Identification of molecular mechanisms of chronic inflammatory responses

Ph.D. Thesis

By

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DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Identification of molecular mechanisms of chronic inflammatory responses"** in the partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEER, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from February, 2015 to September, 2018 under the supervision of Dr. Mirza Saqib Baig, Associate Professor, BSBE, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Signature of the student with date
(MANSI SRIVASTAVA)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Signature of Thesis Supervisor with date
(Dr. MIRZA SAQIB BAIG)

MANSI SRIVASTAVA has successfully given her Ph.D. Oral Examination held on **April 29, 2019**

Signature of Chairperson (OEB) Date:	Signature of External Examiner Date:	Signature(s) of Thesis Supervisor(s) Date:
Signature of PSPC Member #1 Date:	Signature of PSPC Member #2 Date:	Signature of Convener, DPGC Date:

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Date:

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Identification of molecular mechanisms of chronic inflammatory responses"** submitted to Indian Institute of Technology Indore (IITI) by **Ms. Mansi Srivastava** is for the award of the degree of **Doctor of Philosophy**. This work reported herein is original and has not been submitted so far, in part or full, for any other degree or diploma of any other University.

_____

Signature

Dr. Mirza S Baig

Thesis Supervisor

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This Thesis is

Dedicated to

My Beloved Family

MANSI SRIVASTAVA

List of publications and awards

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1. Srivastava M, Saqib U, Banerjee S, Wary K, Kizil B, Muthu K, Baig MS. Inhibition of TIRAP-c-Jun interaction as a novel therapeutic strategy for AP1-mediated inflammatory responses. **Int. Immunopharmacol.** 2019,71:188-197.

2. Srivastava M, Baig MS. NOS1 mediates AP1 nuclear translocation and inflammatory response. **Biomed. Pharmacother.** 2018,102: 839-847.

3. Srivastava M, Uzma S, Naim A, Roy A, Liu D, Bhatnagar D, Ravinder R, Baig MS. The TLR4-NOS1-AP1 signaling axis regulates macrophage polarization. **Inflamm. Res.** 2016, 66(4): 323-334.

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2. Baig MS, Dongfang Liu, Kannan Muthu, Anjali Roy, Uzma Saqib, Adnan Naim, Syed M. Faisal, **Srivastava M**, Rohit Saluja. Heterotrimeric complex of p38 MAPK, PKCδ, and TIRAP is required for AP1 mediated inflammatory response. **Int. Immunopharmacol.** 2017.48:211-2018.

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ACRONYMS

AAM	Alternatively Activated Macrophages
AF594	Alexa-Fluor 594
AKT	Activating Protein Kinase
AP1	Activator Protein 1
APC	Antigen Presenting Cells
ARG1	Arginase 1
ATF2	Activating Transcription Factor 2
BALF	Bronchoalveolar Lavage Fluid
BMDM	Bone marrow Derived Macrophages
BtK	Bruton Tyrosine Kinase
bZIP	Basic leucine Zipper Domain
C/EBPα	CCAT/Enhancer Binding Protein alpha
cAMP	cyclin Adenosine Monophosphate
CAPRI	Critical Assessment of Prediction of interactions
CCL	Chemokine Ligands
CD	Cluster of Differentiation
CDC	Common Dendritic Cell progenitor
Chi3l	Chitinase like protein-3
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
Co-IP	Co-Immunoprecipitation
COPD	Chronic Obstructive Pulmonary Disease
COX2	Cyclo-oxygenase
CREB	cAMP Responsive Element Binding Protein
CSF1	Colony Stimulating Factor-1
CXCL	Chemokine C-X-C Ligand
DAF-FM	4-Amino-5-methylamino-2',7'-difluorofluorescein

DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
ECM	Extra Cellular Membrane
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal Regulated Kinases
FAD	Flavin Adenine Dinucleotide
FDA	Food and Drug Administration
FFT	Fourier Transform
FITC	Fluorescein Isothiocyanate
FMN	Flavin Mononucleotide
Fra-1	Fos-Related Antigen-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMCSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte Macrophage Progenitor
HIV1	Human Immunodeficiency Virus 1
HSC	Hematopoietic Stem Cells
HUVEC	Human Vascular Endothelial Cells
IAEC	Institutional Animal Ethics Committee
IFNγ	Interferon Gamma
IKB	Inhibitor of kappa-B
IL	interleukin
IL-4R	lnterleukin-4 Receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF8	Interferon Regulatory Factor -8
JAK	Janus activated Kinases
JNK	c-Jun N terminal Kinase
KCs	Kupffer Cells
KLF4	Kruppel Like Factor4

LPS	Lipopolysaccharide
MAL	MyD88 Like adaptor Protein
MAPK	Mitogen Activated Protein Kinases
M-CFU	Monocyte-Colony Stimulating Factor
MCP	Mast cell Progenitor
M-CSF	Macrophage-Colony Stimulating Factor
MD	Molecular Dynamics
MDP	Macrophage Dendritic Cell Progenitor
MIP1-α	Macrophage Inflammatory Protein 1-alpha
mIR	micro RNA
MMP1	Matrix Metalloproteinase-1
MRC-1	Mannose Receptor C-1
Mtb	Mycobacterium Tuberculosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor-kappa B
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSCLC	Non-Small Cell Lung Cancer
PAMP	Pathogen Associated Molecular Pattern
PDB	Protein Data Bank
PDGF	Platelet Derived Growth Factor
PHD2	Prolyl-hydroxylase 2
PI3K	Phosphatidylinositol-3 Kinase
Pkcδ	Protein Kinase C delta
PMA	Phorbol 12-myristate 13-acetate
PPARγ	Peroxisome proliferator-activated receptor gamma
PRR	Pathogen Recognition Receptor
q-RTPCR	Quantitative Real Time PCR
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSV	Respiratory Syncytial Virus

SARS	Severe Acute Respiratory Virus
SOCS	Suppressor of Cytokine Signaling
STAT1	Signal Transducer and Activator of Transcription
TBST	Tris Buffer Saline and Tween 20
TF	Transcription Factor
TFRC	Transferrin Receptor Protein 1
TGFβ	Transforming Growth Factor beta
Th1	T helper Cell 1
TIR	Toll Interleukin 1 Receptor
TLR4	Toll like Receptor
TNFα	Tumor Necrosis Factor alpha
TRAF	TNF Receptor Associated Factor
TRE	TPA response element
TRIM	1-(2-Trifluoromethylphenyl) imidazole

NOMENCLATURE

ml	milli litre
mM	milli molar
ng	nano gram
nM	nano molar
nS	nano second
µL	micro litre
µM	micro molar
ºC	degree centrigrade

Preface

Inflammation is a defense response of the immune system that protects the body from deleterious effects of invading pathogens. Macrophages play an indispensable role in the innate immune system by clearing the pathogens. However unchecked inflammatory reactions arising in macrophages give rise to chronic inflammatory disorders, such as cardiovascular diseases, autoimmune diseases, cancer, and sepsis. Nitric oxide is robustly released from macrophages during inflammatory reactions. Recently, the role of NOS1 has been appreciated in the inflammatory cascade employed in macrophages.

The research conducted in this thesis provides novel insights into the involvement of NOS1 in inflammation through TLR4 signaling pathway in macrophages. The study shows that NOS1 inhibition perturbs the activation and nuclear translocation of AP1 transcription factor during endotoxin shock in macrophages. This in turn negatively regulates the AP1 mediated cytokine gene expression and ultimately leads to reduced inflammatory response. In our study, we identified a novel interaction between inflammatory mediators TIRAP and c-Jun (AP1 subunit) in LPS stimulated macrophages. Targeted disruption of TIRAP and c-Jun interaction is proposed as an efficient therapeutic strategy to combat the severity of inflammation caused by LPS.

We used repurposing of already known drugs to inhibit TIRAP-Jun interaction. Out of five potential drug candidates, we found that Gefitinib could significantly inhibit the interface between TIRAP and c-jun. Thereby, providing promising platform for anti-inflammatory therapy. In summary, this work has investigated the molecular targets involved during chronic inflammation and proposes drug repurposing as an efficient strategy for inflammatory responses generated during endotoxin shock in macrophage.

1. Introduction

1.1 Innate Immunity

The human immune system is appreciated as a defense system that protects the body from foreign invaders such as bacteria and viruses (Schultz and Grieder, 1987; Yatim and Lakkis, 2015). It can be divided into two broad categories: Innate and adaptive immune system. Innate immunity is the first line of defense to the invading pathogens and quickly recognizes and responds to the stimulus in a non-specific manner, while the adaptive immunity recognizes explicitly the pathogen based on the memory generated against previously encountered infection, allowing a stronger immune response to repetitive infection (Charles A Janeway et al., 2001; Dempsey et al., 2003).

Recognition of innate immune response dates back to 1908 by Ilya Mechnikov who received the noble prize for the same (Merien, 2016a). As a critical component of immune system, innate immune response provides anatomical barrier to infection in form of skin epithelia, respiratory tract, gastrointestinal tract etc and in form of and physiological barriers such as inflammation, cytokine secretion, phagocytosis and complement activation (Turvey and Broide, 2010; Uthaisangsook et al., 2002). The cellular component of innate immunity such as neutrophils, mast cells, NK cells, dendritic cells and monocytes/macrophages are crucial for mediating the response and play an important role in protection against infectious invaders (Gasteiger et al., 2017). However, uncontrolled activation of innate immune response is associated with inflammatory disorders leading to life threating state in the host (Elliott et al., 2014; Kamada and Rogler, 2016). One of the first signs of the onset of innate immunity is inflammation, which signals the immune cells to accommodate at the site of infection (Newton and Dixit, 2012a; Xiao, 2017; Xu and Larbi, 2018). The majority of innate immune cells recognize pathogen-associated molecular patterns (PAMP) through their pattern recognition receptors (PRR) and induces the inflammatory signalling (Akira et al., 2006a). At the site of inflammation, the newly recruited immune cells secrete

chemokines to attract other immune cells to generate a collective immune response against the invading pathogen, which is called the inflammatory response (Sokol and Luster, 2015).

1.1.1 Inflammation

Inflammation is a biological phenomenon that caters the defense response of the immune system to the invading pathogen such as bacteria and virus that perturb the integrity of healthy tissue (Matzinger, 2002). It encompasses a range of physiological and pathological aspects of immunity and aims towards the resolution of damaged tissue and restoring the homeostasis (Soehnlein and Lindbom, 2010). Infection and tissue injury instigates inflammatory reactions that initiate the recruitment of blood cells such as leukocytes and plasma proteins at the site of damage (Medzhitov, 2008). Vertebrates display an enormous network of inflammatory mediators that provide the protection to infected organs. Inflammation is associated with multiple complex disorders such as cardiovascular diseases, autoimmune diseases, cancer and sepsis (Murakami and Hirano, 2012). Migration of inflammatory molecules to the site of infection is facilitated by a significant increase in the blood flows, vascular permeability and vasodilation.

1.1.2 Physiological aspects of Inflammation

Inflammation is generally of two types; acute or chronic, which depends on the type of stimulus as well as the defense machinery which deals with it. Acute inflammation as the name suggests is quick to happen and relatively quick to last, generally ranging from minutes to few days (Pedersen and Kehlet, 1996). Acute inflammatory response is triggered by the infection caused due to invading pathogens that further activate the immune machinery at the site of infection (Hamidzadeh et al., 2017). First line of defense is provided locally by plasma proteins and neutrophils that are instantly recruited to the site of infection (Selders et al., 2017). Neutrophil trafficking is the major signal of

acute inflammation, which itself results after anaphylatoxins are released at the site of inflammation (Selders et al., 2017). This, in turn stimulates mast cells to release histamine, serotonin and prostaglandins causing blood vessels to expand (vasodilation) and become highly permeable. This attracts neutrophils to migrate to the affected tissue through the capillary wall (diapedesis) and respond to the stimuli. The visible effect of acute inflammation is seen by pus formation, swelling, redness and pain at the site of the external stimuli (Jaeschke and Hasegawa, 2006). Activated neutrophils eliminate the bacterial components by secreting reactive oxygen species (ROS) and reactive nitrogen species (RNS) from their granules causing a potential damage to both the pathogen as well as the host tissue (Huang et al., 2015). Inability of the acute inflammatory response to eliminate the pathogen further results in activation of monocytes and macrophages leading to a chronic inflammatory response, that give rise to granulomas that engulf and destroy the pathogen to provide protection to the host tissue (Ingersoll et al., 2011). Successful elimination of the bacterial invaders is accompanied by resolution of the damage caused to the host tissue after the acute inflammatory response. Tissue-resident monocytes and macrophages give rise to anti-inflammatory growth factors after prolonged damage induced by the leukocytes (Ogle et al., 2016). Chronic inflammation occurs when the cause of inflammation is persistent, as seen in particular viral infections and hypersensitivity reactions (Murakami and Hirano, 2012). The defense army of chronic inflammation is different than that of acute inflammation, with more on-site lymphocytes and macrophages. Also, the chronic inflammation leads to many severe implications like vascular proliferation, fibrosis, and tissue destruction as shown in Fig 1.1.

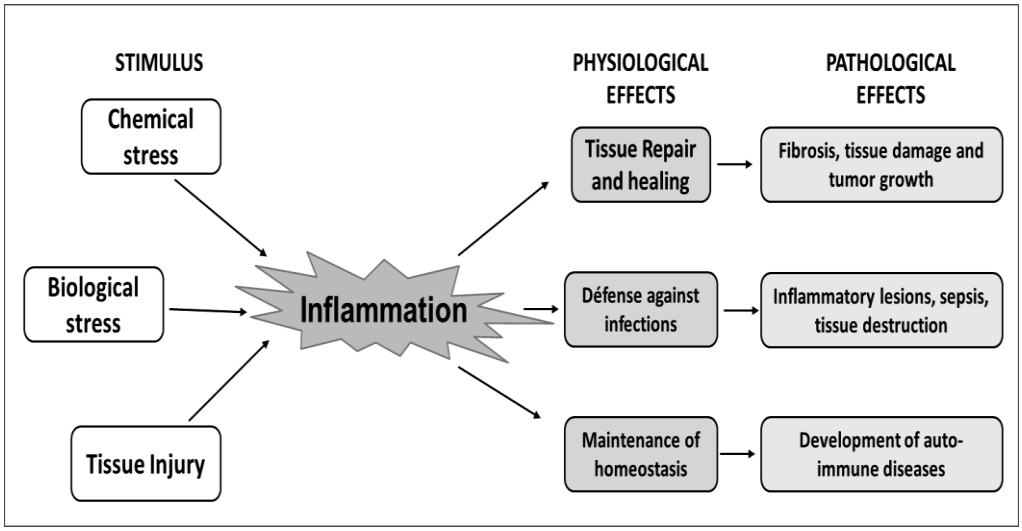


Fig 1.1. Physiological and pathological functions of inflammation

1.1.3 Disease consequences of inflammation

Chronic state of inflammation is associated with multiple inflammatory disorders such as pulmonary disease, arthritis, cardiovascular disease, encephalitis, diabetes, inflammatory bowel disease (Fig 1.2). According to the U.S. Centers for Disease Control and Prevention, 63% of deaths worldwide account for the inflammatory disorders (Prasad and Aggarwal, 2014). Inflammation is triggered by the various biological factors such as bacterial, viral, parasitic infections. Endotoxin shock-induced lung injury is the primary cause of mortality during systemic inflammation (Matthay and Zemans, 2011). Acute lung injury has contributed to substantial mortality in adults and children suffering from sepsis (Dahlem et al., 2007; Erickson et al., 2009; Randolph, 2009). Lack of understanding of the precise mechanism underlying the septic lung injury attributes to poor therapeutic remedies for the disease. Although sepsis generates whole-body inflammatory reaction leading to multiorgan failure, the first organ that observes the damage is lungs (Varisco, 2011). Inflammation-mediated damage activates inflammatory cells in the lungs such as macrophages and neutrophils that release free oxygen and nitrogen radicals and cytokines to orchestrate the signalling pathway.

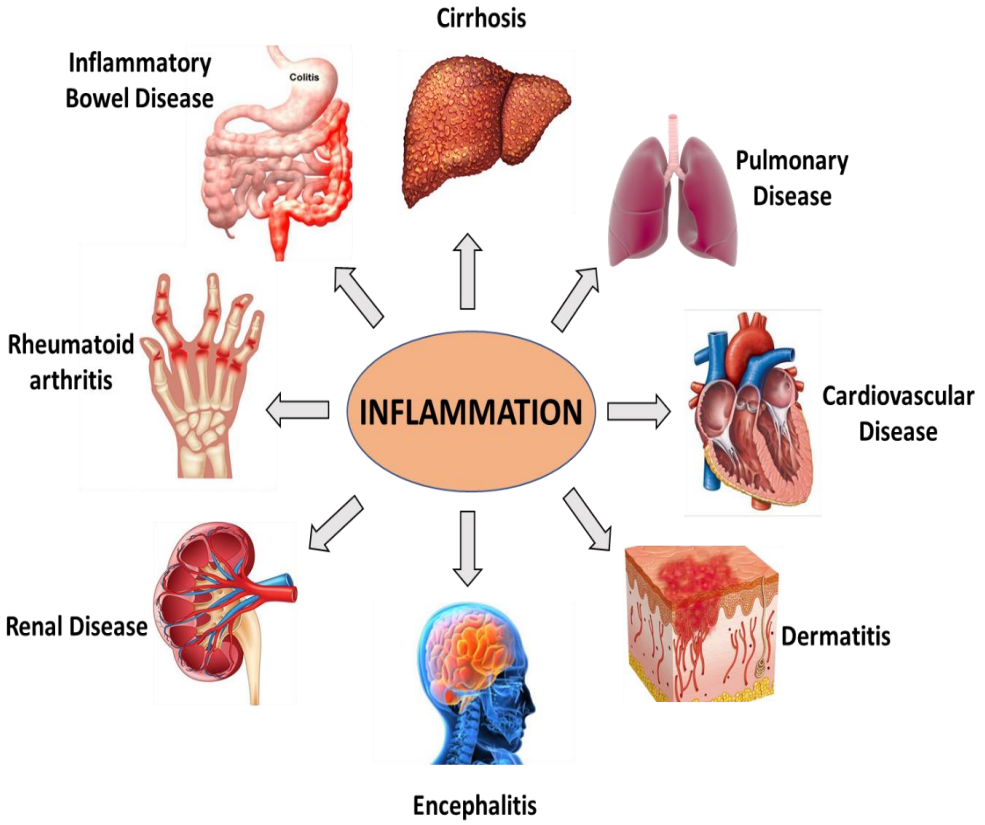


Fig 1.2. Diseases associated with inflammation

Chronic inflammatory disorders such as sepsis is caused by prolonged exposure to endotoxin shock. It affects more than 30 million people worldwide leading to 6 million deaths every year including 3 million newborns and 1.2 million children (Napolitano, 2018). Despite the recent advancement in the clinical treatment of sepsis, it is attributed as a global epidemiological burden (Álvaro-Meca et al., 2018). Sepsis is a state of abnormally enhanced systemic inflammatory reaction of the host that causes multiorgan failure and death. Abrupt nitric oxide production has been implicated with the severity of septic injury. Several lines of evidences have recorded the contribution of NOS2 derived nitric oxide in cellular toxicity during septic shock (Takatani et al., 2018; Winkler et al., 2017). However, role of NOS1 has not been well elucidated in context of sepsis-associated inflammatory responses. (Chandra et al., 2006; Fink, 2014; Kirkebøen and Strand, 1999). This study emphasizes on the role of NOS1 in sepsis and the associated mechanism that leads to activation of inflammatory cascades in macrophages.

1.2 Monocyte and Macrophage

In late 19th century, Elie Metchnikoff, the father of innate immunity, described the role of specialized phagocytic cells, macrophages, and macrophages, in the host response to injury, inflammation, infection and tissue repair (Gordon, 2008; Merien, 2016b; Tauber, 2003). Immune response to pathogens is mediated by a diverse group of cells known as white blood cells or leukocytes that circulate through the blood and lymphatic system and are recruited to the site of infection with distinct functional characteristics (Shi and Pamer, 2011). Approximately 5-10% of peripheral leukocytes subgroups in blood are composed of monocytes and macrophages that originate from the hematopoietic stem cells and vary in their nuclear morphology, granularity and gene expression profiles (Sica et al., 2015; Wynn et al., 2013).

1.2.1 Monocyte and macrophage lineage

Ebert and Florey in 1939, for the first time, demonstrated that monocytes circulating in blood, spleen and bone marrow migrate into tissues and become tissue-resident macrophages during inflammation (Ebert and Florey, 1939). However, recent studies demonstrated the variation in functional and morphological characteristics of these cells suggesting that monocytes are not the only source of macrophage origin (Epelman et al., 2014a). Interestingly, a recent study has led to a new insights on the origin of tissue macrophages in the embryonic yolk sac and not from the circulating monocytes (Davies et al., 2013; Epelman et al., 2014b; Hashimoto et al., 2013a). The study suggests the presence of a pool of embryonically derived and adult-derived macrophages in each tissue, thereby implying a different route of macrophage origin. The majority of monocyte-derived macrophages possess a short life within their resident tissues and are functionally designed to contribute host defense, while the embryonic-derived macrophages assist in tissue remodeling (Divangahi et al., 2015; Galli et al., 2011; Saijo and Glass, 2011; Sheng et al., 2015) However, the ability of distinctly originated macrophages to switch between their functional roles is still unclear.

Diversity in the lineage and common progenitor of monocyte and macrophage reflects its functional and phenotypic heterogeneity in different microenvironments (van Furth and Cohn, 1968). Monocytes are mononuclear phagocytes that originate through proliferation and differentiation of the common myeloid progenitor cells in the bone marrow as monoblast that further differentiate into promonocytes and then into mature monocytes, after which they are released into blood circulation followed by their recruitment at the site of injury or infection (Swirski et al., 2014). Hematopoietic stem cells (HSCs), differentiate further into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMP further diverge into mast cell progenitor (MCP), Granulocyte-macrophage progenitor (GMP) and macrophage-dendritic cell progenitor (MDP) that upon differentiation lose their multipotency and self-renewal capacity (Swirski et al., 2014).

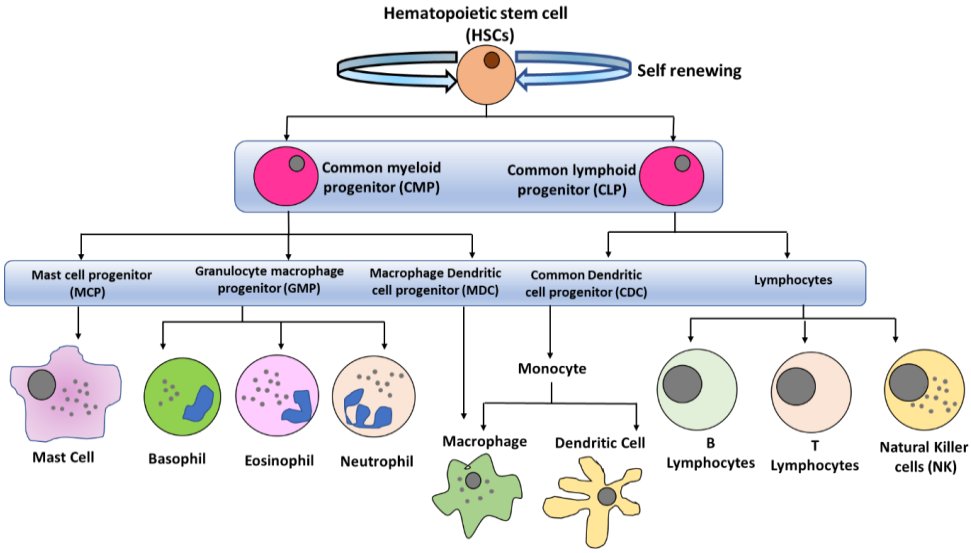


Fig 1.3. Lymphoid and myeloid differentiation from hematopoietic stem cells (HSCs)

Studies using mouse transplantation model demonstrated that mature monocytes differentiated into macrophages in lungs and liver (Kennedy and Abkowitz, 1998). CLP cells terminally give rise to lymphocytes and common dendritic cell progenitor (CDC). Differentiated cells possess distinct functional roles in their microenvironment (Fig 1.3).

Monocyte and macrophage lineage commitment occurs under tight transcriptional control. Several studies have shown the insufficient proliferation of mononuclear phagocyte cells in essential transcription factor knock out mice (Paul et al., 2015). Among the transcription factors that shape hematopoiesis, PU.1 is the master regulator of myeloid and lymphoid differentiation. Early myeloid lineage commitment is controlled majorly by PU.1 (Dakic et al., 2005; Iwasaki and Akashi, 2007; Iwasaki et al., 2005). Studies utilizing PU.1 knock out mice have provided evidences for a significantly impaired number of B cells, T cells, monocytes and macrophages, suggesting that PU.1 is indispensable throughout the myeloid differentiation cascade (Nerlov and Graf, 1998; Pahl et al., 1993). Transcription factors other than PU.1 also contribute in lineage determination, and decisions between particular lineages require transcription factor co-operation (Lichanska et al., 1999). Among the most important factors that promote monocyte production are C/EBPα, which facilitates the CMP to GMP transition interferon regulatory factor 8 (IRF8), which interacts with PU.1 to favor monocyte/macrophage over granulocyte differentiation; Krüppel-like factor 4 (KLF4), which is essential to the generation of inflammatory Ly-6C^{high} monocytes (Alder et al., 2008); and the orphan nuclear hormone receptor NR4A1/Nur77 which generates Ly6C^{low} monocytes (Hanna et al., 2011).

Central to the innate immune system, monocytes represent the effector cell population that possess pathogen recognition receptors (PRR) on their surface. PRRs recognize and bind to the pathogen-associated molecular pattern (PAMPs) (Jang et al., 2015; Mogensen, 2009). Toll-like receptors (TLRs) are kind of PRRs expressed on monocytes and macrophages that bind to specific PAMP on microbial surface. Migration of monocytes into tissues and differentiation into inflammatory macrophages is primarily determined by the external cues and PAMPs (Akira et al., 2006b; Mogensen, 2009). Monocytes are defined into three major population based on the expression of markers CD14 and CD16 (Stansfield and Ingram, 2015). Approximately 90% of human monocytes are defined as classical population equipped with high CD14 and lacking CD16, the second intermediate subset is composed of intermediate CD14 and CD16 while the third subset population possesses low CD14 and

high CD16 expression known as the non-classical subset (Boyette et al., 2017; Ziegler-Heitbrock, 2015). The classical and intermediate Subset represent inflammatory monocytes while the non-classical monocytes and reside majorly in the blood vessels (Wildgruber et al., 2016; Ziegler-Heitbrock, 2015). Based on the environmental cues, the inflammatory monocytes modify their phenotypic characteristics.

The proliferation of phagocyte lineage cells is controlled by the hematopoietic growth factor CSF-1 (colony stimulating factor-1), the receptor for which is encoded by the c-fms oncogene expressed on the monocyte and macrophages (McClanahan et al., 1993; Pierce et al., 1990). Studies on mice lacking a functional Csfl gene suggest an essential role of Csfl in development of monocytes (Sherr, 1990). IL-34 and M-CSF have been explored as the predominant ligands for Csflr and contribute largely to the development of mononuclear phagocyte lineage. Differentiation of monocytes into M1 or M2 macrophages is dependent on the exposure of monocytes to the cytokines such as GM-CSF and IL-4 respectively (Guimarães-Costa et al., 2017; Sallusto and Lanzavecchia, 1994). These studies suggest that monocytes are phenotypically programmed by the specific environmental stimulus. Activation of monocytes by IFNγ and LPS activates inflammatory M1 macrophages that further mediate the Th1 response. However, IL-4 induction is associated with the M2 macrophage polarization that promotes tissue repair and resolution of inflammation (Casella et al., 2016; Hao et al., 2017).

Interestingly, GMPs expressing PU.1 impedes the effect of transcription factor C/EBPα that programs monocytes into neutrophil differentiation, instead induces the macrophage-specific transcription factors such as Erg-1 and Erg-2 (Laslo et al., 2006; Ziegler-Heitbrock, 2015). On the contrary, high expression of PU.1 initiates DC induction from monocytes and inhibits the macrophage promoting transcription factors c-Maf and MafB (Bakri et al., 2005). A similar negative regulation of MafB on PU.1 activation has been reported in the HSPCs (Sarrazin et al., 2009). Furthermore, combinatorial effect of transcription factors with the specific cytokine receptors has emerged as an important factor in determining the monocyte lineage population. In this

context, a combination of MafB and c-Maf downregulates the M-CSF derived macrophages (Sarrazin et al., 2009). Other transcription factors such as Runx3 and Id2 have been shown to modulate the TGFβ in monocyte lineage cells (Zenke and Hieronymus, 2006). These studies suggest a crucial role of transcription factors in determining the fate of hematopoietic stem progenitor cells.

In vitro studies on a population of mice peritoneal macrophages exhibit their proliferation efficiency in presence of CSF-1 (Stanley et al., 1978; Stewart et al., 1975). Immature macrophage lineage cells develop into macrophage colony forming cells (M-CFU) in response to CSF-1. M-CFU further proliferates to give rise to mature macrophage populations that possess microbicidal properties. Murine myeloid progenitor cells have been identified based on the expression of cell-specific markers such as ER-MP12, ER-MP20, ER-MP58 and Ly6C that delineate its fate after maturation. Particularly, the myeloid lineage cells derived from bone marrow is characterized by ER-MP12^{high} and ER-MP20^{low} phenotype and are responsive to CSF-1(de Bruijn et al., 1994). Early stage myeloid progenitor cells express ER-MP58 that marks the myeloid committed cells from other bone marrow-derived progenitor cells(Ling et al., 1997), however after maturation into mature macrophages the expression of ER-MP58 is diminished suggesting its significance in the early stage of myeloid lineage commitment. Inflammatory macrophages, however, express high ER-MP20(Hanna et al., 2011).

There are many cell types that participate in inflammatory reactions, however macrophages are the most active mediators of inflammation during chronic inflammatory responses. Macrophages play an indispensable role in the innate immune system by clearing the pathogens and debris from the tissues through phagocytosis and initiation of inflammatory signalling cascade to get rid of the invading pathogens (Gordon and Martinez, 2010). Macrophages express pattern recognition receptors (PRRs) which are functional components of their phagocytic machinery and inflammatory circuit. PRRs recognize and bind to the pathogen-associated molecular pattern (PAMPs). Toll-like receptors (TLRs) are kind of PRRs expressed on macrophage that bind to specific

PAMP on microbial surface. The concept of classical and alternatively activated macrophages known as M1 and M2 display distinct phenotypes based on specific environmental stimuli and have been interestingly studied (Murray and Wynn, 2011).

Macrophages evolved as the phagocytic cells of the innate immune system that differentiate from the circulating monocytes released from the bone marrow into distinct body tissues protecting host by clearance of microbial and dead cells. Monocytes and macrophages have a vital role in maintaining tissue homeostasis and immunity. However, their contribution to disease pathologies suggests their relevance as attractive therapeutic targets. Macrophages guard the body of any microbial attack by shielding the deleterious effect of pathogenic stimulus triggering the immune response (Kelly and O'Neill, 2015; Plüddemann et al., 2011; Wynn et al., 2013). Therefore, the role of macrophages in innate immunity is of central importance. Macrophages exhibit a variety of subtypes depending on the type of resident tissues such as lungs, liver, bone and brain. Functional flexibility of macrophages allows them to regulate multiple signalling cascades resulting in either tissue damage or repair (Dunster, 2016). Deregulated macrophage behavior gives rise to chronic inflammation and unresolved wounds. Therefore, it is important to conceptualize the macrophage behavior during inflammation that drives therapeutic approaches for immunotherapy.

Macrophage lineage cells are responsive to CSF-1, however, its receptor c-Fms in absent in other myeloid and lymphoid lineages (Sherr et al., 1985). Therefore c-Fms is considered a macrophage lineage cell-specific marker that may be employed for identification of macrophage lineage cells in tissues from other progenitor cells. Heterogeneity of monocyte and macrophage population is delineated based on the differential expression of cell surface markers such as CD14 and CD16. A classical subset of human monocytes are characterized by $CD14^{high}$ and $CD16^{low}$and are marked by high expression of Lymphocyte antigen 6 complex (Ly6C) and chemokine (C-C motif) receptor 2 (CCR2) (Geissmann et al., 2003a; Passlick et al., 1989). Monocyte exhibit enormous plasticity and continue to proliferate and mature in the blood and during this process, they are recruited into resident tissues as heterogenous population

maintained by GM-CSF (Hashimoto et al., 2013b; Sunderkötter et al., 2004; Yona et al., 2013). It is therefore fascinating to understand the differentiation pattern of monocyte lineage cells under different environmental cues that further maintains the entire myeloid and lineage population.

1.2.2 Mouse and human macrophages

A comparative analysis of human and mouse leukocyte populations illustrates remarkable differences. Mice white blood cell population is composed of 75-90% lymphocyte, 10-25% neutrophils and only 2% of monocytes. On the contrary, humans possess 50-70% lymphocytes, 20-40% neutrophil and 10% of monocyte population(Zschaler et al., 2014). M1 or M2 macrophage subsets are identified based on presence of surface markers and differential gene expression profiles in human and mice. In this context, mouse M2 macrophages are marked by the expression of Chi3l3 (Ym1), Relma (Fizz1), matrix metalloproteinase-1 (MMP1) and arginase 1 (Arg1) which are absent in humans. While human-specific markers such as Chi3l2 (Ykl39), KLF4, fibrinoligase and platelet-derived growth factor C (PDGFC) are absent in mice (Jablonski et al., 2015).

