IMMUNOMODULATORY ACTIVITIES OF RNA SPECIES DERIVED FROM COMMENSAL AND PATHOGENIC BACTERIA

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ABSTRACT

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Bacterial RNAs are recognized by various types of immune sensors. Here, we aimed to investigate the differential immune activation mediated by RNAs purified from commensal or pathogenic bacteria. For this, total RNAs and/or individual ribosomal RNAs (5S, 16S and 23S) were isolated from two commensal bacteria, Lactobacillus *salivarious and Lactobacillus fermentum* and two pathogens, *Listeria monocytogenes, and Streptococcus pyogenes*. Bacterial RNA species isolated from pathogens induced stronger pro-inflammatory cytokine production in human peripheral blood mononuclear cells (hPBMCs) and triggered activation of NF-κB/AP-1 in HEK-Blue cells expressing TLR3 or TLR7 receptors. Conversely, significant amount of type I IFN production was induced following delivery of commensal RNAs (total RNAs and 23S rRNAs), but not pathogenic RNAs, to cytosol. Moreover dsRNA content of commensal derived RNAs was shown to be higher than pathogen derived RNAs. These findings suggest the involvement of cytosolic dsRNA sensors like RIG-I and MDA-5 in commensal but not pathogen-derived RNA recognition. Data further showed that the major type I IFN producing

cells responding to commensal RNAs were monocytes but not plasmacytoid dendritic cells (pDCs). Interferogenic activity of commensal origin RNAs was also tested in human monocyte cell line THP-1, confirming the results obtained using primary monocytes. In conclusion, our data implicate that RNAs from commensals and pathogens are recognized differentially by the immune system to initiate either a type I interferon or a pro-inflammatory cytokine dominated immune response.

Keywords: commensal, pathogen, dsRNA, RIG-I/MDA-5, TLR3/TLR7

ÖZ

KOMMENSAL VE PATOJEN BAKTERİLERDEN ELDE EDİLEN RNA TÜRLERİNİN İMMÜNOMODÜLATÖR AKTİVİTELERİ

Kayaoğlu, Başak

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Bakteriyel RNA`lar çeşitli immün reseptörler tarafından tanınırlar. Bu tezde, kommensal veya patojenlerden elde edilen RNA`ların neden olduğu diferansiyel immün aktivasyonu araştırmayı amaçladık. Bunun için, iki kommensal bakteri *Lactobacillus salivarious, Lactobacillus fermentum*`dan ve iki patojenik bakteri, *Listeria monocytogenes, Streptococcus pyogenes*`dan total RNA`ların ve/veya ribozomal RNA`ların (5S, 16S ve 23S) izalasyonu yapıldı. Patojenlerden izole edilen RNA türleri, insan periferik kan mononükleer hücrelerinde (hPBMC`ler) daha güçlü pro-inflamatuvar sitokin üretimini indükledi ve bu RNA`lar TLR3 veya TLR7 reseptörünü ifade eden HEK-Blue hücrelerinde NF-κB/AP-1`in aktivasyonunu tetikledi. Bunun aksine, patojen RNA`ların değil, kommensal RNA`ların (total RNA`lar ve 23S rRNA`lar) sitozole iletilmesinden sonra, tip I İFN üretimini önemli miktarda arttırdığı görüldü. Ayrıca komensallardan izole edilen RNA`ların çift zincirli RNA (dsRNA) içeriklerinin, patojenlerden izole edilen RNA`larınkine oranla

daha yüksek olduğu gösterildi. Bu bulgular, RIG-I ve MDA-5 gibi sitosolik dsRNA sensörlerinin patojenlerden değil, kommensallardan elde edilen RNA`ların tanınmasına katkı sağladığını önermektedir. Veriler ayrıca, kommensal RNA`laryanıt olarak interferon üreten ana hücrelerin plazmositoid dendritik hücrelerin (pDC`ler) değil, monositler olduğunu göstermektedir. Komensal orijinli RNA`ların interferojenik aktivitesi, insan monosit hücre hattı THP-1`de de test edilerek, primer monositler kullanılarak elde edilen sonuçlar teyit edildi. Sonuç olarak, verilerimiz, kommensallardan ve patojenlerden elde edilen RNA`larının immün sistem tarafından farklı şekilde tanınarak ya tip I interferon ya da pro-inflamatuvar sitokin ağırlıklı bir immün yanıtı uyardıklarını göstermektedir.

Anahtar Kelimeler: Kommensal, patojen, dsRNA, RIG-I/MDA-5, TLR3/TLR7

To my family

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophorsis
AIM2	Activator protein 1
AP-1	Absent in melanoma 2
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing CARD
ATP	Adenosine triphosphate
BacRNA	Bacterial RNA
BCR	B cell receptor
Bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
Cgamp	Cyclic guanosine monophosphate-adenosine monophosphate
cGAS	Cyclic GMP-AMP synthase
CpG	Unmethylated cytosine-phosphate-guanosine motifs
DAMP	Danger/damage associated molecular pattern
DC	Dendritic Cells
DDX	DEAD-Box helicase
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked-Immunosorbent Assay
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
hPBMC	Human peripheral blood mononuclear cell
HRP	horseradish peroxidase
HSC	Hematopoietic stem cells
HSV	Herpes Simplex Virus
IFIT	Interferon induced protein with tetratricopeptide repeats
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IP-10	Interferon gamma-induced protein 10
IRF	Interferon-regulatory factor
ISG	Interferon-stimulated genes
ISRE	IFN-stimulated response element
LGP2	Laboratory of Genetics and Physiology
LPS	Lipopolysaccharide
MAVS	Mitochondrial antiviral-signaling protein
MDA-5	Melanoma Differentiation-Associated protein 5
Mdc	Myeloid Denditic Cells
MHC	Major histocompatibility complex
miRNA	MicroRNA
MyD88	Myeloid differentiation factor-88
NF-κB	Nuclear factor-kappa B
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain like receptor
NOD	Nucleotide-binding oligomerization domain
Nt	Nucleotide
OAS1	Oligoadenylate synthetase 1
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
pDC	Plasmacytoid Dendritic Cell
PKR	dsRNA-activated protein kinase R
PMA	Phorbol 12-myristate 13-acetate
PNPP	Para-nitrophenyl pyro phosphate
PolyI:C	Polyriboinosinic polyribocytidylic acid
PolyU	Single stranded poly-uridine
PRR	Pattern recognition receptor
PYHIN	HIN domain-containing
R848	Resiquimod
RIG-I	Retinoic acid-induced gene-I
RLH	Retinoic acid-induced gene-I like helicase
RLR	Retinoic acid-induced gene-I like receptor
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RWB	RNA Wash Buffer
SA-ALP	Alkaline phosphatase conjugated streptavidin
SEAP	Soluble alkaline phosphatase
siRNA	Small Interfering RNA
ssRNA	single stranded RNA

STING	Stimulator of Interferon genes
TAE	Tris-base, Acetic Acid, EDTA
TBK1	TANK-binding kinase 1
TBS-T	Tris-buffered saline, 0.1% Tween 20
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
TRAF	TNF receptor associated factor
TREG	Regulatory T cells
TRIF	TIR domain containing adaptor inducing IFN-β
tRNA	Transfer RNA
TSB	Tryptic soy broth
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Immune System

1.1.1 Immune Recognition and Regulation

Immune system constitutes defense mechanisms that protect the organism from invading pathogens. The first step of this protection is the recognition of foreign (non-self) molecules in order to initiate effector functions of immune system. In addition to non-self, altered-self molecules expressed in virus infected or tumor cells are recognized by specialized sensors of immune cells (Houghton, 1994). This immunological recognition triggers several signaling pathways that can eliminate the infection and neutralize the virulence factors of these pathogens (Medzhitov & Janeway, 1997). Innate and adaptive immune systems are the two arms of immunity working together in harmony to provide the most effective defense against infections (Dunkelberger & Song, 2010). Upon first exposure to a pathogen, innate immune mechanisms provide the first line of defense that produce rapid and general immune responses, whereas the development of highly specific antigen-directed adaptive immune effector responses requires several days, Immune system can also cause harm to the host unless it is kept under control. Proper immune response could be achieved when host's immune system maintains the balance between effector functions and immunomodulation. Any improper activation of the immune system or any compromise in immune regulation may cause severe conditions such as allergy or autoimmunity (Maizels, 2005).

1.1.2 Cells of the Immune System

All blood cell types are produced by the proliferation and differentiation of pluripotent hematopoietic stem cells (HSCs). Common lymphoid and common myeloid progenitors are the two main progenitors produced during the differentiation of HSCs. Myeloid progenitors give rise to granulocytes (neutrophils, eosinophils and basophils), macrophages, erythrocytes and megakaryocytes, whereas lymphoid progenitors generate T lymphocytes, B lymphocytes and natural killer (NK) cells (Kondo, 2010). Both myeloid and lymphoid progenitors can give rise to dendritic cells (DCs) which are classified as myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), respectively (Collin et al., 2013). Macrophages, granulocytes and DCs are collectively called phagocytes since they can engulf extracellular content including the pathogens. Macrophages and neutrophils perform phagocytosis to kill engulfed pathogens in tissues whereas DCs ingest the material taken up by phagocytosis, process and present antigens to T lymphocytes. B cells can recognize antigens as such through their surface expressed immunoglobulins. However, T cells require presentation of peptides derived from antigens in association with MHC Class I or II expressed on infected cells or antigen presenting cells (APCs), respectively. B cell maturation takes place in bone marrow and mature B cells enter into circulation. On the other hand, immature T cells travel to thymus to complete their maturation (Zúñiga-Pflücker, 2004). Following their maturation, both T and B cells circulate between blood stream and peripheral lymphoid organs. Peripheral lymphoid organs such as spleen and lymph nodes have different compartments where lymphocytes separately reside in. For example, antigen presentation between APCs (mostly DCs) and T cells occur in T cell zone. Another cell type of lymphoid lineage is natural killer cells which recognize stressed/infected host cells. For example, NK cells can detect downregulation of MHC Class I molecules on virus infected cells and kill these cells through a mechanism known as “missing-self recognition” (Raulet, 2006).

1.2 Innate Immunity and Pattern Recognition

Innate immunity includes barrier function of epithelial cells, complement system, killing function of phagocytes and inflammatory responses initiated by innate immune cells. Epithelial cells provide both physical and chemical protection against infections. Tight junctions between epithelial cells are an example of physical barrier that prevents the entry of pathogens. Microbiota also contributes to first line defense against invaders since microbiota combat with pathogens for space and nutrients (Murphy & Weaver, 2016). If the first line defense is breached, activation of complement system and secretion of antimicrobial peptides occur immediately to restrain infection. Initiation of inflammatory response and recruitment of phagocytes to the site of infection can be considered as a second line of defense. Inflammatory responses initiated by cells of innate immune system occur upon recognition of non-self molecules by immune receptors. Molecular structures conserved among microbial species are recognized by a wide variety of pattern recognition receptors (PRRs). Conserved structural motifs of pathogens such as bacterial cell wall components or nucleic acids are collectively known as pathogen-associated molecular patterns PAMPs (Broz & Monack, 2013). Although the PRRs are mainly responsible for the sensing of these PAMPs, endogenous molecules released from damages cells can also be detected by PRRs. These molecules that are released in response to stress, tissue damage and necrosis are called damaged-associated molecular patterns (DAMPs) (Takeuchi & Akira, 2010). Pattern recognition receptors are generally classified into four groups based on their localization and function in the cell. These groups include extracellular-soluble receptors (such as mannose-binding protein), membrane-bound phagocytic sensors (such as Dectin-1), membrane-bound signaling receptors and cytosolic signaling receptors (Ranjan et al., 2009). Following pattern recognition, both membrane-bound and cytosolic signaling receptors can induce downstream signaling cascades and consequently help establishment of an inflammatory response.

Membrane-bound signaling PRRs are composed of family members of Toll-like receptors (TLRs). TLRs can be localized on cell surface or endosomal compartments depending on the ligand. Cell-membrane associated TLRs generally recognize bacterial cell-wall derived components (for eg. TLR4 recognizes gram negative outer membrane component, lipopolysaccharide) whereas endosomal TLRs are specialized in nucleic acid recognition (TLR3, 7/8 and TLR9 recognize dsRNA, ssRNA and CpG dinucleotide rich DNA, respectively) (Kawai & Akira, 2006). Cytosolic PRRs include RIG-I like receptors (RLRs), NOD-like receptors (NLRs), HIN domain-containing (PYHIN) family members and wide variety of cytosolic dsDNA sensors (such as cGAS, AIM2 and IFI16) (Broz & Monack, 2013). Different PRRs and their cognate ligands are listed in Table 1.1.

Table 1.1 PRRs and their ligands (Adapted from Takeuch and Akira, 2010)

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Activation of pattern recognition receptors is important for the establishment of inflammatory state since downstream signaling events of these PRRs can upregulate the transcription of genes involved in inflammatory responses. Upon ligand

recognition of TLRs, MyD88 and/or TRIF dependent signaling pathways can be initiated with the aid of several other adaptor proteins. These signaling events consequently induce phosphorylation and translocation of transcription factors such as NF-κB and/or IRF3/IRF7, resulting in production of either pro-inflammatory cytokines or type I interferons (IFN), respectively (Wu & Chen, 2014). Furthermore, IRF3 dependent type I IFN production can also occur through RIG-I like receptor (RLR) signaling via another adaptor protein called MAVS (mitochondrial antiviral-signaling protein) or through the dsDNA sensor cGAS and the adaptor STING (Ablasser et al., 2013). Although NOD-like receptor (NLR) signaling is independent of phosphorylation and translocation of NF-κB and/or IRF3/IRF7, several of these NLRs are required for the caspase-1 activating inflammasome formation and further processing and secretion of pro-inflammatory cytokines such as IL-1β and IL-18 (Schroder & Tschopp, 2010). Moreover, absence in melanoma 2 (AIM2), which is a pyrin and HIN domain-containing (PYHIN) protein family member, can also trigger caspase-1 activating inflammasome formation upon recognition of dsDNA molecules in cytosol (Hong et al., 2011). Although AIM2 and IFI16 are members of the same family, in addition to inflammasome activation, IFI16 can also induce production of IFN-β upon recognition of viral DNA motifs (Unterholzner et al., 2010). PRRs and their associated signaling pathways are summarized in Figure 1.

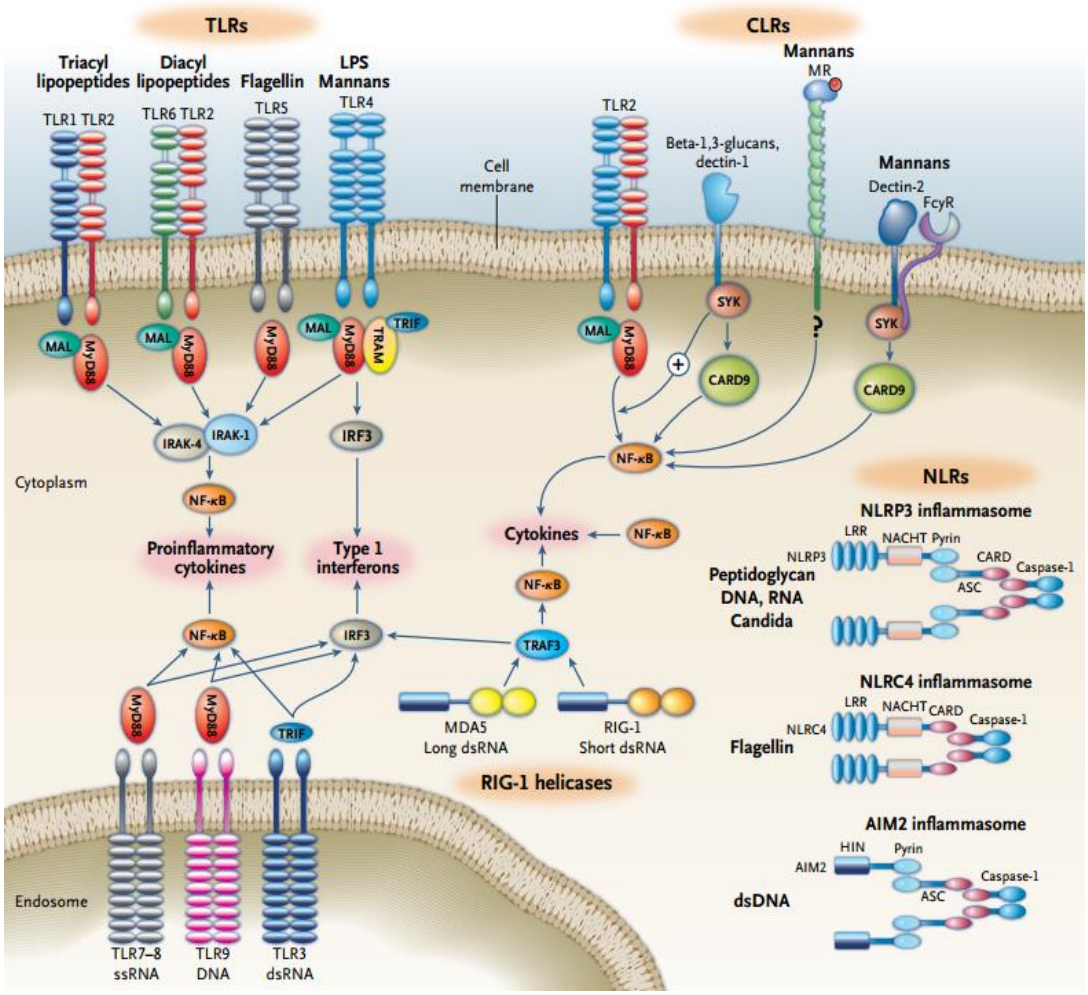

Figure 1.1 Pattern recognition receptor (PRR) signaling pathways (Adapted from Schwartz et al., 2011)

1.2.1 Nucleic Acid Sensing Mechanism

Nucleic acid sensing by endosomal and cytosolic PRRs constitute the major defense strategy against infections. Detection of non-self nucleic acids depends on several factors including nucleic acid structure, base modifications, nucleotide sequence and subcellular localization. For example, viral replication can generate blunt ended short dsRNA molecule with a 5’ triphosphate motif which can be recognized by cytosolic RNA sensors. Endogenous RNA molecules like mRNA found in cytosol can evade recognition by these sensors since posttranscriptional modifications such as 5’-end

capping with 7-methyl guanosine masks this 5’ triphosphate motif (Leung & Amarasinghe, 2016). Moreover, presence of dsDNA (derived from host, virus or bacteria) outside of nucleus indicates danger and there are several sensors functioning for the detection of dsDNA molecules. Under normal circumstances, small amounts of dsDNA generated by cellular damage and leak into the cytosol are degraded by special nucleases to prevent aberrant activation by dsDNA sensors (Grieves et al., 2015). Thus, such sensors are only specifically activated in the case of microbial invasion and/or massive cell death. However, continuous supply and/or defective clearance of autologous nucleic acids can cause accumulation of these molecules in the cytosol and/or extracellular compartments. Uncontrolled recognition of these accumulated self nucleic acids can cause severe conditions such as autoimmune and autoinflammatory disorders (Roers et al., 2016).

1.2.1.1 DNA Sensing

Recognition of Unmethylated CpG DNA and MyD88 Dependent Signaling

TLR9 was the first PRR identified that was shown to specifically recognize DNA. TLR9 recognizes hypomethylated CpG dinucleotide-rich DNA that is enriched in bacterial but not mammalian DNA (Hemmi et al., 2000). Recognition of hypomethylated CpG rich DNA takes place in the endosomal compartment and upon ligand recognition, signaling is initiated through Myd88, IRAK4 and TRAF6 (Gursel et al., 2006), resulting in activation of NF-κB (pro-inflammatory cytokines) or IRF7 (type I IFNs), depending on the nature of the DNA being recognized (Kawai et al., 2004). For example, CpG oligonucleotides (ODN) capable of forming higher-order structures (named as D- or A-type CpG ODN) are retained in early endosomes and trigger IRF7-dependent type I interferons, whereas CpG ODNs that do not form higher order aggregated structures (named as B- or K-type CpG ODN) localize to late endosomes and initiate NF-KB dependent pro-inflammatory cytokine secretion (Gürsel et al., 2002, Gursel et al., 2006)

Detection of Cytosolic DNA by RNA Polymerase III

B form dsDNA in cytosol can be recognized by RNA polymerase III in a sequence specific manner (AT rich region). Upon recognition, it is transcribed into dsRNA containing 5' triphosphate end. Since it is a common ligand for RIG-I, MAVS dependent type I IFN production is triggered following RIG-I pathway activation (Chiu et al., 2009, Ablasser et al., 2009).

STING-Activating Cytosolic DNA Sensors

Stimulator of interferon genes (STING) function as an ER membrane associated adaptor protein in cytosolic dsDNA-specific type I IFN signaling and is a direct sensor of cyclic dinucleotides such as cyclic-di-GMP (Burdette et al., 2011). Upon activation of upstream cytosolic DNA sensors, STING directly interacts with TBK1 resulting in phosphorylation of IRF3 and induction of type I IFNs. The major cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS) catalyzes production of a newly defined second messenger, cyclic GMP-AMP (c-GAMP) from GTP and ATP, following binding to cytosolic dsDNA As such synthesized cGAMP is then recognized by STING and the STING-TBK1-IRF3 signaling pathway is triggered (Wu et al., 2013, Sun et al., 2013). In addition to cGAS, several other cytosolic DNA sensors such as DDX41, IFI16, DNA-PK and MRE 11 have been shown to function in a STING-dependent manner. However, cGAS/STING pathway constitutes the dominant cytosolic DNA sensing pathway in most cell types (Bhat & Fitzgerald, 2014).

