-4. Further to this observation, EMSA studies revealed that the sequence with the insertion binds the nuclear extracts containing the regulatory proteins with higher avidity compared to the wild type oligonucleotides that does not carry the insertion. Although C/EBP beta binds to the region containing the inserted sequence, the nuclear factor ELISA study showed that the presence or absence of the insertion does not change the binding, suggesting that C/EBP is not the transcription factor responsible for the enhancer activity of the insertion sequence. Interestingly, in contrast to our observation in the reporter system, IL-4 decreased the binding of C/EBP beta in the ELISA experiment. Collectively, these data implicate that the differential binding of other transcription factor (or factors) may be responsible for the action of this inserted 10 base pair sequence. These factors

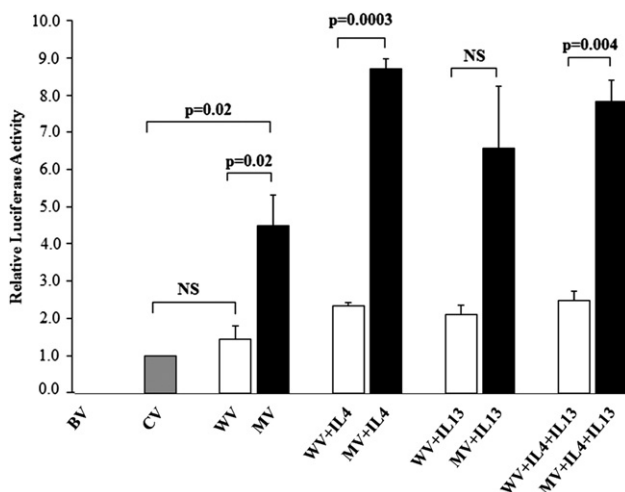


Figure 1 Promoter reporter assay. A549 cells were transiently transfected with wild type reporter vector (WV) or mutant type reporter vector (MV). Reporter activity was normalized relative to the activity of pGL3 control vector. No promoter activity was observed after transfection with the basic vector, therefore only the results of transfection with the control vector are shown. Reporter activity was significantly higher in A549 cells transiently transfected with control reporter vector containing the ten base pair insertion than the constructs bearing the wild type allele. (BV = pGL3 Basic vector without a promoter, CV = pGL3 control vector with a SV40 promoter).

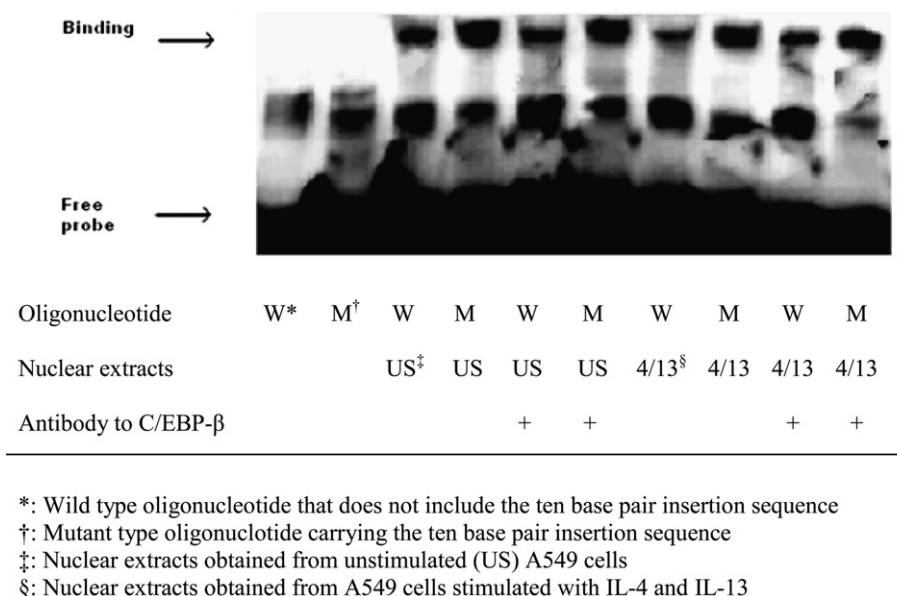


Figure 2 EMSA experiment. Nuclear proteins from A549 cells, unstimulated or stimulated with IL-4 and IL-13, bind to the probes carrying the insertion sequence (M) with more avidity than those probes without the insertion (W). Antibody to C/EBP-β failed to induce a supershift.

remain to be determined. It should also be noted that the functional analyses are carried out in A549 cells which belong to an alveolar cell line. Even though A549 cells have been used in numerous studies to investigate various pathological and physiological aspects of lung diseases,²⁷ they do not truly represent the cells of the airways which are the major players in lung function. Therefore, even though we describe functions related to this insertion, we do not know whether expression of this gene is actually related to the phenotype of lung function.

In addition to our study, four studies have previously investigated the association between the genotypic variant of the *AMCase* gene and asthma. The promoter SNPs that we report here have previously been reported by Bierbaum

et al.¹⁷ They also failed to show any association between these SNPs and asthma phenotypes in German children. Chatterjee et al.¹⁹ on the other hand, have reported significant associations with 2 SNPs in the part of the promoter that goes 1000 bp further up to the 5' region that was selected in our study. One of these SNPs rs3806448 is associated both with asthma and IgE in the Indo-Aryan population. This particular SNP was in linkage disequilibrium with the SNPs used in our study. Therefore, one can assume that we would have failed to find an association with this SNP even if we had done our analysis to include that far in the 5' of the promoter. To further support the discrepancy, Wu et al.²⁰ also failed to find any association with rs3806448 in a North American population of asthmatic children. Similarly, rs10494132 was found to be associated with IgE by Chatterjee et al. but not by Wu et al. In fact, Wu et al., in a comprehensive analysis, failed to show any association between asthma and any of the 24 SNPs in the *AMCase* gene that they studied.

The insertion that we report in our study was only detected in a recent study by Seibold et al.¹⁸ in a population of African American, Puerto Rican, and Mexicans. However, they did not report any association with this particular SNP. In our study, we report two discrepant findings in two different populations. In a population of Turkish asthmatic children we found that the presence of insertion was associated with lower FEV₁%, a finding which was supported by the results of the logistic regression analysis that showed that the insertion was in fact significantly and independently associated with lower FEV₁%. In a North American population composed of mild and severe asthmatics, the same analysis has showed that the same insertion was associated with higher FEV₁%.

Our study may be criticized for attempting to replicate the findings from a pediatric population in a population composed of adults. Since the presence of insertion was associated with lower FEV₁ in the pediatric group, we

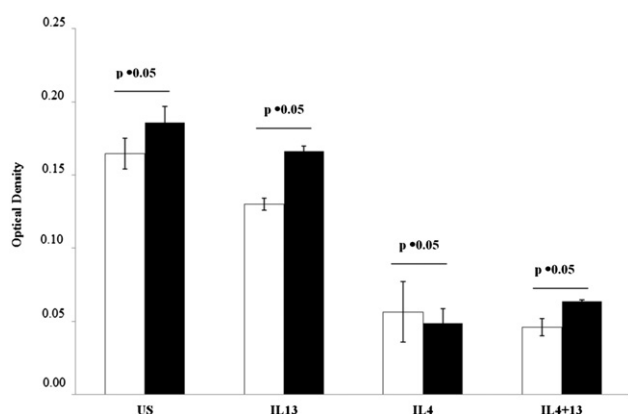


Figure 3 Custom designed C/EBP transcription factor ELISA. Plates are initially coated with biotin labeled double stranded oligos with and without the insertion followed by incubation nuclear extracts obtained from A549 cells. Optical density is measured after incubation with C/EBP beta antibody. Empty bars represent wild type probes without the insertion and black bars represent mutant probes with the insertion.

