REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

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ANKARA
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APPROVAL PAGE

Effect of Calcium Carbonate Encapsulation on the Oral Activity of CpG Oligodeoxynucleotides and Assesment of the Response of Human Monocytes to TLR Ligands

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This thesis study has been approved and accepted as a PhD dissertation in "Tumor Biology and Immunology program " by the assesment committee, whose members are listed below, on 12 March 2018.

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This dissertation has been approved by the above committee in conformity tothe relatedissues of Hacettepe University Graduate Education and Examination Regulation.

Institute Manager 13 Mart 2018

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YAYIMLAMA VE FİKRİ MÜLKİYET HAKLARI BEYANI

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with Dicle Güç, M.D., Ph.D. and Dennis Klinman, M.D., Ph.D. and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences .

Neslihan Kayraklıoğlu

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my advisors Prof. Dr. Dicle Güç and Dr. Dennis M. Klinman for their care, support, trust, patience and guidance. I am grateful for all the knowledge they shared and the values they taught. I cannot thank them enough for helping me to become a scientist and to develop as a person.

I feel lucky to be a part of the MD/PhD program and Basic Oncology Department at Hacettepe University. I would like to express my gratitude to the members of my PhD thesis committee, Prof. Dr. İhsan Gürsel and Prof. Dr. Lale Doğan for their support and feedbacks. I cannot thank enough to Betül Gök for being so eager to help. I would also like to express my heartfelt thanks to Assoc. Dr. Aytekin Akyol for broadening my perspective on life and science, Dr. Stuart Yuspa and Dr. Aykut Üren for their support and encouragement at the very beginning of this journey.

I am really grateful to members of the Klinman group: Begüm Han Horuluoğlu, Emilie Goguet, Defne Bayik and Debra Tross for their friendship, support, share of knowledge and help. I would like to specially thank Kathy Parker, who helped me develop my lab skills at the very beginning and contributed to a part of this thesis with Julia Scheiermann. I am grateful to Roberta Matthai and Kathleen Noer for all the sorting that they have done, to the NIH Blood Bank staff for the samples they provided and to NCI Frederick animal care staff for their help and guidance.

Last but not least, I want to thank my family for their life-long support, encouragement and sacrifice. I would like to express my deepest love to my parents Suna and Nihat; also to my sisters Nihan and Aslıhan. I would like to express my heartfelt gratitude to Engin Kayraklıoğlu. Without his constant love and patience, I couldn't have completed this thesis. I would like to dedicate this thesis to him.

I was financially supported by NIH as part of the NIH-Hacettepe University individual Graduate Partnership Program (GPP) was supported by the NIH intramural funds.

ABSTRACT

Kayraklıoğlu, N. Effect of calcium carbonate encapsulation on the oral activity of CpG oligodeoxynucleotides and assessment of the response of human monocytes to TLR ligands. Hacettepe University Institute of Health Sciences, Ph.D. Thesis in Tumor Biology and Immunology, Ankara, 2018. Toll-like receptor (TLR) ligands modulate immune responses and have been used in immunotherapy. Clinical trials designed to evaluate the utility of CpG ODN, a synthetic TLR9 ligand, relied on parenteral routes of administration. Therapy would be simplified if these ODN could be delivered orally. My thesis compared the activity of orally delivered free and calcium carbonate encapsulated CpG ODN in murine models of acute colitis, LPS-induced inflammation and *L. monocytogenes* infection in comparison with parenteral CpG ODN. Results indicate that orally delivered CpG ODN induce gastrointestinal rather than systemic immunity and that calcium carbonate encapsulation does not significantly alter this behavior. The TLR7/8 agonist R848 was identified as a more effective oral immune modifier for protection from enteric and systemic *L. monocytogenes* infection. The differential response of human monocytes to TLR7 and TLR8 agonists was investigated. While both agonists induced monocytes to differentiate into macrophages, their activity was somewhat different. TLR8 but not TLR7 stimulation led to the production of pro-inflammatory cytokines. TLR7 ligation blocked TLR8 but not TLR2 or 4 cytokine responses in a dose and time dependent manner. TLR2 and 4 ligations also blocked TLR8 dependent cytokine responses. The effects of complex TLR ligand stimulation on monocytes was investigated using heat-killed bacteria. *L. monocytogenes* and *E. coli* stimulation induced monocytes to differentiate into suppressive macrophages characterized by high IL-10 secretion that reduced pro-inflammatory cytokine induction by *L. rhamnosus*. Efforts are underway to identify the suppressive molecule(s) present in *E. coli* lipid and protein extracts that could be of therapeutic value.

Keywords: Oral delivery, TLR interactions, monocyte-derived macrophages

ÖZET

Kayraklıoğlu, N. Kalsiyum karbonat kapsüllemesinin CpG oligodeoksinükleotidlerin oral etkinliği üzerine etkisi ve insan monositlerinin TLR ligandlarına yanıtının değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Tümör Biyolojisi ve İmmünolojisi Doktora Tezi, Ankara, 2018. Toll benzeri reseptör (TLR) ligandları, immün yanıtları modüle eder ve immünoterapi olarak kullanılmaktadırlar. Sentetik TLR9 ligandı, CpG ODN'nin terapötik kullanımını değerlendirmek üzere tasarlanan klinik çalışmalar, parenteral yolla uygulanmalarına dayanır. Ancak oral yolla tedavi gerçekleştirilebilirse uygulama kolaylaşacaktır. Benim tezimde, oral yolla verilen serbest ve kalsiyum karbonat ile kapsüllenmiş CpG ODN'nin etkinliği, parenteral olarak uygulanan CpG ODN ile karşılaştırılmalı olarak; akut kolit, LPS ile tetiklenmiş inflamasyon ve *L. monocytogenes* enfeksiyon modellerinde araştırılmıştır. Sonuçlar oral yolla verilen CpG ODN'nin sistemik bağışıklıktansa, gastrointestinal bağışıklığı indüklediğini ve kalsiyum karbonat kapsüllemesinin bu davranışı önemli ölçüde değiştirmediğini göstermiştir. TLR7 / 8 ligandı R848, enterik ve sistemik *L. monocytogenes* enfeksiyonundan korunmak için daha etkili bir oral immün modülator olarak tanımlanmıştır. TLR7 ve TLR8 yanıtları spesifik agonistler kullanılarak insan monositleri üzerinde incelendi. Her iki agonist de, monositten makrofaja farklılaşmayı indüklerken, sadece TLR8 agonisti pro-inflamatuvar sitokin salınımına neden olabilmiştir. TLR7 ligasyonu, doza ve zamana bağlı olarak TLR8 sitokin yanıtını bloke ederken TLR2 ve 4'e etki etmedi. TLR2 ve 4 ligasyonları da sonraki TLR8 sitokin yanıtını engelledi. Kompleks TLR ligand uyarımlarının monositlere etkileri ısı ile öldürülmüş bakteriler kullanılarak araştırıldı. Monositlerin *L. monocytogenes* ve *E. coli* ile uyarımı, *L. rhamnosus* tarafından indüklenen pro-inflamatuvar sitokinleri azaltabilen, yüksek IL-10 salınımlı süpresif makrofajlara farklılaşmalarına neden oldu. Terapötik kullanim potansiyeline sahip olabilecek *E. coli* lipid ve protein ekstraktlarında bulunan süpresif molekül(ler)in tanımlanması için çalışmalar devam etmektedir.

Anahtar Kelimeler: Oral uygulanım, TLR etkileşimleri, monosit-kökenli makrofaj

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LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
Ag	Antigen
ACUC	Animal Care and Use Committee
AMP	Adenosine monophosphate
AP-1	Activator protein 1
APC	Antigen presenting cell
Arg1	Arginase 1
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BSA	Bovine serum albumin
Ca	Calcium
CaCl	Calcium chloride
cAMP	Cyclic AMP
CaP	Calcium phosphate
CARD	Caspase activation and recruitment domain
CBER	Center for Biologics Evaluation and Research
CD	Cluster of differentiation
CFU	Colony forming unit
CLR	C-type lectin receptors
cMOP	Common monocyte progenitor
CpG	Unmethylated cytosine-guanosine motifs
CREB	Cyclic AMP-responsive element binding protein

CSF	Colony stimulating factor
CT	Cholera toxin
CTL	Cytotoxic T cells
CCL/CXCL	CXC-chemokine ligand
DAI	Disease activity index
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DSS	Dextran sodium sulphate
EC	*E. coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	Enzyme linked-immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	US Food and Drug Administration
FDG	18-Fluro-deoxy-Glucose

FITC	Fluorescein isothiocyanate
FIZZ	Found in inflammatory zone 1
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GIS	Gastrointestinal system
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GSK	Glycogen synthase kinase
HEK 293	Human embryonic kidney cells
HLA-DR	Human leukocyte antigen – antigen D related
HMGB1	High mobility group box 1 protein
HPLC	High-performance liquid chromatography
Hsp	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
i.g	Intragastric
IL	Interleukin
i.m	Intramuscular
IMQ	Imiquimod
IMZ	Imidazoquinoline
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
IRM	Immune response modifier

i.v	Intravenous
IP-10	Interferon gamma-induced protein 10
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
IκB	Inhibitor kappa B
IKK	Inhibitor kappa B kinase
JAK	Janus Kinase
JNK	C-Jun N-terminal kinase
LB	Lysogeny broth
LBP	LPS binding protein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LM	*L. monocytogenes*
LN	Lymph node
LR	*L. rhamnosus*
LRR	Leucine-rich repeats
LTA	Lipotheicoic Acid
mAb	Monoclonal antibody
MALT	Mucosa-associated lymphoid tissue
M-CSF	Macrophage colony stimulating factor-1
mDC	Myeloid dendritic cell
MeOH	Methanol
MFI	Mean fluorescence intensity
MAPK	Mitogen activated protein
MD-2	Myeloid differentiation factor 2
MDA5	Melanoma Differentiation-Associated protein 5

MDP	Macrophage and DC precursor
MDSC	Myeloid-derived suppressor cells
MHC	Major Histocompatibility Complex
MIP1α	Macrophage Inflammatory Protein 1 alpha
MLN	Mesenteric lymph nodes
MMP	Matrix metalloprotease
MMR	Macrophage mannose receptor
MMTV	Mouse mammary tumor virus
moDC	Monocyte-derived dendritic cells
MPLA	Monophosphoryl lipid A
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MTBE	Methyl tert-butyl ether
MyD88	Myeloid differentiation primary response gene 88
NCI	National Cancer Institute
NF-κB	Nuclear factor-kappa B
NCI	National Cancer Institute
NK	Natural killer cell
Nle	Non-LEE-encoded
NLR	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding Oligomerization Domain
ODN	Oligodeoxynucleotide
ODNcap	Calcium carbonate encapsulated ODN
OVA	Ovalbumin

PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PGE	Prostaglandin
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
p.o	Per oral
poly(I:C)	Polyinosinic-polycytidylic acid
PNPP	p-nitrophenyl phosphate
PO-	Phosphodiester backbone
PP	Peyer's patches
PRRs	Pattern recognition receptors
PS-	Phosphorothioate backbone
PSA	Polysaccharide A
RIG	Retinoic acid-inducible gene
RLR	RIG-I-Like Receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RPS	Ribosomal Protein S
RSV	respiratory syncytial virus
s.c.	Subcutaneous
siRNA	Small interfering RNA
SOCS	Suppressive of cytokine signaling

SR	Scavenger receptor
ssDNA	Single stranded DNA
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription
Sup	Suppressive
TAK	TGF-β activated kinase
TAM	Tumor associated macrophage
TBK	TANK-binding kinase
TCR	T-cell receptor
Th	T-helper
TIR	Toll/IL-1 receptor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing IFN-β
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor
VLP	Virus-like particle

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1. INTRODUCTION

TLR ligands modulate immune responses and have great potential for treating immune related conditions including cancer, infection, autoimmunity and auto-inflammatory diseases (1, 2). Therefore their effects on the immune system should be carefully studied. General motivations of this thesis are to; i) better understand the effects TLR agonists on local and systemic immune regulation ii) improve the immune response initiated with TLR agonists by different delivery methods and combination strategies and, iii) clarify the response of human monocyte to specific TLR agonists alone and in combination.

CpG ODN have been investigated extensively in pre-clinical and clinical studies. (3, 4). The therapeutic use of CpG ODN would be enhanced and simplified if it they could be delivered orally. One of the specific aims of this thesis was to investigate the effects of orally delivered CpG ODN on immunity and determine whether calcium carbonate encapsulation would improve their local and systemic activity. Initial studies were done to understand the behavior of calcium carbonate encapsulated CpG ODN *in vitro*. The activity of orally administered free and encapsulated CpG ODN ODN was evaluated in murine models of acute colitis, LPS-induced inflammation and *L. monocytogenes* infection models in comparison with intraperitoneally administered CpG ODN. To improve immune activation, oral CpG ODN was combined with different TLR7/8 agonists. Effects of orally delivered TLR7/8 agonist, R848, were further investigated in enteric and systemic *L. monocytogenes* challenge models.

TLR ligands activate monocytes and stimulates them to differentiate into macrophages (5). Differential effects of TLR7 and 8 ligands on the immunity was being revealed as specific ligands became available (6). Monocytes as an important part of the mononuclear phagocyte system, express both receptors however, effects of the TLR7 or 8 specific ligands on these cells was not known. The second specific aim of this thesis was to evaluate and compare the effects of TLR7 and TLR8 ligands on monocyte activation and differentiation into macrophages. The cytokine response of the monocytes was evaluated and then the phenotype and function of the resultant macrophages were compared.

The third aim was to understand the effect of TLR agonist combinations and the ability of TLRs to regulate one another. This element of the thesis focused on the combination of TLR7 and TLR8 ligands in human monocytes in a time and dose dependent manner. TLR2 and TLR4 agonist combinations were compared with TLR7 and TLR8 agonists. The effects of complex mixtures of TLR ligands (those expressed by various heat-killed bacteria) on monocytes was also investigated. This included assessment of monocyte cytokine production and differentiation into macrophage of different phenotypes and functions. Heat killed *E. coli* was found to induce a suppressive phenotype and function among macrophages in an LPS independent manner. To identify what was driving this effect, lipid extracts derived from *E. coli* were evaluated on human monocytes.

2. GENERAL INFORMATION

2.1. An Overview Of The Immune System

Throughout the evolution, organisms face the threat of infections that can invade their cells and lead to the end of their life (7). In addition to physical barriers such as the skin and epithelial surfaces of the gastrointestinal, genitourinary and respiratory tract, a more sophisticated defense system was needed to protect the life of the organism. The immune system evolved to protect the "self" and ensure continuity of the species (8).

The immune system is generally categorized into two major parts: innate (or non-specific) and adaptive (or specific/acquired). The innate immune system evolved first, about 700 million years ago (7). Today, it represents the only immune system in lower level organisms such as plants and invertebrates. While highly effective against initial infection, the innate immune defense does not provide long-term memory. Thus, repeated exposure to the same pathogen triggered an innate immune response that started 'from scratch' with the recognition of the pathogen by the receptors, then phagocytosis and secretion of anti-microbial peptides and cytokines. This process could harm surrounding self tissues because the released cytokines and the non-specificity of innate receptors sometimes resulted in the recognition of and damage to self (9). Perhaps in response to persistent infection, the adaptive immune system evolved about 450 million years ago with the emergence of the first jawed vertebrates (7). Components of adaptive immunity provide for long-term memory. Moreover, their receptors are not fixed in the genome, meaning that each cell go through a rearrangement process that made them unique to a specific antigen (10, 11). This makes the response of the adaptive immunity slow, but precise and highly effective.

The innate and adaptive immune systems work collaboratively to provide optimal protection to "self" (12). Adaptive immunity relies on the availability of antigen to trigger a response. Antigen recognition can be accomplished via the recognition of an extracellular microbe by antibodies (humoral immunity) produced by B cells . The resultant antigen-antibody complex can result in i) neutralization of the microbe, ii) opsonization and phagocytosis by the innate immune system cells, iii)

direct killing, and/or iv) activation of the complement system to promote phagocytosis, inflammation and lysis of the microbe (11, 13). This process can proceed quickly if specific antibodies are already present in the serum (due to previous exposure to the pathogen). In some cases, however, the microbe invades into cells and proliferates in an environment shielded from circulating antibodies. To handle this threat, all nucleated cells are capable of breaking down foreign antigens and presenting them on their cell surface in association with Major Histocompatibility Complex I (MHC-I) receptors. Recognition of these complexes allows recognition and killing of infected cells by cytotoxic T lymphocytes (CTL) (11).

Optimizing the host response to rapidly proliferating microbe involves a melding of the innate and adaptive arms of the immune system. Cells of the innate immunity rapidly recognize and phagocytize microbes. This not only helps eliminate dangerous pathogens but provides immune cells with a source of antigens from the pathogen that can be presented in the context of Major Histocompatibility Complex II (MHC-II) receptors (11). Cells that are particularly effective in this role are called professional antigen presenting cells (APC), which include dendritic cells, macrophages and B cells. APCs are a vital connection between innate and adaptive immunity. In addition to antigen presentation, they also express co-stimulatory signals that promote the proliferation and differentiation of cells in the adaptive immune system. This supports a feed-forward mechanism that substantially improves the overall immune response. In turn, adaptive immunity fine tunes the innate immune response, protecting self from the further damage that could result from continuous/non-specific inflammation (11).

2.2. The Innate Immune System

The innate immune system provides a critical line of defense against invasive pathogens. It has four main components; physical barriers, cells, circulating recognition proteins and cytokines/chemokines (11).

Physical barriers can be considered the first line of defense. This includes the skin and the mucosal epithelial cells. Although they are classified as physical barriers,

their role in defense is not just being a passive barrier, they actively secrete antimicrobial peptides such as defensins and cathelicidins (11).

Cells of the innate immune system fall into a few broad categories, including phagocytes, dendritic cells and innate lymphoid cells (11). Phagocytes are the first line of defense against the organisms that breach the epithelial barriers. Their duties include recognizing and ingesting microbes and recruiting other cells to the site of infection by releasing cytokines and chemokines (14). Neutrophils respond to these signals and reach the infection site within a few hours where they start ingesting microbes followed by releasing granules containing digestive enzymes (15). Macrophages are the most efficient phagocytes. They can produce reactive oxygen and nitrogen species that aid in the digestion of microbes (16). They also produce large amounts of inflammatory cytokines that support induction of an adaptive immune response (17). Finally, they present antigens to T cells, a function that will be discussed in detail later in this chapter.

Dendritic cells (DC) are subdivided into two major categories; plasmacytoid dendritic cells (pDC) and conventional dendritic cells (cDC) (18). pDCs normally express low amounts of MHC II protein but once they are stimulated by exposure to foreign nucleic acids they secrete large amounts of interferons and become capable of antigen presentation (19). cDCs are the most important antigen presenters and play a key role in linking innate and adaptive immunity. Unlike macrophages, after the engulfment of the microbe, DCs degrade their cargo slowly and can control lysosomal degradation to preserve peptides for T cell recognition (20). Activated DCs then migrate to the lymph nodes to interact with the T cells and present these microbial peptides loaded onto MHC I or MHC II (20).

Innate lymphoid cells (ILC) are the most recently identified group of cells of the innate immune system. They are originated from common lymphoid progenitor but unlike T and B lymphocytes, they don’t express RAG gene therefore they are not antigen specific (21). Rather than lymphoid tissues, ILCs reside mostly in the parenchymal tissues and contribute to the immune regulation, especially at the mucosal surfaces (22). ILCs are classified into 3 categories depending on their cytokine profile and the transcription factors required during the development. ILC1

are charachterized with high IFNγ and TNFα secretion (23). They contribute to protection against intracellular pathogens. ILC2 produce type 2 cytokines such as IL-4, IL-5, IL-13, capable of activating eosinophils, macrophages and smooth muscle of the intestine. They are important for resistance against helmints (22). ILC3 cells produce IL-17, IL-22 and TNFα, induce antimicrobial peptides therefore contribute to immunity against extracellular pathogens (22). Natural killer (NK) cells are the oldest known ILC discovered in 1975 (24). Similar to ILC1, they produce high amounts of IFNγ (25). They are capable of recognizing infected or distressed cells and killing them by releasing granzymes into the cytoplasm of the target (26).

The innate immune system produces soluble recognition proteins that include natural antibodies, components of the complement system, pentraxins, collectins and ficolins (11, 27). These molecules recognize microbial ligands and enhance their clearance. The complement system is composed of several plasma proteins that are activated in sequence after recognition of the microbe both as alone or antibody-bound. The resulting complement cascade culminates in a membrane attack complex forming on the microbial surface which leads to its lysis (28).

Cytokines have been described as “the language of immune cells” as many of the stimulatory, regulatory and communication functions among immune cells are mediated through them (29). Chemokines are chemoattractant cytokines that play major roles in recruiting, trafficking, development and maintenance of immune cells (30). **Table 2.1** summarizes the functions and characteristics of cytokines of the innate immune system.

Table 2.1. Cytokines of the innate immunity (Modified from Abbas *et al*., 2012 (11)).

Cytokines	Principal cell source	Biologic effects
TNF-α	Macrophages, DC, T cells	Local inflammation, neutrophil activation, apoptosis induction, tumor growth inhibition.
IL-1	Macrophages, endothelial cells, some epithelial cells	Endothelial celactivation (inflammation, coagulation); Hypothalamus: fever Liver: synthesis of acute phase proteins
Chemokines	Macrophages, endothelial cells, T cells, fibroblasts, platelets	Chemotaxis, activation, migration of leukocytes into tissues
IL-12	Macrophages, dendritic cells	Activation of NK cells, induction of T_H1 differentiation and cytotoxicity.
IFN-α/β)	IFN-α: macrophages IFN-β: fibroblasts	Antiviral state, increased Class I MHC expression, NK cell activation
IL-10	Macrophages, T cells (mainly regulatory T cells)	Inhibition of IL-12 production by macrophages and DC, suppression of T cell responses.
IL-6	Macrophages, endothelial cells, T cells	Proliferation of antibody-producing cells by B cells, T and B cell growth an differentiation.
IL-15	Macrophages, others	CD8 memory T cell homeostasis, stimulation and proliferation of NK and NK T cells
IL-18	Macrophages	IFNγ synthesis by NK cells and T cells
IL-23	Macrophages, dendritic cells	Maintenance of IL-17 producing T cells
IL-27	Macrophages, dendritic cells	Th1 differentiation, IFNγ synthesis by NK cells

DC: Dendritic cells, NK: Natural killer cells

2.3. Pattern Recognition Receptors

Cells of the innate immune system use receptors known as 'Pattern Recognition Receptors' (PRRs) to recognize microbes. PRRs bind to evolutionary conserved 'Pathogen-Associated Molecular Patterns' (PAMPs) that are expressed by many different microorganisms. PAMPs include molecules on the surface of bacteria (such as cell wall or membrane components) or in its cytoplasm (such as single stranded or double stranded RNA or DNA molecules). This makes it possible for innate immune cells to recognize multiples types of microorganism without the need for receptors specific/unique to any given pathogen. PRRs are germline encoded, non-clonal and highly conserved among various species (31).

PRRs can also recognize damage-associated molecular patterns (DAMP) that are products of stressed, damaged, necrotic or apoptotic cells. Recognition of DAMPs alerts the immune system about danger to the body and therefore play a crucial role in tissue repair, homeostasis and defense (32).

Similar to PAMPs, PRRs can be found on the surface of the immune cells or within intracellular compartments (i.e. cytosol, endosomal membranes) depending on what is being recognized. Retinoic acid inducible gene-1 (RIG-1) Like Receptors (RLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) are found in the cytoplasm. NLRs recognize viral and bacterial components such as peptidoglycan-derived products, while members RLRs such as RIG-I and MDA5 recognize viral nucleic acids (33, 34).

There are several other types of PRR: C-type lectin receptors (CLRs), scavenger receptors and Toll-Like Receptors (TLRs). All of these are found either on the cell surface or within endosomal membranes. Members if the CLR family such as Dectin-1 Dectin-2, Mannose receptor, DC-SIGN and Mincle recognize a variety of carbohydrate groups on the surface of bacteria and fungi (35, 36). Scavenger receptors constitute a heterogeneous family of receptors capable of recognizing DAMPs such as unmodified endogenous proteins and lipoproteins as well as PAMPs such as bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (37). TLRs are the best-characterized family of the PRRs that consist of 10-12 members (depending on the

species). They recognize a diversity of microbial components including cell wall products, flagellin, dsDNA, ssRNA, and unmethylated CpG DNA (38).

2.3.1. Toll-Like receptors

As one of the most ancient receptors expressed by cells of the innate immune system, Toll like receptors are found in both vertebrates and invertebrates (39). Toll receptors were initially discovered in *Drosophila melanogaster* (40) with mammalian homologs being identified in 1997 (41, 42).

As members of IL-1R family, TLRs contain an leucine-rich ectodomain (LRR) that mediates PAMP recognition, a transmembrane component and Toll-IL-1 receptor (TIR) domains that are intracellular and serve to activate downstream signaling pathways (1). Depending on what PAMP they recognize, TLRs can be located either on the surface membrane or intracellularly.

To date, there are twelve TLR types identified in mice (TLR 1-9 and TLR 11-13) and ten in humans (TLR 1-10). TLR4 was the first characterized human TLR (1997) and initially named hToll. With the discovery of additional TLRs in 1998, the nomenclature was revised (42, 43). TLRs 1, 2, 4, 5, 6 and 10 are located on the surface membrane where they detect various PAMPs on the surface of the microbes. TLRs 3, 7, 8, 9, 11, 12 and13 are located intracellularly within endosomal compartments where they recognize nucleic acids derived from engulfed microbes.

Although TLRs recognize different PAMPs, they use similar signaling pathways to induce the production of pro-inflammatory cytokines and/or Type I Interferons. Two major pathways have been described for TLR signaling; myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF) dependent pathways. Most of the TLRs (1, 2, 4, 5, 6, 7, 8, 9, 13) use MyD88-dependent signaling whereas TLR3 use TRIF-dependent signaling. TLR4 can use both pathways (**Figure 2.1**) (43).

Ligand recognition by TLRs (other than the TLR3) results in recruitment of the IL-1 receptor–associated kinase IRAK4 by the MyD88 adaptor protein (44, 45). IRAK4 then phosphorylates IRAK2 and IRAK1. Phosphorylated IRAK1/2 interacts

with tumor necrosis factor-receptor-associated factor6 (TRAF6) which then activates transforming growth factor-β-activated kinase 1 (TAK1). TAK1 activation leads to the phosphorylation of IκB kinase (IKK) and subsequent recruitment of NF-κB to the nucleus. TAK1 also activates several MAPKs; ERK (extracellular-signal-regulated kinase), JNK (c-jun N-terminal kinase) and p38 which results in recruitment of AP-1 and cyclic AMP-responsive element binding protein (CREB). After recruitment to the nucleus, these transcription factors promote the expression of pro-inflammatory cytokines including IL-6 and TNFα. Activation of TLR 7 and 9 also results in Type-I IFN release through the interaction of IRAK1/2 with TRAF3 instead of TRAF6 which results in activation of Interferon regulatory factor 7 (IRF7) (43, 46, 47).

Ligation of TLR3 (or TLR4) with its ligand results in recruitment of TRAF3 by the adaptor protein, TRIF. TRAF3 activates TANK-binding kinase 1 (TBK1) and IκKε resulting in the phosphorylation of IRF3 and IRF7. Phosphorylated IRF3 and IRF7 translocate to the nucleus and induce type I IFNs and IFN-inducible genes (46).

capability by TLR2 is its ability to form heterodimers with TLR1, TLR6, TLR10 and certain non-TLR molecules (such as CD36, Dectin-1). The TLR2/1 heterodimer recognizes triacyl lipopepdtides (synthetic ligand: Pam3Cys4) whereas TLR2/6 recognizes diacyl lipopeptides (synthetic ligand: Pam2Cys4) (52-56).

TLR4 recognizes LPS present on gram negative bacteria and some other PAMPs including the envelope proteins of respiratory syncytial virus (RSV), mouse mammary tumor virus (MMTV) and pneumolysin from gram positive *S. pneumoniae* (57-59). TLR4 forms a heterodimer with myeloid differentiation factor 2 (MD-2) (58). Recognition of LPS does not involve direct binding. First, LPS binds to the LPS binding protein (LBP) then LBP transfers the LPS onto CD14 which can be either soluble or GPI-anchored. CD14 then presents the LPS to TLR4-MD2 heterodimer (60). Like TLR2, TLR4 also can recognize endogenous DAMPs like hyaluron, fibrinogen, Hsp 60,70 and HMGB-1 (50, 51, 61, 62).

TLR5 recognizes flagellin, a protein that is polymerized to form flagellar filaments which are essential for bacterial motility (63, 64). Flagellin can be found in both gram negative and positive bacteria and its structure is highly conserved (65). Other than motility, flagellin is used for adhesion and invasion by bacteria. Therefore, its recognition by the immune system is crucial (66).

expressed by pDCs and to some extent, monocytes, macrophages and B cells. TLR8 agonist stimulation results in prominent induction of proinflammatory cytokines (TNF, IL-12, MIP-1α) and less IFN-β (74), TLR7 agonist stimulation results in increased IFN-α and IFN-regulated genes (IP-10) from human PBMC (6). Although human and mouse TLRs 1-7 and 9 recognize similar ligands in both species, TLR8 does not (75). Mouse TLR8 was considered to be non-functional, as TLR7 deficient mice do not respond to stimulation with TLR7/8 ligands (76, 77). However HEK 293 cells transfected with mouse TLR8 as well as PBMC from TLR7 -/- mice did respond to stimulation with TLR8 ligands when combined with poly d(T) (78). This response was not observed in MyD88 -/- mice suggesting that mouse TLR8 might be functional. Also, DCs of TLR8 -/- mice overexpressed TLR7 and were hyper-responsive to TLR7 ligand. Finally, TLR8 KO mice developed an autoimmune phenotype whereas dual TLR8/TLR7 KO mice did not, suggesting that mouse TLR8 might have some effect on TLR7 function (79, 80).

TLR9 recognizes ssDNA containing unmethylated CpG motifs (CpG DNA) that are present at high frequency in bacterial and viral genomes (81, 82). TLR9 recognition is dependent on both the sequence and methylation status of the DNA; TLR9 does not recognize unmethylated CpG motifs in which the sequence has been switched to GC or is highly methylated. Thus, mammalian DNA is generally protected from recognition by TLR9 due to the high frequency of methylated CpG dimers (83). Like other intracellular TLRs, TLR9 requires endosomal acidification for activation. Proteolytic cleavage of TLR9 is required for recruitment of the MyD88 adaptor protein (84). Mouse and human cells differ in their expression of TLR9. In mice, B cells, monocytes, macrophages and dendritic cells all express TLR9 whereas in humans only pDCs and B cells do so (3). As a result of TLR9 activation, innate immune responses are enhanced by the production of pro-inflammatory cytokines and IFN-related genes through the activation of NF-κB and IRFs (85, 86). TLR9 stimulation induces Th1-biased adaptive immunity, improves antigen presentation, and adaptive immune responses if administered with an antigen (87-89). Other than CpG DNA (and synthetic CpG oligodeoxynucleotides), TLR9 also recognizes natural hemozoin of plasmodium (90). Although TLR9 recognition was considered to be specific for unmethylated CpG DNA, there us evidence suggesting that TLR9 responds to

profilin found on *Toxoplasma gondii* (97, 98). TLR13 can activate NF-κB and IRF7 upon ligand recognition via recognition of vesicular stomatitis virus (an ssRNA virus) and a ribosomal subunit of bacterial RNA (23S rRNA) (99, 100).

2.4. CpG Oligodeoxynucleotides

CpG oligodeoxynucleotides (CpG ODN) are synthetic oligodeoxynucleotides that contain unmethylated CpG motifs. CpG ODN mimic the activity of the CpG DNA (**Figure 2.4**). CpG ODNs are classified into three main classes depending on their sequence and backbone properties; D (also known as A) type, K (also known as B) type and C type ODNs. Less well-charachterized CpG ODN sequences such as; P-ODN, Y shaped ODN, and X shaped ODN will not be discussed here (101-103).

D type CpG ODN consist of phosphodiester backbone with a central CpG motif flanked by a palindromic sequence and phosphorothioate (PS) linked poly(G) sequences the 3' and 5' ends (104). Because of its mixed molecular structure (palindromes and poly-G tails), D-ODNs form G-tetrads and become aggregated. D-ODNs are recognized at the early endosome and recruit MyD88 to induce IRF7 and NF-κB resulting in secretion of IFN-α from pDCs and IFN-γ from NK cells as well as increased co-stimulatory molecules on pDCs and the B cells. Because of the aggregate formation in solution and resulting batch-to batch variation, D-ODNs were not initially considered suitable for use in clinical trials. However, by packaging D-ODNs into stable virus like particles (VLP), D-ODNs showed strong immunotherapeutic effects in preclinical and clinical studies (105-107).

K-type CpG ODN consist of a fully PS backbone and typically express more than one CpG motif. The PS backbone provides protection from nucleases that enhance *in vivo* half-life. Unlike D-ODNs, they don't form aggregates and aren't retained in the early endosome. Instead they quickly move to the late endosomes and result in robust increase of pro-inflammatory cytokines through activation of NF-κB. K-ODNs are very effective at stimulating splenocytes and PBMCs to secrete TNFα, IL-6, IL-12 and IFN-γ (108-110). K-ODNs, increase the expression of CD69 and CD25 and the production of IgM by B-cells. They induce pDC maturation by increasing co-stimulatory molecules like CD83 and CD40 but do not elicit IFN-α

secretion, unlike the D-ODNs (111). Their ability to activate innate and adaptive immunity was established in various *in vivo* disease models of infection, allergy and cancer (88, 112-115). Clinical trials using K-ODNs for the prevention/treatment of those diseases are ongoing (**Table 2.2**) (4).

Table 2.2. CpG ODNs as vaccine adjuvants (Adopted from Bode *et al.,* 2011 (3)).

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.

2.5. Effect Of Orally Delivered CpG ODN On The Immune System

Parenteral routes of drug delivery have been used in the treatment of many diseases however, the compliance of the patients to parenteral drugs is not as good as orally administered drugs (122). The availability of a drug's oral delivery is particularly important in the treatment of chronic diseases, prophylaxis for healthy people with a known risk, or treatment of early stage disease.

Considering the harsh environment of the gastrointestinal tract such as the strong acidic environment of the stomach (pH 1.5-3.5), bile acids and salts, high amounts of nucleases and other enzymes in the intestines as well as in the liver, CpG ODNs effect could be decreased substantially (122). Results from pharmacokinetic studies with orally delivered antisense ODN show that PS-backboned ODNs undergo extensive degradation in the intestinal tract and liver (123).

There are only few studies investigating the effect of orally administered CpG ODNs in mice. This limited human clinical trials with CpG ODN to the most effective parenteral routes of administration (such as i.v, i.p, sc, and im). However, CpG ODN therapy is most effective when initiated early in the disease process and given repeatedly for a long time (124). Such treatment would be more feasible if ODNs were delivered orally rather than by injection.

Systemically delivered CpG ODN can protect mice from infection, an effect seen with doses as low as 20 μg (112). However, Ray *et al.* showed that mice treated orally with CpG ODN were protected from local or systemic challenge with *L. monocytogenes* when higher doses (100-200 μg) were used (125). Gastric administration of CpG ODN in mice induced the release of CC chemokines, RANTES and MIP-1, and the CXC chemokine, IP-10 thereby increased the infiltration of the immune cells to the murine gastric mucosa which then resulted in reduced *H. pylori* colonization (126).

When co-administered orally with antigen, CpG ODN promoted systemic and mucosal immune responses to both weak (HBsAg) and strong (TT) protein antigens in mice, suggesting that CpG ODN were good candidates for oral vaccine adjuvants

(127). CpG ODNs augmented mucosal (IgA in intestinal washings and feces) as well as systemic (IgG in serum, T-cell proliferation) immune responses against pseudorabies live attenuated virus (PRV) in newborn piglets (128). In those studies, CpG ODN stimulated both Th1 (CTL, IgG2) and Th2 (IgG1, IgA) responses when delivered orally. Oral CpG ODN induced OVA specific local and systemic IgG2 and IFNγ responses in both young and aged mice while Cholera toxin (CT) was not able to do so in aged mice (129). Kitagaki *et al.* reported that oral administration of CpG ODNs alone did not modulate IgG1 or IgG2c however, co-administration of CpG ODN with antigen significantly suppressed antigen-specific IgG1 and enhanced antigen-specific IgG2c production in airway inflammation model and reduced eosinophilic airway inflammation (130).

Those studies show that orally-delivered CpG ODN can induce mucosal immunity and have an effect on systemic immunity when co-delivered with antigen. Further studies are needed to understand the possible mechanisms by which CpG ODN impact local and systemic immunity.

2.6. Calcium Carbonate Encapsulation Method For Oral Delivery Of ODNs

One of the methods for improving the activity of orally delivered synthetic ODNs was described by Wang *et al* (131). This method was originally discovered by Chowdhury *et al.* for improving the efficacy of gene therapies without using viral vectors (132-134). It is a similar technique to previously-described calcium phosphate (CaP) precipitation with the addition of carbonate in the apatite crystal (135). The presence of carbonate prevents the growth of crystals therefore the size of the carbonate-apatite/DNA nanoparticles remains smaller than CaP/DNA (133).

This preparation method is relatively easy and inexpensive. The only required materials are CaCl, DMEM media and ODN. Negatively charged nucleotides easily bind to positively charged Ca to form calcium a carbonate apatite-DNA complex when mixed with a buffered media.