Mouse and human macrophages differ significantly in arginine metabolism. Enormous controversy exists in context of inducible nitric oxide synthase (iNOS) expression in human and mice. iNOS is the key enzyme that metabolizes arginine to L-citrulline and produces large amounts of nitric oxide (NO). Evidences support the expression of iNOS in mice upon stimulation with LPS and IFNγ but not in humans., suggesting an alternative source of nitric oxide production in humans (Murray and Wynn, 2011; Schneemann and Schoeden, 2007; Schneemann and Schoedon, 2002). Lack of iNOS in human macrophage counterparts remains a puzzling paradigm in macrophage biology. The possible reason to this mystery can be related to the fact that some studies have been performed in vitro on monocyte-derived macrophages while others have been validated directly on tissue macrophages, and both sources of macrophages may behave differently under different environmental stimulus.

Absence of epigenetic regulation is proposed as a defining factor for the lack of human iNOS expression (Murray and Wynn, 2011). Synthesis of cofactor required for iNOS, tetrahydrobiopterin occurs in mice macrophage but not in humans. Moreover, induction of human macrophages with the iNOS cofactor failed to initiate NO production (Denis, 1994). However, conflicting reports suggest a significant iNOS expression in certain disease models with a less defined mechanism of iNOS expression (MacMicking et al., 1997; Weinberg, 1998a). A combination of stimulating factors such as IFNγ and IL-4 or IL-4 and CD-23 promotes NO production in human macrophage. Evidence from studies on human macrophages suggest activation of human macrophages by interferons α and ß triggers iNOS expression. Arginine acts as substrate to arginase-1 that metabolizes it into L-ornithine and urea. M2 macrophages in mice show higher expression of Arg-1 while diminishing the synthesis of iNOS in M1 macrophages (Munder et al., 1998).

Another line of difference in human and murine macrophages exist in the pattern of expression of arginase type-1. While humans constitutively express arginase type -1 in neutrophil granules contributing to antimicrobial characteristics by metabolizing arginine. However, there is no evidence of expression of arginase type-1 in resting leukocyte population in mice. IL-4 and IL-13 act as primary stimulants to induce arginase type-1 expression in mice but not in humans (Munder et al., 2005). In patients suffering from trauma, mononuclear phagocytes show elevated expression of arginase type-1.

Presence of common markers in human and mouse monocytes present a line of similarity between the two species. $CD14^{+}CD16^{+}$ human monocytes and $CD115^{+}$Ly-$6C^{lo}$ mouse monocytes share common characteristic owing to the similar expression pattern of CCR1 and CCR2 on $CD16^{-}$ human and Ly-$6C^{+}$ mouse monocytes (Geissmann et al., 2003b; Tacke et al., 2007). Moreover, CX3CR1 is significantly upregulated on $CD16^{+}$ human and Ly-$6C^{lo}$ mouse monocytes. Monocyte subsets in mice and human also share a conserved expression of CD43, CD11a and CD26L. Further, human monocytes are widely identified by the CD16 marker which has also been reported in murine $6C^{lo}$, but not Ly-$6C^{+}$, monocytes (Santiago-Raber et al.,

2009). In addition, both mouse Ly-6C^{lo} monocytes and human CD16 monocytes have higher expression of CD11c (Auffray et al., 2007; Sunderkötter et al., 2004; Swirski et al., 2014). Similar expression pattern of surface markers still need to be elucidated in different subsets of monocyte and macrophages (Serbina et al., 2008).

In vitro production of macrophages is driven by providing external macrophage colony stimulating factors (M-CSF) in culture medium from the bone marrow. However, the circulating population varies from the tissue-resident macrophages due to different source of stem cell progenitors (Epelman et al., 2014b). These reasons account for differences in the NO production ability by the cells. Bone marrow-derived macrophages from mice and the human in vitro macrophages behave differently in response to LPS or IFNγ (Bogdan, 2001a; Schneemann et al., 1993; Weinberg, 1998b). Human macrophages in this regard are less sensitive to activation by LPS as compared to the mouse counterparts. A number of research suggest that human macrophage activation might be accelerated by stimulus other than LPS or IFNγ (Bronte and Zanovello, 2005; Schneemann and Schoedon, 2002). In vitro activated human macrophages respond late to LPS over the bone marrow-derived macrophage (Geelhaar-Karsch et al., 2013).

1.2.3 Macrophage activation

Macrophages respond to the environmental stimulus and exhibit two extreme activation pathways, the classical macrophage activation that leads to the abundance of inflammatory M1 macrophages that possess bactericidal properties and clear the pathogen. The other type is the alternative macrophage activation which is induced by IL-4/IL-13 or growth factors such as TGF-β, that promote tissue healing and inflammation resolving M2 macrophages. LPS and IFN induce M1 macrophages (Fig 1.4).

1.2.3.1 Classical macrophage activation

Critical markers for M1 macrophages include IL12^{high}, IL23^{high} and IL10^{low} (Martinez and Gordon, 2014). Other factors that induce M1 macrophages include IL1β, TNFα and GM-CSF. Classical activation of macrophages is associated with Th1 response. M1 macrophages are characterized by high iNOS/NOS2 production leading to abundance of nitric oxide that acts as a critical mediator of inflammatory reaction.

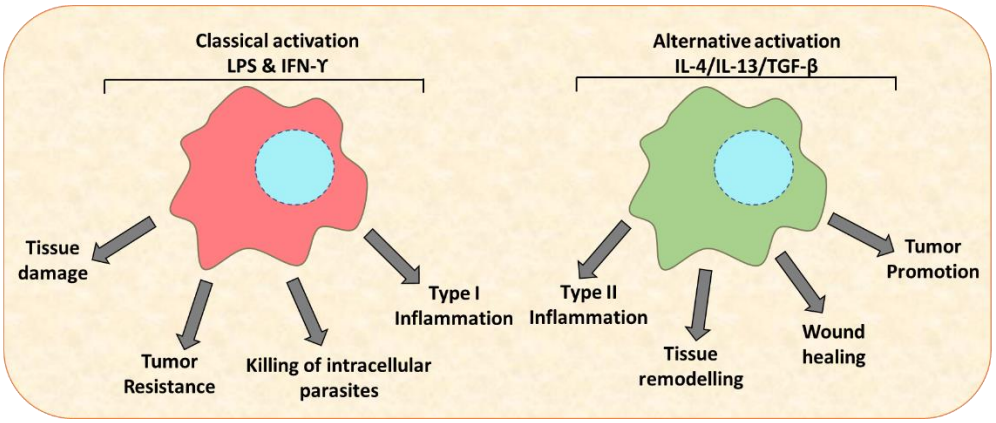


Fig 1.4. Classical versus alternate macrophage activation

M1 macrophages display diminished expression of mannose receptor and Fc receptor for IgG (FcαR) II and a higher expression of active MMP1 and MMP9. Classically activated M1 macrophages are essential for elimination of toxic bacterial agents from the body. However, it is important that the inflammatory reaction once initiated must be resolved and prevented from prolonged activation as it might damage the tissues and result in chronic inflammatory disorders. It is therefore important to maintain a balance between pro and anti-inflammatory reaction originating in macrophages. Classical macrophage activation is characterized by proinflammatory cytokine secretion, phagocytosis and antigen presentation. Naïve T cell activation into Th1 cells by IL12 secretion is another critical function of classically activated macrophages.

Lipopolysaccharide from gram-negative bacteria initiates inflammatory signalling cascade in macrophages, thereby activating the TLR4/NFkB pathway, which directs the macrophage phenotype towards M1 (pro-inflammatory characteristics). Downstream of the TLR4 signalling cascade, NFkB activation occurs via its p50/p65 subunit which in turn induces the expression of pro-inflammatory cytokines (Kitamura et al., 2000). While at the same time elevated expression of IkB gene in the nucleus, suppresses the expression of NFkBp50/p50 homodimer that is known to induce the anti-inflammatory cytokines (Bonizzi and Karin, 2004) which results in classical activation of macrophages. This interesting regulation of macrophage switching is based on the activation status of NFKb, either in form of p50/p65 subunit which is a mark for M1 macrophage phenotype or p50/p50 subunit which balances the excess pro-inflammatory cytokine production and shifts the phenotype to anti-inflammatory M2.

In addition to the NFkB mediated regulation of macrophages, another mechanism depending on STAT1 phosphorylation, downstream of TLR4 activation has been well appreciated for determining the classical activation of macrophages (Luu et al., 2014). STAT1 phosphorylation enhances the expression of downstream expression of pro-inflammatory cytokines such as TNFα. These results suggest a dynamic mechanism existing within the highly orchestrated signalling event in macrophage, which can switch the macrophage phenotype to M1 or M2.

NF-kB activation is dependent on the trimeric complex inhibitor of kappa B kinase (IKK) which is composed of two kinases IKKα, IKKβ and a regulatory protein IKKγ. Stimulatory signals originating from TLR4 receptor lead to the phosphorylation of kinase IKKβ which further phosphorylates and activates inhibitor of kappa B (IkB). Functional IkB activates downstream degradation of IkB causing the release of NFkB p65-p50 heterodimer release from NFkB-IkB complex. Macrophage release of functional NFkB leads to its nuclear translocation where it binds to the promoter region of pro-inflammatory genes. Transcriptional activation of inflammatory genes polarizes the macrophages to M1 phenotype (Wang et al., 2014a).

Another interesting mechanism of classical activation manifesting NFkB subunits is via p50 homodimer regulation. TLR4 induced signals in response to LPS activate the NFkB pathway via inhibitor IKKB complex, leading to p105 proteolysis generating p50 homodimers Despite lacking the transactivation potential, p50 homodimers activate proinflammatory gene transcription with aid of Bcl3 as coactivator. IKKB phosphorylation of p105 triggers release of Tpl2, another kinase that further activates downstream Erk kinase. Activation of NFkB and Erk MAP kinases induces expression of proinflammatory gene in response to microbial LPS stimulation thereby promoting macrophage switch toward M1 phenotype by release of cytokine genes. Macrophage polarization is therefore dependent on transcription factor NFkB and IKK complex that regulates the polarized state of stimulated macrophages.

Central to the classical activation of macrophagesAP1 transcription factor is one of the key modulators of inflammation composed of Jun, Fos and ATF2 family of proteins. Among the members of AP1 family, Jun can homodimerize or heterodimerize with either Fos or ATF2. However, Fos and ATF2 can only form heterodimer with Jun and do not possess homodimerization ability. In addition, some members of the activating transcription factor and cAMP response element-binding protein families also dimerize with the core members of the AP-1 family to regulate a broad variety of inflammatory genes by binding to their promoter and enhancer regions Recently AP1 mediated mechanism of macrophage switch has been proposed that shed light on an important regulatory mechanism of macrophage phenotype (Srivastava and Baig, 2018). AP1 subunits Fos and Jun together form the active AP1 dimer that activate the transcription of proinflammatory genes polarizing the macrophage towards M1 phenotype. In addition to the major transcription factors, inflammatory signalling arising due to classical activation also activates the resident macrophages to stimulate p38 MAP kinase pathway to perpetuate the inflammatory response. IL4 plays an important role in promoting M2 phenotype in macrophages. Interestingly, studies on thioglycollate stimulated peritoneal macrophages show that IL4 induction

activates STAT6 and PI3K pathways along with activation of p38 MAP kinase via phosphorylation.

1.2.3.2 Alternative macrophage activation

Macrophage activation is a highly dynamic event that display features of anti-inflammatory M2 phenotype with an elevated expression of IL10 and lower IL12 and IL23. Alternative macrophage activation creates a pool of macrophages that express M2 phenotype and downregulate the inflammatory reactions. Placental and alveolar derived macrophages represent a naturally occurring alternatively activated macrophages and act as first line of defense to invading pathogen without employing a Th1 response. IL4 along with IL13 that are the major cytokines of Th2 immune response and glucocorticoids are major inducers of alternatively activated macrophages. Studies have shown that macrophages stimulated with IL4 and IL13 cease pro-inflammatory cytokine production and antigen presentation to T cells (Doyle et al., 1994). M2 macrophages utilize arginine and metabolize it into ornithine and polyamines that support tissue regeneration and repair (Bashir et al., 2016; Murray and Wynn, 2011; Wang et al., 2014b).

Alternative activation of macrophages predominantly involved in wound healing and tissue repair after inflammatory lesions in affected tissues. Production of growth-promoting factors such as TGFβ and PDGF aid in the formation of extracellular matrix are hallmark features of M2 macrophages. In addition, high expression of mannose and galactose receptors further dampen the damage caused by infiltrating M1 macrophages by diminishing the expression of proinflammatory IL12 and IL23 cytokines. Various forms of alternatively activated macrophages (AAM) exist in humans (Murray and Wynn, 2011). IL4 is associated with alternative macrophage activation owing to suppressed TNFα and IL6 expression in macrophages. Alternative macrophages are subdivided into three groups M2a, M2b and M2c based on the cell-specific expression of markers and receptors. M2 polarized macrophages inhibit the downstream response of NFkB and STAT1 that are potent regulators of inflammatory reactions. M2a macrophages are profibrotic in nature and are characterized by production of CCL17, CCL18, CCL22 and

CCL24. M2b macrophages are abundant in IL10 and reduced IL12 and IL23 gene expression profiles. Lastly M2c macrophages exhibit enormous expression of CXCL13, CCL16 and CCL18, helps in inflammation suppression and neovascularization. Reprogramming macrophages from classical to alternatively activated states assist in resolution of inflammatory outburst in injured tissues. Proinflammatory M1 macrophages while repolarizing into M2 enhance the expression of M2 markers chitinase-like 3 (YM1) and arginase 1 (ARG1) and transferrin receptor (TFRC) during the resolution phase of inflammation

Several pathological conditions show high polarization towards alternatively activated macrophages leading to onset and offset of inflammation (Mantovani et al., 2002). Studies have shown that the p50 subunit of NFkB is a major regulator of alternative macrophage activation. Studies show that NFkB p50 promotes RNA Pol II recruitment of M2 promoting genes such as CCL17 and Arg1 while limiting its access to the M1 promoting genes such as iNOS, TNFα and IFNβ (Porta et al., 2009). These results have a strong indication towards regulatory activity of p50 homodimer towards M2 tolerant phenotype in-vitro and in-vivo. Previous studies on peripheral blood of sepsis patients have shown accumulation of p50 homodimer in the macrophages, indicating that p50 is indeed an important player in the inflammatory signalling (Ziegler-Heitbrock, 2001). Transcriptional profiling of tumor-associated macrophages characterized as M2 polarized macrophages also showed NFKB p50 accumulation (Biswas et al., 2006), thus indicating that p50 NFkB has a significant role in suppression of inflammation and it can be used as a major therapeutic target for controlling acute and chronic inflammatory disorders.

M2 macrophage-associated genes are up-regulated by histone demethylase JMJD3 induced by IL-4 in mouse macrophages which modulates chromatin modifications to promote expression of M2 genes and inhibit M1 genes. miR-155 was recently identified as targeting the IL-13Rα1 subunit, thus decreasing a set of M2 genes in human macrophages (Martinez-Nunez et al., 2011). In mouse macrophages, Arginase1 gene contains STAT6 response elements upstream of promoter region, which is transcriptionally regulated by IL13 and IL4 (Dzik, 2014; Munder, 2009). Furthermore, inhibition of p38 MAP kinase

pharmacologically or by gene silencing diminishes the upregulated M2 marker response in peritoneal macrophages suggesting that that p38 MAP kinase is involved IL4 signalling to mediate the expression of M2 markers (Jimenez-Garcia et al., 2015). IL4 induction in macrophages, activates protein tyrosine kinase (JAK) that causes subsequent STAT6 phosphorylation and its translocation to nucleus to induce expression of its target genes.

In M2 macrophages, IL4 induction up-regulates Arg1 expression through STAT6 activation (Gray et al., 2005). In context of JAK/STAT6 mediated regulation of macrophage phenotype, studies on thioglycollate stimulated peritoneal macrophages report IL4 dependent p38 MAP kinase activation. This results in STAT6 activation via phosphorylation. Possible mechanism of M2 polarization was determined by assessing the involvement of JAK/STAT6 signalling pathway in promoting M2 marker expression. IL4 signalling drives STAT6 phosphorylation via JAKs, further regulating M2 markers expression levels. The study first confirmed the increased phosphorylation of STAT6 upon IL4 induction followed by decrease in Arg1, Fizz1 and YM1 expression in presence of JAK inhibitor (JAK-I). This study provides interesting clues on the involvement of JAK/STAT6 mediated regulation of M2 macrophage phenotype. Moreover, macrophage M2 phenotype was confirmed by upregulation of M2 markers Arg1, Fizz1, YM1 and IRF4 and shown to be dependent on p38 MAP kinase pathway (Jimenez-Garcia et al., 2015). Thus, activation of p38 MAP kinase significantly triggers the M2 phenotype. Moreover, the same study also revealed that inhibition of p38 MAP kinase abolishes STAT6 and Akt phosphorylation implying that p38 MAP kinase acts upstream of these two pathways.

Interestingly studies have emphasized on p38 MAP kinase-dependent IL4 activity (Canfield et al., 2005). To understand the molecular mechanism of p38 and PI3K pathway in activating M2 markers, specific inhibitors of p38 MAP kinase were used which significantly inhibited the IL4 induced Arg1, YM1, Fizz1, IRF4 and SOCS1 mRNA and protein expression along with diminished Arginase1 activity, while PI3K inhibitor only partially inhibited the expression of M2 markers (Canfield et al., 2005). IL4 signalling also induces other MAP kinases such as ERK and JNK (Moore et al., 2002) apart from p38. However,

no significant difference was observed in the expression levels of M2 markers Arg1 and YM1 in presence of ERK and JNK inhibitor (PD98059 and SP600125) confirming p38 MAP kinase-dependent IL4 signalling and its critical involvement in M2 macrophage switching. Moreover, si-RNA mediated knock down of p38 MAPKα impeded its phosphorylation and subsequently Arg1 mRNA and protein levels (Canfield et al., 2005).

In addition to the in vitro model of M2 macrophage polarization, the study also reported the involvement of p38 MAP kinase in regulating M2 phenotype in a chitin-based in vivo model.Phosphatidylinositol-3-kinase (PI3K) is a molecule downstream of TLR4 triggering inflammatory response by activating protein kinase Akt via PIP3 (phosphatidylinositol-3,4,5 triphosphate). Macrophage polarizes to M1 or M2 phenotype through Akt2 or Akt1 kinase respectively based on microRNA mediated mechanism. miR-155 is a major player in macrophage polarization via PI3K/Akt pathway. Akt2 activates the expression of miR-155 which enhances NFkB and dampens SOCS1 expression. Consequently, impeded SOCS1 results in switch of macrophage phenotype to M1 by generating higher amount of gene products NOS2 and TNFα. Contrary to Akt2, Akt1 downregulates, miR-155 resulting in higher expression of M2 genes IL4 and Arg1. Macrophage M1 to M2 plasticity is tightly governed by coordinated action between Akt1 and Akt2 kinases which determines the overall inflammatory response in macrophages.

In addition to above inflammatory mediators, Kruppel like factor (KLF4) is another transcription factor that regulates macrophage polarization towards M2 phenotype induced by IL4 and IL13 stimuli. Reports by Liao et al. suggest that KLF4 along with STAT6 exerts additive effect on M2 macrophage polarization by sequestering the co-activators of NFkB activation. Macrophages lacking KLF4 were prone to M1 phenotype demonstrating pro-inflammatory characteristics along with enhanced bactericidal activity. Furthermore, mice bearing myeloid-specific deletion of KLF4 exhibited delayed wound healing and were predisposed to developing diet-induced obesity, glucose intolerance, and insulin resistance. Collectively, these data identify KLF4 as what we believe to be a novel regulator of macrophage polarization.

Alternatively activated M2 macrophages are activated by IL-4 and IL-13 through STAT6 activation involving the IL-4 receptor alpha (IL-4Rα) whereas IL-10 activate M2 macrophages via STAT3 pathway through IL-10 receptor (IL-10R) (Wang et al., 2014c). In addition, IL-33, Th2 cell-associated cytokine induces M2 polarization and amplifies the anti-inflammatory effect of IL-13 by up-regulating CCL17, CCCL24 and Arg-1(Kurowska-Stolarska et al., 2009). Among the stimulators that promote M2 phenotype, IL21 also orchestrates the anti-inflammatory M2 macrophages secreted by activated Th2 cells. Macrophages activated through alternative pathway are profibrotic and pro-angiogenic in nature and therefore promote regeneration of the damaged tissue (Braga et al., 2015; Murray and Wynn, 2011; Wang et al., 2014b).

1.2.4 Macrophage Plasticity

Cellular plasticity was originally associated with stem cells that possess the ability to differentiate into cell types with multiple functions. Monocyte and macrophage lineage cells are equipped with plasticity and flexibility and switch into heterogenous functional phenotypes based on environmental cues (Mantovani et al., 2007; Sica and Mantovani, 2012). Polarized macrophage switch between two subsets in vitro and in vivo. Changes in the microenvironment alters the functional phenotype of macrophages by the process of cellular reprogramming. Dynamic polarization of macrophages into proinflammatory M1 or anti-inflammatory M2 provokes inflammatory cascade or terminates inflammatory reaction respectively (Zhou et al., 2014).

Macrophage polarization to M1 or M2 is dependent on type 1 helper T-cell (Th1)/type 2 helper T-cell (Th2) response. Th1 cells secrete TNFα and IFNγ that polarize the macrophages towards M1 phenotype that show microbicidal activity by excessive production of IL12. On the contrary, Th2 secreted cytokines such as IL-4 and IL-10 induce M2 macrophages that support wound healing. Polarization event of macrophages is most critical for developing a healthy and balanced innate immune response. While at one instance, M2 (anti-inflammatory) phenotype is essential to control the excess inflammation,

on the other hand, a prolonged M2 phenotype can also compromise the antiviral and antibacterial immune response. At the end of inflammatory reaction, if the macrophage phenotype does not switch from M1 to M2 it prolongs the production of pro-inflammatory cytokines which damages the tissues and results in chronic inflammatory disorders (Cavaillon and Adib-Conquy, 2006). Polarization of macrophages from M1 to M2 promotes growth healing conditions. However the M2 phenotype is switched under tumor-induced inflammation, which also results in tumor progression (Rakoff-Nahoum, 2006).

Existence of both M1 and M2 macrophages in the same microenvironment has been reported in allergic and alcoholic hepatitis conditions. Macrophages exist in M1 or M2 phenotypic states, residing in different tissues and organs of body. In this regard, colony stimulating factors play a critical role in determining macrophage phenotype, particularly M-CSF and GM-CSF (Hamilton and Achuthan, 2013). Experimental evidences suggest the association of GM-CSF with classical M1 activation while that of M-CSF with alternative M2 activation (Akagawa et al., 2006)(Fleetwood et al., 2007). Stimulation of macrophages with LPS+IFNγ and cultured with GM-CSF induced production of IL12 and IL23 but not of IL10, which polarizes macrophage towards M1 phenotype. On the contrary, significant amount of IL10 production was observed by M-CSF cultured macrophages under similar microenvironment and stimulation which marks the M2 polarization of macrophages (Verreck et al., 2004). Higher levels of pro-inflammatory cytokines such as TNFα, IL6 and IL1β were also observed by GM-CSF as compared with M-CSF in the same study indicating differential involvement of GM-CSF and M-CSF in regulating the polarization events associated with monocyte-derived macrophages.

Another critical factor determining macrophage polarization is the crosstalk in the major regulatory pathway IRF/STAT that governs the switch of M1 to M2 macrophages. As shown in Fig.1.4, IFN-y and LPS stimulate classical activation of macrophages. The stimulus is enhanced via TLR 4 and IFN-y receptor, thereby leading to the activation of downstream transcription factors STAT1 , IRF3 and NF-kB. Activation of STAT1 and NFkB results in M1

phenotype with inflammatory and cytotoxic characteristics. Whereas STAT3 activation via IL10 promotes M2 phenotype, resulting in tissue healing and proliferation. Within the M1 activation state SOCS3 protein is activated through the downstream signalling events. IL10 promotes M2 macrophage phenotype by formation of NFkB p50 homodimer. NFkB being the key transcription factor in the M1 phenotype regulates the expression of genes encoding for proinflammatory cytokines such as TNF-α, IL12, IL23, IL1-β and Cox2.

Atherosclerotic lesions involve the accumulation of apolipoprotein B containing lipoproteins in the walls of artery leading to inflammation. Mechanism underlying the polarization of macrophages during atherosclerotic progression has been well reviewed (Peled and Fisher, 2014). Plaques resulting from atherosclerotic lesions are rich in both M1 and M2 macrophages with M1 macrophages directing the progression of disease while M2 macrophages in the regression of disease. Previous studies indicate the possibility that M1 macrophages are replaced by M2 macrophages during regression of atherosclerosis (Llodra et al., 2004).

The concept of macrophage plasticity underlies the effect of stimulants such as LPS and IFNγ that initiate switching of macrophages towards M1 phenotype accelerating the production of proinflammatory mediators. This further develops a positive feedback loop turning on the inflammatory cascades. Anti-inflammatory cytokines, such as IL-10,TGFβ and IL-4 direct the macrophages to exhibit M2 phenotype, that amplifies the anti-inflammatory signalling pathways (Mantovani et al., 2007). Macrophages after reprogramming to M1 functional state, activate cellular immune response that promotes Th0 cell differentiation into Th1 cells or cytotoxic T-lymphocytes, that actively kill the invading bacterial and viral particles. On the contrary, macrophage polarization towards M2 activates humoral immune response and stimulates Th0 cells differentiation into Th2 cells that aids in B cell development and production of antibodies .

Plasticity of macrophages in two varying phenotypes not only alters their functionality but also modulates other effector cells of immune cells such as T

cells and B cell, that are essential for eliminating the toxic pathogen. Negative regulators such as the members of SOCS family regulate the STAT-mediated macrophage programming under the influence of environmental factors. M2 activating cytokines such as IL-4 and IL-13 act as positive regulators of SOCS1 and SOCS3 that foster the inhibitory action on STAT1 and STAT3 respectively (Liu et al., 2008; Whyte et al., 2011). A network of transcription factors orchestrates macrophage polarization in parallel with STAT/SOCS pathway. Interestingly, STAT6 associates with and synergizes the effect of PPARγ and KLF4 (Cao et al., 2010; Liao et al., 2011; Szanto et al., 2010). PPARγ (Odegaard et al., 2007) and PPARδ (Odegaard et al., 2008) regulate the M2 macrophage expressed genes. KLF4 is an essential transcription factor expressed in M2 macrophages that cooperates with STAT6 to induce the transcription of M2 genes and suppress M1 genes regulated by NF-kB. IL-4 the major regulator of M2 macrophages, activates c-Myc gene in humans and controls macrophage polarization towards M2 phenotype (Pello et al., 2012).

1.2.5 Macrophage plasticity in Health and Diseases

Macrophages are the most crucial cells of the immune system with enormous plasticity that enables them to prevent the progression of many diseases. Macrophages reside in different organs and perform the phenotypic function in response to the environmental stimulants. Among these, alveolar macrophages in lungs, adipose macrophages in adipose tissues, Langerhans cells in skin, osteoclast in bone tissues, peritoneal macrophages in peritoneal cavity and microglia in brain acquire distinct phenotypes in the tissue. The ability of immune system to resolve various disease states depends on the extent of macrophage plasticity. Imbalance in plasticity of macrophages might negatively affect the immune system and lead to onset of number of diseases such as bronchial asthma, arthritis, atherosclerosis and cancer. Therefore, it is important to understand the underlying mechanism of macrophage plasticity that determines macrophage functions in a healthy immune system. However, imbalance in the phenotypic polarization of macrophage results in multiple diseases that compromise the immune system (Fig 1.5).

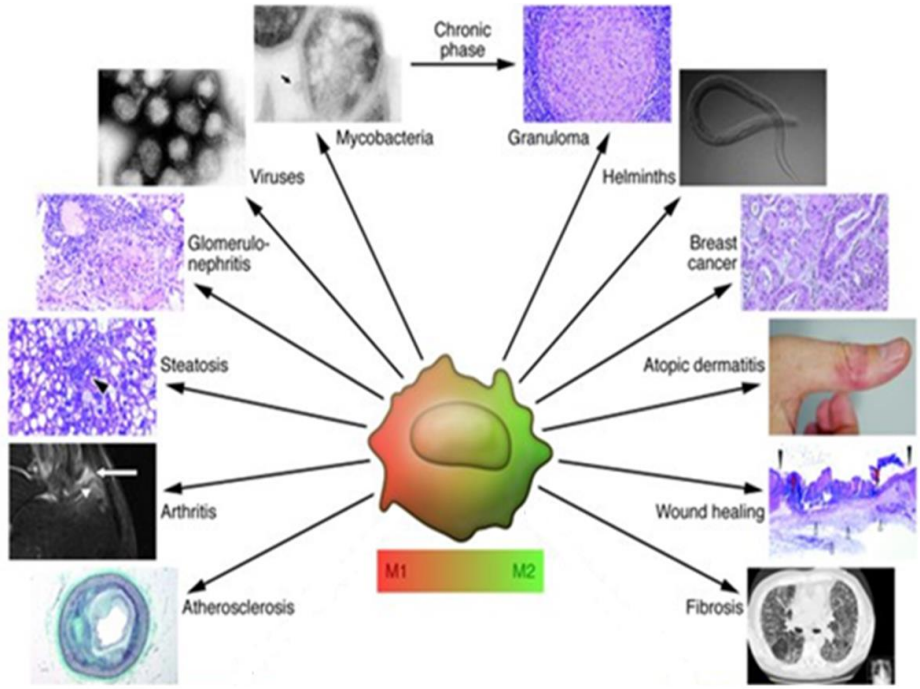


Fig 1.5. Diseases associated with macrophage plasticity
(*J. Clin. Invest.* 2012;122(3):787-795)

Macrophage challenged by bacterial pathogens reprogram into M1 phenotype in early stage of infection. Activated macrophages the produce inflammatory mediators such as NO, IL-1 and TNF that kill the invading bacteria and clear the tissue microenvironment from toxins (Yona et al., 2013). Studies show that infection by *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Salmonella typhi* activate M1 macrophage in a similar way and aid in combating the pathogenesis of severe disease state resulting into sepsis (Sica and Mantovani, 2012). Plasticity of macrophages is well appreciated in patients with typhoid fever, that show polarization of macrophages form inflammatory M1 to wound healing M2 to protect the host from excessive damage caused by infection.

Plasticity of macrophages is also associated with viral infections. M2 macrophages predominantly acquire the influenza virus-infected tissues that clear the apoptotic cells and assist in resolution of inflammatory damage. In

severe acute respiratory syndrome (SARS) infection, M2 macrophages protect the host from long-term fibrosis by activating the STAT pathway. However, both M1 and M2 type macrophages have been reported during the onset of respiratory syncytial virus (RSV) induced bronchiolitis. M2 macrophage activated STAT6 pathway inhibits the inflammation and reduces the epithelial damage in affected tissues.

Tissue-resident macrophages play a critical role in chronic viral diseases. For instance, Kupffer cells (KCs) in liver, display anti-inflammatory characteristics by utilizing the scavenger receptors expressed on their surface and clear the apoptotic hepatocytes from the tissues. On the contrary, the resident macrophages also at as detrimental to immune health by acting as the major reservoir for HIV-1 infection and induces HIV-1 that leads to neurological damage.

Fibrosis, a disease condition characterized by excessive ECM formation is widely associated with chronic inflammatory injury due to rapid proliferation of myofibroblasts that are unresolved by macrophages(Braga et al., 2015; Wick et al., 2010; Wynn and Ramalingam, 2012); Wick et al., 2010; Wynn and Ramalingam, 2012). M1 macrophages infiltrate the affected tissues and release high amounts of cytokines such as IL-12, IL1-β, iNOS, MCP-1,CCR-2 and MMPs that degrade the extra ECM and preventing the tissue injury(Das et al., 2015; Sica and Mantovani, 2012). M2 macrophage-derived TGFβ promote pro-fibrotic activity and development of severe fibrosis (Labonte et al., 2014). As a therapeutic strategy, M2 macrophages have been targeted to regulate their pro-fibrotic properties.

Among the chronic disorders, asthma has been the most complex chronic inflammatory disease. Allergic asthma involves M2 macrophage recruitment to the affected lungs along with Th2 lymphocytes, mast cells and eosinophils that orchestrate the allergic asthma (Locksley, 2010; Murdoch and Lloyd, 2010). Pulmonary macrophages from asthmatic patients are implicated in pathogenesis of disease as they contribute to the production of cytokines and chemokines that in turn recruit eosinophil and basophils that worsens the disease state(Bang et al., 2011). Evidences report high number of M2

macrophages in patients with allergic asthma. The studies suggest a critical involvement of M2 macrophages in severe disease condition. Although research in macrophage polarization provides a growing evidence for involvement of M1 phenotype that induces oxidative DNA damage and leads to inflammation. Therefore, it is important to regulate the balance of M1 and M2 macrophages to resolve the complication of clinical asthma.

Dynamic plasticity has been observed among macrophages in models of ischemic heart disease (Swirski et al., 2009). In particular, the canonical NF-kB pathway is activated in macrophages from a murine model of hind limb ischemia, deficient for the oxygen sensor prolyl-hydroxylase PHD2 that further lead to activation of M2 macrophages (Takeda et al., 2011). The study presents an interesting link between oxygen deficiency and induction of pro-arteriogenic M2 macrophages.

