DIFFERENTIAL ACTIVATION OF IMMUNE CELLS BY COMMENSAL VERSUS PATHOGEN-DERIVED BACTERIAL RNA

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

DIFFERENTIAL ACTIVATION OF IMMUNE CELLS BY COMMENSAL VERSUS PATHOGEN-DERIVED BACTERIAL RNA

Ozcan, Mine

M.Sc., Department of Biology

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Immunological mechanisms contributing to distinguishing signals derived from commensal versus pathogenic bacteria is an active area of research and recent evidence suggests that commensal and pathogens may express different variants of pathogen associated molecular patterns (PAMP). In this thesis, we propose that as a major member of PAMP, bacterial RNAs derived from commensal and pathogens may have distinct immunostimulatory activities due to differentially recognition by the host immune system. In order to test this hypothesis, RNAs derived from two *bona fide* commensal bacteria, *Lactobacillus salivarious*, *Lactobacillus fermentum*, one commensal strain of *Enterococcus faecium*, one virulent clinical isolate of *Enterococcus faecium* and 2 strict pathogens, *Listeria monocytogenes*, *Streptococcus pyogenes* were used. The immunostimulatory activities of bacterial RNAs (bacRNA) were compared in *in vitro* and *in vivo* experiments. Human PBMCs, purified human neutrophils, and 2 distinct reporter cell lines stably expressing the endosomal ssRNA sensor TLR7 or the cytosolic sensors RIG-I and MDA-5 were stimulated with various doses of human

commensal or pathogen-derived purified RNAs as such or following their complexation with the transfection reagent Lipofectamine 2000 or the anti-microbial peptide LL37. Since bacterial RNA was previously shown to be a signature of microbial vitality (a VitaPAMP), we also tested the vaccine adjuvant activities of commensal versus pathogen derived bacRNAs in mice immunized with the model antigen OVA. The results indicate that commensal derived bacRNAs trigger a response dominated by Type I IFN production whereas those of pathogenic origin induce proinflammatory cytokine secretion that can also support Th1 development. Collectively, our findings suggest that commensals and pathogens may possess RNAs with sufficiently distinct structural features enabling their discrimination by immune cells.

Keywords: Bacterial RNA, commensal, pathogen, RIG-I/MDA-5, TLR7

ÖZ

KOMMENSAL VE PATOJEN BAKTERİLERDEN ELDE EDİLEN BAKTERİYEL RNALAR TARAFINDAN BAĞIŞIKLIK HÜCRELERİNİN FARKLI AKTİVASYONU

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Bağışıklık sistemine katkıda bulunan kommensal ve patojen bakterilerden elde edilen ayırt edici sinyaller aktif bir çalışma alanıdır ve son bulgular kommensal ve patojenlerin farklı formlarda patojene bağlı moleküler motifler ifade edebildiğini göstermektedir. Bu tezde, kommensal ve patojenden elde edilen bakteriyel RNAların patojene bağlı moleküler motiflerden biri olarak bağışıklık sistemini farklı olarak aktive edici özelliklere sahip olduğu ve konağın bağışıklık sistemi tarafından farklı olarak tanınmakta olduğu hipotezi test edilmiştir. Bu hipotezi denemek için 2 hakiki insal kommensal bakteri suşu olan *Lactobacillus salivarious*, *Lactobacillus fermentum*, bir kommensal suş *Enterococcus faecium*, bir virulent klinik izolat *Enterococcus faecium* ve 2 mutlak patojen, *Listeria monocytogenes,* ve *Streptococcus pyogenes* suşlarının RNA'ları kullanılmıştır. Bakteriyel RNAların bağışıklık sistemi üzerindeki stimulan aktivitesi *in vitro* ve *in vivo* deneylerde denenmiştir. İnsan periferik mononükleer kan hücreleri, insan nötrofilleri ve endosomal ssRNA sensörü TLR7 ve sitoplazmik

sensörleri RIG-I ve MDA-5'i stabil olarak ifade eden 2 farklı hücre hattı insan kommensal ve patojen bakterilerinden elde edilen RNAların farklı dozlarıyla serbest halde veya lipofectamine 2000 ya da anti-mikrobiyal peptit LL37 ile birlikte stimüle edilmiştir. Bakteriyel RNA mikrobiyal canlılığın (VitaPAMP) göstergesi olarak rapor edildiği için, kommensal ve patojen bakteriyel RNAlarının aşı adjuvantı aktivitesi farede model antijen OVA kullanılarak test edilmiştir. Sonuçlar göstermektedir ki kommensalden elde edilen bakteriyel RNA'lar, tip I interferon ağırlıklı bir immün yanıt göstermekte, buna karşın, patojenden elde edilen bakteriyel RNA'lar ise proenflamatuar sitokinlerinin salımını tetiklerken Th1 gelişimini desteklemektedir. Bulgularımıza göre kommensal ve patojen bakteri RNAlarının farklı yapısal özellikleri bağışıklık sistemi hücreleri tarafından ayrımının yapılmasına sebep olabilir.

Anahtar kelimeler: Bakteriyel RNA, kommensal, patojen, RIG-I/MDA-5, TLR7

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
AIM2	Absent in melanoma 2
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing CARD
BacRNA	bacterial RNA
BCR	B cell receptor
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cGAMP	cyclic GMP-AMP
CpG	Unmethylated cytosine-phosphate-guaniosine motifs
DAI	DNA-dependent activator of IFN regulatory factor
DAMP	Danger/damage associated molecular pattern
DC	Dendritic cell
dsRNA	double stranded RNA
ELISA	Enzyme Linked-Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
hPBMC	Human peripheral blood mononuclear cell
IFN	Interferon
IFNβ	Interferon Beta
Ig	Immunoglobulin
IL	Interleukin
IP 10	Interferon gamma-induced protein 10
IRF	Interferon-regulatory factor
ISRE	IFN-stimulated response element
LPS	Lipopolysaccharide
MDA-5	Melanoma Differentiation-Associated protein 5
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor-88
Mϕ	Macrophages

NF-κB	Nuclear factor- kappa B
NK	Natural Killer Cell
NLR	Nucleotide-binding oligomerization domain like receptors
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate Buffered Saline
PNPP	Para-nitrophenyl pyro phosphate
poly I:C	Polyriboinosinic polyribocytidylic acid
PRR	Pattern recognition receptor
R848	Resiquimod
RIG-I	Retinoic acid-inducible gene-I
RLH	Retinoic acid inducible gene I (RIG-I)-like helicase
RLR	Retinoic acid-inducible gene-I like receptor
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SEAP	Soluble alkaline phosphatase
ssRNA	single stranded RNA
STING	Stimulator of Interferon genes
TAE	Tris-base, Acetic Acid, EDTA
TcR	T cell receptor
TH1	T helper type 1
TH17	T helper type 17
TH2	T helper type 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREG	Regulatory T cells
TRIF	TIR domain containing adaptor inducing IFN-β
TSB	Tryptic soy broth

CHAPTER 1

INTRODUCTION

1.1 Immune System

1.1.1 How immune system works

Immune system is composed of a variety of effector cells and molecules that protect the body from infectious agents. In order to protect from pathogens, immune system has to carry out four main tasks. First, foreign agent needs to be recognized by the help of white blood cells of innate immune system and lymphocytes of the adaptive immune system. Altered self and non-self is discriminated by the immune system (Medzhitov *et al.*, 2002). This process is called immunological recognition. Second, immune effector functions provide instant response to contain and destroy infection (Medzhitov, 2007). The control mechanism to keep immune response under control and to prevent self-damage is called immune regulatory mechanism (Sakaguchi *et al.*, 2008). Lastly, adaptive immune system generates an immunological memory to protect against recurrent infections. It provides immediate and stronger response against any subsequent infection to that agent (Pulendran *et al.*, 2006).

Physical and chemical barriers are first line of defense against invading infectious agents. For instance, tight junctions that hold epithelial cells together form a seal against external environment to prevent pathogen entry (Peterson *et al.*, 2014) whereas mucosa in the gut acts as a barrier against invaders (Ashida *et al.*, 2012). When this line of defense is breached, innate immune response is activated immediately after exposure to

an infectious agent and this response is nonspecific. Innate arm of the immune system is specialized in recognition of certain pathogen associated non-self molecules and some of these cells readily ingest and kill microbes. If the ongoing infection cannot be cleared, adaptive immune response is initiated by the help of antigen presenting cells (APCs) such as the dendritic cells, macrophages and monocytes (Guermonprez *et al.,* 2002). An antigen (Ag) is any substance that is capable to inducing an adaptive immune response. Most Ags are processed and presented by APCs to T cells and T and B cells participate in generation of Ag specific adaptive immune response. Adaptive immunity is a delayed response, taking days rather than hours and it is highly Ag specific, being more effective than the fast acting but non-specific innate immunity (Flajnik *et al.,* 2004). To summarize, together with the first line of defense against invading microbes, innate and adaptive arms the immune system form a highly complex network of recognition and effector functions that collectively provide protection against invading pathogens.

1.1.2 Cells of the immune system

When common progenitor stem cells in bone marrow differentiate, they give rise to all blood cells (Orkin, 2000). Hematopoietic stem cells are divided into two major lineages: Common myeloid progenitors give rise to megakaryocytes, erythrocytes, granulocytes and macrophages whereas common lymphoid progenitors generate T cells, B cells and natural killer (NK) cells (Iwasaki *et al.*, 2007) (Klug *et al.*, 1998). It is thought that dendritic cells may originate from both progenitors (Yang *et al.*, 2005). Most of the immune cells complete their maturation in bone marrow or in tissues they home to. In contrast, immature T lymphocytes migrate to thymus and complete their maturation there (Bhan *et al.*, 1980). Following maturation, both T and B lymphocytes enter bloodstream and circulate between blood and secondary lymphoid tissues whereas other leukocytes such as macrophages become tissue resident. APCs migrate to secondary lymphoid tissues via lymphatic vessels where they are in close contact with T and B cells (Janeway *et al.*, 2008).

1.1.3 Innate Immune system and Pathogen recognition receptors (PRRs)

The first cells that encounter an invading pathogen that has breached the physical barriers are tissue resident macrophages and DCs. Such resident cells release and produce bactericidal agents to kill the microorganisms that are ingested. Following this initial encounter, the resident cells are activated to release cytokines and chemokines that establish a state of inflammation and recruit other cells to the site of infection.

Pathogen recognition (or sensing the signals of danger/damage) depends on the expression of specialized receptors that interact with pathogen-derived or damage-associated molecules. Such molecules of "danger" can be derived from the pathogens themselves and are known as pathogen associated molecular patterns (PAMPs) and/or they can be released from damaged cells and are collectively known as damage associated molecular patterns (DAMPs). PAMPs and DAMPs are recognized by the pattern recognition receptors (PRRs) that are expressed by immune cells. The PRRs of the innate immune system can be classified into 3 large classes with distinct functions: First, there are phagocytic receptors that induce ingestion of the recognized pathogens. A second group of soluble or secreted PRRs such as the ficolins, recognize unusual glycosylation patterns and can activate complement or participate in opsonization of pathogens. The third and the largest group of PRRs are receptors involved both in recognition and signaling. These include the Toll-like receptor family (TLRs), Retinoic acid inducible gene I (RIG-I)-like helicase (RLHs) or RIG-like receptors (for eg., RIG-I and MDA-5), Nucleotide-binding oligomerization domain (NOD)-like receptors (NOD-like receptor family) and C-Type lectin receptors (for eg., Dectin-1) (Janeway *et al.*, 2008). Regular patterns of molecular structures (PAMPS and DAMPS) are recognized by these set of receptors. For instance, LPS is recognized by Toll-like receptor 4 and unmetylated cytosine-phosphate-guanine dinucleotide rich DNAs (CpG) are recognized by the endosomal receptor TLR9. A list of certain PRRs and their ligands are given in **Table 1**.

Table 1 Main Pattern Recognition Receptors and their ligands (Adapted from Lee and Kim, 2007)

Families	Proteins	Major ligands (or activators)	Major cell types[a]
TLRs[c]	TLR1	Triacyl lipopeptides from bacteria and mycobacteria	MΦ, cDC, neutrophil, mast cells
	TLR2	LTA from gram-positive bacteria, yeast zymosan, lipopeptides (Pam_3CSK_4, MALP2), lipoarabinomannan from mycobacteria	MΦ, cDC, neutrophil, mast cell
	TLR3	Viral dsRNA, poly(I:C)	cDC, MΦ (mouse), endo/epithelial cells
	TLR4	LPS from gram-negative bacteria, mannan from *Candida albicans*, GIPLs from *Trypanosoma*, viral envelope proteins from RSV and MMTV	MΦ, cDC, neutrophil, mast cell, eosinophil
	TLR5	Bacterial flagellin	Monocyte, cDC, iEC
	TLR6	Diacyl lipopeptides from *Mycoplasma*, LTA from gram-positive bacteria, yeast zymosan	Monocyte, mast cell, cDC, neutrophil
	TLR7	ssRNA from RNA viruses, imiquimod, resiquimod (R848), synthetic polyU RNA, certain siRNAs	pDC, neutrophil, eosinophil
	TLR8	Resiquimod (R848), viral ssRNA	Monocyte, cDC, mast cell, neutrophil
	TLR9	Bacterial and viral CpG DNA, hemozoin from *Plasmodium*	pDC, NK cell, eosinophil, neutrophil
	TLR10	–	pDC, B cell
	TLR11	Profilin-like molecule from *Toxoplasma gondii*, unknown ligand(s) from uropathogenic bacteria	MΦ, epithelial cell
CARD helicases	RIG-I	In vitro transcribed dsRNA, RNA from various RNA viruses	Ub (IFN inducible)
	MDA5	Poly(I:C), RNA from EMCV	Ub (IFN inducible)
NODs	NOD1	iE-DAP	MΦ, DC, iEC
	NOD2	MDP	MΦ, DC, Paneth cell
NALPs	NALP1	Cell rupture	MΦ, PBL
	NALP1b	Anthrax lethal toxin	MΦ
	NALP2	-	Ub
	NALP3	Bacterial mRNA, extracellular ATP, uric-acid crystals, R848, *Listeria monocytogenes*, *Staphylococcus aureus*	MΦ, PBL
	NALP12	-	MΦ, eosinophil

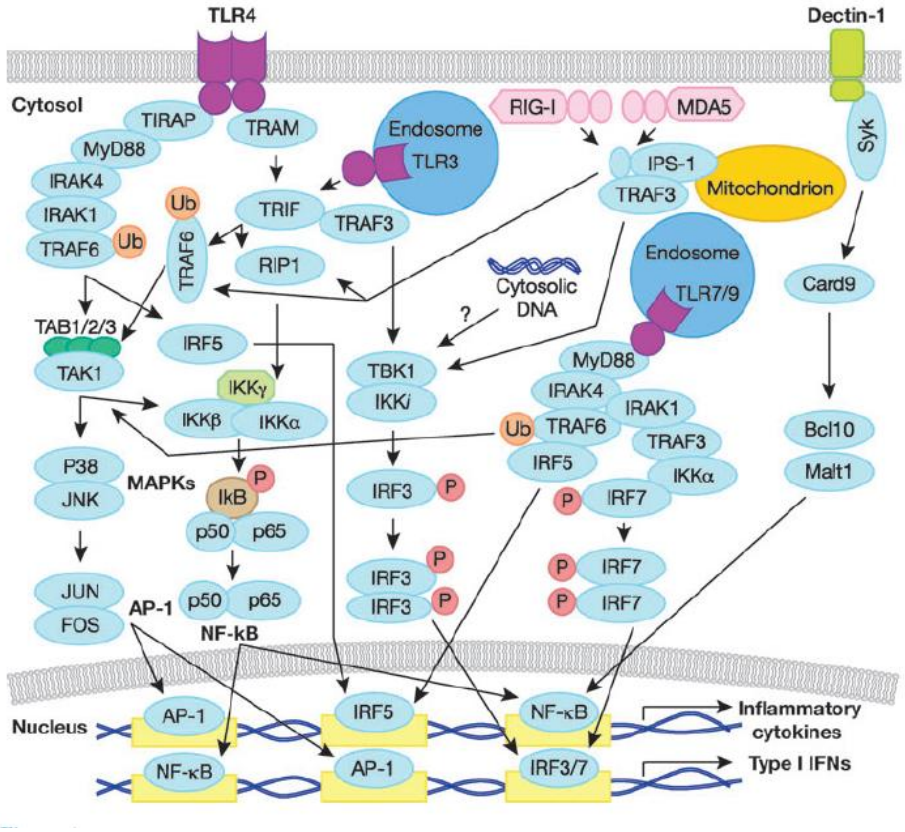


Figure 1 The signaling cascades of main PRRs (Adapted from Lee and Kim, 2007)

1.1.4 Nucleic acid sensors

Besides functioning in the storage and translation of genetic information, nucleic acids are pathogen associated molecular patterns that are recognized by the host. Therefore, discrimination of self and non-self nucleic acids is very crucial. Host and microbe nucleic acid discrimination is based on sequence, structural features and spatial compartmentalization. For instance, bacterial RNA (bacRNA) is recognized as non-self owing to the decreased extent of nucleotide modifications in prokaryotes, additional nucleotide modifications in prokaryotes that are not present in eukaryotes and specific sequence patterns of bacRNA (Eberle *et al.*, 2009) (Dalpke *et al.*, 2012).

Pattern recognition receptors that recognize nucleic acids can be classified according to their location; some of the main cytoplasmic and endolysosomal receptors are RIG-I, MDA-5, Cyclic GMP-AMP synthase and TLR3, TLR7, TLR8 and TLR9 receptors. TLR family function mostly in immune cells, such as DCs, macrophages, and B cells; endosomal TLRs monitor lumen of endosomes and lysosomes in order to detect different forms of nucleic acids of non-self origin. On the other hand, cytosolic nucleic acid sensors detect nucleic acids in the cytoplasm of almost all cell types (Wu *et al.*, 2014). In addition, only in neutrophils, cytoplasmic receptors RIG-I and MDA-5 are stored in secretory vesicles and can fuse with plasma membrane. Therefore RIG-I and MDA-5 is both cytoplasmic and cell surface associated for neutrophils (Berger *et al.*, 2012).

1.1.4.1 DNA sensors

TLR9

Toll-like receptor 9 (TLR9) has been shown to recognize hypomethylated DNA that is rich in CG dinucleotides (Hemmi *et al.*, 2000). Hypomethylated CG dinucleotides are frequent in bacterial DNA. In contrast eukaryotic DNA contains highly methylated CG motifs (Kumagai *et al.*, 2013). Bacterial DNA mediated TLR9 stimulation can be mimicked by synthetic CpG ODNs and can be abrogated upon GC motif delivery (Krieg *et al.*, 1995). However, in 2008 Haas and colleagues stated that dependency on CG dinucleotide sequence can mainly be observed for synthetic phosphorothioate backbones, but might be less crucial for natural backbones. In order to discriminate bacterial and mammalian DNA efficiently, metyhlation and compartmentalization are also important features that are used (Dalpke *et al.*, 2012).

RNA polymerase III/RIG-I pathway

Cytosolic B form of double stranded DNA can be transcribed into 5'-triphosphate and double-stranded secondary structure containing RNA by RNA polymerase III and the transcribed RNA can be recognized by RIG-I and induce Type-I IFN production through MAVS (Chiu *et al.,* 2009), (Ablasser *et al.,* 2009).

Cyclic GMP-AMP synthase (cGAS)

cGAS is a cytoplasmic DNA sensor. Upon binding of DNA, it is activated to catalyze production of cyclic GMP-AMP (cGAMP) from ATP and GTP. cGAMP as a second messenger activates stimulator of IFN genes (STING) which is a direct sensor of cyclic dinucleotides and an important adaptor for Type I IFN induction by cytosolic DNA. Following overexpression of cGAS, IRF3 is activated and IFN-β production is induced (STING-dependent) (Wu *et al.,* 2014).

DNA-dependent activator of IFN regulatory factors (DAI)

DAI is a cytoplasmic sensor of double stranded DNA. Upon binding, its association with IRF3 and TBK1 increases. Over-expression of DAI results in DNA-mediated Type I IFN secretion (Takaoka *et al.,* 2007).

Absent in melanoma 2 (AIM2)

AIM2 recognizes cytoplasmic DNA by its oligonucleotide/oligosaccharide-binding domain and interacts with apoptosis-associated speck-like protein containing CARD (ASC) via its pyrin domain to activate caspase-1. Interaction of AIM2 with ASC results in pyroptosis. Also, activated caspase-1 processes the inactive precursors of IL1β and IL18 into mature cytokines (Fernandes-Alnemri *et al.,* 2009). In figure 2, PRRs that recognize RNA and DNA is shown and in table 2, PRRs that are sensors of RNA are outlined.