Wang *et. al* was the first to show an immunomodulatory ODN's effect using the calcium carbonate encapsulation method. They named these ODN nanoparticles ODNcap. ODNcap were resistant to incubation with simulated gastric juice (pH:1.7) for 4 hours followed by a gradual degradation through 16 hours, whereas free ODNs were being degraded within 1 hour. Other conditions such as strong alkali (pH:12), autoclaving, or DNAse treatment also did not decrease the stability of the ODNcap (131). The ability of CpG ODNcap to stimulate mouse splenocytes was similar to that of free CpG ODN. Following oral delivery, fluorescent labeled ODNcap was detectable in the macrophage (identified as CD11b+) population of the Peyer's patches while free ODN was not detectable. CpG ODNcap were significantly more effective than free CpG ODNs at inducing IFNγ expression in the Peyer's patches when delivered orally. Furthermore, when a suppressive ODN, iSG3, was encapsulated and given daily for 70 days, that treatment prevented mice from developing chemically-induced atopic dermatitis whereas CpG ODNcap treatment aggravated the disease.

2.7. TLR7/8 Ligands

TLR7 and TLR8 can be activated by viral ssRNAs from influenza A virus, vesicular stomatitis virus, human immunodeficiency virus type 1 and Coxsackie B virus as well as by bacterial ssRNAs (99, 136). TLR8 and TLR7 can also be activated by endogenous RNAs released from dead or dying cells and by short interfering RNAs (siRNAs) (137). Thus, these two receptors can contribute to autoimmune diseases such as systemic lupus erythematosus (138).

The exact nucleotide sequences for TLR7 or TLR8 recognition is not yet known. However several studies show that TLR7 recognizes Guanosine whereas TLR8 recognize Uridine rich ssRNA (139). These two receptors are also activated by certain chemical compounds like imidazoquinolins and their base analogs (140).

TLR8 exists as a pre-formed dimer independent of any ligand (141). Upon ligand binding, conformational changes occur that lead the two TIR domains to come closer to each other, thereby initiating signaling (**Figure 2.5**). TLR8 binds degradation products of ssRNA at two distinct sites; the first site prefers uridine mononucleotide which can induce weak receptor activation alone. The second site binds small

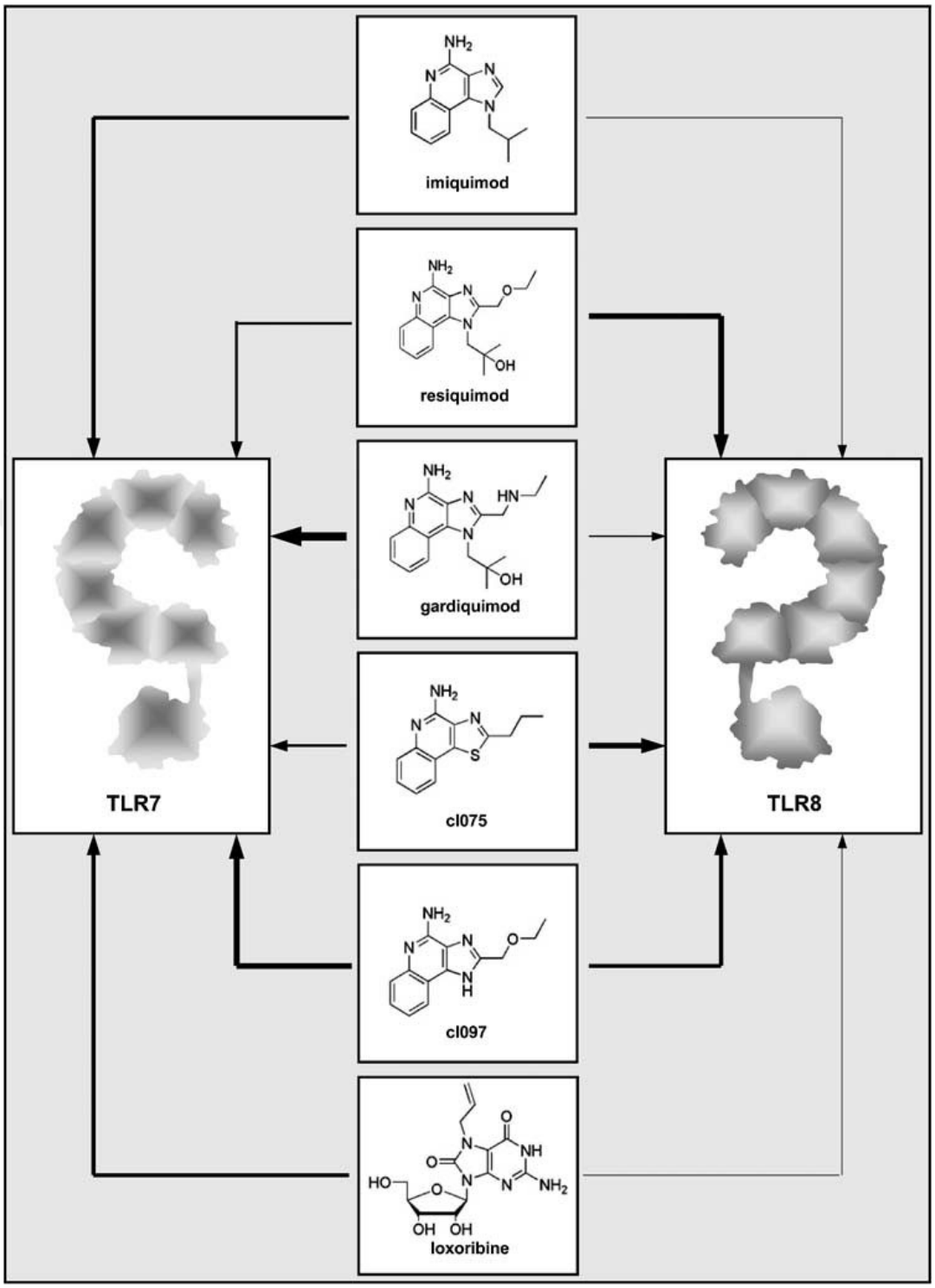

Figure 2.7. Chemical agonists of TLR7 and TLR8 (Adapted from Schön, 2008 (147)).

Various TLR7/8 agonists are undergoing clinical trial for the treatment of asthma, rhinitis, HIV, Hepatitis B and C infections as well as various cancers with or

without combinations with chemo/radiotherapy (US. National Library of Medicine clinical trials database).

2.8. Monocytes

Monocytes are myeloid-derived cells that along with macrophages and dendritic cells constitute the mononuclear phagocyte system (MPS) (149, 150). Cells of the MPS are capable of phagocytosing infectious agents and hence are an important component of the host's innate defense system. They also play a role in tissue homeostasis and support adaptive immune responses (149). Monocytes are an important part of the MPS and they influence the progression of cancer, infection, inflammatory and autoimmune diseases (151). Monocytes have been evolutionarily conserved among vertebrates, constituting ~4% and 10% of the nucleated cells in blood among mice and human respectively (152). Apart from the circulation, considerable numbers of monocytes pool in the spleen and lungs from which they can mobilize as needed (153, 154).

Monocytes are derived from myeloid precursor cells in the fetal liver during embryogenesis and from bone marrow after birth (152, 155). Macrophage and DC precursor cells (MDP) were identified as common progenitors of MPS (152, 155, 156). Common monocyte progenitors (cMOP) were later identified in mice as distinct from MDP based on the absence of CD135 and were shown to give rise to monocytes but not DCs (152). Colony stimulating factor-1 (CSF1 or M-CSF) is required for the development and survival of monocytes, with severe monocytopenia being present in mice deficient in M-CSF or its receptor, CSF-1R (157, 158).

Monocytes derived from cMOP can be divided into two broad categories in mice; $Ly6C^{+}$ $CCR2^{high}$ or classical (further divided into $Ly6C^{high}$ and $Ly6C^{mid}$) and $Ly6C^{low}$ $CX3CR1^{high}$ or non-classical monocytes (159, 160). Classical monocytes have a short half-life (20 hours) and are recruited to sites of inflammation where they differentiate into peripheral mononuclear phagocytes. Non-classical monocytes have a longer half-life (5 days) and their main function is to survey endothelial integrity (152).

Human monocytes are characterized by the expression of different surface markers than found in mice; CD14 (co-receptor for TLR4) and CD16 (FcγRIII). Human monocytes can also be broadly classified into 2 categories; $CD14^{+}$ and $CD14^{low}$. $CD14^{+}$ monocytes are then sub-divided into $CD14^{+}$ $CD16^{-}$ or classical and $CD14^{+}$ $CD16^{+}$ or intermediate monocytes (161, 162). Classical ($CD14^{+}CD16^{-}$) monocytes are the largest subset of monocytes (80-95%) followed by intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{low}$ $CD16^{+}$, constituting 2-8% and 2-11% respectively (163, 164). Similar to mouse classical monocytes, $CD14^{+}$ human monocytes are $CCR2^{high}$ and hence are easily recruited to sites of inflammation where they secrete pro-inflammatory cytokines and differentiate into phagocytes (155). They also replenish resident peripheral monocyte–derived cells under steady-state conditions. Non-classical human monocytes ($CD14^{low}$ $CD16^{+}$) patrol endothelial integrity by crawling on the endothelium mediated by LFA-1 and CX3CL1 and secrete pro-inflammatory cytokines in response to danger signals such as DNA, RNA particles (164-167).

2.9. Macrophages

Élie Metchnikoff, the father of cellular immunology, first discovered cells consuming pathogens and described that event as “phagocytosis”(168). He observed two cells with phagocytic ability and named them microphages (neutrophils) and macrophages, meaning “the big eaters” in Greek (169, 170). Since then, macrophages were described as tissue resident cells that phagocytose pathogens making them a major part of the innate immune system (16). Other than their role in immune defense, macrophages play important roles in tissue homeostasis, the regulation of metabolism, wound healing and remodeling and influence the pathogenesis of certain inflammatory diseases (171-174).

Macrophages were considered to be terminally-differentiated blood monocytes. However recent fate mapping studies in mice revealed that a majority of tissue resident macrophages are derived from the yolk sac or fetal liver (175). Embryo-derived macrophages seed throughout the body and into organs (176). These macrophages have high self-renewal capacity and maintain their frequency under steady state conditions (177, 178). Intestinal macrophages are an exception, being

derived solely from blood monocytes. They have shorter half-life than other tissue resident macrophages (176, 179).

Monocytes are another source of tissue macrophages and are particularly important during inflammatory responses (176). Inflammation rapidly attracts classical monocytes ($Ly6C^{+}$ $CCR2^{high}$) into tissues through the release of chemoattractants (e.g. CCL-2, M-CSF). After extravasation, monocytes respond to various stimuli in the inflamed tissue. Their maturational plasticity makes monocytes well-suited as a source for the rapid replacement of tissue macrophages during inflammation and resolution. Depending on the type of the stimuli, monocytes can differentiate into different types of macrophage having distinct functions (171, 172). Macrophage polarization is a highly dynamic process. Moreover, as these differentiated cells eventually die, they are replaced by new monocytes whose activity may differ, as determined by conditions and factors released at that time. A useful classification distinguishes between two major macrophage populations: M1-like (inflammatory or classical) vs M2-like (suppressive or alternative) macrophages (180).

2.9.1. M1 and M2 Macrophages

M1 and M2 macrophages are at opposite ends of what may actually be a continuum of macrophage phenotypes (181).

The activation of classical macrophages leads to pathogen killing and the release of pro-inflammatory cytokines that attract cytotoxic T lymphocytes (182). Multiple stimuli can induce the activation of classical macrophages (also called M1 polarization) including IFNγ, GM-CSF, TLR and NLR signaling (182). The later agents involve the recognition of intracellular pathogens, bacterial cell wall components, lipoproteins, and TNFα, although the precise contribution of each factor to M1 polarization is unclear and varies in the inflammatory context (183, 184). NF-κB is a major contributor of M1 pathway that induces pro-inflammatory cytokine transcription. STAT1 activation is another major regulator of the M1 pathway, up-regulating the expression of many M1-related genes including iNOS, CXCL9, CXCL10 and CXCL11 (183, 184). IRF5 was found to play a central role in M1 polarization through knock-out studies in which the elimination or IRF5 resulted in a

loss of M1 polarization. IRF5 induces effector cytokines such as IL-12, IL-23 and TNFα (185). M1 macrophages have high iNOS expression leading to the high NO production necessary for pathogen killing (152). M1 macrophages also express high amounts of co-stimulatory molecules such as CD80, CD86 and MHC II indicating their ability to present antigens to T cells although these markers are not specific since they can also be found on M2 macrophages (183, 184).

M2 macrophages were initially identified as alternatively activated macrophages because they functioned differently from classic macrophages. M2 macrophages are regulatory cells that function in wound healing, tissue repair and in suppressing ongoing inflammation that can cause harm to the host if left unchecked (182). M2 differentiation is triggered by signals provided by parasites, fungi, apoptic cells, IL-4, IL-13, PGE2, IL-10, glucocorticoids and M-CSF (183, 184, 186). IL-4 and IL-13 activate the JAK–STAT pathway, leading to the activation of STAT6, which is a major regulator of the M2 pathway together with PPARγ, PPARδ, IRF4 and JMJD3 transcription factors (187). The contribution of JAK/STAT was established in studies of knock-out mice that resulted in a complete loss of M2 polarization (184, 188). PGE2 induces cAMP signaling which activates the CREB pathway which in turn up-regulates IL-10 and Arg1 expression (189). IL-10 activates STAT3 which mediates the inhibition of NO and pro-inflammatory cytokine expression such as TNFα, IL-12, IL-1β and IFNγ (190, 191). Similar to IL-10, glucocorticoids also inhibit pro-inflammatory cytokines by directly interacting with transcription factors such as NF-κB or AP-1 (or their binding to cognate receptors) (192-194). M-CSF stimulation results in downregulation of human leukocyte antigen (HLA) members and increased cell cycle proteins (184, 195). M2 macrophages express less iNOS but more Arginase 1 than M1 macrophages which makes them produce less NO and more L-ornithine thereby they promoting wound healing, suppressing T cell responses and having a reduced ability to kill pathogens (186). Commonly used M2-markers are FIZZ1, Ym1, macrophage mannose receptor (MMR, CD206), C-type lectin receptor DC-SIGN (CD209), scavenger receptors SR-A (CD204) and CD163 (186, 196-201).

2.9.2. Tumor Associated Macrophages

Tumor-associated macrophages (TAM) are a heterogeneous group of cells that reside in the tumor microenvironment (202). The origin of TAMs was thought to be circulating $Ly6G^{+}/CCR2^{+}$ classical monocytes or myeloid derived suppressor cells (MDCSs) recruited into the tumor microenvironment by chemoattractants released by the tumors (such as CCL2, VEGF or M-CSF) (203-206). However, tissue-resident macrophages of embryonic origin were also found to contribute to the TAM pool together with cells recruited from the circulation (152, 207). Their ability to enhance tumor cell proliferation, invasion and metastasis, in part by inhibiting T cell-mediated anti-tumor immune responses and/or stimulating angiogenesis, make them important tumor-promoting cells together with myeloid derived suppressor cells and regulatory T cells (208).

The activation status of TAMs is influenced by stimuli derived from immune or stromal cells in their immediate environment and thus can be highly heterogeneous (209, 210). TAMs can initially contribute to the elimination of cancer cells. Yet as the tumor progresses, it can increase production of TGFβ and CSF-1 causing phenotypic and functional changes that bias TAMs towards M2-like activity (211, 212). Hypoxia is another factor causing TAM heterogeneity with more hypoxic areas containing more M2 like TAMs (213, 214). TAMs in large tumors typically express the pan-macrophage marker CD68, scavenger receptors CD163 and CD204 and mannose receptor CD206. TAMs at the latest stages of cancer express low IL-12, TNFα and NO but high levels of IL-6, IL-10 and TGF-β which inhibit cytotoxic T cell activation, induce the generation/recruitment of Tregs, and support tumor cell survival (180, 215-218). Another mechanism of T cell suppression is through cellular interactions via PD-L1 and PD-L2 immune checkpoint proteins (219). Their amino acid metabolism through Arginase-1 activation causes metabolic starvation of T cells that interferes with effector functions (220). In addition to their effect on the immune milieu, TAMs can release growth factors (e.g. EGF) that promote vascular development and proteolytic enzymes (e.g. MMP9) that digest extracellular matrix thereby facilitate tumor metastasis (221-223). In these ways, TAMs support tumor

involved in the recruitment of monocytes into tumors. Although antibodies targeting CCR2/CCL2 were encouraging in pre-clinical studies, so far results from phase I/II clinical studies with or without chemotherapy was not successful to induce partial or complete remission (231-234). TAMs are an important source of VEGF and other angiogenic factors (211). VEGF both promotes angiogenesis and up-regulates CSF-1R thereby facilitating monocyte recruitment to the tumor (205, 211). Anti-VEGF therapies were found to cause hypoxia that induced a compensatory recruitment of monocytes thereby resulting in treatment resistance in pre-clinical studies (235). However, a dual antibody against VEGF and Angiopoetin-2 showed impressive anti-tumoral activity by converting M2-like TAMs into anti-tumoral M1 macrophages (236). COX1/2 blockade by aspirin can also reduce cancer frequency and metastasis (237). PGE2 is an immunosuppressive molecule that induces mMDSC development and M2 polarization of macrophages (238). Decreasing the amount of PGE2 produced by TAMs may be one of the mechanisms by which aspirin lowers tumor frequency. Other examples of therapies that target TAMs include checkpoint inhibitors that eliminate suppressive functions and trabeculin as an anti-proliferative agent (211).

Rather than depleting the TAMs or blocking their suppressive activity, another approach is to induce the TAMs to become tumoricidal M1-like macrophages. Treatment with recombinant IFNγ, a classical M1-inducing stimulus, improved clinical outcomes in patients with ovarian cancer (239). Various TLR agonists also induced M1 differentiation of TAMs in pre-clinical studies. Treatment with the TLR3 ligand poly I:C in a Lewis lung carcinoma mouse model resulted in tumor regression by converting TAMs to tumoricidal macrophages that produced pro-inflammatory cytokines (240). Intra-tumoral delivery of the TLR7/8 agonist 3M-052 increased the local frequency of M1 macrophages and provided systemic antitumor immunity (241). In another study intratumoral delivery of the TLR7 agonist 1V270 resulted in an increased M1/M2 ratio in the tumor microenvironment (242). The TLR9 agonist CpG ODN in combination with anti-IL-10 antibody triggered a phenotype switch of TAMs from M2 to M1 and rejection of large tumors (243). Agonistic anti-CD40 antibody treatment of mice with pancreatic cancer showed reduced tumor volumes by converting M2 macrophages into M1 based on increased CD86 and MHC class II expression (244). In a phase I study of patients with advanced pancreatic ductal

adenocarcinoma, CD40 agonist treatment in combination with gemcitabine resulted increased pro-inflammatory cytokine levels in the serum and decreased 18-Fluro-deoxy-Glucose (FDG) uptake by the primary tumor (245). Tumor cells express CD47 which prevents them from phagocytosis by interacting macrophage SIRPα (246). Antibodies targeting CD47 induce antibody dependent cellular phagocytosis (ADCP) of tumor cells by TAMs and subsequently their polarization into M1 macrophages (247).

2.10. Effects Of TLR Agonists On Human Monocytes And Macrophages

Monocytes and macrophages help form the first line of defense against pathogens and therefore express wide variety of PRRs. Human monocytes express TLRs 1, 2, 4, 5, 6, 7 and 8 with expression of TLR3 and 9 being induced when monocytes differentiate into dendritic cells *in vitro* (moDC) (248, 249).

As previously discussed, TLR signaling activates NF- κB, a major M1 regulator that induces the release of pro-inflammatory cytokines and IRFs that are also key regulators of the M1 pathway. These induce the release of Type I IFNs which in turn activate STAT1 (187). Regulation of TLR signaling is very important since unregulated immune activation may result in tissue destruction and toxicity. P38α MAPK has an important role in regulating TLR signaling (250). In a feed-back loop, p38α blocks TAK1 activity through phosphorylation of its components (251). P38α MAPK and ERK1/2 phosphorylates CREB and triggers CREB-dependent gene transcription including IL-10 (252). As discussed before, IL-10 is one of the major signals for M2 pathway (253). IL-10 works through JAK family members and activates STAT3 which in turn induces the synthesis of the suppressor of cytokine synthesis 3 protein (SOCS3) (182). Taken together, TLR ligand stimulation results in complex signaling pathways and could result in different monocyte and macrophage responses.

LPS, a gram - bacteria specific PAMP, was one of the first stimuli identified as an M1-inducer as it activates pro-inflammatory mediators. However repeated exposure to LPS generates tolerant macrophages that over-express genes associated with pathogen recognition and clearance but low amounts of pro-inflammatory genes.

This response was attributed to regulation by p38 MAPK signaling (254). Desensitized macrophages also expressed higher levels of the p50 subunit of the NF-κB complex whose homodimerization impairs p65/p50-dependent functions like STAT1 phosphorylation and IFNβ production (255). These studies suggest that LPS could generate both inflammatory and tolerant macrophages.

TLR2 ligands are common in both gram + and – bacteria as well as in fungi, helminths and some virus. This receptor plays a crucial role in innate immune defense as evidenced by increased infection susceptibility in TLR2-/- mice (256, 257). Evidence has accumulated that shown TLR2 stimulated monocytes and macrophages can exercise suppressive functions. *Mycobacterium tuberculosis* LprG stimulation resulted in the inhibition of MHC-II antigen processing macrophages through the TLR2-mediated PI3K/Akt pathway (258, 259). The TLR1/2 ligand Pam3CSK4 induced human monocytes to differentiate into suppressive macrophages that were highly endocytic but had little ability to kill tumor cells or inhibit T cell proliferation or induce Th2 differentiation (260). Another study identified ligand-specific differences in TLR1/2 signaling on monocytes. Human beta defensin-3 (hBD3) an antimicrobial protein that works through TLR1/2 induced expression of the co-stimulatory molecules CD80, CD86 and CD40 by human monocytes whereas Pam3CSK4, another TLR1/2 ligand, down-regulated co-stimulatory receptors by inducing high amounts of IL-10 (261, 262). Failure of IL-10 secretion with hBD3 was attributed to the lack of non-canonical NF-κB pathway involvement (261).

TLR8 stimulation stimulates the production of TNFα, IL-12 and IL-10 by human monocytes and mDCs (6). By comparison, its close relative TLR7 does not cause significant cytokine production by monocytes but instead stimulates pDCs to secrete Type I IFNs (6). A specific monocyte subset, $CD14^{dim}$ $CD16^{+}$ monocytes (intermediate monocytes) have a patrolling duty that is dependent on TLR7 and/or 8 recognitions. TLR7 and 8 ligands both induced significant amounts of pro-inflammatory cytokine production by $CD14^{dim}$ monocytes while other TLR agonists did not (166). The TLR7/8 agonist R848 induced expression of FcγR I and IIa while decreasing the inhibitory receptor FcγR IIb in human classical monocytes and bone marrow derived macrophages. In mice, this process was dependent on TLR7 (263).

TLR8 but not TLR7 agonists induce Granzyme B in human monocytes and improve antibody dependent cellular cytotoxicity (ADCC). Granzyme B induction was further improved by FcγR ligation when combined with TLR8 stimulation suggesting that TLR8 agonists could be combined with FcγR -based therapies of cancer (264).

IFN-γ was shown to synergize with TLR agonists by increasing their expression and enhancing TLR-induced NF-κB activation (265). IFN-γ and TLR agonist combinations improved macrophage tumoricidal activity and production of NO and pro-inflammatory cytokines while decreasing IL-10 (266, 267). Suppression of TLR-mediated IL-10 secretion was through induction of glycogen synthase kinase-3 (GSK3) and decreased MAPK activation which resulted in suppression of IL-10 inducing factors; CREB and AP-1 (266). The combination of IFN-γ with TLR agonists could improve macrophage mediated cancer immune therapy.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Cell Culture Media and Standard Solution Components

RPMI-1640 without L-glutamine (Catalog # 12-167F), FCS (Catalog # 14-502F), 1X PBS (Catalog # 17-516F), 10X PBS (Catalog # 17-516Q), were purchased from Lonza, USA. Sodium Pyruvate (100x; 100 mM; Catalog # 11360-070), L-glutamine (100x; 200 mM; Catalog # 25030-081), HEPES (100x; 1M Catalog # 15630080), Non-essential amino acids solution (100x, Cat# 11140050) and Pen Strep (100x; 5,000 units/ml Penicillin and 5,000 µg/ml Streptomycin; Catalog # 25070-063) were supplied from ThermoFisher Scientific, USA; BSA (Catalog # BSA-50) from Rockland Immunochemicals, USA, and Tween-20 (Catalog # P1379) from Sigma-Aldrich, USA.

3.1.2. Preparation of Complete RPMI Media

In 500 ml RPMI-1640 media; 5 ml HEPES (10 mM final concentration), 5 ml Sodium Pyruvate, (1mM final concentration), 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock), 5 ml L-Glutamine, (2 mM final concentration) added for Complete RPMI preparation (also called RPMI Reg).

- For 2%; 10 ml, for 5%; 25 ml, for 10%; 50 ml of heat-inactivated FCS (56^{o}C for 1 hour) added.

3.1.3. Preparation of Oligo RPMI Media

In 500 ml RPMI-1640 media; 5 ml Pen Strep (Penicillin; 50 U/ml, Streptomycin; 50 ug/ml final concentration), 5 ml HEPES (10 mM final concentration), 5 ml Sodium Pyruvate, (1mM final concentration), 5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x concentrate stock), 5 ml L-Glutamine, (2 mM final concentration) added for Oligo RPMI preparation (also called RPMI-Oligo).

- For 2%; 10 ml, for 5%; 25 ml, for 10%; 50 ml of heat-inactivated FCS (65ºC for 1 hour) added.

3.1.4. Oligonucleotides

All synthetic ODNs used in this thesis were phosphorothioate (PS) backboned and were synthesized at the Center for Biologics Evaluation and Research (CBER) core facility. Nucleotide sequences are shown below:

1555 (15mer) : 5'-GCTAGACGTTAGCGT-3'

1466 (10mer) : 5'-TCAACGTTGA-3'

1612 (15mer) : 5'- GCTAGAGCTTAGGCT-3'

A151 (24mer) : 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'

3.1.5. TLR Ligands

Receptor specificities, catalog numbers, vendors and standard working concentrations of TLR ligands used in stimulation experiments are listed in **Table 3.1.** Concentrations are also mentioned in each experiment figure's legend.

Table 3.1. TLR ligands

Name	Specificity	Catalog #	Company	Working Concentration
PGN-EB	TLR2	tlrl-pgneb	Invivogen (USA)	1 ug/ml
PGN-BS	TLR2	tlrl-pgnb3	Invivogen (USA)	1 ug/ml
Pam3CSK4	TLR1/2	tlrl-pms	Invivogen (USA)	1 ug/ml

Name	Specificity	Catalog #	Company	Working Concentration
LPS-B5 (*E. coli* 055:B5)	TLR4	tlrl-b5lps	Invivogen (USA)	1 µg/ml
LPS-EB-Ultrapure (*E. coli* O111:B4)	TLR4	tlrl-3pelps	Invivogen (USA)	1 µg/ml
R848	TLR7/8	tlrl-r848-5	Invivogen (USA)	3 µg/ml
CL-075	TLR8	tlrl-c75	Invivogen (USA)	300 ng/ml
TL8-056	TLR8	tlrl-tl8506	Invivogen (USA)	100 ng/ml
3M-055*	TLR7	N/A	3M Drug Delivery Systems, USA	3 µM
Imiquimod	TLR7	tlrl-imqs	Invivogen (USA)	1 ug/ml
Gardiquimod	TLR7	tlrl-gdqs	Invivogen (USA)	1 µg/ml
Loxoribine	TLR7	tlrl-lox	Invivogen (USA)	300 µM
CL264	TLR7	tlrl-c264e	Invivogen (USA)	3 µg/ml

Name	Specificity	Catalog #	Company	Working Concentration
CL307	TLR7	tlrl-c307	Invivogen (USA)	1 µg/ml

*3M-055 was a gift from Dr. John Vasilakos.

3.1.6. Heat-Killed Bacteria

Heat-killed bacteria was purchased from Invivogen (USA). **Table 3.2** shows the catalog numbers and concentrations used in this thesis. For lipid extraction studies, bacteria were grown in the lab and was heat-killed as described in the methods section.

Table 3.2. Heat killed bacteria

Name	Cat #	Concentration
E. coli	tlrl-hkeb2	10^8 bacteria/ml culture
L. monocytogenes	tlrl-hklm	10^8 bacteria/ml culture
H. pylori	tlrl-hkhp	10^8 bacteria/ml culture
L. rhamnosus	tlrl-hklr	10^7 bacteria/ml culture
S. typhimurium	tlrl-hkst	10^8 bacteria/ml culture
S. epidermidis	tlrl-hkse	10^7 bacteria/ml culture

3.1.7. Recombinant Cytokines

Human recombinant cytokines were used at a concentration of 50-500 ng/ml. All the reagents shown in **Table 3.3** was purchased from Miltenyi Biotec (Germany).

Table 3.3. Recombinant Cytokines

Name	Catalog #
Human IFNγ, research grade	130-096-873
Human M-CSF, premium grade	130-096-492
Human TNFα, premium grade	130-094-562

3.1.8. Flow Cytometry Reagents and Instruments

Fluorescence-conjugated antibodies used for the flow cytometric analysis of the cells are listed in **Table 3.4**. Dilutions were made in staining buffer (2%BSA in PBS). For endocytosis assays, Dextran, Alexa Fluor 488®, 3,000 MW, Anionic (ThermoFisher Scientific, USA) particles were used. LSR II SORP or Fortessa machines and FACSDiva V8.0.1 (BD Biosciences, USA) software used for data acquisition. Data was analyzed by using FlowJo v10 (BD Biosciences, USA) software.

Table 3.4. Flow Cytometry antibodies

Name	Clone	Catalog #	Company	Dilution
Anti-human Mature Macrophage	eBio25F9 (25F9)	14-0115-82	Thermo Fisher, USA	1:25
Anti-human CD14	MΦP9	560349	BD Pharmingen, USA	1:25
Anti-human CD14	M5E2	301830	Biolegend, USA	1:25

Name	Clone	Catalog #	Company	Dilution
Anti-human HLA-DR	TU36	555561 559868	BD Pharmingen, USA	1:10
Anti-human CD163	GHI/61	333618 333608 333614 333612	Biolegend, USA	1:25
Anti-human CD200R1	380525	FAB3414	R & D Systems	1:10
Anti-human CD206	15-2	321126 321122 321104	Biolegend, USA	1:25
Anti-human CD80	2D10	305218 305232	Biolegend, USA	1:25
Anti-human CD86	IT2.2	305422 305420	Biolegend, USA	1:25
Anti-mouse CD45	30-F11	103106 103138 103139 103132 103128 103126 103116 103114 103108 103112	Biolegend, USA	1:100
Anti-mouse CD3	145-2C11	100312 100330 100328 100320	Biolegend, USA	1:100

Name	Clone	Catalog #	Company	Dilution
Anti-mouse CD19	6D5	115512 115540 115506 115523 115508 115520 115534	Biolegend, USA	1:100
Anti-mouse CD11b	ICRF44	301324 301334 301332 301350	Biolegend, USA	1:100
Anti-mouse F4/80	BM8	123116 123124 123114	Biolegend, USA	1:100
Anti-mouse CD11c	N418	117310 117306 117308	Biolegend, USA	1:100
Anti-mouse IL-6	MP5-20F3	562050	BD Pharmingen	1:100
Anti-mouse IL-12	C17.8	554479	eBioscience	1:100
Anti-mouse TNFα	MP6-XT22	554419 561041	BD Pharmingen	1:100
Anti-mouse IFNγ	XMG1.2	561040 562020	BD Pharmingen	1:100
Anti-mouse IgM	R6-60.2	562033	BD Pharmingen	1:100

3.1.9. ELISA Reagents and Instruments

Immulon 2HB microtiter plates purchased from ThermoFisher Scientific (USA). 1 x PBS supplemented with 0.05% Tween 20 used as washing buffer. All recombinant proteins were purchased from R & D (USA). The specific information of types, clones, brands and working concentrations of ELISA antibodies were shown in **Table 3.5**. Phosphatase-streptavidin was purchased from BD Biosciences (USA) and used 1:5000 diluted in blocking buffer (2% BSA in PBS). PNPP substrate was purchased from Southern Biotech (USA). Spectra Max M5 Microplate Reader, and SoftMax Pro Acquisition and Analysis Software (both Molecular Devices, USA) were used for data acquisition.

Table 3.5. Antibodies used in ELISA

Antigen	Clone	Catalog #	Company	Dilution
Mouse IL-6 coating	MP5-20F3	554400	BD Pharmingen (USA)	1:200
Mouse IL-6 detection	MP5-32C11	554402	BD Pharmingen (USA)	1:500
Mouse IL-12 coating	C15.6	551219	BD Pharmingen (USA)	1:100
Mouse IL-12 detection	C17.8	554476	BD Pharmingen (USA)	1:1000
Mouse TNFα coating	TN3-19.12	MAB410	R & D (USA)	1:100
Mouse TNFα detection	Polyclonal goat IgG	BAF410	R & D (USA)	1:500
Mouse IFNγ coating	N/A	551881 (51-2525KZ)	BD Pharmingen (USA)	1:400
Mouse IFNγ detection	N/A	551881 (51-1818KA)	BD Pharmingen (USA)	1:1000
Human IL-6 coating	6708	MAB206	R & D (USA)	1:200
Human IL-6 detection	Polyclonal goat IgG	BAF206	R & D (USA)	1:1000
Human IL-10 coating	JES3-19F1	554704	BD Pharmingen (USA)	1:200
Human IL-10 detection	12G8	M-011B	Endogen	1:1000
Human TNFα coating	28401	MAB210	R & D (USA)	1:200
Human TNFα detection	Polyclonal goat IgG	BAF210	R & D (USA)	1:1000

N/A: Not available

For detection of Human IL-12, Human IFNα, Human IFNβ, Human IFNγ, Mouse IFNα, Mouse IFNβ, Quantikine ELISA kits from R& D (USA) used according to manufacturer's instructions.

3.2. Methods

3.2.1. Animals

6 to 14-week old wild type adult male or female BALB/c, C57BL/6 mice were housed in NCI-Frederick Animal Facility under controlled ambient conditions (22 ±2 °C) regulated with 12-hour light and 12-hour dark cycles. They were provided with unlimited access of food and water unless otherwise is indicated. All procedures conducted were in compliance with the ethical guidelines for the care and use of laboratory animals and approved by Animal Care and Use Committee (ACUC) of the National Institutes of Health (See **APPENDIX-6** for ethics committee approval documents).

3.2.2. Preparation of the ODNcap

CpG ODNcap were synthesized following the protocol provided by Wang *et al.* (131) shown in **Figure 3.1** by using phosphorothioate ODNs under endotoxin-free conditions. Protocol is described as follows:

- 2 mg ODN was dissolved in 1 ml endotoxin-free water
- 53 ml of 1 M $CaCl_2$ was mixed with ODN under constant stirring and incubated at 37 °C 20'.
- 345 ml hi-glucose DMEM media added into the mixture and incubated at 37 °C 20'.
- After 20' incubation at 37 °C, the solution was centrifuged at 3000 g for 20' and the amount of non-encapsulated ODN measured in the supernatant. Encapsulation efficiency was usually about 60-70%. Non-encapsulated ODN amount subtracted from the initial ODN and the

made on the skin and then peritoneal membrane was exposed by tearing the skin further until abdomen is completely visible. With sterile scissors, a horizontal incision was made on the central part of the peritoneal membrane then two additional incisions made through the left and right sides. Spleen was found on the left side of the mouse, with a sterile plier, spleen was removed carefully with extra attention made for not getting surrounding fat tissue. Harvested spleen was put in a 6-cm petri dish with pre-warmed RPMI-Oligo media supplemented with 2% FCS. Spleen is then mashed on top of a 70 μm cell strainer (Falcon) by using the plunger of a 1 ml syringe. ~10-20 ml media is added on top of the strainer during the mashing process to collect the strained cells into the 50 ml tube. Collected cells were centrifuged at 400 g for 10 minutes. Cells were washed one more time and then counted with KX-21N Automated Hematology Analyzer. Cells then brought to the desired concentration with RPMI-Oligo media supplemented with 5% FCS. Single cell suspensions were cultured at 10^6 cell/ml concentration in 24 well plates (Corning) in 1 ml RPMI-Oligo media supplemented with 5% FCS with addition of the desired stimulants.

3.2.4. Isolation of the mesenteric lymph nodes, appendiceal lymph nodes and Peyer's patches

As explained above for spleen harvesting, abdomen is revealed by cutting the peritoneal membrane. Then the intestines are exposed. Mesenteric lymph nodes (MLN) are found in the connective/fat tissue (mesentery) around the small intestines. They are most visible near the branches of the mesenteric artery, usually 6-8 lymph nodes are collected from each mouse. Peyer's patches (PP) and appendix collection was done after MLN collection because these organs are not found in a sterile environment. First, caecum was located as the most dilated part of the intestines. Appendiceal lymph nodes are visible as white patches on the wall of the caecum. About 2-3 lymph node is collected with attention paid to not get any intestinal contents. PP are located on the wall of the small intestine, most visible around the jejunum as white bulbs. Starting from the jejunum through the duodenum, all the PPs visible were collected (usually about 8-12 PPs/mouse). Again, special attention is paid for not getting the intestinal content. All of these collected lymph tissues of the gastrointestinal tract was put in 6 cm petri dishes filled with pre-warmed RPMI 2%-

Oligo then mashed in top of a 70 μm cell strainer (Falcon) to obtain single cell suspensions as described for splenocyte collection. Collected cells were centrifuged at 400 g for 10 minutes. Cells were washed one more time and then counted with KX-21N Automated Hematology Analyzer. Cells then brought to the desired concentration with RPMI-Oligo media supplemented with 5% FCS to be cultured as described for splenocytes.