Macrophage plasticity plays a predominant role in the pathogenesis of chronic sepsis, characterized by abrupt inflammatory response to bacterial infection affecting major organs of body. Severe septic shock acts as leading cause of death in patients with the disorder. Macrophage play a critical role in the affected microenvironment of sepsis that develop an immunosuppressive state by polarizing towards M2 phenotype. Therefore, unraveling the mechanism of macrophage plasticity in sepsis is important to develop therapeutic strategy for disease resolution.

Chronic obstructive pulmonary disease (COPD) is a global epidemic, characterized by inflammatory airway obstruction and loss of alveolar tissue thereby causing reduced respiratory surface area. It is marked by elevated accumulation of macrophages in airway epithelium during severity. Various MMPs secreted by macrophages (MMP 1,9,12) are involved in the pathogenesis of the disease. There is high expression of pro-inflammatory cytokines such as IL1β, IL6, IL8 and TNFα (Bucchioni et al., 2003; Daldegan et al., 2005; Sapey et al., 2009) in the lungs which are markers of M1 macrophage polarization. However a contradictory study in COPD patients also suggest involvement of M2 Macrophages (Shaykhiev et al., 2009).

Macrophage plasticity is closely associated with number of diseases and it requires precise regulation in order to balance the inflammatory response.

2. Review of Literature

Inflammation is a defense response of the immune system that protects the body from deleterious effects of invading pathogens. Mammalian Toll-like receptors (TLRs) function as sensors of infection and induce the activation of innate and adaptive immune responses (Akira and Takeda, 2004; Akira et al., 2001; Drexler and Foxwell, 2010). Upon recognition of conserved pathogen-associated molecular products, TLRs activate host defense response by mediating the activation of immune cells. Macrophages play an indispensable role in the innate immune system by clearing the pathogen(Ariel et al., 2012). However, unchecked inflammatory reactions arising in macrophages give rise to chronic inflammatory disorders, such as cardiovascular diseases, autoimmune diseases, cancer, and sepsis. (Arango Duque and Descoteaux, 2014a; Hamidzadeh et al., 2017). Therefore, it is important to identify the inflammatory mechanisms that participate in the initiation and maintenance of inflammatory lesions.

Nitric oxide synthase (NOS) are the class of enzymes that produce the smallest signalling molecule Nitric Oxide (NO). NOS utilize L-arginine as substrate and convert it to citrulline by using molecular oxygen and cofactors nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and release nitric oxide in the process (Alderton et al., 2001a; Knowles and Moncada, 1994; Nathan and Xie, 1994).

2.1 Nitric Oxide Synthases (NOS)

There are three reported isoforms of NOS: NOS1 (nNOS or neuronal NOS), NOS2 (iNOS or inducible NOS) and NOS 3 (eNOS or endothelial NOS). (Alderton et al., 2001b). All three isoforms utilize L-arginine as substrate and convert it into L-citrulline to produce nitric oxide (Fig 2.1).

Among the NOS isoforms, NOS2 (iNOS or inducible NOS) and NOS3 (eNOS or endothelial NOS) have been appreciated well as the critical mediators of inflammation (Cirino et al., 2003a; Gray et al., 2018; Ying and Hofseth, 2007). However, the involvement of NOS1 (nNOS or neuronal NOS) in inflammation is not well understood. Recently, the role of NOS1 has been appreciated in the inflammatory cascade employed in macrophages (Baig et al., 2015a). The study reports that NOS1 derived nitric oxide is crucial for transcriptional activity of NF-kB through inhibition of suppressor of cytokine signalling-1 (SOCS1). In continuation of this finding, we have investigated the mechanism by which the NOS1 regulates the transcriptional activity of another important transcription factor which is involved in the expression of various proinflammatory cytokines.

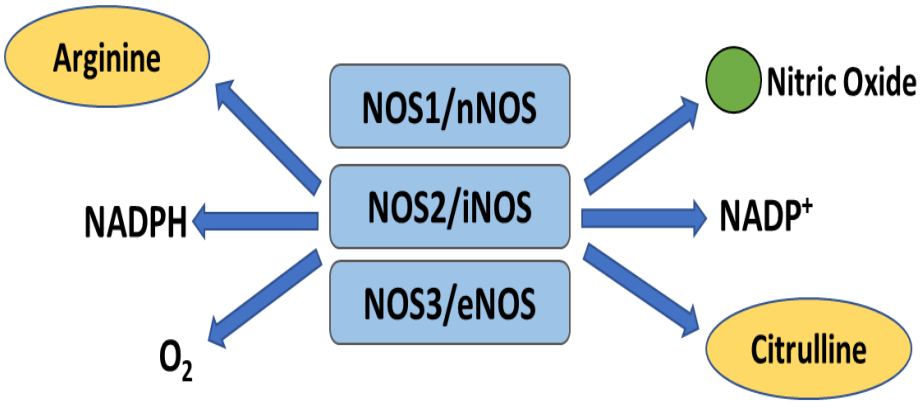


Fig 2.1. Isoforms of nitric oxide synthase

2.1.1 Isoforms of Nitric Oxide Synthases

Neuronal nitric oxide synthase (NOS1) has been reported with constitutive expression in the central and peripheral nervous system. The major function of NO produced by NOS1 include synaptic plasticity, smooth muscle relaxation and vasodilation (Brophy et al., 2000). Inflammatory aspects of NOS1 in macrophages have been recently reported on mice lacking NOS1, NOS2 and NOS3 respectively (Baig et al., 2015a). The study suggests interesting paradigm about the involvement of NOS1 in the pathogenesis of septic reactions by targeting nitrosation and degradation of inflammatory suppressor molecule SOCS1.Thereby abrogating the suppressing effects of SOCS1 on the downstream mediators. This key finding has paved new path for the study of NOS1 in inflammatory response generate by macrophages during chronic bacterial infections (Baig et al., 2015a). Inducible nitric oxide synthase (NOS2) contributes mainly to the production of NO upon microbial attack and activation of TLR4 receptors in macrophages (Suschek et al., 2004). It has unprecedented role in amplifying the pathophysiology of chronic inflammatory diseases such as sepsis (Kröncke et al., 1998; Suschek et al., 2004).

The third isoform endothelial nitric oxide synthase (NOS3) is predominantly expressed in endothelial cells and has a vasoprotective role in maintaining the homeostasis of endothelial cells. It regulates the blood pressure and assists in blood vessel dilation and therefore poses numerous anti-atherosclerotic effects (Albrecht et al., 2003; Bucci et al., 2005; Cirino et al., 2003b). It was initially reported that nNOS and eNOS are constitutively expressed in the neurons and endothelial cells respectively, maintaining synaptic plasticity and blood vessel dilation upon signals activation. However, recent studies have emphasized expression of eNOS and nNOS under the influence of inflammatory signals apart from the physiological conditions prevailing within the cellular environment (Dudzinski et al., 2006; Förstermann et al., 1998; Tsutsui et al., 2010).

Inflammatory reactions arise with a coordinated release of chemical mediators such as cytokines, chemokines and inflammatory mediators such as histamines and prostaglandins. Cytokines secreted from the activated macrophages such as IL-10, TGFβ, IL-4 and IL-13 suppress the release of NO from iNOS providing a balance of pro and anti-inflammatory response generated from macrophage (Bogdan et al., 1994; Doyle et al., 1994; Vodovotz et al., 1993; Zamora et al., 2000a). Synthesis of nitric oxide from NOS occurs in a calmodulin-dependent pathway. A concurrent increase in the intracellular calcium facilitates calmodulin binding to nNOS and eNOS. nNOS alternatively exhibits calmodulin binding at even low intracellular calcium owing to a different amino acid structure of calmodulin binding site (Cho et al., 1992; Hemmens and Mayer, 1998). Characteristics of various isoforms of human NOS are listed in table below:

Table 2.1 Properties of NOS isoforms

Properties	**NOS1**	**NOS2**	**NOS3**
Cell Source	Neurons, macrophages	Macrophages, Neutrophils	Endothelial cells
Protein Size (kDa)	160	131	144
Gene length (kB)	160	37	21
Number of amino acids	1554	1153	1203
Number of exons	29	26	26
Mechanism of activation	Calmodulin dependent	Calmodulin dependent	Calmodulin dependent
Chromosomal location	12 q24.1.2	17 cen-q11.2 or q11.2–q12	7 q35–36

2.1.2 Functions of NOS

In mammals NO can be produced by three isoforms of NOS, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) (Förstermann and Sessa, 2012a; Lange et al., 2010). NOS2 and NOS3 have been well established as potential mediators of inflammatory response. However, less is known about the role of NOS1 in inflammation. Nitric oxide is a potent gaseous signalling molecule that plays versatile roles in the innate and adaptive immune system (Bogdan, 2001b). Inducible nitric oxide synthase produces majority of nitric oxide in macrophages upon microbial and cytokine stimulation(Zamora et al., 2000a). It was initially reported that nNOS and eNOS are constitutively expressed in the neurons and endothelial cells respectively, maintaining synaptic plasticity and blood vessel dilation upon signals activation. However, recent studies have emphasized expression of eNOS and nNOS under the influence of inflammatory signals apart from the physiological conditions prevailing within the cellular environment (Dudzinski et al., 2006; Förstermann et al., 1998; Tsutsui et al., 2010). Interesting studies on triple NOS null mice, where all three NOS genes are completely perturbed lead to interesting insights on the crucial role of NOS in pathogenesis of various disorders such as cardiovascular diseases, renal remodeling and pulmonary fibrosis (Tsutsui et al., 2015). The results highlight the role of NOS isoform in disease pathogenesis of different organs.

2.1.3 Role of Nitric oxide in Inflammation

Nitric oxide (NO) is the key signalling molecule produced by Nitric oxide synthase and plays crucial role in immune defense, pathogenesis of inflammation and neurotransmission (Wallace, 2005). Overproduction of NO in the immune cells causes aberrant activation of immune response that leads to inflammatory lesions in the affected tissues (MacMicking et al., 1997; Sharma et al., 2007).

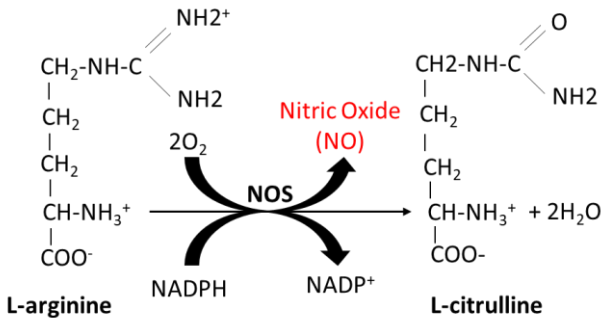


Fig 2.2. Mechanism of nitric oxide synthesis

NO-mediated inflammatory disorders have been reported in lungs, gut and joints and in excessive abundance may also lead to multiple organ failure (Bogdan, 2001a; Lyons, 1995; Winkler et al., 2017). NO facilitates the release of inflammatory mediators such as cytokines and chemokines from the effector cells of immune system such as mast cells, macrophages and endothelial cells (Bogdan et al., 2000; Laskin et al., 1994). Being a free radical with an unshared electron, NO readily forms new complexes with thiol groups in proteins such as haemoglobin and glutathione. That account for its bioavailability in the bloodstream targeting distant cells. Attributed to its gaseous form, NO is highly permeable through the membrane and diffuses between cells to activate paracrine signalling events (Murad, 2011). Exposure to endotoxins activates nitric oxide synthases (NOS) in cells. This results in the production of NO that aids in the generation of immune response and inflammation. Overproduction of NO in immune cells can cause massive damage to the host. Respiratory disorders such as asthma are characterized by increased expression of NOS2 and produce abundant NO within the respiratory epithelial and immune cells. NO then acts as a signalling mediator and activates other immune cells to produce inflammatory cytokines. Dysregulated NO production along with proinflammatory mediators increases the severity of the disease (Ashutosh, 2000; Ghosh and Erzurum, 2011; Prado et al., 2011). Therefore, strategies to control NO production using targeted drugs has emerged as an effective therapy to treat chronic inflammatory diseases (Wong and Lerner, 2015).

Table 2.2 Biological functions of nitric oxide

Source of nitric oxide	Biological Functions
Macrophages, microglia, astroglia, keratinocytes, mesangial cells	Necrosis or fibrosis of the parenchyma
Macrophages, microglia, neutrophils, eosinophils, fibroblasts, endothelial cells, epithelial cells	Killing or reduced replication of infectious agents (viruses, bacteria, protozoa, fungi and helminths)
Macrophages, eosinophils	Killing or growth inhibition of tumor cells
Macrophages, T cells, endothelial cells, fibroblasts	Up- and downregulation, e.g., of: IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, IFN-γ, TNF TGF-β, G-CSF, M-CSF, VEGF, MIP-1α, MIP-2, MCP-1
Macrophages ('suppressor phenotype')	Immunoregulatory functions Inhibition of T and B cell proliferation, leukocyte recruitment (adhesion, extravasation, chemotaxis)

2.1.4 Role of NOS1 (Neuronal NOS) in inflammation

Although NOS2 has remained the focus of studies involving NO production and inflammation. However in the last decade, key research outcomes have proposed the active participation of NOS1 in the pathogenesis of inflammatory response originating in cell types other than neurons such as skeletal muscle, smooth muscles, cardiac muscles and importantly in the macrophages (Enkhbaatar et al., 2003, 2009; Gocan et al., 2000). Imbalance in the expression of NOS1 in neurons distorts the physiology of neuronal cells causing neurodegenerative disorders such as multiple sclerosis, Parkinson's diseases and Alzheimer's (Steinert et al., 2010). In the course of inflammatory reactions, studies have proven that NOS1 has a prominent impact on the initiation of systemic inflammation in septic mice model (Duma et al., 2011). The results from the study demonstrate that NOS1 derived nitric oxide is an essential signalling molecule. Both the pharmacological inhibition and generic

deletion of NOS1 reduced the inflammatory response and protected the mice from septic lethality (Baig et al., 2015a; Duma et al., 2011). NOS1 has exhibited a crucial role in generating early response to bacterial challenge (Lange et al., 2010, 2011). Strikingly, NO produced from NOS1 in skeletal muscles modulates the vascular responsiveness during sepsis (Gocan et al., 2000).

2.1.5 Recent advances on NOS1 in inflammation

Studies on NOS1 knock out mice have provided exciting evidences towards its crucial involvement in pathophysiology of inflammatory disorders. As evident by the enhanced resistance of NOS1 knock out mice to the septic lethality, implying that NOS1 deficiency not only impedes the proinflammatory cytokine expression but also aids in phenotypic switch of macrophages from proinflammatory M1 to anti-inflammatory M2 (Baig et al., 2015a; Srivastava et al., 2017). Suppressor molecule SOCS1 that degrades p65 and inhibits the activity of NF-Kb has been attributed as the direct target of NOS1. Inhibition of p65/p50 heterodimer by SOCS1 results in accumulation of less potent p50/p50 homodimer that confers an anti-inflammatory phenotype in macrophages. However, in presence of NOS1, the basal expression of SOCS1 is disrupted that cause activation of p65/p50 subunits that bind to and regulate the expression of inflammatory cytokine genes. This causes an abrupt increase in the inflammatory response to the pathogen which in chronic condition may damage the host tissue and lead to inflammatory disorders.

2.2. Suppressor of Cytokine Signalling (SOCS1)

Inflammatory responses arising within macrophages are adequately balanced by a series of negative regulators that are expressed in response to inflammatory outburst. SOCS1 (Suppressor of cytokine signalling 1) is one such intracellular protein that shuts down the signalling by overall attenuation of the inflammatory cytokines (Fujimoto and Naka, 2010; Kinjyo et al., 2002). Structural studies on mouse and human SOCS1 protein suggests the presence of 212 and 211 amino acids respectively, having an identity close to 95-99% (Starr et al., 1997).

2.2.1 Structural domains of SOCS proteins

SOCS family proteins were initially identified as the inhibitors of JAK/STAT pathway and have been associated with cell survival and differentiation (Alexander et al., 1999; Croker et al., 2008; Liau et al., 2018). Interestingly, analysis of mice with impaired SOCS1 has shown its essential involvement in negative regulation of IFN-γ signalling during T cell development (Krebs and Hilton, 2001). SOCS are encoded by family of eight members, (cytokine-inducible SH2 protein (CIS) and SOCS1–7) (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). All members of SOCS family are composed of three domains: A C-terminal SOCS box responsible for proteasomal degradation and a central SH2 domain that provides the binding specificity with the target proteins and a N-terminal kinase inhibitory region (KIR) (Krebs and Hilton, 2001; Naka et al., 1997). Among the SOCS family, SOCS1, SOCS2 and SOCS3 are well characterized for their functional roles in inflammatory suppression, however less is known about SOCS4, SOCS5, SOCS6 and SOCS7 owing to their non-responsive behaviour during cytokine stimulation (Hilton et al., 1998).

2.2.2 Role of SOCS1 in inflammation

Studies on SOCS1 inhibitory potential suggests that SOCS1 targets JAK2 degradation by binding to its tyrosine (Tyr-1007) (Nicholson and Hilton, 1998; Yasukawa et al., 1999). Further studies revealed that SOCS1 also inhibits STAT1 by direct binding to interferon receptors (Fenner et al., 2006; Qing et al., 2005). SOCS1 mediated protection from inflammatory damage is evident from the study on SOCS1 knock out mice that succumb to the autoinflammatory disorders resulting from abrupt IFN-γ signalling (Alexander et al., 1999; Starr et al., 1998). However, dysregulated SOCS1 signalling has been linked with multiple primary cancers such as myeloid leukemia, breast cancer, ovarian cancer and breast cancer(Chen et al., 2003; Sutherland et al., 2004). The immunomodulatory functions of SOCS1 have been explored in context of several pathologies such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and cancer (Cooper et al., 2010; Isomäki et al., 2007; Tsao et al., 2008; Vandenbroeck et al., 2012). Studies show that

SOCS1 deficient mice administered with IFNs die in neonatal stage within 3 weeks post birth due to systemic inflammatory response (Alexander et al., 1999; Marine et al., 1999) suggesting a crucial role of SOCS1 in the regulation of IFN signalling. The major cause of death in SOCS1 deficient mice is attributed to multiple organ failure and aberrant T cell activation. During hepatic inflammation, SOCS1 deficiency increases the sensitivity of hepatocyte to inflammatory cytokines leading to abrupt activation of hepatic lymphocytes that worsens the inflammation-induced damage to cells (Alexander et al., 1999; Naka et al., 2001). Therefore, SOCS1 expression is crucial for suppression of hepatic inflammation in mice.

Presence of STAT1 binding site in the promoter of SOCS1 gene implicates activation of SOCS1 transcription by inducing interferon regulatory factor-1 (IRF-1). Translational regulation of SOCS1 is marked by the loss of SOCS box in the protein structure of SOCS, that drastically reduce its expression. Interestingly, pretreatment with proteasome inhibitor recovers its expression implying that SOCS box protects SOCS1 from proteasomal degradation (Narazaki et al., 1998). Findings that illustrate the presence of STAT binding site in SOCS1 promoter, show that it can be activated by the cytokines such as IFN-γ, IL-2, IL-4 and IL-6 through JAK-STAT pathway (Davey et al., 2006; Dickensheets et al., 2007; Sporri et al., 2001; Starr et al., 1997). SOCS1 downregulates JAK-STAT pathway by competitive binding to JAK catalytic domain and inhibits STAT binding to JAK thereby abrogating the signalling response.

Inflammatory reaction elicited by bacterial LPS poses a fatal effect on the immune system, the major target being the effector cells macrophages. Production of reactive oxygen and nitrogen species and inflammatory cytokines such as IL1β and TNFα from LPS activated macrophages ameliorates the inflammatory response (Berczi, 1998). LPS induced binding of TLR4 with MD2 recruits MyD88 to their cytoplasmic domain followed by recruitment of serine/threonine kinase IL-1R-associated kinase (IRAK), that undergoes phosphorylation and dissociates with the receptor complex and associates with another factor called TNF receptor-associated factor (TRAF)-

6. The complex association of series of kinases and adaptor proteins leads to activation of downstream MAP kinases such as JNK and p38 and essential transcription factors such as NF-kB and AP1 (Akira et al., 2001; Medzhitov et al., 1998). However, NF-kB and p38 activation have been reported even in the absence of MyD88, proposing the existence of a MyD88 independent pathway for activation of the TLR4 mediated signalling (Kawai et al., 2001).

Moreover, the inhibitory effects of SOCS1 have been reported in LPS induced macrophage activation (Kinjyo et al., 2002). Results from this study stated that SOCS1 knock out mice were more susceptible to LPS induced endotoxin shock compared to their wild-type counterparts. In accordance with the observation, LPS induced nitric oxide synthesis and TNFα production was strikingly enhanced in SOCS1 knock out macrophages. SOCS1 expression is therefore essential to balance the detrimental effect of excessive cytokine production that is detrimental to the health of host immune system (Cross et al., 1995; Danner et al., 1991). It is of paramount interest for the immunologist to explore the measures taken by immune cells to nullify the endotoxin challenge. Some pioneer literature suggests that downregulation of TLR4 receptor might be one of the potential route taken up by macrophages to rescue from excessive inflammatory damage (Nomura et al., 2000).

Induction of SOCS1 in macrophages occurs majorly through recognition of bacterial LPS that activate TLR4 and CpG oligonucleotide that activate TLR9 (Dalpke et al., 2001; Kinjyo et al., 2002; Nakagawa et al., 2002a). Once activated it regulates the IFN-γ mediated signalling as indicated by the prolonged activation of IFN-γ induced STAT1 in absence of SOCS1. Owing to the immune protective aspects of SOCS1, it has been suitably implicated in the regulation of IFN induced cytotoxicity and minimizes the cytokine-mediated damage to host. In addition to this, recently SOCS1 has been considered a potential player in modulating macrophage phenotypic changes (Yoshimura et al., 2007; Zhou et al., 2017).

2.2.3 Role of SOCS1 in macrophage polarization

Crucial roles of SOCS1 have been reported in context of macrophage polarization. SOCS1 suppresses M1 macrophage activation by blocking the TLR4 mediated NF-kB signalling pathway. SOCS1 utilizes its SOCS box to cause ubiquitin-mediated proteasomal degradation of p65 subunit of NF-kB and the adaptor molecule Mal/TIRAP (Kinjyo et al., 2002; Mansell et al., 2006a; Nakagawa et al., 2002a; Ryo et al., 2003; Strebovsky et al., 2012). Furthermore, analysis of M1 macrophages from SOCS1 knock out mice displayed upregulation of nitric oxide and inflammatory cytokines IL6, IL1 (Baetz et al., 2004; Whyte et al., 2011). SOCS1 levels are itself regulated within the LPS challenged macrophages by its methylation state. To balance the inflammatory requirements in the cellular environment. In line with this, the SOCS1 promoter undergoes hypermethylation that result in its subsequent downregulation allowing the persistence of inflammatory signals arising from the expression of proinflammatory cytokines (Cheng et al., 2014).

Regulatory mechanism occurring with the aid of micro RNAs allow precise functioning of innate immunity. miR-155 has been identified as a negative regulator of SOCS1 that causes its degradation in M1 activated macrophages and ameliorates the inflammatory response (Androulidaki et al., 2009; Ma et al., 2017; O'Connell et al., 2007). SOCS1 regulation by miR-155 therefore potentiates its role in promoting M1 macrophage phenotype. Inhibition of miR-155 has expectedly given rise to tumor promoting M2 macrophages thereby suggesting that miR-155 mediated regulation of SOCS1 is a novel mechanism of maintaining balance between proinflammatory M1 and anti-inflammatory M2 macrophages (Jiang et al., 2016). With these findings, SOCS1 is considered as an important molecule in maintaining the M1-M2 balance that assist in protecting the integrity of a healthy immune system (Wilson, 2014; Zhou et al., 2017).

2.3 Mal (MyD88 adaptor like)/ TIRAP

Inflammatory signals that arise in response to pathogenic stimuli, are recognized by TLR4 receptor on macrophages. The signals are the transmitted through a series of receptor-associated proteins such as MyD88, IRAK and TRAF6. The activated receptors in turn orchestrate the signal to adaptor molecule Mal/TIRAP (Toll/interleukin-1 receptor domain-containing adapter protein) that are involved in the pathogenesis of inflammatory disorders such as rheumatoid arthritis (Sacre et al., 2007a, 2007b). Studies have demonstrated crucial role of TIRAP in LPS induced activation of IRF-3 that amplifies the intensity of inflammatory response (Shinobu et al., 2002a).TIRAP potentiates the inflammatory circuit by recruiting downstream kinases that activate the downstream transcription factors such as NFkB p65 that bind and upregulate the expression of proinflammatory genes (Horng et al., 2001). TIRAP undergoes phosphorylation by Btk kinase making it a target for degradation by SOCS1 (Gabhann et al., 2014; Gray et al., 2006; Mansell et al., 2006a). SOCS1 mediated regulation of TIRAP is necessary to control the overexpression of inflammatory genes. TIRAP expression is closely associated with the inflammatory events arising due to activation of both TLR2 and TLR4 pathways (Yamamoto et al., 2002). Studies have provided insights on the interaction of TIRAP with TRAF6 that facilitate NFkB activation leading to high expression of inflammatory genes (Mansell et al., 2004; Verstak et al., 2009).

2.3.1 Structural domains of Mal/TIRAP

TIRAP is characterized with a N-terminal phosphoinositide (PI)-binding domain (PBD) that is essential for membrane binding, followed by a TIR domain which is required for its interaction with TLR2 and TLR4 receptors (Fitzgerald et al., 2001; Kagan and Medzhitov, 2006; Valkov et al., 2011). The TIR domain of TIRAP serves as a bridge to interact with MyD88 which triggers the recruitment of other kinases that subsequently facilitate nuclear translocation of NF-kB (Lin et al., 2012; Yamamoto et al., 2002). However, recent studies have reported MyD88 independent role of TIRAP in

mediating the inflammatory response (Bernard and O'Neill, 2013). The multifunctional role of TIRAP highlight its role as an integral component of inflammatory cascade.

2.3.2 Role of TIRAP in inflammation

Inflammation within the cellular environment results from a cooperative action of various signalling mediators activated downstream of the membrane receptors. Transcription factors NF-kB, AP1 and STAT1 receive the activating signals and translocate into nucleus to bind to their target genes. Recently, a heterotrimeric complex has been identified composed of kinase p38, pkcδ and adaptor protein TIRAP. TIRAP acts as a bridge and brings p38 and pkcδ in close proximity thereby allowing phosphorylation of p38 by pkcδ. Together this complex activates the transcription factor AP1 mediating the expression of AP1 responsive genes (Baig et al., 2017a). Disruption of this complex might provide novel therapeutic strategies to diminish the severity of inflammatory reactions. Reportedly, a defective response to bacterial invaders has been observed in TIRAP knock out mice, displaying early mortality and higher bacterial load in the lungs (Jeyaseelan et al., 2005). Studies on alveolar macrophages from TIRAP knock out mice infected with B. pertussis showed impaired production of proinflammatory cytokines. Subsequently the mice succumbed to death due to heavy bacterial load in the lungs and lack of appropriate signalling events in the alveolar macrophages (Bernard et al., 2015). This finding highlighted the importance of TIRAP in intracellular survival of alveolar macrophages and catering protection from bacterial infection.

TIRAP has been recognized as a "bridging adaptor" where it facilitates the binding of MyD88 to the TLRs (Aviello et al., 2014; Bernard and O'Neill, 2013). In addition, to the bridging component, TIRAP is also linked with the TLR induced expression of IL-10 through activation of CREB (Mellett et al., 2011). It is evident from the studies on TIRAP-deficient bone marrow-derived macrophages, that fail to respond to TLR2 and TLR4 ligands with respect to the expression of CREB induced genes such as IL-10. In addition to its

modulating effect on cytokine expression, TIRAP has proved to be an essential player in the IFN-γ receptor signalling, that subsequently leads to p38 activation which is an important requirement to kill intracellular Mycobacterium tuberculosis (Mtb). The findings from previous literature, support the significance of TIRAP as immunomodulatory adaptor protein with multifunctional roles within and outside the TLR signalling pathways.

Regulatory functions of SOCS1 are also observed with important adaptor proteins of inflammatory pathways such as Mal/TIRAP (Mansell et al., 2006a). Upon activation, TLR4 receptor signals Btk tyrosine kinase to phosphorylate TIRAP and subsequently generate a binding site for SH2 domain of SOCS1 (Fujimoto and Naka, 2010). Phosphorylated TIRAP then becomes a target for SOCS1 that causes its ubiquitin-dependent proteasomal degradation and hampers the downstream TIRAP dependent NF-Kb Activation (Mansell et al., 2006a). Suppression of inflammation by SOCS1 was mediated by the blockade of kinases such as JNK and p38 that are essential for activation of inflammatory mediators down the signalling pathway.

2.4 Activator Protein 1 (AP1)

Activator protein 1 (AP1) family of transcription factor was recognized in 1987 as the first mammalian sequence-specific transcription factor (Bohmann et al., 1987; Karin et al., 1997). Among AP1 family, Jun (consist of c-Jun, Jun B and Jun-D), Fos (consist of c-Fos, FosB, Fra-1 and Fra-2) and ATF2 (consist of ATFa, ATF2 and ATF3) that share structural similarities and form heterodimers (Curran and Franza, 1988; Wisdom, 1999).

2.4.1 Structure of AP1

Structurally, each member of AP1 family is composed of three functional domains, a carboxy-terminal leucine zipper, basic DNA binding domain and an amino-terminal transactivation domain. Subunits of AP1 are capable of forming homo or hetero dimers within the family and with other transcription

factors containing basic leucine zipper (bZip). c-Jun protein predominantly forms heterodimer with either Fos or ATF2 and can also exist as homodimers, while Fos and ATF2 cannot make stable homodimers (Curran and Franza, 1988). Transactivation potential of Jun and Fos proteins have been widely explored. Jun, Fos and FosB possess strong transactivation potential while Fra-1, Fra-2, Jun-B and Jun-D exhibit weak transactivation potential. Binding to the target DNA is mediated by the basic DNA binding domain that tethers to target sequence known as 12-O-tetradecanoyl-13-phorbol acetate-response element (TGACTCA, TRE). Transcriptional regulation by AP1 is mediated by the presence of consensus TRE sites in the target genes also known as TPA-responsive elements (Angel et al., 1987; Foletta et al., 1998; Lee et al., 1987).Recently a novel AP-1 binding site has been recognized containing methylated CpG DNA known as meAP-1. These sites are preferentially occupied by AP-1 heterodimers in the promoters of transcriptionally active AP-1 target genes (Gustems et al., 2014). Together these subunits belong to a group of structurally related family of transcription factors that possess an evolutionary conserved basic leucine zipper (bZip) domain (Angel et al., 1987; Hess et al., 2004). Importantly, the leucine zipper mediates dimerization of AP1 subunits that further facilitates binding with target DNA. AP1 subunits have been referred as the proto-oncogenes owing to their high sequence homology to retroviral coded oncogenic proteins and their active involvement in tumorigenic transformation of target cells (Kim et al., 2003; Shaulian and Karin, 2002; van Straaten et al., 1983). Each subunit of AP1 is differently regulated in cellular environment and have distinct functional relevance in pathology of inflammatory diseases.

Functional heterodimers of AP1 have crucial regulatory functions in response to pathogenic stimulus detected by immune system. Interesting observations in genetically modified mice emphasize on the role of AP-1 in neoplastic transformation of cells (Suzuki et al., 1994; Young et al., 1999). However, the relative abundance of AP1 dimers and composition of dimeric subunits largely determine the cellular response generated by AP1 activation. Regulated activation of AP1 subunits depends on the transcriptional and translational control of genes encoding the AP1 subunits. MAP kinases such as JNK (Jun

N-terminal kinase) are activated by a cascade of MAPK signalling, that directs its translocation into nucleus and targets Jun for phosphorylation (Ser 63/73) with its N-terminal transactivation domain (Das and Muniyappa, 2010; Johnson and Nakamura, 2007; Wisdom, 1999). Activated Jun thereby exhibits enhanced transactivation potential to drive the transcription of inflammatory genes. AP1 regulates cellular processes such as differentiation, proliferation and apoptosis (Shaulian and Karin, 2001, 2002). However, evidences report that Jun B and Jun D suppress the lymphocyte proliferation suggesting that different AP1 subunits might pose a varying impact on cellular physiology (Hess et al., 2004; Passegué and Wagner, 2000).

2.4.2 AP1 in Inflammation

Chronic inflammatory diseases, such as sepsis, rheumatoid arthritis, inflammatory bowel disease and psoriasis result from dysregulated activity of transcription factors that control inflammatory gene expression (Zenz et al., 2008). AP1 subunits have multiple functions including cell proliferation, differentiation and tumorigenic transformation that make it a versatile transcription factor (Angel and Karin, 1991; Eferl and Wagner, 2003). AP1 complex is dynamically activated during bacterial and viral infections and regulate the inflammatory cascade (Seo et al., 2004; Xie et al., 2005). Effector cells of immune system such as macrophages and dendritic cell respond to the stimulus and activate the Toll-like receptors that signals the downstream transcription factor such as AP1 and NFkB leading to robust production of inflammatory cytokines. Inflammatory signalling is triggered by upregulated expression of cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6, IL1β, TNFα, CD40L and chemokines such as MIP1α that are majorly regulated by the activity of AP1 transcription factor (Eferl and Wagner, 2003; Schonthaler et al., 2011; Zenz et al., 2008).

Studies on murine model of *C. pneumoniae* infection have identified active involvement of Fos subunit of AP1 in the disease pathogenesis (Huang et al., 2008; Miller et al., 1998). Transcriptional regulation by AP1 is mediated through binding of AP1 to the promoters of inflammatory mediators such as

IL8 independent of other transcription factors such as NF-κB (Balasubramanian et al., 2003; Yeo et al., 2004). Reportedly, infections arising from Streptococcus have been linked with interaction of AP1 with other potent transcription factors (Vallejo et al., 2000).However the functional aspects of AP1 in inflammatory disorders still remains elusive.