Table 4 Characteristics of the adult population with asthma.

	Mild asthma <i>n</i> = 317 <i>n</i> (%)	Severe asthma <i>n</i> = 145 <i>n</i> (%)	<i>P</i>
Age (years) ^a	30 (±0.57)	44 (±1.12)	< 0.0001
Gender			
Male	114 (36)	56 (39)	>0.05
Female	203 (64)	89 (61)	
Race			
Caucasian	215 (68)	86 (59)	>0.05
African American	65 (20)	43 (30)	
Asian	10 (3)	7 (5)	
Other/Mixed	27 (9)	9 (6)	
Ethnicity			
Hispanic	25 (8)	10 (7)	>0.05
Non-Hispanic	292 (92)	135 (93)	
FEV ₁ (% predicted) ^a	90 (±0.80)	64 (±1.33)	< 0.0001
ICS use	28 (9)	145 (100)	< 0.0001
Genotype ^b			
Wild	237 (75)	108 (75)	>0.05
Heterozygote	68 (21)	33 (23)	
Mutant	12 (4)	3 (2)	

^a Mean (Standard error of the mean).

^b Genotype frequencies of 10 the base pair insertion in the 5'UTR region of exon 2.

hypothesized that it may be associated with the severity of the disease as lower FEV₁ is a major determinant of asthma severity. However, severe asthma is quite rare in children. Therefore, in order to obtain a robust finding regarding the association of the SNP with asthma severity we chose an adult population containing a significant number of patients with severe asthma.

There are two major differences between our initial and replication cohorts: they are of different genetic backgrounds and they are of different age groups. There may be several reasons to account for the discrepant results reported in the literature and here. One explanation would be differences in the genetic backgrounds of the populations studied. The presence of polymorphisms in the other regions of the AMCase gene or variants in other genes causing epistatic effects may also be operative in underlying the differences.

In addition to age and ethnic differences, another factor could be the differences in the environmental exposures as several studies have shown that the response of the genes can vary significantly depending on the environmental exposures. In one of the best examples of the gene environment interaction, when the results of the asthma and endotoxin study were stratified according to the endotoxin exposure it was shown that the C allele of the CD14-159 C/T SNP was associated with a higher risk of allergic sensitization at low levels of endotoxin exposure and lower risk at high levels of exposure.^{28,29} This observation was later substantiated by two independent studies.^{30,31} In the *in-vitro* environment, we have shown that the two genotypes respond differently to increasing concentrations of endotoxin.³² Therefore, there is reason to believe that the effects of the genetic variants may vary significantly depending on the environmental chitin exposure, as well. To make the issue even more complicated, a recent study by Wu et al. have shown that high mold exposure significantly modified the relation between 3 SNPs in *CHIT1* (rs2486953, rs4950936,

rs1417149) and severe exacerbations, even though the same SNPs showed no association in their original study where the environmental exposures have not been taken into account.³³ This observation suggests that the association between the chitinase genes and environmental exposure can go even beyond environmental chitin exposure and may be influenced by mold exposure, as well.

A third factor to account for the discrepant results between the two populations of this study can be the age of the studied populations. Expression patterns of genes may be under epigenetic influences and thus may change with age. Recently, Munthe-Kaas et al. have shown that CD14 methylation increased significantly from age 2–10 years, and that the level of methylation was inversely correlated with sCD14 levels at 10 years.³⁴ They have also shown that even though 3 SNPs were associated with sCD14 levels within the first two years of life, only one was associated at 10 years. Therefore, the differences that we observed between the two populations, one pediatric and the other adult, can be partially due to the differences in the age of the study cohorts and be modified by epigenetic factors. Finally, as we have extensively discussed, the two populations are different with respect to many variables including their ethnic backgrounds and environmental exposures. Even though we have chosen an adult population of patients with severe asthma in order to obtain a robust finding regarding the association of the SNP with asthma severity, it would definitely be desirable and increase the strength of our findings if we could do the same comparison in a well matched pediatric population with severe asthma.

Our study has weaknesses. Firstly, it would be extremely helpful to have the measures of the environmental chitin exposure or, in light of recent studies, mold exposure levels of our population. Secondly, even though we have shown that the insertion increases the expression of the reporter gene, we do not have any evidence that it changes the

protein levels in the lungs of the asthmatics and that it is directly associated with measures of lung function. Similarly, we do not have evidence that it affects the tissue levels of infiltrating eosinophils.

To conclude, our study shows that a ten base pair insertion in the second exon in the 5'UTR region of the AMCase gene may modify the gene expression and thus may affect the severity of asthma. However, its effects may be different in different populations and may be modified by various environmental exposures and demographics of the population under study.

Acknowledgment

This study was supported by Hacettepe University Scientific Research Fund grants #02 02 101 020, #01402 01 002, and #01 402 01 004.

Conflict of interest

All the authors declare that there is no conflict of interest.