Inflammasome Activation by Cytosolic DNA Sensors

Apart from the cytosolic DNA sensors involved in type I IFN production, a small number of DNA sensors have been known to trigger inflammasome assembly. For example, AIM2 is a PHYIN family member than can detect the presence of long molecules of dsDNA (more than 80 bps) in cytosol (Jin et al., 2012). Following ligand recognition, pyrin domain of AIM2 interacts with adaptor protein ASC

resulting in inflammasome assembly and subsequent secretion of pro-inflammatory cytokines IL-1β and IL-18 (Hornung et al., 2009). Another PHYIN family member, IFI16 can also induce inflammasome pathway in response to viral DNA in nucleus in addition to its role in type I IFN production. (Kerur et al., 2011). Moreover, NLRP3 inflammasome is known to be activated following recognition of oxidized mitochondrial DNA and RNA:DNA hybrids (Shimada et al., 2012, Vanaja et al., 2014). PRRs that recognize DNA molecules are outlined in figure 1.2.

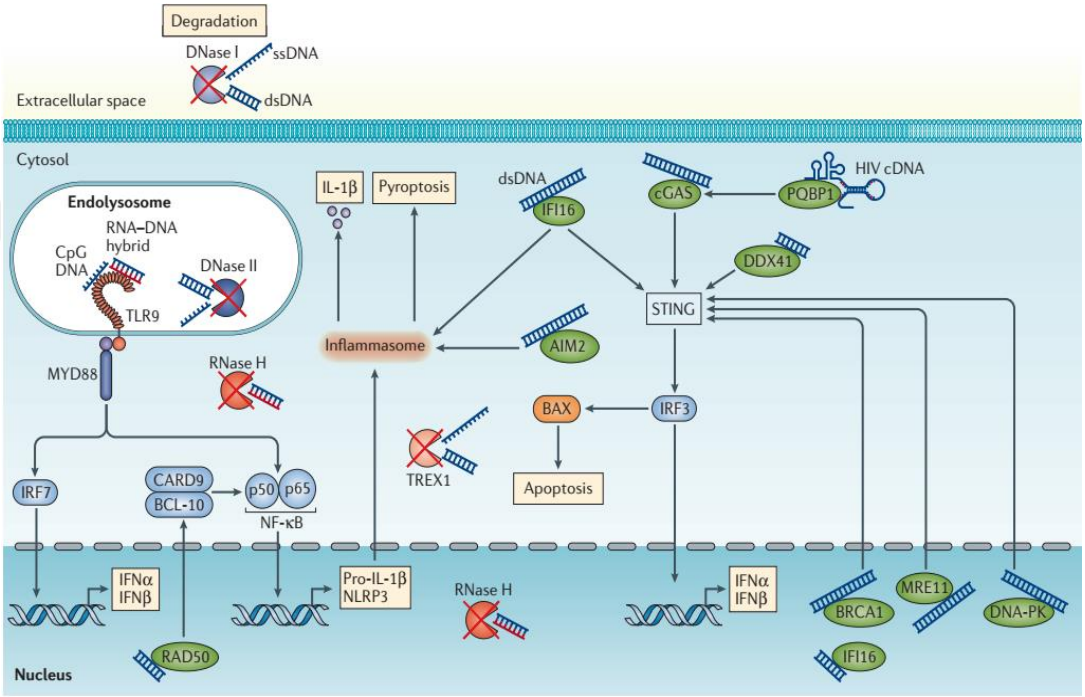

Figure 1.2 Immune sensing receptors of DNA (Adapted from Schlee & Hartmann, 2016)

1.2.1.2 RNA Sensing

TLR Mediated RNA Sensing

RNA molecules are recognized by several endosomal TLRs (TLR3, TLR7/TLR8 and mouse TLR13). Ligands taken up from extracellular environment and/or released within endosomal compartments are recognized by these TLRs. TLR3 is expressed in several type of cells such as macrophages, dendritic cells, natural killer cells and

intestinal epithelial cells (Murphy & Weaver, 2016b). Independent from sequence, TLR3 can detect the presence of double stranded RNA structures like the genome of dsRNA viruses and synthetic analogs of dsRNA (polyI:C). TLR7 and TLR8 can be activated following recognition of single stranded RNAs and synthetic chemical analogs such as imidazoquinoline derivatives (Cervantes et al., 2012). While expression of TLR8 is seen in monocytes/macrophages and myeloid dendritic cells (mDCs), TLR7 is predominantly expressed in plasmacytoid dendritic cells (pDCs) which are the major producers of type I IFNs (Hornung et al., 2002). Moreover, it has been shown that mouse TLR13 can respond to bacterial 23S ribosomal RNAs in a sequence specific manner (Li & Chen, 2012).

Ligand binding to leucine-rich repeats (LRRs) of endosomal TLRs induces homodimerization through the interaction of TIR (Toll-IL-1 receptor) domains of two identical TLRs. Dimerization results in the recruitment of adaptor proteins involved in downstream signaling cascades. Endosomal TLRs other than TLR3 signal through an adaptor protein called Myd88. Production of type I IFNs via activation of IRF7 and pro-inflammatory cytokine production through NF-κB activation occurs in a MyD88 and TRAF6 dependent manner (Takeda & Akira, 2004). TRIF is the adaptor protein involved in TLR3 signaling pathway. This adaptor activates both TRAF3 and TRAF6, resulting in activation of NF-κB and IRF3/IRF7, respectively (Takeuchi & Akira, 2010). In summary, ligand recognition through endosomal TLRs can induce both pro-inflammatory cytokine production and type I IFN production.

RLR-Mediated RNA Recognition

DExD/H box helicase family members; RIG-I, MDA-5 and LGP2 are collectively called RIG-I like receptors (RLRs). These sensors have important functions in viral infections. RIG-I and MDA-5 can activate type I IFN signaling upon ligand recognition. RIG-I recognize short dsRNA molecule with blunt ends, ssRNA and dsRNA containing 5’ triphosphate or diphosphate moieties, whereas MDA-5

internally binds to long molecules of dsRNA (Reikine et al., 2014). In contrast, LGP2 functions in regulation of RIG-I and MDA-5 since this sensor does not contain a CARD domain required for triggering anti-viral signaling. In other words, upon binding to termini of dsRNA, LGP2 negatively regulates RIG-I and synergistically activates MDA-5 signaling pathway (X. Li et al., 2009, Bruns & Horvath, 2015). Following ligand recognition through RIG-I or MDA-5, these sensors interact with mitochondrial antiviral-signaling protein (MAVS), an adaptor required for the initiation of downstream IFN signaling (Kawai & Akira, 2008).

Inflammasome Assembly Upon RNA Sensing

Activation of NLRP3 inflammasome following recognition of viral and bacterial RNAs have been reported by several groups (Kanneganti et al., 2006, Wang et al., 2014). DHX33, which is a DExD/H-box RNA helicase family member, has been shown to bind dsRNA in cytosol and interact with NLRP3 resulting in inflammasome assembly (Mitoma et al., 2013). Additionally, recognition of dsRNA in cytosol via RIG-I and MDA-5 can trigger MAVS-dependent activation of NLRP3 inflammasome via membrane permeabilization and K^+ efflux (Franchi et al., 2014). Apart from indirect activation of NLRP3 inflammasome by certain RNA species, RIG-I can directly interact with adaptor protein ASC to initiate caspase-1 dependent inflammasome assembly (Poeck et al., 2010). Figure 1.3 summarizes the signaling pathways involved in RNA sensing. Table 1.2 presents a list of known RNA sensors, their cellular localization and the ligands they recognize.

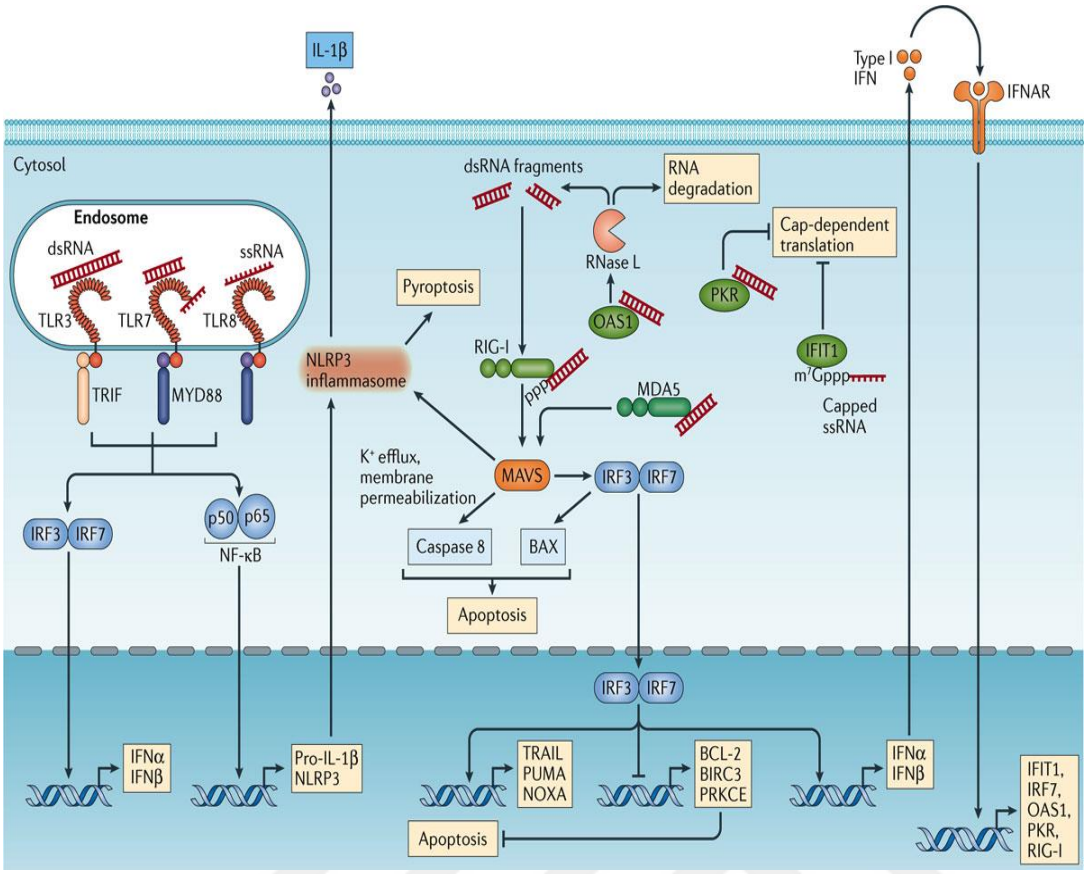

Figure 1.3 RNA sensors and associated signal transduction pathways (Adapted from Schlee & Hartmann, 2016)

Table 1.2 RNA specific nucleic acid sensors, their cellular localization and cognate ligands.

PRRs	Localization	Ligand
TLR3	Endosomal	dsRNA (>35 bps)
TLR7	Endosomal	ssRNA, short dsRNA
TLR8	Endosomal	ssRNA, short dsRNA
TLR13 (specific to mouse)	Endosomal	Bacterial 23S rRNA with "CGGAAAGACC" motif
RIG-I	Cytosolic	5'ppp moeity of ssRNA and/or dsRNA
MDA-5	Cytosolic	dsRNA (>300 bps)
LGP2	Cytosolic	dsRNA
DHX33	Cytosolic	dsRNA
NLRP3	Cytosolic	ssRNA, dsRNA, bacterial mRNA and RNA:DNA hybrid
OAS1	Cytosolic	dsRNA
PKR	Cytosolic	dsRNA
IFIT1	Cytosolic	5'ppp moeity of ssRNA

1.3 Interaction between the Immune System and Microbiome

Colonization with commensals starts immediately after birth and a relatively stable microbiome is established within the first 2-3 years of life. Commensal microorganisms populate mainly mucosal surfaces (gastrointestinal, respiratory and

urogenital tracts) and skin (Maynard et al., 2012). Complex and dynamic interaction between immune system and microbiome is required for the development and proper functioning of immune system (Figure 1.4). For example, commensal bacteria colonized in gut lumen help to prevent intestinal infections by competing with pathogens for food and space or by activating epithelial and resident immune cells (Abt & Artis, 2013). In addition, microbiome benefits from host by providing the basic needs of shelter and nutrients.

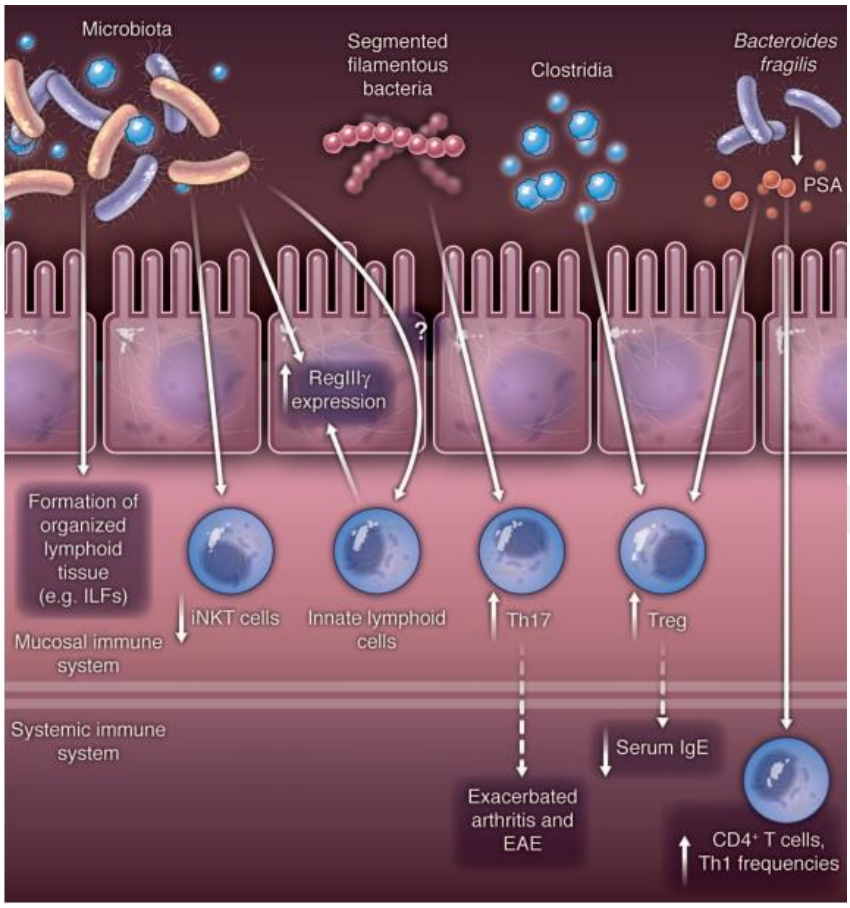

Figure 1.4 How microbiota shapes host immunity (Adapted from Hooper et al., 2012)

Although commensals contain molecular patterns similar to pathogens that can be recognized by PRRs, discrimination between commensal and pathogens prevents induction of detrimental immune responses against microbiome (Sansonetti &

Medzhitov, 2009). The exact mechanism behind this discrimination is not completely understood. Several hypotheses were proposed that may explain this phenomenon. One such hypothesis states that PAMPs-derived from commensals are recognized less efficiently by PRRs. In other words, some modifications of these PAMPs suppress their immune stimulatory activity. For example, a difference in the number of acyl groups added to lipid A, a major component of gram negative bacterial LPS, can change the agonistic effect of this PAMP (Sansonetti, 2011). Second hypothesis focuses on the immunosuppressive effect of microbiome, and several published reports are in support of this hypothesis. For example, commensal bacteria have been shown to downregulate inflammatory responses by promoting regulatory T cells and IL-10 producing macrophages (Sun et al., 2015). Furthermore, it has been known that microbiome-derived metabolites have anti-inflammatory properties (Lopez et al., 2014). The last hypothesis suggests that the requirement for a second (danger) signal to induce a proper immune response is not fulfilled by commensal microorganisms.

In contrast to the general belief that microbiome exerts immunosuppressive effects, recent reports indicate that commensals are indispensable in initiation of systemic antiviral immunity. It has been reported that germ-free mice or antibiotic-treated mice fail to establish a proper antiviral response and are more susceptible to viral infections compared to mice with healthy microbiota (Abt et al., 2012 , Ganal et al., 2012). In this context, activation of interferon signaling by commensal-derived molecules has been shown to play a major role in establishment of anti-viral immunity (Li et al., 2011, Weiss et al., 2012, Kawashima et al., 2013). However, the identity of commensal-derived molecules that trigger this Type I dominated anti-viral response has not been explored. Figure 1.5 summarizes the requirement of microbiota in proper effector function of immune system against virus infections. In summary, microbiota has an indisputable influence on the immune system's development, homeostasis and establishment of proper effector functions.

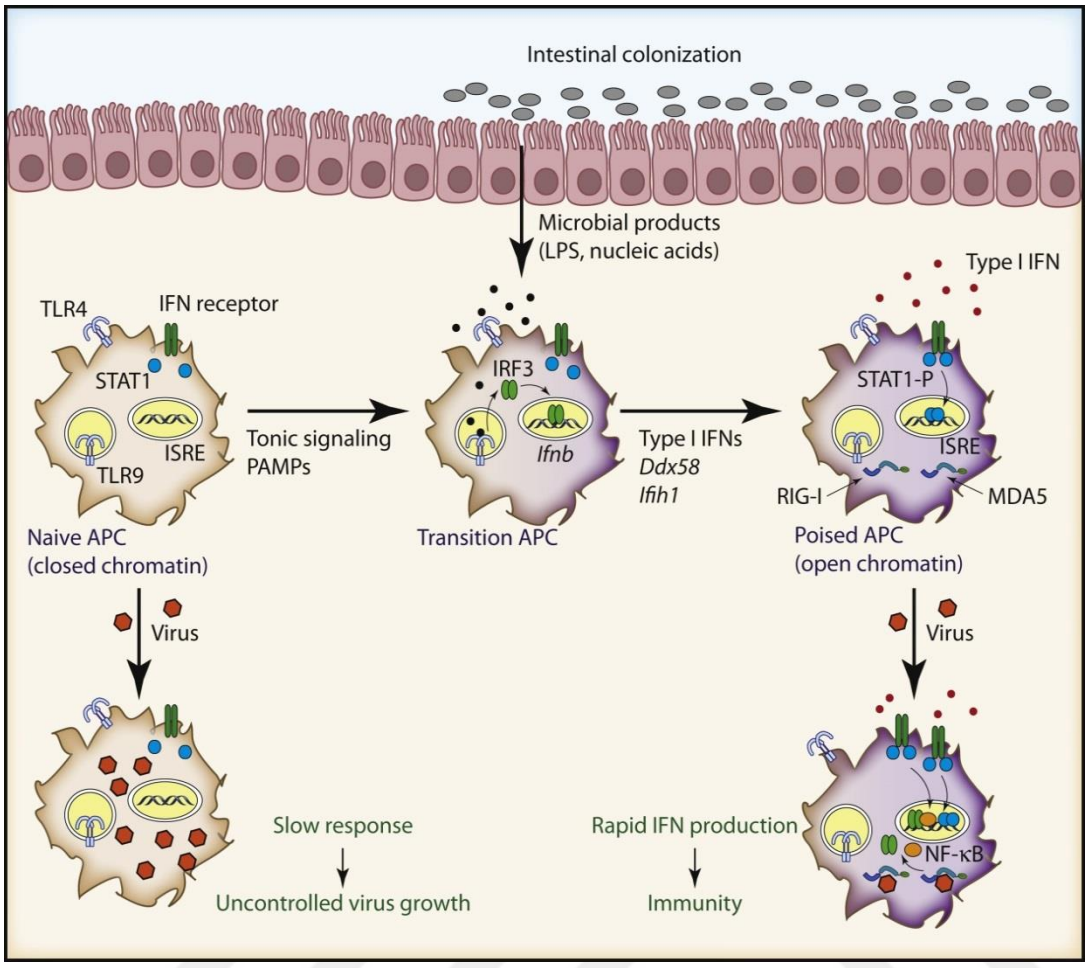

Figure 1.5 Tonic type I IFN signaling poises innate immune defense against viruses (Adapted from McAleer and Kolls, 2012)

1.4 Aim of the Study

Pathogen-associated molecular patterns (PAMPs) expressed by commensal and pathogenic bacteria have been previously shown to trigger distinct immune responses. However, how the immune system discriminates commensals from pathogens is still unclear and there are several contradictory publications, suggesting that more work in this field is required for clarification. . Therefore, we aimed to investigate the differential immunostimulatory activities of bacterial RNAs isolated from commensals and pathogens as RNA molecules constitute one of the most

important PAMPs. For this, RNAs were isolated from two commensal bacteria (*Lactobacillus salivarious* and *Lactobacillus fermentum*) and two pathogens (*Listeria monocytogenes* and *Streptococcus pyogenes*) and their immune stimulatory activities were compared in various assay systems. Preliminary results showed that RNAs of pathogen and commensal bacteria can trigger either pro-inflammatory cytokine or type I IFN dominated immune response, respectively. To examine the involvement of different RNA species on this differential activation, individual ribosomal RNAs (5S, 16S and 23S) were also isolated and tested for pro-inflammatory cytokine production or type I interferon production in vitro in cell lines stably expressing dsRNA or ssRNA sensors. Furthermore, since commensal RNAs specifically induced type I IFN production in human blood, herein, we also tried to identify the major cell type involved in this response using a cell-depletion based approach. Results implicate the complexity of the mechanisms involved in differential activation of immune system in response to commensal and pathogen derived RNAs, paving the way for more focused future studies.