Signals activating AP1 include release of cytokines, chemokines and growth factors from immune cells of innate or adaptive immune system. Additionally, stress- responsive, mitogen-activated protein kinases, such as Jun-N-terminal kinases, leads to an activation of AP-1(Papachristou et al., 2003; Wagner and Nebreda, 2009). Functional aspects of AP1 have been remarkably reported in transgenic mice with altered AP1 genes, suggesting the activity of AP1 in maintaining the homeostasis of various tissues (Schonthaler et al., 2011). Among the AP1 subunits, c-Jun and JunB have been implicated in the developmental process, deletion of which leads to perturbed embryonic development (Hilberg et al., 1993; Schorpp-Kistner et al., 1999). AP1 plays a predominant role in the initiation and maintenance of inflammatory response generated by macrophages (Tengku-Muhammad et al., 2000). It has been associated with the pathogenesis of several inflammatory disorders. Studies on murine macrophage cell line J774.1.2 suggest that exposure of cells to LPS, TNF-alpha, IFN-gamma and IL-1 enhanced the expression of AP1 subunits c-Jun, JunB and Fos, but not JunD, implying that not all AP1 members actively participate in the initiation of pathogenic response (Tengku-Muhammad et al., 2000). Transcription factors such as AP1 and NF-κB control the immunomodulatory functions of inflammatory genes. There is a cohort of MAP kinases and adaptor proteins that regulate the activation of TFs. One such heterotrimeric complex has been recently reported comprising of Protein kinase C delta type (PKCδ), Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein (TIRAP), and p38 proteins that assemble in close proximity and activate the downstream transcription factor AP1 that then regulates the expression of inflammatory cytokines IL-12 and IL-23 (Baig et al., 2017a). Abrogation of the complex has been proposed as a potential strategy to control the severity of inflammatory response.

Furthermore, inflammatory activities of AP1 have been explored in activated macrophages and dendritic cells that lead to enhanced expression of IL-23 through TLR4 and MyD88 pathway (Liu et al., 2009). Studies on alveolar macrophages reveal that endotoxin shock such as LPS induces lung injury and mortality by upregulating the Fos-related antigen-1 (Fra-1) transcription factor through cooperative induction of NF-Kb and c-Jun subunit of AP1 (Mishra et al., 2016).

2.4.3 Nuclear translocation of AP1

Nuclear translocation of AP1 is a critical event that impacts the transcriptional regulation of inflammatory genes. Previous studies have shown that leucine zipper facilitates the nuclear translocation of AP1 by mediating the interaction between Fos and Jun subunits (Chida et al., 1999a). Rapid import of AP1 subunits is mediated by the strong nuclear localization signal located in Jun. While Fos employs a novel transportin as a nuclear import receptor for its nuclear accumulation (Arnold et al., 2006). However less is known about the dynamic regulation of c-Fos within the cell. Research has shown that monomeric c-Fos is evenly distributed in the cell, heterodimerization with c-Jun dramatically increases its nuclear abundance suggesting important role of heterodimers in regulating the intracellular dynamics and distribution of AP1 in the cellular environment (Malnou et al., 2010). Thus, there exist a finely controlled nuclear translocation of AP1 that determine the outcome of transcription factor activation.

2.5 Therapeutic strategy for inflammatory diseases

Recent discoveries about repurposed drugs have emerged as an attractive platform to solve the bottlenecks in the development of wide range of therapeutics. A precise understanding of the molecular basis of disease offers opportunity to design agents that can specifically target the faulty gene. However, development of a novel drug requires extensive amount of time and money with low success rate. Therefore, repurposing the existing drugs for treatment of the cause other than the one they were originally designed fastens

the drug development process effectively (Corsello et al., 2017). An interesting article records the advantages of teaching new tricks to old drugs that could save both cost and time of drug development (Nosengo, 2016). Several drugs approved as repurposed drugs for varying diseases have been listed recently (Corsello et al., 2017). To list a few, antiemetic thalidomide is utilized for treatment of multiple myeloma as a repurposed drug (Palumbo et al., 2008). Moreover, cyclooxygenase inhibitor aspirin is repurposed for treatment of colorectal cancer (Flossmann et al., 2007). Additionally, an antiepileptic drug topiramate was recently reported to be repurposed for its efficacy to treat inflammatory bowel disease (IBD) or in general for any gastrointestinal tract related inflammatory disorder (Dudley et al., 2011).

In last few years, much interest has been developed in the area of repurposed drugs. Gefitinib belongs to the family of drugs that have been utilized as analgesics and anti-inflammatory agents other than their anti-cancer properties. The mechanism of action lies in their ability to inhibit TNFα production and T cell proliferation (Tobe et al., 2001). Remarkably, Gefitinib that was originally designed to treat non-small cell lung cancer (NSCLC), has been explored as a immunomodulatory drug (Brooks, 2013). Reports from the repurposing studies strongly imply its potential as a drug for treatment of inflammatory disorders (Brooks, 2013; Hur et al., 2007). Gefitinib has also been characterized in non-cancerous TNFα mediated autoimmune disorders by blocking the interaction of TNFα with TNF receptor (Bradley, 2008; Ueno et al., 2005). Chronic inflammatory diseases such as Crohn's disease exhibit high level of TNF-α production that increases the risk of development of colorectal cancer. Research suggests that Gefitinib might be a useful repurposed drug for treatment of Crohn's disease (Tigno-Aranjuez et al., 2010). Rheumatoid arthritis is another chronic autoimmune disorder characterized by elevated expression of TNF-α and interleukin-1 (IL-1) by synovial fluid macrophages (Dayer, 2002). Several TNF-α blockers such as infliximab, adalimumab and etanercept have been employed to narrow down the severity of inflammatory disorders but have demonstrated weak treatment efficacy (Curtis and Singh, 2011). In this context, intriguing evidences suggest a targeted inhibition of both TNF-α and IL-1 by Gefitinib making it a more effective drug candidate for rheumatoid arthritis (Mitsos et al., 2009). Synovial inflammation marked

by elevated inflammatory cytokines TNF-α and IL-1 and erosion of joint cartilages are predominant symptoms of Osteoarthritis (OA), also known as degenerative arthritis. Use of Gefitinib as an anti-inflammatory therapy may provide advanced therapy for the disease treatment (Moryl et al., 2006).

Chronic inflammation affects the integrity of host immune system and results in compromised immune response. Bronchial asthma is a chronic inflammatory disorder characterized by infiltration of lymphocytes, macrophages and mast cells with prominent thickening of bronchial wall and fibrosis (Cohn et al., 2004; Nakagome and Nagata, 2011). Studies show that severity of bronchial damage is directly related to the level of EGFR expression that in turn activates multiple signalling cascades such as Ras MAP kinase, STAT pathway and PI3K/Akt pathway (Amishima et al., 1998; Hur et al., 2007). These studies provide significant insight on the use of Gefitinib as a tyrosine kinase (EGFR) inhibitor to combat the pathogenesis of asthma. Studies on ova sensitized mice pretreated with Gefitinib displayed higher expression of anti-inflammatory cytokines IL-4 and IL-13 and reduction in the inflammatory cell count in bronchoalveolar lavage fluid (BALF). This was in accordance with the decreased EGFR expression and diminished Akt phosphorylation suggesting that Gefitinib might have a potential role in asthma treatment by possible inhibition of EGFR and PI3K/Akt pathway (Hur et al., 2007).

So far, all studies on repurposing of Gefitinib have emphasized its role as an inhibitor of inflammatory cytokines by blocking the EGFR tyrosine kinase receptor in the target cells. However, a precise target of Gefitinib has not yet been elucidated in the signalling pathways activated in response to chronic bacterial infections that lead to sepsis. In this study, a novel anti-inflammatory property of Gefitinib is proposed in septic mice model. The study provides further clarity on the underlying mechanism that determines the mode of action of Gefitinib in macrophages from LPS injected mice. The inflammatory mediators that participate in the signalling induced by TLR4 receptor include neuronal nitric oxide synthase (nNOS or NOS1) that targets SOCS1 for degradation. Due to SOCS1 degradation, TIRAP/Mal which is one of the proteins targeted by SOCS1 for degradation is thereby elevated within the

macrophages. TIRAP is an important adaptor protein that has multiple roles other than bridging MyD88 for signalling (Bernard and O'Neill, 2013). This study shows that TIRAP once activated downstream of TLR4 pathway, interacts with the AP1 subunit Jun which then gets phosphorylated. Activated Jun subsequently forms heterodimers with the other AP1 family subunits Fos and ATF2 and translocate to nucleus to induce the expression of target genes. This study provides first evidence for the TIRAP and Jun interaction and proposes that therapeutic inhibition of the two proteins by Gefitinib might be an enthralling strategy to control the pathogenesis of sepsis. The results from this study show significant reduction of inflammatory cytokines in septic mice pretreated with Gefitinib. Repurposing Gefitinib for the anti-inflammatory role in sepsis is a novel approach to overcome the deleterious effect of the disease.

Scope and plan of thesis

This objective of this thesis was to investigate the detailed molecular mechanism of chronic inflammation. Nitric oxide synthases (NOS) are enzymes that actively participate in the inflammation driven disorders. (Alderton et al., 2001b). Previous literature has highlighted the significance of NOS2 and NOS3 during inflammatory triggers. (Cirino et al., 2003a; Gray et al., 2018; Ying and Hofseth, 2007). However, NOS1 remains yet to be explored in this context. A recent report suggesting the involvement of NOS1 in inflammatory responses in macrophages was an exciting finding (Baig et al., 2015a). This led us to further expand the studies on the in depth molecular mechanisms of inflammation mediated through NOS1.

In continuation of this finding, we have investigated the mechanism by which NOS1 regulates AP1 transcription factor. AP1 activation and nuclear translocation is critical for the transcriptional regulation of inflammatory cytokines. This study emphasizes the mechanism by which NOS1 modulates the expression of inflammatory mediators SOCS1 and TIRAP that lead to the activation and nuclear translocation of c-Jun subunit of AP1. These events ultimately determine the onset and severity of inflammatory responses.

Broadly, following aims have been covered in this thesis:

AIM 1: It deals with the basic understanding of the involvement of NOS1 derived nitric oxide in the development of systemic inflammatory response. Here we have investigated the role of NOS1 during early triggers of inflammatory responses in macrophages. We have also analysed that the other two isoforms of NOS, i.e. NOS2 and NOS3 do not contribute to the early signals of inflammation.

AIM 2: Here we have explored the mechanism of NOS1 mediated inflammatory responses. We show that NOS1 regulates TIRAP-Jun interaction in endotoxin induced macrophages. Furthermore, TIRAP-Jun interaction is essential for AP-1 mediated inflammatory responses.

AIM 3: It deals with the therapeutic part of the study. Our findings illustrate that TIRAP-Jun interaction is a novel therapeutic strategy for sepsis. To conduct this study, we have repurposed Gefitinib as an anti-inflammatory drug for endotoxin shock mediated inflammatory responses. Therefore, our study offers efficient strategy for inflammatory disorders using repurposed drugs.

AIM 4: In this aim, we have explored a unique mechanism involving TLR4-NOS1-AP1 that together regulate the inflammatory responses in macrophages. Here we show that AP1 dimerization pattern is differentially regulated through NOS1 activation during LPS infection. This further provides novel insight into NOS1 mediated regulation of AP1 transcription factor.

Altogether, the study presents both mechanistic as well as therapeutic approach to inflammatory responses. Understanding of these mechanisms is essential to balance the detrimental outcomes of uncontrolled inflammatory responses that lead to chronic diseases.

3. Material and Methods

3.1 Cell culture

Mouse RAW 264.2.3 macrophages, human THP-1 macrophages and L929 cells were purchased from the National Centre for Cell Science (NCCS), Pune, India. RAW 264.2.3 macrophages were cultured in Dulbecco's minimal essential medium (DMEM) (Catalog No. 11965092, Gibco, California, U.S.) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Catalog No. 10270106, Invitrogen, California, U.S.) along with 100 U/ml penicillin and 100 µg/ml streptomycin (Catalog No. 15140122, Invitrogen, California, U.S.). THP-1 cells were cultured in RPMI media (Catalog No. A1049101, Gibco, California, U.S.) supplemented with 10% heat-inactivated FBS along with 100 U/ml penicillin and 100 µg/ml streptomycin (Catalog No. 15140122, Invitrogen, California, U.S.) along with 50 µM β-mercaptoethanol (Catalog No. M3148, Sigma, St. Louis, Missouri, U.S.). THP-1 cells were differentiated into macrophages using 25 ng of phorbol 12-myristate 13-acetate (PMA) (Catalog No. P8139, Sigma, St. Louis, Missouri, U.S.) in the culture media for 3 days. Subsequently, PMA-treated media was replaced with fresh RPMI media before adding LPS (Catalog No. L2630, Sigma, St. Louis, Missouri, U.S.) and 1-(2-trifluoromethylphenyl) imidazole (TRIM) (Catalog No. T7313, Sigma Aldrich, St. Louis, Missouri, U.S.). Cells were incubated in a humidified incubator with 5% CO_2 at 37^oC. Prior to seeding, cell counting was performed on a haemocytometer using Trypan Blue dye (Catalog No. 15250061, Thermo Fisher, M.A, U.S). Cells were seeded in tissue culture plates at a confluency of 60-70% and were treated with LPS, TRIM or Gefitinib for various for various experiments.

Bone marrow-derived macrophages (BMDM) were isolated from femur of Swiss albino mice. Bones were collected in 1X sterile PBS and sterilized by 70% ethanol (Catalog No. 180077, M.P. Biomedicals, C.A., U.S.). For extracting the cells, bones were cut from both ends and the cells were flushed into fresh DMEM media using 26 mm needle and 5 ml syringe in 50 ml tubes.

Media containing the cell suspension was then centrifuged at 1500 rpm for 15 min and cell pellet was obtained. Supernatant media was discarded, and the cell pellet was resuspended in DMEM media supplemented with 10% FBS (Catalog No. 10270106, Invitrogen, California, U.S.) and 100 U/ml penicillin and 100 µg/ml streptomycin (Catalog No. 15140122, Invitrogen, California, U.S.) in 140 mm culture dishes sterilized by radiation. 20% % L929- cell conditioned media was added as a source of M-CSF for 72 hours. Fresh media containing 10% L929-cell conditioned media was exchanged following the third day of culture. Before starting the experiment, all cells were synchronized to same growth phase by culturing the cells in serum-free DMEM medium overnight. Adherent BMDM were removed from the surface of culture plate by sucking the cells in an 18-gauge needle and syringe using 1 mM EDTA. Once the BMDM appear in suspension, they were collected in 50 ml falcon tubes containing DMEM medium. Cell suspension was centrifuged and BMDM were obtained in the pellet and plated on multi-well plates for various experiments. Prior to treatment, fresh DMEM media containing 10% FBS was added to the differentiated macrophages. Cells were incubated in a humidified incubator with 5% CO2 at 37°C.

3.2 Nitrite oxide measurement in macrophages

Measurement of nitric oxide was performed in a cell-based assay using DAF-FM (Catalog No. D23844, Life Technologies, Rockford, U.S.) to stain cells that produce nitric oxide. For this assay, BMDM were seeded on coverslips in a six-well plate in DMEM medium. Stimulation was done with LPS (250 ng/ml) and TRIM (100 nM) or LPS alone up to 1 h. Post-stimulation, media was removed, and cells were washed with 1X PBS. DAF-FM was first dissolved in high-quality DMSO (Catalog No. MB058, HiMedia, Maharashtra, India) to make a 7mM stock. Cells were then incubated with DAF-FM diacetate diluted to a final concentration of 10µM in Milli-Q water for 20 min at 37°C. Post incubation, excess dye was removed by washing the cells with 1X PBS. 4% formaldehyde (Catalog No. 30525-89-4, Loba Chemie, Maharashtra, India) was used for fixing the cells for 1 h at room temperature. Fixed cells were mounted on to slides in an inverted position with DAPI

containing mounting media (Catalog No. 62248, Thermo Fisher Scientific, M.A., U.S.). Stained cells dried for 30 min at room temperature. Visualization of nitric oxide produced in cells was done by confocal imaging using Olympus confocal laser scanning microscope.

Detection of nitrite, a stable metabolite of nitric oxide, was done using Griess reagent (Catalog No. G4410, Sigma-Aldrich, St. Louis, Missouri, U.S.) as per manufacturer's instructions. BMDM were seeded in 12-well plates and number of cells/ml were determined using haemocytometer. Cells were plated at a density of 10^4 cells/well. Prior to treatment, serum-free DMEM medium was added to the wells. Cells were then treated with LPS (Catalog No. L2630, Sigma, St. Louis, Missouri, U.S.) and 1-(2-trifluoromethylphenyl) imidazole (TRIM) (Catalog No. T7313, Sigma Aldrich, St. Louis, Missouri, U.S.) up to 2 h. After incubation, 50 µl of supernatant media was collected from each well and mixed with equal volume of Griess reagent in a 96 well plate. A nitrite standard reference was prepared by dissolving sodium nitrite (Catalog No. 146015, Thomas Bakers, Maharashtra, India) in DMEM media to a final concentration of 100 µM to compare the nitrite from each sample with a standard nitrite concentration. Plate was left for 30 min at room temperature without disturbance. Samples were added in 96 well plates in triplicates and equal volumes were maintained in each well. The absorbance of samples was measured at 570 nm in Synergy H1 Bio-Tek microplate reader and nitrite (µM) was estimated per milligram of total protein in each sample.

3.3 Quantitative Real-time PCR

Cytokine expression was studied in different conditions using quantitative real-time PCR. Post-treatment, cells were lysed in Trizol reagent (Catalog No. 9109, Takara, Shiga, Japan). For RNA extraction, 100 µl chloroform (Catalog No. 67-66-3, Avantor) was added in each sample, shaken vigorously and allowed for phase separation by leaving the samples at room temperature for 5 min. All samples were then centrifuged at 12000 rpm for 10 min at 4ºC. Aqueous layer present in supernatant was collected in fresh tubes and equal volume of isopropanol (Catalog No. 67-63-0, Thomas Baker, Maharashtra, India) was added to each sample and incubated at room temperature for 10

min. Samples were centrifuged at 13000 rpm for 30 min at 4ºC to obtain the RNA pellet followed by 75% ethanol wash. Samples were subjected to centrifugation at 13000 rpm for 15 min at 4ºC and purified RNA was obtained in the pellet. RNA was dissolved in nuclease-free water (Catalog No. G4635A, GCC Biotech, West Bengal, India). Concentration of RNA was estimated using Nanodrop. cDNA synthesis was performed in the subsequent step using cDNA synthesis kit (Catalog No. 4368814, Thermo Fisher Scientific, M.A., U.S). Equal amount of RNA was taken from each sample for cDNA synthesis. cDNA samples were used for quantitative real-time PCR using Sybr Green (Catalog No. A25742, Thermo Fisher Scientific, M.A., U.S.). CT values of target gene compared to that of the housekeeping gene (GAPDH) were used to quantify gene expression in each sample. Primer sets used for each gene are described in Table 3.1 below (m: mouse primers; h: human primers).

Table 3.1 List of primers used for q-RT PCR

Gene Name	Forward Primer	Reverse Primer
mIL12	AAGAGCAGTAGCAGTTCCCC	GTTGGGCAGGTGACATCCTC
mIL23	ACCAGCGGGACATATGAATCT	AGACCTTGGCGGATCCTTTG
mTNFα	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG
mIFNγ	AAAGAGATAATCTGGCTCTGC	GCTCTGAGACAATGAACGCT
mMIP1-α	TCAGCACCATGAAGGTCTCCAC	CTCAGGCATTTAGTTCCAGCT
mM-CSF	GACTTCATGCCAGATTGCC	GGTGGCTTTAGGGTACAGG
mFizz-1	ACTTCTTGCCAATCCAGCTAAC	CAAGCACACCCAGTAGCAGT
mArg-1	ACATTGGCTTGCGAGACGTA	ATCACCTTGCCAATCCCCAG
hMRC-1	TCTTTTACGAGAAGTTGGGGTCAG	ATCATTCCGTTCACCAGAGGG
hKLF-4	GCCCCTCGGGCGGCTTCGTGG	CGTACTCGCTGCCAGGGGCG
mGAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
hGAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

3.4 Immunoblotting

For immunoblotting with BMDM, cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer (Catalog no. 89900, Thermo Fisher Scientific, M.A., U.S.) containing protease and phosphatase inhibitor tablet (Catalog no. 88669, Thermo Fisher Scientific, M.A., U.S.). Lung tissues were first snap chilled in liquid nitrogen and the dried tissue pieces were crushed in RIPA buffer. RIPA lysed samples were kept on ice and subjected to vigorous vortexing for 10 sec and kept on ice again. This process was repeated 3 times up to 5 min. After cell shearing, samples were centrifuged at 12000 rpm for 10 min at 4^{o}C.

Supernatant containing the protein was collected in fresh tubes and concentration was estimated by using Bradford reagent (Catalog No. 500-0006, Biorad, C.A., U.S.). Equal volume of protein from each sample was separated on 7% or 10% SDS-PAGE depending on the molecular weight of protein to be separated and blotted onto nitrocellulose membrane and later visualized using Ponceau stain (Catalog No. P7170, Sigma Aldrich, M.A., U.S) and stain was removed by TBST wash. This was followed by blocking with 5% skimmed milk (Catalog No. 170-6404, Biorad, C.A., U.S). Blocker was removed from membrane by washing with TBST buffer 3 times on a rocker at room temperature. Samples were then probed with primary antibodies (1:1000) for the protein of interest. Following primary antibodies were obtained from Santa Cruz Biotechnology, California, U.S: Phospho-NOS1 (Catalog no. sc-19826), NOS2 (Catalog no. sc-7271), NOS3 (Catalog no. sc-376751), TIRAP(Catalog no. sc-31309), Phospho-c-Jun (Catalog no. sc-53182), c-Jun (Catalog no. sc-74543), Fos (Catalog no. sc-52), ATF2 (Catalog no. sc-187), β-Actin (Catalog no. sc-4778). SOCS1 antibody was obtained from Cell Signalling Technology (CST, M.A., U.S.) while HDAC antibody was procured from Bethyl laboratories (Catalog No. A300-713A-T). Membrane was cleared off the excess primary antibody by TBST wash 3 times. Samples were then probed with secondary antibody (1:10,000) diluted in TBST buffer for 1 h at room temperature. Following secondary antibodies were obtained from Santa Cruz Biotechnology for this study: Donkey anti-mouse-HRP (sc-2318), mouse anti-rabbit-HRP (sc-2357) and Donkey anti-

Goat (sc-2020). Subsequently, the excess secondary antibody was washed off using TBST 3 times and blots were exposed to chemiluminescence substrate (Catalog No. 1068701, Serva, US.). Blots were developed and detected on a Gel doc machine.

3.5 Co-immunoprecipitation

BMDM treated with LPS (250 ng/ml) and TRIM (100 nM) were washed in 1X PBS after treatment and lysed in RIPA buffer diluted with the Tris–HCl base buffer (pH 7.5). Proteins were extracted from each sample and concentration was estimated using Bradford reagent (Catalog No. 500-0006, Biorad, C.A., U.S.). An equal amount of protein (250 μg) from each sample was immunoprecipitated with Jun antibody overnight with shaking at 4^{o}C. The next day, samples were pulled down with protein A/G plus agarose beads (Catalog No. sc-2003, Santa Cruz Biotechnology) by gentle shaking at 4^{o}C for 1 h. Jun bound proteins were then pulled down along with beads by centrifugation at 6000 rpm at 4^{o}C for 5 min and washed with base buffer 3 times to remove the unbound beads from the sample. Samples were then mixed with the loading dye and heated to 80^{o}C and resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane. This was followed by incubation with primary antibody for TIRAP for 1 h at room temperature. Excess primary antibody was removed by TBST wash. Subsequently, membrane was incubated with HRP tagged secondary antibody, mouse anti-rabbit (sc-2357). This was proceeded by TBST washing 3 times and blots were captured on Gel doc machine.

3.6 Confocal microscopy

BMDM were seeded on coverslips placed in six-well plates. Macrophage activation was attained by stimulation with LPS (250 ng/ml) and TRIM (100 nM). For confocal analysis, cells were washed with 1X PBS and fixed with 4% paraformaldehyde (Catalog No. 30552-89-4, Loba Chemie) for 15 min at room temperature and permeabilized with 0.1% Triton-X-100 (Catalog No. 9002-93-1, Thomas Bakers, Maharashtra, India) for 10 min at room temperature without shaking the samples. Cells were then blocked with 5% BSA (Catalog

no. 199896, Maharashtra, India) in 1X PBS for 1 h at room temperature by gentle shaking. Blocking solution was removed by TBST wash 3 times at room temperature. This was followed by incubation with primary antibodies (1:400 dilution in TBST). Post-primary antibody incubation, cells were stained with secondary antibody FITC-conjugated donkey anti-mouse (Santa Cruz) and Alexa Fluor 594 conjugated goat anti-rabbit (Santa Cruz), for 1 h at room temperature. Following primary antibodies were obtained from Santa Cruz Biotechnology: Fos (sc-52), ATF2(sc-187), c-Jun (sc-74543) and TIRAP (sc-31309). Nuclear counterstaining was done using DAPI present in the mounting media (Catalog No. 62248, Thermo Fisher Scientific, M.A., U.S.) according to the manufacturer's instructions. Stained cells were analysed by Olympus confocal laser scanning microscopy.

3.7 Flow cytometry

Flow cytometry analysis was performed on BMDM seeded in six-well plates with a cell density of 10^7 cells/well. Post LPS and TRIM treatment, cells were collected by centrifugation and resuspended in 1X PBS. Fixation of cells was done in 4% formaldehyde for 10 min at 37 °C followed by snap chill on ice for 1 min. Cells were then permeabilized by adding 90% methanol (Catalog no. 67-56-1, Advent Chembio, India) on ice. Post permeabilization, cells were repeatedly washed in incubation buffer (0.5 g bovine serum albumin in 100 ml 1X PBS). 100 µl of NOS2 primary antibody (Santa Cruz Biotechnology; 200µg/ml) at a concentration of 1µg/10^6 cells was added to the cell pellet for 1 h at room temperature. Unbound primary antibody was removed by washing in incubation buffer. The cell pellet was resuspended in FITC conjugated secondary antibody for NOS1 (Donkey anti-mouse-FITC; Santa Cruz Biotechnology) for 30 min at room temperature. Cells were then washed with incubation buffer. After final washing, cells were resuspended in 1X PBS and analysed on a flow cytometer (BD LSR Fortessa). NOS2 protein expression in LPS and TRIM treated macrophages was analysed by the intensity of FITC signal from a cellular population of each sample.

3.8 In-vivo studies

Animal handling was conducted in accordance with the regulations of animal house facility at Acropolis College, Indore. Swiss albino mice were acquired from the Veterinary College, Mhow and housed at the animal house facility at Acropolis College. All experiments included 8-12 week old male mice each weighing 28-30 g. The animal study was approved by the Institutional Animal Ethics Committee (IAEC) of Acropolis Institute of Pharmaceutical Education and research and conducted in accordance with the policies of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. Mice were kept under standard condition of temperature (20-25°C) and relative humidity (55-60 %) with a 14:10 h of light and dark cycle and had ad libitum access to purified water and diet of dry pellets.

Involvement of NOS1 in endotoxin-induced lethality was studied by determining the mice survivability in presence and absence of NOS1 inhibitor TRIM. Endotoxin shock was induced in mice by intraperitoneal injections of 30 mg/kg LPS. Lethal endpoints were analyzed up to 72 h, after which the remaining animals were euthanized by anesthesia followed by cervical dislocation. Survivability was reported for the control and experimental group of mice. For histopathological observations, mice were sacrificed, and lungs were removed 8 h after treatment. Lungs were fixed in 10% formalin and paraffin-embedded sections were stained with hematoxylin and eosin. Morphology of lung sections was observed under light microscope at 20X resolution.

To determine the therapeutic efficacy of Gefitinib in a murine model of sepsis, the mice were acclimatized in the animal house facility at Acropolis pharmacy college, Indore in a pathogen-free vivarium and segregated randomly into five groups (two control and three experimental) with seven mice in each group. Briefly, Gefitinib (A10422-100, Adooq Biosciences) was dissolved in 1:9 solution of DMSO and 1X PBS. Gefitinib solution (40 mg/kg body weight of mice) was intraperitoneally injected in all mice belonging to only Gefitinib and Gefitinib along with LPS group in a 0.5 ml solution per mice. Two control groups were assigned, one group

composed of wild-type animals without any injection. Second control group mice received the solvent vehicle alone (0.5 ml of 1:9 ratio of DMSO and 1X PBS).

Post 1 h Gefitinib administration, LPS (*E. coli* B100) diluted in 0.5ml of 1X PBS was intraperitoneally injected in Gefitinib along with LPS group of mice (30 mg/kg body weight of animals). The therapeutic efficacy of Gefitinib was demonstrated by observing the mortality of mice in the experimental group compared to the control group up to 72 h. Post 72 h, mice that survived the endotoxin shock, were euthanized by anesthesia using chloroform followed by cervical dislocation. To study the protective effect of Gefitinib on lung infection, left and right lungs were harvested for Hematoxylin and eosin (H&E) staining or immunohistochemistry with antibodies for TIRAP and c-Jun.

3.9 Immunohistochemistry

This method has been previously described (Zhou and Moore, 2017). In brief, paraffin-embedded thin (6-8 μm) lung sections on slides were incubated in xylene for 15 min at room temperature. Post xylene incubation, sections were transferred to 100% ethanol for 15 min, followed by incubation in 95%, 70% and 50% ethanol for 5 min each. Lung sections were then rinsed in distilled water and placed in 1X PBS. Subsequently, each section was boiled to 90ºC for 10 min in Tris-EDTA (pH 9.0). Each section was then blocked in 3% BSA in TBST for 30 min at room temperature, followed by rinsing in TBST. Staining of primary antibody (TIRAP and c-Jun, 1:400) was done for 1 h, followed by secondary antibody FITC-conjugated donkey anti-mouse (Santa Cruz) and Alexa Fluor 594 conjugated goat anti-rabbit (Santa Cruz) for another 1 h at room temperature. All samples were washed in TBST post-primary and secondary antibody staining. Lung sections were mounted using DAPI mounting media (Sigma) by placing the coverslip over the tissue. Stained sections were visualized using Olympus confocal laser scanning microscope.

3.10 Molecular modeling

The three-dimensional (3D) structure of c-Jun (residues 201 to 256) was elucidated on the basis of highest sequence identity among other templates as

given by the Modbase program (Pieper et al., 2014). The structure of pre-mRNA-processing-splicing factor 8 (4KIT; Chain C) from the Protein Data Bank (PDB) was our template of choice. The coordinates for the c-Jun segment were further assigned through pairwise sequence alignment by the Modbase program followed by the construction of 3D models of the target sequence. The chosen model was minimized and subjected to molecular docking with TIRAP. The crystal structure of the TIR domain of TIRAP available in PDB (3UB2) was used for the molecular docking studies using the ZDOCK server (Pierce et al., 2014). ZDOCK is a protein-protein docking program used to generate rigid-body docking conformations. We used the default parameters of docking including a blind docking run in order to perform a non-biased docking. ZDOCK is a highly validated docking program and has among the best-performing algorithms in the Critical Assessment of Prediction of Interactions (CAPRI) (Janin et al., 2003), a community-wide project assessing the accuracy of protein-protein docking algorithms. ZDOCK employs a fast Fourier transform (FFT) correlation-based method, which performs a systematic search in the six-dimensional space created by 3 rotational and 3 translational degrees of freedom. Docking conformations are predicted based on the desolvation and electrostatics contributions to the complex formation as well as the pairwise shape complementarity. It searches all possible binding modes in the translational and rotational space between the two proteins and evaluates each pose using an energy-based scoring function. Finally, we collected multiple high scoring conformations of the binary complex, out of which the best scoring one was selected for the Molecular Dynamic simulation.

The docked complex of c-Jun with TIRAP was subjected to molecular dynamic simulation to determine the stability and structural transition of the complex using GROMACS 5.1.2 suite (Hess et al., 2008; Van Der Spoel et al., 2005). Hydrogen atoms were added to the complex and the topology was generated by assigning proper geometrical parameters according to gromos54a8 force field. The complex was then settled in a cubic box where the edge of the box from the molecule was set to 1.0 nm in all directions. SPC216 water model was used to solvate the box based on Periodic boundary conditions. Total charge was neutralized, and the system was minimized by steepest descent algorithm up to a maximum of 50,000 steps and a convergence tolerance of 1000 kJ mol-1 nm-1.

Before the production step of the molecular dynamics (MD) run, two different methods for position restrain: NVT (constant number of particles, volume and temperature) and NPT (constant number of particles, pressure and temperature) were used to equilibrate the system for 2000 ps (2 ns). In both the cases LINCS (Hess et al., 1997) holonomic constraints were used for bonded parameters and SETTLE (Miyamoto and Kollman, 1992) was used for constraining the water geometry. Particle Mesh Ewald (PME)(Kawata and Nagashima, 2001) coulomb type was used for long-range electrostatics with a PME order of 4 and maintaining the Fourier spacing by 0.16. In addition, the V- rescale temperature coupling was used to retain the temperature at 300 K for both protein and non-protein coupling groups. In NPT equilibration step, Parrinello–Rahman pressure coupling was introduced which includes the isotropic coupling type to maintain a uniform scaling of box vectors with 1.0 bar as the reference pressure. Production MD run was carried out for 100 ns timescale for TIRAP-c-Jun complexes using the above-mentioned protocol. MD simulation was performed using NVIDIA Tesla K20M GPU with Intel E5-2640Vz processor. The parameters used for MD simulation can also be referred from our previous work(Muthu et al., 2015).