References

- Fuhrman JA, Piessens WF. Chitin synthesis and sheath morphogenesis in *Brugia malayi* microfilariae. *Mol Biochem Parasitol* 1985;17:93–104.
- Debono M, Gordee RS. Antibiotics that inhibit fungal cell wall development. *Annu Rev Microbiol* 1994;48:471–97.
- Shahabuddin M, Kaslow DC. Plasmodium: parasite chitinase and its role in malaria transmission. *Exp Parasitol* 1994;79:85–8.
- Merzendorfer H, Zimoch L. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J Exp Biol* 2003;206:4393–412.
- Renkema GH, Boot RG, Muijsers AO, Donker-Koopman WE, Aerts JM. Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. *J Biol Chem* 1995;270:2198–202.
- Bleau G, Massicotte F, Merlen Y, Boisvert C. Mammalian chitinase-like proteins. *EXS* 1999;87:211–2.
- Saito A, Ozaki K, Fujiwara T, Nakamura Y, Tanigami A. Isolation and mapping of a human lung-specific gene, TSA1902, encoding a novel chitinase family member. *Gene* 1999;239:325–31.
- Owhashi M, Arita H, Hayai N. Identification of a novel eosinophil chemotactic cytokine (ECF-L) as a chitinase family protein. *J Biol Chem* 2000;275:1279–86.
- Funkhouser JD, Aronson Jr NN. Chitinase family GH18: evolutionary insights from the genomic history of a diverse protein family. *BMC Evol Biol* 2007;7:96.
- Boot RG, Blommaert EF, Swart E, Ghauharali-van der Vlugt K, Bijl N, Moe C, Place A, Aerts JM. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. *J Biol Chem* 2001;276:6770–8.
- Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elias JA. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004;304:1678–82.
- Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, Dziura JD, Reed J, Coyle AJ, Kiener P, Cullen M, Grandsaigne M, Dombret MC, Aubier M, Pretolani M, Elias JA. A chitinase-like protein in the lung and circulation of patients with severe asthma. *N Engl J Med* 2007;357:2016–27.
- Ober C, Tan Z, Sun Y, Possick JD, Pan L, Nicolae R, Radford S, Parry RR, Heinzmann A, Deichmann KA, Lester LA, Gern JE, Lemanske RF, Nicolae DL, Elias JA, Chupp GL. Effect of variation in *CHI3L1* on serum YKL-40 level, risk of asthma, and lung function. *N Engl J Med* 2008;358:1682–91.
- Elias JA, Homer RJ, Hamid Q, Lee CG. Chitinases and chitinase-like proteins in T(H)2 inflammation and asthma. *J Allergy Clin Immunol* 2005;116:497–500.
- Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007;447:92–6.
- Seibold MA, Donnelly S, Solon M, Innes A, Woodruff PG, Boot RG, Burchard EG, Fahy JV. Chitotriosidase is the primary active chitinase in the human lung and is modulated by genotype and smoking habit. *J Allergy Clin Immunol* 2008;122:944–50.
- Bierbaum S, Nickel R, Koch A, Lau S, Deichmann KA, Wahn U, Superti-Furga A, Heinzmann A. Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. *Am J Respir Crit Care Med* 2005;172:505–9.
- Seibold MA, Reese TA, Choudhry S, Salam MT, Beckman K, Eng C, Atakilit A, Meade K, Lenoir M, Watson HG, Thyne S, Kumar R, Weiss KB, Grammer LC, Avila P, Schleimer RP, Fahy JV, Rodriguez-Santana J, Rodriguez-Cintrón W, Boot RG, Sheppard D, Gilliland FD, Locksley RM, Burchard EG. Differential enzymatic activity of common haplotypic versions of the human acidic mammalian chitinase protein. *J Biol Chem* 2009;284:19650–8.
- Chatterjee R, Batra J, Das S, Sharma SK, Ghosh B. Genetic association of acidic mammalian chitinase with atopic asthma and serum total IgE levels. *J Allergy Clin Immunol* 2008;122:202–8. 208.e1–7.
- Wu AC, Lasky-Su J, Rogers CA, Klanderman BJ, Litonjua A. Polymorphisms of chitinases are not associated with asthma. *J Allergy Clin Immunol* 2010;125:754–7. 757.e1–757.e2.
- Sackesen C, Karaaslan C, Keskin O, Tokol N, Tahan F, Civelek E, Soyer OU, Adalioglu G, Tuncer A, Birben E, Oner C, Kalayci O. The effect of polymorphisms at the CD14 promoter and the TLR4 gene on asthma phenotypes in Turkish children with asthma. *Allergy* 2005;60:1485–92.
- Kalayci O, Birben E, Sackesen C, Keskin O, Tahan F, Wechsler ME, Civelek E, Soyer OU, Adalioglu G, Tuncer A, Israel E, Lilly C. ALOX5 promoter genotype, asthma severity and LTC production by eosinophils. *Allergy* 2006;61:97–103.
- Ercan H, Birben E, Dizdar EA, Keskin O, Karaaslan C, Soyer OU, Dut R, Sackesen C, Besler T, Kalayci O. Oxidative stress and genetic and epidemiologic determinants of oxidant injury in childhood asthma. *J Allergy Clin Immunol* 2006;118:1097–104.
- Global Initiative for Asthma. *Global strategy for asthma management and prevention (revised ed)*. Bethesda (MD): National Institutes of Health, National Heart Lung and Blood Institute; 2002.
- Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, Mitchell EA, Pearce N, Sibbald B, Stewart AW, Strachan D, Weiland SK, Williams HC. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995;8:483–91.
- Bush A, Menzies-Gow A. Phenotypic differences between pediatric and adult asthma. *Proc Am Thorac Soc* 2009;6(8):712–9.
- Bonner K, Kariyawasam HH, Ali FR, Clark P, Kay AB. Expression of functional receptor activity modifying protein 1 by airway epithelial cells with dysregulation in asthma. *J Allergy Clin Immunol* 2010;126(6):1277–83.
- Braun Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP,

- Schierl R, Renz H, Nowak D, von Mutius E, Allergy and Endotoxin Study Team. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; **347**:869–77.
29. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, Nowak D, Martinez FD, Allergy and Endotoxin Alex Study Team. Opposite effects of CD 14/-260 on serum IgE levels in children raised in different environments. *J Allergy Clin Immunol* 2005; **116**:601–7.
30. Simpson A, John SL, Jury F, Niven R, Woodcock A, Ollier WE, Custovic A. Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. *Am J Respir Crit Care Med* 2006; **174**:386–92.
31. Williams LK, McPhee RA, Ownby DR, Peterson EL, James M, Zoratti EM, Johnson CC. Gene-environment interactions with CD14 C-260T and their relationship to total serum IgE levels in adults. *J Allergy Clin Immunol* 2006; **118**:851–7.
32. Sackesen C, Birben E, Soyer OU, Sahiner UM, Yavuz TS, Civelek E, Karabulut E, Akdis M, Akdis CA, Kalayci O. The effect of CD14 C159T polymorphism on *in vitro* IgE synthesis and cytokine production by PBMC from children with asthma. *Allergy* 2011; **66**(1):48–57.
33. Wu AC, Lasky-Su J, Rogers CA, Klanderman BJ, Litonjua AA. Fungal exposure modulates the effect of polymorphisms of chitinases on emergency department visits and hospitalizations. *Am J Respir Crit Care Med* 2010; **182**(7):884–9.
34. Munthe-Kaas MC, Torjussen TM, Gervin K, Lødrup Carlsen KC, Carlsen KH, Granum B, Hjorthaug HS, Undlien D, Lyle R. CD14 polymorphisms and serum CD14 levels through childhood: a role for gene methylation? *J Allergy Clin Immunol* 2010; **125**:1361–8.

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis^{*[5]}

Received for publication, March 22, 2010, and in revised form, June 16, 2010. Published, JBC Papers in Press, July 14, 2010, DOI 10.1074/jbc.M110.125948

Fuat C. Yagci^{†1}, Ozlem Aslan^{§1}, Mayda Gursel[¶], Gizem Tincer[‡], Yasemin Özdamar[§], Kutay Karatepe[‡], K. Can Akcali[‡], and Ihsan Gursel^{†2}

From the [†]Biotherapeutic Oligonucleotide Laboratory, Department of Molecular Biology and Genetics, [¶]Merkez Lojmanlari, Bilkent University, 06800 Ankara, Turkey, and the [§]Ministry of Health, Ulucanlar Eye Research and Education Hospital, 06240 Ankara, Turkey

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

DNA and RNA are the essential components of all living organisms. Accumulated evidence strongly suggests that these nucleic acids have multiple and complex effects on the immune system and are more than a blueprint of life (1, 2). On one hand, due to their high unmethylated CpG motif frequency, bacterial DNAs are recognized as “non-self” via TLR9 (Toll-like receptor 9) and trigger an innate immune response characterized by the proliferation and maturation of B cells, natural killer cells, and plasmacytoid dendritic cells and the secretion of T-helper

1-type cytokines, chemokines, and/or multivalent immunoglobulins (3–8). On the other hand, telomeric regions of mammalian chromosomes contain suppressive TTAGGG motifs that can inhibit several TLR-dependent and TLR-independent Th1-mediated immune responses. Of note, these motifs are underrepresented in the prokaryotic genome. Synthetic single-stranded oligodeoxynucleotides (ODN)³ containing repetitive TTAGGG motifs mimic this effect (1, 9–11). Previous studies revealed that deleterious inflammatory responses to a host can be down-regulated by suppressive ODN. *In vitro*, suppressive ODN inhibits the production of several proinflammatory cytokines and chemokines induced by bacteria (1, 12–14). Furthermore, *in vivo* suppressive ODN administration reduces the frequency and severity of several autoimmune and inflammatory diseases such as arthritis, systemic lupus erythematosus, pulmonary inflammation, toxic shock, silicosis, and experimental autoimmune encephalomyelitis (10, 15–21).