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

2.1.1 Chemicals

Agarose, lysozyme, chloroform, ethylenediaminetetraacetic acid (EDTA) disodium salt solution (0.5M), and phenol solution (saturated with 0.1M citrate buffer, pH 4.3 ± 0.2) were purchased from Sigma (USA). Proteinase K isolated from *Tritirachium album* was purchased from Merck Millipore (Germany). SeaPlaque Agarose (low melting) was from Lonza (Switzerland). Tris-base, molecular biology grade 10X Tris-Acetate-EDTA (TAE) buffer, RNase Away decontamination solution and Tween 20 detergent were obtained from Thermo Fisher Scientific (USA). Total RNA isolations from bacteria were performed using Trizol Reagent (Thermo Fisher Scientific, USA) and impurities and degraded products in total RNAs were removed by RNA Clean and Concentrator-25 (Zymo Research, USA). Quick-Load 10 kb DNA Ladder and ssRNA Ladder were purchased from NEB, UK. Preparations of buffers and other solutions used in experiments are described in Appendices.

2.1.2 Antibodies and Related Reagent

Monoclonal capture and detection antibodies used in cytokine ELISAs, alkaline phosphatase conjugated streptavidin (SA-ALP) and recombinant proteins were purchased from Mabtech (USA). Para-nitrophenyl phosphate disodium salt (PNPP; substrate for alkaline phosphatase), and substrate buffer for PNPP were obtained from Thermo Fisher Scientific (USA). 96-well ELISA plates were purchased from SPL Life Sciences (Korea). Depletion or enrichment of specific cell types was performed using magnetic cell isolation and separation kits from Miltenyi Biotec

(Germany). Dot blot assays were performed using J2 anti-dsRNA monoclonal antibody from Scicons (Hungary), horseradish peroxidase (HRP) conjugated secondary antibody from R&D Systems (USA) and Pierce ECL Western Blotting Substrate kit from Thermo Fisher Scientific (USA).

Table 2.1 Fluorochrome Conjugated Antibodies

Antibody	Fluorochrome	Company
Anti-human CD303	FITC	MiltenyBiotec, Germany
Anti-human CD123	PerCP/Cy5.5	Biolegend, USA
Anti-human CD14	FITC	Biolegend, USA
Anti-human TNF-α	PE	Biolegend, USA
Anti-human IP-10	PE	BD Bioscience, USA

2.1.3 Pattern Recognition Receptor (PRR) Ligands

High molecular weight Polyinosinic-polycytidylic (pI:C), 5’ triphosphate double stranded RNA (5’ppp dsRNA), single stranded polyU (pU), Resiquimod (R848) and HSV-60 were obtained from Invivogen, USA. CpG ODN D35 was synthesized by Alpha DNA (Canada) and had the following immunostimulator sequence: GGtgcatcgatgcaggggGG (capital letters indicate phosphorothioate backbone while lower case letters indicate phosphodiester backbone.). Lipofectamine 2000 (Thermo Fisher Scientific, USA) was used to deliver the ligands to the cytosol.

2.1.4 Cell Lines and Cell Culture Media

Reporter cell lines used in this study (HEK-Blue hTLR3, HEK-Blue hTLR7 and THP1-Blue ISG cells) were from Invivogen (USA). Normocin, Zeocin, Blasticidin and Quanti-Blue were purchased from Invivogen (USA) for maintenance or reporter function of these cell lines. Gibco RPMI Media 1640 supplemented with L-Glutamine and Opti-MEM Reduced Serum Media were obtained from Thermo Fisher Scientific (USA). HEPES Buffer (1M), sodium pyruvate, non-essential amino

acids, Penicillin/ Streptomycin and Fetal Bovine Serum (FBS) were purchased from Lonza (Switzerland). Molecular biology grade water and phosphate buffered saline (PBS) were obtained from Biological Industries (USA). Human PBMC isolations were performed using Lympho-Paque cell separation medium (Genaxxon, Germany). Fixation medium A and Permeabilization Medium B (Thermo Fisher Scientific, USA) were used in cell fixation and intracellular cytokine staining protocols. Preparations of cell culture media are given in appendices.

2.1.5 Bacteria Strains and Bacterial Culture Media

In this study, RNAs of two commensal bacteria and two pathogenic bacteria were used. Commensal bacterial strains were *Lactobacillus salivarious* (isolated from infant feces) and *Lactobacillus fermentum* (isolated from infant feces) and pathogenic strains were *Listeria monocytogenes* (ATCC35152) and *Streptococcus pyogenes* (NCTC12696/ATCC19615). MRS Agar and MRS broth media (Conda, Spain) were employed to culture commensal bacterial strains. *Listeria monocytogenes* was grown in Tryptone Soy Broth (TSB) media which is composed of tryptone, soytone (Conda, Spain) and NaCl (Thermo Fisher Scientific, USA) or grown on Tryptone Soy Agar (TSA) plates which additionally contains bacto agar (Conda, Spain). *Streptococcus pyogenes* was cultured in C medium which is composed of proteose peptone, yeast extract (Conda, Spain), K2HPO4, $MgSO_4$ and NaCl (Thermo Fisher Scientific, USA) or cultured on C Agar plates which additionally include bacto agar (Conda, Spain).

2.2 Methods

2.2.1 Bacterial Growth

Lactobacillus salivarious (LS) and *Lactobacillus fermentum* (LF) were grown overnight at 37°C on MRS agar plates. *Listeria monocytogenes* (LM) and *Streptococcus pyogenes* (SP) were grown overnight at 37°C on Tryptone Soy agar and C agar plates, respectively. Single colonies on agar plates were inoculated into

5 ml of corresponding medium and incubated at 37℃ overnight at 130 rpm. After overnight growth, 1 ml of bacterial cultures were diluted with 9 ml of fresh media and incubated until the culture reached the mid-log phase. The incubation time required to reach mid-log phase for each bacteria species were determined by another MSc study conducted previously in our laboratory.

Table 2.2 Incubation time points to reach mid-log phase for each bacterial strain.

Abbreviation	Name of Bacterial Strain	Incubation Time (min)
LS	*Lactobacillus salivarious*	150
LF	*Lactobacillus fermentum*	150
LM	*Listeria monocytogenes*	180
SP	*Streptococcus pyogenes*	240

2.2.2 RNA Isolation from Bacteria

2.2.2.1 Total RNA Isolation

Bacterial cell pellets were obtained by centrifugation of 10 ml of cultures at mid-log phase at 3044 xg for 30 minutes and decanting the supernatants. Cells were re-suspended in 300 µl of lysis buffer containing 30 mM Tris-HCl (pH 8.0), 1mM EDTA and 15mg/ml lysozyme. Then cell suspensions were transferred into a 1.5 ml Eppendorf tube and 15 µl of proteinase K (20 mg/ml) was added to each tube. Enzymatic digestions were continued at 37℃ for 30 minutes. Following incubation, tubes were centrifuged at 3880 xg (4℃ for 15 min) and supernatants were removed. After this point, tubes were kept on ice to protect RNA integrity. Pellets were completely dissolved in 1 ml of Trizol reagent and 200 µl of chloroform was added to each tube. Vigorous shaking was performed for about 15 seconds until trizol and chloroform formed a homogeneous mixture. Then, tubes were incubated for 3 min at room temperature and centrifuged at 14,000 rpm for 15 min at 4℃. This centrifugation step separated the mixture into phases. The upper aqueous phase containing the RNA molecules was collected into fresh tubes. Equal volume of

isopropanol was added and homogeneity was obtained by gentle rotation. Tubes were incubated for 10 minutes at room temperature and then centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatants were decanted and RNA pellets were washed with 1 ml pre-chilled 70% ethanol and tubes were centrifuged at 8,000 rpm for 6 minutes. Ethanol was discarded and washing step was repeated with 1 ml of pre-chilled 100% ethanol. Following removal of ethanol, RNA pellets were left to air dry for about 10 minutes. Final pellets were dissolved in 100 µl of RNase & DNase free water and quantification-quality analyses were performed using Biodrop, UK. RNA samples were stored at -80°C until further use.

2.2.2.2 Purification of Total RNA

In order to remove degraded products, RNA clean & concentrator-25 kit from Zymo-research was used. 25 µg RNA samples were diluted in RNase/DNase free water to obtain 50 µl volume of solution. Two volumes of RNA binding buffer (100 µl) was mixed with each RNA sample. Then, 150 µl of 100% ethanol was added to each mixture. After obtaining a homogeneous mixture, samples were applied to columns and centrifuged at 14,000 g for 1 minute. Flow through was discarded and 300 µl of RNA wash buffer (RWB) was added to each column. Centrifugation was repeated at 14,000 g for 30 seconds. Contaminating DNA was removed from RNA samples by DNase I treatment as follows; 10 µl of DNase, 10 µl of DNase Reaction Buffer (10X) and 80 µl of RWB were mixed and applied to each column. Columns were incubated at 37°C for 30 minutes and centrifuged at 14,000 xg for 30 seconds. Flow through was discarded from collection tubes, 400 µl of RNA prep buffer was applied to each column and centrifuged at 14,000 xg for 1 minute. Washing step was repeated twice with 400 µl of RWB as mentioned above. In order to elute the remaining RWB, columns were centrifuged for 2 minutes without addition of any solution. After this step, fresh tubes were placed under the columns, 30 µl of RNase/DNase free water was added to each column and were incubated for 1 minute at room temperature followed by centrifugation at 10,000 xg for 30 seconds. Tubes

containing the eluates were labeled and stored at -80°C after quantification/quality analyses were performed as indicated previously.

2.2.2.3 Isolation of Ribosomal RNA Subunits

One percent agarose gel was prepared with TAE (1X) and ethidium bromide (EtBr) was added to the gel solution shortly before pouring the gel. 10 µg of RNA samples were mixed with 6X loading dye, loaded into wells and the gel was run under constant voltage, 80V, for 1-3 hours depending on the target subunit. Agarose gel was run by using the runVIEW gel system (Cleaver Scientific, UK) which enabled the monitoring of running samples in real-time. When RNA molecules were separated from each other, a piece of agarose gel in front of the band belonging to our interest of ribosomal RNA subunit was cut and removed. The through was filled with 1% low melting agarose and incubated for 1 minute for completion of gelation. Then electrophoresis was resumed until the RNA fragment of interest entered into the low melting agarose. Band of RNA fragment in low melting agarose was then removed and transferred into an eppendorf tube. 500 µl of TAE (1X) was added to tube and incubated at 65°C until agarose completely dissolved. Total incubation time was restricted not to exceed 5 min to prevent the degradation of RNA. An equal volume of phenol (pH 4.3) was added to the RNA containing solution and mixed vigorously. The sample was centrifuged at 10,000 xg for 10 minutes at 4°C and the upper aqueous phase was collected. In order to precipitate RNA, aqueous phase was mixed with 0.1 volume of sodium acetate (pH 4.7) and 2.5 volume of pre-chilled 100% ethanol and incubated overnight at -20°C. The sample was then centrifuged at 16,000 xg for 45 minutes at 4°C and ethanol-salt mixture was decanted. Pre-chilled 70% ethanol was added to wash the pellet and centrifuged at 7,500 xg for 6 minutes at 4°C. Washing step was repeated with 100% ethanol and the final pellet was left to air dry for 10 minutes. The RNA pellet was dissolved in an appropriate volume of RNase & DNase free dH_2O followed by quantitation. Samples were stored as explained previously until further use.

2.2.2.4 Visualization of RNAs

One percent agarose gel containing EtBr was used to visualize RNA samples. 10 kb DNA ladder and ssRNA ladder were used as controls. DNA ladder mixture was prepared by mixing 1 µl DNA ladder, 1 µl 6X loading dye and 4 µl RNase & DNase free dH_2O. In order to prepare ssRNA ladder mixture, 5 µl RNA loading buffer (2X), 3 µl RNase & DNase free dH_2O and 2 µl ssRNA ladder were mixed and incubated at 65°C for 5 minutes to denature the secondary structures of ssRNA ladder. ssRNA ladder mixture was placed on ice immediately to prevent re-formation of 2° structures. RNA samples were mixed with loading dye (6X) in a 5:1 ratio. Ladder mixtures and RNA samples were loaded into wells and the gel was run under constant voltage, 80V, for an hour. RNA bands were imaged under a UV trans-illuminator.

2.2.3 Cell Culture

2.2.3.1 Cell Lines

2.2.3.1.1 HEK-Blue hTLR3 & hLTR7 Cells

HEK-Blue hTLR3 and HEK-Blue hTLR7 cells are human embryonic kidney cells stably expressing two plasmids: one plasmid encodes for secreted embryonic alkaline phosphatase (SEAP) and the other one encodes for hTLR3 or hTLR7 proteins, respectively. SEAP production occurs under the control of IFN-β minimal promoter fused to 5 NF-κB and AP-1 binding sites. In other words, stimulation of cells with corresponding ligands (dsRNA for hTLR3 or ssRNA for hTLR7) activates NF-κB and AP-1 and results in SEAP secretion into the culture medium. Secreted SEAP levels can be detected using the colorimetric SEAP-specific Quanti-Blue detection medium. HEK-Blue hTLR3 and HEK-Blue hTLR7 cells were cultured in sterile petri dishes in RPMI 1640 medium supplemented with 10% FBS and Normocin (100 µg/ml). Normocin was used to prevent mycoplasma infections. Zeocin (100 µg/ml) and blasticidin (30 µg/ml) were also used to select cells that maintained plasmids

encoding SEAP and hTLR3/hTLR7, respectively. Cells were passaged twice a week and were not allowed to exceed 80% confluency.

2.2.3.1.2 THP-1 & THP-1-Blue ISG Cells

THP-1 cells (human leukemic monocytes), were used as such or following priming with PMA. Cells were maintained in T25 flasks in RPMI 1640 medium supplemented with 10% FBS and Normocin (100 μg/ml). THP-1 cells were inoculated at a concentration of 3×10^5 cells/ml and cell concentrations were kept below 1×10^6 cells/ml.

THP1-Blue ISG cells are engineered to monitor activation of interferon regulatory factor (IRF) upon stimulation with type I interferons. In this study, THP1-Blue ISG cells were used to detect the amounts of type I IFNs secreted into culture supernatants of stimulated normal THP-1 cells. THP-Blue ISG cells were inoculated at a concentration of 7×10^5 cells/ml and cell concentrations were kept below 2×10^6 cells/ml.

2.2.3.2 Isolation of Human Peripheral Blood Mononuclear Cells (hPBMC) from Whole Blood

Experiments involving human participants have been approved by Ethics Committee of Bilkent University. Blood samples from healthy donors were collected in 10 ml blood collection tubes coated with EDTA as an anti-coagulation agent (BD Bioscience, USA). For better separation, blood samples were diluted 1:1 with PBS (1X) in a 50 ml falcon tube. Diluted blood samples were carefully layered onto lympho-paque density separation medium at a ratio of 2:1. Tubes were then centrifuged at 1700 rpm for 30 minutes at room temperature with the break off, in order to keep the separated layers intact. The thin cloudy layer formed between the lympho-paque density medium and the uppermost plasma consisting of peripheral blood mononuclear cells (PBMC) was collected with a sterile Pasteur pipette and

transferred to a new falcon tube. hPBMCs, were washed twice with wash medium (RPMI 1640 with 2% FBS containing) and final pellet was re-suspended in 1 ml of complete medium (RPMI 1640 containing 10% FBS).

2.2.3.3 Monocyte and pDC Depletion from hPBMC by Magnetic Cell Separation Kit

10^7 hPBMCs was pelleted by centrifugation at 300 xg for 10 minutes and supernatant was aspirated. For monocyte depletion, cell pellet was re-suspended in 80 µl of MACS buffer (Appendix A) and 20 µl of anti-CD14 Microbeads. Microbeads were allowed to bind to $CD14^+$ cells at +4°C for 20 minutes. Following the incubation period, cells were washed in 10 ml of cold MACS buffer and centrifuged at 300 xg for 10 minutes at +4°C. Meanwhile an MS column was placed on the magnet and rinsed twice with 1 ml of MACS buffer. Cell pellet was re-suspended in 500 µl of MACS buffer and loaded onto the prepared column, and allowed to penetrate the column matrix with the help of gravity. Unlabeled cells that passed through the column were collected while rinsing the column with 1 ml of MACS buffer three times. These cells represent hPBMCs depleted of monocytes. The column was then separated from the magnet and monocytes were extruded in 1 ml of MACS buffer. Monocyte-enriched and monocyte-depleted cells were centrifuged at 300 xg for 10 minutes and re-suspended in 1 ml of RPMI 1640 complete medium (10% FBS). For the pDC depletion studies, anti-CD304 pDC selecting microbeads were used. The protocol for pDC depletion was essentially the same as described above.

2.2.3.4 Cell Counting

Twenty µl of sample was taken from cells suspended in 1 ml media and transferred into 10 ml of filtered isotonic solution. 20 µl of diluted cells were acquired in a flow cytometer and cells were gated according to forward and side scatters. Cell number in the original solution was calculated using the formula:

Number of cells in 1 ml = Number of cells in gated area × 50 × 500

2.2.4 Investigation of immunomodulatory Activities of Bacterial RNA species

2.2.4.1 Stimulation of Cells in vitro

2.2.4.1.1 Stimulation for ELISA and SEAP Production Assay

Stimulations were carried out in complete RPMI medium (5% FBS) in 96-well flat bottom plates in a final volume of 200 µl. THP-1 cells were used either as such or after overnight priming with PMA (20 ng/ml). In order to increase transfection efficiency, stimulants were prepared in Opti-MEM serum-free medium. Lipofectamine 2000 to RNA ratios were 1:1 and 1:3 for 1 µg/ml and 3 µg/ml RNA, respectively. Cell suspensions (180 µl) and stimulants (20 µl) were mixed in wells and incubated for 24 hour at 37°C, 5% CO_2 incubator. At the end of 24 hour incubation, culture supernatants were collected and stored at -20°C for further applications.

Table 2.3 Cell concentrations used in stimulations

Cell Type	Cell Concentration
hPBMC	2×10^6 cells/ml
HEK-Blue hTLR3/hTLR7 Cells	3×10^5 cells/ml
THP-1 & THP-1-Blue ISG Cells	5×10^5 cells/ml

2.2.4.1.2 Stimulation for Intracellular Cytokine Staining

Stimulations for intracellular cytokine staining for TNF-α performed with hPBMC (1×10^6 cells/ml) treated with Brefeldin A (1000X diluted). Cells were stimulated with controls and total RNAs as explained previously and incubated for 6 hour at 37°C, 5% CO_2 incubator. Stimulations for intracellular IP-10 staining, hPBMCs (1×10^6 cells/ml) were stimulated with total RNAs as such and incubated for 5 hour at 37°C, 5% CO_2 incubator. At the end of 5 hour incubation, cells were treated with Brefeldin A (1000X diluted) and incubated for 3 more hours. At the end of these incubations, cells were fixed for further staining.

Table 2.4 Ligands used in stimulations

Ligand	Working Concentration	Target PRR
PolyI:C	10 μg/ml	TLR3,RIG-I and MDA-5
5'ppp-dsRNA	1 μg/ml	RIG-I
R848	5 μg/ml	TLR7/8
PolyU	10 μg/ml	TLR8
HSV-60	5 μg/ml	Cytosolic DNA Sensors (CDS)
LPS	1 μg/ml	TLR4
D35 CpG ODN (20 mer) Sequence: GGtgcatcgatgcaggggGG	3 μM	TLR9

2.2.4.2 Cytokine determination by Enzyme Linked-Immunosorbent Assay (ELISA)

Amount of cytokines secreted into culture supernatants from stimulated cells were determined by cytokine ELISA. SPL immunoplates were coated with 50 μl of monoclonal capture antibody specific to the target cytokine. Capture antibody was diluted in PBS and all of the dilutions recommended for plate coating were performed according to manufacturer's protocol (Mabtech, USA). Following overnight coating at +4°C, capture antibody was discarded by gentle flicking of plates. In order to prevent nonspecific binding, wells were treated with 200 μl of blocking buffer (Appendix A) and incubated for two hours at room temperature. Plates were washed 5 times with 200 μl of ELISA wash buffer (Appendix A) for each well and incubated for 3 minutes after each washing. Plates were air-dried and 50 μl of cell supernatants or 50 μl of recombinant cytokine solutions (prepared by two-fold serial dilution) were added to corresponding wells. Plates were incubated for two hours at room temperature and washed as described above. Biotinylated

detection antibody was prepared in T cell buffer (Appendix A), added to wells and left for overnight incubation at +4°C. Alkaline phosphatase conjugated streptavidin was prepared in T cell buffer one hour prior to use (1:1000 dilution). After washing steps, 50 µl of streptavidin solution was transferred into wells and incubated for 1 hour at room temperature. Plates were washed as described previously with an additional rinsing step with distilled water. For color development, 50 µl of PNPP substrate was added to each well and absorbance at 405 nm was measured and recorded at various time intervals. Colorimetric measurements were recorded on a Multiskan FC Microplate Photometer (Thermo Fisher Scientific (USA)).