3.11 Computer-assisted screening

Computer-aided virtual screen was performed using commercially available Discovery Studio 4.1.3 Program (www.accelrys.com). Only the TIRAP structure from the binary complex was used as the starting point for the screen. This was followed by picking an active site grid from the list of sites/spheres given by the program. Out of the total 10 site points provided by the program, we found site 2 encompassing the c-Jun binding site as obtained in the protein-protein docking above. Hence, we went ahead with site 2 for ligand screening. We utilized FDA approved database available in the DrugBank (https://www.drugbank.ca/) for repurposing known drugs for anti-inflammatory activity. The LibDock program in Discovery Studio was used to dock the DrugBank compounds to TIRAP. The docked conformations of the resulting compounds were scored utilizing an intensive scoring analysis using the Score Ligand Poses functionality. Various empirical, force-field and knowledge-based scoring functions (LigScore1-

Dreiding; LigScore2-Dreiding) implemented in Discovery Studio were used to evaluate the best-docked poses. Resulting poses were automatically saved as SD files and analyzed in Discovery studio. Finally, the compounds were sorted based on the highest (1) LigScore1-Dreiding (2) LigScore2-Dreiding and (3) LibDock energy scores as well their 3-dimensional conformations in the TIRAP binding site.

3.12 Statistical Analysis

Quantification of immunoblots from all experiments was performed using the Image J software. The data has been analyzed using Student's paired t-test to compare the mean difference between all pairs of groups employed for statistical analysis. The data has been expressed as mean ± SEM and values up to $p < 0.05$ have been considered significant. Mice survivability was calculated using GraphPad (Prism 6.0) and plotted as percent survival with respect to hours post LPS injection.

4. Results Chapter 1

NOS1 derived nitric oxide is an essential triggering signal for the development of systemic inflammatory response

4.1.1. NOS1 deficiency protects mice from LPS induced injury

The inflammatory response to infection or tissue injury facilitates initiation, maintenance as well as resolution of the inflammatory response to protect the host against infectious challenges(Newton and Dixit, 2012b). Macrophages play a central role in mediating the inflammatory response by activating the cascade of transcription factors that further regulate the expression of cytokine genes leading to outburst of inflammatory reactions (Ariel et al., 2012; Fujiwara and Kobayashi, 2005; Ivashkiv, 2011). Balance between the prolonged activation and resolution of inflammation determines the fate of inflammatory response towards tissue repair or damage (Fujiwara and Kobayashi, 2005; Newton and Dixit, 2012b). Inflammation is marked by a robust release of potent signaling molecule nitric oxide (NO) produced by a vital enzyme of the immune cells nitric oxide synthase (NOS) to eliminate the damage caused by pathogen (Wallace, 2005). Early studies have identified three isoforms of NOS, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) (Alderton et al., 2001b; Stuehr, 1999). Considerable advances illustrating the involvement of NOS2 and NOS3 in inflammatory processes have established their role as important mediator of inflammation (Cirino et al., 2003a; Salvemini and Marino, 1998). However, role of NOS1 in inflammation is not well elucidated. This study highlights the potential of NOS1 in modulating the inflammatory response in macrophages.

Previous studies on NOS1 mediated inflammatory responses lead us to determine the role of NOS1 in endotoxin shock induced lung injury in mice. We used lethal LPS dose (30 mg/kg) to induce endotoxin shock in mice. Another group of mice was administered with NOS1 inhibitor TRIM (25 mg/kg) 1 h prior to LPS. Control group of mice (n=7 per group) that received the solvent solution (DMSO+PBS) survived. However, in LPS injected mice, 72% mortality was observed post 24 h. At the end of 72 h, only 14% mice survived from the endotoxin shock (Fig. 4.1.1A). On the contrary, mice injected with NOS1 inhibitor TRIM, 1 h prior to LPS, exhibited only 29% mortality up to 24 h and 64% mice survived up to 72 h which is significantly higher than 14% survival of the LPS group of mice. (Fig 4.1.1 A). Lipopolysaccharide (LPS) which is a component of the outer wall of gram-negative bacteria, elicits strong immune response in animals (Fang et al., 2004; Opal, 2010).

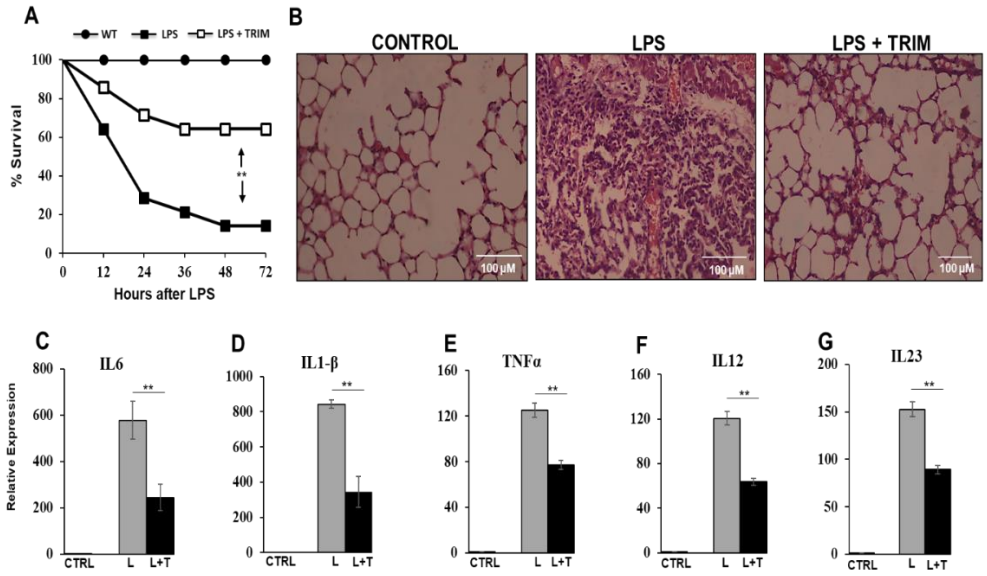


Fig 4.1.1. NOS1 deficiency protects mice from LPS induced injury. Swiss albino mice were challenged with LPS (30 mg/kg) alone or with TRIM (25 mg/kg) 1 h prior to LPS. (A) Survival of control mice, LPS injected mice and LPS+TRIM injected mice compiled from at least 3 independent experiments using 10 mice per group. (A) Survival was monitored for the indicated time points. (B) Lungs were harvested from each group of mice 8 h post i.p. LPS and TRIM challenge. Lung sections were prepared and stained with H&E to visualize lung morphology using light microscope (bar, 100 µM). Representative q-RT PCR for cytokine mRNA expression from lung tissues for (C) IL-6, (D) IL1-β (E) TNF-α, (F) IL-12 and (G) IL-23. All data are representative of three independent experiments, presented as mean $\pm$ SD, Significance in all experiments was determined using Student's t-test **P<0.002.

We used lethal dose of LPS (30 mg/kg) to model the septic mice injury for our studies. To study the involvement of NOS1 in septic lung injury, we analyzed three groups of mice: first group with control animals (WT), second group injected with only LPS and third group that received both LPS along with NOS1 inhibitor TRIM injections. Here we observed a conserved lung morphology with intact pulmonary capillaries in WT mice, which deteriorated adversely in lungs of LPS injected mice (Fig 4.1.1 B). LPS induced lung injury was in accordance with the previously reported observations (Asti et al., 2000; Baig et al., 2015a).

Interestingly, mice injected with NOS1 inhibitor 1-(2-Trifluoromethylphenyl) imidazole, TRIM (Handy et al., 1995), 1 h prior to LPS demonstrated a remarkable recovery from the LPS induced lung injury. The data strongly implicates the involvement of NOS1 in LPS induced lung damage and systemic inflammation in animals suggesting that administration of TRIM acted as a protective mechanism against septic shock injury. After evaluation of the lung morphology, we next determined the cellular mediators of lung deterioration in LPS injected mice. Cytokines are critical modulators of immune response in inflamed tissue (Chaudhry et al., 2013; Schulte et al., 2013). Cytokines are produced during the inflammatory reactions and activates other immune cells in the affected tissues. Inflammation associated cytokines majorly include IL6, IL1β and TNFα are produced by variety of cell types but the predominant source include the inflammatory macrophages at the site of infection(Kushner, 1993). We therefore determined the changes in cytokine gene expression in septic mice model. We utilized three groups of mice, WT, LPS administered and LPS along with TRIM injected mice group for this study. Lung tissues were extracted post LPS and TRIM dosing and analyzed for cytokine expression.

Interleukin 6 (IL6) is an inflammatory cytokine produced at the site of infection and assists in the development of acute and chronic inflammatory response (Gabay, 2006; Tanaka et al., 2014). In addition, IL6 promotes differentiation of naïve $CD4^{+}$T cells thereby linking innate immunity with adaptive immunity (Tanaka et al., 2014). This prompted us to analyze the

expression of IL6 in mice aroused with LPS shock. Our findings indicate that IL6 is elevated in lungs of LPS administered mice compared to the WT mice. However, mice that received TRIM injection prior to LPS failed to produce high levels of IL6, thereby suggesting that NOS1 inhibition negatively modulates the expression of IL6 (Fig 4.1.1 C).

Among the cytokine family, IL1 members are primarily associated with chronic inflammatory disorders (Dinarello, 2011; Ren and Torres, 2009). Evidences demonstrate the involvement of IL1β in endotoxin shock mediated induction and maintenance of inflammation. Studies on neonatal endotoxin shock, have demonstrated involvement of serum IL1β as a critical mediator of inflammation that can be utilized as a therapeutic marker for evaluation of the disease severity (Kurt et al., 2007). This lead us to determine the expression of IL1β in all the experimental groups of mice. Our results show that IL1β is strongly elicited in response to LPS exposure to mice compared to the WT mice. However, the response impeded drastically in mice injected with NOS1 inhibitor TRIM (Fig 4.1.1 D).

The data indicated that NOS1 has a significant role in modulating the critical cytokines of immune system. In this study we focussed on cytokines that enhance the inflammatory responses in macrophages. TNFα is another robust cytokine that is rapidly released after exposure to bacterial LPS in inflamed tissues (Parameswaran and Patial, 2010). TNFα amplifies the inflammatory response by promoting the expression of NF-kB and essential MAP kinases of the signalling pathway in macrophages (Newton and Dixit, 2012b; Vujanovic, 2011). We therefore investigated the changes in the mRNA expression of TNFα upon LPS stimulation. We observed an advancement in the TNFα expression after 1 h of LPS stimulation. Next, we analyzed the effect of NOS1 inhibition on TNFα expression. It was interesting to observe that TNFα expression dropped in cells treated with NOS1 inhibitor TRIM prior to LPS (Fig 4.1.1 E). Collectively, the data suggests that NOS1 has an indispensable role in modulating the expression of proinflammatory cytokines that determine the severity of the inflammatory response in mice.

We observed the cytokine expression of IL-12 and IL-23 cytokines which are critical mediators of inflammation (Fig. 4.1.1 F, G). With LPS induction there was a sharp increase in the expression which depreciated with NOS1 inhibition, suggesting that NOS1 is the key player in regulating the expression of important inflammatory cytokines.

4.1.2 Pro-inflammatory cytokine response to LPS are diminished with NOS1 inhibition in macrophages.

Overexpression of proinflammatory cytokines plays critical role in promoting inflammatory tissue injury during endotoxin shock (Schulte et al., 2013). To test whether NOS1 is a determining factor for altering the macrophage transitions, we further examined the effects of pharmacological NOS1 inhibitor (TRIM) on the cytokine production in different cell line models in-vitro. The study was conducted primary cells (Bone marrow-derived macrophages) were obtained from Swiss albino mice. In addition, two macrophage cell lines: THP-1 and Raw 264.2.3 were used for this study. Cells were stimulated with LPS (250 ng/ml) up to 4 h in presence or absence of NOS1 inhibitor TRIM.

In accordance with our previous data delineating NOS1 inhibition in protection from endotoxin injury, we observed that NOS1 inhibition using TRIM displayed significantly decreased cytokine responses 4 h post-LPS treatment compared with the control in all three models of macrophages (Fig. 4.1.2). Bone marrow-derived macrophages (BMDM) are appreciated as a potential model to study functional attributes of macrophages (Roberts et al., 2015; Yang et al., 2016). A recent report suggests that BMDM decreases mycobacterium infection by augmenting the NO production (Yang et al., 2016).In this study we examined the LPS induced proinflammatory cytokine gene regulation and their varied expression upon NOS1 inhibition by pharmacological inhibitor TRIM in bone marrow-derived macrophages. Proinflammatory cytokines IL6 and IL1-β were highly expressed with LPS challenge in a time-dependent manner (Fig.4.1.2 A, B). LPS stimulation up to

2 h significantly increased cytokine expression, which however decreased in presence of TRIM. Cytokines expression was remarkably augmented up to 4 h of LPS challenge. Similar to this observation, TNF-α gene expression also markedly upregulated upon LPS induction with a steady increase up to 4 h (Fig.4.1.2 C). Strikingly, TNF-α gene exhibited diminished expression with the depletion of NOS1 in macrophages by TRIM. Results demonstrated a rapid increase in TNF-α expression within 1 h of LPS stimulation with a constant increase up to 4 h.

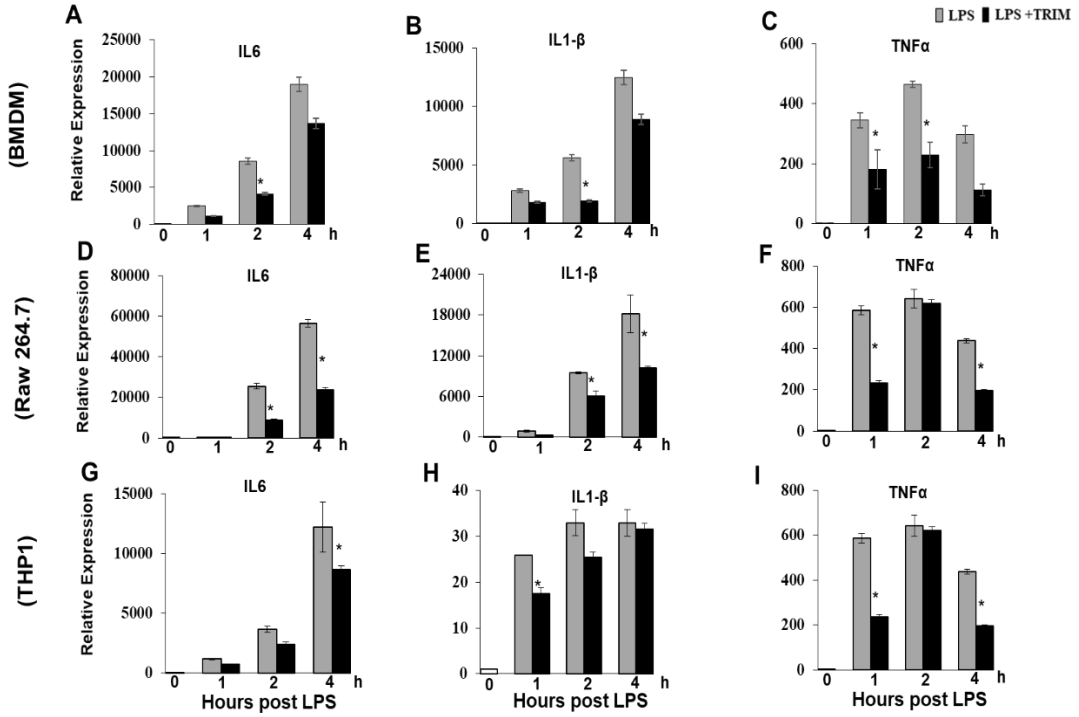


Fig 4.1.2. Pro-inflammatory cytokine response to LPS are diminished with NOS1 inhibition in macrophages. Macrophages treated with LPS (250 ng/ml) or TRIM (100 nM) 1 h before LPS were subjected to q- RT PCR after indicated time points. Quantitative m-RNA expression of cytokine genes in BMDM (A) IL-6, (B) IL1-β, (C) TNF-α, in Raw-264.2.3 macrophages (D) IL-6, (E) IL1-β, (F) TNF-α and in THP-1 cells (G) IL-6, (H) IL1-β, (I) TNF-α. All data are representative of three independent experiments, presented as mean $\pm$ SD, Significance in all experiments was determined using Student's t-test, *P<0.005, **P<0.002.

Subsequently, NOS1 suppression by TRIM notably declined cytokine expression suggesting a NOS1 dependent upregulation of proinflammatory

cytokine that further orchestrates robust inflammatory signals in bone marrow-derived macrophages.

Our next model of study was Raw macrophages where we estimated the expression of inflammatory cytokines (IL6, IL1-β and TNF-α) after stimulation with LPS and TRIM (Fig. 4.1.2 D, E, F). The results were in accordance with the BMDM cytokine expression suggesting the crucial role of NOS1 in inflammatory cytokine production from macrophages. THP1 is a human monocytic cell line derived from monocytic leukemia patient (Tsuchiya et al., 1980). THP-1 cells are widely used as model to study monocyte to macrophage differentiation mechanism and to examine the macrophage-related inflammatory processes. THP-1 cells respond to the signals induced by LPS or IFN-γ and differentiate readily into proinflammatory macrophages(Bosshart and Heinzelmann, 2016). We therefore utilized THP-1 as our model to study the associated inflammatory cytokine responses induced by LPS up to 4 h. Results from this study demonstrated significant reductions in mRNA for an array of cytokines (IL6, IL1-β and TNF-α) after treatment with NOS1-specific inhibitor TRIM (1-(2- trifluoromethylphenyl) imidazole) as compared to the LPS treated cells. Unstimulated cells failed to express the inflammatory cytokines (Fig. 4.1.2 G, H, I). Results from all three macrophages indicate the LPS stimulation amplifies the cytokine response and NOS1 inhibition suppresses this response leading to decreased inflammatory signalling.

4.1.3 NOS1 is activated in macrophages after LPS treatment and is required for rapid NO production

Nitric oxide synthase (NOS) has been well identified as a crucial enzyme that produces the potent signalling molecule, nitric oxide (NO) and plays a central role in mediating the inflammatory reactions arising due to pathogenic damage to body (Alderton et al., 2001b; Coleman, 2001; Förstermann and Sessa, 2012b; Korhonen et al., 2005; Wallace, 2005). Three isoforms of NOS are recognized based on the cell type expressed which includes neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS

(eNOS or NOS3) (Förstermann and Sessa, 2012b; Zamora et al., 2000b). Inflammatory processes have been associated with NOS2 and NOS3, however considerably less is investigated about the role of NOS1 in pathophysiology of inflammatory diseases and endotoxin shock (Cirino et al., 2003a; Kröncke et al., 1998; Zamora et al., 2000b). Our previous study elaborated the involvement of NOS1 derived nitric oxide in advancing the inflammatory reaction via NF-kB pathway. Further to this study, NOS1 activity was analyzed as a principal source of inflammation during early stage of pathogenic stimulus.

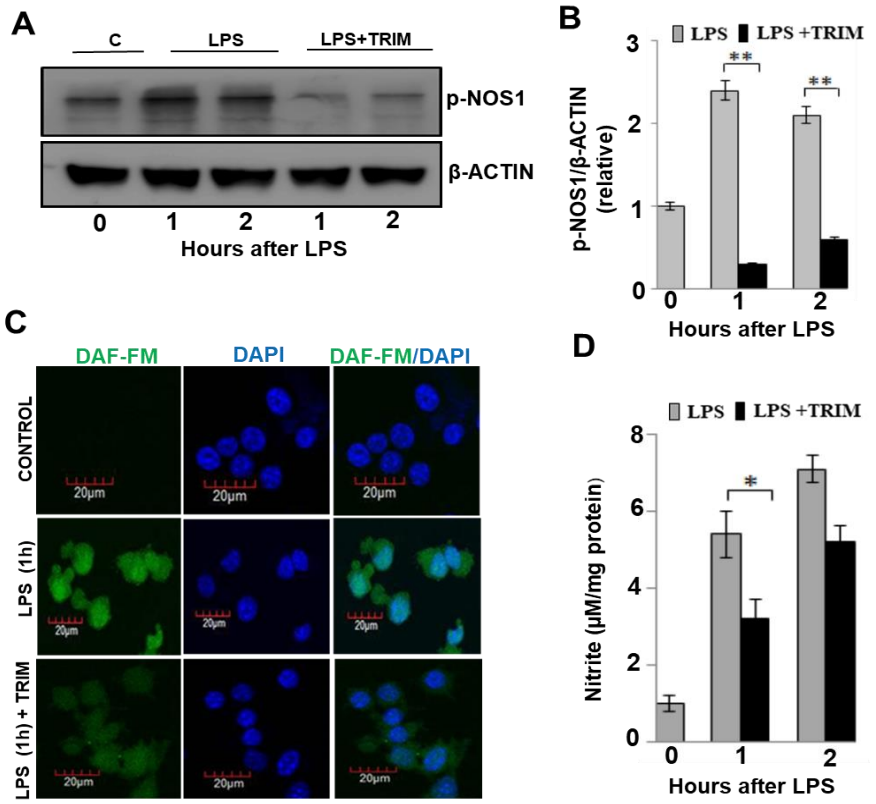


Fig 4.1.3. NOS1 is activated in macrophages after LPS treatment and is required for rapid NO production. (A) BMDM stimulated with LPS (250 ng/ml) or TRIM (100 nM) 1 h before LPS were assayed by immunoblot for phosphorylation of NOS1 which correlates with its enzymatic activation up to 2 h. (B) Densitometry analysis of phospho-NOS1 as in (A), normalized to actin, presented as mean ± SEM of quantitation of three independent experiments. **P < 0.002 (Student's t-test). (C) Nitric oxide production in macrophages (BMDM) was detected using DAF-FM staining, detected on confocal microscope (D) Indirect nitrite accumulation in the supernatant of cultured BMDM was measured using Greiss reagent on a microplate reader, before or after LPS stimulation (250 ng/ml). All data are representative of three independent experiments presented as mean ± SD. P values in (B) and (D) were determined by Student's t-test; *<P0.05, **<P0.005.

To validate the role of NOS1, bone marrow-derived macrophages (BMDM) were challenged with LPS (250 ng/ml) with or without NOS1 inhibitor TRIM and phospho-NOS1 expression was analyzed up to 2 h. The observations from this study demonstrated phospho-NOS1 expression up to 2 h of LPS stimulation which was evidently diminished with effect of NOS1 inhibitor TRIM on macrophages (Fig 4.1.3 A). Quantitative analysis of the phospho-NOS1 expression represents the fold increase in phospho-NOS1 protein expression compared to the expression in unstimulated macrophages (Fig 4.1.3 B).

We next determined the nitric oxide produced by the macrophages to ascertain the activity of NOS1. This was performed in a cell-based assay using DAF-FM staining. Level of nitric oxide was visualized by staining cells with DAF-FM with the intensity of DAF-FM signals as the measure of nitric oxide produced by macrophages. DAF-FM diacetate is a non-fluorescent and cell permeable dye which readily combines with the available nitric oxide inside the cells. Cellular esterases mediate deacetylation of DAF-FM diacetate and convert it into fluorescent benzotriazole derivatives which are fluorescently detected on a microplate reader. Fluorescence from each sample corresponds to the level of nitric oxide produced by the macrophages. The data from DAF-FM staining suggest a robust increase in nitric oxide from macrophages in 1 h of LPS stimulation, which however decline with the presence of TRIM (Fig 4.1.3 C)

NOS1 activity was further validated by the measurement of nitric oxide produced by macrophages stimulated with LPS either in presence or absence of NOS1 inhibitor TRIM. An indirect measurement of NO production was performed by detecting nitrite as the stable metabolite in the supernatant of cultured macrophages using Greiss reagent. Briefly, BMDM were stimulated with LPS (250 ng/ml) up to 2 h, and the media from the macrophages was collected for calorimetric analysis with Greiss reagent. The results depicted the presence of nitric oxide at 1 h of LPS stimulation with a steady increase up to

2 h (Fig. 3.3 D). Strikingly, level of nitric oxide produced by macrophages was significantly impeded in presence of NOS1 inhibitor TRIM (Fig 4.1.3 D).

4.1.4 NOS2 and NOS3 do not contribute to early inflammatory response in macrophages

NOS2, which is most appreciated for its potent involvement in inflammatory outburst is induced upon the pathogen recognition and therefore appears at later stages of inflammatory signalling (Zamora et al., 2000b). However, it is less clear whether it is involved in early inflammatory responses in macrophages. After investigating the activation of NOS1 in early phase of endotoxin shock in macrophages, we next examined the activation and contribution of NOS2 in producing nitric oxide which is required for the downstream inflammatory response. We determined the presence of NOS2 mRNA in bone marrow-derived macrophages stimulated with LPS up to 12 h. The data illustrates absence of NOS2 expression up to initial 2 h of LPS trigger and commences 4 h onwards with a potent induction up to 12 h (Fig 4.1.4 A). Interestingly, induction with TRIM 1 h prior to LPS was able to inhibit NOS2 mRNA expression indicating that TRIM poses an inhibitory action on NOS2 isoform in macrophages.

To further rule out the possibility of NOS2 involvement in early stages of inflammation, NOS2 protein expression was analysed in macrophages stimulated with LPS and TRIM. Expectedly, NOS2 protein also could not be detected in macrophages up to 2 h of LPS load and started to appear from 4 h LPS treatment onwards following an increasing expression pattern up to 12 h (Fig. 4.1.4 B). In accordance with the mRNA expression, NOS2 protein expression also depreciated in presence of TRIM (Fig 4.1.4 B).

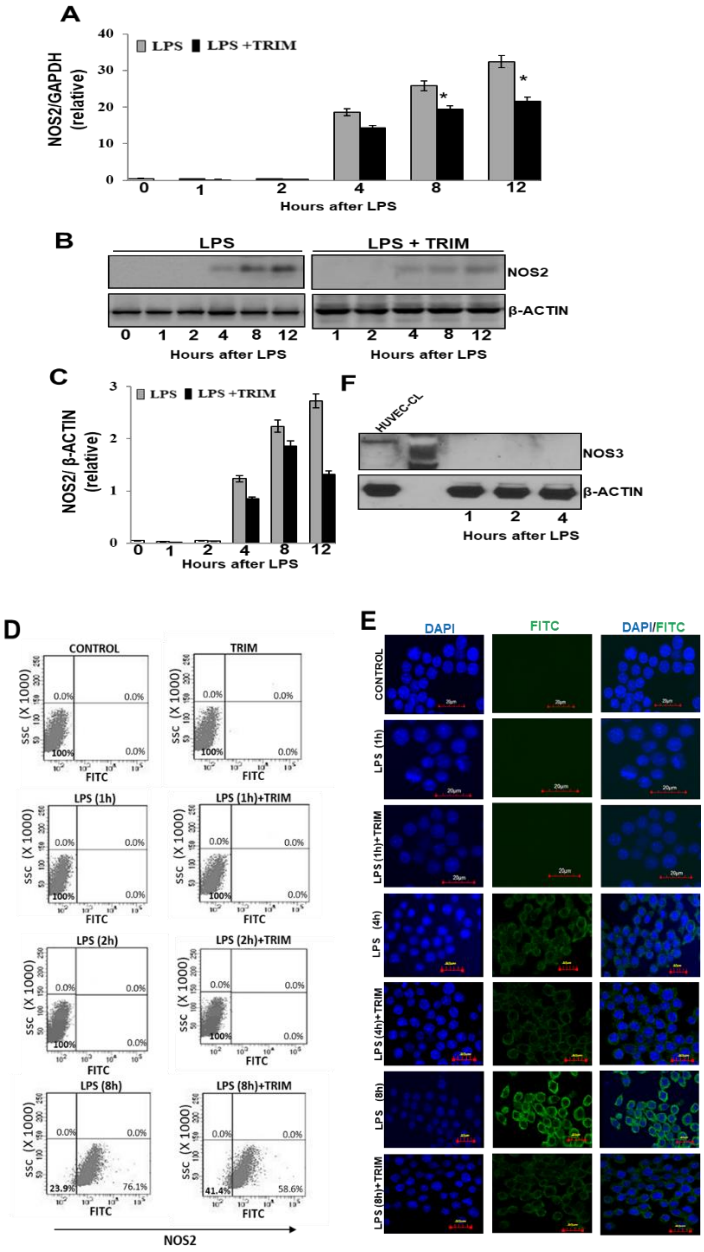


Fig 4.1.4. NOS2 and NOS3 do not contribute to early inflammatory response in macrophages. BMDM stimulated with LPS (250 ng/ml) or TRIM (100 nM) 1 h before LPS were analyzed for (A) NOS2 mRNA expression using quantitative real time-PCR (B) NOS2 protein up to indicated time points (C) relative quantification of NOS2 protein as in (B) normalized to actin, presented as mean ± SEM of quantitation of three independent experiments. *P < 0.005 (Student's t-test) (D) Flow cytometry analysis of NOS2 protein (FITC) expressing BMDM population up to 8 h LPS stimulation (E) Confocal microscopic analysis after fixation and immunostaining for NOS2 (FITC), nuclei were counterstained with DAPI and representative images from three independent experiments are shown (bar, 20 μM). (E) NOS3 protein expression in macrophages treated with LPS up to 4 h and in human vascular endothelial cells (HUVEC) as a positive control. Data are representative of three independent experiments; all are presented as mean ± SD.

Quantitative representation of NOS2 protein indicate presence of NOS only after 4 h of LPS exposure and a continuous rise up to 1 h (Fig 4.1.4 C). Furthermore, NOS2 protein in macrophage population activated with LPS was examined by flow cytometry by staining NOS2 with FITC and analyzed for the intensity of FITC in stimulated cells.

In accordance with NOS2 protein expression data by immunoblotting, macrophage population triggered with LPS for 1 h did not produce any signal for NOS2 expressing population similar to the unstimulated population as evident by flow cytometry analysis (Fig 4.1.4 D). TRIM alone did not trigger any NOS2 signals from the macrophages, however a significant rise in NOS2 expressing population was observed in cells exposed to LPS for 8 h, demonstrating a robust increase in NOS2 only at later stage of LPS induction (Fig 4.1.4 D).

Further confirmation to the activation of NOS2 protein expression in macrophages was provided by staining NOS2 with FITC in Raw macrophages and analyzing the expression using confocal microscopy. Macrophages were stimulated with LPS for 1 h did not produce NOS2 which was evident by the absence of FITC signals from the macrophages (Fig 4.1.4 E). NOS2 expression was clearly detectable only after 4 h of endotoxin shock and it increases gradually up to 8 h of stimulation, which is in accordance with q-RT PCR and immunoblot data implying a later stage induction of NOS2 in macrophages. TRIM treatment prior to LPS, inhibited NOS2 expression in macrophages which was observed at 4 h and 8 h of stimulation (Fig 4.1.4 E).

We also determined the absence of NOS3 expression in macrophages stimulated with LPS up to 4 h (Fig 4.1.4 F), which provides a clear insight that there was no NOS3 expression detected in macrophages upon LPS stimulation. A positive control was included in this study using human vascular endothelial cells (HUVEC) which are known to produce NOS3 (endothelial NOS) robustly. NOS3 expression was expectedly observed in HUVEC cells.

Together the data suggests that NOS1 is the primary source of nitric oxide during early inflammatory signals in macrophages. NOS2 however, is induced only at later stages of inflammation resulting in an outburst of inflammatory mediators. We therefore confirm a regulatory function of NOS1 alone in initiating and commencing the inflammatory signals during early onset of inflammatory reactions. Importantly, NOS2 does not contribute to early inflammatory responses in macrophages. A critical step further contributes to regulatory function in the downstream inflammatory cascade.

Results *Chapter 2*

TIRAP-Jun interaction is essential for early stage inflammatory response of AP1 mediated signaling

4.2.1 LPS stimulated macrophages show elevated expression of phospho-NOS1, TIRAP and c-Jun while diminished expression of SOCS1

Inflammatory responses originating in macrophages are balanced by multiple negative regulators that aid in the maintenance of healthy immune system. SOCS1 (suppressor of cytokine signalling) are a group of intracellular proteins that negatively regulate the cascade of inflammatory signalling by suppressing the cytokines induced by TLR4 pathway (Kinjyo et al., 2002; Krebs and Hilton, 2001; Nakagawa et al., 2002b; Yoshimura et al., 2005). Inflammatory signals arise in response to the bacterial invasion, recognized by TLR4 receptors that orchestrates the entire cascade by activating the key adaptor proteins such as TIRAP (Toll/interleukin-1 receptor domain-containing adapter protein). TIRAP potentiates the inflammatory circuit by recruiting downstream kinases that activate the downstream transcription factors such as NFkB p65 that bind and upregulate the expression of proinflammatory genes (Shinobu et al., 2002b; Yamamoto et al., 2002). As a critical effector of TLR4 signalling, TIRAP itself undergoes phosphorylation by Btk (Bruton's tyrosine kinase) which then interacts with SOCS1 resulting in the polyubiquitination and proteasomal degradation (Gray et al., 2006; Mansell et al., 2006b).

However, absence of SOCS1 regulation, amplifies TIRAP mediated p65 phosphorylation causing its transactivation and a significant upregulation in the inflammatory gene expression (Piao et al., 2008). The studies suggest a primary role of SOCS1 in suppressing TIRAP mediated inflammatory trigger initiated via Btk. A similar mode of Btk facilitates phosphorylation of p65 on

serine 536 and promotes its transactivation (Doyle et al., 2005; Ní Gabhann et al., 2014). Evidences from previous reports have explored the mechanism of TIRAP regulation and its impact on inflammatory cascades. LPS stimulation up to 1 h promotes nitrosation and degradation of SOCS1 leading to a subsequent upregulation of TIRAP due to insufficient expression of the negative regulator SOCS1 (Baig et al., 2015b).

