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2016

**TLR2-dependent type I interferon induction
in human monocytes and its relevance
to *Staphylococcus aureus* infections**

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& IMMUNOLOGY

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**School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute,
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Abstract

Even though *Staphylococcus aureus* is a part of healthy microflora, it is also a significant human pathogen causing mild as well as life-threatening infections. Furthermore, an increasing incidence of antibiotic resistant strains within the community comprises a major health risk for patients worldwide. Although there is an extensive research on *S. aureus* pathogenicity and immune evasion, our understanding of protective immunity to *S. aureus* is limited. So far, attempts for vaccine development against *S. aureus* were not successful; hence, new insights into the interaction between immune cells and *S. aureus* are needed. Type I interferons (IFNs) are potent immunomodulators of both innate and adaptive immunity, with a well-defined anti-viral role, yet their role during bacterial infections remains less understood. In addition, studies in mice lacking type I IFN receptor showed contradictory results, especially for *S. aureus* infections. Toll-like receptor 2 (TLR2) is known to be the key receptor for recognition of Gram-positive bacteria, including *S. aureus*. *S. aureus* possesses a wide repertoire of immunostimulatory molecules, such as lipoteichoic acid (LTA), which triggers strong proinflammatory response. However, whether human TLR2 mediates type I IFNs in response to *S. aureus* remains unclear, as does the impact of type I IFNs on the outcome of *S. aureus* infection in human cells.

Our results revealed that both LTA and *S. aureus* stimulated stronger induction of type I IFNs in human monocytes compared to monocyte-derived macrophages. THP-1 monocytes were also more efficient at killing of *S. aureus* than THP-1-derived macrophages. In addition, we observed that bacterial killing and type I IFNs were TLR2-dependent and that stimulation with IFN α decreased killing capacity of THP-1 monocytes. In human primary monocytes, type I IFNs required endocytosis and endosomal maturation, and both type I IFNs and TNF α were dependent on Mal and MyD88 adaptors, the myddosome, TRAF6 ubiquitin ligase, and TAK1 kinase during *S. aureus* infection. Furthermore, *S. aureus*-stimulated type I IFNs were dependent on activity of both canonical IKK β and noncanonical TBK1/IKK ϵ , leading to transcription of IFN β . Surprisingly, throughout the experiments, we noted differences between Malp2 and LTA signaling, although these ligands are believed to similarly stimulate TLR2 in mouse cells. In addition, we investigated signaling components of other physiologically relevant cytokines, IL-1 β and IL-10, during *S. aureus* infection. Our data showed that LTA alone and *S. aureus* triggered IL-1 β release in a TLR2-dependent manner, whereas *S. aureus*-mediated IL-10 induction was TLR2-independent in human monocytes.

Overall, our results reveal that human monocytes have an intrinsic ability to kill *S. aureus* and to produce type I IFNs via a novel endosomal TLR2 pathway following bacterial infection. Furthermore, we suspect that type I IFNs might represent a mechanism by which *S. aureus* evades bacterial killing in human monocytes, and thus promote dissemination of the bacteria during infection. Lastly, IFN α might contribute to increased pathogenesis during bacterial superinfection after preceding viral infections.

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Table of Content

Declaration i

Abstract iii

Acknowledgement v

Table of Content.....vii

List of Figures xi

List of Tables.....xiv

List of Abbreviationsxv

1. Introduction..... 1

 1.1 The immune system 3

 1.2 The innate immune system..... 3

 1.2.1 Neutrophils..... 4

 1.2.2 Monocytes..... 4

 1.2.3 Macrophages..... 5

 1.2.4 Dendritic cells 6

 1.3 Pattern recognition receptors..... 6

 1.3.1 Toll-like receptors..... 7

 1.3.2 RIG-I-like receptors..... 15

 1.3.3 C-type lectin-like receptors 16

 1.3.4 NOD-like receptors..... 18

 1.3.5 AIM2-like receptors..... 19

 1.4 DNA sensors 21

 1.5 The adaptive immune system 23

 1.6 Mediators of immune responses 24

 1.6.1 Interleukins..... 24

 1.6.2 Chemokines 26

 1.6.3 Tumor necrosis factor alpha..... 27

 1.6.4 Type I interferons 30

 1.7 *Staphylococcus aureus* and its interaction with the immune system..... 34

 1.7.1 *Staphylococcus aureus* 34

 1.7.2 *S. aureus* and innate immunity 36

 1.7.3 *S. aureus* and adaptive immunity..... 44

 1.7.4 *S. aureus* and vaccine development..... 45

 1.8 Aims..... 47

2. Materials and Methods	49
2.1 Materials	51
2.1.1 General chemicals and plastic.....	51
2.1.2 Cells and cell culture	51
2.1.3 Bacteria and bacterial culture	53
2.1.4 siRNA and NEON® transfection system.....	54
2.1.5 Enzyme-linked immunosorbent assay (ELISA)	54
2.1.6 Quantitative real time polymerase chain reaction (qRT-PCR)	55
2.1.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis ..	55
2.2 Methods.....	56
2.2.1 Cell culture	56
2.2.2 Peripheral Blood Mononuclear Cells (PBMCs)	57
2.2.3 Bacterial culture	58
2.2.4 siRNA silencing	59
2.2.5 LDH assay	60
2.2.6 MTT assay.....	60
2.2.7 ELISA.....	60
2.2.8 HEK-Blue™ IFNα/β bioassay	61
2.2.9 qRT-PCR.....	62
2.2.10 Western Blot (WB) analysis.....	63
2.2.11 Statistical analysis	64
3. Result I: <i>Staphylococcus aureus</i> stimulates type I interferons in human monocytes.....	65
3.1 Introduction	67
3.2 Results.....	69
3.2.1 Immune responses to TLR2 ligands in human lung epithelial cells.....	69
3.2.2 Differentiation of human monocytes alters their responses to TLR ligands	75
3.2.3 Differentiation of human monocytes alters their responses to <i>S. aureus</i>	82
3.3 Discussion.....	91
3.3.1 Immune responses to TLR2 ligands in human lung epithelial cells.....	91
3.3.2 Differentiation of human monocytes alters their responses to TLR ligands	91
3.3.3 Differentiation of human monocytes alters their responses to <i>S. aureus</i>	92
4. Result II: The ability of THP-1 monocytes to kill <i>S. aureus</i>	95
4.1 Introduction	97
4.2 Results	99

4.2.1 <i>S. aureus</i> survival is affected by THP-1 differentiation	99
4.2.2 Blocking the endosomal pathway enhances killing of <i>S. aureus</i> in THP-1.....	
monocytes	104
4.3 Discussion.....	111
4.3.1 <i>S. aureus</i> survival is affected by THP-1 differentiation	111
4.2.2 Blocking the endosomal pathway enhances killing of <i>S. aureus</i> in THP-1.....	
monocytes	112
5. Result III: Dissection of <i>S. aureus</i>-mediated type I IFN pathway in human monocytes ...	115
5.1 Introduction	117
5.2 Results	121
5.2.1 TLR2 is required for IFN β and TNF α induction by <i>S. aureus</i> in human	
monocytes	121
5.2.2 TLR2-stimulated type I IFNs is dependent on the endosomal pathway	129
5.2.3 Role of adaptor proteins in TLR2-induced type I IFN pathway	141
5.2.4 Role of myddosome in TLR2-induced type I IFN pathway	149
5.2.5 TRAF6 and TAK1 are involved in TLR2-stimulated type I IFN induction.....	156
5.2.6 Role of canonical and noncanonical IKK kinases in TLR2-dependent type.....	
I IFNs	163
5.2.7 TLR2 activation upregulates IRF7 expression.....	172
5.3 Discussion.....	177
5.3.1 TLR2 is required for IFN β and TNF α induction by <i>S. aureus</i> in human.....	
monocytes	177
5.3.2 TLR2-stimulated type I IFNs is dependent on the endosomal pathway	178
5.3.3 Role of adaptor proteins in TLR2-induced type I IFN pathway	181
5.3.4 Role of myddosome in TLR2-induced type I IFN pathway	183
5.3.5 TRAF6 and TAK1 are involved in TLR2-stimulated type I IFN induction.....	184
5.3.6 Role of canonical and noncanonical IKK kinases in TLR2-dependent type.....	
I IFNs	186
5.3.7 TLR2 activation upregulates IRF7 expression.....	188
6. Result IV: Mechanisms of <i>S. aureus</i>-mediated IL-1β and IL-10 induction in human.....	191
monocytes	191
6.1 Introduction	193
6.2 Results	195
6.2.1 TLR2-mediated induction of IL-1 β in human monocytes.....	195

6.2.2 *S. aureus*-stimulated IL-10 induction in human monocytes 204

6.3 Discussion..... 212

6.3.1 TLR2-mediated induction of IL-1 β in human monocytes..... 212

6.3.2 TLR2-mediated induction of IL-10 in human monocytes..... 215

7. Final discussion 219

References..... 231

List of Figures

Figure 1.1: Mouse and human TLR signaling pathways.....	14
Figure 1.2: Human RLRs and CLRs signaling pathways.	17
Figure 1.3: Human NLRs and AIM2 inflammasome signaling pathways.....	20
Figure 1.4: Cytosolic DNA receptors involved in IFN β induction.	22
Figure 1.5: Human TNF α signaling.	29
Figure 1.6: Type I IFN signaling.	33
Figure 1.7: Recognition of <i>S. aureus</i> by innate immune system in mice and humans.	39
Figure 1.8: Phagocytosis and mechanisms of microbial killing.....	42
Figure 3.1: Responses to TLR ligands in human lung epithelial cells.	71
Figure 3.2: Responses of human lung epithelial cells to TLR ligands are time-dependent.	72
Figure 3.3: Human lung epithelial cells have limited responsiveness to TLR2 and TLR4.....	73
Figure 3.4: Responses to TLR ligands in THP-1 monocytes.....	74
Figure 3.5: PMA treatment of THP-1 monocytes alters their responses to TLR ligands.....	78
Figure 3.6: Differentiation of THP-1 cells alters their responses to TLR ligands.....	79
Figure 3.7: Differentiation of CD14+ monocytes alters their responses to TLR ligands.	80
Figure 3.8: Responses of CD14+ monocytes to purified <i>S. aureus</i> -LTA.	81
Figure 3.9: PMA treatment of THP-1 monocytes alters their responses during infection.....	85
Figure 3.10: Differentiation of CD14+ monocytes alters their responses during infection.....	86
Figure 3.11: Cytokine responses to live <i>S. aureus</i> in THP-1 monocytes.	87
Figure 3.12: Cytokine responses to live <i>S. aureus</i> in CD14+ monocytes.....	88
Figure 3.13: IFN β and TNF α mRNA induction in human monocytes.	89
Figure 3.14: TLR2, TLR4 and MD2 mRNA induction in human monocytes.	90
Figure 4.1: Killing of <i>S. aureus</i> by THP-1 cells is affected by differentiation.....	101
Figure 4.2: <i>S. aureus</i> can survive within THP-1-derived macrophages.....	102
Figure 4.3: Killing of <i>S. aureus</i> is TLR2-dependent in THP-1 monocytes.....	103
Figure 4.4: Inhibition of dynamin enhances killing of <i>S. aureus</i> in THP-1 monocytes.	107
Figure 4.5: Inhibition of Syk kinase enhances killing of <i>S. aureus</i> in THP-1 monocytes.	108
Figure 4.6: Blocking endosomal processing enhances killing of <i>S. aureus</i> in THP-1.....	109
Figure 4.7: Exogenous IFN α impairs killing of <i>S. aureus</i> in THP-1 monocytes.	110
Figure 5.1: TLR2 is required for LTA-induced type I IFN and TNF α responses in human.....	123
Figure 5.2: TLR2 is required for Malp2-induced type I IFN and TNF α responses in THP-1.....	124
Figure 5.3: TLR2 is required for <i>S. aureus</i> -induced type I IFN and TNF α responses in human.....	125
Figure 5.4: Response to TLR2 ligands is impaired in THP-1 TLR2 knockout cells.....	126
Figure 5.5: Responses to live <i>S. aureus</i> in THP-1 TLR2 knockout cells.	127
Figure 5.6: CD14 co-receptor is required for <i>S. aureus</i> -, LTA- and LPS-induced type I IFN.....	128
Figure 5.7: Inhibitors of the endosomal pathway and mechanism of their action.	132
Figure 5.8: Inhibition of dynamin impairs <i>S. aureus</i> - and LTA-induced type I IFN induction.....	133
Figure 5.9: Syk kinase is required for LTA-induced type I IFN induction in human.....	134
Figure 5.10: Syk kinase is not required for Malp2-induced type I IFN induction in THP-1.....	135

Figure 5.11: Syk kinase is required for <i>S. aureus</i> -induced type I IFN induction in human.....	monocytes.....	136
Figure 5.12: Inhibition of actin polymerization impairs <i>S. aureus</i> -induced type I IFN.....	induction in THP-1 monocytes.....	137
Figure 5.13: Endosomal processing is required for LTA-induced type I IFN induction in.....	human monocytes.....	138
Figure 5.14: Endosomal processing is required for <i>S. aureus</i> -induced type I IFN induction.....	in THP-1 monocytes.....	139
Figure 5.15: Endosomal processing is required for <i>S. aureus</i> -induced type I IFN and TNF α	induction in CD14+ monocytes.....	140
Figure 5.16: VIPER impairs LTA-induced type I IFN and TNF α responses in THP-1.....	monocytes.....	144
Figure 5.17: VIPER impairs LTA-induced type I IFN and TNF α responses in CD14+.....	monocytes.....	145
Figure 5.18: <i>S. aureus</i> -induced type I IFN and TNF α responses are VIPER sensitive.....	in human monocytes.....	146
Figure 5.19: Mal is required for LTA- and <i>S. aureus</i> -induced type I IFN and TNF α	responses in THP-1 monocytes.....	147
Figure 5.20: TRIF is required for Malp2-induced type I IFN in THP-1 monocytes.....		148
Figure 5.21: MyD88 is required for LTA-induced type I IFN and TNF α responses in THP-1.....	monocytes.....	151
Figure 5.22: IRAK2 is required for LTA-induced type I IFN and TNF α responses in THP-1.....	monocytes.....	152
Figure 5.23: IRAK-1/4 is required for LTA-induced type I IFN and TNF α responses in human.....	monocytes.....	153
Figure 5.24: IRAK-1/4 is not required for Malp2-induced type I IFN and TNF α responses.....	in THP-1 monocytes.....	154
Figure 5.25: IRAK-1/4 is required for and <i>S. aureus</i> -induced type I IFN and TNF α responses.....	in human monocytes.....	155
Figure 5.26: TRAF6 is required for LTA-induced type I IFN and TNF α induction in THP-1.....	monocytes.....	158
Figure 5.27: STING is not required for TLR2-induced type I IFN and TNF α induction in THP-1.....	monocytes.....	159
Figure 5.28: TAK1 is required for LTA-induced type I IFN and TNF α induction in human.....	monocytes.....	160
Figure 5.29: TAK1 is required for Malp2-induced type I IFN and TNF α responses in THP-1.....	monocytes.....	161
Figure 5.30: TAK1 is required for <i>S. aureus</i> -induced type I IFN and TNF α induction in.....	monocytes.....	162
Figure 5.31: IKK β kinase is required for LTA-induced type I IFN and TNF α responses.....	in human monocytes.....	166
Figure 5.32: IKK β kinase is required for <i>S. aureus</i> -induced type I IFN and TNF α responses.....	in monocytes.....	167
Figure 5.33: Distinct involvement of IKK-related kinases in LTA-induced responses.....	between THP-1 and CD14+ monocytes.....	168
Figure 5.34: Role for AMPKs in Malp2-induced type I IFN induction in THP-1.....	monocytes.....	169
Figure 5.35: Distinct involvement of IKK-related kinases in <i>S. aureus</i> -induced responses.....	between THP-1 and CD14+ monocytes.....	170
Figure 5.36: Inhibition of IKK-related kinases upregulates p65 phosphorylation in THP-1.....	monocytes.....	171

Figure 5.37: TLR2 activation upregulates IRF7 transcription THP-1 monocytes.....	174
Figure 5.38: TLR2 is required for LTA-stimulated induction of IRF7 protein in THP-1..... monocytes.....	175
Figure 5.39: IRF7 is required for TLR2 -induced TNF α in THP-1 monocytes.	176
Figure 5.40: Endosomal TLR2-dependent type I IFN induction in human monocytes.	190
Figure 6.1: TLR2 is required for LTA- and <i>S. aureus</i> -induced IL-1 β induction in human..... monocytes.....	197
Figure 6.2: IL-1 β response to TLR2 ligands and <i>S. aureus</i> is impaired in TLR2 knockout..... THP-1 monocytes.	198
Figure 6.3: Endosomal processing is not required for IL-1 β induction in response to..... <i>S. aureus</i> and LTA in human monocytes.	199
Figure 6.4: Role of Syk kinase in LTA- and <i>S. aureus</i> -stimulated IL-1 β induction in human..... monocytes.....	200
Figure 6.5: IL-1 β induction is VIPER sensitive in human monocytes.....	201
Figure 6.6: IL-1 β induction requires Ikk β in human monocytes.	202
Figure 6.7: IL-1 β induction in human monocytes does not require IKK-related kinases..... in THP-1 monocytes.	203
Figure 6.8: TLR2 is not required for <i>S. aureus</i> -induced IL-10 induction in human..... monocytes.....	206
Figure 6. 9: IL-10 response to TLR2 ligands and live <i>S. aureus</i> is not impaired in TLR2..... knockout cells.....	207
Figure 6.10: Inhibition of endosomal processing or Syk kinase activity impairs IL-10 induction.... in response to <i>S. aureus</i> in time-dependent manner in human monocytes.	208
Figure 6.11: IL-10 induction is sensitive to VIPER in CD14+ monocytes but not in THP-1..... monocytes.....	209
Figure 6.12: IL-10 induction requires Ikk β kinase in human monocytes.	210
Figure 6.13: IL-10 induction does not require IKK-related kinases in THP-1 monocytes.....	211
Figure 6.14: Mechanisms of <i>S. aureus</i> -mediated IL-1 β induction in human monocytes..... compared to mouse macrophages.	214
Figure 6.15: Mechanism of <i>S. aureus</i> -mediated IL-10 induction in human monocytes..... compared to mouse macrophages.	217
Figure 7.1: Malp2 and LTA signaling pathways in THP-1 monocytes.....	227

List of Tables

Table 1.1: Summary of human TLR receptors and their ligands..... 10

Table 1.2: Summary of expression and function of IRF family members. 31

Table 2.1: Common buffers and their composition. 51

Table 2.2: Overview of ligands and their receptors. 52

Table 2.3: IC50 (nM) values for inhibition of kinases by MRT67307 and MRT199665.....
compounds 53

Table 2.4: List of inhibitors, mechanism of action and supplier. 53

Table 2.5: Overview of siRNA sequences. 54

Table 2.6: Overview of primer sequences. 55

Table 2.7: List of phosphatase inhibitors. 63

List of Abbreviations

AIM2	Absent In Melanoma 2
Akt	Protein kinase B (PKB)
ALR	AIM2-like receptor
AMPK	AMP-activated protein kinase
AP1	Activator protein 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosin triphosphate
Bcl-10	B-cell lymphoma/leukemia 10
BCR	B-cell receptor
BIR	Baculovirus inhibitor repeats
BMDM	Bone marrow-derived macrophage
BRSK	Brain-specific kinase
CARD	Caspase activation and recruitment domain
Cas9	CRISPR-associated gene 9
Caspase	Cysteine-aspartic proteases
CBP	CREB-binding protein
CD	Cluster of differentiation
CFU	Colony-forming unit
cDC	Conventional DC
c-di-AMP	Cyclic diadenylate monophosphate
c-di-GMP	Cyclic diguanylate monophosphate
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
ClfB	Clumping factor B
CLR	C-type lectin receptor
CNS	Central nervous system
CREB	Cyclic AMP-responsive element-binding protein
CRISPR	Clustered Regularly Interspersed Palindromic Repeats
CTL	Cytotoxic T cell
DAI	DNA-dependent activator of IFN-regulatory factor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell

DC-SIGN	DC-specific intracellular adhesion molecule grabbing nonintegrin
DD	Death domain
DExD/H	Aspartate-Glutamate-any amino acid-Aspartate/Histidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECACC	European collection of animal cell cultures
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FLICE	FADD-like IL-1 β -converting enzyme
FLIP	FLICE inhibitory protein
FnBP	Fibronectin binding protein
FOXP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HEK	Human embryonic kidney
HIN	Hematopoietic IFN-inducible nuclear antigens with 200 amino acid repeat
Hla	Hemolysin alpha
HPV18	Human papillomavirus 18
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
IE-DAP	Meso-diaminopimelic acid
IFI16	IFN-inducible myeloid differentiation transcriptional activator
IFN	Interferon
IFNAR	Type I IFN receptor
I κ B	Inhibitor of κ B
IKK	I κ B kinase
IL	Interleukin
IP-10	IFN gamma-induced protein 10
IRAK	Interleukin-1 receptor-associated kinase
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element

ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LBP	Lipopolysaccharide binding protein
LGP2	Laboratory of genetics and physiology 2
LPDC	Lamina propria DC
LPS	Lipopolysaccharide
LRR	Leucine-rich motif
LTA	Lipoteichoic acid
LukAB	Leukocidin AB
Mal	MyD88-adaptor-like protein (TIRAP)
Malp2	Macrophage-activating lipopeptide 2
MALT	Mucosa associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MARK	Microtubule affinity-regulating kinase
MAVS	Mitochondrial antiviral signaling protein
MCP	Monocyte chemoattractant protein
MD2	Myeloid differentiation factor 2
MDA5	Melanoma differentiation-associated protein 5
mDC	Myeloid DC
MDP	Muramyl dipeptide
MELK	Maternal embryonic leucine zipper kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MNDA	Myeloid cell nuclear differentiation antigen
MR	Mannose receptor
mRNA	Messenger RNA
MRSA	Methicillin-resistant <i>S. aureus</i>
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
mTOR	Mechanistic target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP1	NAK-associated protein 1

NEMO	NFκB essential modifier
NET	Neutrophil extracellular trap
NFκB	Nuclear factor κB
NIK	NFκB inducing kinase
NK	Natural killer
NKT	Natural killer T cell
NLR	NOD-like receptor
NLRP	NOD-like receptor protein
NLS	Nuclear localization sequence
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NUAK	NUAK family SNF1-like kinase
ODN	Oligodeoxynucleotide
Pam3Csk4	S-(2,3-bispalmitoyloxypropyl)-N-palmitoyl-Cys-Lys-Lys-Lys-Lys
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PGN	Peptidoglycan
PI3K	Phosphatidylinositol 3 kinase
pIRF	Phosphorylated IFN regulatory factor
PKC	Protein kinase C-δ
PMN	Polymorphonuclear cell
poly(dA:dT)	Polydeoxyadenylic:polydeoxythymidylic acid sodium salt
poly(I:C)	Polyinosinic:polycytidylic acid
POP3	Pyrin only protein 3
PVL	Panton-Valentine leukocidin
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain family member 1
qRT-PCR	Quantitative Real Time PCR
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene 1
RIP	Receptor-interacting protein

RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SARM	Sterile α - and armadillo-motif-containing protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SIK	Salt-inducible kinase
siRNA	Small interfering RNA
STING	Stimulator of IFN genes
STX	Staphyloxanthin
Syk	Spleen tyrosine kinase
TAB	TAK1-binding protein
TAK1	TGF β -activated kinase 1
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TGF β	Transforming growth factor beta
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
TNFR	TNF receptor
TRADD	TNFR-associated death domain
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule (TICAM2)
TRIF	TIR-domain-containing adaptor protein inducing IFN β (TICAM1)
UNC93B1	Unc-93 homolog B1
VACV	Vaccinia virus
VIPER	Viral inhibitory peptide of TLR4
VRSA	Vancomycin-resistant <i>S. aureus</i>
VSV	Vesicular stomatitis virus
WTA	Wall teichoic acids

1. Introduction

1.1 The immune system

Immunity is the ability of an organism to fight invading pathogens and to maintain intrinsic homeostasis. The immune system has developed into a complex network that can detect pathogens and distinguish them from the body's own tissues. However, pathogens are able to adapt quickly to avoid detection and neutralization by the components of the immune system. Hence, selective pressure of host-pathogen coevolution gave rise to multiple levels of the host immune defense mechanisms. The vertebrate immune system evolved into specific and nonspecific arms [1]. The nonspecific components act either as physical barriers or as nonspecific eliminators of pathogens. The specific components, on the other hand, adapt to the pathogens and generate pathogen-specific immunity, thus competing with the evolution of pathogens on the cellular level. Classically, the immune system is divided into innate and adaptive immunity [1]. Typically, the innate immune system was viewed as a primitive and non-specific defense mechanism that operates before the more complex adaptive immune response could take over. In recent years, however, there has been a great focus on understanding innate as well as adaptive immune mechanisms and the latest discoveries have changed historical views on the role of the innate immune system. The innate immunity has emerged as a critical regulator of immune responses detecting and eliminating invading pathogens, recruiting immune cells to the site of infection as well as priming cells of the adaptive immune system [1].

1.2 The innate immune system

The innate immune system is the first line of defense, which initiates rapid protection against pathogens. However, it does not render long-term immunity against the same pathogen. Invading pathogens are first met with physical, chemical and biological barriers including skin, mucus containing enzymes, low pH and gut microflora, and the complement cascade, which enhances the recognition of the pathogens. Innate immune responses are mediated by cells of both hematopoietic and non-hematopoietic origin such as monocytes, macrophages, epithelial and endothelial cells, respectively. The effector cells of the immune system are derived from a common hematopoietic progenitor cell and its maturation gives rise to cells of both innate and adaptive immunity. The cell-mediated innate immunity is mainly dependent on phagocytes that are capable of ingestion of foreign particles, bacteria and dead cells. Phagocytes can be "professional" and "non-professional" depending on their biological ability to detect and to ingest bacteria [2]. The professional phagocytes include neutrophils, monocytes, macrophages and dendritic cells, and they actively migrate to the site of infection, ingest opsonized bacteria, present an antigen to T cells and trigger the adaptive immune

response. On the other hand, the non-professional phagocytes such as epithelial, endothelial cells and fibroblasts have limited ability of migration and phagocytosis but are able to produce cytokines in response to pathogens. Therefore, both non-professional and professional phagocytes can be involved in mediating the innate immune responses. In our study, we focus on events during the early innate immune response, which involves recognition of pathogens and initial production of cytokines.

1.2.1 Neutrophils

Neutrophils, basophils and eosinophils form a family of polymorphonuclear cells (PMNs) characterized by their distinctive lobed nuclei and the presence of granules in their cytoplasm. Neutrophils are the most abundant type of leukocyte cells in human blood. They are short-lived, highly mobile and during the acute phase of infection, they are the first inflammatory cells in the site of infection. During the early stages of bacterial infection, neutrophils immediately migrate through the blood vessels to the site of infection following chemotactic signals such as IL-8 or CCL5. Neutrophils are well equipped for the fight with pathogens through phagocytosis, release of granules containing antimicrobial enzymes and generation of neutrophil extracellular traps. In 2004, Brinkmann and colleagues observed that activation of neutrophils caused the release of web-like structures of DNA, which they termed neutrophil extracellular traps (NETs) [3]. The NETs were made of chromatin fibres and serine proteases that trapped and killed extracellular microbes independently of phagocytosis. Hence, NETs might serve an important barrier preventing the spread of extracellular pathogens. In addition, neutrophils produce large amounts of cytokines, which stimulates phagocytic activity of monocytes and macrophages and intracellular killing of bacteria [4].

1.2.2 Monocytes

Monocytes are derived from monoblastic cells that differentiate from hematopoietic stem cells in the bone marrow. Monocytes circulate in the bloodstream, from which they eventually migrate to other tissues and differentiate into tissue-resident macrophages or dendritic cells. The main duties of monocytes, monocyte-derived macrophages and dendritic cells are phagocytosis of pathogens, antigen presentation and activation of adaptive immunity, and lastly production of inflammatory cytokines such as tumor necrosis factor alpha (TNF α) and interleukins (ILs). It has been established that monocytes can be categorized into three groups according to the expression of cell surface markers [5]. The classical monocytes are characterized by high expression of CD14 receptor (CD14⁺⁺CD16⁻). The intermediate monocytes show high expression of CD14 and low expression of CD16 receptor (CD14⁺⁺CD16⁺).

Lastly, the non-classical monocytes display low expression of CD14 but have high expression of CD16 receptor (CD14⁺CD16⁺⁺).

The classical monocytes (CD14⁺⁺CD16⁻) are considered as inflammatory mediators. They are characterized by additional expression of CCR2, and their migration is strongly dependent on MCP-1 secreted by resident macrophages [6]. The non-classical monocytes (CD14⁺CD16⁺⁺) express high levels of CX3CR1 but do not express CCR2 or CD62L and they depend on CX3CL1 (also known as fractalkine) for their attraction and recruitment to the endothelial surfaces [7]. Cros and colleagues suggested that non-classical monocytes were patrolling the blood vessels and were involved in the innate surveillance of tissues [8]. Interestingly, non-classical monocytes are weak phagocytes and cytokine producers in response to cell-surface Toll-like receptors (TLR). Instead, they selectively produce proinflammatory cytokines in response to viruses and nucleic acids via endosomal TLR7/8 [8]. The intermediate monocytes (CD14⁺⁺CD16⁺) have the highest expression of major histocompatibility complex (MHC) class II molecules, whereas non-classical monocytes are characterized by high expression of cytoskeletal re-arrangement genes, inflammatory cytokines, and CD294 [6]. In addition, intermediate monocytes express receptors involved in reparative processes and they are abundantly present in the bone marrow [9]. Hence, blood monocytes are a mixture of at least three subpopulations with distinct roles in the immune system. However, due to confusions in the nomenclature of monocytes, it is still difficult to characterize individual subsets of monocytes.

1.2.3 Macrophages

Macrophages are derived from progenitor monocyte cells, which express the cell surface marker CD14. After activation, monocytes migrate to the designated tissue and differentiate into tissue-specific resident macrophages that express a wide variety of immune receptors including pattern recognition receptors. Apart from their function in phagocytosis and cytokine production, activated macrophages also act as antigen presenting cells (APCs). APCs are essential for an efficient adaptive immune response since the function of CD4⁺T cells depends on APCs. Processed antigen is presented on the surface of APCs via MHC class II molecules and is recognized by T cell receptor (TCR) on the surface of CD4⁺ T cells. The interaction between the antigen and TCR together with expression of additional co-stimulatory molecules activates CD4⁺ T cell function and thus adaptive immune response. Macrophages can be categorized into three types of macrophages based on their cytokine profile. M1 macrophages are conventionally activated macrophages that secrete high levels

of proinflammatory cytokines such as TNF α , IL-12, and IL-23, which modulate Th1 and Th17 cell responses. In addition, M1 macrophages produce large amounts of the inflammatory cytokine IL-1 β . M2 macrophages, on the other hand, produce anti-inflammatory cytokines that recruit Th2 and regulatory T cells to the site of infection. M2 macrophages are also involved in wound healing and tissue repair by production of metalloproteinases and growth factors. During the later stages of infection, macrophages can develop into regulatory macrophages and produce anti-inflammatory IL-10, which antagonizes the proinflammatory cytokines and reduces the inflammation [1].

1.2.4 Dendritic cells

Similar to macrophages, dendritic cells (DCs) are also derived from monocytes. DCs are highly specialized APCs, which are characterized by the expression of the cell surface marker CD11c. DCs also express a wide range of pattern recognition receptors on their cell surface and in the endosomes. Recognition of pathogen by naïve DCs leads to their maturation and migration to the lymph nodes. Mature DCs express MHC class II as well as a variety of co-stimulatory molecules, all of which are required for activation of CD4⁺ T cells. After activation, T cells differentiate into helper and cytotoxic T cells, which further modulate adaptive immune responses. B cells, on the other hand, express B cell receptor (BCR), which mediates recognition of antigen and its maturation into antibody-producing plasmacytoid cells, or into memory B cells. DCs can be divided into two subtypes. Conventional DCs (cDCs) function as the main APCs expressing Toll-like receptor (TLR) 2 and TLR4 and producing high amounts of IL-12, which matures T cells into Th1 cells [10]. Plasmacytoid DCs (pDCs) are characterized by expression of TLR7 and TLR9, by which they recognize nucleic acids [11]. It has been shown that pDCs constitutively express interferon regulatory factor 7 (IRF7). Therefore, upon recognition of nucleic acids, pDCs rapidly respond by production of antiviral effector proteins known as type I interferons (IFNs) [11].

1.3 Pattern recognition receptors

The innate immune response is triggered by recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). PRRs are germline-encoded receptors that are abundantly expressed by immune as well as non-immune cells. PAMPs are evolutionary conserved components of pathogens that are essential for their survival but are distinct from “self” [1]. PAMPs include bacterial carbohydrates (lipoteichoic acid, lipopolysaccharide), proteins (flagellin) and nucleic acids (viral dsRNA, bacterial DNA). Recently, a new class of antigens was described and termed “damage or danger-associated

molecular patterns" (DAMPs). DAMPs are cellular components, such as uric acid or heat shock proteins, released during cell damage, which act as endogenous stress signals [12]. Even though the repertoire of structures recognized by PRRs is limited, immediate recognition and protection delays the spread of pathogens before an adaptive response is mobilized. Upon recognition of PAMPs, PRRs trigger a signaling cascade that leads to the activation of two major classes of transcription factors: nuclear factor κ B (NF κ B) and IRFs, which initiate transcription of immune genes. Whereas NF κ B is a master regulator of proinflammatory responses with a role in induction of proinflammatory cytokines, IRFs provide an antiviral response by inducing type I IFN production.

Since the first hypothesis proposing the existence of PRRs was made by Janeway in 1989 [13], there has been an increasing body of research that resulted in the discovery of at least five PRR subfamilies. PRR families consist of Toll-like receptors (TLRs) [14], RIG-I-like receptors (RLRs) [14], C-type lectin-like receptors (CLRs) [15], NOD-like receptors (NLRs) [14], and lastly AIM2-like receptors (ALRs) [16].

1.3.1 Toll-like receptors

Toll-like receptors (TLRs) are prototype PRR recognizing both PAMPs and DAMPs. They are the most conserved components of the immune system since their homologs can be found in genomes across all three kingdoms of life. TLRs and Interleukin-1 receptors (IL-1R) share a Toll-IL-1 receptor (TIR) domain and they form a family known as the IL-1R/TLR superfamily. IL-1R type 1 was the first member identified in 1980 [17]. In 1991, the cytosolic domain of IL-1R was shown to be homologous to *Drosophila melanogaster* protein Toll [18]. While the inflammatory effects of IL-1 have been already shown, the only known function for Toll was its role in promoting dorsoventral polarity during embryo development [19]. Later, IL-1R and Toll were shown to have a common amino acid motif essential for NF κ B binding, suggesting that this signaling cascade might be used in the development of fly embryo as well as in the proinflammatory responses in mammals [20].

TLRs are transmembrane proteins located on the plasma membrane or in the endosomal compartments. They consist of three domains: an extracellular leucine-rich repeat domain, a transmembrane domain comprising ~20 uncharged amino acids and an intracellular TIR domain. The TIR domain mediates the interaction with cytosolic adaptor proteins, which trigger the signal transduction. The extracellular domain contains 22-29-residue long tandem copies of hydrophobic leucine-rich motifs (LRRs) which form a three dimensional loop [21]. Upon activation of TLRs with the ligands, TLR proteins form an m-shaped hetero- or homo-

dimers bringing the two intracellular domains into the close proximity, which triggers the downstream signaling cascade via their respective adaptor proteins [21].

1.3.1.1 TLRs in humans and mice

Most mammals have between ten to fifteen TLRs. So far, ten TLRs have been identified in humans, thirteen in mice and homologs to some of these TLRs have been found in other mammalian species. Mice express additional TLRs 11, 12 and 13 that are not present in the human genome; however, their TLR10 gene was disrupted by insertion of a retrovirus, leading to the creation of a pseudogene. Consequently, murine TLRs might have redundant or unique functions; therefore, the translation of the findings from mice to humans can be limited.

Human TLR2 plays an important role in innate immunity. TLR2 recognizes a wide range of PAMPs derived from various pathogens including bacteria, fungi, parasites or viruses, and upon recognition of its ligand, it induces strong proinflammatory responses. TLR2 forms a heterodimer with TLR1 to recognize triacylated lipopeptides such as S-(2,3-bisphosphatidyl)-N-palmitoyl-Cys-Lys-Lys-Lys-Lys (Pam3Csk4) produced by *E. coli* and other Gram-negative bacteria. Alternatively, TLR2 can heterodimerize with TLR6 to recognize diacylated lipopeptides, including macrophage-activating lipopeptide 2 (Malp2) found in mycoplasma and lipoteichoic acid (LTA) present in the cell wall of Gram-positive bacteria. Additionally, TLR2 can form heterodimers with non-TLR molecules such as CD14, CD36, and dectin-1 and recognize other structures. CD36, a class II scavenger receptor, is expressed on the surface of immune cells acting as co-receptor for TLR2-TLR6 dimers and enhancing the proinflammatory response [22]. CD14, a glycosylphosphatidylinositol (GPI)-linked protein-containing LRRs, has been involved in the recognition of diacylated lipopeptides and acts synergistically with TLR2. Dectin-1, a C-type lectin, binds fungal β -glucan and induces proinflammatory responses in cooperation with TLR2 [23]. In addition, TLR2 alone or as a dimer with as yet unknown molecules can recognize other ligands including peptidoglycan (PGN) from Gram-positive bacteria, porin from *Neisseria*, zymosan from *Saccharomyces*, GPI-mucin from *Trypanosoma*, GPI from *Plasmodium*, and hemagglutinin from measles virus [24]. TLR2 is expressed by immune cells, including monocytes, macrophages, dendritic cells, neutrophils, B and T cells as well as by non-immune cells such as epithelial cells in the respiratory tract, renal corpuscles and keratinocytes.

TLR4 is a crucial receptor in immune responses to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. It has been shown that TLR4 itself binds LPS weakly but once TLR4 forms a complex with MD2, MD2 potentiates its

signaling and induces a strong inflammatory response to LPS [25]. In addition, LPS-binding protein (LBP) detects LPS and binds to the TLR2-MD2 complex via CD14 co-receptor, thus initiating TLR4 signaling [26]. Apart from LPS, TLR4 has been implicated in the recognition of respiratory syncytial virus (RSV) and mouse mammary tumor virus envelope proteins [24]. TLR4 is expressed mainly in monocytes and placenta. TLR5 recognizes conserved residues in flagellin, a major component of bacterial flagella [27]. TLR5 is expressed by intestinal epithelial cells, thus indicating its role in gut protection. Accordingly, high expression levels of TLR5 have been found in lamina propria DCs (LPDCs). In addition, TLR5 has been implicated in IgA production by LPDCs and differentiation of Th1 and Th17 cells, thus linking innate and adaptive immune responses [28]. TLR10 is the last identified cell-surface TLR family member yet without a defined function. TLR10 is located in the same genomic cluster as TLR1 and TLR6, all of which probably arose from a duplication event [29]. It has been proposed that TLR2 subfamily evolved under a purifying selection; hence, maintenance of TLR10 might imply its distinct and important function [30]. TLR10 has been shown to recognize triacylated lipopeptides and to share a wide range of agonists with TLR1 but not with TLR6 [31]. Similar to TLR1 and TLR6, TLR10 requires TLR2 for ligand recognition and downstream signaling. TLR10-TLR2 heterodimer has been shown to physically interact in phagosomes and recruit MyD88 [31]; however, the exact downstream signaling remains unknown. TLR10 is highly expressed in B cells, suggesting its role in B cell function.

TLR3, TLR7, TLR8 and TLR9 are all expressed in endosomes where they recognize viral nucleic acids and trigger type I IFNs as well as proinflammatory responses. TLR3 recognizes double stranded RNA (dsRNA), a major component of many viruses including reovirus and RSV, and synthetic polyinosinic:polycytidylic acid (poly(I:C)) [24]. TLR3 is expressed by conventional DCs (cDCs) and macrophages as well as by non-immune cells such as fibroblasts and epithelial cells [24]. Upon activation, TLR3 triggers transcription of both type I IFNs and proinflammatory cytokines. In some cases, however, TLR3-mediated responses have been shown to mediate pathogenesis rather than protection during infection with viruses, such as influenza A [32]. TLR7 recognizes derivatives of imidazoquinoline (R848) and thiazoloquinoline (CL075). TLR7 is highly expressed by plasmacytoid DCs (pDCs) and it rapidly triggers type I IFN response in response to viral infection [33]. TLR8 is phylogenetically the most related to TLR7. While TLR7 recognizes viral single stranded RNA (ssRNA) derived from VSV and influenza A virus, TLR8 recognizes both viral and bacterial ssRNA [34]. Human TLR8 is expressed in various tissues and is found to be upregulated upon bacterial infections in monocytes. Even though murine TLR8 has been long believed to be nonfunctional, a recent study suggested that TLR8

had an important role in regulation of mouse TLR7 expression and prevention of spontaneous autoimmunity [35]. TLR9 recognizes CpG-rich hypomethylated DNA motifs, which are present in bacteria and viruses but are rare in mammals [36]. TLR9 is expressed by pDCs, which are the main source of type I IFNs in response to DNA viruses such as HSV [37], or to synthetic CpG oligodeoxynucleotides (ODNs). In addition to human TLRs, mice express TLR11, TLR12 and TLR13. Whereas mouse TLR11 has been implicated in the recognition of uropathogenic bacteria [38], a TLR11-TLR12 heterodimer has been shown to detect profilin from *Toxoplasma gondii* [39]. Lastly, mouse TLR13 has been reported to specifically recognize ribosomal RNA from *Streptococcus pyogenes* in endosomes [40].

Collectively, TLRs are the most versatile PRRs sensing a wide repertoire of PAMPs (Table 1.1). TLRs are one of the first receptors that recognize invading pathogens and consequently contribute to activation of adaptive immunity. Accordingly, polymorphisms in TLRs and downstream signaling components have been associated with susceptibility to infections, predisposition to inflammatory diseases and vulnerability to cancer [41].

TLR	Location	Ligand	Prototype ligand
TLR2-TLR1	cell surface	triacylated lipopeptide	Pam3Csk4
TLR2-TLR6	cell surface	diacylated lipopeptide	Malp2
TLR2-TLR10	cell surface/endosome	triacylated lipopeptide	N/A
TLR2-dectin 1	cell surface	β -glucan	zymosan
TLR4	cell surface/endosome	LPS	LPS
TLR3	endosome	dsRNA	poly(I:C), poly(A:U)
TLR7/8	endosome	ssRNA, imidazoquinoline	R848, CL075
TLR9	endosome	CpG DNA	CpG-ODN

Table 1.1: Summary of human TLR receptors and their ligands. N/A – not available.

1.3.1.2 TIR adaptor proteins

TLR signaling involves a family of five TIR-domain-containing adaptor proteins, which transmit signals from TLRs to downstream kinases that ultimately trigger the activation of transcription factors such as NF κ B and IRFs. TLR signaling is transduced via TIR-TIR domain interactions between the cytosolic domain of TLRs and the adaptor proteins. TIR adaptor proteins include myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like protein (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2) and sterile α - and armadillo-motif-containing protein (SARM). The adaptor proteins are selectively recruited to their respective TLR and induce signaling cascades depending on the type of TLR dimer after PAMP encounter. MyD88 is utilized by all TLRs except for TLR3, and drives NF κ B as well as IRF activation. Upon activation of a TLR receptor,

MyD88 mediates assembly of a signaling complex termed the myddosome, which facilitates activation of downstream kinases. The myddosome is composed of six MyD88 molecules, four molecules of IRAK4 and four IRAK1 or IRAK2 molecules, and is self-assembled via interaction among their death domains. Mal is recruited to TLR2 and TLR4, and functions as a sorting adaptor for MyD88. TRIF is used by TLR3 as its only adaptor protein, and by TLR4, and leads to the activation of IRF3, NFκB and MAPK induction. TRAM serves as a link between TLR4 and TRIF; however, there is recent evidence for the role of TRAM and TRIF in TLR2 signaling [42, 43]. Recently, Kagan (2012) proposed the term “sorting adaptors” for Mal and TRAM adaptor proteins. These adaptors contain a localization domain, which places them to the site of signal transduction prior to any microbial encounter, consequently, the sorting adaptors define the signaling pathways activated from the specific location [44]. Lastly, SARM is the most evolutionary conserved adaptor protein of all five; however, our understanding of its function is still limited. In mouse, SARM has been associated with stress-induced neuronal cell death and immune responses in the CNS [45]. Additionally, SARM has been linked to the cell death of mouse axons [46] and to mitochondria-induced cell destruction in sensory neurons [47]. Recently, it has been reported that SARM is required for RANTES RNA transcription after TLR and nucleic acid sensors in mouse macrophages, suggesting its role in immune responses [48]. In humans, SARM has been identified as a negative regulator of TRIF-dependent signaling, transcription factor activation and gene induction [49]. Collectively, these findings demonstrate distinct roles of SARM in TLR signaling compared to the other TIR adaptors.

TLR4 signals through both MyD88- and TRIF-mediated pathways by recruiting Mal and TRAM adaptors to the receptor [Fig. 1.1]. In resting cells, MyD88 and TRIF are scattered in the cytosol, whereas Mal and TRAM are attached to the plasma membrane consistent with their function as sorting adaptors [50]. Upon LPS recognition, Mal interacts with TLR4 and initiates signaling by delivering MyD88 to TLR4, which leads to NFκB and p38 mitogen-activated protein kinase (MAPK) activation [51]. Subsequently, a TRAM myristoylation site is phosphorylated by protein kinase Cε, thus releasing TRAM from the plasma membrane [52]. TRAM then binds to TRIF, triggers translocation of the TLR4 signaling complex into endosomes, and activates IRF3 and the second wave of NFκB and MAPK signaling [53].

TLR7/8 and TLR9 signaling pathways were believed to require only MyD88 adaptor until recently, Bonham and colleagues demonstrated that Mal was required in signaling from both surface and endosomal TLRs apart from TLR3 [54]. Upon TLR activation, Mal is directed to different organelles according to the lipid targets on its phosphoinositide-binding domain

and triggers assembly of myddosome [54]. In addition, it has been shown that TRAM has a role in TLR7 signaling and its myristoylation is required for TLR7-mediated gene induction [55]. Collectively, these new findings clearly demonstrate that our understanding of TLR signaling is still limited. The posttranslational modifications can license adaptors for novel interactions and functions; hence, the relation between receptor and adaptor is more complex than we thought.

1.3.1.3 TLR-mediated signaling pathways

Classically, TLR-stimulated immune pathways are divided into MyD88-dependent (TRIF-independent) and MyD88-independent (TRIF-dependent). The TIR domain of MyD88 has been shown to interact with the intracellular TIR domain of every TLR except for TLR3, whereas the MyD88 death domain (DD) interacts with the DD of IL-1 receptor associated kinases (IRAKs) [56]. Upon activation of a TLR receptor, MyD88 DD interacts with IRAKs DD, thus forming the myddosome. The myddosome mediates phosphorylation of IRAK1 and IRAK2 by IRAK4 inducing their autophosphorylation activity [57]. The phosphorylation of IRAKs results in dissociation of IRAKs from the receptor complex and subsequent activation of TRAF6. TRAF6 is an E3 ubiquitin ligase that generates lysine 63-linked polyubiquitin chains linked to itself and to other substrates such as transforming growth factor β (TGF β)-activated kinase 1 (TAK1). TRAF6 polyubiquitin chains bind to the TAK1-binding protein 1 (TAB1), TAB2 and TAB3, which induces conformational changes licensing TAK1 for its autophosphorylation [58]. TAK1 subsequently activate inhibitor of κ B (I κ B) kinase (IKK) complex. The IKK complex consists of two catalytic subunits IKK α and IKK β and a regulatory subunit IKK γ (also known as NF κ B essential modifier, NEMO), all of which catalyze I κ B phosphorylation and degradation. Consequently, NF κ B is released from suppression by I κ B and translocates to the nucleus, where it induces transcription of proinflammatory cytokines. In addition, TRAF6 signaling leads to the activation of p38 mitogen-activated protein kinase (MAPK), c-JUN N-terminal kinase (JNK), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1) transcription factors, all of which promote upregulation of proinflammatory cytokines.

Endosomal TLR signaling results in the induction of proinflammatory cytokines via NF κ B as well as a type I IFN cytokines via IRFs. Signaling from TLR3 and TLR4 leads to the recruitment of TRAF6 to TRIF via receptor-interacting protein 1 (RIP1) and TNFR-associated death domain (TRADD) complex [Fig. 1.1]. The complex then facilitates TAK1 activation and downstream signaling, leading to induction of proinflammatory cytokines [59]. Alternatively, TRIF recruits the E3 ubiquitin ligase TRAF3, thus mediating TRAF3 activation. Polyubiquitinated

TRAF3 activates noncanonical IKKs or so-called IKK-related kinases TBK1 and IKK ϵ , which phosphorylates IRF3 [60]. TBK1/IKK ϵ are possibly activated by TRIF, which interacts with NAK-associated protein 1 (NAP1) and TRAF3 [61], while TBK1/IKK ϵ function is inhibited by TRAF associated NF κ B activator (TANK) [62]. Phosphorylated IRF3 forms homodimers, which translocates to the nucleus, binds to the promoters containing interferon-stimulated response element (ISRE) and induces transcription of antiviral genes such as IP-10, RANTES and type I IFNs.

Upon viral infection, endosomal TLRs recognize viral nucleic acids and trigger type I IFN responses via IRFs. Out of nine IRF members, IRF1, IRF3, IRF5, IRF7 and IRF8 are directly involved in TLR signaling [24]. IRF3 is triggered upon TLR3 or TLR4 activation and was described previously. IRF7 is highly expressed in pDCs, and upon TLR7/8 and TLR9 activation, rapidly triggers production of IFN α . MyD88, TRAF6, IRAK1 and IRAK4 form a complex, which activates NF κ B and induces transcription of antiviral cytokines [Fig. 1.1]. Alternatively, the MyD88-TRAF6-IRAKs complex associates with IKK α and triggers IKK α activation, IKK α then directly phosphorylates IRF7, which induces induction of type I IFNs [63]. Alternatively, IRF7 can be activated via the phosphatidylinositol 3 kinase (PI3K)-Akt pathway. PI3K-Akt pathway is activated by various TLR ligands and functions as a positive as well as a negative regulator of TLR signaling depending on the cell type [64]. IRF1 was historically the first IRF discovered and activates type I IFNs in myeloid DCs (mDCs) and macrophages. Upon TLR9 activation, IRF1 is activated through a MyD88-IRAK1 axis [65]. IFN γ has been shown to potentiate TLR9-mediated IRF1 signaling, leading to rapid translocation of IRF1 to the nucleus and induction of IFN β gene transcription [66]. Additionally, IRF1 has been implicated in the development of NK-cells through IL-15 induction, suggesting that TLR9 might induce both NK and T cell responses [67]. Similar to IRF1, IRF5 also interacts with MyD88 and TRAF6 upon TLR7 or TLR9 activation. Phosphorylated IRF5 is translocated to the nucleus activating transcription of proinflammatory cytokines including TNF α and IL-6 [68]. IRF5 has been shown to play a role in M1 macrophage polarization and subsequent shaping of Th1 and Th17 responses following LPS stimulation [69]. Additionally, IRF5-mediated IFN β has been implicated in protection during *Candida albicans* infection after its recognition by dectin-1 in DCs [70]. IRF4 has been shown to antagonize IRF5 functions by competing with IRF5 for MyD88 binding [71]. Lastly, IRF8 is involved in TLR4/TLR9-MyD88 pathways and enhances production of proinflammatory cytokines by interaction with and activation of TRAF6 in cDCs [72].

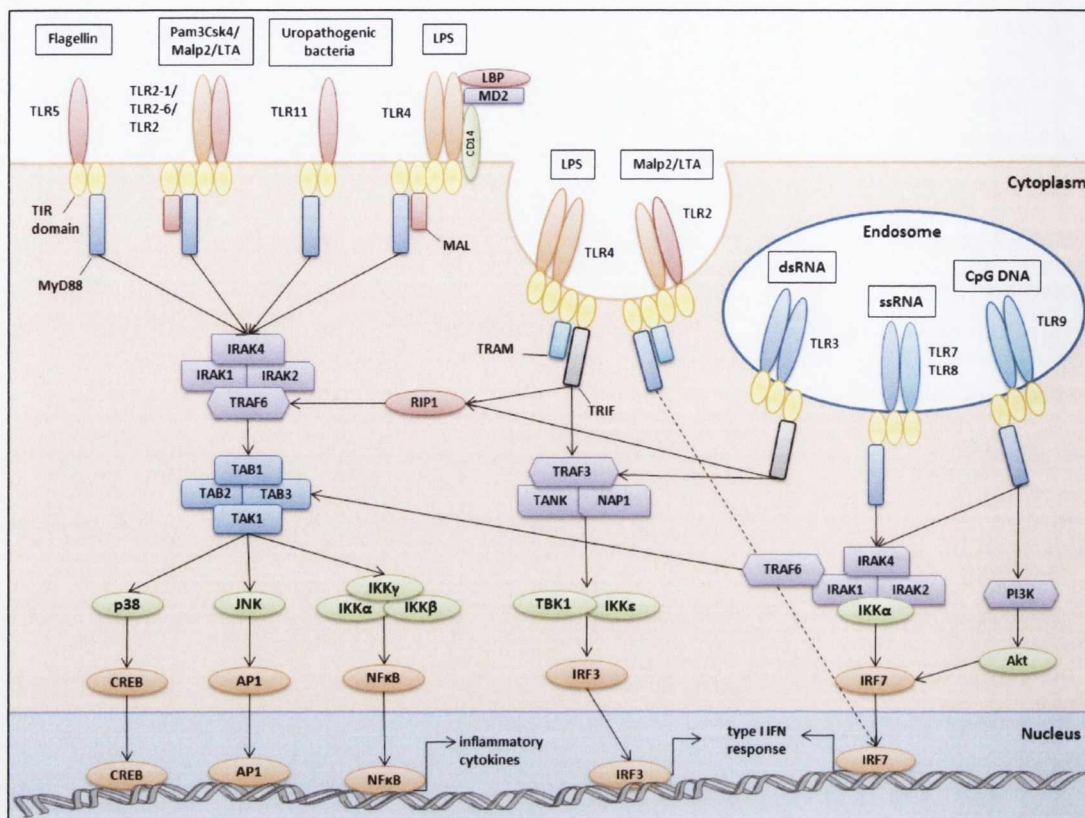


Figure 1.1: Mouse and human TLR signaling pathways.

TLRs are a major class of PRRs that play a key role in sensing PAMPs. TLR5, TLR11, TLR2-TLR1 and TLR2-TLR6 heterodimers recognize ligands on the cell surface, while TLR3, TLR7, TLR8 and TLR9 sense ligands in the endosomes. TLR4 detect its ligand on the cell surface and consequently translocates to the endosome, where it initiates a second wave of immune signaling. Similar to TLR4, TLR2 has been shown to translocate to the endosome and trigger type I IFN induction via IRF7 (dashed arrow) [42]. Upon ligand recognition on the cell surface, TLR receptors dimerize and initiate signal transduction via TIR-TIR domain interactions between TLRs and TIR domain adaptor proteins (MyD88 and Mal, or TRAM and TRIF). Myddosome complex is assembled via DD interactions between MyD88 and IRAKs, mediating activation of IRAKs. Signal is then transmitted from IRAKs to TRAF6, which in turn activates TAK1 and MAPKs. Subsequently, the kinases phosphorylate CREB, AP1 and NfκB transcription factors, leading to upregulation of proinflammatory cytokines [24]. Signaling from endosomal TLR3 and TLR4 activates TRAF3, which phosphorylates IRF3 via TBK1/IKKε and leads to the induction of type I IFNs. TLR7/8 and TLR9 stimulation leads to the activation IKKα via MyD88-IRAK-TRAF6 complex, which then recruits IRF1, IRF5 or IRF7, and induces both proinflammatory and type I IFN responses. Signaling from TLR9 also activates PI3K-Akt axis and induces the type I IFNs upregulation via IRF7 [24].

1.3.2 RIG-I-like receptors

After the discovery of endosomal TLRs, which recognized viral nucleic acids, the detection of some viruses remained puzzling. Firstly, expression of endosomal TLRs is limited to a number of cells such as dendritic cells; however, other cell types have been shown to respond to viral RNA [73]. Secondly, endosomal TLRs can detect nucleic acids only within the endosomal compartments, whereas replication of viruses occurs mainly within the cytosol or the nucleus. Consequently, another class of cytoplasmic PRRs involved in the recognition of viral RNA was discovered and termed as retinoic acid inducible gene-1 (RIG-I)-like receptors (RLRs).

The RLR family comprises three members: retinoic acid inducible gene-1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). All three proteins belong to the DExD/H family, which contains RNA helicases. Therefore, RLRs can directly interact with double stranded RNA (dsRNA) that is present in RNA viruses as a part of their genome or as an intermediate during their replication. RIG-I has been shown to bind short dsRNA containing 5'triphosphate present in Sendai virus, influenza [74], HCV and VSV [75] genomes. In addition, it has been demonstrated that RIG-I can also bind 5'diphosphate found in reovirus-infected cells [76]. MDA5, on other hand, recognizes long RNA duplexes present in Picornaviruses [77] and in synthetic poly(I:C). RLRs are the major sensors for viral infection in conventional DCs, macrophages and fibroblasts.

Upon recognition of viral RNA, both RIG-I and MDA5 induce downstream signaling cascades through their N-terminal caspase activation and recruitment domain (CARD) [Fig. 1.2 A]. The RLRs' CARD interacts with the CARD present in mitochondrial antiviral signaling protein (MAVS, also known as IPS1). This interaction induces IRF3 phosphorylation via TRAF3 and TBK1/IKK ϵ , which leads to the induction of type I IFNs. MAVS can also interact with Fas-associated protein with death domain (FADD) and TRAF6, leading to NF κ B activation. Lastly, LGP2 contains an RNA binding domain but lacks CARD found in RIG-I and MDA5, and thus LGP2 is unable to mediate downstream RLR signaling. In addition, there is evidence that LGP2 can act as a positive [78] as well as a negative regulator of RLRs [79].

1.3.3 C-type lectin-like receptors

CLRs are transmembrane proteins characterized by C-type lectin domain and they are implicated in antifungal immunity [80]. CLRs recognize carbohydrate structures found in fungal walls such as mannose and β -glucose. The CLRs include mannose receptor, dectin-1, dectin-2 and DC-specific intracellular adhesion molecule grabbing nonintegrin (DC-SIGN). Mannose receptor (MR) is present on the surface of macrophages and DCs and links innate and adaptive immunity. MR recognizes mannose motifs on the surface of microorganisms, including *Candida albicans* and *Leishmania donovani* [81]. Dectin-1 is a transmembrane receptor that contains an intracellular immunoreceptor tyrosine-based activation motif (ITAM)-like motif and an extracellular single C-type lectin-like domain recognizing β -glucans present in fungi, some bacteria and plants. ITAM-like motif mediates cellular responses including phagocytosis, respiratory burst, DC maturation, and cytokine production. Dectin-1 is expressed by macrophages, neutrophils and DCs, and is known to couple with spleen tyrosine kinase (Syk) [Fig. 1.2 B]. Upon activation, Syk kinase phosphorylates protein kinase C- δ (PKC- δ) and activates assembly of Card9 complex, leading to NF κ B activation and production of proinflammatory cytokines including IL-1 β , IL-6 and TNF α [82]. Dectin-1 has been shown to interact with TLR2 and induce a MyD88-dependent proinflammatory response during *C. albicans* infection [23]. In addition, dectin-1 has been shown to activate IRF5 transcription factor and induce protective type I IFN response during infection with *C. albicans* [70].

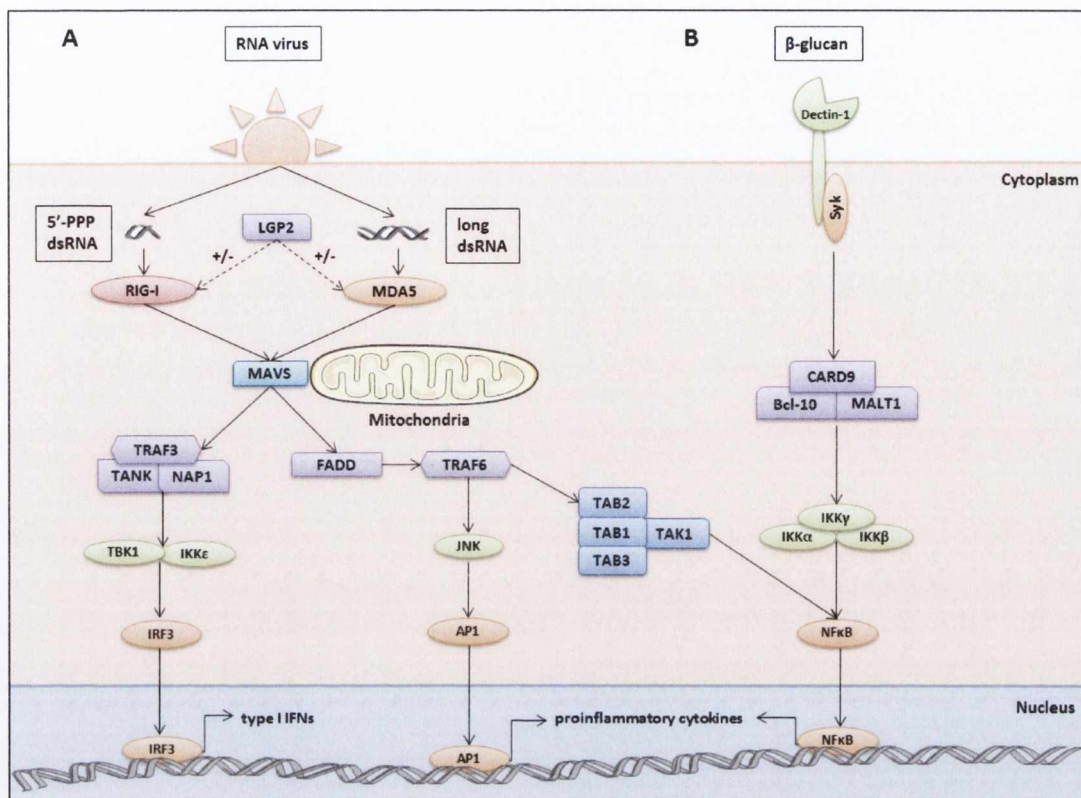


Figure 1.2: Human RLRs and CLR signaling pathways.

A: RLRs: Upon viral infection, RIG-I recognizes short 5'-PPP dsRNA, whereas long dsRNA fragments are sensed by MDA5. Both RIG-I and MDA5 transduce a signal to mitochondrial protein MAVS via CARD-CARD interactions. MAVS then activates IRF3 through a TRAF3-TBK1/IKKε signaling axis, leading to upregulation of type I IFNs. Alternatively, MAVS triggers NFkB and AP1 activation through FADD-TRAF6 signaling cascade inducing transcription of proinflammatory cytokines [14]. LGP2 act as a positive and/or negative regulator of MDA5 and RIG-I signaling (dashed arrows). **B: CLR:** Upon fungal infection, dectin-1 recognizes β-glucan and couples with Syk kinase, leading to NFkB activation via a CARD9-Bcl10-Malt1 complex, which activates IKK complex, thus NFkB-dependent transcription of proinflammatory genes [24].

1.3.4 NOD-like receptors

The nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are cytosolic receptors that recognize bacterial PAMPs and cellular DAMPs. NLRs constitute a major class of intracellular PRRs that regulate inflammatory and apoptotic responses. Until now, more than 20 NLRs have been identified in mammals, with expression found in many cell types. All NLRs share a three-domain structure: a central NOD (also known as NACHT domain), mediating nucleotide binding; a C-terminal leucine-rich repeat (LRR) responsible for ligand binding; and an interacting N-terminal domain consisting of a caspase activation and recruitment domain (CARD), a pyrin domain (PYD), or an acidic transactivating domain or baculovirus inhibitor repeats (BIRs). Similar to the TIR domain, CARD and PYD domains mediate homotypic interactions with the downstream effector proteins. According to their phylogenetic relationship, NLRs can be divided into NOD, NLRP and IPAF subfamilies.

The NOD subfamily members NOD1 and NOD2 recognize bacterial peptidoglycan (PGN), an essential component of bacterial cell walls. NOD1 senses meso-diaminopimelic acid (IE-DAP) found in all Gram-negative and some Gram-positive bacteria, while NOD2 recognizes intracellular muramyl dipeptide (MDP) found in most of bacteria including *S. aureus* [83]. Upon intracellular recognition of the ligand, NOD1 and NOD2 recruit receptor-interacting protein 2 (RIP2) via CARD-CARD interactions and activate TAK1 kinase [84]. Activation of TAK1 leads to induction of NF κ B and MAPK and expression of inflammatory cytokines including IL-6, IL-8 and RANTES [Fig. 1.3 A]. Interestingly, mutations in *NOD2* gene have been associated with susceptibility to Crohn's disease by altering the recognition of microbial antigens by NOD2 or constitutive activation of NF κ B [85].

The NLRP subfamily comprises 14 members, which are characterized by the PYD domain with an important function during inflammation. NLRP3 is involved in the inflammatory responses against both Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes* and *Neisseria gonorrhoeae* [86, 87], and is the best-characterized receptor for activation of an inflammasome. In addition, stress signals such as aluminum salts, silica crystals [88] and basic calcium phosphate crystals [89] have been shown to activate NLRP3. The inflammasome is an oligomeric protein complex consisting of pro-caspase 1, NLRP3 and the apoptosis-associated speck-like protein containing a CARD (ASC) adapter. The inflammasome is responsible for the activation of inflammatory responses by maturation of pro-IL-1 β and pro-IL-18, and the induction of pyroptosis, a programmed cell death during inflammation [90]. The production of mature IL-1 β and IL-18 always requires two signals.

The first signal comes from activated PRRs such as TLR4 activated by LPS, and initiates pro-IL-1 β and pro-IL-18 gene transcription. The second signal such as ATP activates an inflammasome to recruit and cleave inactive pro-caspase 1 in the inflammasome complex to an active form, which cleaves pro-IL-1 β and pro-IL-18 into the mature and functional cytokines.

1.3.5 AIM2-like receptors

Lastly, AIM2-like receptors (ALRs) are a group of DNA innate immune receptors that has been recently identified [16]. ALRs belong to the PYHIN protein family which is characterized by a pyrin domain (PYD) and one or two hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeat (HIN) domains [91]. The HIN domain contains conserved repeats, which facilitates DNA binding. Only five PYHIN proteins have been described in humans, whereas up to 13 PYHIN proteins have been found in mice. The human PYHIN family involves absent in myeloma 2 (AIM2), interferon-inducible myeloid differentiation transcriptional activator or gamma-interferon-inducible protein I χ -16 (IFI16), myeloid cell nuclear differentiation antigen (MNDA), pyrin and HIN domain family member 1 (PYHIN1), and pyrin only protein 3 (POP3).

Unlike other PYHINs, AIM2 lacks a nuclear localization sequence (NLS); thus, it has been associated with recognition of pathogen DNA in the cytosol. AIM2 has been shown to bind synthetic dsDNA poly(dA:dT) through its HIN domain and initiate formation of the inflammasome [Fig. 1.3 B; 92]. In addition, activation of the AIM2 inflammasome was found to be important during vaccinia virus and mouse cytomegalovirus infections as well as during infection with Gram-negative cytosolic bacterium *Francisella tularensis* [93]. IFI16 has been implicated in the recognition of both nuclear and cytosolic viruses thanks to acetylation status of its NLS, which modulates IFI16 cellular localization of IFI16 [94]. Upon recognition of dsDNA, IFI16 triggers signaling cascade via STING-TBK1, leading to activation of IRF3 and transcription of IFN β [Fig. 1.4;16]. In addition, IFI16 has been shown to act as a restriction factor for some viruses, including human papillomavirus 18 (HPV18) via epigenetic modifications on viral promoters [95]. Lastly, similar to AIM2, IFI16 has been found to trigger inflammasome activation by interacting with ASC in response to dsDNA of large viruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV) [96]. However, future work is needed to elucidate the functions of ALRs and PYHIN proteins during viral infections as well as their interaction with and contribution to other signaling pathways.

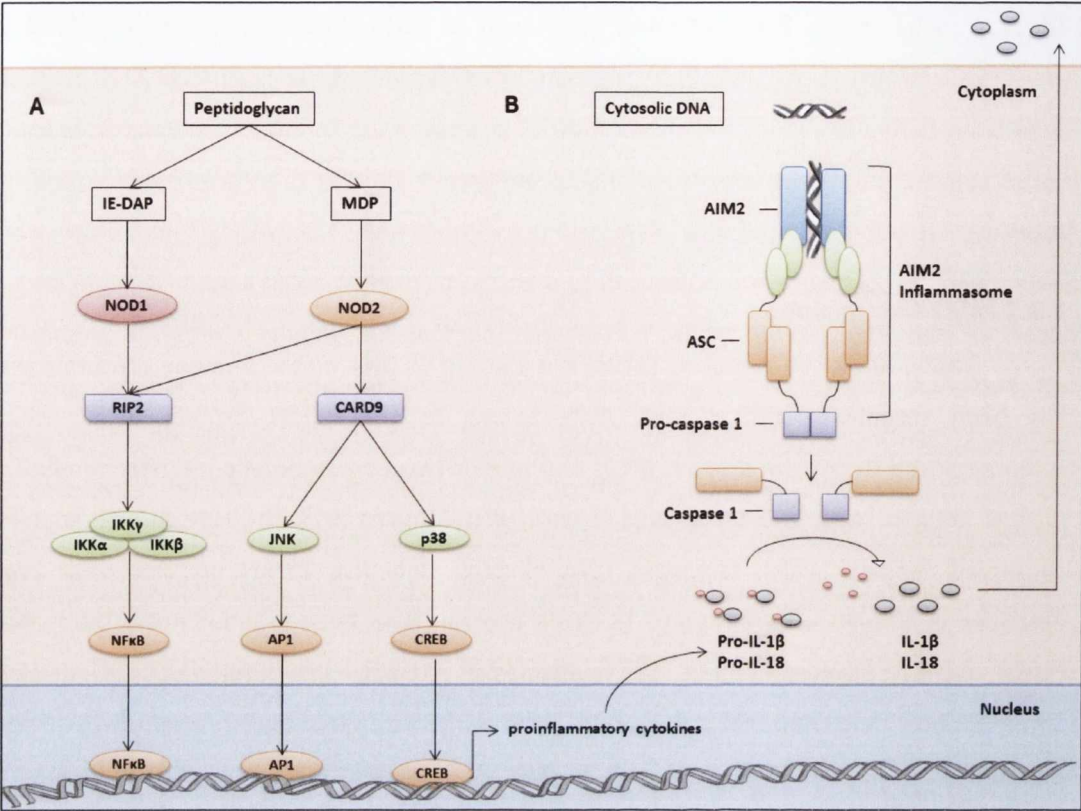


Figure 1.3: Human NLRs and AIM2 inflammasome signaling pathways.