3.2.5. Bone Marrow isolation and culture

6-12-week-old BALB/c mice were euthanized by cervical dislocation. Skin of the abdominal wall was soaked in 70% etOH in water. A small horizontal incision was made on the lower half of the abdominal wall skin. Skin was teared by pulling until the legs were exposed completely. Both legs were cut from the sacroiliac joint then surrounding leg muscles were removed using scissors and tissue paper. Femur and tibia cut on the both ends then each bone washed with 10 ml RPMI Oligo supplemented with 2% FCS using a 23 G needle and 10 ml syringe. After completing the volume to 50 ml with media, cells centrifuged at 400 g for 10 minutes. After straining cells through a 70 μm cell strainer (Falcon), cells were washed one more time and then counted with KX-21N Automated Hematology Analyzer. Cells then brought to the desired concentration with RPMI-Oligo media supplemented with 5% FCS. Single cell suspensions were cultured at 10^6 cell/ml concentration in 24 well plates (Corning) in 1 ml RPMI-Oligo media supplemented with 5% FCS with addition of the desired stimulants.

3.2.6. Blood sampling and serum collection from the mice

Blood collection was done from submandibular vein. A 4-5 mm Lancet was punched halfway between the ear and the mandible with enough pressure to produce a small incision. Drops of blood was collected into 1.5 ml mictotubes. Tubes were incubated in 37 C for 20-30 minutes to be clotted, then centrifuged at 13500 rpm for 10 minutes on a tabletop centrifuge. Clear serum was collected into new set of microtubes and stored at -20 C until use.

3.2.7. Staining for Surface and Intracellular markers for flow cytometry analysis

Staining of the human samples were done on the 96 well-plates they were cultured on or in 1.5 ml microtubes. Plates were centrifuged at 400 g for 5 minutes, 1,5 ml microtubes were centrifuged at 5200 rpm for 2 minutes. Cells were then re-suspended in 50 µl staining buffer containing the fluorescent labeled antibodies in the indicated dilutions (as shown on **Table 3.4**) were incubated on 4 °C, at dark for 30 minutes. Samples were washed with 150 – 1000 µl of staining buffer and centrifuged at 400 *g* for 5 minutes. Pellets were resuspended in 50 µl of Fc. Cells were incubated for 15 minutes at room temperature in the dark and then washed with 150 – 1000 µl of staining buffer as previously described.

For intracellular staining, fixed cells were resuspended in 50 µl Permeabilization Buffer B, containing desired fluorescence-conjugated antibodies in the indicated dilutions shown on **Table 3.4**. Cells were incubated for 20 minutes at room temperature in the dark and washed with 150 – 1000 µl of staining buffer as previously described. Finally, cells were resuspended with 200 – 600 µl of staining buffer. To use as control for monocyte stimulation experiments, $5x10^4$-$4x10^5$ HLA-DR^+ monocytes were fixed at Day 0 and then stained simultaneously with the stimulated cells.

For mouse sample staining an additional Fc Blocking step was required in the beginning. Cells were incubated with 100 µl staining buffer containing 1:1000 diluted Fc Block (BD Biosciences, USA) at 4 °C for 10 minutes. After washing, cells were incubated with 50 ul staining buffer containing 1:100 diluted fluorescent-labeled surface antibodies for 30 minutes on ice. Samples were washed as previously described and then incubated with 100 ul of BD fixation/permeabilization solution for 15 minutes at room temperature in the dark for fixation.

If intracellular staining is desired, cells were incubated with 1x BD Perm/Wash buffer for 15 minutes at 4 °C in the dark and then washed with 1x BD Perm/wash buffer once. Permeabilized cells then stained with fluorescent-labeled antibodies for

intracellular antigens/cytokines or appropriate isotype controls diluted in 1x BD Perm/wash buffer. Finally, cells were re-suspended with 300-500 µl staining buffer.

Single stained samples were prepared with UltraBeads (eBioscience, USA) for each fluorophore used in the staining of the samples. Unstained controls were prepared from fixed elutriated monocytes or mouse splenocytes. These controls were used to set Voltages and compensations adjusted by using these single stained beads and unstained cells. Acquired event number was between 1,000-20,000 for human samples and 20,000-100,000 for mouse samples.

3.2.8. *In vivo* administration of CpG ODNcap

10 µg CpG ODNcap in 100 µl of PBS was administered by gastric gavage to BALB/c mice for 3 or 10 consecutive days. As control 100 µl PBS was administered orally to one group of mice. In the end, mice were bled terminally (as explained before) then sacrificed. Peyer's Patches, mesenteric lymph nodes and spleens harvested and single cell suspensions were prepared as described before. Single cell suspensions were cultured in 6 well plates at a density of $5x10^5$ cell/ml in the presence of Brefeldin A for 12 hours in RPMI-Oligo media supplemented with 5% FCS. Cells are detached from the plates by pipetting up and down and collected into 1.5 ml mictrotubes. Cells were then incubated with Fc Block and then stained with fluorescent-labeled anti-CD45 antibody as described before. Cells were fixed, permeabilized then stained with cytokine specific antibodies or proper isotype controls and run on LSR II SORP as described before.

3.2.9. Acute colitis model

9-10-week-old female C57/B6Ncr mice (purchased from Jackson Laboratories, USA) received 2% DSS (MW: 36,000-50,000 kDa, Gojira Fine Chemicals, USA) in drinking water for 8 consecutive days. 25 µg of CpG ODN, CpG ODNcap or PBS as control was administered by gastric gavage in a final volume of 400 µl. Treatments started one day before the DSS initiation given and every other day until the study ended (on day 12). Of note, in this experiment, free CpG ODN was prepared in 1.5% sodium bicarbonate buffer (prepared in PBS) while CpG ODNcap

concentration ~10^9 CFU/ml). For oral challenge studies, more concentrated stocks were needed and prepared as described here: A vial was thawed and streaked onto a BHI agar plates (BD Difco). After an overnight culture (~16 hours) at 37 °C, colonies were visible. One colony is selected and inoculated into 5 ml BHI medium (BD Bacto) in a shaking incubator at 37 °C 180 rpm. After reaching the log phase (in ~3 hours, OD600= 0.5-0.6) 5 ml bacteria media transferred in 100 ml BHI media and growth overnight in a shaking incubator at 37 °C 180 rpm. 100 ml bacteria then transferred into 2 L BHI media and growth until the log phase. Bacteria centrifuged for 30 min at 3000 g then re-suspended with BHI medium with 20% glycerol aiming a concentration of 10^{11} CFU/ml and put in cryopreserve vials. 10 µl from a vial then plated in 10-fold serial dilutions on BHI agar plates to determine the exact concentration/vial.

For heat killing, same growth steps were done but instead of centrifugation, first the bacteria amount is determined by plating on BHI agar plates in 10-fold serial dilutions then, bacteria were autoclaved for 20 min at 121 °C. Centrifugation was done after autoclave step. Killed bacteria pellet then re-suspended in ddH_2O, aiming a concentration of 10^{11} bacteria/ml and put in cryopreserve vials. 100 µl from a vial then plated in 5 BHI agar plates and also 100 µl was inoculated in 5 ml BHI medium to make sure the bacteria are dead. No growth was observed in 7 days.

E. coli: *E. coli* serotype O111:B4 (purchased from ATCC, catalog # 33780) was cultured in LB medium (BD Bacto) and heat-killed as described for *L. monocytogenes*. Frozen heat-killed E. coli vials (100 mg/vial) then sent to the Mass Spectrometry/HPLC core facility (NCI, Frecerick, MD, USA) for lipid extraction.

3.2.14. Bacteria challenge

For oral challenge, *L. monocytogenes* EGD strain was grown as described above. For i.p challenge, frozen stock from the Klinman lab (NCI, Frecerick, MD, USA) used. 8-10-week-old BALB/c mice were treated with 100 µg of free or encapsulated CpG ODN either i.p. or orally. 3 days later, mice were challenged i.p. with 3-5x 10^3 CFU or orally with 2-6 x 10^9 CFU in 200 µl PBS. For long-term treatment experiments, 100 µg of free or encapsulated CpG ODN or PBS were administered orally for 7 consecutive days then bacteria challenge was done i.p 1 day

after the last oral treatment. As control, 100 ug CpG ODN was administered 3 days before the bacteria challenge. 4 days after the challenge mice were sacrificed, spleen and liver harvested as described. Each organ was weighed. For livers, total weight was measured then a portion about 100-200 mg was cut and weighed. Liver portion and the spleen mashed on top of a 70 μm cell strainer (Falcon) by using PBS with 0.1% Tween 20. Spleen homogenates were prepared in 10 ml, liver portions were prepared in 5 ml. From those homogenates, 10-fold dilutions prepared (starting from undiluted, 1:10, 1:100) and 100 μl from diluted samples were plated on BHI agar plates. Colonies were counted the next day. CFU/gram of organ was calculated by this formula:

[Colony count x dilution factor x 10 (to get /ml) x 10 for spleen, 5 for liver (to get per organ or per liver portion)]/gram of the organ (for spleen), gram of the liver portion (for liver)

3.2.15. Extraction of *E. coli* lipids

Isolation of *E. coli* lipids were done by Stephen D. Fox at the protein characterization laboratory of the HPLC/Mass Spectrometry Core (NCI, Frederick, MD, USA). Extraction was done by MTBE/MeOH standard protocol. Each 100 mg vials yielded about 5 mg of lipid material. One aliquot was dried under nitrogen (Bulk extract), remaining extract was fractioned on silica into three sub-fractions; A) neutral lipids, B) glycolipids, C) phospholipids. The three fractions were dried under nitrogen and together with the bulk extract, sent to Klinman Lab Lab (NCI, Frederick, MD, USA) for stimulation of the human monocytes.

3.2.16. Collection and culture of elutriated human cells

Elutriated monocytes were obtained from healthy volunteers who gave written consent to participate in an Institutional Review Board approved study (National Institutes of Health, Bethesda, MD, USA). Samples were collected in NIH Blood bank then shipped within 2-3 hours of collection to Klinman Lab (NCI, Frederick, MD, USA). Upon arrival, elutriated monocytes were immediately washed with pre-warmed 20 ml RPMI supplemented with 2% FCS then cultured overnight in 2% FCS

supplemented RPMI at a density of $2x10^6$ cells/ml media in T-175 or T-75 tissue culture flasks.

3.2.17. Sorting of HLA-DR$^+$ monocytes

Overnight cultured elutriated monocytes collected and passed through 70 μm cell strainer (Falcon, USA). Cell count determined by KX-21N Automated Hematology Analyzer (Sysmex, USA). Cells were centrifuged at 400 g for 10 minutes and then re-suspended with sorting buffer (2.5% FCS containing PBS). From that mix, 1 μl of cells separated for each single stained controls and unstained control in 1.5 ml microtubes. For single stained controls,10 μl sorting buffer with 0.5 μl anti-CD14 Brilliant Violet 450 + 0.5 μl anti-CD14 V450 antibodies or 1 μl anti-HLA-DR antibody added on 1 ul of unstained cells. The remaining sample then stained with addition of Brilliant Violet V421- and V450-conjugated anti-human CD14, and PE-conjugated anti-human HLA-DR antibodies. Staining was done for 30 minutes on 4 °C. The volume of sorting solution and antibody amounts were determined according to the cell number shown in the table.

Table 3.7. Buffer volumes and antibody amounts.

Cell #	**Sorting Buffer**	**anti-CD14 antibody (each)**	**anti-HLA-DR antibody**
< 75 million	180 μl	15 μl	60 μl
75 – 150 million	240 μl	20 μl	80 μl
> 150 million	300 μl	25 μl	100 μl

After the staining period, cells were washed with 10 ml Sorting Buffer, centrifuged at 400 g for 5 minutes then re-suspended in 1 ml sorting buffer. Single stained cells and unstained cells were washed with 200 μl sorting buffer, centrifuged at 5200 rpm for 2.5 minutes on tabletop centrifuge and re-suspended in 200 μl sorting buffer. Monocytes identified as; CD14^{bright}HLA-DR$^+$ cells and were purified by sorting using FACSAria II (BD Bioscience, USA) at the CCR-Frederick Flow

Cytometry Core (NVI, Frederick, MD, USA). Sorted cells were collected in collection buffer (20% FCS containing RPMI-1640).

3.2.18. Stimulation of HLA-DR$^+$ monocytes and phenotype analysis

1x10^5 purified monocytes were cultured in flat bottomed 96-well plates in 200 µl RPMI-1640 supplemented with 2% FCS. Cells were stimulated with the TLR agonists (**Table 3.1**), Heat killed bacteria (**Table 3.2**), lipid extracts of *E. coli* or with 500 ng/ml recombinant human cytokines (**Table 3.3**) (250 ng/ml for IFNγ) either alone or in various combinations as detailed in each experiment. For assessment of the macrophage differentiation 3-6 day, for measurement of the monocyte response to the stimulants, 24 hour cultures were done. After 3-6 days, differentiation of monocytes into M1- or M2-like macrophages was determined by using fluorescence-conjugated surface antibodies such as; anti-25F9 (as a pan-macrophage marker), anti-CD163, anti-CD206 and anti-CD200R1 (as M2-macrophage markers) (5, 260).

3.2.19. Endocytosis assay of the monocyte differentiated macrophages

Three sets of HLA-DR$^+$ monocytes differentiated with the stimulants for 3-6 days as described above. Afterwards, 96 well plates containing the differentiated cells were centrifuged at 400 g for 5 minutes. Supernatants were discarded (or collected for ELISA) then cells in 2 plates were incubated with 50 µg/ml of Dextran-Alexa Fluor 488 for 45 minutes in 50 µl of RPMI Reg supplemented with 2% FCS; one plate at 37 °C, one plate at 4 °C. Third plate was re-suspended with 50 µl of RPMI Reg supplemented with 2% FCS without the dextran (for background). The uptake rate was determined by measuring the Mean Fluorescence Intensity (MFI) by flow cytometry. Real uptake values were determined by subtracting the MFI at 4 °C incubation from 37 °C incubation (5, 260).

3.2.20. Cytokine detection by ELISA

Supernatants from all stimulation studies were collected by centrifugation at 400 g for 5-10 minutes and stored at -20 °C until use. Immulon 2HB microtiter plates were coated with 50 µl/well with diluted anti-cytokine antibodies in PBS (**Table 3.5**),

for 4 hours at room temperature or overnight at 4 °C. Plates were blocked with 200 µl blocking buffer for 2 hours at room temperature or overnight at 4 °C. Plates were washed with washing buffer three times for three minutes/each then rinsed three times with ddH_2O. Supernatant (either undiluted or diluted in blocking buffer) or standard loading volume was 50 µl. Standards were prepared with 3-fold serially diluted recombinant proteins (in blocking buffer starting from 50-250 ng/ml depending on the cytokine and the cell type). After incubation at room temperature for 2 hours or at 4 °C overnight, plates were washed as previously described. Plates were incubated with biotin-conjugated detection antibodies (prepared in blocking buffer) at room temperature for 2 hours. After another washing step, plates were incubated with 1:5000 diluted phosphatase-streptavidin in blocking buffer for 30-45 minutes. After a washing step, 70 µl of PNPP substrate was added to the wells and plates were kept at dark. Optical density readings at 405 nm wavelength were acquired at multiple time points (usually from 30 minutes to overnight).

3.2.21. Statistics

Statistical analyses were performed using GraphPad Prism 7.01 and R. Differences between the groups were assessed by One-way ANOVA followed by Tukey's tests to control for type I errors when more than two groups were compared. Student's two-tailed t-tests was used when only two groups were being compared.

For *L. monocytogenes* challenge studies, statistical analyses were performed on log-transformed data of the bacteria counts. Longitudinal DIA and weight loss data were analyzed using a linear mixed-effects repeated measures regression model which takes into account within-mouse correlated responses over time by NIH statistician Dr. Gregory Alvord. For those analyses, the F-statistic (from ANOVA) was used to determine treatment group differences.

4. RESULTS

4.1. Calcium Carbonate Encapsulated CpG ODN Retain Biological Activity *In Vitro*.

CpG ODN stimulate the immune system, resulting in increased immune activation, cell migration and expression of signaling molecules within 30 minutes that increases over time (111). Overall pro-inflammatory cytokine secretion peaks around 10 hours (85, 111). To determine whether CpG ODN activity would be altered by calcium carbonate encapsulation, splenocytes obtained from BALB/c mice were stimulated with equal amounts of ODN either encapsulated in calcium carbonate (CpG ODNcap) or delivered in free form. Stimulations were done for 24 and 48 hours to detect possible time-dependent differences in activation between free and encapsulated CpG ODN. As widely accepted metrics of CpG activation (85, 111), amounts of IL-6, TNFα, IL-12 and IFNγ in the culture supernatants were monitored by ELISA. As expected, 24 hours of stimulation with CpG ODN resulted in increased IL-6, TNFα and IL-12 production by splenocytes (**Figure 4.1**). The same effect was observed following CpG ODNcap stimulation ($p<0.001$). With prolonged stimulation (48 hours), cytokine levels remained high with the addition of significantly increased IFNγ levels detected from both CpG ODN and CpG ODNcap groups. IL-6, IL-12 and IFNγ levels were equivalent in the two stimulation groups whereas TNFα was higher with CpG ODNcap at both 24 and 48 hours. These results show that calcium carbonate encapsulation does not reduce the stimulatory activity of the CpG ODN.

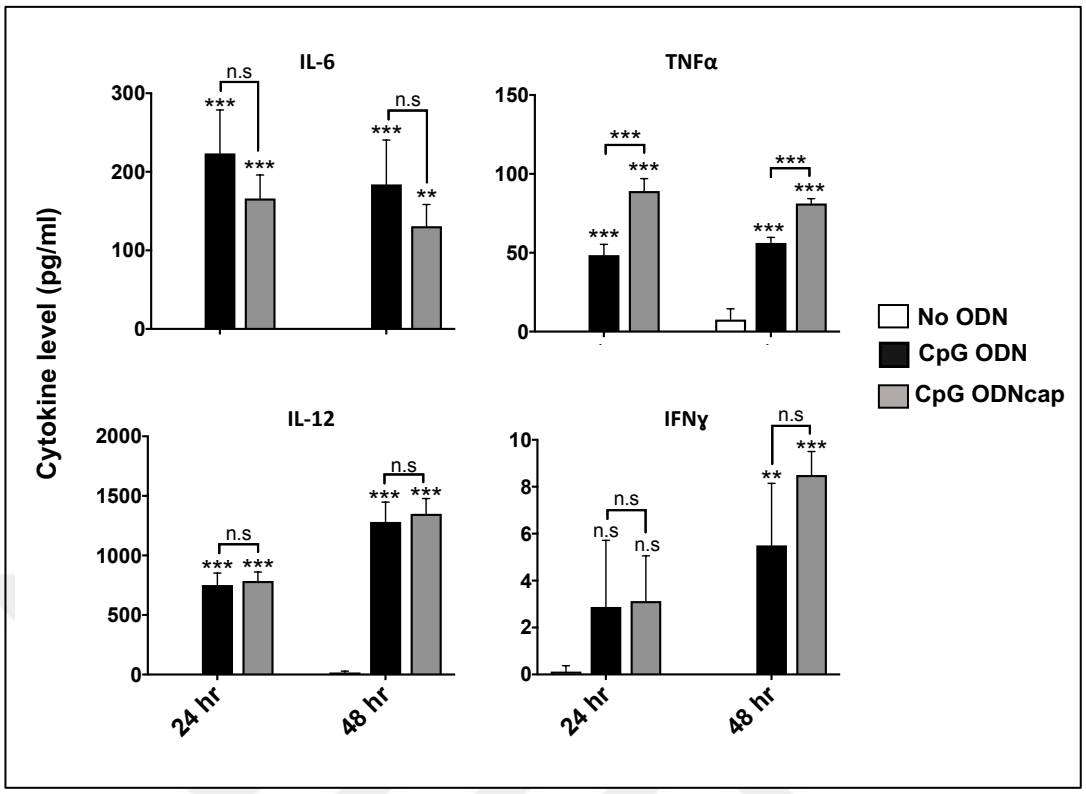

Figure 4.1. In vitro response of spleen cells to CpG ODNcap. Splenocytes of BALB/c mice (n=4) were stimulated with 6 μg/ml of free or encapsulated CpG ODN for 24 and 48 hours. Cytokine levels in culture supernatants were measured by ELISA. **; $p<0.01$, ***; $p<0.001$ vs unstimulated controls.

To check that the stimulatory activity of the CpG ODNcap was due to its CpG ODN component rather than an off-target effect related to the calcium carbonate, ODNcap containing control GpC ODN motifs were added to BALB/c splenocytes. As shown in **Figure 4.2**, while encapsulated CpG ODN 1555 induced pro-inflammatory cytokine production from the splenocytes, encapsulated control ODN 1612 did not cause detectable cytokine release at 2 μg/ml or 6 μg/ml concentrations both at 24 hours and 48 hours of stimulation. CpG ODN 1555 stimulation caused significantly higher cytokine induction when compared to the GpC ODN 1612. This result suggests that the activation induced by the CpG ODNcap is dependent on the CpG motifs within the ODNcap.

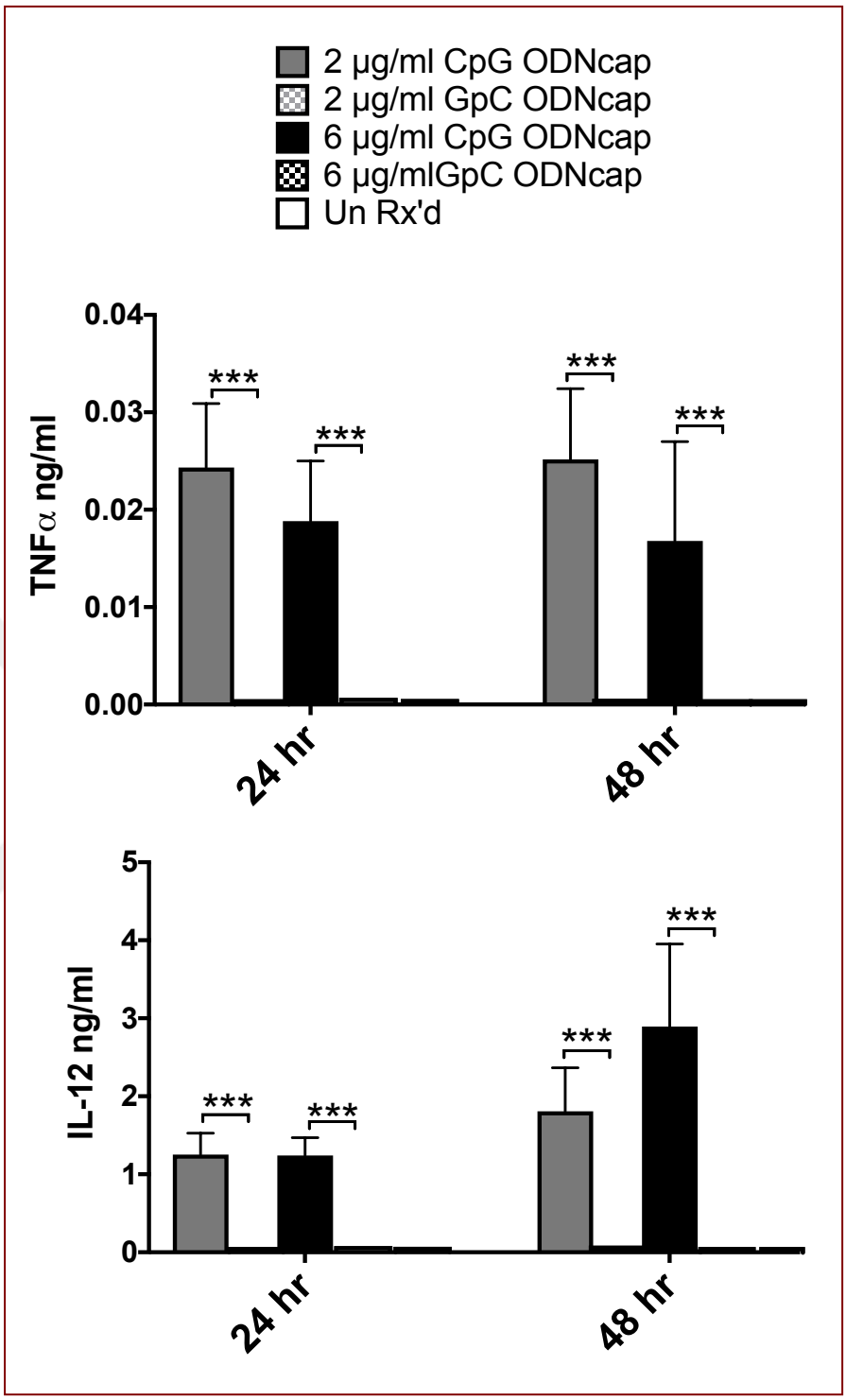

Figure 4.2. In vitro response of spleen cells to CpG ODNcap versus GpC ODNcap. Splenocytes of BALB/c mice (triplicate analysis, 2 experiments) were stimulated with 2 µg/ml or 6 µg/ml of encapsulated CpG ODN 1555 or encapsulated GpC ODN 1612 for 24 and 48 hours. Cytokine levels in culture supernatants were measured by ELISA. ***; $p<0.001$ vs unstimulated controls.

A151, a synthetic ODN containing suppressive TTAGGG motifs, can inhibit the cytokine response initiated by the CpG ODN (119). To test the encapsulation effect on the suppressive activity of A151, BALB/c splenocytes were stimulated with equal concentrations of CpG ODN and A151 for 48 hours. As expected, A151 blocked the IL-6 release from CpG ODN stimulated cells by ~90% and IL-12 release by ~50% (**Figure 4.3**). Similar to this finding, encapsulated A151 blocked the IL-6 release by ~90% and IL-12 release by ~60% from CpG ODNcap stimulated cells. These results altogether suggest that calcium carbonate encapsulation does not reduce the activity of either suppressive or stimulatory ODNs.

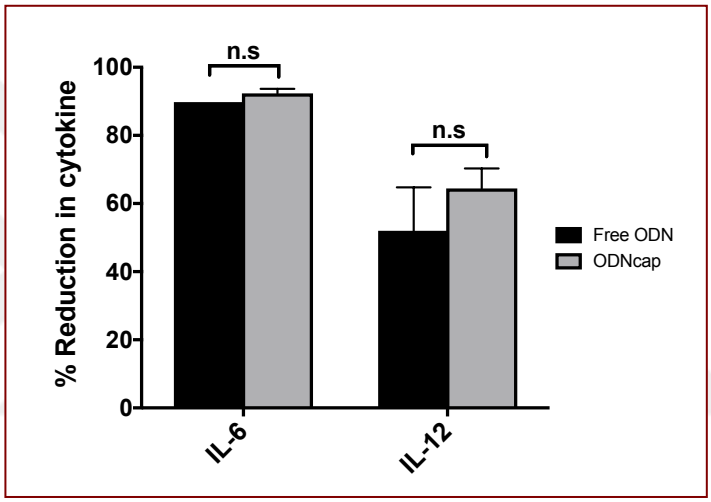

Figure 4.3. A151 inhibition of CpG ODN induced activation. Splenocytes of BALB/c mouse were stimulated with 3 μM of free CpG ODN plus 3 μM of free A151 (Black bars) or equal amounts of encapsulated CpG ODN plus encapsulated A151 (Grey bars) for 48 hours. Cytokine levels in culture supernatants were measured by ELISA. Bar graphs show the % decrease of cytokine with free or encapsulated A151 when compared to the CpG ODN or CpG ODNcap alone.

4.2. CpG ODN And CpG ODNcap Activate Same Type Of Immune Cells

CpG ODN is recognized by TLR9 (269). The immune cells in mice that express TLR9 are B cells, monocytes, macrophages and dendritic cells (111). These TLR9 expressing cells are primarily responsible for CpG ODN-induced inflammation. TLR9 is expressed intracellularly on endosomal membranes. Therefore, binding and internalization of the ODN is not dependent on TLR9 expression. Calcium-carbonate encapsulation creates large aggregates of CpG ODNs (~100-200 nm) (131). Thus,

their association with immune cells might differ from free CpG ODN. To identify the cell types associated with ODNcaps and whether this differs from free ODNs, Cy5 or FITC labeled CpG ODN and CpG ODNcap were incubated with BALB/c splenocytes for 90 minutes at 37 °C. Because of their size, unbound ODNcaps were found in the pellet after centrifugation. To separate the unbound fluorescent labeled ODN or ODNcap in the culture, CD45+ cells were isolated using magnetic beads after incubation. Flow cytometric analysis revealed differences in the cells that were positive for ODN depending on their fluorescent tag. 90-100% of the CD45+ cells, macrophages (CD45+ F4/80+), B cells (CD45+ CD19+) and T cells (CD45+ CD3+) were positive for Cy5-labeled ODN or ODNcap. About ~70% of the CD45+ cells were positive for FITC-ODN whereas only ~40% of them were positive for FITC-ODNcap. B cells and macrophages had the highest FITC-labeled ODN or ODNcap percentage and the T cells had the lowest. FITC-ODNcap percentage was significantly less than FITC-ODN in all cell types analyzed (**Figure 4.4**). These results suggest that the association of ODN or ODNcap with cells is dependent on its fluorescent tag.

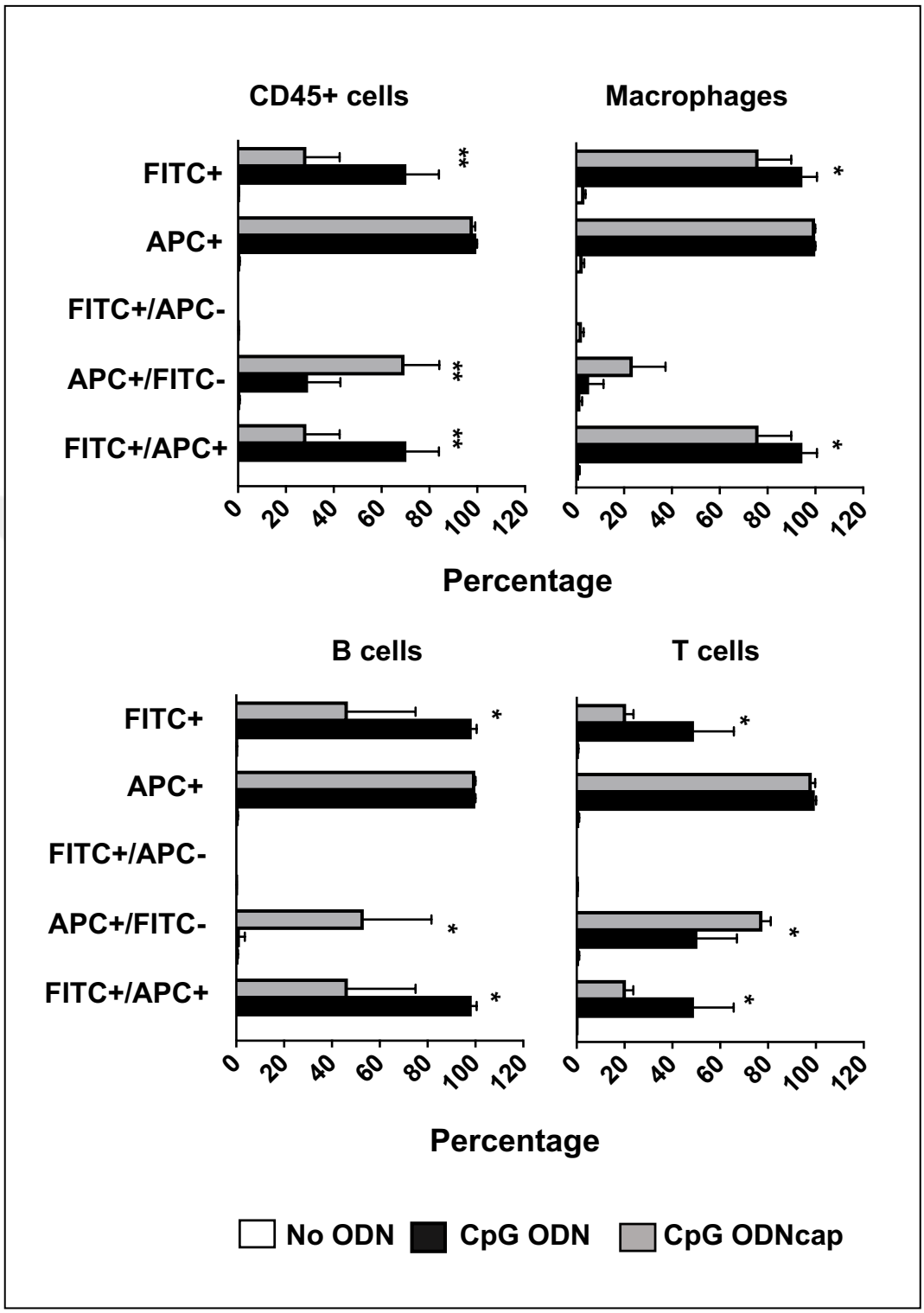

Figure 4.4. Association of Cy5 or FITC labeled CpG ODN and ODNcap with different immune cells *in vitro.* Splenocytes incubated with Cy5 or FITC-labeled CpG ODN and CpG ODNcap for 90 minutes at 37 °C. Macrophages were identified as CD45+ F4/80+, B cells as CD45+ CD19+, T cells as CD45+ CD3+. Bar graphs show percentages of immune cells positive for FITC and/or APC (Cy5) determined by flow cytometry. Data is the sum of 3-4 independently analyzed experiments.

To determine whether the cells that take up these aggregates are also activated by CpG ODNcap, the response of the cells cultured with fluorescent-labeled CpG ODNcap was examined. Splenocytes from BALB/c mice were stimulated for 20 hours with 1 μM of Cy5 or FITC labeled CpG ODN or CpG ODNcap. To monitor cytokine release, Brefeldin A was added for the final 5 hours. Similar to what was shown in **Figure 4.5** ~100% of the cells were positive for Cy5-ODN or ODNcap whereas ~70% of the cells were positive for FITC-ODN and ~50% for FITC ODNcap even after 20 hours of incubation. Unlabeled CpG ODN and ODNcap triggered IL-6 production from a similar fraction of cells (~4-5%). Cytokine producing cells were all positive for FITC or Cy5 labeled ODN and ODNcap however, slight differences were observed in the cytokine production amounts depending on the fluorophore used to tag the CpG ODN.

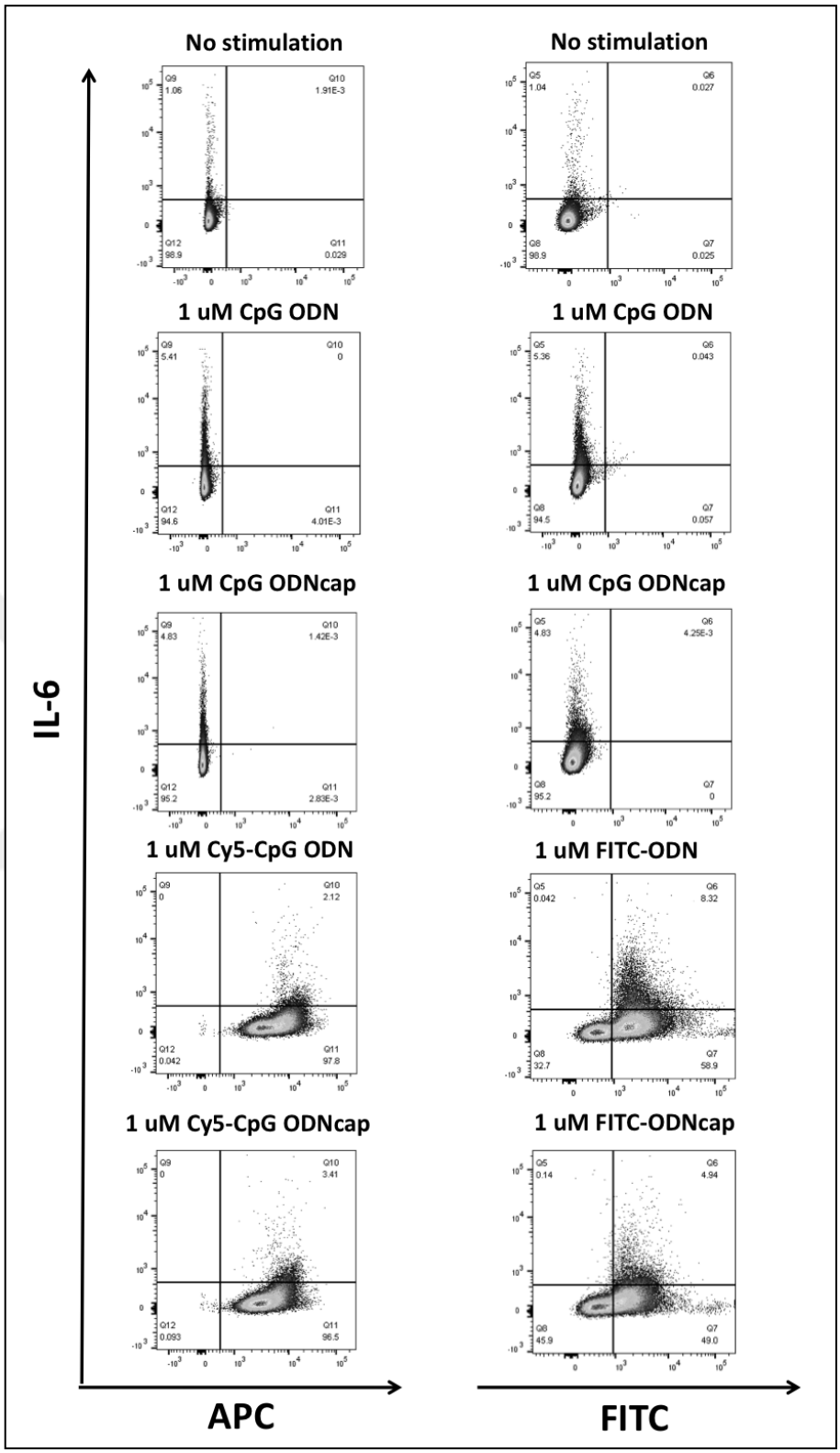

Figure 4.5. Activation of splenocytes with Cy5 or FITC labeled CpG ODN and ODNcap *in vitro*. Representative dot plots show IL-6 production after 20-hour incubation of splenocytes with 1 μM Cy5 and FITC CpG ODN or CpG ODNcaps with Brefeldin A addition on the final 5 hours.