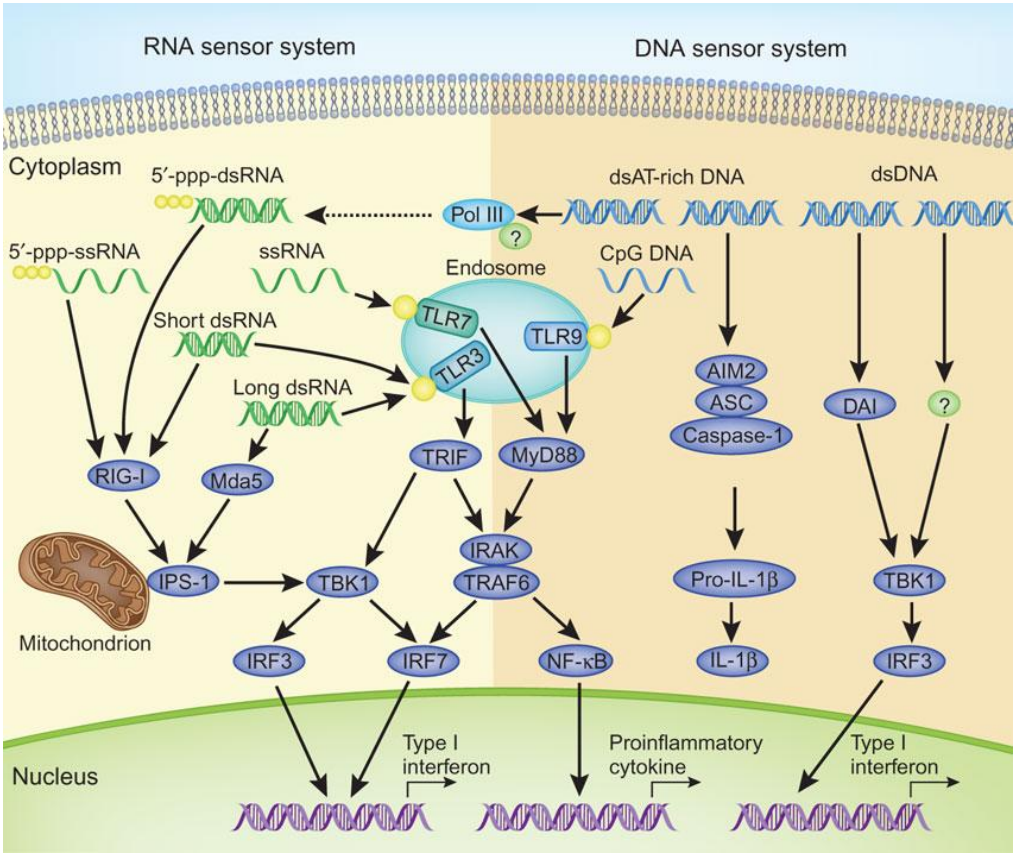


Figure 2 Nucleic acid sensors (Adapted from Cao, 2009)

Table 2 PRRs that have shown to recognize RNA

PRRs	**Localization**	**Ligands**	**Cell type specific expression**
TLR3	endolysosomal	dsRNA	mDC,T, NK cells
TLR7	endolysosomal	ssRNA and short dsRNA	MΦ/monocytes, Bcells, pDCs
TLR8	endolysosomal	ssRNA short dsRNA	monocytes, MΦ, mDCs, neutrophils
TLR13	endolysosomal	23S rRNA specific seq. "CGGAAAGACC"	DC, MΦ, Bcells
RIG-I	cytosolic	5'PPP ds RNA(~19bp)	immune and non-immune cells, neutrophils
MDA-5	cytosolic	long ds RNA	immune and non-immune cells, neutrophils
NLRP3	cytosolic	ssRNA, dsRNA, bacterial mRNA	BMDCs, BMDMs and epithelial cells

1.1.4.2 RNA sensors

Recognition of cytosolic RNA (RIG-I, MDA-5 and NLRP3)

TLRs that recognize nucleic acids (TLR3, 7, 8 and 9) are located within endosomes. Therefore, their ligand binding domains face the lumen of the endosome. The expression of these TLRs is restricted to immune cells. The endosomal localization ensures that such TLRs remain "ignorant" of the nucleic acids of non-host origin that may be found in the cytoplasm but respond to those that are internalized.

A distinct set of cytoplasmic receptors are more broadly expressed and are dedicated to sense foreign nucleic acids in the cytosol. RIG-I and MDA-5 are recognized as bacterial/viral RNA sensor and meet these criteria (*Wu et al.,* 2014). Bacterial RNA was shown to enter the cytosol where it is recognized by cytosolic RNA receptors. RIG-I detects 5' triphosporylated dsRNA and panhandle-like secondary structures. Bacterial RNA, unlike eukaryotic RNA, is not capped and contains 5' triphosporylated RNA and therefore can be recognized by RIG-I (Hagmann *et al.,* 2013). In addition, MDA-5 recognizes long dsRNAs (Wu *et al.,* 2014). As a result of RIG-I and MDA-5 activation, Type I IFNs and proinflammatory cytokines are secreted.

NLRP3 inflammasome is a multi-protein complex consisting of procaspase-1, adaptor molecule ASC and a sensor protein Nlrp3, which mediates autocatalytic self-cleavage of caspase 1 into an active enzyme. The activation of Nlrp3 inflammasome depends upon two signals; first is the priming signal to activate NF-κB by a PAMP and the other is required for cleavage of pro-IL1β into IL1β via the activation caspase-1. Bacterial RNA can induce activation of NLRP3 inflammasome in human monocytes without a priming signal. Following recognition in cytoplasm, caspase-1 is activated and IL1β is secreted (Eigenbrod *et al.,* 2012),(Kanneganti *et al.,* 2007) (Franchi *et al.,* 2009) (Davis *et al.,* 2011).

Recognition of RNA by endosomal TLRs

TLR3, TLR7, TLR8, TLR9 and TLR13 (only in mice) recognize nucleic acids in endosomal compartments. These receptors monitor endolysosomal lumen for pathogen derived nucleic acids, and their signaling is via two different pathway; TLR3 mediated recognition recruits the adaptor TRIF whereas TLR7, TLR8, TLR9 and TLR13 signaling operates through the adaptor protein MyD88. Both TRIF and MyD88 dependent pathways result in activation of the transcription pathways NF-κB. Depending on the identity of the TLR receptors involved, TRIF-dependent IRF3 and MyD88-dependent IRF7 activation can also occur (Wu *et al.*, 2014). TLR3 recognizes long stretches of dsRNA (Kumagai *et al.*, 2013) independent of their sequence (Dalpke *et al.*, 2012). TLR7 and TLR8 (TLR8 is functional only in humans but not in mice) are phylogenetically close TLRs and they both recognize single stranded RNA (ssRNA), imidazoquinoline derivatives such as R848, short dsRNA and bacterial RNA (Blasius *et al.*, 2010), (Gantier *et al.*, 2008) (Cervantes *et al.*, 2012). TLR13 (only in mice) recognizes 23S ribosomal RNA (rRNA) specific sequence "CGGAAAGACC" (Hochrein *et al.*, 2013), (Hidmark *et al.*, 2012) (Li *et al.*, 2012). Following recognition of their ligands, TLRs induce expression of proinflammatory cytokines and Type I IFNs (Figure 3).

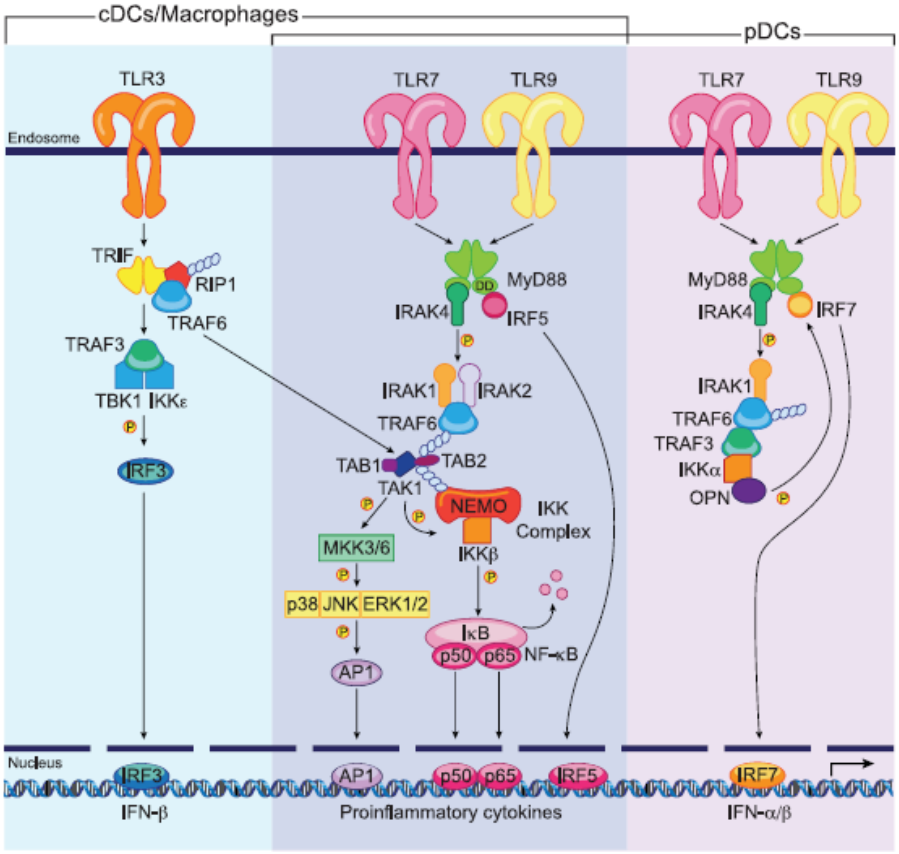


Figure 3 Intracellular TLR signaling pathways activate 3 distinct transcription factors (NF-κB, IRF-3 and IRF-7) resulting in transcription of Type I IFNs and pro-inflammatory cytokines. (Adapted from Blasius *et al.*, 2010)

1.1.5 Adaptive immune system

If an infection is not cleared by members of the innate immune system, adaptive immune system needs to be activated. Adaptive immune system is instructed by innate arm of immunity through antigen-presenting cells providing 2 signals to T cells: processed antigen derived peptides presented in the context of MHC molecules and co-stimulatory signals (for eg., CD80 and CD86) (Janeway *et al.*, 2002). In the absence of antigen, naïve T cells circulate between blood, secondary lymphoid organs and the lymphatics. When T cells come across with a specific antigen presented by APCs in secondary

lymphoid organs, they proliferate and differentiate into effector cells. Cytotoxic T lymphocytes differentiate from naïve CD8+ T cells following recognition of pathogen derived peptides presented with MHC Class I molecules expressed on the surface of APCs. Once fully activated, these cells can kill infected or tumor cells that express the same antigenic peptides that triggered their initial activation. On the other hand, CD4+ T cells that recognizes peptides presented by MHC Class II expressing APCs can differentiate into different subtypes of T helper cells depending on the combination of cytokines that are present in the environment during their differentiation (Figure 4). For instance, if there is IFNγ and IL12 in the milieu, upon recognition of a pathogen derived peptide presented by APCs with MHC Class II molecules, naïve CD4+ T cells differentiate into Th1 cells that provide help to other cells of the immune system to eliminate intracellular pathogens (Walport *et al.,* 2008 and Linterman *et al.,* 2009).

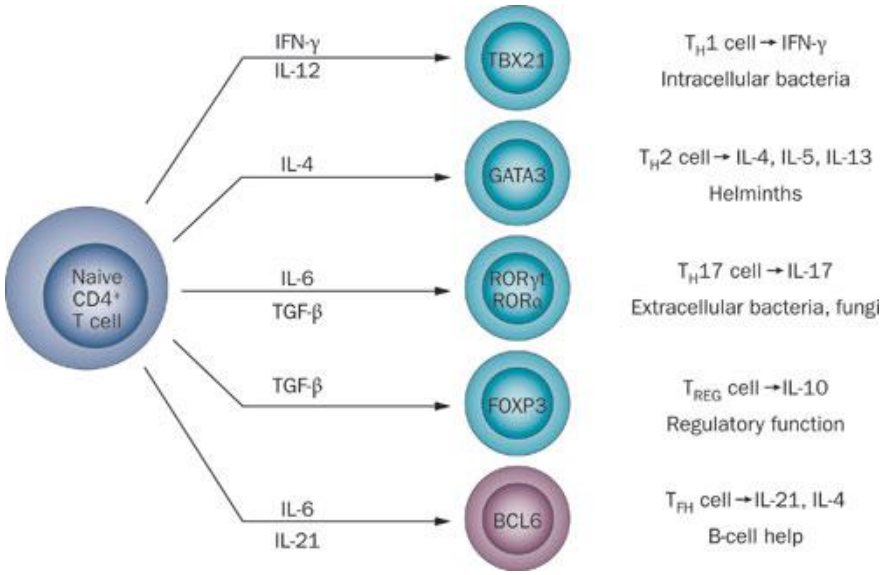


Figure 4 Differentiation of CD4+ T cells into different subtypes(Adapted from Linterman *et al.,* 2009)

B cells are also very crucial in adaptive immunity. There is no MHC restriction for B cells. They are activated by direct recognition of antigen via the B cell receptor (BCR). However, without T cell help, activated B cells differentiate into IgM secreting B cells (Geisburger *et al.*, 2006). In order to achieve class switching, T cell help is required so that B cells can differentiate into plasma cells having the ability to secrete other classes and subclasses of antibodies (IgG, IgE and IgA) (Figure 5) (Czyzewska-Buczyńska *et al.*, 2010). In addition, a small percent of T and B cells provide immunological memory.

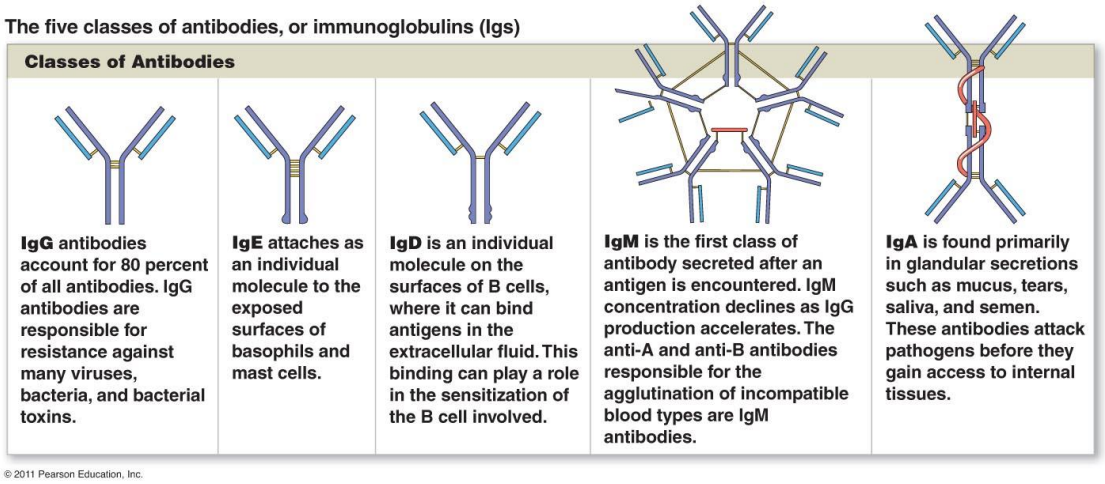


Figure 5 Classes of antibodies and their major functions (Retrieved from http://www.highlands.edu/academics/divisions/scipe/biology/faculty/harnden/2122/images/abclasses.jpg, n.d.)

1.2 Effect of microbiome on immune system

Both innate and adaptive immune system evolved to tolerate microbial symbionts and identify and kill microbial pathogens (Sansonetti *et al.*, 2009). The colon of the gastrointestinal tract solely contains 10^{14} microorganisms which is 10 times more than the total number of human cells that make up the body. The associated metagenome contains a global number of genes 100 times more than the human genome (Sansonetti, 2011). The microbiota in humans and higher primates is composed of a large population

of various bacterial species present in oral cavity, upper respiratory tract, the gastrointestinal tract, the urogenital tract and on the skin. The major benefits of the gut microbiota to the host are to release nutrients from food such as hydrolysis of complex sugars and synthesis of certain vitamins, and formation of a barrier against microbial pathogens (Sansonetti, 2004). In figure 6, effect of microbiota on immune system is shown.

Extent of colonization of the gut and other tissues by commensal bacteria is a shared feature among metazoans. However, the extent of colonization has increased during evolution in vertebrates as a result of emergence of new organs that could provide a niche for growth (Ley *et al.,* 2008). This relationship requires a controlled system which is characterized by the ability to efficiently discriminate between continuously resident microbiota, with which a homeostatic balance is formed and the invasive pathogens, which must be eliminated. Tolerance of the host immune system against commensal microbes is mediated by immune cells, gut epithelium and microbes themselves. In general, there is an active suppression of the host immune response by commensals. For example, *Lactobacillus casei* downregulates the E3-SCF ubiquitin ligase subunit Roc-1 and several components of the proteasome complex, preventing IκB degradation and hence NF-κB activation (Tien *et al.,* 2006). Commensals can also evade immune recognition. For instance, lipid A (a constituent of LPS in Gram negative bacteria) of Bacteroidetes species in the gut is pentacylated. This form of lipid A is a poor agonist of TLR4 (the LPS receptor) (Munford *et al.,* 2006). Moreover, commensal bacteria can regulate immune responses by maintaining an important proportion of resident macrophages and dendritic cells in immaturity and by initiating a balance between the regulatory T cells and inflammatory lymphocytes (i.e. Th1 and Th17 cells) (Sansonetti *et al.,* 2009)

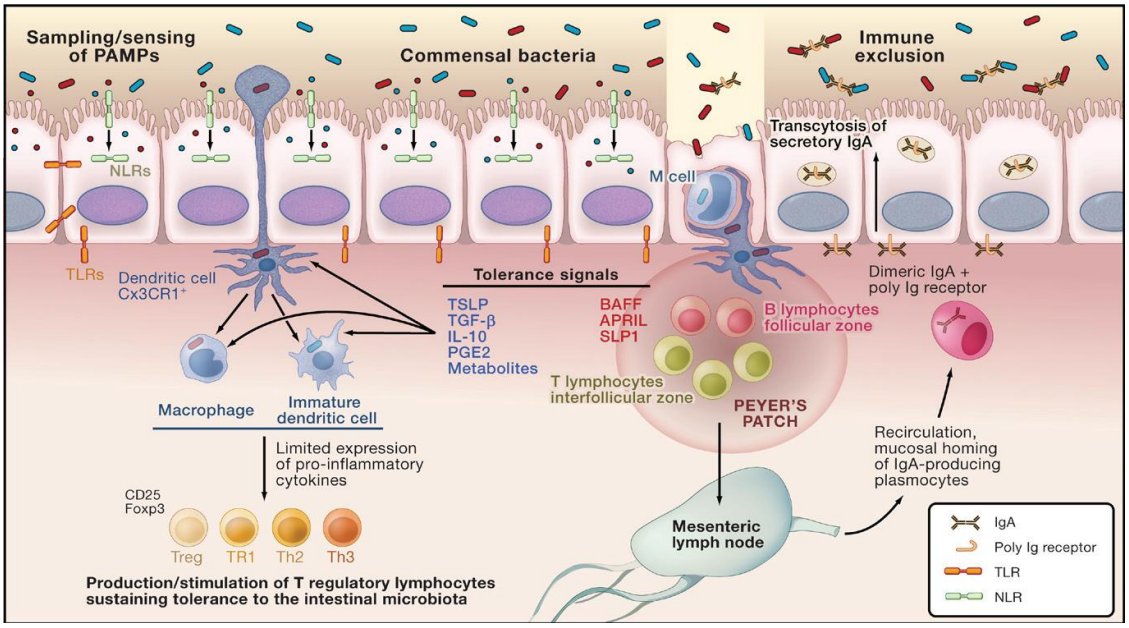


Figure 6 Effect of microbiota on immune system (Adapted from Sansonetti and Medzhitov, 2009).

Microbial pathogens on the other hand, do not follow the rules of commensal bacteria. They impose their own signaling which results in a strong immune response against them.

Pathogen recognition concept was first hypothesized by Medzhitov and Janeway in 1997. According to this, "various PAMPs recognized by cognate PRRs expressed on APCs induce the expression of co-stimulatory molecules of the B7 family, thus signaling the presence of pathogens and allowing activation of lymphocytes specific for antigens derived from the pathogens. PRRs that are strategically expressed on the effector cells of the innate immune system induce the expression of corresponding sets of effector cytokines, which in turn direct the induction of the appropriate effector mechanisms in the adaptive immune response." However, this PAMP-PRR based distinction of self from non-self does not cover the differential sensing of commensal versus pathogenic bacteria. According to the "Janeway paradigm" since both commensals and pathogens

express PAMPS, they should be recognized by identical PRRs. In fact, the cell wall component peptidoglycan, nonmethylated DNA, LPS and several other structures are shared prokaryotic patterns of commensals and pathogens that are recognized by PRRs. Yet, the story must be more complicated since despite these shared patterns, pathogens and commensals can induce differential immune activation.