A: NLRs: During infection with Gram-positive bacteria, bacterial peptidoglycan is cleaved into smaller fragments such as meso-diaminopimelic acid (IE-DAP) and intracellular muramyl dipeptide (MDP). Consequently, NOD1 recognizes IE-DAP, leading to NF κ B activation via RIP2 and IKK complex. NOD2 senses MDP and activates AP1 and CREB transcription factors via CARD9 and MAPKs, leading to an upregulation of proinflammatory cytokines [84]. **B: AIM2 inflammasome:** The release of mature IL-1 β and IL-18 is tightly regulated and requires two signals. The first signal comes from activated PRRs and results in the transcription of immature pro-IL-1 β and pro-IL-18. The second signal comes after recognition of cytosolic DNA by a DNA sensor such as AIM2. Activated AIM2 triggers formation of the inflammasome, a multiprotein complex consisting of AIM2, ASC and pro-caspase 1. Activation of the inflammasome causes cleavage of pro-caspase 1 into mature caspase 1, which in turn cleaves pro-IL-1 β and pro-IL-18 into their active forms [92].

1.4 DNA sensors

Even though cytosolic DNA was known to be immunostimulatory, the nature of the cytosolic DNA sensor remained unknown. Up until 2007, the only known receptor for recognition of viral DNA was endosomal TLR9. Consequently, it was shown that mice lacking stimulator of interferon genes (STING) were defective in IFN β production following dsDNA and HSV-1 challenge and STING was identified as the key regulator of DNA-induced type I IFN responses [97]. STING is a transmembrane protein in endoplasmic reticulum that directly binds both TBK1 and IRF3 in the cytosol. STING mediates phosphorylation of IRF3 by the kinase activity of TBK1 by bringing them into a close proximity, thus leading to IRF3 phosphorylation and induction of type I IFNs [98]. In addition, other molecules have been shown to detect DNA and trigger STING activation, including cGAMP, IFI16, DDX41, DNA-dependent activator of IFN-regulatory factors (DAI) and RNA polymerase III. Cyclic GMP-AMP, also known as cGAMP, is an endogenous secondary messenger, a hybrid of cyclic diguanylate (c-di-GMP) and cyclic diadenylate monophosphate (c-di-AMP) [99]. cGAMP is synthesized by a cyclic GMP-AMP synthase (cGAS), a member of nucleotidyl transferases [100]. cGAS directly binds DNA and catalyzes the reaction between GMP and AMP creating cGAMP molecules, which then mediate STING-dependent transcription of type I IFNs [Fig. 1.4]. Furthermore, it has been shown that cGAMP generated in virus-infected cells can spread through gap junctions to non-infected cells, and thus prime neighboring cells for antiviral response [101].

Apart from antiviral immunity, STING-mediated DNA recognition has been implicated in immune responses during bacterial and parasitic infections. However, STING-induced responses during these infections had an ambiguous effect on the outcome of infection. It has been reported that Gram-positive intracellular *Listeria monocytogenes* can activate STING by production of bacterial cyclic di-AMP and induce production of MCP-1 and MCP-3 chemokines [102]. Consequently, STING deficient monocytes have defects in migration, leading to increased bacterial burden in mice livers during *L. monocytogenes* infection, thus suggesting a role for STING in cell migration. In addition, it has been shown that extracellular DNA from *Mycobacterium tuberculosis* activates STING and enhance type I IFN response in macrophages, leading to increased pathogenesis during infection, hence implying a negative effect of STING activation during mycobacterial infections [103]. In addition, genomes of parasitic *Plasmodium falciparum* and *P. berghei* contain AT-rich stem-loop DNA motifs, which have been shown to induce type I IFN production via the STING-TBK1-IRF3 axis. Consequently, mice deficient in type I IFN responses have been resistant to cerebral malaria induced by *P. berghei*, indicating a negative role of STING in pathogenesis during *Plasmodium* infection [104].

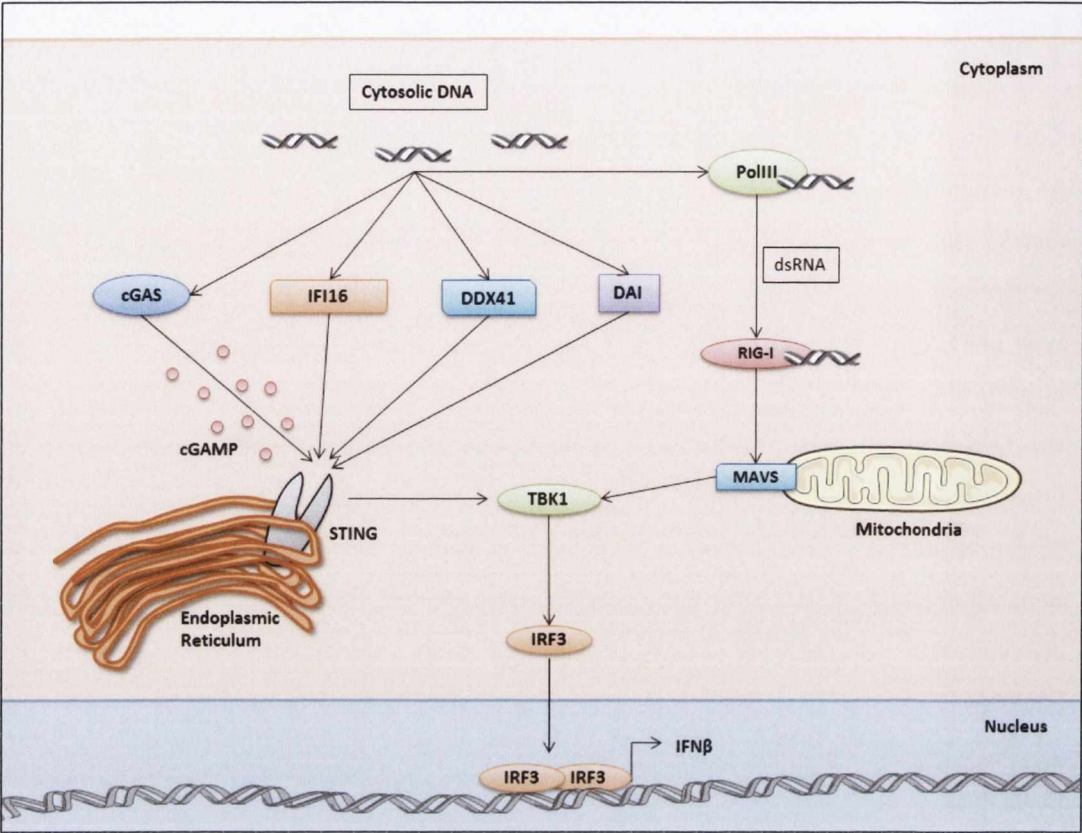


Figure 1.4: Cytosolic DNA receptors involved in IFNβ induction.

Recently, multiple DNA receptors activating STING-dependent pathway have been identified. Upon DNA recognition, the DNA sensors trigger STING, which then mediates phosphorylation of IRF3 by TBK1 and induces transcription of IFNβ mRNA. DNA is recognized by cGAS, which catalyses an enzymatic reaction between GMP and AMP molecules resulting in production of a second messenger cGAMP. cGAMP then activates STING and leads to IFNβ mRNA transcription via TBK1-IRF3 axis [100]. The molecular mechanisms of STING activation by IFI16, DDX41 and DAI are less defined. Pol III initiates a STING-independent pathway through the transcription of poly(dA:dT) to dsRNA, which is then recognized by RIG-I initiating IFNβ transcription via MAVS [14].

1.5 The adaptive immune system

Recognition of pathogens by PRRs on APCs leads to signaling cascades culminating in the production of cytokines, expression of antigen-specific receptor and antigen nonspecific co-stimulatory molecules on the membrane of APCs. Consequently, recognition of receptors and co-stimulatory molecules activates cells of adaptive immune system. The adaptive immune response comprises highly specialized cells and processes that eliminate and prevent the growth of pathogens [1]. In contrast to the innate immune response, adaptive immunity results in immunological memory after an initial encounter with a pathogen, leading to enhanced and accelerated responses upon subsequent confrontation with the same pathogen. The adaptive immune response is mediated by a subset of leukocytes known as lymphocytes, including B and T cells.

There are many subsets of T lymphocytes with distinct functions but they are all characterized by expression of a unique T cell receptor (TCR) on their surface. Helper T cells (Th) express the CD4 marker and shape immune responses by directing other immune cells. Naïve Th cells are activated upon recognition of antigens presented to them by MHC class II molecules on APCs. The maturation of Th cells is completed only after recognition of the second signal, which is mediated by interaction between co-stimulatory molecules on APCs with their corresponding receptors on Th cells. One of the best characterized co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 and CD86 on the membrane of APCs. The Th cells then proliferate and differentiate into two subtypes of Th cells according to the type of infection. The Th1 response is aimed against intracellular bacteria, viruses and protozoa. It is triggered in response to IL-2 and IL-12 cytokines and consequently results in the production of high amounts of type II IFN, such as IFN γ , which activates macrophages. Th2 cells are efficient against extracellular bacteria, toxins and helminths. They produce high levels of IL-4, IL-5 and IL-13 cytokines, which activate eosinophils and B cell isotype switching and production of antibodies [1]. Upon resolution of infection, however, only a few CD4⁺ cells remain as memory T cells. Cytotoxic T cells eliminate virus-infected and tumor cells and cause transplant rejection. They express CD8 molecule on their surface and recognize antigens presented by MHC class I molecules on the surface of all nucleated cells. Like Th cells, most of the cytotoxic T cells will die after the resolution of infection. Memory T cells are long-lived antigen specific T cells that rapidly expand into large numbers of effector T cells upon re-infection with the same pathogen. Memory T cells express either CD4 or CD8 molecules on their cell surface. Regulatory T cells maintain immunological tolerance by suppressing T cell-mediated immunity against pathogens by producing IL-10, and by eliminating

autoreactive T cells. Regulatory T cells are characterized by expression of FOXP3 transcription factor. Lastly, natural killer T cells (NKTs) bridge innate and adaptive immune responses. They recognize antigens presented by CD1d and upon activation, they produce cytokines and cytolytic molecules in order to eliminate pathogens.

B cells are the major cells that produce antibodies. Naïve B cells can be activated in a T-cell dependent or independent manner but activation always requires two signals. B cells express a unique B-cell receptor (BCR) on their surface that recognizes and binds a particular antigen. Once a B cell recognizes a specific antigen and receives an additional signal from Th cells, it differentiates into a plasma cell which produces antigen-specific antibodies [1]. About 10% of plasma cells will become long-lived antigen-specific memory B cells. These cells are primed to produce pathogen-specific antibodies and if a pathogen re-infects the host, they quickly eliminate it. This immunological memory is the basis for most vaccines.

1.6 Mediators of immune responses

As discussed previously, upon recognition of PAMPs, PRRs trigger signaling cascades that leads to the production of cytokines via activation of NF κ B and IRFs. NF κ B is a master regulator of inflammatory responses with a role in transcription of interleukins, chemokines and TNF α . IRFs on the other hand, provide an antiviral response by inducing type I IFN production.

1.6.1 Interleukins

Interleukins (ILs) are a heterogeneous family of secreted proteins that mediate communication among leukocytes. The majority of interleukins are produced by immune cells including monocytes, macrophages, and CD4⁺ T cells. Additionally, non-immune cells are able to produce interleukins to certain extent in response to immune stimulation. Interleukins were clustered into several families based on their sequence homology, receptor chain similarities and functional properties.

IL-1 family comprises IL-1 α and IL-1 β cytokines with similar biological properties and IL-1 receptor antagonist (IL-1Ra), which inhibits IL-1 signaling. Whereas IL-1 β is translated into an inactive form and its activation is tightly regulated, IL-1 α is produced in a biologically active form. Maturation of pro-IL-1 β is mediated by caspase-1, which together with NLRP3 and ASC forms a complex called the inflammasome. IL-1 is an endogenous pyrogen and major proinflammatory cytokine with diverse effects on proliferation, differentiation and function

of many innate cells [105]. The inflammasome mediates activation of caspases, leading to IL-1 β release and induction of pyroptosis, a programmed cell death during inflammation.

IL-6 is pleiotropic cytokine induced during immune responses. IL-6 is secreted by immune cells such as macrophages and T cells and by non-immune cells including endothelial cells and fibroblasts. IL-6 promotes T-cell proliferation, B-cell differentiation and production of IgG, IgA, and IgM [106]. In addition, IL-6 can act as an anti-inflammatory cytokine by inhibition of TNF α and IL-1 function and by induction of IL-1Ra and IL-10. It has been shown that mice deficient in IL-6 have impaired survival after *Streptococcus pneumoniae* infection [107]. Consequently, IL-6 was required for downregulation of cytokines in the lungs, which contributes to the host defence.

IL-10 is an important anti-inflammatory cytokine that regulates several aspects of immune responses. IL-10 is produced by monocytes, macrophages, dendritic cells, natural killer cells, T and B cells. IL-10 inhibits the synthesis of a number of cytokines, including IFN γ , TNF α , and GM-CSF produced by activated macrophages and Th cells. IL-10 downregulates the expression of MHC class II and costimulatory molecules on the surface of APCs, and thus suppress T cell activation [108]. In contrast to its inhibitory effects on T cells, IL-10 enhances B cell survival, proliferation, and antibody production [108].

The IL-12 family includes IL-12 itself, IL-23, IL-27, and IL-35, all of which display cell-type specific functions and use distinct receptor chains. IL-12 consists of p35 and p40 subunits and is produced by activated monocytes, macrophages, neutrophils, microglia, and DCs [109]. IL-12 mediates Th1 cell development and their maintenance by induction of IFN γ production, and also enhances cytotoxic activity of NK cells. IL-23 shares the p40 subunit with IL-12 and is expressed mainly by phagocytic cells in peripheral tissues such as skin, intestine and lungs [110]. The receptor for IL-23 can be found on activated and memory T cells, NK cells, eosinophils, APCs, and epithelial cells. Importantly, genetic variants of the IL-23R have been associated with autoimmune and chronic inflammatory disorders including inflammatory bowel disease and psoriasis [111]. It has been shown that IL-23 stimulation of intestinal leukocytes induces IL-17 and IFN γ production by innate lymphoid cells and consequently causes intestinal pathology. IL-27 is expressed by APCs and endothelial cells and has important immunoregulatory functions. IL-27 promotes differentiation of naïve T cells into the Th1 cells by induction of Th1-specific transcription factor T-bet and by inhibition of Th2-specific transcription factor GATA-3 [112]. In addition, IL-27 antagonizes the development of Th17 cells by suppression of IL-6-mediated T cell proliferation [113].

The IL-17 family is a group of structurally distinct cytokines. IL-17A is the founding member of the family; originally referred to as IL-17. IL-17A is a proinflammatory cytokine, which is expressed by activated CD4⁺ Th17 cells and to a lesser extent by other cells, including CD8⁺ T cells, $\gamma\delta$ T cells, NK cells, and neutrophils [114]. Human naive T cells produce maximum amounts of IL-17A only after stimulation with IL-1 β , IL-6, IL-23, and TGF β . IL-17A acts on a variety of cells, which respond to IL-17 by expression of proinflammatory cytokines and chemokines, thus attracting monocytes and neutrophils to the site of infection. IL-17A has been involved in several inflammatory disorders, including rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), and allergic diseases, such as asthma and atopic dermatitis [114]. IL-17A can form a homodimer or a heterodimer with IL-17F and both dimers can bind the same receptor. Similar to IL-17A, IL-17F acts on many cell types and induces proinflammatory cytokines and chemokines. Expression of other family members is restricted to non-immune cells and their function is less clear [108].

1.6.2 Chemokines

Chemokines are a large family of secreted proteins, which are classified into four subfamilies according to the presence and number of amino acids between their N-terminal cysteine residues, i.e. CXC, CC, CX₃C and XC [115]. Chemokines signal via G-protein-coupled transmembrane receptors, which are classified according to the chemokines they bind, i.e. CXCR, CCR, CX₃CR and XCR [116]. Chemokines are secreted by both immune and non-immune cells and they have heterogeneous roles in immune responses. Some chemokines are considered proinflammatory since they are induced during immune responses and they are responsible for attracting other immune cells to the site of infection. Other chemokines are involved in regulating homeostatic functions, apoptotic pathways or angiogenesis [115].

CCL5 also known as RANTES (regulated on activation, normal T cell expressed and secreted) is a CC motif chemokine, which is chemotactic for T cells, eosinophils and basophils, thus recruiting leukocytes to the site of inflammation. CCL5 is expressed later after T cell activation and functions as a ligand for the chemokine receptor chemokine receptor 5 (CCR5). It has been reported that RANTES, along with related chemokines MIP-1 α and MIP-1 β , acts as major HIV-suppressive factors produced by CD8⁺ T cells and other immune cells [117].

CXCL10, also known as interferon gamma-induced protein 10 (IP-10) is a CXC motif chemokine, which is secreted by monocytes, endothelial cells and fibroblasts in response to stimulation with LPS, type I and type II IFNs [118]. IP-10 functions as a chemoattractant for monocytes, macrophages, NK cells and dendritic cells, and has been implicated in the

generation of effector T cells and T cell adhesion to endothelial cells [119]. In addition, IP-10 has antitumor activity and plays a role in inhibition of bone marrow colony formation and angiogenesis [120].

1.6.3 Tumor necrosis factor alpha

The tumor necrosis factor (TNF) superfamily is a group of cytokines with an extreme spectrum of bioactivities. TNF was originally found for its cytotoxic effects on tumor cells and its ability to cause necrosis of tumours [121]. TNF is considered as a major mediator and regulator of immune responses. TNF α is produced as a transmembrane protein, which becomes soluble after its proteolytic cleavage by the TNF alpha converting enzyme [122]. Transmembrane TNF α is expressed by macrophages, T lymphocytes and by non-immune cells including mast cells, endothelial cells, fibroblasts, and in neuronal tissue. Soluble TNF α mediates immune responses in distant tissues by interaction with its designated receptor. Engagement of the receptor triggers distinct and overlapping signal transduction pathways. These signalling cascades lead to distinct cellular responses including cell death, cell survival, cell differentiation and migration of cells [123].

TNF α binds to type 1 and 2 TNF receptors (TNFR1/2, also known as CD120a/b), which are differentially expressed on cells and tissues. Whereas most human cells and tissues express TNFR1, TNFR2 expression is restricted to immune cells. TNFR1 facilitates both inflammatory and cell death pathways, whereas less characterized TNFR2 signalling has been associated with tissue repair and angiogenesis [123]. After binding of TNF α to the receptor, TNFR1 recruits TNFR-associated death domain (TRADD) protein [Fig. 1.5 A; 124]. TRADD then interacts with receptor interacting protein 1 (RIP1) via interaction between their death domains [125], and with TNFR-associated factor 2 (TRAF2), an E3 ubiquitin ligase [126]. When the TRADD-RIP1-TRAF2 complex is released from the receptor, it activates distinct mitogen activated protein kinases (MAPKs). A serine/threonine kinase RIP1 then mediates activation of TAK1, which phosphorylates and activates IKK complex, leading to degradation of I κ B, release of NF κ B and induction of gene transcription [127]. Alternatively, the TRADD-RIP1-TRAF2 complex activates c-Jun N-terminal kinases (JNKs) and p38 MAPKs, which phosphorylates the transcription factor activating protein 1 (AP1). Phosphorylated AP1 then interacts with cAMP response element (CREB)-binding protein (CBP)/p300 and initiates gene transcription. Lastly, TNFR1 signaling can induce a signalling cascade, which mediates a programmed cell death. In this case, TRADD binds to Fas-associated protein with death domain (FADD) and recruits pro-caspase 8 [123]. The TRADD-FADD complex mediates

maturation of caspase 8, which then initiates apoptosis through cleavage and activation of pro-caspase 3. It has been shown that TNFR1-mediated activation of caspases can be inhibited by FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (FLIP). FLIP is upregulated by NF κ B in response to TNFR1 stimulation and antagonizes Fas ligand and TNF α mediated activation of caspases [128]. In addition, TNFR1 activates other less described signalling cascades including ERK-1/2 and PI3K-Akt signaling, both of which are associated with cell survival and proliferation. TNFR2-mediated signaling is less described however, it has been shown that it can act as a receptor for the transmembrane TNF α and lead to activation of NF κ B via IKK complex [Fig. 1.5 B].

The major function of TNF α is its role in immune defence to bacterial, viral and parasitic infections [123]. Endothelial cells respond to TNF α by a number of changes, which result in increased leukocyte adhesion and transendothelial migration of leukocytes to the site of infection. It has been reported that mice deficient in TNFR1 are resistant to LPS- and *S. aureus* enterotoxin B-induced septic shock; however, they are highly susceptible to *Listeria monocytogenes* infection [129]. This opposing effect of TNF α highlights its dual roles during infection. In addition, TNFR1 is widely distributed on the airway epithelium and it has been shown to directly interact with *S. aureus* protein A. Consequently, a signaling pathway triggered by protein A led to upregulation of NF κ B and AP1 transcription factors and increased pathogenesis during staphylococcal pneumonia [130].

Even though TNF α is essential for physiological responses during infection, excessive production of TNF α has been associated with chronic inflammatory diseases. It has been shown that TNF α plays a critical role in regulating leukocyte trafficking during autoimmune diseases such as RA [131]. Consequently, treatment with neutralizing TNF α antibodies reduces the number of inflammatory cells in RA joints by decreasing levels of chemokines and improving RA symptoms. Likewise, TNF α antagonists are efficiently used for the treatment of IBD, such as Crohn's disease, confirming a central role for TNF α in the inflammatory cascade [132].

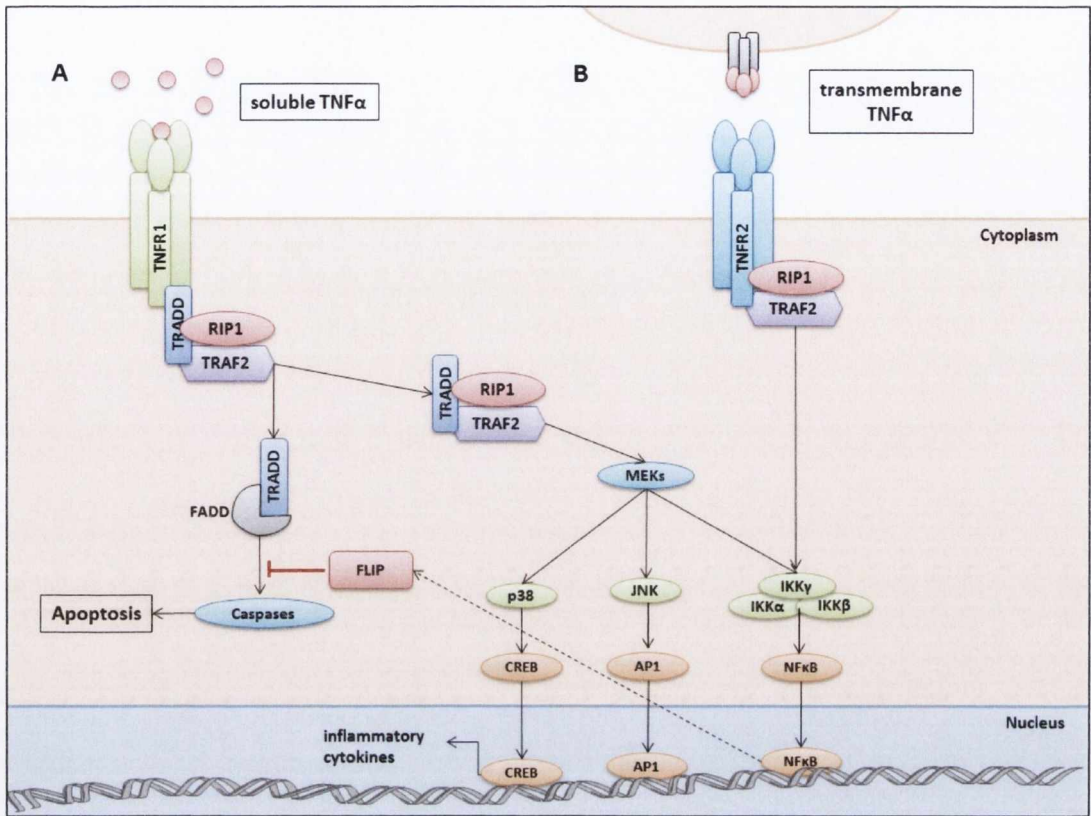


Figure 1.5: Human TNFα signaling.

TNFα is produced as a transmembrane precursor and is activated after proteolytic cleavage in response to immune stimuli. **A:** Soluble TNFα binds to TNFR1, which recruits TNFR-associated-death-domain protein (TRADD). TRADD forms a complex with RIP1 and TRAF2, which activates distinct mitogen activated protein kinases (MAPKs), leading to activation of NFκB, AP1 and CREB transcription factors. TRADD can also form complex with Fas-associated protein with death domain (FADD) and caspases, and thus mediate apoptosis. Caspase activation can be inhibited by FLIP, whose transcription is upregulated by NFκB activation (dashed arrow). **B:** Transmembrane TNFα binds to TNFR2 and leads to activation of IKK complex and NFκB-dependent gene transcription.

1.6.4 Type I interferons

Originally, interferons (IFNs) were identified for their ability to interfere with viral replication, however nowadays IFNs are known to facilitate versatile functions in the immune system. There are three main groups of IFNs with distinct functions. The type I IFNs are secreted by cells in response to various infections and under conditions including cancer and autoimmunity. Type I IFNs consist of multiple subtypes of IFN α , a single IFN β and other less characterized members such as IFN ϵ , IFN κ , and IFN ω [133]. The type II IFNs comprise of a single IFN γ , which signals through heterodimeric IFN gamma receptor. IFN γ is crucial for the full activation of macrophage function in bacterial killing and for enhancement of cytokine production. Lastly, recently recognized type III IFN family includes IFN λ 1, 2 and 3 (also known as IL-29, IL-28A and IL-28B) and signals through a receptor consisting of IL-28R and IL-10R2 chains [134]. The type III IFNs have antiviral function since they have been shown to inhibit replication of hepatitis B and C viruses [135].

The type I IFNs have three main functions in the immune system [136]. Firstly, type I IFNs induce antiviral and antimicrobial mechanisms in infected cells as well as neighboring cells, and thus limit the spread of infectious agents. Secondly, they modulate the innate immune responses by promoting antigen presentation and natural killer functions while inhibiting production of pro-inflammatory cytokines. Lastly, they activate the adaptive immune system by promoting the development of T and B cells and immunological memory. Whereas type I IFNs can be protective during acute viral infection, there is a growing body of evidence for both protective and pathogenic roles during bacterial infections [137].

Both IFN α and IFN β signal through the same heterodimeric IFN α receptor (IFNAR) consisting of two chains [138]. Activation of IFNAR leads to a signaling cascade through a canonical JAK-STAT pathway [136]. Janus kinases (JAK) Tyk2 and JAK1 are constitutively associated with the IFNAR1-IFNAR2 complex [Fig. 1.6]. Upon activation of IFNAR, JAK activates signal transducer and activator of transcription (STAT) 1 and STAT2. Phosphorylated STAT1-STAT2 proteins then associate with IRF9 to form an IFN-stimulated gene factor 3 (ISGF3) [139]. ISGF3 acts as a transcription factor which translocates to the nucleus, binds to IFN-stimulated response elements (ISRE) within promoters of IFN-stimulated genes (ISGs) and induces their expression. ISGs are effector genes that are upregulated in response to type I IFNs and orchestrate the immune response during infection. Immune cells are able to respond to low levels of IFN β within a short time after the onset of infection. This priming of cells is a result of high basal expression of STAT1 and IRF9, which is maintained through an autocrine loop

driven by basal production of IFN β in response to commensal bacteria [140]. The priming of immune cells allows them to respond rapidly to infectious agents through the production of the antiviral ISGs.

The IRF family of transcription factors has functionally diverse roles in the regulation of the immune system. Mammalian IRF family comprises nine members, which are implicated in immunoregulation of PRR signaling, cell differentiation, regulation of cell cycle, apoptosis and suppression of tumor growth [141]. IRFs have been shown to interact with multiple components of the TLR signaling cascade highlighting their role in innate immune responses. The expression of individual IRFs is cell type dependent as is their function (Table 1.2). IRF1 was the first IRF discovered when it was shown that IRF1 bound to an enhancer element in the IFN β promoter [142]. IRF2 was discovered shortly afterwards and it was implicated in the inhibition of type I IFNs by antagonizing IRF1 function [143]. IRF3 and IRF7 are considered as the key regulators of type I IFN gene expression in response to viral and bacterial infections, however, the function of other IRFs remains poorly defined.

IRF	Expression	Key function	Reference
IRF1	constitutive, IFN γ -inducible	• promotes antibacterial and antiviral immunity • promotes Th1 cell responses	[65], [150-154]
IRF2	constitutive	• attenuates type I IFN responses • promotes Th1 cell responses	[143], [155]
IRF3	constitutive	• promotes antibacterial and antiviral immunity	[156-158]
IRF4	constitutive, TLR- inducible	• controls T and B cell functions and DCs development	[159-161]
IRF5	constitutive, IFN γ -/TLR-inducible	• promotes inflammation and antibacterial immunity	[68-70], [162]
IRF6	constitutive	• promotes keratinocyte differentiation	[163]
IRF7	constitutive, type I IFN-inducible	• promotes antibacterial and antiviral immunity	[11], [42], [152], [156-158]
IRF8	constitutive, IFN γ -inducible	• promotes Th1 cell responses	[160-161]
IRF9	constitutive	• mediates type I IFN responses	[139]

Table 1.2: Summary of expression and function of IRF family members.

Innate immune cells such as monocytes, macrophages and dendritic cells can produce both IFN α and IFN β upon recognition of PAMPs by surface, endosomal or cytosolic PRRs. Whereas most cells can upregulate IFN β in response to bacterial cell wall components via the endosomal TLR2 or TLR4, IFN α is induced mainly upon recognition of viral DNA in pDCs [144]. Even though the type I IFNs signal through the same receptor complex, there is as little as 30 % amino acid sequence identity between the two cytokines. Consequently, there are some differences distinguishing them from each other [138]. Both IFN α and IFN β share roles in antiviral responses; however, there are some functions exhibited exclusively by IFN β . It has

been shown that IFN β is responsible for tumor suppression by induction of apoptosis [145] and is efficient in therapy of multiple sclerosis [146]. Recombinant IFN α , on the other hand, is used for the treatment of hepatitis C [147]. The differences between their function were partially explained when it emerged that IFN β could specifically ligate to IFNAR1 and trigger gene expression independently of JAK-STAT signaling [138]. Importantly, the signaling pathway of IFN α and IFN β induction seems to be different. During infection, IFN β transcription is first induced via the TBK1-IRF3 axis [Fig. 1.6 A]. IFN β then signals through IFNAR in an autocrine and paracrine loop, leading to activation of the canonical JAK-STAT pathway and upregulation of ISGs, including IRF7 [Fig. 1.6 B]. Similar to IRF3, IRF7 is present in the cytoplasm and once it is activated, IRF7 translocates to the nucleus and induces transcription of IFN α and IFN β [Fig. 1.6 C]. It has been shown that TBK1 is the main kinase responsible for IRF3/7 phosphorylation and IFN β production [148]. Whereas IRF3 can strongly induce expression of the IFNB gene, it has no effect on IFNA gene transcription. IRF7, on the other hand, is a potent inducer of expression of both IFNA and IFNB genes. This dependency suggests that a forward feedback loop exists by which the initial upregulation of IFN β is mediated by IRF3 and the late phase of type I IFN induction is mediated by IRF7, which itself is induced by type I IFNs [149].

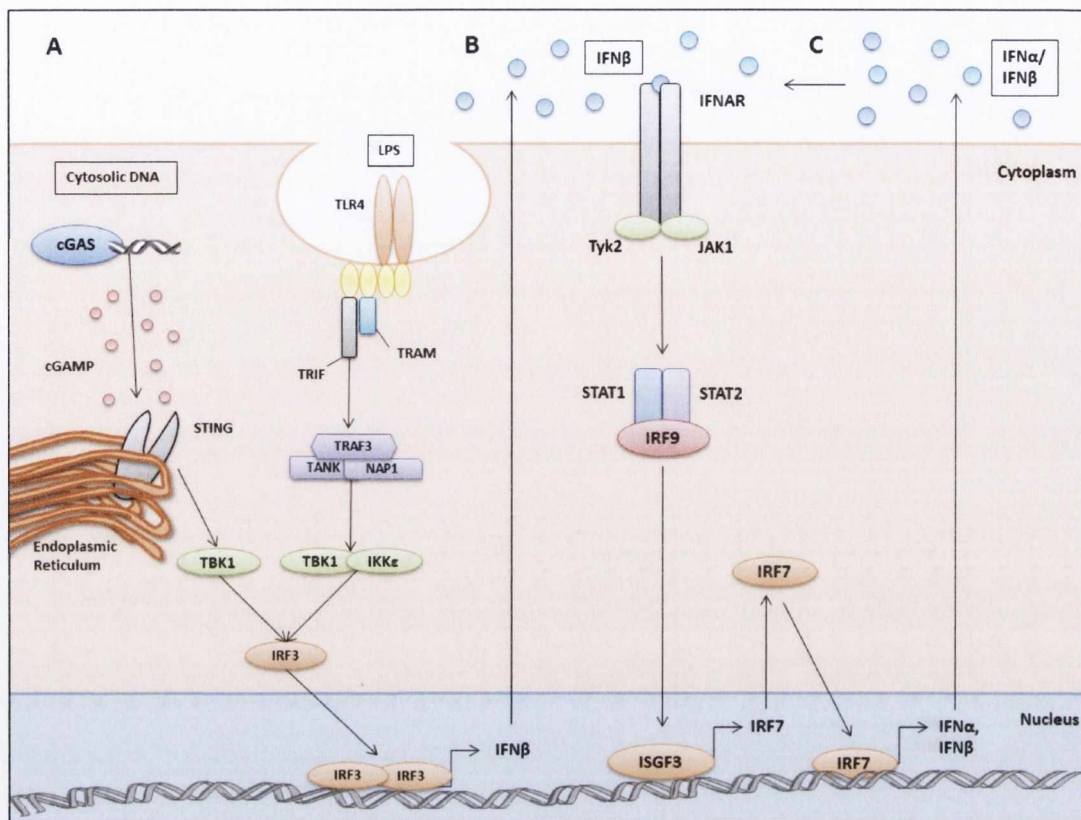


Figure 1.6: Type I IFN signaling.

Induction of type I IFNs is a multistep process, in which IFN β mRNA is induced by PAMPs followed by a positive feedback loop via upregulation of IRF7. **A:** IFN β transcription is induced in response to cytosolic DNA or microbial PAMPs such as LPS. Cytosolic DNA is recognized by and binds to cGAS, which produces the second messenger cGAMP via enzymatic reaction. cGAMP then triggers dimerization of STING in the endoplasmic reticulum. Activated STING recruits TBK1 and IRF3 and facilitates phosphorylation of IRF3. Phosphorylated IRF3 dimerizes, translocates to the nucleus and trigger transcription of IFN β mRNA [100]. Alternatively, IFN β mRNA is induced in response to LPS via endosomal TLR4 pathway [24]. **B:** Mature IFN β protein binds to IFNAR causing phosphorylation of STAT1/2 proteins via JAK kinases. Activated STAT proteins form a complex with IRF9, known as ISGF3, which translocates to the nucleus and trigger transcription of ISGs, including IRF7 [139]. **C:** Phosphorylated IRF7 serves as transcription factor for IFN α mRNA expression. IFN α protein is secreted and is recognized by IFNAR, thus creating a positive feedback loop [149].

1.7 *Staphylococcus aureus* and its interaction with the immune system

1.7.1 *Staphylococcus aureus*

Staphylococcus aureus is a commensal bacterium exceptionally well adapted to humans. It is chronically carried by about one fifth of the population in the upper respiratory tract and on the skin [164]. In addition, about two thirds of the population can host *S. aureus* intermittently. In healthy individuals, commensal *S. aureus* does not normally causes serious complication, instead it contributes to healthy microflora. Additionally, multiple microbial and host factors promote the development and maintenance of the carrier state, but the precise mechanisms remain unclear. In compromised individuals, *S. aureus* is a significant human pathogen causing a wide range of infections from minor skin infections to more severe infections, including sepsis, pneumonia, osteomyelitis and endocarditis [165]. *S. aureus* frequently infects surgical wounds and medical implants, thus being a leading cause of nosocomial infections. The primary site of infection is often a minor scratch in the skin followed by extensive tissue destruction and necrosis at the site of infection. The severity of infections became especially threatening due to the increasing prevalence of methicillin-resistant *S. aureus* strains (MRSA) in hospitals as well as within the community. MRSA is clinically the most dangerous strain since a single genetic element confers resistance to the most frequently prescribed β -lactam antibiotics such as penicillins, cephalosporins and carbapenems [166]. MRSA was reported only six months after the first introduction of methicillin in 1960. Nowadays, MRSA is the most frequently isolated antibiotic-resistant pathogen in hospitals worldwide. There are studies showing that hospital-acquired MRSA infections increase morbidity, the risk of mortality and costs for both the patients and the hospitals. Horizontal transfer of DNA among bacterial strains or species plays an important role in spreading the resistance elements. Consequently, in 2002, *S. aureus* strain resistant to vancomycin (VRSA), an antibiotic that is used to treat MRSA infections, was isolated from patients co-infected with vancomycin-resistant enterococci [167]. Hence, new approaches are urgently needed in the prevention, treatment and elimination of this rogue bacterium.

S. aureus harbours a formidable array of virulence factors including toxins, adhesins and immunomodulatory proteins. Successful infection is a result of host immune competences and a combination of two or more virulence factors since a single virulence factor is rarely sufficient to elicit the infection. Virulence factors contribute to the development of infections by promoting bacterial adhesion to cells and tissues, evading host immune defences and causing damage of cells and tissues by secretion of proteases and exotoxins [168]. The most studied and significant toxins are pore-forming toxins, including secreted hemolysin alpha

(Hla), Pantone-Valentine leukocidin (PVL), leukocidin AB (LukAB) and gamma hemolysins. Hla has been identified as a critical effector molecule essential for *S. aureus* survival within macrophages [169]. PVL has been associated with necrotizing pneumonia; however, its involvement in other types of infections remains controversial [170]. Recently described LukAB has been shown to kill human phagocytes *in vitro* and to facilitate *S. aureus* escape from phagocytes, suggesting its role in bacterial survival and persistence [171]. In addition, carotenoid pigment staphyloxanthin (STX) has been reported to facilitate *S. aureus* survival within neutrophils due to its antioxidant activity [172]. Consequently, loss of STX led to reduced virulence of *S. aureus* and conversely to improved clearance of bacterium in a mouse model of sepsis [172]. Once *S. aureus* survives the unfavourable conditions within phagocytes, it escapes into the cytoplasm causing host cell death and release of bacteria into the extracellular space where they can replicate and infect other cells.

The success of *S. aureus* as an invading pathogen is due to its ability to adhere to the host cells and tissues. The bacterial adhesion is mediated by cell wall-associated proteins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). *S. aureus* expresses up to 20 potential MSCRAMMs, which facilitate binding to the host extracellular matrix proteins such as fibrinogen, fibronectin and collagen. The major staphylococcal adhesins are fibronectin binding proteins (FnBPs), which mediate binding of *S. aureus* to non-professional phagocytes such as bovine mammary gland epithelial cells, endothelial cells, fibroblasts, osteoblasts and keratinocytes [173, 174]. Interaction between FnBPs and β 1-integrins on the cell surface has been associated with internalization of *S. aureus* by non-phagocytic cells [175]. In contrast to non-professional phagocytes, professional phagocytes have been shown to internalize microbial pathogens via β 2-integrin [176]. In addition, β 3-integrins have been reported to cooperate with TLR2 in sensing viral and bacterial components [177]. Consequently, β 3-integrins can boost TLR2-mediated proinflammatory and type I IFN responses in a MyD88- and IRAK4- dependent fashion [177]. Furthermore, it has been shown that *S. aureus* cell wall teichoic acids (WTA) are important for establishment of nasal colonization via binding to the scavenger receptor [178]. Clumping factor B (ClfB) has been reported to bind to keratinocytes in the extracellular matrix of the host cells [179]. Finally, yet importantly, it has been shown that staphylococcal cell wall protein A binds to TNFR1, and thus induces expression of proinflammatory cytokines causing a significant damage in the lungs [130].

1.7.2 *S. aureus* and innate immunity

S. aureus is a pyogenic bacterium that is recognized by monocytes and macrophages. Monocytes and macrophages consequently produce inflammatory cytokines and chemokines that attract neutrophils to the site of infection and kill the pathogenic bacteria. However, *S. aureus* is equipped with a number of strategies of tissue invasion and phagocyte evasion. For many years, *S. aureus* was regarded as an extracellular pathogen that damages host cells after adhesion to their extracellular matrix [180]. However, recent studies have shown that *S. aureus* is able to invade a variety of mammalian cells [181] and persist inside phagocytes including neutrophils [182].

1.7.2.1 Recognition of *S. aureus* by innate immune cells

Recognition of invading bacteria is crucial for an efficient induction of immune responses. The bacterial cell wall is a rich source of antigens that are recognized by PRRs. Typical antigens of Gram-positive bacteria are peptidoglycans (PGN), lipoteichoic acids (LTA) and secreted toxins, while a prototype antigen of Gram-negative bacteria is lipopolysaccharide (LPS). It has been elucidated that TLR4 is the major receptor for LPS recognition, whereas TLR2 plays a key role in recognition of Gram-positive bacteria [183].

Despite an extensive literature about *S. aureus*, surprisingly little is known about the signaling pathways by which *S. aureus* triggers innate and adaptive immune responses in human cells. Most of the research conducted on *S. aureus* pathogenesis uses mouse cells and mouse models of infection; however, mice are not natural hosts of human *S. aureus* isolates, therefore mice have to be genetically altered prior to *S. aureus* infections [184]. It has been demonstrated that mice deficient in TLR2 and MyD88 are protected against *S. aureus* induced death, thus implying a key role of the TLR2-MyD88 pathway in *S. aureus* recognition [185]. Studies in IRAK4 deficient mice showed impaired proinflammatory responses upon peptidoglycan stimulation and *S. aureus* infection [186]. In addition, the same authors demonstrated that Mal and TRAF6 were involved in the signaling cascade in mouse BMDMs [186]. Importantly, clinical data from patients with MyD88 and IRAK4 deficiencies revealed a protective role of TLR2 signaling during recurrent bacterial infections in humans [187, 188]. Interestingly, patients with these deficiencies suffered mainly from upper respiratory tract infections caused by Gram-positive bacteria, including *S. aureus* or *Pseudomonas aeruginosa*, but not from viral, parasitical or fungal infections. These results demonstrate that TLR2-MyD88-IRAK4 signaling is crucial for the induction of proinflammatory responses upon *S. aureus* infection in mouse and human cells [Fig. 1.7 A].

Hence, TLR2 is recognized as the key cell surface receptor for recognition of *S. aureus* cell wall components. Upon detection of pathogens, TLRs homo- or hetero-dimerize in order to activate downstream signaling cascades. However, in the case of *S. aureus* recognition, an involvement of other TLRs or co-receptors remains speculative. This might be a coincidence of several factors found across the literature, including a diversity of *S. aureus* antigens, impure ligand preparations or distinct cell types used in studies. At first, a TLR2-TLR6 heterodimer was implicated in the recognition of *S. aureus*, however macrophages from TLR6 deficient mice could still produce TNF α after PGN stimulation, suggesting that TLR6 is not necessary for PGN recognition [189]. Later, it was shown that recognition of pure PGN is TLR2-independent, whereas purified LTA is sensed synergistically through a TLR2-TLR1 heterodimer, indicating that PGN might be recognized by intracellular receptors such as NLRs [190]. Additionally, a scavenger receptor CD36 was shown to play a role in LTA recognition and *S. aureus* phagocytosis. CD36 deficient mice were susceptible to *S. aureus* infection and had impaired responses after LTA and Malp2 but not after Pam3Csk4 and zymosan stimulation [22]. Since Malp2 and LTA are both diacylated lipopeptides and CD36 had an additive effect on NF κ B activation, it has been proposed that CD36 might enhance diacylglyceride signaling via TLR2-TLR6 heterodimer [22]. Furthermore, CD14, a key molecule in TLR4-LPS signaling, has been shown to interact with LTA derived from both *Staphylococci* and *Streptococci* [191]. LTA recognition was enhanced by LBP and CD14 in a TLR2-dependent but TLR4-independent manner, suggesting that LBP and CD14 might contribute to the pathogenesis caused by clinically significant Gram-positive bacteria [191]. On the other hand, CD14 failed to protect mice from *S. aureus*-induced sepsis, indicating that CD14 does not have a role during septic shock [192]. Lastly, a member of cytosolic NLR family NOD2 has been shown to detect PGN derived from both Gram-negative and Gram-positive bacteria through recognition of the PGN-derived ligand muramyl dipeptide (MDP) [193]. Moreover, mutations in *NOD2* gene have been associated with Crohn's disease and cells derived from these patients failed to detect MDP [85]. It has been shown that *S. aureus* can persist intracellularly in both phagocytic and non-phagocytic cells, therefore NOD2 can access PGN from the cytoplasm-dwelling *S. aureus* and trigger NOD2-dependent immune responses. Additionally, NOD2 has a co-stimulatory role in *S. aureus* recognition as MDP has been shown to lead to a robust cytokine secretion and enhanced DC maturation in mice [Fig. 1.7 B;194]. This shows that cooperation of multiple receptors with the central role of TLR2 is necessary for efficient recognition of *S. aureus* and induction of immune responses during bacterial infection.

Finally, it has been shown that phagosomal processing is required for maturation of *S. aureus* antigens and induction of immune responses [195]. Once inside a phagosome, bacterial nucleic acids can be released and can be recognized by endosomal TLRs. Accordingly, endosomal TLR8 has been shown to detect staphylococcal ssRNA and trigger MyD88-dependent activation of IRF5, thus leading to IFN β and IL-12 induction in human primary monocytes [162]. Interestingly, the same authors suggested an inhibitory role of surface TLR2 on this pathway [Fig. 1.7 C]. In addition, mice DCs have been shown to actively phagocytose *S. aureus* and sense staphylococcal DNA via endosomal TLR9 and MyD88, leading to IRF1-dependent activation of type I IFNs [Fig. 1.7 D;153].

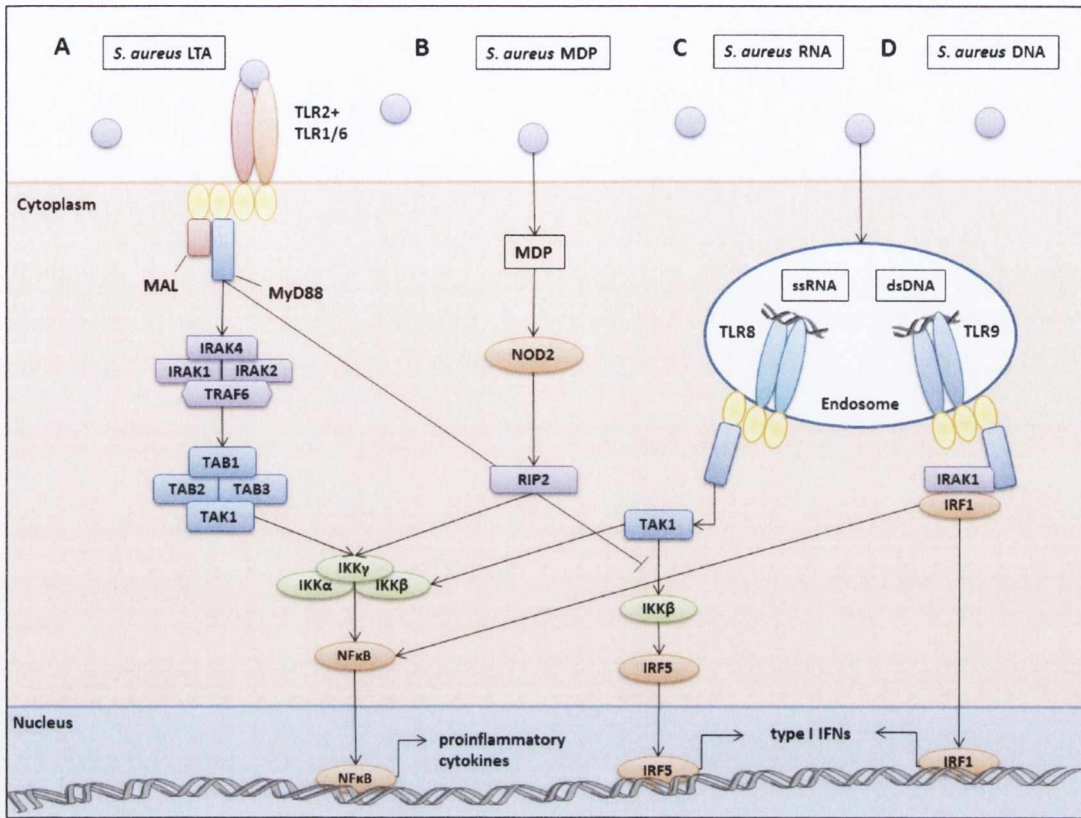


Figure 1.7: Recognition of *S. aureus* by innate immune system in mice and humans.

A: The whole bacterium is recognized by surface TLR2, which recruits MyD88 and Mal adaptor proteins. MyD88 triggers assembly of the myddosome and activation of TRAF6 via kinase activity of IRAK4. TRAF6 mediates activation of TAK1, leading to phosphorylation of IKK complex. IKK complex facilitates activation of NfκB and upregulation of proinflammatory cytokines [186]. **B:** Fragments of peptidoglycan (MDP) from a cytoplasm-dwelling *S. aureus* is detected by NOD2. NOD2 triggers RIP2 activation, which in turn activates IKK complex, leading to NfκB activation and induction of proinflammatory cytokines [194]. **C:** Staphylococcal ssRNA is sensed by endosomal TLR8 and activates TAK1. TAK1 kinase activity then phosphorylates IKKβ, which triggers IRF5-dependent IFNβ transcription. The TAK1/IKKβ activation can be inhibited by signaling from surface TLR2 after recognition of staphylococcal lipoproteins. In addition, TAK1 induces IKK complex, which triggers NfκB activation and transcription of inflammatory genes [162]. **D:** Staphylococcal dsDNA from endocytosed *S. aureus* can be detected by TLR9 and activate IRF1 via MyD88-IRAK1 complex, which triggers both proinflammatory and type I IFN responses [153].

1.7.2.2 Mechanisms of microbial degradation

Professional phagocytes, such as neutrophils, macrophages and dendritic cells, are well equipped for phagocytosis of invading microbes and efficient degradation of ingested microbes. Accordingly, professional phagocytes have higher basal phagocytic activity since internalization of microorganisms occurs via a so called “zipper mechanism”. In this process, bacteria express specific surface proteins that bind to receptors on the host cell membrane [Fig. 1.8 A]. This interaction induces signaling cascades, leading to reorganization of actin cytoskeleton and internalization of the bacterium [Fig. 1.8 B]. Both polymorphonuclear (PMN) and mononuclear phagocytes are important components of host immune defenses against invading bacteria. Both phagocytes create a microenvironment inhospitable to most bacteria by producing oxidizing and reducing agents within their phagosomes. Additionally, PMN phagocytes are equipped with granules containing catalytic and noncatalytic antimicrobial peptides, which fuse with microbe-containing phagosomes and further impair survival of the ingested microbe [Fig. 1.8 C]. Recently, a new defense mechanism against extracellular pathogens has been described. Upon activation, neutrophils have been shown to release web-like structures of DNA termed as neutrophil extracellular traps (NETs) [3]. The NETS contain chromatin fibres and serine proteases, which trap and kill extracellular microbes independently of phagocytosis.

However, the most powerful antimicrobial systems are the NADPH phagocyte oxidase and inducible nitric oxide synthase (iNOS) pathways, which generate superoxide and nitric oxide radicals also referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively. NADPH phagocyte oxidase and iNOS are separate enzyme complexes with independent regulation, however, they often function together and they are both dependent on NADPH and molecular oxygen [196]. Both PMN and mononuclear phagocytes express the NADPH oxidase and iNOS but neutrophils produce more ROS while macrophages produce higher amounts of RNS. During infection, phagocytes are primed to produce ROS immediately, whereas RNS production requires *de novo* protein synthesis [Fig. 1.8 C]. In addition, inflammatory cytokines such as TNF α and GM-CSF, have been shown to prime the NADPH oxidase activity via a mechanism involving PI3K [197]. It has been also shown that cytokines such as IFN γ , and microbial products, including LPS, can induce transcription of genes encoding components of the phagocyte superoxide system and enhance neutrophil and macrophage oxidative metabolism [198]. Induction of iNOS transcription involves signaling cascades comprising NF κ B [199] and JAK-STAT-IRF1 [150], which are triggered in response to stimulation

with microbial products such as LPS, or proinflammatory cytokines, including type I IFNs, IL-1 β and TNF α .

Both ROS and RNS can interact with numerous targets in a microbial cell including thiols, metal centers, protein tyrosines, nucleotide bases and lipids [200]. It has been reported that low concentration of ROS cause DNA damage, whereas high concentrations act on several bacterial targets [201]. Oxidative attack of DNA is dependent on iron causing production of hydroxyl and ferryl radicals, while sugar modifications of DNA can result in strand breaks. Proteins undergo a range of oxidative modifications in particular at cysteine, methionine, tyrosine, phenylalanine and tryptophan residues [196]. The antimicrobial activities of RNS are more complex than ROS actions and are dependent on the local redox environment [202]. Nitric oxide radicals have been shown to block bacterial DNA replication via zinc mobilization from metalloproteinases and to inhibit bacterial respiration [203]. Furthermore, nitric oxide and tyrosyl radicals can inhibit ribonucleotide reductases, and thus indirectly limit synthesis and repair of DNA [204].

Apart from antimicrobial functions, both ROS and RNS have other important roles in the host. Both ROS and RNS are significant signal transducers in processes including neurotransmission, phagocyte activation, cell proliferation and apoptosis. Nitric oxide signaling in the cardiovascular system is a vital factor of vascular homeostasis. However, ROS and RNS can also cause tissue damage and injury because superoxide and nitric oxide radicals attack not only microbes but also host cells and tissues. Lastly, ROS and RNS have central roles in regulation of inflammatory responses since they limit the damage caused by neutrophils to host tissues.

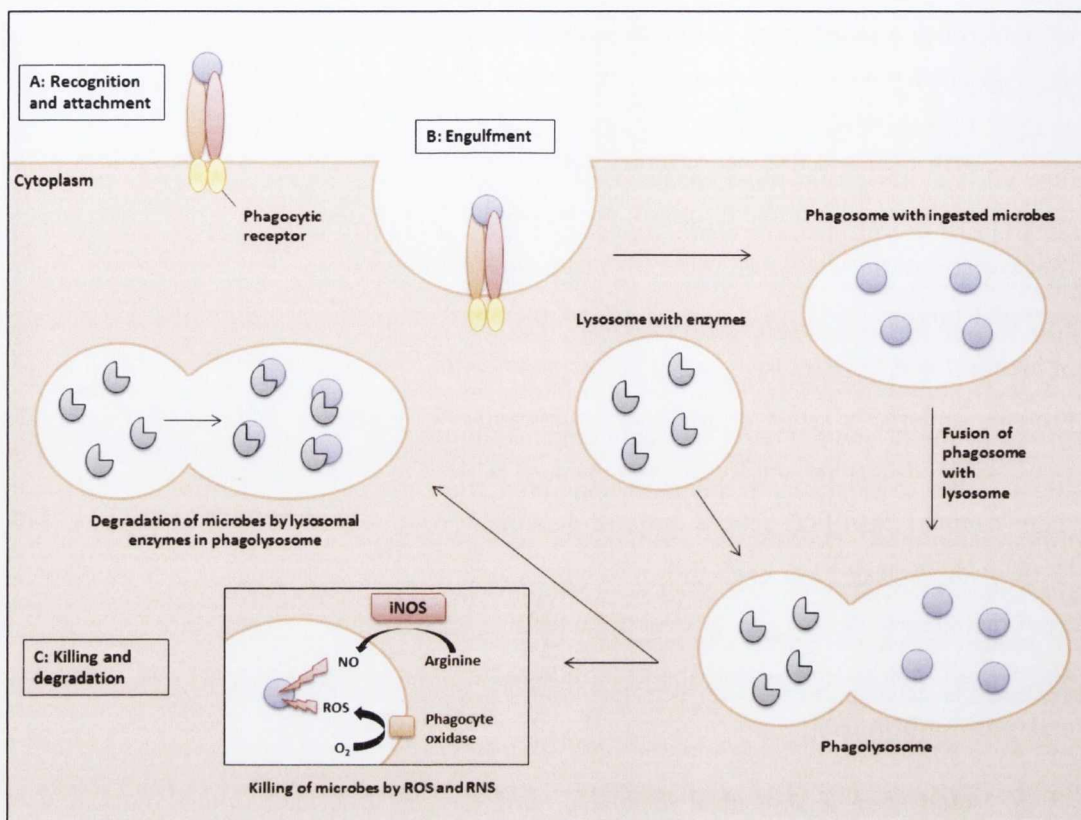


Figure 1.8: Phagocytosis and mechanisms of microbial killing

A: Phagocytosis is initiated by recognition of a microbe by surface receptors expressed on phagocytes. **B:** Activation of receptors activates actin filaments in the phagocyte membrane resulting in structural changes at the site of the attachment, formation of cytoplasmic pseudopods around the microbe and its engulfment into a phagocytic vacuole. Phagosome then fuses with one or more lysosomes containing proteolytic enzymes and form a phagolysosome. **C:** The microbe is killed by reactive oxygen species (ROS) and nitric metabolites (NO), which are produced in activated phagocytes during the respiratory burst in the presence of NADPH oxidase and nitric oxide synthase (iNOS). Alternatively, the microbe is degraded by the release of antimicrobial enzymes from lysosomal granules.

1.7.2.3 Microbial evasion and subversion of innate responses

Despite potent antimicrobial mechanisms of phagocytes, pathogenic microorganisms have evolved strategies of subverting or resisting the effects of ROS and RNS. In general, microbes can evade ROS and RNS actions simply by avoiding uptake by phagocytes through numerous mechanisms including evasion of pathogen recognition by receptors or disruption of phagocyte signaling pathways. Alternatively, microbial enzymes have been shown to suppress the respiratory burst and the subsequent production of ROS [205], or to convert ROS to less toxic species. For example, it has been shown that *S. aureus* carotenoid pigment staphyloxanthin (STX) serves as an antioxidant and protects the bacterium from phagocytic killing [172]. Additionally, microbes can avoid toxic ROS by compartmentalizing iron stores and controlling iron uptake [196]. Accordingly, bacteria deficient in iron storage proteins are more susceptible to oxidative stress, suggesting that iron homeostasis is an important factor in bacterial susceptibility to RNS and ROS [206]. Importantly, oxidative or nitrosative stress induce transcription of bacterial regulatory genes, which controls expression of genes involved in resistance to stress and in damage repair. For example, *S. aureus* transcriptional factor sigma B (σ^B) plays a central role in the stress responses and persistence *in vivo* [207] by induction of enhanced pigmentation by STX, thus increased resistance to H₂O₂ [208]. Moreover, σ^B down-regulates the *agr* expression, which controls the expression of several virulence factors including toxin α -hemolysin, a critical molecule in *S. aureus* survival within macrophages [169]. The ability to repair DNA damage caused by ROS and RNS is an important factor of resistance to microbial killing. Inhibition of DNA replication induces SOS response, which includes transcription of stress genes involved in DNA repair functions [203].

In addition, autophagy has an important role in the host defence against intracellular bacteria. Autophagy is a homeostatic process in eukaryotic cells critical for their survival. Autophagosomes are double-membrane vacuoles containing damaged components of cytosol which are digested after fusion with lysosomes [209]. During infection, a microorganism can be sequestered into autophagosomes which then facilitate lysosomal destruction of the bacteria. Consequently, some microorganisms have evolved mechanisms of subversion of autophagic processes by delaying autophagosomal maturation and fusion with lysosomes, thus creating a niche within the autophagosome [209]. For example, *S. aureus* has been shown to localize into autophagosomes and inhibit lysosomal function in HeLa cells, indicating a role of autophagy in facilitating intracellular survival of *S. aureus* in non-professional phagocytes [210]. Furthermore, it has been demonstrated that *S. aureus* survives within autophagosomes

by inhibition of autophagosomal maturation in mouse DCs, implicating the role of autophagy in survival and persistence in professional phagocytes [211].

1.7.3 *S. aureus* and adaptive immunity

The activation of innate immunity culminates in the activation of macrophages, recruitment of neutrophils to the site of infection and elimination of the pathogen. Importantly, activation of TLR signaling leads to maturation of DCs and induction of adaptive immune responses [212]. However, *S. aureus* is known for its ability to asymptotically colonize the nasal cavity and skin of many people. Long-term exposure to microbes induces adaptive immune responses, however there is limited evidence of *S. aureus* influence on systemic antibody responses in humans [213]. Understanding the interaction between *S. aureus* and T cells during colonization is of major interest for T cell manipulation during treatment or for vaccine development.

There have not been many studies addressing the role of T helper cells during *S. aureus* colonization in humans, however there is a growing evidence for their importance during *S. aureus* infections. Early colonization of the intestine by *S. aureus* has been associated with increased serum levels of IL-4 and IL-10, suggesting a shaping effect of microbiota on T helper cells [214]. Hence, it has been suggested that early microbiota might modulate the risk of developing inflammatory conditions such as allergy within the following years. Accordingly, airway exposure to *S. aureus* enterotoxins has been linked to the development of asthma by induction of local Th1/Th17 responses [215]. However, the persistent activation of T cells following the constant exposure to antigens might result in the loss of the ability of T cells to respond to these antigens (anergy) [216]. The establishment of T cell anergy to superantigens inhibits MHC Class II/TCR presentation of processed antigens, and thus development of antigen-specific effector and memory cells [216].

The ability of *S. aureus* to evade innate immune defences and manipulate the adaptive immune responses surely contributes to the lack of an effective immunological memory. Invasive *S. aureus* infections have been associated with decreased transcription of adaptive immunity genes, while expression of myeloid and innate immunity genes was enhanced [217]. Immunomodulatory IL-10 has been shown to reduce development of human proinflammatory Th1/Th17 responses and this inhibitory effect was less dramatic when the antigen-presenting cells were DCs [218]. Stimulation of naïve human T cells with heat-killed *S. aureus* gave rise to antigen-specific populations of proinflammatory Th1 and Th17 cells [219]. However, re-stimulation of these *S. aureus*-specific Th17 cells resulted in a switch to an anti-

inflammatory phenotype accompanied with reduced IL-17 and increased IL-10 production [219].

Such immunomodulatory effects might be consequences of the constant interaction between *S. aureus* and the innate immune system, which polarize T cell production and memory responses. To support this hypothesis, it has been shown that staphylococcal peptidoglycans triggered TLR2-mediated IL-10 production, which resulted in downregulation of superantigen-induced T cells and contributed to prevention of toxic shock syndrome [220]. Immunosuppression may be seen as a defensive adaptation against a proinflammatory “cytokine storm” mediated by massive T cell activation in response to staphylococcal superantigens. Remarkably, despite the high prevalence of superantigens in the clinical isolates, the incidence of *S. aureus*-induced toxic shock syndrome was diagnosed more sporadically than *S. aureus*-induced bacteraemia [221]. It has been shown that regulatory T cells express IL-10 in response to lower concentrations of superantigen than is required for IFN γ production, indicating a mechanism for bacterial evasion through the induction of regulatory T cells [222]. However, it has been shown that high serum levels of IL-10 during *S. aureus* bacteraemia strongly correlates with increased mortality of patients, while low IL-1 β levels correlates with prolonged bacteraemia [223]. In mice, *S. aureus* colonization seems to be mediated by an immunosuppression and specific proinflammatory Th17 responses [224]. Interestingly, a group of patients with hyper-IgE syndrome suffer from an increased susceptibility to lung and skin infections caused by *S. aureus* [225]. These patients have a mutation in STAT3, a key transcription factor in the development of Th17 cells; hence, they are unable to produce Th17 cells during infections, indicating a critical role of the Th17 pathway in immunity against *S. aureus* in humans [226].

1.7.4 *S. aureus* and vaccine development

Successful vaccines have efficiently reduced mortality caused by *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* infections, however an effective vaccine for prevention of *S. aureus* infections remains elusive. To date, no vaccine conferring protective immunity against *S. aureus* has successfully passed human clinical trials despite successful preclinical mouse studies [227]. This failure is most likely due to several factors that altogether make the development of efficient vaccine problematic. *S. aureus* is a common bacterium found in human microflora and about 20 % of the population carries *S. aureus* permanently in their upper respiratory tract and on the skin. The long-term exposure to *S. aureus* has most likely immunomodulatory effects on the innate and adaptive immune

responses that potentially influence vaccine-induced immunity. Moreover, there is a limited understanding of the human protective immunity against *S. aureus* apart from the realization that the immune responses in humans are different from those of most animals, especially mice [227]. In addition, *S. aureus* is equipped with numerous mechanisms for evasion from the innate immune defenses as well as for modulation of the adaptive immune responses.

So far, the attempts to produce efficient vaccine based on the production of antibodies against *S. aureus* have failed. It is possible that the wrong antigen or their combinations were chosen, that the antibodies failed to induce activity of neutrophils, or that the antibodies induced inhibitory responses [228]. Moreover, animal models failed to predict efficacy of vaccines in humans, hence a deeper understanding of *S. aureus* interactions with the human immune system is needed. It is now emerging that cell-mediated immunity and Th17 cells play critical roles in immune defenses against *S. aureus*. Accordingly, patients with impaired cell-mediated immunity, HIV patients, and patients with defects in IFN γ production or Th17 responses suffer from increased incidence of *S. aureus* infections [229]. Interestingly, influenza A has been shown to inhibit Th17-mediated host defences in mice via induction of type I IFNs, leading to impaired clearance of *S. aureus* and increased mortality [230]. Consequently, stimulation of IL-17 and IL-22 production resulted in enhanced clearance of *S. aureus* and reduced mortality. Importantly, highly virulent community-associated MRSA was found to induce type I IFNs and inhibit Th17 cell responses in mice lungs [231]. Thus, these studies suggest a central role of Th17 cells in protective responses during *S. aureus* infection and indicate a role for type I IFNs in modulation of these responses. Furthermore, it has been shown that most people have already protective antibodies against *S. aureus* in their blood thanks to the long-term interaction between *S. aureus* and their immune system [229]. Therefore, it is possible that current vaccines cannot provide more protection than is naturally available. However, if the design of vaccines was focused on a narrow-spectrum of diseases, such as *S. aureus* pneumonia caused by staphylococcal toxins, the vaccine development could be based on induction of neutralizing antibodies against α -toxin and Panton-Valentine leukocidin. Even though the vaccines based on Th17 responses might constitute a new direction for vaccine development, the questions concerning the duration of Th17 cell memory remains unclear [229]. Hence, instead of disease prevention, the new vaccines should focus on reduction of disease severity, morbidity and mortality during *S. aureus* infections. In any case, there is a need for efficient *S. aureus* vaccine or an alternative treatment of infections due to the increasing prevalence of antibiotic resistant strains within the community.

1.8 Aims

Pattern recognition receptors (PRRs) recognize conserved microbial structures and trigger a cascade of immune responses that culminate in microbial clearance and induction of adaptive immune responses via production of cytokines and type I interferons (IFNs). Type I IFNs are potent immunomodulators of both innate and adaptive immunity, with a well-defined anti-viral role, yet their role during bacterial infections remains unsolved. *Staphylococcus aureus* is a part of healthy microflora as well as a significant cause of infections in humans. Although there is an extensive research on *S. aureus* pathogenicity and immune evasion, the role of type I IFNs during infection remains controversial. It has been shown that TLR2 is a major PRR for recognition of *S. aureus*, however little is known about the ability of *S. aureus* to induce type I IFNs in human cells. Furthermore, the nature of signaling components in *S. aureus*-mediated type I IFN signaling remains unclear, as does the role of type I IFNs on the outcome of *S. aureus* infection in human cells.

Thus, the aims of this project are:

1. To establish whether *Staphylococcus aureus* stimulates type I IFN induction in human cells.
2. To investigate the ability of human cells to kill *S. aureus in vitro*.
3. To dissect molecular mechanisms of the type I IFN pathway in response to TLR2 ligands compared to *S. aureus* in human cells.
4. To investigate induction of other relevant cytokines during *S. aureus* infection in human cells.

2. Materials and Methods

2.1 Materials

2.1.1 General chemicals and plastic

General chemicals were purchased from Sigma-Aldrich (Arklow, Ireland). General plastic was purchased from Cruinn Diagnostics (Dublin, Ireland), Grenier Bio-One (Stonehouse, UK) and VWR International Ltd. (Dublin, Ireland). General buffers and their composition are listed in Table 2.1.

Buffer	Recipe	
10x phosphate buffered saline (PBS)	• 1.45 M • 34 mM • 227 mM	NaCl NaH ₂ PO ₄ Na ₂ HPO ₄
10 x tris buffered saline (TBS)	• 1.37 M • 250 mM • 27 mM	NaCl Tris KCl pH 7.5
10x running buffer	• 250 mM • 1.92 M • 35 mM	Tris glycine SDS
10x transfer buffer	• 250 mM • 1.92 mM	Tris glycine
1x transfer buffer	• 10 % • 20 %	10x transfer buffer methanol (v/v)
3x sample buffer (SB)	• 50 mM • 10 % • 2 % • 0.1 %	0.5 M Tris, pH 6.8 glycerol (v/v) SDS (w/v) bromphenol blue (w/v) + inhibitors (Table 2.7)

Table 2.1: Common buffers and their composition.

2.1.2 Cells and cell culture

THP-1 and A549 cells were purchased from European Collection of Cell Culture (ECACC). BEAS-2B cells were a kind gift from Dr. Sinead Miggin (National University of Ireland Maynooth, Ireland). HEK-Blue™ IFN-alpha/beta cells were a kind gift from Prof. Luke O'Neill (Trinity College Dublin, Ireland). CRISPR TLR2 knockout THP-1 cell clones A5 and D4 were a kind gift from Prof. Dr. med. Veit Hornung (University Hospital of Bonn, Germany). Primary blood mononuclear cells (PBMCs) were obtained from Irish Blood Transfusion Centre in the form of buffy coats. Ethical Approval was obtained from the Faculty of Health Sciences Ethics Committee for experiments involving PBMCs.

2.1.2.1 Cell culture

Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 were purchased from Gibco® (Dublin, Ireland). Phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), trypsin-EDTA (TE), sodium pyruvate, penicillin-streptomycin, gentamicin and ciprofloxacin were purchased from Sigma-Aldrich (Arklow, Ireland). Blastidin, normocin™

and zeocinTM were purchased from Invivogen (CA, USA). Cell culture plastics were purchased from Fisher Scientific Inc. (Dublin, Ireland). LymphoprepTM was purchased from Axis-Shield Density Gradient Media (Oslo, Norway). 10x Red Lysis Buffer, CD14+ microbeads and LS columns for MACS cell separation were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.1.2.2 Cell culture ligands

CL075, low molecular weight (LMW) poly(I:C), Pam3Csk4 and purified LTA-SA were purchased from Invivogen (CA, USA). Malp2 and ultrapure LPS were purchased from Enzo Life Sciences Ltd. (Exeter, UK). Human interferon alpha (IFN α) was obtained from PBL Assay Science (NJ, USA). Vaccinia virus (VACV) dsDNA 70mer was synthesized as two complementary strands that were purchased from Integrated DNA Technology (Iowa, USA). The single strands were mixed in 1:1 ratio; the mixture was ligated by boiling at 95°C for 5 min and then cooling to room temperature. For an overview of ligands and their receptors, refer to table 2.2.