We further explored the impact of NOS1 on SOCS1 and TIRAP expression in macrophages. BMDM were stimulated with LPS (250 ng/ml) and TRIM (100 nM), 1 h prior to LPS starting from 5 min up to 120 min. Post-treatment, cells were harvested for immunoblot to determine the expression of SOCS1 and TIRAP (Fig 4.2.1.1).

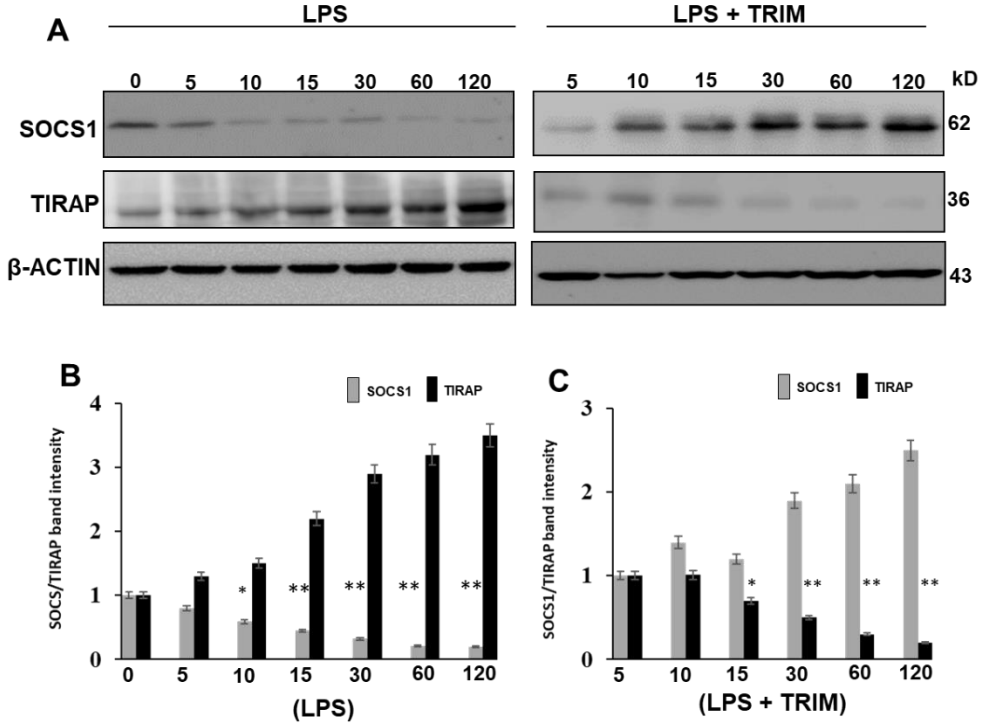


Fig. 4.2.1.1. NOS1 diminishes SOCS1 while up-regulates TIRAP expression in LPS stimulated macrophages. BMDM stimulated with LPS (250 ng/ml) or TRIM (100 nM) 1 h prior to LPS were analyzed by immunoblot for (A) SOCS1 and TIRAP expression up to indicated time points. NOS1 mediated regulation of SOCS1 and TIRAP was analyzed in BMDM treated with TRIM (100 nM) 1 h prior to LPS. Densitometry analysis of SOCS1 and TIRAP as in (A), normalized to actin, presented as mean ± SEM of quantitation of three independent experiments, *p<0.003, **P < 0.002 determined using Student's t-test.

We observed a sharp decrease in SOCS1 expression starting from 5 min LPS stimulation onwards, with a complete absence up to 120 min. Interestingly, SOCS1 expression recovered in TRIM treated macrophages, implying that NOS1 derived nitric oxide mediates nitrosation and degradation during LPS challenge, which however is not observed with NOS1 inhibition (Fig. 4.2.1.1 A). At the same time points, we also detected TIRAP protein expression through immunoblot. Results indicated that TIRAP expression up-regulated gradually from 5 min LPS stimulation up to 120 min. However, upon TRIM treatment prior to LPS, TIRAP expression was diminished in macrophages (Fig 4.2.1.1 A). Presence of SOCS1 during NOS1 inhibition might be the reason for TIRAP degradation, implying a reverse correlation between SOCS1 and TIRAP in LPS stimulated macrophages. Relative quantification of SOCS1 and TIRAP in LPS treated macrophages and LPS along with TRIM treated macrophages represent the reverse effect of the two proteins in presence and absence of NOS1 (Fig 4.2.1.1 B-C).

The constitutive isoform of nitric oxide synthase, NOS1 plays a prominent role in systemic inflammatory response during endotoxin shock. We next intended to examine the regulation of important molecules of TLR4 signalling pathway through NOS1. We first determined the expression of activated NOS1(phospho NOS1) in LPS challenged BMDMs. In accordance with the previous reported data, macrophages stimulated with LPS for 1 h, exhibited significant increase in phospho-NOS1 expression, which decreased in presence of NOS1 inhibitor TRIM (Fig 4.2.1.2 A). Relative quantification represents the fold change in the expression of phospho NOS1in macrophages (Fig 4.2.1.2 B).

The signaling events originating downstream of the TLR4 receptor are coordinated by adaptors MyD88 and Mal/TIRAP. TLR4 induced signalling events are tightly regulated to protect the host from excessive inflammatory injury. Negative regulators such as suppressor of cytokine signalling (SOCS) are family of proteins that check the alarming onset of inflammatory signalling by targeting the degradation of critical effectors such as TIRAP (Kinjyo et al., 2002; Liau et al., 2018; Nakagawa et al., 2002b). TIRAP is a critical adaptor molecule in mediating the NFkB dependent inflammatory response. Negative

regulation by SOCS1 interrupts the TIRAP-dependent NFkB p65 phosphorylation and transactivation (Mansell et al., 2006a). Therefore, SOCS1 dependent TIRAP degradation suppresses the inflammatory response and provides a rigid check on the severity of inflammatory reactions. These studies identify TIRAP as a target of SOCS1 and further regulates the inflammatory response.

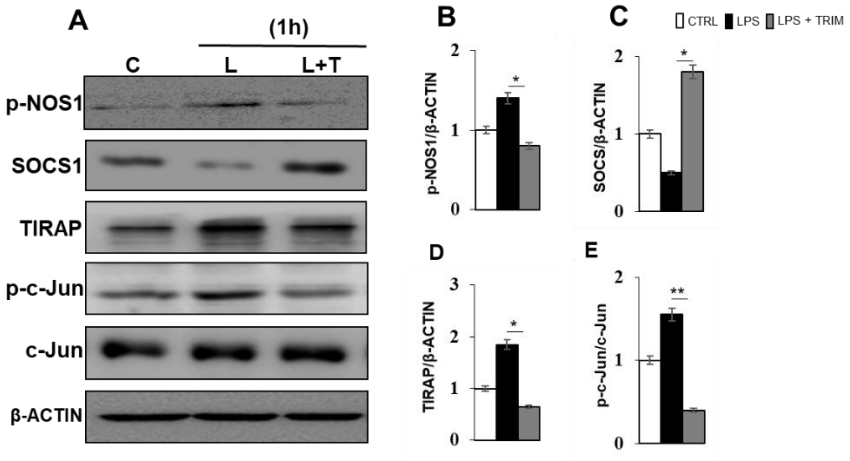


Fig. 4.2.1.2. LPS stimulated macrophages show elevated expression of phospho-NOS1, TIRAP and c-Jun while diminished expression of SOCS1. (A) BMDM were stimulated with LPS (250 ng/ml) for 1 h and TRIM (100 nM) 1 h prior to LPS and analyzed by immunoblot for phospho-NOS1, SOCS1, TIRAP, phospho-c-Jun and total c-Jun. Densitometry analysis of (B) phospho-NOS1, (C) SOCS1, (D) TIRAP and (E) phospho-c-Jun as represented in (A), normalized to actin, presented as mean ± SEM of quantitation of three independent experiments, *p<0.005, **P < 0.002 determined using Student's t-test.

To further explore NOS1 mediated regulation of SOCS1, we determined expression of SOCS1 in activated macrophages. In this study, we show that LPS activated BMDM show diminished SOCS1 expression after 1h LPS exposure which increases in presence of NOS1 inhibitor TRIM (Fig 4.2.1.2 A). Relative quantification of the SOCS1 immunoblot represents the relative fold change compared to the unstimulated macrophages (Fig 4.2.1.2 C). TIRAP expression was detected in the similar conditions of LPS and TRIM stimulation in macrophages. Results indicate that TIRAP is abundantly expressed after 1 h LPS stimulation, however, it goes down in presence of

TRIM, which was in accordance with our previous data (Fig 4.2.1.2 A). Quantification of TIRAP immunoblot relative to actin is represented (Fig 4.2.1.2 D). The data suggest that absence of SOCS1 during LPS trigger sustains TIRAP expression and subsequently amplifies the inflammatory signaling. Results from our study emphasize on SOCS1 mediated negative regulation of TIRAP in 1 h of LPS challenge in bone marrow-derived macrophages. Therefore, the relative expressions of phospho-NOS1, SOCS1 and TIRAP during LPS stimulation provide interesting regulatory mechanism during inflammatory cycles in macrophages.

Moving further, we aimed to determine whether NOS1 mediated regulation of SOCS1 and TIRAP have a substantial effect on the expression of AP-1 subunit c-Jun. AP1 transcription factor is essential in orchestrating the TLR4 signaling pathway. An integral component of AP1, c-jun is a highly dynamic subunit that when phosphorylated forms heterodimer with other AP1 subunits, Fos and ATF2. Activated heterodimer then translocates into the nucleus and ameliorates the cytokine gene expression and subsequent inflammatory response. Therefore, we checked the expression of phospho-c-Jun in macrophages stimulated with LPS for 1 h. It was interesting to observe that LPS stimulation abundantly increases phospho-c-Jun expression which got diminished in presence on NOS1 inhibitor TRIM (Fig 4.2.1.2 A). Total c-Jun expression and actin remained unaltered during any stimulation. Relative quantification of phospho-c-Jun compared to total c-Jun represents the immunoblot data accordingly (Fig 4.2.1.2 E). The results implied that NOS1 facilitated c-Jun activation through SOCS1 and TIRAP.

4.2.2 TIRAP immunoprecipitates with c-Jun in LPS stimulated macrophages

Initiation and maintenance of TLR4 signal transduction pathway requires adaptor protein TIRAP that plays an indispensable role in regulating the transactivation of essential transcription factor NFkB and AP1 (Baig et al., 2017a; Mansell et al., 2006a). Previous studies have uncovered the TIRAP

dependent NFkB p65 transactivation that further amplify the proinflammatory cytokine gene expression (Mansell et al., 2006a). Based on the previous findings, we anticipated the involvement of TIRAP in transactivation of AP1 transcription factor as well. To address the possibility, we performed co-immunoprecipitation (Co-IP) in LPS induced bone marrow-derived macrophages. BMDM treated with LPS and TRIM were subjected to co-immunoprecipitation with c-Jun. Immunoprecipitated fractions were pulled down and immunoblotted with TIRAP antibody. The results suggest that TIRAP immunoblotted with c-Jun post LPS stimulation. Therefore, suggesting a positive interaction between TIRAP and Jun within 1 h of LPS trigger in BMDM (Fig 4.2.2 A). Interestingly, with the presence of NOS1 inhibitor TRIM, the interaction between TIRAP and c-Jun was lost as is evident with the absence of TIRAP immunoblot implying that NOS1 potentiates the interaction of TIRAP and c-Jun. Total c-Jun expression did not show any change upon LPS or TRIM stimulation. Relative quantification of TIRAP immunoblotted with c-Jun precipitated samples depicts an increase in their interaction upon LPS, which is significantly down-regulated in presence of NOS1 inhibitor TRIM (Fig 4.2.2 B).

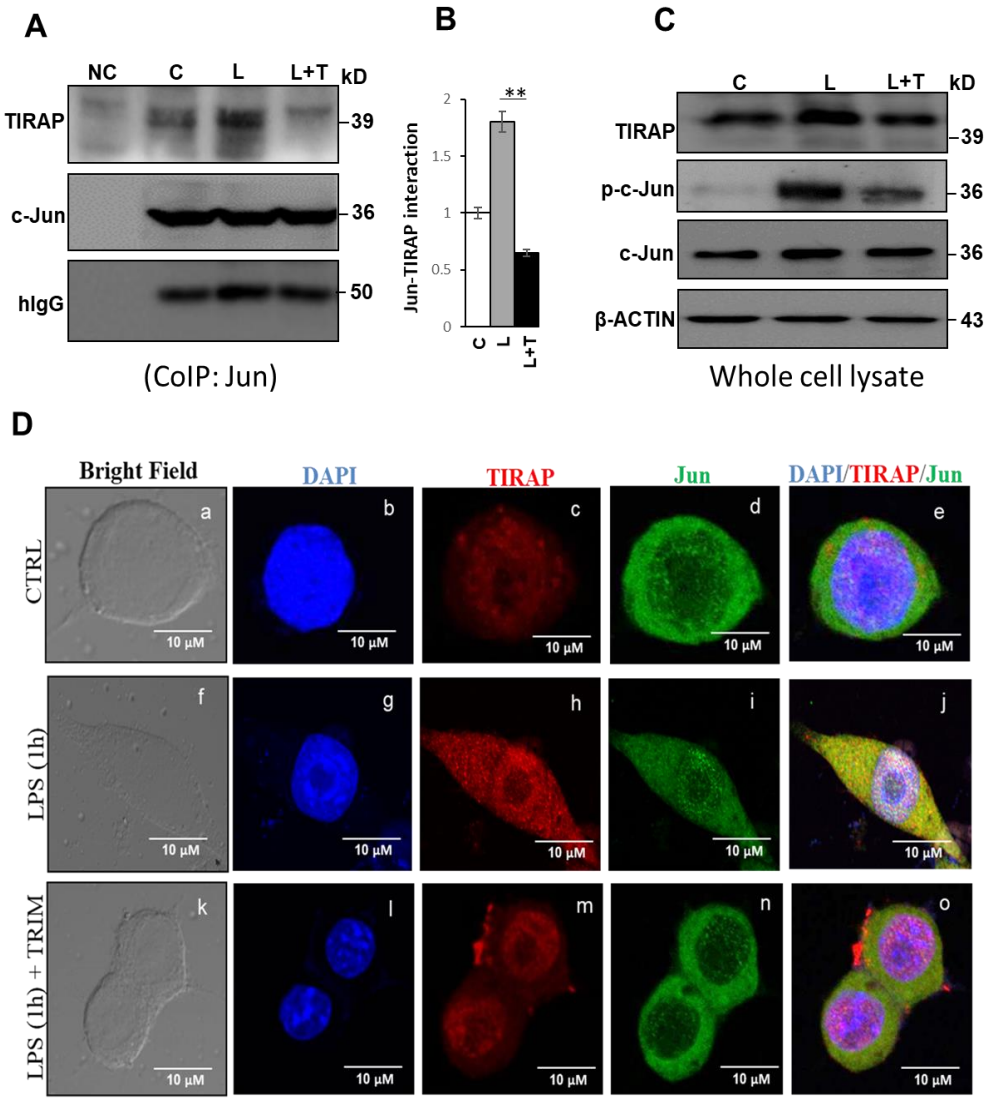


Fig 4.2.2. TIRAP immunoprecipitates with c-Jun in LPS stimulated macrophages. BMDM extracted from Swiss albino mice were stimulated with LPS (250 ng/ml) for 1 h in presence or absence of TRIM (100 nM). Proteins were harvested and subjected to co-immunoprecipitation with either TIRAP or c-Jun post LPS treatment. (A) Representative immunoblots for co-immunoprecipitation with TIRAP and subsequently probed with antibody for c-Jun to study possible interaction. (B) Densitometric analysis of TIRAP-Jun interaction normalized to actin. (C) Whole cell lysate from LPS stimulated BMDM were subjected to immunoblot to analyze the expression of TIRAP, phospho-c-Jun and total-c-Jun (F). (D) BMDM were fixed and immunostained for TIRAP (Alexa-fluor 594; red) and c-Jun (FITC; green) to determine the cellular interaction of both proteins. Nuclei were counterstained with DAPI and representative images from three independent experiments are shown (bar, 10 µM, 60X resolution). All data are representative of three independent experiments, presented as mean $\pm$ SD,**P<0.02 (Student's t-test).

The results from Jun Co-IP suggested a potential interaction between TIRAP and Jun that was abolished with the presence of NOS1 inhibitor TRIM. We also performed immunoblotting on whole cell lysates without any prior immunoprecipitation with either protein. BMDM stimulated with LPS and TRIM were checked for TIRAP and phospho-c-Jun expression. We observed an increase in TIRAP expression after 1 h LPS trigger, which was diminished upon TRIM treatment and was in accordance with our previous data (Fig 4.2.2 C). However, observation with phospho-c-Jun expression in BMDM showed an increase in expression with LPS stimulation which degraded in presence of TRIM. This was our key finding, where we determined that TIRAP interacts with c-Jun leading to its transactivation and therefore facilitating the AP-1 mediated inflammatory responses.

The above data signify LPS mediated positive interaction between TIRAP and Jun proteins in macrophages. NOS1 inhibition by addition of pharmacological inhibitor TRIM, inhibits this interaction suggesting that LPS induced NOS1 plays a critical role in facilitating the interaction between the two signaling proteins that play a vital role in initiation and progression of inflammatory responses.

To further confirm the cellular interaction between TIRAP and c-Jun in endotoxin-induced environment, confocal analysis was performed. BMDM were stimulated with LPS (250 ng/ml) and TRIM (100 nM). Post-stimulation, cells were fixed and stained for TIRAP (Alexa Fluor 594) and Jun (FITC) and visualized by confocal microscopy (Fig 4.2.2 D). Results from confocal analysis reveal the absence of TIRAP and c-Jun interaction in the control macrophages. However we observed that TIRAP and c-Jun exhibited upregulated nuclear expression and increased interaction in LPS stimulated macrophages. Interestingly, NOS1 inhibition potentially inhibited the TIRAP protein expression and loss of interaction between TIRAP and c-Jun. The data reveals a novel mechanism of NOS1 mediated TIRAP and Jun interaction in LPS activated BMDM.

This study confirms that LPS facilitates the interaction of key signaling molecules TIRAP and c-Jun. c-Jun transactivation via TIRAP provokes the immune machinery in cells and amplifies the inflammatory response through AP-1 transcription factor. The present study uncovers a novel mechanism of TIRAP mediated Jun transactivation in macrophages.

4.2.3 NOS1 mediates nuclear translocation of AP1 subunits in macrophages

Inflammatory signaling activates key transcription factors including activator protein-1 (AP1) that extensively transduces the downstream signals by regulating the proinflammatory cytokine genes in nucleus. AP1 is a dimeric transcription factor that is composed to three subunits Fos, Jun and ATF2, with Jun having the ability to dimerize with either Fos or ATF2 through the leucine zipper domain (Kyriakis, 1999). Active dimers of AP1 translocate into nucleus to induce the transcription of inflammatory genes leading to downstream inflammatory response against infection or tissue damage. Nuclear translocation of AP-1 is a crucial event in mediating its downstream regulation on target genes. Therefore, to understand the regulation of AP1 nuclear translocation by NOS1, BMDM stimulated with LPS in presence or absence of TRIM were subjected to visualization of individual subunit nuclear translocation by confocal microscopic analysis by staining Jun with FITC while Fos and ATF2 with Alexa fluor 594. The results indicate a rapid nuclear translocation of all three subunits including Fos (Fig 4.2.3.1 A), Jun (Fig 4.2.3.1 B) and ATF2 (Fig 4.2.3.1 C) post 1 h of LPS exposure. Interestingly, nuclear translocation of all subunits was hampered upon NOS1 inhibition by TRIM (Fig 4.2.3.1 A, B, and C). The impeded nuclear translocation provides evidence for NOS1 mediated regulation of AP-1 subunits nuclear translocation.

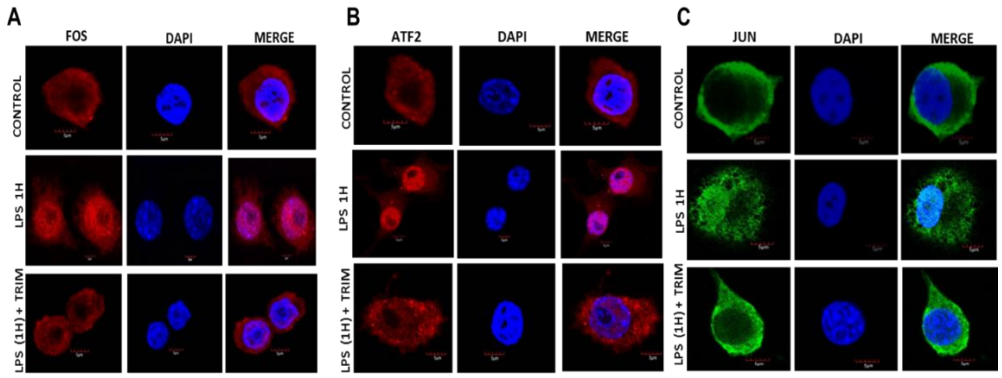


Fig 4.2.3.1. NOS1 promotes nuclear localization of AP1 subunits. BMDM were treated with LPS (250 ng/ml) with or without TRIM (100 nM) for 1h and fixed for confocal analysis. Samples were probed with antibodies for either ATF2 (A), Fos (B) or Jun (C). Cellular localization of each subunit was studied by staining Jun, Fos, and ATF2 with secondary antibodies: Jun tagged with FITC (green); Fos and ATF2 tagged with Alexa Fluor 594 (red). Nucleus was counterstained with DAPI (Blue). Data are representative of three independent experiments.

The results provide an interesting insight into NOS1 mediated regulation of AP1 nuclear translocation that is a crucial determining factor for the downstream inflammatory response. Absence of AP-1 in nucleus will subsequently abrogate the transcription of AP-1 related genes and therefore negatively regulate the inflammatory cascade in response to pathogen

In order to confirm the effect of NOS1 on nuclear translocation of AP1, nuclear and cytoplasmic fractions of bone marrow-derived macrophages were isolated and immunoblotted for Fos, Jun and ATF2 proteins. BMDM were stimulated with LPS and TRIM for 1 h and subjected to immunoblots. The rationale behind the study was to check the precise abundance of nuclear and cytoplasmic proportions of AP-1 subunits during LPS and TRIM treatment.

Immunoblots with cytoplasmic protein fractions, revealed that upon LPS stimulation, there was loss of protein expression of Fos, c-Jun and ATF2 from the cytoplasm compared to the unstimulated macrophages (Fig 4.2.3.2B). In presence of TRIM, the cytoplasmic protein expression of all three subunits recovered in the cytoplasm. This data implied that NOS1 inhibition increased the abundance of AP-1 subunits in the cytoplasm.

At the same time, observations from nuclear protein fractions provide evidence for an increase in the nuclear content of all three AP1 subunits (Fos, c-Jun and ATF2) in macrophages. However, when pretreated with NOS1 inhibitor TRIM, the nuclear protein expression was diminished which could be correlated to an increase in the protein expression in the cytoplasmic counterparts (Fig 4.2.3.2B). Markedly, the whole cell lysate expression of all three subunits remained unaltered with LPS stimulation in presence or absence of TRIM (Fig 4.2.3.2A).

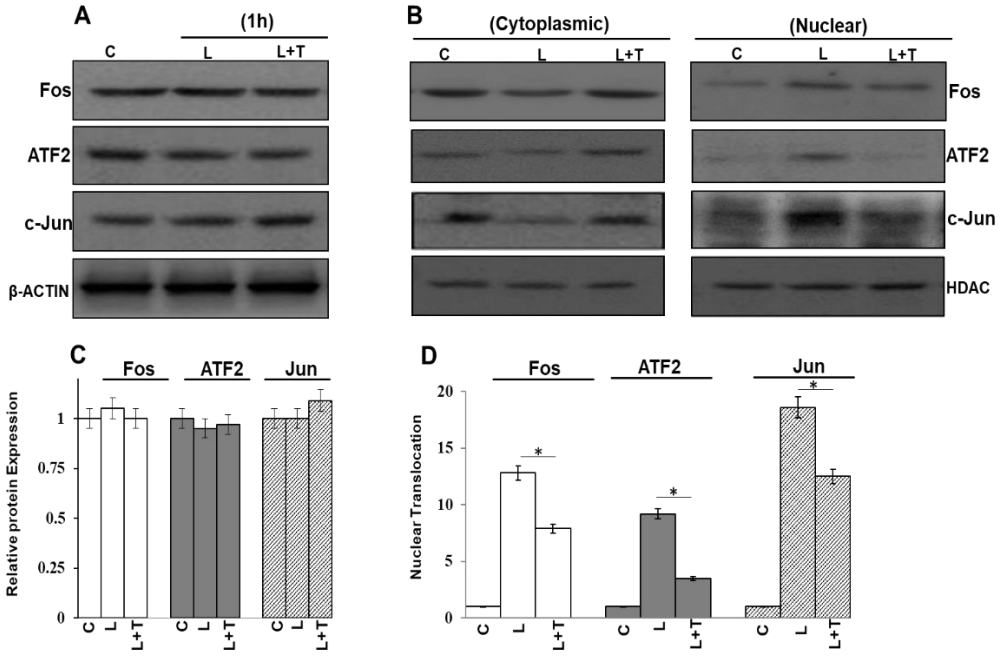


Fig 4.2.3.2. Nuclear to cytoplasmic shuttling of AP1 subunits is negatively regulated by NOS1 inhibition. BMDM extracted from Swiss albino mice were stimulated with LPS (250 ng/ml) for 1 h in presence or absence of TRIM (100 nM). Proteins were harvested and subjected to (A) immunoblot of whole cell lysate expression for Fos, ATF2, and Jun along with β-actin as a loading control (B) Quantification of protein levels from whole cell lysates probed for Fos, ATF2, and Jun from respective immunoblots (C) Nuclear and cytoplasmic fractions of protein extract from LPS and TRIM stimulated BMDM were analyzed by immunoblot using antibody recognizing Fos, Jun, and ATF2 protein. HDAC and β-actin are used as a loading control for nuclear and cytoplasmic fractions respectively (D) Quantification of protein levels from nuclear fractions probed for Fos, Jun, and ATF2 from respective immunoblots (G). All data are representative of three independent experiments, presented as mean ± SD. P values were determined by Student's T-test; *$P < 0.05$.

Quantification of immunoblots from whole cell lysates indicate no change in the expression of AP-1 subunits after LPS and TRIM treatment (Fig 4.2.3.2C). Relative quantification from nuclear and protein fractions demonstrate the changes in nuclear translocation after LPS and TRIM treatment. Results represent an increase in nuclear expression of Fos, ATF2 and c-Jun after LPS stimulation and decrease after TRIM treatment (Fig 4.2.3.2D). Together the results provide significant insight about the regulation of nuclear translocation of AP-1 transcription factor in presence of NOS1.

4.2.4 NOS1 inhibition suppresses AP1-mediated gene expression

Inflammatory signalling in macrophages is a tightly regulated event that prompts the production of proinflammatory cytokines upon infectious challenges. Engagement of Toll-like receptor-4 (TLR-4) with LPS is associated with activation of key transcription factors such as NFkB and AP1. Downstream effect of the active NF-κB and AP1 pathway (Bhatt and Ghosh, 2014; Khalaf et al., 2010), propagate the pathogenic signals into cellular response by rapid production of proinflammatory cytokines including IL12, IL23, TNF-α and IFN-γ (Liu et al., 2009; Yao et al., 1997).

Macrophages play a predominant role in mediating the cellular immunity. To understand the involvement of NOS1 in perpetuating the inflammatory response. We analyzed the expression of inflammatory cytokines in LPS stimulated macrophages. For this study, bone marrow-derived macrophages were stimulated with LPS (250 ng/ml) for 1 h with or without the presence of NOS1 inhibitor TRIM (100 nM). Cells were lysed to extract the mRNA and subjected to quantitative real-time PCR to determine cytokine expression. In accordance with the in-vivo data, the results demonstrated sharp increment in the cytokines IL12, IL23, INF-γ, MIP1-α, M-CSF and TNF-α upon LPS stimulation compared to the control macrophages (Fig. 4.2.4 A-F). Role of NOS1 in transcriptional regulation of the cytokine genes was determined with

the observed decrease in the cytokine expression in presence of NOS1 inhibitor TRIM (Fig 4.2.4 A-F).

Among the inflammatory cytokines, IL12 family of cytokines are comprised of 4 members IL12, IL23, IL27 and IL35 and are key players in regulation of macrophage and T cell response (Gee et al., 2009; Sun et al., 2015). Among the IL12 members, IL12, IL23 and IL27 are secreted by macrophages and activated antigen presenting cells (APC) during antigen presentation to T cells. IL12 and IL23 cytokines link the innate and adaptive immunity by priming naïve CD4 $^{+}$T cells into cytokine-producing Th cells thereby inducing effector immune response (Steinman, 2006; Sun et al., 2015). In addition, these cytokines also activate the proinflammatory pathways for generating appropriate response to infection. Therefore, the role of IL12 and IL23 is essential for immune function of macrophages (Arango Duque and Descoteaux, 2014b; Unanue et al., 1976). Several line of evidences suggest that LPS mediated endotoxin shock effectively induces the expression of IL12 and IL23 in mice (Liu et al., 2009; Saito et al., 2006).

In accordance with previous reports, we observed a significant increase in expression of IL12 and IL23 post 8 h of LPS administration in mice. Nonetheless mice that received TRIM illustrated, a considerable decrease in the cytokine expression in the lung tissue. This suggested that, IL12 and IL23 are implicated in endotoxin shock and are regulated by the NOS1 in macrophages. Our data revealed an increase in expression of IL12 and IL23, which decreased significantly in presence of TRIM (Fig 4.2.4 A and B).

IFN-γ is an integral cytokine of the immune response to viral infections (Hoeksema et al., 2015; Wang et al., 2018). Pioneer studies have reported robust production of IFN-γ in LPS primed macrophages that lead to inflammatory immune response (Fultz et al., 1993). Reportedly, mice susceptible to CLP-induced septic shock facilitates IFN-γ production leading to elevated immune response (Romero et al., 2010). Furthermore, expression of IFN-γ regulated by NF-κB and AP1 in a concomitant manner was determined in LPS stimulated macrophages. We found that upon LPS stimulation, there was an increment in the IFN-γ production by macrophages,

while a significant decrease was observed upon pretreatment with NOS1 inhibitor TRIM (Fig 4.2.4 C).

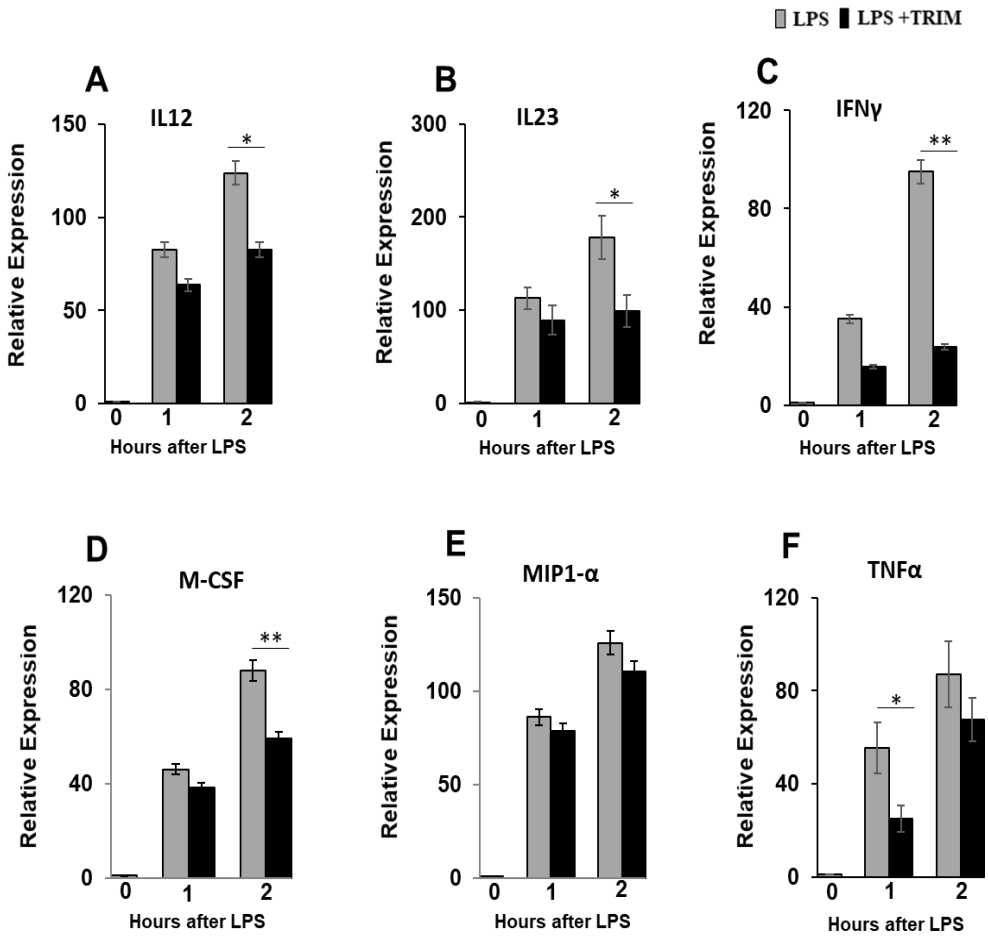


Fig 4.2.4. NOS1 inhibition suppresses AP1-mediated gene expression. Quantitative Real-time PCR analysis of cytokine mRNA expression of (A) IL12, (B) IL23, (C) IFN-γ, (D) M-CSF, (E) MIP1-α and (F) TNFα in LPS- stimulated BMDMs in the presence and absence of NOS1 inhibitor TRIM (100 nM). Data are representative of three independent experiments; all are presented as mean ± SD. P values were determined by Student's T-test; *P<0.05, **P<0.0005.