How does the immune system discriminate pathogen versus commensal microorganisms and respond accordingly? Sansonetti (2011) proposed three hypotheses to explain this phenomenon: First is whether PAMPs from pathogenic microorganisms are more agonistic to PRRs than those of commensal microorganisms. For example, α and ε-Proteobacteriaceae express flagellin molecules that are poor agonists of TLR5 when compared to β and γ- Proteobacteriaceae. Their flagellin sequences include amino acid differences in TLR5 recognition site which provides an escape from TLR5 recognition (Andersen-Nissen *et al.,* 2005) It is also possible that discrimination may be based on combination of PAMPs that is recognized by a combination of PRRs. Sansonetti's second hypothesis states that symbionts or their products can dampen the innate immunity. *Lactobacillus casei is* a probiotic microorganism that antagonizes induction of proinflammatory cytokines by lowering the capacity of a cell to degrade I-κB and activate NF-κB when cells are infected with Shigella (Tien *et al.,* 2006). Even if reports show that symbionts can antagonize proinflammatory signals, the big picture is more complicated. In the gut, homeostasis is maintained between symbiotic microorganisms and epithelium; microorganisms are contained in the lumen and embedded in the upper portion of mucus layer where antimicrobial molecules are also embedded. The epithelial cells are exposed to some of the bacterial PAMPs and induce and maintain regulatory T cells in lamina propria. When this homeostasis is disrupted (dysbiosis), commensal bacteria induce immune activation. Therefore, in the limits of homeostasis, commensals are tolerogenic but upon epithelial damage, inflammatory response can be observed. Third hypothesis of Sansonetti is "second or danger signal". Since "Janeway paradigm" is not sufficient to delineate pathogens, a revised and improved concept incorporating the signals associated with expression of pathogenicity should be considered. Pathogenic as well as nonpathogenic bacteria have mobile and

accessory genetic elements that code for functions related with bacterial fitness and adaptation (i.e. colonization capacity) (Dobrindt *et al.,* 2010). Some species of bacteria include both pathogenic and commensal variants. For example, Enterococcus faecium is a native gut commensal bacteria but pathogenic forms are emerging as the cause of multidrug-resistant hospital acquired infection. Lebreton and colleagues (2013) examined the genome sequences of these to understand bacterial adaptations that result in turning of a "friend into foe". They observed that changes in the genome is related with changes in human behavior; first lineage branching was seen at the time of urbanization of humans and domestication of animals and second bifurcation was observed at the time of introduction of antibiotics. Therefore, in order to adapt to changes in their habitat, commensals gain additional genetic elements conferring additional antibiotic resistance and virulence. Also, it was reported that many antibiotic-resistant clinical isolate of this strain carry mobile DNA elements of foreign origin and this mobile DNA elements contain pathogenicity islands that harbor a number of virulence associated genes (i.e. genes that confer vancomycin resistance, three plasmids that provide resistance to other antibiotics and insertion sequences) (Paulsen *et al.,* 2003), (Shankar *et al.,* 2002). How multiple antibiotic resistant enteroccus infections occur was explained in a few steps; commensal strains of enterococci which do not have antibiotic resistance plasmids and pathogenicity islands are found in a healthy gut flora. Upon antibiotic administration, susceptible strains of bacteria are eliminated. Multiple antibiotic resistant enterococci can colonize in the gut since they have the acquired genes that confer their colonization in the gut; surface proteins that enable attachment to host cells, carbohydrate metabolism, bile acid hydrolase and other properties to survive in that habitat and infect the host (Gilmore *et al.,* 2003)

1.3 Aim of the study

Immunological mechanisms contributing to distinguishing signals derived from commensal versus pathogenic bacteria is an active area of research and recent evidence suggests that commensal and pathogens may express different variants of pathogen

associated molecular patterns (PAMP). We hypothesized that as a major member of PAMP, bacterial RNA (bacRNA) originating from commensals versus pathogens may possess distinct immunostimulatory activities, enabling their discrimination by the immune system. To test this, two *bona fide* commensal bacteria, *Lactobacillus salivarious*, *Lactobacillus fermentum*, one commensal strain of *Enterococcus faecium*, one virulent clinical isolate of *Enterococcus faecium* and 2 strict pathogens, *Listeria monocytogenes*, *Streptococcus pyogenes* were used. Bacterial RNAs were isolated from these commensal and pathogen bacteria and their differential immunostimulatory activities were compared in various *in vitro* and *in vivo* assays. The results suggest that the immune system can indeed discriminate bacRNAs of commensal and pathogen origin and generate a response dominated by chronic type I IFN production or pro-inflammatory cytokine production, respectively. These findings may be of interest since the immunomodulatory properties of PAMPs derived from commensal bacteria are currently unclear.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1 Bacteria Strains and bacterial culture media

The bacterial strains used in this study and their sources are described in Table 3. Commensal bacteria strains; LS, LF and CEF were a kind gift from Prof. Dr. Ihsan Gursel (Bilkent University, Molecular Biology and Genetics Department). Pathogenic bacterial strains EF, LM and SP were a kind gift from Assoc. Prof. Dr. Orhan Bedir (GATA Department of Medical Microbiology). MRS medium and MRS agar were purchased from CONDA, Spain and prepared according to the manufacturer's protocol. TSB medium consisted of 1.5 % Tryptone (Conda, Spain), 0.5 % Soytone (Conda, Spain) and 0.5 % NaCl (Fisher Scientific, USA) and the pH was adjusted to 7.3. Tryptic soy agar (TSA) was composed of 1.5 % Bacto agar (Conda, Spain) per liter. C medium contained 0.5 % Proteose peptone #3 (Conda, Spain), 1.5 % Yeast extract (Conda, Spain), 10 mM K_2HPO_4, 0.4 mM $MgSO_4$, 17 mM NaCl, and the final pH was adjusted to 7.5. To prepare C agar, Bacto agar was added to a final concentration of 1.4 % (w/v).

Table 3 Bacteria strains, their sources and bacterial culture media

Abbreviation of bacteria	Name of the bacteria strain	Source	Bacterial Culture Media
LS	*Lactobacillus salivarious*	Infant feces	MRS medium
LF	*Lactobacillus fermentum*	Infant feces	MRS medium
CEF	*Enterococcus faecium*	Infant feces	MRS medium
EF	*Enterococcus faecium*	Clinical isolate *	MRS medium
LM	*Listeria monocytogenes*	ATCC 35152	Tryptic soy broth(TSB)
SP	*Streptococcus pyogenes*	NCTC 12696/ATCC 19615	C medium

* from GATA Department of Medical Microbiology

2.1.2 Cell culture media, Buffers and other solutions

RPMI 1640 cell culture media, Penicillin/ Streptomycin, HEPES, Na pyruvate, Non-essential amino acid solution and L-Glutamine were purchased from Hyclone, USA. DNase RNase free distilled water, Fetal Bovine Serum (FBS) and Phosphate Buffered Saline (PBS) were from Lonza, Switzerland. Tris-Acetate-EDTA (TAE) 10X solution and Tween 20 were from Fisher Scientific, USA. Opti-MEM Reduced Serum Media was obtained from Life Technologies, USA. Cell culture media, buffers and solutions used in experiments are described in Appendices.

2.1.3 Reagents and Reporter Cell lines

DNase I RNase-free, RNase I DNase-free, Loading dye (6X), Proteinase K and RNase away were from Thermo Scientific, USA. Lysozyme, Tris-base, Molecular biology grade Ethylenediaminetetraacetic acid (EDTA) disodium salt solution, Hydrochloric acid (HCl) and Dextran from *Leuconostoc mesenteroides* (average mol wt 150,000) were from Sigma, USA. Trypan Blue Dye and Lymphocyte separation medium were from Lonza, Switzerland. Lipofectamine 2000 was obtained from Invitrogen.1 kb DNA ladder and ssRNA ladder were from NEB, UK. HEK-Blue hTLR7 cells, B16-Blue IFN-α/β cells, Normocin, Zeocin, Blasticidin and QUANTI-Blue were purchased from Invivogen, USA. Cell fixation and permabilization were performed using the Fixation and Permeabilization Medium A and Medium B from Invitrogen, USA. RNA Clean & Concentrator-25 columns were obtained from Zymoresearch, USA.

2.1.4 Toll like Receptor (TLR) Ligands, CpG oligonucleotides (ODNs) and Peptides

TLR ligands and ODNs that are used in this study were endotoxin and protein-free (Table 4). Sequences of ODN used (5'→3') are given as the single letter nucleotides and bases shown in capital letters represent phosphorothioate whereas those in lower case demonstrate phosphodiester linkages. OVA used as a model antigen was purchased from Anaspec, USA. Antimicrobial peptide LL37 was from Anaspec, USA.

Table 4 TLR ligands and ODNs used in this study

K23 CpG ODN:TCGAGCGTTCTC-	Alpha DNA, Canada
D35 CpG ODN:GGtgcatcgatgcaggggGG	Alpha DNA, Canada
R848	Invivogen, USA
Poly(I:C)	Amersham, UK
LPS isolated from *E. coli*	Sigma, USA

2.1.5 Antibodies and related Reagents

Cytokine ELISA assays were performed using monoclonal capture and detection (biotinylayed) antibodies, streptavidin-alkalane phosphatase (ALP) conjugates purchased from Biolegend or Mabtech, USA. For antigen specific antibody measurements, immunoglobulin ELISA was performed using ALP-conjugated goat anti-mouse total IgG, IgG1 and IgG2c from Southern Biotech, USA. *p*-nitrophenyl phosphate di-sodium salt (PNPP) substrate, Diethanolamine substrate buffer, Immulon 1B and Immulon 2HB plates were from Thermo Scientific, USA. All fluorescent-dye conjugated antibodies cytometric assays were from Biolegend, USA. Sytox Orange and Syto Green Nucleic Acid stains were obtained from Life technologies, USA. Cytometric Bead Array (CBA) Human Soluble Protein Flex Set System was purchased from BD Biosciences, USA.

2.2 Methods

2.2.1 Establishment of Bacterial Growth Curves

Mid-log phase growth of bacteria is the optimum time point at which bacterial RNA is isolated. Therefore, growth curves of individual bacterial strains were determined in preliminary experiments. For this, both indirect and direct measurements were used. First, bacterial strains were recovered from glycerol stocks, transferred into liquid growth media and cultured for 24 hrs at 37 $^{\circ}$C with shaking (130 rpm). The cultures were then diluted to 1% using the corresponding growth media. For indirect measurement, growth of 1% diluted bacterial culture was monitored by recording OD values at 600 nm on an hourly basis using a spectrometer (Thermo Scientific, USA) until an OD of 1 was reached (usually corresponds to early steady state for most cultures). For direct measurement, single colonies were counted from agar plates. For this, bacterial cultures were diluted to a concentration of 10^{-5} and 10^{-6}, and were spreaded on agar plates on an hourly basis and incubated at 37 $^{\circ}$ C for 24 hours. Following the end of the incubation period, the numbers of colonies were determined from each plate.

2.2.2 Bacterial RNA Isolation

Bacterial strains were grown overnight (~16 hrs) at 37 $^{\circ}$C, 130 rpm shaking. Then, cultures were diluted 1/10X and incubated further until they reached their corresponding mid-log phase.10 ml of each culture was then centrifuged at 6000 rpm (15 min) to pellet the bacterial cells. Meanwhile, bacterial lysis buffer was prepared as described in Appendix A. Bacterial pellets were re-suspended in 200 µl of bacterial lysis buffer plus 15 µl Proteinase K (20 mg/ml) per tube and incubated 10 min at room temperature by vortexing for 10 seconds every other 2 minutes of the incubation. Lysis buffer was removed by centrifugation (6000 rpm for 15 min, +4°C). Supernatants containing the lysis buffer were discarded and the resultant pellets were resuspended in 1 ml Trizol per tube by vigorous shaking until complete homogenization. Homogenates were transferred into 1.5 ml eppendorf tubes, followed by 200 µl chloroform addition, vigorously shaking

and incubation for 3 min at room temperature. Next, tubes were centrifuged at 14,000 rpm for 15 min at +4°C and the clear supernatant phase containing the RNA was collected without disturbing other layers. 500 µl isopropanol was then added onto this clear phase, mixed and incubated at room temperature for 10 min. Following centrifugation at 14,000 rpm for 10 min (4°C), supernatants were discarded and RNA pellets were washed twice with 75% EtOH and then with 99.8% EtOH (centrifugation at 8000 rpm, 7 min, 4°C). RNA pellets were dried under laminar flow for 10 min, re-suspended in 20 µl DNase-RNase free water and concentration of each sample was determined by Nanodrop.

To clean and concentrate bacterial RNA, RNA clean and concentrator columns-25 (Zymoresearch, USA) were used. For this, 100 µl of RNA binding buffer (RBB) was added to 50 µl of RNA sample (~25 µg) and mixed. 150 µl molecular biology grade EtOH was then added to the mixture followed by transfer to the column placed over a collection tube. After centrifugation at 11,390 rpm for 1 min, the flow through was discarded, 300 µl RNA wash buffer (RWB) was added to each column, and the above mentioned centrifugation step was repeated to discard the flow through. Contaminating DNA was removed by the addition of 25 µl RNase free DNase I (1U/µl), 25 µl 10X reaction buffer, 200 µl RWB to each columns and samples were incubated at 37 °C for 20 min. Columns were centrifuged at 11,390 rpm (14,000g), 30 sec, the flow throughs were discarded, followed by two consecutive washing steps with 400 µl RNA Prep buffer and and 400 µl RWB, respectively. Columns were then centrifuged at 11,390 rpm for 2 min to get rid of any excess RWB. To elute the purified RNA, 30 µl DNase-RNase free water was added directly onto the column matrix, incubated for 1 min at room temperature, followed by centrifugation at 9,630 rpm (10,000g) for 30 sec. This step was repeated once more to ensure complete elution from the column. The purity and concentration of the eluted RNA was determined by Nanodrop and samples were stored at -80 °C until further use.

2.2.3 Characterization of bacterial RNA by Agarose Gel Electrophoresis

In order to analyze the integrity of the purified bacterial RNAs, 3 µg of bacterial RNA was mixed with 6X loading dye, and loaded onto the wells of a 1% agarose gel containing 0.5 µg/ml Acridine orange in 1X Tris-Acetate-EDTA solution. 1kb DNA ladder was prepared by mixing 4 µl DNase-RNase free water, 1µl 6X loading dye, 1µl DNA ladder. ssRNA ladder was prepared by mixing 2µl ssRNA ladder, 3 µl DNase-RNase free water, 5 µl 2X RNA loading buffer, followed by heating at 65 $^{\circ}$C for 5 min and chilling on ice prior to loading . Samples were then run at 80 V for 40 min using 1X TAE Buffer. The agarose gels were visualized in real-time using the RunVIEW horizontal gel apparatus equipped with blue LED transilluminator (Cleaver Scientific, UK).

2.2.4 Cell Culture

2.2.4.1 Cell Lines and Culture Conditions

Thp-1 cells

Thp-1, human monocytic cell line was cultured in complete RPMI 1640 containing 10% heat inactivated FBS and cells were passaged every 2 to 3 days. Cells were maintained at a concentration of 2 - 4 x 10^5 viable cells/ml and the maximum cell concentration was kept below 1 x 10^6 cells/ml.

B16-Blue IFN-α/β cells

B16-Blue IFN-α/β cells, expressing the cytoplasmic RNA sensors (i.e. RIG-I and MDA-5), allow for the detection of bioactive murine Type I IFNs by monitoring the activation of the JAK/STAT/ISGF3 pathway and/or interferon regulatory transcription factor-3 (IRF3) pathway. These reporter cells stably express a soluble alkaline phosphatase (SEAP) reporter gene that works under the control of the IFN-α/β-inducible IFN-

stimulated gene (ISG54) promoter enhanced by a multimeric IFN-stimulated response element (ISRE). The reporter cells were maintained and subcultured in complete RPMI 1640 containing 10% FBS, 100 μg/ml Normocin and 100 μg/ml Zeocin (required to maintain the plasmid coding for secreted alkaline phosphatase) as selective antibiotic and the cells were passaged when 70-80% of confluency was reached.

HEK-Blue hTLR7 cells

HEK-Blue hTLR7 cells express hTLR7 receptor which is a sensor of ssRNA, also express SEAP reporter gene that is placed under control of IFNβ promoter fused to five NF-κB and AP-1 binding sites. Upon ligand recognition, NF-κB and AP-1 is activated to induce production of SEAP that is quantitated from culture supernatants. Cells were maintained and subcultured in complete RPMI 1640 containing 10% FBS, 100 μg/ml Normocin, 100 μg/ml Zeocin(required to maintain the plasmid coding for secreted alkaline phosphatase) and 100 μg/ml Blasticidin(required to maintain the plasmid coding for hTLR7) as selective antibiotic and cells were passaged when 70-80% confluency was reached.

2.2.4.2 Culture of Primary Cells

Preparation of Spleenocytes from mouse

All female BALB/c (8-12 weeks old) mice used in the *in vitro* stimulation experiments were housed at the Animal House Facility in Molecular Biology and Genetics Department, Bilkent University. Animal handling and all experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC). To harvest splenocytes, mice were sacrificed via cervical dislocation, spleens were removed and placed into 35 cm petri dishes containing 3 ml of complete RPMI 1640 supplemented with 5% FBS. Solid organs were mashed with the back of a sterile syringe plunger, and the, homogeneous single cell suspensions were transferred to 15 ml falcon tubes. Cells were washed 2 times in 15 ml of complete RPMI 1640 by, centrifugation at

1700 rpm for 10 minutes. Final cell, pellets were suspended in 1 ml complete RPMI and counted (described in Section 2.2.4.3).

Human Peripheral Blood Mononuclear Cell (hPBMC) Isolation from Whole Blood

Blood samples from healthy donors were collected into anti-coagulant containing (i.e. Na-citrate coated) tubes and then all the blood from one donor was pooled in a 50 ml falcon tube. After determination of the volume of blood, the volume of Lymphocyte Separation Medium to be used was calculated accordingly (for 2 volumes of blood, 1 volume of Lymphocyte Separation Medium was used). Therefore, 1 volume of Lymphocyte Separation Medium was placed into a 50 ml falcon tube, then 2 volumes of blood was carefully layered onto the separation medium at a 45° angle without disturbing the layers. Samples were centrifuged at 1700 rpm for 30 min at room temperature, without brake. Following centrifugation, the cloudy buffy coat layer residing between the plasma and the density gradient medium was collected using a sterile plastic Pasteur pipette and transferred to a clean 50 ml falcon tube. Cells were washed twice in 50 ml of complete RPMI 1640 by centrifugation at 1700 rpm, 10 min. Supernatants were aspirated and the resultant pellets were resuspended in 1 ml complete RPMI 1640. The cells were counted as given in Section 2.2.4.3.

Isolation of Human Neutrophils from Whole Blood

Following hPBMC isolation, all upper layers were aspirated until the red blood cell (RBC) and granulocyte pellet was reached. The remaining pellet was resuspended in a volume of PBS equal to the original volume of blood. This mixture was then mixed with an equal volume of dextran/saline (3% dextran in PBS) and incubated in an upright position about 20 minutes at room temperature to allow formation of a clean interface between granulocytes and RBCs by gravitation. The upper layer containing the granulocytes was aspirated by a pipette, transferred to a clean tube and cells were washed with 15 ml complete RPMI 160 by centrifugation at 250g for 10 min, +4 °C.

Supernatant was aspirated and discarded, and the residual red blood cells were lysed following, resuspension in 2ml of ACK lysis buffer. Cells were centrifuged at 250g for 10 minutes, supernatant was removed with a pipette and the final cell pellet was resuspended in 1 ml RPMI 1640 containing 5% FBS. Cells were counted and adjusted to 320,000 cells/ml for the netosis assay and to 1 $x10^6$ cells/ml for ELISA.

2.2.4.3 Cell counting

hPBMC, mouse splenocytes and cell lines were resuspended in 1 ml cell culture media. 20 µl of each preparation was diluted 1/10 X and 10 µl of this diluted cell preparation was mixed with an equal volume of trypan blue (0.4 %) The mixture was loaded onto a hematocytometer by capillary action. Cells in 4 corners (containing 16 small squares) with 1 mm^2 area were counted under a light microscope and the cell number was calculated according to the formula given below:

$$\frac{\text{Total cell number in 4 big squares x Dilution factor x } 10^4}{4} = \text{number of cells per ml}$$

2.2.5 Determination of Immunostimulatory Activity of Bacterial RNA

2.2.5.1 In vitro stimulation of Cells with bacterial RNA

Bacterial RNA was isolated and purified as described in Section 2.2.2. All bacterial RNAs were delivered as such using three different doses (0.3 µg/ml, 1 µg/ml, 3 µg/ml\ final concentration) or upon transfection with transfection reagent Lipofectamine2000 (0.3 µg/ml, 1 µg/ml, 3 µg/ml). Bacterial RNA was also complexed with LL-37 in a ratio of 1:9 (w/w), 20 µl/ well in molecular biology grade PBS 15 min prior to stimulation. All stimulation experiments were done in duplicate in a 96-well plate. Bacterial RNAs that were delivered free were diluted to the desired concentration with RPMI 1640, 5%

FBS in a volume of 20 µl per well. In order to transfect RNAs, first, lipofectamine 2000 was mixed with 20 µl/well Optimem reduced serum media and incubated for 15 min. Then, this was mixed with bacterial RNAs in the desired concentration, incubated for 30 minutes and added to the wells. Working cell concentration was adjusted to 2 x 10^6 cells/ml for ELISA and flow cytometric analysis.