Ligand	Receptor	Company
SA-LTA	TLR2	Invivogen
Pam3Csk4	TLR2-TLR1	Invivogen
Malp2	TLR2-TLR6	Enzo Life Sciences
LMW poly(I:C)	TLR3	Invivogen
LPS	TLR4	Enzo Life Sciences
CL075	TLR7/8	Invivogen
70mer	STING	Integrated DNA Technology
IFN α	IFNAR1/2	PBL Assay Science

Table 2.2: Overview of ligands and their receptors.

2.1.2.3 Cell culture reagents

Phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Arklow, Ireland). Lipofectamine[®] 2000 was purchased from InvitrogenTM (Dublin, Ireland). PierceTM LDH Cytotoxicity Assay Kit was purchased from Thermo Fisher Scientific Inc. (Dublin, Ireland). QUANTI-BlueTM was purchased from Invivogen (CA, USA).

2.1.2.4 Cell culture inhibitors

Bafilomycin A1, Cytochalasin D, Dynasore and Piceatannol were obtained from Sigma-Aldrich (Arklow, Ireland). Neutralizing human anti-TLR2 antibody and IgG1 isotype control were purchased from eBioscience (San Diego, USA). Eritoran tetrasodium/E5564 was obtained from Eisai Co., Ltd. (Tokyo, Japan). VIPER and CP7 peptides were synthesized by Imgenex/Novus Biologicals (Abingdon, UK). (5Z)-Zeaenol, (5Z)-7-Oxozeaenol and IRAK-1/4 Inhibitor were purchased from Merck Millipore (MA, USA). BI605906, MRT67307 and MRT199665

compounds were a kind gift from Prof. Philip Cohen (University of Dundee, UK; Table 2.3). All inhibitors are listed in Table 2.4.

	MRT67307	MRT199665
AMPK α 1/ α 2	810	10
MARK1	27	2
MARK2	52	2
MARK3	36	3
MARK4	41	2
SIK1	250	110
SIK2	67	12
SIK3	430	43
NUAK1	230	3
NUAK2	2600	120
BRSK1	>10 000	>10 000
BRSK2	10 000	10 000
MELK	900	29
TBK1	19	5400
IKK ϵ	160	7700

Table 2.3: IC50 (nM) values for inhibition of kinases by MRT67307 and MRT199665 compounds ^[232].

Inhibitor	Mechanism of action	Supplier
TLR2 antibody	antagonize Toll-like receptor 2	eBioscience
IgG1	TLR2 antibody isotype control	
Eritoran	binds to CD14, and thus blocks TLR4 signalling and trafficking of TLR4 into endosome	Eisai Co., Ltd
Dynasore	targets dynamin, and thus inhibits dynamin-dependent endocytosis	Sigma-Aldrich
Piceatannol	inhibits non-receptor Syk kinase, and thus blocks Syk-mediated endocytosis	
Cytochalasin D	prevents actin polymerization, and disrupts actin polymers	
Bafilomycin A1	inhibits vacuolar H ⁺ ATPase, thus fusion of endosomes with lysosomes	
VIPER	blocks interactions between TLR4 and adaptors Mal/TRAM, and thus inhibits TLR4 signaling	Novus Biologicals
CP7	VIPER control peptide	
IRAK-1/4 Inhibitor	inhibitor of interleukin-1 receptor-associated kinases 1 and 4	Merck Millipore
(5Z)-7-Oxozeaenol	inhibitor of TAK1 activity preventing IL-1-induced JNK and p38 phosphorylation	
(5Z)-Zeaenol	negative control for TAK1 inhibitor	
BI605906 ^[233]	small molecule inhibitor of the Ikk β kinase	Prof. Philip Cohen, University of Dundee, UK
MRT67307 ^[232]	small molecule inhibitor of the I κ B kinase (IKK)-related kinases and AMP-activated protein kinases (AMPK) family	
MRT199665 ^[232]	small molecule inhibitor of the AMP-activated protein kinases (AMPK) family	

Table 2.4: List of inhibitors, mechanism of action and supplier.

2.1.3 Bacteria and bacterial culture

Staphylococcus aureus strain SH1000 described in [208] was a kind gift from Dr. Rachel McLoughlin (Trinity College Dublin, Ireland). Briefly, SH1000 is derived from 8325-4 parental strain that carries a small deletion in rsbU. This locus encodes a positive regulator of σ^B activity

that controls stress responses important for *S. aureus* survival and that might contribute to virulence. SH1000 carries reconstituted *rsbU*⁺ locus that alters its phenotype when compared to 8325-4 parental strain including production of the orange pigment staphyloxanthin, decreased levels of alpha toxin, expression of *asp23*, and increased resistance to H₂O₂ [208]. Tryptic soy agar (TSA; per 1 L: 15 g casein peptone (pancreatic), 5 g soy peptone (pancreatic), 5 g sodium chloride, 15 g Agar) for *S. aureus* culture was purchased from Sigma-Aldrich (Arklow, Ireland). Plastics for bacterial culture were purchased from VWR International Ltd., and Fisher Scientific Inc. (Dublin, Ireland).

2.1.4 siRNA and NEON® transfection system

The NEON® transfection system and kit were purchased from Invitrogen™/Fisher Scientific Inc. (Dublin, Ireland). Custom siRNAs were synthesized by Qiagen (Netherlands) and the sequences are listed in Table 2.5. SMARTpool ON-TARGETplus TICAM2 (TRAM) and TIRAP (MAL) siRNA, and ON-TARGETplus Non-targeting Pool were purchased from Dharmacon/Thermo Fisher Scientific Inc. (Dublin, Ireland). SMARTpool ON-TARGETplus TICAM1 (TRIF) siRNA was a kind gift from Dr. Emma Creagh (Trinity College Dublin).

Target gene		Sequence 5'---3'
human IRAK2	sense	GCAACGUCAAGAGCUCUAATT
	antisense	UUAGAGCUCUUGACGUUGCTG
human IRF7	sense	GCCUCUAUGACGACAUCGA
	antisense	UCGAUGUCGUCAUAGAGGC
human MyD88	sense	CUGGAACAGACAAACUAUCTT
	antisense	GAUAGUUUGUCUGUCCAGTT
human STING	sense	GCAUCAAGGAUCGGGUUUATT
	antisense	UAAACCCGAUCCUUGAUGCCA
human TRAF3	sense	AAGAUUCGCGACUACAAUUTT
	antisense	AAUUGUAGUCGCGAAUCUUCC
human TRAF6	sense	CCACGAAGAGAUAAUGGAUTT
	antisense	AUCCAUAUCUCUUCGUGGTT

Table 2.5: Overview of siRNA sequences.

2.1.5 Enzyme-linked immunosorbent assay (ELISA)

The IFNα ELISA kit was purchased from MABTECH (Buro Deutschland, Germany). The CCL5 (RANTES), CXCL10 (IP-10), IL-1β/IL-1F2, IL-6, IL-10 and TNFα ELISA kits were purchased from R&D SYSTEMS Europe Ltd. (MI, USA). BD OptEIA™ TMB Substrate Reagent Set was purchased from BD Biosciences (Oxford, UK). Stop solution was composed of 2 M H₂SO₄. High binding 96-well plates were purchased from SPL Life Sciences Ltd. (Gyeonggi-do, Korea). Medium binding 96-well plates were purchased from Greiner Bio-One (Stonehouse, UK).

2.1.6 Quantitative real time polymerase chain reaction (qRT-PCR)

The High Pure RNA Isolation kit was obtained from Roche (Burgess Hill, UK). dNTPs were purchased from New England Biolabs Inc. (MA, USA). M-MLV reverse transcriptase and 5x M-MLV buffer were purchased from Promega (WI, USA). SensiFAST™ SYBR Lo-ROX Sybr Green was purchased from Bioline Reagents Ltd. (London, UK). Random hexamer and primers were synthesized by IDT (Iowa, USA). The primer sequences are listed in Table 2.6.

Gene		Sequence 5'---3'
human β -actin	Fwd	CGCGAGAAGATGACCCAGATC
	Rev	GCCAGAGGCGTACAGGGATA
human IFN β	Fwd	CTTTGCTCTGGCACAACAGG
	Rev	GTGGAGAAGCACAAACAGGAGA
human IRF7	Fwd	CACTAACGACAGGCGAGGC
	Rev	CTTGGGGAGGGTGACAGGTA
human IRAK2	Fwd	CGGGAAGCCATTCTGCTTCA
	Rev	GGGTGGCAGCATCTAAGACA
human MAL (TIRAP)	Fwd	AGCTGTCATGCGTTATCTGC
	Rev	TCACTCCAATTTCCGGGGTC
human MD2	Fwd	AAGGGAGAGACTGTGAATACAACAA
	Rev	GGCTCCCAGAAATAGCTTCAAC
human MyD88	Fwd	ACCCAGCATTGAGGAGGATTG
	Rev	GAAGGCATCGAAACGCTCAG
human STING	Fwd	ACTGTGGGGTGCCTGATAAC
	Rev	TGGCAAACAAAGTCTGCAAG
human TNF α	Fwd	GCCCATGTTGTAGCAAACCC
	Rev	TATCTCTCAGCTCCACGCCA
human TLR2	Fwd	TCTGTAGCAACTGGCTTAGTTCA
	Rev	TGGCCACAGAGGAGTCTCTTA
human TLR4	Fwd	CGGAGGCCATTATGCTATGT
	Rev	TCCCTTCCTCCTTTTCCCTA
human TRAF3	Fwd	CTCACAAGTGCAGCGTCCAG
	Rev	GCTCCACTCCTTCAGCAGGT
human TRAM (TICAM2)	Fwd	TTCCTGCCCTCTTTCTCTCTC
	Rev	AACATCTCTTCCACGCTCTGA

Table 2.6: Overview of primer sequences.

2.1.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Glycine, trizma base (Tris), sodium dodecyl sulphate (SDS), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), bromophenol blue, tween 20® and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Dublin, Ireland). Protogel® acrylamide was purchased from National Diagnostics (Hessle, UK). Dithiothreitol (DTT), PageRuler™ Plus Prestained Protein Ladder, 3MM Whatmann® filter papers and polyvinylidene fluoride (PVDF) membrane were purchased from Fisher Scientific Inc. (Dublin, Ireland). EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL) was purchased from Merck Millipore (MA, USA). NF κ B p65 (C-20) and TRAF6 (C-20) antibodies were purchased from Santa Cruz Biotechnology Ltd. (Heidelberg, Germany). IRF-3 antibody (Cat. no. 18781) was purchased

from ImmunoBiological Laboratories Inc. (MN, USA). IRF7, phospho-IRF-3 (Ser396, 4D4G), phospho-NF- κ B p65 (Ser536) (93H1) and TRIF antibodies were purchased from Cell Signaling Technology® (MA, USA). β -actin primary and anti-Rabbit IgG-Peroxidase secondary antibodies were purchased from Sigma-Aldrich (Dublin, Ireland). The IR Dye® anti-mouse secondary antibody for the Odyssey Infrared Imaging System was purchased from Li-COR Biosciences (Cambridge, UK).

2.2 Methods

2.2.1 Cell culture

Cells were handled under aseptic conditions in a laminar flow hood and all reagents were sterile.

2.2.1.1 Maintaining THP-1 cells

The human monocytic THP-1 cell line was grown in RPMI 1640 medium containing 2 mM L-Glutamine, 10% (v/v) FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. THP-1 CRISPR knockout cells were grown in RPMI 1640 medium containing 2 mM L-Glutamine, 10% (v/v) FCS, 10 μ g/ml ciprofloxacin and 1 mM sodium pyruvate. Cells were kept at 37°C with 5% CO₂ and were split every 2-3 days.

2.2.1.2 Maintaining A549 and BEAS-2 cells

The human lung epithelial cell lines A549 and BEAS-2 were grown in DMEM medium containing 2 mM L-Glutamine, 10% (v/v) FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were kept at 37°C with 5% CO₂ and were split every 2-3 days upon reaching 80% confluency. Cells were split by removing medium and washing the cells with 1x PBS and adding 1x TE. Complete DMEM was added once the cells were in suspension. Cells were harvested by centrifugation at 100 acceleration due to gravity (g) for 5 min at room temperature. The supernatant was removed; cells were re-suspended in fresh DMEM, counted using a haemocytometer and split accordingly.

2.2.1.3 Maintaining HEK-Blue™ IFN α / β cells

The human HEK-Blue™ IFN α / β cell line was grown in DMEM medium containing 2 mM L-Glutamine, 10% (v/v) FCS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 30 μ g/ml blasticidin, 100 μ g/ml normocin™ and 100 μ g/ml zeocin™ (growth medium). Cells were kept at 37°C with 5% CO₂ and were split every 3 days upon reaching 75% confluency. Cells were split by removing medium and washing the cells with 1x PBS and adding 1x TE. DMEM was added once the cells were in suspension. Cells were harvested by centrifugation at 100 g for 5 min at room temperature. The supernatant was removed, cells were re-suspended in fresh DMEM,

counted using a haemocytometer and split accordingly. For the HEK-Blue IFN- α/β bioassay, DMEM Test medium containing 2 mM L-Glutamine, 10% (v/v) FCS, 50 U/ml Penicillin, 50 μ g/ml Streptomycin, 100 μ g/ml NormocinTM was used.

2.2.1.4 Treatment and stimulation of cells

Cells were counted using a haemocytometer and were seeded accordingly. In some cases, cells were treated with 100 nM PMA 24 hr (THP-1) or 50 ng/ml GM-CSF (CD14+) 6 days prior to treatment. Cells were treated with inhibitor 1 hr prior to stimulation or infection. Inhibitors were diluted in water or serum free media (SFM) and were added directly onto cells at appropriate concentrations. TLR ligands were diluted in water or SFM and were added directly onto cells at appropriate concentration. VACV 70mer and LMW poly(I:C) were transfected using Lipofectamine[®] 2000. For every ml of cells, a master mix containing 20 μ l SFM and 1 μ l of Lipofectamine was made and was allowed to stand for 5 min at room temperature. The appropriate amount of ligand was added into 20 μ l of SFM. The master mix was divided between ligand containing and ligand-free SFM. The mixture was allowed to stand for 20 min at room temperature before it was added to the cells.

2.2.2 Peripheral Blood Mononuclear Cells (PBMCs)

2.2.2.1 PBMCs isolation

Blood for PBMC isolation was obtained from the Irish Blood Transfusion Service in the form of buffy coat packs. The blood was diluted at the ratio of 1:7 with 1x sterile PBS. Lymphoprep was dispensed at a volume 15 ml into a 50 ml falcon tube. 35 ml of the diluted blood was slowly added on top of lymphoprep by letting the blood flow along the inner side of the tube using sterile Pasteur pipette, making sure the blood flowed at a steady and slow rate, to ensure that two distinct layers were present in the tube. The tubes were then centrifuged at 400 g for 30 min at 16°C with the brake turned off. Four distinct phases were formed in the tube and the PBMCs were collected using a Pasteur pipette from the interface between lymphoprep and the serum. The PBMCs were washed in 1x sterile PBS and centrifuged at 400 g for 5 min with the brake on. The cells were re-suspended in 1x Red Lysis Buffer and incubated for 10 min at room temperature. The reaction was ceased by adding the equivalent volume of complete media. Cells were re-suspended in complete media and were counted using haemocytometer.

2.2.2.2 CD14+ monocyte isolation

Primary monocytes were isolated from the PBMCs by positive selection using Miltenyi Biotec CD14+ microbeads and LS columns following the manufacturer's protocol. Briefly, PBMCs were

counted using a haemocytometer and required volume of PBMCs was centrifuged at 400 g for 5 min. Supernatant was discarded and cells were re-suspended in 100 μ l of buffer (0.05% BSA (w/v), 2mM EDTA, 1x PBS) per 10^7 cells. CD14+ microbeads were added to the cell suspension at 8 μ l per 10^7 cells and the mixture was incubated for 15 min at 4°C. Cells were washed by adding 1 ml of buffer per 10^7 cells and were centrifuged at 400 g for 5 min. Supernatant was discarded and cells were re-suspended in 500 μ l of buffer per 10^8 cells. LS column was placed into MACS Separator and was rinsed with 3 ml of buffer. Once the column reservoir was empty, the cell suspension was placed onto the column. Column was washed 3x with 3 ml of buffer and after the final wash, the pass through effluent was clear. Column was then removed from MACS Separator and was placed into a sterilin. The magnetically labeled cells were flushed out in 5 ml of buffer by a firm push of the plunger into the sterilin. CD14+ cells were centrifuged at 400 g for 5 min and were re-suspended in complete medium. CD14+ cells were counted using haemocytometer and were seeded accordingly.

2.2.2.3 Maintaining CD14+ monocytes

The isolated primary CD14+ monocytes were kept in RPMI 1640 medium GlutaMAX™ containing 10% (v/v) FCS, 50 μ g/ml Gentamicin. In some cases, CD14+ monocytes were differentiated into monocyte-derived macrophages by adding GM-CSF (50 ng/ml) to the culture medium. Cells were incubated for 6 days and medium was replenished every 2 days. For infection studies, cells were kept in RPMI 1640 medium GlutaMAX™ containing 10% (v/v) FCS without antibiotics. In all cases, cells were kept at 37°C with 5% CO₂.

2.2.3 Bacterial culture

Bacterial stocks were stored at -80°C or were kept on ice at all times. *S. aureus* was streaked on fresh TSA agar plate using sterile inoculation loop and plates were incubated overnight at 37°C. The next day, *S. aureus* was harvested into sterile PBS and absorbance was measured on a Biospectrometer® (Eppendorf). Absorbance of bacteria was adjusted to OD₆₀₀=0.43-0.45, which was previously estimated to contain 4×10^8 CFU/ml.

2.2.3.1 Bacterial infection *in vitro*

For *S. aureus* infection assays, cells were seeded on a 24-well plate (1×10^6 cells/ml) in media without FCS and antibiotics. Cells were infected with live *S. aureus* at a multiplicity of infection (MOI) of 1, 10 or 100 and cells were incubated at 37°C with 5% CO₂. After 2 hr of infection, gentamicin (200 μ g/ml) was added into the media followed by incubation for indicated times and subsequent collection of cells and supernatants by centrifugation at 100 g for 5 min.

All experiments were performed in triplicate wells for each condition and repeated at least three times.

2.2.3.2 Total bacterial killing assay

For bacterial killing, cells were seeded on a 24-well plate (1×10^6 cells/ml) in media without FCS and antibiotics. Cells were infected with live *S. aureus* at an MOI of 100 and cells were incubated at 37°C with 5% CO₂. At indicated times after infection, cells and supernatants were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100 for 10 min at room temperature. The supernatant was re-introduced into well and mixed with the cell lysate. Serial dilutions of the suspensions were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. All experiments were performed in duplicate wells for each condition and repeated at least three times.

2.2.3.3 Gentamicin protection assay

For protection assay, cells were seeded on 24-well plate (1×10^6 cells/ml) in media without FCS and antibiotics. Cells were infected with live *S. aureus* at an MOI of 100 and cells were incubated at 37°C with 5% CO₂. Gentamicin (200 µg/ml) was added after 30 min of infection and this was considered as the time zero. The cells were further incubated for indicated times followed by collection of cells and supernatants by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100 for 10 min at room temperature. Serial dilutions of the cell lysates were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. Bacterial survival was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. All experiments were performed in duplicate wells for each condition and repeated at least three times.

2.2.4 siRNA silencing

RNA silencing was performed using a Neon® Transfection System (Invitrogen™). THP-1 cells were washed in sterile 1x PBS and counted using a haemocytometer. 2×10^6 cells were transferred into a sterilin tube and centrifuged at 100 g for 5 min at room temperature. Supernatant was discarded, cells were re-suspended in 115 µl R Buffer and 100 pmol siRNA was added to the cell suspension. Cells were collected in a 100 µl NEON tip using a NEON pipette. The pipette was placed into the NEON electroporation chamber filled with 3 ml

E2 buffer. THP-1 cells were electroporated using pre-set program #15 with the following setting: pulse voltage: 1300 V, pulse width: 20 ms, pulse number: 2. Alternatively, CD14⁺ monocytes were electroporated using pre-set program #9 with the following setting: pulse voltage: 1400 V, pulse width: 30 ms, pulse number 1. After electroporation, cells were dispensed into pre-warmed RPMI 1640 medium without antibiotics and plated into 24-well plate. Cells were incubated at 37°C with 5% CO₂ for approximately 42 hr and then were stimulated for further 6 hr. All experiments were performed in duplicate wells for each condition and repeated at least three times.

2.2.5 LDH assay

LDH assay was performed using Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Briefly, 10x Lysis buffer was added to untreated cells as a positive control. Supernatants were harvested by centrifugation at 100 g for 5 min and collected. 50 µl of the positive and test samples were plated into 96 well-plate and 50 µl of LDH reaction buffer was added. Reaction plate was kept in the dark and was incubated until positive sample turned red. The reaction was stopped by addition of 50 µl of Stop solution and the absorbance was measured at 490 nm and 680 nm on a Multiskan™ FC Microplate Photometer (Thermo Scientific™) using SkanIt software 2.5.1. The data was normalized to lysed untreated and unstimulated cells. All experiments were performed in duplicate or triplicate wells for each condition and repeated at least three times.

2.2.6 MTT assay

Suspension cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and 50 µl of MTT solution was added to each well. Cells were incubated with MTT for 2 hr at 37°C with 5% CO₂. After 2 hr, unmetabolized MTT was carefully removed and metabolized MTT (formazan) was re-suspended in 50 µl DMSO. Absorbance was measured at 620 nm on a Multiskan™ FC Microplate Photometer (Thermo Scientific™) using SkanIt software 2.5.1. The data was normalized to untreated and unstimulated cells. All experiments were performed in triplicate wells for each condition and repeated at least three times.

2.2.7 ELISA

RANTES, IP-10, IL-1β, IL-6, IL-10 and TNFα protein concentrations in supernatants were detected using the appropriate R&D ELISA kit following the manufacturer's instructions and corresponding lot-specific Certificate of Analysis (C of A). IFNα protein concentrations were detected using MABTECH ELISA kit following the manufacturer's instructions and corresponding concentrations. Briefly, high binding ELISA plate was coated with 50 µl/well

of capture antibody in 1x PBS for 2 hr at room temperature. Plate was washed 3x with ELISA wash buffer (1x PBS/0.05% (v/v) Tween) and dried thoroughly. Non-specific protein binding was blocked with 50 µl/well Reagent Diluent (1% BSA/1x PBS) for 1 hr at room temperature followed by 3x wash with ELISA wash buffer and dried thoroughly. Standard was performed in triplicates followed by 1:2 serial dilution including blank wells containing reagent diluent alone. Supernatant were plated with appropriate dilutions in reagent diluent in the total volume of 50 µl per well and the plate was incubated overnight at 4°C. The following day, the plate was washed 3x and dried thoroughly and 50 µl/well detection antibody was added in reagent diluent. The plate was incubated for 2 hr at room temperature followed by washing and drying. 50 µl/well streptavidin-HRP in reagent diluent was added to the plate and was incubated in the dark for 30 min followed by washing and drying. Lastly, 50 µl of 1:1 mixture of TMB substrate solution was added to each well until the standard curve was visible and the reaction was stopped with 25 µl of stop solution. The plate was read using a VersaMax ELISA Microplate Reader (Molecular Devices) at absorbance 450 nm and 540 nm. The data were analyzed using the SoftMax Pro v4.8 software. All experiments were performed in duplicate or triplicate wells for each condition and repeated at least three times.

2.2.8 HEK-Blue™ IFN α / β bioassay

HEK-Blue IFN- α / β bioassay protocol for measuring bioactive type I IFN was obtained from Prof. Søren Paludan and Prof. Martin Jakobsen (Aarhus University, Denmark). HEK-Blue IFN- α / β cells have reconstituted type I IFN signaling thanks to stable expression of STAT2 and IRF9. Presence of type I IFNs activates the JAK-STAT pathway and induces expression of the reporter gene expressing secreted embryonic alkaline phosphatase (SEAP) under the control of IFN-inducible ISG54 promoter. In the presence of type I IFNs, SEAP is secreted into the medium and quantified by QUANTI-Blue. Briefly, supernatants from previous experiments were plated into tissue culture 96 well plate with appropriate dilutions in the total volume of 50 µl in the test medium (DMEM, 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml normocin). The standard curve was performed in duplicates and comprised of human IFN α with the highest concentration of 100 U/ml followed by 1:2 serial dilution, and blank wells containing medium alone. HEK-Blue IFN- α / β cells were harvested, counted using a haemocytometer and 2.8×10^5 cells/ml were added to wells containing supernatant, standard and blank. The plate was incubated for 20-24 hr at 37°C with 5% CO₂. SEAP was detected using 50 µl of HEK-Blue IFN- α / β supernatant and 150 µl of pre-warmed QUANTI-Blue solution. Cells were incubated for 5-30 min at 37°C until the standard row became visible. Absorbance was measured at 620 nm on a Multiskan™ FC Microplate Photometer (Thermo Scientific™) using

Skant software 2.5.1. All experiments were performed in duplicate or triplicate wells for each condition and repeated at least three times.

2.2.9 qRT-PCR

2.2.9.1 RNA isolation

Total RNA was isolated from cells using Roche High Pure RNA isolation kit following the manufacturer's instructions. Briefly, cells were re-suspended in 100 µl of 1x PBS followed by adding 200 µl of Lysis/Binding buffer and vortexed for 15 s and spun down briefly. In some cases, the suspension was stored at -80°C until needed or processed for RNA isolation. The sample was transferred onto a High Pure Filter Tube placed in collection tube and centrifuged at 8 000 g for 30 s. The flow through was discarded and tube was placed back onto the collection tube. Each sample was then incubated with 50 µl of mixture containing 5 µl of DNase I and 45 µl of DNase I Incubation buffer. After 15 min incubation, 500 µl of Wash buffer I was placed to the reservoir and centrifuged at 8 000 g for 30 s. The flow through was discarded followed by wash with 500 µl of Wash buffer II and centrifuged at 8 000 g for 30 s. The flow through was discarded and tube placed back onto collection tube followed second wash with 200 µl of Wash buffer II and centrifuged at 13 000 g for 2 min. The collection tube was discarded and Filter Tube was placed into RNase free 1.5 ml microcentrifuge tube. In order to elute RNA, 50 µl of Elution buffer was added to the upper reservoir of the Filter Tube. After 1 min incubation, the tubes were centrifuged at 8 000 g for 1 min. The eluted RNA was kept on ice or stored at -80°C until cDNA synthesis was carried out.

2.2.9.2 cDNA synthesis

cDNA was synthesized from total RNA by reverse transcription. A master mix was prepared consisting of 2 µl 5x M-MLV Buffer, 2 µl dNTPs (2.5 mM each), 0.5 µl Random hexamer (1 µg/ml), 0.25 µl RNaseOUT and 0.25 µl M-MLV reverse transcriptase enzyme. Master Mix was gently mixed, centrifuged briefly and 5 µl of master mix was dispensed into PCR tubes. 5 µl of RNA was added to each tube to bring the total volume to 10 µl. The tubes were centrifuged briefly to ensure that all their contents were at the bottom of the tubes. The RT PCR reactions were run in a Mastercycler Gradient Thermal Cycler (Eppendorf) under the following program: 25°C for 15 min, 42°C for 40 min, and 95°C for 3 min. The cDNA samples were stored at -20°C and used for qRT-PCR.

2.2.9.3 qRT-PCR

A master mix was prepared consisting of 4 µl Sybr Green, 3 µl H₂O, 0.5 µl Forward primer (5 pmol/µl) and 0.5 µl Reverse primer (5 pmol/µl). Master mix was gently mixed, centrifuged

briefly and 8 μ l of master mix was dispensed into a 96-well plate for Real Time PCR. 2 μ l of cDNA was added to each well. The plate was covered with adhesive slip and was centrifuged gently to ensure all the contents were at the bottom of the well. The plate was run in a Mx3000P qPCR machine (Stratagene) under Fast 2 Step program with the following steps: 95°C for 2 min, and then 40 cycles of 95°C for 10 s and 60°C for 30 s. Results were analyzed using comparative C_t method and values were normalized to untreated samples. All experiments were performed in duplicate or triplicate wells for each condition and repeated at least three times.

2.2.10 Western Blot (WB) analysis

2.2.10.1 Lysate preparation

THP-1 cells were harvested by centrifugation at 100 g for 5 min at 4°C in 1.5 ml tubes. The supernatants were discarded and cells were washed in ice-cold 1x PBS. Cells were centrifuged and PBS was removed. 65 μ l of 3x sample buffer containing freshly added DTT (1 mM), mixture of inhibitors (Table 2.7), and benzonase (25 units) to remove DNA, was added to cells. The samples were then incubated 5 min on ice, boiled for 5 min at 99°C followed by centrifugation to remove condensation and incubated on ice for further 5 min. The samples were kept on ice at all times and stored at -80°C.

Inhibitor	Target	Concentration
aprotinin	serine proteases	2 μ g/ml
phenylmethanesulfonyl fluoride (PMSF)	serine/cysteine proteases	10 mM
sodium β -glycerophosphate (BGP)	serine/threonine phosphatases	20 mM
sodium fluoride (NaF)	serine/threonine phosphatases	1 mM
sodium orthovanadate (Na_3VO_4)	tyrosine/alkaline phosphatases	1 mM
sodium pyrophosphate	serine/threonine phosphatases	20 mM

Table 2.7: List of phosphatase inhibitors.

2.2.10.2 SDS-PAGE

SDS-PAGE mini gels were made using Bio-Rad apparatus. The 10% acrylamide 1.5 mm resolving gels were made using 4.1 ml dH_2O , 3.3 ml 30% acrylamide, 2.5 ml 1.5 M Tris-HCl pH 8.8, 100 μ l 10% (v/v) SDS, 50 μ l 10% (v/v) APS, and 5 μ l TEMED. The top of the resolving gel was covered with isopropanol and allowed to solidify. The isopropanol was removed and 5% stacking gel made of 1.7 ml dH_2O , 0.5 ml acrylamide, 0.75 ml 0.5 M Tris HCl H 6.8, 30 μ l 10% (v/v) SDS, 30 μ l 10% (v/v) APS, and 3 μ l TEMED was poured on top of the resolving gel. The comb was inserted and the gel was left to solidify. A pre-stained molecular weight marker was loaded to the first well and the gel ran at 25 mA per gel for 2 hr in 1x running buffer.

2.2.10.3 Semi-dry transfer

The resolved proteins were transferred to 0.2 μ m PVDF membrane using Biometra semi-dry transfer system (Göttingen, Germany). The PVDF membrane was activated in methanol prior to transfer. 3MM Whatman® filter papers were cut and soaked in 1x transfer buffer. A transfer sandwich was made by laying three sheets of filter paper on the surface of semi-dry machine followed by activated membrane, SDS-PAGE gel and covered with an additional layer of three filter papers. The semi-dry transfer ran at 75 mA per gel for 90 min.

2.2.10.4 Detection

The PVDF membrane was blocked for non-specific protein binding by incubation in 3% (w/v) BSA and 1x TBS/0.1% (v/v) Tween for 1 hr. The membrane was incubated overnight at 4°C in primary antibody diluted in 3 ml 1% (w/v) BSA/TBS-Tween, and 0.5% (v/v) sodium azide. The membrane was then washed four times in TBS-Tween for 1 hr. The appropriate secondary antibody was added to 3% (w/v) BSA/TBS-Tween and was incubated for 1 hr followed by washing the membrane four times in TBS-Tween for further 1 hr. The membrane was developed using Chemiluminescent HRP Substrate on ChemiDoc™ MP System (Bio-Rad) and the images were compiled using Image Lab 5.0 software. The β -actin protein was visualized using IRDye® anti-mouse secondary antibody on Odyssey Infrared Imaging System (Li-COR).

2.2.11 Statistical analysis

Statistical analysis was performed using paired Student *t* test or two-way ANOVA with Bonferroni posttest in a GraphPad Prism® version 5.01.

3. Result I: *Staphylococcus aureus* stimulates type I interferons in human monocytes

3.1 Introduction

Staphylococcus aureus is a common human commensal bacterium, which is carried by about 20 % of the population in the upper respiratory tract and on the skin, and up to 60 % of the population carries *S. aureus* intermittently [234]. The presence of *S. aureus* on healthy individuals is not associated with health complications since microbial as well as host factors contribute to the development and maintenance of the *S. aureus* carrier state. On the other hand, *S. aureus* is a major human pathogen causing skin and soft tissue infections, pneumonia, and superinfection following influenza infections. The severity of *S. aureus* infections is further elevated by the appearance of strains resistant to antibiotics, such as methicillin and vancomycin. The vaccine development is further complicated due to the long-term interaction between *S. aureus* and human immune cells; therefore, the induction of long-term protective immunity against *S. aureus* is less feasible. In addition, the mechanisms of *S. aureus* recognition as well as the nature of signaling components in response to *S. aureus* remain poorly described in humans cells.

The innate immune responses are triggered after recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs are germline-encoded receptors that are expressed by both immune and non-immune cells. PAMPs are evolutionary conserved components of pathogens that are essential for their survival, such as components of the cell wall (lipopolysaccharide, lipoteichoic acid), proteins (flagellin) and nucleic acids (viral dsRNA, bacterial DNA) [1]. Upon recognition of PAMPs, PRRs trigger a signaling cascade that leads to the activation of two major classes of transcription factors: nuclear factor κ B (NF κ B) and interferon regulatory factors (IRFs). NF κ B is a master regulator of proinflammatory responses with a role in transcription of cytokines such as IL-6, IL-1 β , RANTES and TNF α . IRFs, on the other hands, provide antiviral responses by induction of type I IFNs. The type I IFNs family consists of multiple subtypes of IFN α , a single IFN β and other less characterized members such as IFN ϵ , IFN κ , and IFN ω [133]. Type I IFNs signal through the heterodimeric IFNAR1-IFNAR2 complex, leading to the transcription of ISGs. Even though all type I IFNs signal through the same receptor complex, the means of type I IFN induction are distinct [138]. For example, it has been shown that IFN α is induced mainly during viral infections, whereas IFN β is triggered in response to bacterial LPS. Furthermore, it has been reported that all nucleated cells can produce type I IFNs in response to viral infection, which is mediated by intracellular nucleic acid sensors such as cytoplasmic RLRs or endoplasmic TLRs [144]. Lung epithelial cells are in direct contact with various pathogens including both viruses

and bacteria; however, their ability to produce type I IFNs in response to bacterial infection remains poorly understood.

In contrast to the epithelial cells, monocytes are mobile immune cells that produce inflammatory cytokines upon recognition of various pathogens and activate adaptive immune responses. In addition, activated monocytes can differentiate into macrophages and further modulate adaptive immune responses. The drug-induced differentiation of THP-1 monocytes into macrophages has been extensively used in studies of macrophages role in inflammatory diseases. However, differentiation-inducing reagents such as phorbol 12-myristate 13-acetate (PMA) have been shown to stimulate induction of various cytokine genes in THP-1 cells including IL-1 β , IL-8 and TNF α [235]. On the other hand, PMA treatment induced upregulation of CD14, a surface marker of macrophages, while expression of TLR2 and TLR4 remained unchanged [235]. It has been also shown that LPS stimulation induced higher levels of TNF α protein in THP-1-derived macrophages than in THP-1 monocytes [236]. Consequently, LPS-induced translocation of NF κ B occurred more rapidly in THP-1-derived macrophages than in THP-1 monocytes, leading to increased NF κ B levels in the nucleus of THP-1-derived macrophages, thus priming the cells for rapid secretion of proinflammatory cytokines such as TNF α [236]. In addition, professional phagocytes have been shown to express the greatest variety of TLR mRNAs and PMA treatment of THP-1 monocytes increased their sensitivity to LPS and enhanced production of TNF α when compared to PMA untreated THP-1 monocytes [237]. Moreover, PMA-induced differentiation significantly upregulated expression of TLR4, CD14, MD2, and MyD88 mRNA compared to naïve THP-1 monocytes [237].

To date, most of the research on *S. aureus* infections is based on mouse models; however, there are considerable differences between the hosts. Importantly, mouse is not a natural host of human *S. aureus* isolates [184] and distinct mechanisms contribute to activation of protective immunity [227]. In previous studies, we showed that TLR2 stimulation led to the induction of type I IFNs in mouse bone marrow derived macrophages (BMDMs) [42]. Furthermore, the IFN β induction was dependent on TRAM adaptor protein in response to *Staphylococcus aureus* and herpes virus infection *in vitro*. However, the ability of human cells to produce type I IFNs in response to TLR2 ligand stimulation or *S. aureus* infection remains poorly described. Hence, the aim of this chapter is to assess the ability of various human cell lines to produce TLR2-dependent type I IFNs.

3.2 Results

3.2.1 Immune responses to TLR2 ligands in human lung epithelial cells

The goal of this chapter was to investigate whether TLR2 ligands and Gram-positive bacteria, such as *Staphylococcus aureus*, stimulate type I IFN production in human cells. Therefore, in order to assess the induction of proinflammatory cytokines and type I IFNs, we tested ability of human cell lines to produce cytokines after stimulation with TLR ligands and infection with live *S. aureus*.

Macrophage-activating lipopeptide-2 (Malp2) was first isolated from *Mycoplasma fermentans*, and is a typical ligand recognized by a TLR2-TLR6 heterodimer. Pam3Csk4 is a synthetic triacylated lipopeptide that mimics the amino terminus of bacterial lipopeptides, which is sensed by a TLR2-TLR1 heterodimer. Lastly, lipoteichoic acid (LTA) is a major immunostimulatory component of Gram-positive bacteria. LTA stimulates immune cells through TLR2 and its recognition involves additional lipopolysaccharide (LPS)-binding protein (LBP) and CD14 co-receptor [191]. LPS is, on the other hand, the principal component of Gram-negative bacteria activating innate immune cells. LPS recognition is mediated by TLR4 and involves LBP and CD14, which interact with a TLR4-MD2 complex [238]. Polyinosinic-polycytidylic acid (poly (I:C)) is a synthetic analogue of double-stranded RNA that is recognized by endosomal TLR3 and induces activation of proinflammatory and type I IFN pathways. Due to the variety among TLR2 ligands, all three ligands were tested in order to investigate differences among TLR2 pathways while LPS and poly (I:C) were used as stimuli independent of TLR2 signaling. Lastly, activation of immune cells was examined in the context of *S. aureus* infection. *S. aureus* is a Gram-positive bacterium, which possesses many immunostimulatory molecules, such as LTA; however, the nature of signaling components, leading to the activation of immune cells in humans remains less described.

The degree of immune activation was measured at the transcription level by assessing mRNA expression of tumor necrosis factor α (TNF α), interferon α (IFN α) and IFN β using quantitative Real-Time PCR (qRT-PCR). The bioactivity of type I IFNs was assessed by HEK-Blue IFN α/β bioassay, which measured both IFN α and IFN β proteins. The secretion of IL-1 β , IL-10, IL-6, CCL5 (also known as regulated on activation, normal T cell expressed and secreted (RANTES)), CXCL10 (also known as IFN gamma-induced protein 10 (IP-10)), and TNF α cytokines was measured by ELISA to assess the degree of immune activation.

Initially, immune responses in human lung epithelial cell lines, A549 and BEAS-2B, were measured. Although lung epithelial cells are in direct contact with pathogens, Malp2,

Pam3Csk4 and LPS stimulation failed to induce IFN β or TNF α mRNA in A549 cells and only slightly upregulated IFN α mRNA [Fig. 3.1 A-C]. In BEAS-2B cells, both Malp2 and Pam3Csk4 failed to induce IFN α , IFN β or TNF α mRNA; however, LPS stimulation upregulated IFN β mRNA expression. This result suggests that lung epithelial cells do not produce type I IFNs or TNF α in response to TLR2 stimulation; therefore, production of other cytokines was measured. In order to determine the kinetics of immune responses in A549 and BEAS-2B cells, RANTES induction was measured 6 and 24 hr after stimulation. In both cell lines, stimulation with all ligands induced time-dependent RANTES production with a peak of responses 24 hr after stimulation [Fig. 3.2 A-B]. Malp2 and Pam3Csk4 induced RANTES protein weakly, while transfected poly(I:C) strongly upregulated RANTES production in both A549 and BEAS-2B cells. Lipofectamine alone did not affect RANTES production, thus excluding nonspecific activation of the pathway by the transfection reagent. Interestingly, untransfected poly(I:C) did not induce RANTES protein in A549 cells, whereas it upregulated RANTES production in BEAS-2B cells, suggesting a lack of functional TLR3 signaling pathway in A549 cells.

Next, a dose response to TLR ligands was measured in A549 and BEAS-2B cells. Consistent with previous results, Malp2 and Pam3Csk4 induced RANTES, IP-10 and IL-6 cytokines weakly in both cell lines [Fig. 3.3 A-B]. Stimulation with higher concentration of either TLR2 ligand had little effect on increasing cytokine production. Interestingly, basal levels of IL-6 protein were much higher in BEAS-2B than A549 cells. Similarly to TLR2 stimulation, LPS stimulation did not upregulate RANTES, IP-10 or IL-6 cytokines. In both A549 and BEAS-2B cells, transfected poly(I:C) dose-dependently upregulated RANTES, IP-10 and IL-6 cytokines [Fig. 3.3 A-C]; however, higher concentration of poly(I:C) had adverse effects on viability of the cells [data not shown]. These observations show that lung epithelial cell lines can serve as cell models for RNA-mediated responses.

Lastly, activation of innate immune cells was investigated in human monocytic THP-1 cell line. THP-1 cells were seeded at two densities and were stimulated with TLR ligands. IFN β mRNA was equally upregulated in sparsely and densely seeded cells after both TLR2 and TLR4 stimulation [Fig. 3.4 A]. The degree of TNF α mRNA expression increased with higher number of cells upon Malp2, Pam3Csk4 and LPS stimulation [Fig. 3.4 B]. This finding suggests that cell density is an important factor for activation of TNF α amongst immune cells. Importantly, this result demonstrates that THP-1 monocytes are the cells of interest for further investigation of the type I IFN induction in human cells.

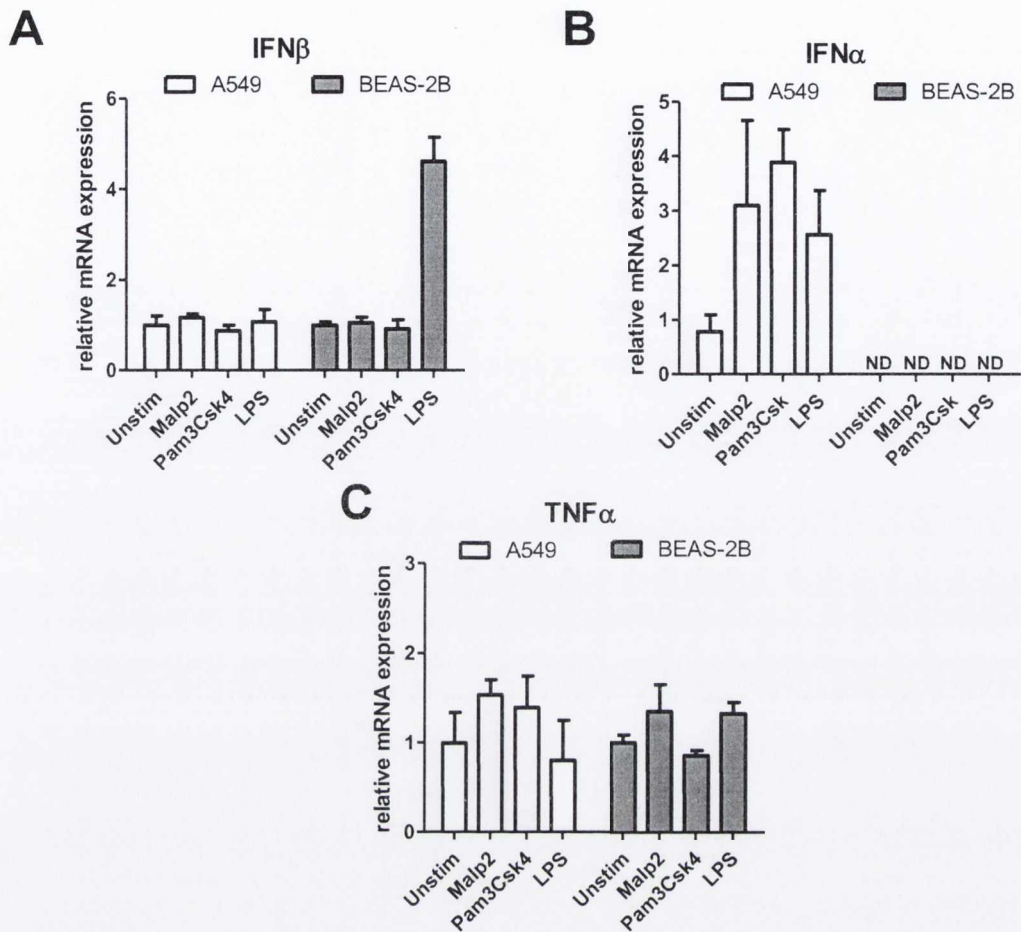


Figure 3.1: Responses to TLR ligands in human lung epithelial cells.

A549 and BEAS-2B cells were seeded at 1×10^5 cells/ml into 24-well plate 24 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml), Pam3Csk4 (5 μ g/ml), and LPS (100 ng/ml) for 3 hr. Cells were harvested and RNA was extracted followed by RT PCR. IFN β (A), IFN α (B) and TNF α (C) mRNA was measured by qRT-PCR. Data are relative to unstimulated sample and normalized to the levels of β -actin. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

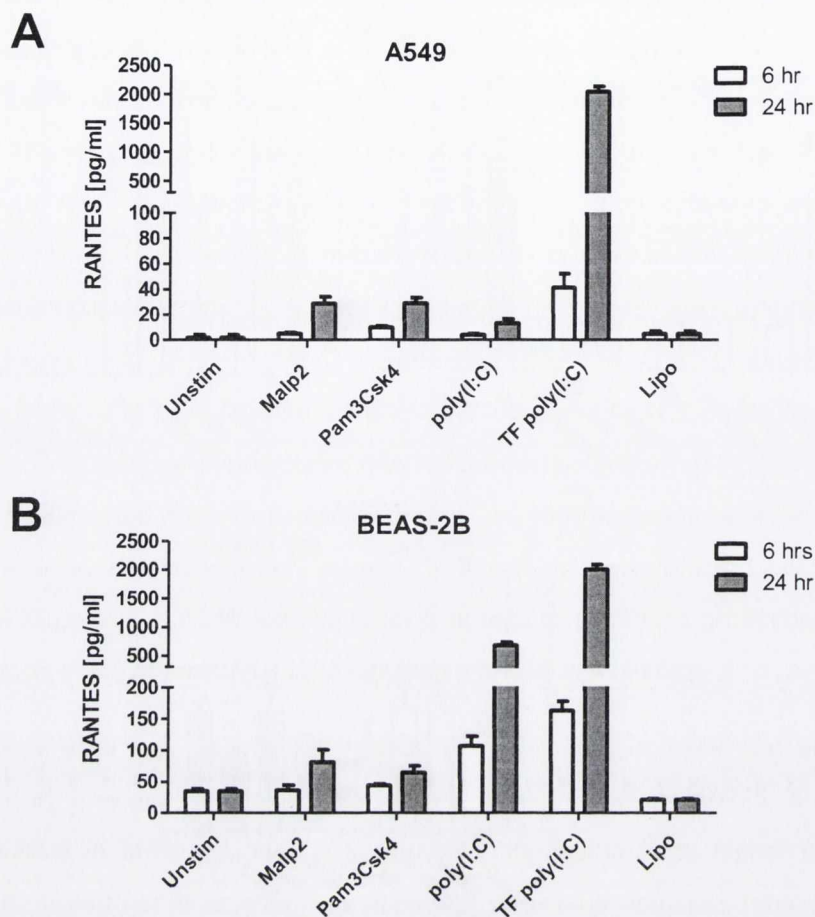


Figure 3.2: Responses of human lung epithelial cells to TLR ligands are time-dependent.

A549 (A) and BEAS-2B (B) cells were seeded at 1×10^5 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml), Pam3Csk4 (2.5 μ g/ml), untransfected or transfected poly(I:C) (25 μ g/ml) using Lipofectamine[®] 2000, and LPS (100 ng/ml) for 6 or 24 hr. Supernatants were collected, and RANTES protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

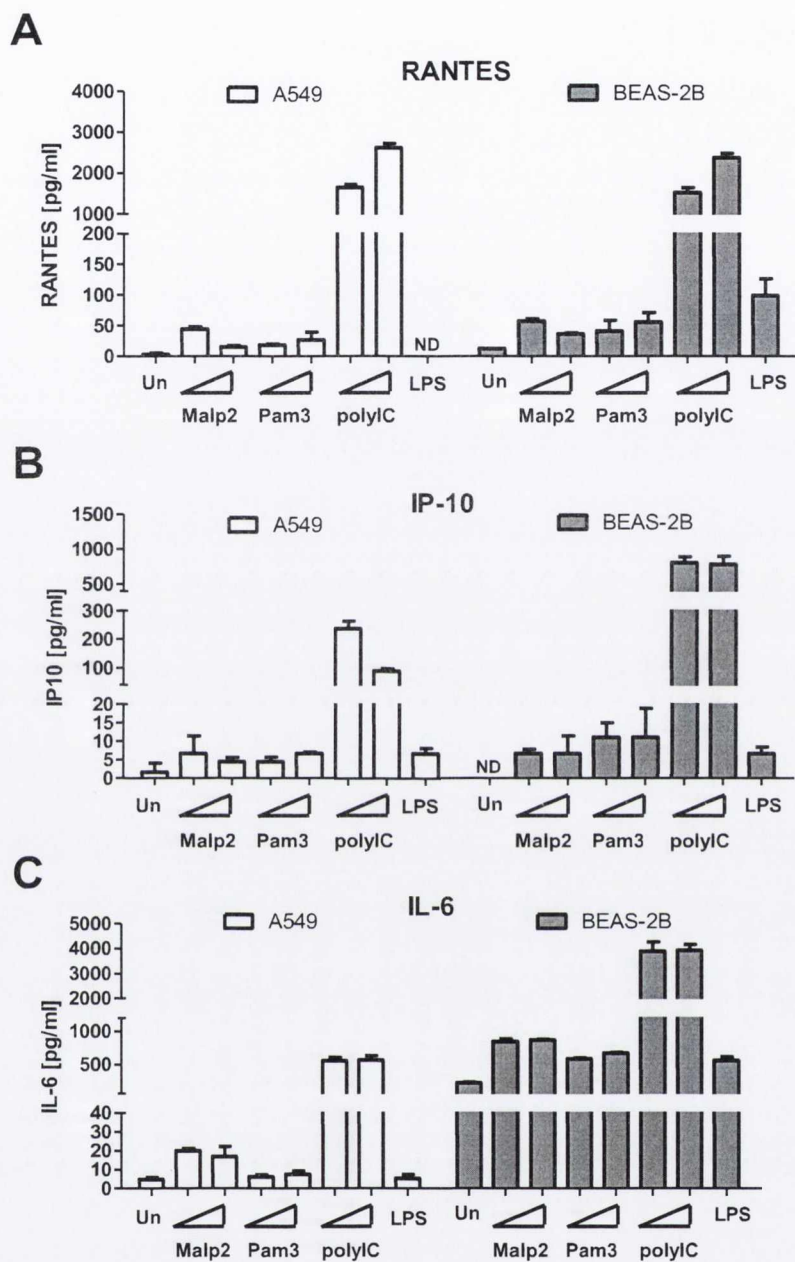


Figure 3.3: Human lung epithelial cells have limited responsiveness to TLR2 and TLR4 ligands.

A549 and BEAS-2B cells were seeded at 1×10^5 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were stimulated with increasing concentration of Malp2 (50/100 ng/ml), Pam3Csk4 (2.5/5 μ g/ml), transfected with poly(I:C) (25/50 μ g/ml) using Lipofectamine[®] 2000, and LPS (100 ng/ml) for 24 hr. Supernatants were collected, and RANTES (A), IP-10 (B) and IL-6 (C) proteins were measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

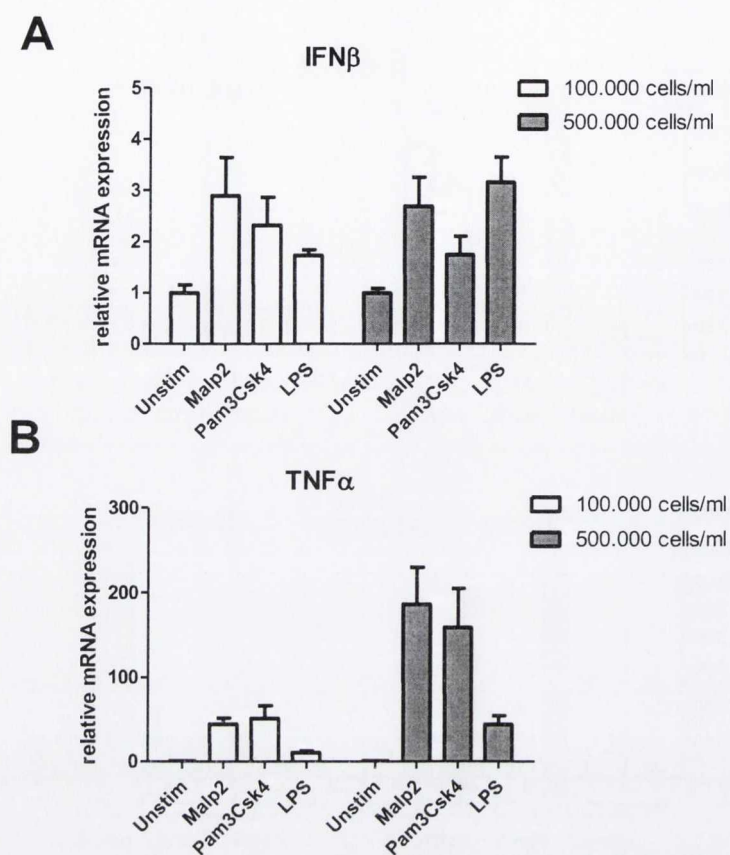


Figure 3.4: Responses to TLR ligands in THP-1 monocytes.

THP-1 cells were seeded at 1×10^5 or 5×10^5 cells/ml into 24-well plate 24 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml), Pam3Csk4 (2.5 μ g/ml), and LPS (100 ng/ml) for 3 hr. Cells were harvested by centrifugation at 100 g for 5 min. RNA was extracted followed by RT-PCR and IFN β (A) and TNF α (B) mRNA was measured by qRT-PCR. Data are relative to unstimulated sample and normalized to the levels of β -actin. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

3.2.2 Differentiation of human monocytes alters their responses to TLR ligands

Monocytes are mobile innate immune cells, which can differentiate into tissue-specific macrophages in response to immune signals. Both monocytes and macrophages produce proinflammatory cytokines and chemokines and play an important role in elimination of invading pathogens. It has been noted that monocytes can undergo differentiation into macrophages after treatment with chemical agents, such as phorbol 12-myristate 13-acetate (PMA), retinoic acid and cytokines, such as IFN γ [235]. However, differentiation-inducing agents have been shown, themselves, to alter cytokine responses of the cells to immune stimulation [235]. Therefore, we first measured cytokine production in both monocytes and monocyte-derived macrophages in response to stimulation with TLR ligands. In order to differentiate monocytes into macrophages, THP-1 cells were treated with PMA for 2 days and human primary CD14 $^{+}$ monocytes were treated with GM-CSF for 5 days before they were stimulated with TLR ligands.

In THP-1 monocytes, Malp2-induced IFN β mRNA was upregulated 1 hr after stimulation and maintained the same level of induction 3 hr after stimulation [Fig. 3.5 A]. In THP-1-derived macrophages, Malp2-stimulated IFN β expression was induced 3 hr after stimulation; however, the degree of IFN β activation was much lower in THP-1-derived macrophages than in THP-1 monocytes. LPS-stimulated IFN β mRNA was induced 1 hr after stimulation and dropped to the basal levels 3 hr after stimulation in THP-1 monocytes [Fig. 3.5 A]. In THP-1-derived macrophages, LPS-induced IFN β mRNA was mildly induced after 1 hr and was still upregulated 3 hr after stimulation. Malp2- and LPS-stimulated TNF α mRNA was slightly upregulated 1 hr after stimulation and mRNA expression further increased 3 hr after stimulation in THP-1-derived macrophages [Fig. 3.5 B]. In THP-1 monocytes, however, there was a peak of TNF α mRNA induction 1 hr after Malp2 and LPS stimulation followed by a sharp decrease in TNF α expression. These observations confirm that differentiation of THP-1 cells with PMA considerably influences TLR-mediated IFN β and TNF α responses. In addition, these results demonstrate that the kinetics of immune responses in THP-1 monocytes and THP-1-derived macrophages is different.

In order to investigate differences in the immune activation of THP-1 monocytes compared to THP-1-derived macrophages, both cell types were stimulated with a range of TLR2 ligands (Malp2, Pam3Csk4 and LTA) alongside with a single TLR4 ligand (LPS), and type I IFN bioactivity, TNF α , IP-10 and RANTES proteins were measured. In THP-1-derived macrophages, TLR2 stimulation hardly induced any IFN α/β bioactivity, while LPS-mediated

IFN α/β bioactivity was strongly upregulated [3.6 A]. In THP-1 monocytes, TLR2 stimulation induced strong dose-dependent IFN α/β bioactivity, whereas LPS did not stimulate IFN α/β bioactivity. Similarly to IFN α/β bioactivity, IP-10 protein was strongly upregulated after LPS stimulation in THP-1-derived macrophages, while TLR2 ligands stimulated IP-10 production in THP-1 monocytes [3.6 B]. TNF α protein was dose-dependently upregulated in both THP-1-derived macrophages and THP-1 monocytes; however, the degree of TNF α production was greater in THP-1-derived macrophages when compared to THP-1 monocytes [3.6 C]. TNF α protein was strongly upregulated after LPS stimulation in THP-1-derived macrophages, whereas the degree of TNF α production was very low in THP-1 monocytes when compared to THP-1-derived macrophages. Similarly to TNF α protein, RANTES protein was induced in a dose-dependent manner after stimulation with TLR2 ligands or LPS and stronger production was observed in THP-1-derived macrophages when compared to THP-1 monocytes [3.6 D]. These results demonstrate that distinct signaling pathways operate in THP-1-derived macrophages and THP-1 monocytes. In addition, we observed two types of immune activation. The first type of immune responses was represented by type I IFNs and IP-10 cytokines, whereas the second type comprised TNF α and RANTES responses.

Importantly, the findings obtained in THP-1 monocytes were compared to the immune responses in human CD14⁺ monocytes isolated from peripheral blood mononuclear cells (PBMCs). Of interest, a significant variability in the degree of immune activation was observed among individual blood donors; however, the trend of responses was consistent throughout the experiment. Similarly to THP-1 cells, the induction of proinflammatory cytokines and type I IFNs in response to TLR ligands was measured in both CD14⁺ monocytes (CD14+GM-CSF-) and monocyte-derived macrophages (CD14+GM-CSF+). In monocyte-derived macrophages, TLR2 ligands induced low IFN α/β bioactivity, while there was a dose-dependent induction of IFN α/β bioactivity in monocytes [Fig. 3.7 A]. TLR4 stimulation upregulated IFN α/β bioactivity in both cell types; however, the degree of type I IFN activation was stronger in monocyte-derived macrophages than in monocytes. TNF α protein was induced in a dose-dependent manner in response to TLR2 and TLR4 ligands in both cell types; however, the amount of TNF α protein was greater in monocyte-derived macrophages than in monocytes [Fig. 3.7 B]. Induction of IL-1 β protein, a marker of inflammation, was also upregulated in both cell types and stronger IL-1 β protein production was observed in monocyte-derived macrophages than in monocytes [Fig. 3.7 C]. The cytotoxic effects of TLR ligands on cell viability were measured by MTT assay and no adverse effects of TLR stimulation on viability of cells were observed when compared to unstimulated and untreated cells [Fig. 3.7 D]. In order to determine the

kinetics of cytokine induction in CD14⁺ monocytes, a time course and a dose response to LTA was performed. For both IFN α/β bioactivity and TNF α protein induction, there was a time-dependent response with a peak 18 hr after stimulation [Fig. 3.8 A-B]. There was no dose-dependent effect of LTA on protein induction; however, the lower concentrations of LTA had toxic effects on the cell viability when compared to the highest concentration of LTA [Fig. 3.8 C].

These results show that differentiation of human monocytes into monocytes-derived macrophages alters induction of type I IFNs and proinflammatory cytokines in response to TLR stimulation. Collectively, these results highlight the differences in the TLR signaling between monocytes and monocyte-derived macrophages, which might be due to their distinct functions during infection. Hence, a model cell lines should be chosen carefully when studying innate immune responses.

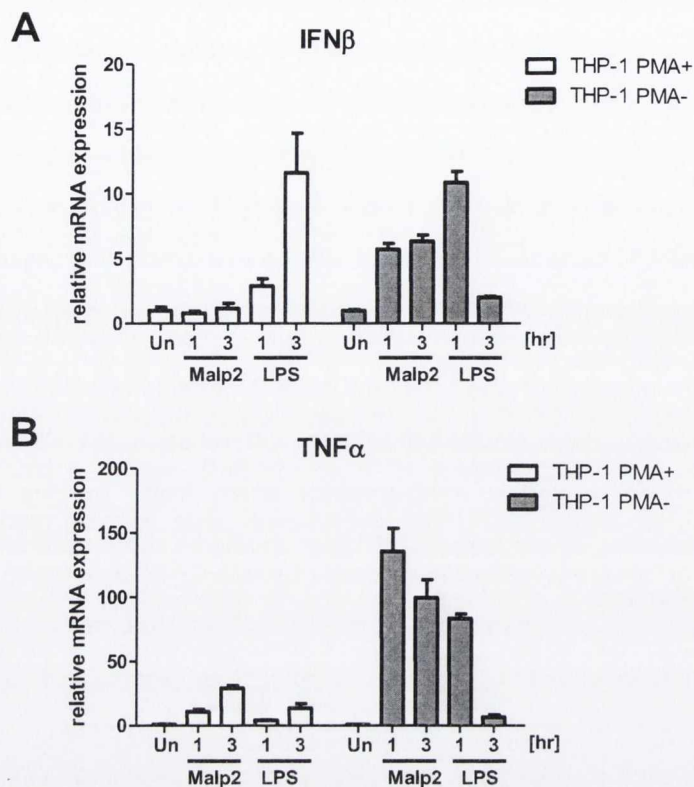


Figure 3.5: PMA treatment of THP-1 monocytes alters their responses to TLR ligands.

THP-1 cells were seeded at 5×10^5 cells/ml into 24-well plate 24 hr prior to stimulation and differentiated with PMA (100 nM) or left untreated. Cells were stimulated with Malp2 (50 ng/ml), and LPS (100 ng/ml) for 1 or 3 hr. Cells were harvested by centrifugation at 100 g for 5 min, and RNA was extracted followed by RT-PCR. IFN β (A) and TNF α (B) mRNA was measured by qRT-PCR. Data are relative to unstimulated sample and normalized to the levels of β -actin. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

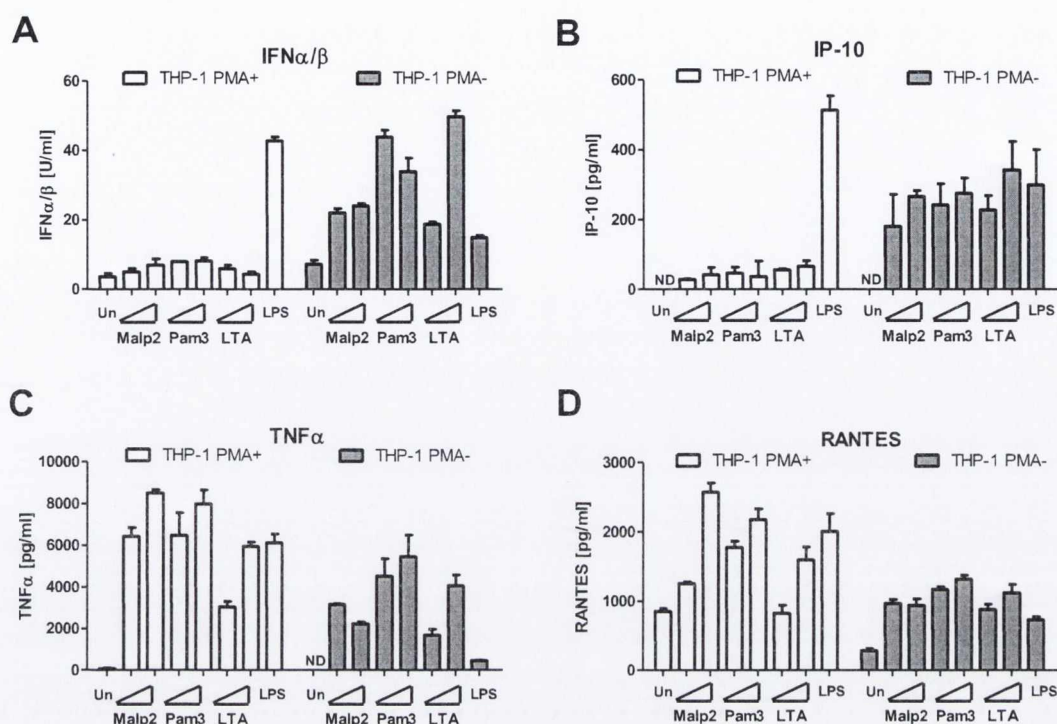


Figure 3.6: Differentiation of THP-1 cells alters their responses to TLR ligands.

THP-1 cells were seeded at 5×10^5 cells/ml into 96-well plate 24 hr prior to stimulation and differentiated with PMA (100 nM) or or left untreated. Cells were stimulated with increasing concentration of Malp2 (50/100 ng/ml), Pam3Csk4 (2.5/5 μ g/ml), LTA (1.25/2.5 μ g/ml), and LPS (100 ng/ml) for 6 hr. Cells were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B), IP-10 (C) and RANTES (D) proteins were measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

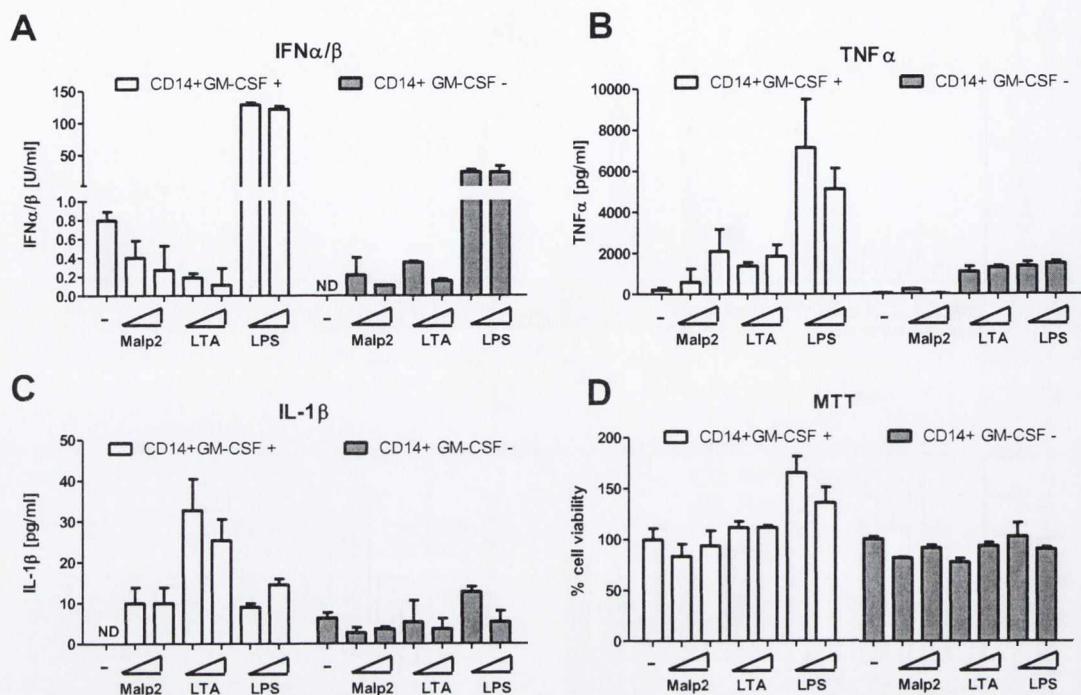


Figure 3.7: Differentiation of CD14+ monocytes alters their responses to TLR ligands.

CD14+ PBMCs were seeded at 8×10^5 cells/ml into 96-well plate and differentiated with GM-CSF (50 ng/ml) for 6 days or left untreated. Cells were stimulated with increasing concentration of Malp2 (25/50 ng/ml), LTA (1.25/2.5 μ g/ml), and LPS (10/100 ng/ml) for 6 hr. Cells were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B) and IL-1 β (C) proteins were measured by standard ELISA with appropriate dilutions. Cell viability (D) was measured by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

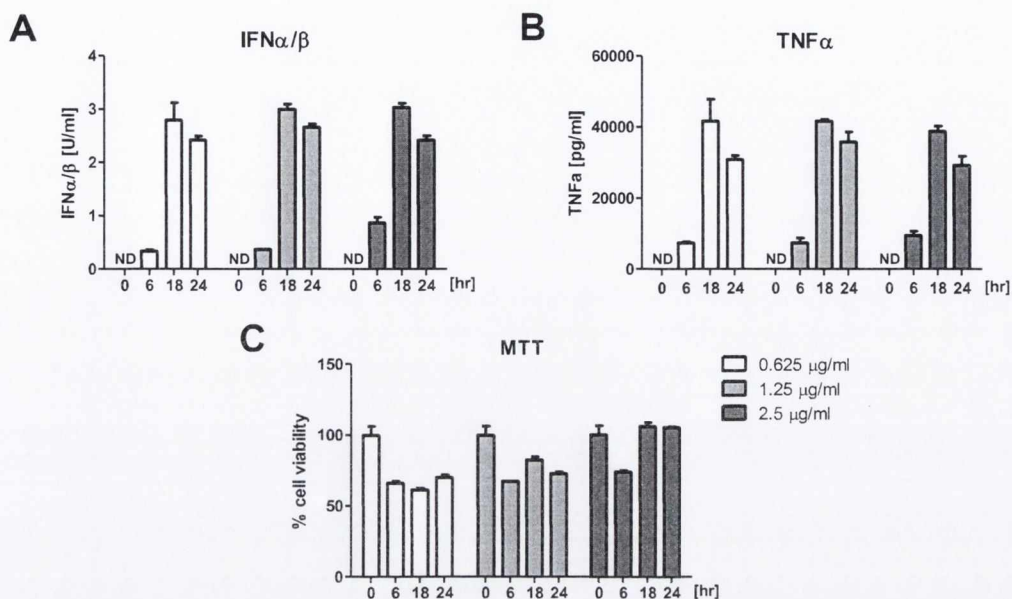


Figure 3.8: Responses of CD14+ monocytes to purified *S. aureus*-LTA.