Macrophages are activated in response to invading pathogen and play a predominant role in inflammatory reactions. Differentiation of macrophages from unprimed macrophages is a critical event that determines the intensity of inflammatory response. Macrophage colony-stimulating factor (M-CSF) is an essential growth factor for macrophages that participates in the inflammatory reactions during endotoxin shock (Fixe and Praloran, 1998; Ogiku et al., 2011; Popova et al., 2011). In our studies, we observed that BMDM treated with LPS produce more M-CSF compared to unstimulated macrophages. Interestingly, M-CSF expression was depreciated in macrophages treated with TRIM prior to LPS (Fig 4.2.4 D).

Macrophage inflammatory protein 1 alpha (MIP1-α) is a low molecular weight chemokine protein that is involved in proinflammatory responses in macrophages. Interestingly, MIP1-α is actively produced by macrophages upon endotoxin induction and is critical for immune response to infection. It is also implicated in other immune functions such as leukocyte chemotaxis and recruitment of inflammatory cells to the site of infection (Cook, 1996, 1988, 1993). Here, we recorded an increase in MIP1-α expression upon LPS stimulation which was observed to decrease in presence of NOS1 inhibitor TRIM (Fig 4.2.4 E)

Among the inflammatory cytokines, tumor necrosis factor alpha (TNF-α) is strongly associated with inflammatory reactions during infection. Evidences have shown elevated expression of TNF-α in acute and chronic inflammatory disorders such as endotoxin shock, rheumatoid arthritis and inflammatory bowel disease (Bradley, 2008; Popa et al., 2007). This led us to determine the effect of LPS induced NOS1 on TNF-α production. We found that TNF-α expression was upregulated with LPS stimulation in BMDM and was down-regulated in presence of TRIM (Fig 4.2.4 F).

The data suggests that LPS stimulated macrophages exhibit dynamic changes in inflammatory cytokine production under the influence NOS1 activated downstream of TLR4 signalling pathway. Studies show that transcriptional control of the cytokines in macrophages are the primary effector response of systemic inflammation. Uncontrolled expression of cytokines might pose

deleterious immune responses that could compromise the organ integrity. Therefore, a precise control of the cytokine production is required for the generation of balanced immune response in host. Our study determined that NOS1 activated by endotoxin shock in macrophages is a detrimental factor in inducing inflammatory cytokines. However, an in-depth understanding is required to ascertain the mechanism of the changes in inflammatory cytokine expression.

4.2.5 NOS1-SOCS1-TIRAP axis regulate AP1 activity during inflammation

In this study, we have determined the molecular mechanism of NOS1 mediated inflammatory responses in macrophages. The hypothesis is based on the activation of TLR4 signalling pathway upon LPS stimulation. This further orchestrates the signals by downstream activation of NOS1, which is the focus of this study. Investigation on the role of NOS1 during such responses provides novel insight to this work.

NOS1 targets SOCS1 degradation by nitrosation and has been reported previously (Baig et al., 2015a). TIRAP, which is one of the targets of SOCS1 for proteasomal degradation is protected in absence of SOCS1. This in turn allows TIRAP to transactivate c-Jun subunit of AP-1. Once activated, c-Jun forms heterodimers with other two subunits of AP-1, Fos and ATF2. The active dimers (Fos-Jun or ATF2-Jun) then translocate into nucleus to bind to their target genes. In absence of TIRAP, the abundance of active AP-1 heterodimers will be low, thereby reducing the inflammatory response to LPS (Fig 4.2.5).

The work from this thesis has focussed on TLR4 signalling pathway in response to endotoxin shock. We have identified the interaction of TIRAP and c-Jun as a novel mechanism that leads to transactivation of c-Jun thereby allowing the AP-1 transcription factor to translocate into nucleus and regulate the activity of its target genes. Therefore, we aimed to proceed with this

mechanism and utilize TIRAP-Jun as a therapeutic complex, abrogation of which might be a potential therapy for inflammatory disorders.

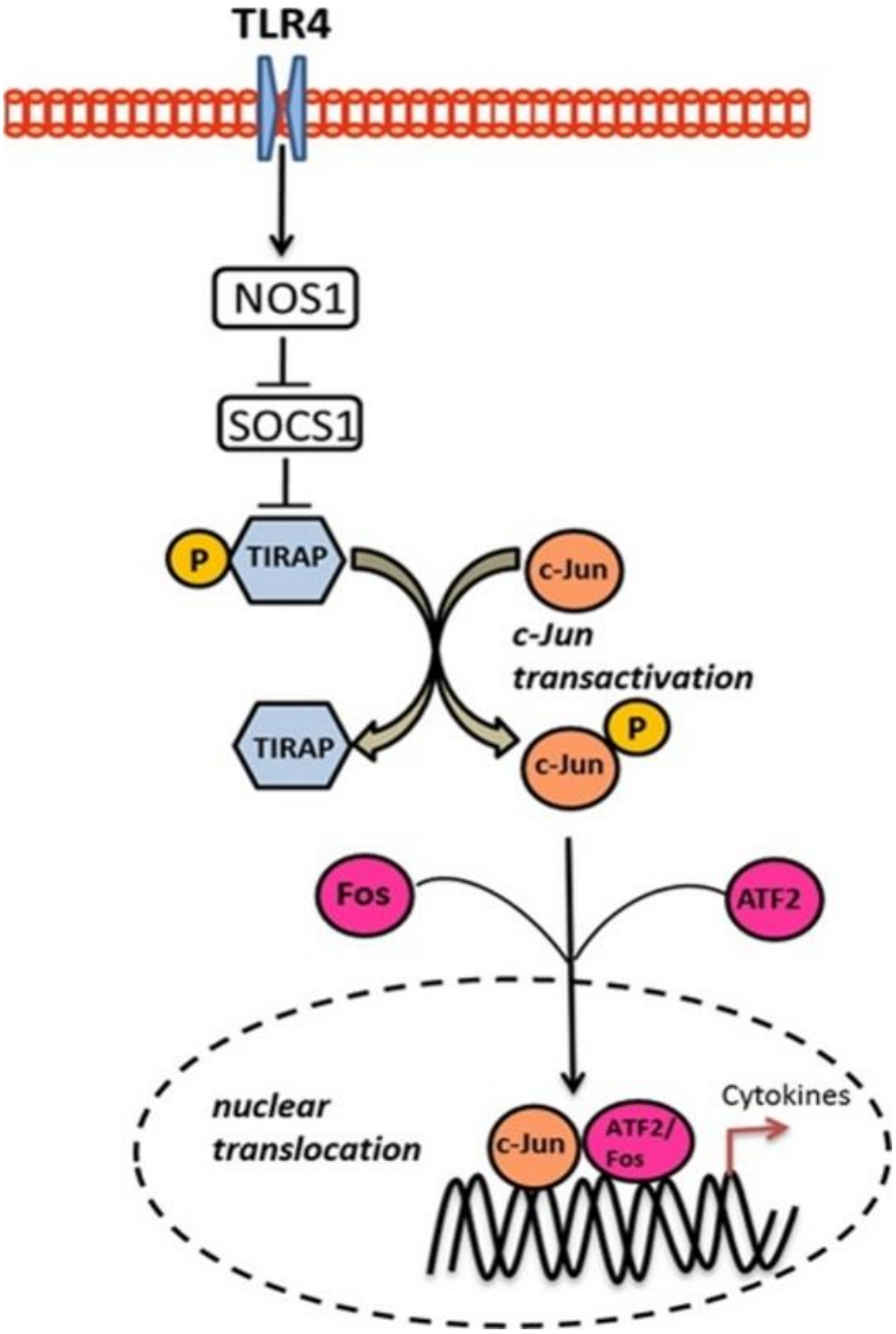


Fig 4.2.5. NOS1-SOCS1-TIRAP axis regulate AP1 activity during inflammatory response

Targeting TIRAP-Jun interaction as a novel therapeutic strategy for sepsis

4.3.1 *Molecular dynamics* (MD) *simulation* of TIRAP-c-Jun complex

The 3D structure of c-Jun (Residues 201 to 256) is not available in the PDB. Hence, we modeled the structure using Modbase program(Pieper et al., 2014). The template structure of pre-mRNA-processing-splicing factor 8 with the highest sequence identity of about 40% with the query sequence was selected for homology modeling by the server. The resulting top conformation was retained for the docking run. Molecular docking between c-Jun and TIRAP has been done in order to understand whether and where the two interact. Hence, a blind docking run was initiated using ZDOCK server (Pierce et al., 2014), where we omitted the residue specification in order to have a bias-free relative conformation of the two partners in the complex. The top conformation of the docked complex was used for further analysis (Fig 4.3.1.1 A and B). Most of the times, the docking programs accurately predict the docked conformations of the binding partners; however, this is not true every time. Hence, we ran a molecular dynamics simulation of the binary complex in order to understand and validate the docking results.

The c-Jun-TIRAP complex has been used as a starting point for the MD run. The MD simulations were done for a longer time in order to fully replicate the original conformations of the complex. The backbone RMSD profile of TIRAP in complex with c-Jun was analysed (Fig 4.3.1.1 C and D). The TIRAP and c-Jun complex were equilibrated well after 50ns production MD run till the end of simulation with the RMSD values ranging from 1.00 to 1.15nm. This clearly shows that the complex is consistently stable as evidenced by the lower RMSD difference (1.5Å) between the initial and final conformations (Fig 4.3.1.1 C and D).

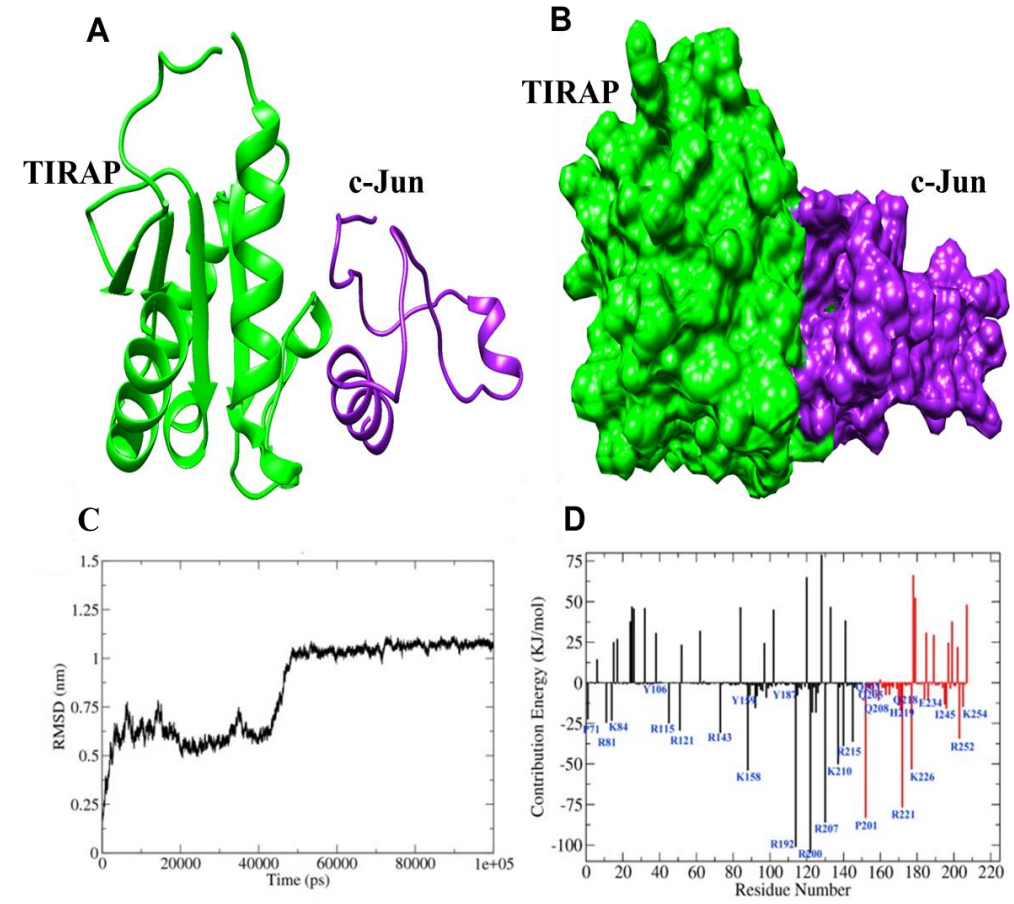
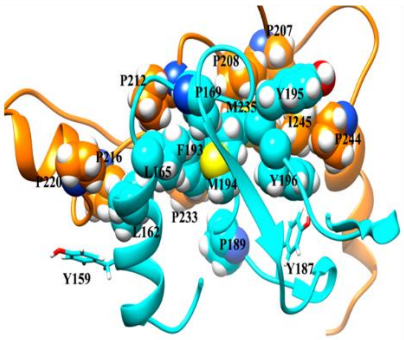


Fig 4.3.1.1. Docked complex of TIRAP and c-Jun retrieved from ZDOCK server. (A) The green ribbons indicate TIRAP, while the purple ribbons indicate c-Jun structure. (B) Surface view structure of TIRAP and c-Jun representing the proximity of two proteins in 3D space. (C) The stability and residual energy analysis from molecular dynamics simulation. The 100ns backbone RMSD profile of TIRAP and c-Jun. D) The residual contribution energy of TIRAP (Black) and c-Jun (Red) showing the complex stability nature.

Further, the residual contribution energy of the TIRAP and c-Jun complex was analysed using MMPBSA calculation. The results show that the residues P71, R81, K84, R115, Y106, R121, R143, K158, Y159, Y187, R192, R200, R207, K210 and R215 of TIRAP and P201 Q203, Q205, Q208, H219, Q218, R221, K226, I245, R252 and K254 of c-Jun have the lowest binding energy and hence much contribute for the complex stability (Fig 4.3.1.2 A and B). The lowest energy conformer was retrieved from the trajectory and its residual

interaction analysis shows that residues; R192, Q163, E193, M194, E172, L165, Y159, R184 and E190 of TIRAP form stable hydrogen-bond interactions with many c-Jun residues including E234, Q218, M235, Q203, H219, Q223, Q205, E251, Q214, E248 etc (Fig 4.3.1.2 A and B). In addition, an array of hydrophobic interactions was also formed between TIRAP residues L162, P169, P189, F193, M194, Y195, Y196 and c-Jun residues; P216, P220, P212, P233, P208, P207, P244, L244, I245, M235 (Fig 4.3.1.2 A and B). All these hydrogen bonds and hydrophobic interactions collectively contribute to the stability of TIRAP-c-Jun complex. Based on RMSD and energy of the complex along with consistent interacting residues, TIRAP- c-Jun complex structure seems to be quite stable in a time-dependent manner and hence validates our docking model of the TIRAP-c-Jun complex.

A



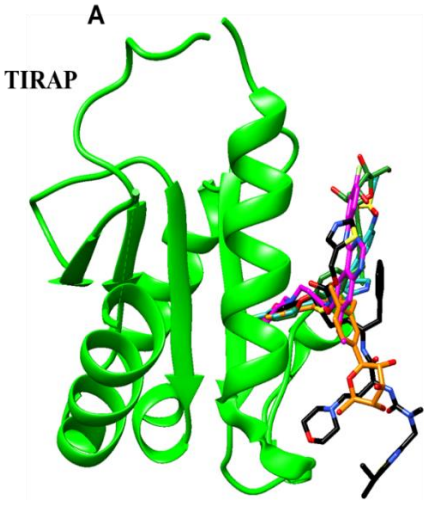
B

Type of interaction	Residues in TIRAP	Residues in c-Jun
Lowest binding energy leading to complex stability	P71, R81, K84, R115, Y106, R121, R143, K158, Y159, Y187, R192, R200, R207, K210, R215	P201 Q203, Q205, Q208, H219, Q218, R221, K226, I245, R252 and K254
Stable hydrogen bond interaction	R192, Q163, E193, M194, E172, L165, Y159, R184, E190	E234, Q218, M235, Q203, H219, Q223, Q205, E251, Q214, E248
Hydrophobic interaction	L162, P169, P189, F193, M194, Y195, Y196	P216, P220, P212, P233, P208, P207, P244, L244, I245, M235

Fig 4.3.1.2. Residual interactions between TIRAP-Jun interface retrieved from ZDOCK server. The residual interaction of TIRAP (color: Cyan) in complex with c-Jun (color: Orange). Here, the structural stability obtained through series of hydrophobic interaction and represented in sphere model whereas the phosphorylation site represented as stick model with hetero atom type. (B) Table summarizing the interacting residues between TIRAP and c-Jun.

4.3.2 TIRAP structure docked by the DrugBank compounds

After validation of the complex stability by MD, we initiated the virtual screen against TIRAP. The docking-based virtual screen was carried out using the three-dimensional TIRAP structure. The LibDock program was used as mentioned above. However, this time we were careful to select the active site which encompasses the c-Jun binding site. This is because we wanted to target that area on TIRAP structure responsible for c-Jun binding. This would, in turn, enable us to retrieve compounds binding at the TIRAP site meant for c-Jun binding, thereby inhibiting the binary interaction. The docking run resulted in a total of 9557 confirmations from 1877 drugs deposited in the FDA database. The top conformations of screened drugs were individually analysed based on their docking energies and binding modes. The docked conformations of these 5 compounds in the TIRAP binding site (meant to bind c-Jun) (Fig 4.3.2 A). As observed in the figure, all the selected drugs bind TIRAP in an overlapping fashion or in close vicinity, indicating consistent binding at the TIRAP structure. Different scoring functions of drugs are summarized (Fig 4.3.2 B).



B

Drug	Drug Bank ID	Lig Score1-Drieding	Lig Score2-Drieding	Lib Dock Score
Vemurafenib	DB08881	5.24	4.73	68.35
Gefitinib	DB00317	5.03	4.93	108.25
Cobicistat	DB09065	5.02	6.6	137.53
Empaglifloin	DB09038	5.01	4.88	72.49
Canagliflozin	DB08907	4.98	4.45	63.38

Fig 4.3.2. TIRAP structure docked by the Drug Bank compounds. (A) The top 5 docked compounds (sticks in 5 different colors) were selected after analyzing their docked scores as well as conformations in the TIRAP structure (green ribbons). (B) Table summarizing scoring functions of top five drugs.

4.3.3 Gefitinib interrupts the interaction between TIRAP and c-Jun in LPS stimulated macrophages

A wide variety of therapeutic drugs act as potential therapy for chronic inflammatory disorders. In this study we focussed on TIRAP and c-Jun complex as a targets for suppressing downstream inflammatory signalling. Using computational screening, five potential drugs (Vemurafenib, Gefitinib, Canagliflozin, Empagliflozin and Cobicistat) were identified with high dock score and inhibited the interface between TIRAP and Jun. To validate the in-vitro efficacy of the drugs, co-immunoprecipitation was performed using TIRAP and c-Jun antibody as described previously. Briefly, BMDM were stimulated either with LPS or LPS along with 1 h prior treatment with the screened drugs. LPS and drug treated samples were co-immunoprecipitated with TIRAP and pulled down by agarose beads. Immune complex consisting of TIRAP antibody was then immunoblotted with c-Jun to check for the modulating effects on TIRAP-Jun interaction. Co-IP data illustrated that out of five drugs, Gefitinib possessed maximum inhibitory efficacy to disrupt the interaction between TIRAP and c-Jun (Fig 4.3.3 A). Relative quantification of TIRAP and Jun co-immunoprecipitation data demonstrate that in presence of Gefitinib, TIRAP immunoprecipitates with Jun with lower efficiency (Fig 4.3.3 B). While other drugs did not exhibit a profound effect on the interaction of the two proteins.

We therefore aimed to proceed with Gefitinib for our further investigations. In order to validate the in-vitro efficacy of Gefitinib, bone marrow derived macrophages were induced by LPS and Gefitinib and subsequently stained with TIRAP and Jun antibody and counter stained with fluorescent tagged secondary antibodies (TIRAP-Alexa-fluor 594 and c-Jun-FITC). Confocal microscopy was used to determine the interaction between the two proteins. The data reveals that unstimulated macrophages do not favour the interaction of the two proteins. However, triggering the macrophages with LPS induces interaction between the two proteins. Strikingly, the confocal analysis from macrophages treated with Gefitinib prior to LPS exhibited suppressed TIRAP-Jun interaction (Fig 4.3.3 C).

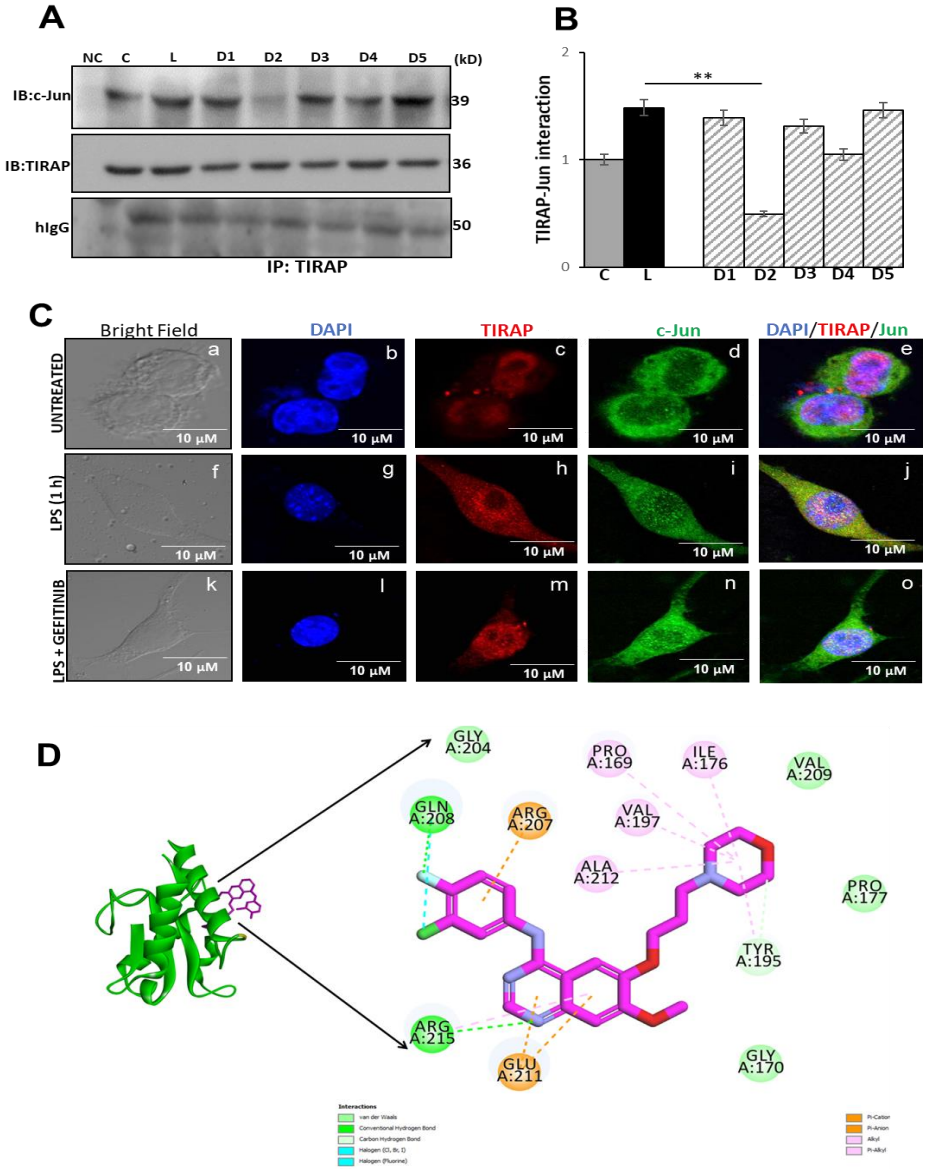


Fig 4.3.3. Gefitinib interrupts the interaction between TIRAP and c-Jun in LPS stimulated macrophages. BMDM extracted from swiss albino mice were stimulated with LPS (250 ng/ml) for 1 h in presence of five drugs (D1: Vemurafenib, D2: Gefitinib, D3: Cobicistat, D4: Empagliflozin and D5: Canagliflozin). Proteins were harvested and subjected to (A) co-immunoprecipitation with TIRAP post LPS treatment. (B) Densitometric analysis for TIRAP and c-Jun interaction as in (A), normalized to actin. (C) BMDM stimulated with LPS (250 ng/ml) in presence of Gefitinib (1 µM) were stained with TIRAP (Alexa-fluor 594; red) and c-Jun (FITC; green) to determine the cellular interaction of both proteins. Nuclei were counterstained with DAPI and representative images from three independent experiments are shown (bar, 10 µM, 60X resolution). (D) The overall structure of TIRAP (green ribbons) bound to Gefitinib (magenta) at the c-Jun binding site (Left). The zoomed 2-Dimentional view of Gefitinib (magenta atom color)-TIRAP (colored spheres) atomic interactions. The detailed information of color-coding of the interactions is given at the bottom of the figure.

Gefitinib was shown to have an inhibitory effect on TIRAP-c-Jun binding as indicated by the pull-down assays. Hence, we further zoomed the 2-D binding interactions between Gefitinib and TIRAP (Fig 4.3.3 D). It could be observed that Gefitinib binds with TIRAP with an array of non-covalent interactions including hydrogen-bonds, hydrophobic, wander Waals, halogen bonds and multiple pi-interactions. We compared the binding modes of Gefitinib and c-Jun with TIRAP and found many analogies in their mode of interaction. Gefitinib is expected to bind TIRAP at the c-Jun binding site and hence was found to be making several similar interactions with TIRAP as that those made by c-Jun. For example, the interaction with TIRAP involving its residues R215, Q208, R207, K210 etc seem to be conserved among c-Jun and Gefintinib (Fig 4.3.3 D). The strong in silico and in-vitro binding could be attributed to these non-covalent bonds present between Gefitinib and TIRAP which are eventually responsible for disrupting the TIRAP- c-Jun interaction.

4.3.4 Gefitinib increases survivability in mice induced with endotoxin shock

In order to validate the anti-inflammatory property of Gefitinib, three groups of mice were used. The control group without any stimulation, the second group that received LPS injection and third group that received Gefitinib prior to LPS. We recorded the activity of mice after 8 h of LPS and Gefitinib treatment. The control group of mice were active and healthy without any signs of infection. Expectedly, the endotoxin-induced mice demonstrated morbid condition and did not show any physical activity and were near mortality. Surprisingly, the mice that received Gefitinib prior to LPS retained their health and did not succumb to endotoxin infection (Fig 4.3.4 A). This was the first evidence where we observed that gefitinib exerted anti-inflammatory property in mice subjected to lethal endotoxin shock.

In the next experiment, mice subjected to endotoxin shock by intraperitoneal lethal LPS dose (30 mg/kg), were examined for survival when injected with Gefitinib. All the mice in control group (n=7 per group) that were injected the solvent solution (DMSO+PBS) survived. However, mice belonging to the

experimental group, that received only LPS showed 62% mortality mice after 24 h period, the survival percentage was recorded 15% at the end of the 72 h period of septic shock (Fig 4.3.4 B). In contrast, the group of mice that were injected with Gefitinib along with LPS, illustrated higher survival ratio of 76% within 24 h which decreased to 76% to 36 h. However, the survival was stable to 69% as recorded up to 72 h period (Fig 4.3.4 B). The survival graph demonstrates the protective efficacy of Gefitinib on mice subjected to LPS shock. This highlights the potential anti-inflammatory properties of Gefitinib in septic mice model.

To study the effect of Gefitinib on the lung morphology of LPS injected mice, lung tissues were harvested and sections were HE stained to observe the changes in the healthy tissue. Light microscope was used to capture the morphology under 20X magnification. The control group of mice (DMSO+PBS) depicted preserved epithelium lining of airways and intact pulmonary capillaries and alveolar septa. In contrast, the lungs from septic mice displayed distorted epithelium lining due to infiltration of neutrophils and lymphocytes. Alveolar septum of infected mice persisted with mucus and thickened airway lining (Fig 4.3.4 C). In contrast, the lung tissue from Gefitinib pre-treated mice showed recovery to the damage caused by septic shock as depicted by control group mice. Recovery in Gefitinib injected mice were marked by intact epithelium lining and normal alveolar septum (Fig 4.3.4 C). Together, these observations indicate the protective efficacy of Gefitinib on LPS infected mice providing novel mechanism towards therapeutic application.

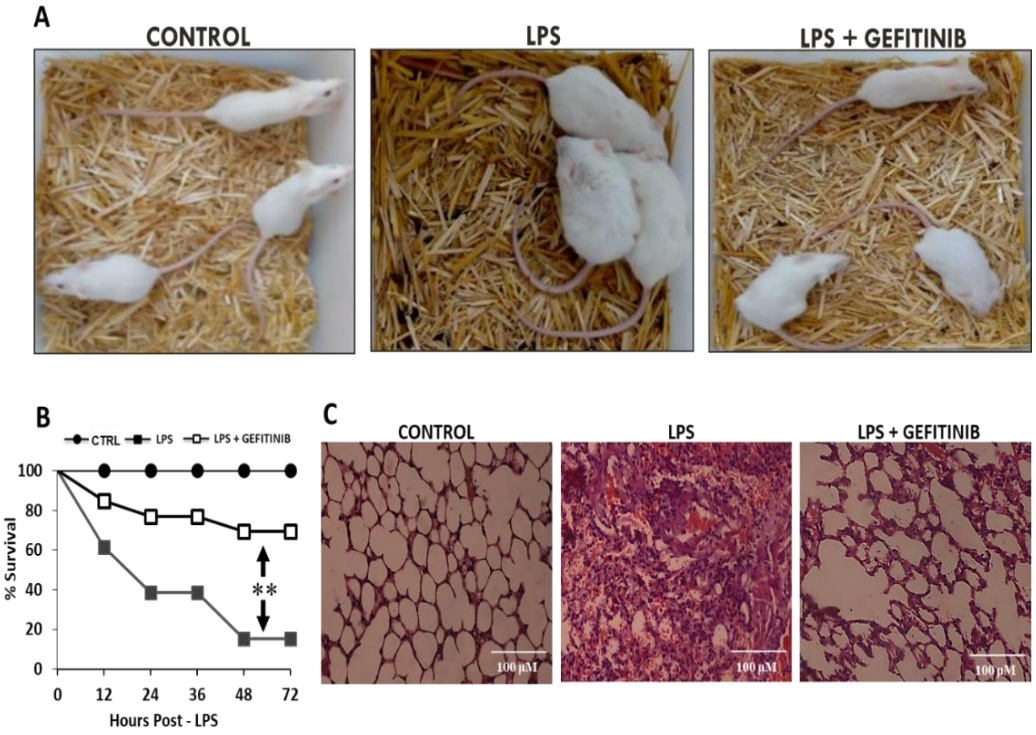


***Fig 4.3.4 Gefitinib protects mice from LPS induced injury*.** Swiss albino mice were challenged with LPS (30 mg/kg) alone or with Gefitinib (40 mg/kg), 1 h prior to LPS. (A) Mice activity was recorded 8 h post LPS and Gefitinib injection. (B) Survival of control mice, LPS injected mice and LPS+Gefitinib injected mice compiled from at least 3 independent experiments using 10 mice per group. Survival was monitored for the indicated time points. (B) Lungs were harvested from each group of mice 8 h post i.p. LPS and Gefitinib injections. Lung sections were prepared and stained with H&E to visualize lung morphology using light microscope (bar, 100 µM).

4.3.5 Gefitinib impedes TIRAP-Jun interaction in endotoxin-induced mice

In order to further validate the anti-inflammatory properties of Gefitinib, we next assessed the presence of TIRAP and Jun interaction in endotoxin-induced mice. Mice were administered with LPS (30 mg/kg) and Gefitinib (40 mg/kg) 1 h prior to LPS. After 8 h, lung tissues from all groups of mice were extracted and sections were prepared to stain for TIRAP and Jun. Similar to the in-vitro data, lung sections derived from the wild-type mice revealed a lack of TIRAP and Jun interaction. However, lung tissues from LPS injected mice

demonstrated positive interaction between TIRAP and Jun. Another group of mice pretreated with Gefitinib prior to LPS showed abolished TIRAP and c-Jun interaction (Fig 4.3.5 A). Together the data suggests that Gefitinib can impede TIRAP-Jun interaction and is therefore proposed as anti-inflammatory drug.

Anti-inflammatory drugs target the suppression of key inflammatory cytokines such as IL12, IL23, INF-γ, MIP1-α, M-CSF and TNF-α, leading to end point modulation of inflammatory reaction. Our previous data on the Gefitinib effect on septic mice, led us to identify the downstream expression of AP1 induced inflammatory cytokines in LPS injected septic mice in presence and absence of Gefitinib. Briefly, group of mice were injected with LPS alone and another group was pre-treated with Gefitinib and then induced with endotoxin shock to check the effect on cytokine gene expression. In accordance with previously described LPS induced cytokine expression, we observed an increase in the cytokines in lung tissue derived from LPS injected mice compared to the control mice. Remarkably, in mice that received Gefitinib prior to LPS injection there was a significant reduction in the cytokine expression (Fig 4.3.5 B-G).