Having observed that the fluorescent-tagged ODN or ODNcap gave different results in terms of binding and activation, unlabeled CpG ODN or CpG ODNcap were used to stimulate splenocytes which were then analyzed by flow cytometry to identify the cell types responsible for cytokine production as shown in **Figure 4.1**. As described before, B cells, T cells and macrophages were identified by their surface markers. Cytokine release from these different immune cell populations revealed that B cells stimulated with free or encapsulated ODN increased their production of IL-6 and IgM approximately by ~5 fold ($p. <0.05$, **Figure 4.6**) macrophage responded with a ~2-4 fold increase in IL-12 and IL-6 production following either type of stimulation ($p. <0.01$, **Figure 4.6**). The response of both cell types to free vs encapsulated CpG ODN was equivalent. Although TLR9 negative T cells were able to associate with labeled free or encapsulated CpG ODN as previously shown in **Figure 4.4**, they failed to produce cytokine following incubation with CpG ODN or CpG ODNcap. These results confirm that binding/uptake of CpG ODNcap is non-selective but that cell activation in response to free or encapsulated CpG ODN was dependent on TLR9 expression.

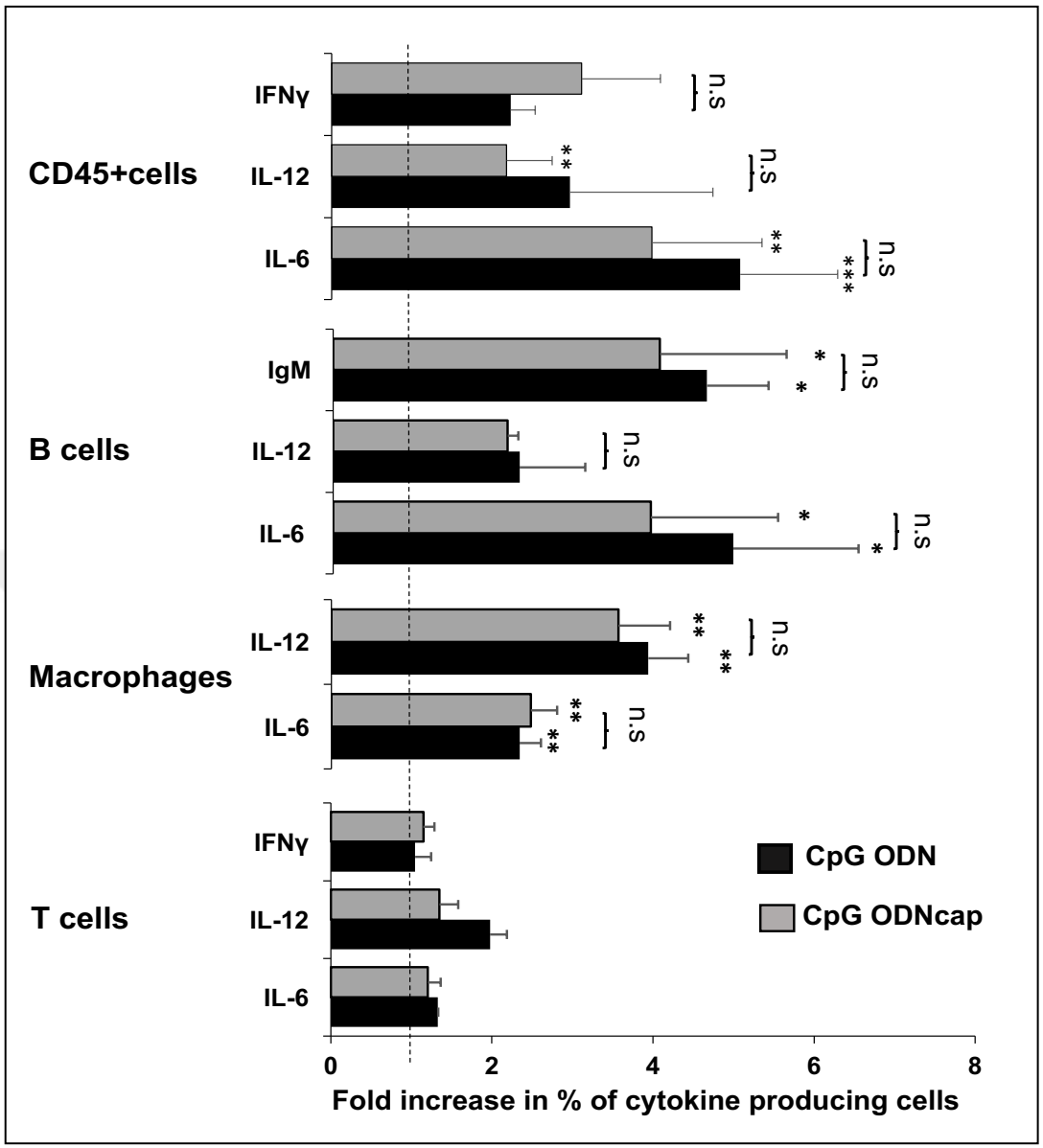

Figure 4.6. In vitro response of lymphocytes and macrophage to CpG ODNcap. BALB/c spleen cells were cultured with 1 μM of CpG ODN or CpG ODNcap for 24 hours with Brefeldin A addition on the final 5 hours. Data show the fold increase in percentage of IL-6, IL-12 and/or IgM producing cells compared to unstimulated controls (dashed line). Results represent the mean + SD of 2-4 independent experiments. *; p <.05, **; p<0.01 vs unstimulated cells.

4.3. Orally Administered CpG ODNcap Localizes To Gastrointestinal System Associated Lymphoid Tissues.

Wang *et. al.* showed that orally delivered 6FAM-labeled ODNcap was taken up specifically by CD11b+ cells (identified as macrophages) in the Peyer's patches (131). To verify this finding and determine whether ODNcaps were present in other

lymphoid organs within the gastrointestinal tract, mice were fed 25 μg of FITC and 25 μg Cy5-labeled CpG ODNcap or PBS as control. Feeding was done 90 minutes, 4.5 hours, 14 hours and 42 hours before sacrifice. The amount of Cy5 labeled ODNcap peaked at 90 minutes in the Peyer's patches and appendix and then decreased over time. ODN levels in the mesenteric lymph nodes peaked around 4.5 hours suggesting that immune cells might be migrating from the lymphoid organs of the intestinal wall, explaining why more time was required for ODNcap to be found in the MLN compared with the Peyer's patches and appendix. By 14 hours, ODNcap was not detectable in GI associated lymphoid organs. The uptake of these ODN by immune cells was analyzed by using surface markers for T cells (CD3+), B cells (CD19+), myeloid cells (CD11b+) and macrophages (F4/80+). Unlike what Wang *et. al.* observed, all cell types analyzed were positive for fluorescent-labeled ODNcap. This finding is in concordance with the in vitro association studies presented earlier in **Figure 4.4** that all the cell types can associate with the CpG ODNcap. Signal from FITC-labeled ODNcap was not detectable in any other organ analyzed (**Supplementary Figure A**). In a preliminary experiment, FITC and Cy5 labeled ODNcap were incubated with splenocytes and CD45+ cells isolated by magnetic sorting and transferred into another group of mice by i.v. injection. Mice were sacrificed at different time points to track the labeled ODN-containing cells over time. FITC signal was not detectable in blood, spleen or mesenteric lymph nodes, whereas Cy5 signal was detectable in all three organs in a time-dependent manner (**Supplementary Figure B**). These findings suggest that the FITC signal was being lost *in vivo* and was unsuitable for tracking ODNcap.

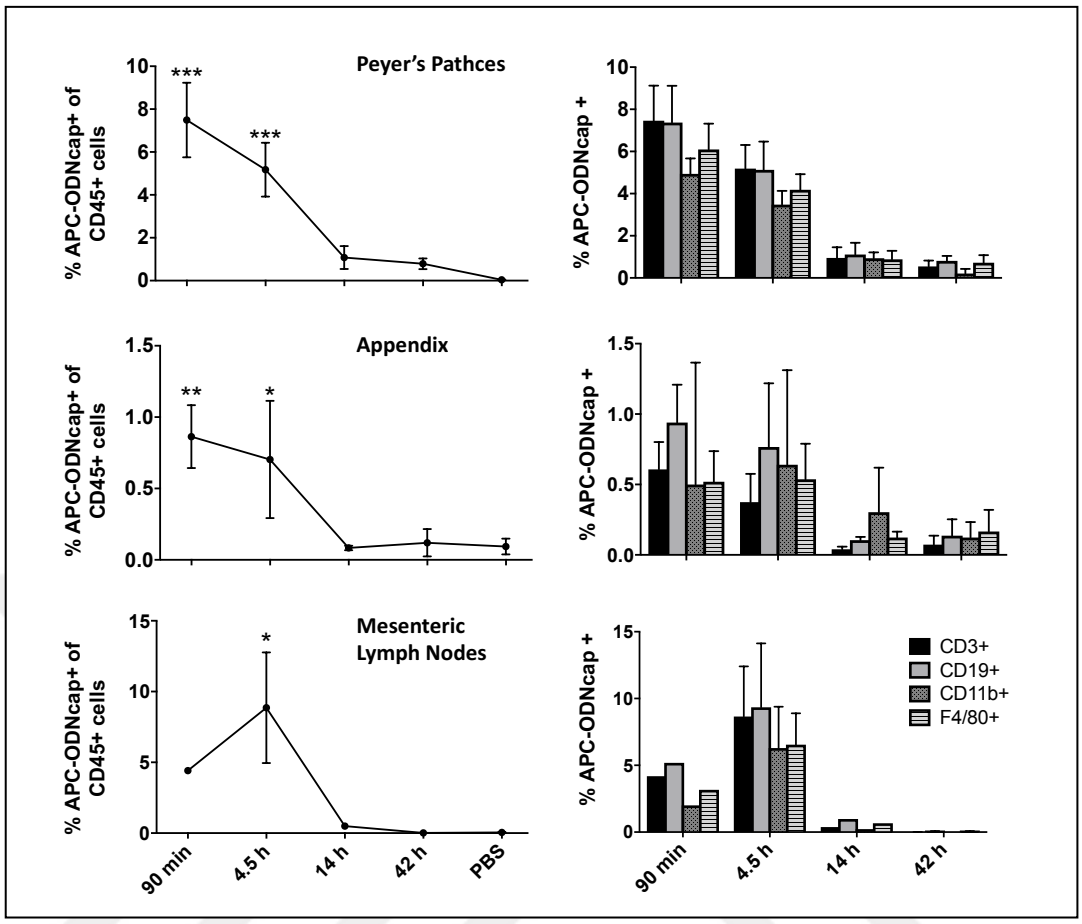

Figure 4.7. *In vivo* localization of labeled CpG ODNcap. BALB/c mice (n=3/time point) were treated with 25 μg of FITC and 25 μg Cy5-labeled CpG ODNcap or PBS as control. After indicated time points, mice were sacrificed and lymphoid organs harvested. Fluorescence positive cell percentages of live CD45+ cells and specific cell types: T cells (CD45+ CD3+), B cells (CD45+ CD19+), Myeloid cells (CD45+ CD11b+) and macrophages (CD45+ F4/80+) detected by Flow cytometry. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs PBS control.

Wang *et al*. described that IFNγ production was increased in the Peyer's patches following three days of oral CpG ODNcap administration (131). To verify that observation and determine whether other sites were also responding, 10 μg of CpG ODNcap was delivered by gastric gavage to BALB/c mice for 3 or 10 consecutive days. Terminal blood collection was done to obtain serum samples and then cells were harvested from the Peyer's patches, mesenteric lymph nodes and spleen. Single cell suspensions were prepared and immediately cultured with Brefeldin A to detect ongoing cytokine secretion. 3 days of CpG ODNcap administration triggered cells in the Peyer's patches to secrete IL-6, IL-12 and IFNγ ($p<0.05$, **Figure 4.8**). When CpG ODNcap treatment continued for 10 days, activation in mesenteric lymph nodes was

also detectable. However, no change in splenocyte activation was detected after 3 or 10 days of ODN administration (**Figure 4.8**). Serum analysis of the mice did not show any detectable cytokine response (not shown). These results suggest that orally administered CpG ODNcap can activate gastrointestinal system associated lymphoid tissue (GALT) immunity but might not be effective for systemic immunity.

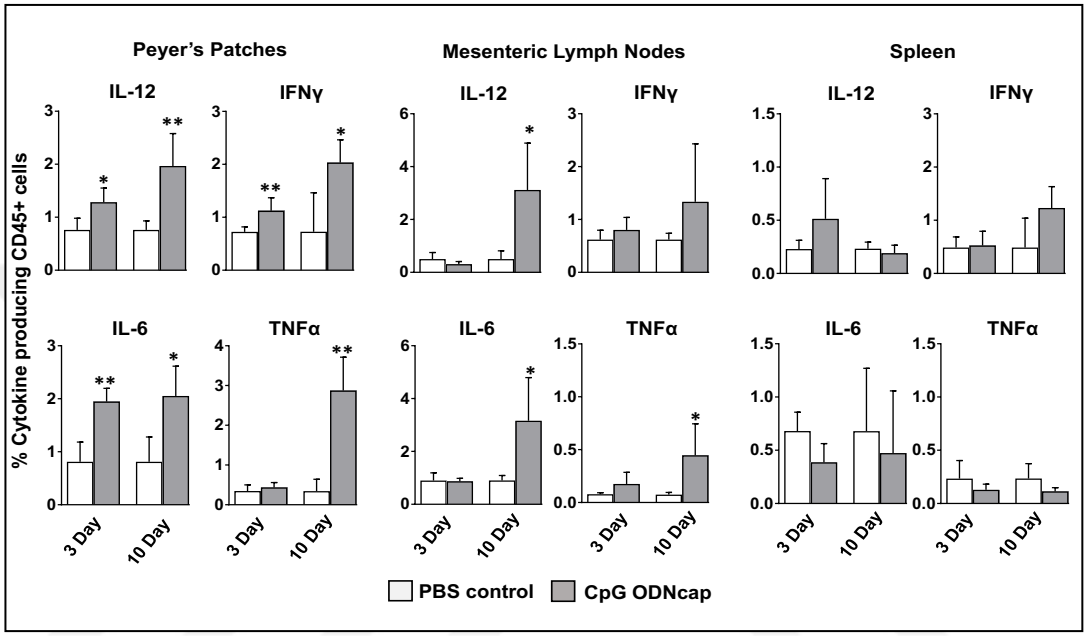

Figure 4.8. Activation of lymphoid organs after oral administration of CpG ODNcap. BALB/c mice were treated with 10 μg of CpG ODNcap or PBS by gastric gavage for 3 or 10 consecutive days. The percentage of CD45+ cells secreting IL-12, IFNγ, IL-6 and TNFα in the Peyer's Patches (left), mesenteric lymph nodes (middle) and spleen (right) is shown. Data represent the mean + SD of 3-8 mice/group from 3 independent experiments. *; $p<0.05$, **; $p<0.01$ vs PBS control.

4.4. Orally Delivered Free Or Encapsulated CpG ODN Exacerbates DSS-Induced Colitis.

The above findings suggest that orally administered CpG ODNcap stimulates the immune cells of the GALT. To confirm this finding in an inflammation model involving the gastrointestinal system, a well described murine model of chemically-induced colitis was used. Dextran sodium sulphate (DSS) disrupts the colonic epithelium thereby facilitating the invasion of intestinal microbes through the mucosa, leading to inflammation (268, 270). As a result of epithelial disruption and inflammation, disease with symptoms including diarrhea, weight loss and rectal bleeding occurs. Previous studies showed that systemically delivered CpG ODN

exacerbated the severity of DSS-induced colitis by enhancing Th1 responses (271-273).

As shown in **Figure 4.9A**, inflammation was generated by delivering 2% DSS in drinking water to C57/Bl6 mice for 8 days. This was replaced with normal house water for the remaining 5 days. Animals were gavaged with 25 μg of free or encapsulated CpG ODN or PBS as control. Treatments were done every other day, starting the day before initial DSS administration and continuing until the end of the study (2 weeks). Disease activity and weight measurement were done daily as described in the methods section. Inflammation started around day 3 after DSS administration as seen by and increase in the Disease Activity Index score (**Figure 4.9C**). During the induction phase of disease (days 3-8), orally delivered free or encapsulated ODN significantly worsened colitis when compared to the PBS group ($p < 0.01$, **Figure 4.9 B and C**). CpG ODN and CpG ODNcap treatment also slowed recovery during the resolution phase of disease (days 9-12, note the difference in recovery slope, $p<0.01$, **Figure 4.9C**). Consistent with this longer and more severe course of disease, colon length was significantly reduced in the ODN and ODNcap treated mice than those treated with DSS alone ($p < 0.05$, **Figure 4.9D**). There was no difference in weight loss, disease severity, or colon shortening between mice treated with free vs encapsulated CpG ODN.

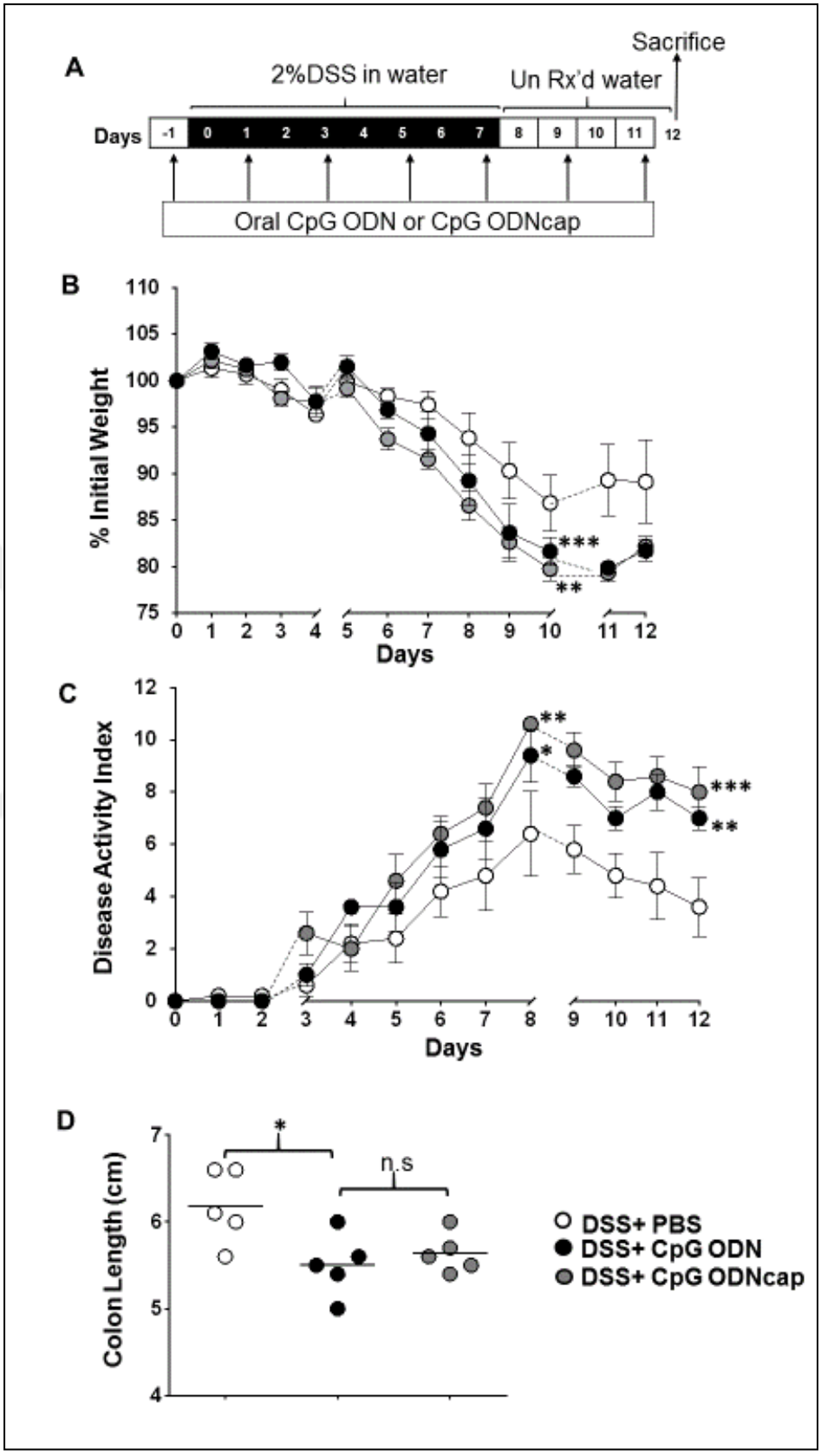

Figure 4.9. Effect of CpG ODNcap on DSS-induced colitis. A) C57Bl/6 mice received 2% DSS for 8 days followed by regular water for 5 days. Mice were treated

with 25 μg of CpG ODNcap or free CpG ODN by gavage every other day. B) Animals were monitored for weight loss and C) disease activity over time. D) Colon lengths were measured from the ileocecal junction to rectum on day 12. Data represent the mean ± SE of 5 mice/group. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs DSS treated controls. Note: This experiment was repeated using 1% DSS administration and yielded similar results. There was no difference in any disease parameter between mice treated with CpG ODNcap vs free CpG ODN in either experiment.

One cm portions of distal colons from each mouse were cultured for 24 hours and the supernatant evaluated for cytokine content (described in the methods section in detail). As shown in the left panel of **Figure 4.10**, IL-6 levels were significantly higher in CpG ODN and CpG ODNcap treated mice colons when compared to the control. In contrast, IL-6 levels were not increased in the serum of CpG ODN or CpG ODNcap treated mice (**Figure 4.10**, right panel). In both panels, there was no significant difference observed between CpG ODN and CpG ODNcap treated mice IL-6 levels. These findings are consistent with the earlier observations showing that CpG ODNcap were effective on GALT but had little effect on systemic immunity. Overall experiments with DSS-colitis model confirmed the previous findings suggesting that CpG ODNcap activate GALT and can manipulate disease progression of an inflammatory disease confined to the gastrointestinal system.

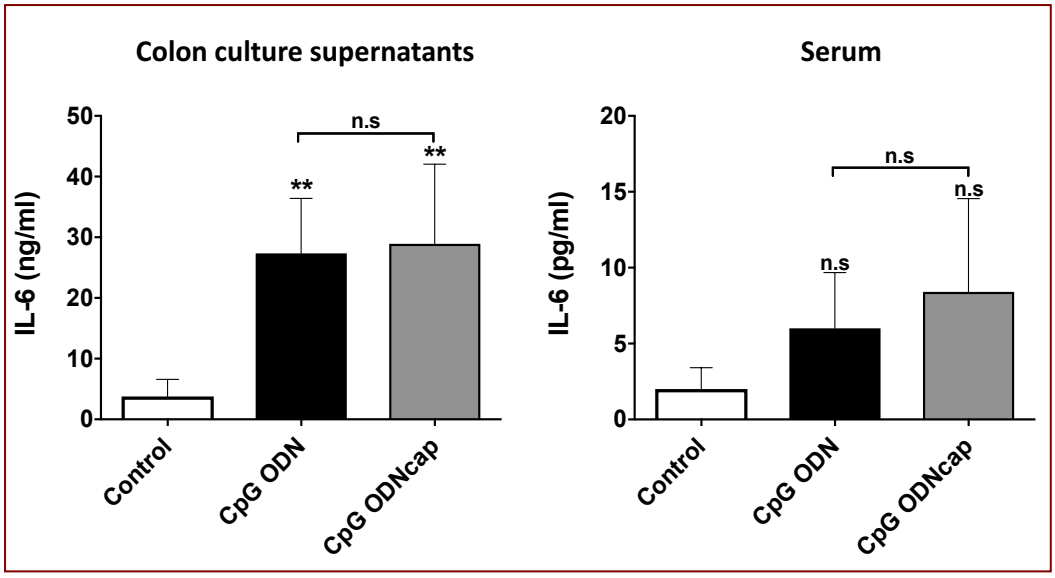

Figure 4.10. Effect of CpG ODNcap on colons and the serum of the mice with colitis. IL-6 levels from 24-hour cultured 1 cm distal colon portions (Left panel) and from serums collected at the end of the study (Right panel) measured by ELISA. Data represent the mean ± SD of 5 mice/group. *; $p<0.05$, **; $p<0.01$ vs PBS control.

4.5. Orally Delivered Free Or Encapsulated CpG ODN Does Not Increase Systemic Inflammation Induced By LPS.

Previous studies show that orally administered CpG ODNcap had no effect on spleen cell activation or serum cytokine levels (**Figure 4.8**), suggesting that their effect might be limited to the GALT. The DSS colitis model confirmed that CpG ODNcaps were active on the GALT but failed to detect any change in cytokine levels in the serum, suggesting no effect on systemic immunity. There is considerable data showing that parenterally delivered CpG ODN increase host sensitivity to systemic immune challenge (120, 274, 275) Thus, oral ODNcap might also increase host sensitivity to systemic immune challenge. To examine this possibility, CpG ODN, CpG ODNcap and PBS as control was delivered by gastric gavage to BALB/c mice 3 hours before a sublethal LPS challenge (Described in **Figure 4.11A**). Consistent with earlier reports, systemic delivery of CpG ODN significantly increased the IL-6 (**Figure 4.11B**) and TNFα (**Figure 4.11C**) response elicited by LPS (10, 11). In contrast, neither CpG ODNcap nor orally delivered free CpG ODN were able to increase LPS induced IL-6 or TNFα production significantly (**Figure 4.11B, C** respectively). These findings suggest that parenteral delivery of CpG ODN triggers a systemic immune response much more effectively than orally administered free or encapsulated ODN.

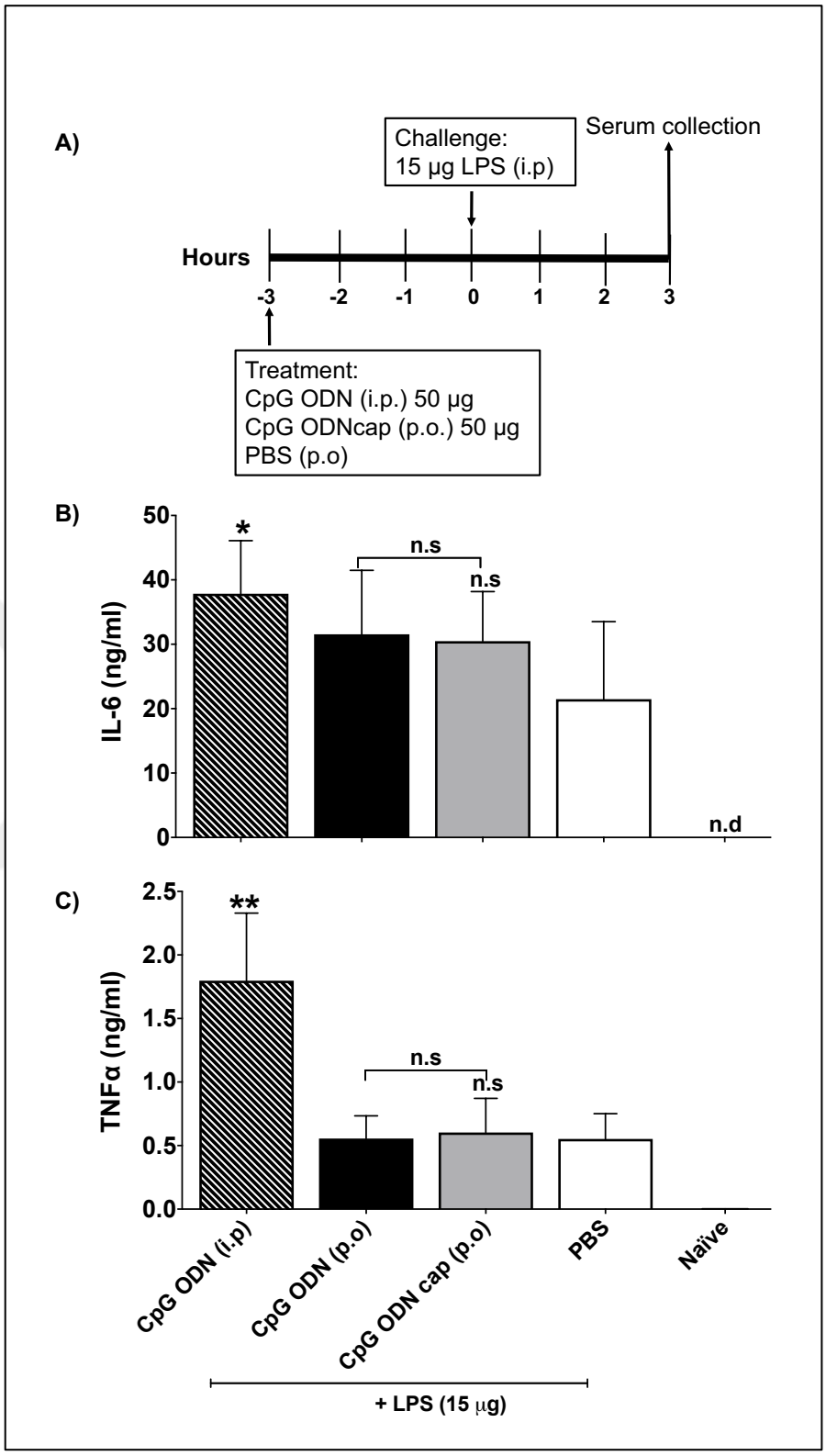

Figure 4.11. Effect of oral CpG ODNcap on LPS-induced cytokine production *in vivo*. 50 µg of CpG ODN or CpG ODNcap was delivered i.p. or by oral gavage to BALB/c mice, 3 hours later the mice were challenged i.p. with 15 µg of LPS (A). Data represent the mean + SD serum IL-6 (B) and TNFα (C) levels of 5 mice/group performed 3 hours after LPS injection. *; $p<0.05$, **; $p<0.01$

4.6. Comparison Of Oral CpG ODNcap Vs Free CpG ODN In Conferring Protection Against Local And Systemic *L. monocytogenes* Infection.

To further evaluate local and systemic responses elicited by orally administered CpG ODNcap, *L. monocytogenes* infection studies were conducted. This is a sensitive and readily quantifiable model in which both local and systemic responses can be evaluated. *L. monocytogenes* is a gram positive facultative anaerobe rod that is transmitted by the oral route (276). Once the bacteria reach the intestines they can penetrate the mucosal tissue directly via engulfment by enterocytes or indirectly via active penetration of the Peyer's patches (277-279). Subsequently, the bacteria migrate to the mesenteric lymph nodes, spleen and liver to cause a systemic infection (276). GALT immunity could thus play an important role in controlling the severity of *L. monocytogenes* infection.

Previous studies showed that prior parenteral treatment with CpG ODN protected mice from a diverse array of systemic pathogen challenges including *L. monocytogenes* (112, 125, 280, 281). As demonstrated in **Figure 4.12A**, mice were treated orally with CpG ODN or CpG ODNcap and protection was compared to CpG ODN administered i.p. Free CpG ODN in bicarbonate buffer was also administered orally to another group of mice. Three days after the treatments, intragastric challenge with *L. monocytogenes* was accomplished by gavage. Previous studies showed that maximal bacterial proliferation occurred in the liver and spleen about 4 days after the gastric challenge with *L. monocytogenes* (125). Therefore, mice were sacrificed on day 4 post-challenge. The extent of bacterial growth was monitored in the liver (one of the dominant sites of *L. monocytogenes* replication) (**Figure 4.12B**). Consistent with previous evidence, gastric administration of CpG ODN activated GI immunity and protects the mice from listeria infection. Oral CpG ODN matched the efficacy of parenteral delivery in terms of improving host resistance to pathogen delivered to the gut (**Figure 4.12B**). However, neither CpG ODNcap nor free CpG ODN delivered in bicarbonate buffer was able to improve the effect of orally administered free CpG ODN (in PBS).

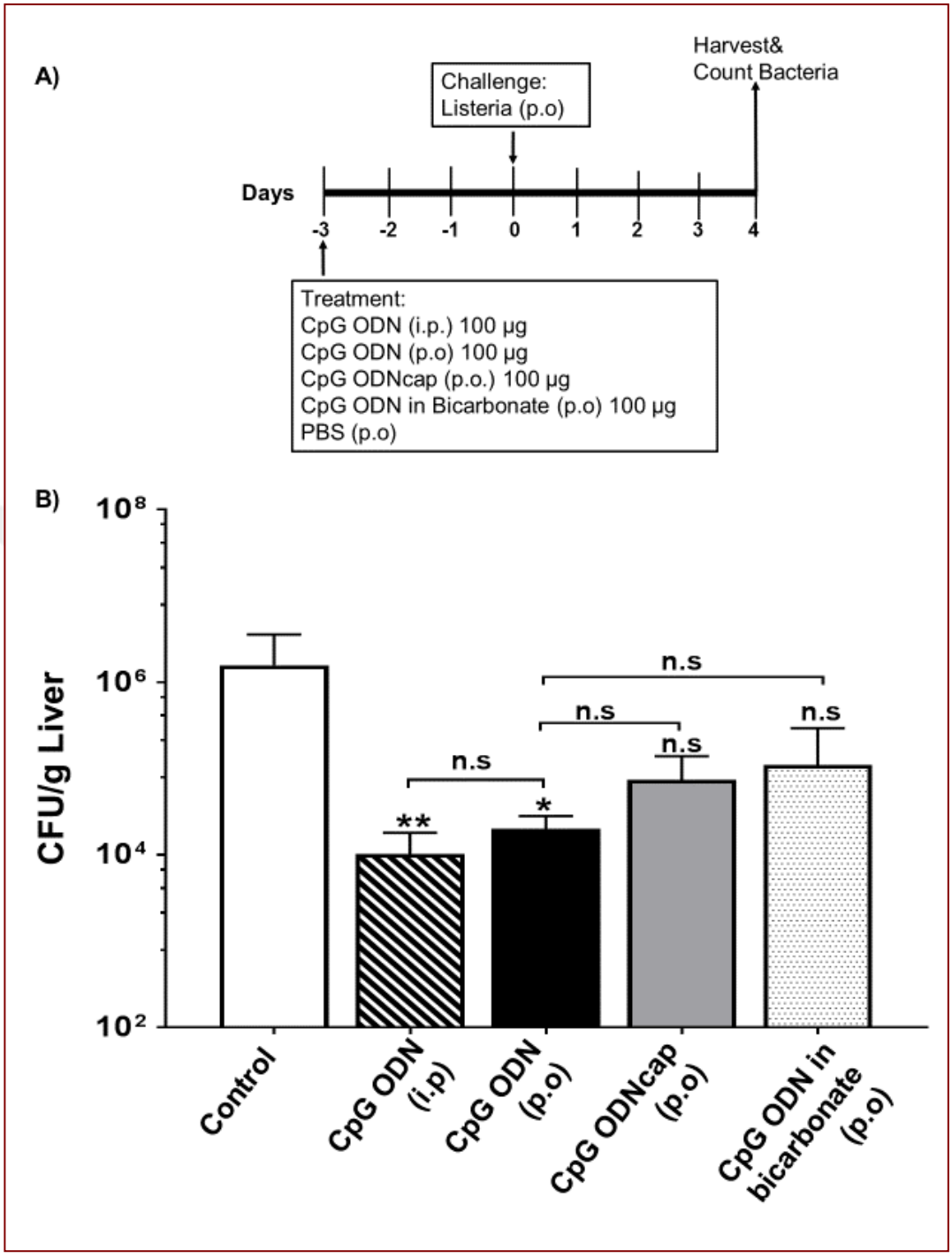

Figure 4.12. Effect of oral CpG ODNcap on host resistance to intragastric *L. monocytogenes* challenge. BALB/c mice were treated parenterally (i.p.) or orally with 100 µg of free CpG ODN in PBS or in bicarbonate buffer or equal amounts of encapsulated CpG ODN. Animals were challenged with 6 x 109 CFU intragastrically (A). Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 5 mice/group. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs PBS treated controls

The success of free CpG ODN in conferring protection against GI infection by *L. monocytogenes* led me to further investigate its effect in a systemic challenge model. Systemic delivery of *L. monocytogenes* results in severe infection. Previous studies showed that parenteral CpG ODN pre-treatment protected against that route of challenge (112, 125). Using the same experimental groups as described above, mice were challenged by i.p injection of *L. monocytogenes*. Consistent with previous reports, parenteral treatment with CpG ODN reduced bacterial load following systemic *L. monocytogenes* challenge by nearly 4 orders of magnitude ($p < .001$, **Figure 4.13A**). A significant reduction in pathogen load was also observed in mice treated orally with CpG ODN ($p < 0.05$, **Figure 4.13A**). Parenteral CpG ODN treatment was much more effective than oral CpG ODN ($p<0.01$, **Figure 4.13A**). As noted previously, CpG ODNcap did not improve the oral activity of CpG ODN.