CD14+ PBMCs were seeded at 8×10^5 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were stimulated with increasing concentration of LTA (0.625/1.25/2.5 $\mu\text{g/ml}$) for 6, 18 or 24 hr. Cells were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; and TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Cell viability (C) was measured by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

3.2.3 Differentiation of human monocytes alters their responses to *S. aureus*

Previously, we have established that human lung epithelial cells responded weakly to stimulation with TLR2 ligands while cytokine responses were upregulated after stimulation with nucleic acids. On the other hand, we showed that monocytes induced strong proinflammatory and type I IFN responses after TLR2 stimulation. Furthermore, we noted that the degree of type I IFN activation was greater in monocytes than in monocyte-derived macrophages. Next, we measured upregulation of cytokines during bacterial infection as a biological means of immune cell activation. *S. aureus* is a Gram-positive bacterium that possesses a number of immunostimulatory molecules, which activates a variety of immune cells. Even though the nature of signaling pathways remains unclear in human cells, it is known that TLR2 significantly contributes to the recognition of *S. aureus* in both human and mice cells [191]. Having established the model cells for assessing TLR2-stimulated type I IFN responses, particularly THP-1 and CD14⁺ monocytes, the degree of immune activation upon *S. aureus* infection was measured.

Initially, both monocytes and monocyte-derived macrophages were infected with *S. aureus* at different multiplicities of infection (MOI) for 6 and 18 hr. The induction of IFN α / β bioactivity and TNF α protein were dose- and time-dependent in both THP-1 monocytes and THP-1-derived macrophages [Fig. 3.9 A-D]. Infection with *S. aureus* at an MOI of 10 did not induce considerable amounts of IFN α / β bioactivity 6 or 18 hr after infection in either cell type [Fig. 3.9 A-B]. Infection with *S. aureus* at an MOI of 100 induced strong IFN α / β bioactivity in a time-dependent manner in both cell types; however, the degree of IFN α / β bioactivity was higher in THP-1 monocytes when compared to THP-1-derived macrophages. TNF α protein was strongly upregulated after infection with *S. aureus* at an MOI of 10 and 100 in THP-1-derived macrophages, while in THP-1 monocytes TNF α production was strongly induced only after infection with *S. aureus* at an MOI of 100 [Fig. 3.9 C-D]. In contrast to IFN α / β bioactivity, the degree of TNF α production was higher in THP-1-derived macrophages than in THP-1 monocytes. In CD14⁺ cells, *S. aureus* also induced dose- and time-dependent IFN α / β bioactivity and TNF α production [Fig. 3.10 A-D]. Infection with *S. aureus* at an MOI of 1 induced greater IFN α / β bioactivity in CD14⁺ monocytes than in CD14⁺-derived macrophages during *S. aureus* infection [Fig. 3.10 A-B]. Infection with *S. aureus* at an MOI of 10 did not induce IFN α / β bioactivity 6 hr after infection in either cell type; however, IFN α / β bioactivity was upregulated 18 hr after infection and the degree of IFN α / β bioactivity was higher in CD14⁺ monocytes than in CD14⁺-derived macrophages. TNF α protein was strongly upregulated during infection with *S. aureus* at an MOI of 1 and 10 in CD14⁺ monocytes, while only an MOI

of 10 stimulated TNF α production 18 hr after infection in CD14 $^{+}$ monocyte-derived macrophages [Fig. 3.10 C-D]. Lastly, no upregulation of IFN α/β bioactivity or TNF α production was observed in human lung epithelial cell lines A549 and BEAS-2B after infection with *S. aureus* [data not shown]. This result demonstrates that human monocytes can produce type I IFNs in response to bacterial infection *in vitro*. In addition, this result suggests that the recognition of *S. aureus* is more sensitive in CD14 $^{+}$ monocytes than in THP-1 monocytes. In CD14 $^{+}$ monocytes, the highest degree of type I IFN activation was achieved by infection with *S. aureus* at an MOI of 1 for 18 hr [Fig. 3.10 B]. In THP-1 monocytes, MOI of 100 induced strong type I IFN response both 6 and 18 hr after infection [Fig. 3.9 A, B].

Because biological responses change over time, both THP-1 monocytes and CD14 $^{+}$ monocytes were infected with *S. aureus* and immune responses were measured at several times during infection. Cell viability was quantified by measuring a release of lactate dehydrogenase (LDH) into the media from damaged cells using LDH assay kit. Consistent with our previous results, infection with *S. aureus* at an MOI of 10 induced very low immune responses in THP-1 monocytes [Fig. 3.11 A-D]. However, infection with *S. aureus* at an MOI of 100 potentially induced IFN α/β bioactivity with a peak 24 hr after infection [Fig. 3.11 A]. TNF α protein was strongly upregulated after infection with *S. aureus* at an MOI of 100 with two peaks of TNF α induction 3 and 24 hr after infection [Fig. 3.11 B]. IL-1 β protein induction by *S. aureus* at an MOI of 100 correlated with LDH release and increased sharply 18 hr after infection [Fig. 3.11 C, E]. IL-10 protein was hardly induced in response to *S. aureus* at an MOI of 100 in THP-1 monocytes [Fig. 3.11 D]. In CD14 $^{+}$ monocytes, infection with *S. aureus* at an MOI of 10 induced strong immune activation while LDH release by cells was comparable after infection with *S. aureus* at an MOI of 1 and 10 [Fig. 3.12 A-D]. IFN α/β bioactivity and TNF α protein were strongly upregulated 18 hr after infection with *S. aureus* at an MOI of 1 and stayed high 24 hr after infection [Fig. 3.12 A-B]. IL-1 β protein was upregulated dose- and time-dependently with the peak of responses 6 hr after *S. aureus* infection [Fig. 3.12 C]. In contrast to THP-1 monocytes, IL-10 protein was strongly induced 18 and 24 hr after *S. aureus* infection [Fig. 3.12 D]. LDH release, however, did not correlate with IL-1 β release since the levels of LDH were high from the onset of *S. aureus* infection [Fig. 3.12 E]. These results suggest that proinflammatory responses are more tightly regulated in CD14 $^{+}$ than in THP-1 monocytes.

In order to further investigate differences in response to *S. aureus* between monocytes and monocyte-derived macrophages, the degree of cytokine transcription was assessed. In contrast to protein measurements, mRNA analysis revealed increased induction

of IFN β expression 6 hr after *S. aureus* infection in monocyte-derived macrophages when compared to THP-1 and CD14+ monocytes [Fig. 3.13 A, C]. In addition, TNF α expression was highly upregulated in both THP-1 and CD14+ monocytes compared to monocyte-derived macrophages 6 hr after *S. aureus* infection [Fig. 3.13 B, D]. This might suggest different kinetics of immune responses in monocytes compared to monocyte-derived macrophages. In addition, the basal expression of the innate immune receptors changed during differentiation of cells from monocytes into monocyte-derived macrophages. In CD14+ monocytes, there was no difference in MD2 expression, while both TLR2 and TLR4 were upregulated when compared to monocyte-derived macrophages [Fig. 3.14 B]. Accordingly, CD14+ monocytes and monocyte-derived macrophages were responding to both types of ligands. In THP-1 cells, the levels of TLR2 and TLR4 expression were comparable; however, there was considerable upregulation of MD2 expression [Fig. 3.14 A]. MD2 is a co-receptor essential for LPS-induced TLR4 responses; therefore, MD2 upregulation might have primed THP-1 derived macrophages for TLR4-mediated responses, whereas TLR2 responses stayed unaffected.

Hence, our results demonstrate that drug-induced differentiation of human monocytes into monocyte-derived macrophages significantly modifies their immune responses to TLR ligands as well as to live *S. aureus*. The priming of cells towards particular immune response is most likely due to an altered expression of receptors and co-receptors on the cell surface. Moreover, we noted that proinflammatory responses were more tightly regulated in primary than in THP-1 monocytes in terms of IL-10 production stressing the complexity of the immune system in the human body.

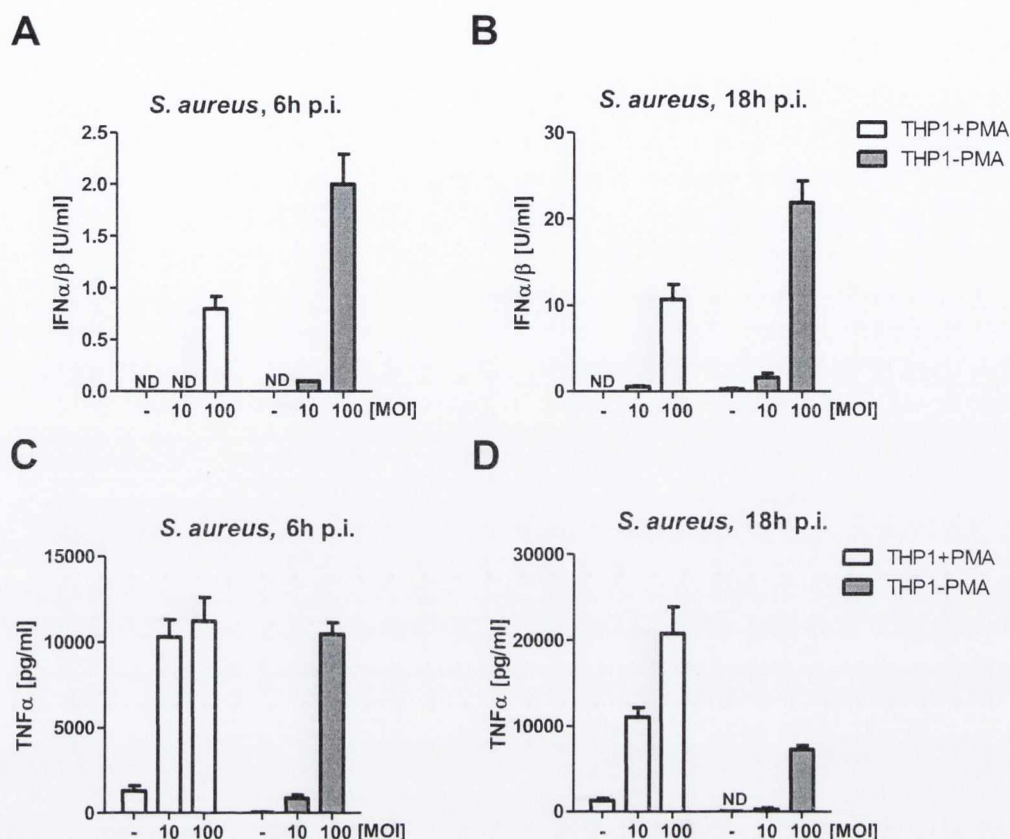


Figure 3.9: PMA treatment of THP-1 monocytes alters their responses during infection with *S. aureus*.

THP-1 cells were seeded at 1×10^6 cells/ml into 24-well plate 24 hr prior to infection and differentiated with PMA (100 nM) or prior to infection and left untreated. Cells were infected with *S. aureus* strain SH1000 at an MOI of 10 or 100 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 or 18 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; and TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

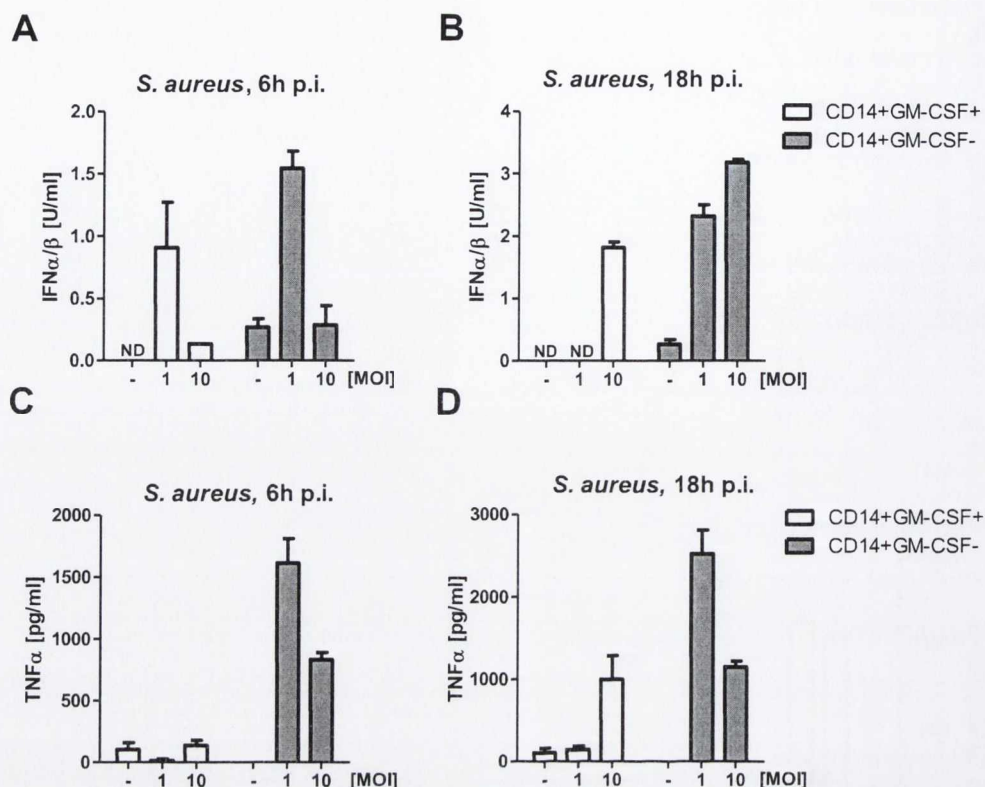


Figure 3.10: Differentiation of CD14+ monocytes alters their responses during infection with *S. aureus*.

CD14+ PBMCs were seeded at 0.8×10^6 cells/ml into 24-well plate and differentiated with GM-CSF (50 ng/ml) for 6 days or left untreated. Cells were infected with *S. aureus* strain SH1000 at an MOI of 1 or 10 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 or 18 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; and TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

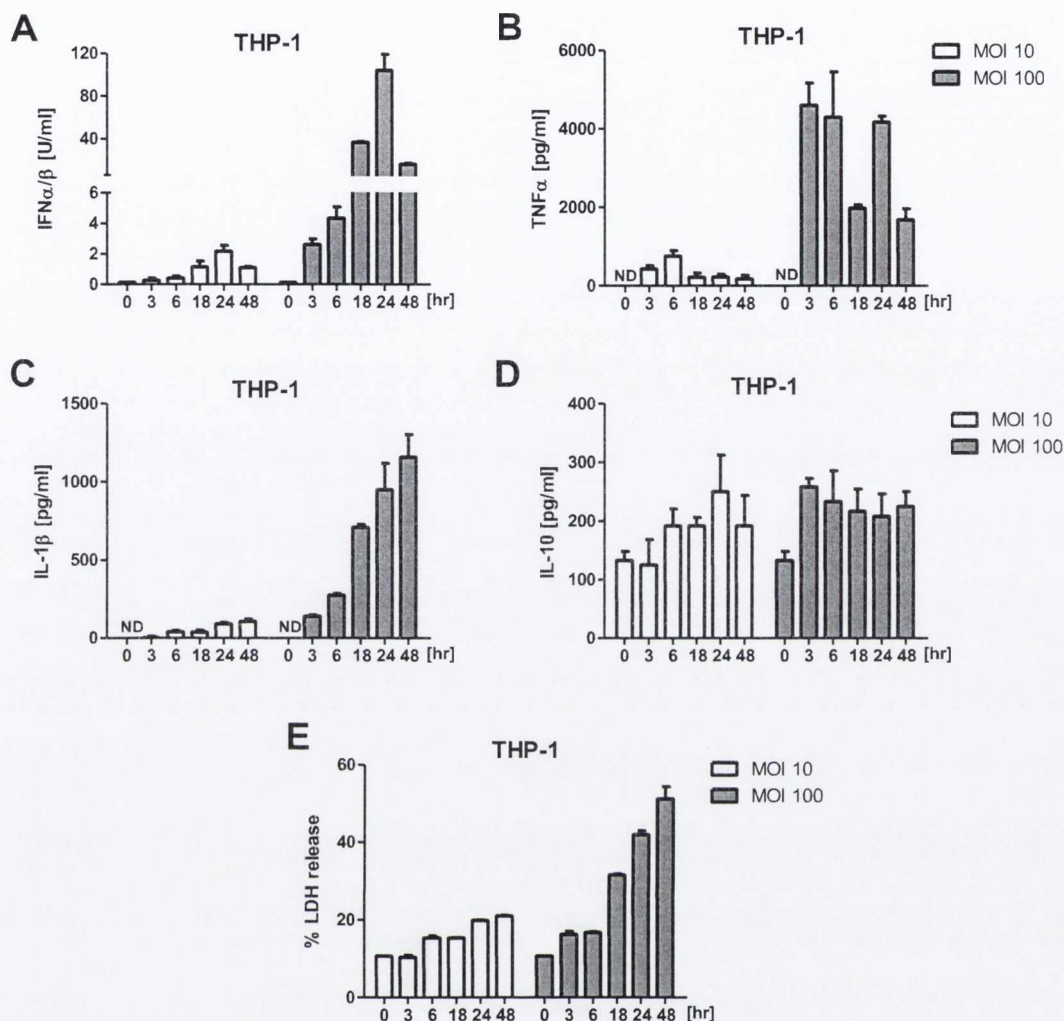


Figure 3.11: Cytokine responses to live *S. aureus* in THP-1 monocytes.

THP-1 cells were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 10 or 100 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 3, 6, 18, 24 or 48 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B), IL-1 β (C) and IL-10 (D) proteins were measured by standard ELISA with appropriate dilutions. Cell viability (E) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

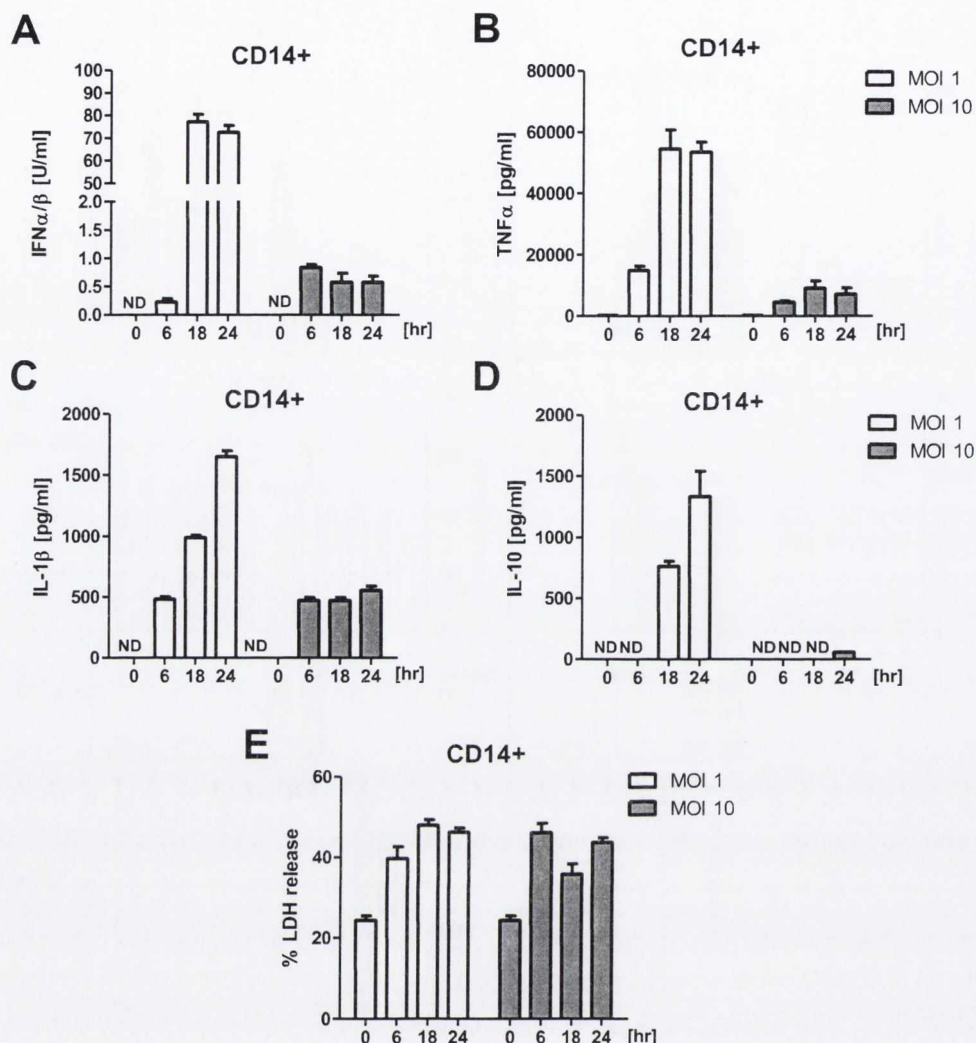


Figure 3.12: Cytokine responses to live *S. aureus* in CD14+ monocytes.

CD14+ PBMCs were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 1 or 10 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 3, 6, 18 or 24 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B), IL-10 (C) and IL-1 β (D) proteins were measured by standard ELISA with appropriate dilutions. Cell viability (E) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected.

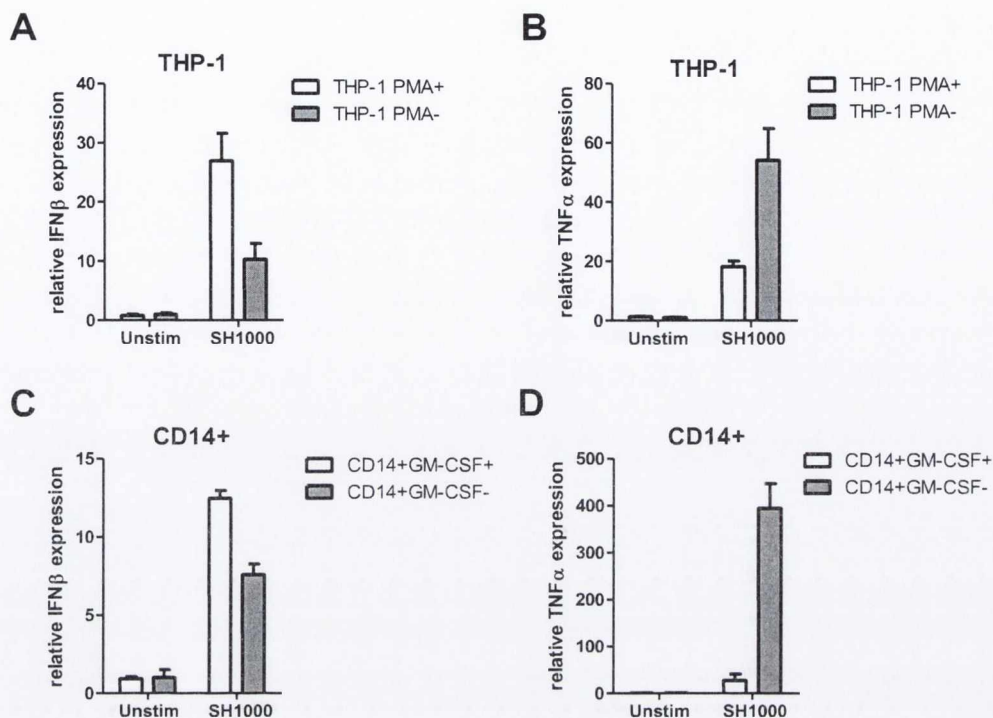


Figure 3.13: IFNβ and TNFα mRNA induction in human monocytes.

THP-1 cells (A-B) or CD14+ monocytes (C-D) were seeded at 1×10^6 cells/ml into 24-well plate prior to stimulation and differentiated with PMA (100 nM, A-B) for 24 hr, GM-CSF (50 ng/ml, C-D) for 6 days or left untreated. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-B) or at an MOI of 1 (C-D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-B) or 18 hr (C-D) followed by collection of cells by centrifugation at 100 g for 5 min. Total RNA was extracted followed by RT-PCR and IFNβ (A, C) and TNFα (B, D) mRNA was measured by qRT-PCR. Data are relative to unstimulated and undifferentiated sample and normalized to the levels of β-actin. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

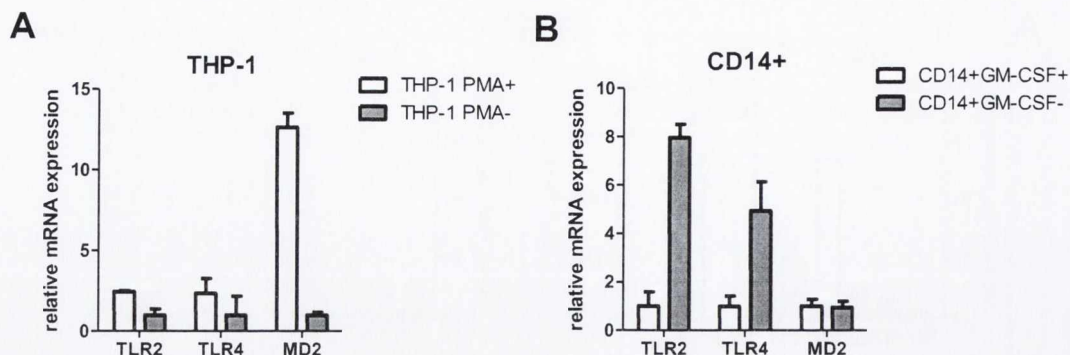


Figure 3.14: TLR2, TLR4 and MD2 mRNA induction in human monocytes.

THP-1 cells (A) or CD14+ monocytes (B) were seeded at 1×10^6 cells/ml into 24-well plate 24-well plate prior to stimulation and differentiated with PMA (100 nM, A) for 24 hr, GM-CSF (50 ng/ml, B) for 6 days or left untreated. Cells were harvested by centrifugation at 100 g for 5 min. Total RNA was extracted followed by RT PCR, and TLR2, TLR4 and MD2 mRNA was measured by qRT-PCR. Data are relative to unstimulated and undifferentiated (A) or unstimulated and differentiated sample (B) and normalized to the levels of β -actin. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

3.3 Discussion

3.3.1 Immune responses to TLR2 ligands in human lung epithelial cells

Stimulation of TLRs with microbial products triggers diverse immune responses depending on the type of stimulation and the cell type. Hence, in order to study TLR2-mediated type I IFN response in human cells, it was first necessary to establish a model cell line. Lung epithelial cells constitute an important protective layer between the outer and the inner body environment. In addition, they are capable of cytokine production and, to a lesser extent, of phagocytosis. Therefore, we first assessed the degree of cytokine release by lung epithelial cells after stimulation with TLR ligands. However, we observed weak production of inflammatory cytokines and type I IFNs by lung epithelial cells in response to both TLR2 and TLR4 ligands. On the other hand, we noted that production of all cytokines was significantly upregulated upon stimulation with synthetic dsRNA, which was recognized by intracellular nucleic acid sensors. Hence, our data suggest that lung epithelial cells are prone to sense nucleic acids generated during viral infection. This is consistent with observations that all nucleated cells can produce type I IFNs upon viral infection through intracellular nucleic acid sensors such as endosomal TLRs or cytosolic RLRs [144]. On the other hand, we observed impaired release of cytokines in response to extracellular RNA in A549 cells, suggesting a lack of TLR3 signaling in these cells. However, the expression levels of TLRs, such as TLR1, TLR2, TLR3, TLR4 and TLR6, were not assessed in the lung epithelial cells. Hence, the lack of or low expression of the receptors might be responsible for the low production of proinflammatory cytokines and/or type I IFNs in these cells. In the future, it will be interesting to measure mRNA levels of TLRs in these cells. Monocytes, on the other hand, play an important role during early stages of innate immunity. Monocytes recognize pathogens, mature into macrophages and DCs, and trigger adaptive immune responses. Accordingly, we observed that TLR2 and TLR4 stimulation potently induced both proinflammatory cytokines and type I IFNs in THP-1 monocytes, indicating that THP-1 monocytes can produce type I IFNs in response to microbial pathogens.

3.3.2 Differentiation of human monocytes alters their responses to TLR ligands

After recognition of pathogen, naïve monocytes morphologically differentiate into mature macrophages in the site of infection. THP-1 monocytes can undergo differentiation *in vitro* upon incubation with chemicals, such as PMA or cytokines [235]. The drug-induced differentiation of monocytes has been a useful model for studying macrophage functions in laboratory conditions. However, it has been shown that differentiation of THP-1 monocytes also alters their responses to immune stimulation [236]. Hence, in order to characterize TLR2-

stimulated immune responses, we first investigated the degree of immune activation in both THP-1 monocytes and THP-1-derived macrophages. Consistent with the reported effect of PMA treatment on immune responses, we observed sharp differences between the THP-1 cell subsets at mRNA expression as well as cytokine production. Expression of IFN β and TNF α mRNA was upregulated upon both TLR2 and TLR4 stimuli; however, the kinetics of immune activation was different between the THP-1 cell subsets. While mRNA expression was upregulated early after stimulation in THP-1 monocytes, there was a delayed upregulation of mRNA in THP-1-derived macrophages, suggesting that the immune activation of cells changes over time. This might also imply that THP-1-derived macrophages and THP-1 monocytes have different functions during immune responses.

In order to assess differences in TLR2 signaling, we stimulated cells with distinct TLR2 ligands. However, we did not observe any discrepancies in the immune activation among the ligands. Interestingly, we noted two classes of immune responses. The first group of responses comprised type I IFNs and IP-10 proteins, whereas the second group was characterized by proinflammatory TNF α and RANTES cytokines. Consistent with the report that THP-1-derived macrophages are primed for LPS responses [236], we observed that TLR4 stimulation upregulated proinflammatory and type I IFNs responses in THP-1-derived macrophages. However, we did not observe priming of THP-1-derived macrophages for type I IFN induction in responses to TLR2 stimulation; instead, we noted that THP-1 monocytes were primed for TLR2-stimulated type I IFN production. Hence, our results further show that THP-1-derived macrophages and THP-1 monocytes respond differently to TLR stimulation. Importantly, we measured immune responses in human primary monocytes and observed similar pattern of cytokine production as in THP-1 monocytes. Collectively, our observations demonstrate that monocytes and monocyte-derived macrophages are differentially primed for immune responses according to the type of TLR stimulation.

3.3.3 Differentiation of human monocytes alters their responses to *S. aureus*

S. aureus is a significant human pathogen causing skin and soft tissue infections, pneumonia, and is a leading cause of nosocomial infections. Due to the nature of infections, most of the research conducted on *S. aureus* pathogenicity focuses primarily on epithelial cells, keratinocytes and osteoblasts. However, relatively little is known about the role of human monocytes and macrophages during infections caused by *S. aureus*.

In order to study immune responses to *S. aureus in vitro*, we investigated the degree of immune activation during *S. aureus* infection in both human monocytes and monocyte-

derived macrophages. Consistent with our previous results with LTA, we observed a bias of THP-1 monocytes towards type I IFN production, while THP-1-derived macrophages were skewed towards production of proinflammatory cytokines during *S. aureus* infection. In primary monocytes, we noted a greater degree of type I IFN and proinflammatory cytokine production in monocytes than in monocytes-derived macrophages during *S. aureus* infection. Our results demonstrate that differentiation of human monocytes can significantly affect immune responses to *S. aureus*, a biologically relevant pathogen. Moreover, we noted considerable differences in the production of anti-inflammatory cytokine IL-10 between THP-1 and primary monocytes. While IL-10 was barely induced in THP-1 monocytes, it was a strongly upregulated in primary monocytes during *S. aureus* infection. Hence, our results show that proinflammatory responses are tightly regulated in primary monocytes, whereas THP-1 monocytes lack the brake of proinflammatory responses.

Interestingly, we observed different pattern of immune activation at mRNA level than at protein level. Our results show that activation of immune cells is more complex in the context of bacterial infection than after pure ligand stimulation. First, the immune responses might be regulated at the posttranscriptional level; therefore, mRNA expression does not correlate with the protein levels. Secondly, different kinetics of immune responses can take place in monocytes compared to monocyte-derived macrophages. Lastly, it is known that some bacteria can manipulate host immune responses; hence, *S. aureus* could actively alter activation of immune responses. In addition, we observed altered basal expression of the innate immune genes after differentiation of monocytes into monocyte-derived macrophages. Interestingly, the gene expression differed between THP-1 and primary monocytes. In line with Park and colleagues [235], we observed upregulation of MD2 expression, while expression of TLR2 and TLR4 was equal in THP-1 monocytes. Since MD2 is a co-receptor essential for LPS-induced TLR4 response, upregulation of MD2 could explain the enhancement of inflammatory responses after LPS stimulation in THP-1 derived macrophages. In primary monocytes, we observed upregulation of both TLR2 and TLR4; accordingly, both monocytes and monocyte-derived macrophages produced cytokines in response to TLR2 and TLR4 stimulation. Thus, the bias towards proinflammatory or type I IFN responses in primary cells might be a result of upregulation of other molecules, which affect immune responses.

Collectively, our results demonstrate that human monocytes are able to produce type I IFNs in response to TLR2 stimulation and *S. aureus* infection. In addition, we showed that the degree of type I IFN induction depends on the differentiation state of the cells. However,

if the differentiation of monocytes has also impact on elimination of pathogens remains unclear, as does the role of type I IFNs during these processes. Hence, in the following chapter, we will examine the ability of THP-1 monocytes and THP-1 monocyte-derived macrophages to kill *S. aureus*. Furthermore, we will address the role of TLR2 and IFN α on the killing activities of THP-1 cells.

4. Result II: The ability of THP-1 monocytes to kill *S. aureus*

4.1 Introduction

For many years, *S. aureus* was regarded as an extracellular pathogen damaging host cells after its adhesion to the extracellular matrix of the cells [180]. However, recent studies have demonstrated that *S. aureus* is able to invade a variety of nonprofessional phagocytes, such as epithelial cells [181], and can survive inside professional phagocytes, including neutrophils and monocytes [182].

S. aureus possess an array of adhesins termed “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs), mediating bacterial adhesion to the host cells and tissues. The most important MSCRAMMs during *S. aureus* invasion are fibronectin-binding proteins (FnBPs) [180]. Interestingly, professional phagocytes have been shown to internalize *S. aureus* via its binding to $\beta 2$ -integrin, whereas *S. aureus* binds to $\beta 1$ -integrin on the surface of non-phagocytic cells and triggers its uptake [176]. This suggests that internalization of *S. aureus* occurs by receptor-mediated endocytosis.

In non-professional phagocytes, studies have shown the ability of *S. aureus* to trigger endocytic processes, to evade phagosomal degradation and to induce apoptosis of cells. It has been reported that inhibition of actin polymerization by cytochalasin D decreased numbers of intracellular *S. aureus* in human osteoblasts [174]. Likewise, inhibition of clathrin-dependent receptor-mediated endocytosis led to lower numbers of intracellular *S. aureus* in human osteoblasts compared to control cells [174]. These results suggest that actin filaments and clathrin-dependent receptor-mediated endocytosis are important during the uptake of *S. aureus* by non-professional phagocytes. Some bacteria, such as *Salmonella* and *Yersinia*, have been show to interact with the host cell receptors and to trigger their own internalization [239, 240]. In addition, the invasion of eukaryotic cells by these bacteria was dependent on activation of tyrosine phosphorylation cascades [239]. Accordingly, inhibition of protein tyrosine kinase activity reduced uptake of *S. aureus* by epithelial cells compared to control cells [173]. These findings suggest that activation of signal transduction cascades plays an important role in internalization of *S. aureus* by non-phagocytic cells.

Most of the laboratory strains of *S. aureus* carry a small deletion in the *rsbU* locus. *rsbU* is a phosphatase that positively regulates transcription factor sigma B (σ^B), which in turn downregulates *agr* expression. The *agr* locus regulates *S. aureus* virulence while σ^B plays a central role in stress responses and persistence of *S. aureus in vivo* [207]. SH1000 is an isogenic variant of laboratory strain 8325-4 with restored functions of *rsbU* locus. This reversion caused enhanced pigmentation by orange pigment staphyloxanthin (STX), decreased

levels of α -toxin and increased resistance to H_2O_2 [208]. In addition, it has been shown that SH1000 was efficiently phagocytosed by THP-1 and human umbilical vein endothelial cells (HUVEC) compared to the parental strain 8325-4 [241]. In addition, intracellular growth of SH1000 in both HUVEC and THP-1 cells was greater than intracellular growth of the parental strain. Using confocal and electron microscopy, SH1000 was confined to phagolysosomes 3 and 24 hr after infection in THP-1 phagocytes, while in HUVEC cells a small amount of bacteria was also found in the cytosol. STX has been reported to protect *S. aureus* against reactive oxygen species (ROS)-dependent cell defense mechanisms [242]. Accordingly, SH1000 with restored STX activity gained higher resistance to H_2O_2 than the parental strain. Inhibition of STX biosynthesis by BPH-652 inhibitor did not affect bacterial growth in the medium; however, it significantly reduced intracellular growth of SH1000 in THP-1 cells [241]. This result demonstrates that STX provides a primary defence mechanism against oxidative damage. In addition, this study demonstrates the ability of THP-1 monocytes to phagocytose *S. aureus* and the ability of *S. aureus* to survive within THP-1 monocytes.

Unlike non-professional phagocytes, professional phagocytes, such as neutrophils, macrophages and dendritic cells, are able to engulf microbes. Accordingly, professional phagocytes have higher basal phagocytic activity and the internalization process involves formation of pseudopods by the cells. Polymorphonuclear leukocytes (PMN) provide an important means of the host defense against bacteria, such as *S. aureus*. Granules containing antimicrobial peptides and NADPH oxidase generating reactive oxygen species create an inhospitable microenvironment in the phagosomes. However, it has been shown that *S. aureus* can survive bactericidal attack within phagosomes of neutrophils and that these phagosomes contain viable intracellular bacteria [182]. It has been reported that *S. aureus* is able to survive within monocytes [241] as well as macrophages [169] and that persistence of *S. aureus* within professional phagocytes may contribute to the dissemination of the bacterium from the primary focus of infection to systemic loci.

So far, the research conducted on the survival of *S. aureus* within professional phagocytes or killing of *S. aureus* by professional phagocytes focused primarily on neutrophils and, to a lesser extent, on macrophages. However, little is known about the interaction between *S. aureus* and monocytes. Hence, the goal of this chapter is to assess the ability of THP-1 monocytes and THP-1-derived macrophages to kill *S. aureus in vitro*. Additionally, we will examine the role of *S. aureus*-stimulated type I IFNs on the killing activity of THP-1 cells.

4.2 Results

4.2.1 *S. aureus* survival is affected by THP-1 differentiation

Both monocytes and macrophages are professional phagocytes, which play important roles in elimination of invading pathogens. Our previous data showed that both monocytes and monocytes-derived macrophages produce proinflammatory cytokines and type I IFNs during *S. aureus* infection. Moreover, we noticed differences between the activation of immune responses between the cell subsets. These data suggest that there might be functional differences between monocytes and monocytes-derived macrophages during *S. aureus* infection. Hence, we investigated the killing ability of the THP-1 cell subsets, and the role of TLR2 and IFN α on the killing ability of THP-1 cells.

First, THP-1 monocytes and THP-1-derived macrophages were infected with *S. aureus* at multiplicity of infection (MOI) of 100 for 30 min, 3 and 6 hr, and the colony-forming units (CFU)/ml of bacteria in the supernatant and the cells combined were determined for each time point. The ability of bacteria to survive and consequently the capacity of cells to kill bacteria was determined as the percentage reduction in the number of CFU at indicated time from to the number of CFU at the time zero. The load of bacteria 30 min after infection was comparable between the THP-1 monocytes and THP-1-derived macrophages demonstrating that the initial load of bacteria was equal between the cell types [Fig. 4.1 A]. Interestingly, both THP-1 cell types were highly efficient at killing of *S. aureus* as the number of remaining bacteria dropped dramatically 3 and 6 hr after infection when compared to the initial number of bacteria. However, the number of remaining bacteria was significantly lower in THP-1 monocytes when compared to THP-1-derived macrophages both 3 and 6 after infection [Fig. 4.1 A]. Additionally, in order to confirm cell viability and ability to produce cytokines, LDH release, TNF α and IFN α/β bioactivity were measured. In terms of viability, THP-1-derived macrophages were more susceptible to cell damage during *S. aureus* infection when compared to THP-1 monocytes [Fig. 4.1 B]. Consistent with results from our previous chapter, TNF α protein was upregulated in THP-1-derived macrophages when compared to THP-1 monocytes, whereas induction IFN α/β bioactivity was greater in THP-1 monocytes when compared to THP-1-derived macrophages [Fig. 4.1 C-D]. This result suggests that THP-1 monocytes have better potential of killing *S. aureus* than THP-1-derived macrophages.

In order to explore the mechanism of *S. aureus* survival within THP-1-derived macrophages, a gentamicin protection assay was performed. In this case, any extracellular bacteria were killed by gentamicin and were washed away so that only intracellular bacteria

would survive. The bacterial survival was determined as the percentage of the number of CFU remaining in the wells containing bacteria and cells at a certain time after infection compared to the number of CFU in the wells containing bacteria and cells at time zero. Surprisingly, *S. aureus* was able to survive and to multiply within the THP-1-derived macrophages, while *S. aureus* was efficiently killed by THP-1 monocytes 3 hr after infection [Fig. 4.2 A]. THP-1-derived macrophages were able to kill intracellular *S. aureus* 6 hr after infection yet significantly less than THP-1 monocytes. LDH release and TNF α protein were increased in THP-1-derived macrophages when compared to THP-1 monocytes [Fig. 4.2 B-C]. IFN α/β bioactivity, on the other hand, was increased in THP-1 monocytes when compared to THP-1-derived macrophages [Fig. 4.2 D]. These findings demonstrate that killing of *S. aureus* occurs more rapidly in THP-1 monocytes than in THP-1-derived macrophages. In addition, *S. aureus* can multiply within and resist its killing by THP-1-derived macrophages *in vitro*.

Lastly, the involvement of TLR2 in *S. aureus* recognition and killing was investigated using CRISPR/Cas9-generated THP-1 TLR2 knockout monocytes [243]. Interestingly, the TLR2 knockout monocytes were significantly less efficient in clearing bacteria 3 hr after infection than wild type monocytes [Fig. 4.3 A]. However, the percentage of remaining bacteria was almost equal 6 hr after infection between the two cell types. In addition, TLR2 knockout cells released higher amounts of LDH compared to wild type cells, suggesting greater vulnerability of TLR2 knockout cells during *S. aureus* infection [Fig. 4.3 B]. Consistent with the role of TLR2 in the activation of innate immune responses, TNF α protein and IFN α/β bioactivity were strongly impaired in TLR2 deficient cells when compared to wild type THP-1 monocytes [Fig. 4.3 C-D]. This result suggests that TLR2 might be partially required for *S. aureus* endocytosis by THP-1 monocytes *in vitro*.

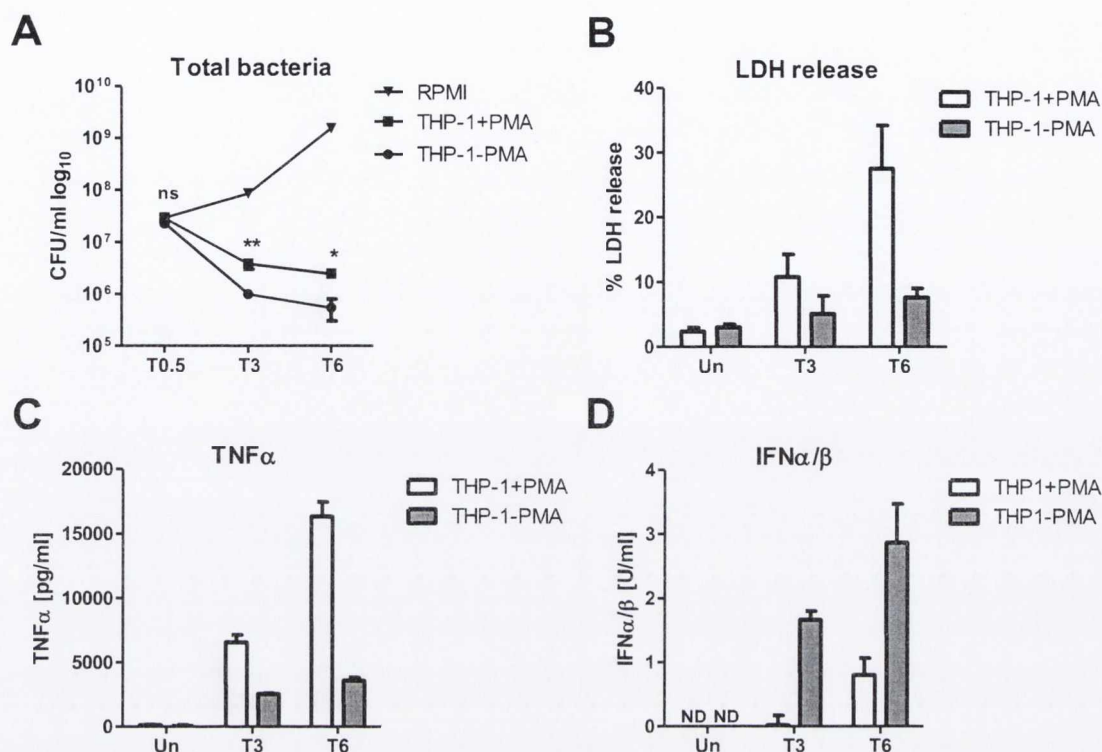


Figure 4.1: Killing of *S. aureus* by THP-1 cells is affected by differentiation.

THP-1 cells were seeded at 1×10^6 cells/ml into 24-well plate 24 hr prior to infection and differentiated with PMA (100 nM) or prior to the infection and left undifferentiated in RPMI without antibiotics. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 3 or 6 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNF α (C) protein was measured by standard ELISA with appropriate dilutions; and IFN α / β bioactivity (D) was assayed using HEK Blue IFN α / β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, ** $p < 0.01$ when compared to cells treated with PMA as determined by two-way ANOVA with a Bonferroni posttest.

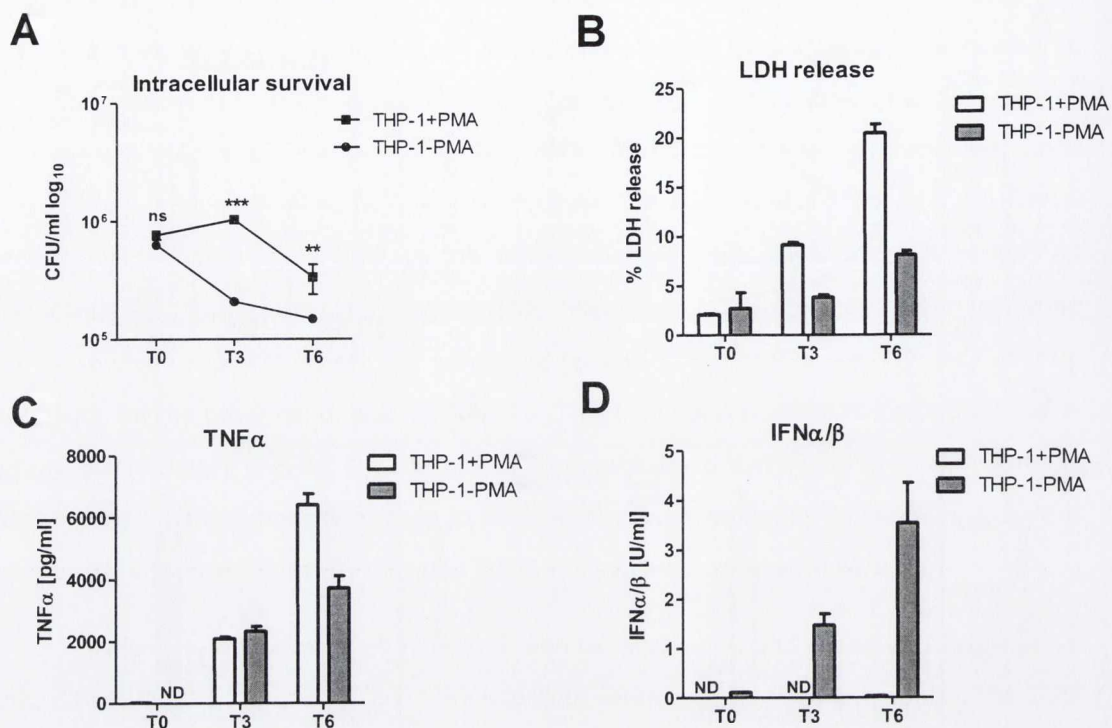


Figure 4.2: *S. aureus* can survive within THP-1-derived macrophages.

THP-1 cells were seeded at 1×10^6 cells/ml into 24-well plate 24 hr prior to infection and differentiated with PMA (100 nM) or prior to the infection and left undifferentiated in RPMI without antibiotics. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100, gentamicin (200 μ g/ml) was added after 30 min and this was considered as time zero. Cells were further incubated for 15 min (T0), 3 hr (T3) or 6 hr (T6). At the indicated times, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. Serial dilutions of the cell lysates were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The intracellular survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay. TNF α (C) protein was measured by standard ELISA with appropriate dilutions; and IFN α / β bioactivity (D) was assayed using HEK Blue IFN α / β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$ when compared to cells treated with PMA as determined by two-way ANOVA with a Bonferroni posttest.

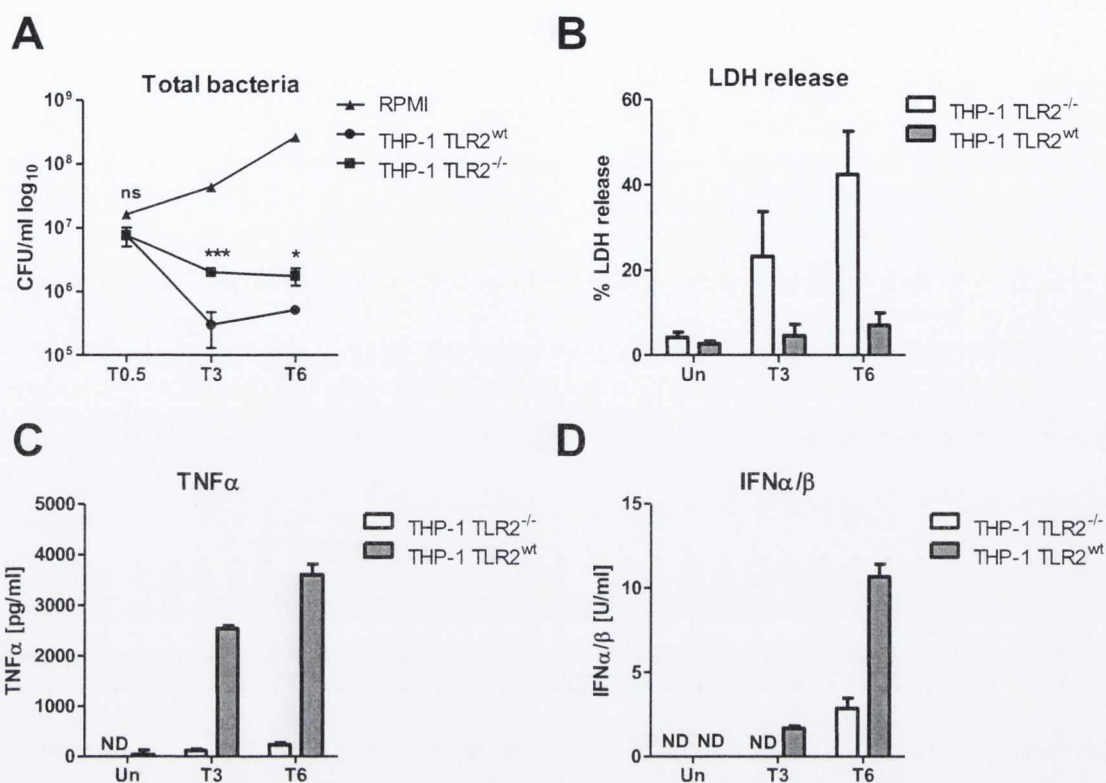


Figure 4.3: Killing of *S. aureus* is TLR2-dependent in THP-1 monocytes.

Wild type THP-1 cells and THP-1 TLR2 CRISPR knockout cell clone A5 were seeded at 0.5×10^6 cells/ml into 24-well plate prior to infection in RPMI without antibiotics. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 3 or 6 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNFα (C) protein was measured by standard ELISA with appropriate dilutions; and IFNα/β bioactivity (D) was assayed using HEK Blue IFNα/β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, *** $p < 0.001$ when compared to infected wild type cells as determined by two-way ANOVA with a Bonferroni posttest.

4.2.2 Blocking the endosomal pathway enhances killing of *S. aureus* in THP-1 monocytes

In order to investigate the mechanisms involved in the killing of *S. aureus*, a variety of inhibitors of an endosomal pathway was tested. First, an effect of dynasore on the ability of cells to clear bacteria was examined.

Dynasore is a potent inhibitor of endocytic pathways, which are dynamin-dependent [244]. Dynamin plays an important role during clathrin-mediated endosomal formation and is required for budding of membrane, formation of vesicles and their scission. Dynasore has been shown to interfere with GTPase activity of dynamin, thus blocking vesicle formation and leading to accumulation of half and fully formed vesicles. Surprisingly, THP-1 monocytes treated with dynasore had considerably increased ability to kill *S. aureus* 6 and 18 hr after infection when compared to DMSO-treated THP-1 monocytes [Fig. 4.4 A]. Furthermore, incubation of *S. aureus* with dynasore or DMSO did not affect its viability since the CFUs from the wells containing *S. aureus* and media+dynasore, media+DMSO or media alone were comparable [data not shown]. However, treatment of cells with dynasore had toxic effects on cell viability since the LDH release increased over time when compared to DMSO-treated cells [Fig. 4.4 B]. TNF α protein induction was not affected after dynasore treatment, whereas IFN α / β bioactivity was dramatically reduced after dynasore treatment when compared to DMSO-treated THP-1 monocytes [Fig. 4.4 C-D].

Next, a role of spleen tyrosine (Syk) kinase in the bacterial killing was tested using Syk kinase inhibitor piceatannol. The non-receptor tyrosine kinase Syk has been shown to regulate TLR4-mediated endocytosis [245]. Nonetheless, the role of Syk or Syk-mediated endocytosis during TLR2-dependent endocytosis and *S. aureus* infection are not known. Remarkably, THP-1 monocytes treated with piceatannol had significantly lower percentage of remaining bacteria at 6 hr after infection when compared to DMSO-treated THP-1 monocytes [Fig. 4.5 A]. Moreover, incubation of *S. aureus* with piceatannol or DMSO did not affect its survival since the CFUs from the wells containing *S. aureus* and media+piceatannol, media+DMSO or media alone were comparable [data not shown]. Treatment with piceatannol was not toxic to cells at earlier time points; however, it was toxic 18 hr after infection when compared to DMSO treated cells [Fig. 4.5 B]. There was no effect of piceatannol on TNF α protein production, whereas IFN α / β bioactivity was impaired at all time points after piceatannol treatment when compared to DMSO-treated THP-1 monocytes [Fig. 4.5 C-D].

Lastly, an effect of bafilomycin A1, a highly specific inhibitor of vacuolar H⁺ ATPase, was examined. Bafilomycin A1 leads to increased pH in phagosomes, thus blocking its fusion with lysosomes, a process essential for antigen maturation. Treatment with bafilomycin A1 significantly enhanced killing of *S. aureus* 3 and 6 hr after infection when compared to cells treated with DMSO [Fig. 4.6 A]. Furthermore, incubation of *S. aureus* with bafilomycin A1 or DMSO did not affect its survival since the CFUs from the wells containing *S. aureus* and media+bafilomycin A1, media+DMSO or media alone were comparable [data not shown]. Bafilomycin A1 and DMSO treatment had similar effects on cell viability since LDH release was equal at all time points after infection [Fig. 4.6 B]. TNF α protein induction was not affected after bafilomycin A1 treatment; however, IFN α / β bioactivity was noticeably impaired after bafilomycin A1 treatment when compared to DMSO-treated THP-1 monocytes [Fig. 4.6 C-D]. Collectively, these results suggest that manipulation with the endosomal pathway might represent a means, by which *S. aureus* escapes its killing by innate immune cells, and that type I IFNs might play a role in this process since the inhibitors also reduced IFN α / β bioactivity.

Hence, an effect of type I IFNs on the ability of THP-1 monocytes to kill *S. aureus* was assessed. For this purpose, THP-1 monocytes were stimulated with recombinant IFN α for approximately 18 hr before *S. aureus* infection in order to activate interferon-stimulated genes. Noticeably, THP-1 monocytes stimulated with IFN α had significantly reduced ability to kill *S. aureus in vitro* when compared to naïve THP-1 monocytes [Fig. 4.7 A]. In addition, combination of IFN α and *S. aureus* infection had adverse effects on cell viability when compared to untreated THP-1 monocytes or THP-1 monocytes stimulated with IFN α or *S. aureus* alone [Fig. 4.7 B, data not shown]. IFN α stimulation impaired *S. aureus*-mediated TNF α production, while *S. aureus*-stimulated IFN α / β bioactivity was upregulated when compared to naïve THP-1 monocytes [Fig. 4.7 C-D]. This result demonstrates that upregulation of interferon-stimulated genes or direct effects of IFN α significantly impair the ability of THP-1 monocytes to kill *S. aureus in vitro*. Moreover, the combination of IFN α stimulation and *S. aureus* infection severely reduces viability of THP-1 monocytes.

Collectively, our data demonstrate that THP-1 monocytes can kill *S. aureus* more efficiently and rapidly than THP-1-derived macrophages. Furthermore, *S. aureus* is able to resist killing and multiply within THP-1-derived macrophages. We also observed that killing of *S. aureus* by THP-1 monocytes was partially TLR2-dependent. Furthermore, we noted that inhibition of the endosomal pathway significantly enhanced bacterial killing and that the endosomal pathway was involved in the type I IFN induction in response to *S. aureus*.

In addition, stimulation with recombinant IFN α impaired the ability of THP-1 monocytes to kill *S. aureus*, and thus enhanced survival of *S. aureus* within THP-1 monocytes. Altogether, our findings suggest that *S. aureus*-stimulated type I IFNs might represent a means by which *S. aureus* manipulates host immune responses and delays its killing within phagosome.

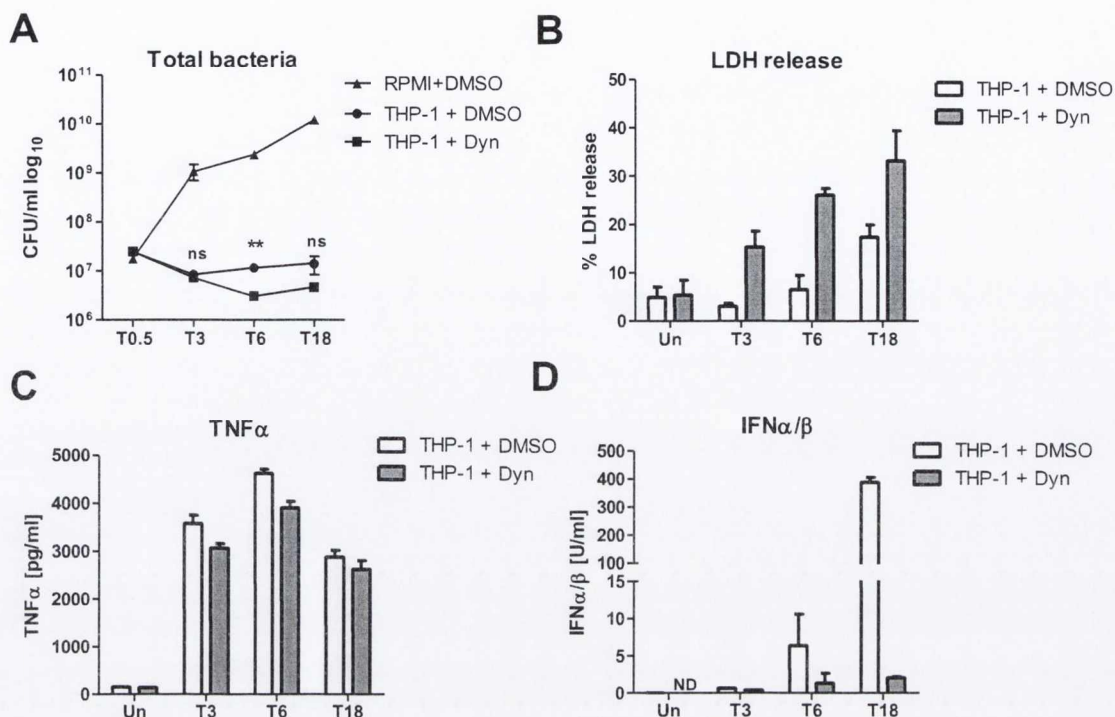


Figure 4.4: Inhibition of dynamin enhances killing of *S. aureus* in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 24-well plate prior to infection in RPMI without antibiotics. Cells were pre-treated with dynasore (20 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 3, 6 or 18 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar, plates followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNFα (C) protein was measured by standard ELISA with appropriate dilutions; and IFNα/β bioactivity (D) was assayed using HEK Blue IFNα/β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$, when compared with infected cells treated with DMSO as determined by two-way ANOVA with a Bonferroni posttest.

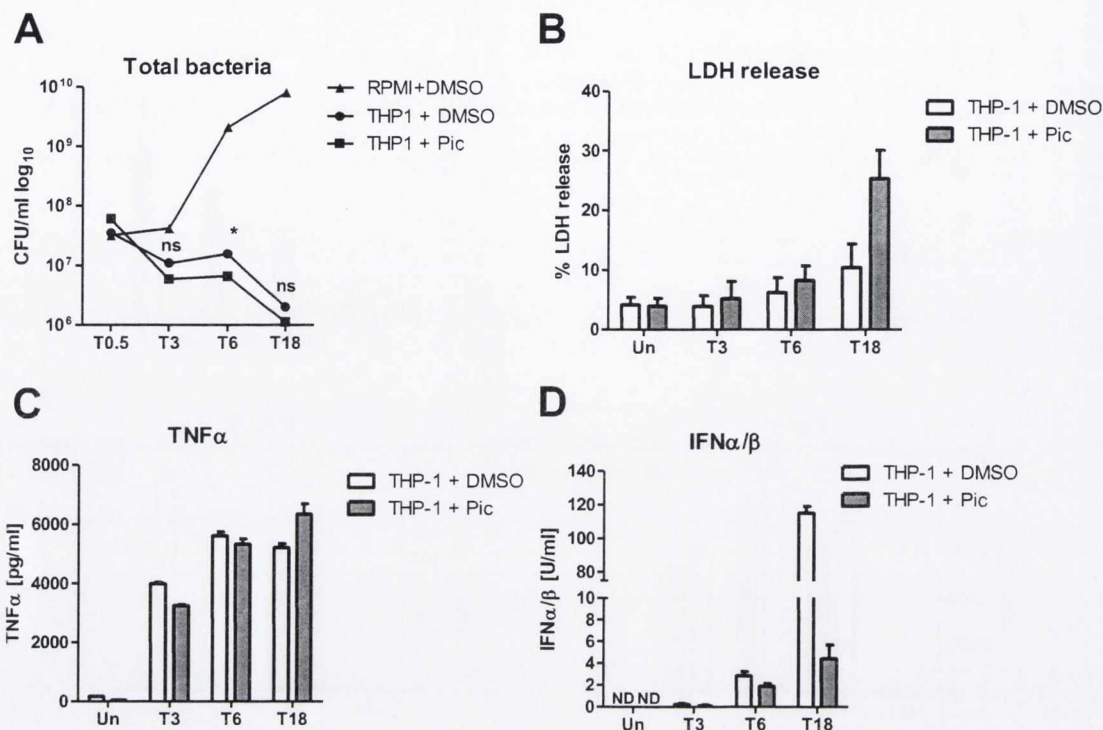


Figure 4.5: Inhibition of Syk kinase enhances killing of *S. aureus* in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 24-well plate prior to infection in RPMI without antibiotics. Cells were pre-treated with piceatannol (20 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 3, 6 or 18 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar plates followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNF α (C) protein was measured by standard ELISA with appropriate dilutions; and IFN α/β bioactivity (D) was assayed using HEK Blue IFN α/β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$ when compared with infected cells treated with DMSO as determined by two-way ANOVA with a Bonferroni posttest.

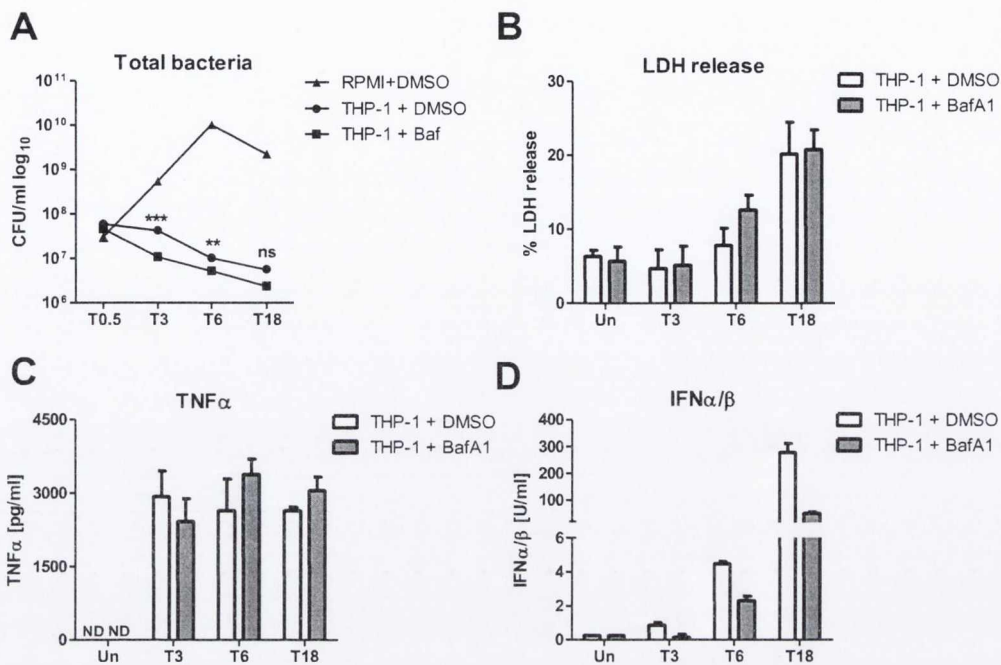


Figure 4.6: Blocking endosomal processing enhances killing of *S. aureus* in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 24-well plate prior to the infection in RPMI without antibiotics. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 3, 6 or 18 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNF α (C) protein was measured by standard ELISA with appropriate dilutions; and IFN α / β bioactivity (D) was assayed using HEK Blue IFN α / β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$, *** $p < 0.001$, when compared with infected cells treated with DMSO as determined by two-way ANOVA with a Bonferroni posttest.

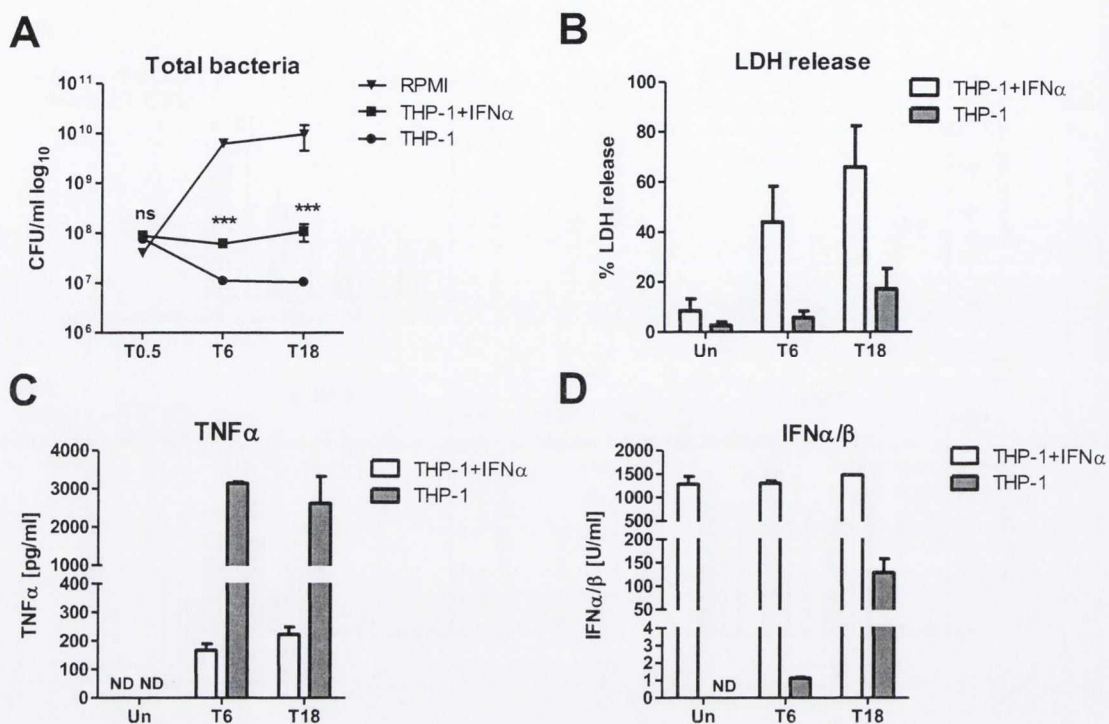


Figure 4.7: Exogenous IFN α impairs killing of *S. aureus* in THP-1 monocytes.

THP-1 cells were seeded at 0.25×10^6 cells/ml into 24-well plate 24 hr prior to the infection in RPMI without antibiotics and stimulated with IFN α (10 000 U/ml) or left untreated. Cells were infected with live *S. aureus* strain SH1000 at an MOI of 100 for 0.5, 6 or 18 hr. At the time point, cells were harvested by centrifugation at 100 g for 5 min. Supernatants were collected and cells were lysed with 0.1% Triton X-100. The supernatant was reintroduced into well and mixed with the cell lysate. Serial dilutions of the suspension were plated onto tryptic soy agar followed by incubation at 37°C and the number of CFU/milliliter was counted the next day. The total bacterial survival (A) was determined as the percentage reduction in the number of bacteria at indicated time from the number of bacteria at the time zero. Cell viability (B) was quantified by LDH cytotoxicity assay; TNF α (C) protein was measured by standard ELISA with appropriate dilutions; and IFN α /β bioactivity (D) was assayed using HEK Blue IFN α /β bioassay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ***p<0.001, when compared with untreated infected cells as determined by two-way ANOVA with a Bonferroni posttest.

4.3 Discussion

4.3.1 *S. aureus* survival is affected by THP-1 differentiation

The goal of this chapter was to investigate the ability of THP-1 monocytes and THP-1-derived macrophages to kill *S. aureus* and the effect of type I IFNs on bacterial clearance. So far, most of the research on the survival of *S. aureus* within or the killing of *S. aureus* by phagocytes has focused on neutrophils. Indeed, PMNs are well equipped for elimination of ingested bacteria. It has been reported that human neutrophils could phagocytose microbes more efficiently than monocytes even though the intracellular killing capacity of both cell types was equal [246]. Therefore, the authors concluded that monocytes are less bactericidal than neutrophils because they are less phagocytic. However, the degree of phagocytosis by monocytes was still considerable, thus indicating that monocytes also contribute to the host innate defenses against bacteria. In addition, it has been shown that THP-1 monocytes efficiently phagocytose *S. aureus* strain SH1000 and that this strain survives intracellularly in phagolysosomes during infection of THP-1 monocytes [241]. However, the literature on interaction between *S. aureus* and THP-1 monocytes is limited. Hence, we investigated the ability of THP-1 monocytes and THP-1-derived macrophages to kill *S. aureus in vitro*. In addition, we examined the role of TLR2, IFN α and the endosomal pathway in the clearance of *S. aureus* by THP-1 monocytes *in vitro*.