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

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

^{*} This work was supported by Scientific and Technological Research Council of Turkey (TUBITAK) Grants SBAG106S102, SBAG108S316, EU/FP6/Marie Curie, and EU/FP7 UNAM REGPOT 203953.

^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1 and 2.

[†] Both authors contributed equally to this work.

² To whom correspondence should be addressed. Tel.: 90-312-290-2408; Fax: 90-312-266-5097; E-mail: ihsangursel@bilkent.edu.tr.

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

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

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

EXPERIMENTAL PROCEDURES

Materials—All cell culture medium components were from HyClone. Cytokine pairs for ELISAs were from Endogen. LPS (isolated from *Escherichia coli*) was obtained from Sigma. The phosphorothioate-modified suppressive ODN A151 (24-mer, 5'-(TTAGGG)₄-3') and control ODN (24-mer, 5'-(TTACCC)₄-3') were obtained from Alpha DNA (Montreal, Canada). TRIzol G (AppliChem GmbH, Darmstadt, Germany) was used for RNA isolation. cDNAs were synthesized using a DyNAmo™ cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. DyNAzyme™ PCR Master Mix was used for PCRs.

Maintenance of Animals—Adult female BALB/c mice and adult New Zealand rabbits were used for the experiments. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled conditions at 22 °C with 12-h light and 12-h dark cycles. They were provided with unlimited access of food and water.

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

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

Cytokine and IgM ELISAs and NO Assays—Immulon 2 HB microtiter plates (Thermo Scientific) were coated with anti-cytokine or anti-IgM antibodies (BD Pharmingen) and then blocked with PBS and 1% BSA (1, 34). Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine antibody followed by phosphatase-streptavidin (Perbio), whereas bound IgM was detected using phosphatase-conjugated anti-IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) as described (1). Nitric oxide detection by the Griess method was conducted on murine peritoneal exudate cells (10⁶/ml) after 12–36 h of *ex vivo* incubation as described by the supplier (Promega).

Analysis of Cell-surface Molecule Expression by FACS—2 × 10⁶ spleen cells/ml were isolated from 24-h post-treated mice. Cells were washed, fixed, and co-stained with one of the phycoerythrin-labeled anti-CD40, anti-CD86, and anti-ICAM-1 and FITC-labeled cell-specific antibodies (*i.e.* CD11c for dendritic cells, CD11b for macrophages, and B220 for B cells (BD Pharmingen)) for 30 min at room temperature. Following washing, they were studied using a FACSCalibur (BD Biosciences) and analyzed with CellQuest Pro software.

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

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis

TABLE 1

Oligonucleotide PCR primers used in mouse or rabbit experiments

m, mouse; rb, rabbit.

Primer	Forward	Reverse	Product bp
mβ-actin ^a	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA	450
mIP10 ^a	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT	127
miNOS ^a	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	95
mMIP1α ^b	ACCATGACACTCTGCAACCA	AGGCATTGAGTTCCAGGTCA	238
mIL-5 ^a	AGCACAGTGGTGAAGAGACCTT	TCCAATGCATAGCTGGTGATT	117
mIL-15 ^a	CATCCATCTCGTCTACTTGTGT	CATCTATCCAGTTGGCCTCTGT	126
mIL-18 ^b	GATCAAAGTGCCAGTGAACC	ACAAACCTTCCCACCTAAC	384
mMCP1 ^b	AGGTCCTGTCATGCTTCTG	TCTGGACCCATTCTTCTTG	249
mMIP3α ^b	CGTCTGCTCTTCTTGTCTTT	CCTTTTACCCAGTTCTGCT	250
mCXCL16 ^b	CCTTGTCTCTTGCCTTCTTC	GGTTGGGTGTGCTCTTGTGT	384
mMIP1β ^b	CCAGCTCTGTGCAACCTAA	CTGTCTGCCTCTTTTGGTCA	250
rbGAPDH ^c	TCACCATCTTCCAGGAGCGA	CACAATGCCGAAGTGGTCGT	319
rbIL-6 ^c	GCTCCTGGTGGTGGCTAC	GGGTGGCTTCTTCATTCAA	450
rbIL-1β ^c	GCCGATGGTCCAATTACAT	ACAAGACCTGCCGAAGCT	121

^a Taken from Ref. 43.

^b In house-designed primers.

^c Taken from Ref. 44.

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

RESULTS

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

This study was performed with 82 mice and 26 rabbits. Initial experiments were conducted to optimize the induction of EIU (supplement Fig. 1). For the mouse experiments, systemic administration of LPS doses between 25 and 100 μg/mouse were sufficient to induce uveitic eyes within 24 h as judged by clinical and histopathological investigations (supplement Figs. 1 and 2). For the rabbit experiments, intraocular 100-μg LPS injection was found to be optimal to induce EIU. Following local or intraperitoneal LPS and/or suppressive ODN administration, rabbit and mouse eyes were removed, and RNAs from the irises, vitreous, and corneas of the rabbit eyes were obtained. PCR was run with the cDNA from each sample, and the mRNA levels of IL-6, IL-15, IP10 (interferon-γ-inducible protein 10), iNOS, MIP1α, IL-18, MIP3α, CXCL16 (CXC chemokine ligand 16), MIP1β, and IL-1β were monitored. In addition, 24 h post-LPS and/or A151 treatment, splenocyte suspensions were incubated *ex vivo* for 6–24 h, and IgM, IL-6, IL-10, IL-12, and IFNγ levels from the supernatants were determined by ELISA. FACS analyses were conducted on spleen cells to monitor co-stimulatory/surface marker molecule expressions.

The results indicated that in rabbits, suppressive ODN administered before or after 100-μg LPS treatment or co-injected with LPS significantly down-regulated the expression of IL-1β message from the iris (Fig. 1A). In cornea, IL-6 was down-regulated when suppressive ODN was given before or simultaneously with LPS administration (Fig. 1B). There was no sig-