Repeated systemic treatment with CpG ODN was shown to increase both the duration and magnitude of host resistance to infection (124). To determine whether repeated oral delivery of CpG ODN could duplicate this effect, mice were treated with free or encapsulated CpG ODN by gavage for 7 consecutive days and then challenged i.p. with *L. monocytogenes* a day after the last treatment. As seen in **Figure 4.13B**, repeated oral treatment failed to achieve the level of protection conferred by a single parenteral dose of CpG ODN ($p<0.05$, **Figure 4.13B**). Again, repetitive administration of CpG ODNcap was not more effective than repetitive orally administered free CpG ODN.

These results suggest that the calcium carbonate encapsulation does not enhance the activity of orally delivered CpG ODN and that free CpG ODN has some level of activity when delivered orally, particularly on GALT immunity. The following studies were done to investigate whether co-administering oral CpG ODN with other agents might improve systemic immunity.

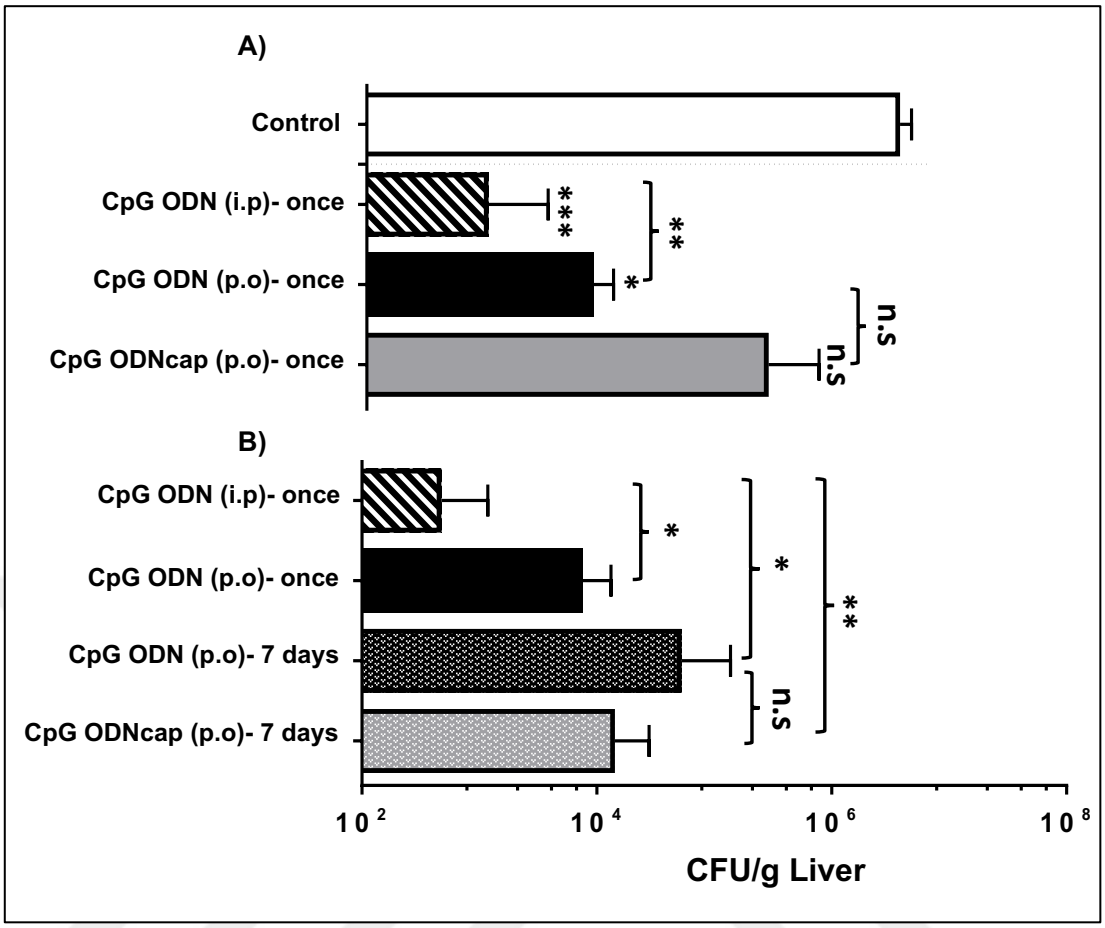

Figure 4.13. Effect of oral CpG ODNcap on host resistance to systemic *L. monocytogenes* challenge. BALB/c mice were treated parenterally (i.p.) or orally with 100 μg of free or encapsulated CpG ODN. Animals were challenged i.p. with 5 x 103 CFU of *L. monocytogenes*. Mice were treated once with all the reagents (A) or for 7 consecutive days orally with free or encapsulated CpG ODN or once i.p with free CpG ODN before challenge (B). Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 4-5 mice/group. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs PBS treated controls (A) or i.p CpG ODN (B).

4.7. R848 Is A Promising Immune Modifier For Oral Delivery

Different immune cell types express different TLRs. There is accumulating evidence that TLR agonist combinations may interact on and between cell types to regulate each other's effect. One example of such synergy was observed between TLR9 and TLR7/8 agonists. TLR7/8 and TLR9 agonists can both eliminate small tumors when used as monotherapy. Co-administration of TLR7/8 and 9 agonists generated an improved anti-tumor response capable of eradicating large, established tumors that neither agent alone could destroy (282).

To improve the immune response elicited by orally-delivered free CpG ODN, a combination strategy examining different TLR 7/8 agonists was pursued. The TLR7/8 agonists R848 (which is water soluble), 3M-052 and 3M-058 (which are lipophilic) were all examined in this experiment. An effective dose of CpG ODN (100 μg) was combined with 50 μg of the each TLR7/8 agonists and the mice then challenged orally with *L. monocytogenes* as described before (**Figure 4.12A**). The response to combination therapy was compared to orally delivered CpG ODN monotherapy. As seen in **Figure 4.14**, R848 and CpG ODN combination was significantly more protective than the CpG ODN administered alone ($p<0.05$). However, this improvement was not observed when CpG ODN was combined with the other TLR7/8 agonists, 3M-52 or 3M-058.

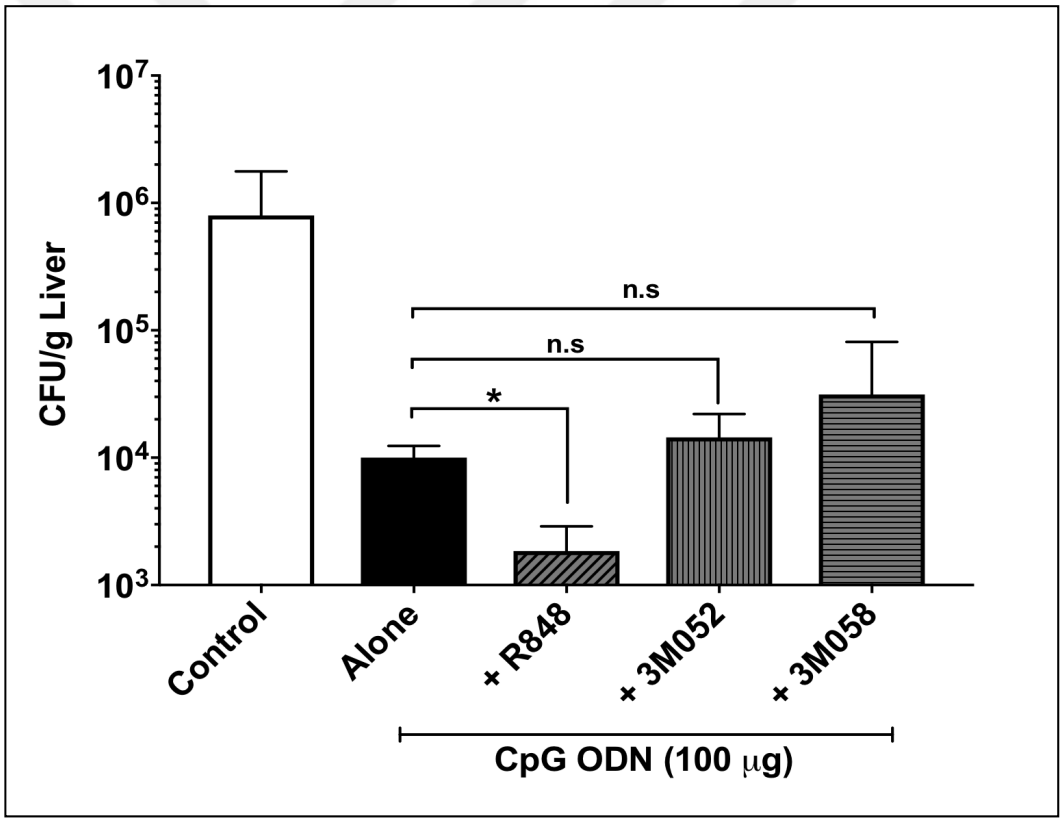

Figure 4.14. Effect of oral CpG ODN and TLR7/8 agonist combination on host resistance to intragastric *L. monocytogenes* challenge. BALB/c mice were treated orally with 100 μg of free CpG ODN or 50 μg of TLR7/8 agonists R848, 3M-052 or 3M-058. Animals were challenged with 6 x 10^9 CFU *L. monocytogenes* intragastrically. Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 5 mice/group. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ vs CpG ODN treated mice.

After observing that R848 could be suitable for oral therapy, its protective effect was further investigated. A dose study with R848 was done ranging from 50 µg to 0.5 µg. Mice were challenged systemically or orally with *L. monocytogenes* as described before (**Figure 4.12A**). A significant reduction in bacteria counts in the livers observed at 50 µg of R848 dose in both orally or systemically challenged mice ($p<0.05$ **Figure 4.15**).

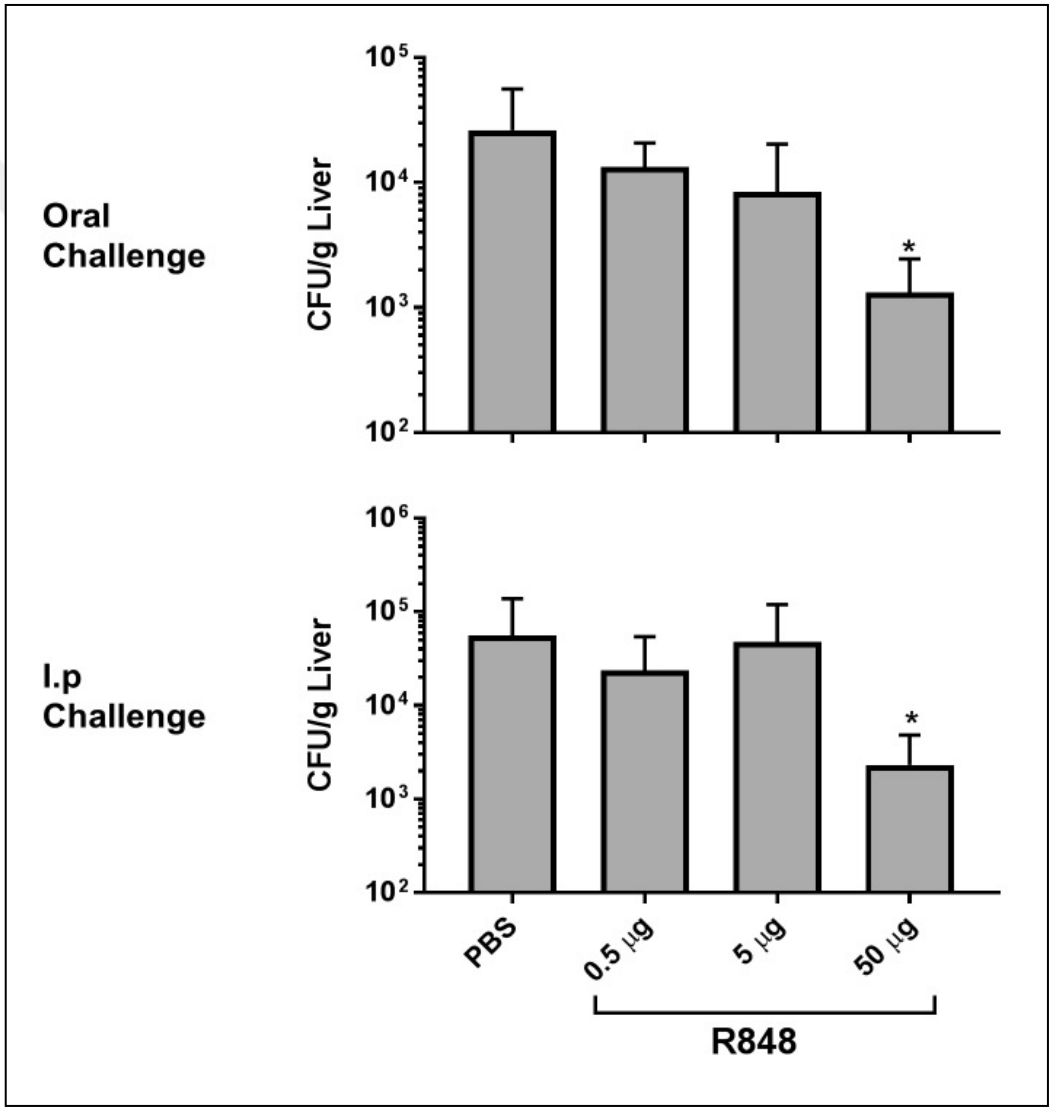

Figure 4.15. Effect of oral R848 on host resistance to intragastric and systemic *L. monocytogenes* challenge. BALB/c mice were treated orally with 50 µg of R848. Animals were challenged with 6 x 10^9 CFU *L. monocytogenes* intragastrically (upper panel) or 5x103 intraperitoneally (lower panel). Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 5 mice/group. *; $p<0.05$ vs PBS control.

Systemic CpG ODN is protective from systemic *L. monocytogenes* infection during the period from 3 to 14 days after the treatment (112, 124). The protection covers the first 2 day period of susceptibility if the treatment was given repeatedly (124). To determine the protection interval, mice were pre-treated with an effective dose of R848 (50 μg) at 28, 21, 14, 7, 3, 1 day before and in one group, simultaneously (d0) with systemic *L. monocytogenes* challenge. As shown in **Figure 4.16**, orally delivered R848 is protective from systemic *L. monocytogenes* infection when given one week to one day before the challenge. These results suggest that effects of oral R848 is quicker but might be going away faster than systemic CpG ODN treatment.

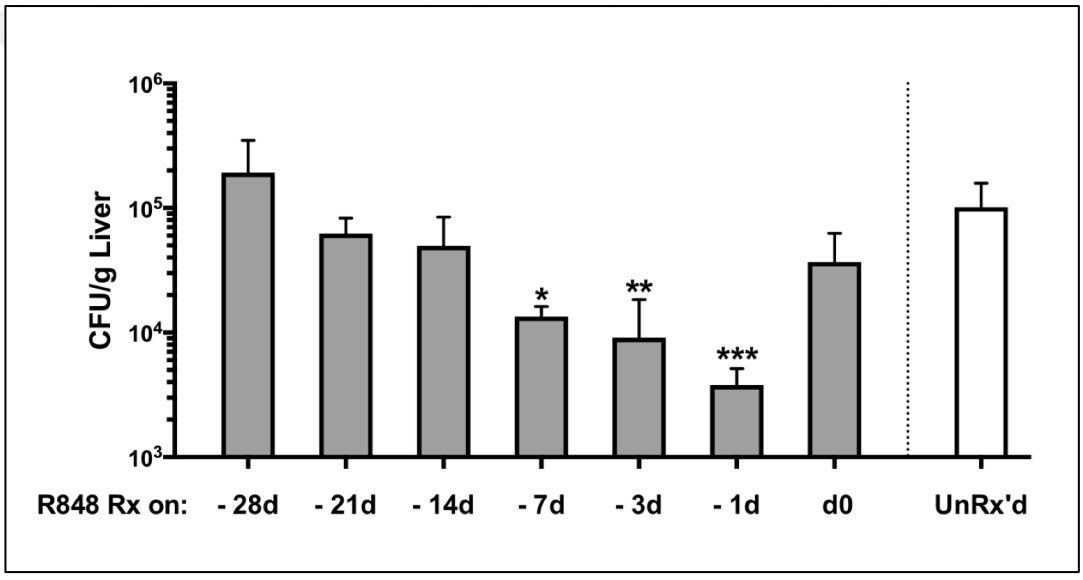

Figure 4.16. Protection interval of oral R848 treatment on systemic *L. monocytogenes* challenge. BALB/c mice were treated orally with 50 μg of R848 28, 21, 14, 7, 3, 1 days before and simultaneously (d0) with $10x10^3$ CFU *L. monoctytogenes* intraperitoneally. Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 3-4 mice/group. *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ vs UnRx'd control.

4.8. CpG ODN And R848 Do Not Synergistically Protect Against Local or Systemic *L. monocytogenes* Challenge

A better effect was observed with the combination of R848 when compared to CpG ODN alone on oral *L. monocytogenes* challenge as seen in **Figure 4.14**. The effect of TLR7/8 agonists on the *L. monocytogenes* was not known when the initial experiment was conducted. The aim was to find out whether immune activation could

be improved by oral administration of TLR7/8 and TLR9 agonists and to test the different TLR7/8 agonists available for use. Based on the results of the initial experiment, I decided to test the activity of the optimal concentration of R848 (50 μg) alone. Consistent with previous results (**Figure 4.14**), the combination of R848 with CpG ODN was significantly better than CpG ODN alone ($p<0.01$, **Figure 4.17** upper panel). However, R848 alone was already highly effective and combining it with CpG ODN did not further improve this effect. The next experiment examined the lowered dose of R848 (25 μg). The outcome was similar: the combination was better than CpG ODN alone ($p<0.01$, **Figure 4.17** middle panel) but not different than 25 μg R848 alone. Finally, a 10 μg dose of R848 was evaluated. Both CpG ODN and R848 alone had the same activity as the combination (**Figure 4.17** lower panel).

These results suggest that combining R848 with CpG ODN did not provide improved protection from *L. monocytogenes* oral challenge. To find out if the result would be different in a more severe infection model, a systemic challenge experiment was done with the lowest effective dose of R848 (10 μg) and the CpG ODN (**Figure 4.18**). Both treatments alone were effective, causing 4 orders of magnitude decrease in the bacteria counts in the liver. However, as seen in the previous results with the oral challenge experiments, the combination effect was similar to the effect of each agonist alone.

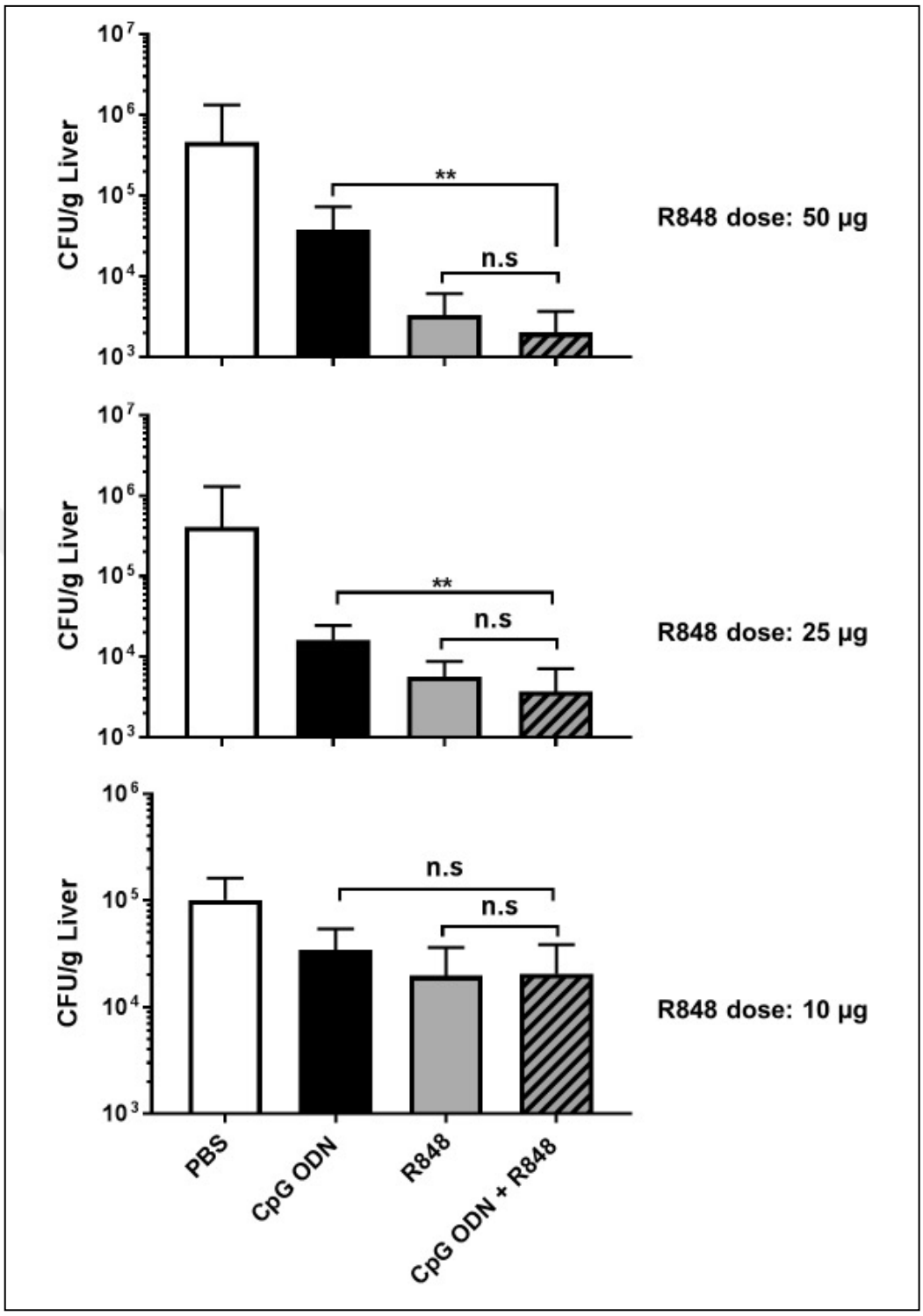

Figure 4.17. Effect of oral CpG ODN and R848 combination on host resistance to intragastric *L. monocytogenes* challenge. BALB/c mice were treated orally with 100 µg CpG ODN alone or combined with 50 µg (upper panel), 25 µg (middle panel) or 10 µg (lower panel) of R848 (in the same solution prepared in PBS). Animals were challenged with 6-9x 10^9 CFU *L. monocytogenes* intragastrically. Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 5 mice/group. *; $p<0.05$ **; $p<0.01$ vs combination

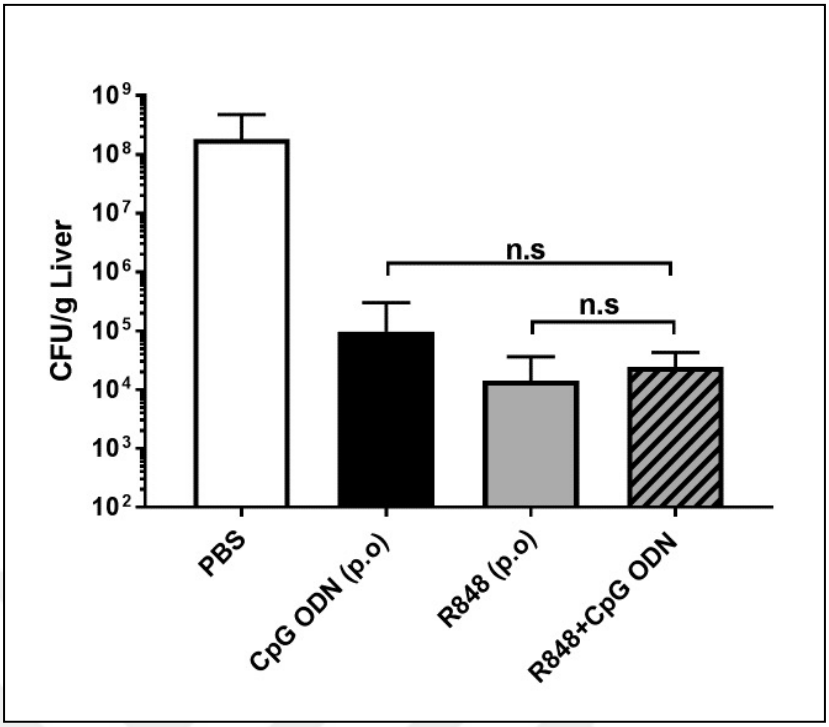

Figure 4.18. Effect of oral CpG ODN and R848 combination on host resistance to systemic *L. monocytogenes* challenge. BALB/c mice were treated orally with 100 µg CpG ODN alone or combined with 10 µg of R848. Animals were challenged with 5x 10^3 CFU *L. monocytogenes* intraperitoneally. Data show the mean + SD of bacterial counts (CFU) per gram of liver harvested 4 days after challenge from 5 mice/group.

These results altogether show that i) R848 could be considered as a candidate for orally-administrable immune modifier for the protection from infections ii) there is no significant synergism observed between TLR7/8 and TLR9 agonists in the bacterial infection prophylaxis model.

R848 functions through TLR7 in mice, whereas in human it activates both TLR7 and TLR8. To clarify the role of TLR7 vs TLR8 mediated immune regulation in humans, classical monocytes are selected as target cells expressing both receptors.

4.9. TLR 7 And 8 Agonists Induce Human Monocytes To Differentiation Into Macrophages.

Monocytes and myeloid derived suppressor cells (MDSCs) have the ability to differentiate into distinct types of macrophage (broadly classified as M1 or M2) depending on the stimuli to which they are exposed (182, 188). This feature renders monocytes and MDSCs suitable targets for immunotherapy (283). Our lab and others

have shown that TLR7 induces mMDSC to differentiate preferentially into tumoricidal M1 macrophages (282, 284) as does the TLR7/8 agonist R848 (5).

Classical human monocytes express both TLR7 and TLR8 (166). However, the role of TLR7 and TLR8 in monocyte activation and differentiation has not been characterized. To investigate this, human monocytes (CD14+, HLA DR+) were obtained from healthy donors and stimulated with the TLR7 selective agonist 3M-055, the TLR8 agonist CL075 or the dual agonist R848 for 3-6 days. Macrophage phenotypes were assessed by using the general macrophage surface marker 25F9 combined with markers associated with M2-phenotype; scavenger receptor CD163, mannose receptor CD206 and inhibitory receptor CD200R. When left untreated, only ~15% of the monocytes differentiated into macrophages (25F9+) whereas with M-CSF and IFNγ stimulation ~80% and ~40% of the monocytes differentiated, respectively. As expected, M-CSF stimulation generated a primarily M2-like phenotype (classified as CD163+CD206+CD200R+) whereas IFNγ stimulation resulted in a more M1-like phenotype (CD163- CD206-) (**Figure 4.19B**).

TLR7, 8 and 7/8 dual agonists also induced significant macrophage differentiation (about 50-60%), when compared to untreated controls ($p<0.001$, **Figure 4.19A**). TLR 7, 8 or 7/8 dual stimulation resulted in a more mixed phenotype than the M1 or M2 controls (IFNγ and M-CSF). All three agonists significantly increased the frequency of CD200R- but also CD206+ macrophages when compared to controls. TLR7 and TLR8-differentiated macrophages were distinct in terms of their expression of CD163; TLR7 agonist stimulation produced primarily CD163- whereas TLR8 or TLR7/8 agonist stimulation produced mostly CD163+ macrophages (**Figure 4.19B**). The M1:M2 ratio was calculated to analyze and quantify the dominant phenotype in these cultures. As shown in (**Figure 4.19C**), IFNγ induced more CD163-, CD206- and CD200R- macrophages than CD163+, CD206+, CD200R+ macrophages (~54 vs 13, 4-fold respectively) indicating a strong M1-dominancy. In contrast, M-CSF stimulation generated CD163+, CD206+ and CD200R+ dominated cultures (~12, 36, 5-fold respectively). TLR7 stimulation induced ~2.8 and 3.4-fold more CD163- and CD200R- macrophages than CD163+, CD200R+ macrophages, and yet generated CD206+ dominated cultures (~7.7-fold). TLR8 stimulation induced

~2.6-fold CD200R- than CD200R+ macrophages, with mixed CD163 expression (M1:M2 ratio; 1.9). Like TLR7 agonists, TLR8 agonists also induced CD206 dominant cultures (~5.9-fold). TLR7/8 dual agonist stimulation resulted in a more mixed population; ~1.6-fold induction of CD200R-, ~1.7-fold CD163- macrophages over CD200R+ and CD163+ macrophages respectively, while still inducing a slightly more dominant CD206+ population (2.6-fold).

These results suggest that TLR7-differentiated macrophages were slightly more M1-dominant then TLR8-differentiated macrophages which were a more mixed population. Both groups increase the frequency of CD206+ macrophages when compared to untreated monocytes, and had 5 to 7-fold induction of CD206+ cells over CD206-. TLR7/8 dual agonist-differentiated macrophages were phenotypically more similar to the TLR8-differentiated macrophages confirming the previous publications claiming that R848 activity on human cells having more TLR8 preference than TLR7 (147).

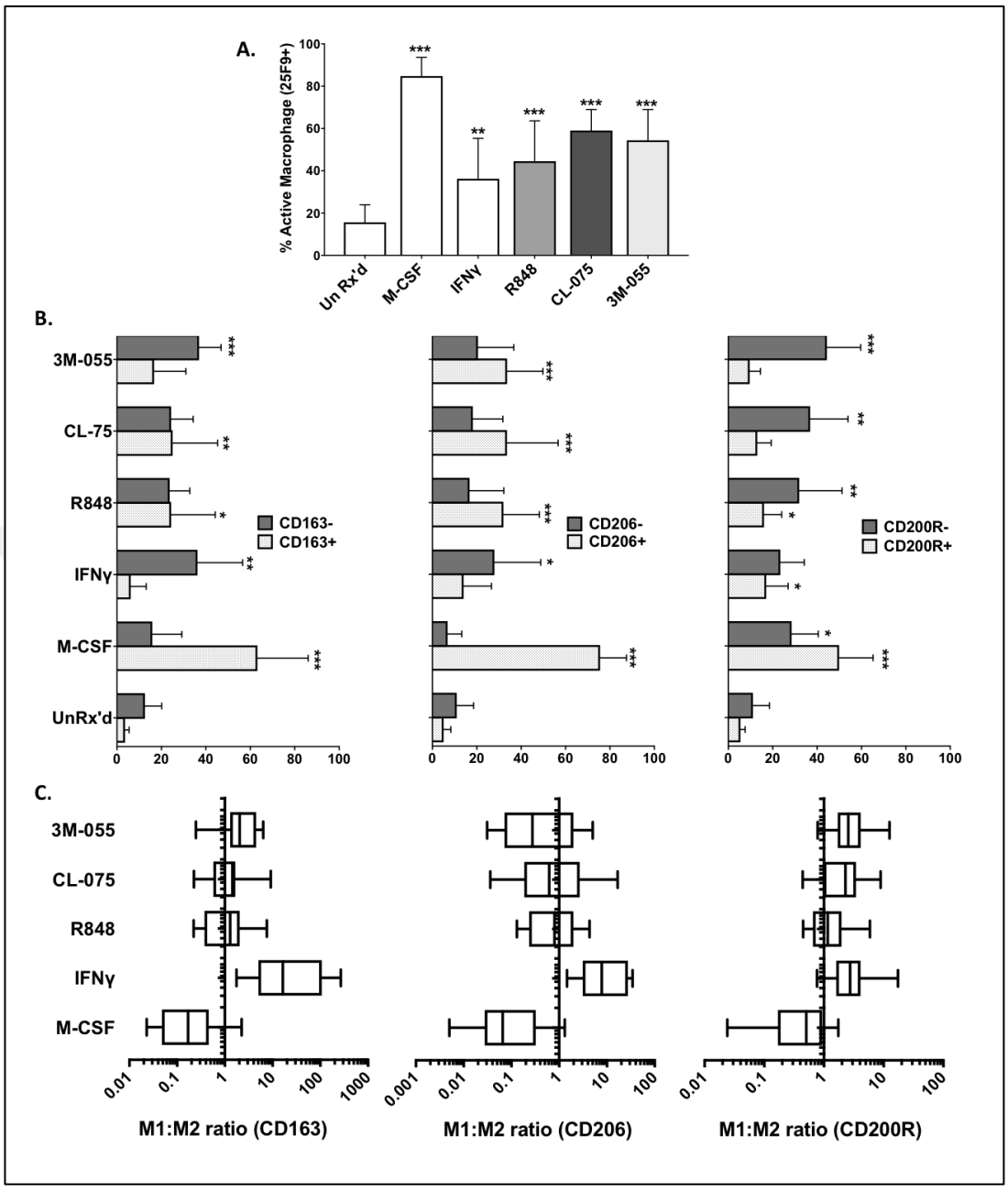

Figure 4.19. Phenotypic analysis of TLR7, 8 or 7/8-differentiated human macrophages. CD14+ HLA DR+ human monocytes were differentiated with TLR7/8 dual agonist R848 (3 μg/ml), TLR7 agonist 3M-055 (3 μM), TLR8 agonist CL075 (300 ng/ml), M-CSF and IFNγ (both 500 ng/ml) or left untreated for 3-6 days. A) Percentage of differentiated macrophages (25F9+) B) Percentage of 25F9+ CD163+/-, 25F9+ CD200R+/-, 25F9+ CD206+/- cells C) M1:M2 ratio defined by the expression of CD163, CD206 and CD200R as M2-macrophage markers. Mean ±SD of 10-16 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ compared to UnRx'd.

TLR7 and TLR8 differentiated macrophages showed a more mixed phenotype when compared to IFNγ-differentiated (M1-like) or M-CSF-differentiated (M2-like)

macrophages. Both agonists supported the generation of CD200R- as well as CD206+ macrophages that can be described as M1 and M2-like respectively. Because of the discrepancy between phenotypes of the resultant macrophages, a functional assay was performed to determine if those cells behave more like M1 or M2 macrophages. Endocytic ability, which is a prominent M2-like macrophage function, was evaluated by assessing the uptake of fluorescent-labeled dextran particles. Macrophage generated after 5 days in culture with the indicated stimulants were incubated with fluorescent-labelled particles for 45 minutes. Mean Fluorescent Intensity (MFI) values were calculated as described in the methods section. As expected, M2-dominated cultures from M-CSF-differentiated macrophages were highly endocytic ($p<0.001$, **Figure 4.20**) as opposed to IFNγ–differentiated M1-dominant macrophages. TLR7, 8 or 7/8- differentiated macrophages did not have significant endocytosis ability when compared to untreated controls. These data suggest that although they promote the differentiation of phenotypically M2- like macrophages (CD206+ and CD163+ on TLR8-differentiated), TLR7 and TLR8-differentiated macrophages do not have prominent M2-like function.

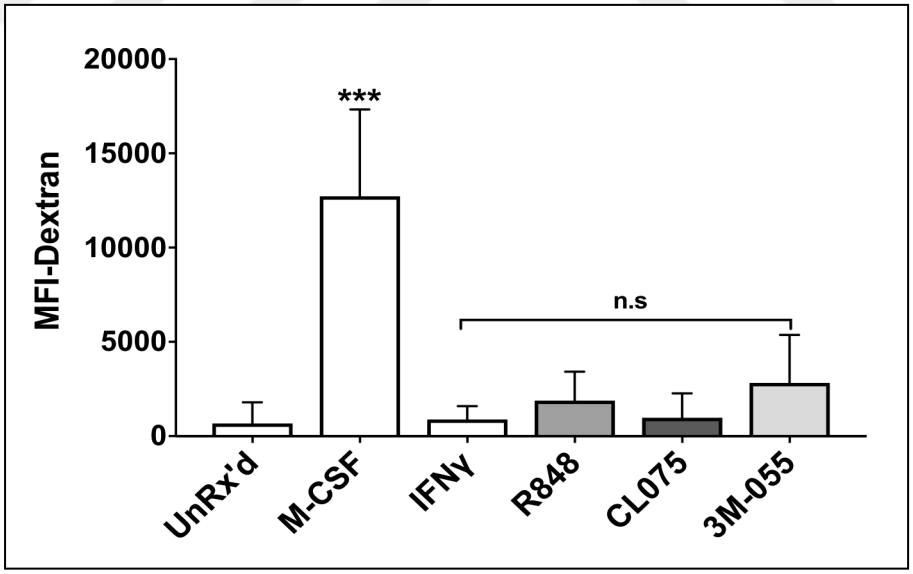

Figure 4.20. Endocytosis capability of the TLR7, 8 or 7/8-differentiated macrophages. Mean MFI value of fluorescent labeled-dextran treated macrophages generated by TLR7/8 dual agonist R848 (3 μg/ml), TLR7 agonist 3M-055 (3 μM), TLR8 agonist CL075 (300 ng/ml), M-CSF and IFNγ (both 500 ng/ml). Mean ±SD of 6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ compared to UnRx'd.

4.10. TLR8 But Not TLR7 Stimulation Induces Pro-Inflammatory Cytokine Production From Monocytes.

Human monocytes responded to TLR7 and TLR8 specific stimulation by differentiating into macrophages with a mixed phenotype. Studies show that stimulation of a mixed human PBMC population with TLR8 agonists results in the prominent release of TNFα mainly from monocytes and mDCs, whereas TLR7 stimulation results in Type-I interferon secretion mainly from pDCs (6). To further characterize the TLR7 vs TLR8 specific responses of human monocytes, cytokine production after TLR7, 8 or 7/8 dual agonist- stimulation was assessed. For these studies, the same ligand types and doses that induced optimal macrophage differentiation were used. Consistent with the previous studies, agonists that have TLR8 activity (CL075 and R848) induced significant amounts of pro-inflammatory cytokine production (TNFα, IL-12 and IL-6) as well as IL-10 (**Figure 4.21**). TLR7 agonist stimulation did not induce significant pro-inflammatory cytokine release. IL-10 was the only cytokine significantly induced via TLR7 ($p<0.05$, **Figure 4.21**) but the amount of IL-10 was lower than in cultures stimulated via TLR8. None of the agonists induced Type I IFN production (IFNα and IFNβ) from monocytes.