Table 2.5 Antibodies and recombinants used in cytokine ELISA

	Working Concentration of Coating Antibody	Working Concentration of Detection Antibody	Initial Concentration of Recombinant Protein
Human IL-1β	2µg/ml	1µg/ml	20ng/ml
Human Pan-α	4µg/ml	1µg/ml	20ng/ml
Human IL-29	2µg/ml	1µg/ml	5ng/ml

2.2.4.3 Quantification SEAP Secretion from Reporter Cell Lines

The SEAP substrate Quanti-Blue powder was dissolved in 100 ml of molecular biology grade water. 180 µl of warm Quanti-Blue solution and 20 µl of cell culture supernatant were mixed and transferred into 96 well plates. Plates were incubated at 37°C in the dark and color development was followed at 645 nm.

2.2.4.4 Cell Fixation

hPBMCs (~1 x 10^7) were centrifuged at 400 xg for 10 minutes and supernatant was decanted. While vortexing, 100 µl of Fixation Medium A (4% paraformaldehyde) was added dropwise to prevent clumping. Tubes were incubated at room temperature for 15 min and cells were washed in 1 ml of FACS buffer (Appendix A) two times. Fixed cells were either directly used for cell surface staining or re-suspended in 1 ml FACS buffer and stored at +4°C.

2.2.4.5 Cell Surface Marker Staining

Pelleted cells were re-suspended in 100 µl of fluorescent dye conjugated antibody solution (1µg/ml) and incubated for 30 minutes in the dark. Labeled cells were washed twice with FACS buffer and resultant cell pellet was dissolved in 300 µl FACS buffer. A minimum of 10,000 events were acquired using a BD Accuri C6 Flow cytometer.

2.2.4.6 Intracellular Cytokine Staining

Pelleted cells were re-suspended in 100 µl of fluorescent dye conjugated antibody (1µg/ml) containing Permeabilization Medium B and incubated for 30 minutes protected from light. Labeled cells were washed twice with FACS buffer and final cell pellet was dissolved in 300 µl FACS buffer. A minimum of 10,000 events were acquired using Flow cytometer.

2.2.5 Determination of dsRNA Content of Commensal versus Pathogen RNAs by Dot Blot Assay

Dot blot assay was performed with bacterial total RNA, ribosomal RNA subunits, polyI:C (positive control) and polyU (negative control). 1 µl from each sample was blotted on circles drawn on nitrocellulose membrane and left to air dry for a few minutes. RNA samples were fixed on nitrocellulose by exposing the membrane to

UV light for 2 minutes. In order to prevent nonspecific binding, membranes were treated with blocking buffer containing 1% BSA for 1 hour on a rocker. Following the incubation, blocking buffer was discarded and the membrane was washed three times with wash buffer (TBS-T) for 10 minutes. J2 (anti-dsRNA) antibody was diluted in 0.1% BSA containing TBS-T at a 1:1000 ratio and used as primary antibody. Membrane was incubated with J2 antibody solution for an hour on a rocker and washed as described above. For detection, membrane was treated with 1:1000 diluted secondary antibody (HRP conjugated anti-IgG) solution for an hour and washed as described previously. Image development was achieved with the help of ECL (chemiluminescent substrate of HRP) and Bio-Rad (USA) gel imaging system.

2.2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6. Kruskal-Wallis Dunn's test was conducted to compare IFN responses of hPBMC versus pDC or monocyte depleted hPBMC. Unpaired t-test was conducted to compare naïve (untreated) versus treatment groups. *, **, *** indicate $p<0.05$, $p<0.01$, $p<0.001$, respectively.

CHAPTER 3

RESULTS & DISCUSSION

3.1 Visualization of Bacterial RNA Species by Agarose Gel Electrophoresis

Total RNAs were isolated from mid-log phase bacteria with Trizol and purified with Zymo-research RNA Clean & Concentrator. Ribosomal RNAs were isolated form agarose gels as explained previously. Quality and quantity of bacterial RNAs were determined using Biodrop. 260/280 ratio in between 1.8 to 2.2 and 260/230 ratio in between 2.0 to 2.2 were considered as of sufficient purity. Furthermore, integrity and absence of DNA contamination were confirmed by agarose gel electrophoresis (Figure 3.1). Expected sizes of ribosomal RNAs for each bacteria were similar; ~120 nt for 5S, ~1,500 nt for 16S and ~ 2,900 nt for 23S rRNAs (Data was obtained from Nucleotide BLAST, NCBI). 23S ribosomal RNA isolation of sufficiently high purity was not successful as the samples contained an additional band corresponding to 16S rRNA. Because of the close proximity between bands of 16S and 23S rRNAs, cross contamination might be observed in 23S rRNAs samples. In order to fix this problem, isolation of 23S rRNA was performed using the Zymo-clean Gel RNA recovery kit (Zymo-research, USA). However, contamination problem could not be overcome in the 23S rRNA samples isolated using Zymo-clean Gel RNA recovery kit. Furthermore, we could obtain only very low concentrations of 23S rRNA which was not enough to carry out stimulation experiments. For these reasons, all ribosomal RNAs were isolated by low melting agarose method for further applications. Although the 23S rRNA was not completely pure, it was nevertheless highly enriched and therefore was used as such.

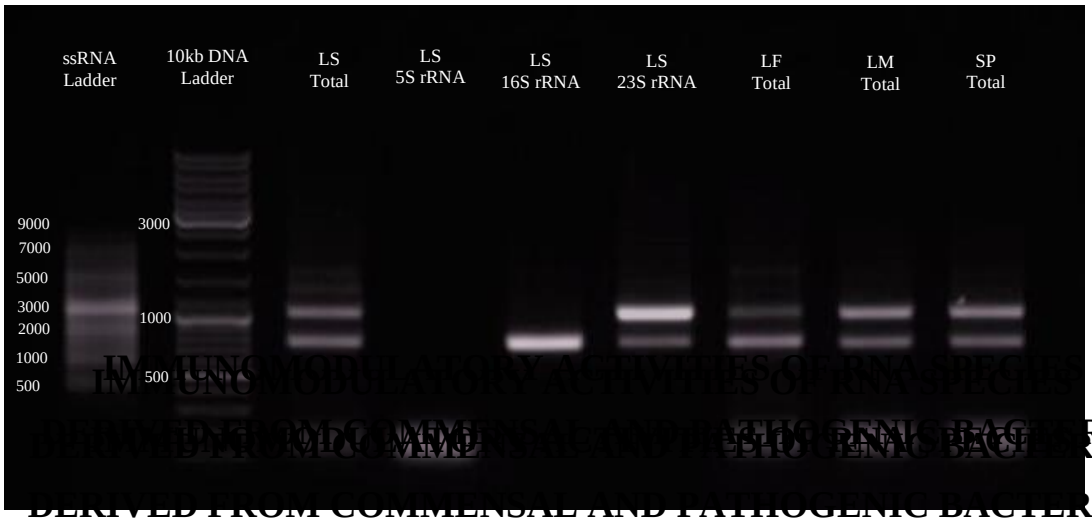

Figure 3.1 Visualization of bacterial RNAs on a 1% agarose gel containing EtBr. 1µg of bacterial RNA species (total RNAs derived from both commensal (LS&LF) and pathogen (LM&SP), 5S, 16S and 23S rRNA of LS isolated from AGE) were electrophoresed at 80V for 1h in order to examine the integrity and purity of each RNA species. ssRNA ladder and 10 kb DNA ladder were used as molecular size markers.

3.2 Determination of NF-κB and AP-1 Activation in HEK-Blue hTLR3 and HEK-Blue hTLR7 Cells upon Stimulation with Commensal and Pathogen Derived Bacterial RNAs

Previous work from our lab (MSc thesis by Mine Ozcan) suggested that pathogen-derived RNAs triggered significantly higher levels of pro-inflammatory cytokine production from human PBMCs compared to commensal derived RNAs. To assess the identity of pattern recognition receptors that might be involved in such a response, we tested the immunostimulatory activity of total and rRNA subunits purified from 2 commensal (LS and LF) versus 2 pathogens (LM, SP) in HEK-Blue cells stably expressing hTLR3 or hTLR7 receptors.

Since the endosomal TLRs specific to RNA molecules can recognize a variety of structures, we speculated the involvement of these TLRs in the differential activation of immune system by commensal and pathogen derived bacterial RNAs. Since the bacterial RNAs are a mixed population composed of single and double stranded RNAs (Kawashima et al., 2013), the first hypothesis was the recognition of bacRNAs by both of these endosomal TLRs; TLR3 and TLR7. In addition to total RNAs, immune responses to individual ribosomal RNAs (5S, 16S and 23S) were also investigated as the majority of bacterial RNAs consist of ribosomal RNAs (Ciulla et al., 2010). Moreover, it is known that 16S rRNA sequencing is a widely used technique for the identification and classification of bacteria (Janda & Abbott, 2007) and 23S rRNA of bacteria contain a motif that is recognized by mouse TLR13 (Xiao-Dong Li & Chen, 2012). On the basis of this knowledge, it was speculated that these bacterial rRNAs might have differential immunostimulatory activity in human immune cells. Thereby HEK-Blue cells expressing hTLR3 or hTLR7 were stimulated with all bacterial RNA species and NK-κB/AP-1 activation was assessed based on NF-κB-dependent SEAP production. 3 μg/ml bacterial RNAs were mixed with Lipofectamine 2000 (at a ratio of 3:1) prior to stimulation in order to protect RNA molecules from nuclease attack and enhance their delivery to cells. Optimum dose of bacRNA and lipofectamine 2000 ratio used in stimulations were determined in another MSc study previously conducted in our lab. PolyI:C and R848 were used as positive controls for TLR3 and TLR7, respectively.

Total RNAs and 5S rRNAs derived from pathogens (LM&SP) specifically induced comparable levels of NF-κB and AP-1 activation in both HEK-Blue hTLR3 and HEK-Blue hTLR7 cells (Figure 3.3 and 3.4). 16S rRNA of pathogens triggered low levels of TLR3 or TLR7 dependent NF-κB activation whereas all RNA species originating from commensals and the 23S rRNA of pathogenic bacteria failed to activate NF-κB and AP-1. In other words, pathogenic RNAs, but not the commensal derived RNAs, might contain some structural motifs that are recognized by these endosomal TLRs and consequently activate the NF-κB pathway. Since total RNA

and the 5S rRNAs of pathogens showed similar activity, it was concluded that the 5S rRNAs might be the main RNA species in total RNA samples which triggered the NF-κB pathway through TLR3/TLR7 signaling.

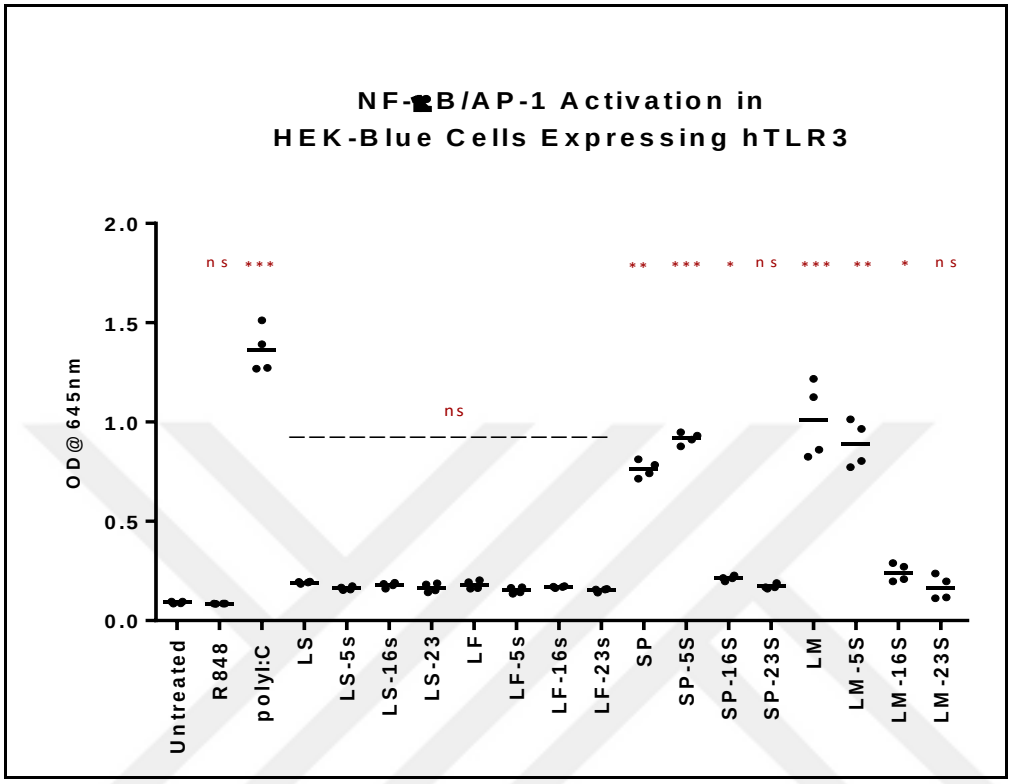

Figure 3.2 NF-κB and AP-1 activation in HEK-Blue hTLR3 cells upon stimulation with commensal and pathogen originated bacterial RNAs. 3×10^5cells/ml HEK-Blue hTLR3 cells were tranfected with 3 µg/ml of each RNA species with Lipofectamine 2000. Culture supernatants were collected after 24 hours and SEAP production induced by NF-κB and AP-1 activation was determined using Quanti-Blue reagent. OD_{645} represents quantification of SEAP in supernatants. Each experiment was repeated twice. Kruskal-Wallis Dunn's test was conducted for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

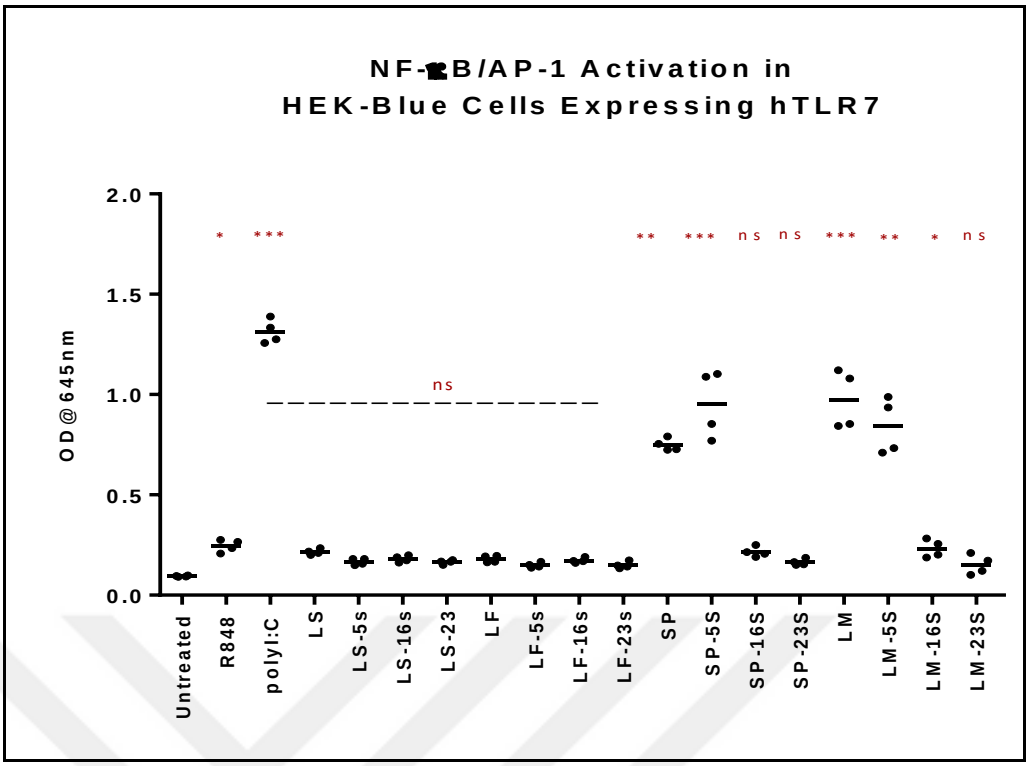

Figure 3.3 NF-κB and AP-1 activation in HEK-Blue hTLR7 cells upon stimulation with commensal and pathogen originated bacterial RNAs. 3×10^5cells/ml HEK-Blue hTLR7 cells were transfected with 3 µg/ml of each RNA species with Lipofectamine 2000. Culture supernatants were collected after 24 hours and SEAP production induced by NF-κB and AP-1 activation was determined by using Quanti-Blue reagent. OD_{645} represents quantification of SEAP in supernatants. Each experiment was repeated twice. Kruskal-Wallis Dunn's test was conducted for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

Although our results suggest that only the pathogen but not the commensal-derived total RNAs and the 5S rRNA had TLR3 and TLR7-dependent NF-κB inducing activity, there still remains the possibility that commensal-derived RNAs might be also be recognized through these receptors. TLR3 and TLR7-dependent signaling can activate the NF-κB and/or the IRF3/IRF7 transcription factors, resulting either in pro-inflammatory cytokine or type I interferon production, respectively. TLR7

signaling occur via the adaptor protein MyD88 which consequently induces TRAF6 activation (Gay et al.,2014). NF-κB and IRF7 phosphorylation can occur through TRAF6 for this TLR. Furthermore, TLR3 signaling pathway uses the adaptor protein TRIF which activates TRAF3 and TRAF6. Signaling through TRAF6 in TLR3 signaling pathway activates NF-κB and pro-inflammatory cytokine production while activation of TRAF3 induces phosphorylation and translocation of IRF3/IRF7. In summary, TLR signaling can trigger both pro-inflammatory cytokine and type I interferon production and factors contributing to the preference for these downstream pathways has not been completely understood. In our experimental system, activation of the IRF signaling pathway and TLR3/TLR7 driven IFN response cannot be monitored since HEK-Blue cells don't have constitutive IRF expression. Therefore our results suggest that commensal derived RNAs fail to induce TLR3 and TLR7 dependent NF-κB activation but these findings does not preclude the possibility that commensal derived RNAs could be recognized by TLR3 and TLR7 and activate IRF dependent type I interferon production.

The differential activation of NF-κB through TLR3/TLR7 signaling upon stimulation with RNAs exclusively derived from pathogens might be explained by the complexity of ligand recognition for these TLRs. In general, it is stated that TLR3 recognizes double stranded RNAs while TLR7 recognizes single stranded RNAs. However, ligand recognition by these receptors is more diverse and occurs in a complex manner. TLR3 detects endocytosed dsRNAs with a critical length of around 40 bps (Dalpke & Helm, 2012). This prerequisite prevents the recognition of several RNA species such as miRNA, siRNA and secondary structures of tRNA. Bacterial and viral single stranded RNAs are recognized by TLR7 and TLR8 in both sequence dependent and independent manner (Hornung et al., 2008). Although TLR7/TLR8 preferentially recognize single stranded RNA structures, it has been showed that short double stranded RNAs such as siRNAs can be also detected by TLR7 in a sequence dependent manner. Furthermore, presence of chemical modification on RNA molecules prevents the activation and downstream signaling by TLR7

(Robbins et al., 2007). In the light of this knowledge, it can be said that commensal derived RNAs may not possess any of the structures required for TLR3/TLR7 dependent NK-κB activation, while pathogen derived RNAs have one or more of these required structures. In addition to the complexity in ligand recognition, the factors contributing to the decision to activate the NF-κB and/or the IRF3/7 pathways are not completely understood. Involvement of additional receptors/signaling counterparts might be required for this preference. The reason why commensal RNAs cannot induce NF-κB pathway might be related with requirement of additional signaling events driving this pathway.

3.3 Differential Activities of Commensal versus Pathogen-DerivedRNA Species on Human Peripheral Blood Mononuclear Cells

Cytokines especially coming from innate immune cells like monocytes and dendritic cell are one of the most important indicators of an immune response. The type of cytokines secreted can be indicative of the identity of the pattern recognition receptors involved in ligand recognition. In order to consolidate our previous findings, hPBMCs were stimulated with each bacterial RNA species the following cytokine concentrations were determined from culture supernatants: IFNα and IP-10 as signature cytokines of nucleic acid sensing pathways, IL-1β as a readout for inflammasome activation and TNFα as an NF-κB-dependent pro-inflammatory cytokine.