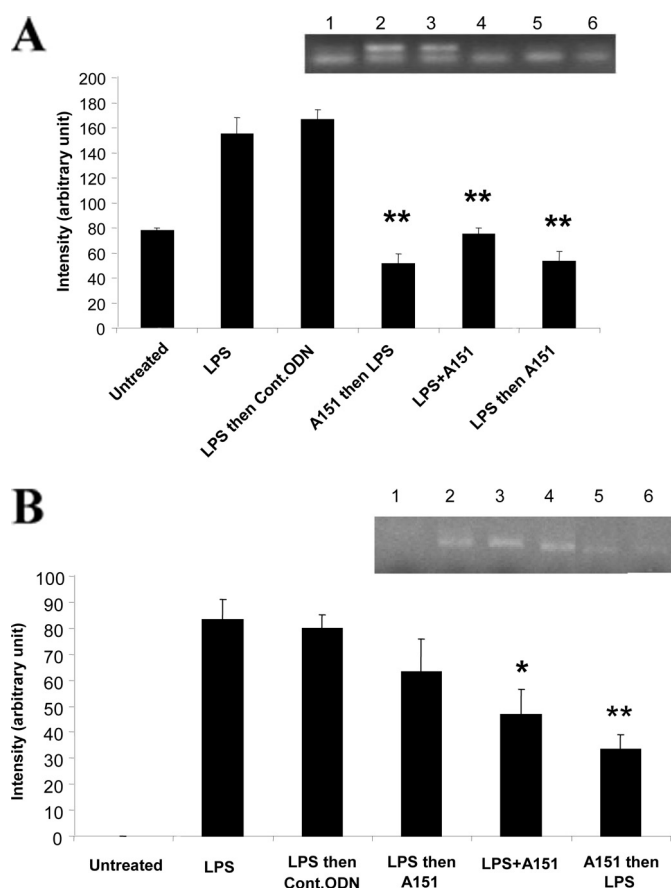


FIGURE 1. Suppressive A151 ODN administration after LPS challenge significantly down-regulates IL-1β and IL-6 expression in the iris and cornea, respectively. Rabbits were injected intraocularly with 100 μg of LPS and 250 μg of suppressive ODN. The average of densitometric measurements of four animals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

nificant inhibitory effect when A151 was given post-LPS treatment. In all these experiments, the mRNA message reduction was suppressive ODN-dependent because control ODN administration did not show any benefit for alleviation of LPS reactivity.

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis

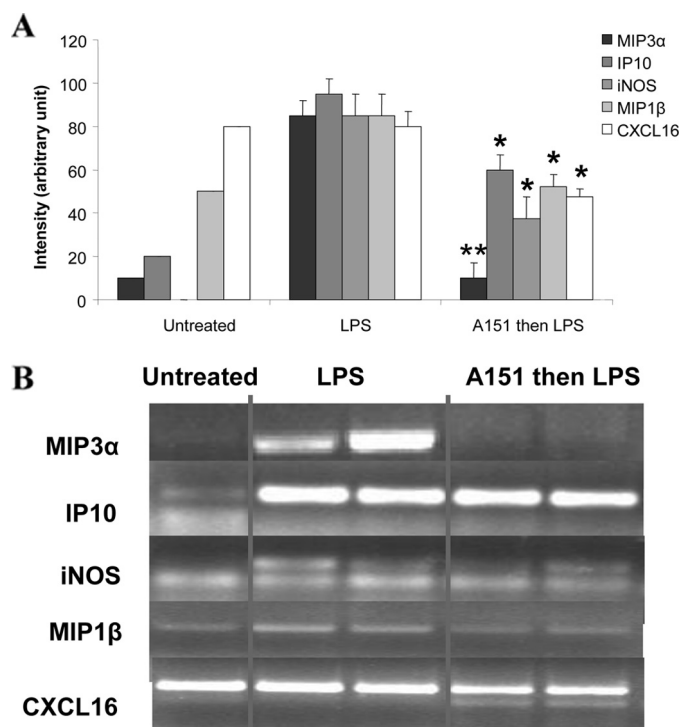


FIGURE 2. A, suppressive A151 ODN treatment after 100- μ g LPS challenge significantly down-regulates IP10, MIP3 α , iNOS, MIP1 β , and CXCL16 expression levels in the eyes of mice. Mice were injected intraperitoneally with 100 μ g of LPS and 250 μ g of suppressive ODN and killed 18 h after injection. *, $p < 0.05$; **, $p < 0.01$ between LPS-treated and LPS + A151 ODN-co-administered groups. B, representative gel image.

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

The results showed that when 250 μ g of suppressive ODN was administered before LPS injection (2 h), it significantly down-regulated the expression of IP10, iNOS, MIP3 α , CXCL16, and MIP1 β in the 100- μ g LPS-injected mouse EIU model (Figs. 2, A and B). Other cytokines such as MIP1 α and IL-18 also showed substantial but insignificant down-regulation at these doses (data not shown). To understand the systemic effect of suppressive A151, IL-6 secreted from murine splenocytes after *ex vivo* incubation for up to 24 h in culture was monitored by ELISA (Fig. 3). Our results revealed that suppressive ODN was able to reduce $>65\%$ of the secreted IL-6 (430 ± 70 and 135 ± 55 ng/ml for LPS and A151 + LPS groups, respectively). Co-administration of suppressive ODN with LPS significantly decreased cytokine mRNA levels *in vivo* or cytokine production in *ex vivo* spleen cells ($p < 0.01$) (Figs. 2 and 3). These effects were attributable to the activity of suppressive motifs because control ODN did not reduce the cytokine production elicited by co-administered LPS (Figs. 1–3).

The (TTAGGG)₄ multimers inhibited LPS-dependent up-regulation of co-stimulatory and surface marker molecules on

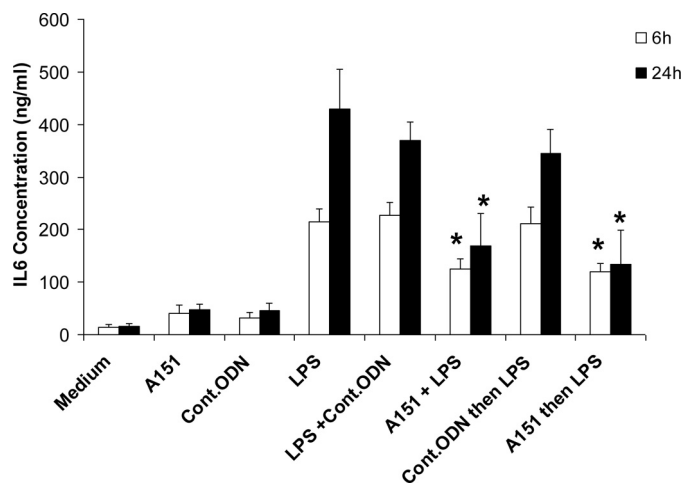


FIGURE 3. Suppressive A151 ODN administration significantly suppresses IL-6 release from murine splenocytes. Mice were injected intraperitoneally with 100 μ g of LPS and 250 μ g of suppressive or control ODN as further indicated. Spleen cells were removed (24 h post injection) and incubated 6–24 h, and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

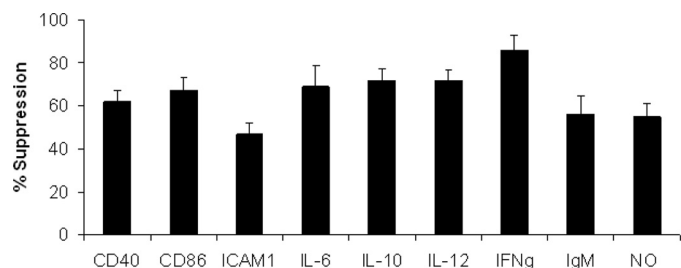


FIGURE 4. Inhibitory effect of suppressive ODN on LPS-mediated immune activation. The levels of CD40, CD86, and ICAM-1 expression (mean fluorescence intensity) were determined by FACS 24 h after *in vivo* injection of LPS or LPS plus A151 (50 μ g of LPS and 250 μ g of ODN). IL-6, IL-10, IL-12, IFN γ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

antigen-presenting cells (CD40, CD86, and ICAM-1), IgM production by B cells, and NO release from peritoneal macrophages ($p < 0.01$) (Fig. 4). Furthermore, co-administration of LPS (50 μ g) with A151 ODN (250 μ g) inhibited $>65\%$ of several immunoregulatory and inflammatory cytokines (*i.e.* IL-6, IL-10, and IL-12; $p < 0.001$) (Fig. 4). This reduction reached $>85\%$ for IFN γ (176 ± 29 and 26 ± 15 ng/ml for LPS and A151 + LPS groups, respectively).