For all *in vitro* stimulations, the total volume of cells plus stimulants was kept at 200 µl (20 µl /well stimulants and 180 µl/well cell suspensions). Following incubation for 24 hours, supernatants were collected and stored at -20°C until use of cytokine ELISA. For intracellular cytokine staining, cells were stimulated with bacterial RNAs for 5+2 hours (brefeldin A was added for the last 2 h of incubation) and collected from the wells in 100 µl PBS and were stained as described in section 2.2.5.3.

Lipofectamine 2000 concentration was selected to be 1 µl for 1 µg of RNA for the doses of bacterial RNA 0.3 µg/ml and 1 µg/ml; however for the highest dose of RNA (3 µg/ml); 1µg/ml lipofectamine 2000 was used.

Working cell concentration of HEK-Blue hTLR7 cells and B16-Blue IFN-α/β cells were adjusted to 3x10^5 cells/ml. Both of these cell lines were stimulated in a total volume of 200 µl (20 µl /well stimulants and 180 µl/well cell suspensions in RPMI 1640 medium supplemented with 10% FBS and normocin). Following 24 hours of incubation at 37°C, 5% CO2 incubator, supernatants were transferred to a new 96-well flat bottom plate. Meanwhile, Quanti-Blue, which is the detection medium for SEAP, was prepared according to the manufacturer's protocol and pre-warmed at 37°C water bath. 20 µl of each supernatant collected as described above was mixed with 180 µl per well of Quanti-Blue in a 96 well plate and incubated at 37°C for 24 hours. After 24 hours, OD was measured at 655nm using a Thermo Scientific multiplate reader.

For assays involving neutrophils, cell number was adjusted to 320,000 cells/ml (netosis) or to 1 x10^6 cells/ml for ELISA. For netosis assay, cells (100 µl, 80,000 cells/well) and all stimulants (100 µl) including the positive control PMA (25 nM) were added to wells,

incubated at 37°C for 4 hours, followed by staining with 50 µl Syto green and Sytox orange mixture (4 µM final concentration). Possible neutrophil extracellular trap formation (NETs) was visualized using the Floid Cell Imaging Station, Life Technologies, USA. Neutrophils were also stimulated at a concentration of 1 $x10^6$ cells/ml for the detection of IL8 from the 24 hour incubated stimulated cells' supernatants (Elisa protocol is described in Section 2.2.5.2)

All experiments included positive controls. The concentrations and identities of these stimulants are summarized in Table 5.

Table 5 Concentration of stimulants used in this study

Stimulant	Concentration used	PRR that recognize corresponding ligand
K 23	1 µM	TLR9
D35	3 µM	TLR9
R848	3 µg/ml	TLR7
Poly(I:C)	10 µg/ml	TLR3
5'PPP	1 µg/ml	RIG-I

2.2.5.2 Determination of Cytokine Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

In order to detect cytokine production, supernatants of the cells that were stimulated as described in Section 2.2.5.1 were used. First, Immulon 2HB plates were coated with 50 µl of the monoclonal antibody of interest in PBS and plates were incubated overnight at +4 °C. Then, capture antibody was discarded by inversion of plates, 200 µl Blocking buffer (Appendices) was added to wells to block nonspecific binding, and plates were incubated at room temperature for 2 hours. Washing was performed with the ELISA

wash buffer (Appendices) 5 times as follows: All wells were filled completely with the wash buffer and incubated for 5 min. This was repeated 5 times. Next, all plates were washed with distilled water 4 times. The described washing step was employed in all ELISAs described in this thesis and will be summarized as "plates were washed" henceforth. After the washing step, plates were dried, 50 µl of culture supernatants and 50 µl of serially diluted (2-fold in PBS) recombinant cytokine standards were added onto wells and incubated 2 hours at room temperature. Plates were washed and dried as previously described, biotinylated detection antibody was diluted in T cell buffer (Appendix A) and added onto wells and all samples were incubated overnight at +4 $^{\circ}$C. Capture and detection antibodies were diluted according to the manufacturer's protocol (Biolegend and Mabtech, USA). Plates were washed and dried as before. Streptavidin-ALP was prepared the night before (1/1000 dilution in T cell buffer) and was added 50 µl per well, followed by incubation for 1h at room temperature. After washing and drying, plates were developed using 50 µl of PNPP substrate addition. OD at 405 nm was measured at defined intervals by using an ELISA plate reader, Multiskan FC Microplate Photometer, Thermo Scientific, USA.

2.2.5.3 Flow cytometric analysis of samples

Cell fixation

Cells that were stimulated in Section 2.2.5.1 were transferred to U-bottom plates and centrifuged at 1700 rpm, 10 min. Supernatants were flicked out, 100 µl Fixation Medium A was added onto wells, cell pellets were suspended, incubated for 15 min at room temperature. Samples were then washed 2 times by addition of 150 µl FACS buffer (Appendix A) onto wells and centrifugation at 1700 rpm, 10 minutes. Fixed cells were resuspended in 200 µl FACS buffer and kept at + 4 $^{\circ}$C until further use.

Cell Surface and Intracellular Cytokine Staining

Fixed or live cells (in 96 well U-bottom plates) were centrifuged at 1700 rpm, 10 min, supernatants were discarded, and cells were resuspended in 100 µl Permeabilization Medium B containing 1 µg/ml of fluorescently labeled antibodies cocktail and incubated 30 min in dark. Following washing 2 times with 200 µl FACS buffer as described above, samples were resuspended in 150 µl FACS buffer. Antibodies used for intracellular staining were: anti-human IP10 PE, and anti-human CD14 FITC.

Cytometric Bead Array (CBA)

In this study, TNF-α, IL1β, IP-10, IL8 and IFNα CBA human soluble protein flex sets were used. 1 µl per sample of capture beads and detection bead was diluted with 50 µl per sample capture bead diluents and PE detection reagent diluent, respectively. Standard solutions were serially diluted. 50 µl of hPBMC culture supernatants that were stimulated with bacterial RNA for 24 hours were mixed with 50 µl mixed capture beads and incubated for 1h at room temperature. Then, 50 µl of mixed PE detection reagent was added onto wells. This sandwich complex consisting of capture bead, analyte and detection reagent was incubated for 2 h at room temperature. Following incubation, samples were washed with 250 µl/ well wash buffer and centrifuged at 200 g for 5 min; resuspended in 200 µl/well wash buffer and samples were acquired on the flow cytometer.

2.2.6 Vaccination study

2.2.6.1 Animal maintenance

For vaccination studies female C57BL/6 (6-8 weeks old) mice were used. Animals were housed at the Animal House Facility in Molecular Biology and Genetics Department, Bilkent University and maintained under controlled ambient conditions (22ºC ± 2) regulated with 12 hours of light and dark cycles, and unlimited access to food and water.

Animal handling and experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC).

2.2.6.2 Immunization with OVA adjuvanted with bacterial RNA

6-8 weeks old C57BL/6 mice were immunized intraperitoneally in prime and boost regimen on days 0 and 14 with 100 ul of PBS, OVA or OVA mixed with various adjuvant formulations as described below. On days 13 and 28, blood samples were collected into glass tubes from tail veins by introducing a 1mm cut using a scalpel blade. Afterwards, sera were prepared as described in Section 2.2.6.3.

Table 6 Dose of components used in vaccination study

Components	**Dose**
OVA	7.5 μg/mouse
RNA	20 μg /mouse
K23	10 μg /mouse
Poly(I:C)	10 μg /mouse

2.2.6.3 Preparation of Sera

Blood samples that were taken into glass tubes were tightly capped with parafilm and incubated in a water bath at 37 $^{\circ}$C for 2h. After clot formation, sera were transferred to eppendorfs and centrifuged at 8000 rpm for 10 min. Supernatants were collected, transferred to 96 well plates and stored at -20 $^{\circ}$C until use.

2.2.6.4 IgG ELISA

In order to determine antigen specific total IgG, IgG1 and IgG2c levels in blood of immunized mice, IgG ELISA was performed from the collected sera. For this, immulon

1B plates were coated with 50 µl of OVA per well (7.5 µg/ml diluted in PBS), overnight at +4 ^{0}C. Blocking was performed using 200 µl/well blocking buffer (Appendix A) and incubation at room temperature for 3h. Plates were washed and dried as described in the Section 2.2.5.2. Sera that were collected were diluted in 2% BSA in PBS and 50 µl of 1/8 diluted sera were added to the top wells and then 5-fold serially diluted. Plates were incubated overnight at + 4 $^{\circ}$C, washed with wash buffer and distilled water, dried and 50 ul goat anti mouse total IgG/ALP, goat anti mouse IgG1/ALP or goat anti-mouse IgG2c/ALP (1/1000 diluted in T cell buffer the night before) were added to wells. Following 3 hours of incubation at room temperature, plates were washed and PNPP substrate was introduced. Color development was measured by recording OD values at 405 nm using an ELISA plate reader, Multiskan FC Microplate Photometer, Thermo Scientific, USA.

2.2.7 Statistical Analysis

Statistical analysis for the treatment groups were conducted by IBM SPSS17 software. Student`s t-test was conducted between control (or naïve) versus treatment groups (two-tailed unpaired comparison). P values that were <0.05 were considered as statistically significant throughout these studies when compared to untreated samples.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Determination of Growth Curve of Bacterial Species

RNA from all bacterial species were isolated from mid-log growth suspension cultures. To determine the time point of mid-log growth phase, growth curves of bacterial strains were studied. For this, growth curve of bacterial strains were assessed by both direct and indirect measurements. Figure 3.1 and Figure 3.2 show the growth curves determined according to the indirect and direct measurements, respectively. Typical bacterial growth curve is formed by 3 distinct phases: lag, exponential (log) and stationary phase that collectively constitute an S-shape curve (Cooper, 1991). The OD600 nm-based indirect measurements generated growth curves with ill-defined distinct phases (Figure 7). To better estimate a more accurate time point of mid-log phase, a direct measurement-based approach was used (Figure 8). This generated more reliable growth curves with distinct phases and the time point of mid-log growth was estimated to be 2.5h for LS and LF, 3h for CEF and LM and 4h for EF and SP. Therefore, based on this data, the above mentioned time points were used to isolate RNA from the corresponding bacterial species.

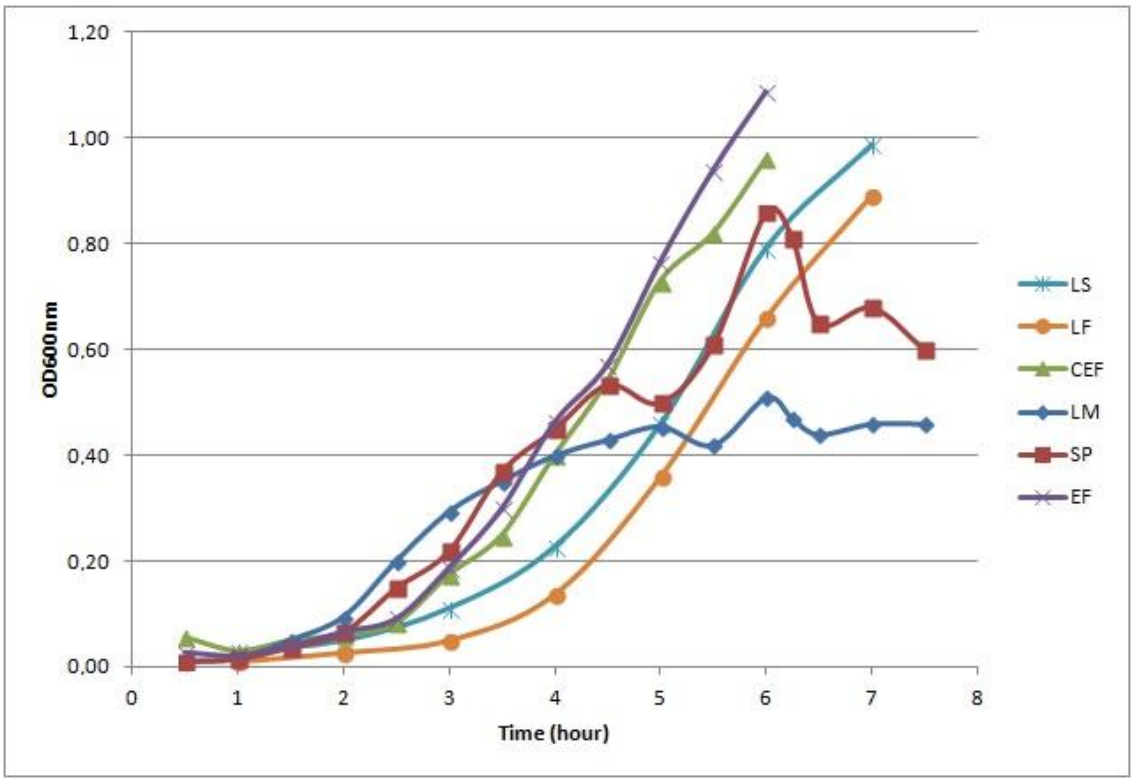


Figure 7 Growth Curve of Bacterial Strains (Indirect measurement). Growth of 1% diluted corresponding bacterial cultures was monitored by recording OD values at 600 nm on an hourly basis.

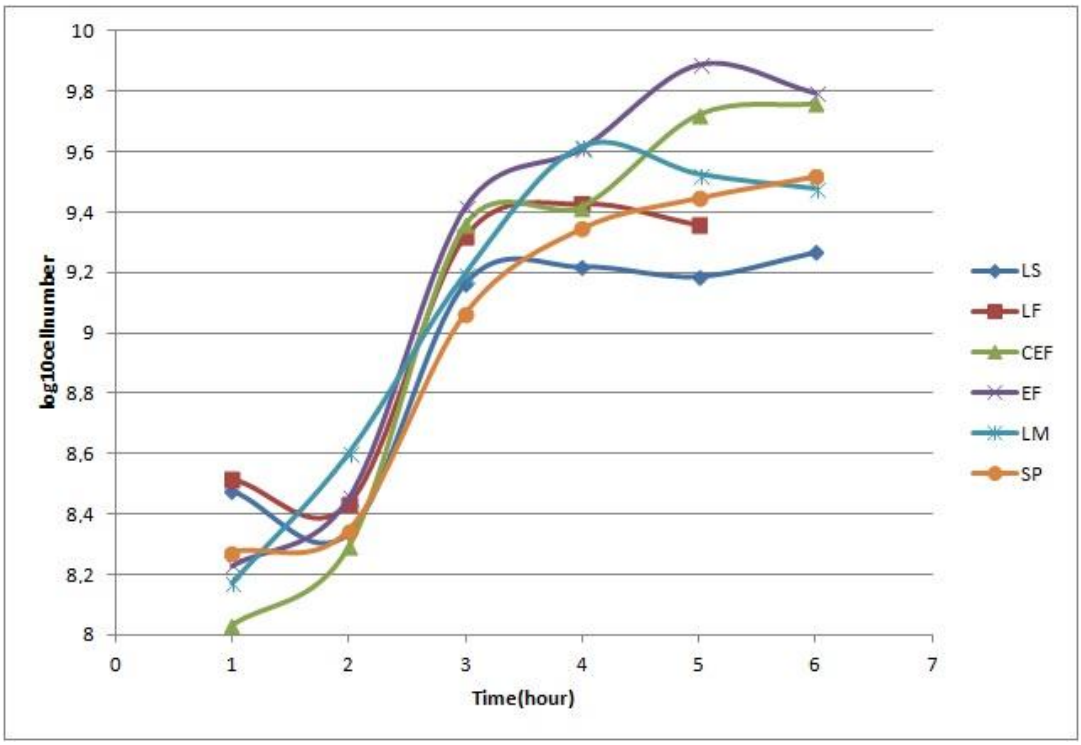


Figure 8 Growth Curve of Bacterial Strains (Direct Measurement). Growth of 1% diluted corresponding bacterial cultures was monitored following dilution to a concentration of 10^{-5} and 10^{-6} and then spreading on agar plates on an hourly basis. Plates were incubated at 37ºC for 24 hours and the number of colonies were determined from each plate.

3.2 Characterization of bacterial RNAs by Agarose Gel Electrophoresis Stained with Acridine orange

Acridine orange is a cationic dye that stains nucleic acids. It either binds electrostatically to phosphate groups of single stranded molecules and fluoresce red at 650 nm, or intercalate into double stranded molecules and fluoresce green at about 525 nm (Re, 2005). The reason why we used acridine orange to stain the RNAs in gels was to observe the predominant conformations (single versus double stranded) of the isolated nucleic acids. Following bacterial RNA isolation, column purification and DNase I treatment, 3 µg of bacterial RNAs were run on 1% agarose gel containing acridine orange (0.5 µg/ml in TAE). Expected bands corresponding to the 23S (~2904 bases), 16S (~1542 bases) and 5S rRNAs (~121 bases) were present with no apparent degradation (Figure 9,

Melnikov *et al.*, 2012). Evidence from literature suggests that commensal derived bacteria contain more dsRNA than pathogenic bacteria (Kawashima *et al.*, 2013). However, using the acridine orange staining protocol, it was not possible to discriminate the predominant RNA conformation of the samples since all bands emitted both green and red signals (Figure 9), suggesting the presence of single stranded and double stranded regions in all.

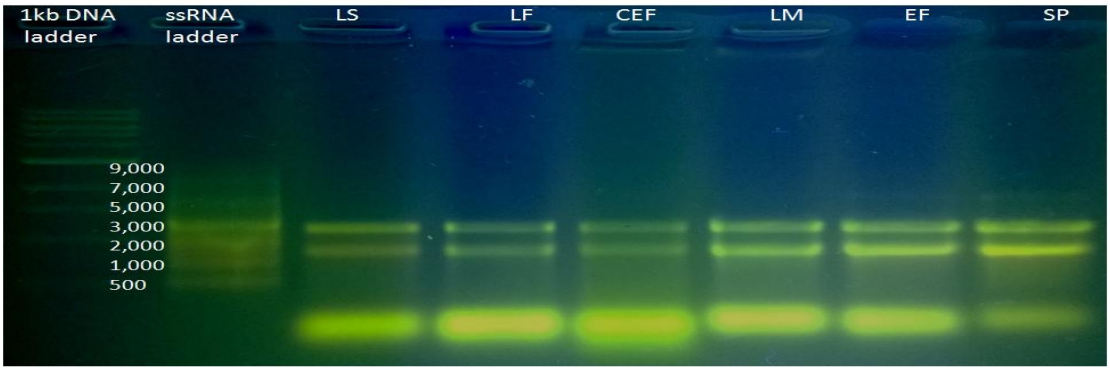


Figure 9 Integrity and purity of bacterial RNAs isolated from three commensal (LS, LF and CEF) and 3 pathogens (LM, EF and SP) were determined on a 1% agarose gel containing 0.5 µg/ml Acridine orange. Each well contained 3 µg of bacterial RNA. The ssRNA ladder consists of 7 RNA molecules produced by in vitro transcription of a mixture of 7 linear DNA templates. The ladder sizes are: 9000, 7000, 5000, 3000, 2000, 1000 and 500 bases.

3.3 Immunostimulatory Activities of Bacterial RNAs in hPBMC

To test whether the immune system could discriminate and respond differentially to commensal versus pathogen derived bacterial RNA, hPBMCs were isolated as described in Section 2.2.4.2 and then were stimulated with bacterial RNAs. For this, RNAs were delivered as such (0.3 µg/ml, 1 µg/ml and 3 µg/ml) or were transfected to the cytosol using the lipofectamine 2000 transfection reagent (three doses; 0.3 µg/ml, 1 µg/ml and 3 µg/ml). 24 hours later, cytokine levels were measured by ELISA from culture supernatants. All experiments also included positive control ligands for various TLR

receptors (LPS for TLR4, dsRNA mimic poly(I:C) for TLR3, R848 for TLR7 and/or K and D type CpG ODN for TLR9) and negative control for lipofectamine 2000 alone.

Certain PAMPs trigger a signaling cascade that promotes gene transcription by nuclear factor-kB (NF-kB). One such downstream target is IL6, a major proinflammatory cytokine produced following TLR-mediated recognition of PAMPs by the cells of the innate immune system (Dörner, 2006). Therefore it was of interest to compare IL6 levels of the cells that were stimulated either with commensal or pathogen derived bacterial RNA.

In preliminary experiments, bacterial RNAs used in the stimulation experiments were not column purified since the 260/280 and 260/230 ratios were of sufficient quality (both ratios were around 2.0) and there were no bands indicative of degradation on agarose gels. However, when as such purified bacterial RNAs were used in stimulation experiments, cytokine production failed to correlate with increasing doses of the bacterial RNAs used (Figure 10 and 11 for untransfected and transfected samples, respectively). Especially, when the maximum dose of RNA was used (3 μg/ml) an abrupt decrease in the level of cytokine production was observed (Figure 10 and 11).