3.3.1 Pro-inflammatory Cytokine Responses to RNA Species

Since the major downstream event of NF-κB activation is pro-inflammatory cytokine production (Takeuchi & Akira, 2001), we decided to concentrate on the pivotal pro-inflammatory cytokine TNFα. For this, human PBMCs were stimulated with RNAs of pathogen or commensal origin and TNF-α production was assessed by intracellular cytokine staining.. Since this experimental setup requires stimulation of cells in a large volume (1 ml), necessitating the use of high concentrations of RNA,

the activities of individual RNA species other than the 5S component could not be determined due to the limited amounts that can be purified. Hence, TNFα-inducing activities of total RNAs and 5S rRNAs of commensal and pathogen origin were compared in cells treated with Brefeldin A (an agent that prevents cytokine secretion and aids accumulation of it within producing cells). Results showed that TNF-α production can be induced by the positive control LPS and total RNAs derived from pathogens (Figure 3.4; upper panels for PBMCs isolated from 2 different subjects). The immune stimulatory activities of commensal derived total RNAs were substantially lower. Majority of TNF-α producers for all ligands tested were CD14 positive cells, consistent with monocyte phenotype. However, when the contribution of 5S rRNAs were analyzed, to our surprise, results were contradictory to our expectations and 5S rRNAs of commensals but not the pathogens triggered highest levels of this cytokine from monocytes. Human monocytes are known to human monocytes express TLR8 but lack TLR3 and TLR7 expression (Bekeredjian-Ding et al., 2006). Furthermore, these cells also express cytosolic RNA sensors RIG-I and MDA5 that can detect dsRNA structures (Broz & Monack, 2013). TLR8 senses uridine rich ssRNA sequences and short degradation products of RNAs (Tanji et al., 2015). It is a possibility that commensal 5S rRNAs could be enriched in uridines that trigger TLR8-dependent TNF-α production only when used at a high enough concentration to exceed a threshold level (i.e in 5S purified fraction). The amount of such U-rich sequences might remain below a threshold due to dilution effect when total rRNAs were employed. In contrast, such sequences might be enriched in 16S and/or 23S rRNAs of pathogens and remain low in pathogen 5S rRNA. Further experiments are required to identify the receptors that might be involved in discrimination of RNAs of pathogen and commensal origin in human cells.

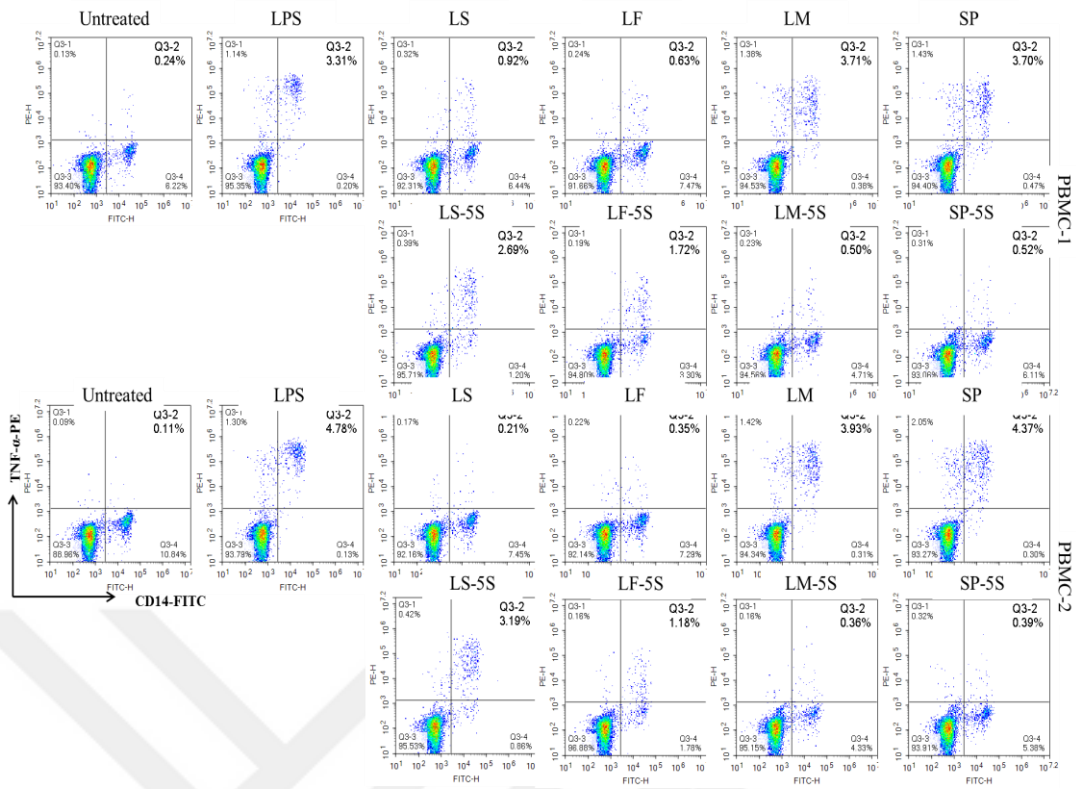

Figure 3.4 TNF-α production from $CD14^+$ hPBMC in response to bacterial total and 5S rRNAs derived from commensal vs pathogens. $2x10^6$/ml hPBMCs were stimulated with total or 5S rRNAs (via transfection with Lipofectamine 2000; 1 µg/ml) for 6 hour in the presence of Brefeldin A. At the end of 6 hour incubation, cells were fixed, permeabilized and stained with anti-TNF-α-PE and anti-CD14-FITC antibodies. 10,000 events were acquired for flow cytometric analyses.

3.3.2 IL-1β Cytokine Responses to RNA Species

Cytosolic dsRNA and bacterial RNA are known to activate the NLRP3 inflammasome pathway (Sha et al., 2014). However, whether commensal versus pathogen derived RNAs have differences in triggering inflammasome activation is not known. For this reason, hPBMCs were stimulated with total or individual rRNA species and production of IL-1β was assessed from culture supernatants as a read-out of inflammasome activation. Significant amount of IL-1β production was only

observed in cells stimulated with total bacterial RNAs (Figure 3.5). When responses to commensal and pathogen derived total RNAs were compared, it can be said that total RNAs of pathogens (LM&SP) induced stronger IL-β production (~ 4-fold higher response with respect to commensal derived total RNAs). Individual ribosomal RNAs derived from both commensal and pathogens failed to reproduce the IL-β triggering activity of total RNAs. Sha, W. et al. (2014) showed that induction of NLRP3 inflammasome by several types of bacterial RNAs is possible. However they also showed that while bacteria derived total RNAs and mRNAs could activate the NLRP3 inflammasome pathway, bacterial rRNAs failed to induce this response. It is possible that such an explanation can also be valid for the low levels of response obtained when individual rRNA species were employed. In other words, our results also are in support of the finding that inflammasome triggering activity of bacterial RNAs is ribosomal RNA–independent. In summary, total RNAs derived from pathogenic bacteria might be potent activators of both NF-κB and inflammasome pathways whereas the 5S rRNAs of pathogens lack the ability to activate the inflammasome pathway.

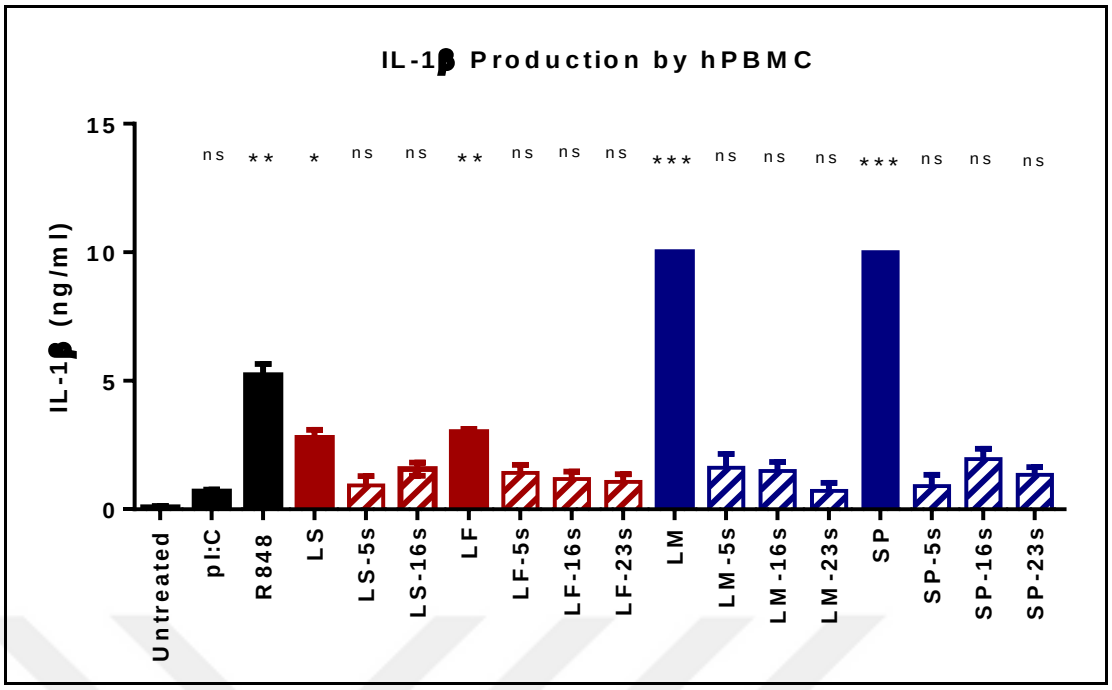

Figure 3.5 IL-1β response of hPBMCs to bacterial RNAs. 2×106 cells/ml was stimulated with 1 µg/ml of RNAs via transfection with Lipofectamine 2000. Amount of secreted IL-1β was determined from culture supernatant collected after 24 hour incubation by ELISA. Results are the average of 4 PBMCs ± SD. Kruskal-Wallis Dunn's test was applied to compare each treatment group to untreated (naïve). (*p<0.05, **p<0.01, ***p<0.001).

3.3.3 Type I IFN Responses to RNA Species

Previous MSc study conducted by Mine Özcan showed that INF-γ inducible protein (IP-10, CXCL10) production can be induced by bacterial RNAs derived from commensals, but not of pathogens. IP-10, which is an important chemokine in IFN signaling pathway, are expressed by monocytes. This protein can be induced upon stimulation with type I and II IFNs. Thus, production of this chemokine by commensal derived RNAs indicate the activation of IFN signaling by these RNAs. To confirm these findings on IP-10 data, we repeated the experiment with total RNAs of commensal and pathogenic bacteria. Since the secretion of IP-10 occurs

indirectly (depends on the initial secretion of type I IFNs), Brefeldin A, which prevents cytokine secretion from cells, was applied to cells after 5 hour stimulation with bacRNAs. Following Brefeldin A addition, cells were incubated for 3 more hours to allow for intracellular IP-10 accumulation. Results showed that production of IP-10 was initiated following stimulation with commensal but not pathogen derived total RNAs (Figure 3.6). These results suggested that type I and/or type II IFN signaling was triggered by commensal derived total RNAs and IP-10 production occurred indirectly under the influence of these interferons.

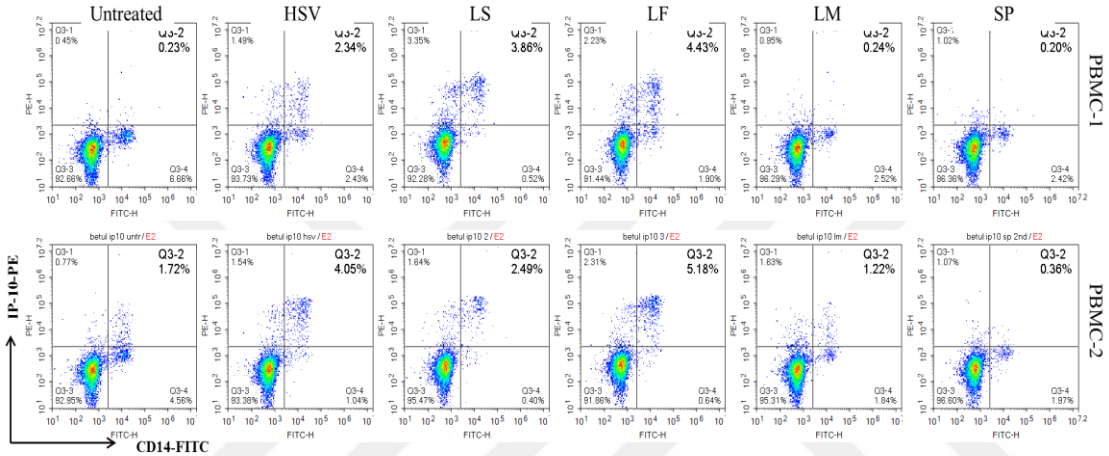

Figure 3.6 IP-10 production from $CD14^{+}$ hPBMC in response to bacterial total RNAs derived from commensal vs pathogens. $2x10^{6}$/ml hPBMCs were stimulated with total RNAs (via transfection with Lipofectamine 2000) for 5 hour. Following 5 hour incubation, cells were treated with Brefeldin A and incubated for 3 additional hours. At the end of incubations, cells were fixed, permeabilized and stained with IP-10-PE and CD14-FITC antibodies. 10,000 events were acquired for flow cytometric analyses.

Next, we assessed type I IFN production from hPBMCs stimulated with bacterial RNA species. Type I IFN family consists of a single IFN-β and 14 different subtypes of IFN-α. These cytokines play a major role in antiviral immunity, mainly by interfering with viral proliferation (Pichlmair et al., 2007). Production of these potent

cytokines generally occurs as a result of recognition of a PAMP that viruses possess such as dsRNA. Recognition of dsRNA via cytosolic sensors RIG-I and MDA-5 induces the production of type I IFNs and the establishment of an antiviral state in the cell (Loo & Gale, 2011).

Recent studies showed that commensal bacteria derived-products modulate immune responses against pathogens. Several groups claimed that microbiota provides a steady-state level of type I IFN production upon recognition of commensal derived products via immune sensor. These products might stimulate peripheral immune cells after translocation and facilitate the rapid immune response in the case of pathogen involvement (Clarke et al., 2010). They can also stimulate epithelial cells which secrete potent molecules into circulation and these secreted products might prime peripheral immune cells for possible pathogen encounter (Abt & Artis, 2013). In addition, it has been shown that dysregulated microbiota increase susceptibility to several infections and inflammatory diseases (Abt et al., 2012b), (McAleer et al., 2012).

How commensals trigger Type I IFNs is unclear and there are conflicting studies conducted to investigate the underlying mechanism. Kawashima and colleagues (2013) claim that type I IFN response to commensal derived dsRNAs occurs in a TLR3 dependent manner while another group published data that indicates the involvement of cytosolic RNA sensors and MAVS in type I IFN response to enriched dsRNA sequences present in commensal bacteria (Li et al., 2011). In addition to dsRNA in commensal bacteria, it has been claimed that RNA of *Listeria monocytogenes* induces type I interferon response in a RIG-I and MAVS dependent manner in several types of immune cells (Hagmann et al., 2013). Furthermore, several groups showed TLR7 and TLR8 dependent type I IFN response to single stranded bacterial RNAs (Gantier et al., 2008), (Eberle et al., 2009), (J. L. Cervantes et al., 2011), (Deshmukh et al., 2011), (Dalpke & Helm, 2012), In summary, in order

to broaden our knowledge in this field, type I IFN responses to both commensal and pathogen derived RNA species were investigated.

Results showed that IFN-α secretion occurred upon stimulation with commensal (LS&LF) derived RNAs, but not with pathogen derived RNAs (Figure 3.7). This type I IFN response was observed when commensal derived RNAs were delivered to the cytosol by means of Lipofectamine 2000 complexation. In other words, RNA species had to gain access to the cytosol to trigger an interferon response. Under physiological conditions, it is possible that cationic anti-microbial peptides can act as transfection reagents and carry commensal-derived RNAs to the cytosol of immune cells (Wan et al., 2014).

Type I IFN production was also observed upon stimulation with ribosomal RNAs, although the observed responses were not as potent as those obtained with total RNAs. Specifically, 23S rRNAs of commensal bacteria induced the highest levels of IFN-α among the tested rRNA species (Figure 3.7), suggesting the presence of a specific immune-stimulating sequence and/or secondary structures that preferentially stimulate type I IFNs. Since these findings were of interest, we decided to investigate the type I IFN inducing activity of commensal RNAs in detail.

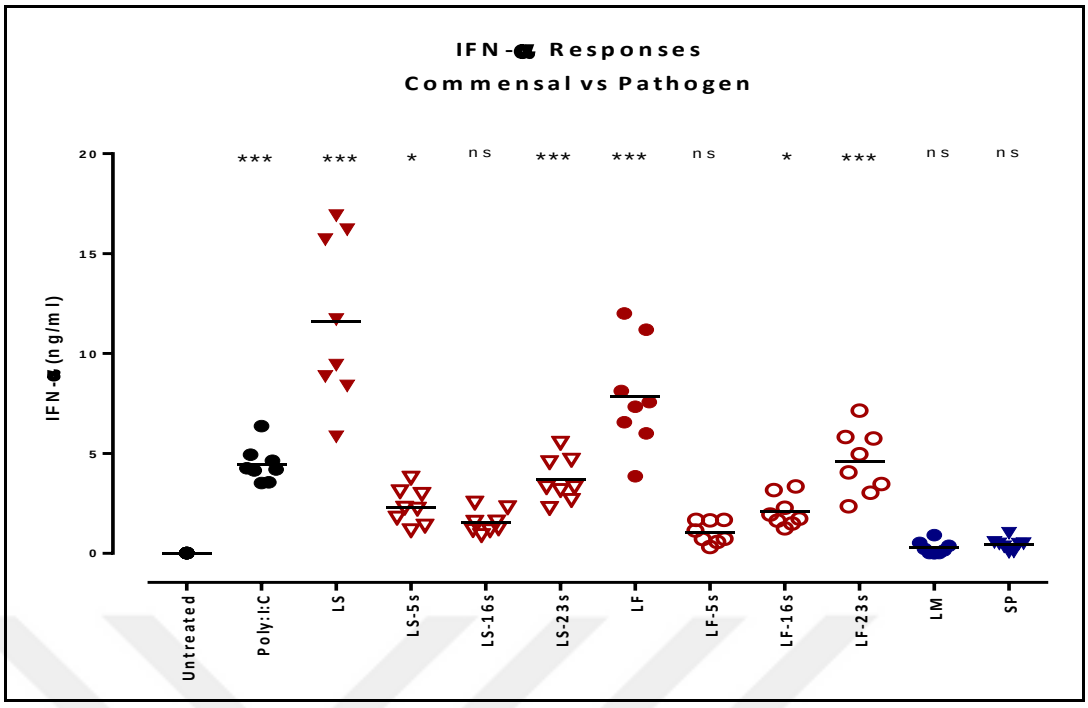

Figure 3.7 IFN-α response of hPBMC to bacterial RNAs. 2×106 cells/ml was stimulated with 1 µg/ml of RNA via transfection with Lipofectamine 2000. Amount of secreted IFN-α was determined from culture supernatant collected after 24 hour incubation by ELISA. Individual results from 4 different PBMCs are displayed. Kruskal-Wallis Dunn's test was used to compare each treatment group to the untreated (naïve) control. (*p<0.05, **p<0.01, ***p<0.001)

3.4 Detection of IFN Responses to Commensal Derived Ribosomal RNAs in Combination

Type I IFN response to total RNAs was stronger than both positive controls and individual rRNAs. Morever, 23S rRNA of LS&LF induced stronger IFN-α production when the responses to individual rRNA subunits were compared with each other. To determine whether rRNAs synergized to trigger a type I IFN response equivalent to those obtained with total RNAs, rRNA subunits were used in various combinations. Synergy occurs when an overall effect of more than one substance is

greater than the sum of effects of each substance. Thus, in this case, the overall effect of each ribosomal RNA on type I IFN response might be higher than the sum of the effects of each rRNA alone. To test this hypothesis, commensal derived rRNA was used in combinations to stimulate hPBMCs. For this, stimulation groups consisted of either single subunits (5S, 16S and 23S) or or combinations of subunits (5S+16S, 5S+23S, 16S+23S, 5S+16S+23S) were tried for both LS and LF (Figure 3.8 and Figure 3.9).

For both commensal bacteria, combination groups did not show any synergistic effect. None of the combinations could surpass the group with 23S rRNA alone althoughgroups with 23S rRNAs yielded higher type I IFN response than groups devoid of 23S rRNA. For example, the '16S+23S' groups for both LS&LF did not show a stronger response obtained with 23S rRNA alone, suggesting the absence of any synergy. In summary, although our results suggest that 23S commensal rRNAs possess interferonegenic activity, the combination trial did not answer the question as to why total RNAs induced stronger IFN response than combinations of ribosomal RNAs. It may be possible that other types of RNAs like mRNA, tRNA or non-coding RNAs may also have functions in immune recognition and type I IFN response.