DISCUSSION

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

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis

Compared with local injection of (intraocular) LPS and/or LPS- and control ODN-treated rabbits, suppressive ODN-administrated animals exhibited reduced levels of IL-1 β and IL-6 expression in the iris and cornea, respectively. In the mouse model, the results revealed that pretreatment with 250 μ g of suppressive ODN reduced the expression of IP10, iNOS, MIP1 α , IL-18, MIP3 α , CXCL16, and MIP1 β in 100- μ g LPS-injected mice. In another experiment, with different doses of suppressive ODN and LPS, suppressive ODN also down-regulated the expression of MCP1, which is an important chemokine for monocyte chemoattraction (data not shown). The suppressive action of this class of ODN was not only on the mRNA levels of several Th1-type cytokines and chemokines but also on the secreted protein level. ELISA experiments showed that suppressive ODN pre- and post-treatments significantly diminished IL-6 secretion at 6 and 24 h; simultaneous administration of suppressive ODN also reduced IL-6 production.

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

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

to down-regulate sustained/pathologic microbe-associated molecular pattern-induced immunity. In conclusion, we have provided evidence that suppressive A151 ODN is able to significantly reduce the ocular inflammatory responses in both rabbit and murine EIU models.

Acknowledgments—We thank Tamer Kahraman and Erdem Eriki for excellent technical support and Burcu C. Insal for assistance during animal procedures. We greatly appreciate Dr. Onder Bozdogan for guidance during histopathological investigations.

REFERENCES

- Gursel, I., Gursel, M., Yamada, H., Ishii, K. J., Takeshita, F., and Klinman, D. M. (2003) *J. Immunol.* **171**, 1393–1400
- Ishii, K. J., and Akira, S. (2005) *Int. J. Cancer* **117**, 517–523
- Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) *J. Immunol.* **148**, 4072–4076
- Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2879–2883
- Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W., and Wagner, H. (1998) *Eur. J. Immunol.* **28**, 2045–2054
- Krieg, A. M. (2000) *Curr. Opin. Immunol.* **12**, 35–43
- Yamada, H., Gursel, I., Takeshita, F., Conover, J., Ishii, K. J., Gursel, M., Takeshita, S., and Klinman, D. M. (2002) *J. Immunol.* **169**, 5590–5594
- Klinman, D. M., Klaschik, S., Sato, T., and Tross, D. (2009) *Adv. Drug Deliv. Rev.* **61**, 248–255
- Klinman, D. M., Zeuner, R., Yamada, H., Gursel, M., Currie, D., and Gursel, I. (2003) *Ann. N.Y. Acad. Sci.* **1002**, 112–123
- Shirota, H., Gursel, I., Gursel, M., and Klinman, D. M. (2005) *J. Immunol.* **174**, 4579–4583
- Hu, D., Su, X., Sun, R., Yang, G., Wang, H., Ren, J., Sun, L., Wu, X., Hu, X., Yu, Y., and Wang, L. (2009) *Mol. Immunol.* **46**, 1387–1396
- Zhu, F. G., Reich, C. F., and Pisetsky, D. S. (2002) *J. Leukocyte Biol.* **71**, 686–694
- Shirota, H., Gursel, M., and Klinman, D. M. (2004) *J. Immunol.* **173**, 5002–5007
- Klinman, D. M., Tross, D., Klaschik, S., Shirota, H., and Sato, T. (2009) *Ann. N.Y. Acad. Sci.* **1175**, 80–88
- Zeuner, R. A., Ishii, K. J., Lizak, M. J., Gursel, I., Yamada, H., Klinman, D. M., and Verthelyi, D. (2002) *Arthritis Rheum.* **46**, 2219–2224
- Ho, P. P., Fontoura, P., Ruiz, P. J., Steinman, L., and Garren, H. (2003) *J. Immunol.* **171**, 4920–4926
- Zeuner, R. A., Verthelyi, D., Gursel, M., Ishii, K. J., and Klinman, D. M. (2003) *Arthritis Rheum.* **48**, 1701–1707
- Dong, L., Ito, S., Ishii, K. J., and Klinman, D. M. (2004) *Arthritis Rheum.* **50**, 1686–1689
- Dong, L., Ito, S., Ishii, K. J., and Klinman, D. M. (2005) *Arthritis Rheum.* **52**, 651–658
- Ho, P. P., Fontoura, P., Platten, M., Sobel, R. A., DeVoss, J. J., Lee, L. Y., Kidd, B. A., Tomooka, B. H., Capers, J., Agrawal, A., Gupta, R., Zernik, J., Yee, M. K., Lee, B. J., Garren, H., Robinson, W. H., and Steinman, L. (2005) *J. Immunol.* **175**, 6226–6234
- Sato, T., Shimozato, T., Alvord, W. G., and Klinman, D. M. (2008) *J. Immunol.* **180**, 7648–7654
- Rothova, A., Suttorp-van Schulten, M. S., Frits Treffers, W., and Kijlstra, A. (1996) *Br. J. Ophthalmol.* **80**, 332–336
- Durrani, O. M., Tehrani, N. N., Marr, J. E., Moradi, P., Stavrou, P., and Murray, P. I. (2004) *Br. J. Ophthalmol.* **88**, 1159–1162
- Sande, P. H., Fernandez, D. C., Aldana Marcos, H. J., Chianelli, M. S., Aisemberg, J., Silberman, D. M., Sáenz, D. A., and Rosenstein, R. E. (2008) *Am J. Pathol.* **173**, 1702–1713
- Hafezi-Moghadam, A., Noda, K., Almulki, L., Iliaki, E. F., Poulaki, V., Thomas, K. L., Nakazawa, T., Hisatomi, T., Miller, J. W., and Gragoudas, E. S. (2007) *FASEB J.* **21**, 464–474
- Nagai, N., Oike, Y., Noda, K., Urano, T., Kubota, Y., Ozawa, Y., Shinoda,