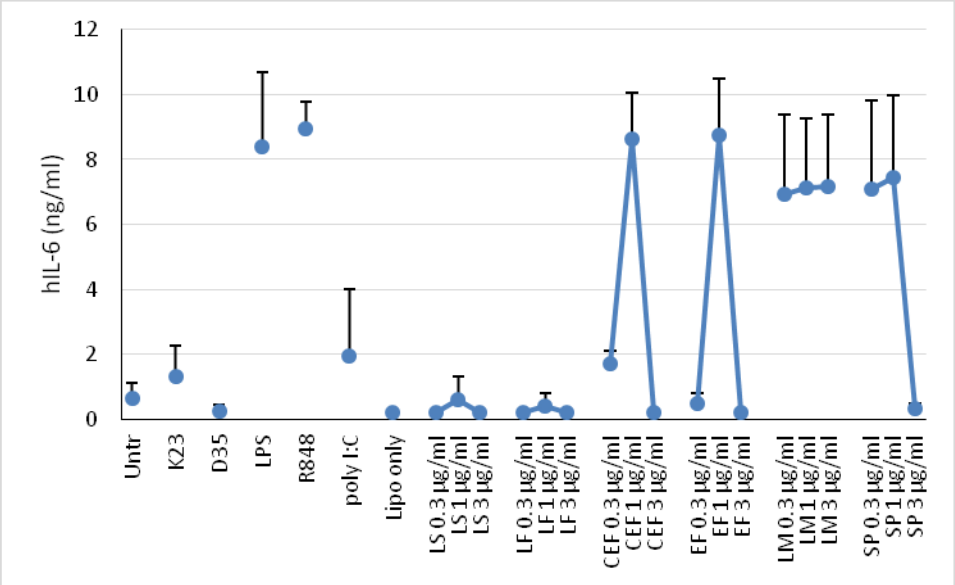


Figure 10 IL-6 production from hPBMC stimulated with Trizol purified bacterial RNAs. $2x10^6$/ml PBMC were stimulated in duplicate with 0.3, 1, or 3 µg/ml of bacterial RNAs for 24 h and cytokine production was assessed from culture supernatants by ELISA. Results show the average IL-6 response (ng/ml) of 3 different PBMC samples ± S.D.

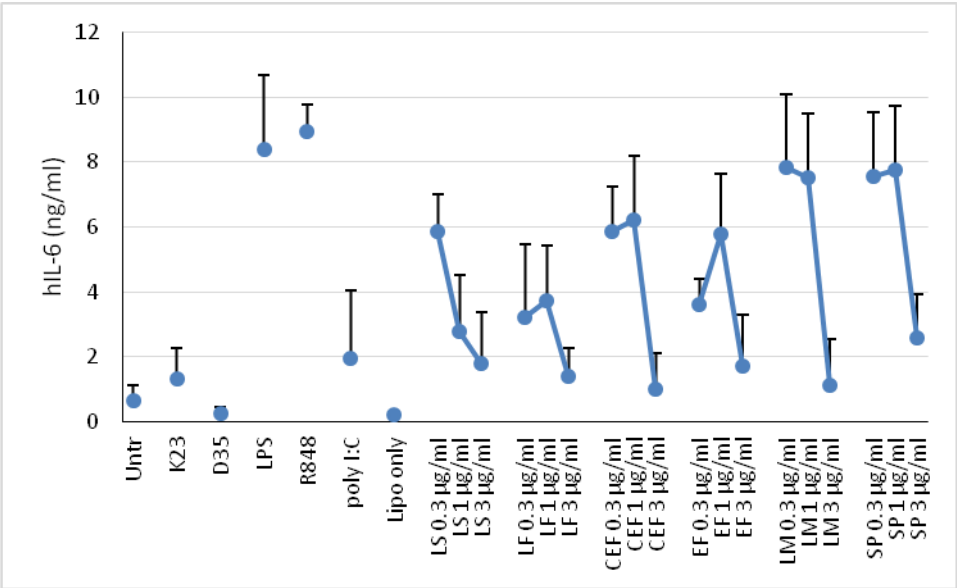


Figure 11 IL-6 production from hPBMC stimulated with Trizol purified and Lipofectamine 2000 transfected bacterial RNAs. $2x10^6$/ml PBMC were stimulated in duplicate with 0.3, 1, or 3 µg/ml of bacterial RNAs (complexed with the transfection reagent) for 24 h and cytokine production was assessed from culture supernatants by ELISA. Results show the average IL-6 response (ng/ml) of 3 different PBMC samples ± S.D.

Since these results suggested the presence of a contaminant in the isolated bacterial RNAs that interfered with cytokine production at higher concentrations, the purification method was modified to include an additional column-based enrichment technique that would exclude the suspected inhibitor from the final product.

Henceforth, the isolated bacterial RNAs were column purified and used as such in *in vitro* and *in vivo* experiments. Figure 12 demonstrates that the column purified bacterial RNAs triggered a dose dependent IL-6 production from hPBMCs. The 3µg/ml dose was no longer inhibitory, implying that the suspected inhibitor was eliminated following column purification. Therefore it was now possible to compare the immunostimulatory properties of bacterial RNAs. Figure 12 shows that commensal purified RNAs (LS, LF and CEF) failed to induce IL-6 production from hPBMC in their free form but activated cells to produce moderate levels of this cytokine only after transfection into the cytosol.

In contrast, RNAs of the obligate pathogens LM and SP triggered significantly higher levels of IL-6 even in the absence of transfection. Furthermore, even the lowest dose (0.3 μg/ml) was potent enough to induce a response that was comparable to the positive control LPS. Interestingly, RNA prurified from the virulent strain of *Enterococcus faecium* (EF) showed a similar pattern of IL-6 stimulation when compared to its non-virulent counterpart (i.e commensal CEF). Enterococci, such as the *E. faecium* are prominent members of the intestinal microbiota and are considered as commensals. However, enterococci are also known to be the causative agents of antibiotic resistant hospital infections (Lebreton *et al.*, 2013). Such virulent strains acquire mobile DNA elements from the environment (such as the pathogenicity islands) conferring infectivity and virulence and thus make the transition from a commensal to a pathogen (Paulsen *et al.*, 2003). Considering that the pool of total RNA consists predominantly of ribosomal RNAs (~80%), it would be plausible to assume that the majority of RNAs isolated from the commensal or the pathogenic form of *E. faecalis* would be identical except for the transcripts encoded by the acquired pathogenicity island genes. In this context, we hypothesize that the commensal purified RNA (CEF) and the RNA extracted from commensal-turned-into-pathogen (EF) would be perceived as similar by the immune system. Data presented in Figure 12 and those that are presented in the following pages of this thesis are in support of this hypothesis.

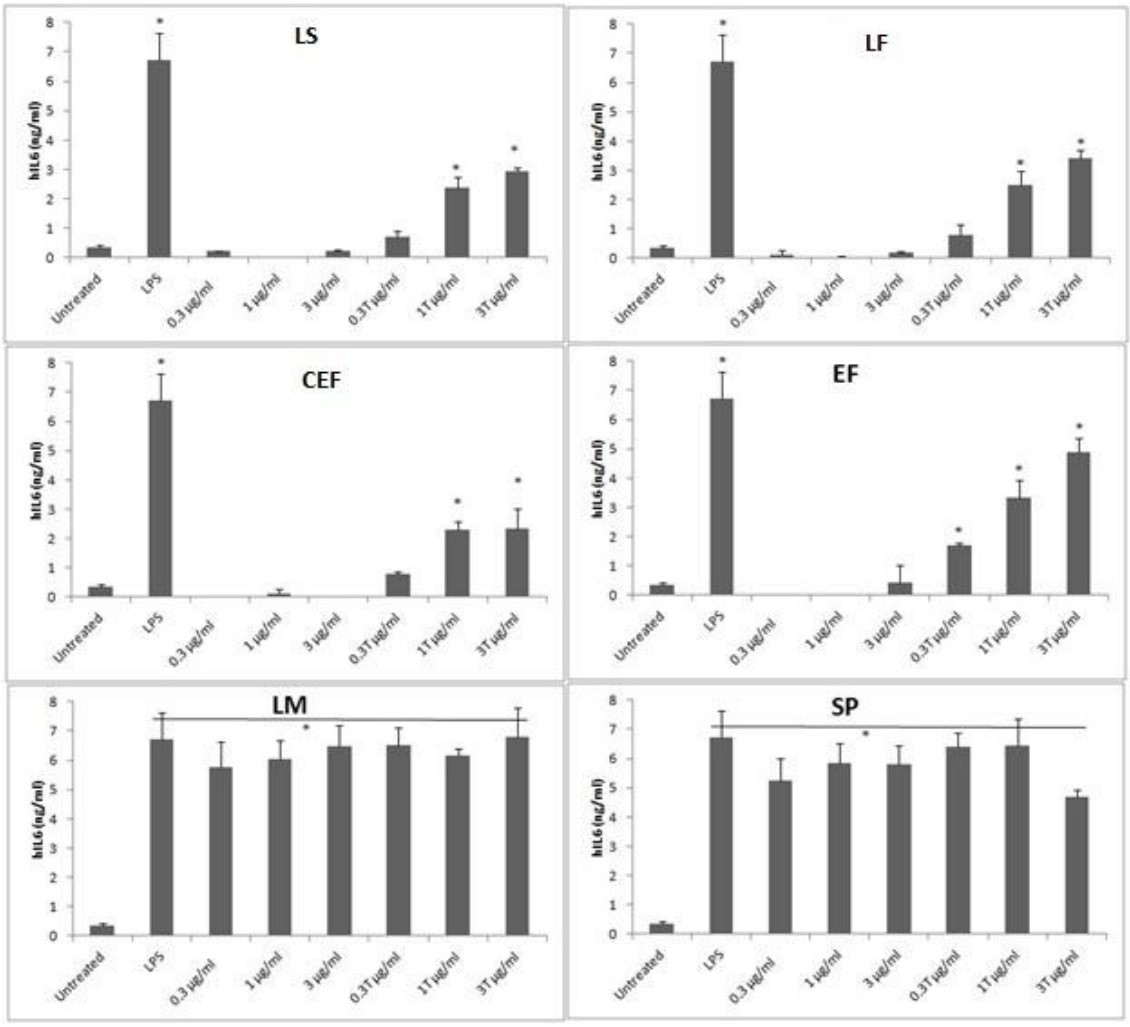


Figure 12 IL-6 production from hPBMC stimulated with Trizol plus column purified bacterial RNAs. $2x10^6$/ml PBMC were stimulated in duplicate with 0.3, 1, or 3 µg/ml of bacterial RNAs (as such or transfected with Lipofectamine 2000; T indicates transfected samples) for 24 h and cytokine production was assessed from culture supernatants by ELISA. Results show the average IL-6 response (ng/ml) of 3-5 different PBMC samples + S.D. * indicates $P<0.05$.

To ascertain that the observed response (in this case IL6 levels) was only the result of bacterial RNA stimulation and not due to other contaminating substances, stimulation experiments were repeated with samples treated with RNAse. For this, RNAse I was chosen as this enzyme completely digests RNA to mononucleotides (Meador *et al.*,

1990), unlike RNase A which leaves residual oligoribonucleotides after digestion (Robberson *et al.*, 1972). Figure 13 demonstrates that IL6 production was lost following stimulation of cells with bacRNAs treated with RNAse I. These results confirm that the observed response was specific to the prokaryotic RNA and not to other contaminants that would trigger an RNA-independent response.

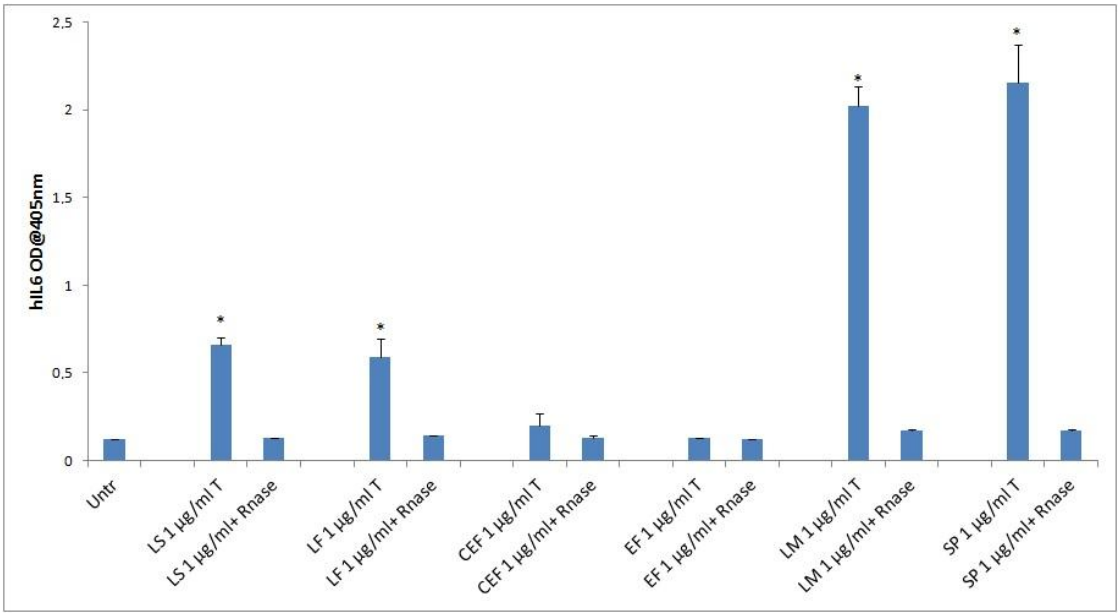


Figure 13 IL-6 production from hPBMC stimulated with bacterial RNAs that were untreated or treated with RNAse I. $2x10^6$/ml PBMC were stimulated in duplicate with 1 µg/ml of bacterial RNAs for 24 h and cytokine production was assessed from culture supernatants by ELISA. Results show the average IL-6 response (ng/ml) of 3 different PBMC samples + S.D. * indicates $P<0.05$.

In addition to the TLR and C-type lectin receptors that reside on the plasma membrane or are located in endosomes, a distinct set of PRRs also reside in the cytosol. Among these, the nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins (Takeuchi and Akira, 2012) assemble cytosolic protein complexes called inflammasomes following recognition of cytosolic danger signals. Intracellular delivery of RNAs isolated from *Escherichia coli*, gram positive and gram negative bacteria have been shown to activate caspase-1 via the Nlrp3 inflammasome, resulting in secretion of

active IL-1β (Kanneganti *et al.*, 2006), (Kanneganti *et al.*, 2007), (Eigenbrod *et al.*, 2012). However, whether all bacterial RNAs can activate the Nlrp3 inflammasome equally or are there differences in the magnitude of response triggered by commensal vs pathogen derived RNAs are still unknown. To address this issue, human PBMCs were stimulated with bacRNAs and active IL1β secretion (an indicator of inflammasome activation) was assessed from culture supernatants. As it can be seen in Figure 14, commensal derived bacterial RNAs induced low levels of IL1β only after transfection. In contrast, pathogen derived bacterial RNAs (LM and SP) activated inflammasome even when delivered extracellularly. The observed IL1β response doubled when LM and SP RNAs were delivered into the cytosol. The commensal and pathogenic strains of *E. faecium* (CEF and EF, respectively) generated a response similar to those seen with other commensal RNAs.

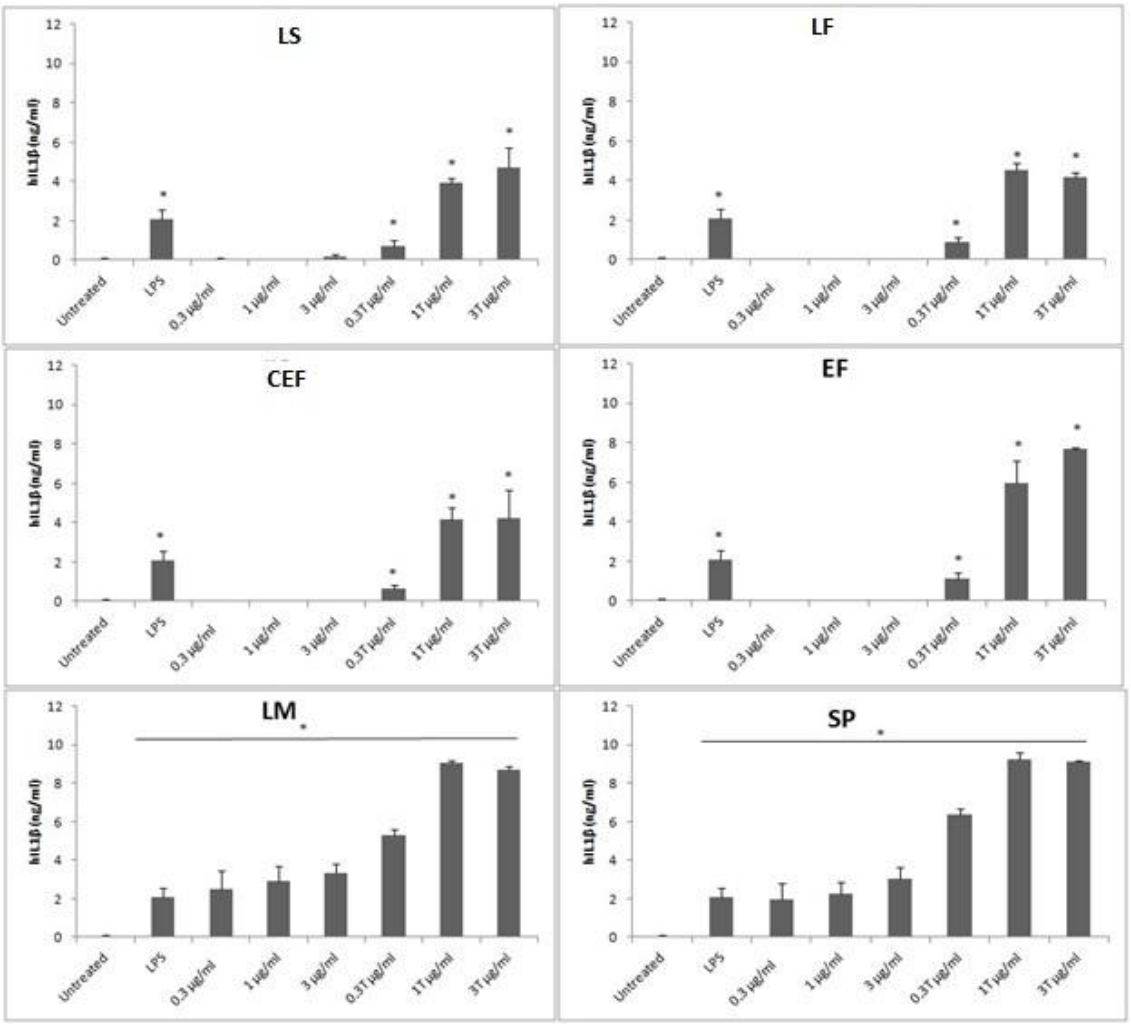


Figure 14 IL1β production from hPBMC stimulated with bacterial RNAs. $2x10^6$/ml hPBMC were stimulated with 0.3, 1 or 3 µg/ml of bacterial RNAs in duplicate (as such or delivered with Lipofectamine 2000, T stands for transfected samples) for 24 hours and cytokines levels were determined from culture supernatants by ELISA. Results represent IL1β response (ng/ml) of 3-5 different PBMC samples ± S.D. * shows $P<0.05$.

In summary, these findings suggest that bacterial RNA from all species can trigger inflammasome activation upon delivery into the cytosol but only the strict pathogen RNAs can do so when added extracellularly. Moreover, pathogen derived RNAs (LM and SP) are more potent activators of inflammasome.

To confirm that bacRNAs activate inflammasome in monocytes, we repeated the above experiment using the Thp-1 human monocytic cell line. Human peripheral blood monocytes express constitutively activated caspase-1 and hence do not require priming for release of IL1β (Netea *et al.,* 2009). In contrast, Thp-1 cells require priming to induce transcription of caspase-1 and pro-IL1β and then the 2^{nd} signal that would activate the inflammasome. Therefore, Thp-1 cells were first primed with 20 ng/ml PMA overnight at 37 °C. Following differentiation, cells were stimulated with bacterial RNAs (free, transfected and/or RNAse I treated), incubated for 8 hrs and supernatants were collected to assess the levels of secreted IL1β by ELISA. Preliminary experiments conducted without priming resulted in no observable ILβ production. Figure 15 shows that in contrast to hPBMC results, all bacRNAs were capable of inducing equivalent levels of IL1β secretion from primed Thp-1 cells, but only after delivery to the cytosol. This effect was RNA-dependent since treatment with RNAse I abolished the response. This difference suggests that the mechanism of bacRNA detection is more intricate in hPBMCs, representing a mixed cell population that express a wider variety of sensors. Further experiments are required to address whether highly purified peripheral blood monocytes would respond differentially to commensal vs pathogen derived RNAs and if additional cellular internalization mechanisms are at play that would direct pathogen derived but not commensal RNAs into the cytosol. In this context, it is also imperative to carry out structural analyses of bacterial ribosomal RNAs of pathogen and commensal origin to highlight possible differences (i.e content of single stranded and duplex regions, nature of the degradation products following exposure to serum nucleases, interaction of these products with serum proteins, 5'-ppp content of RNAs, etc.) that would allow the immune system to discriminate commensals as "self" and pathogens as "non-self".