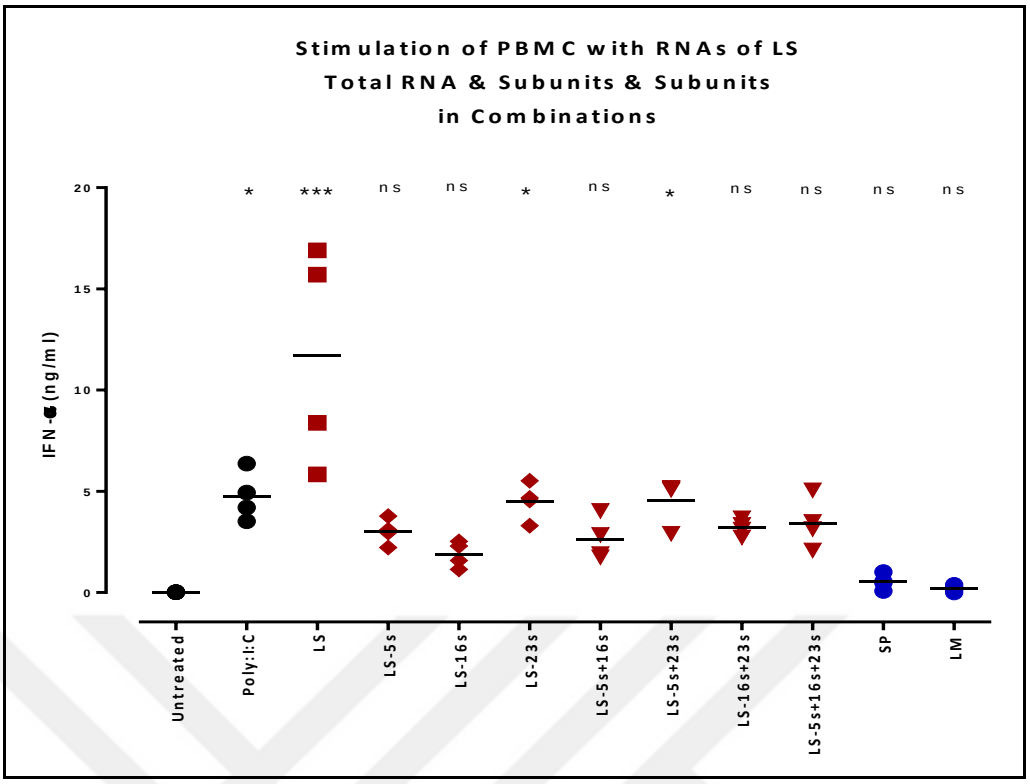

Figure 3.8 Investigation of synergistic immunostimulatory activity of ribosomal RNA subunits of Lactobacillus salivarious when used in different combinations. 2×106 PBMCs/ml were stimulated with 1 µg/ml of RNAs in total via transfection with Lipofectamine 2000. IFN-α production was determined from culture supernatants collected 24 hours after stimulation by ELISA. Individual results from 2 different PBMCs are displayed. Kruskal-Wallis Dunn's test was performed for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

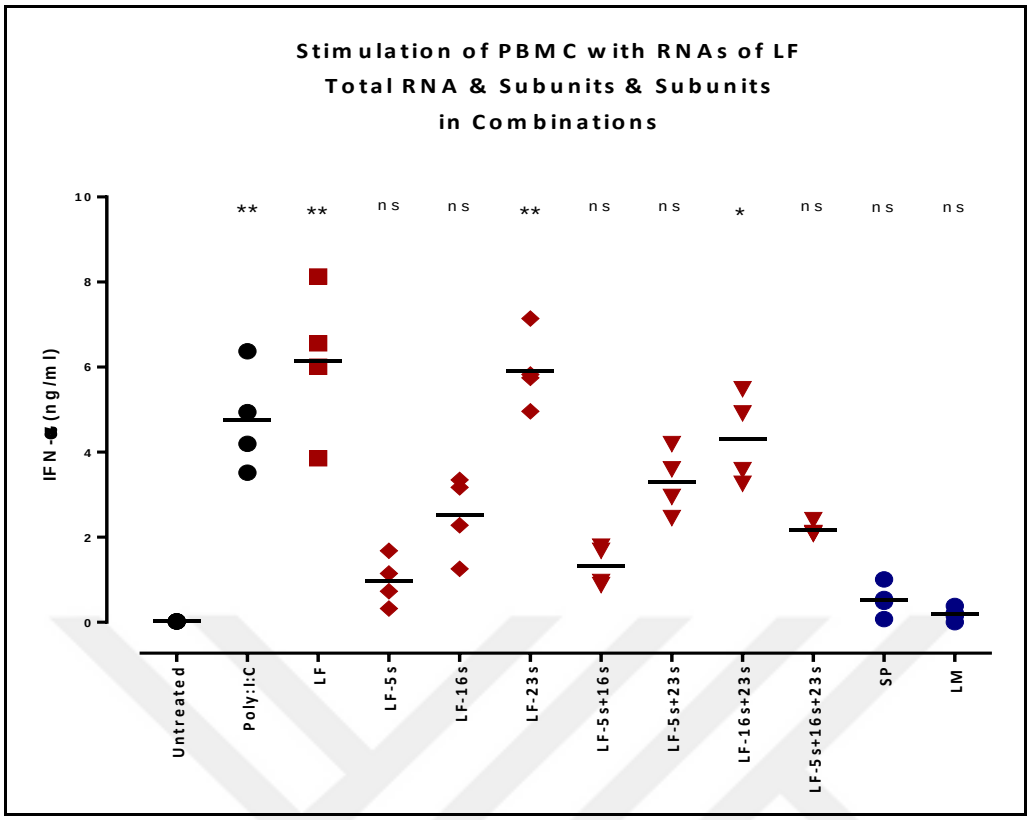

Figure 3.9 Investigation of possible synergistic immunostimulatory activity of ribosomal RNA subunits of Lactobacillus fermentum when used in different combinations. 2×106 PBMCs/ml were stimulated with 1 µg/ml of RNA in total via transfection with Lipofectamine 2000. IFN-α production was determined from culture supernatants collected 24 hour after stimulation by ELISA. Individual results from 2 different PBMCs are displayed. Kruskal-Wallis Dunn's test was performed for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

3.5 Contribution of Plasmacytoid Dendritic Cells (pDCs) and Monocytes in IFN Production as a Result of Bacterial RNA Stimulation

Preliminary results showed that bacterial RNAs originating from commensals but not pathogens induced potent IFN-α response. Although plasmacytoid dendritic cells (pDCs) are the major producers of Type I IFNs, monocytes also secrete these cytokines (Lee & Kim, 2007), (Gary-Gouy et al., 2002). Major RNA sensors

expressed in pDCs include TLR7 and RIG-I (Szabo et al., 2014). In monocytes expression of TLR8, RIG-I and MDA-5 has been described (Lee & Kim, 2007). To determine which cell type contributed most to the observed type I IFN production in response to commensal derived total and ribosomal RNAs, PBMCs were either depleted of pDCs or monocytes and IFN responses of both depleted and not-depleted hPBMCs were determined following stimulation.

3.5.1 Effect of pDC Depletion on Type I & III IFN production following stimulation with bacterial RNAs

Plasmacytoid dendritic cells are considered to be the major type I IFN producers in blood, although they constitute a very small fraction of total blood leukocytes (generally less than 1% of hPBMCs). pDCs can be defined mainly by surface expression of CD303 (BDCA-2), CD304 (BDCA-4) and CD123 (Narbutt et al., 2004). For depletion experiments, a magnetic cell separation kit specific to pDCs was used. In this system, pDCs were targeted by magnetically labelled anti-CD304 antibodies. Both cell fractions (pDC depleted and pDC enriched) were collected and cells were fixed for cell surface staining. Efficiency of depletion was confirmed by staining with fluorochrome conjugated anti-CD123 and anti-CD303 antibodies. Cell surface staining was also performed with pDC enriched fraction in order to develop proper gating strategy for the pDC population. Depletion efficiency was ~70% (0.9 % pDC in undepleted sample vs 0.2% in the depleted PBMC; Figure 3.10), a reduction high enough to diminish any response that may be pDC-dependent.

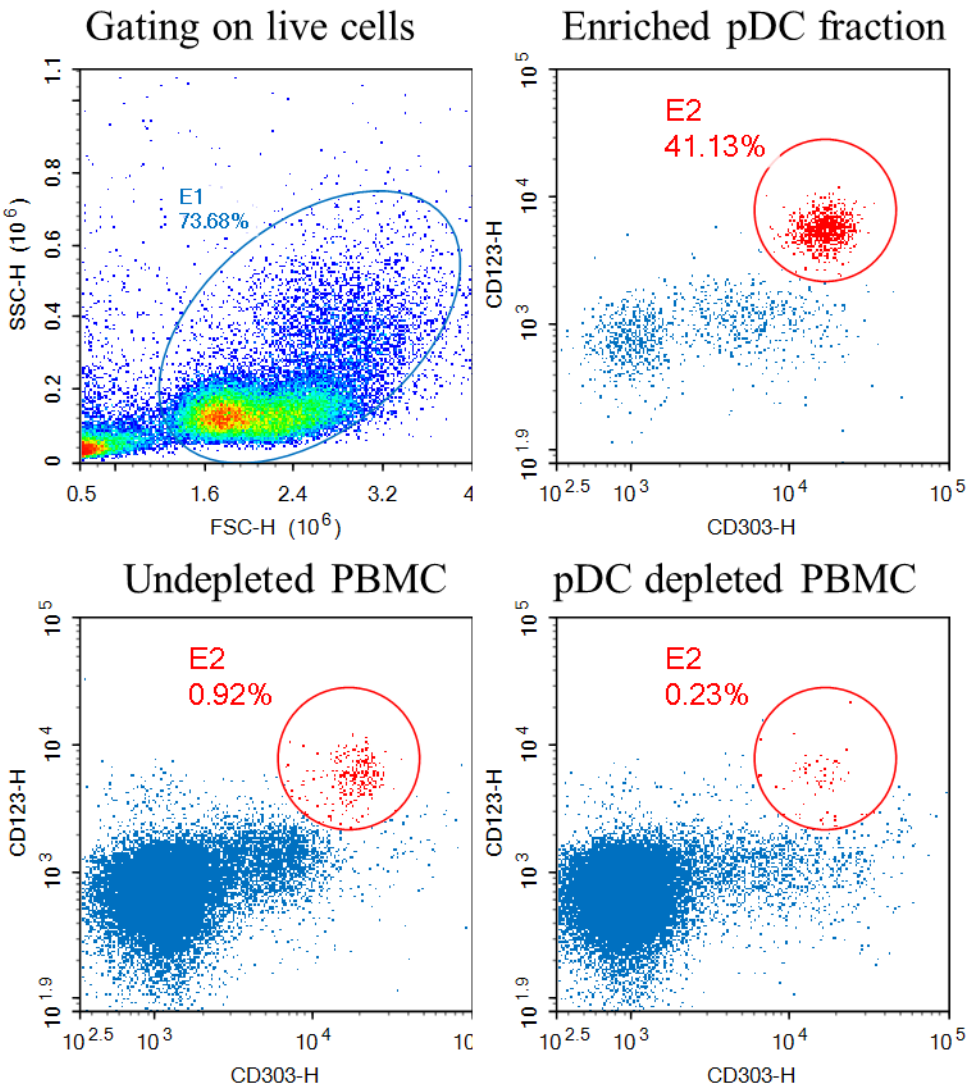

Figure 3.10 Gating strategy and illustration of pDC depletion. After depletion of pDC population from hPBMC by CD304 microbeads, efficiency of depletion was confirmed following cell surface staining with two pDC markers; CD303 and CD123. Cells in E2 gate correspond to CD123/CD304 double positive pDCs. Results are representative of 8 different PBMCs. Analysis was performed using an Accuri C6 flow cytometer.

The effeciency of pDC depletion was confirmed using the type I IFN inducing CpG ODN D35. This specific D35 ODN exclusively targets pDCs and triggers high levels

of type I IFN production only from pDCs (Gursel et al., 2013). Consistent with pDC depletion yield, there was a drastic reduction in D35-dependent type I IFN production from pDC depleted PBMCs (~ 70% decrease) (Figure 3.11). In contrast, pDC depletion had a very modest effect when response to commensal RNAs was analyzed. However, this decrease was not drastic and not valid for each RNA species. These findings suggest that pDCs do not constitute the major cell source responding to bacterial RNAs and another cell type might be the major contributor to type I IFN response to commensal derived RNAs.

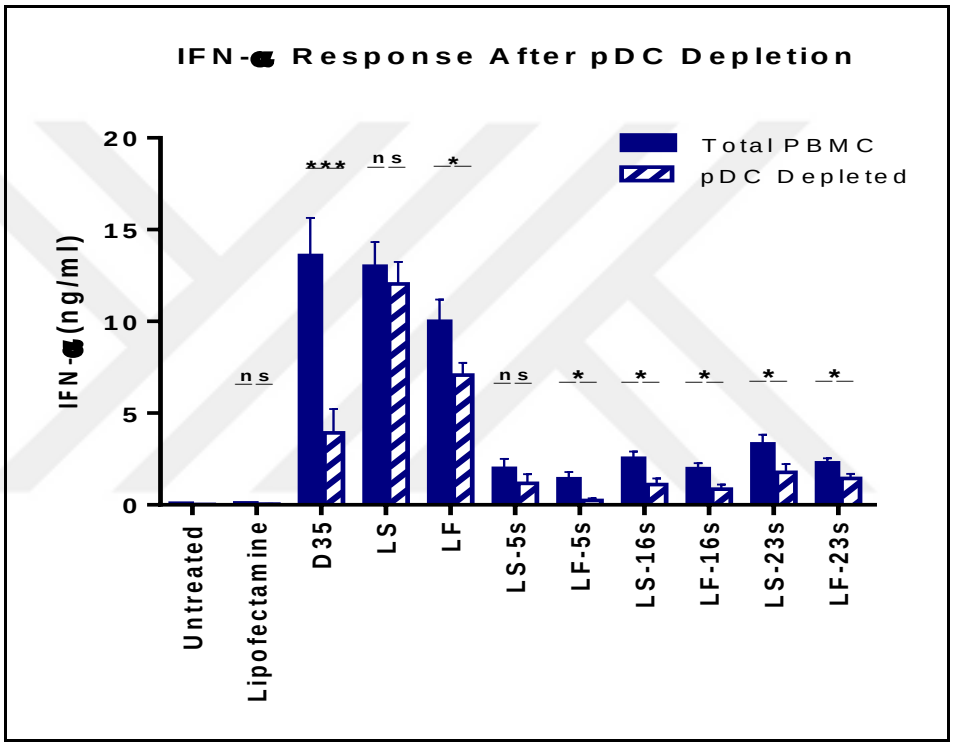

Figure 3.11 Change in the Type I IFN response after depletion of pDC population from hPBMCs. Both untouched hPBMC and pDC depleted hPBMC (2×106 cells/ml) were stimulated side by side with 1 µg/ml of RNA via transfection with Lipofectamine 2000. Amount of secreted IFN-α was determined from culture supernatant collected 24 hour after stimulation using ELISA. Results represent the average of 8 PBMCs ± SD. Unpaired t-test was used for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

Type III IFNs are a recently identified group of cytokines consisting of four members (IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4) (Egli et al., 2014). Regulation and effector function of this IFN group is closely linked to type I IFNs. Wide variety of stimuli can induce expression of λ interferons in several different cell types (Wack et al., 2015). The most distinctive feature of type III IFNs is their selective targeting of mucosal epithelial cells. Similar to type I IFNs, λ interferons have significant functions in the establishment of antiviral protection (Lazea et al., 2015).

Based on this knowledge on type III IFNs, production of IFN-λ1, one of the family members, was also investigated after stimulation with RNAs of commensal bacteria. In addition, contribution of pDC population on this response was determined. The results showed that RNAs of commensal bacteria induced type III IFN similar to results obtained with type I IFN (Figure 3.12). Thus, it can be concluded that commensal derived bacterial RNAs can initiate type III interferon production in hPBMC. Furthermore, depletion of pDCs did not influence IFN-λ1 production significantly. These results prompted us to analyze the involvement of another major IFN producer in blood, the monocytes in IFN response to RNAs of commensal bacteria.

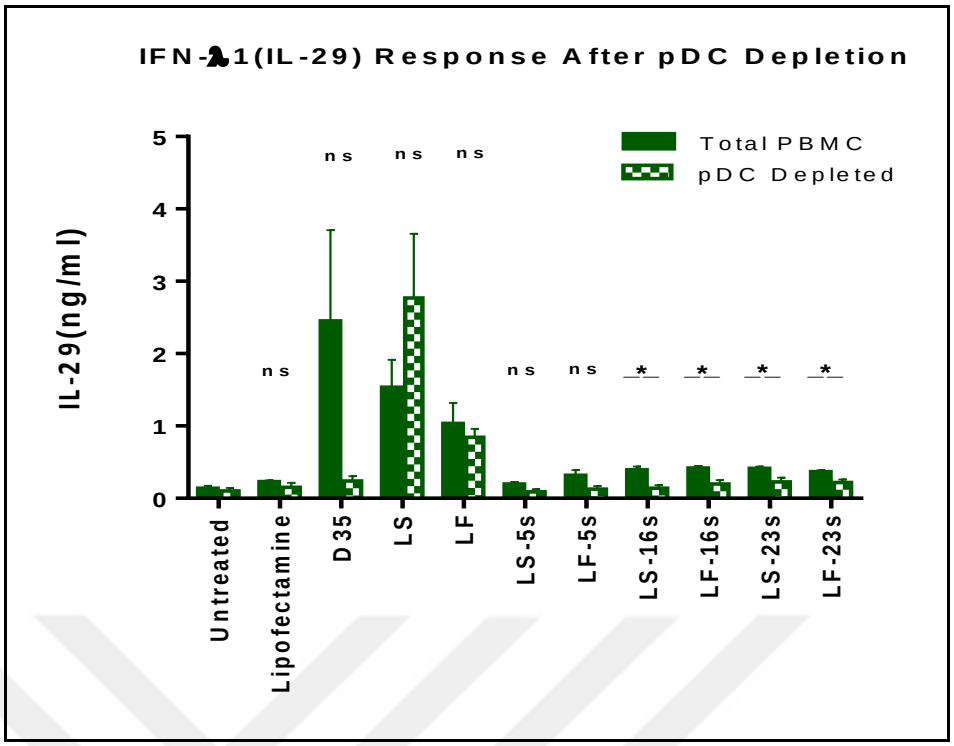

Figure 3.12 Change in the Type III IFN response after depletion of pDC population from hPBMCs. Both untouched hPBMC and pDC depleted hPBMC (2×106 cells/ml) were stimulated side by side with 1 µg/ml of RNA via transfection with Lipofectamine 2000. IFN-λ1 (IL-29) production was determined from culture supernatant collected 24 hour after incubation using ELISA. Results represent the average of 8 PBMCs ± SD. Unpaired t-test was used for each comparison. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

3.5.2 Effect of Monocyte Depletion on Type I & III IFN production following stimulation with bacterial RNAs

Monocytes are the second most important group of cells that has a function in type I IFN response. They constitute about 10% of hPBMCs and they can be identified by their distinct forward and side scatter profile as well as cell surface expression of CD14. In depletion assay, monocytes were targeted by anti-CD14 antibody conjugated magnetic microbeads. Both cell fractions (depleted and enriched) were collected and cells were fixed for cell surface staining. Depletion was confirmed by

staining with fluorochrome conjugated anti-CD14 antibody. Staining of monocyte enriched fraction was also performed in order to confirm proper gating strategy. Depletion efficiency was calculated to be more than 95% (Figure 3.13).

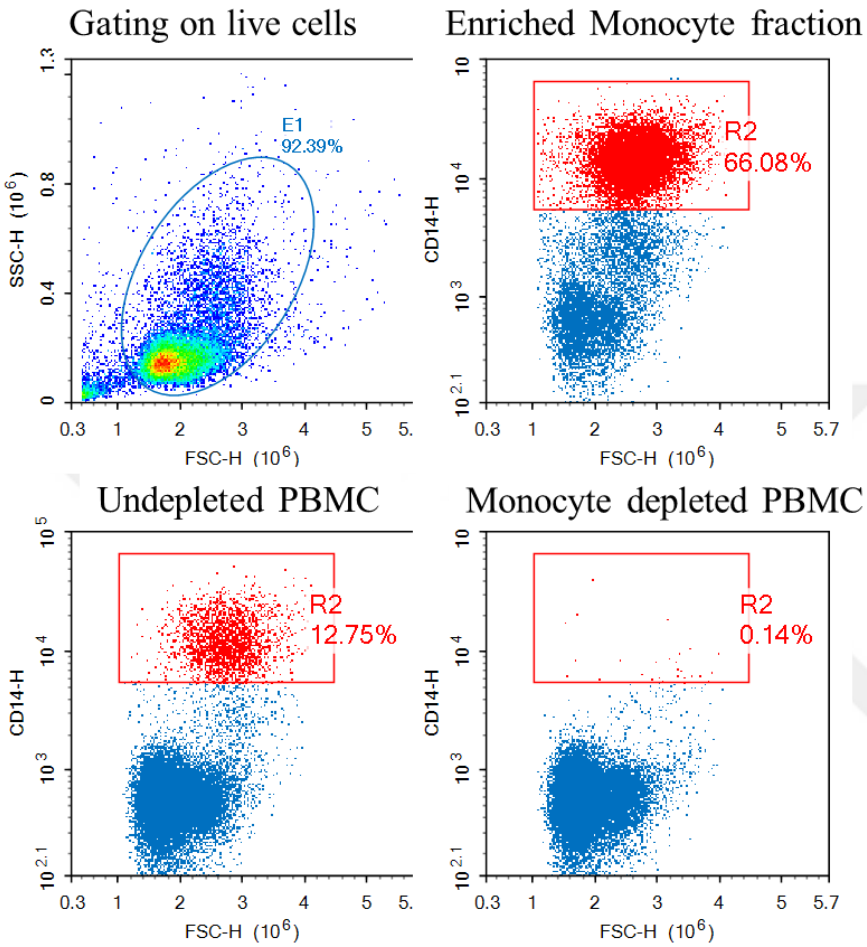

Figure 3.13 Gating strategy and illustration of monocyte depletion. After depletion of monocytes from hPBMC1 by CD14 microbeads, depletion efficiency was confirmed following staining for the cell surface marker selectively expressed on monocytes (CD14). Results are representative of 4 different PBMCs Analysis was performed using an Accuri C6 flow cytometer.