Cytokine responses to other TLR7 and TLR8 agonists were also assessed to determine whether these findings were generalizable. A dose study was done with the TLR7 specific agonists imiquimod, gardiquimod, loxoribine, CL-264, CL-307 and the TLR8 specific agonist; TL8-506. Consistent with results described above, TL8-056 was the only agonist that induced significant TNFα, IL-6 and IL-10 secretion (**Figure 4.22**). Of the TLR7 agonists; gardiquimod induced IL-6 and IL-10 while loxoribine induced only IL-6 (and only at the highest concentration). The concentration of these cytokines was considerably smaller than that elicited by TLR8 stimulation.

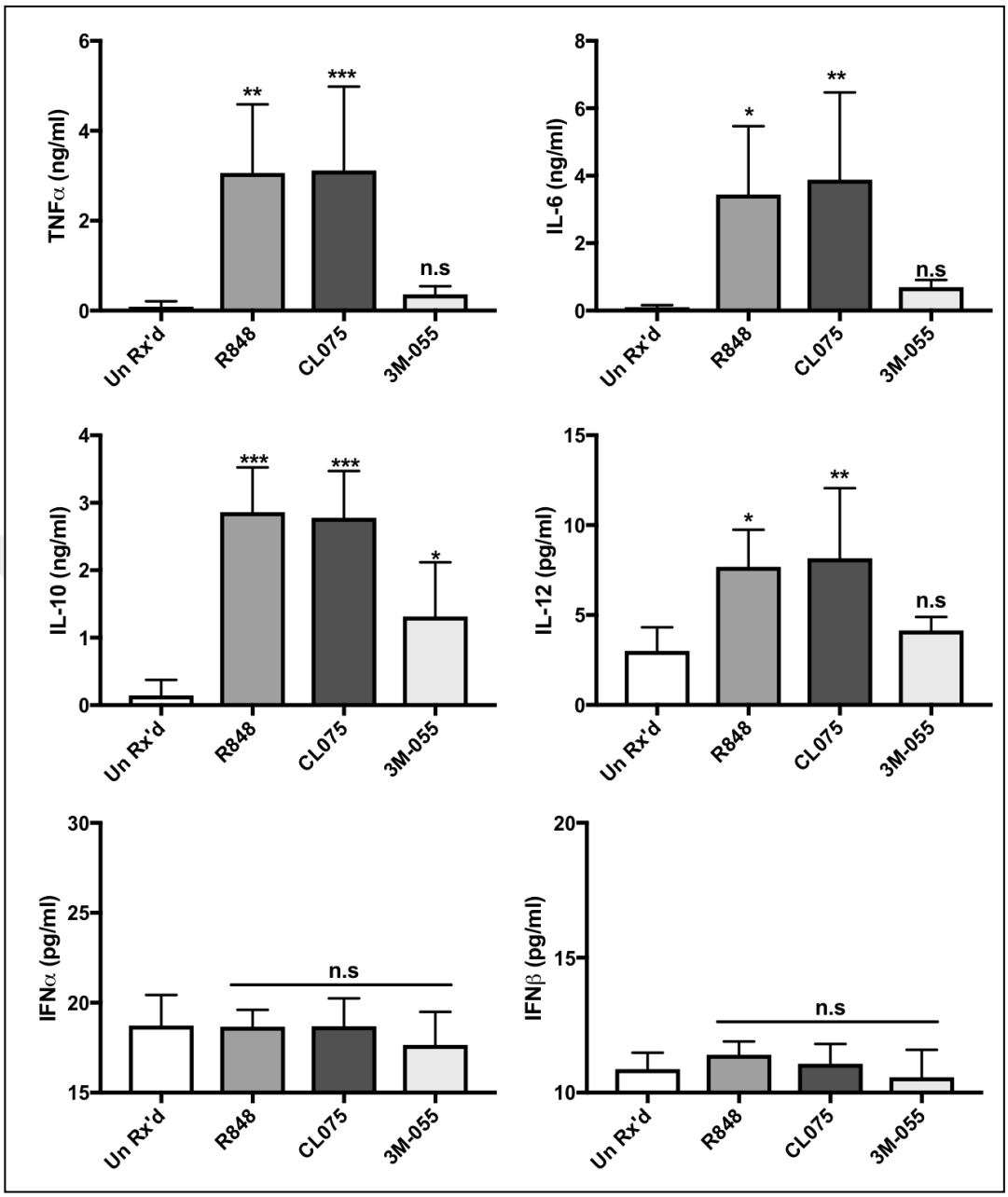

Figure 4.21. Cytokine response of monocytes to TLR7 or TLR8 stimulation. TNFα, IL-6, IL-10, IL-12, IFNα, IFNβ amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. R848 (TLR7/8 dual agonist, 3 μg/ml) 3M-055 (TLR7 agonist, 3 μM), CL075 (TLR8 agonist, 300 ng/ml) used. Mean ±SD of 4-6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ compared to UnRx'd.

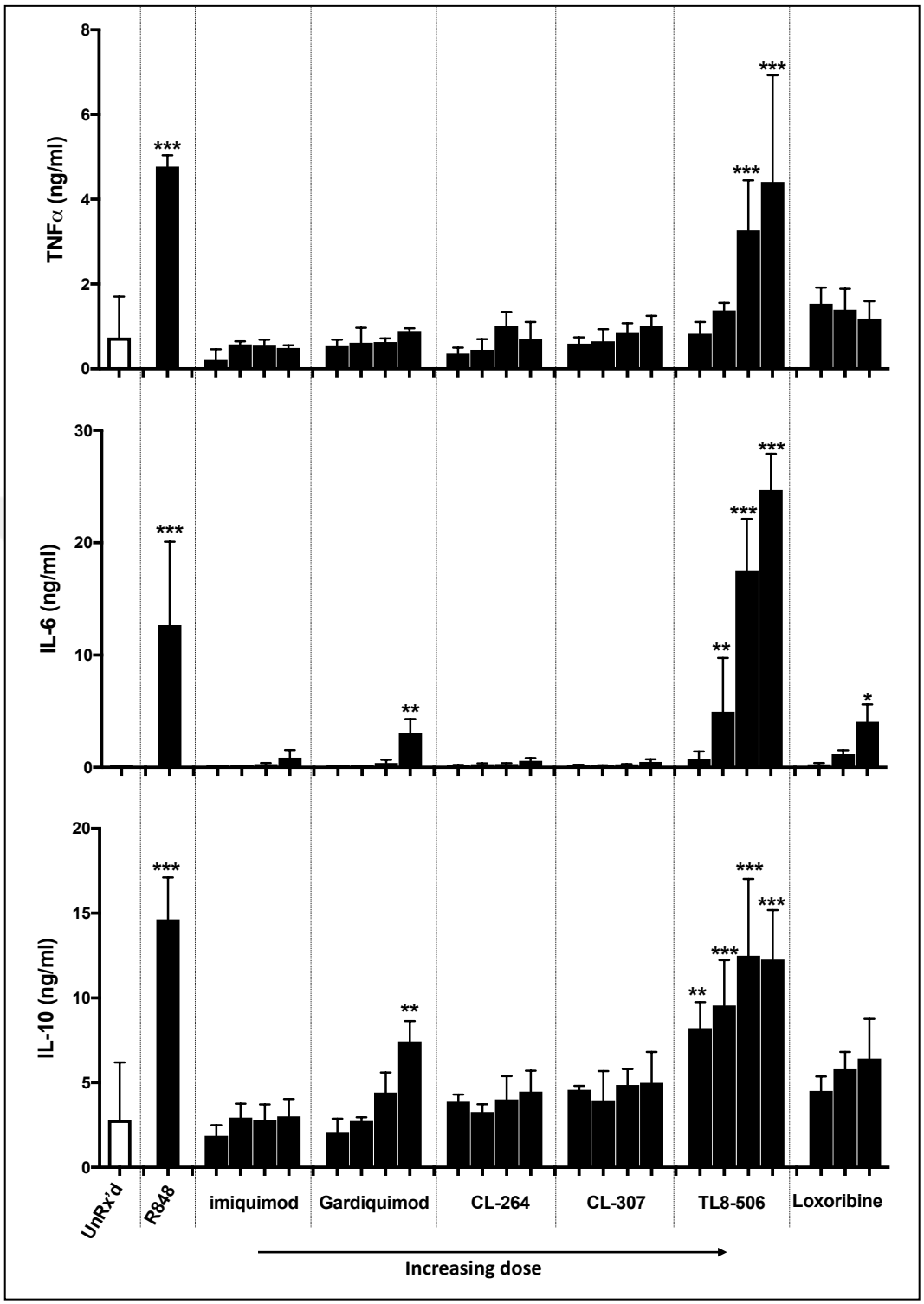

Figure 4.22. Cytokine response of monocytes to various TLR7 or TLR8 ligands. 3-fold dilutions of imiquimod (0.3-10 μg/ml), gardiquimod (0.3-10 μg/ml), CL-264 (1-30 μg/ml), CL-307 (0.1-3 μg/ml), TL8-506 (10-300 ng/ml), loxoribine (0.1 mM-1 mM) and R848 (3 μg/ml) used. TNFα, IL-6 and IL-10 amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. Mean ±SD of 4 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ compared to UnRx'd.

4.11. TLR7 Activation Regulates The Response To TLR8 Engagement

The interaction between these two closely related TLRs was investigated in some of the previous studies. HEK 293 cells transfected with TLR7 were responsive to TLR7 agonists but when co-transfected with TLR7 and TLR8, they become unresponsive to TLR7 ligand stimulation. Interestingly, mouse TLR8 (which is considered to be inactive) also inhibited the activation of both human and mouse TLR7 when co-transfected into HEK 293 cells (285). Another study shows that TLR8 -/- mice were hyper responsive to TLR7 ligation (79). These studies suggest that presence of TLR8 blocks TLR7 responsiveness. Yet this phenomenon was never documented under physiologic conditions. As human monocytes express both receptors, I concluded they would be suitable in which targets to test the interaction between TLR7 and TLR8.

Monocytes were stimulated for 24 hours with TLR7, TLR8, TLR7/8 or TLR7 plus TLR8 agonists. The same ligand types and doses that induced optimal macrophage differentiation were used in these studies. To overcome the challenge of variability between the response of individual human donors (e.g. some donors does not respond as strong as others, independent of the stimuli) cytokine values were normalized against a constant stimulus in each group (*L. rhamnosus* stimulation selected as they induce broad cytokine responses in most of the donors). As shown in (**Figure 4.23**), agonists with TLR8 activity elicited the production of significant amounts of cytokine. However, simultaneous stimulation via TLR7 and TLR8 induced significantly less cytokine release. This reduction was of larger magnitude among the TLR8-characteristic pro-inflammatory cytokines; TNFα and IL-12 whereas the reduction in IL-6 and IL-10 levels was not significant (**Figure 4.23**). A further study was done to find out if this behavior was dose-dependent. Increased doses of 3M-055 (0 to 10 μM) were combined with CL075 (0 to 3 μg/ml, equals 12 μM) and cytokine production assessed after 24 hours. As shown in (**Figure 4.24**), TNFα amounts were maximized at 0.3 μg/ml of CL075 and the production of this cytokine decreased as more 3M-055 was added during culture ($p<0.05$ at 3 μM and 10 μM of 3M-055). IL-10 and IL-6 was maximized at lower doses of CL075 and remained steady up to 3

μg/ml. Reductions in IL-10 and IL-6 observed at 3M-055 concentrations of 3 or 10 μM ($p<0.05$).

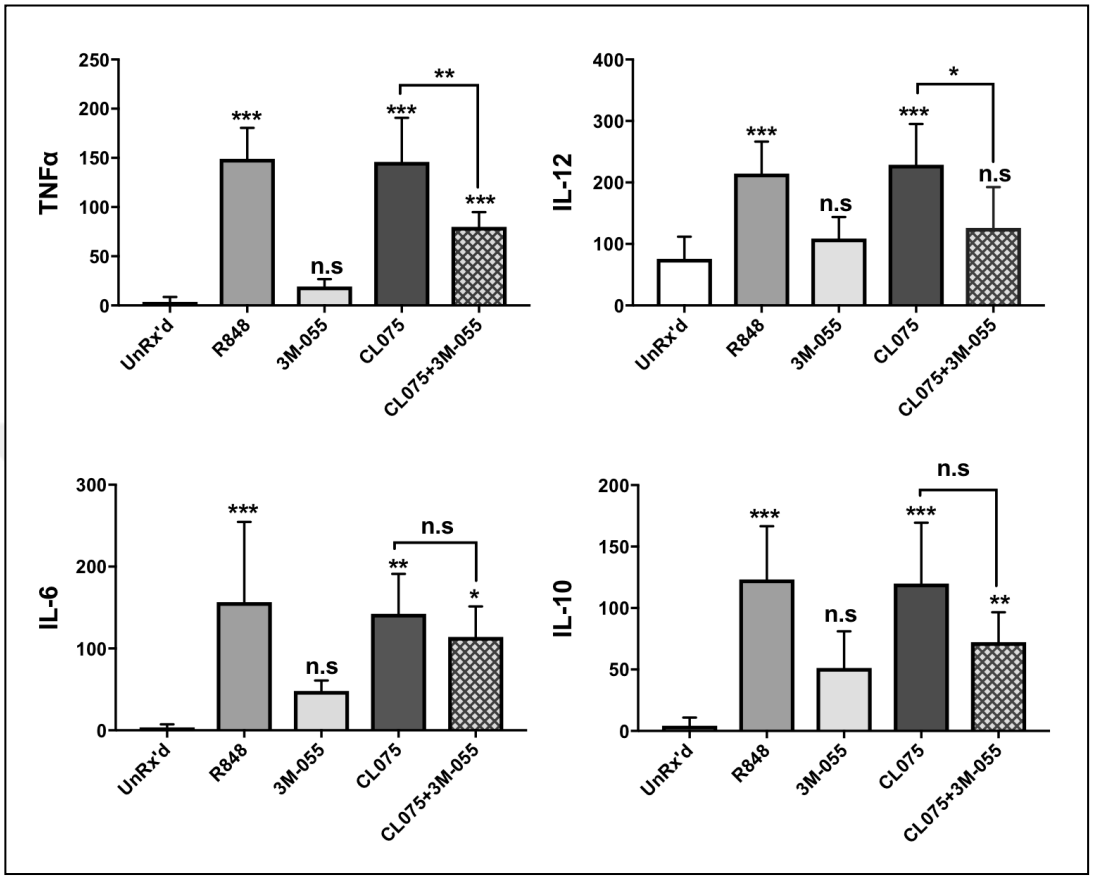

Figure 4.23. Effect of TLR7 agonist on TLR8 cytokine response. TNFα, IL-6, IL-10 and IL-12 amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. R848 (TLR7/8 dual agonist, 3 μg/ml) 3M-055 (TLR7 agonist, 3 μM), CL075 (TLR8 agonist, 300 ng/ml), and combination of 3M-055 (3 μM) plus CL075 (300 ng/ml) used. Mean ±SD of normalized values from 4-6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$ compared to UnRx'd,

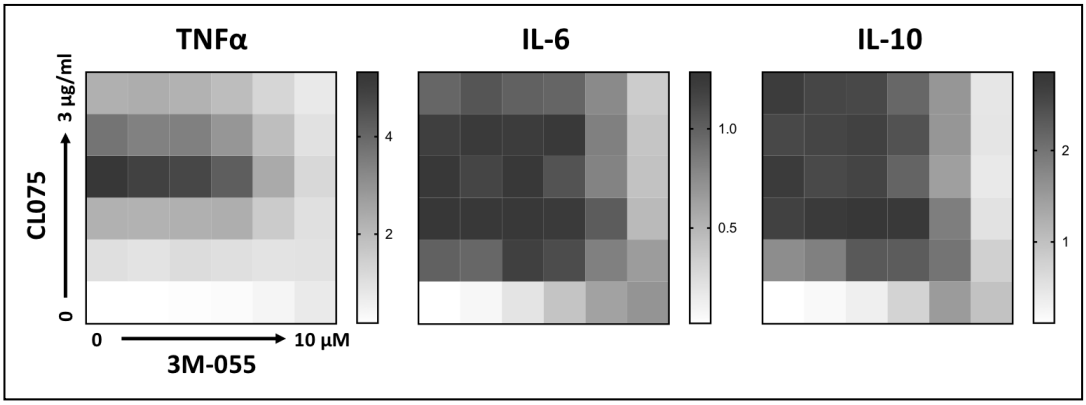

Figure 4.24. Dose-dependent effect of TLR7 agonist on TLR8 cytokine response. 0-0.1-0.3-1-3-10 μM of 3M-055 was combined with 0-0.03-0.1-0.3-1-3 μg/ml CL075. TNFα, IL-6 and IL-10 amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. Heat-map graphs represent the mean values from 3 independently analyzed donors.

Reasons of the effect of 3M-055 on CL075 could be due to physical interactions at the receptor level or regulation of downstream pathways. There is also a possibility that these effects were specific only to 3M-055 or CL075. To further investigate these issues, a time- dependent co-stimulation study was done with another TLR7 agonist, imiquimod (IMQ), and TLR8 agonist, TL8-506. Monocytes were stimulated with TLR7, TLR8 or both agonists simultaneously or with a 3-hour delay. TNFα, a characteristic TLR8 cytokine, was quantified. As shown in (**Figure 4.25A),** 3-hour pre-treatment with 3M-055 resulted in a significant reduction in the subsequent response to TLR8 stimulation. Pre-treatment with 3M-055 yielded an ~ 80% and 65% reduction whereas simultaneous treatment resulted in ~15% and 10% reduction when compared to CL075 and TL8-506 stimulated groups respectively (**Figure 4.25C**). Another TLR7 agonist, imiquimod also caused a significant reduction in TNFα levels when given 3 hours before the TLR8 agonist (**Figure 4.25B**). The magnitude of the reduction was ~90% and 80% when given 3 hours before, whereas simultaneous stimulation resulted in ~10% and 25% reduction from CL075 and TL8-506 stimulated groups respectively (**Figure 4.25C).**

These results indicate that this blocking effect was more prominent with increased amounts of TLR7 agonist and when TLR7 agonists were given before TLR8 agonists, suggesting that this effect could be attributable to either physical interactions or the regulation of downstream pathways. To differentiate between these possibilities, the relationship between TLR7 ligands and other MyD88-dependent TLRs was investigated. The TLR4 agonist lipopolysaccharide (LPS) and TLR2 agonist peptidoglycan (PGN) were tested in combination with TLR7 agonists, 3M-055 and imiquimod in a time-dependent manner, as described previously. As shown in (**Figure 4.26**), pre-treatment with either TLR7 agonists did not result in a reduction in TNFα release by TLR2 (**Figure 4.26A)** or TLR4 (**Figure 4.26B**) stimulation. On the contrary, an improved response was observed when 3M-055 was co-administered with those agonists. These results suggest that the blocking effect of a TLR7 agonist on the

TNFα response to TLR8 stimulation is not related to an interference with the common pathways but specific to TLR8 signaling either at the receptor level or downstream.

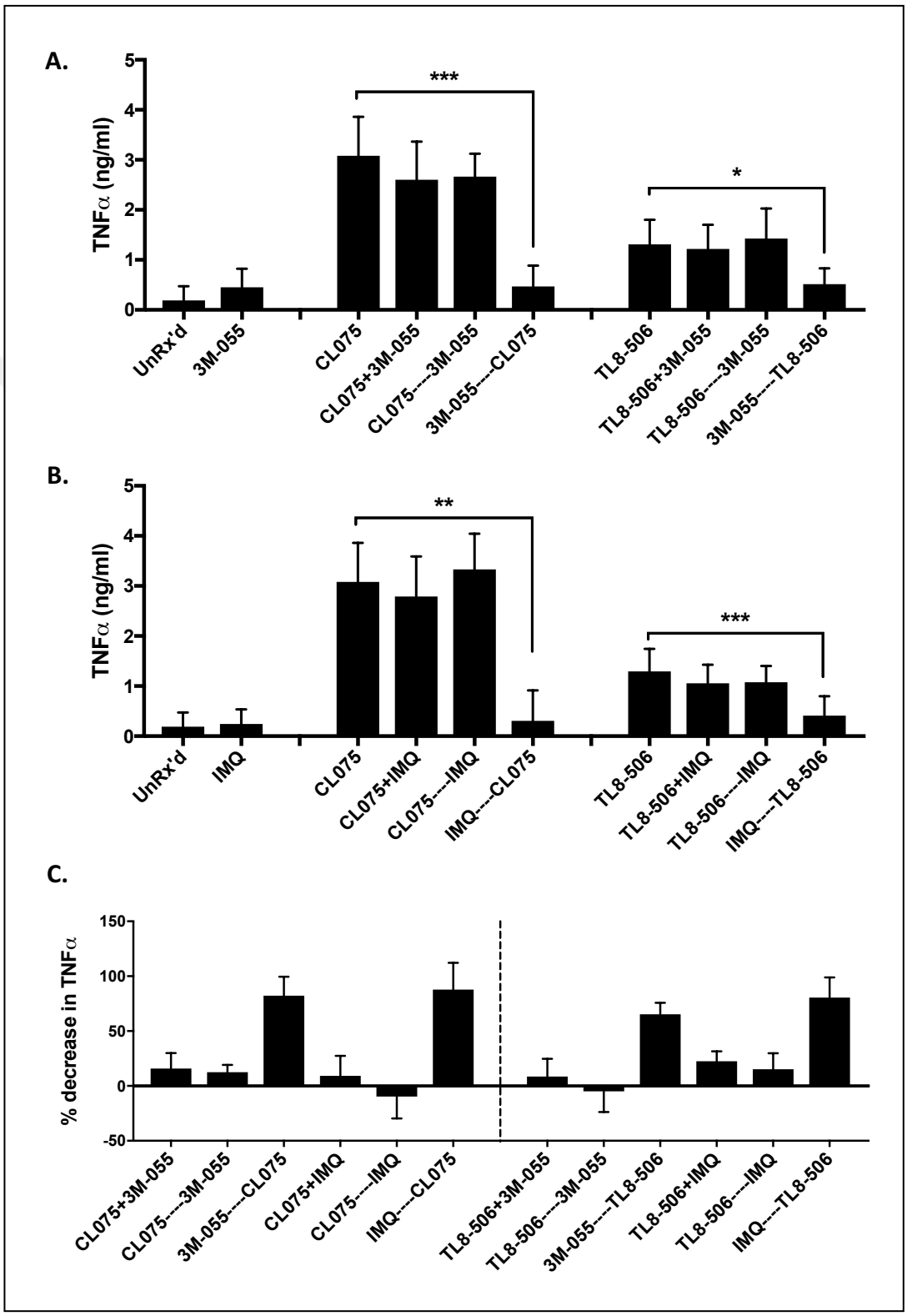

Figure 4.25. Time dependent effect of different TLR7 agonists on TLR8 cytokine response. 3M-055 (TLR7 agonist, 3 μM), CL075 (TLR8 agonist, 300 ng/ml), Imiquimod (IMQ, TLR7 agonist, 1 μg/ml), TL8-506 (TLR8 agonist, 100 ng/ml) used. Simultaneous stimulations were indicated with "+". Subsequent stimulations were indicated as first stimuli ---- second stimuli (3-hour time interval). TNFα amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. A) The effect of 3M-055 on CL075 and TL8-506 B) The effect of imiquimod on CL075 and TL8-506 C) % decrease in cytokine levels with TLR7 agonist combinations compared to the TLR8 agonists alone. Mean ±SD from 4-6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$.

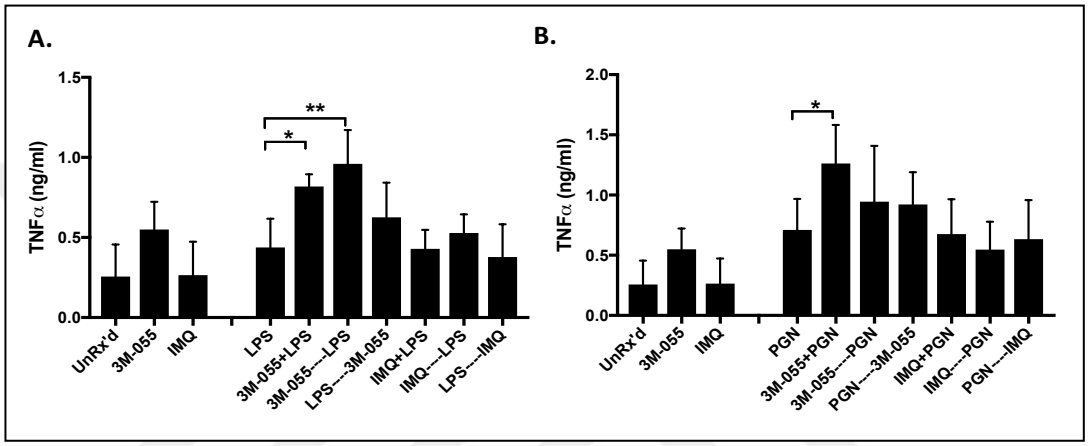

Figure 4.26. Effect of TLR7 agonists on TLR2 and TLR4 agonist response. 3M-055 (TLR7 agonist, 3 μM), Imiquimod (TLR7 agonist, 1 μg/ml), LPS (TLR4 agonist, 1 μg/ml) and PGN (TLR2 agonist, 1 μg/ml) used. Simultaneous stimulations were indicated with "+". Subsequent stimulations were indicated as first stimuli ---- second stimuli (3-hour time interval). TNFα amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. A) The effect of 3M-055 and imiquimod on LPS B) The effect of 3M-055 and imiquimod on PGN. Mean ±SD from 4-6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$.

4.12. TLR2 And TLR4 Activation Regulate The Response To TLR8

As described above, TLR7 agonists block TNFα production elicited via activation of TLR8 but not TLR2 or TLR4. To determine whether TLR2 or TLR4 could affect the TLR8 response, a time-dependent co-stimulation study was done. As shown in (**Figure 4.27A),** LPS pre-treatment inhibited the activation induced by subsequent TLR8 agonist stimulation (as reflected by a ~65% reduction in TNFα (**Figure 4.27C**). However, when the TLR8 agonist was given first, subsequent LPS treatment further increased TNFα production by ~35-45% (**Figure 4.27A, C).** Similar to LPS, PGN pre-treatment also reduced the secretion of TNFα following TLR8

stimulation by ~55% (**Figure 4.27B, D).** When given after the TLR8 agonist, however, PGN increased TNFα levels significantly by ~30-35% (**Figure 4.27B, D).**

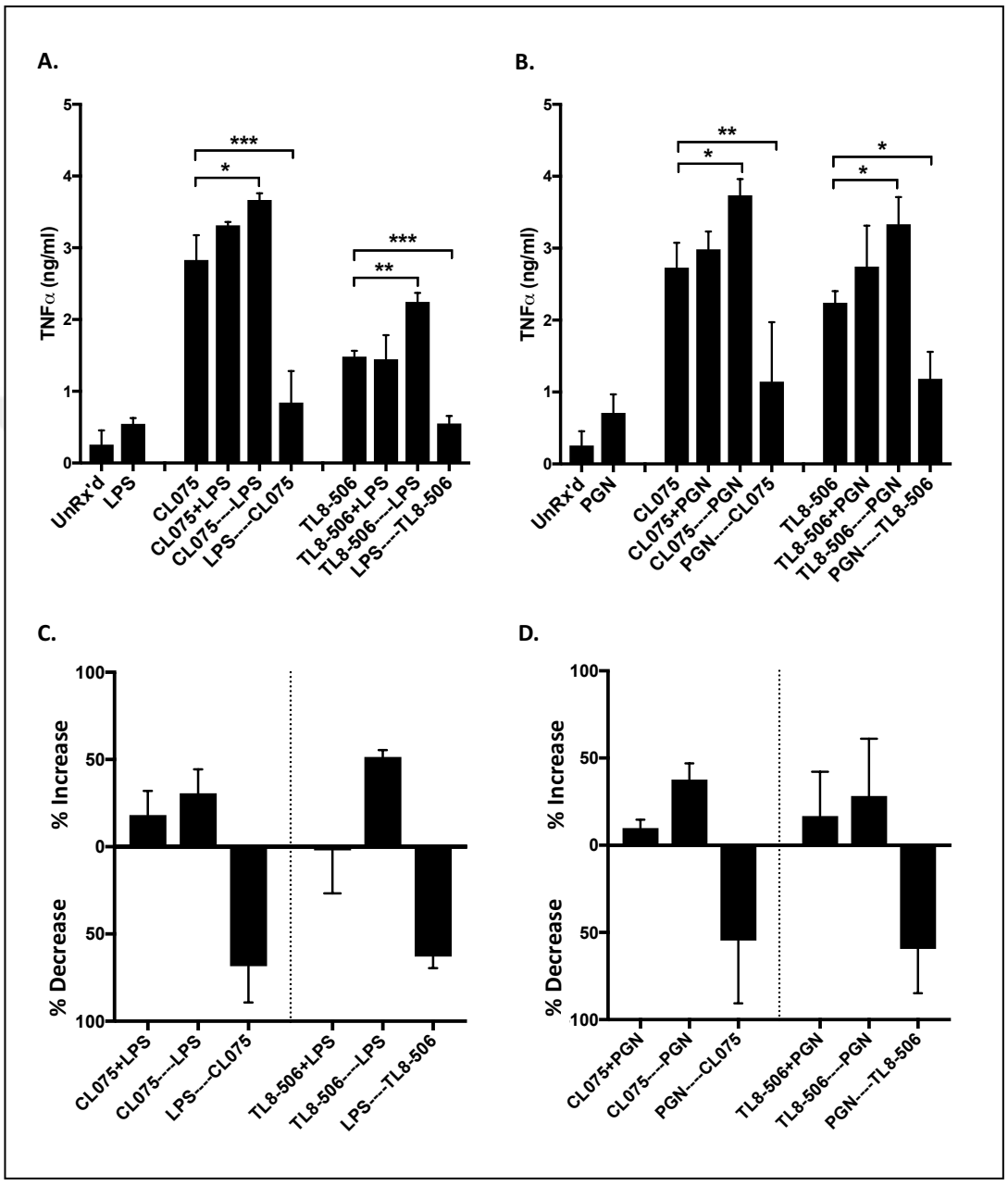

Figure 4.27. Effect of TLR2 and TLR4 agonists on TLR8 response. CL075 (TLR8 agonist, 300 ng/ml), TL8-506 (TLR8 agonist, 100 ng/ml), LPS (TLR4 agonist, 1 μg/ml) and PGN (TLR2 agonist, 1 μg/ml) used. Simultaneous stimulations were indicated with "+". Subsequent stimulations were indicated as first stimuli ---- second stimuli (3-hour time interval). TNFα amounts in the supernatants of 24-hour cultured monocytes measured by ELISA. A) The effect of LPS on CL075 and TL8-506 B) The effect of PGN on CL075 and TL8-506 C, D) % decrease in TNFα levels with LPS (C) and PGN (D) combinations compared to the TLR8 agonists alone. Mean ±SD from 4-6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$.

These results showed that TLR8 TNFα response is strongly affected by previous TLR stimulation by TLRs 7, 2 and 4. However, pre-treatment with TLR8 agonist does not affect the subsequent TNFα response to TLR 2 or 4 stimulations. While studies to understand the relationship between these TLRs, we then assessed the effect of multiple TLR ligand-containing heat killed bacteria effect on human monocytes.

4.13. Monocytes Respond Differentially To Heat-Killed Bacterial Stimulation

Gram positive and negative organisms express some surface TLR ligands in common (e.g., PGN and lipoproteins) whereas other ligands (e.g., LPS) are exclusive to gram - while others (e.g., lipoteichoic acid) are limited to gram + bacteria. The specific contribution of each TLR to the response induced by intact bacteria is poorly understood. Several studies showed that gram + organisms induced robust TNFα and IL-12 secretion whereas gram – bacteria induced more IL-6, IL-10, and IL-8 dominated responses (286, 287). Yet no general rule for predicting the response based on gram + or – classification has been identified, and there might be other molecules or combinations of molecules responsible for the observed differences in response.

We initially investigated the response of human monocytes to several different gram + or - heat-killed bacteria. Exposure to these heat-killed bacteria induced the monocytes to differentiate into macrophage with any of several different phenotypes (**Figure 4.28A**). As shown in (**Figure 4.28B),** *L. rhamnosus* and *S. epidermidis* favored the generation of an M1 associated phenotype whereas *E. coli, H. pylori* and *L. monocytogenes* preferentially generated M2-like macrophage (based on the expression of CD163). Endocytosis assays were conducted to assess the functionality of the resultant macrophages. Consistent with the predicted phenotype, *L. rhamnosus* and *S. epidermidis*-differentiated macrophages lacked endocytic ability whereas phenotypically M2-dominant cultures of *E. coli, H. pylori* and *L. monocytogenes*-differentiated macrophages expressed significant endocytic activity as determined by increased dextran particle uptake compared to untreated controls (**Figure 4.28C**). The most dominant M1 differentiation was achieved by *L. rhamnosus* stimulated and the

most dominant M2 differentiation by *E. coli* and *H. pylori* stimulation. *L. rhamnosus* and *E. coli* were therefore selected for further studies of cytokine production as inducers of M1 and M2 macrophage, respectively. The activity of *L. monocytogenes*, a gram + bacterium that also generated M2-dominant cultures, was also analyzed.

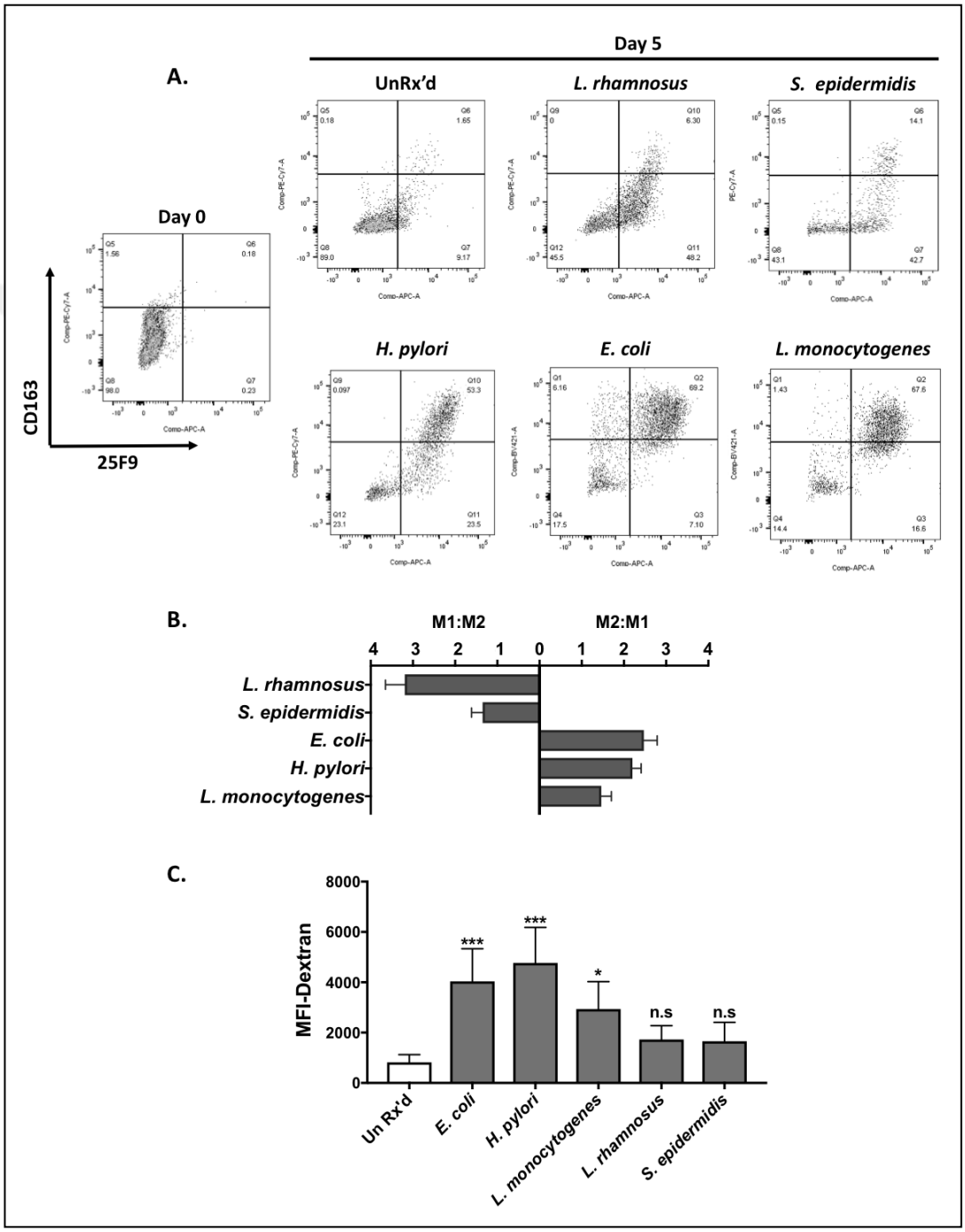

Figure 4.28. Heat killed bacteria differentially induce monocyte differentiation into macrophage. Monocytes stimulated with heat killed *L. rhamnosus* (10^7/ml), *S. epidermidis* (10^7/ml), *H. pylori* (10^8/ml), *E. coli* (10^8/ml), *L. monocytogenes* (10^8/ml)

for 5 days. A) Representative dot plots showing 25F9 and CD163 expression B) Shows ratio of M1 (25F9+CD163-) and M2 (25F9+CD163-) macrophages, (n=15) C) Mean MFI value of fluorescent labeled-Dextran treated macrophages (n=6). *; p<0.05, ***; p<0.001 compared to UnRx'd.