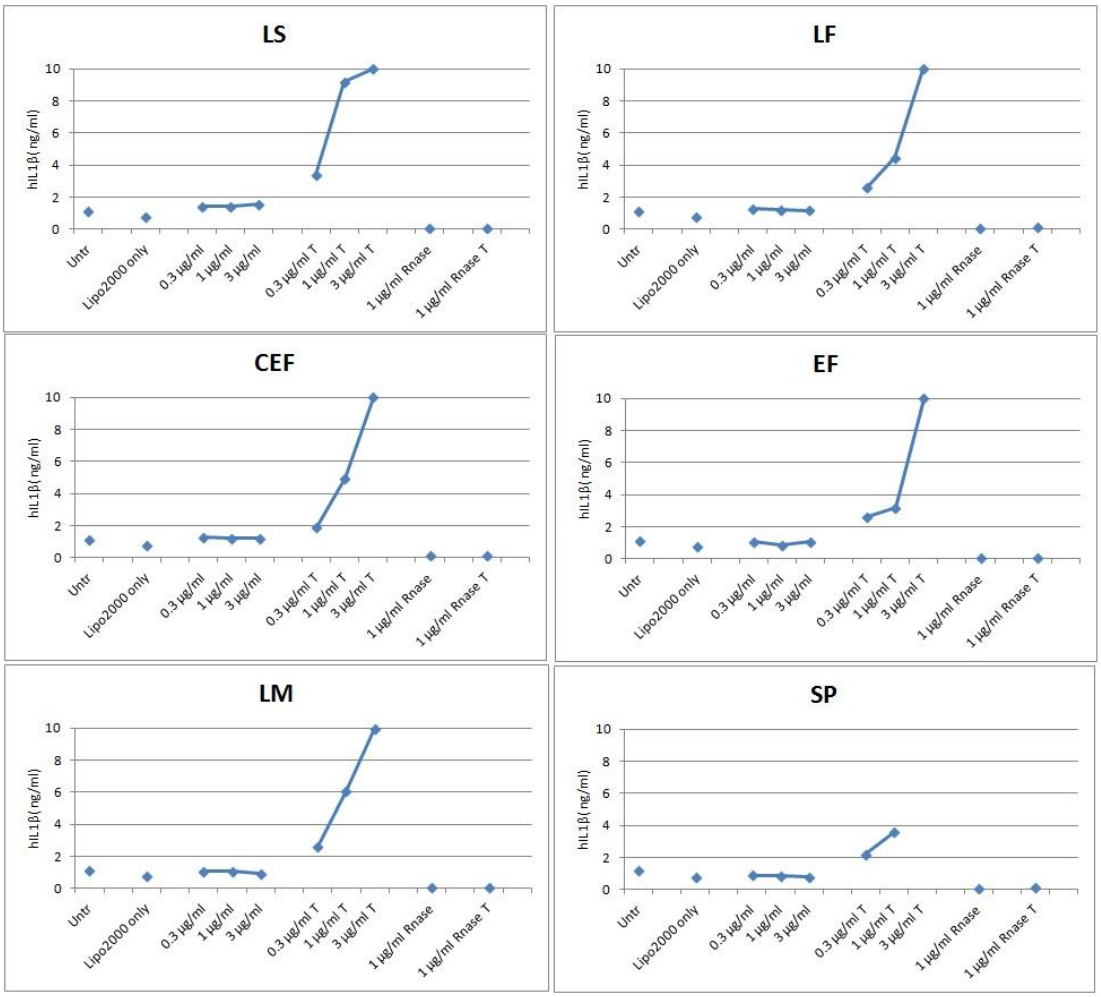


Figure 15 IL1β production from Thp-1 cells primed with PMA (20 ng/ml) and stimulated with bacterial RNAs. $0.4x10^6$/ml Thp-1 cells were stimulated with 0.3, 1 or 3 µg/ml of free or transfected bacterial RNAs for 8 hours (T indicates transfected samples) and cytokine level was assessed from culture supernatants by ELISA.

3.4 Detection of Type I IFN response induced by commensal and pathogen derived bacterial RNAs

Microbiota has been recently shown to play a crucial role in establishment of anti-viral immunity. Specifically, depletion of microbiota prior to a viral infection severely impaired the protective type I IFN response (McAleer *et al.*, 2012), (Abt *et al.*, 2012), (Ganal *et al.*, 2012). It was suggested that microbiota-derived product(s) induced low levels of type I IFNs leading to tonic type I IFN receptor-mediated signaling in mononuclear phagocytes, thereby preparing these cells for efficient viral recognition. Work by Kawashima and colleagues in 2013 furthered these observations and demonstrated that dsRNA in commensal but not pathogenic bacteria triggered interferon-β production in a TLR3-dependent manner. However, our understanding of bacRNA recognition with respect to type I IFN production is very incomplete since there are several conflicting publications in the field. For example, Hagmann and colleagues, in 2013 claim that triphosphorylated RNA of the pathogen LM activates a RIG-I dependent, TLR independent, type I IFN induction when transfected into human monocytes, epithelial cells or hepatocytes. In contrast, Li and colleagues in 2011 demonstrated commensal RNA to be a RIG-I ligand, inducing IFNβ in a mitochondrial antiviral signaling protein (MAVS) dependent manner. Whereas Gratz N. *et al.* (2011) claimed S. pyogenes-derived RNA as the principle IFNβ inducer in conventional DCs, Deshmukh, S. D et al. (2011) showed that the recognition of group B Streptococcus and other Gram-positive bacterial RNA was ssRNA dependent and occured through TLR7. Lastly, Dalpke, A., & Helm, M. (2012), Gantier, M. P. *et al.* (2008) and Eberle, F *et al.* (2009) all pointed to the importance of TLR7-mediated sensing of bacRNA in production of interferonα. Considering the above mentioned confusion in the field, we designed experiments to determine the pattern of IFNα and IP-10 (a chemokine induced by IFNα/β production from hPBMCs stimulated with commensal and pathogen RNAs. We also used 2 reporter cell lines stably expressing the RNA sensors RIG-I/MDA5 or TLR7 to address the role of these receptors in recognition of commensal versus pathogen RNAs. Finally, we focused on isolated human neutrophils as an unusual cell

type that reportedly express RIG-I, MDA-5 and the ssRNA sensor TLR8 receptor in secretory vesicles as well as on their surface (Berger *et al.*, 2012). The following sections summarize data from these studies.

3.4.1 IFN response of hPBMC to bacterial RNAs

Human PBMCs were stimulated with bacRNAs and the generated IFNα levels were measured by ELISA (described in section 2.2.5.1). Results (Figure 16) show that all commensal derived bacterial RNAs and the commensal-turned-into pathogen (EF) triggered IFNα secretion upon transfection (mostly in their optimum dose 1μg/ml). In contrast, pathogen derived RNAs (LM and SP) were ineffective. These results are consistent with Kawashima and colleagues findings in 2013, that commensal but not pathogen RNAs induce type I IFNs in hPBMC.

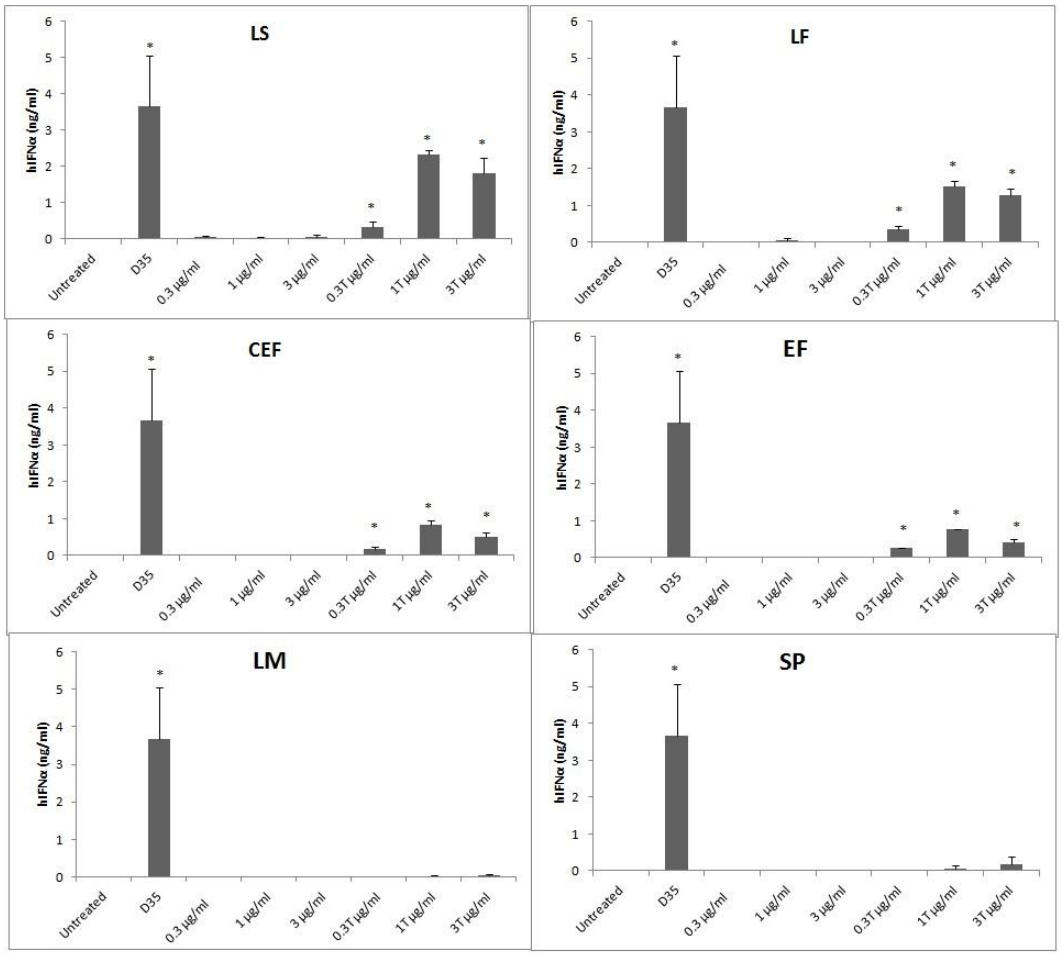
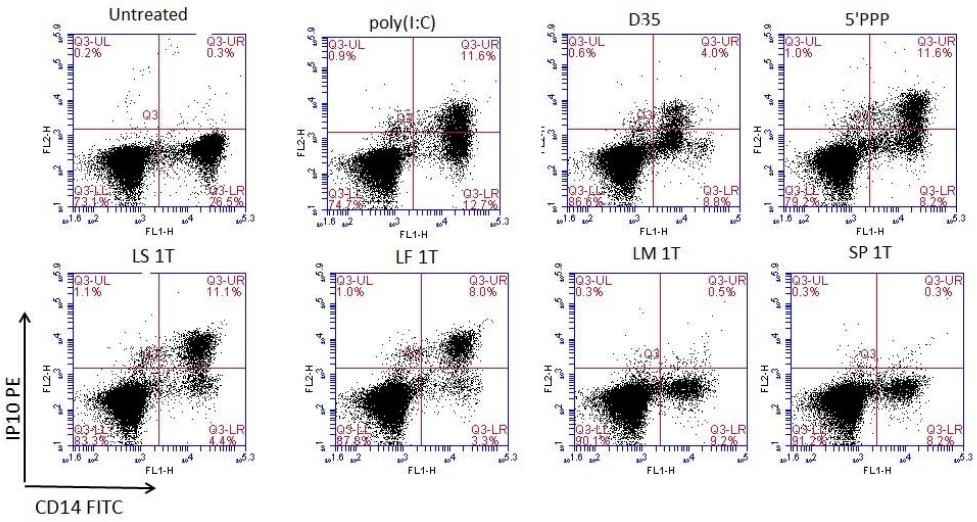


Figure 16 IFNα production from hPBMC stimulated with bacterial RNAs. $2x10^6$/ml PBMC were stimulated with 0.3, 1 or 3 µg/ml of bacterial RNAs in duplicate (as such or delivered with Lipofectamine 2000, T stands for transfected samples) for 24 hours and cytokines levels were determined from culture supernatants by ELISA. Results represent IFNα response (ng/ml) of 3-5 different PBMC samples ± S.D. * shows $P<0.05$.

3.4.2 IP10 response of hPBMC to bacterial RNAs

IP10 is a chemokine that is also considered as a biomarker of IFN activity (Rose *et al.*, 2013). Therefore, production of IP10 was assessed from bacRNA stimulated hPBMCs using intracellular cytokine staining and flow cytometry. For this, cells were stimulated with bacRNAs for the first 5 h to allow for secretion of type I interferons followed by brefeldin A supplementation for an additional last 2 h of incubation (this blocks secretion and causes accumulation of the chemokine within cells). Cells were then fixed, permeabilized and stained for IP10 and CD14 (monocyte marker) expression. Figure 3.17 A shows flow cytometric dot plots of % of IP10 producers in hPBMCs. All IP10 was produced by CD14+ monocytes. The percent of double positive cells (i.e. monocytes that secrete IP10) were 11.6%, 4.0%, and 11.6% for the positive controls poly(I:C), D35 and 5'PPP-dsRNA, respectively. Commensal derived transfected RNAs were as equally effective as the positive controls (11.1 % for LS and 8.0 % for LF). Pathogen derived transfected RNAs induced no IP10 production (0.5 % and 0.3% for LM and SP, respectively). Figure 3.17 B summarizes the average data accumulated using the flow cytometric analysis and demostrates the absence of response for untransfected samples. These results are in support of the IFN data and suggest that the immune system can discriminate bacterial RNAs of commensal and pathogen origin. It is plausible that the commensal RNAs serve as the major microbiota-derived products that trigger the tonic type I IFN response mentioned in references (McAleer *et al.*, 2012), (Abt *et al.*, 2012), (Ganal *et al.*, 2012).

A



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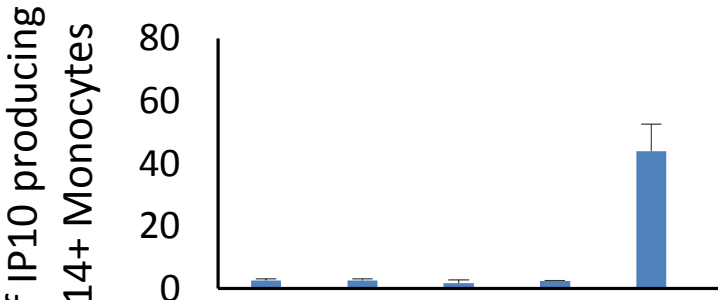

Figure 17 Flow cytometric analysis of IP10 production from CD14+ monocytes stimulated with bacRNAs (1 µg/ml). (A) 2x106/ml hPBMC were stimulated with bacterial RNAs for 5+2 hours (brefeldin A was added for the last 2 h of incubation) and the cells were then fixed, permeabilized and stained for IP10 and CD14 expression. IP10 (y-axis) and CD14 (x-axis) expression was determined using fluorescently labeled antibodies. Cells were analyzed using Flow Cytometer. Results are representative of 3 PBMCs stimulated with similar results. (B) Average results demonstrate the % of IP10 producing CD14+ monocytes (average of 3 different hPBMC ± S.D. Note that untransfected samples generated no IP10).

3.4.3 Confirmation of overall hPBMC results using Cytometric Bead Array (CBA)

In summary, the hPBMC data suggested that pathogen RNAs could directly (without the need of transfection) trigger a pro-inflammatory response without any type I IFN production, whereas commensal RNAs required delivery into the cytosol to induce both a pro-inflammatory and a type I interferon-dominated response. To confirm these findings, we took advantage of the highly sensitive cytometric bead array technology to assess the production of 6 different cytokines (IL8, IFNα, IL6, IP10, IL1β and TNFα) simultaneously in hPBMC stimulated with free or transfected forms of one commensal RNA (LS) and one pathogen RNA (LM). The findings are presented in Figure 18.

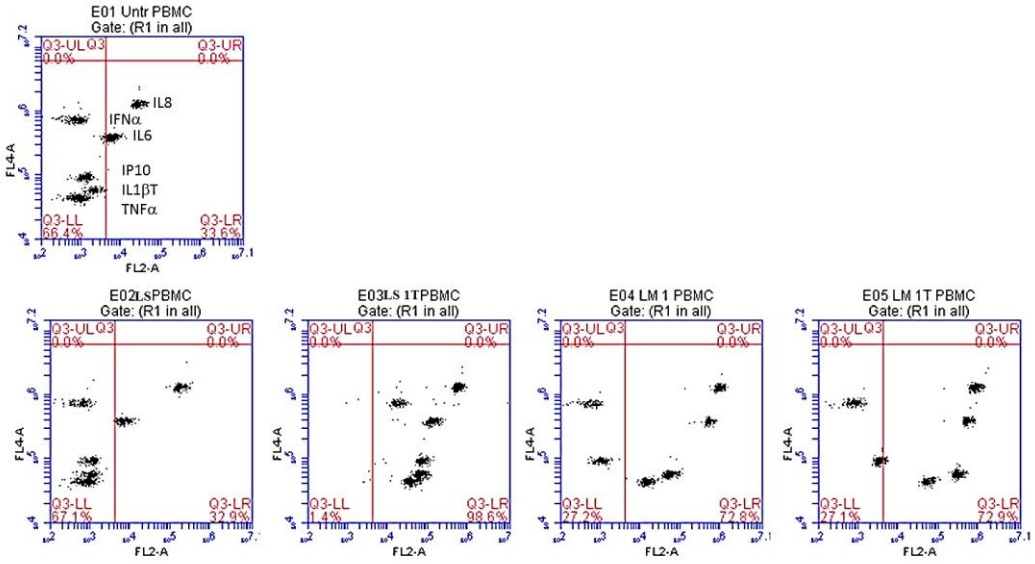

Figure 18 Cytometric Bead Array of hPBMC sitmulated with free or transfected forms of one commensal RNA (LS) and one pathogen RNA (LM) (1µg/ml). Y-axis indicates internal fluorescent intensity of capture beads associated with each cytokine and X-axis is is a measure of amount of cytokines being produced (a shift towards the right correlates with higher concentration).

The CBA data confirms that commensal RNA (LS) had no stimulatory effect when used as such. Transfection generated a response characterized by marked production of IL8, IL6, IFNα, IP-10, IL1β. In contrast, pathogen RNA (LM) induced secretion of pro-inflammatory cytokines IL8, IL6, IL1β and TNFα with or without transfection. There was no measurable IFNα production in response to free or transfected LM RNA and only a slight increase in the level of IP-10 when transfected. These results are consistent with previous data presented in this thesis.

3.4.4 IFN-α/β response of the B16 Blue IFN-α/β reporter cell line to stimulation with bacterial RNAs

The B16 Blue IFN-α/β reporter cell line expresses the cytoplasmic receptors RIG-I and MDA-5 that recognize short 5' triphosphate containing dsRNAs and long dsRNAs, respectively. They do not express any of the TLRs. Recognition through RIG-I/MDA-5 leads to activation of JAK/STAT/ISGF3 pathway and/or interferon regulatory transcription factor-3 (IRF3) pathways. A soluble alkaline phosphatase (SEAP) reporter gene that works under the control of the IFN-α/β-inducible IFN-stimulated gene (ISG54) promoter enhanced by a multimeric IFN-stimulated response element (ISRE) allows for quantitation of this response following the addition of SEAP substrate to culture supernatants. Figure 19 shows that transfected commensal derived RNAs (LS and LF) are potent inducers of Type I IFNs in B16 cells, suggesting direct involvement of RIG-I/MDA-5 in this recognition. However, unlike hPBMC data, CEF RNA failed to show an IFN response and SP RNA induced significantly higher levels of Type I IFN secretion compared to other pathogen RNAs. B16 cells are of mouse origin and thus express murine RIG-I and MDA-5. It is conceivable that murine and human nucleic acid sensors have evolved to discriminate features present in their own commensals and pathogens and thus the B16 reporter cell line might not be ideal for study of human commensal and pathogen RNAs.

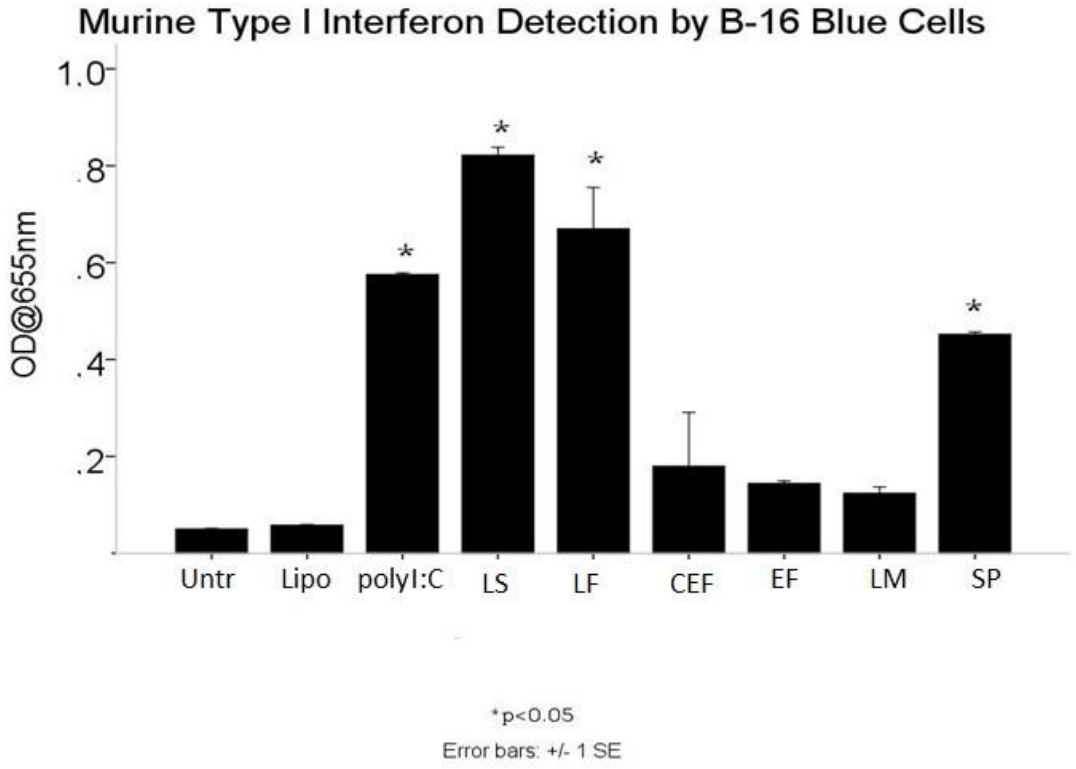

Figure 19 IFN-α/β response of B16 Blue IFN-α/β reporter cell line stimulated with transfected bacterial RNAs. $0.3x10^6$/ml B16 Blue IFN-α/β reporter cells were stimulated with 3μg/ml of Lipofectamine 2000 transfected bacterial RNAs for 24 h and IFN-α/β levels were determined by incubation of 20 μl of culture supernatants with 180 μl of Quanti- Blue (detection medium for SEAP) for 24 h. Results represent IFN-α/β levels (OD at 655 nm) of samples ± S.D. * shows $P<0.05$.