Remarkably, we observed that both THP-1 monocytes and THP-1-derived macrophages efficiently killed *S. aureus*; however, THP-1 monocytes eliminated *S. aureus* more rapidly than THP-1-derived macrophages. We also noted a correlation between LDH release and increased TNF α production by THP-1-derived macrophages, suggesting that the upregulation of proinflammatory response might contribute to pathogenesis during *S. aureus* infection. Indeed, it has been shown that interaction between staphylococcal protein A and TNFR1 induces TNF α production causing increased pathogenesis during *S. aureus* infection [130]. In addition, we observed greater degree of type I IFN induction in THP-1 monocytes than in THP-1-derived macrophages, which is consistent with our previous findings in the previous chapter. Hence, our results demonstrate that THP-1 monocytes as well as THP-1-derived macrophages are able to kill *S. aureus*. However, THP-1 monocytes kill *S. aureus* more rapidly and produce higher amounts of type I IFNs, whereas THP-1-derived macrophages produce more TNF α , which can contribute to increased pathogenicity during *S. aureus* infection. In the future experiments, it would be interesting to address the effects of TNF α stimulation on the killing capacity of THP-1 monocytes and on their viability during infection.

It has been shown that *S. aureus* can persist inside monocytes [241] as well as inside macrophages [169]. In line with these studies, we observed that *S. aureus* survived and multiplied within THP-1-derived macrophages during early stages of infection. In addition, we observed that THP-1-derived macrophages were not able to kill intracellular *S. aureus* as efficiently as THP-1 monocytes during later stages of infection. Hence, our data show that although both THP-1 cell subsets phagocytose *S. aureus*, the bacterium can resist its killing within phagosomes of THP-1-derived macrophages, while it is killed in THP-1 monocytes.

Previous studies have demonstrated that both TLR2 and MyD88 are important for the control of *S. aureus* infection in mouse peritoneal macrophages [185]. In addition, it has been shown that TLR2 co-localizes in phagosomes containing *S. aureus* in mouse RAW macrophages [247]. Using TLR2 knockout THP-1 monocytes, we observed that TLR2 knockout monocytes killed fewer bacteria during early infection than wild type monocytes; however, the differences in killing was less distinct at later stages of infection. Hence, our result reveals a delayed uptake of bacteria in the absence of TLR2, suggesting a role of TLR2 in endocytosis of *S. aureus* by THP-1 monocytes. However, further studies are needed in order to elucidate the role of TLR2 in *S. aureus* phagocytosis in monocytes. This could be achieved by confocal microscopy using fluorescently label bacteria.

4.2.2 Blocking the endosomal pathway enhances killing of *S. aureus* in THP-1 monocytes

Phagosomes play an essential role during infection by providing a microenvironment, in which bacteria is killed and digested. However, many pathogens have evolved mechanisms of resistance to phagolysosomal killing or evasion from phagosomes. Therefore, we examined a role of the endosomal pathway in the killing of *S. aureus* by blocking several steps during phagocytosis in THP-1 monocytes.

Dynamin plays an important role during clathrin-mediated endosomal formation in macrophages and is required for budding of membrane, formation of vesicles and their scission. Dynasore has been shown to block vesicle formation by interfering with its GTPase activity, leading to accumulation of immature vesicles. In addition, it has been reported that inhibition of clathrin-dependent endocytosis led to decreased numbers of intracellular *S. aureus* in human osteoblast [174]. Accordingly, we observed that inhibition of dynamin-dependent endocytosis reduced the number of remaining *S. aureus* in THP-1 monocytes. However, our result shows that the decrease in *S. aureus* numbers is due to an enhanced capacity of bacterial killing by THP-1 monocytes, while in osteoblasts the reduction is a result

of impaired endocytosis. This observation implies that distinct mechanisms of bacterial killing occur in non-professional and professional phagocytes. Collectively, these results suggest that clathrin- and dynamin-dependent endocytosis is involved in *S. aureus* endocytosis by THP-1 monocytes; however, immature vesicles are recognized and *S. aureus* is killed by the cells.

It has been noted that tyrosine phosphorylation cascade is required for invasion of non-professional phagocytes by some bacteria [239]. Accordingly, inhibition of tyrosine kinase activity by genistein reduced uptake of *S. aureus* by bovine mammary gland epithelial cell line [173]. In addition, the non-receptor tyrosine kinase Syk has been involved in LPS-dependent translocation of a TLR4-CD14 complex into endosomes [245]. However, the function of Syk in *S. aureus* endocytosis is unclear in human monocytes; therefore, we examined the effect of Syk kinase inhibitor on bacterial killing. Notably, we observed that more bacteria were killed after inhibition of Syk kinase, suggesting a role of Syk in *S. aureus* killing by THP-1 monocytes.

Finally, it has been shown that phagosomal acidification is required for maturation of staphylococcal antigens and activation of TLR signaling [195]. Hence, we investigated a role of the endosomal processing in the killing of *S. aureus* by blocking vacuolar H⁺ ATPase, whose function is required for the maturation of endosomes. Remarkably, we noted that fewer bacteria survived after blocking phagosomal acidification in THP-1 monocytes. Importantly, we did not observe toxic effects of the inhibitors or vehicle control on the growth of bacteria, indicating that the reduction in number of bacteria was due to altered functions of cells. Thus, our results suggest that in an immature endosome *S. aureus* might be recognized and eliminated by the cell. However, when *S. aureus* reaches mature endosomes, it might delay its killing by the cells.

In contrast to the lack of effect of inhibitors on bacterial viability, dynasore and Syk kinase inhibitor had adverse effects on viability of THP-1 monocytes. Consequently, cell death might have caused release of phagosomal enzymes into the media and killing of extracellular bacteria. However, we did not observe any difference in TNF α production between inhibitor- and control-treated cells, suggesting that cells were still able to produce TNF α . Treatment with bafilomycin A1 had equal effects on cell viability as vehicle control and caused the most significant enhancement in bacterial killing. Therefore, our results suggest that *S. aureus* is able to manipulate the endosomal pathway as a means of its evasion; however, the mechanism remains unclear. It will be interesting to explore changes in cellular localization of *S. aureus* during infection after blocking the endosomal pathway by confocal microscopy

of fluorescently labeled bacteria within the cell compartments. In all cases, inhibition of the endosomal pathway did not affect induction of TNF α protein; however, it greatly impaired induction of type I IFNs in response to *S. aureus* infection. Thus, our results suggest that proinflammatory response does not contribute to killing of *S. aureus*, and that type I IFNs might represent a means how *S. aureus* manipulates its fate within the THP-1 monocytes.

Bacterial superinfections after viral lung infections are well-documented phenomena; however, the mechanisms by which they occur remain poorly studied. It has been shown that *S. aureus* activates type I IFN signaling and this contributes to its virulence in mice and human airway epithelial cells [231]. A major component of the immune response to influenza is the activation of type I IFN production. Accordingly, mice infected with both influenza A and *S. aureus* had higher mortality, greater lung damage and increased bacterial density than mice infected with *S. aureus* alone [248]. Therefore, we investigated the effect of recombinant IFN α on the outcome of infection with *S. aureus*. Consistent with the reported negative effect of type I IFNs on the outcome of bacterial infection, we observed that THP-1 monocytes had significantly impaired capacity to kill *S. aureus* after IFN α stimulation. In addition, we noted that the combination of IFN α stimulation and *S. aureus* infection had adverse effects on viability of THP-1 monocytes. Hence, our results suggest that IFN α induction by viral agents may play a detrimental role during bacterial superinfections following influenza infection.

Collectively, our data demonstrate that both THP-1 monocytes and THP-1-derived macrophages are able to phagocytose and kill *S. aureus in vitro*. Moreover, THP-1 monocytes eliminate *S. aureus* more rapidly than THP-1-derived macrophages. Our result also demonstrates that slower killing of *S. aureus* is most likely due to *S. aureus* ability to resist immediate killing within THP-1-derived macrophages. In addition, our data suggests that the early phagocytosis of *S. aureus* partially requires TLR2 since both phagocytosis and killing of *S. aureus* are delayed in the absence of TLR2. Furthermore, we demonstrated that inhibition of the endosomal pathway leads to enhanced bacterial killing. We also show that the endosomal pathway is involved in type I IFN production in response to *S. aureus* and that its inhibition leads to reduction of type I IFNs. Importantly, we showed that stimulation with recombinant IFN α impaired killing capacity of THP-1 monocytes *in vitro*. This might provide an insight into the mechanisms of host susceptibility to bacterial infections after preceding viral infections. Moreover, our findings suggest that *S. aureus*-stimulated type I IFNs might represent a means of *S. aureus* manipulation with the host immune responses.

5. Result III: Dissection of *S. aureus*-mediated type I IFN pathway in human monocytes

5.1 Introduction

Upon detection of PAMPs, TLRs activate a signaling cascade, which culminates in the production of proinflammatory cytokines and type I IFNs. Human TLR2 recognizes a wide range of PAMPs derived from various pathogens including bacteria, fungi, parasites or viruses. TLR2 can form heterodimers with TLR1, TLR6, with non-TLR molecules including CD14, CD36, dectin-1, and with other yet unknown molecules. Such promiscuity of TLR2 results in its ability to recognize typical as well as unusual bacterial PAMPs.

Upon recognition of PAMPs, TLRs use adaptor proteins in order to transduce a signal to downstream kinases, which activate transcription factors such as NF κ B and IRFs. TLR2 typically utilizes MyD88 and MAL adaptor proteins; however, there is an evidence that TLR2 can also employ TRAM and TRIF in the downstream signaling cascade [42, 43]. Typically, upon activation of surface TLRs, MyD88 recruits IRAK4, IRAK1 and IRAK2 and forms a signaling complex termed the myddosome. The myddosome mediates phosphorylation of IRAKs resulting in activation of TRAF6. TRAF6 then ubiquitinates TAK1-binding (TAB) proteins, leading to TAK1 phosphorylation and activation of IKK complex consisting of IKK α , IKK β and IKK γ subunits. IKK complex mediates NF κ B activation, translocation to the nucleus and transcription of proinflammatory cytokines. In addition, TAK1 can activate p38 mitogen-activated protein kinase (MAPK) and c-JUN N-terminal kinase (JNK) leading to the induction of transcription factors, such as cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1), and further upregulation of proinflammatory cytokines.

On the other hand, activation of endosomal TLRs results in the induction of both proinflammatory cytokines and type I IFNs. Upon dimerization of TLR3 or TLR4, TRIF adaptor protein and TRAF3 ubiquitin ligase mediate phosphorylation and activation of IRF3 via noncanonical IKKs or so-called IKK-related kinases TBK1 and IKK ϵ . Phosphorylated IRF3 dimerizes and translocates to the nucleus, where it binds promoters containing interferon-stimulated response element (ISRE), and thus induces expression of antiviral genes such as IP-10, RANTES and type I IFNs. Upon TLR7/8 and TLR9 activation, IKK α rapidly induces production of IFN α in pDCs. IKK α binds to the complex containing MyD88, TRAF6 and IRAK-1/4 and kinase activity of IRAK1 activates IKK α . Phosphorylated IKK α translocates to the nucleus and induce transcription of IFN α [63].

There is a growing body of evidence that as well as TLR3, TLR4, TLR7/8 and TLR9, TLR2 can also mediate type I IFN induction. In 2009, Barbalat and colleagues reported that VACV induced type I IFNs via TLR2- and MyD88-dependent activation of IRF3 and IRF7

in inflammatory monocytes [157]. Another piece of evidence showed that TLR2-mediated type I IFN via MyD88, IRF1 and IRF7, and required internalization and endolysosomal processing [152]. Furthermore, a TLR2-IFN β pathway was associated with *Listeria monocytogenes* infection and was dependent on bacterial internalization, TRIF, IRF3 and IRF7 in mouse macrophages derived from the peritoneal cavity [158]. In addition, our previous work revealed that TLR2-mediated type I IFNs was Mal- and TRAM-dependent in mouse bone marrow derived macrophages (BMDMs). Consequently, infection of BMDMs lacking these adaptors with herpesvirus or *S. aureus* led to an impaired IFN β production [42]. Furthermore, TRAM was responsible for trafficking of TLR2-MyD88 complex into endosomes and IRF7 was responsible for IFN β induction in HEK293 cells. Collectively, these results demonstrate that TLR2 is able to stimulate type I IFNs with involvement of MyD88 and IRFs in the pathway. However, the individual components of TLR2-mediated type I IFN pathway are cell-type specific.

The cell wall of Gram-positive bacteria is a rich source of PAMPs such as peptidoglycan (PGN) or lipoteichoic acids (LTA) that are recognized by PRRs. Despite an extensive literature about *S. aureus*, little is known about the signaling pathways by which *S. aureus* triggers innate immune responses in human cells. To date, most of the research on cytokine induction was based on mouse models of *S. aureus* infection; however, there are considerable differences between the infection of mice and humans. First, mice are not natural host of human *S. aureus* isolates; therefore, genetic alterations of mice are required in order to sensitize mice for infection [184]. Second, while the induction of protective immunity in mice is based on opsonic antibodies, the mechanisms of adaptive immunity in humans are still unclear [227]. Lastly, the activation of the inflammasome, a marker of the inflammatory responses, is different between mice and humans. While it requires two signals in mouse macrophages, only one signal is necessary for the inflammasome induction in human monocytes [251]. Hence, the results obtained from mouse studies should be compared to and be verified in human systems. It has been demonstrated that mice deficient in TLR2 and MyD88 are protected against *S. aureus* induced sepsis, implicating a key role for TLR2 in *S. aureus* recognition and function of MyD88 in downstream signaling [185]. Studies in BMDMs further showed the involvement of Mal, IRAK4 and TRAF6 in the signaling cascade, leading to inflammatory responses after PGN stimulation [186]. Importantly, clinical data from patients with MyD88 and IRAK4 deficiencies revealed a protective role of TLR2- IRAK4 signaling during recurrent bacterial infections in humans [187, 188]. These results demonstrate that

TLR2-MyD88-IRAK4 signaling pathway involving Mal and TRAF6 is implicated in the induction of proinflammatory responses upon *S. aureus* infection.

Even though TLR2 is the key surface receptor for *S. aureus* recognition, the contribution of other receptors and co-receptors remains ambiguous. Moreover, most of the available literature is contradictory, which might be due to the diversity of *S. aureus* antigens, impure ligand preparations and distinct cell types used in studies. Both TLR2-dependent and TLR2-independent pathways have been linked to the recognition of *S. aureus*. TLR2-TLR1 heterodimer [190] and scavenger receptor CD36 [22] have been implicated in the LTA sensing, whereas TLR2-TLR6 heterodimer [189] and NOD2 [190] have been associated with PGN recognition. In addition, NOD2 has been shown to recognize staphylococcal muramyl dipeptide (MDP) in the cytosol [193]. Interestingly, mutations in *NOD2* gene have been associated with Crohn's disease and cells of these patients failed to detect PGN or MDP by NOD2 [85]. In addition, CD14 has been shown to interact with staphylococcal and streptococcal LTA whose recognition was enhanced by LBP and CD14 in a TLR2-dependent manner [191]. The variety of *S. aureus* PAMPs implicates that its recognition is most likely mediated by multiple receptors with a central role of TLR2 in cooperation with various co-receptors.

In addition, evidence about the role of type I IFNs in *S. aureus*-mediated pathogenesis is accumulating. However, the research conducted on the function of type I IFNs during *S. aureus* infection is often controversial. In a mouse model of post-haemorrhage *S. aureus* pneumonia, it has been shown that treatment with CpG DNA induced TNF α , IFN β and IL-12 transcription activity and increased cytokine production correlated with decreased mortality of mice [252]. Furthermore, it has been reported that treatment with recombinant IFN α protected human lung epithelial cells against α -toxin-induced cell death during *S. aureus* infection [253]. Interestingly, type I IFN induction has been shown to require phagosomal degradation since *S. aureus* induced low amounts of IFN β in myeloid phagocytes *in vitro* or during cutaneous infection *in vivo* [254]. However, using a strain sensitive to lysozyme, *S. aureus* was able to induce strong IFN β response in mice DCs, which was MyD88-dependent but independent of TLRs. Importantly, treatment with recombinant IFN β enhanced clearance of *S. aureus* by DCs *in vitro* and reduced bacterial load in cutaneous lesions *in vivo* [254].

In contrast to the protective role of type I IFNs, it has been reported that mice deficient in type I IFN receptor (*Ifnar*) and *Tlr9* had improved clearance of *S. aureus* from the lungs [153]. Interestingly, enhanced clearance of *S. aureus* correlated with decreased type

I IFN and TNF α production [153]. Likewise, *Ifnar*^{-/-} mice exhibited reduced mortality during *S. aureus* pulmonary infection compared to their wild type littermates and their survival was accompanied with reduced levels of type I IFNs in the bronchoalveolar lavage [231]. Furthermore, it has been shown that *S. aureus* strain 502A induced an excessive type I IFN production via NOD2 and IRF5 [255]. Remarkably, 502A-induced type I IFNs upregulation correlated with increased mortality, greater destruction of the lung and significant increase in proinflammatory response in mice [255].

Collectively, these studies demonstrate that the role of type I IFNs in *S. aureus* infection is highly variable and depends on many factors, including *S. aureus* strain, nature of the host and cell type. Consequently, the function of type I IFNs during *S. aureus* infection remains poorly described. Previously, we showed that inhibition of the endosomal pathway enhanced clearing of *S. aureus* by THP-1 monocytes, and treatment with recombinant IFN α had a detrimental effect on the ability of THP-1 monocytes to clear *S. aureus in vitro*. Hence, we further investigated the nature of signaling components involved in *S. aureus*-mediated type I IFN signaling pathway in human monocytes.

5.2 Results

5.2.1 TLR2 is required for IFN β and TNF α induction by *S. aureus* in human monocytes

In previous chapters, we measured immune responses to TLR2 ligands and *S. aureus* infection in human cells. Consequently, we identified THP-1 monocytes and primary CD14⁺ monocytes as the model cell lines for studying TLR2-induced type I IFNs. In addition, we showed that THP-1 monocytes efficiently killed *S. aureus in vitro* and TLR2 was partially required for the clearance of live *S. aureus* in THP-1 monocytes. Hence, in order to study the contribution of TLR2 in type I IFN pathway, an effect of neutralizing TLR2 antibody (α TLR2) was investigated in human monocytes. Of interest, we observed a significant variability in cytokine induction among individual blood donors; however, the effect of inhibitors was consistent throughout the experiments.

First, THP-1 and CD14⁺ monocytes were stimulated with purified *S. aureus* lipoteichoic acid (LTA), a major immunostimulatory component of Gram-positive bacteria, and immune responses were measured after neutralizing TLR2 function. Treatment with α TLR2 antibody significantly impaired LTA-induced IFN α/β bioactivity and TNF α production when compared to isotype control treated THP-1 and CD14⁺ monocytes [Fig. 5.1 A-B, D-E]. Neither α TLR2 antibody nor isotype control had adverse effects on cell viability [Fig. 5.1 C, F]. Additionally, THP-1 monocytes were stimulated with macrophage-activating lipopeptide-2 (Malp2), an established ligand engaging a TLR2-TLR6 heterodimer. Accordingly, Malp2-stimulated IFN α/β bioactivity and TNF α induction were diminished after blocking TLR2 function without having toxic effects on cell viability when compared to THP-1 monocytes treated with isotype control [Fig. 5.2 A-C]. Next, an effect of TLR2 neutralizing antibody was investigated on *S. aureus*-induced cytokines. Accordingly, treatment with α TLR2 antibody significantly decreased *S. aureus*-induced IFN α/β bioactivity and TNF α production in both THP-1 and CD14⁺ monocytes when compared to isotype control treated monocytes [Fig. 5.3 A-B, D-E]. Moreover, the combination of α TLR2 antibody and *S. aureus* infection did not have adverse effects on cell viability [Fig. 5.3 C, F]. These observations suggest that recognition of LTA, Malp2 and *S. aureus* is mediated by and dependent on TLR2 in human monocytes.

In order to confirm the requirement for TLR2 in type I IFN induction, we employed THP-1 monocytes lacking functional TLR2 generated by the CRISPR-Cas9 technique [243]. First, responses to TLR ligands were tested in two independent TLR2 knockout clones A5 and D4 and degree of immune activation was compared with wild type THP-1 monocytes. Consistent with the effect of α TLR2 antibody, LTA- and Malp2-mediated IFN α/β bioactivity and TNF α protein

were abolished in TLR2 knockout monocytes when compared to wild type monocytes [Fig. 5.4 A-B]. In order to exclude off-target effects of Cas9 enzyme, CL075 was employed to stimulate cells as a ligand independent of TLR2 signaling. CL075 is a thiazoloquinolone derivative that stimulates production of IFN α protein through TLR7, and induction of TNF α protein via TLR8 in human monocytes. While CL075-stimulated IFN α / β bioactivity was unaffected in clone A5, impairment was observed in clone D4 after CL075 stimulation when compared to wild type monocytes [Fig. 5.4 A]. This result suggests that off-target effects might have occurred during genome editing process in clone D4. TNF α protein was not induced in wild type THP-1 monocytes or TLR2 knockout cells after CL075 stimulation [Fig. 5.4 B]. Lastly, TLR2 knockout and wild type monocytes were challenged with *S. aureus* at two multiplicities of infection (MOIs). Accordingly, both IFN α / β bioactivity and TNF α induction were significantly impaired after *S. aureus* infection in TLR knockout monocytes when compared to wild type THP-1 monocytes [Fig. 5.5 A, C]. However, IFN α induction was significantly upregulated upon *S. aureus* infection in TLR2 knockout monocytes when compared to wild type THP-1 monocytes [Fig. 5.5 B]. Importantly, infection with *S. aureus* had toxic effects on viability of TLR2 knockout monocytes when compared to wild type THP-1 monocytes [Fig. 4.7 B]. These findings confirm that TLR2 is a key PRR for recognition of *S. aureus*. Furthermore, these results demonstrate that IFN β and TNF α induction is TLR2-dependent, whereas IFN α induction is independent of TLR2 during *S. aureus* infection in THP-1 monocytes.

It has been noted that LTA activates TLR2 through LBP and CD14 co-receptor, a mechanism similar to TLR4 signaling [191]. Eritoran is an analogue of lipid A, which inhibits LPS-induced signaling by competing with lipid A for binding to CD14 [238]. In addition, CD14 has been shown to regulate TLR4 internalization and its trafficking into endosomes; however, its role in TLR2 signaling is not fully elucidated. Therefore, an effect of eritoran on TLR2-induced signalling was tested. Accordingly to its role in TLR4 signaling, LPS-induced IFN α / β bioactivity and TNF α production were impaired after eritoran treatment when compared to untreated THP-1 monocytes confirming an efficacy of eritoran in THP-1 cells [Fig. 5.6 A, D]. Treatment with eritoran also reduced LTA- and *S. aureus*-induced IFN α / β bioactivity and TNF α induction in a dose-dependent manner when compared to untreated THP-1 monocytes [Fig. 5.6 B-C, E-F]. These results suggest that recognition of LTA and *S. aureus* by TLR2 also involves CD14 co-receptor.

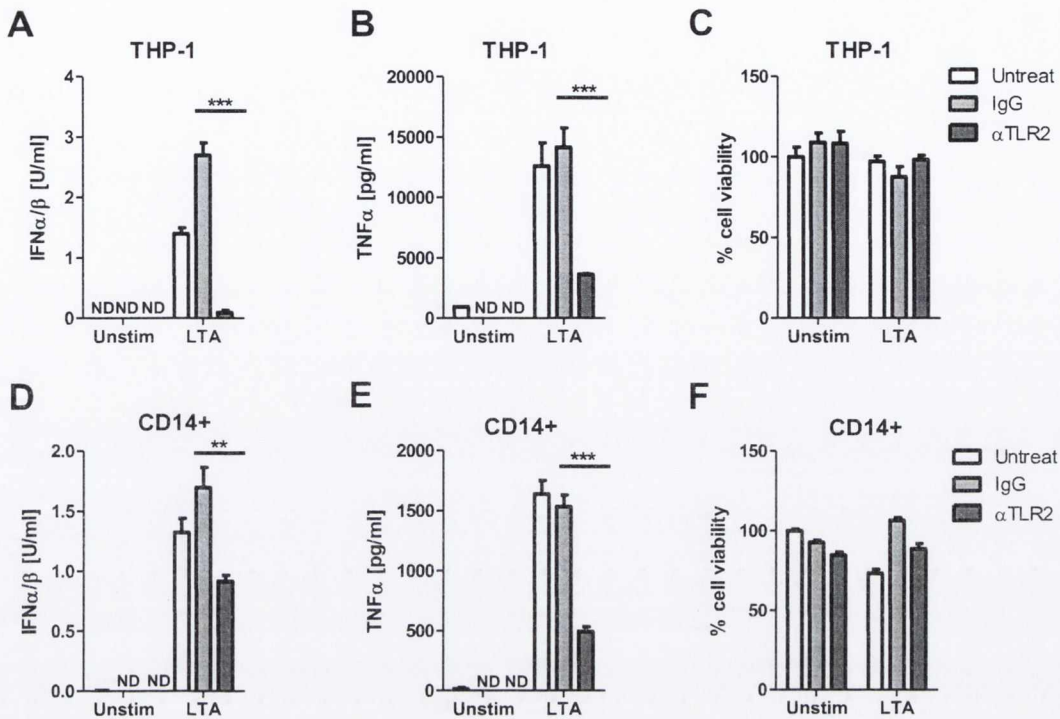


Figure 5.1: TLR2 is required for LTA-induced type I IFN and TNF α responses in human monocytes.

THP-1 cells (A-C) or CD14+ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with α TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvest by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A,D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with stimulated samples treated with isotype control as determined by Student t test.

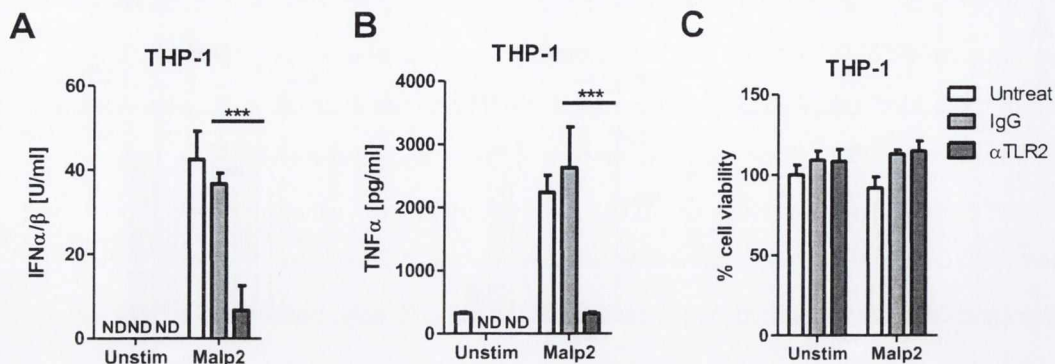


Figure 5.2: TLR2 is required for Malp2-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with anti-TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml) for 6 hr. Supernatants were harvest by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, *** $p < 0.001$, when compared with stimulated samples treated with isotype control as determined by Student t test.

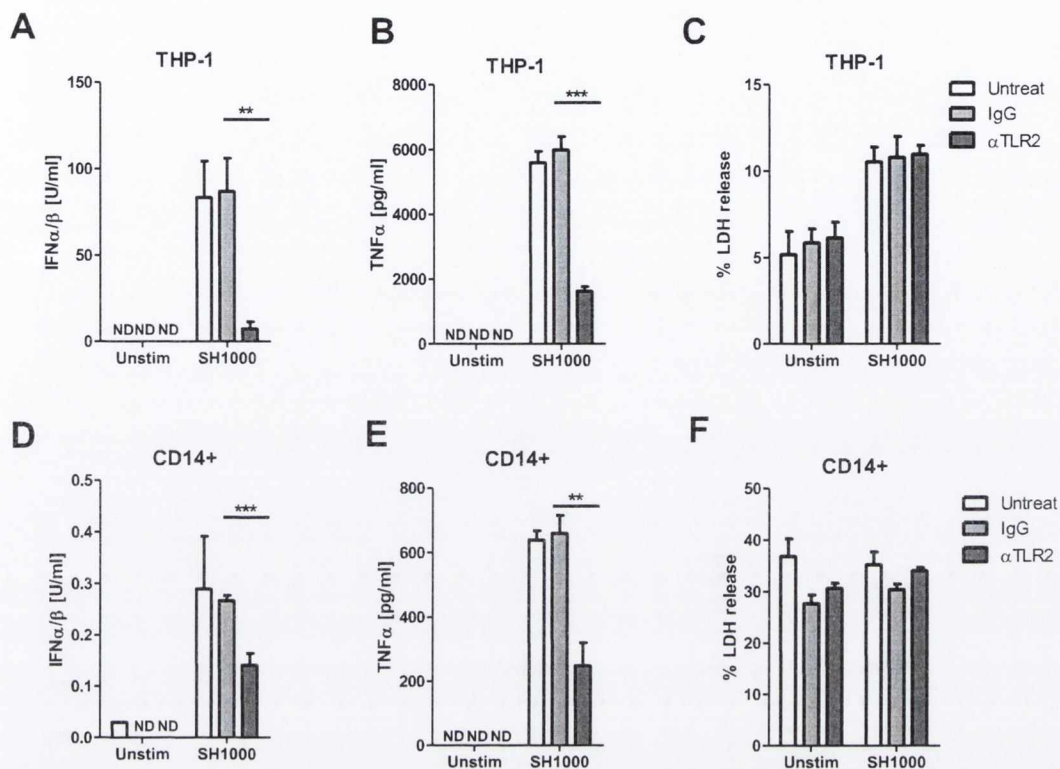


Figure 5.3: TLR2 is required for *S. aureus*-induced type I IFN and TNF α responses in human monocytes.

THP-1 cells (A-C) or CD14⁺ monocytes (D-F) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with α TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-C) or an MOI of 1 (D-F) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-C) or 18 hr (D-F) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with isotype control as determined by Student *t* test.

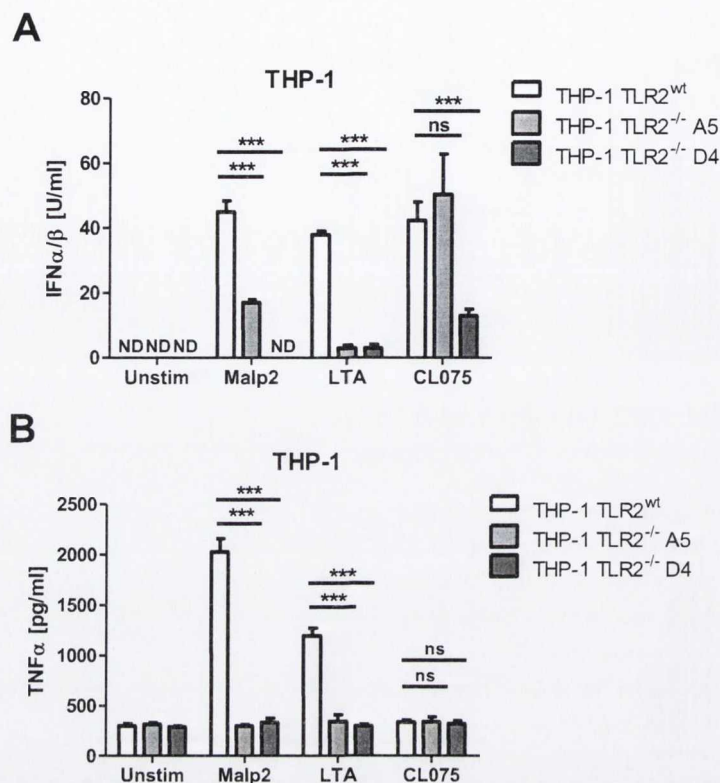


Figure 5.4: Response to TLR2 ligands is impaired in THP-1 TLR2 knockout cells.

Wild type THP-1 cells and THP-1 TLR2 CRISPR knockout cell clones A5 and D4 were seeded at 0.5×10^6 cells/ml in 96-well plate 24 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml), LTA (2.5 μ g/ml), or CL075 (2.5 μ g/ml) for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay, and TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$, when compared with stimulated wild type cells as determined by Student t test.

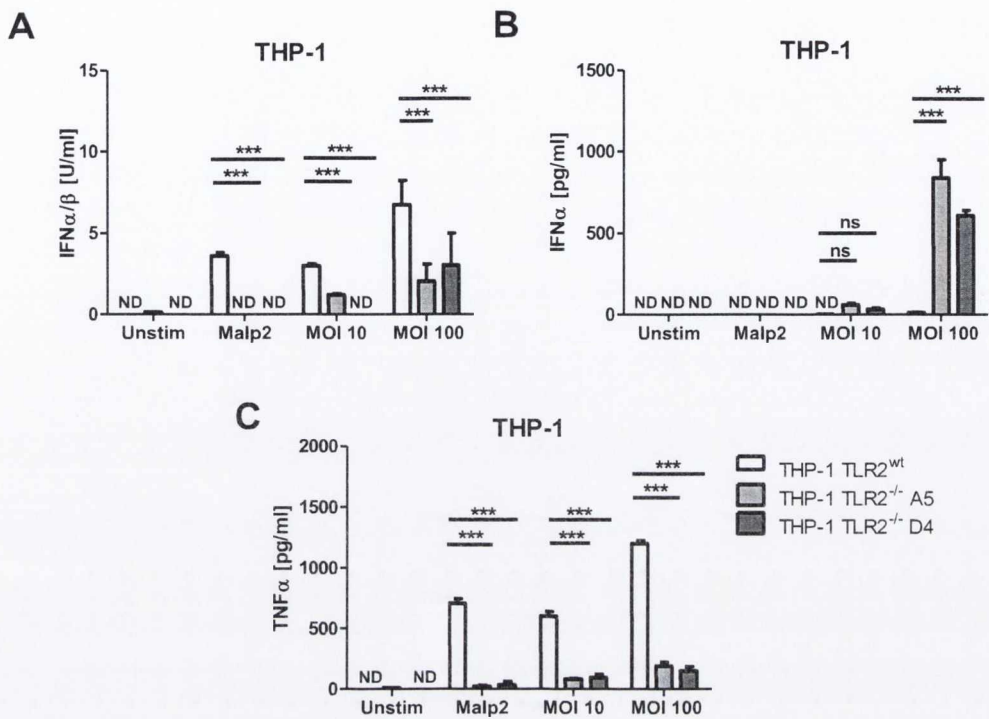


Figure 5.5: Responses to live *S. aureus* in THP-1 TLR2 knockout cells.

Wild type THP-1 cells and THP-1 TLR2 CRISPR knockout cell clones A5 and D4 were seeded at 1×10^6 cells/ml into 24-well plate prior to infection. Cells were stimulated with Malp2 (50 ng/ml) or infected with *S. aureus* strain SH1000 at an MOI of 10 or 100 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; IFN α (B), and TNF α (C) proteins were measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$, when compared with stimulated or infected wild type cells as determined by Student *t* test.

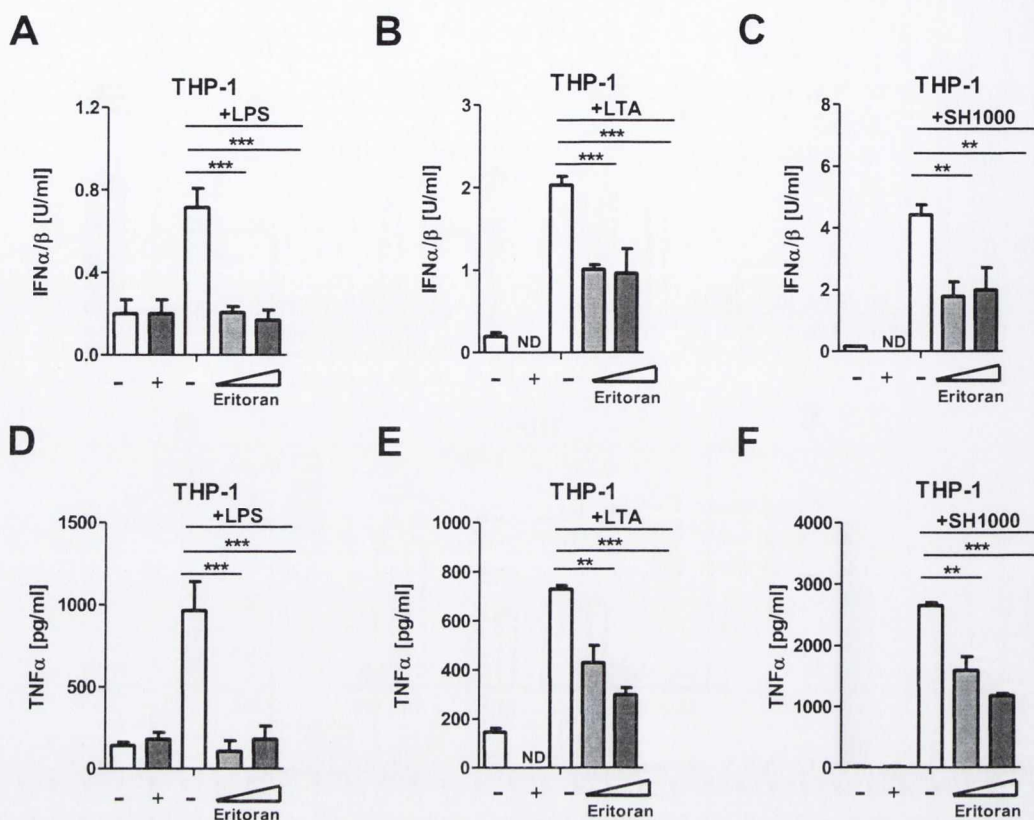


Figure 5.6: CD14 co-receptor is required for *S. aureus*, LTA- and LPS-induced type I IFN and TNFα responses.

THP-1 cells were seeded at 5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation (A-B, D-E) or at 1×10^6 cells/ml into 24-well plate prior to infection (C, F). Cells were pre-treated with increasing concentration of eritoran (2.5/5 μ g/ml) for 1 hr prior to stimulation. Cells were stimulated with LPS (100 ng/ml, A, D), LTA (2.5 μ g/ml, B, E) or infected with *S. aureus* strain SH1000 at an MOI of 100 for 2 hr (C, F) followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFNα/β bioactivity (A-C) was assayed using HEK Blue IFNα/β bioassay; and TNFα (D-E) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected or stimulated untreated cells as determined by Student *t* test.

5.2.2 TLR2-stimulated type I IFNs is dependent on the endosomal pathway

Endosomes are important compartments mediating transport of molecules within cells. Internalized molecules are transported from plasma membrane to lysosomes for their degradation or they are recycled back to the plasma membrane. Professional phagocytes are equipped with multiple strategies for elimination of intracellular microbes, including production of reactive oxygen species (ROS) and nitric oxide (NO). It has been shown that *S. aureus* requires endosomal processing before it can trigger immune responses [195]. Furthermore, signaling from endosomal TLR2 has been reported to stimulate type I IFNs in mouse BMDMs [42]. In addition, we have shown that inhibition of the endosomal pathway leads to enhanced bacterial killing and decreased type I IFN production. Therefore, we examined an effect of inhibitors of the endosomal pathway on TLR2-induced cytokine production in human monocytes [Fig. 5.7].

First, an effect of dynasore, an inhibitor of dynamin, on bacterial-induced immune activation was tested. It has been shown that treatment with dynasore leads to accumulation of U-shaped and O-shaped vesicles attached to the membrane [Fig. 5.7 A; 244]. Treatment with dynasore impaired LTA- and *S. aureus*-stimulated IFN α/β bioactivity in a dose-dependent manner when compared to DMSO-treated THP-1 monocytes [Fig 5.8 A, D]. No effect of dynasore treatment on TNF α production was observed when compared to DMSO-treated THP-1 monocytes [Fig 5.8 B, E]. However, dynasore treatment had adverse effects on cell viability even at the lowest concentrations when compared to DMSO treated THP-1 monocytes [Fig 5.8 C, F]. This data suggests for the first time that TLR2-mediated induction of type I IFNs is dependent on the endosomal pathway in THP-1 monocytes.

Next, the involvement of spleen tyrosine kinase (Syk) in TLR2-mediated type I IFNs was investigated. The non-receptor Syk kinase has been shown to regulate TLR4 endocytosis [245] as well as to modulate signaling by inhibiting MyD88-dependent pathways [256]. This implicates that Syk kinase can function at multiple steps of TLR signaling, thus being a promising target for modulation of TLR signaling. Hence, the effect of Syk kinase inhibitor piceatannol was examined on TLR2-stimulated responses. Treatment with piceatannol dose-dependently reduced LTA-stimulated IFN α/β bioactivity, while TNF α production remained unchanged when compared to DMSO-treated THP-1 monocytes [Fig 5.9 A-B]. Similarly to THP-1 monocytes, LTA-induced IFN α/β bioactivity was impaired in CD14 $^{+}$ monocytes, whereas TNF α induction was not affected after piceatannol treatment when compared to DMSO-treated monocytes [Fig 5.9 D-E]. However, treatment with piceatannol had toxic effects on cell

viability in both THP-1 and CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig 5.9 C, F]. In contrast to LTA, Malp2-induced IFN α / β bioactivity was not inhibited after piceatannol treatment, while Malp2-stimulated TNF α induction remained unaffected when compared to DMSO-treated THP-1 monocytes [Fig 5.10 A-C]. This suggests that differences between LTA and Malp2 signaling exist. Importantly, IFN α / β bioactivity was reduced in both THP-1 and CD14⁺ monocytes upon *S. aureus* infection after piceatannol treatment when compared to DMSO-treated monocytes [Fig 5.11 A, D]. Piceatannol treatment did not affect TNF α induction in THP-1 monocytes after *S. aureus* infection; however, impairment of TNF α production was observed in CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig 5.11 B, E]. In the context of infection, treatment with piceatannol had similar effect on cell viability as treatment with DMSO [Fig 5.11 C, F]. Collectively, these results suggest that Syk kinase is involved in type I IFN signaling upon LTA and *S. aureus* challenge in human monocytes. On the other hand, Syk kinase is redundant for proinflammatory response in THP-1 monocytes, whereas it is involved in CD14⁺ monocytes during *S. aureus* infection. Lastly, Syk kinase is redundant for Malp2-induced type I IFNs and proinflammatory cytokines in THP-1 monocytes.

Next, an effect of cytochalasin D on TLR2-mediated immune responses was examined. Cytochalasin D is a fungal toxin that prevents actin monomers from polymerization and inhibits F-actin polymerization, and thus rapidly blocks movement of vesicles within the cell [Fig. 5.7 C]. Treatment with cytochalasin D potently blocked LTA- and *S. aureus*-mediated IFN α / β bioactivity in dose dependent manner when compared to THP-1 monocytes treated with DMSO [Fig 5.12 A, D]. Even though treatment with cytochalasin D had adverse impact on cell viability, no effect of cytochalasin D treatment on TNF α induction was observed when compared to DMSO-treated THP-1 monocytes [Fig 5.12 B-C, E-F]. This result shows that induction of type I IFNs in response to LTA and *S. aureus* challenge requires an intact endosomal pathway.

Previously, it has been shown that the endosomal processing plays a central role in TLR2-induced type I IFNs in HEK293 cells [42]. Bafilomycin A1 is a highly specific inhibitor of the vacuolar H⁺ ATPase [Fig. 5.7 D]. Inhibition of ATPase activity leads to increased pH in phagosomes, thus preventing its fusion with lysosome, which is then required for antigen maturation [195]. Hence, an effect of bafilomycin A1 on TLR2-mediated responses was assessed. In line with our results from previous chapter, bafilomycin A1 treatment impaired LTA-stimulated IFN α / β bioactivity, whereas no effect on TNF α induction was detected when

compared to THP-1 and CD14⁺ monocytes treated with DMSO [Fig 5.13 A-B, D-E]. Furthermore, bafilomycin A1 did not have adverse effects on cell viability when compared to DMSO treated monocytes [Fig 5.13 C, F]. Similarly to LTA, Malp2-induced IFN α / β bioactivity was impaired after bafilomycin A1 treatment without affecting TNF α induction when compared to DMSO treated THP-1 monocytes [data not shown]. In order to explore an effect of bafilomycin A1 during *S. aureus* infection, a time course was performed in both THP-1 and CD14⁺ monocytes. In THP-1 monocytes, bafilomycin A1 potentially blocked *S. aureus*-induced IFN α / β bioactivity throughout the whole experiment when compared to DMSO treated THP-1 monocytes [Fig 5.14 A]. *S. aureus*-mediated TNF α induction was not affected except for 24 hr after infection when TNF α induction was decreased in bafilomycin A1-treated cells when compared to DMSO-treated THP-1 monocytes [Fig 5.14 B]. At the latest time after infection, the combined effects of bafilomycin A1 and *S. aureus* infection proved to be toxic to cells [Fig 5.14 C]. In CD14⁺ monocytes, bafilomycin A1 also inhibited *S. aureus*-induced IFN α / β bioactivity throughout the whole experiment when compared to monocytes treated with DMSO [Fig 5.15 A]. In contrast to THP-1 monocytes, a significant reduction in *S. aureus*-mediated TNF α induction was observed at all times after infection when compared to DMSO-treated CD14⁺ monocytes [Fig 5.15 B]. Furthermore, bafilomycin A1 treatment increased LDH release when compared to DMSO-treated monocytes; however, the initial levels of LDH were already high in untreated CD14⁺ monocytes [Fig 5.15 C]. These findings demonstrate role of the endosomal processing in LTA-mediated type I IFN induction in human monocytes. In addition, these findings show that the endosomal pathway is important for *S. aureus* induced type I IFNs in human monocytes. However, in the terms of proinflammatory responses, striking differences exist between THP-1 and CD14⁺ monocytes, suggesting a greater complexity of signaling pathways in primary monocytes. Generally, inhibitors of the endosomal pathway had adverse effects on cell viability indicating that the endosomal formation is an important process in monocytes and that inhibition of the endosomal pathway is not well tolerated by cells.

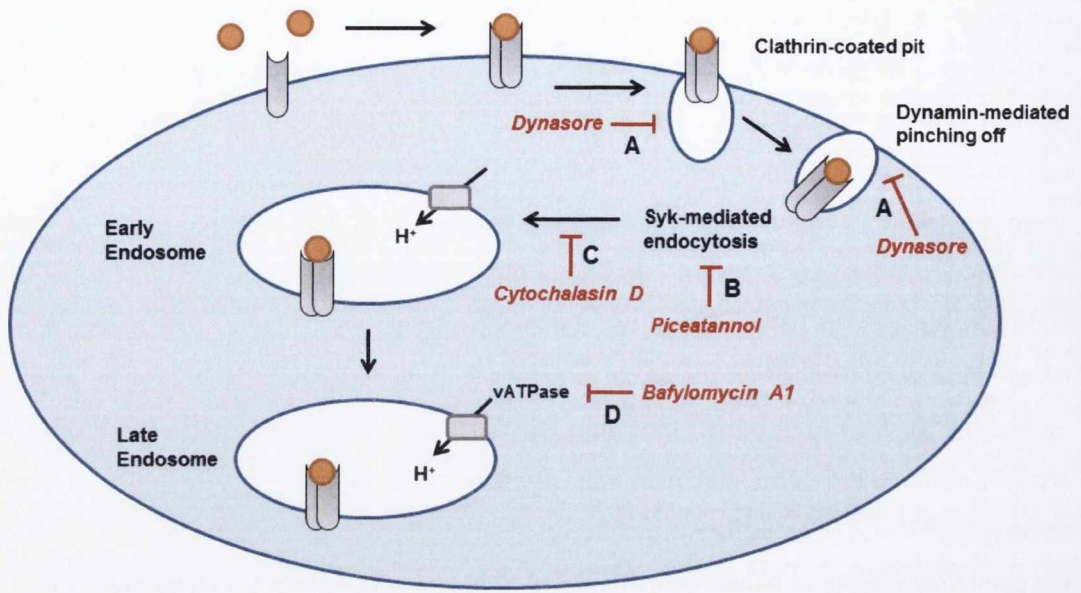


Figure 5.7: Inhibitors of the endosomal pathway and mechanism of their action.

Pathogen is recognized by PRR, induces its dimerization and activation of downstream signaling cascade. In some cases, pathogen-receptor complex is internalized by clathrin-mediated endocytosis, a process that can be inhibited by dynasore. Dynasore blocks formation of vesicles and their scission, leading to accumulation of half and fully formed vesicles (A). Next, endosomes are trafficked into the early endosome. The Syk kinase inhibitor piceatannol blocks Syk-mediated endocytosis (B); and the endocytosis process is further blocked by cytochalasin D, which blocks actin polymerization (C). Endosomes then mature by decreasing their pH within the phagosome, and fuse with lysosomes to mediate degradation of their content. Endosomal acidification can be blocked by bafilomycin A1, which inhibits activity of vacuolar H⁺ ATPase thus endosomal maturation (D).

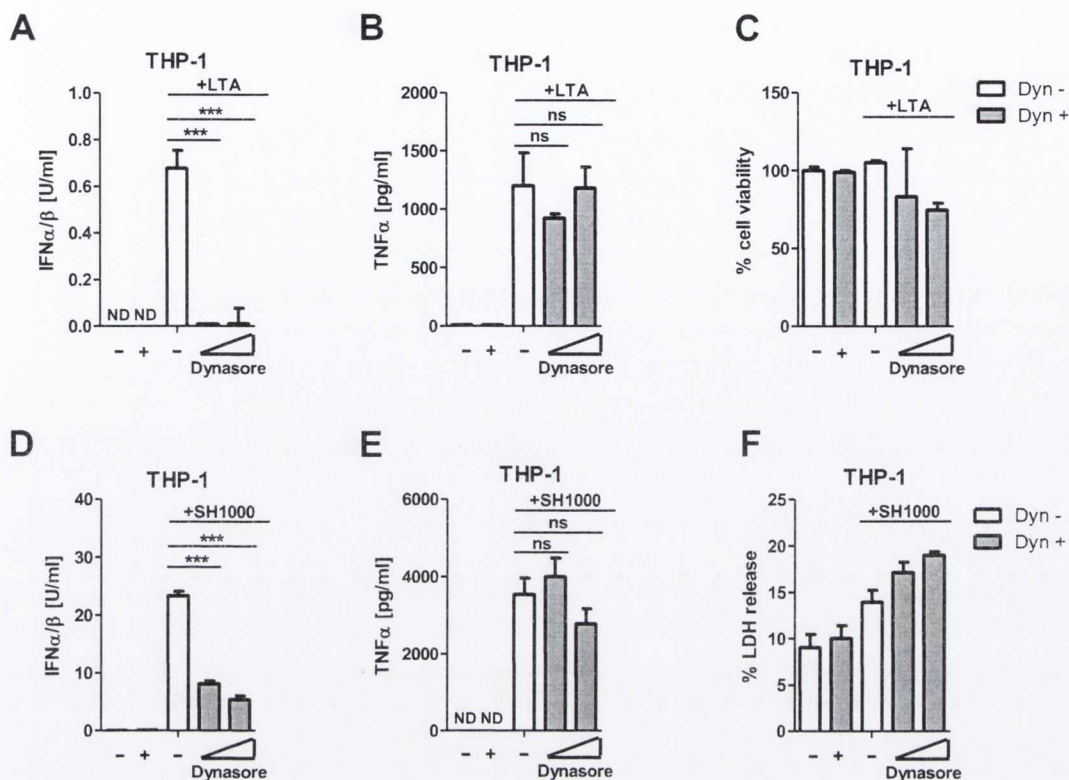


Figure 5.8: Inhibition of dynamin impairs *S. aureus*- and LTA-induced type I IFN induction in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation (A-C) or at 1×10^6 cells/ml into 24-well plate prior infection (D-F). Cells were pre-treated with increasing concentration of dynasore (10/20 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or infected with *S. aureus* strain SH1000 at an MOI of 100 for 2 hr (D-F) followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability was quantified by MTT assay (C) or by LDH cytotoxicity assay (F). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$ compared with stimulated samples treated with DMSO as determined by Student *t* test.

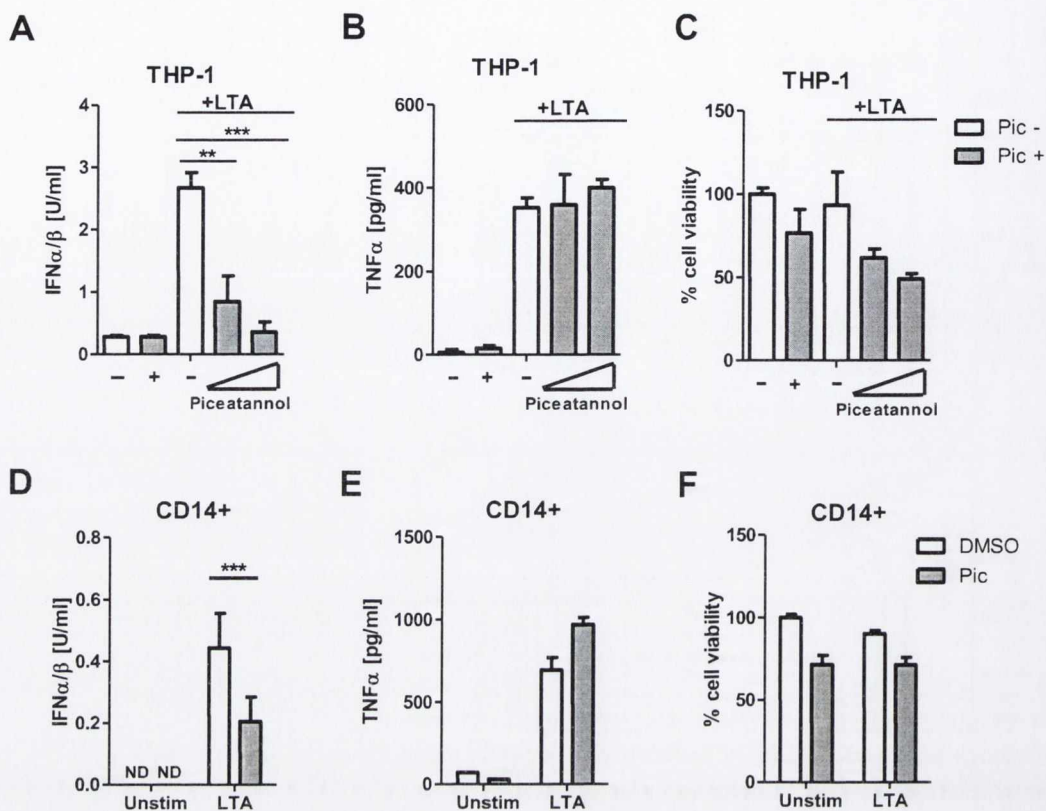


Figure 5.9: Syk kinase is required for LTA-induced type I IFN induction in human monocytes.

THP-1 cells (A-C) or CD14⁺ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with increasing concentration (20/40 μ M, A-C) or single concentration (20 μ M, D-F) of piceatannol or equivalent amount of DMSO 1 hr prior to stimulation. Cells stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability was quantified by MTT assay (C, F). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$ compared with stimulated samples treated with DMSO as determined by Student *t* test.

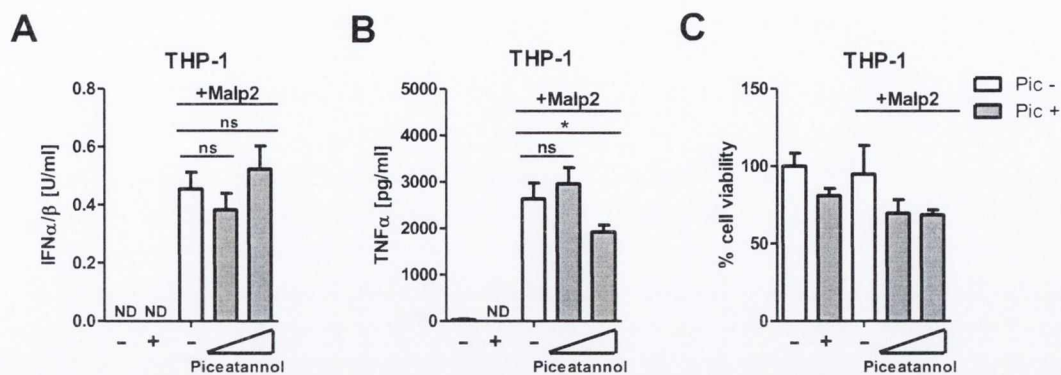


Figure 5.10: Syk kinase is not required for Malp2-induced type I IFN induction in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation (D-F). Cells were pre-treated with increasing concentration of piceatannol (20/40 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml) for 6 hr. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions, and cell viability was quantified by MTT assay (C). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, * $p < 0.05$ compared with stimulated samples treated with DMSO as determined by Student t test.

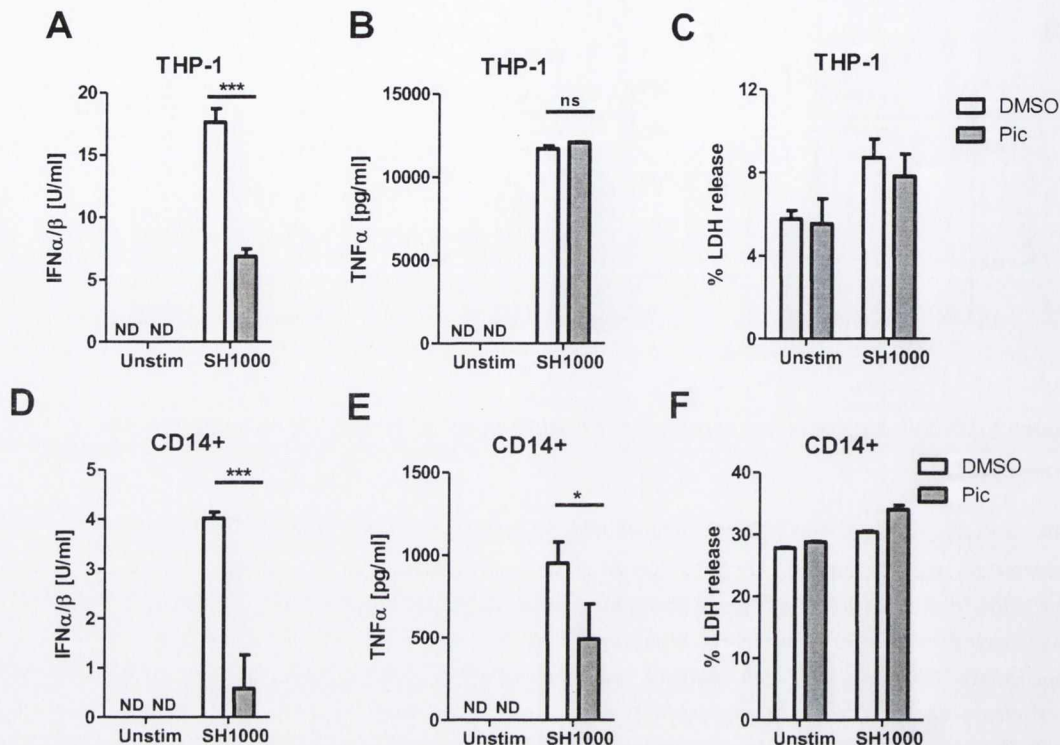


Figure 5.11: *Sy*k kinase is required for *S. aureus*-induced type I IFN induction in human monocytes.

THP-1 cells (A-C) or CD14⁺ monocytes (D-F) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with piceatannol (20 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-C) or at an MOI of 1 (D-F) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-C) or 18 hr (D-F) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, D) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C,F) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, *** $p < 0.001$ when compared with infected samples treated with DMSO as determined by Student *t* test.

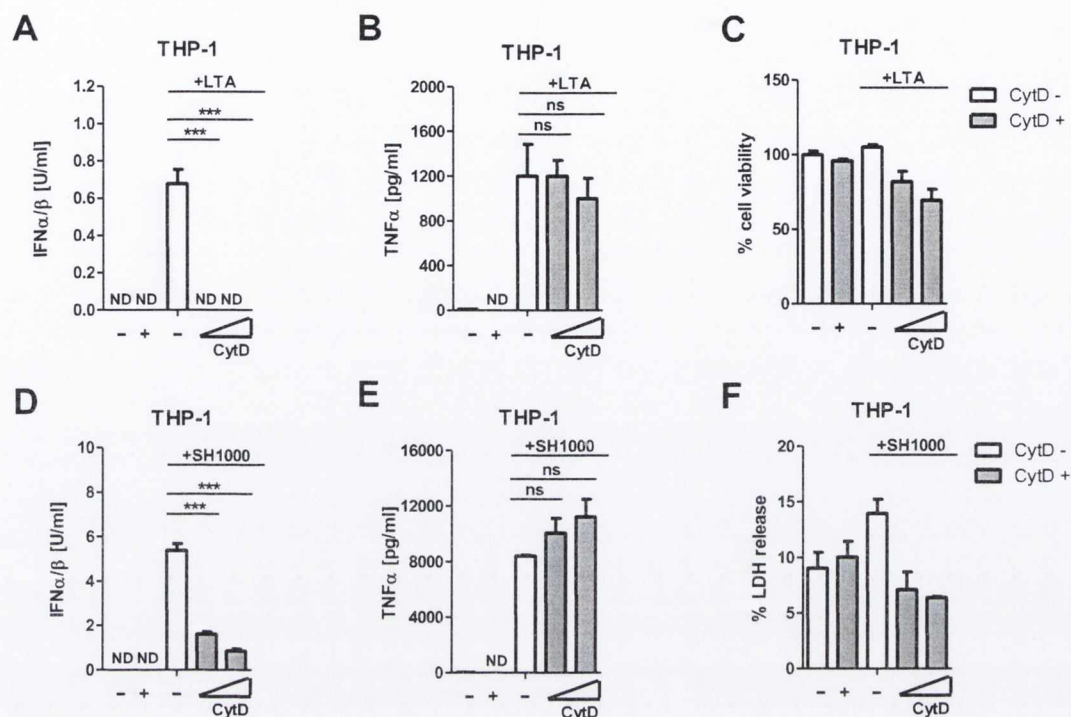


Figure 5.12: Inhibition of actin polymerization impairs *S. aureus*-induced type I IFN induction in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation (A-C) or at 1×10^6 cells/ml into 24-well plate prior infection (D-F). Cells were pre-treated with increasing concentration of cytochalasin D (10/20 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or infected with *S. aureus* strain SH1000 at an MOI of 100 for 2 hr (D-F) followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability was quantified by MTT assay (C) or by LDH cytotoxicity assay (F). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$ compared with stimulated samples treated with DMSO as determined by Student *t* test.

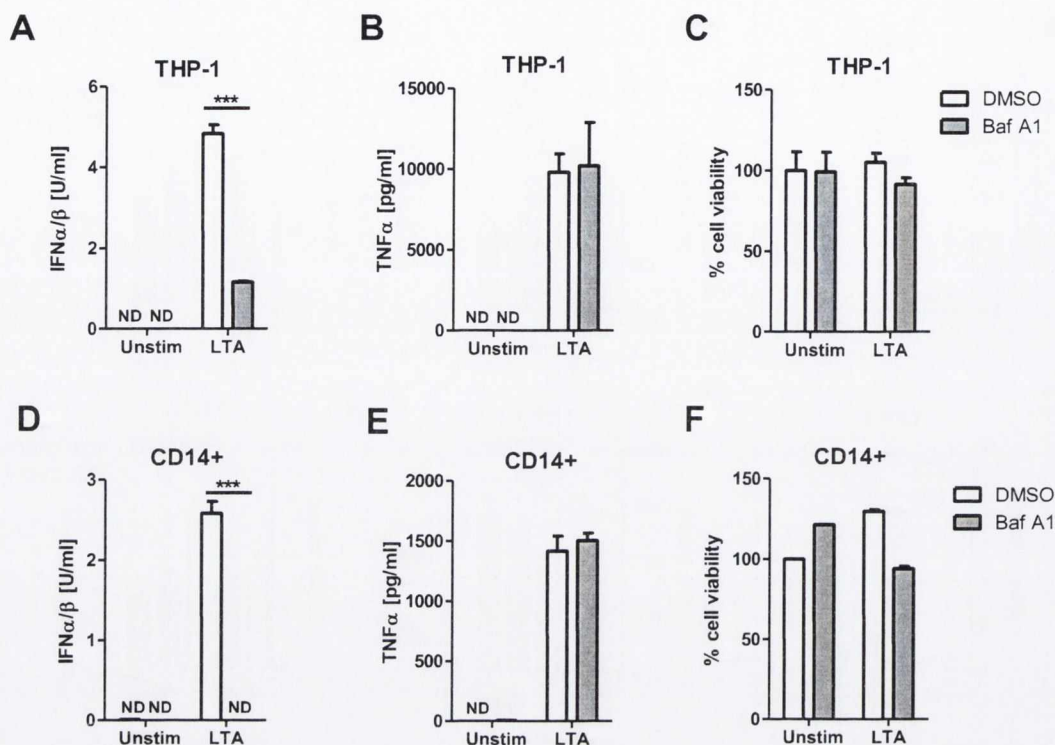


Figure 5.13: Endosomal processing is required for LTA-induced type I IFN induction in human monocytes.

THP-1 cells (A-C) or CD14⁺ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, D) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$ compared with stimulated samples treated with DMSO as determined by Student t test.

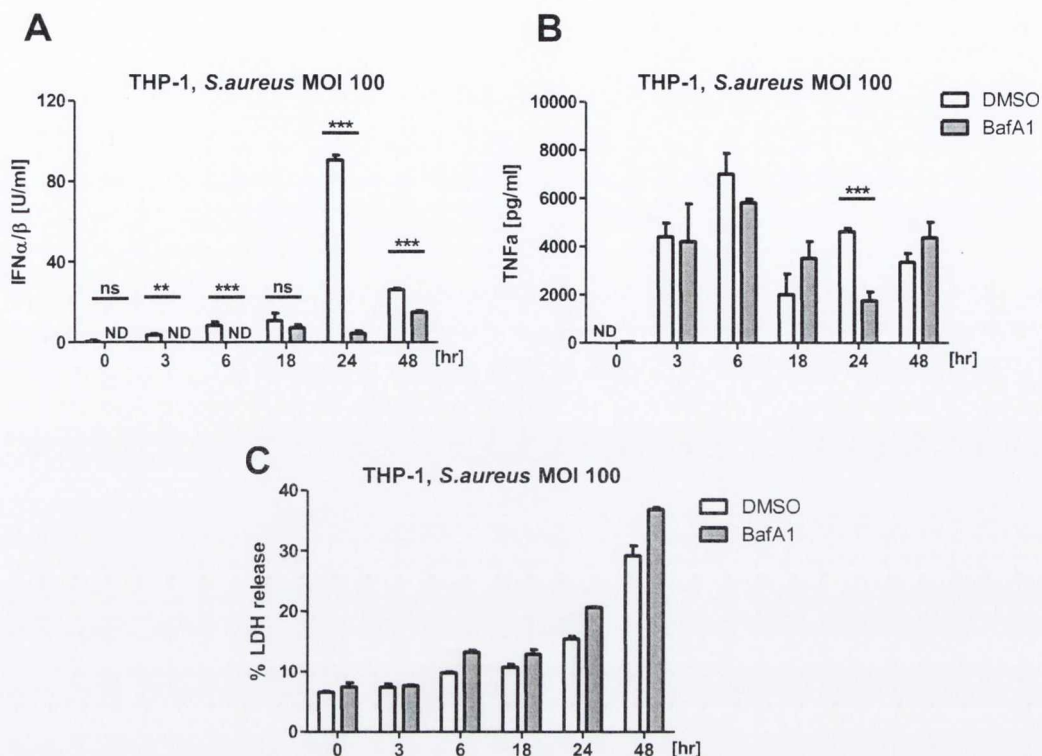


Figure 5.14: Endosomal processing is required for *S. aureus*-induced type I IFN induction in THP-1 monocytes.

THP-1 cells were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 3, 6, 18, 24 and 48 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B) and IL-1 β (C) proteins were measured by standard ELISA with appropriate dilutions, and cell viability (D) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$ compared with infected samples treated with DMSO as determined by Student *t* test.

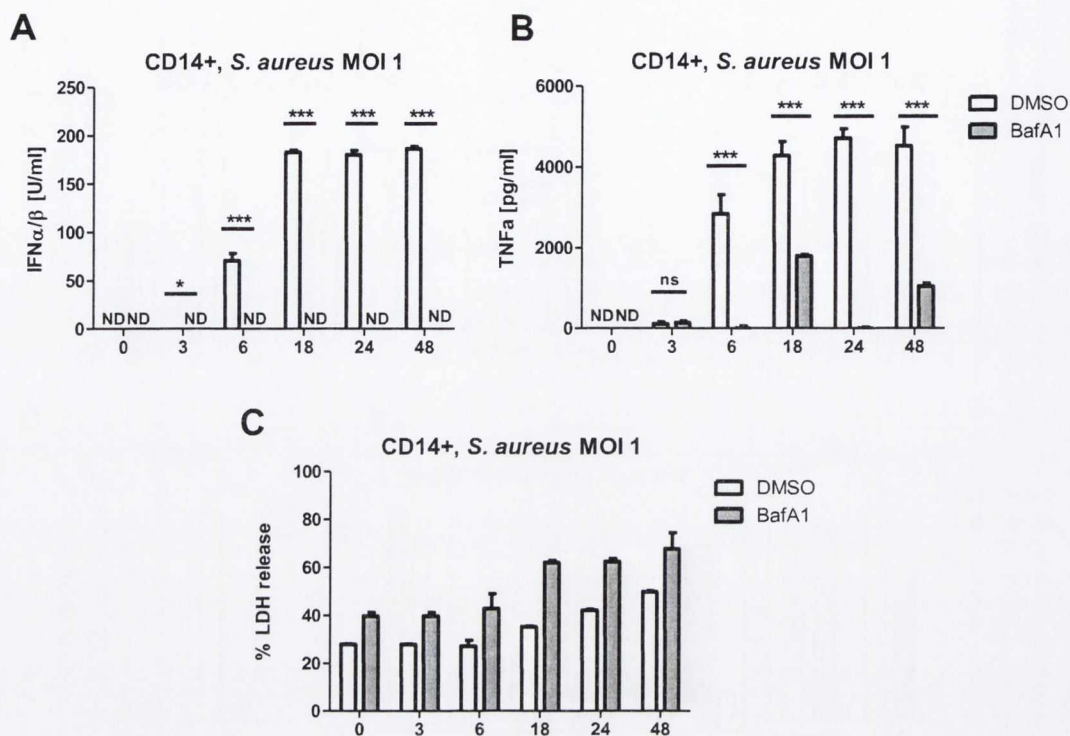


Figure 5.15: Endosomal processing is required for *S. aureus*-induced type I IFN and TNF α induction in CD14+ monocytes.