As seen in (**Figure 4.29**), 24-hour stimulation of monocytes with heat killed *E. coli, L. rhamnosus* and *L. monocytogenes* induced different cytokine profiles. All three bacteria induced significant IL-6 production. *L. rhamnosus* induced TNFα and IL-12 whereas *E. coli* did not. Rather, IL-10 was significantly induced in *E. coli* treated cultures. *L. monocytogenes* also did not induce production of TNFα or IL-12 but like *E. coli* induced significant amounts of IL-10.

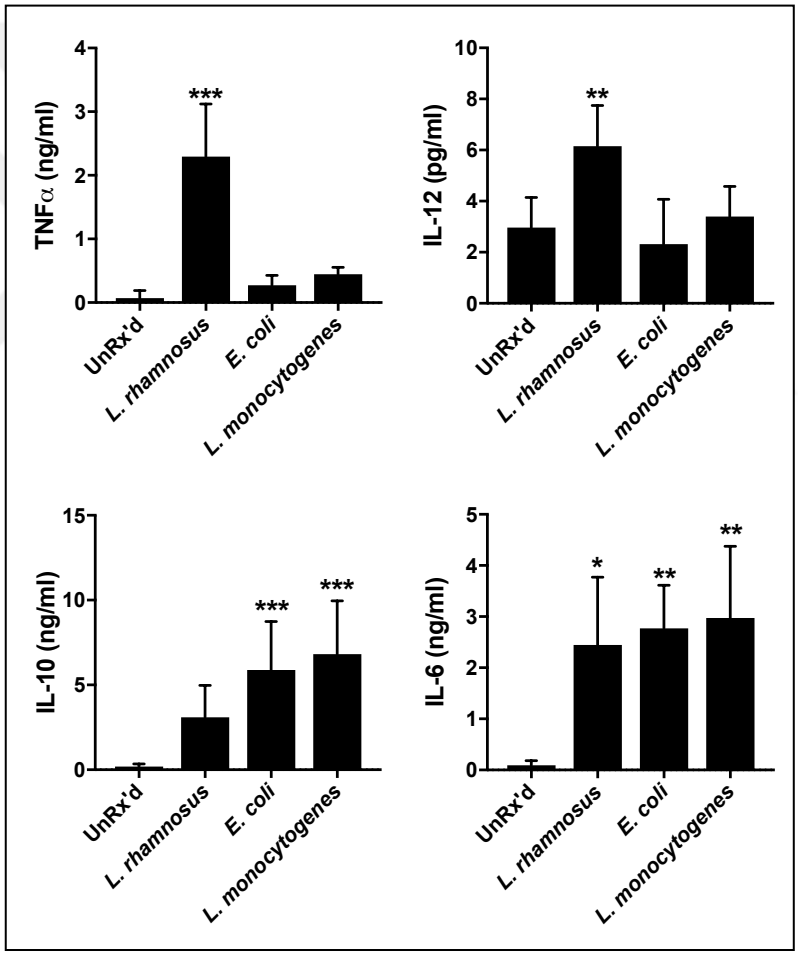

Figure 4.29. Heat killed bacteria induce different cytokine profiles from monocytes. Monocytes stimulated with heat killed *L. rhamnosus* (10^7/ml), *E. coli* (10^8/ml) and *L. monocytogenes* (10^8/ml) for 24 hours. TNFα, IL-12, IL-10 and IL-6 amounts were measured by ELISA. Mean ±SD from 6 independently analyzed donors, *; p<0.05 **; p<0.01 ***; p<0.001 compared to UnRx'd.

These results show that *L. rhamnosus* induces a strong pro-inflammatory response characterized by TNFα and IL-12 secretion accompanied by the differentiation of monocytes into phenotypically and functionally M1-like macrophage whereas *E. coli* and *L. monocytogenes* induced a prominent IL-10 response and the preferential generation of M2-like macrophages.

4.14. Heat Killed *E. coli* And *L. monocytogenes* Can Influence The Production Of Pro-Inflammatory Cytokines Induced By *L. rhamnosus*

After observing the differences in the cytokine profile induced by different strains of heat killed bacteria when cultured with monocytes, combination studies were done to assess which response was dominant. *E. coli* and *L. monocytogenes* themselves were weak inducers of TNFα and IL-12 but strong inducers of IL-10 therefore, their effect on *L. rhamnosus* was assessed. As shown in (**Figure 4.30A**), the TNFα and IL-12 secretion elicited by *L. rhamnosus* (LR) were significantly induced when *E. coli* (EC) or *L. monocytogenes* (LM) were added during culture. In contrast, the IL-10 production induced by *E. coli* and *L. monocytogenes* was not significantly inhibited by the addition of *L. rhamnosus*.

The cytokine profile induced by stimulation of human monocytes with *E. coli* was previously investigated (288), but the effect of adding *E. coli* to cultures stimulated by *L. rhamnosus* was not shown before. Although *L. monocytogenes* had the similar effect, the behavior of the *E. coli* might be attributable to the presence of the LPS. To address that possibility, monocytes were co-cultured with LPS derived from *E. coli* (O111:B4) alone or in combination with *L. rhamnosus* or *L. monocytogenes.* As shown in **(Figure 4.30B)** unlike *E. coli*, LPS addition did not have an effect on the TNFα, IL-12 or IL-10 levels induced by *L. rhamnosus*. The cytokine response induced by *L. monocytogenes* was also unaffected by LPS addition.

These results suggest that *E. coli* and *L. monocytogenes* both induce a suppressive response by the monocytes as evidenced by the resultant macrophage phenotype, cytokine profile and the reduction effect on the pro-inflammatory response of *L. rhamnosus*. This behavior cannot be attributable to LPS, since *L. monocytogenes*

does not produce LPS and LPS did not decrease the amount of pro-inflammatory cytokines in *L. rhamnosus* stimulated monocyte cultures.

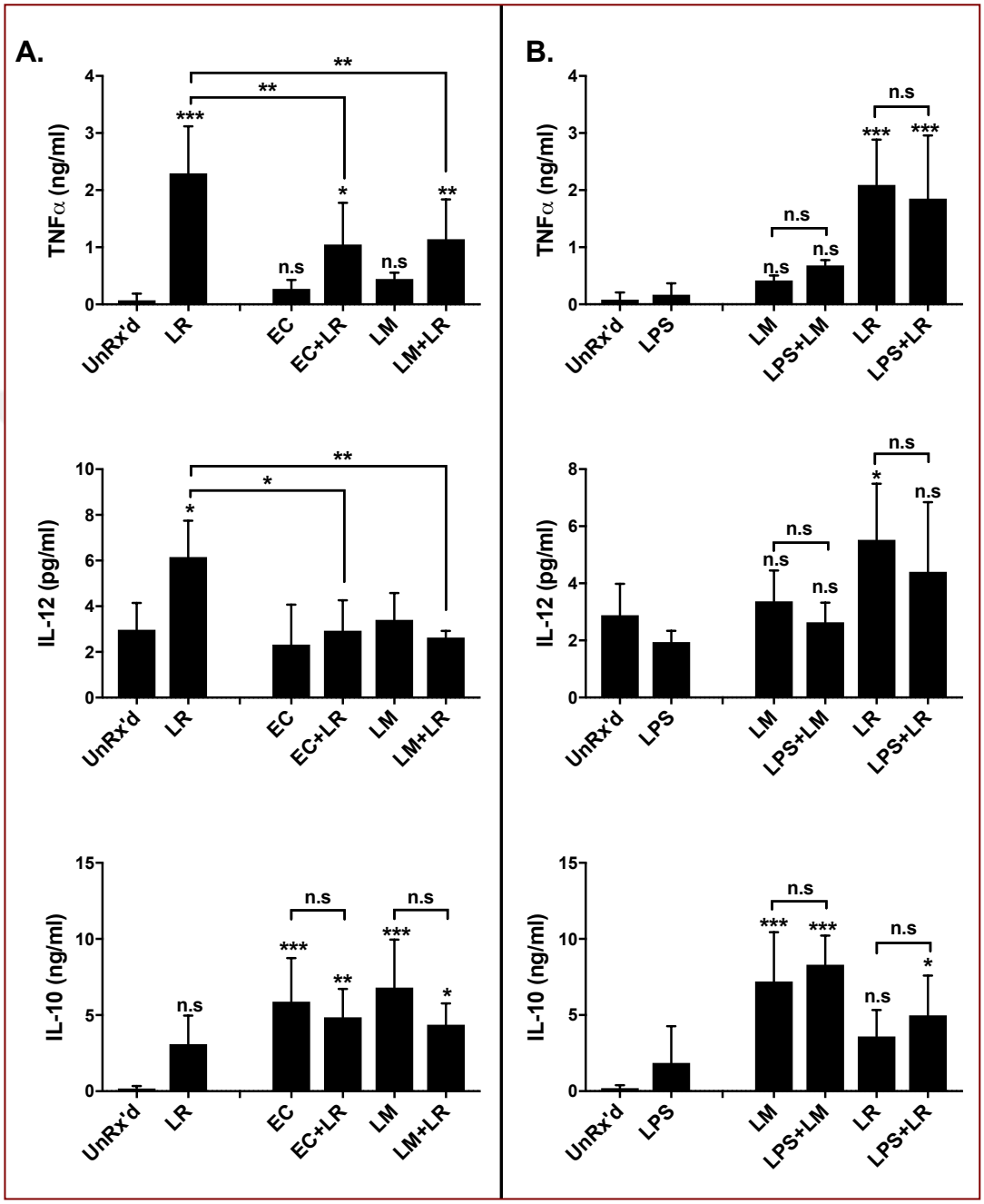

Figure 4.30. Heat killed *E. coli* and *L. monocytogenes* regulates pro-inflammatory cytokine induction by *L. rhamnosus*. Monocytes stimulated with heat killed *L. rhamnosus* (LR, 10^7/ml), *E. coli* (EC, 10^8/ml), *L. monocytogenes* (LM, 10^8/ml), LPS (1 μg/ml) alone or in combinations for 24 hours. TNFα, IL-12, IL-10 and IL-6 amounts were measured by ELISA. Mean ±SD from 6 independently analyzed donors, *; $p<0.05$ **; $p<0.01$ ***; $p<0.001$.

4.15. Lipid Isolates From *E. coli* Do Not Recapitulate The Effect Of The Whole Bacteria

To identify molecules that might be responsible of the suppressive function of the *E. coli*, a preliminary experiment was done. To separate membrane particles from intracellular small molecules, a vial of heat-killed *E. coli* was sonicated for 1 minutes then centrifuged for 20 min at 13000 g (**Figure 4.31A**). The supernatant was collected and the pellet was re-suspended in sterile PBS. The whole bacterial prep, supernatant and re-suspended pellet were tested at 10-fold serial dilutions for their ability to stimulate monocytes. Phenotype analysis of the resultant macrophage on day 5 revealed that stimulation with the resuspended pellet mimicked the response to the initial bacterial prep in terms of the resultant CD200R and CD163 expression profile (**Figure 4.31B**). An increase in CD200R+ cell frequency accompanied the use of increased amounts of bacteria from the initial prep or the cell pellet (**Figure 4.31B**). This result suggests that the cell wall contains the molecule(s) responsible of the stimulation induced by *E. coli* rather than small or soluble molecules in the supernatant.

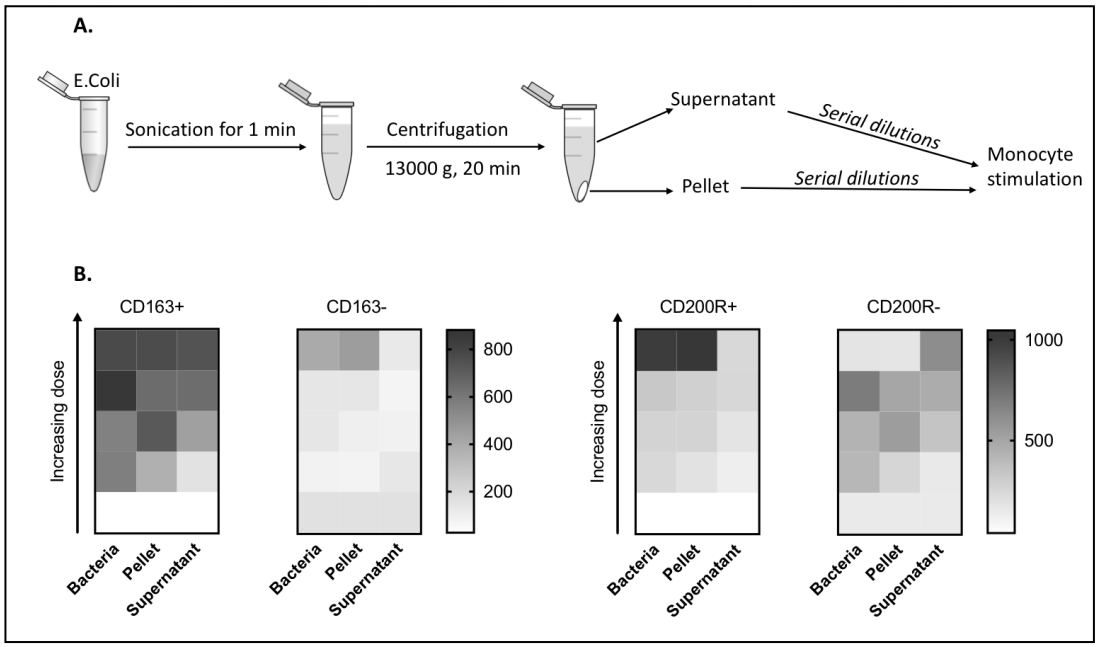

Figure 4.31. Cell wall particles of *E. coli* replicates the whole bacteria effect. A) Shows the separation protocol of the *E. coli* cell wall particles from small molecules. B) Representative heat-map graph shows the number of 25F9 expressing CD163+,

CD163- , CD200R+, CD200R- cells. Monocytes were stimulated 10^5 to 10^8 heat-killed *E. coli*/ml and pellet or supernatants of the corresponding amounts of bacteria.

To more precisely identify the molecules present in the cell wall of *E. coli* involved in mediating the induction of M2-like suppressive macrophage, a collaboration was initiated with Dr. Andresson Thorkell (Mass Spectrometry Core Facility, NCI Frederick, MD, USA). Heat killed *E. coli* samples were sent to Dr. Thorkell for lipid isolation as described in the Methods section. A bulk extract was generated from which neutral lipids, glycolipids and phospholipids were extracted. The response of monocyte to stimulation with each lipid product was evaluated. As shown in (**Figure 4.32A**), bulk lipids, glycolipids and phospholipids induced some monocyte differentiation whereas neutral lipids had no effect. Bulk lipids at 0.1- 10 μg/ml and phospholipids at 1 μg/ml concentration induced significant macrophage differentiation (**Figure 4.32A**, $p<0.05$). All lipid products were toxic at 100 μg/ml dose (viability assessments were also done, not shown). However, unlike whole bacteria, none of the lipid extracts induced increased CD200R expression by differentiation monocytes (**Figure 4.32C**). Although the number of CD163- cells was similar, CD163+ cells characteristic of the M2-dominant phenotype generated by of *E. coli*-differentiated macrophages were not generated in large numbers by these extracts (**Figure 4.32B**).

The cytokine inducing capacities of lipid extracts was then evaluated. As expected, 24-hour of stimulation with heat killed *E. coli* resulted in increased IL-10, IL-6 and to a lesser extent TNFα productions (**Figure 4.33**). Bulk lipids activated monocytes to secrete IL-6 at the highest dose (100 μg/ml) which was previously shown to be toxic (**Figure 4.32A**). Phospholipids induced IL-6 and to some degree IL-10 production but at cytokine levels far below those observed in *E. coli* stimulated monocyte cultures. Glycolipids and neutral lipids increased TNFα to some degree but IL-6 and IL-10 levels were minimal (**Figure 4.33**).

These studies suggest that the lipid component of *E. coli* can induce monocytes to secrete IL-6 and differentiate into a mixed population of macrophages. Phospholipids were the most active lipid subtype however, but like the bulk lipid were

unable to replicate the cytokine profile or the resultant macrophage phenotype of whole bacteria stimulated monocytes.

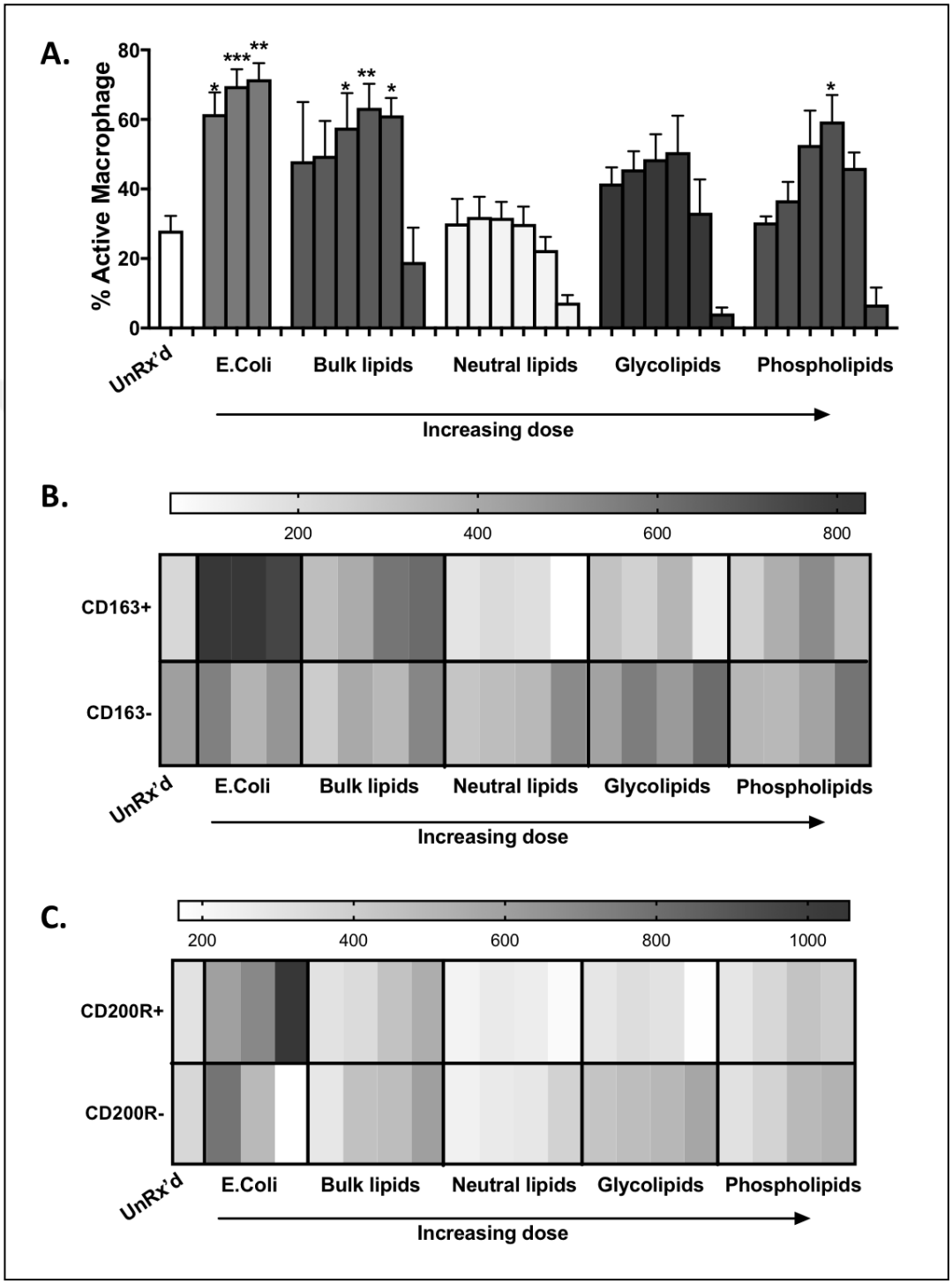

Figure 4.32. Lipid extract stimulation effect on monocyte differentiation. Monocytes stimulated with heat killed *E. coli* 10^6 to 10^8/ml, bulk lipids, neutral lipids, glycolipids, phospholipids (1 ng/ml to 100 μg/ml) for 5 days. A) Bar graphs show active macrophage percentages as 25F9+ cells (lipid concentrations: 1 ng/ml to 100

μg/ml). B) Heat-map graph shows the average of 25F9 expressing CD163+, CD163- C) CD200R+, CD200R- cell counts. (lipid concentrations: 10 ng/ml to 10 μg/ml). Mean ±SD from 6 independently analyzed donors. *; p<0.05 **; p<0.01 ***; p<0.001.

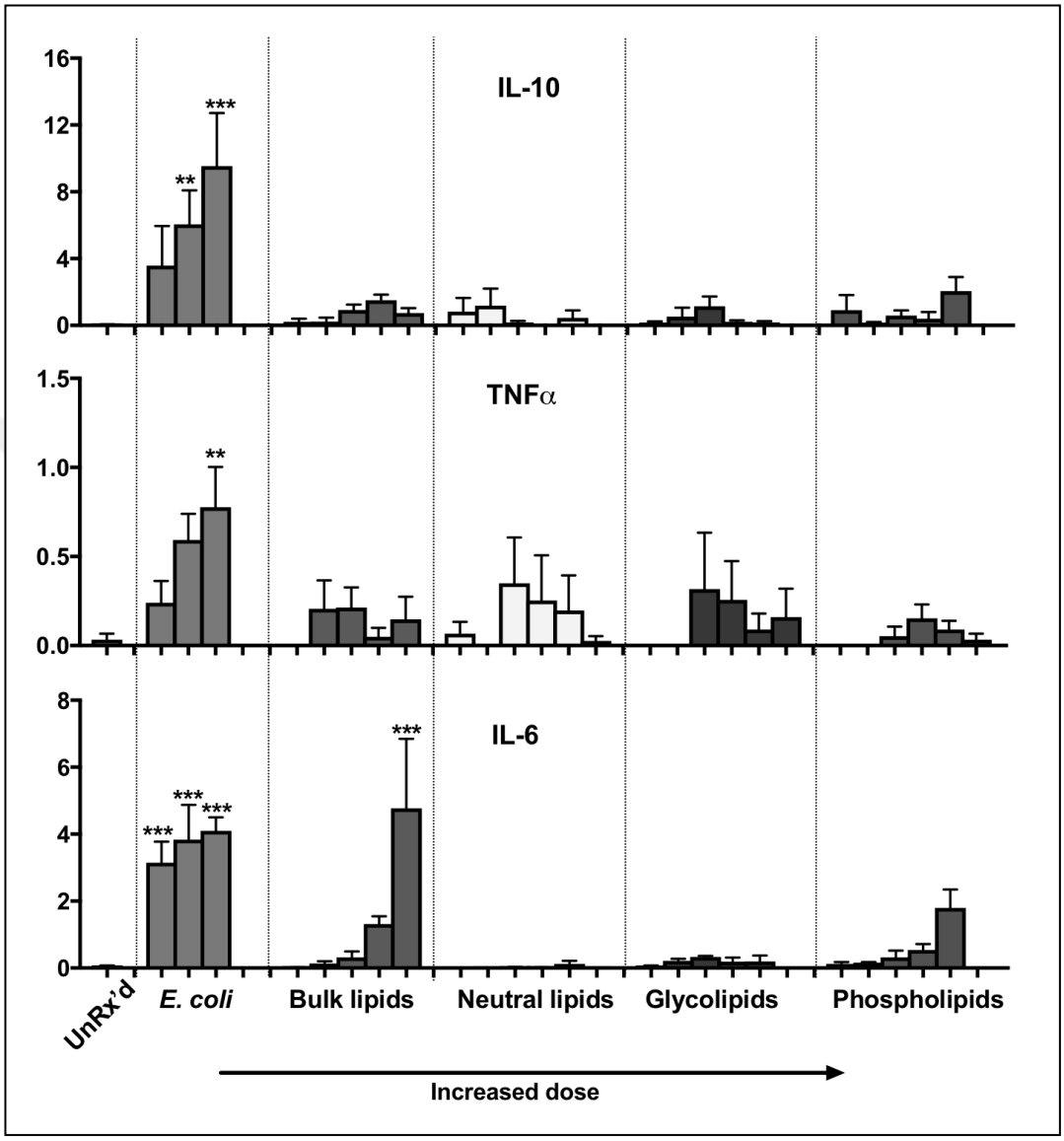

Figure 4.33. Lipid extract stimulation effect on monocyte cytokine response. Monocytes stimulated with heat killed *E. coli* 10^6 to 10^8/ml, bulk lipids, neutral lipids, glycolipids, phospholipids (1 ng/ml to 100 μg/ml) for 24 hours. Bar graphs show TNFα, IL-10 and IL-6 levels in the supernatants measured by ELISA. Mean ±SD from 3 independently analyzed donors. *; p<0.05 **; p<0.01 ***; p<0.001.

5. DISCUSSION

CpG ODN mimic the ability of CpG DNA to interact with TLR9 and trigger a pro-inflammatory cytokine and immunoglobulin response that improves both innate and adaptive immunity (111). The effect of CpG ODN was examined in various *in vivo* disease models of infection, allergy and cancer (88, 112-115). Pre-clinical studies show that immunomodulatory ODN treatment was improved by repetitive administration especially starting at the early stages of disease (124, 289). Clinical trials involving CpG ODNs were performed using systemically administered K-type CpG ODNs that have a full PS backbone to protect them from serum nucleases and improve *in vivo* half-life (290). If oral delivery of these ODN was an option it would widen their therapeutic utility in humans, as repeated injection is not a readily acceptable strategy for the long term treatment of chronic diseases. However oral administration is problematic due to exposure to gastric acids, digestive enzymes, bile salts, hepatic metabolism and possible effects of the microbiome (291, 292). Few studies examined the utility of orally administered immunomodulatory ODNs. There was some evidence in the literature showing that CpG ODNs can influence immunity when delivered orally if used at higher doses than those typically delivered systemically (eg, by i.p, s.c. or i.m treatment ((125, 126, 129, 130)). The outcome of those studies suggested that orally delivered CpG ODN could alter GI immunity but that to achieve a strong systemic immune response need to be co-administered with an antigen.

In an effort to improve the bioavailability of orally administered ODNs, Wang *et al.* described a method to encapsulate ODNs within calcium carbonate nanoparticles (131). Their study showed that such particles (ODNcap) were resistant to simulated gastric acid and nuclease incubation *in vitro*. Furthermore, they showed that long term oral administration of ODNcap had a systemic effect on disease severity in a murine model of chemically induced atopic dermatitis (131). While those results were promising, the published work failed to include free ODN as a control group in the disease model and thus failed to establish the effectiveness of the encapsulation method.

My thesis carefully compared the effects of oral ODNcap to free ODN delivered orally or systemically on GI and systemic immunity. Initial studies measured the activity of ODNcap *in vitro.* CpG ODN 1555 was used in most of the studies since it is highly effective on mouse cells and the outcome is easily measurable by cytokine production. Consistent with Wang *et al.,* the *in vitro* activity of CpG ODNcap was similar to free CpG ODN in that both induced IL-6, IL-12, and TNFα production at 24 hours and IFNγ at 48 hours (**Figure 4.1**). No difference was observed between the effects of CpG ODN vs CpG ODNcap at 24 or 48 hours except for TNFα, which was more strongly induced by CpG ODNcap at both time points. This difference could be an off-target effect caused by the calcium which acts as a secondary messenger and was shown to regulate TNFα and IL-6 release in response to IgE stimulation of the mast cells through IKKβ activation (293). Control ODNs that do not contain unmethylated CpG motifs did not have any significant effect on any of the cytokines studied (**Figure 4.2**) suggesting that calcium in the ODNcap material does not activate immune cells by itself. However, the possibility that calcium increases the activity of CpG ODN remains. The effect of calcium carbonate encapsulation on other types of ODNs could be assessed to find out if this method had a general effect on all immunomodulatory ODNs. Consistent with Wang *et al*., Suppressive ODN was also active when encapsulated in vitro, as evidenced by the decrease on the activation of CpG ODNcap (**Figure 4.3**).

To identify the immune cells responding to CpG ODNcap, fluorescent-labeled CpG ODN was used. *In vitro* co-localization studies showed contrasting results. Two fluorescent labels, Cy5 and FITC, gave different results; Cy5 labeled free and encapsulated ODN bound to nearly 100% of all cells whereas FITC labeled CpG ODN and CpG ODNcap bound to 70% and 40% of the leukocytes, respectively. Previously published studies with FITC-labeled CpG ODN yielded inconsistent results. One showed that about 50% of cells bound ODN (incubated at 4 °C) whereas only 20% internalized the labeled material (when incubated at 37 °C). In contrast, another study found that surface bound and internalized CpG ODN did not differ (294, 295). In my study, incubation was done at 37 °C. Therefore results cover both surface bound and internalized ODN. Cell specific associations were also dependent on the fluorescent

labeled used. Cy5 labeled CpG ODN and ODNcap associated with all cells in a non-specific manner whereas FITC labeled CpG ODN and ODN cap showed selectivity for macrophages and B cells but not T cells. This finding is partially in concordance with a previous study involving human PBMC, albeit with a different type of CpG ODN (295). As TLR9 is located intracellularly, CpG ODN internalization does not involve surface membrane TLR9 expression. PS-ODN cellular uptake was mediated via receptor-like mechanisms at low concentrations and pinocytosis at higher concentrations (296). Thus, it is not surprising that ODN uptake was non-specific. Wang *et al.* used a suppressive type of ODN labeled with 6-FAM for uptake study and their results indicate that both free and encapsulated ODN was taken up by all cell types; 35% of CD11b+ cells, %12-13 of CD19+ and CD3+ cells were positive for the free ODN whereas, 59% of CD11b+, 77% of CD3+, 48% of CD19+ cells were positive for the ODNcap (131). Given this disparity in results, the question of what percentage of cells bind and internalize CpG ODN and ODNcap still remains. Fluorescence microscopy was attempted to quantify fluorescent labeled leukocytes however those efforts was not successful due to the equipment issues will not be discussed further.

Due to the discrepancies observed using fluorescent-labeled ODN to study binding and uptake (**Figure 4.5**), non-labeled CpG ODN and ODNcap were used to stimulate and monitor cytokine production by different cell populations (**Figure 4.6**). As expected, TLR9 expressing B cells and macrophages responded to both agents whereas T cells did not.

Although all leukocytes are able to take up ODNcap *in vitro*, Wang *et al.* claimed that only macrophages in the Peyer's patches take up orally delivered ODNcaps *in vivo* (131). In contrast to those results, we observed that all cell types were able to associate with labeled CpG ODNcap in the Peyer's patches (as well as in appendiceal and mesenteric lymph nodes). Of note, both the fluorophores and type of ODN used in the two studies differed: we used Cy5 labeled CpG ODN 1555 while Wang *et al.* used 6-FAM labeled suppressive ODN (iSG3). In our hands, FITC labeled CpG ODNcap could not be detected in any organ after oral delivery (**Supplementary Figure A**). Furthermore, splenocytes incubated with FITC labeled CpG ODNcap could not be detected when injected i.v to other mice whereas Cy5-CpG ODNcap

associated cells could be detected in a time-dependent manner. These findings suggest that Cy5 is more suitable for *in vivo* tracking studies (**Supplementary Figure B**). Previous studies show that the FITC signal is highly pH dependent, weakens in acidic pH, and is prone to bleaching. These drawbacks might explain the weak signals we observed (297, 298). There is always a possibility that the Cy5 signal is an artefact due to intestinal contents, however we did see a time dependent change in the percentage of the Cy5+ cells that peaked at 90 minutes in the Peyer's patches and appendix then a gradual decrease. By comparison, increased numbers of labeled cells in the mesenteric lymph nodes peaked at 4.5 hours and then gradually declined, suggesting that the signal does come from cells following their routes through lymphatics rather than intestinal content (**Figure 4.7**).

Expanding on the *in vivo* findings of Wang *et al.* showing that IFNγ mRNA expression increased in Peyer's patches following 3 days of CpG ODNcap treatment, we observed that immune activation in the mesenteric lymph nodes could be detected when treatment duration was prolonged to 10 days (**Figure 4.8**). Consistent with an ability to activate GI immunity, we found that orally delivered CpG ODNcap modulated disease activity in a murine model of inflammatory colitis (**Figure 4.9**). CpG ODN was previously shown to exacerbate DSS induced colitis symptoms when given i.p (271-273). In my work, we observed that orally delivered free CpG ODN also did so. Note that in this study free CpG ODN was given in bicarbonate buffer to protect it from gastric acid, however we subsequently observed that that bicarbonate did not improve the activity of orally delivered CpG ODN in a bacterial challenge model (**Figure 4.12**). We did not repeat the gastric acid incubation experiment performed by Wang *et al.* showing that naked ODN was not resistant to gastric acid (degrading within an hour) whereas ODNcap resisted degradation for up to 16 hours (131). However, previous pharmacodynamics studies with orally delivered PS-ODNs showed that they were stable in the stomach up to 3 hours while extensive degradation occured in the small intestine (123).

Although Wang *et al.* reported that long term CpG ODNcap treatment increased the severity of atopic dermatitis in mice, our findings in multiple experimental settings suggested that ODNcap had little effect on systemic immunity.

CpG ODNcap did not increased the severity of atopic dermatitis in mice, our findings in multiple experimental settings suggested that ODNcap had little effect on systemic immunity. CpG ODNcap did not increase cytokine levels in the spleen or serum, consistent with its dominant effect being limited to GI immunity. We selected disease models in which the efficacy of systemically delivered CpG ODN was previous established (112, 120, 124, 125). In this context, no group has ever shown that systemic CpG ODN effect atopic dermatitis (the model used by Wang *et al.*). We were unable to detect any systemic effect of orally delivered free or encapsulated CpG ODN in a murine model of LPS-induced inflammation (**Figure 4.11**) and activity was minimal in a model of systemic *L. monocytogenes* challenge model (despite a prolonged treatment arm) (**Figure 4.13**). Yet the same treatment was effective against localized infection induced by i.g delivery of the same pathogen (**Figure 4.12**). In summary, the first part of my thesis confirmed that calcium carbonate encapsulation did not alter the immunomodulatory activity of ODN and that orally delivered ODNcap could detected in the Peyer's patches. We extended those findings and identified other GALT tissue in which ODNcap could be detected. We then corrected the one major oversight of the original work by Wang *et al.*: failure to include free ODN as a control in the disease model. By comparing the activity of encapsulated vs free CpG ODN (delivered either orally or intraperitoneally) in multiple experimental models, we concluded that calcium carbonate encapsulation did not enhance the activity of orally delivered ODNs.

We did find that oral delivery of CpG ODN was as effective as systemic treatment in the prevention of infection following gastric challenge with *L. monocytogenes*. Confirming results by Ray *et al.*, orally delivered CpG ODN therapy was an effective prophylactic against mucosal infection (125). More studies are needed to identify the exact mechanism of the CpG ODN uptake in the intestine to further improve the delivery strategy. Intestinal epithelial cells (IEC) express TLR9 on their apical and basolateral surface (299). While activation through basolateral TLR9 results in NF-κB activation, apical TLR9 ligation does not (300). Furthermore, apical TLR9 ligation results in tolerance to a second microbial stimulus delivered to either the apical or basolateral surface, suggesting a role of TLR9 in the GI tolerance (300). Another potentially relelvant cell type in the intestinal epithelium is the M cell which is located

on the dome of mucosal lymphoid tissues (such as Peyer's patches) (301). Different than IEC, M cells have fewer lysosomes and lack a mucous glycocalyx on their surface and basolateral extracellular space that surrounds interdigitated lymphoid cells (302). M cells sample intestinal material through transcytosis via endocytosis of clathrin-coated vesicles, actin-dependent phagocytosis and fluid-phase pinocytosis to the lymphoid cells underneath (303). As we and others detected ODNcap in the Peyer's patches, GI immune activation might involve sampling of luminal contents by M cells (131, 304). However the role of apical TLR9 expressed on IEC could be a negative factor, decreasing the activation initiated by orally delivered CpG ODN. Targeting the M cells could improve the activity of orally delivered immunomodulatory molecules (305, 306).

On the other hand, IECs do not respond to apical or basolateral stimulation by R848 and do not have such inhibitory activity like apical TLR9 (300). Our studies showed a better response to orally delivered R848 when compared to CpG ODN in mice challenged with enteric *L. monocytogenes*. Oral R848 was also effective at reducing systemic *L. monocytogenes* challenge suggesting that it could be a better mucosal prophylactic agent than CpG ODN. This could reflect the higher stability of R848 in the GI tract or a stronger effect on GALT (including IEC as discussed above). R848 treatment did not cause any visible side effects in mouse however, in a phase IIa clinical trial, oral R848 reduced viral titers of chronic hepatitis C virus in patients but only at doses associated with a high frequency of adverse events related to systemic cytokine secretion (such as fever, nausea, shivering and lymphophenia) (307). Considering these adverse effects, we thought it reasonable to investigate the activity of other TLR7 agonists. To that end, 3M052 and 3M058 did not improve the activity of CpG ODN while R848 did, suggesting that their molecular structures (insoluble in physiological solutions) make the latter TLR7 agonists unsuitable for oral delivery (**Figure 4.14**). Another study shows that oral R848 induced expression of the antimicrobial protein Reg3γ in the ileum while other TLR7/8 agonists such as imiquimod, gardiquimod and CL907 did not (308). While this finding does not exclude the possibility that other TLR7/8 agonists might protect against infection, it does lowers that possibility. I plan to identify the cells and the lymphoid organs activated by oral R848 (and other TLR7 ligands) in future studies.