Mammalian Telomeric DNA Suppresses Endotoxin-induced Uveitis

- H., Koto, T., Shinoda, K., Inoue, M., Tsubota, K., Yamashiro, K., Suda, T., and Ishida, S. (2005) *Invest. Ophthalmol. Vis. Sci.* **46**, 2925–2931
27. Hoekzema, R., Verhagen, C., van Haren, M., and Kijlstra, A. (1992) *Invest. Ophthalmol. Vis. Sci.* **33**, 532–539
28. Ohta, K., Kikuchi, T., Miyahara, T., and Yoshimura, N. (2005) *Exp. Eye Res.* **80**, 401–412
29. Koizumi, K., Poulaki, V., Doehmen, S., Welsandt, G., Radetzky, S., Lappas, A., Kociok, N., Kirchhof, B., and Joussen, A. M. (2003) *Invest. Ophthalmol. Vis. Sci.* **44**, 2184–2191
30. Mo, J. S., Matsukawa, A., Ohkawara, S., and Yoshinaga, M. (1999) *Exp. Eye Res.* **68**, 333–340
31. Mandai, M., Yoshimura, N., Yoshida, M., Iwaki, M., and Honda, Y. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**, 3673–3680
32. Lajavardi, L., Bochot, A., Camelo, S., Goldenberg, B., Naud, M. C., Behar-Cohen, F., Fattal, E., and de Kozak, Y. (2007) *Invest. Ophthalmol. Vis. Sci.* **48**, 3230–3238
33. Chan, C. C., Caspi, R. R., Ni, M., Leake, W. C., Wiggert, B., Chader, G. J., and Nussenblatt, R. B. (1990) *J. Autoimmun.* **3**, 247–255
34. Gursel, I., Gursel, M., Ishii, K. J., and Klinman, D. M. (2001) *J. Immunol.* **167**, 3324–3328
35. Dunn, J. P. (2004) *Curr. Opin. Ophthalmol.* **15**, 293–298
36. Moorthy, R. S., Mermoud, A., Baerveldt, G., Minckler, D. S., Lee, P. P., and Rao, N. A. (1997) *Surv. Ophthalmol.* **41**, 361–394
37. Lightman, S. (1997) *Eye* **11**, 222–226
38. Avunduk, M. C., Avunduk, A. M., Oztekin, E., Baltaci, A. K., Ozyazgan, Y., and Mogolkoc, R. (2004) *Exp. Eye Res.* **79**, 357–365
39. Adamus, G., Burrows, G. G., Vandenbark, A. A., and Offner, H. (2006) *Invest. Ophthalmol. Vis. Sci.* **47**, 2555–2561
40. Fujimoto, C., Klinman, D. M., Shi, G., Yin, H., Vistica, B. P., Lovaas, J. D., Wawrousek, E. F., Igarashi, T., Chan, C. C., and Gery, I. (2009) *Clin. Exp. Immunol.* **156**, 528–534
41. Rudolph, K. L., Chang, S., Lee, H. W., Blasco, M., Gottlieb, G. J., Greider, C., and DePinho, R. A. (1999) *Cell* **96**, 701–712
42. Hackett, J. A., Feldser, D. M., and Greider, C. W. (2001) *Cell* **106**, 275–286
43. Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. (2001) *Methods* **25**, 386–401
44. Sobajima, S., Shimer, A. L., Chadderdon, R. C., Kompel, J. F., Kim, J. S., Gilbertson, L. G., and Kang, J. D. (2005) *Spine J.* **5**, 14–23

Permissions to the Copyrighted Materials

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tinoer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979480865385
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Journal of Allergy and Clinical Immunology
Licensed content title	Interleukins, from 1 to 37, and interferon- γ : Receptors, functions, and roles in diseases
Licensed content author	Mübeccel Akdis, Simone Burgler, Reto Crameri, Thomas Eiwegger, Hiroyuki Fujita, Enrique Gomez, Sven Klunker, Norbert Meyer, Liam O'Mahony, Oscar Palomares, Claudio Rhyner, Nadia Quaked, Anna Schaffartzik, Willem Van De Veen, Sabine Zeller, Maya Zimmermann, Cezmi A. Akdis
Licensed content date	March 2011
Licensed content volume number	127
Licensed content issue number	3
Number of pages	91
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979481145424
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Developmental & Comparative Immunology
Licensed content title	Chemokines
Licensed content author	Kerry J Laing, Christopher J Secombes
Licensed content date	3 May 2004
Licensed content volume number	28
Licensed content issue number	5
Number of pages	18
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD



RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979331059859
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Immunology
Licensed content title	The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors
Licensed content author	Taro Kawai, Shizuo Akira
Licensed content date	Apr 20, 2010
Type of Use	reuse in a thesis/dissertation
Volume number	11
Issue number	5
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
High-res required	no
Figures	Figure 1 and Figure 2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979340479553
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Biochemical and Biophysical Research Communications
Licensed content title	Toll-like receptors and innate immunity
Licensed content author	Himanshu Kumar,Taro Kawai,Shizuo Akira
Licensed content date	30 October 2009
Licensed content volume number	388
Licensed content issue number	4
Number of pages	5
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD



[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979340724351
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Cellular & Molecular Immunology
Licensed content title	C-type lectin receptor-induced NF- κ B activation in innate immune and inflammatory responses
Licensed content author	Lara M Kingeter and Xin Lin
Licensed content date	Jan 16, 2012
Type of Use	reuse in a thesis/dissertation
Volume number	9
Issue number	2
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD



[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979340951462
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Current Opinion in Immunology
Licensed content title	NOD-like receptors (NLRs): bona fide intracellular microbial sensors
Licensed content author	Michael H Shaw,Thornik Reimer,Yun-Gi Kim,Gabriel Nuñez
Licensed content date	August 2008
Licensed content volume number	20
Licensed content issue number	4
Number of pages	6
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD



[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979341115697
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Immunology
Licensed content title	Inflammasomes: far beyond inflammation
Licensed content author	Jorge Henao-Mejia, Eran Elinav, Till Strowig, Richard A Flavell
Licensed content date	Mar 19, 2012
Type of Use	reuse in a thesis/dissertation
Volume number	13
Issue number	4
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979350057091
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Cell
Licensed content title	The Inflammasomes
Licensed content author	Kate Schroder, Jurg Tschopp
Licensed content date	19 March 2010
Licensed content volume number	140
Licensed content issue number	6
Number of pages	12
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979350357937
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Immunology
Licensed content title	Cooperation of Toll-like receptor signals in innate immune defence
Licensed content author	Giorgio Trinchieri and Alan Sher
Licensed content date	Mar 1, 2007
Type of Use	reuse in a thesis/dissertation
Volume number	7
Issue number	3
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
High-res required	no
Figures	Figures 1 and 4
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3053301282272
License date	Dec 20, 2012
Licensed content publisher	Elsevier
Licensed content publication	Advanced Drug Delivery Reviews
Licensed content title	CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases
Licensed content author	Dennis M. Klinman, Sven Klaschik, Takashi Sato, Debbie Tross
Licensed content date	28 March 2009
Licensed content volume number	61
Licensed content issue number	3
Number of pages	8
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Jan 2013
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979381302565
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Ultramicroscopy
Licensed content title	Higher order structure of short immunostimulatory oligonucleotides studied by atomic force microscopy
Licensed content author	Dionne C.G. Klein,Eicke Latz,Terje Espevik,Bjørn T. Stokke
Licensed content date	May 2010
Licensed content volume number	110
Licensed content issue number	6
Number of pages	5
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD



[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979410119946
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Drug Discovery
Licensed content title	Therapeutic potential of Toll-like receptor 9 activation
Licensed content author	Arthur M. Krieg
Licensed content date	Jun 1, 2006
Type of Use	reuse in a thesis/dissertation
Volume number	5
Issue number	6
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Box 2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and John Wiley and Sons ("John Wiley and Sons"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979410415448
License date	Aug 31, 2012
Licensed content publisher	John Wiley and Sons
Licensed content publication	Immunology
Book title	None
Licensed content author	Makiya Nishikawa,Mitsuhiro Matono,Sakulrat Rattanakit,Nao Matsuoka,Yoshinobu Takakura
Licensed content date	Jan 23, 2008
Start page	247
End page	255
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1a
Will you be translating?	No
Order reference number	None
Total	0.00 USD