CD14+ monocytes were seeded at 1×10^6 cells/ml into 24-well plate 24 hr prior to the infection. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 1 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 3, 6, 18, 24 and 48 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B) and IL-1 β (C) proteins were measured by standard ELISA with appropriate dilutions, and cell viability (D) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, *** $p < 0.001$ compared with infected samples treated with DMSO as determined by Student *t* test.

5.2.3 Role of adaptor proteins in TLR2-induced type I IFN pathway

TLR signaling requires adaptor proteins, which mediate signal transduction and activation of downstream signaling cascades. It has been proposed that TLR endosomal signaling is facilitated by sorting adaptors Mal or TRAM, which define the TLR signaling pathway activated from the specific location [44]. Our studies identified a potent inhibitor of Mal and TRAM in TLR4 signaling termed VIPER [257]. VIPER is a viral inhibitory peptide derived from vaccinia virus (VACV) protein A46, which inhibits TLR4 signaling. VIPER was shown to associate with Mal and TRAM but not with MyD88 or TRIF, and thus disrupting receptor:adaptor interactions [258]. Moreover, VIPER was shown to inhibit TLR2-mediated type I IFN induction in HEK293 cells and requirement for Mal and TRAM in TLR2-IFN β pathway was confirmed in mouse BMDMs [42]. In order to examine the requirement for Mal and/or TRAM adaptors in human monocytes during *S. aureus*-mediated type I IFN pathway, an effect of VIPER peptide was tested and compared to an effect of its control peptide CP7.

First, sensitivity of TLR2-mediated responses to VIPER treatment was investigated in THP-1 monocytes and THP-1-derived macrophages. In THP-1-derived macrophages, VIPER treatment significantly impaired LPS-induced IFN α/β bioactivity compared to CP7 treated cells, while no IFN α/β bioactivity was detected in THP-1 monocytes [Fig. 5.16 A, C]. Treatment with VIPER significantly impaired TNF α protein after LPS stimulation in both THP-1-derived macrophages and in THP-1 monocytes when compared to CP7-treated cells [Fig. 5.16 B, D]. These results are consistent with the effects of VIPER treatment on LPS-stimulated TNF α and IFN β induction in mouse BMDMs [42]. Importantly, this result confirms the efficacy of VIPER treatment in THP-1 cells. Surprisingly, the sensitivity of IFN α/β bioactivity to VIPER treatment significantly varied between TLR2 stimuli in THP-1 monocytes. Whereas no effect of VIPER treatment was observed on Malp2-induced IFN α/β bioactivity, significant impairment was detected upon LTA stimulation when compared to CP7-treated THP-1 monocytes [Fig. 5.16 A]. LTA-induced TNF α production was also significantly impaired after VIPER treatment, while no effect of VIPER treatment was observed on Malp2-stimulated TNF α induction when compared to THP-1 monocytes treated with CP7 [Fig. 5.16 B]. No induction of IFN α/β bioactivity was detected upon TLR2 stimuli in THP-1-derived macrophages [Fig. 5.16 C]. Treatment with VIPER significantly impaired Malp2-induced TNF α protein in THP-1-derived macrophages, while no effect on TNF α induction was observed upon LTA stimulation when compared to CP7-treated THP-1-derived macrophages [Fig. 5.16 D]. These results suggest that whereas Malp2-induced type I IFNs and TNF α protein require adaptor proteins other than Mal or TRAM, LTA signaling involves Mal and/or TRAM adaptors in both type I IFN and TNF α

induction. These findings also demonstrate that differentiation of THP-1 cells alters TLR2 pathways, whereas TLR4 signaling remains unaffected.

Next, the impact of VIPER treatment on type I IFNs and TNF α protein was tested in CD14⁺ monocytes. Interestingly, LTA-induced IFN α / β bioactivity and TNF α protein were significantly inhibited after VIPER treatment when compared to CP7-treated CD14⁺ monocytes [Fig. 5.17 A-B]. However, VIPER treatment did not affect Malp2-stimulated IFN α / β bioactivity or TNF α protein when compared to CP7-treated CD14⁺ monocytes [Fig. 5.17 D-E]. Neither VIPER nor CP7 treatment had negative effect on cell viability when compared to untreated CD14⁺ monocytes [Fig. 5.17 C, F]. These findings suggest that considerable differences between LTA and Malp2 signaling exist also in human monocytes.

Importantly, the effect of VIPER treatment on IFN α / β bioactivity and TNF α induction was assessed upon *S. aureus* infection in THP-1 and CD14⁺ monocytes. Treatment with VIPER significantly impaired *S. aureus*-induced IFN α / β bioactivity and TNF α induction when compared to CP7-treated monocytes [Fig. 5.18 A-D]. This result suggests that Mal and/or TRAM are required for induction of type I IFN and TNF α signaling during *S. aureus* infection in human monocytes. Previously, we demonstrated that LTA and Malp2 responses differed after VIPER treatment; however, LTA- and *S. aureus*-induced responses were identical after VIPER treatment in human monocytes. This reveals that LTA, but not Malp2, model the TLR2-dependent responses to *S. aureus*.

Since VIPER interacts with both Mal and TRAM, the requirement for a single adaptor protein remained ambiguous. Therefore, small interfering RNA (siRNA) against Mal and TRAM were employed in THP-1 monocytes. Notably, silencing of Mal adaptor protein impaired LTA-, Malp2-, LPS- and *S. aureus*-stimulated IFN α / β bioactivity when compared to THP-1 cells transfected with negative siRNA [Fig. 5.19 A]. Malp2-induced TNF α protein was not affected by Mal silencing, while silencing of Mal reduced LTA-, LPS- and *S. aureus*-stimulated TNF α induction when compared to THP-1 cells transfected with negative siRNA [Fig. 5.19 B]. Silencing of TRAM did not affect LTA- or *S. aureus*-induced IFN α / β bioactivity; however, Malp2- and LPS-stimulated IFN α / β bioactivity was impaired after TRAM silencing when compared to THP-1 cells transfected with negative siRNA [Fig. 5.19 D]. In addition, silencing of TRAM did not affect TNF α induction upon all stimuli when compared to THP-1 cells transfected with negative siRNA [Fig. 5.19 E]. The degree of Mal and TRAM silencing was confirmed by assessing mRNA expression levels and it was consistent throughout the experiments [Fig. 5.19 C, F]. Unfortunately, attempts for RNA silencing in CD14⁺ were not

successful [data not shown]. However, regarding the similar effects of VIPER in THP-1 and CD14⁺ monocytes, these results suggest that Mal plays a key role in type I IFN and TNF α signaling upon LTA and *S. aureus* infection.

Recently, both TRAM and TRIF adaptor proteins have been implicated in TLR2-mediated RANTES and IFN β induction in mouse BMDMs [43]. TRIF is typically involved in TLR3 and TLR4 signaling from endosomes; however, its role in TLR2 signaling is not yet described. Hence, an effect of TRIF silencing on TLR2-mediated immune response was assessed in THP-1 monocytes. Surprisingly, silencing of TRIF impaired LTA- and Malp2-induced IFN α/β bioactivity, while TNF α induction remained unaffected when compared to THP-1 cells transfected with negative siRNA [Fig. 5.20 A-B]. Silencing of TRIF was confirmed by assessing protein level by immunoblot and it was consistent throughout the experiments [Fig. 5.20 C]. This result suggests that TRIF might play a partial role in the induction of type I IFNs in response to Malp2 and LTA.

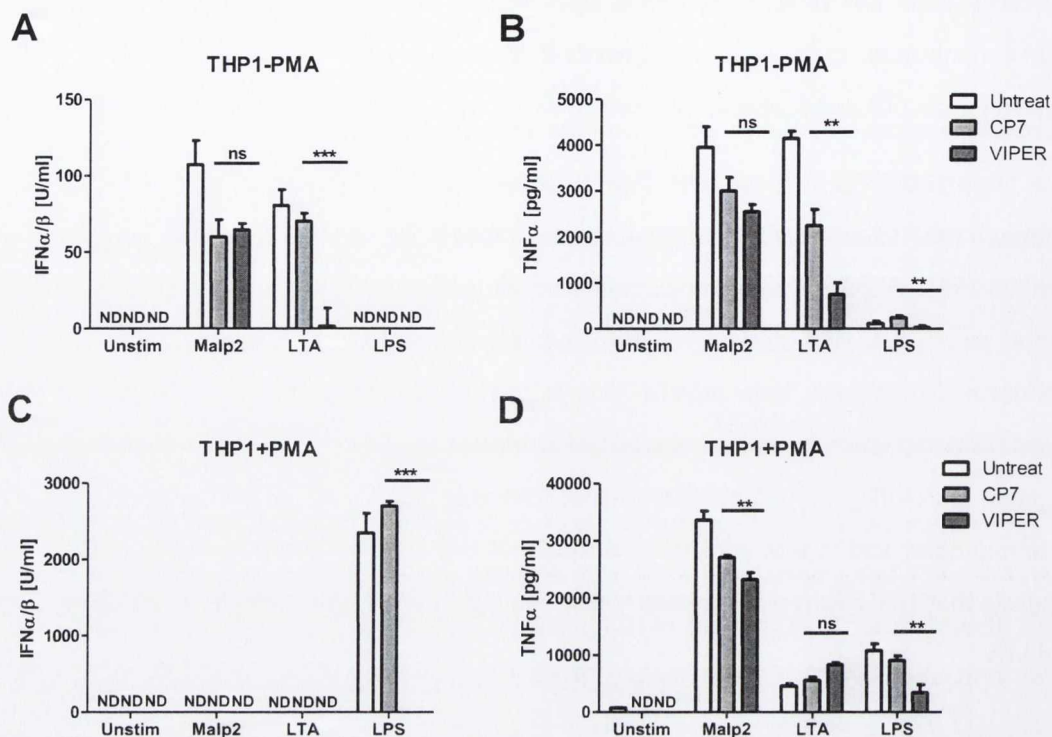


Figure 5.16: VIPER impairs LTA-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation and were differentiated with PMA (100 nM, C, D) or left untreated (A, B). Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) or left untreated 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml), LTA (2.5 μ g/ml), and LPS (100 ng/ml) for 6 hr. Supernatants were harvest by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, C) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, D) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$, *** $p < 0.001$ compared with stimulated samples treated with control peptide CP7 as determined by Student t test.

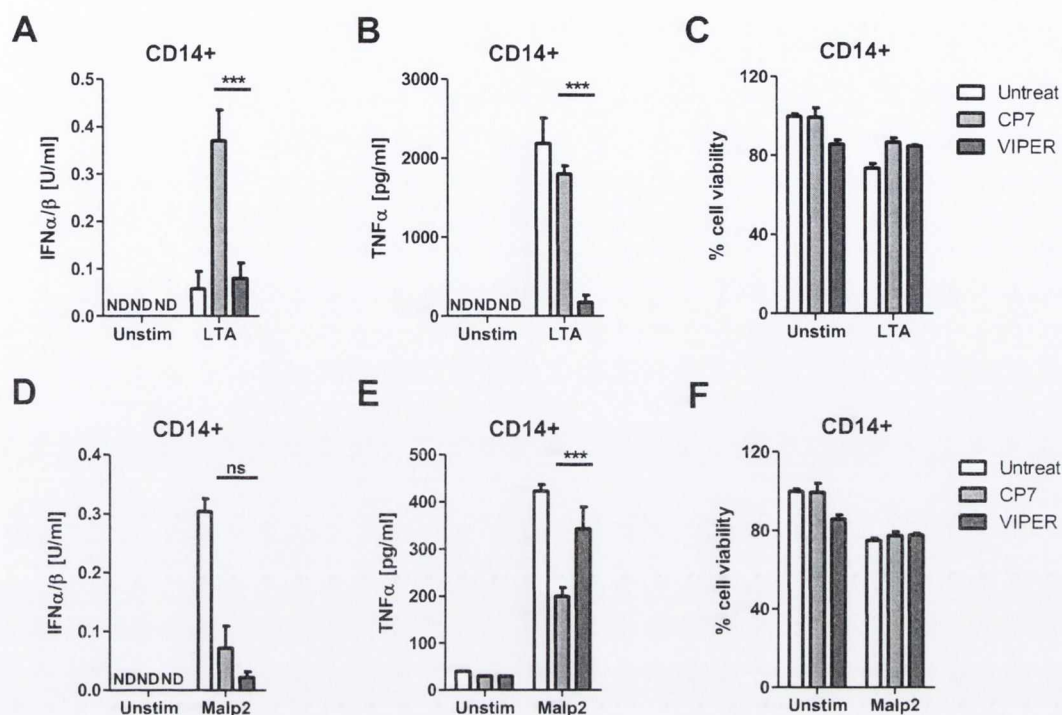


Figure 5.17: VIPER impairs LTA-induced type I IFN and TNF α responses in CD14+ monocytes.

CD14+ monocytes were seeded at 0.8×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) or left untreated 1 hr prior to infection. Cells were stimulated with LTA (2.5 μ g/ml, A-C) or Malp2 (50 ng/ml, D-F) for 18 hr. Supernatants were harvest by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions. Cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$ *** $p < 0.001$, when compared with stimulated samples treated with CP7 as determined by Student t test.

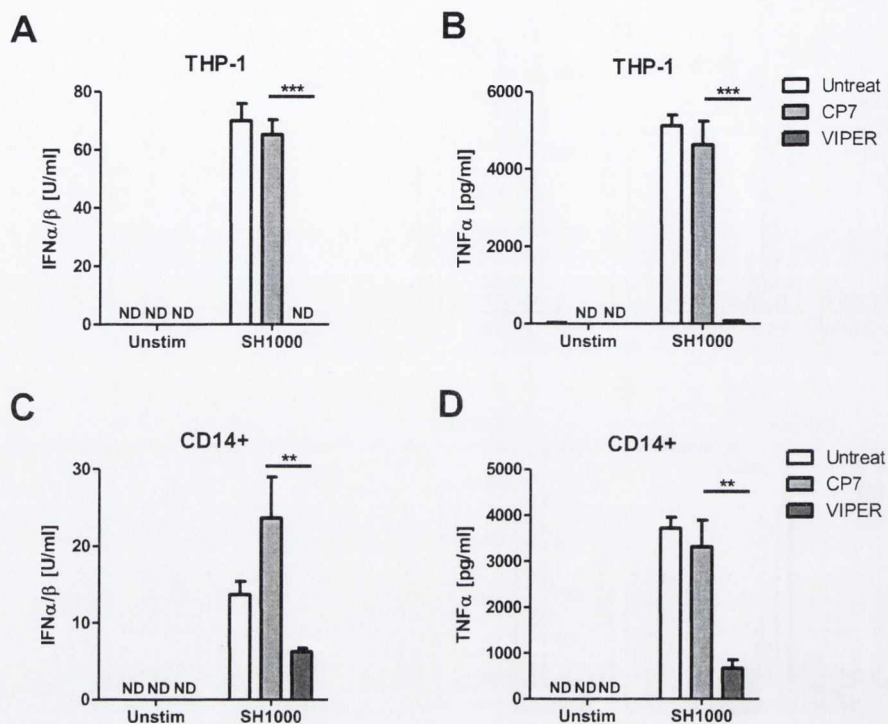


Figure 5.18: *S. aureus*-induced type I IFN and TNF α responses are VIPER sensitive in human monocytes.

THP-1 cells (A-B) or CD14⁺ monocytes (C-D) were seeded at 1×10^6 cells/ml into 24-well plate prior to infection. Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) 1 hr prior to infection or left untreated. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-B) or at an MOI of 1 (C-D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-B) or 18 hr (C-D) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, C) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, D) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$ *** $p < 0.001$, when compared with infected samples treated with CP7 as determined by Student *t* test.

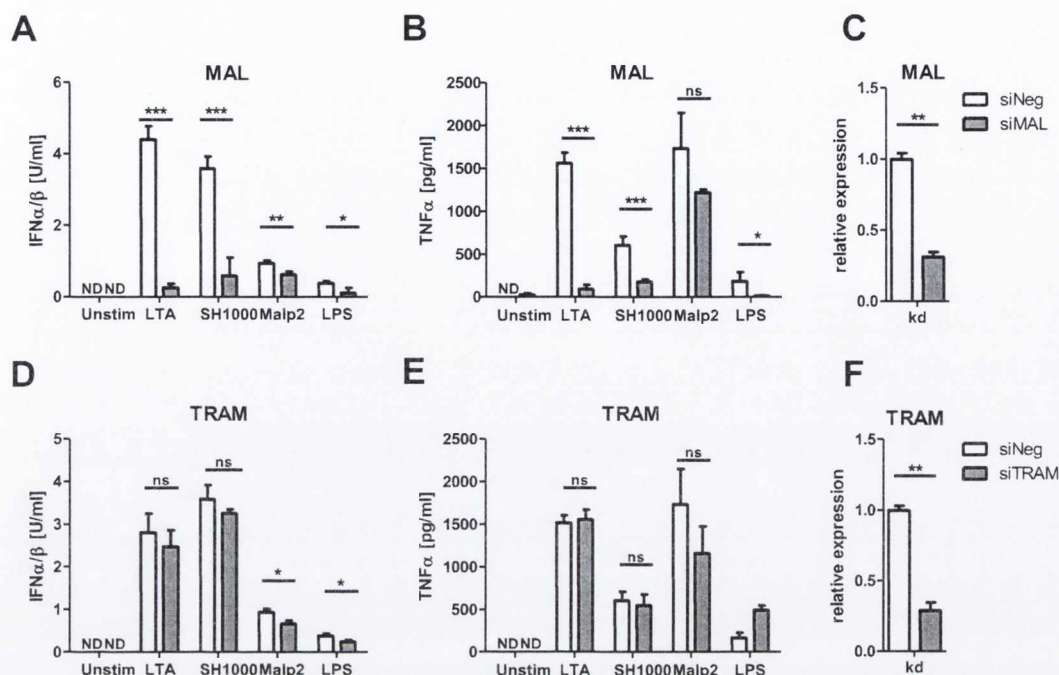


Figure 5.19: Mal is required for LTA- and *S. aureus*-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with MAL (100 pmol, A-C), TRAM (200 pmol, D-F) alongside with negative SMARTpool siRNA (200 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr of incubation, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml), LPS (100 ng/ml) or infected with *S. aureus* strain SH1000 at an MOI of 10 for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, D) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions. Knockdown (C, F) was confirmed by qRT-PCR using gene specific primers. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, kd - knockdown, ns - not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with stimulated samples transfected with negative siRNA as determined by Student *t* test.

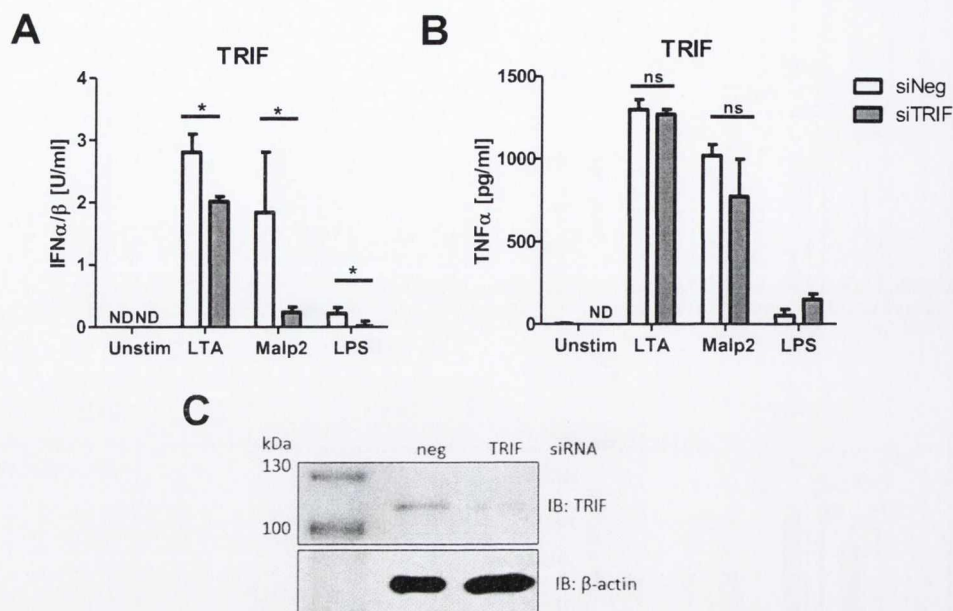


Figure 5.20: TRIF is required for Malp2-induced type I IFN in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with TRIF (100 pmol) alongside with negative SMARTpool siRNA (100 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr of incubation, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml) or LPS (100 ng/ml). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Knockdown was confirmed by immunoblotting using TRIF specific antibody (C). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, *** $p < 0.001$, when compared with stimulated samples transfected with negative siRNA as determined by Student t test.

5.2.4 Role of myddosome in TLR2-induced type I IFN pathway

The myddosome is a signaling protein complex that plays a key role in transmission of innate immune signals from receptors to downstream signaling molecules. The myddosome is composed of six molecules of MyD88, four molecules of IRAK4 and four IRAK1 or IRAK2 molecules. Upon activation of a TIR-domain containing receptor complex, the complex is self-assembled, facilitating IRAKs phosphorylation and activation of downstream molecules such as TRAF6 [57]. Furthermore, it has been reported that patients deficient in MyD88 and IRAK4 are susceptible to infections caused by Gram-positive bacteria including *S. aureus* [187, 188]. Hence, the involvement of myddosome components in TLR2-mediated type I IFNs was investigated in human monocytes using RNA silencing and an inhibitor of IRAKs kinase activity.

First, the roles of MyD88, a key component of the myddosome, and IRAK2 were tested using a siRNA approach. Silencing of MyD88 impaired LTA- and LPS-stimulated IFN α/β bioactivity and TNF α induction, while no effect on Malp2-induced responses was observed after MyD88 silencing when compared to THP-1 cells transfected with negative siRNA [Fig. 5.21 A-B]. Silencing of IRAK2 impaired LTA- but not Malp2- or LPS-stimulated IFN α/β bioactivity when compared to THP-1 cells transfected with negative siRNA [Fig. 5.22 A]. TNF α induction was decreased upon LTA and LPS but not Malp2 stimulation after IRAK2 silencing when compared to THP-1 cells transfected with negative siRNA [Fig. 5.22 B]. The degree of MyD88 and IRAK2 silencing was confirmed by assessing mRNA expression levels and was consistent throughout the experiments [Fig. 5.21 C, 5.22 C]. This result suggests that LTA uses a conventional MyD88-dependent pathway in type I IFN and TNF α responses, whereas Malp2 might be using a yet unknown pathway in THP-1 monocytes. This result also suggests the involvement of IRAK2 in LTA-stimulated IFN α/β bioactivity and TNF α production, while IRAK2 is redundant for Malp2-induced responses in THP-1 monocytes.

Next, a role of IRAK1 and IRAK4 in TLR2-induced signaling was tested in human monocytes using an inhibitor, which blocks both IRAK1 and IRAK4 kinase activities since an inhibitor of a single IRAK kinase is not available. Treatment with IRAK-1/4 inhibitor significantly decreased LTA-stimulated IFN α/β bioactivity and TNF α induction in a dose-dependent manner when compared to DMSO-treated THP-1 monocytes [Fig. 5.23 A-B]. Similarly, LTA-stimulated IFN α/β bioactivity and TNF α induction were impaired after IRAK-1/4 inhibitor treatment in CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig. 5.23 D-E]. Treatment with IRAK-1/4 inhibitor did not have adverse effects on cell viability when compared to DMSO- treated monocytes [Fig. 5.23 C, F]. Surprisingly, Malp2-induced

IFN α / β bioactivity and TNF α induction were impaired after treatment with the highest concentration of IRAK-1/4 inhibitor when compared to DMSO-treated THP-1 monocytes [Fig. 5.24 A-B]. Treatment with IRAK-1/4 inhibitor did not affect cell viability after Malp2 stimulation when compared to DMSO-treated THP-1 monocytes [Fig. 5.24 C]. Notably, both IFN α / β bioactivity and TNF α induction were significantly impaired after treatment with IRAK-1/4 inhibitor during *S. aureus* infection in THP-1 and CD14⁺ monocytes when compared to monocytes treated with DMSO [Fig. 5.25 A-D]. These results confirm a role for IRAK1 and/or IRAK4 in LTA- and *S. aureus*-mediated type I IFN and TNF α induction. Collectively, these results strongly suggest that the myddosome is involved in LTA- and *S. aureus*-mediated type I IFN and TNF α induction. Furthermore, these results reveal that stimulation with Malp2 triggers an unconventional TLR2 signaling pathway, which is MyD88 and IRAK2-independent but it partially involves IRAK1 and/or IRAK4.

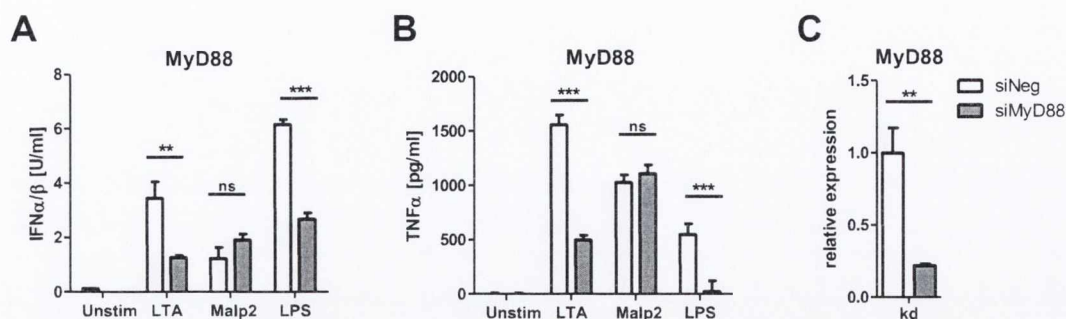


Figure 5.21: MyD88 is required for LTA-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with MyD88 (100 pmol) alongside with negative Qiagen siRNA (100 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr of incubation, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml), LPS (100 ng/ml) or infected with *S. aureus* strain SH1000 at an MOI of 10 for 6 hr. Gentamicin (200 μ g/ml) was added 2 hr after infection. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Knockdown (C) was confirmed by qRT-PCR using MyD88 specific primers. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, kd - knockdown, ns - not significant, **p<0.01, ***p<0.001 when compared with stimulated samples transfected with negative siRNA as determined by Student *t* test.

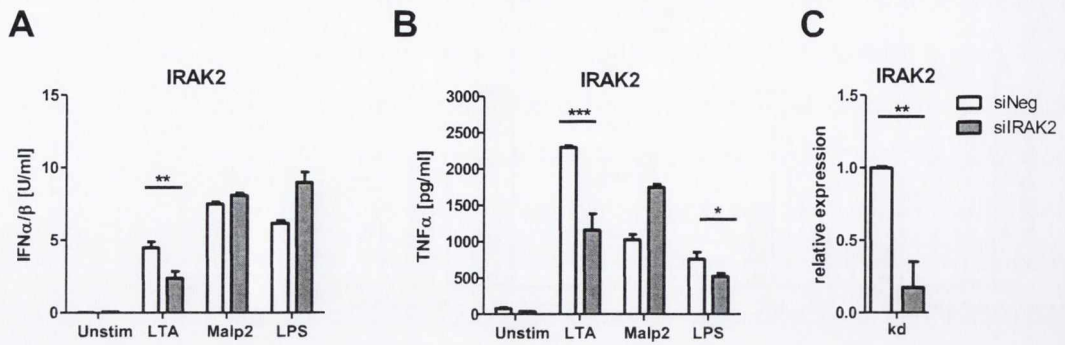


Figure 5.22: IRAK2 is required for LTA-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with IRAK2 (100 pmol) alongside with negative Qiagen siRNA (100 pmol) using NEON[®] electroporation transfection system at setting #15. After 40 hr of incubation, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml), LPS (100 ng/ml) or infected with *S. aureus* strain SH1000 at an MOI of 10 for 6 hr. Gentamicin (200 μ g/ml) was added 2 hr after infection. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Knockdown (C) was confirmed by qRT-PCR using IRAK2 specific primers. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, kd - knockdown, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with stimulated samples transfected with negative siRNA as determined by Student *t* test.

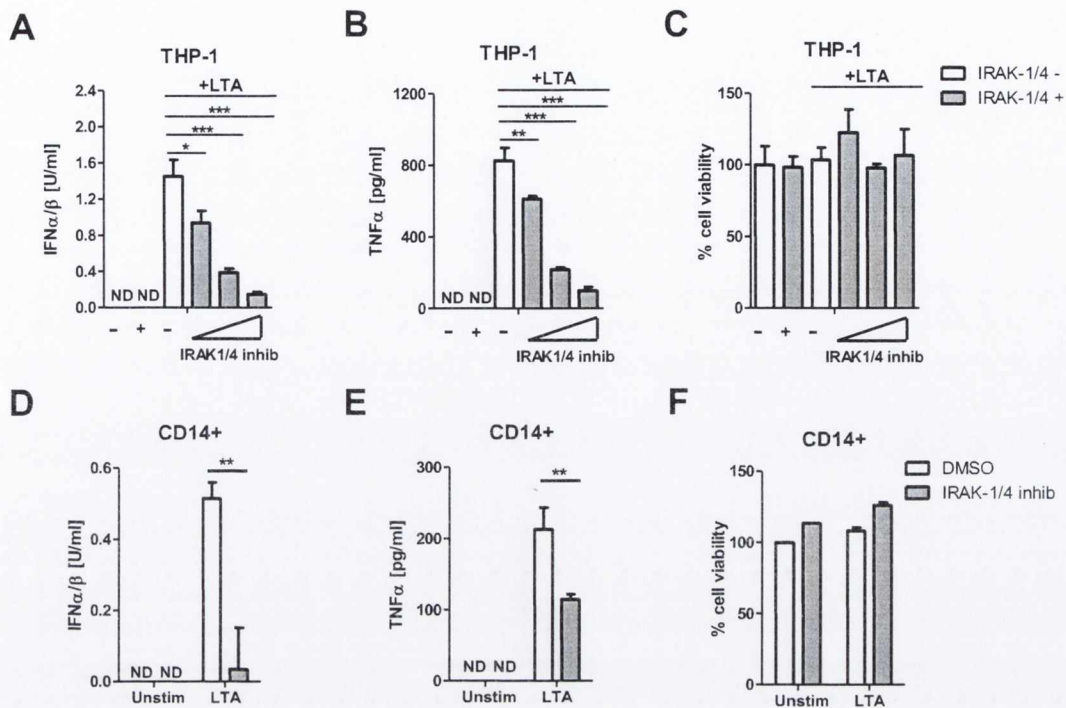


Figure 5.23: IRAK-1/4 is required for LTA-induced type I IFN and TNF α responses in human monocytes.

THP-1 cells (A-C) or CD14 $^{+}$ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation. Cells were pre-treated with increasing concentration (0.1/1/2 μ M, A-C) or single concentration (2 μ M, D-E) of IRAK-1/4 inhibitor or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with stimulated samples treated with DMSO as determined by Student t test.

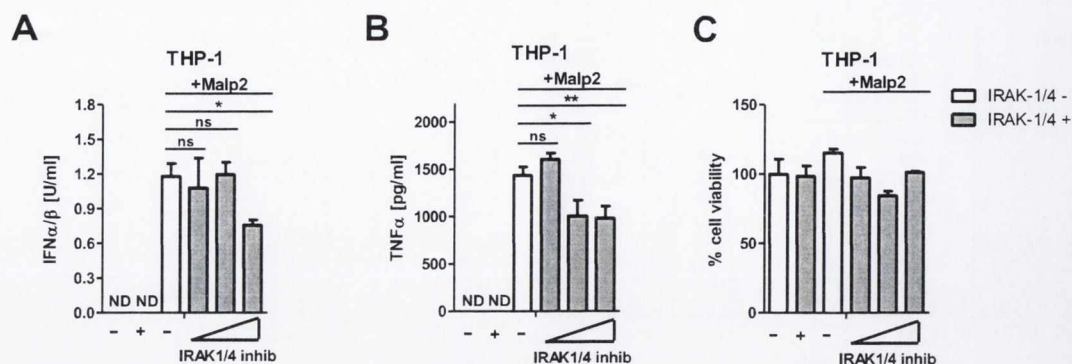


Figure 5.24: IRAK-1/4 is not required for Malp2-induced type I IFN and TNF α responses in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation. Cells were pre-treated with increasing concentration of IRAK-1/4 inhibitor (0.1/1/2 μ M, A-C) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml) for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, ** $p < 0.01$, when compared with stimulated samples treated with DMSO as determined by Student t test.

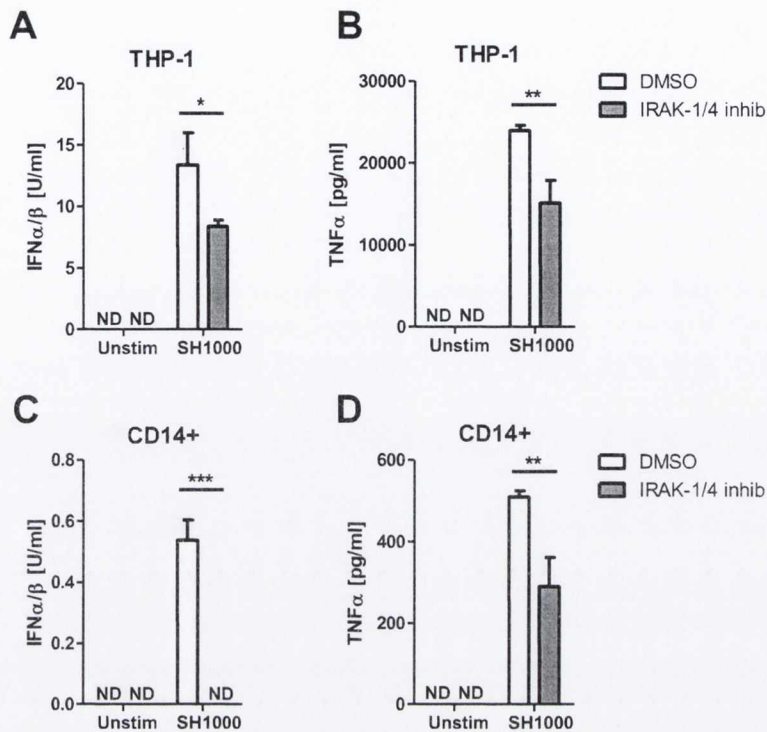


Figure 5.25: IRAK-1/4 is required for and *S. aureus*-induced type I IFN and TNFα responses in human monocytes.

THP-1 cells (A-B) or CD14⁺ monocytes (C-D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with IRAK-1/4 inhibitor (2 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-B) or at an MOI of 1 (C-D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-B) or 18 hr (C-D) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFNα/β bioactivity (A, C) was assayed using HEK Blue IFNα/β bioassay; TNFα (B, D) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with infected samples treated with DMSO as determined by Student *t* test.

5.2.5 TRAF6 and TAK1 are involved in TLR2-stimulated type I IFN induction

Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) were originally discovered as adaptor proteins, which linked TNF receptor to signaling pathways [259]. Only later, they were also associated with innate immune signaling as signal transducers of Toll/interleukin-1 family members. TRAF proteins family members are ubiquitin E3 ligases playing essential roles in activating downstream kinases such as TAK1 or TBK1. It has been shown that TRAF3 activates IRF3 transcription factor, thus inducing type I IFNs [260], while TRAF6 is involved in MyD88 signaling, leading to production of proinflammatory cytokines. In addition, TRAF6 is implicated in type I IFN induction as a part of a MyD88-IKK α complex, where its ubiquitin activity is required for direct IKK α activation [63]. TRAF6 ubiquitin activity induces TAK1-binding proteins (TABs), which recruit transforming growth factor β (TGF β)-activated kinase 1 (TAK1). TAK1 then undergoes conformational changes, leading to induction of its kinase activity and activation of IKK protein complex. IKK complex then activates NF κ B, which induces transcription of proinflammatory cytokines and contributes to IFN β induction [58]. Apart from IKK activation, TAK1 also targets MAP kinases, including ERK, p38 and JNK, in response to signals from multiple immune receptors such as TLRs, TCR, BCR, and TNFR [261]. In addition, TAK1, NF κ B and IKK signaling were implicated in the regulatory T cells development in the thymus [262] and were found indispensable for maintaining the peripheral regulatory T cells [263]. Hence, we sought to examine the roles of TRAF3 and TRAF6 in TLR2-mediated type I IFN response in THP-1 monocytes using siRNA approach. In addition, an effect of an inhibitor of TAK1 activity, (5Z)-7-Oxozeaenol, on TLR2-mediated signaling pathway was examined in human monocytes. (5Z)-7-Oxozeaenol has been shown to inhibit TAK1-mediated JNK and p38 phosphorylation, whereas related compound (5Z)-Zeaenol served as a negative control for TAK1 inhibition studies.

TRAF3 silencing did not have any effect on LTA- or Malp2-stimulated IFN α / β bioactivity when compared to THP-1 cells transfected with negative siRNA [Fig. 5.26 A]. Interestingly, TNF α induction was significantly impaired after Malp2-stimulation in TRAF3 silenced cells, while TRAF3 silencing had no effect on LTA-stimulated TNF α induction when compared to THP-1 cells transfected with negative siRNA [Fig. 5.26 B]. LPS-stimulated IFN α / β bioactivity was significantly decreased in TRAF3 silenced cells, whereas TNF α induction was not affected after LPS stimulation when compared to THP-1 cells transfected with negative siRNA [Fig. 5.26 A-B]. In contrast to TRAF3 silencing, LTA- and Pam3Csk4-induced IFN α / β bioactivity and TNF α protein were impaired after TRAF6 silencing when compared to THP-1 cells transfected with negative siRNA [Fig. 5.26 D-E]. The degree of TRAF3 silencing was

confirmed by assessing mRNA expression levels since the TRAF3-specific antibody was not reliable and TRAF6 silencing was confirmed on protein level by immunoblot. In both cases, silencing was consistent throughout the experiments [Fig. 5.26 C, F]. This finding reveals involvement of TRAF3 in Malp2-induced TNF α induction, which could be a part of the unconventional signaling pathway triggered by Malp2 in THP-1 monocytes. Moreover, these results show the central role of TRAF6 in mediating TLR2 signaling in human monocytes.

STING is a key component of DNA-mediated type I IFNs by facilitating IRF3 activation [97]. Recently, there has been evidence that STING might be also involved in Pam3Csk4-induced IFN β protein induction in mouse BMDMs [264]. Hence, the involvement of STING in TLR2 signaling was investigated in THP-1 monocytes. However, there was no effect on LTA- or Malp2-induced IFN α/β bioactivity and TNF α production after STING silencing when compared to THP-1 cells transfected with negative siRNA [Fig. 5.27 A-B]. IFN α/β bioactivity was significantly impaired after 70mer stimulation in STING silenced cells when compared to THP-1 cells transfected with negative siRNA, thus confirming the functional effects of STING silencing [Fig. 5.27 A]. The degree of STING silencing was confirmed by assessing mRNA expression levels and was consistent throughout the experiments [Fig. 5.27 C]. The result show that STING is not likely involved in LTA or Malp2 signaling in THP-1 monocytes.

Treatment with TAK1 inhibitor significantly inhibited LTA-stimulated IFN α/β bioactivity and TNF α induction in a dose-dependent manner when compared to THP-1 monocytes treated with the TAK1 inhibitor control [Fig. 5.28 A-B]. Moreover, LTA-stimulated IFN α/β bioactivity and TNF α protein were impaired after treatment with TAK1 inhibitor but not with TAK1 inhibitor control in CD14 $^{+}$ monocytes [Fig. 5.28 D-E]. TAK1 inhibitor and TAK1 inhibitor control did not have adverse effects on cell viability when compared to DMSO-treated monocytes [Fig. 5.28 C, F]. Similarly to LTA stimulation, Malp2-induced IFN α/β bioactivity and TNF α induction were inhibited in a dose-dependent manner after TAK1 inhibitor treatment, while it did not affect cell viability when compared to DMSO-treated THP-1 monocytes [Fig. 5.29 A-C]. Importantly, treatment with TAK1 inhibitor significantly impaired IFN α/β bioactivity and TNF α induction upon *S. aureus* infection in THP-1 and CD14 $^{+}$ monocytes when compared to monocytes treated with TAK1 inhibitor control [Fig. 5.30 A-B, D-E]. In the context of infection, TAK1 inhibitor treatment slightly decreased LDH release from both THP-1 and CD14 $^{+}$ monocytes when compared to control treated monocytes [Fig. 5.30 C, F]. These results demonstrate that TAK1 activity plays a key role in the *S. aureus*-stimulated TLR2 signaling in human monocytes.

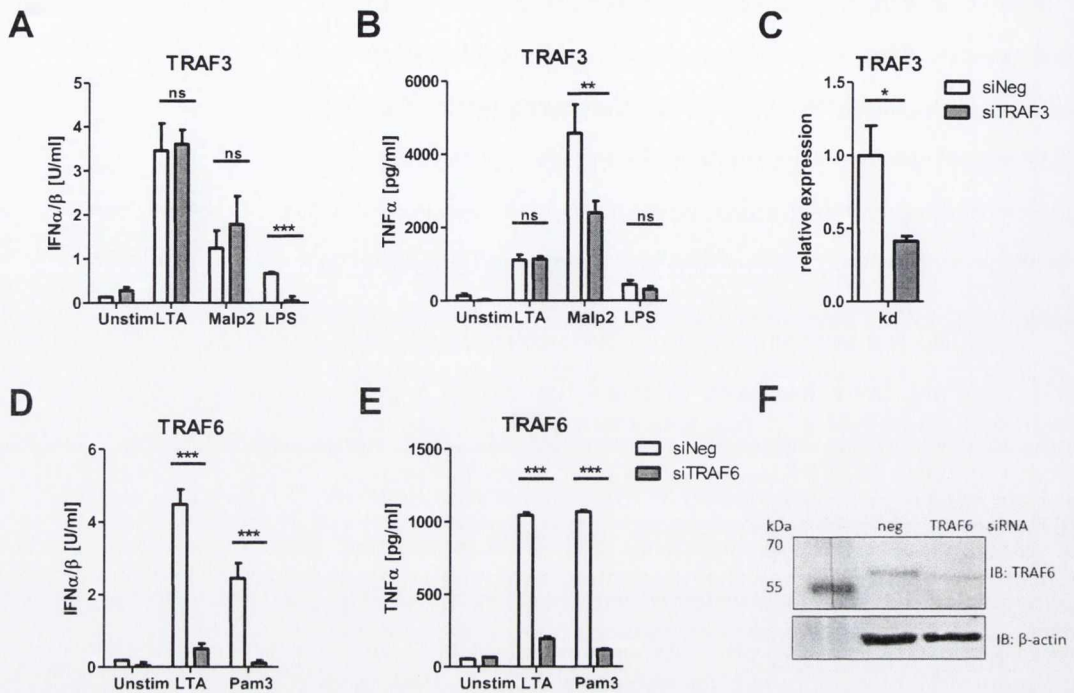


Figure 5.26: TRAF6 is required for LTA-induced type I IFN and TNF α induction in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with TRAF3 (100 pmol, A, B), TRAF6 (100 pmol, D, E) alongside with negative Qiagen siRNA (100 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml), Pam3CSK4 (2.5 μ g/ml) or LPS (100 ng/ml) for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, D) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions. Knockdown was confirmed by qRT-PCR using TRAF3 specific primers (C) or by immunoblotting using TRAF6 specific antibody (F). Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, kd - knockdown, ns - not significant, ** $p < 0.01$, *** $p < 0.001$, when compared with stimulated samples transfected with negative siRNA.

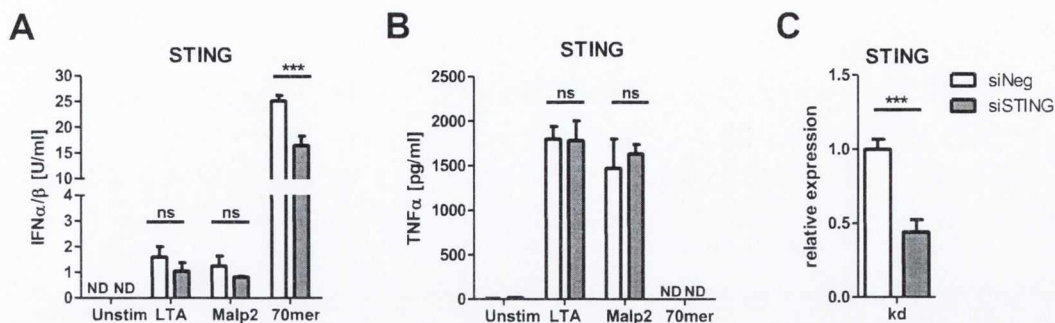


Figure 5.27: STING is not required for TLR2-induced type I IFN and TNF α induction in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with STING (100 pmol) alongside with negative Qiagen siRNA (100 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr, cells were stimulated with LTA (2.5 μ g/ml), Malp2 (50 ng/ml) or transfected with VACV 70mer (1 μ g/ml) using Lipofectamine[®] 2000 for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Knockdown (C) was confirmed by qRT-PCR using STING specific primers. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, kd - knockdown, ns - not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with stimulated samples transfected with negative siRNA.

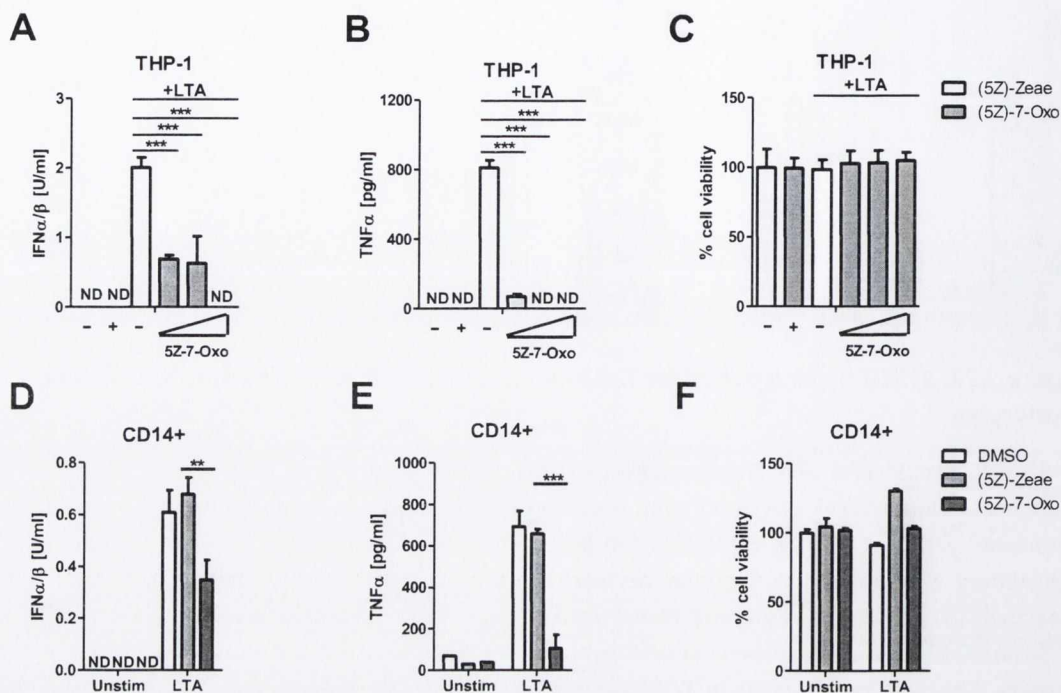


Figure 5.28: TAK1 is required for LTA-induced type I IFN and TNF α induction in human monocytes.

THP-1 cells (A-C) or CD14 $^{+}$ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with increasing concentration (0.1/1/2 μ M, 5Z-7-Oxo, A-C), or single concentration (2 μ M, 5Z-7-Oxo, D-F) of TAK1 inhibitor, single concentration of TAK1 inhibitor control (2 μ M, 5Z-7-Zeae) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A, D) was assayed using HEK Blue IFN α / β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with stimulated samples treated with DMSO.

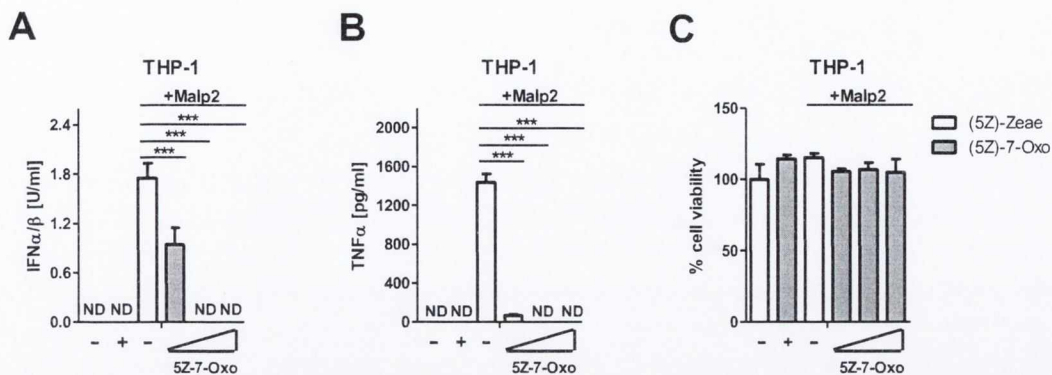


Figure 5.29: TAK1 is required for Malp2-induced type I IFN and TNFα responses in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation. Cells were pre-treated with increasing concentration of TAK1 inhibitor (0.1/1/2 μ M, 5Z-7-Oxo), single concentration of TAK1 inhibitor control (2 μ M, 5Z-7-Zeae) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml) for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFNα/β bioactivity (A) was assayed using HEK Blue IFNα/β bioassay; TNFα (B) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns – not significant, *** $p < 0.001$, when compared with stimulated samples treated with DMSO.

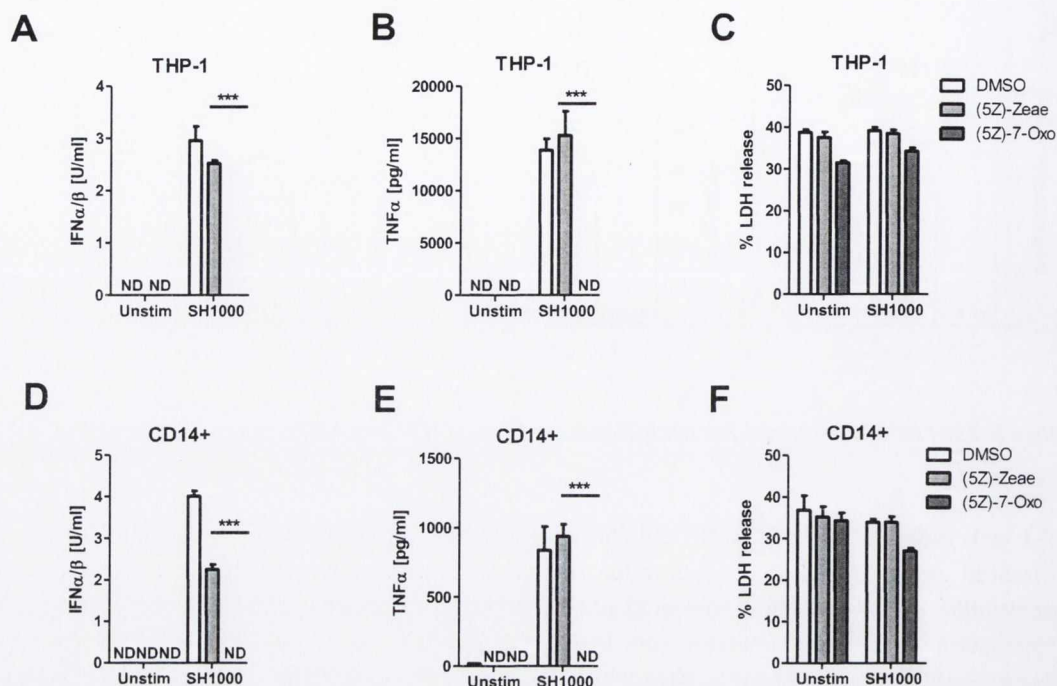


Figure 5.30: TAK1 is required for *S. aureus*-induced type I IFN and TNF α induction in monocytes.

THP-1 cells (A-C) or CD14⁺ monocytes (D-F) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with TAK1 inhibitor (1 μ M, 5Z-7-Oxo), TAK1 inhibitor control (1 μ M, 5Z-7-Zee) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 for 6 hr (A-C) or at an MOI of 1 for 18 hr (D-F). Gentamicin (200 μ g/ml) was added 2 hr after infection. Supernatants were harvest by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C,F) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, *** $p < 0.001$ when compared with infected samples treated with DMSO.

5.2.6 Role of canonical and noncanonical IKK kinases in TLR2-dependent type I IFNs

One of the consequences of TAK1 activation is the stimulation of I κ B kinase (IKK) kinase activity. The IKK complex consists of two catalytic subunits IKK α and IKK β , and a regulatory subunit IKK γ . The IKK complex catalyzes degradation of inhibitor of κ B (I κ B), which leads to release of NF κ B from its suppression by I κ B and transcription of proinflammatory cytokines. Therefore, an effect of a small molecule inhibitor of IKK β (BI605906) on TLR2-mediated immune responses was tested in human monocytes.

Interestingly, LTA-stimulated IFN α/β bioactivity and TNF α induction were impaired in a dose-dependent manner after treatment with IKK β inhibitor when compared to DMSO-treated THP-1 monocytes [Fig. 5.31 A-B]. In addition, LTA-induced IFN α/β bioactivity and TNF α were significantly decreased after treatment with IKK β inhibitor when compared to DMSO treated CD14 $^{+}$ monocytes [Fig. 5.31 D-E]. Cell viability was not affected after treatment with the IKK β inhibitor when compared to DMSO-treated monocytes [Fig. 5.31 C, F]. Noticeably, IKK β inhibitor significantly inhibited IFN α/β bioactivity and TNF α induction upon *S. aureus* infection in THP-1 and CD14 $^{+}$ monocytes when compared to DMSO treated monocytes [Fig. 5.32 A-B, D-E]. Moreover, treatment with IKK β inhibitor was not toxic to cells in the context of *S. aureus* infection [Fig. 5.32 C, F]. These results show that IKK β plays a central role in *S. aureus*-mediated TLR2 responses in both THP-1 and CD14 $^{+}$ monocytes.

In order to investigate the involvement of the noncanonical IKKs or so called IKK-related kinases, TBK1 and IKK ϵ , in TLR2-mediated type I IFN signaling in human monocytes, small molecule inhibitors of the IKK-related kinases were employed [232]. MRT67307 compound has been identified as a potent inhibitor of TBK1 and IKK ϵ ; however, it has also been shown that MRT67307 also inhibit members of the AMP-activated protein kinase (AMPK) subfamily. MRT67307 inhibited TBK1/IKK ϵ to the same extent as microtubule affinity-regulating kinases (MARKs), salt-inducible kinases (SIKs) and NUAK family SNF1-like kinases (NUAKs), while it did not inhibit brain-specific kinases (BSKs) and only weakly inhibited maternal embryonic leucine zipper kinase (MELK) and AMPK itself (Table 2.3). In contrast to MRT67307, MRT199665 compound proved to be an inhibitor of activity of most members of AMPK subfamily without blocking IKK-related kinases when compared to its effect on BSKs [232]. Therefore, in our studies, MRT67307 compound was used as inhibitor of both AMP- and IKK-related kinases, whereas MRT199665 compound was employed to assess the effects of MRT67307 on IKK-related kinases compared to its effect on AMP-related kinases. Thus, the difference between the two inhibitors represents the effect on IKK-related kinases.

Treatment with MRT67307 significantly impaired LTA-induced IFN α/β bioactivity when compared to DMSO- or MRT199665-treated THP-1 monocytes [Fig. 5.33 A]. LTA-stimulated TNF α induction was significantly upregulated after MRT67307 treatment when compared to DMSO- or MRT199665-treated THP-1 monocytes [Fig. 5.33 B]. However, there was no difference in LTA-stimulated IFN α/β bioactivity and TNF α induction between DMSO- and MRT199665-treated THP-1 monocytes. In CD14⁺ monocytes, MRT199665 treatment significantly impaired LTA-induced IFN α/β bioactivity when compared to MRT67307- or DMSO-treated monocytes [Fig. 5.33 D]. No change in LTA-stimulated IFN α/β bioactivity was observed after MRT67307 treatment when compared to DMSO-treated cells. In contrast to THP-1 monocytes, LTA-induced TNF α protein was significantly inhibited after MRT67307 and MRT199665 treatment in CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig. 5.33 E]. Importantly, treatment of human monocytes with MRT67307 or MRT199665 compound did not affect cell viability when compared to DMSO treated monocytes [Fig. 5.33 C, F]. This result suggests that IKK-related kinases are involved in LTA-dependent type I IFN response and that they negatively affect TNF α induction in THP-1 monocytes. In CD14⁺ monocytes, kinases other than AMP- and IKK-related kinases are involved in LTA-stimulated type I IFNs, while TNF α induction is partially dependent on AMPKs and partially on other kinases.

The effect of small molecule inhibitors of IKK-related kinases was further tested upon Malp2 and LPS stimulation in THP-1 monocytes. Malp2- and LPS-induced IFN α/β bioactivity was significantly impaired after MRT199665 treatment when compared to DMSO- or MRT67307-treated THP-1 monocytes [Fig. 5.34 A]. No inhibition of IFN α/β bioactivity was observed after MRT67307 treatment when compared to DMSO-treated cells. Treatment with MRT67307 significantly upregulated Malp2- and LPS-stimulated TNF α protein when compared to DMSO- or MRT199665-treated THP-1 monocytes [Fig. 5.34 B]. There was no difference in TNF α induction between DMSO- and MRT199665-treated THP-1 monocytes. In the initial screen, we noticed that type I IFNs and IP-10 chemokine followed the same pattern of protein induction after TLR2 and TLR4 stimulation; therefore, we measured IP-10 as an additional cytokine readout. Treatment with MRT67307 and MRT199665 significantly decreased Malp2- and LPS-induced IP-10 production when compared to DMSO-treated THP-1 monocytes [Fig. 5.34 C]. There was no difference in IP-10 induction between MRT67307- and MRT199665-treated THP-1 monocytes. Treatment with MRT67307 and MRT199665 compounds did not influence cell viability when compared to DMSO-treated THP-1 monocytes [Fig. 5.34 D]. These results suggest that kinases other than AMP- and IKK-related kinases are involved in Malp2-

and LPS-mediated type I IFN induction. Furthermore, IKK-related kinases might negatively regulate canonical IKKs, thus induction of proinflammatory cytokines. Lastly, members of AMPK family might be involved in TLR2- and TLR4-stimulated IP-10 induction.

In the context of *S. aureus* infection, treatment with MRT67307 significantly inhibited IFN α/β bioactivity when compared to DMSO- or MRT199665-treated THP-1 monocytes [Fig. 5.35 A]. No difference in *S. aureus*-induced IFN α/β bioactivity was observed after MRT199665 treatment when compared to DMSO-treated THP-1 monocytes. Treatment with MRT67307 and MRT199665 slightly upregulated *S. aureus*-stimulated TNF α induction compared to DMSO-treated THP-1 monocytes [Fig. 5.35 B]. There was no difference in TNF α induction between MRT67307- and MRT199665-treated THP-1 monocytes. In CD14⁺ monocytes, *S. aureus*-induced IFN α/β bioactivity was dramatically upregulated after MRT67307 treatment when compared to DMSO- and MRT199665-treated monocytes [Fig. 5.35 C]. No change in *S. aureus*-stimulated IFN α/β bioactivity was observed after MRT199665 treatment when compared to DMSO-treated CD14⁺ monocytes. Treatment with MRT199665 considerably inhibited *S. aureus*-induced TNF α production in CD14⁺ monocytes when compared to DMSO- and MRT67307-treated monocytes [Fig. 5.35 D]. No difference in TNF α induction was observed between DMSO- and MRT67307-treated CD14⁺ monocytes. These results show that IKK-related kinases are required for type I IFN induction, while they negatively regulate proinflammatory cytokines during *S. aureus* infection in THP-1 monocytes. In CD14⁺ monocytes, IKK related kinases negatively regulate canonical IKK kinases in type I IFN induction during *S. aureus* infection. Moreover, proinflammatory cytokines require kinases other than AMP- and IKK-related kinases during *S. aureus* infection. Collectively, these results demonstrate that distinct kinases and mechanisms are present in THP-1 and CD14⁺ monocytes after LTA stimulation and *S. aureus* infection.

Lastly, in order to test a crosstalk between canonical and noncanonical IKK kinases, the degree of NF κ B activation was assessed by immunoblot. THP-1 monocytes were treated with MRT67307 small molecule inhibitor or with a vehicle control DMSO, and the degree of p65 phosphorylation, a subunit of NF κ B, was examined. Interestingly, treatment with MRT67307 dramatically increased phosphorylation of p65 when compared to untreated or DMSO-treated cells [Fig. 5.36]. In addition, DMSO treatment increased the level of p65 phosphorylation when compared to untreated THP-1 monocytes. This result demonstrates that IKK-related kinases might negatively regulate canonical IKK kinases in resting cells, thus keeping proinflammatory cytokines under control.

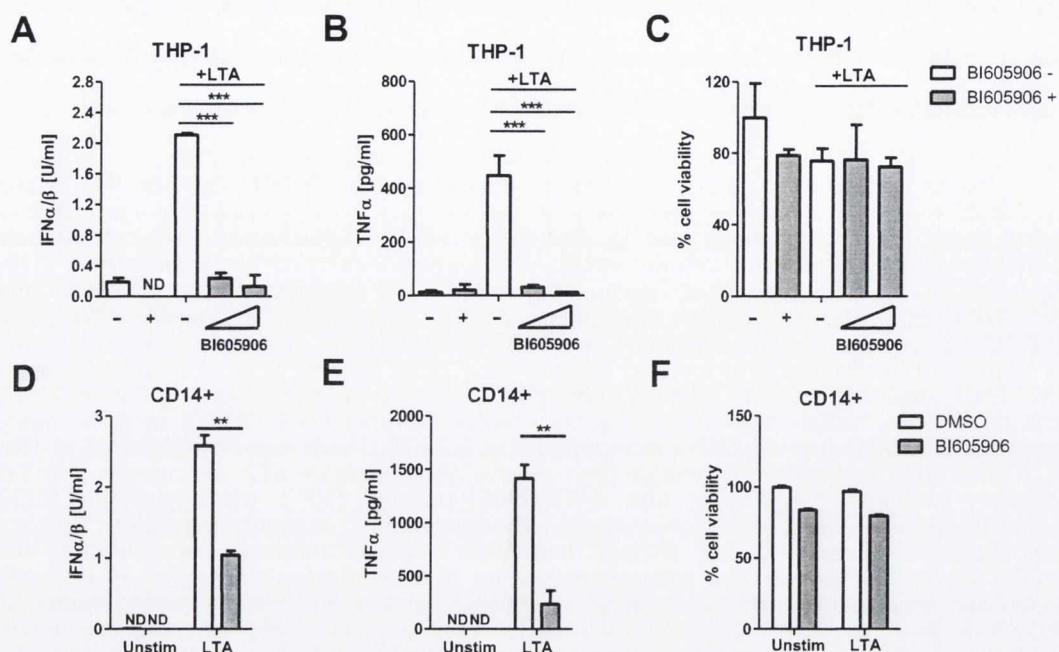


Figure 5.31: IKK β kinase is required for LTA-induced type I IFN and TNF α responses in human monocytes.

THP-1 cells (A-C) or CD14 $^{+}$ monocytes (D-F) at 0.5×10^6 cells/ml were seeded into 96-well plate 24 hr prior stimulation. Cells were pre-treated with increasing concentration of BI605906 (1/10 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions. Cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$ *** $p < 0.001$, when compared with stimulated samples treated with DMSO as determined by Student t test.

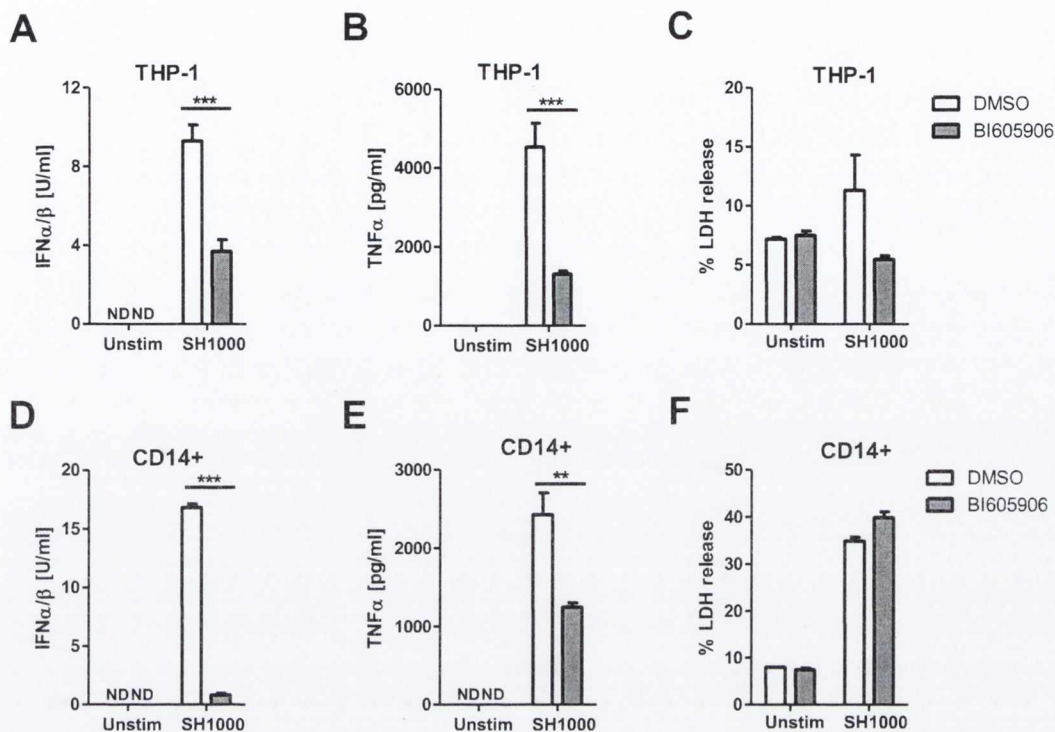


Figure 5.32: IKK β kinase is required for *S. aureus*-induced type I IFN and TNF α responses in monocytes.

THP-1 cells (A-C) or CD14 $^{+}$ monocytes (D-F) at 1×10^6 cells/ml were seeded into 24-well plate prior to infection. Cells were pre-treated with BI605906 (10 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A-C) or at an MOI of 1 (D-F) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-C) or 18 hr (D-F) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions, and cell viability (C, F) was quantified by LDH cytotoxicity assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, ** $p < 0.01$ *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

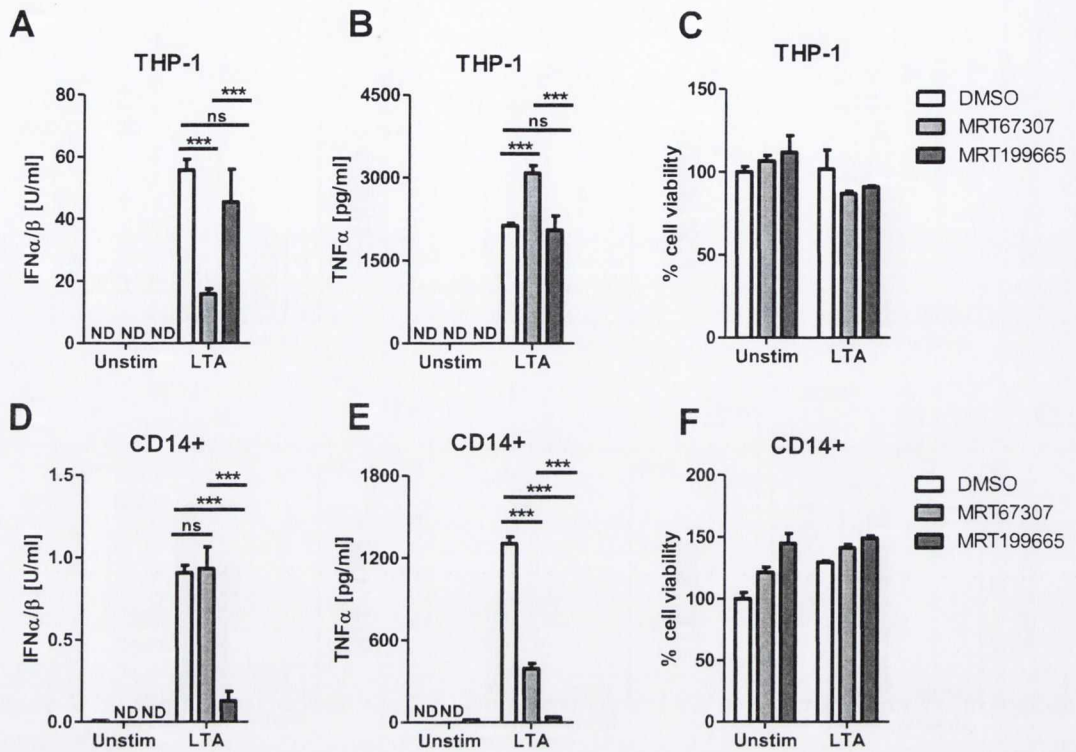


Figure 5.33: Distinct involvement of IKK-related kinases in LTA-induced responses between THP-1 and CD14+ monocytes.

THP-1 cells (A-C) or CD14+ monocytes (D-F) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior stimulation. Cells were pre-treated with MRT67307 (2 μ M), MRT199665 (1 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A-C) or 18 hr (D-F). Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, D) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, E) protein was measured by standard ELISA with appropriate dilutions. Cell viability (C, F) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, when comparing DMSO- and MRT67307-, DMSO- and MRT199665-, or MRT67307- and MRT199665-treated and infected samples as determined by two-way ANOVA with a Bonferroni posttest..