3.5 Detection of commensal and pathogen bacterial RNA activity on HEK-Blue hTLR7 cell line

HEK-Blue hTLR7 cell line expresses the ssRNA sensor hTLR7. These cells also express the SEAP reporter gene which is placed under the control of the IFNβ minimal promoter fused to five NF-kB and AP-1-binding sites. TLR7-mediated ligand recognition activates NF-kB and AP-1 which induce the production of SEAP that can be quantitated from culture supernatants. Transfected commensal and pathogen derived RNAs were used in this sytem and the results are shown in Figure 20. Pathogen RNAs (LM and SP)

triggered significantly higher NF-kB and AP-1 activation than commensal derived RNAs and the EF RNA. These results are consistent with higher proinflammatory cytokine production seen in hPBMC samples and may suggest that pathogen RNAs contain a higher percentage of ssRNA. In fact, Kawashima et.al's work in 2013 also support this view.

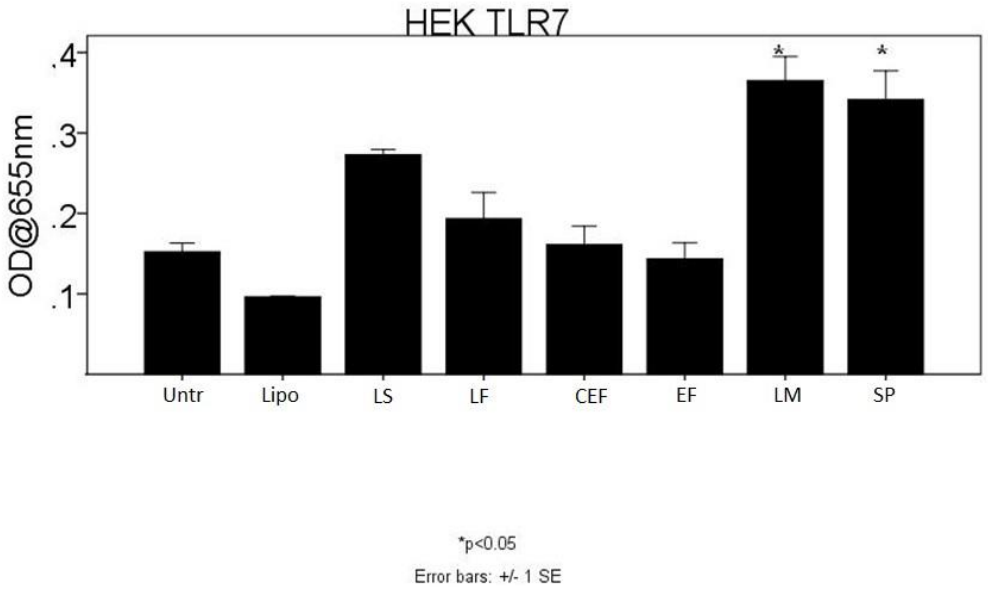

Figure 20 NF-kB and AP-1 activation in HEK Blue TLR7 cells stimulated with bacterial RNAs. $0.3x10^6$/ml HEK TLR7 cell line were stimulated with 3µg/ml of Lipofectamine 2000 transfected bacterial RNA for 24 h and stimulatory activity of bacterial RNAs was determined by incubation of 20 µl of culture supernatants and 180 µl of Quanti- Blue (detection medium for SEAP) for 24 h. Results represent stimulatory activity of bacterial RNAs (OD at 655 nm) ± S.D. * shows $P<0.05$.

3.6 Detection of bacterial RNA activity on human neutrophils and Netosis assay

Neutrophils correspond to the major fraction of circulating leukocytes and are the first line of cellular defence against microbes. Furthermore, they express the nucleic acid sensors RIG-I, MDA-5 and TLR8 both in secretory vesicles, in the cytoplasm and on the plasma membrane. Thus it is possible that neutrophils express the receptors to recognize

RNA both in cytoplasm and on the cell surface. (Berger *et al.*, 2012). Neutrophils also eliminate pathogens by phagocytosis and secrete chemokines such as IL8 (CXCL8). IL8 is a chemokine that attracts cells to the site of infection, contributing to neutrophil activation (Mahalingam *et al.*,, 1999). Neutrophils also respond to certain pathogenic organisms and inflammatory stimuli by releasing neutrophil extracellular traps (NETs) by undergoing a specific type of cell death called as netosis. The NETs consist of DNA decorated with antimicrobial proteins/peptides and serve to capture and kill microbes (Brinkmann *et al.*, 2004, Urban *et al.*, 2006). How neutrophils respond to RNA is still unclear. There are only 2 studies on this subject with completely opposing results: Work by Berger *et. al* (2012) showed that neutrophil associated RIG-I recognized the ligand 5'triphosphate dsRNA and siRNA but did not trigger these cells to produce TNFα. In contrast, Tammassia *et al.* in 2008 demonstrated that neutrophils responded to transfected poly(I:C) by upregulating a number of genes involved in anti-viral defense. Information on bacRNA recognition by human neutrophils is nonexistent. In light of the above information, we investigated the response of human neutrophils stimulated with the bacRNAs. For this, using purified neutrophils, we tested whether bacRNAs would trigger NET formation or IL-8 secretion from these cells.

Neutrophils were first purified from human blood as described in Section 2.2.4.2. Figure 21 (A) shows that the isolated cells consisted of > 95% neutrophils as evidenced by the typical horse shoe shaped nuclear staining patterns (using the cell membrane permeable nucleic acid dye SytoGreen). Purified cells were then stimulated with bacRNAs or the NET-inducer phorbolmyristate (PMA) for 4 h. Possible NET formations were assessed following staining of cells with the cell permeable SytoGreen and the cell impermeable SytoxOrange dyes. The latter dye is excluded from live cells but stains DNA present in dead cells or in NETs. None of the bacRNAs induced NET formation (representative pictures for LS RNA and LM RNA are given in Figure 21 (B) and 21 (C)) whereas the positive control PMA led to extensive NET formation (Figure 21)D)).

A

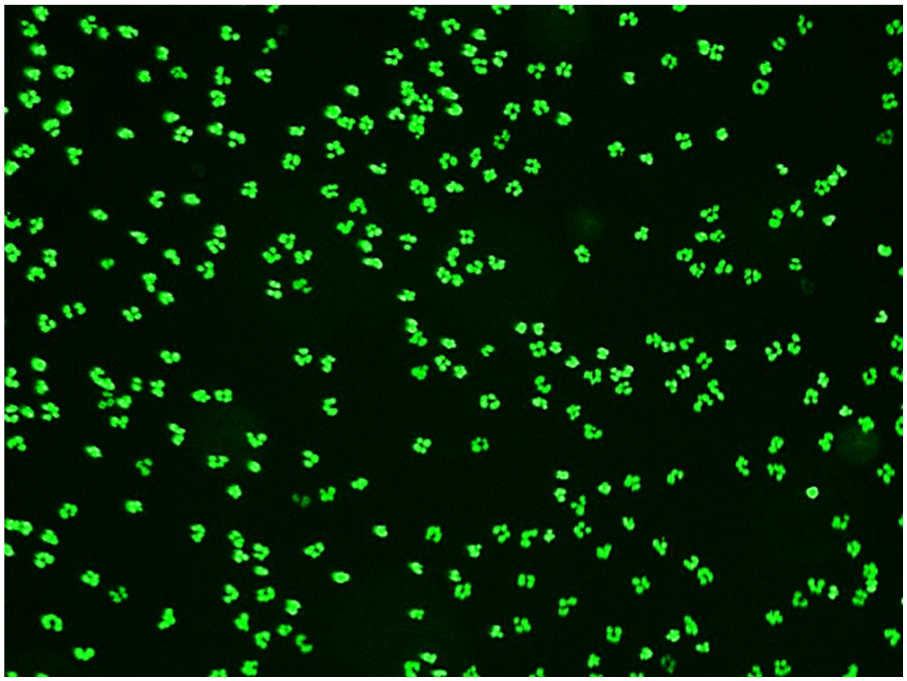

Figure 21 Netosis assay of neutrophils that were stimulated with bacterial RNAs or with PMA. 320,000/ml human neutrophils were stimulated with PMA (25 nM) or 1 μg/ml bacterial RNA for 4 h and stained with SytoGreen and SytoxOrange (4 μM final concentrations). Samples were visualized using the Floid Cell imaging station. (A) Freshly isolated neutrophils stained only with SytoGreen. (B), (C) and (D) are samples stimulated with LS RNA, LM RNA and PMA, respectively.

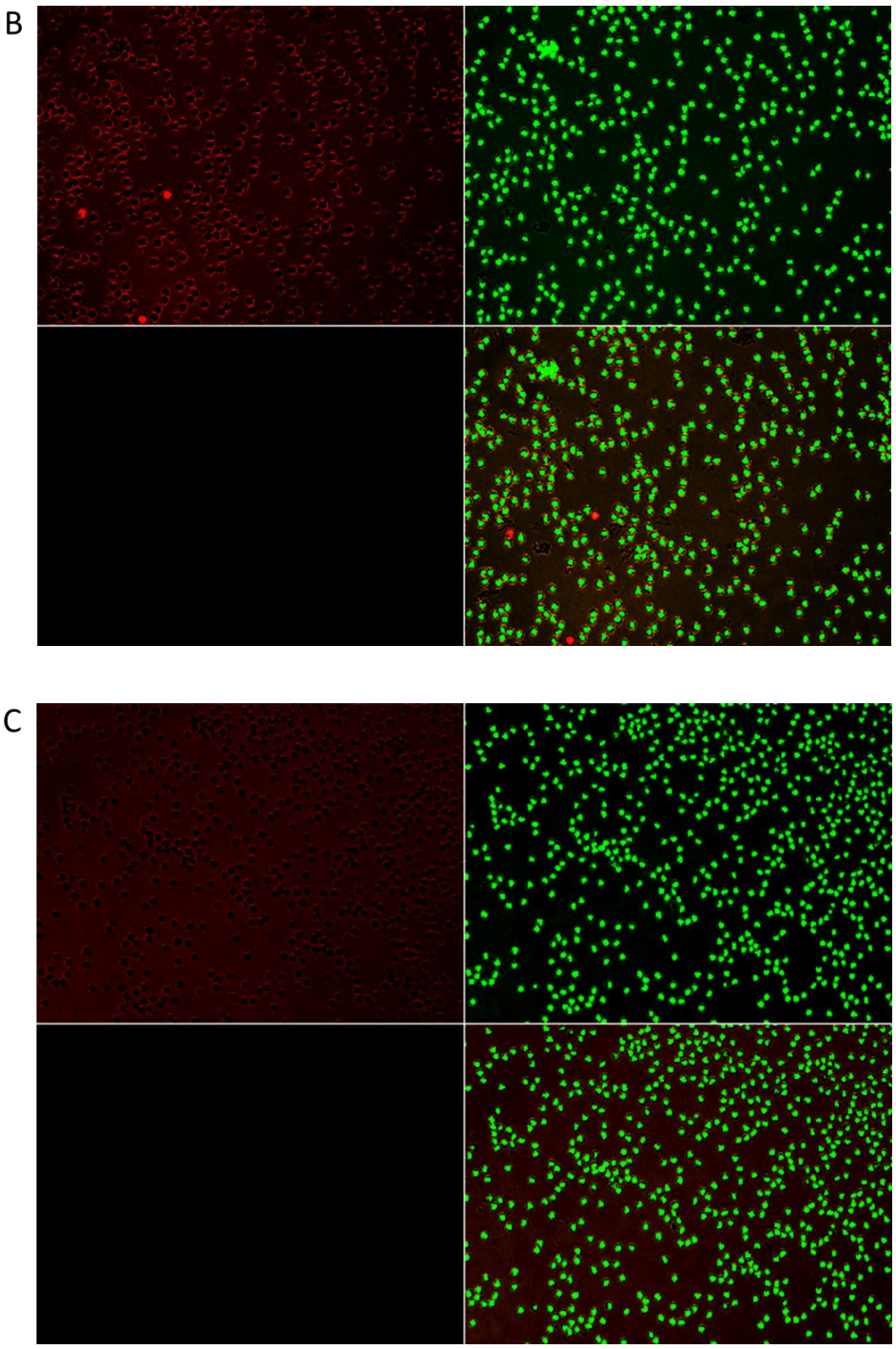

Figure 21(Continued)

D

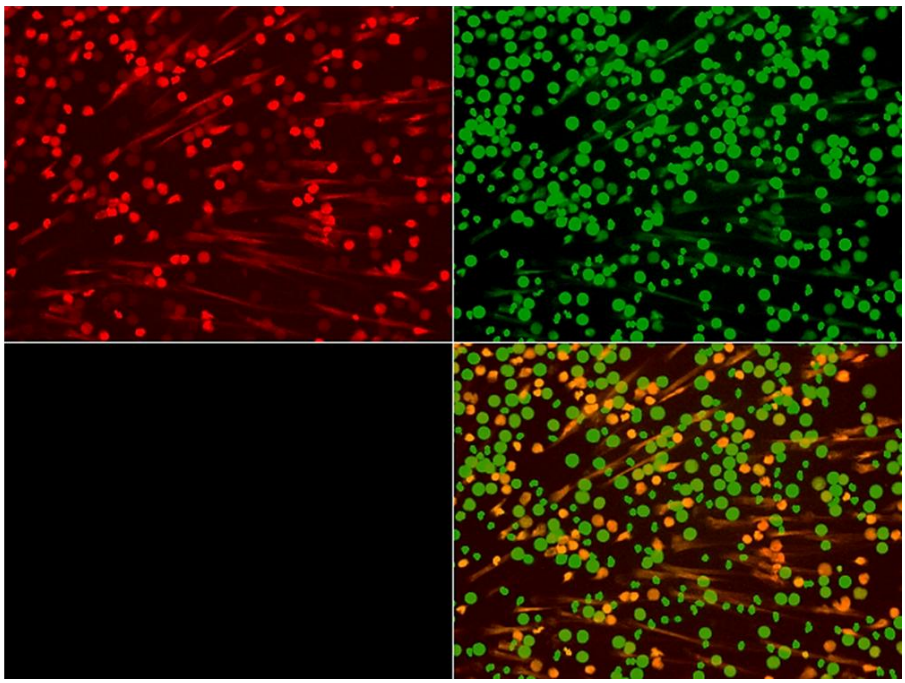

Figure 21 (Continued)

Next, culture supernatants of neutrophils stimulated for 24 hours with bacterial RNAs were assayed for IL8 production by ELISA. Figure 22 shows that only pathogen derived RNAs (LM and SP) induced an IL-8 response in neutrophils. Externally added pathogen RNAs were equally effective as the transfected samples. In contrast, commensal RNAs induced no such response even when transfected. These results suggest that at least in neutrophils RIG-I/MDA-5 and/or TLR8 might be involved in discrimination of commensal versus pathogen RNAs.

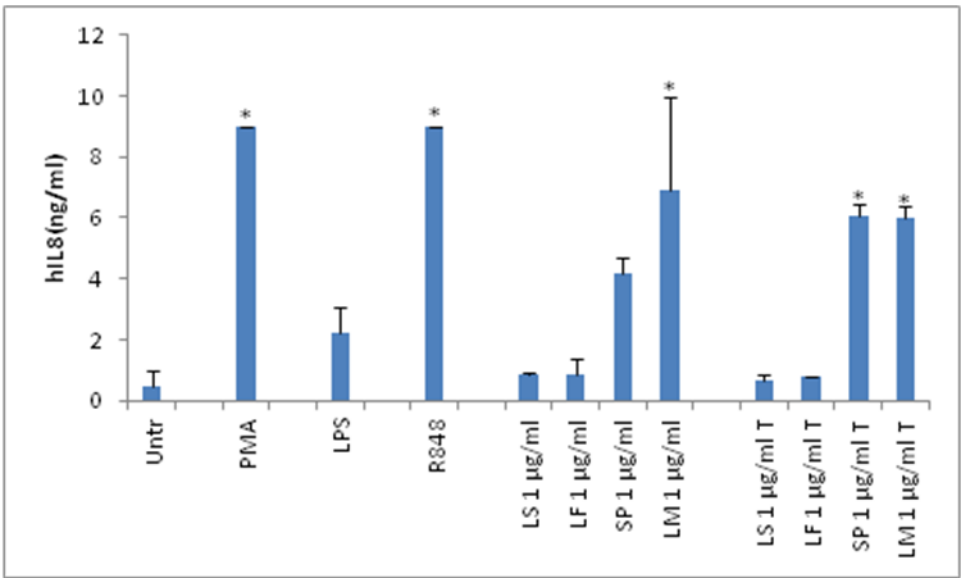

Figure 22 IL8 production from human neutrophils stimulated with bacterial RNAs. $1x10^6$/ml neutrophils were stimulated with 1µg/ml of bacterial RNAs in duplicate (free or delivered with Lipofectamine 2000, T indicates transfection) for 24 hours and cytokine levels were assessed by ELISA. Result shows IL8 response (ng/ml) of neutrophils ± S.D. * shows $P<0.05$.

3.7 Effect of bacterial RNA/antimicrobial peptide LL-37 complexes on murine spleen cells and hPBMCs

Results presented so far in this thesis proved that commensal RNAs showed an effect only when they were transfected, questioning the relevance of these findings in an in vivo setting where transfection would not be a possibility. However, *in vivo*, alternative delivery routes for nucleic acids exists. Of these, cationic peptide mediated delivery of nucleic acids into cells is of special importance. One such example is the human cathelicidin LL-37, a cationic antimicrobial peptide secreted by a variety of cells (Agerberth *et al.*, 2000). Self-DNA can form complexes with this cationic peptide and such complexes were shown to activate plasmacytoid dendritic cells (pDC) to produce IFNα (Lande *et al.*, 2007). LL-37 can also bind self-RNA released from dying cells,

and in this form activate DCs to secrete Type I IFNs through a TLR7/TLR8 mediated pathway (Ganguly *et al.*, 2009). One more paper demonstrated that this cationic peptide redirects trafficking of polyI:C and can enhance TLR3-mediated signaling (Singh *et al.*, 2013). Thus, as a proof of concept, we stimulated mouse splenocytes with bacterial RNAs complexed with LL-37 and checked whether commensal RNAs associated with this anti-microbial peptide could stimulate cells in the absence of transfection. Figure 23 demonstrates that complexation with LL-37 greatly improves the recognition of commensal RNAs but not the pathogen RNAs (LM and SP).

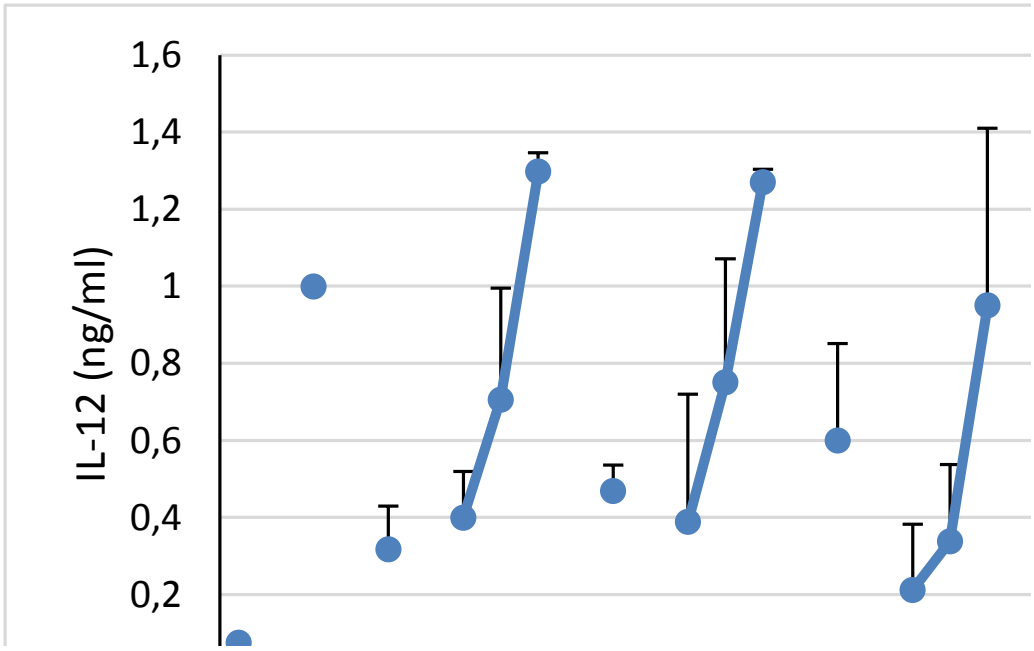

Figure 23 IL-12 secretion from mouse splenocytes stimulated with 0.3 µg/ml transfected bacterial RNA (T indicates transfection) and 0.3, 1, 3µg/ml bacterial RNA complexed with LL-37 (in 1:9 ratio for 20 minutes before delivery) for 24 hours. Cytokine levels were measured by ELISA. Result represents average IL12 response (ng/ml) of 2 mice ± S.D.