Responses to poly:I:C (TLR3 ligand) and R848 (TLR7 ligand) were not affected significantly in monocyte depleted hPBMC (Figure 3.14 and 3.15). Since

conventional DCs and pDCs express high levels of TLR3 and TLR7, respectively, cytokine production from these cells remained intact. In contrast, monocyte depletion drastically diminished both type I and type III IFN responses to commensal derived bacterial RNAs (Figure 3.14 and 3.15). Furthermore, for most RNA species, interferon response was completely lost in monocyte depleted samples. These results support the previous findings that monocyte population is the main source of IP-10 response to total RNAs of commensals. In summary, these findings suggest that monocytes are the main source of interferon produced in response to commensal RNAs and pDC population has a modest contribution to this response. Since human monocytes lack expression of TLR3 and TLR7 but express the RNA sensors TLR8, RIG-I and MDA-5, these results also suggest the involvement of these latter receptors in bacterial RNA recognition.

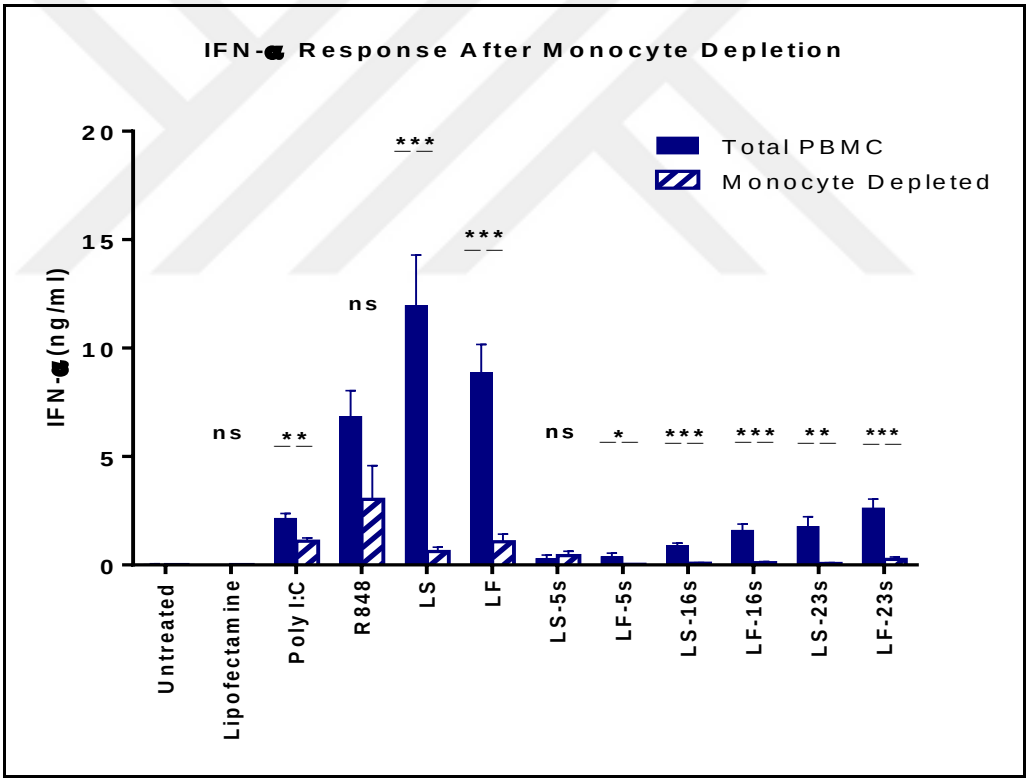

Figure 3.14 Change in the Type I IFN response after depletion of monocyte population from hPBMCs. Both untouched hPBMC and monocye depleted hPBMC

(2×106 cells/ml) were stimulated side by side with 1 µg/ml of RNA via transfection with Lipofectamine 2000. Amount of secreted IFN-α was determined from culture supernatants collected 24 hour after stimulation by ELISA. Results represent the average of 4 PBMCs ± SD. Unpaired t-test was used for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

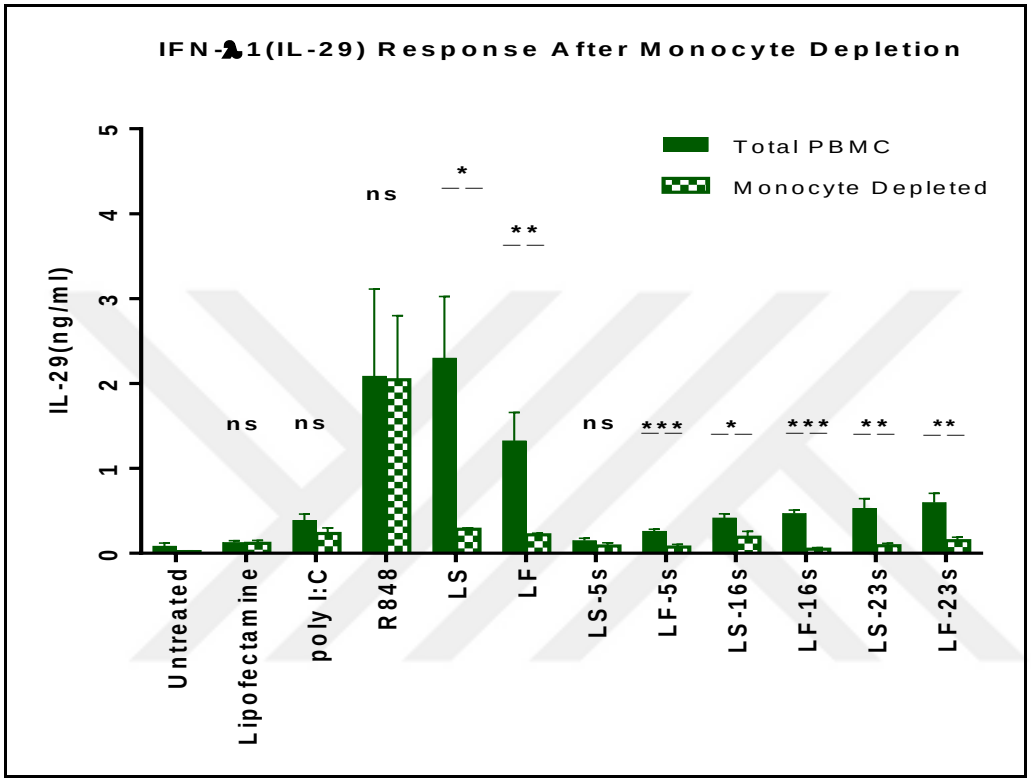

Figure 3.15 Change in the Type III IFN response after depletion of monocyte population from hPBMCs. Both untouched hPBMC and monocyte depleted hPBMC (2×106 cells/ml) were stimulated side by side with 1 µg/ml of RNA via transfection with Lipofectamine 2000. IFN-λ1 (IL-29) production was determined from culture supernatant collected 24 hour after stimulation by ELISA. Results represent the average of 4 PBMCs ± SD. Unpaired t-test was used for each comparison. (*p<0.05, **p<0.01, ***p<0.001)

3.6 Investigation of IFN responses in THP1 cells upon Stimulation with Commensal Derived Ribosomal RNAs

Since our findings demonstrated that monocytes are the main source of interferon produced upon stimulation with bacterial RNAs obtained from commensal bacteria, we wanted to verify these results in THP-1 human leukemic monocyte cells. Bacterial RNA induced type I and III IFN responses were investigated in null and PMA differentiated THP1 cells. For this, we used THP-1-Blue ISG cells which allowed direct monitoring of interferon signaling pathway. These cells stably express an IRF-inducible SEAP reporter construct and allows the monitoring of IRF activation by determining the activity of SEAP in culture supernatants. In other words, SEAP production, which depended on IRF activation, was determined to obtain information about IFN signaling pathway initiating capabilities of commensal RNAs. In this cell system, IRF activation reflects overall interferon response and is not specific to one group of IFN.

Human blood monocytes do not require additional priming signals for their effector functions, whereas THP-1 cells require priming for their full differentiation and establishment of certain effector functions. PMA treatment generally upregulates gene expression, so that a stronger immune response can be obtained with a secondary stimulus (Park et al., 2007).

Since it has been showed that distinctive features of bacterial RNAs might provide priming function (Koski et al., 2004), immunostimulatory effects of commensal RNAs were determined in both PMA differentiated and naïve THP-1-Blue ISG cells.

3.6.1 Activation of interferon signaling pathways in THP-1-Blue ISG Cells in response to bacterial RNAs

Results showed that commensal derived RNAs are potent activators of IFN signaling pathway in THP1-Blue ISG cells. Such a response could be initiated in the absence

of PMA priming (Figure 3. 16). While total RNAs showed strong IRF activation, 5S rRNAs had no significant effect on (except for LF 23S) IFN signaling pathway. When the responses to known ligands were examined, it was observed that R848 and HSV can also induced IFN response in the naïve THP1-Blue ISG cells, suggesting involvement of TLR7/8 and DNA sensing pathways, respectively.

Since priming is known to upregulate the expression of cytosolic RNA sensors RIG-I and MDA5 but downregulates TLR7 expression (a phenotype closely mimicking human monocytes), the above experiment was repeated using primed THP1-Blue ISG cells. Priming was confirmed by change in cellular morphology and following the response to certain ligands. For example, as expected, in primed cells R848 response was abolished (due to down regulation of TLR7 expression) while responses to cytosolic DNA and RNA ligands drastically increased (Figure 3.17). . Furthermore, IFN signaling pathway was also activated by 16S and 23S rRNAs of commensal bacteria. . Collectively, these results indicate that primed THP-1 cells do not express TLR7 (as evidenced by nonresponsiveness to R848), but highly express RIG-I (based on the strong response to RIG-I ligand 5’ ppp RNA), suggesting that cytosolic RNA sensors RIG-I and MDA5 might in fact be the major receptors recognizing commensal RNAs and their subunits.

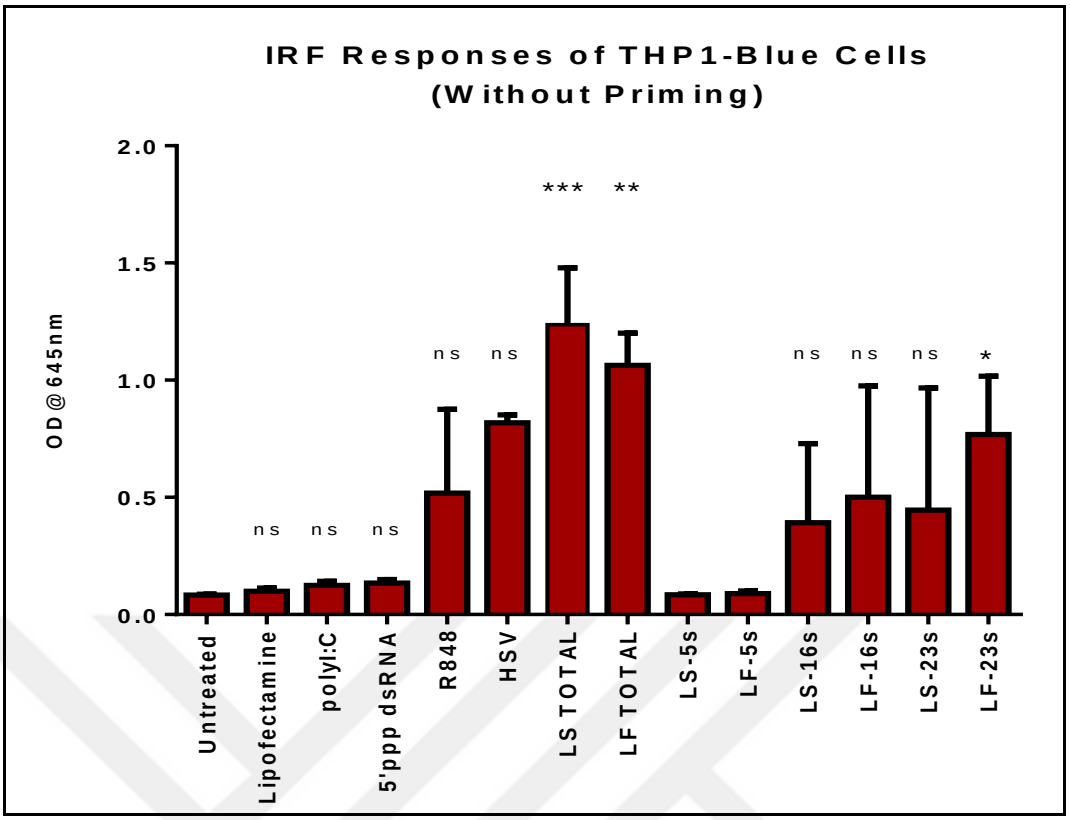

Figure 3.16 Quantification of IRF-induced SEAP production in naïve THP-1-Blue ISG cells upon stimulation with bacterial RNAs. THP1-Blue ISG cells were cultured at a concentration 5×105 cells/ml and stimulated with 1 µg/ml of RNA via transfection with Lipofectamine 2000. 24 h after stimulation, 20 µl of culture supernatant was mixed with 180 µl Quanti-Blue as a read out for SEAP. OD measurement was taken at 645 nm after 3 hours incubation at 37°C in the dark. Each experiment was repeated twice. Kruskal-Wallis Dunn's test was conducted to compare each treatment groups to untreated group. (*p<0.05, **p<0.01, ***p<0.001)

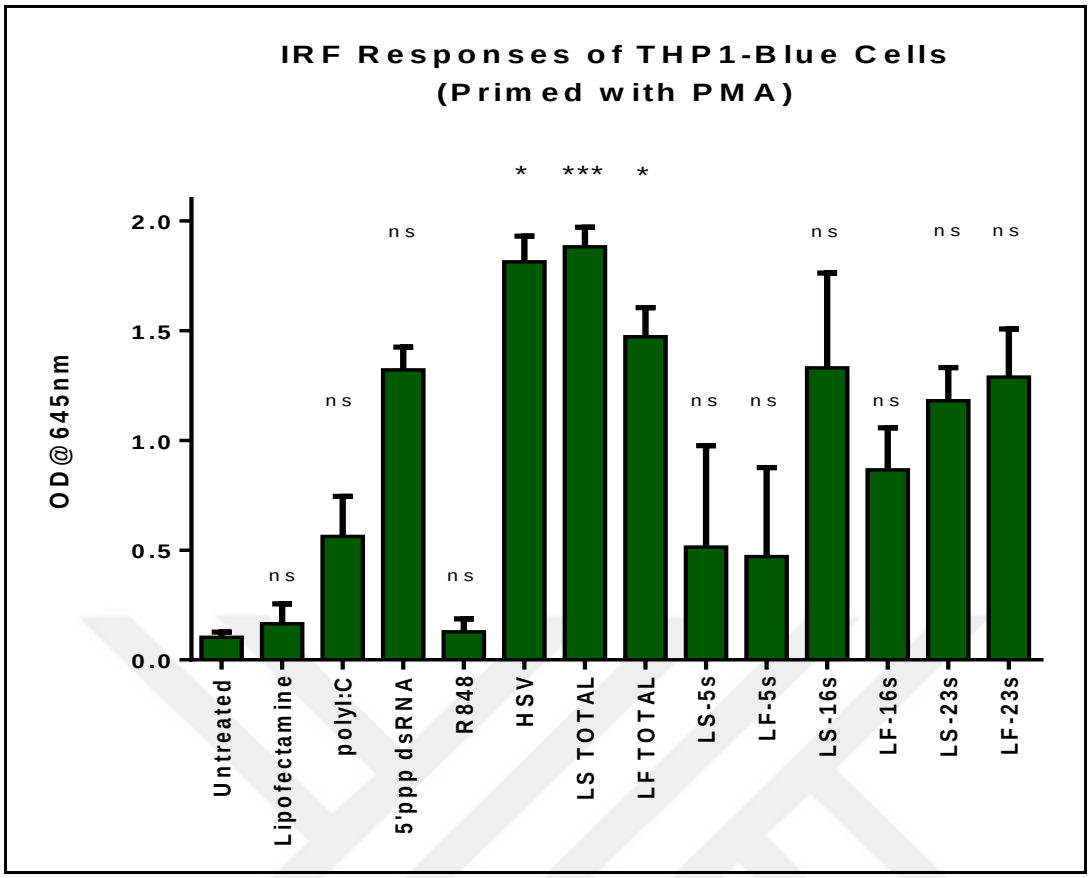

Figure 3.17 Quantification of IRF-induced SEAP production in PMA-primed THP-1-Blue ISG cells upon stimulation with bacterial RNAs. After overnight priming with PMA (20ng/ml), THP1-Blue ISG cells (5×105 cells/ml) were stimulated with 1 µg/ml of RNA via transfection with Lipofectamine 2000. 24 h after stimulation, 20 µl of culture supernatant was mixed with 180 µl Quanti-Blue as a read out for SEAP. OD measurement was taken at 645 nm after 1 hour incubation at 37°C in the dark. Each experiment was conducted two times. Kruskal-Wallis Dunn's test was conducted to compare each treatment groups to untreated group. (*p<0.05, **p<0.01, ***p<0.001)

3.6.2 Determination of IFN Response in Conventional THP-1 Cells

Results using the engineered THP-1 cells showed that commensal RNAs triggered a potent IFN response. Since as a future plan we had decided to knock down individual

RNA sensors in normal THP-1 cells to identify the receptors involved in commensal RNA recognition, the above experiments were also repeated in conventional THP-1 cells. IFN response to commensal RNA stimulation was examined using 2 different methods: First, presence of IFNs in THP-1 culture supernatants was monitored indirectly using the THP-1-Blue ISG reporter cells. Since the THP-1 cells produce IFNs, addition of such culture supernatants onto reporter cells would trigger IRF activation and SEAP production, which can then be quantified (Figure 3.18). The second method was based on quantitation of λ interferon production from THP-1 culture supernatants by ELISA (direct method; Figure 3.19). As can be seen from Figures 3.17, 3.18 and 3.19, total RNAs triggered strong type I and III IFN response from THP-1 cells. Among the subunits tested, significant response only to the 23S rRNA component of LF was observed.