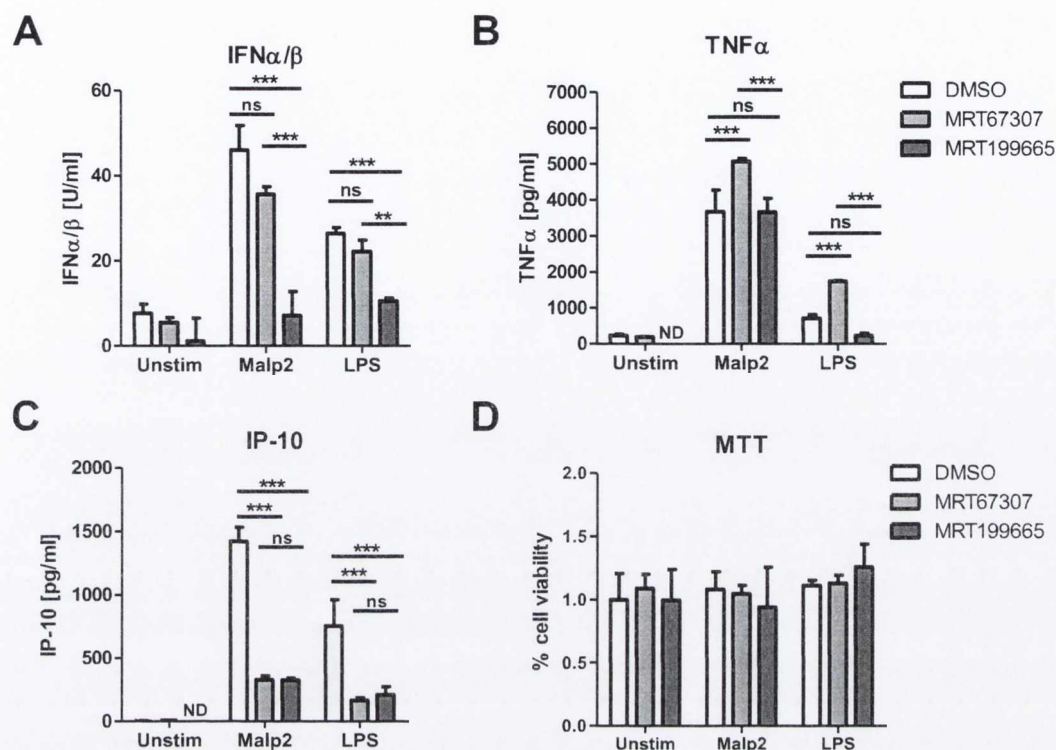


Figure 5.34: Role for AMPKs in Malp2-induced type I IFN induction in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with MRT67307 (2 μ M), MRT199665 (1 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with Malp2 (50 ng/ml) or LPS (100 ng/ml) for 6 hr. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A) was assayed using HEK Blue IFN α/β bioassay; TNF α (B) and IP-10 (C) proteins were measured by standard ELISA with appropriate dilutions, and cell viability (D) was quantified by MTT assay. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns – not significant, ** $p < 0.01$, *** $p < 0.001$, when comparing DMSO- and MRT67307-, DMSO- and MRT199665-, or MRT67307- and MRT199665-treated and infected samples as determined by two-way ANOVA with a Bonferroni posttest.

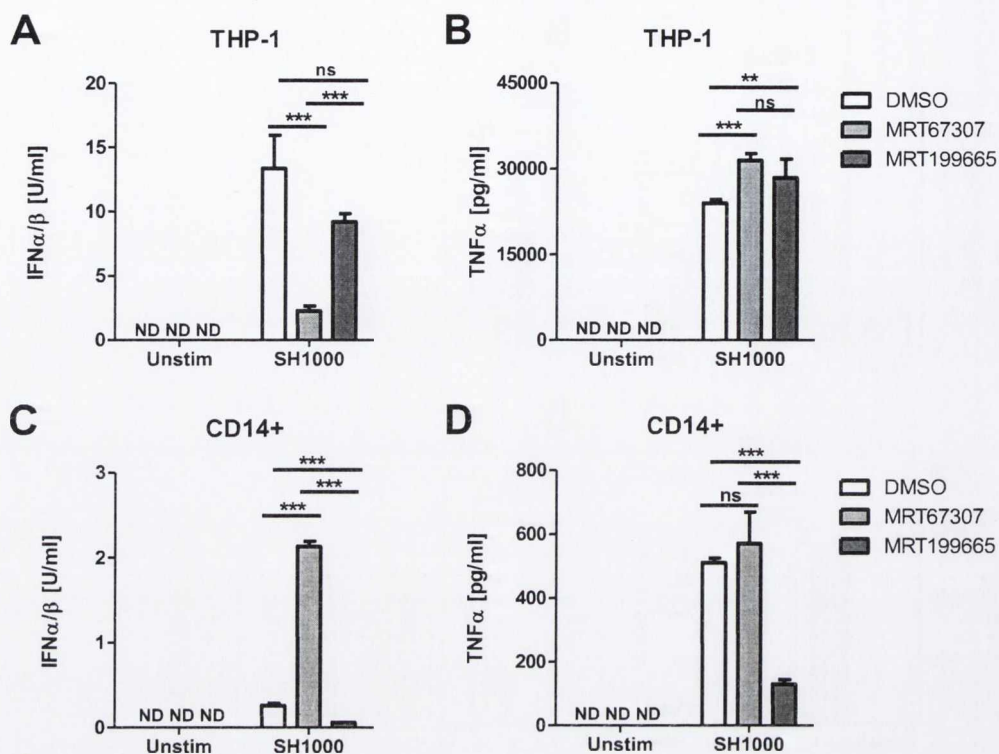


Figure 5.35: Distinct involvement of IKK-related kinases in *S. aureus*-induced responses between THP-1 and CD14+ monocytes.

THP-1 cells (A, B) or CD14+ monocytes (C, D) were seeded at 1×10^6 cells/ml into 24-well plate prior to infection. Cells were pre-treated with MRT67307 (2 μ M), MRT199665 (1 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A, B) or at an MOI of 1 (C, D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A-B) or 18 hr (C-D) followed by collection of supernatants by centrifugation at 100 g for 5 min. IFN α/β bioactivity (A, C) was assayed using HEK Blue IFN α/β bioassay; TNF α (B, D) protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when comparing DMSO- and MRT67307-, DMSO- and MRT199665-, or MRT67307- and MRT199665-treated and infected samples as determined by two-way ANOVA with a Bonferroni posttest.

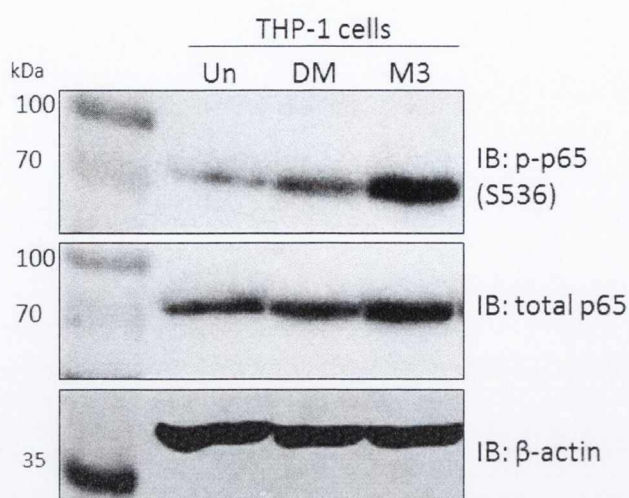


Figure 5.36: Inhibition of IKK-related kinases upregulates p65 phosphorylation in THP-1 monocytes.

THP-1 cells were seeded at 1×10^6 into 12-well plate. Cells were pre-treated with MRT67307 (2 μ M, M3), equivalent amount of DMSO (DM) or left untreated (Un) for 1 hr. Cells were harvest by centrifugation at 100 g for 5 min at 4°C. Cells were lysed in 60 μ l 3x sample buffer containing inhibitors. Lysates were incubated 5 min on ice, boiled for 5 min at 99°C and stored at -80 °C until needed. The protein levels of phosphorylated p65 (pp65), total p65 (tp65) and β -actin were analyzed by immunoblotting using 10 % gels and 0.2 μ m PVDF membrane. Data are representative of three independent experiments.

5.2.7 TLR2 activation upregulates IRF7 expression

Our previous study suggested that TLR2-mediated type I IFN induction required IRF7 transcription factor in HEK293 cells [42]. In addition, IKK-related kinases are involved in phosphorylation of IRF3 in numerous signaling pathways, leading to type I IFN responses. In order to determine whether IRF7 and/or IRF3 were involved in human TLR2-IFN β pathway, the induction of IRF3 phosphorylation (pIRF3) and IRF7 transcription was investigated by immunoblot. THP-1 cells were stimulated with VACV 70mer, Malp2, LTA, and *S. aureus* for 3, 6, 9 and 24 hr and protein levels were measured. VACV 70mer was used as a positive control since it induces both IRF3 phosphorylation and IRF7 transcription.

Accordingly, 70mer induced pIRF3 3 hr after stimulation followed by its decline to the basal level 24 hr after stimulation [Fig 5.37 A]. IRF7 expression was upregulated 6 hr after 70mer stimulation and peaked 24 hr after stimulation. Although THP-1 monocytes produced total IRF3 protein, no activation of IRF3 was detected upon Malp2, LTA or *S. aureus* stimulation [Fig. 5.37 B-D]. IRF7 expression was detected 6 and 9 hr after Malp2 and LTA stimulation and went down 24 hr after stimulation. *S. aureus* infection induced IRF7 expression 9 hr after infection with the peak at 24 hr after infection. This result clearly demonstrate that IRF3 is not involved in TLR2-mediated type I IFN induction, and that IRF7 might be involved in the second wave of type I IFNs in THP-1 monocytes. Consequently, other IRFs might be involved in the TLR2-mediated type I IFN signaling upon TLR2 activation.

Next, in order to confirm the role of TLR2 in IRF7 upregulation, IRF7 expression was assessed in TLR2 knockout THP-1 monocytes. Consistent with previous results, IRF3 was not phosphorylated after LTA stimulation in both wild type and TLR2 knockout cells [Fig. 5.38 A]. IRF7 expression was upregulated 6 hr after LTA stimulation with the peak at 24 hr after stimulation in wild type THP-1 monocytes, while it was absent in TLR2 deficient cells. IRF7 expression and IRF3 phosphorylation remained unaffected after 70mer stimulation in both wild type THP-1 monocytes and TLR2 knockout cells [Fig. 5.38 B]. This result further confirms that IRF7 expression is dependent on TLR2 signaling.

Lastly, the effect of IRF7 silencing on LTA- and *S. aureus*-stimulated responses was investigated in THP-1 monocytes. Surprisingly, LTA- and *S. aureus*-induced IFN α/β bioactivity was not affected by IRF7 silencing, whereas 70mer-induced IFN α/β bioactivity was significantly impaired when compared to THP-1 monocytes transfected with negative siRNA [Fig. 5.39 A]. However, TNF α induction was impaired after LTA and *S. aureus* stimulation in IRF7-silenced monocytes when compared to THP-1 monocytes transfected with negative siRNA [Fig. 5.39 B].

The degree of IRF7 knockdown was confirmed by assessing mRNA level and was consistent during the experiments [Fig. 5.39 C]. The result suggests that IRF7 is involved in inflammatory responses after TLR2 stimulation, and that other IRFs might contribute to TLR2-mediated type I IFN responses in THP-1 monocytes.

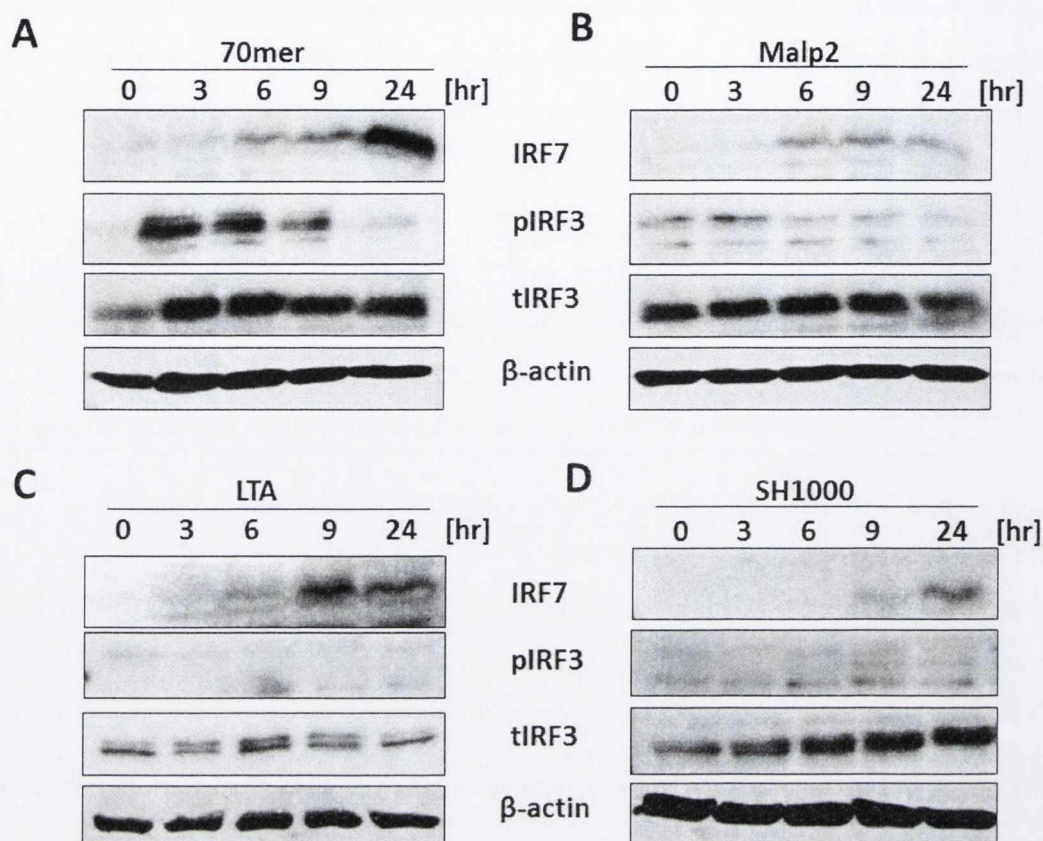


Figure 5.37: TLR2 activation upregulates IRF7 transcription THP-1 monocytes.

THP-1 cells were seeded at 1×10^6 into 12-well plate. Cells were stimulated with VACV 70mer (1 $\mu\text{g/ml}$) using Lipofectamine® 2000, Malp2 (50 $\mu\text{g/ml}$), LTA (2.5 $\mu\text{g/ml}$) or infected with *S. aureus* strain SH1000 at an MOI of 100 for 2 hr followed by gentamicin (200 $\mu\text{g/ml}$) treatment. The cells were further incubated for the total of 3, 6, 9 or 24 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. Cells were lysed in 60 μl 3x sample buffer containing inhibitors. Lysates were incubated 5 min on ice, boiled for 5 min at 99°C and stored at -80 °C until needed. The protein levels of IRF7, phosphorylated IRF3 (pIRF3), IRF3 (tIRF3) and β-actin were analyzed by immunoblotting using 10 % gels and 0.2 μm PVDF membrane. Data are representative of three independent experiments.

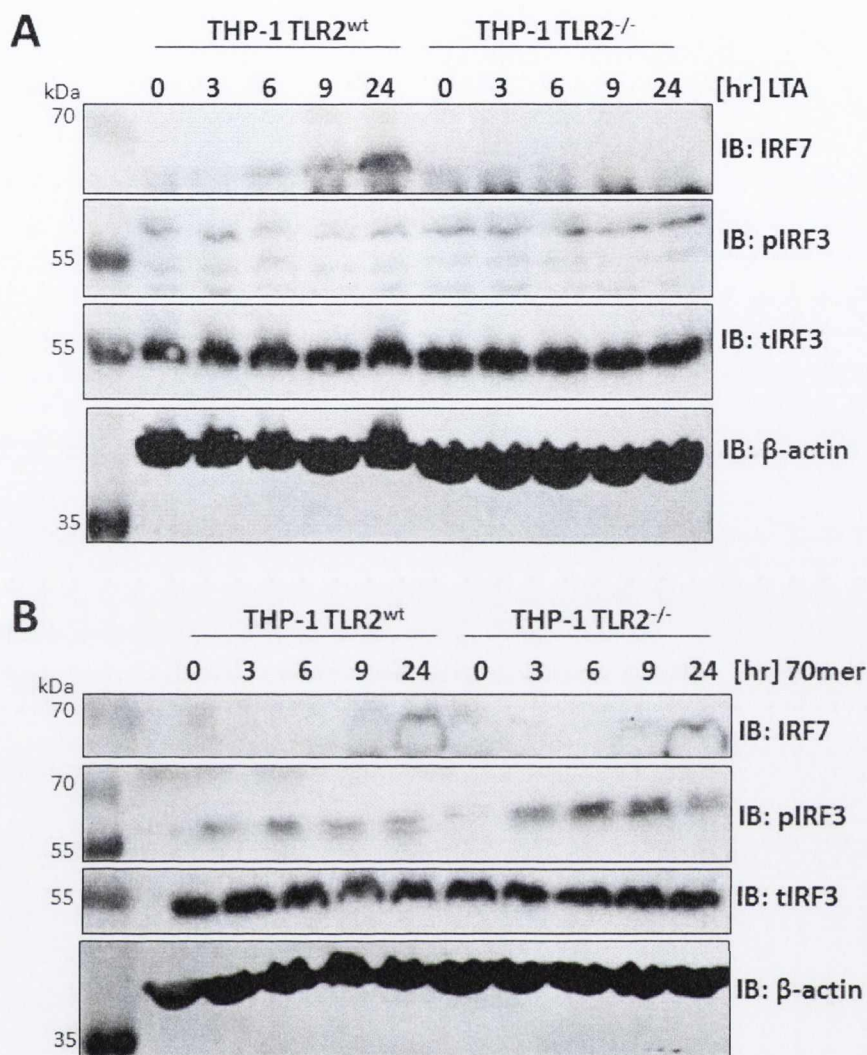


Figure 5.38: TLR2 is required for LTA-stimulated induction of IRF7 protein in THP-1 monocytes.

THP-1 cells and THP-1 TLR2 CRISPR knockout cell clone A5 were seeded at 1×10^6 into 12-well plate. Cells were stimulated with LTA (2.5 $\mu\text{g/ml}$) or VACV 70mer (1 $\mu\text{g/ml}$) using Lipofectamine® 2000. Cells were harvest by centrifugation at 100 g for 5 min at 4°C. Cells were lysed in 60 μl 3x sample buffer containing inhibitors. Lysates were incubated 5 min on ice, boiled for 5 min at 99°C and stored at -80 °C until needed. The protein levels of IRF7, phosphorylated IRF3 (pIRF3), IRF3 (tIRF3) and β -actin were analyzed by immunoblotting using 10 % gels and 0.2 μm PVDF membrane. Data are representative of three independent experiments.

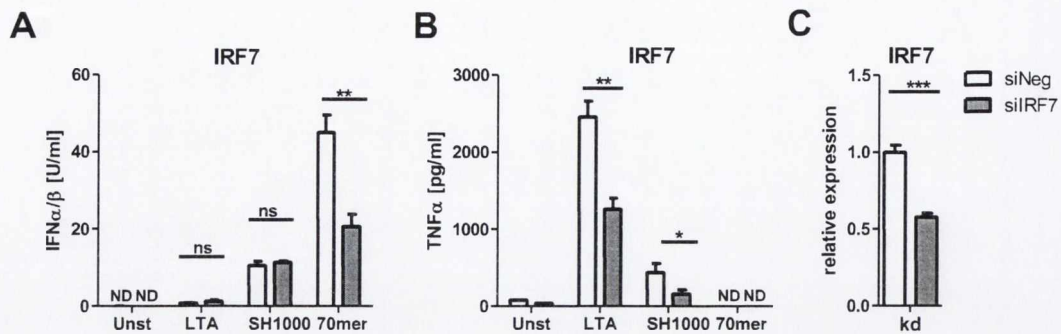


Figure 5.39: IRF7 is required for TLR2 -induced TNF α in THP-1 monocytes.

THP-1 cells (2×10^6 cells) were transfected with IRF7 (200 pmol) alongside with negative Qiagen siRNA (200 pmol) using NEON[®] electroporation transfection system at setting #15. After 42 hr, cells were stimulated with LTA (2.5 μ g/ml), transfected with VACV 70mer (1 μ g/ml) using Lipofectamine[®] 2000 or infected with *S. aureus* strain SH1000 at an MOI of 10 for 6 hr. Gentamicin (200 μ g/ml) was added 2 hr after infection. Supernatants were harvested by centrifugation at 100 g for 5 min. IFN α / β bioactivity (A) was assayed using HEK Blue IFN α / β bioassay; TNF α (B) protein was measured by standard ELISA with appropriate dilutions. Knockdown (C) was confirmed by qRT-PCR using IRF7 specific primers. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with stimulated samples transfected with negative siRNA as determined by Student *t* test.

5.3 Discussion

5.3.1 TLR2 is required for IFN β and TNF α induction by *S. aureus* in human monocytes

TLR2 is a pattern recognition receptor (PRR), which recognizes a broad range of bacterial PAMPs. In mouse BMDMs, TLR2 has been already implicated in the recognition of *S. aureus* *in vitro* and *in vivo* [185, 186]. In addition, it has been shown that LBP and CD14 are involved in recognition of staphylococcal LTA by human monocytes [191]. Furthermore, in our previous chapter, we showed that THP-1 monocytes efficiently killed *S. aureus* *in vitro* and this was partially TLR2 dependent. Hence, we sought to examine the degree by which TLR2 contributes to type I IFN production in human monocytes. Consistent with its broad role in innate immune signaling, we observed that TLR2 was indispensable for induction of both type I IFNs and proinflammatory cytokines upon LTA stimulation in THP-1 and human primary monocytes as well as for Malp2-induced responses in THP-1 monocytes. Importantly, we noted that TLR2 was strongly required for induction of both cytokines during *S. aureus* infection in human monocytes. Hence, our results support a central role of TLR2 in the recognition of *S. aureus* and activation of innate immune responses in human monocytes.

In order to exclude non-specific effects of the TLR2 neutralizing antibody, we employed TLR2 knockout THP-1 monocytes generated by CRISPR-Cas9 technique. Consistent with experiments using neutralizing TLR2 antibody, we observed that LTA- and Malp2-stimulated type I IFNs and proinflammatory cytokines were diminished in the absence of TLR2. However, there are concerns that the CRISPR-Cas9 technique, by which TLR2 knockout cells were generated, might lead to off target effects [265]. In fact, off-target effects possibly occurred during genome editing process in clone D4 since we observed impaired type I IFN induction after stimulation with TLR7/8 ligand. Hence, in other experiments we focused on clone A5, whose responses were comparable to wild type THP-1 monocytes. In the context of *S. aureus* infection, we noted that induction of IFN β and TNF α was strongly dependent on TLR2, whereas induction of IFN α was upregulated in the absence of TLR2. Therefore, an alternative pathway might be responsible for induction of IFN α in the absence of TLR2. Indeed, it has been shown that IFN α production required endosomal processing and TLR7 or TLR9 signaling in response to staphylococcal nucleic acids in human plasmacytoid DCs [266]. In addition, *S. aureus* has been shown to produce ssRNA that was recognized by macrophages independently of endosomal TLRs but still required MyD88 and UNC93B1 [267]. UNC93B1 is an endoplasmic reticulum (ER) protein, which is involved in trafficking of TLR7 and TLR9 from the ER into the endolysosomes [268]. However, in our model of endosomal TLR2

signaling we suppose that TLR2 moves to the endosome from the plasma membrane, which is consistent with the reported co-localization of TLR2 with MyD88 and TRAM in endosome after Malp2 stimulation [42]. Therefore, it is unlikely that UNC93B1 play a role in TLR2-mediated type I IFN pathway. In addition, we observed IFN α induction only in TLR2 knockout monocytes, suggesting that *S. aureus* is recognized by other receptors, such as endosomal TLRs or cytosolic RLRs and NLRs, and that TLR2 signaling can inhibit IFN α induction. In addition, infection of TLR2 knockout monocytes with *S. aureus* had deleterious effects on cell viability. Together with our results showing that IFN α stimulation negatively affected bacterial killing and cell viability, we suggest that IFN α has a detrimental role during *S. aureus* infection. Hence, our results show that TLR2 is the primary receptor for recognition of *S. aureus*, which triggers production of IFN β and could inhibit IFN α transcription. However, in the absence of TLR2, other receptors such as TLR8 or TLR9, recognize *S. aureus* nucleic acids and induce IFN α transcription.

Even though CD14 has been shown to regulate TLR4 internalization and trafficking into the endosomes, its role during TLR2-mediated recognition of *S. aureus* remains ambiguous. It has been reported that LTA is recognized by TLR2 in cooperation with LBP and CD14 in human monocytes [191]. Using eritoran, an analogue of lipid A, we confirmed that CD14 is required for TLR4-induced type I IFNs and proinflammatory cytokines in THP-1 monocytes [238]. Furthermore, we observed that CD14 was involved in TLR2-mediated induction of proinflammatory cytokines and type I IFNs after LTA stimulation and *S. aureus* infection in THP-1 monocytes. Hence, our result shows that TLR2 and CD14 cooperate during LTA recognition in order to trigger downstream signaling in THP-1 monocytes. This is consistent with recent NMR study, which demonstrated that both LPS and LTA were able to bind to the rim of CD14 hydrophobic pocket [269]. In addition, CD14 had a synergistic effect on LTA- and LPS-stimulated NF κ B activation, whereas MDP showed little synergy between CD14 and TLR2 [269]. However, we cannot rule out interaction between TLR2 and other co-receptors during recognition of *S. aureus* cell wall components; hence, it will be interesting to examine the ability of TLR2 to dimerize with other molecules such as CD36, or with TLR2 itself.

5.3.2 TLR2-stimulated type I IFNs is dependent on the endosomal pathway

Endosomes are important compartments, which mediates transport of molecules within cells and degradation of intracellular pathogens. Professional phagocytes, such as neutrophils, macrophages and dendritic cells, are equipped with multiple strategies for elimination of intracellular microbes. We have previously shown that THP-1 monocytes

were able to kill *S. aureus in vitro* and the clearance of *S. aureus* was enhanced after inhibition of the endosomal pathway. In addition, we observed impaired type I IFN induction, while proinflammatory cytokines remained unchanged. It is a common belief that proinflammatory cytokines are mediated by surface TLRs, whereas type I IFNs are triggered from endosomal compartments. Exceptionally, TLR4 can trigger both signaling pathways by utilizing distinct adaptor proteins depending on the compartment from which the signaling occurs [53]. Similarly to TLR4, endolysosomal processing has been associated with TLR2-mediated activation of IRF1 and IRF7 without affecting proinflammatory responses [152]. Likewise, we previously showed that endosomal processing was required for TLR2-dependent IRF7 gene reporter transactivation in HEK293 cells [42]. Hence, we further investigated a role of the endosomal pathway in TLR2-stimulated type I IFNs in human monocytes.

First, we investigated the role of dynamin in TLR2-mediated induction of cytokines. Dynamin plays an important role during clathrin-dependent endosomal formation and is responsible for primary steps during endocytosis, including budding of membrane, formation and scission of vesicles. In the previous chapter, we noted that inhibition of dynamin led to enhanced killing of *S. aureus* by THP-1 monocytes. Our results further show that dynasore inhibits type I IFN production after LTA stimulation and *S. aureus* infection, while inflammatory cytokines remain unaffected in THP-1 monocytes. Next, we sought to examine the role of downstream endosomal processes in cytokine production in THP-1 monocytes. Cytochalasin D is a fungal toxin that blocks polymerization of actin monomers. Cytochalasin D can also bind to F-actin polymers, disrupt actin filaments and cause arrest of the cell cycle. In line with previous results using dynasore, we observed that treatment with cytochalasin D impaired type I IFNs after LTA stimulation and *S. aureus* infection, whereas proinflammatory responses remained unchanged. Collectively, our results demonstrate that clathrin-mediated endocytosis and actin polymerization are required for TLR2-mediated type I IFN induction. Therefore, our data strongly suggest that the induction of type I IFNs is mediated via endosomal TLR2 while proinflammatory responses are triggered from surface TLR2.

Spleen tyrosine kinase (Syk) has been implicated in LPS-stimulated translocation of a TLR4-CD14 complex into endosomes [245]. However, an additional Syk-independent pathway that contributes to TLR4 endocytosis was later identified [256]. Furthermore, Syk kinase has been shown to interact with multiple components of TLR signaling and regulate MyD88-TRAF6-TAK1 and TRIF-TRAF3-TBK1 pathways after LPS stimulation [256]. The same

authors also demonstrated that Syk kinase inhibited TAK1-mediated proinflammatory responses after activation from the surface TLR4; however, when the signaling occurred from the endosomes, Syk kinase enhanced TBK1-mediated type I IFN production [256]. In addition, a similar pattern was observed for TLR2-, TLR3-, TLR7/8- and TLR9-mediated responses, suggesting that Syk kinase was a common regulator of TLR pathways [256]. Consistent with findings of Lin and colleagues [256], we observed that Syk kinase was required for LTA-induced type I IFN production, while it was redundant for inflammatory cytokines in THP-1 monocytes. Interestingly, we noted that Syk kinase was redundant for Malp2-stimulated responses in THP-1 monocytes, indicating signaling differences between LTA- and Malp2-induced signaling pathways. In the context of *S. aureus* infection, we observed that Syk kinase was required for induction of type I IFNs in human monocytes. However, we noted that the role of Syk kinase in induction of proinflammatory responses was different between the two types of monocytes during *S. aureus* infection. Whereas Syk was required for proinflammatory cytokines in primary monocytes, it was redundant in THP-1 monocytes during infection. Collectively, our results show that the endosomal pathway is required for LTA- and *S. aureus*-induced type I IFNs in human monocytes, while it is redundant for Malp2 signaling pathway. In the terms of proinflammatory responses, our results suggest that Syk kinase has different roles during LTA and *S. aureus* challenge in THP-1 and primary monocytes.

Bafilomycin A1 is a highly specific inhibitor of the vacuolar H⁺ ATPase. Treatment with bafilomycin A1 leads to increased pH in endosomes and blocking their fusion with lysosomes, a process required for antigen maturation. We previously showed that endosomal processing is required for TLR2-induced type I IFNs in HEK293 cells [42]. Our findings further shows that the endosomal pathway is also required for LTA- and *S. aureus*-mediated type I IFN induction in human monocytes. Similarly to Syk kinase treatment, we noted striking differences in the requirement for endosomal maturation and the induction of proinflammatory responses between the monocytes. While the endosomal processing was not involved in inflammatory responses in THP-1 monocytes, it was required in primary monocytes. This might have been a result of toxic effects of bafilomycin A1 treatment on cell viability; however, we also measured IL-1 β production and we did not observe correlation between cell death and IL-1 β release. Hence, our data suggest that the endosomal processing is required for both TNF α and type I IFN induction in primary monocytes, and that the signaling pathways are more complex in primary than in THP-1 monocytes. Interestingly, in contrast to Syk kinase, we observed that the endosomal processing was also required for Malp2-induced type I IFNs, while it did not affect proinflammatory cytokines. Hence, our data suggest that Malp2 might trigger Syk-

independent endocytosis but endosomal processing is equally required for induction of type I IFNs by LTA and Malp2.

We also noted that inhibitors of the endosomal pathway had adverse effects on viability of THP-1 monocytes, which could result in cell lysis, release of hydrolytic enzymes and alterations in cytokine production. However, we did not observe differences in TNF α protein induction between treated and untreated THP-1 monocytes. Moreover, the cytotoxic effects of bafilomycin A1 treatment were comparable to the effects of vehicle treatment and they occurred during later stages of infection. Therefore, this suggests that the effect of endosomal inhibitors on type I IFN induction was due to an altered function of the cells. Collectively, our data demonstrate that the endosomal pathway is required for type I IFN induction in response to LTA stimulation and *S. aureus* infection in human monocytes, while it is redundant for proinflammatory responses. However, we also observed that inhibition of the endosomal pathway enhanced killing of *S. aureus*, suggesting that *S. aureus* might be using the endosomal pathway as a means of manipulation of the host immune responses. In the future experiments, it will be interesting to see how inhibitors of the endosomal pathway alter localization of *S. aureus* within the cells by using confocal microscopy.

5.3.3 Role of adaptor proteins in TLR2-induced type I IFN pathway

Adaptor proteins are necessary for signal transduction from activated TLRs and induction of downstream signaling cascades. In addition, Mal and TRAM adaptors has been shown to affect TLR pathways according to the cellular location from which they trigger signaling [44]. Previously, we identified VIPER as a viral inhibitor, which disrupts TLR4 signaling by blocking receptor:adaptor interactions, i.e. TLR4:Mal and TLR4:TRAM [258]. Surprisingly, it has been shown that VIPER also inhibited TLR2-mediated type I IFN induction in human HEK293 cells in Mal- and TRAM-dependent manner [42]. Furthermore, TRAM was responsible for trafficking of TLR2-MyD88 complex into the endosomes, indicating that TRAM might act as a sorting adaptor not only for TLR4 but also for TLR2 signaling [42]. Since little is known about TLR2-stimulated type I IFNs in human cells, we examined the effect of VIPER treatment on TLR2 stimulated immune responses in human monocytes.

Consistent with the effects of VIPER treatment in mouse BMDMs [42], we observed that Mal and/or TRAM were required for TLR4-stimulated type I IFNs and proinflammatory cytokines. However, we noted significant differences in the effect of VIPER between Malp2- and LTA-induced responses. Whereas LTA required Mal and/or TRAM adaptors for induction of type I IFNs and proinflammatory cytokines, Malp2 signaling involved adaptor proteins other

than Mal or TRAM. In addition, we observed an effect of VIPER on TLR4-stimulated responses; therefore, we could rule out that VIPER was not functional in THP-1 monocytes. Furthermore, LTA-induced responses were sensitive to VIPER treatment, suggesting that concentration was sufficient to block TLR2-induced signaling. Hence, our result demonstrates that Malp2 and LTA signal via distinct TLR2 pathways. In the context of *S. aureus* infection, we observed that *S. aureus*-mediated downstream signaling also required Mal and/or TRAM, indicating that LTA models *S. aureus*-induced pathways in human monocytes.

The role of Mal in endosomal signaling is supported by a recent report showing that Mal can regulate distinct TLR signaling from different compartments [54]. In this case, Mal is directed to the plasma membrane or into endosomes depending on a lipid moiety recognized by its N-terminal lipid-binding domain [54]. Upon TLR activation, Mal triggers the myddosome assembly at the site determined by its lipid-binding domain, indicating that posttranslational modifications can license adaptors for signaling from diverse cellular compartments, and that Mal can act downstream of most TLRs apart from TLR3 [54]. In addition, the involvement of Mal in *S. aureus*-mediated signaling is consistent with mouse studies showing that Mal is required for induction of proinflammatory cytokines [186]. In support of the role for TRAM in endosomal signaling, a recent study showed that TRAM could mediate signaling from endosomal TLR7 via a physical interaction with the TLR7-MyD88 complex and activate IRF3 [55]. Interestingly, the role of TRAM in TLR7 signaling was cytokine-specific since TRAM silencing led to impaired IFN β and RANTES induction, while not affecting TNF α induction in human macrophages [55]. Moreover, we previously reported that type I IFN induction is dependent on Mal and TRAM in mouse BMDMs; however, TRAM was responsible for trafficking of the TLR2-MyD88 complex into endosomes [42].

Hence, in our study, it was of major interest to distinguish individual adaptor proteins involved in *S. aureus*-mediated signaling in human monocytes. Since VIPER interacts with both Mal and TRAM, and inhibits their interaction with the receptor, we used siRNA against individual adaptor proteins. Remarkably, we observed that Mal had a central role in *S. aureus*-stimulated type I IFN and proinflammatory signaling. However, in contrast to mouse BMDMs, we noted that TRAM had a redundant function in the activation of immune responses in response to *S. aureus* in THP-1 monocytes. In addition, our results further elaborated differences between Malp2 and LTA signaling. While both Mal and TRAM partially required in Malp2 pathway, LTA signaling was dependent solely on Mal. Recently, both TRAM and TRIF adaptor proteins were implicated in TLR2-mediated RANTES induction in mouse BMDMs [43];

however, the role of TRIF in TLR2 signaling in human cells remained poorly described. Our data reveals that TRIF might play a partial role in both LTA- and Malp2-induced type I IFN signaling in THP-1 monocytes.

Collectively, our results demonstrate that Mal plays a crucial role in LTA- and *S. aureus*-stimulated type I IFNs and proinflammatory cytokines in THP-1 monocytes, and that Mal can cooperate with TRIF in downstream signaling leading to type I IFN induction. Malp2 signaling, on the other hand, requires Mal, TRAM and TRIF adaptor proteins for production of type I IFN but all adaptors are redundant in induction of proinflammatory cytokines in THP-1 monocytes.

5.3.4 Role of myddosome in TLR2-induced type I IFN pathway

The myddosome is a multiprotein complex responsible for signal transmission from receptors to downstream kinases. Upon activation of TLRs, the myddosome complex is self-assembled and triggers phosphorylation of IRAKs and activation of downstream molecules, including TRAF6 [57]. Polyubiquitinated TRAF6 then triggers TAK1 phosphorylation leading to activation of IKK complex, which in turn induces translocation of NFκB to the nucleus and transcription of proinflammatory genes [270]. Furthermore, it has been reported that patients deficient in MyD88 and IRAK-4, the components of the myddosome, suffered from increased susceptibility to recurrent infections caused by Gram-positive bacteria, including *S. aureus* [187, 188]. Moreover, both MyD88 and IRAK4 have been implicated in recognition of *S. aureus* by mouse BMDMs [186]; however, the role of the myddosome in the TLR2-mediated type I IFNs in human monocytes remains poorly understood.

In human monocytes, we observed that LTA- and *S. aureus*-stimulated type I IFNs and proinflammatory cytokines required components of the myddosome, including MyD88, IRAK2, IRAK1 and/or IRAK4. Therefore, our data suggest that type I IFN induction is mediated via TLR2 signaling pathway, which involves assembly of the myddosome complex at the endosome membrane. However, we noted that Malp2 stimulation activated a noncanonical pathway, which was MyD88- and IRAK2-independent but partially involved IRAK1 and/or IRAK4. Next, it will be interesting to distinguish, which IRAKs are required for LTA-mediated type I IFNs using siRNA against IRAK1. Additionally, it is appealing to investigate the effect of the IRAK-1/4 inhibitor on phosphorylation status of IRAKs by immunoblot.

5.3.5 TRAF6 and TAK1 are involved in TLR2-stimulated type I IFN induction

Tumor necrosis factor receptor-associated factors (TRAFs) were originally discovered as adaptor proteins linking TNF receptor to TNF α signaling pathway [259]. Only later, TRAFs were also associated with innate immune signaling as signal transducers of Toll/IL-1 family members. TLR4 stimulation has been shown to trigger TRAF3, leading to type I IFN induction via TBK1-IRF3 [260], while TRAF6 mediates MyD88-dependent transcription of inflammatory genes as well as induction of type I IFN via IKK α activation. In addition, it has been shown that Syk kinase can bind TRAF3 and TRAF6 via its distinct domains, and thus differentially affect their polyubiquitination [256]. In the previous subchapter, we showed that inhibition of Syk kinase led to impaired induction of type I IFNs after TLR2 stimulation; hence, we further sought to investigate involvement of TRAF3 and TRAF6 in TLR2-mediated immune responses.

Surprisingly, we observed that TRAF3 was required for Malp2-mediated inflammatory cytokines, whereas induction of type I IFNs was TRAF3 independent. So far, TRAF3 was shown to negatively regulate NF κ B signaling by degradation of NF κ B inducing kinase (NIK), and thus block NF κ B activation [271]. However, Lin and colleagues demonstrated that TRAF3 degradation was not sufficient for NF κ B activation in B-lymphocytes [272]. Although we showed that TRAF3 might be involved in proinflammatory responses upon Malp2 stimulation, the mechanism remains unknown. In addition, TRAF3 might constitute a novel unconventional pathway used by Malp2. Unfortunately, the involvement of TRAF6 in Malp2 signaling remains unclear. However, in previous experiments using VIPER, we observed that Pam3Csk4 followed the same signaling pattern as Malp2. In addition, we observed that Pam3Csk4-induced responses were TRAF6-dependent; therefore, TRAF6 is likely to be also involved in Malp2-stimulated immune responses.

Recently, it has been shown that Mal and TRAM contains a putative TRAF6 binding motif that is crucial for TLR2 and TLR4 mediated proinflammatory responses [273,274,293]. The TRAF6 binding motif was situated in the TIR domain of Mal, suggesting that MyD88 and TRAF6 might compete for binding to Mal [273, 274]. In addition, it has been shown that both Mal and TRAM can directly recruit TRAF6 to the plasma membrane and trigger an alternative signaling pathway. Notably, a single mutation in Mal's TRAF6 binding site has been shown to impair TNF α production upon TLR2 and TLR4 activation demonstrating the importance of Mal-TRAF6 interaction [273]. Likewise, mutation in TRAM's TRAF6 binding motif impaired NF κ B and IFN β gene reporter activity and consequently TNF α and RANTES production upon TLR4 stimulation [274]. Therefore, these findings suggest that Mal and TRAM are not only

bridging adaptors between MyD88 and TRAF6 but they also have a direct function in TRAF6 signaling. In our experiments, we observed that TRAF6 was strongly required for TLR2-mediated type I IFN and proinflammatory responses in THP-1 monocytes. In addition, we noted that Mal had a crucial role in the activation of LTA- and *S. aureus*-mediated immune responses. However, we also observed that the LTA-stimulated signaling required the myddosome signaling complex. Hence, after recognition of LTA, the myddosome might mediate conventional activation of TRAF6 via IRAKs or form a bridge for Mal-TRAF6 interactions and induction of downstream signaling in THP-1 monocytes.

Activation of TRAF6 ubiquitin activity induces TAK1-binding (TAB) proteins, which mediate TAK1 conformational changes leading to phosphorylation of TAK1. Kinase activity of TAK1 then activates IKK protein complex and MAP kinases, p38 and JNK, all of which induce transcription of proinflammatory cytokines [270]. It has been shown that TAK1 phosphorylates IKK β and IKK α subunits of IKK complex, which then phosphorylates each other [76]. The IKK complex catalyzes degradation of I κ B, release of NF κ B and transcription of inflammatory cytokines. In our experiments, we observed that inhibition of TAK1 activity impaired both type I IFNs and proinflammatory cytokines in response to stimulation with both TLR2 ligands and *S. aureus* infection in human monocytes. Hence, our result shows that TAK1 has a central role in TLR2-mediated downstream signaling in human monocytes. So far, there has been a scattered evidence for the role of TAK1 in type I IFN induction and even less evidence for TLR2-mediated induction of type I IFNs. Bruni and colleagues showed that TAK1 is involved downstream of endosomal TLR3 and mediates both type I IFN and proinflammatory responses, and that TAK1 activity is inhibited by IRAK1-IKK ϵ signaling [275]. In contrast to our findings, Bergstrom and colleagues showed that stimulation of the surface TLR2 with FSL-1 or with *S. aureus* blocked staphylococcal ssRNA-mediated IFN β induction via inhibition of TAK1/IKK β downstream of TLR8 in human primary monocytes [162]. However, in our experiments, we did not observe inhibitory effects on type I IFN production after stimulation with LTA or in the context of infection with the whole bacteria. Similar to Malp2, FSL-1 is a synthetic lipoprotein derived from *Mycoplasma salivarium*. Importantly, we previously observed significant signaling differences between Malp2 and LTA pathways. In addition, in our model, we suppose that TLR2-mediated type I IFN signaling comes from endosomes, while the TLR2-dependent inhibition of IFN β was mediated by the surface TLR2. Hence, the discrepancies between the effects of TLR2 signaling on type I IFNs might be a result of the distinct nature of TLR2 ligands used in the studies or the compartments from which the TLR2-mediated signaling occurred.

5.3.6 Role of canonical and noncanonical IKK kinases in TLR2-dependent type I IFNs

Activation of TAK1 by TABs culminates in the induction of canonical IKKs, and MAP kinases p38 and JNK. The canonical IKK complex consists of two catalytic subunits IKK α and IKK β , and a regulatory subunit IKK γ . The IKK complex catalyzes degradation of I κ B, leading to release of NF κ B and transcription of proinflammatory cytokines. It has been reported that TAK1 phosphorylates IKK β and IKK α subunits of the IKK complex, which then phosphorylates each other [76]. However, whether activity of a single IKK subunit is sufficient for induction of type I IFNs remains unclear. Here, we show that IKK β plays an important role in induction of *S. aureus*-stimulated type I IFNs and proinflammatory cytokines in human monocytes. However, we cannot exclude a contribution of IKK α in TLR2-mediated signaling since it has been shown that IKK α can directly phosphorylate IRF7 and induce transcription of IFN α [63]. Hence, it will be interesting to investigate the role of IKK α in TLR2-mediated IFN β induction by siRNA against IKK α since an IKK α inhibitor is not available. In our model, it is possible that IKK β is activated in response to TLR2 stimulation and mediates IFN β transcription, while IKK α might be triggered in response to stimulation from endosomal TLRs and mediate IFN α induction.

Activation of TLRs leads to downstream signaling via canonical and noncanonical IKKs. It is a common consensus that canonical IKKs facilitate degradation of I κ B, and thus induce NF κ B-dependent gene transcription. The noncanonical IKKs or so-called IKK-related kinases TBK1 and IKK ϵ , on the other hand, phosphorylate IRF3 leading to its dimerization, nuclear translocation and transcription of type I IFNs. It was believed that IKK-related kinases were activated solely after ligand recognition until Clark and colleagues demonstrated that activation of IKK-related kinases was a two-step process [233]. First, IKK-related kinases are phosphorylated by activated canonical IKKs licensing them for a second phosphorylation event carried out by their own autocatalytic activity [233]. Moreover, IKK-related kinases are able to phosphorylate canonical IKKs resulting in their reduced activity and decreased transcription of NF κ B-dependent gene [232]. Therefore, these observations strongly suggest that a regulation loop between canonical and noncanonical IKKs exists in order to orchestrate innate immune responses. Hence, we examined the role of noncanonical IKKs in TLR2-mediated type I IFNs using a small molecule inhibitor of IKK-related kinases (MRT67307) [232]. However, it has been shown that MRT67307 also inhibits AMPK subfamily members including AMPK, MARKs, MELK, NUAks and SIKs. Therefore, we used a small molecule inhibitor of AMP-related kinases (MRT199665) as a control inhibitor, which did not inhibit IKK-related kinases

(Table 2.3). Consequently, the sensitivity of type I IFNs and proinflammatory cytokines to the pair of inhibitors represented the effect of IKK-related kinases on the TLR2-mediated signaling.

In our hands, we observed that IKK-related kinases were required for LTA-stimulated type I IFNs in THP-1 monocytes. Surprisingly, we noted that Malp2 stimulation triggered kinases other than AMP- and IKK-related kinases for induction of type I IFNs in THP-1 monocytes. Consistent with Clark and colleagues [233], we noted that inhibition of IKK-related kinases led to enhanced TLR2-stimulated proinflammatory response, indicating a presence of negative regulation loop between noncanonical and canonical IKKs in THP-1 monocytes. Furthermore, we observed enhanced phosphorylation of p65, a subunit of NF κ B, after inhibition of noncanonical IKKs in unstimulated THP-1 monocytes. Therefore, our result suggests that IKK-related kinases negatively regulate canonical IKKs also in resting cells, and thus keeping proinflammatory cytokines under control. In addition, we observed that IKK-related kinases contributed to type I IFN induction during *S. aureus* infection, while they negatively regulated proinflammatory cytokines in THP-1 monocytes, a pattern similar to LTA-stimulated responses. Hence, our results reveal further differences between Malp2 and LTA signaling, and highlight similarities between LTA- and *S. aureus*-stimulated pathways. Whereas both LTA and *S. aureus* signaling triggers type I IFNs via IKK-related kinases, Malp2 induces type I IFN induction via distinct kinases or even via canonical IKKs. On the other hand, both Malp2 and LTA uses similar pathway for induction of proinflammatory cytokines. Furthermore, our results suggest that canonical and noncanonical IKKs are part of an activation feedback loop, by which they regulate each other in order to orchestrate immune responses in THP-1 monocytes.

In human primary monocytes, however, the effect of the small molecule inhibitors on TLR2-mediated responses was strikingly different from the effect observed in THP-1 monocytes. We noted that type I IFN induction was mediated by kinases other than AMP- and IKK-related kinases after LTA stimulation. Furthermore, we noted that inhibition of IKK-related kinases upregulated induction of type I IFNs during *S. aureus* infection, suggesting a negative regulation of canonical IKKs or other kinases by noncanonical IKKs. On the other hand, we observed that AMP-related and other kinases contributed to LTA- and *S. aureus*-stimulated proinflammatory responses. Hence, our results demonstrate that distinct kinases and mechanisms are present in THP-1 and primary monocytes after LTA stimulation and *S. aureus* infection. However, regarding our previous results showing the role of IKK β in downstream

signaling, it is possible that canonical IKKs have a central role in induction of type I IFNs and proinflammatory cytokines in primary monocytes.

5.3.7 TLR2 activation upregulates IRF7 expression

Recent studies suggested a role of various IRFs in TLR2-mediated type I IFN induction. Barbalat and colleagues showed that TLR2 recognized VACV and induced type I IFNs via MyD88-dependent activation of IRF3 and IRF7 in inflammatory monocytes [157]. Another piece of evidence showed that TLR2 required internalization and endolysosomal processing in order to trigger IFN β production via MyD88, IRF1 and IRF7 [152]. The TLR2-IFN β pathway was also associated with *Listeria monocytogenes* infection and IFN β induction required bacterial internalization, TRIF adaptor and IRF3 and IRF7 in mouse macrophages derived from the peritoneal cavity [158]. Our previous work in BMDMs revealed that type I IFNs was Mal- and TRAM-dependent and infection with herpesvirus and *S. aureus* led to impaired IFN β production in BMDMs lacking these adaptors [42]. Furthermore, TRAM was responsible for trafficking of TLR2-MyD88 complex into endosomes followed by transactivation of IRF7 in HEK293 cells [42]. Collectively, these results demonstrate that TLR2 is able to stimulate type I IFNs in mice and human cells; however, TLR2-type I IFN pathway is dependent on the cell type and the nature of the hosts.

Consistent with the role of IRF7 in TLR2-mediated type I IFN induction, we observed upregulation of IRF7 transcription after stimulation with both TLR2 ligands and infection with *S. aureus*, while the phosphorylation status of IRF3 remained unaffected in THP-1 monocytes. Importantly, we observed that LTA-mediated IRF7 transcription was dependent on TLR2 since it was absent in TLR2 knockout cells. In all cases, we noted that IRF7 transcription and IRF3 phosphorylation remained intact in both wild type and TLR2 knockout cells after dsDNA stimulation, and thus confirmed the ability of cells to induce IRFs. In addition, our results clearly show that IRF3 is not involved in TLR2-mediated type I IFNs. On the other hand, IRF7 transcription is TLR2-dependent and IRF7 might play a role during later stages of type I IFN induction. In order to test the requirement of IRF7 in early induction of type I IFNs, we used siRNA against IRF7. Surprisingly, we noted that IRF7 was involved in induction of inflammatory responses after LTA and *S. aureus* stimulation. In addition, silencing of IRF7 did not affect TLR2-stimulated type I IFN induction in THP-1 monocytes. Hence, our results suggest that other IRFs, such as IRF1, IRF5 and IRF8, might be involved in induction of TLR2-mediated type I IFN responses in THP-1 monocytes. In future experiments, it will be interesting to investigate the involvement of other IRFs in early induction of type I IFNs upon TLR2 stimulation.

In summary, our results present a detailed dissection of TLR2 signaling pathway in response to *S. aureus* in human monocytes. In addition, we present evidence that challenge with LTA and *S. aureus* stimulate type I IFN production in human monocytes. We show that induction of both type I IFNs and proinflammatory cytokines by LTA and *S. aureus* is dependent on TLR2 in cooperation with CD14. However, in the absence of TLR2, other receptors, such as endosomal TLR9, can recognize staphylococcal DNA and induce IFN α transcription [Fig. 5.40 C]. *S. aureus*-mediated TNF α production signals via a conventional signaling pathway comprising the myddosome-TRAF6-TAK1 components, leading to activation of canonical IKKs and NF κ B-dependent gene transcription [Fig. 5.40 A]. Our data show that LTA- and *S. aureus*-stimulated type I IFN induction is dependent on clathrin-dependent formation of vesicles, Syk-mediated internalization, actin-dependent trafficking and endosomal maturation, while TNF α production is not affected by inhibition of either of these processes [Fig. 5.40 B]. We show that Mal, MyD88 and partially TRIF adaptor proteins play roles during *S. aureus*-stimulated type I IFN induction from the endosomes. In addition, our results suggest that signaling from the endosome leads to assembly of the myddosome and activation of TRAF6. During both TNF α and type I IFN signaling, literature suggests that Mal is present in the plasma membrane prior to the activation of PRRs [44]. After activation, Mal triggers assembly of the myddosome according to its location or it directly binds to TRAF6 and triggers an alternative signaling pathway [273]. In either case, TRAF6 mediates activation of TAK1 possibly via TABs. Our results further suggest that TAK1 kinase activity induces noncanonical TBK1/IKK ϵ via activation of IKK β or directly via TAK1 kinase activity. The noncanonical IKKs can in turn inhibit canonical IKKs, and thus creating a negative feedback loop. Our results suggest that TBK1/IKK ϵ phosphorylate a yet unknown member of IRF, which induces IFN β transcription. Alternatively, NF κ B could contribute to the initial induction of IFN β transcription. We also show that IRF7 is upregulated in response to TLR2 stimulation; however, this might be a result of the second wave of type I IFN induction. In addition, our results show a possibility that IRF7 can be also involved in TNF α production.

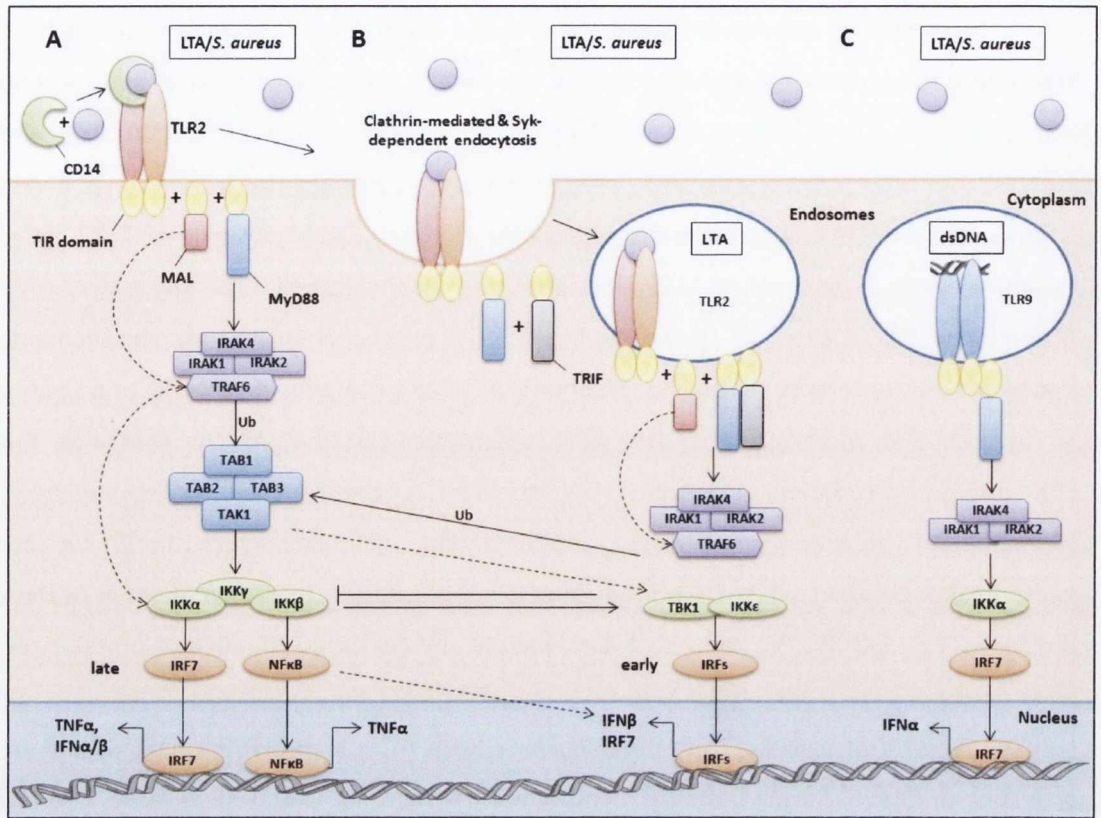


Figure 5.40: Endosomal TLR2-dependent type I IFN induction in human monocytes.

A: *S. aureus* is recognized by TLR2 in cooperation with CD14, which binds LTA. Activated TLR2 recruits Mal and MyD88 adaptor proteins, leading to the assembly of the myddosome. The myddosome mediates activation of TRAF6 via IRAKs kinase activity or alternatively, Mal binds TRAF6 and mediates its activation. TRAF6 then activates TABs, which in turn facilitate TAK1 phosphorylation, leading to IKK complex activation, release of NfκB, and induction of TNFα transcription. **B:** Upon recognition of *S. aureus* by TLR2, the pathogen-receptor complex is internalized via clathrin-dependent and Syk-mediated endocytosis. TLR2 complex in the endosome recruits additional adaptor TRIF and induces assembly of the myddosome and activation of TRAF6. TRAF6 then mediates TAK1 activation via TABs, leading to activation of noncanonical kinases TBK1 and/or IKKε by kinase activity of IKKβ or TAK1. The noncanonical kinases can inhibit canonical IKKs, thus creating a negative feedback loop. TBK1/IKKε kinases phosphorylate a yet unknown IRF, which translocates to the nucleus and induces transcription of IFNβ. Alternatively, activated NfκB can contribute to the initial IFNβ transcription. **C:** In the absence of TLR2, endosomal TLR9 can recognize staphylococcal DNA and activate IKKα via the IRAKs activity within the myddosome. IKKα then directly phosphorylates IRF7, leading to its translocation to the nucleus and induction of IFNα transcription. Ub – ubiquitination, dashed arrows – hypothetical interactions.

6. Result IV: Mechanisms of *S. aureus*-mediated IL-1 β and IL-10 induction in human monocytes

6.1 Introduction

In previous chapters, we have demonstrated that THP-1 monocytes efficiently kill *S. aureus* *in vitro*. In addition, we have investigated *S. aureus*-mediated signaling pathways, leading to TNF α and type I IFN production in human monocytes. However, immune responses are not limited to these cytokines; therefore, in this chapter we explore the induction of other physiologically relevant cytokines, IL-1 β and IL-10, during *S. aureus* infection in human monocytes.

Innate recognition of *S. aureus* activates a wide range of pattern recognition receptors, including the intracellular NOD-like receptor protein 3 (NLRP3). NLRP3, together with proteins ASC and pro-caspase 1, forms a cytoplasmic oligomeric complex known as the inflammasome, which plays a critical role in initiating innate immune responses. Activation of inflammasome culminates in IL-1 β and always requires two signals. The first signal comes from PRRs, such as TLR4 activated by LPS, and initiates pro-IL-1 β gene transcription. The second signal, such as ATP, activates the cleavage of inactive pro-caspase 1 into an active form within the inflammasome. Consequently, active caspase 1 cleaves pro-IL-1 β into mature protein [Fig. 6.14]. It has been shown that *S. aureus* can activate the NLRP3 inflammasome in monocytes and macrophages, leading to caspase 1 activation, IL-1 β secretion and induction of necrotic cell death [276]. *S. aureus* functions as a stimulus for NLRP3 activation; however, the nature of stimulating signals is yet unknown [277]. It has been reported that permeabilization and release of lysosomal protease, cathepsin B, are important mechanisms of the NLRP3 activation during *S. aureus* infection. Consequently, staphylococcal α - and γ -toxins have been shown to permeabilize lysosomal membrane releasing cathepsin B [277]. In addition, α -toxin can permeabilize plasma membrane causing potassium efflux, which triggers inflammasome activation [278]. β -toxin has been involved in staphylococcal escape from phagosomes, thus further contributing to the cathepsin release [279]. In addition, leukotoxin A/B (LukAB) has been shown to kill human primary phagocytes, including neutrophils, monocytes, macrophages, and dendritic cells [171]. Accordingly, purified LukAB has been sufficient to activate caspase 1, to induce IL-1 β secretion, and to cause necrotic cell death in both THP-1 and primary CD14⁺ human monocytes [280]. Moreover, it has been shown that IL-1 β is required for neutrophil recruitment to the site of infection during cutaneous *S. aureus* infection in mice, suggesting a significant contribution of IL-1 β to host defences against *S. aureus* [281].

IL-10 is, on the other hand, an important anti-inflammatory cytokine, which is produced by myeloid and lymphoid cells. During infection, IL-10 inhibits activity of Th1 cells, NK cells, and macrophages, all of which are required for pathogen clearance; however, they can also contribute to tissue damage. It is not surprising that pathogens have evolved mechanisms of manipulating IL-10 production in order to suppress antimicrobial immune responses. It has been observed that *Candida albicans* induces IL-10 production through TLR2-mediated signaling, which consequently leads to survival of regulatory T cells and immunosuppression [282]. This and other reports have linked the down-modulatory effects of TLR2 signaling to the adaptive regulatory T cells. However, Chau and colleagues associated the TLR2-mediated immunoregulatory effects with APCs, including monocytes, macrophages and dendritic cells [220]. In addition, they were unable to detect TLR2 expression on resting human peripheral blood T cells in contrast to mouse T cells. Finally, they proposed that TLR2 triggers canonical IL-10 induction via NF κ B activation followed by apoptosis of APCs cells, thus indirectly inhibiting T cell activation [220]. Interestingly, it has been reported that *S. aureus* peptidoglycan triggered distinct immune responses in monocyte-derived cells [218]. In monocyte-derived macrophages, peptidoglycan triggered predominantly IL-10 production and weak Th17 response, while it induced robust Th1/Th17 response in monocyte-derived dendritic cells [218]. In contrast to proinflammatory responses, the modulatory IL-10 response was CD14- and CD36-independent but required activation of PI3K [218]. These results suggest that polarization of APCs can lead to distinct adaptive immune responses to *S. aureus*. Collectively, it is recognized that TLR2-mediated signalling can contribute to anti-inflammatory responses; however, the type of APC on which TLR2 signaling occurs may be a crucial factor for cytokine production and induction of apoptosis.

Hence, in this chapter we will briefly investigate the signaling components involved in induction of IL-1 β and IL-10 cytokines during *S. aureus* infection in human monocytes. The research on cytokine induction was mainly based on mice models of *S. aureus* infection; however, there are considerable differences between the natures of the hosts. First, mice are not natural host for human *S. aureus* isolates; therefore, genetic alterations of mice are required in order to sensitize mice for infection [184]. Second, whereas the induction of protective immunity in mice is based on opsonic antibodies, the mechanisms of protective immunity in humans are still unclear [227]. Lastly, the activation of the inflammasome requires two signals in mouse macrophages; however, one signal is sufficient for the inflammasome induction in human monocytes [251].

6.2 Results

6.2.1 TLR2-mediated induction of IL-1 β in human monocytes

In previous chapter, we explored *S. aureus*-stimulated signaling pathways leading to the production of TNF α and type I IFNs in human monocytes. However, recognition of *S. aureus* by immune cells triggers production of variety of cytokines in order to orchestrate immune responses against invading pathogens. Prior to immune stimulation, THP-1 monocytes have been shown to express high levels of NLRP3, ASC, and pro-caspase 1, all of which are components of the inflammasome [283]. Therefore, THP-1 monocytes are extensively used in the study of inflammasome activation and mechanism of cell death *in vitro*. In chapter 3, we showed that both LTA alone and *S. aureus* were sufficient to induce IL-1 β release in human monocytes. IL-1 β maturation is an important marker of inflammasome activation; hence, in this subchapter, we will briefly investigate the signalling pathway of IL-1 β release in response to LTA and *S. aureus* in human monocytes. Of interest, we observed a significant variability in cytokine induction among individual blood donors; however, the effect of inhibitors was consistent throughout the experiments.

Initially, the role of TLR2 in release of IL-1 β protein was investigated. Induction of IL-1 β release was significantly impaired in response to LTA stimulation and *S. aureus* infection after blocking TLR2 function in both THP-1 and CD14 $^{+}$ monocytes when compared to monocytes treated with isotype control [Fig. 6.1 A-D]. In addition, IL-1 β release was decreased after Malp2 and LTA stimulation in TLR2 deficient monocytes when compared to wild type THP-1 monocytes [Fig. 6.2 A]. Similarly to the ligand stimulation, *S. aureus*-induced IL-1 β production was severely impaired in the absence of TLR2 when compared to wild type THP-1 monocytes [Fig. 6.2 B]. These results demonstrate that *S. aureus*-stimulated IL-1 β release is TLR2-dependent in human monocytes.

Next, we examined an effect of endosomal inhibitors on IL-1 β release. Treatment with bafilomycin A1 significantly enhanced LTA-induced IL-1 β induction 6 hr after infection when compared to DMSO treated THP-1 monocytes [Fig. 6.3 A]. However, in CD14 $^{+}$ monocytes, bafilomycin A1 treatment did not affect LTA-stimulated IL-1 β release 18 hr after stimulation when compared to DMSO treated monocytes [Fig. 6.3 B]. In the context of *S. aureus* infection, IL-1 β was significantly upregulated 3 hr after infection in bafilomycin A1-treated monocytes when compared to DMSO-treated THP-1 monocytes [Fig. 6.3 C]. Bafilomycin A1 treatment had no effect on *S. aureus*-stimulated IL-1 β production 6 and 18 hr after infection; however, it significantly impaired IL-1 β release 24 and 48 hr after infection when compared to DMSO-

treated THP-1 monocytes [Fig. 6.3 C]. In CD14⁺ monocytes, treatment with bafilomycin A1 significantly upregulated IL-1 β protein release 3 and 6 hr after *S. aureus* infection, while IL-1 β release was significantly impaired 18 hr after infection, and no differences in protein levels were observed 24 and 48 hr after infection when compared to DMSO-treated monocytes [Fig. 6.3 D]. These results demonstrate that the endosomal processing is not required for LTA-stimulated IL-1 β induction in human monocytes. In addition, IL-1 β release is independent of the endosomal processing during early stages of *S. aureus* infection in human monocytes. Therefore, this result suggests that the mechanism of IL-1 β release is different from the type I IFN pathway and is independent of the endosomal pathway.

Treatment with Syk kinase inhibitor, piceatannol, did not affect LTA- and *S. aureus*-induced IL-1 β release 6 hr after challenge in THP-1 monocytes when compared to DMSO-treated THP-1 monocytes [Fig. 6.4 A, C]. However, treatment of CD14⁺ monocytes with piceatannol significantly decreased LTA- and *S. aureus*-stimulated IL-1 β protein 18 hr after challenge when compared to DMSO-treated monocytes [Fig. 6.4 B, D]. This result shows that Syk kinase might play distinct roles during TLR2-stimulated IL-1 β induction in THP-1 and CD14⁺ monocytes. The distinct effect of piceatannol on IL-1 β induction might also reflect different times of incubation. Whereas THP-1 monocytes were incubated for 6 hr, CD14⁺ monocytes were incubated for 18 hr after LTA stimulation or *S. aureus* infection.

On the other hand, treatment with VIPER significantly impaired LTA- and *S. aureus*-induced IL-1 β release in both THP-1 and CD14⁺ monocytes when compared to CP7-treated monocytes [Fig. 6.5 A-D]. This result suggests that Mal and/or TRAM adaptor proteins are involved in TLR2-stimulated IL-1 β protein induction. In addition, LTA- and *S. aureus*-stimulated IL-1 β release was significantly decreased after treatment with IKK β inhibitor in both THP-1 and CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig. 6.6 A-D]. Lastly, treatment with MRT67307 or MRT199665 upregulated LTA- and *S. aureus*-induced IL-1 β release compared to DMSO-treated THP-1 monocytes [Fig. 6.7 A-B]. These results demonstrate that Ikk β is required for IL-1 β release by both THP-1 and CD14⁺ monocytes, and that IKK-related kinases are redundant for IL-1 β release in THP-1 monocytes. Collectively, these results demonstrate that mechanisms of IL-1 β release are similar in THP-1 and CD14⁺ monocytes; however, distinct from induction of type I IFNs.

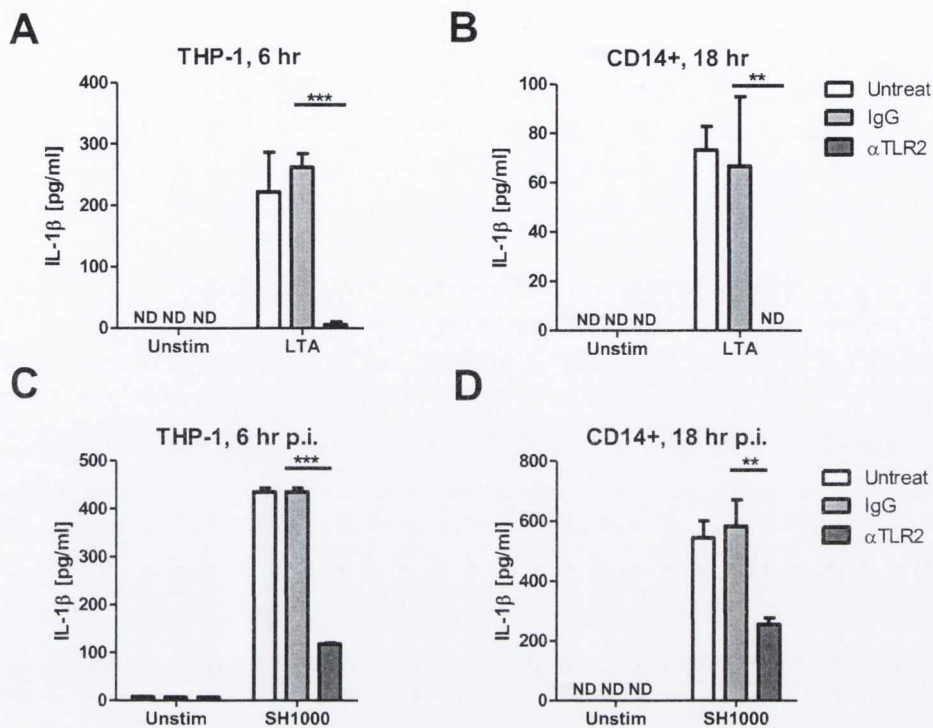


Figure 6.1: TLR2 is required for LTA- and *S. aureus*-induced IL-1 β induction in human monocytes.

A-B: THP-1 cells (A) or CD14⁺ monocytes (B) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with α TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A) or 18 hr (B). **C-D:** THP-1 cells (C) or CD14⁺ monocytes (D) were seeded at 1×10^6 cells/ml into 24-well plate prior to infection. Cells were pre-treated with α TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (C) or at an MOI of 1 (D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (C) or 18 hr (D). **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with isotype control as determined by Student *t* test.

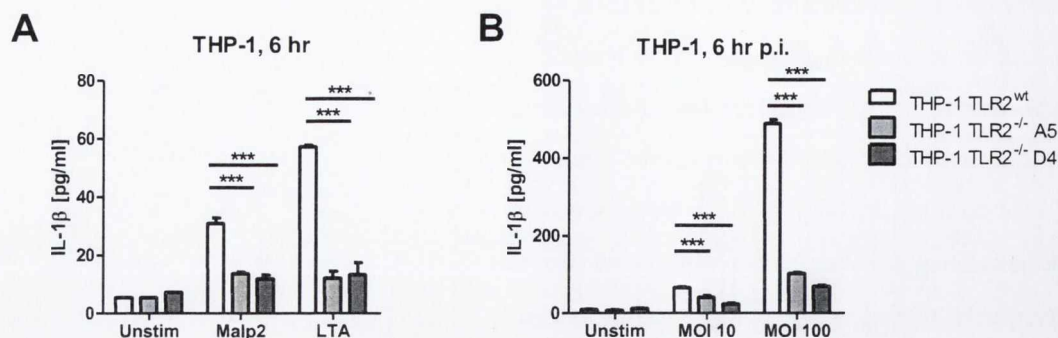


Figure 6.2: IL-1 β response to TLR2 ligands and *S. aureus* is impaired in TLR2 knockout THP-1 monocytes.