License Details

This is a License Agreement between Gizem Tinoer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979410533511
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	Biomaterials
Licensed content title	Biodegradable CpG DNA hydrogels for sustained delivery of doxorubicin and immunostimulatory signals in tumor-bearing mice
Licensed content author	Makiya Nishikawa, Yumiko Mizuno, Kohta Mohri, Nao Matsuoka, Sakurat Rattanakiat, Yuki Takahashi, Hisakage Funabashi, Dan Luo, Yoshinobu Takakura
Licensed content date	January 2011
Licensed content volume number	32
Licensed content issue number	2
Number of pages	7
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD

[← Back](#)



My Orders > Orders > All Orders

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979440924090
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Immunology
Licensed content title	Cancer immunoediting: from immunosurveillance to tumor escape
Licensed content author	Gavin P. Dunn , Allen T. Bruce , Hiroaki Ikeda , Lloyd J. Old and Robert D. Schreiber
Licensed content date	Nov 1, 2002
Type of Use	reuse in a thesis/dissertation
Volume number	3
Issue number	11
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979470922379
License date	Aug 31, 2012
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Drug Discovery
Licensed content title	Strategies in the design of nanoparticles for therapeutic applications
Licensed content author	Robby A. Petros and Joseph M. DeSimone
Licensed content date	Jul 9, 2010
Type of Use	reuse in a thesis/dissertation
Volume number	9
Issue number	8
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Total	0.00 USD



Permission to use Expert Reviews Ltd copyright material

Request from:

- Contact name: Gizem Tinçer
- Publisher/company name:
- Address:
- Telephone/e-mail: gizemtin@gmail.com

Request details:

- Request to use the following content: Table 2 - Expert Review of Vaccines, Feb 2012, Vol. 11, No. 2, Pages 237-256.
- In the following publication: Thesis
- In what media (print/electronic/print & electronic): Print and Electronic
- In the following languages: All

We, Expert Reviews Ltd, grant permission to reuse the material specified above within the publication specified above.

Notes and conditions:

1. This permission is granted free of charge, for one-time use only.
2. Expert Reviews Ltd grant the publisher non-exclusive world rights to publish the content in the publication/website specified above.
3. Expert Reviews Ltd retains copyright ownership of the content.
4. Permission is granted on a one-time basis only. Separate permission is required for any further use or edition.
5. The publisher will make due acknowledgement of the original publication wherever they republish the content: citing the author, content title, publication name and Expert Reviews Ltd as the original publisher.
6. The publisher will not amend, abridge, or otherwise change the content without authorization from Expert Reviews Ltd.
7. Permission does not include any copyrighted material from other sources that may be incorporated within the content.
8. Failure to comply with the conditions above will result in immediate revocation of the permission here granted.

Date: 03/09/2012.....

Permission to use Expert Reviews Ltd copyright material

Request from:

- Contact name: Gizem Tinger
- Publisher/company name:
- Address:
- Telephone/e-mail: gizemtin@gmail.com

Request details:

- Request to use the following content: Table 2 - Expert Review of Vaccines, April 2011, Vol. 10, No. 4, Pages 499-511
- In the following publication: Thesis
- In what media (print/electronic/print & electronic): Print and Electronic
- In the following languages: All

We, Expert Reviews Ltd, grant permission to reuse the material specified above within the publication specified above.

Notes and conditions:

1. This permission is granted free of charge, for one-time use only.
2. Expert Reviews Ltd grant the publisher non-exclusive world rights to publish the content in the publication/website specified above.
3. Expert Reviews Ltd retains copyright ownership of the content.
4. Permission is granted on a one-time basis only. Separate permission is required for any further use or edition.
5. The publisher will make due acknowledgement of the original publication wherever they republish the content: citing the author, content title, publication name and Expert Reviews Ltd as the original publisher.
6. The publisher will not amend, abridge, or otherwise change the content without authorization from Expert Reviews Ltd.
7. Permission does not include any copyrighted material from other sources that may be incorporated within the content.
8. Failure to comply with the conditions above will result in immediate revocation of the permission here granted.

Date: 03/09/2012.....



RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	2979490688529
License date	Aug 31, 2012
Licensed content publisher	Elsevier
Licensed content publication	The Lancet Infectious Diseases
Licensed content title	Targeting of Toll-like receptors: a decade of progress in combating infectious diseases
Licensed content author	Mona Hedayat, Mihai G Netea, Nima Rezaei
Licensed content date	September 2011
Licensed content volume number	11
Licensed content issue number	9
Number of pages	11
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Sep 2012
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD



RightsLink®

[Home](#)

[Account Info](#)

[Help](#)



Title: Advances in avian immunology
—prospects for disease control:
a review

Author: Pete Kaiser

Publication: Avian Pathology

Publisher: Taylor & Francis

Date: Oct 1, 2010

Copyright © 2010 Taylor & Francis

Logged in as:

Gizem Tincer

Account #:
3000330566

[LOGOUT](#)

Thesis/Dissertation Reuse Request

Taylor & Francis is pleased to offer reuses of its content for a thesis or dissertation free of charge contingent on resubmission of permission request if work is published.

[BACK](#)

[CLOSE WINDOW](#)

Copyright © 2012 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#).
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

The Journal of Poultry Science

Japan Poultry Science Association

c/o National Institute of Livestock and Grassland Science, 2 Ikenodai Tsukuba Ibaraki 305-0901, Japan

TEL&FAX +81-29-838-8777 E-mail: jpsa-s@naro.affrc.go.jp

Web site address: <http://jpn-psa.jp/english>

December 10, 2012

Mrs. Gizem TiNCER,
Department of Molecular Biology and Genetics,
Faculty of Science,
Bilkent University

Permission to reprint

The editorial board of the Journal of Poultry Science hereby grants right to
Mrs. Gizem TiNCER to use Table 1 appeared in:

Immunotherapeutic Potential of CpG Oligonucleotides in Chickens

Arshud Dar, Brenda Allan, Susantha Gomis, Andrew Potter and George Mutwiri

The Journal of Poultry Science Vol 46: 69-80, 2009.

to be cited in the PhD thesis:

“Immunostimulatory Mechanisms and Immunotherapeutic Applications of
Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands”

Permission granted by:



Dr. Atsushi Tajima
Editor-in-Chief
The Journal of Poultry Science





RightsLink®

[My Orders](#) > [Orders](#) > [All Orders](#)

License Details

This is a License Agreement between Gizem Tincer ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the [payment terms and conditions](#).

[Get the printable license.](#)

License Number	3042650725812
License date	Dec 05, 2012
Licensed content publisher	Elsevier
Licensed content publication	Biomaterials
Licensed content title	Immunostimulatory activity of polysaccharide-poly(L:G) nanoparticles
Licensed content author	Gizem Tincer, Seda Yerlikaya, Fuat C. Yagci, Tamer Kahraman, Osman M. Atanur, Oktay Erbatur, Ihsan Gurses
Licensed content date	June 2011
Licensed content volume number	32
Licensed content issue number	18
Number of pages	8
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	4
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	None
Title of your thesis/dissertation	Immunostimulatory Mechanisms and Immunotherapeutic Applications of Polysaccharide Nanocarrier Complexed with Nucleic Acid Based TLR Ligands
Expected completion date	Jan 2013
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD
Total	0.00 USD