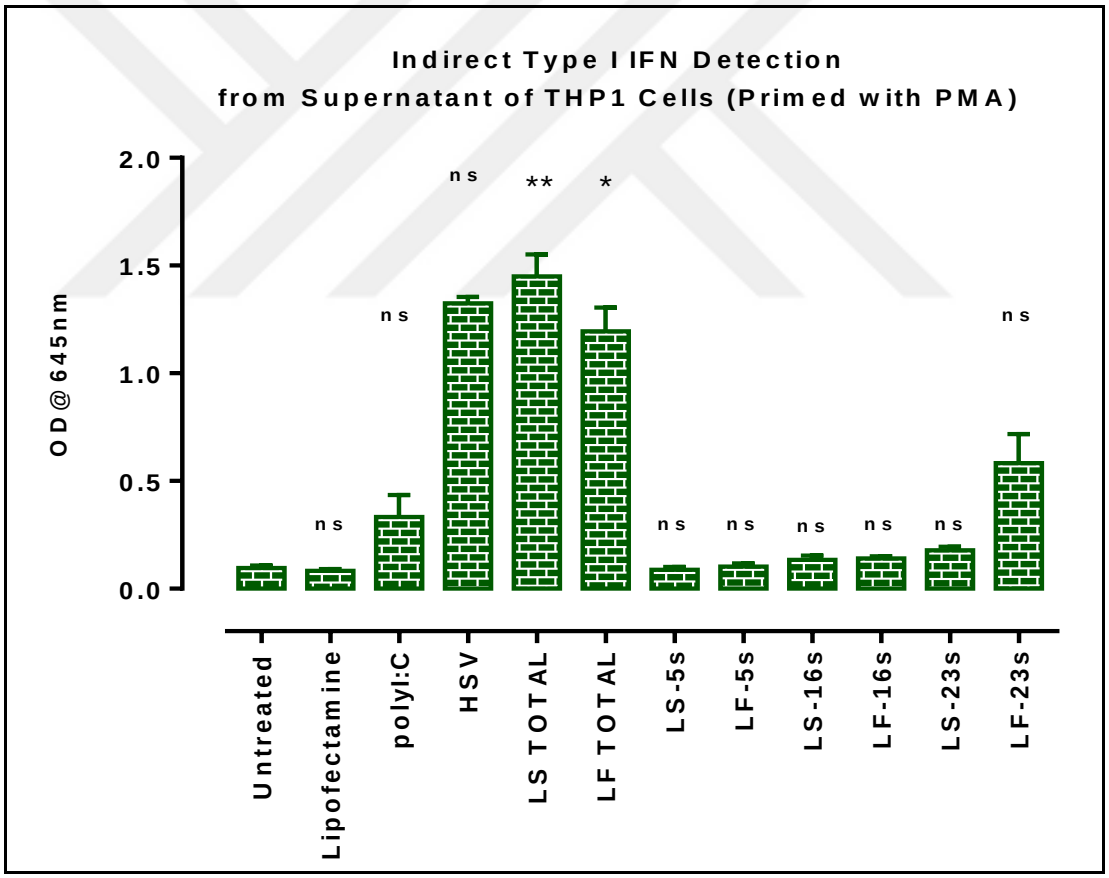

Figure 3.18 Quantification of IRF-induced SEAP production in PMA-primed THP1-Blue ISG cells upon stimulation with culture supernatant. After overnight priming with PMA (20ng/ml), THP1-Blue ISG cells stimulated with 20 µl of PMA-primed conventional THP1 culture supernatant, which was collected at 24 hour after stimulation with bacterial RNAs. 24 h after stimulation, 20 µl of culture supernatant of THP1-Blue ISG cells was mixed with 180 µl Quanti-Blue as a read out for SEAP. OD measurement was taken at 645 nm after 2 hours incubation at 37°C in the dark. Each experiment was performed two times. Kruskal-Wallis Dunn's test was conducted to compare each treatment groups to untreated group. (*p<0.05, **p<0.01, ***p<0.001)

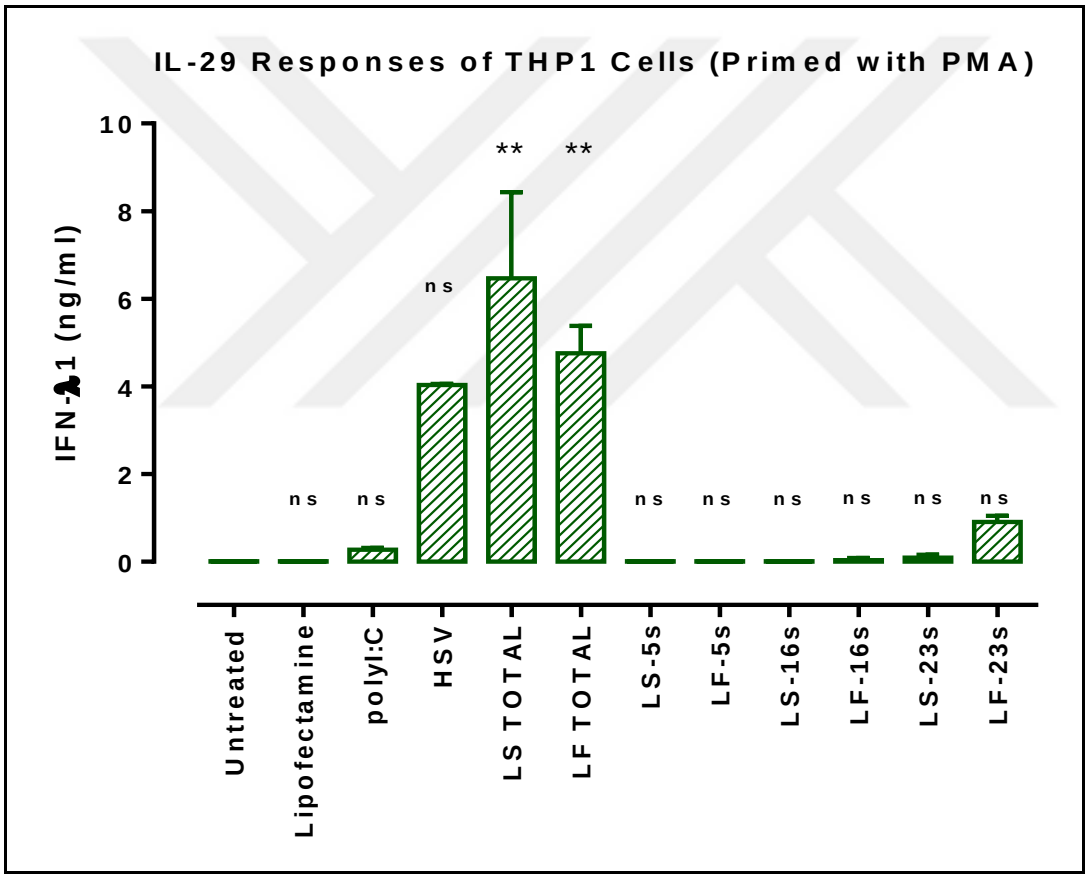

Figure 3.19 Type III IFN responses of conventional, PMA-primed THP1 cells upon stimulation with each RNA species. PMA-primed THP1 cells (5×10^5 cells/ml) were

stimulated with 1 μg/ml of RNA via transfection with Lipofectamine 2000. IFN-λ1 (IL-29) production was determined from culture supernatants collected 24 hour after stimulation using ELISA. Each experiment was repeated twice. Kruskal-Wallis Dunn's test was applied for each comparison. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

3.7 dsRNA Content Determination of Bacterial RNA

Overall results showed that commensal derived RNA activated interferon signaling pathway when they were delivered to cytosol. This indicates the involvement of cytosolic RNA sensors RIG-I and MDA-5. These sensors have important functions in IFN signaling and establishment of antiviral state. Since the ligands of RIG-I and MDA-5 are predominantly double-stranded RNAs, and commensal but not pathogen RNAs preferentially triggered type I interferons, we decided to assess dsRNA contents of commensal vs pathogen RNAs. To investigate the hypothesis that commensal RNAs might be enriched in dsRNA structures, we performed a J2-antibody (dsRNA specific antibody) utilizing-dot blot assay with bacterial RNAs derived from commensal and pathogens. PolyI:C was used as positive control since it is a synthetic double-stranded RNA while polyU was used as a negative control since it is a synthetic single-stranded RNA. Results showed that dsRNA content of commensal RNAs was significantly higher than those found in pathogens (Figure 3.20). This result might explain why commensal derived RNA samples can induce an IFN response while pathogen derived RNAs cannot (i.e detection of dominant dsRNA structures through the cytosolic sensors RIG-I and MDA5).

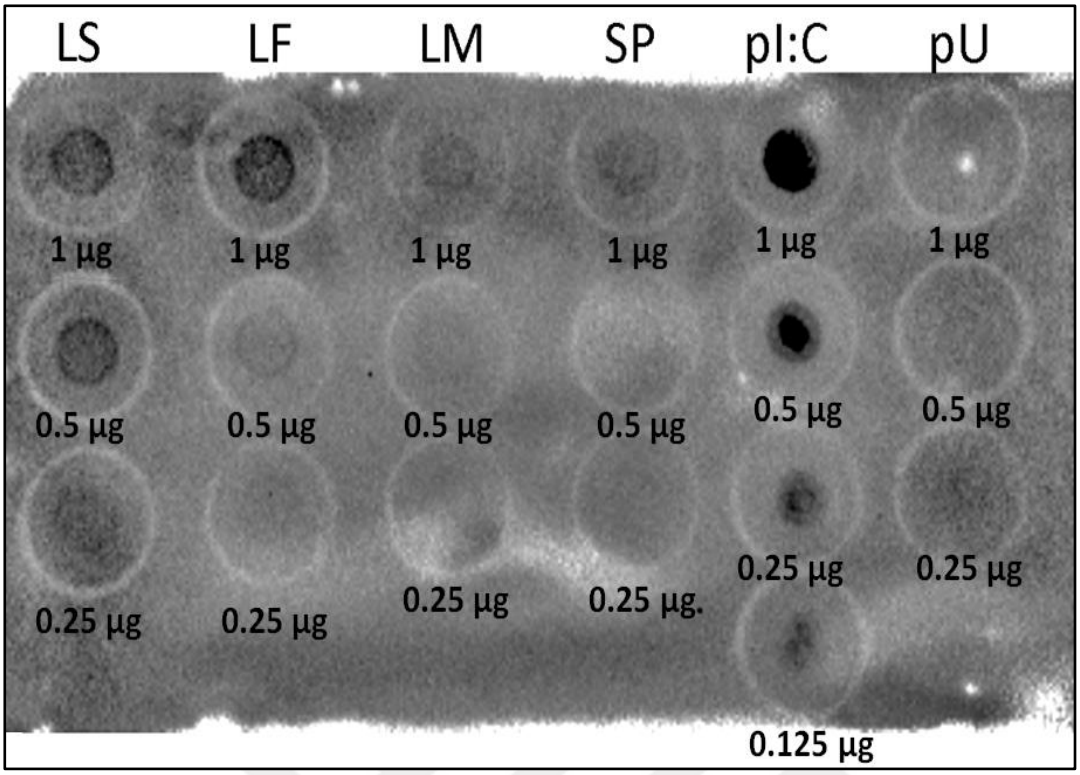

Figure 3.20 Comparison of double stranded RNA contents of commensal versus pathogen originated RNA samples. 1 µl of RNA samples including positive and negative controls were applied on nitrocellulose membrane in different concentrations. After UV crosslinking, membrane was treated with blocking buffer, J2 primary antibody and anti-IgG secondary antibody, respectively. Image was developed via ECL kit and Bio-Rad gel imaging system.

Furthermore, dsRNA contents of ribosomal RNAs (5S, 16S and 23S) of commensals were also assessed using the same assay. Since generally 23S showed stronger IFN responses than other rRNA species, it was expected that dsRNA content of 23S would be higher than the others. However, there was no such detectable difference when different rRNAs were compared with each other (Figure 3.21). These results suggest that in total RNA preparations, dsRNA secondary structures are enriched in commensal RNAs but such structures are destroyed when subunit species are physically separated. However, the ability of 23S rRNA to trigger modest levels of

type I IFNs might be independent of double strandedness and this subunit might possess a motif that could be recognized by an unidentified receptor.

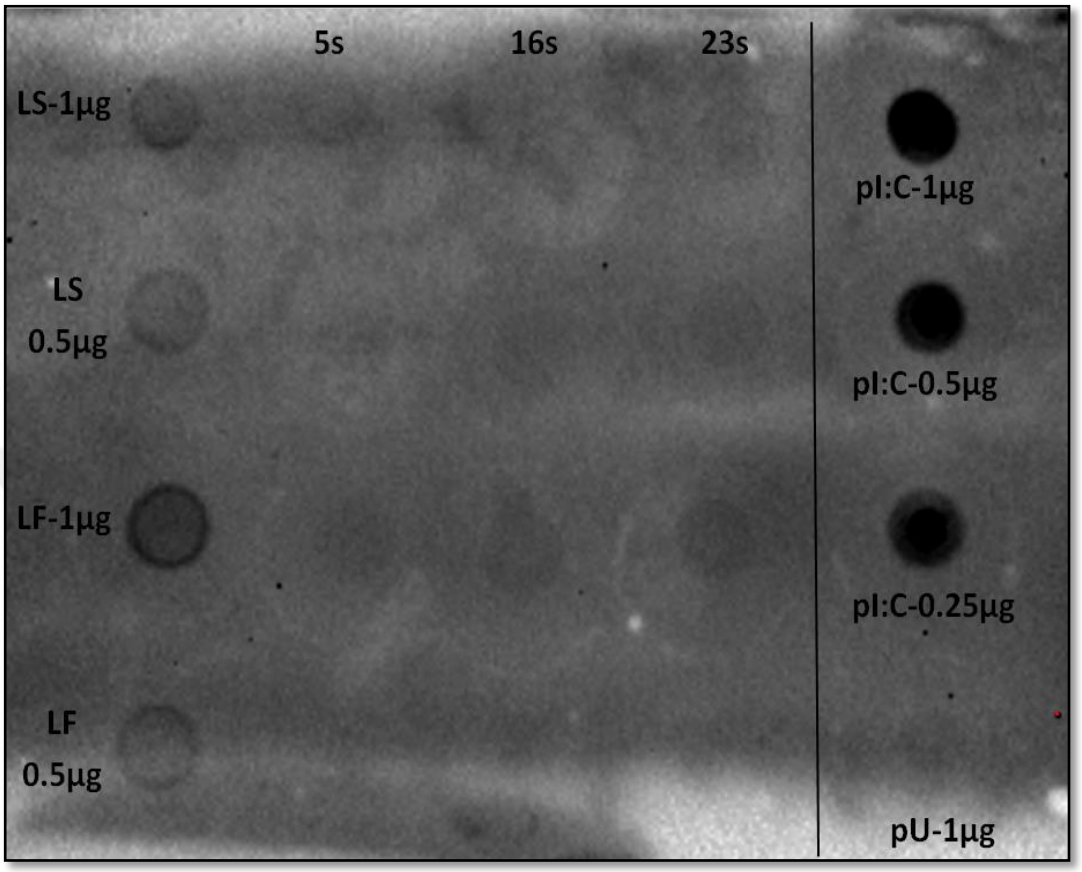

Figure 3.21 Comparison of double stranded RNA contents of commensal derived ribosomal RNA samples. 1 µl of RNA samples including positive and negative controls were applied on nitrocellulose membrane in different concentrations. After UV crosslinking, membrane was treated with blocking buffer, J2 primary antibody and anti-IgG secondary antibody, respectively. Image was developed via ECL kit and Bio-Rad gel imaging system.

CHAPTER 4

CONCLUSION

This thesis intended to investigate mechanisms contributing to differential recognition of the commensal versus pathogen derived RNA species and their abilities to initiate distinctive immune responses. For this, immunostimulatory activities of commensal versus pathogenic RNA species were tested in HEK-Blue cells expressing TLR3 or TLR7, hPBMCs and THP-1 cells.

AP-1/NF-κB activation was observed in HEK-Blue hTLR3 and HEK-Blue hTLR7 cells in response to pathogenic RNAs, but not to commensal RNAs. Production of TNF-α and IL-1β in response to pathogenic RNAs was more robust when compared to commensal RNAs. These results suggest that pro-inflammatory cytokine response is triggered predominantly by pathogen derived RNAs. Data further implicate that this response occurs in a TLR-dependent manner since NF-κB-dependent pro-inflammatory cytokine production occurs following recognition of RNAs by endosomal TLRs (TLR3, TLR7 and TLR8), but not by cytosolic RNA sensors (RIG-I and MDA-5).

When the immunostimulatory activities of individual ribosomal RNAs were tested, it was observed that among the three tested rRNAs, 5S rRNAs of pathogens induced AP-1/NF-κB activation in HEK-Blue hTLR3/hTLR7 cells while they failed to initiate TNF-α production from hPBMCs. Surprisingly, although 5S rRNAs of commensal origin had no detectable AP-1/NF-κB stimulating activity in HEK-Blue hTLR3 and hTLR7 expressing cell lines, they triggered TNF-α production in human PBMCs, although this response was not observed following stimulation with total RNAs of commensals. This finding might be explained by complexity of ligand

recognition and cell-type specific expression of TLRs. For example, the major TNF-α producing cells following bacterial RNA stimulation was the monocytes ($CD14^+$ cells), which express TLR8 but not TLR3 and TLR7. Commensal derived 5S rRNA might possess uridine or guanine rich degradation products that can be recognized via TLR8 whereas those found in pathogens might be recognized specifically by TLR3 and/or TLR7. The question as to why commensal derived total RNA fails to induce TNFα production from hPBMCs as opposed to the results obtained with purified 5S component could be due to the cross-regulation between type I IFNs and TNFα: Recent evidence suggests that IFNα can suppress TNFα by inducing and activating Axl, a receptor tyrosine kinase that induces the expression of a transcriptional repressor of the TNFα promoter (Sharif et al., 2006). Hence, the type I IFN inducing activity of total RNAs derived from commensals could suppress the TNFα stimulating activity found in the 5S component. In contrast to total commensal derived RNAs since the purified 5S rRNAs have no interferonogenic activity, such cross-regulation would not be observed, unmasking the TNFα stimulating activity of this subunit.

Although commensal derived total RNAs failed to induce NF-κB-dependent pro-inflammatory cytokine production, we found that they stimulated significant amounts of type I IFN production. Such a response was not observed with RNAs derived from pathogens. Type I IFN response was induced only when the commensal RNAs were delivered to the cytosol by means of Lipofectamine 2000 complexation, which suggests the involvement of cytosolic RNA sensors in this response. It has been recently shown that the antimicrobial peptide LL-37 can bind to dsRNAs released from necrotic cells and can deliver its associated cargo into the cytosol of immune cells, thereby aiding in recognition through the cytosolic RNA sensors RIG-I and MDA-5 (Zhang et al., 2016). Previous data from our lab had demonstrated that LL-37 could in fact replace Lipofectamine 2000 in experiments using commensal

derived RNAs (MSc thesis by Mine Ozcan). Physiologically, a similar mechanism could be involved in recognition of commensal derived RNAs in human gut. It was proposed that unidentified commensal-derived molecules were of importance in stimulating and activating type I interferon signaling and consequent establishment of anti-viral immunity (McAleer & Kolls, 2012). Herein, we show that commensal derived RNAs could be one of those unidentified commensal-derived molecules contributing to the abovementioned interferonogenic activity. Commensal derived RNAs might have an important function in the gut in triggering tonic levels of type I IFN signaling, which primes the immune system to fight against viral infections in a quick and efficient manner.

Our results further demonstrated that monocyte depletion from hPBMCs drastically diminished both type I and type III IFN response to bacterial RNA species isolated from commensals. This finding, coupled with our results demonstrating that the majority of IP-10 producers responding to stimulation with commensal derived total RNAs were monocytes, implicate that this cell population contributes to commensal-derived RNA recognition and interferon production. Human monocytes do not express TLR3 and TLR7 but they express the ssRNA sensor TLR8 and the dsRNA sensors RIG-I/MDA-5 (Bekeredjian-Ding et al., 2006, Broz & Monack, 2013), suggesting that these receptors might be of interest in recognition of commensal-derived RNAs. Our experiments in primed THP-1 cells, a treatment which is known to downmodulate TLR7/8 but upregulate the expression of RIG-I and MDA-5, demonstrated a significant increase in commensal-derived RNA-induced type I IFN production, implicating involvement of RIG-I and/or MDA-5 as the major sensors. Since RIG-I and MDA-5 specifically recognize dsRNA-rich sequences, we probed the dsRNA contents of pathogen and commensal-derived RNAs with an antibody that specifically binds to dsRNA. Results showed that dsRNA content of commensal RNAs was significantly higher compared to those found in pathogens. These

findings collectively suggest that cytosolic dsRNA sensors RIG-I and MDA-5 might in fact be the main receptors recognizing commensal RNAs and their subunits.

Of the tested commensal derived rRNA species, 23S rRNA induced very modest levels of type I interferon production in primed THP-1 cells whereas the 16S and 5S rRNAs had no such activity. However this response was very weak when compared to the response observed with total commensal RNA. To determine whether combinations of purified rRNAs could reconstitute the interferonogenic activity of total RNA, possible synergy between individual rRNAs was investigated. However, combination groups did not show any synergistic effect on type I IFN response, suggesting that involvement of other RNA species such as mRNA, tRNA or non-coding RNAs could be of major interest in establishment of this response. Furthermore, dsRNA contents of commensal derived rRNAs were also determined. Results showed that dsRNA contents of commensal rRNAs were lower than those of total RNAs and no significant differences were observed among the tested rRNAs. Data implicates that rRNAs, especially 23S rRNA of commensals might possess structural motifs, other than double stranded structures, that can be recognized by an unidentified receptor to initiate very low levels of type I IFNs. However, majority of interferon stimulating RNA species might be of non-ribosomal origin.

In conclusion, bacterial RNAs derived from commensal and pathogens are recognized by different sets of receptors initiating either a type I IFN or a pro-inflammatory cytokine dominated response, respectively. Further studies will concentrate on investigation of the interferon response to other RNA species such as mRNA and knockdown of RIG-I/MDA-5 in primary cells and/or monocyte cell lines.

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APPENDIX A

BUFFERS, SOLUTIONS AND CULTURE MEDIA

Phosphate Buffered Saline (PBS-10X) [pH 6.8]

80 grams NaCl
2 grams KCl
8.01 grams $Na_2HPO_4.2H_2O$
2 grams KH_2PO_4
Complete to 1 lt with dH_2O

Blocking Buffer (ELISA)

500 ml 1X PBS
25 grams BSA (5%)
250 µl Tween20 (0.025%)

ELISA Wash Buffer

500 ml 10X PBS
2.5 ml Tween20
4.5 lt dH_2O

T-cell Buffer (ELISA)

500 ml 1X PBS
25 ml FBS (5%)
250 µl Tween20 (0.025%)

FACS Buffer (PBS-BSA-Na Azide)

500 ml 1X PBS

5 grams BSA (1%)

125 milligrams Na Azide (0.25%)

MACS Buffer

500 ml 1X PBS

2.5 grams BSA (0.5%)

2 mM EDTA

Tris-Buffered Saline (TBS-10X)

24 grams Tris base

88 grams NaCl

900 ml dH_2O

Adjust pH to 7.2-7.4 with HCl, complete to 1 lt with dH_2O

Tris-Buffered Saline with Tween20 (TBS-T)

100 ml 10X TBS

900 ml dH_2O

1 ml Tween (0.1%)

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