Our lab previous found that the combination of TLR7/8 and TLR9 agonists successfully eliminated large tumors whereas either agonist alone could not (282). In my study of this combination, such synergy was not observed in *L. monocytogenes* prophylaxis (**Figure 4.17**). This could be due to the differences between the two models, such as evidence of strong immune suppression in the established tumor model which could require targeting different cells simultaneously. In the prophylaxis model, by comparison, suppression is not present and a combination might not improve outcome. *In vitro* stimulation of bone marrow cells with the combination of R848 and CpG ODN also did not improve cytokine production (data not shown). However this combination strategy might be beneficial if a wider range of microbes needs to be covered by prophylaxis. Further studies with other pathogenic bacteria are needed to clarify whether the protective effect of R848 is generalizable for use as a prophylactic oral agent.

R848 works through TLR7 in mice therefore all its effects in the models used can be attributed to TLR7 activation (76). However in humans R848 activates both TLR7 and TLR8 (76). Efforts to discriminate between the activity of these two closely related receptor began in 2005 when specific agonists for each receptor first became available (6). Yet much remains unknown about the function of these 2 receptors when expressed by the same cell. Monocytes and dendritic cells are the two important cells of the MPS that express both TLR7 and 8 receptors and are, therefore, suitable targets to investigate the roles of TLR7 and TLR8 under physiologic conditions. One report showed that TLR7 and TLR8 both induce dendritic cells to mature, but that only TLR8 agonists induced IL-12 p70 secretion and IFNβ expression whereas both agonists induced secretion of IL-6 (309). Our results on monocytes showed that TLR8 stimulated more IL-12, IL-6, IL-10 and TNFα secretion than TLR7 agonists (**Figure 4.21**). Although stimulation via TLR7 induced only modest cytokine release from monocytes, both agonists were equivalent in their ability to induce monocytes to mature into macrophage (**Figure 4.19**). We observed slight differences in the phenotypes of TLR7 vs TLR8 differentiated macrophages. Yet both agonists generated macrophages that expressed the M2 marker CD206. Surprisingly, the cells developed no significant increase in their ability to endocytose particles (**Figure 4.20**), suggesting that they might not be functioning like M2 macrophages. While expression

of the co-stimulatory receptors CD80 and CD86 is reportedly increased on M1-like macrophages, our preliminary studies did not confirm these results as M-CSF induced macrophages have higher expression of these markers than any of the stimuli used (310, 311).

One interesting finding from my work was the ability of TLR7 ligation to block the TNFα response to subsequent TLR8 stimulation. Previous studies suggest a regulatory role of TLR8 on TLR7 as shown by increased autoimmunity in TLR8 -/- mice due to hyper responsiveness to TLR7 stimulation (79). A later study on TLR8/9-/- mice showed a further increase in autoimmunity indicating that TLR9 as well as TLR8 regulates the activity of TLR7. However different cells were involved: TLR8 controlled dendritic cells whereas TLR9 controlled B cells (80). These studies do not explain the mechanism underlying this phenomenon. HEK293 cells co-transfected with either TLRs 7 and 8 or 7 and 9 did not respond to TLR7 specific stimulation and this response was correlated with the transfection dose of TLR8 and 9 (285). This phenomenon did not occur when cells were co-transfected with TLR2 and 8 or TLR4 and 8 suggesting that the reason is not an effect on the common downstream pathways. The observation that inhibition was dose dependent, combined with immunoprecipitation experiments, suggests that this effect was due to physical interactions between endosomal receptors (285). These studies support the conclusion that the presence of TLR8 or TLR9 regulates TLR7 responsiveness in transgenic models which might explain the lack of cytokine production from the TLR7 stimulated monocytes. However, TLR7 ligation does have an impact on monocyte differentiation into macrophages and a more detailed analysis is required to understand the nature of TLR7 stimulus.

The effect of TLR7 ligation on the response to TLR8 agonists was both dose and time dependent. The time dependent nature of this inhibition suggests a mechanism involving regulation on a common downstream pathway as was previously shown in cross tolerance between TLRs TLR2, 5 and 9 (312, 313). However, TLR7 pre-stimulation did not block subsequent TLR2 or TLR4 responses. To the contrary, an additive response was observed (**Figure 4.26**) suggesting the inhibitory effect of TLR7 ligation is not generalizable to all MyD88 dependent TLRs. One possible

explanation involves competition for the UNC93B1 protein which carries TLRs 3,7,8 and 9 from the endoplasmic reticulum to endolysosomes for ligand recognition. A competition between TLR9 and TLR7 for UNC93B1 in dendritic cells was previously documented, therefore it is possible that similar competition might occur between TLRs 7 and 8 in human monocytes (314). Since we did not detect any cytokine response following TLR7 stimulation, it was not possible to investigate the role of TLR8 regulation on the TLR7 response. There is still the possibility that the TLR7 agonists physically blocks recognition of the TLR8 receptor, consistent with the dose response for TLR7 activity (**Figure 4.24**). Studies using TLR7 and 8 ligand combinations on TLR7 or 8 transfected HEK293 cells are under way to investigate this possibility.

Interestingly TLR2 and TLR4 pre-stimulation also decreased the subsequent TLR8 response whereas TLR8 pre-stimulation did not block activation with TLR2 or 4 agonists (consistent with previous studies showing synergy) (74, 315, 316). This effect could be mediated through the same mechanism responsible for TLR7 induced tolerance (although not through UNC93B1) or could be completely different and needs further investigation. Overall conclusion from our studies is that the TLR8 response of human monocytes is strictly regulated by TLRs 2,4 and 7 stimulation.

To further understand the effect of TLR agonist combinations on human monocytes, I stimulated cells with a variety of gram – and + heat-killed bacteria. Although gram- and + bacteria responses were previously reported to be distinct, our studies revealed differences in the responses are unrelated to gram staining properties (317, 318). *E. coli, H. pylori* and *L. monocytogenes*-differentiated macrophages gave similar results; M2-like differentiation and less pro-inflammatory cytokine release, whereas *L. rhamnosus* and *S. epidermidis* induced M1-like differentiation. Furthermore, *E. coli* or *L. monocytogenes* reduced TNFα and IL-12 release by *L. rhamnosus* suggesting that these two bacteria might contain a molecule that interferes with the pro-inflammatory *L. rhamnosus* response. Identifying such molecule would be a promising approach to treating autoimmune and autoinflammatory diseases. Similar to our findings, other groups showed the suppressive ability of *H. hepaticus* through induction of IL-10 release by intestinal macrophages and Tregs (319, 320). A

large soluble polysaccharide produced by *H. hepaticus* was identified as inducing a CREB-dependent anti-inflammatory gene signature involving IL-10 via the TLR2 pathway (320). Since LPS could also trigger CREB-dependent IL-10 induction, we evaluated the effect of *E. coli* LPS (same strain as the bacteria) on *L. rhamnosus* induced cytokine production (252, 321). However, *E. coli* LPS was not responsible for the increase in IL-10 or suppression of TNFα (**Figure 4.30**). Another bacterial product, polysaccharide A (PSA) produced by *B. fragilis,* was shown to stimulate Tregs to suppress anti-bacterial immune responses through a TLR2 dependent mechanism (322-324). It is possible that the suppressive effect of *E. coli* is TLR2 mediated. As studies continue we plan to define the contribution of specific TLRs to the response induced by *E. coli*. I believe that production of a soluble suppressive molecule by *E. coli* is unlikely since supernatants from sonicated bacteria did not induce M2-like differentiation (**Figure 4.31**). To the contrary, the bacteria pellet, which contains non-soluble particles, mimicked the effects of whole bacteria (**Figure 4.31**). This finding led us to investigate the effects of *E. coli* lipid extracts. Although bulk lipids induced macrophage differentiation, the resultant phenotype differed from that generated by whole bacteria in that only IL-6 was produced, suggesting that it is not lipids from *E. coli* that are responsible for its anti-inflammatory properties (**Figure 4.32**, **Figure 4.33**).

Attaching and effacing pathogens such as enteropathogenic (EPEC) and enterohemorrhagic *E. coli* (EHEC) use a type III secretion system (T3SS) to secrete proteins into host cells (325, 326). One such group of proteins are known as non-LEE-encoded (Nle) effectors target the NF-κB signaling pathway to block immune activation (326, 327). NleB prevents the ubiquitination of TRAF2 and 3 thereby blocks signal transduction upstream of NF-κB (328, 329); NleC and NelD are metalloproteases that disrupt NF-κB and AP-1 activation by the cleaving NF-κB's p65 subunit and JNK, respectively (330, 331); NelE blocks the transport of p65 into the nucleus (332) while NleH1 inhibits the phosphorylation of RPS3 thereby preventing DNA binding of p65-p50 heterodimer (333). These examples show that bacterial-derived proteins use multiple mechanisms to suppress the immune system. Heat killed bacteria would not have an active T3SS therefore Nle proteins are unlikely the cause

of the effect we observe however, it would still worth examining the effects of *E. coli* protein extracts on the immune system.

6. CONCLUSIONS AND FUTURE DIRECTIONS

1- Calcium carbonate encapsulation does not interfere with the activation of the ODN. However, ODNcaps are not an effective method of enhancing the bioavailability of orally delivered CpG ODNs.

2- Orally delivered CpG ODN induce activation of GI immunity and might be useful prophylactically to prevent enteric bacterial infections. However orally delivered ODN have little or no effect on systemic immunity. Further research is needed to identify novel strategies to improve the activity of orally delivered immunomodulatory ODN.

3- The TLR7/8 agonist R848 is an effective oral immunomodulator that can provide protection against enteric and systemic *L. monocytogenes* infection. Further studies with other pathogenic bacteria are needed to determine if R848 can be used to protect broadly from enteric challenge.

4- The combination of R848 and CpG ODN does not provide better protection against enteric or systemic *L. monocytogenes* infection than R848 alone. Whether this combination protects against multi-bacterial infections needs to be investigated.

5- TLR7 and TLR8 agonist stimulation results in different responses from monocytes. Both agonists effectively induce monocytes to differentiate into macrophages, although the phenotypes of the cells arising from these treatment differ slightly. While TLR7-differentiated macrophages were phenotypically M1-like, they did not mount an M1-associated cytokine response. TLR8-differentiated macrophages were a phenotypically more mixed population capable of secreting large amounts of pro and anti-inflammatory cytokines. To identify the regulatory pathways by which TLR7 and TLR8 mediate macrophage differentiation will require the use of sophisticated methods such as RNAseq.

6- The TLR8 mediated cytokine response of monocytes is influenced by activation of TLRs 2,4 and 7.

7- TLR7 ligation blocks TLR8 activation in a dose and time dependent manner. Studies examining the effect of TLR7 and 8 agonists on human TLR7 or 8 transfected HEK293 cells are under way to investigate possible physical interactions at the receptor level. Further studies are needed to clarify if downstream regulatory pathways are involved.

8- Ligation of TLR2 and TLR4 also blocks TLR8 activation in a time dependent manner. Dose- response studies should be performed.

9- *E. coli* and *L. monocytogenes* induce M2-like differentiation of the macrophages with prominent IL-10 and IL-6 secretion whereas *L. rhamnosus* and *S. epidermidis* induce M1-like macrophage differentiation with prominent TNFα and IL-12 production.

10- *E. coli* and *L. monocytogenes* can block TNFα and IL-12 release induced by *L. rhamnosus* stimulation.

11- *E. coli*-derived LPS is not responsible from the suppressive effect on *L. rhamnosus.*

12- The *E. coli* derived molecule mediating this suppression is likely to be non water soluble.

13- *E. coli* lipids can induce macrophage differentiation but the resultant macrophages are less suppressive then *E. coli*-differentiated cells and do not produce IL-10. Ongoing studies are designed to investigate the contribution of *E. coli* derived proteins on this differentiation.

14- The effects of specific TLRs on the differential response of monocytes (cytokine response and differentiation into macrophages) to heat-killed bacteria should be assessed by blocking each TLRs with antibodies.

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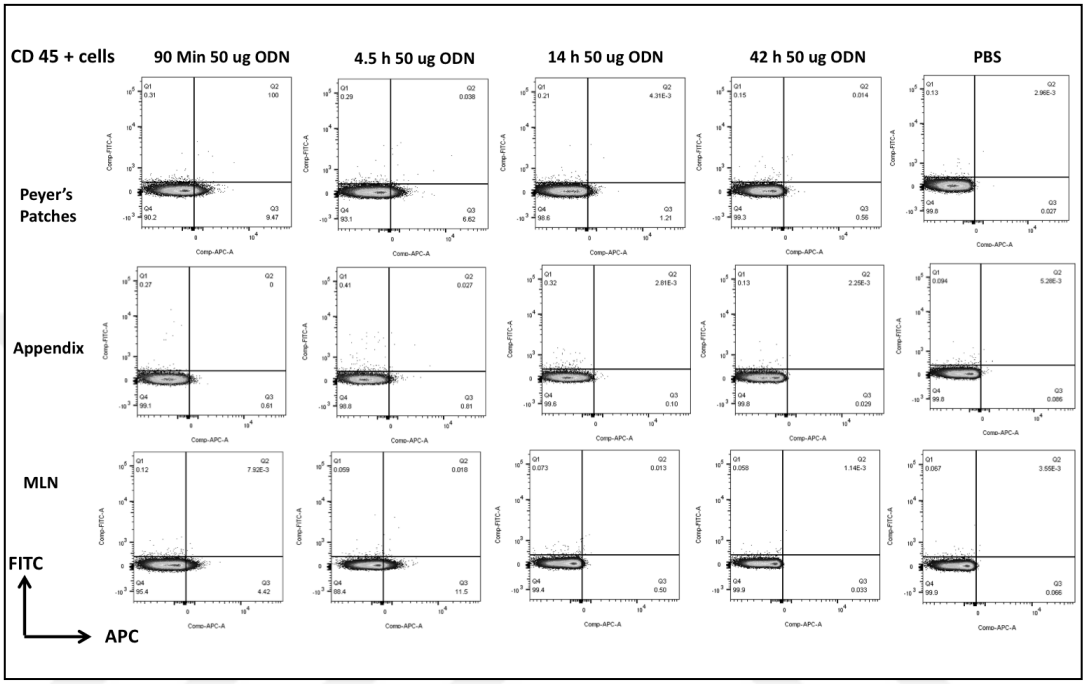
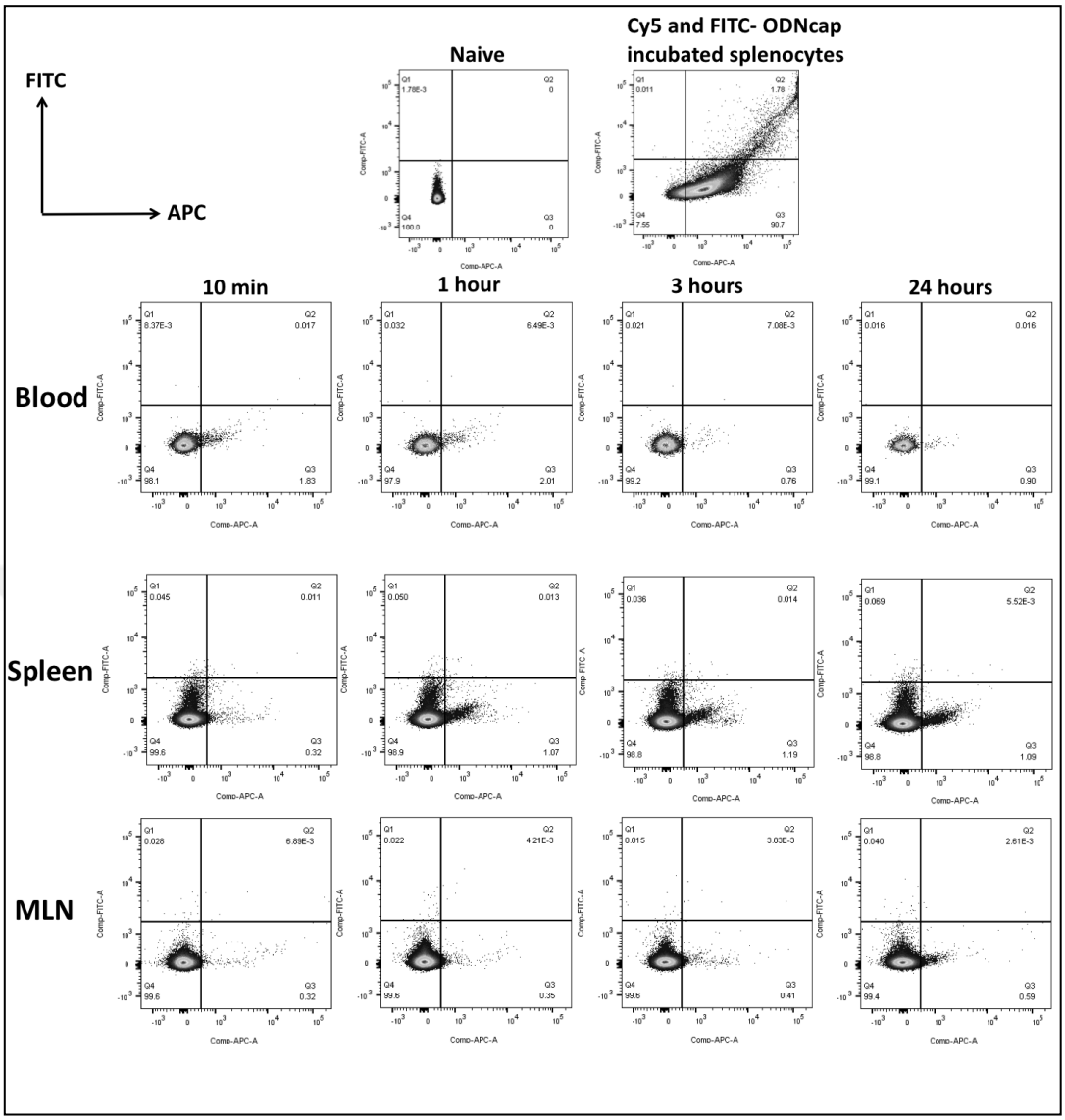
8. APPENDIX

Appendix-1: Supplementary Figures



Supplementary Figure A: *In vivo* localization of labeled CpG ODNcap. BALB/c mice (n=3/time point) were treated with 25 µg of FITC and 25 µg Cy5-labeled CpG ODNcap or PBS as control. After indicated time points, mice were sacrificed and lymphoid organs harvested. Representative dot plots of live, CD45+ cells are shown



Supplementary Figure B *In vivo* tracking of Fluorescent-ODNcap treated splenocytes: 1 uM FITC and 1 uM Cy5 labeled CpG ODNcap were incubated with BALB/c splenocytes for 90 minutes. CD45+ cells were magnetically sorted and 10^7 of them were given to another group of mice by i.v. injection. Mice were sacrificed at different time points to track the labeled ODN-containing cells over time. N=1 mouse/timepoint.

Appendix-2: Publications related to this thesis

Kayraklioglu N, Scheiermann J, Alvord WG, Klinman DM. Effect of Calcium Carbonate Encapsulation on the Activity of Orally Administered CpG Oligonucleotides. *Molecular Therapy Nucleic Acids*. 2017;8:243-249. doi:10.1016/j.omtn.2017.06.015.

Appendix-3: Poster and oral presentations related to this thesis

- **Kayraklioglu N.** Immune modulation with TLR agonists. Cancer and inflammation Program Staff talks. National Cancer Institute, Frederick MD. October 23, 2017 (oral presentation)
- **Kayraklioglu, N.,** Klinman, D.M. Effects of TLR7 and TLR8 agonists on human monocyte activation & differentiation. Cancer immunology and immunotherapy: From conception to delivery. National Institutes of Health, Bethesda, MD, October 12-13, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. Research Festival 2017, National Institutes of Health, Bethesda, MD, September 12-13, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA, September 6-7, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Parker K.H., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. American Association of Immunology 2017 meeting, Washington, DC May 12-17, 2017 (poster presentation).
- **Kayraklioglu, N.,** Klinman, D.M. Effects of TLR7 and TLR8 agonists on human monocyte differentiation. Keystone Symposia: Mononuclear phagocytes in health and disease, Austin, TX. April 30-May 4, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Parker, K.H., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligodeoxynucleotides Fellows & Young Investigators Colloquium, National Cancer Institute, Shady Grove, MD. March 16-17, 2017 (oral presentation).
- **Kayraklioglu N.** Effects of calcium encapsulated oligodeoxynucleotides in murine colitis. Cancer and inflammation Program Staff talks. National Cancer Institute, Frederick MD. October 3, 2016 (oral presentation)
- **Kayraklioglu, N.,** Parker, K.H., Klinman, D.M. Use of encapsulated oligodeoxynucleotides in murine colitis. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA. September 7-8, 2016 (poster presentation)
- Parker, K.H., **Kayraklioglu, N.,** Klinman, D.M. Assesment of calcium nanoparticles containing immunomodulatory oligonucleotides for oral treatment of inflammatory conditions. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA. September 7-8, 2016

Appendix-4: Funding Sources

This work was conducted at Klinman Lab, National Cancer Institute (NCI), National Institutes of Health (NIH) and funded by the Intramural Research Program of the NIH, NCI.

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Wang Y, Yamamoto Y, Shigemori S, Watanabe T, Oshiro K, Wang X, et al. Inhibitory/suppressive oligodeoxynucleotide nanocapsules as simple oral delivery. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International Public License (https://creativecommons.org/licenses/by-nc-nd/4.0/), permitting all non-commercial copy and redistribution without reproduction with the original work is properly cited.

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Author: Christian Bode, Gan Zhao, Folkert Steinhagen, et al
Publication: Expert Review of Vaccines
Publisher: Taylor & Francis
Date: Apr 1, 2011
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Title	Effect of calcium carbonate encapsulation on the oral activity of CpG oligodeoxynucleotides and assessment of the response of human monocytes to TLR ligands.
Instructor name	Dicle Guc
Institution name	Hacettepe University Cancer Institute
Expected presentation date	Mar 2018
Portions	Figure 4
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Licensed Content Author	M P Schön, M Schön
Licensed Content Date	Jan 7, 2008
Licensed Content Volume	27
Licensed Content Issue	2
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Instructor name	Dicle Guc
Institution name	Hacettepe University Cancer Institute
Expected presentation date	Mar 2018
Portions	Figure 1
Requestor Location	Dr. Neslihan Kayraklioglu 108 Monroe St #301 COURTHOUSE, MD 20850 United States Attn: Dr. Neslihan Kayraklioglu
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Author of this Springer Nature content	no
Title	Effect of calcium carbonate encapsulation on the oral activity of CpG oligodeoxynucleotides and assessment of the response of human monocytes to TLR ligands.
Instructor name	Dicle Guc
Institution name	Hacettepe University Cancer Institute
Expected presentation date	Mar 2018
Portions	Figure 6
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Licensed Content Author	Luke A. J. O'Neill, Douglas Golenbock, Andrew G. Bowie
Licensed Content Date	May 17, 2013
Licensed Content Volume	13
Licensed Content Issue	6
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Title	Effect of calcium carbonate encapsulation on the oral activity of CpG oligodeoxynucleotides and assessment of the response of human monocytes to TLR ligands.
Instructor name	Dicle Guc
Institution name	Hacettepe University Cancer Institute
Expected presentation date	Mar 2018
Portions	Figure 1: Mammalian TLR signalling pathways.

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Licensed Content Date	Apr 20, 2010
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Title	Effect of calcium carbonate encapsulation on the oral activity of CpG oligodeoxynucleotides and assessment of the response of human monocytes to TLR ligands.
Instructor name	Dicle Guc
Institution name	Hacettepe University Cancer Institute
Expected presentation date	Mar 2018
Portions	Figures 1 and 2
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Appendix-6: Ethics committee approval of animal studies

DEPARTMENT OF HEALTH & HUMAN SERVICES PUBLIC HEALTH SERVICE

NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE

P.O. Box B
Frederick, Maryland 21702-1201
National Cancer Institute at Frederick

MEMORANDUM

July 14, 2015

TO: Dr. Klinman
Principal Investigator

FROM: National Cancer Institute at Frederick Animal Care and Use Committee

SUBJECT: Animal Study Proposal – **APPROVAL**

Your National Cancer Institute at Frederick Animal Study Proposal **15-438** entitled **"Evaluation of immunologic and inflammatory responses induced by vaccines, antigens and adjuvants,"** was given **APPROVAL** by the National Cancer Institute at Frederick Animal Care and Use Committee [ACUC] on June 18, 2015. Please retain the attached copy of this proposal for your files. Any additional change to this proposal requires the submission of a modification for ACUC review and approval in advance. Please refer to the *ACUC Guidelines Regarding Modifications to Animal Study Proposals at* http://ncifrederick.cancer.gov/Lasp/Acuc/Frederick/Media/Documents/ACUC6.pdf. For additional information on National Cancer Institute at Frederick ACUC procedures, policies, guidelines, forms, training, etc., please visit our website at *http://web.ncifcrf.gov/rtp/lasp/intra/acuc/fred main.asp*

***IMPORTANT NOTE**: To ensure humane care and use at the National Cancer Institute at Frederick, please ensure that all staff working on this study is reminded that all animals are to be **monitored at least daily** for animal health issues. All animal health related issues must be reported in accordance with SOP 2.002 "Animal Health Evaluation." We appreciate your cooperation and commitment to ensure quality animal care at the National Cancer Institute at Frederick.*

FACILITY RESOURCE ISSUES

Please note that you must stay within your authorized cage allocation

Attachment: Approved Animal Study Proposal

Distribution: Perella [Manager]; Barber [Safety]; Smith [Pathology]

Please contact the ACUC Office at 301-846-7544 if you have any questions or concerns

ANIMAL CARE and USE COMMITTEE

DEPARTMENT OF HEALTH & HUMAN SERVICES PUBLIC HEALTH SERVICE

NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE

P.O. Box B
Frederick, Maryland 21702-1201
National Cancer Institute at Frederick

MEMORANDUM

October 7, 2015

TO: Dr. Klinman
Principal Investigator

FROM: National Cancer Institute at Frederick Animal Care and Use Committee

SUBJECT: Animal Study Proposal – **APPROVAL**

Your National Cancer Institute at Frederick Animal Study Proposal **15-459** entitled **"Oligodeoxynucleotides as immune modulators for the prevention/treatment of infectious disease and cancer,"** was given **APPROVAL** by the National Cancer Institute at Frederick Animal Care and Use Committee [ACUC] on September 24, 2015. Please retain the attached copy of this proposal for your files. Any additional change to this proposal requires the submission of a modification for ACUC review and approval in advance. Please refer to the *ACUC Guidelines Regarding Modifications to Animal Study Proposals at* http://ncifrederick.cancer.gov/Lasp/Acuc/Frederick/Media/Documents/ACUC6.pdf. For additional information on National Cancer Institute at Frederick ACUC procedures, policies, guidelines, forms, training, etc., please visit our website at http://web.ncifcrf.gov/rtp/lasp/intra/acuc/fred main.asp

***IMPORTANT NOTE**: To ensure humane care and use at the National Cancer Institute at Frederick, please ensure that all staff working on this study is reminded that all animals are to be **monitored at least daily** for animal health issues. All animal health related issues must be reported in accordance with SOP 2.002 "Animal Health Evaluation." We appreciate your cooperation and commitment to ensure quality animal care at the National Cancer Institute at Frederick.*

FACILITY RESOURCE ISSUES

Please note that you must stay within your authorized cage allocation

Attachment: Approved Animal Study Proposal

Distribution: Perella [Manager]; Barber [Safety]; Smith [Pathology]

Please contact the ACUC Office at 301-846-7544 if you have any questions or concerns

ANIMAL CARE and USE COMMITTEE

DEPARTMENT OF HEALTH & HUMAN SERVICES PUBLIC HEALTH SERVICE

NATIONAL INSTITUTES OF HEALTH
NATIONAL CANCER INSTITUTE

P.O. Box B
Frederick, Maryland 21702-1201
National Cancer Institute at Frederick

MEMORANDUM

November 16, 2015

TO: Dr. Klinman
Principal Investigator

FROM: NCI at Frederick Animal Care and Use Committee

SUBJECT: Animal Study Proposal Modification – **FINAL APPROVAL WITH STIPULATION**

Your NCI at Frederick Animal Study Proposal Modifications **15-438-M2 and 15-459-M1** were given **FINAL APPROVAL with STIPULATION** by the NCI at Frederick Animal Care and Use Committee on November 10, 2015. Please retain the attached copy of this modification for your files. Any additional change to this proposal requires the submission of a modification memorandum. For modification instructions and/or additional information regarding animal care and use, please visit the NCI at Frederick ACUC website at *http://ncifrederick.cancer.gov/Lasp/Acuc/Frederick/Default.aspx*.

***IMPORTANT NOTE**: To ensure humane care and use at the NCI at Frederick, please ensure that all staff working on this study is reminded that all animals are to be **monitored at least daily** for animal health issues. All animal health related issues must be reported in accordance with SOP 2.002 "Animal Health Evaluation." We appreciate your cooperation and commitment to ensure quality animal care at the NCI at Frederick.*

POST-APPROVAL MONITORING STIPULATION**

The NCI at Frederick Animal Care and Use Committee requires post-approval monitoring for this study. The LAM veterinary staff must observe the procedure and verify that all requirements to ensure humane care and use of animals have been fulfilled in accordance with Federal requirements.

****You are not permitted to proceed with the procedure until you have received written verification from the ACUC Office that this requirement has been satisfied.**

Please contact the LAM veterinary staff [301-846-5577] when **Kathy Parker and Neslihan Kayraklioglu are ready to perform cervical dislocation** so that LAM can ensure proficiency.

Attachment: Approved Animal Study Proposal Modification

Distribution: Perella [Facility]; Braden [LAM]; Barber [Safety]; Smith [PHL]

Please contact the ACUC Office at 301-846-7544 if you have any questions or concerns

ANIMAL CARE and USE COMMITTEE

NCI AT FREDERICK ANIMAL CARE AND USE COMMITTEE
Modification to an Approved Animal Study Proposal

ENTERED

15-438-M2
ASP#: 15-459-M1 PI: Kinman ACUC Received: 10/30/15

APPROVAL SIGNATURES:

ACUC Reviewer: Kimberly D. Klarmann 11-07-2015
Signature *Date*

Facility Manager(s):
☐ A Facility Resource Sheet is Attached
☑ A Facility Resource Sheet is Not Required for this Modification

Attending Veterinarian: 11/6/15
Signature *Date*

FINAL APPROVAL:

☑ This modification was reviewed and approved by the DESIGNATED REVIEW process.

☐ This modification was reviewed and approved during a SCHEDULED ACUC MEETING.

ACUC Chair: 11/10/15
Signature *Date*

Please contact the ACUC Office [301-846-7544] if there are any questions or concerns

I have received the following request for designated member review:

15-438 M2 (no IBC)
Evaluation of Immunologic and Inflammatory Responses Induced by Vaccines, Antigens and Adjuvants
Klinman

15-459-M1 (IBC 12-71)
Oligodeoxynucleotides as immune modulators for the prevention/treatment of infectious disease and cancer

I'd like both new fellows on the same 2 protocols and approved to do the same procedures (listed below). Information on each fellow is also provided.

Procedures: Injections (i.m., i.v., s.c., i.p.), challenges, bleed, euthanasia (CO_2 and cervical dislocation)

Name	Kathy Parker	Neslihan Kayraklioglu
Experience	4 years (grad student)	None
ACUC online training	10/5/2015	10/28/2015

LAM will perform post approval monitoring on cervical dislocation.

ANIMAL CARE and USE COMMITTEE

CURRICULUM VITAE

Personal Information

Name:	Neslihan Kayraklioglu
Date of Birth:	8/19/1991
Citizenship:	Turkey
Languages:	Turkish (native) English (fluent) French (basic)
Address:	1050 Boyles Street, Bldg 567, Rm228 Frederick, MD, 21702, U.S.A.
Contact Information:	**turann@mail.nih.gov** **nturan91@gmail.com**

Education

2013-present	Ph.D., (Tumor Biology and Immunology), Hacettepe University, Ankara, Turkey. Current GPA:3.75/4
2015	M.D., Hacettepe University, Ankara, Turkey. High Honor student, GPA:3.57/4

Research Experience

2015-present	Post-doctoral visiting fellow, Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD PI: Dennis Klinman, MD/PhD Projects: Evaluation of calcium encapsulation on the oral activity of CpG Oligonucleotides, effects of TLR 7/8 agonist treatment on the differentiation of human monocytes, assessment of different bacterial stimuli on monocyte differentiation.

Trained in: Cell culture, bacteria culture, multi-color flow cytometry, ELISA. In vivo experience: i.p/i.v injection, gastric gavage, mandibular/retro-orbital/tail vein bleeding, peritoneal macrophage isolation, organ harvest/cell isolation.

2015 Elective research rotation, Georgetown University Lombardi Cancer Research Center
PI: Aykut Üren, MD
Project: Identifying CD99 inhibitors as potential therapy for Ewing sarcoma
Trained in: Cell culture, WST assay

2013 Summer internship, National Cancer Institute, NIH, Bethesda, MD
PI: Stuart Yuspa, MD
Project: Identifying the effect of hypoxia on CLIC4 expression
Trained in: Keratinocyte and skin fibroblast isolation, cell culture, Western Blot, ELISA, MTT assay

2012 Summer Internship, Georgetown University Lombardi Cancer Research Center
PI: Aykut Üren, MD
Project: Creating a Cre-inducible mouse model of Ewing sarcoma
Trained in: Western Blot, PCR, qPCR, cell culture

Clinical Experience

2015 Medical intern, Hacettepe University Hospitals, Ankara, Turkey.

Obligatory 1-year clinical rotation before graduation from medical school with duties similar to first-year medical resident

Teaching & Mentoring Experience

2017 Summer Intern Mentor, National Cancer Institute, NIH
Mentored a medical student in research project: Assessment of TLR7/8 and TLR9 agonist combination effect on infection protection. Gave trainings in scientific techniques such as ELISA, Cell culture, Bacteria culture, In vivo techniques (gastric gavage, i.p injection) and organ harvest.

Publications

Original Articles

- **Kayraklioglu N**, Scheiermann J, Alvord WG, Klinman DM. Effect of Calcium Carbonate Encapsulation on the Activity of Orally Administered CpG Oligonucleotides. *Molecular Therapy Nucleic Acids*. 2017;8:243-249. doi:10.1016/j.omtn.2017.06.015.
- Çelik H, Sciandra M, Flashner B, Gelmez E, **Kayraklıoğlu N**, Allegakoen DV, et al. Clofarabine inhibits Ewing sarcoma growth through a novel molecular mechanism involving direct binding to CD99. Oncogene. 2018 Jan 31;1.

Book Chapter Translations

- Current Diagnosis & Treatment: Neurology, 2nd Ed., McGraw-Hill. Translated chapters; Central nervous system neoplasms (12), paraneoplastic neurologic syndromes (13) to Turkish. 2014

Abstracts& Presentations

- **Kayraklioglu N.** Immune modulation with TLR agonists. Cancer and inflammation Program Staff talks. National Cancer Institute, Frederick MD. October 23, 2017 (oral presentation)
- **Kayraklioglu, N.,** Klinman, D.M. Effects of TLR7 and TLR8 agonists on human monocyte activation & differentiation. Cancer immunology and immunotherapy: From conception to delivery. National Institutes of Health, Bethesda, MD, October 12-13, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. Research Festival 2017, National Institutes of Health, Bethesda, MD, September 12-13, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA, September 6-7, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Alvord, W.G., Parker K.H., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligonucleotides. American Association of Immunology 2017 meeting, Washington, DC May 12-17, 2017 (poster presentation).
- **Kayraklioglu, N.,** Klinman, D.M. Effects of TLR7 and TLR8 agonists on human monocyte differentiation. Keystone Symposia: Mononuclear phagocytes in health and disease, Austin, TX. April 30-May 4, 2017 (poster presentation).
- **Kayraklioglu, N.,** Scheiermann, J., Parker, K.H., Klinman, D.M. Effect of calcium carbonate encapsulation on the activity of orally administered CpG oligodeoxynucleotides Fellows & Young Investigators Colloquium, National Cancer Institute, Shady Grove, MD. March 16-17, 2017 (oral presentation).

- **Kayraklioglu N.** Effects of calcium encapsulated oligodeoxynucleotides in murine colitis. Cancer and inflammation Program Staff talks. National Cancer Institute, Frederick MD. October 3, 2016 (oral presentation)
- **Kayraklioglu, N.,** Parker, K.H., Klinman, D.M. Use of encapsulated oligodeoxynucleotides in murine colitis. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA. September 7-8, 2016 (poster presentation)
- Parker, K.H., **Kayraklioglu, N.,** Klinman, D.M. Assesment of calcium nanoparticles containing immunomodulatory oligonucleotides for oral treatment of inflammatory conditions. Immunology Interest Group Workshop, National Institutes of Health, Leesburg, VA. September 7-8, 2016

Selected Trainings & Workshops

- Translational Research in Clinical Oncology NCI, Bethesda, 2017
- Scientist Teaching Science Online Pedagogy Course, OITE NIH 2017
- Introduction to Principles of Clinical Research, NIH clinical center, 2017
- Grant Writing Workshop, OITE, NIH, 2017
- Teaching in Medical Education, OITE, NIH, 2017
- Statistics Tutorial, NCI, Bethesda, 2016
- Biotech 23 Flow cytometry: Principles and methods of Flow Cytometry, FAES, NIH, 2016
- Education of the Educators Program, Hacettepe University, 2015
- Pediatric Advanced Life Support Course, Hacettepe University, 2014
- Basic Life Support for Healthcare Providers Classroom Course, Hacettepe University, 2014
- PCR Course, Hacettepe University, 2012
- Scientific Publication Workshop, Hacettepe University, 2011
- Biostatistics Course, Hacettepe University, 2010

Professional Memberships

- American Association of Immunology, 2017- Present
- American Association for Cancer Research, 2017- Present
- National Postdoc association, 2017- Present