Wild type THP-1 cells and THP-1 TLR2 CRISPR knockout cell clones A5 and D4 were seeded at 0.5×10^6 cells/ml in 96-well plate 24 hr prior to stimulation (A) or at 1×10^6 cells/ml into 24-well plate prior to the infection (B). Cells were stimulated with Malp2 (50 ng/ml) or LTA (2.5 μ g/ml) for 6 hr (A) or infected with *S. aureus* strain SH1000 at an MOI of 10 or 100 for 2 hr (B) followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, *** $p < 0.001$, when compared with stimulated wild type cells as determined by Student *t* test.

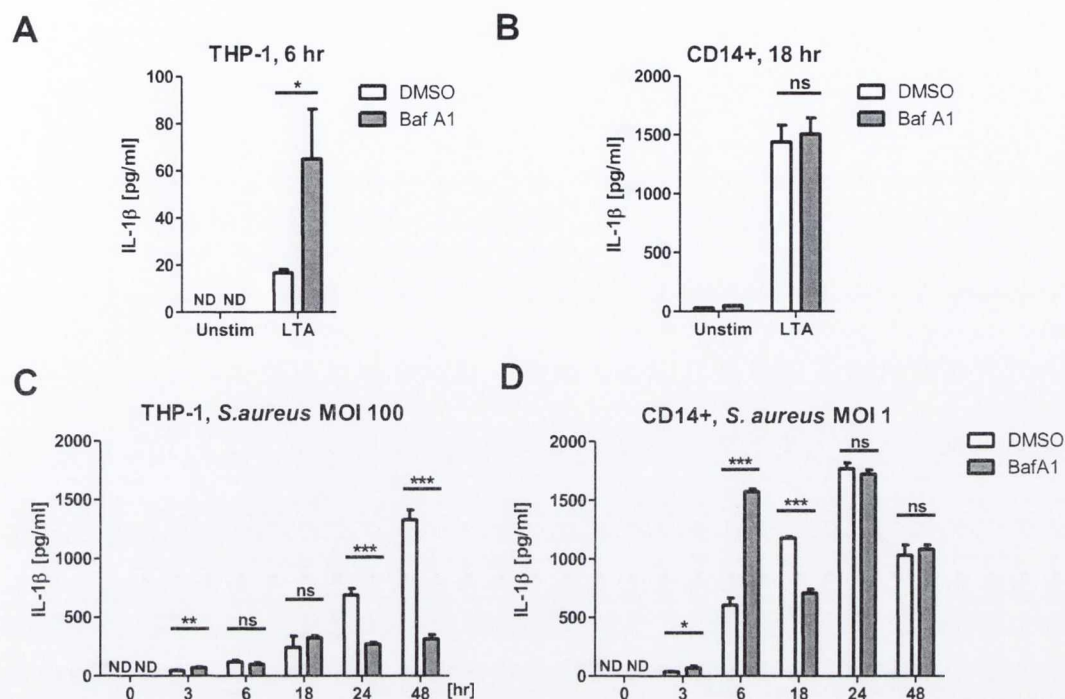


Figure 6.3: Endosomal processing is not required for IL-18 induction in response to *S. aureus* and LTA in human monocytes.

A-B: THP-1 cells (A) or CD14⁺ monocytes (B) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A) or 18 hr (B). **C-D:** THP-1 cells (C) or CD14⁺ monocytes (D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with bafilomycin A1 (100 nM) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (C) or at an MOI of 1 (D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 3, 6, 18, 24 or 48 hr (D-F). **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

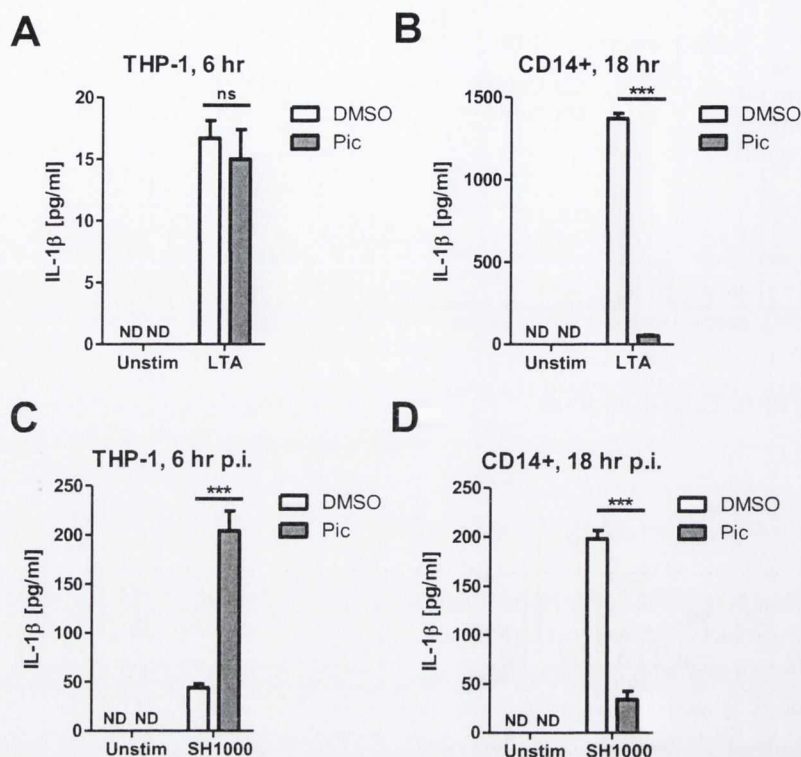


Figure 6.4: Role of Syk kinase in LTA- and *S. aureus*-stimulated IL-1 β induction in human monocytes.

A-B: THP-1 cells (A) or CD14+ monocytes (B) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with piceatannol (20 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A) or 18 hr (B). **C-D:** THP-1 cells (C) or CD14+ monocytes (D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with piceatannol (20 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (C) or at an MOI of 1 (D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (C) or 18 hr (D). **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns – not significant, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

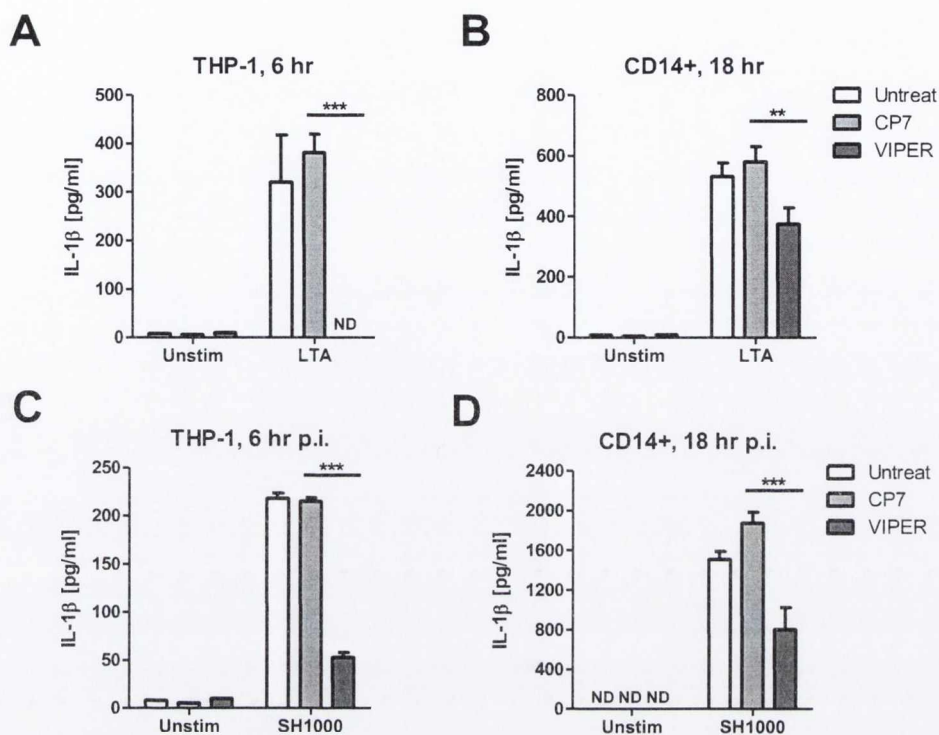


Figure 6.5: IL-1 β induction is VIPER sensitive in human monocytes.

A-B: THP-1 cells (A) or CD14 $^{+}$ monocytes (B) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) or left untreated 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A) or 18 hr (B). **C-D:** THP-1 cells (C) or CD14 $^{+}$ monocytes (D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) or left untreated 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (C) or at an MOI of 1 (D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (C) or 18 hr (D). **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with CP7 as determined by Student *t* test.

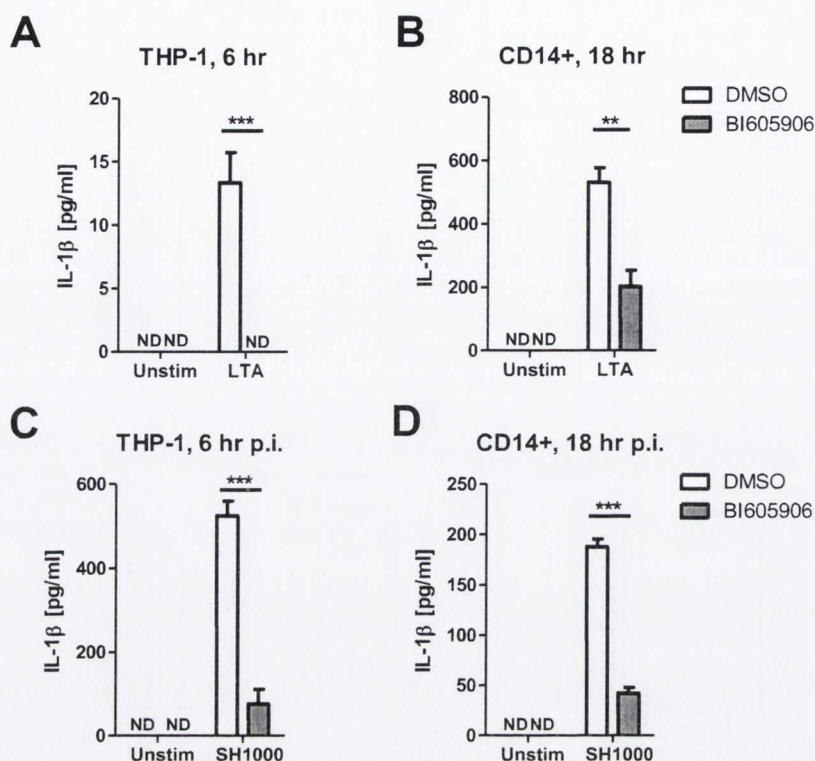


Figure 6.6: IL-1 β induction requires Ikk β in human monocytes.

A-B: THP-1 cells (A) or CD14 $^{+}$ monocytes (B) were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation. Cells were pre-treated with BI605906 (10 μ M) or equivalent amount of DMSO 1 hr prior to stimulation. Cells were stimulated with LTA (2.5 μ g/ml) for 6 hr (A) or 18 hr (B). **C-D:** THP-1 cells (C) or CD14 $^{+}$ monocytes (D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with BI605906 (10 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (C) or at an MOI of 1 (D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (C) or 18 hr (D). **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student t test.

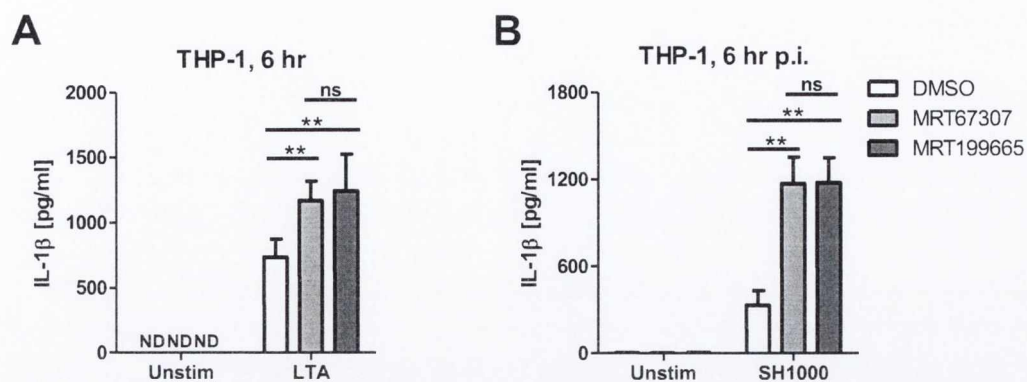


Figure 6.7: IL-1 β induction in human monocytes does not require IKK-related kinases in THP-1 monocytes.

THP-1 cells were seeded at 0.5×10^6 cells/ml into 96-well plate 24 hr prior to stimulation (B) or at 1×10^6 cells/ml into 24-well plate prior to the infection (B). Cells were pre-treated with MRT67307 (2 μ M), MRT199665 (1 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were stimulated with LTA (2.5 μ g/ml, A) for 6 hr or infected with *S. aureus* strain SH1000 at an MOI of 100 (B) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr. **A-D:** Supernatants were harvest by centrifugation at 100 g for 5 min. IL-1 β protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, $**p < 0.01$, when comparing DMSO and MRT67307, DMSO and MRT199665, or MRT67307 and MRT199665 treated infected or stimulated samples as determined by two-way ANOVA with a Bonferroni posttest.

6.2.2 *S. aureus*-stimulated IL-10 induction in human monocytes

IL-10 protein is an important anti-inflammatory cytokine produced by macrophages and dendritic cells during bacterial infections. We have previously shown that IL-10 is produced by CD14⁺ monocytes and to a lesser extent by THP-1 monocytes, during *S. aureus* infection. Therefore, in this subchapter, we will briefly examine the signaling components of *S. aureus*-mediated IL-10 induction in human monocytes. Of interest, we observed a significant variability in cytokine induction among individual blood donors; however, the effect of inhibitors was consistent throughout the experiments.

Surprisingly, treatment with TLR2 neutralizing antibody did not affect *S. aureus*-stimulated IL-10 induction when compared to isotype control treated THP-1 or CD14⁺ monocytes [Fig. 6.8 A-B]. Similarly, LTA-stimulated IL-10 production was not affected in the absence of TLR2, while Malp2-induced IL-10 protein was enhanced when compared to wild type THP-1 monocytes [Fig. 6.9 A]. The absence of TLR2 did not affect IL-10 production after infection with *S. aureus* at an MOI of 10; however, IL-10 protein was significantly upregulated after infection with *S. aureus* at an MOI of 100 when compared to wild type THP-1 monocytes [Fig. 6.9 B]. Importantly, we previously showed the functional effect of the TLR2 absence on type I IFN and proinflammatory responses to ligand stimulation [Fig. 5.4 A-B] as well as to *S. aureus* infection [Fig. 5.5 A-C]. Hence, these results show that TLR2 is not required for IL-10 induction by human monocytes in response to *S. aureus*.

Inhibition of the endosomal pathway showed distinct effects on IL-10 induction between the cell types. In THP-1 monocytes, treatment with either bafilomycin A1 or piceatannol did not affect *S. aureus*-induced IL-10 production when compared to DMSO-treated THP-1 monocytes [Fig. 6.10 A, C]. In contrast to THP-1 monocytes, treatment with either bafilomycin A1 or piceatannol significantly impaired IL-10 induction after *S. aureus* infection in CD14⁺ monocytes when compared to monocytes treated with DMSO [Fig. 6.10 B, D]. This result suggests that IL-10 induction is differentially regulated in THP-1 and CD14⁺ monocytes. VIPER treatment did not impair *S. aureus*-stimulated IL-10 induction in THP-1 monocytes; however, it significantly decreased *S. aureus*-induced IL-10 protein in CD14⁺ monocytes when compared to CP7-treated monocytes [Fig. 6.11 A-B]. This shows that Mal and/or TRAM are not required for IL-10 induction in THP-1 monocytes, whereas they play a role in IL-10 release in CD14⁺ monocytes. On the other hand, inhibition of IKK β impaired *S. aureus*-mediated IL-10 induction in both THP-1 and CD14⁺ monocytes when compared to DMSO-treated monocytes [Fig. 6.12 A-B]. This result suggests that IKK β plays a central role

in signaling pathways in both monocytes. Lastly, treatment with MRT67307 or MRT199665 did not affect *S. aureus*-induced IL-10 protein in THP-1 monocytes when compared to DMSO-treated cells [Fig. 6.13 A]. In CD14⁺ monocytes, treatment with MRT67307 upregulated *S. aureus*-stimulated IL-10 induction compared to MRT199665-treated monocytes [Fig. 6.13 B]. However, IL-10 induction was unaffected after MRT67307 or MRT199665 treatment when compared to DMSO-treated monocytes. These results suggest that IKK related kinases are redundant for IL-10 induction in THP-1 monocytes, while kinases other than AMP- and IKK-related kinases play role in IL-10 production in CD14⁺ monocytes. Collectively, these results demonstrate that IL-10 induction utilizes distinct pathways in THP-1 and CD14⁺ monocytes. The low degree of IL-10 activation in THP-1 monocytes might also imply that IL-10 pathway is significantly altered in THP-1 monocytes compared to CD14⁺ monocytes.

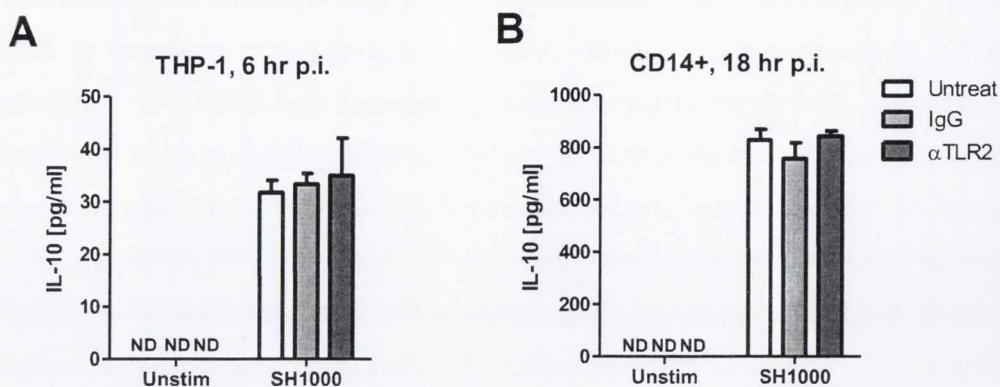


Figure 6.8: TLR2 is not required for *S. aureus*-induced IL-10 induction in human monocytes.

THP-1 cells (A) or CD14⁺ monocytes (B) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with α TLR2 antibody (2.5 μ g/ml), IgG1 isotype control antibody (2.5 μ g/ml) or left untreated 1 hr prior to stimulation. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A) or at an MOI of 1 (B) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A) or 18 hr (B) followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, when compared with stimulated cells treated with isotype control as determined by Student *t* test.

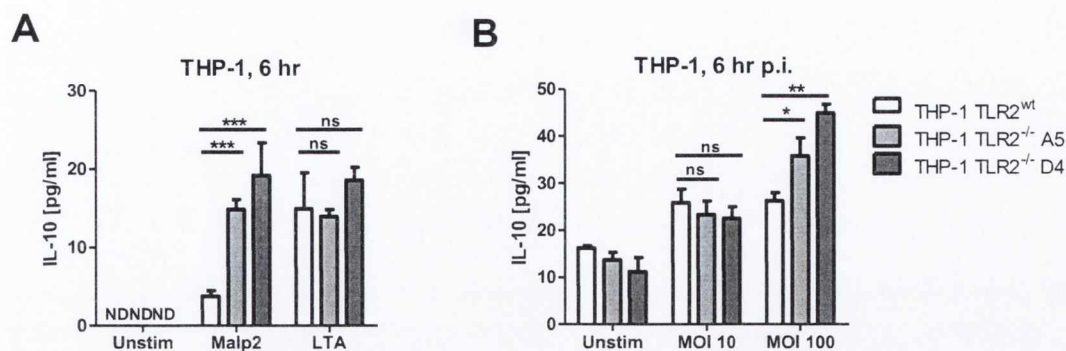


Figure 6. 9: IL-10 response to TLR2 ligands and live *S. aureus* is not impaired in TLR2 knockout cells.

Wild type THP-1 cells and THP-1 TLR2 CRISPR knockout cells A5 and D4 were seeded at 0.5×10^6 cells/ml in 96-well plate 24 hr prior to stimulation (A) or at 1×10^6 cells/ml into 24-well plate prior to the infection (B). Cells were stimulated with Malp2 (50 ng/ml) or LTA (2.5 μ g/ml) for 6 hr (A) or infected with *S. aureus* strain SH1000 at an MOI of 10 or 100 for 2 hr (B) followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, when compared with stimulated wild type cells as determined by Student *t* test.

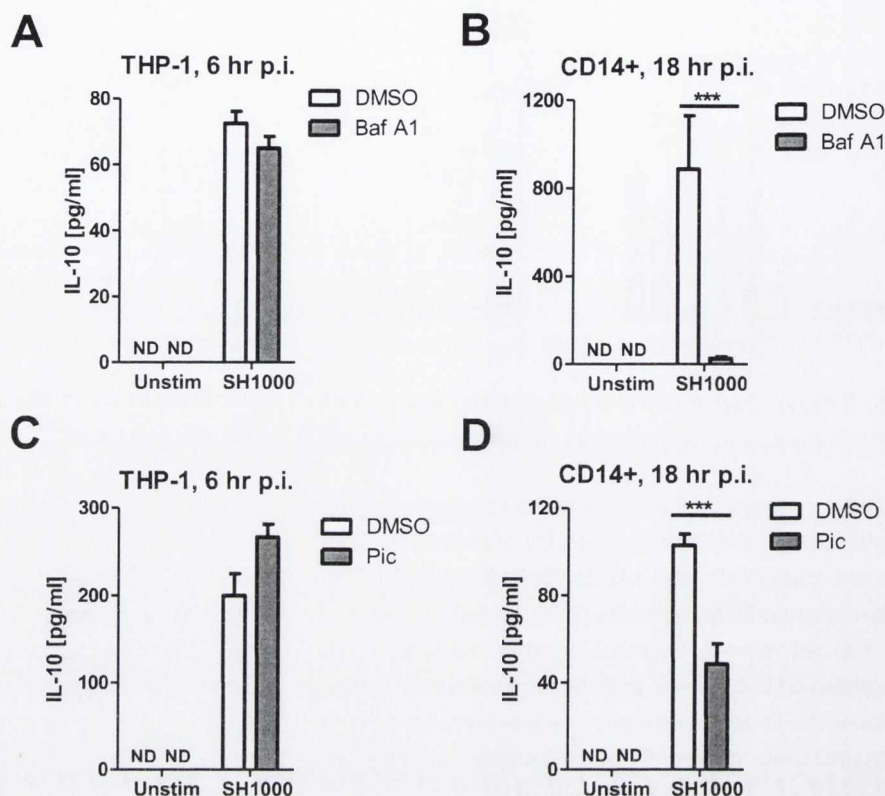


Figure 6.10: Inhibition of endosomal processing or Syk kinase activity impairs IL-10 induction in response to *S. aureus* in time-dependent manner in human monocytes.

THP-1 cells (A, C) or CD14+ monocytes (B, D) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with bafilomycin A1 (100 nM, A-B) or piceatannol (20 μ M, C-D) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A, C) or at an MOI of 1 (B, D) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A, C) or 18 hr (B, D) followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns - not significant, * $p < 0.05$, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

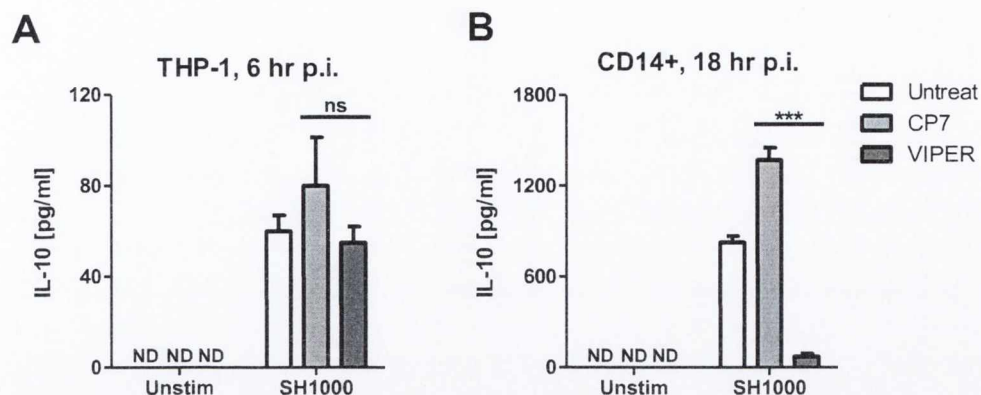


Figure 6.11: IL-10 induction is sensitive to VIPER in CD14+ monocytes but not in THP-1 monocytes.

THP-1 cells (A) or CD14+ monocytes (B) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with VIPER (5 μ M), CP7 (5 μ M) or left untreated 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A) or at an MOI of 1 (B) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A) or 18 hr (B) followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

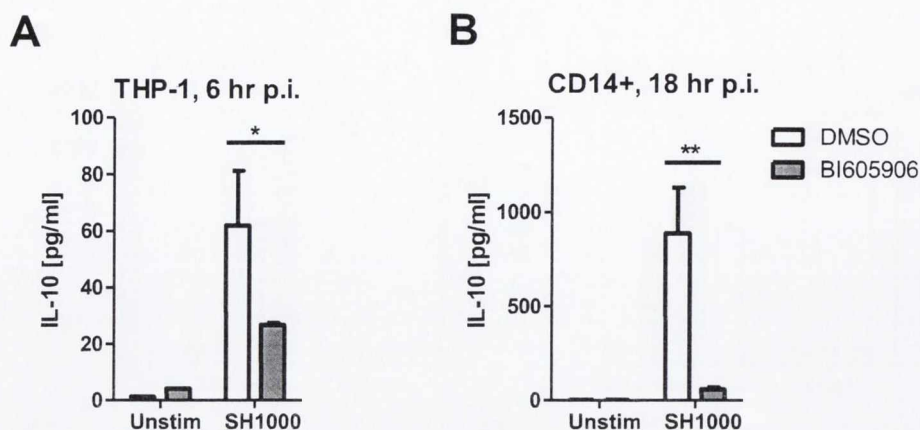


Figure 6.12: IL-10 induction requires *Ikkβ* kinase in human monocytes.

THP-1 cells (A) or CD14⁺ monocytes (B) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with BI605906 (10 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A) or at an MOI of 1 (B) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A) or 18 hr (B) followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ** $p < 0.01$, *** $p < 0.001$, when compared with infected samples treated with DMSO as determined by Student *t* test.

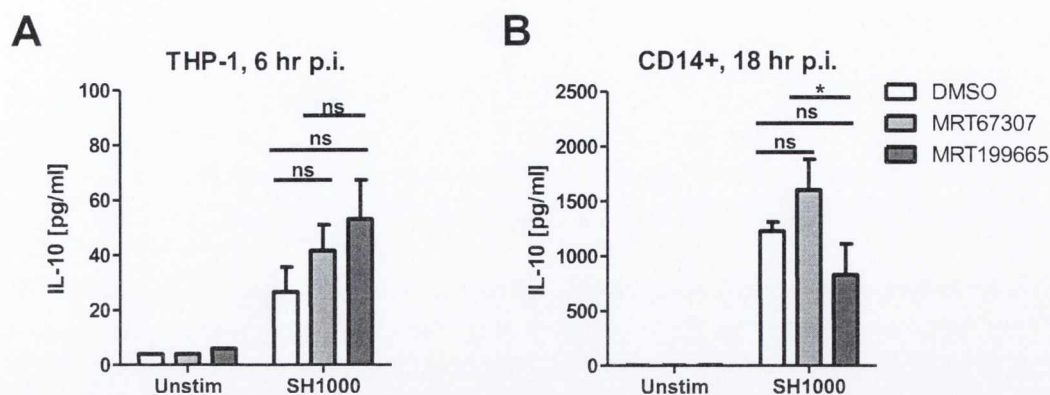


Figure 6.13: IL-10 induction does not require IKK-related kinases in THP-1 monocytes.

THP-1 cells (A) or CD14+ monocytes (B) were seeded at 1×10^6 cells/ml into 24-well plate prior to the infection. Cells were pre-treated with MRT67307 (2 μ M), MRT199665 (1 μ M) or equivalent amount of DMSO 1 hr prior to infection. Cells were infected with *S. aureus* strain SH1000 at an MOI of 100 (A) or at an MOI of 1 (B) for 2 hr followed by gentamicin (200 μ g/ml) treatment. The cells were further incubated for the total of 6 hr (A) or 18 hr (B) followed by collection of supernatants by centrifugation at 100 g for 5 min. IL-10 protein was measured by standard ELISA with appropriate dilutions. Data are mean $\pm$ SD of triplicate samples and are representative of three independent experiments. ND - not detected, ns – not significant, * $p < 0.05$, when comparing DMSO- and MRT67307-, DMSO- and MRT199665-, or MRT67307- and MRT199665-treated and infected samples as determined by two-way ANOVA with a Bonferroni posttest.

6.3 Discussion

6.3.1 TLR2-mediated induction of IL-1 β in human monocytes

IL-1 β is an inflammatory cytokine, whose production is tightly controlled. Secretion of IL-1 β is dependent on activation of a cytoplasmic oligomeric complex known as the inflammasome. The inflammasome comprises of NLRP3, ASC and pro-caspase 1 proteins and IL-1 β release requires two independent signals. The cell wall of Gram-positive bacteria contains many components that are recognized by the innate immune system. However, it is not yet clear whether single cell wall components, such as muramyl dipeptide (MDP) or peptidoglycan (PGN), are sufficient to activate inflammasome and IL-1 β secretion. It has been reported that phagocytosis and degradation of PGN were necessary to activate NLRP3 inflammasome and IL-1 β secretion during *S. aureus* infection of mouse macrophages [284]. In addition, permeabilization of cell membranes and release of lysosomal proteases into the cytosol by staphylococcal toxins has been shown to contribute to inflammasome activation during *S. aureus* infection [277]. Hence, cell wall components together with membrane permeabilization are potent stimuli for the NLRP3 inflammasome activation, IL-1 β secretion and induction of necrotic cell death in mouse macrophages. However, human monocytes have been shown to express high levels of NLRP3, ASC, and pro-caspase 1 prior to the immune stimulation [283]. Accordingly, *S. aureus* leukotoxin LukAB alone was sufficient to activate caspase 1, induce IL-1 β and cause necrotic cell death in THP-1 and human primary monocytes [280].

In line with Melehani and colleagues [280], we observed strong IL-1 β release by monocytes after stimulation with purified *S. aureus* LTA as well as after *S. aureus* infection. Therefore, our data show that recognition of a single cell wall component by TLR2 provides a signal sufficient for inflammasome activation in human monocytes in contrast to mouse macrophages. It has been shown that activation of caspase 1 and release of IL-1 β was dependent on the endosomal processing and was independent of TLR signaling in BMDMs [285]. However, we observed strong dependency between IL-1 β release and TLR2 in human monocytes, indicating signaling differences between mouse and human hosts, and between monocytes and macrophages. In contrast to Shimada [284] and Sokolovska [285], we did not observe requirement for the endosomal processing in IL-1 β induction after LTA stimulation and during early stages of *S. aureus* infection. Even though the phagosomal processing was important for IL-1 β production later during *S. aureus* infection in THP-1 monocytes, it was redundant in primary monocytes. Therefore, our results show that endosomal maturation does not play a major role in IL-1 β release by human monocytes in contrast to mouse BMDMs.

However, we noted that IL-1 β release was dependent on Syk kinase activity in primary monocytes, while Syk kinase function was not required for IL-1 β release in THP-1 monocytes. Hence, our data suggest that Syk kinase might be involved in downstream TLR2 signaling pathways. Alternatively, this might be a result of different requirement for Syk kinase in TLR2-mediated IL-1 β induction over time. Moreover, we observed that LTA- and *S. aureus*-mediated IL-1 β induction was sensitive to VIPER in human monocytes, indicating involvement of Mal and/or TRAM adaptor proteins downstream of TLR2. Finally, we noted that IL-1 β release was dependent on canonical IKK β but not on noncanonical TBK1/IKK ϵ in human monocytes.

In summary, our results present evidence that IL-1 β release is TLR2- and IKK β -dependent in response to LTA and *S. aureus* in human monocytes [Fig. 6.14 A]. In addition, Mal and/or TRAM play a role downstream of TLR2 signaling. Our data further demonstrates that stimulation of human monocytes with staphylococcal lipoprotein alone provides a signal sufficient for both inflammasome activation and IL-1 β release. In addition, there are significant differences in IL-1 β induction between human monocytes and mouse macrophages [Fig. 6.14 B]. IL-1 β plays an important role in the host defence against *S. aureus* by recruiting neutrophils to the site of infection and by facilitating staphylococcal clearance in mice [281]. It has been shown that IL-1 β signaling is required for *S. aureus*-mediated activation of Th17 cells. However, preceding influenza infection significantly attenuates IL-1 β production, decreases Th17 activation and increases mice mortality [286]. Therefore, this study suggests a novel mechanism of increased susceptibility to and a poorer outcome of secondary bacterial infections after preceding influenza infection. Since a major component of immune responses to influenza infection is activation of type I IFN signaling, type I IFNs might mediate the suppression of IL-1 β and attenuation of Th17 cells. Hence, it would be interesting to address the role of type I IFNs on IL-1 β release and IL-17 production during *S. aureus* infection of human monocytes.

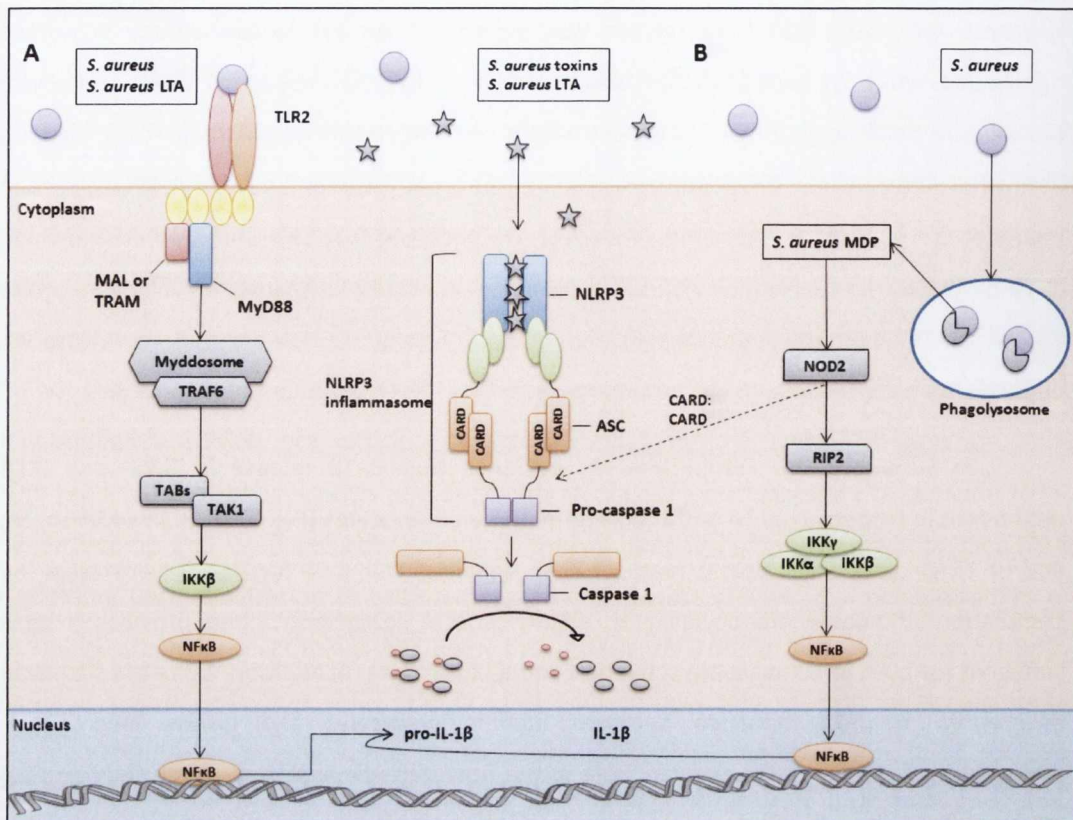


Figure 6.14: Mechanisms of *S. aureus*-mediated IL-1 β induction in human monocytes compared to mouse macrophages.

A: In human monocytes, *S. aureus* lipoteichoic acid (LTA) is recognized by surface TLR2. Activated TLR2 recruits Mal and/or TRAM and presumably MyD88. IKK β is phosphorylated probably by TAK1 kinase, which might be phosphorylated by TAB proteins. TABs might be activated by TRAF6, whose ubiquitin activation might be a result of the myddosome assembly. IKK β induces NF κ B translocation to the nucleus and transcription of pro-IL-1 β . In human monocytes, LTA is sufficient to activate NLRP3 inflammasome and cleavage of pro-caspase 1. Caspase 1 then processes pro-IL-1 β into mature form and its release. During *S. aureus* infection, the bacterium triggers pro-IL-1 β transcription and maturation by cell wall LTA. In addition, IL-1 β maturation is enhanced by bacterial toxins, which further activate NLRP3 inflammasome and activation of caspase 1. **B:** In mouse macrophages, *S. aureus* undergoes phagocytosis and endosomal processing [285]. Peptidoglycan is processed in phagolysosomes into muramyl dipeptide (MDP). MDP leaks into the cytosol or alternatively, *S. aureus* escapes from phagolysosomes. In either case, MDP is recognized by intracellular NOD2 receptor, which triggers NF κ B activation via RIP2-IKK complex thus transcription of pro-IL-1 β . In a separate event, *S. aureus*-produced toxins trigger activation of NLRP3 inflammasome and activation of caspase 1. Alternatively, NOD2 might bind pro-caspase 1 through a CARD:CARD interaction and activate pro-caspase 1 cleavage (dashed arrow) [287]. Active caspase 1 then processes pro-IL-1 β into mature form, which is then released from the cells.

6.3.2 TLR2-mediated induction of IL-10 in human monocytes

Upon recognition of antigens, naïve monocytes mature into APCs and trigger T cell responses, which promote both humoral and cellular immunity. In some cases, microbes, such as *S. aureus*, can manipulate production of IL-10 by macrophages. Consequently, high amounts of IL-10 inhibit innate responses by reducing the release of proinflammatory cytokines, and by decreasing the expression of MHC class II on APCs, thus downregulating T cell activation. It has been reported that TLR2 deficient mouse macrophages have increased resistance to candidiasis due to decreased IL-10 production and diminished generation of regulatory T cells [282]. Furthermore, Pam3Csk4 has been shown to directly enhance proliferation of regulatory T cells resulting in a loss of suppressive phenotype in a MyD88-dependent fashion [288]. In contrast to T cells, *S. aureus* cell wall ligands have been shown to stimulate IL-10 induction and apoptosis in monocytes, thus indirectly inhibiting responses of regulatory T cells to staphylococcal superantigens [220]. In addition, it has been shown that monocyte-derived macrophages produce strong IL-10 in response to peptidoglycan, whereas monocyte-derived dendritic cells induce robust Th1/Th17 responses, suggesting that the type of APCs is an important factor in cytokine production [218]. Furthermore, in contrast to inflammatory responses, TLR2-mediated IL-10 production required PI3K and was independent of CD14 and CD36; hence, the polarization of APCs seems to also modulate signaling pathway [218].

In contrast to studies implicating a role of TLR2 in IL-10 production, we did not observe any requirement for TLR2 in *S. aureus*-induced IL-10 induction in human monocytes. This was consistent for both ligand and *S. aureus* stimulation; moreover, we noted that IL-10 protein was upregulated in the absence of TLR2. This might suggest that other receptor, such as NOD2, can recognize *S. aureus* structures and mediate IL-10 production. In addition, our data suggest that TLR2 signaling might inhibit IL-10 production. Our findings are consistent with a report showing that pro- and anti-inflammatory responses to *S. aureus* are independent of one another in human primary monocytes [289]. It has been shown that anti-inflammatory IL-10 responses were dependent on PI3K-Akt-mTOR and ERK pathways and did not require *S. aureus* internalization [Fig. 6.15 B; 289]. On the other hand, proinflammatory responses were mediated by p38 pathway and were dependent on phagocytosis of *S. aureus*. Hence, the authors proposed that the cell wall of *S. aureus* contains two types of TLR2 ligands inducing distinct immune responses [289].

Next, using inhibitors on downstream signaling, we observed considerable differences in IL-10 production between THP-1 and primary monocytes. Our data show that *S. aureus*-

mediated IL-10 induction does not require endosomal pathway and is independent of Mal/TRAM adaptor proteins in THP-1 monocytes. In primary monocytes, however, we observed that production of IL-10 was dependent on internalization and endosomal maturation of *S. aureus* and that IL-10 induction was mediated via Mal/TRAM adaptor proteins [Fig. 6.15 A]. However, our results are in contrast with the previous report showing that IL-10 is independent of the endosomal pathway in primary monocytes [289]. This might reflect differences between *S. aureus* strains since the time of the infection was equal. While Peres and colleagues used nasal *S. aureus* isolates obtained from patients [289], we used laboratory strain SH1000 [208]. Lastly, we observed that IL-10 production was dependent on canonical IKK β in both THP-1 and primary monocytes, suggesting that IKK β phosphorylation and presumably NF κ B activation are required for production of IL-10 cytokine. On the other hand, our data show that noncanonical IKKs are redundant for IL-10 induction in human monocytes.

In summary, our results present significant differences in IL-10 protein production between THP-1 and primary monocytes. We also noted that the level of IL-10 induction was significantly lower in THP-1 than in primary monocytes, indicating that the IL-10 pathway is altered or defective in THP-1 monocytes. In contrast to THP-1 monocytes, primary monocytes produce significant amounts of IL-10 protein, suggesting that proinflammatory responses are tightly regulated in primary monocytes. We propose that induction of IL-10 protein in human monocytes is triggered by receptor(s) other than TLR2. In contrast to Peres and colleagues [289], our data suggest that endosomal pathway is also required for anti-inflammatory responses in human primary monocytes. We also show that IL-10 induction requires Mal and/or TRAM adaptors; therefore, other TLR receptors, such as TLR4 or endosomal TLRs, might be involved in IL-10 induction. It has been shown that TLR4-dependent TRIF-TRAF3 signaling is involved in IL-10 production in mouse macrophages and mDCs [Fig. 6.15 C; 290]. However, in contrast to human monocytes, it is recognized that TLR2 signaling and TLR2-dependent activation of IKK complex and MAPKs have crucial roles in IL-10 production in mouse macrophages [290]. Lastly, our data show that IKK β and presumably NF κ B play a central role in immune signaling in human cells.

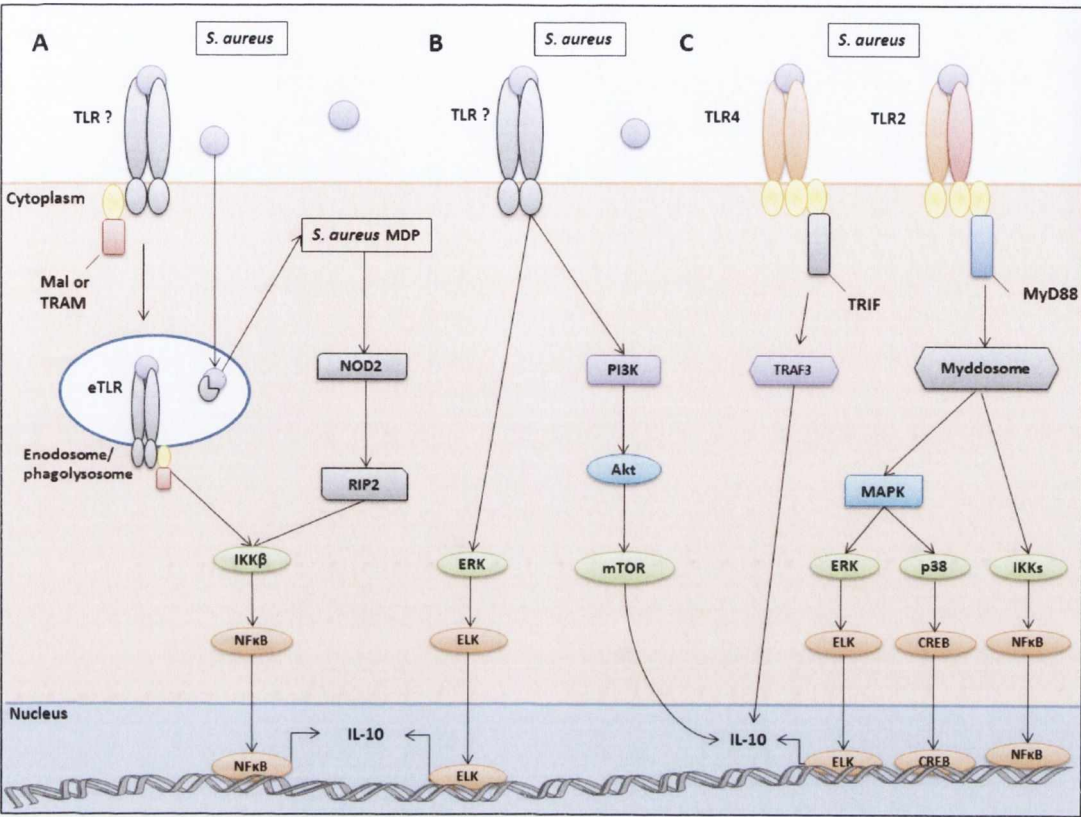


Figure 6.15: Mechanism of *S. aureus*-mediated IL-10 induction in human monocytes compared to mouse macrophages.

A: In human primary monocytes, *S. aureus* cell wall components are recognized by unknown surface TLR or by endosomal TLR receptor after phagocytosis of *S. aureus*. Alternatively, intracellular NOD2 detects muramyl dipeptide (MDP) from phagolysosome or on cytoplasm-dwelling *S. aureus*. IL-10 induction is triggered via IKK β and presumably via NF κ B activation. **B:** In addition, *S. aureus* can also activate PI3K-Akt-mTOR and ERK-ELK signaling pathways via unknown receptor, leading to induction of IL-10 transcription in human monocytes. **C:** In mouse macrophages and mDCs, IL-10 is triggered upon activation of TLR4 in response to bacterial cell wall components via TRIF-TRAF3 signaling pathway. Alternatively, TLR2 detects *S. aureus* and recruits MyD88 in mouse macrophages. MyD88 possibly triggers myddosome assembly and activates downstream signaling, leading to activation of IKK complex and MAPKs, including ERK and p38. Kinases then activate transcription factors NF κ B, ELK and CREB, respectively, and induce IL-10 transcription [290].

7. Final discussion

Staphylococcus aureus is a common commensal bacterium, which is carried by about 20 % of the population permanently in their upper respiratory tract and on their skin [234]. However, *S. aureus* is also an important human pathogen causing significant community-acquired infections as well as life-threatening nosocomial infections. The importance of the carriers is elevated by the appearance of antibiotic resistant strains and by the ease of *S. aureus* transmission within the community. In addition, the long-term exposure of immune cells to *S. aureus* complicates development of vaccines, which often fails to elicit protective immunity against *S. aureus*. Although there is an extensive research on interactions between *S. aureus* and immune system, the research is often conducted in mouse models of *S. aureus* infection. However, there are significant differences between mouse and human immune systems in terms of interactions with *S. aureus*. First, mice are not natural hosts of human *S. aureus* isolates; therefore, genetic alterations of mice are necessary for induction of *S. aureus* carrier state and *S. aureus* infections [184]. Conversely, mice *S. aureus* strain are used in the studies which might not correspond with human strains. Second, while mouse protective immunity against *S. aureus* is based on opsonic antibodies, the mechanisms of protective immunity in humans are still unclear [227]. Lastly, the activation of the inflammasome requires two signals in mouse macrophages; however, only one signal is necessary for the inflammasome induction in human monocytes [251]. Collectively, mice models of *S. aureus* infections are a helpful model for initial studies, however findings obtained from humanized mice might not correspond with the function of the human immune system. Hence, the results should be informative rather than defining and should be verified in human cells, especially regarding vaccine development. Furthermore, *S. aureus* is known to evade its recognition by immune cells as well as to manipulate its fate within the cells. Hence, new insights into the consequences of long-term exposure to *S. aureus* and mechanisms of immune manipulation by *S. aureus* are needed.

Type I IFNs are potent immunomodulators of both innate and adaptive immunity, with a well-known anti-viral role, yet their impact on bacterial infections remains unclear. TLR2 is the key receptor for recognition of Gram-positive bacteria, including *S. aureus*, and after recognition of *S. aureus*, TLR2 mediates strong proinflammatory responses. In addition, evidence for *S. aureus*-mediated induction of type I IFNs is accumulating in both mouse and human cells. However, whether type I IFN induction can be mediated via TLR2 remains less understood, as does the role of type I IFNs during *S. aureus* infection in human cells. Recently, Parker and colleagues described induction of type I IFNs in mouse DCs by *S. aureus* and the effects of type I IFNs varied between two distinct strains of *S. aureus* [255]. Likewise, *S. aureus*

has been shown to induce type I IFNs in human monocytes and macrophages and TLR2 has been identified as a negative regulator of the type I IFNs induction, however the role of type I IFNs remained unclear [162]. Moreover, studies conducted on *S. aureus* infection in mice lacking type I IFN receptor showed contradictory results. Hence, in this project we first examined induction of type I IFNs by *S. aureus* in human cell lines. Secondly, we investigated the ability of human cells to kill *S. aureus in vitro* and the impact of type I IFNs on bacterial killing. Thirdly, we dissected type I IFN signaling pathways in response to TLR2 ligands compared to *S. aureus* in human cells. Lastly, we investigated induction of other physiologically relevant IL-1 β and IL-10 cytokines during *S. aureus* infection.

Here, we present evidence that challenge of cells with both LTA and *S. aureus* stimulates type I IFN production, and that the induction of type I IFNs is cell-type dependent. Using THP-1 and human primary monocytes, we showed that *S. aureus* elicited higher type I IFN production in monocytes compared to monocytes differentiated into macrophages. Lung epithelial cells, on the other hand, did not produce type I IFNs or inflammatory cytokines in response to LTA or *S. aureus* stimulation but they strongly responded to stimulation with nucleic acids. Our findings are consistent with studies showing that all nucleated cells can produce type I IFNs during viral infections through intracellular nucleic acid sensors such as endosomal TLRs or cytosolic RLRs [144]. Collectively, our data shows that lung epithelial cells are physiologically important IFN producers during viral infections, while both monocytes and monocyte-derived macrophages can produce type I IFNs also during bacterial infections. However, we observed significant differences in the degree of immune activation between monocytes and monocyte-derived macrophages, suggesting polarization of their function during *S. aureus* infection.

Further, our data demonstrate that both THP-1 monocytes and THP-1-derived macrophages can kill *S. aureus in vitro*. It has been reported that primary human monocyte-derived macrophages can phagocytose *S. aureus* more efficiently than primary monocytes, PMNs and DCs [294]. However, monocytes and PMNs exhibited higher capacity to kill intracellular bacteria than monocyte-derived macrophages and DCs [294]. This is consistent with our observations that THP-1 monocytes killed live *S. aureus* more rapidly than THP-1-derived macrophages. Our data also suggested that slower killing of *S. aureus* was due to *S. aureus* ability to resist immediate killing in phagosomes within THP-1-derived macrophages. We also observed that upregulation of proinflammatory cytokines correlated with a significant cytotoxicity, which might have led to increased bacterial survival. It has been previously shown

that staphylococcal protein A can directly stimulate TNFR1 and that upregulation of TNF α increases pathogenesis during *S. aureus* infection [130]. Overall, these findings suggest that TNF α might contribute to increased pathogenesis, bacterial survival and dissemination of *S. aureus* during systemic infection. Hence, blocking soluble TNF α or TNFR might help to prevent serious complications during *S. aureus*-induced sepsis.

Several studies have demonstrated that TLR2 is the key receptor for recognition of *S. aureus* in mouse macrophages [185, 186] and that TLR2 and *S. aureus* co-localize in phagosomes within mouse RAW macrophages [247]. Our data further suggests that TLR2 is required during early phagocytosis of *S. aureus* by THP-1 monocytes. We suppose that *S. aureus* can enter the cells via other receptors during later phases of infection such as integrins. Although Parker and colleagues identified intracellular receptors TLR9 and NOD2, respectively, for the induction of type I IFNs in response to *S. aureus*, the recognition receptor for the *S. aureus* entry was not described [153, 255]. On the other hand, Bergstrom and colleagues found that TLR2 stimulation upregulates expression of CD11b/CD18 integrins on both monocytes and granulocytes and thus TLR2 is indirectly responsible for an enhanced phagocytosis of *S. aureus* [162]. It will be interesting to examine expression of these integrins also in our model of *S. aureus* infection in THP-1 monocytes.

Additionally, in the absence of TLR2, we observed increased cell death, suggesting that TLR2 signaling pathway is important for an optimal resolution of *S. aureus* infection. It has been previously reported that endocytosis is important for invasion of non-professional phagocytes by *S. aureus* and that inhibition of endocytic processes leads to reduction of intracellular *S. aureus* [174]. In contrast to these findings, our data suggest that the endosomal pathway plays a role in the survival of *S. aureus* within professional phagocytes such as monocytes. Using distinct inhibitors of the endosomal pathway, we observed enhanced killing of *S. aureus* and decreased production of type I IFNs by THP-1 monocytes. Therefore, *S. aureus* might manipulate the endosomal pathway and thus delay its killing within phagosomes. In addition, type I IFNs might constitute means by which *S. aureus* control its survival within monocytes. In order to address whether type I IFNs are actively induced by *S. aureus* or they are produced by cells as an antibacterial response, it will be of major interest to measure type I IFNs production after stimulation of cells with heat killed *S. aureus*.

Unfortunately, studies addressing the role of type I IFNs during *S. aureus* infection offer contradictory results. In addition, most of the research was conducted in mice models; and thus the role of type I IFNs during *S. aureus* infection in human cells remains poorly

described. It has been reported that secondary bacterial infections after preceding influenza infection can lead to increased mortality in previously healthy individuals. Accordingly, mice infected with both influenza and *S. aureus* had higher mortality, greater lung damage and increased bacterial density than mice infected with *S. aureus* alone [248]. However, the mechanisms by which influenza infection attenuates immune responses to bacterial infections remain less understood. Since the major consequence of influenza infection is the upregulation of type I IFNs, the type I IFNs might predispose individuals to bacterial superinfections. Accordingly, we observed that stimulation with recombinant IFN α significantly impaired killing capacity of THP-1 monocytes and that the combination of IFN α and *S. aureus* infection decreased cell viability. Hence, our results may provide an insight into the mechanisms of host susceptibility to bacterial infections after preceding viral infections and might consequently provide a tool by which we could treat or prevent these complications, for example by blocking soluble type I IFNs.

In order to understand induction of type I IFNs by *S. aureus* in human monocytes, we examined the underlying signaling pathway by using a variety of inhibitors and siRNA. Consistent with the mouse models of *S. aureus* infection [185, 186], our data show that *S. aureus* triggers a conventional TLR2 downstream signaling pathway consisting of Mal-myddosome-TRAF6-TAK1, leading to activation of canonical IKKs and NF κ B-dependent transcription of proinflammatory cytokines, such as TNF α and IL-1 β . Further, we show that induction of type I IFN by *S. aureus* is dependent on TLR2 in cooperation with CD14. Interestingly, we observed upregulation of IFN α transcription in the absence of TLR2, suggesting that TLR2 signaling might inhibit IFN α induction in favor of IFN β . It has been shown that endosomal TLR7 or TLR9 can detect staphylococcal nucleic acids after endosomal maturation and mediate IFN α transcription in human plasmacytoid DCs [266]. In addition, our data showed that *S. aureus* infection after preceding IFN α stimulation led to impaired bacterial killing by, and increased cellular toxicity of, THP-1 monocytes. Since pDCs express low levels of TLR2, endosomal TLRs might induce IFN α transcription via an alternative pathway and contribute to increased pathogenicity during *S. aureus* infection.

Next, we present evidence that *S. aureus*-stimulated type I IFN induction but not TNF α production requires clathrin-dependent formation of vesicles, Syk-mediated internalization, actin-dependent trafficking and endosomal maturation in human monocytes. We also showed that inhibition of the endosomal pathway increased killing of *S. aureus* by THP-1 monocytes. Hence, we suspect that type I IFNs might play a role in *S. aureus* evasion from its killing

in phagosomes within THP-1 monocytes. In addition, we observed that MyD88, Mal and partially TRIF adaptors contributed to *S. aureus*-stimulated type I IFNs from the endosomes. Since Mal and MyD88 adaptors are involved in conventional TLR2 signaling from the plasma membrane, TRIF might be a distinctive feature of the endosomal pathway. In previous studies, we observed that TRAM was responsible for the endosomal induction of IFN β in mouse BMDMs [42]. However, in THP-1 monocytes, we did not observe any functional impact of TRAM silencing on *S. aureus*-mediated signaling, indicating that TRAM has a redundant role in THP-1 monocytes. Similarly to TRAM, TRIF adaptor protein has been associated with TLR2-mediated RANTES induction in mouse BMDMs [43]. Collectively, these data suggest that TRIF might function downstream of TLR2 in both mice and human cells. In addition, TRAM might be utilized by mouse macrophages, whereas Mal might have a role in human monocytes.

Further, our results suggest that signaling from the endosomes leads to the assembly of the myddosome complex and activation of TRAF6. It has been suggested that Mal can trigger myddosome assembly according to its location [44] or it can directly bind TRAF6 and initiate an alternative signaling pathway [273]. In either case, TRAF6 most likely activates TABs, which catalyze TAK1 kinase activity. TAK1 in turn induces activation of IKK β and consequently mediates phosphorylation of noncanonical IKKs. It has been suggested that noncanonical IKKs can inhibit canonical IKKs and form a negative feedback loop [232] and our data support this interaction. Consequently, TBK1/IKK ϵ kinases activate yet unknown IRF(s), which induces transcription of IFN β . Alternatively, IKK β -stimulated NF κ B activation could contribute to the initial IFN β induction. TLR2-mediated IFN β production then leads to IRF7 upregulation during the late phase of type I IFN induction, consistent with the observations that in most cell types, IRF7 needs to be upregulated by type I IFNs in order to induce IRF7-dependent transcription [149]. Therefore, other IRFs, such as IRF1, IRF5 and IRF8, might be involved in early induction of IFN β . In summary, our study presents an evidence for a novel *S. aureus*-stimulated endosomal TLR2 pathway, which culminates in induction of type I IFNs. The downstream signaling components of the endosomal TLR2 pathway involve Mal-TRAF6-TAK1-IKK β /TBK1 and IRF/NF κ B. Similar to our results, Bergstrom and colleagues described that *S. aureus* activate surface TLR2 and endosomal TLR8 triggering induction of TAK1, IKK β and IRF5 in human primary monocytes [162]. However, in their model, TLR2 was acting as a negative regulator of the endosomal TLR8-mediated IRF5 activation and thus IFN β production [162]. In our model, we observed induction of type I IFNs after *S. aureus* infection as well as after LTA stimulation suggesting that LTA alone is able to trigger TLR2-dependent type I IFN production. The degree of type I IFN induction by live *S. aureus* was stronger than LTA stimulation

suggesting that the total levels of type I IFNs might be mediated by additional receptors, such as endosomal TLRs, cytosolic NODs or RLRs. However, we did not test whether LTA can inhibit *S. aureus*-induced type I IFNs and this could be an interesting experiment in the future.

In addition, our results strongly suggest that Malp2 triggers unconventional pathways in THP-1 monocytes, while LTA models *S. aureus*-induced signaling. Even though both Malp2 and LTA are recognized by TLR2, the downstream signaling is considerably different in human monocytes. Whereas LTA-stimulated TLR2 recruits Mal and the myddosome complex, we failed to identify an adaptor protein in Malp2-induced TNF α induction [Fig. 7.1 A]. Instead, we observed that Malp2 stimulation required TRAF3 activation possibly via IRAK-1/4 and TRAF6. We suppose that both LTA and Malp2 similarly stimulate the TRAF6-TAK1-IKK axis, leading to NF κ B-dependent transcription of proinflammatory genes. In terms of type I IFN production, we observed that both LTA and Malp2 required endosomal maturation; however, LTA-stimulated type I IFNs required Syk kinase activity [Fig. 7.1 B]. Furthermore, while LTA required MyD88, Mal and TRIF adaptors, Malp2 stimulation led to the recruitment of Mal, TRAM and TRIF adaptors to the endosome signaling complex. Hence, this suggest that Malp2 can trigger MyD88-independent but TRIF-dependent TLR signaling pathway, a mechanism similar to endosomal TLR3 and TLR4 signaling. Next, we observed that LTA signaled via myddosome-TRAF6-TAK1, whereas Malp2 triggered IRAK1/4 and TRAF6 activation independently of myddosome, possibly via Mal-TRAF6 interaction [273]. LTA-stimulated TRAF6 then mediates activation of TAK1 presumably via TABs, and TAK1 facilitates activation of noncanonical IKKs via phosphorylation of IKK β or directly by TAK1 kinase activity. The non-canonical IKKs most likely trigger transcription of IFN β via not yet identified IRF(s) or via NF κ B. After Malp2 stimulation, however, we suspect that upregulation of IFN β is mediated via direct activation of IRF(s) by TRAF6- IRAK-1/4 complex or indirectly via TAK1-IKKs-NF κ B pathway.

In conclusion, TLR signaling seems to be more complex than we originally thought. First, there are considerable differences in the induction of immune responses among cell types within the body. Secondly, each ligand, bacteria and/or virus might trigger distinct downstream signaling pathways although they activate the same receptor. Thirdly, the differences between mice and human immune systems are significant and they need to be addressed in the studies accordingly. Last but not least, it is difficult to discover new components of the signaling pathways since we don't generally know what we are looking for. Hence new approaches including a broad range of -omics, and a wider acceptance and appreciation of new discoveries might be a way to go.

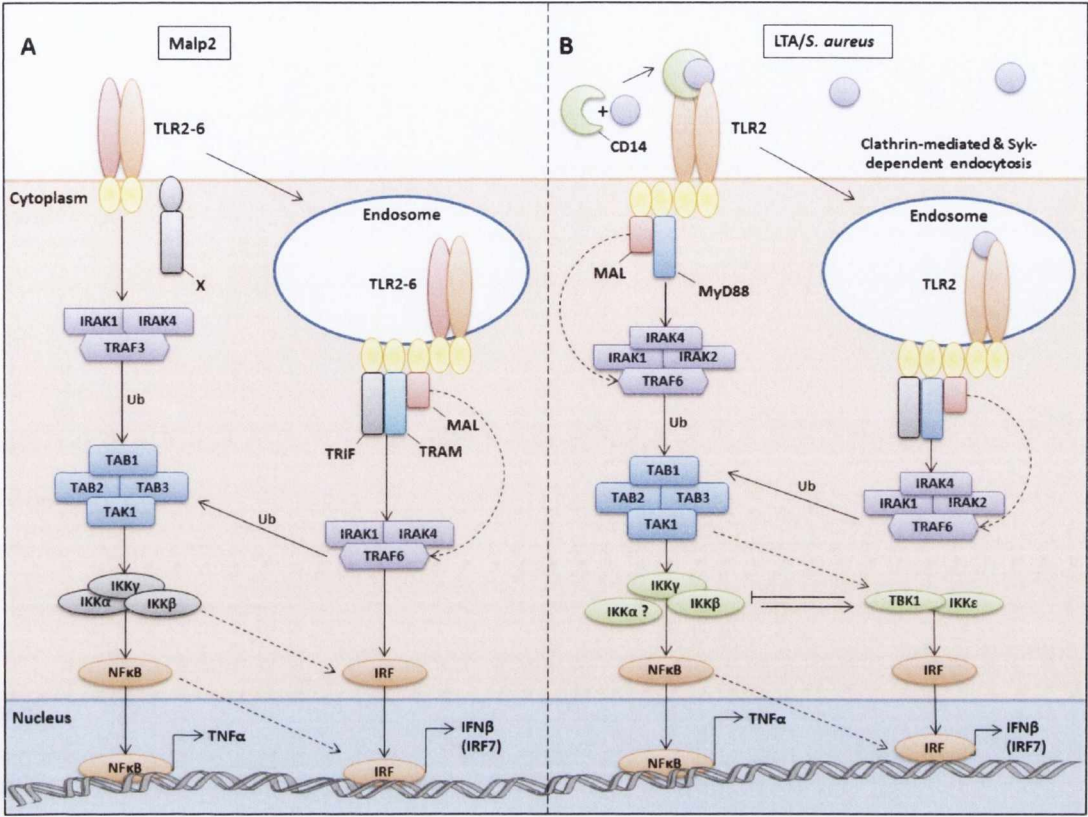


Figure 7.1: Malp2 and LTA signaling pathways in THP-1 monocytes.

A: Malp2 is detected by a surface TLR2-6 heterodimer. After its recognition, TLR2 recruits unknown adaptor X, which activates IRAK-1/4 kinases and TRAF3 ubiquitin ligase. Alternatively, TLR2 might interact with IRAK-1/4 directly and trigger TRAF3. Activated TRAF3 then mediates TAK1 phosphorylation presumably via TABs. TAK1 probably activates the IKK complex, which induces NFκB-dependent transcription of proinflammatory genes. In some cases, Malp2-TLR2 complex translocates to the endosomes, where it recruits Mal, TRAM and TRIF adaptors. The receptor complex then activates IRAK-1/4, thus facilitating TRAF6 activation. TRAF6 then mediates activation of IRF7 and transcription of type I IFNs. Alternatively, TRAF6 can also activate TABs and thus TAK1, which in turn catalyses IKK complex. IKK complex then activates NFκB and IRF7 gene transcription. **B:** Staphylococcal LTA is recognized by CD14 and the LTA-CD14 complex binds to TLR2. Activated TLR2 then triggers a conventional signaling cascade involving Mal-myddosome-TRAF6-TAK1-IKK complex culminating in induction of NFκB-dependent TNFα transcription. In some cases, LTA-TLR2 complex undergoes clathrin-dependent and Syk-mediated endocytosis. LTA-TLR2 complex recruits additional TRIF adaptor and induces assembly of the myddosome and activation of TRAF6. TRAF6 then facilitates TABs-mediated TAK1 activation, leading to phosphorylation of noncanonical kinases TBK1 and/or IKKε by kinase activity of IKKβ or TAK1. The noncanonical kinases can inhibit canonical IKKs, thus creating a negative feedback loop. Activated TBK1/IKKε phosphorylate yet unknown IRF, which translocates to the nucleus and induces transcription of IFNβ. Alternatively, activated NFκB can contribute to the initial IFNβ transcription. Ub – ubiquitination, dashed arrows – hypothetical interactions.

In our study, we were primarily interested in the novel TLR2-mediated type I IFN pathway in human monocytes. For a comparison, we also measured induction of TNF α protein as a cytokine well known to be stimulated by TLR2 pathway. However, activation of immune responses during *S. aureus* infection is not limited to those two cytokines. Therefore, we briefly investigated the signaling pathways of proinflammatory IL-1 β and anti-inflammatory IL-10, cytokines known for their important roles during *S. aureus* infections.

In this study, we present evidence that LTA alone and *S. aureus* are able to induce IL-1 β release in human monocytes. Furthermore, we observed that IL-1 β release was TLR2- and IKK β -dependent and involved Mal and/or TRAM adaptors, and most likely mirrored conventional proinflammatory TLR2 signaling pathway. Interestingly, Gai and colleagues reported that *S. aureus* protein A was able to induce expression of the type II IL-1 receptor (IL-1RII) by monocytes and neutrophils [295]. IL-1RII acts as a soluble decoy receptor and consequently it regulates the biological activity of IL-1 β including induction of inflammatory cytokines and chemokines. Furthermore, high levels of IL-1RII were associated with increased bacterial persistence in septic patients, hence IL-1RII might be a new evasion strategy of *S. aureus* [295]. In our experiments, we observed strong IL-1 β induction after both LTA stimulation and *S. aureus* infection *in vitro*, however the whole blood is a complex system and we might not be able to capture all evasion mechanisms in *in vitro* conditions. On the other hand, it has been reported that IL-1 β is a critical mediator of protective immunity during infection with *Mycobacterium tuberculosis*, while type I IFNs have been associated with disease progression. Interestingly, type I IFNs have been shown to selectively limit the production of IL-1 β in human macrophages and worsen the disease symptoms [291]. Hence, it would be interesting to examine if *S. aureus*-induced type I IFNs could affect IL-1 β production.

In contrast to IL-1 β release, we observed that IL-10 signaling pathway was significantly downregulated in THP-1 monocytes compared to human primary monocytes, suggesting that IL-10 pathway is altered or defective in THP-1 monocytes. In primary human monocytes, IL-10 induction was TLR2-independent; however, it required Mal and/or TRAM adaptor proteins, *S. aureus* internalization, endosomal maturation and IKK β activity. Hence, this data suggests that other TLR receptors, such as TLR4 or endosomal TLRs, might be involved in IL-10 induction in human monocytes. Interestingly, it has been reported that production of type I IFNs was required for LPS-induced upregulation of IL-10 by BMDMs, indicating an indirect anti-inflammatory role of type I IFNs [292]. Therefore, *S. aureus*-induced type I IFNs might influence both arms of immune responses to *S. aureus*. Similarly to *M. tuberculosis* [291], type I IFNs

could decrease IL-1 β production by monocytes delaying activation of other innate immune cells, and thus escape its killing. Secondly, *S. aureus*-mediated type I IFNs could enhance IL-10 production by human monocytes and inhibit proinflammatory responses. Hence, our future work will try to address the impact of *S. aureus*-stimulated type I IFNs on proinflammatory versus anti-inflammatory arms of the innate immunity.

Altogether, we present evidence for *S. aureus*-stimulated type I IFN pathway in human monocytes. We show that both bacterial killing and type I IFNs are TLR2-dependent and require the endosomal pathway in human monocytes, whereas *S. aureus* resist killing within monocyte-derived macrophages. In addition, treatment with recombinant IFN α significantly impairs killing ability of THP-1 monocytes. Hence, we suspect that type I IFNs might contribute to *S. aureus* evasion from its killing. The TLR2-dependent type I IFN signaling involves Mal and MyD88 adaptors, the myddosome, TRAF6 ubiquitin ligase, TAK1 kinase, canonical IKK β and noncanonical TBK1/IKK ϵ kinases, leading to activation of NF κ B and IRF(s) and transcription of TNF α and IFN β . Furthermore, we observed that *S. aureus* stimulates IL-1 β release in a TLR2-dependent manner, while IL-10 production requires other receptors than TLR2. Whether *S. aureus*-mediated type I IFNs directly affect IL-1 β or IL-10 production by human monocytes, and thus further manipulate immune responses remains an open question.

Tschurtschenthaler and colleagues recently described that type I IFN receptor in the intestinal epithelial cells regulates the intestinal microbial ecosystem [296]. Therefore, low level of type I IFN induction might contribute to the regulation of the host-*S. aureus* commensal relationship on the skin and influence the overall microbiota. Hence, type I IFNs might also act as regulators of healthy microbiome. On the other hand, high levels of type I IFNs induced by viral infection seem to have detrimental effect on immune responses during subsequent microbial infections. In this context, blocking of excessive type I IFNs production might help during serious complications caused by invading bacteria. However, efforts to design vaccines based on limiting type I IFNs might not be successful since type I IFNs might act as a double-edged sword.

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