Whether complexation of commensal bacterial RNA with LL37 would replace the need of transfection was also tested on hPBMC. In this experiment, optimum dose of bacterial RNA (1µg/ml) was either transfected with lipofectamine 2000 or complexed with LL37

in a ratio of 1:6 and 1:9. Figure 24 shows that 1:9 ratio of bacterial RNA: LL37 complexes induced IL6 secretion that was nearly as high as transfected samples. Therefore, in the absence of transfection, both commensal and pathogen bacterial RNA can be delivered to the cytosol/endosomes by complexation of anti-microbial peptide LL37 in humans. As a summary, these results suggest that a similar mechanism might be of importance in in vivo recognition of commensal RNAs in humans.

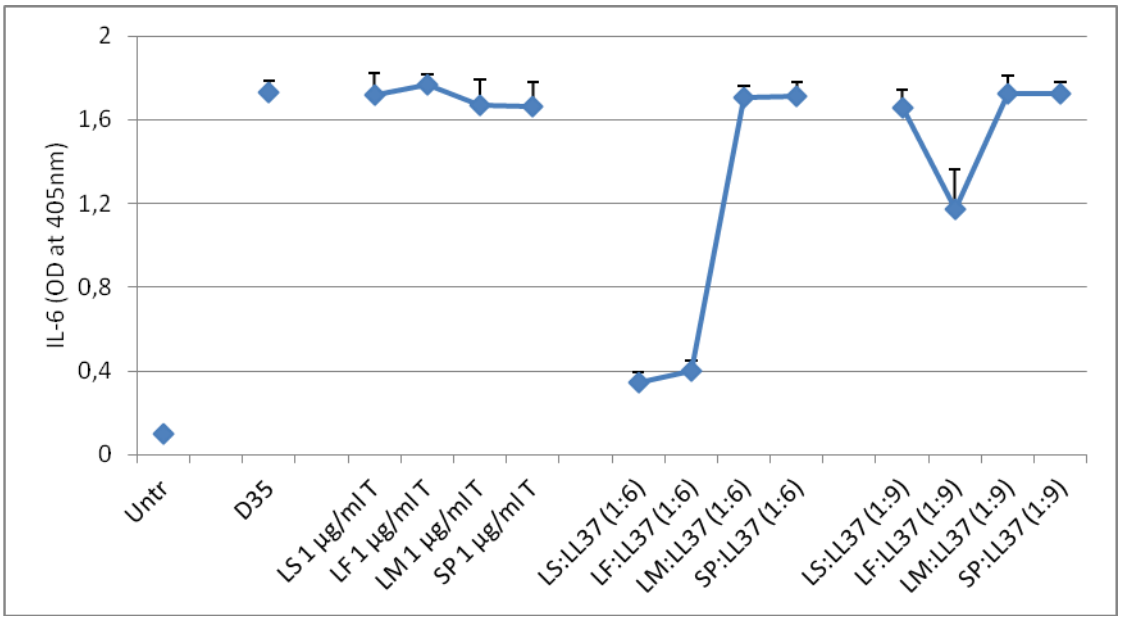

Figure 24 IL6 secretion from hPBMC stimulated with transfected 1µg/ml bacterial RNA (T shows transfection) and bacterial RNAs complexed with LL37 (in a 1:6 and 1:9 ratio prior to stimulation) for 24 hours. Cytokine levels were assessed by ELISA. Result shows average IL6 response (OD at 405nm) of 2 patients ± S.D.

3.8 Vaccination

Live vaccines have been far more effective than killed vaccines (Detmer *et al.*, 2006). Considering the existance of identical PAMPs in both live and killed vaccines, explanation of this phenomenon remains difficult. Recently, one group reported that the mammalian innate immune system can detect microbial viability via detection of

viability-associated PAMPs (so-called Vita-PAMPs). This group demonstrated bacterial RNA to be the major vita-PAMP (Sander *et al.*, 2011), required for potent vaccine activity. Based on this information, we wanted to test the vaccine adjuvant activities of bacRNAs using ovalbumin (OVA) as a model antigen in a prime and boost regimen. For this, C57BL/6 mice were immunized (five animals per group) intraperitoneally with PBS, OVA, or OVA adjuvanted with CpG ODN (K23), dsRNA mimic poly (I:C), LS RNA, LF RNA, LM RNA and SP RNA on days 0 and 14. Sera were collected on days 13 and 28 to determine OVA specific antibody levels generated in immunized mice. Titers of antigen-specific total IgG, IgG1 and IgG2c (IgG2a isotype in C57BL/6 mice) antibody subclasses were measured. Figure 25 and 26 presents the results of the primary and the secondary responses, respectively. Graphs on the left depict the mean OVA-specific IgG subtype levels (raw OD values) as a function of serial dilution. Serial dilutions of sera provides us the information that the response is directly associated with the amount of antibody produced and not just an artifact. Titers were determined using a cut-off value 3 standard deviations above the average OD recordings obtained from naive animals. For each animal the reciprocal serum dilution that generated an OD at or below this level was considered as the antibody titer.

Results show that all adjuvants (except for the CpG ODN K23) were effective in inducing significantly higher levels of OVA-specific total IgG and IgG1. However, when the antigen-specific IgG2c responses were analyzed, a striking difference was observed. For this antibody class, pathogen RNAs significantly boosted OVA-specific IgG2c titer to levels comparable to poly(I:C) adjuvanted group. Commensal RNAs had no such adjuvant effect. These results suggest that pathogen RNAs can trigger a response in vivo similar to poly(I:C) that can sustain Th1 cell development, providing help for Ag-specific IgG2c production. In contrast, commensal RNAs are ineffective Th1 inducers and hence the response is dominated by a mild increase in OVA-specific IgG2c production. In vivo data suggests absence of vigorous recognition for commensal RNAs but a strong response to pathogen RNAs.

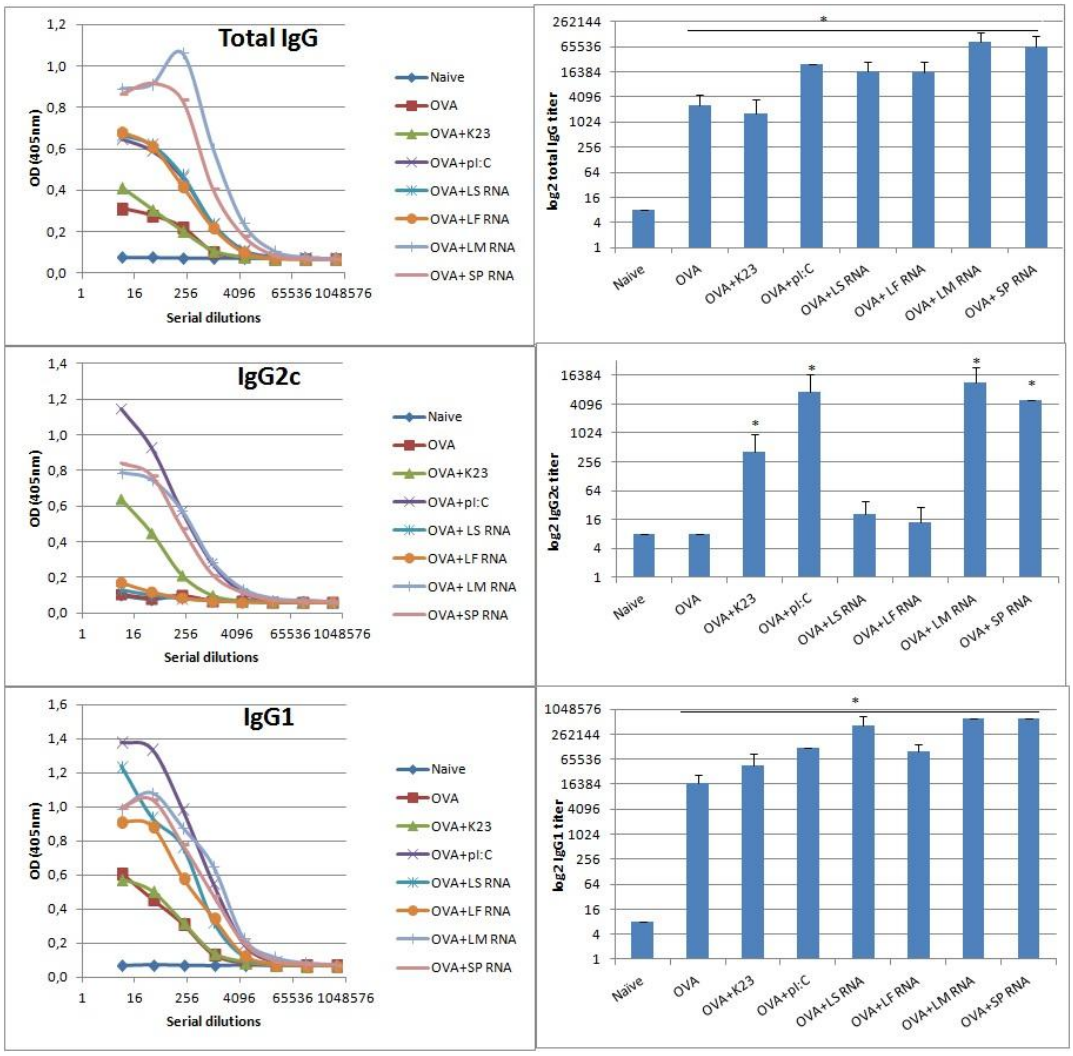

Figure 25 Primary response showing OVA specific Total IgG, IgG2c and IgG1 titers. Results are the average of 5 mice per group. ± S.D. * shows P<0.05.

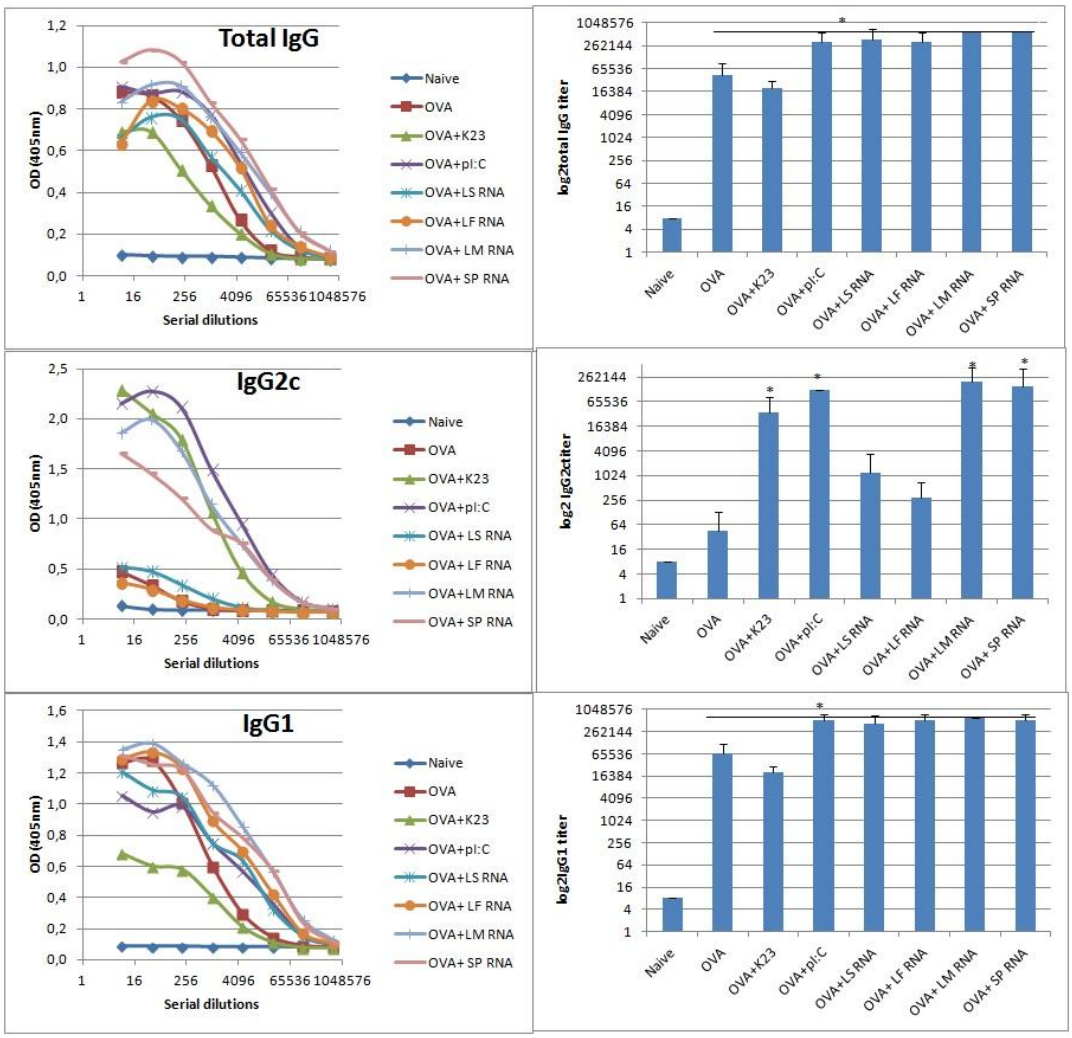

Figure 26 Secondary response showing OVA specific Total IgG, IgG2c and IgG1 titers. Results are the average of 5 mice per group. ± S.D. * shows P<0.05.

CHAPTER 4

CONCLUSION

In this study, we proposed that bacterial RNAs of commensal and pathogen origins may have different immunostimulatory activities resulting in differential activation of immune system. In order to test this hypothesis, human PBMCs, Thp-1 cell line, RIG-I/MDA-5 expressing B16 IFN reporter cell line, endosomal ssRNA receptor TLR7 expressing HEK TLR7 cell line and purified human neutrophils were stimulated with three different doses of bacterial RNA as such or transfected with Lipofectamine 2000. Results showed that strict pathogen derived bacterial RNAs induced higher levels of the proinflammatory cytokine IL6 even in the absence of transfection in hPBMC. The virulent strain of E. faecium showed a similar pattern of response demonstrated by its commensal counterpart.

The ability of commensal and pathogen derived bacRNAs to activate the inflammasome was also tested in hPBMC and Thp-1 cells through quantitation of active IL1β secretion. Commensal derived bacRNAs induced low levels of IL1β production only after transfection. In contrast, pathogen derived bacRNAs induced higher levels of IL1β in hPBMC even when they were delivered as such. The commensal and pathogenic strains of E. faecium generated a response similar to those observed with commensal RNAs, suggesting that the distinction between commensal and pathogen RNAs was largely based on the ribosomal RNAs and not due to pathogenicity island encoded mRNAs. Both commensal and pathogen derived bacRNA triggered similar levels of IL1β in the monocytic Thp-1 cell line. Since hPBMC represents a mixed cell population and it contains various receptors, this difference may stem from unique internalization patterns that may operate in hPBMC but not in the monocytic cell line.

Based on published work demonstrating that commensal derived products/molecules could trigger chronic type I IFN release in the host, we hypothesized that one of these molecules could be the bacRNAs released by dead cells. Therefore, the ability of commensal and pathogen RNAs to trigger IFNα and IP10 production was tested hPBMCs. Findings supported the view that commensal derived RNAs but not the pathogenic bacRNAs induced a Type I IFN response. To determine the possible receptors involved in this recognition, experiments were conducted using reporter cell lines. Stimulation of RIG-I/MDA-5 expressing B16 reporter cell line with bacRNAs showed that LS, LF, and SP RNAs triggered higher levels of Type I IFNs whereas the other bacterial strains did not. The B16 cell line is of mouse origin, and may not reflect an ideal system to compare differences in recognition of human commensal and pathogen bacRNAs. Low level of Type I IFN induction by microbiota was shown to regulate immune system and retain a low level of immune activation against possible antiviral infections (McAleer *et al.,* 2012). Higher levels of dsRNA present in commensal but not pathogenic bacteria was previously shown to trigger IFNβ (Kawashima *et al.,* 2013). Our results support the findings that commensal but not pathogen bacRNA trigger tonic Type I IFN response that is important in regulation of immune system. However, whether dsRNA content is directly responsible for this difference, remains to be determined.

Pathogen RNAs were more stimulatory than commensal derived bacRNA and EF RNA in HEK-Blue TLR7 cell line expressing the ssRNA sensor TLR7. Since ssRNA recognition results in activation of NFκB and AP-1 in this cell line, the results are consistent with hPBMC experiments. Since the results indicate that pathogenic RNAs are more stimulatory in this sytem, indicating higher levels of ssRNA content, our findings indirectly support Kawashima et.al's results (2013) that commensal RNAs contain more dsRNA.

Neutrophils constitute the first line of cellular defence against microbial infections and they express the nucleic acid sensors RIG-I/MDA-5 and TLR8 both in their secretory vesicles, in the cytoplasm and on the plasma membrane.Therefore, how neutrophils

respond to bacRNA was of interest. Upon stimulation of purified human neutrophils with bacRNA, only the pathogen derived bacRNAs induced IL8 secretion, suggesting that pathogen-commensal discrimination is also valid in this cell type. Furthermore, our results demonstrated that neither pathogen nor commensal derived bacRNAs triggered NET formation.

Commensal derived bacRNAs were effective only when transfected into the cytosol. Several papers reported that nucleic acids can form complexes with antimicrobial peptides and activate DCs to secrete Type-I IFN (Lande *et al.,* 2007, Ganguly *et al.,* 2009). Upon complexation of bacRNAs with the antimicrobial peptide LL37, both commensal and pathogen derived bacRNAs were effectively recognized without the need of transfection. These results suggest that a similar mechanism might be responsible for recognition of commensal RNA in humans.

Vaccination experiments in which OVA was used as a model antigen and bacRNAs were employed as adjuvants demonstrated that both commensal and pathogen bacRNAs induced high levels of total IgG and IgG1, indicative of Th-2 based help. However, only the pathogen RNAs boosted Ag-specific IgG2c response, suggestive of Th1 activation through the induction of pro-inflammatory cytokines. Thus, pathogen RNAs induced a similar response as seen with poly(I:C) adjuvanted group whereas commensal RNAs were ineffective in inducing a Th1 response. These results are consistent with the view that Th1 triggering activity, which would be associated with extensive tissue damage is not compatible with symbionts and is mostly restricted for pathogen recognition.

Collectively, our results show that pathogen and commensal derived bacRNA have distinct immunostimulatory activities. Evolutionarily conserved structural differences present in commensal and pathogen RNAs, route of entry of those RNAs and their resistance to plasma nucleases might be important factors that lead to such diverse response.

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APPENDIX A

BUFFERS, SOLUTIONS AND CULTURE MEDIA

Blocking Buffer (ELISA)

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

Bacteria Lysis Buffer

30mM Tris-HCl, pH 8.0
1mM EDTA
15 mg/ml lysozyme

Loading Dye (Agarose gel)

0,009 grams Bromophenol blue
0,009 grams Xylen cyanol
2,8 ml ddH2O
1,2 ml 0,5M EDTA
11 ml glycerol

PBS (Phosphate Buffered Saline) [10x]

80 grams NaCl
2 grams KCl
8,01 grams $Na_2HPO_4 . 2H_2O$
2 grams KH_2PO_4
1 lt with ddH_2O

PBS-BSA-Na azide (FACs Buffer)

500 ml 1x PBS
5g BSA (1%)
125mg (0,25%)

T-cell Buffer (ELISA)

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

Wash Buffer (ELISA)

500 ml 10x PBS
2,5 ml Tween20
4,5 lt ddH_2O

RPMI-1640 (Hyclone)

2 % : 10 ml FBS (FBS = inactivated at 55°C)
5 % : 25 ml FBS
10 % : 50 ml FBS

5 ml Penicillin/Streptomycin (50 μg/ml final conc.)

5 ml HEPES (Biological Industries), (10 mM final conc.)

5 ml Na Pyruvate, (0,11 mg/ml final conc.)

5 ml Non-Essential Amino Acids Solution, (diluted into 1x from 100x conc. stock)

5 ml L-Glutamine, (2 mM final conc.)