AP1 regulated cytokines IL12 and IL23 were highly expressed in LPS induced mice, however their expression depreciated significantly upon Gefitinib treatment (Fig 4.3.5 B and C). To our observation, we found an elevated expression of IFN-γ in LPS triggered mice compared to control. In contrast, mice administered with gefitinib 1 h prior to LPS showed decreased expression of IFN-γ suggesting that NOS1 regulates the inflammatory cytokine production in mice lungs (Fig 4.3.5 D). In continuation to our study on inflammatory mediators, we investigated the production of MIP1-α in mice challenged with endotoxin shock. We observed a remarkable increase in MIP1-α upon stimulation with LPS in mice (Fig 4.3.5 E).

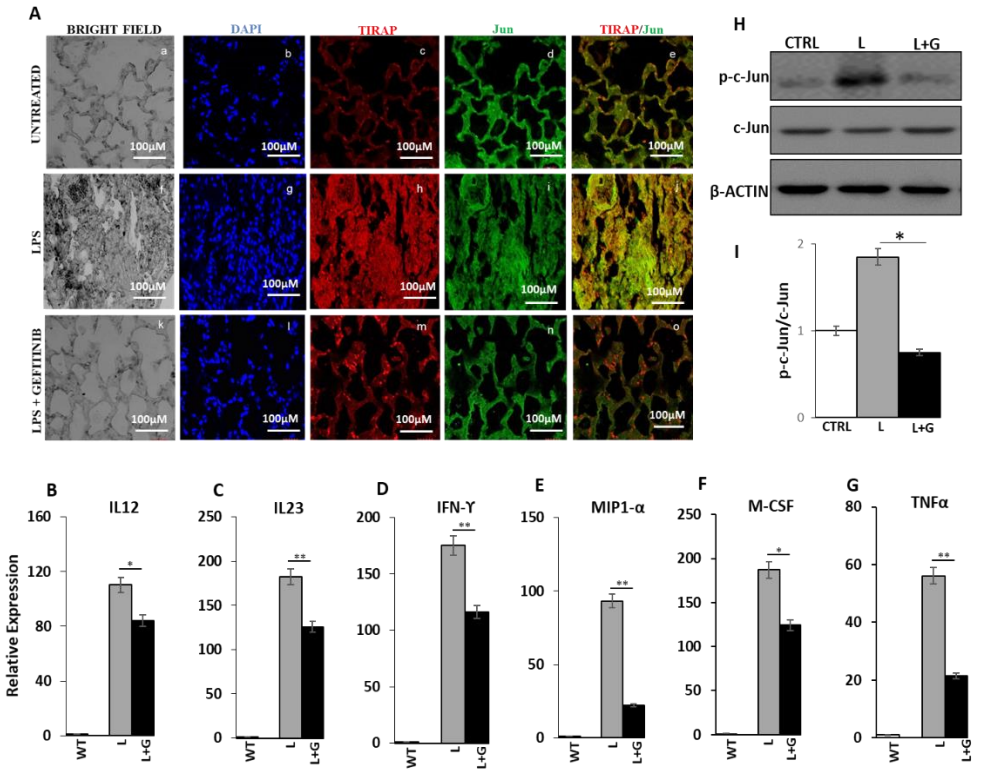


Fig 4.3.5. Gefitinib inhibits TIRAP-Jun interaction and AP1 mediated cytokine gene expression. (A) Swiss albino mice were injected with LPS (30 mg/kg) alone or with Gefitinib (40 mg/kg), 1 h prior to LPS. 8 h post LPS and Gefitinib injection, lungs were harvested, and sections were immunostained with TIRAP (Alexa-fluor 594; red) and c-Jun (FITC; green) to determine the tissue level interaction of both proteins. Nuclei were counterstained with DAPI and representative images from three independent experiments are shown (bar, 100 μM, 20X resolution). Representative q-RT PCR for cytokine mRNA expression from lung tissues for (C) IL-6, (D) IL1-β (E) TNF-α, (F) IL-12 and (G) IL-23. (H) Lung tissues from all groups of mice were subjected to immunoblot for phospho-jun expression with actin as the loading control. (I) Densitometric analysis of phospho-Jun in tissue lysates normalized to actin as represented in (H). All data are representative of three independent experiments, presented as mean ± SD, Significance in all experiments was determined using Student's t-test **P<0.002.

Furthermore, mice that received TRIM prior to LPS showed comparably decreased expression of MIP1-α. In accordance to previous reports, we observed strong induction of MIP1-α in septic mice compared to WT mice. On the contrary, we perceived significant reduction in MIP1-α in TRIM administered mice. The data implicated that NOS1 modulates the expression of MIP1-α in mice triggered with endotoxin shock (Fig 4.3.5 E)

Next, we examined the effect of endotoxin shock on the expression of M-CSF in mice lungs and observed that LPS promotes expression of M-CSF up to 8 h. However, pre-treatment with TRIM had profound inhibitory effect on expression of M-CSF. Thereby suggesting that NOS1 regulates M-CSF and modulates macrophage activation during inflammation (Fig 4.3.5 F). The data strongly implies that Gefitinib administered mice failed to produce high levels of inflammatory cytokines and therefore protected them from LPS induced septic lung injury. We found a significant increment in the TNF-α production in LPS fed mice compared to the WT mice. However, TRIM administration declined TNF-α expression to a remarkable extent. Our study highlights the critical regulation of inflammatory mediators by LPS induced NOS1 in septic mice (Fig. 4.3.5 G).

Primarily our study focussed on AP1 induced inflammatory cytokines that advances the inflammatory response. We therefore examined the effect of Gefitinib on the expression of activated AP1 subunit phospho-c-Jun in different groups of mice. In contrast to the wild-type mice, LPS injected mice exhibited elevated expression of phospho-c-Jun illustrating the role of AP1 during LPS induced inflammatory response. Notably, the expression of phospho-c-Jun was significantly ceased in mice pre-injected with Gefitinib prior to LPS (Fig 4.3.5 H) Quantification of immunoblots represents the Gefitinib induced suppression of phospho-c-Jun expression (Fig 4.3.5 I). The results provide a novel mechanism of Gefitinib induced anti-inflammatory response in mice. Gefitinib is therefore proposed as a potential anti-inflammatory drug that targets TIRAP and Jun interaction thereby impeding the cytokines to suppress the inflammatory outburst.

4.3.6 Gefitinib suppresses AP-1 mediated cytokine gene expression in BMDM

In support of the in-vivo studies, we also analyzed the ability of gefitinib to suppress the AP-1 related cytokine gene expression. BMDM stimulated with LPS and Gefitinib were harvested post-treatment to check for the expression of

cytokines through q-RT PCR. Similar to the in-vivo data, we observed high expression of inflammatory cytokines IL12, IL23, INF-γ, MIP1-α, M-CSF and TNF-α, 8 h after intraperitoneal LPS treatment compared to the control macrophages. However, there was significant decrease in the cytokine expression recorded in presence of Gefitinib (Fig 4.3.6 A-F).

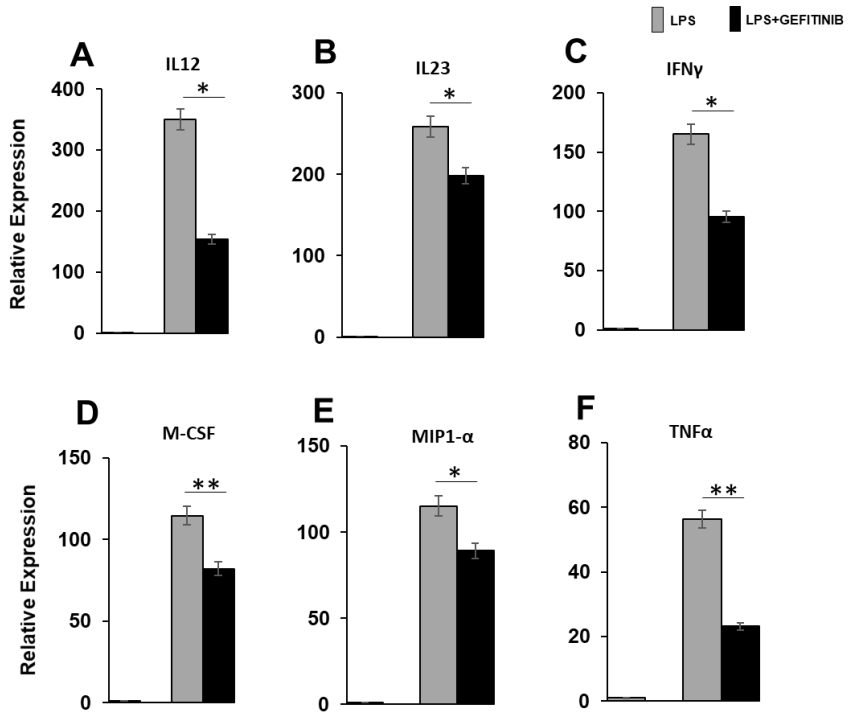


Fig 4.3.6. Gefitinib suppresses AP-1 mediated cytokine gene expression in BMDM. Quantitative Real-time PCR analysis of cytokine mRNA expression of (A) IL12, (B) IL23, (C) IFN-γ, (D) M-CSF, (E) MIP1-α and (F) TNFα in LPS- stimulated BMDMs in the presence and absence of Gefitinib (1 μM). Data are representative of three independent experiments; all are presented as mean ± SD. P values were determined by Student's T-test; *P<0.05, **P<0.0005

The data illustrates the critical involvement of gefitinib in suppressing the AP-1 related cytokine gene expression. In conclusion, the decreased production of IL12, IL23, TNF-α, IFN-γ genes in presence of gefitinib is proposed as a anti-inflammatory therapy in AP-1 mediated inflammatory responses.

Results *Chapter 4*

TLR4-NOS1-AP1 signaling axis regulates macrophage polarization

4.4.1 NOS1 inhibition promotes dimerization of Jun– ATF2 dimer over Fos–Jun dimer in LPS-stimulated macrophages

AP1 protein composed of subunits Fos, Jun and ATF2 belongs to basic leucine zipper family of DNA binding proteins having varying affinities for binding to DNA. A diverse range of functions has been attributed to AP1 complexes composed of distinct dimers assembled in response to various physiological and pathological stimuli. The assembly of AP1 subunits determines their promoter-binding specificity, stability and localization, governing the transcriptional activity of the proinflammatory gene. In response to bacterial infections, macrophages augment the inflammatory signalling cascade by promoting NO production. Since NO derived from NOS1 contributes to early-stage inflammation in macrophages, we analysed the effect of NOS1-derived NO in regulating the activity of AP1 transcriptional factor in the presence and absence of the NOS1 inhibitor. Jun protein is the common subunit which forms a dimer with either Fos or ATF2 that possesses relatively high or low binding efficiency, respectively, for the promoter of target genes.

To examine the effect of NOS1-derived NO on the subsequent dimerization of AP1 subunits, RAW 264.2.3 macrophages were treated with LPS with or without NOS1 inhibitor TRIM for up to 2 h. Cells were then lysed for protein extraction and subjected to co-immunoprecipitation (Co-IP) for each of the subunits and further immunoblotted with the common dimer subunit Jun. The results demonstrate an overall increase in both dimers under LPS stimulation in macrophages. However, the presence of NOS1 inhibitor downregulated the Fos-Jun dimer while sustaining the expression of Jun–ATF2 dimer in THP1 cells macrophages (Fig 4.4.1.1 A) and RAW 264.2.3 (Fig 4.4.1.1 D). The

results suggest a negative regulation of NOS1 on the dimerization of Fos-Jun dimers that have strong affinity for binding to inflammatory genes, while augmenting the Jun–ATF2 dimer that is comparatively less efficient in activating the transcription of inflammatory genes IL-12 and IL-23. Relative quantification of Fos-Jun and Jun–ATF2 dimers in THP1 cells (Fig 4.4.1.1 B and C) and RAW 264.2.3 macrophages (Fig 4.4.1.1 E and F) represents an overall decrease in the Fos-Jun dimer, but an increase in the Jun–ATF2 dimer. Based on the co-immunoprecipitation data, we propose a novel regulatory mechanism for AP1 dimerization that is involved in the inflammatory response to bacterial load in macrophages. The differential regulation of Fos-Jun and Jun–ATF2 dimers by NOS1 provides interesting insight into the therapeutic application of the dimers in targeting chronic inflammatory diseases.

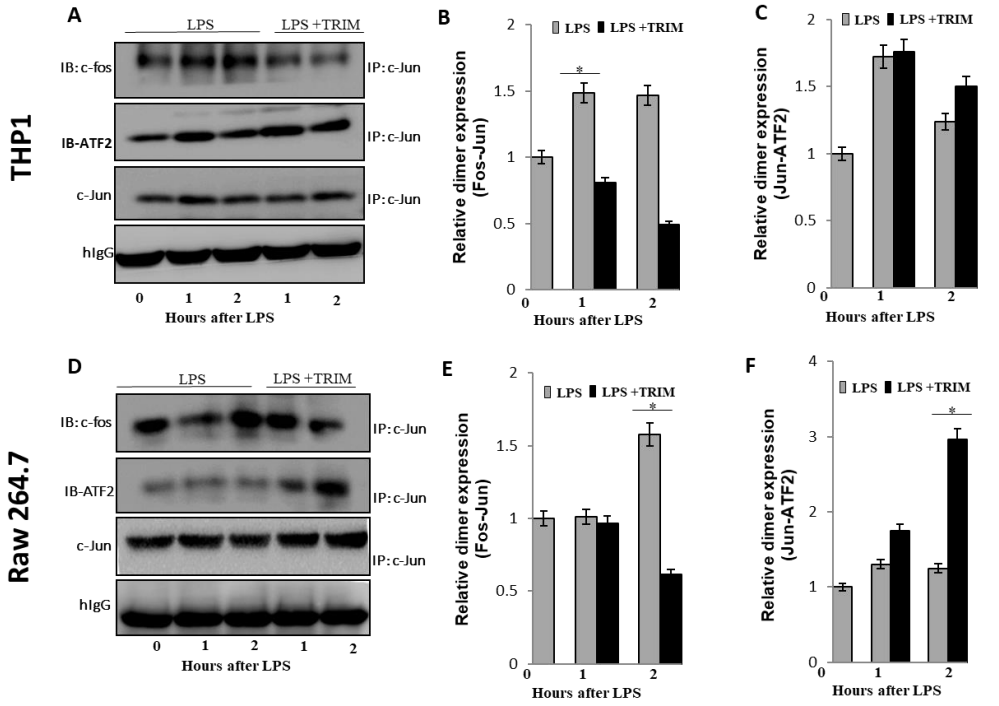


Fig 4.4.1.1 NOS1 inhibition promotes dimerization of Jun–ATF2 dimer over Fos–Jun dimer in LPS-stimulated macrophages. (A) THP-1 and (D) Raw 264.2.3 macrophages were stimulated with LPS (250 ng/ml) with or without TRIM (100 nM) for indicated time points. Lysed cells were immunoprecipitated with c-Jun antibody and pulled down with protein agarose beads to analyze for dimer association and immunoblotted with c-Fos and ATF2 antibody in THP1 cells and Raw 264.2.3 macrophages respectively. Quantitative analysis of relative dimers Fos–Jun and Jun–ATF2 in THP-1 (B and C) and Raw 264.2.3 (E and F) represent the effect of NOS1-derived NO on AP1 dimerization. All data are representative of three independent experiments, P values were determined by Student's T-test; *P<0.05.

At normal cellular conditions, the binding between Jun-Fos and Jun–ATF2 is well known (Chida et al., 1999b). However, we found that the interaction between Jun and ATF2 becomes less profound after a conformational change due to nitrosylation of a cysteine residue at position 351 in the bZIP domain of ATF2. ATF2, Fos and Jun have a single cysteine residue in their bZIP domains. The cysteine residues in both Jun– ATF2 and Jun-Fos complexes face off each other. This suggests their role in a disulfide bond formation possibly strengthening the structural stability of the respective complexes. We investigated whether nitrosylation of these cysteine residues might lead to structural changes affecting binding affinity of the two complexes. We therefore performed molecular modeling studies, wherein the Cys351 residue of ATF2 was manually nitrosylated with a molecule of nitric oxide. We individually truncated ATF2 as well as Jun chains from the rest of the crystal structure to be next used for energy minimization and docking analysis.

To confirm the docking efficiency of the program, we performed docking of the original Jun–Fos complex. The resulting docked complex reproduced the same conformation as in the crystal structure. A good overlay between the crystal and docked structures of the Jun-Fos complex is observed (Fig 4.4.1.2 A). After the docking of nitrosylated ATF2 with Jun, the resulting complex was compared with the original Jun–ATF2 complex. It was found that the complex undergoes substantial structural arrangements with respect to the ATF2 monomer after cysteine nitrosylation, leading to reduced binding between the two (Fig 4.4.1.2 B). For instance, the two cysteine residues which were close together in the unnitrosylated complex became distant in the nitrosylated complex. Also, the overall structure of the binary complex seems to be perturbed due to this nitrosylation event. Similarly, nitrosylation of the Fos bZIP domain Cys154 was done to find out whether a similar nitrosylation event occurs sin the Jun-Fos dimer too. Hence, we performed the same procedure for the molecular modelling of the Jun-Fos complex as done above for Jun–ATF2. There is almost no change in the conformation of the nitrosylated cysteine in Fos, suggesting that it has no role in regulating the

affinity between the two partners (Fig 4.4.1.2 B). Hence, our results confirm a possible role of cysteine nitrosylation in Jun–ATF2 binding.

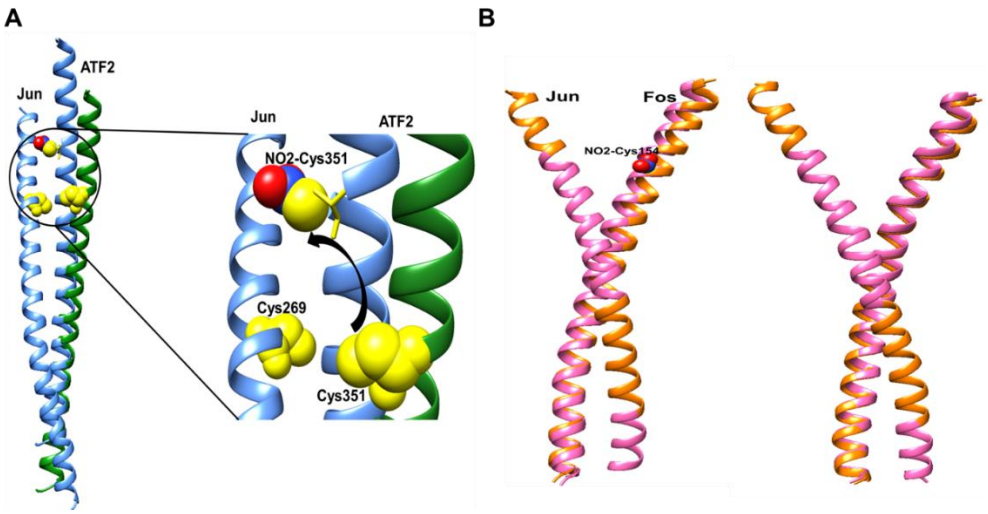


Fig 4.4.1.2. Superimposed structures of crystal and docked structure of the Jun-Fos complex. (A) The overlay of the docked complex (pink ribbons) on the crystal structure (orange ribbons) with negligible root mean square deviation (rmsd) clearly validates the docking protocol and reproducibility of the docking program for Jun–ATF2 docking. (B) Superimposition of docked complexes of unmodified and nitrosylated Jun–ATF2. The left panel shows the overall difference between the structures of unmodified Jun–ATF2 complex (dark green ribbons) and nitrosylated Jun–ATF2 (light blue ribbons). The right panel is the zoomed image of a sub-domain of the superimposed complexes, showing drastic shift in the position of nitrosylated Cys351 residue from its actual unmodified position where it was facing Cys269 from Jun. Jun and ATF2 have been labeled. The nitro group of modified Cys351 is shown as atom colored spheres. The overlay of the unmodified complex (orange ribbons) on the nitrosylated Jun–Fos structure (pink ribbons) clearly indicates the negligible difference between the position of the respective monomers before and after Fos Cys154 nitrosylation, confirming no structural change in the complex. The nitro group of modified Cys154 is shown as atom coloured spheres.

4.4.2 NOS1 inhibition promotes expression of anti-inflammatory cytokines

To test whether NOS1 is a determining factor for altering the macrophage transitions, we further examined the effects of pharmacological NOS1 inhibitor (TRIM) on the cytokine production. We investigated the expression of anti-inflammatory cytokine levels of MRC1 (mannose receptor C, type 1), KLF4 (Kruppel-like factor 4), FIZZ1 (found in the inflammatory zone) and

Arg1 (Arginase1) were detected in THP1 and RAW 264.2.3 macrophages by real-time quantitative PCR (Fig 4.4.2 A-D). Taken together, our data suggest that NOS1 Inhibition promotes anti-inflammatory cytokine expression and thereby suppresses the inflammatory signalling in macrophages. Our data revealed the preferential formation of Fos-Jun dimer over Jun-ATF2 dimer in LPS stimulated macrophages that express NOS1. Therefore, it is anticipated that AP-1 dimerization (Jun-ATF2 over Fos-Jun) leads to high expression of anti-inflammatory cytokines in macrophages challenged with endotoxin shock as represented in the schematic diagram (Fig 4.4.2 E).

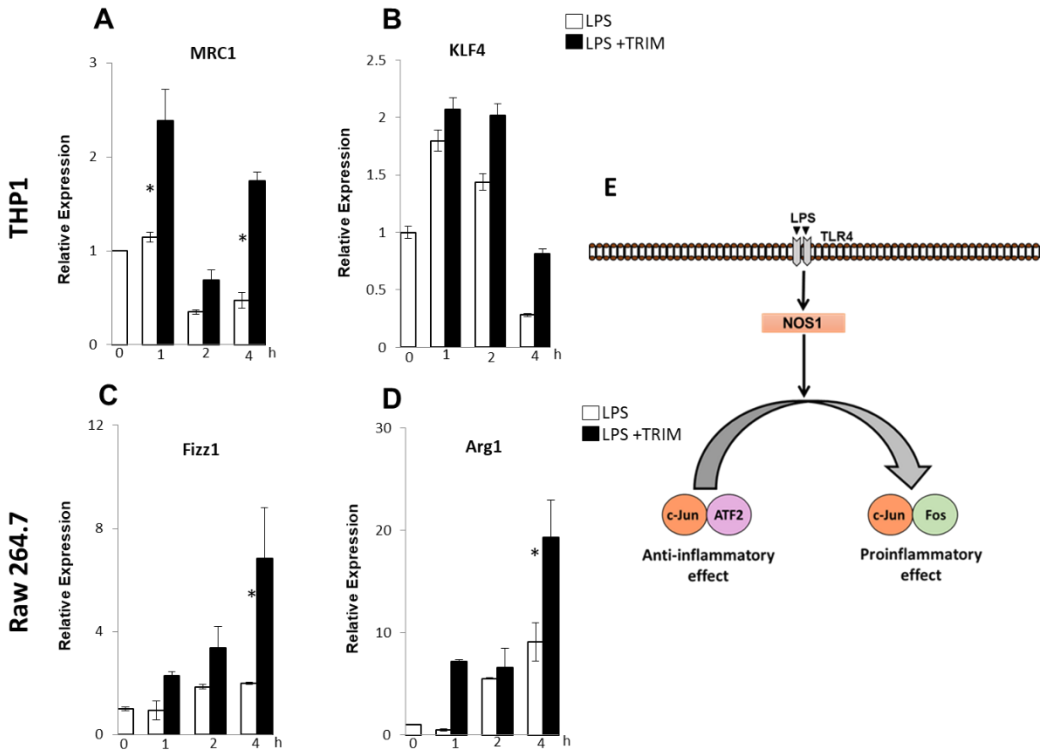


Fig 4.4.2. Anti-inflammatory cytokine responses to LPS are increased in NOS1-inhibited macrophages. Quantitative RT-PCR analysis of anti-inflammatory cytokine (A) MRC1 and (B) KLF4) for THP1 and (C) Fizz1 and (D) Arg1 mRNA expression in RAW 264.2.3 macrophages stimulated with LPS (250 ng/ml) with or without treatment with TRIM (100 nM) before LPS stimulation for indicated time points. (E) Schematic representation of the AP-1 mediated inflammatory response regulated by NOS1. Data are representative of three independent experiments; all are presented as mean ± SD. P values were determined by Student's T test; *P<0.05.

5. Discussion

Inflammation is an inevitable response of the immune system that is finely tuned to expel the invading pathogens and clear the damaged cells from the site of injury. However, an unchecked response might create deleterious consequences which could be fatal to the host during chronic conditions. It is therefore essential to understand the mechanism underlying the onset of inflammation during physiological and pathological responses. Recent advances have shed light on potential targets of inflammation that are indispensable for generation of an adequate response to a pathogen (Baig et al., 2017b; Kuzmich et al., 2017; Roy et al., 2016). Immediately after the invasion of the pathogen, a local inflammatory response is activated that recruits the inflammatory cells such as neutrophils from the circulation to the site of injury. Prompt recognition of the pathogen leads to degranulation of inflammatory mediators from the mast cells and platelets (Thomas and Storey, 2015; Yamanishi and Karasuyama, 2016). This further activates resident macrophages that respond to the proinflammatory mediators and recognize the pathogen-associated molecular patterns (PAMP) through their surface receptors (Dunster, 2016; Fujiwara and Kobayashi, 2005). Inflammatory reactions are augmented by the release of reactive oxygen and reactive nitrogen species that signal the inflammatory response in macrophages.

Nitric oxide synthases (NOS) have been widely studied in the context of pathophysiology associated with inflammation (Alderton et al., 2001b) Among the NOS isoforms : NOS1 (nNOS or neuronal NOS), NOS2 (iNOS or inducible NOS) and NOS3 (eNOS or endothelial NOS), NOS2 and NOS3 have been well appreciated as the critical mediators of inflammation (Cirino et al., 2003a; Gray et al., 2018; Ying and Hofseth, 2007). However, the involvement of NOS1 in inflammation is not well understood. Recently, the role of NOS1 has been reported in the inflammatory cascade employed in macrophages (Baig et al., 2015a). The study has described the crucial role of

NOS1 in nitrosation and degradation of inflammatory mediators such as SOCS1 that in turn fails to suppress the expression of adaptor protein TIRAP. The regulatory effect of NOS1 determines the expression of transcription factor NFkB-p65 that controls the proinflammatory gene expression. This led us to explore further the role of NOS1 in inflammation arising in macrophages.

Chronic inflammatory disorders such as sepsis is caused by exposure to severe endotoxin shock that is unresolved by the immune system. It affects more than 30 million people worldwide leading to 6 million deaths every year including 3 million newborns and 1.2 million children (Napolitano, 2018). Despite the recent advancement in the clinical treatment of sepsis, it is attributed as a global epidemiological burden (Álvaro-Meca et al., 2018). It leads to a state of abnormally enhanced systemic inflammatory reaction of the host that causes multiorgan failure and death. Abrupt nitric oxide production has been implicated with the severity of endotoxin-mediated injury. Several lines of evidence have recorded the contribution of NOS2 derived nitric oxide in cellular toxicity during septic shock (Takatani et al., 2018; Winkler et al., 2017). However, the role of NOS1 has not been well elucidated in the context of endotoxin-associated inflammatory responses. (Chandra et al., 2006; Fink, 2014; Kirkebøen and Strand, 1999). This study emphasizes the role of NOS1 during endotoxin injury and the associated mechanism that leads to activation of inflammatory cascades in macrophages.

In order to examine the role of NOS1 derived NO in sepsis, we used mice model administered with LPS as the endotoxin shock. Compared to the wild-type group of mice, we observed high lethality in the LPS injected mice. However, there was a significant recovery in the survival rate in mice injected with NOS1 inhibitor TRIM, implying that NOS1 has an unprecedented role in LPS induced mortality (Fig 4.1.1A). Lung morphology from LPS group of mice showed high distortion compared to the wild-type mice. On the contrary, mice administered with NOS1 inhibitor TRIM recovered from the endotoxin shock (Fig. 4.1.1B). Our preliminary data suggested the involvement of NOS1 in endotoxin associated lung injury. Cytokines such as IL6, IL1-β IL12, IL23 and TNF-α have been implicated in the pathogenesis of inflammatory disorders (Bradley, 2008; Dinarello, 2011; Tanaka et al., 2014). We therefore

tested the expression of cytokines involved in the inflammatory signalling in absence and presence of NOS1 inhibitor TRIM in the lung tissues of mice. Striking increment in the cytokine production in LPS challenged mice contributed to the lung injury, however, with TRIM administration cytokines expression ceased remarkably suggesting that NOS1 is a central player in the regulation of endpoint cytokines during inflammation (Fig. 4.1.1 C-G). With this study, we provide a novel evidence for the involvement of NOS1 during the LPS induced inflammation.

Further evidence to the involvement of NOS1 mediated NO production in macrophages was confirmed by immunoblot of phospho-NOS1 suggesting its activation in 1 h of LPS stimulation and its inhibition upon treatment with TRIM (Fig 4.1.3 A). In accordance with NOS1 activation, we also measured nitrite production in macrophages post LPS treatment. The data concluded that LPS triggered nitrite production in macrophages up to 2 h and contributes to the inflammatory lesions, which were impeded drastically upon TRIM treatment. Cellular staining with DAF-FM also represented the presence of nitrite in macrophages up to 1 h and a remarkable decrease with TRIM treatment (Fig 4.1.3 C and D). Through this study, we confirmed that NOS1 is activated at an early stage of inflammation in macrophages.

We next ruled out the possibility for the contribution of NOS2 and NOS3 during early hours of LPS infection. BMDM infected with LPS did not express NOS2 up to 2 h of LPS stimulation as evident by the mRNA and protein expression data (Fig 4.1.4 A-C). A cellular assay using flow cytometry also depicted the absence of NOS2 expression in macrophages up to 4 h that begins to appear only after 4 h of LPS infection (Fig. 4.1.4 D). Confocal microscopy using FITC stained NOS2 in LPS aroused macrophages also demonstrated lack of NOS2 expression up to 2 h of LPS stimulation and starts to express only after 4 h of LPS shock (Fig. 4.1.4 E). Our data from this study signified that LPS triggers activation of NOS1 up to 2 h, however during this time NOS2 which is an inducible isoform of NOS fails to be activated in macrophages. We, therefore, anticipate that NO production during early hours of inflammation is predominantly through NOS1 and not NOS2.

TLR4 signalling cascade is tightly regulated by negative regulators SOCS1 which in turn targets TIRAP adaptor protein for degradation. Here we show that under LPS stimulation, SOCS1 expression is diminished while TIRAP expression is upregulated (Fig. 4.2.1.1 A). This data supports the previous reports that NOS1 facilitates nitrosation and degradation of SOCS1, decreasing its abundance in early hours of inflammation. Consequently, we observe an increase in the expression of SOCS1 target, TIRAP. The interesting part of this study involves NOS1 inhibition during endotoxin shock that results in reverse effect on the expression of SOCS1 and TIRAP. This led us to determine the effect of NOS1 on important transcription factors during inflammation.

Activation of NFkB and AP1 transcription factors has been described as major regulatory events in mediating the LPS mediated septic injury (Lawrence, 2009; Zenz et al., 2008). An integral component of AP1, c-jun is a highly dynamic subunit that when phosphorylated forms heterodimer with other AP1 subunits, Fos and ATF2. Our immunoblot analysis suggests that along with increased expression of TIRAP, there is activation of c-Jun after 1 h of LPS infection (Fig. 4.2.1.2 A). However, NOS1 inhibition with TRIM, depreciated both TIRAP and phospho-c-Jun expression. Altogether, the data poses an interesting link between NOS1 activation that in turn transactivates c-Jun after LPS exposure. This provided us the direction for further exploration of the detailed signalling mechanism of c-Jun transactivation.

Our previous understanding of TIRAP mediated p65 transactivation lead us to determine whether TIRAP also modulates activation of c-Jun. We therefore immunoprecipitated TIRAP with c-Jun in LPS stimulated macrophages. Strikingly, we observed that TIRAP immunoprecipitated with c-Jun under LPS shock (Fig. 4.2.2 A). To further confirm the study, we performed in-vitro confocal analysis and checked for TIRAP and Jun interaction during LPS challenge. In accordance with the immunoblot data, we observed a remarkable increase in TIRAP and Jun interaction during in LPS stimulation which was abolished upon treatment with NOS1 inhibitor TRIM (Fig. 4.2.2 D). This suggested that TIRAP and Jun interaction is an important therapeutic complex, abrogation of which will protect the immune cells from uncontrolled

inflammatory lesions. This observation provided a rationale for the activation of c-Jun during LPS stimulation. With inhibition of NOS1, we observed decreased c-Jun activation, which might be due to the abundance of SOCS1 which degrades TIRAP and therefore less transactivation of c-Jun. The confocal analysis also supports the immunoprecipitation data. The data strongly implied that NOS1 mediates TIRAP and c-Jun interaction and thereby activates the AP1 transcription factor.

AP1 transcription factor composed of Fos, Jun, and ATF2 heterodimers translocate into the nucleus to drive the transcription of their target genes such as IL12, IL23, INF-γ, MIP1-α, M-CSF and TNF-α that are ultimate effectors of inflammation. We examined the effect of NOS1 on AP1 nuclear translocation and the inflammatory response downstream of LPS stimulation using confocal microscopy. In accordance with previous observations, AP1 subunits Fos, Jun and ATF2 translocated into the nucleus upon LPS stimulation (Fig. 4.2.3.1 A-C). Surprisingly, we found that the translocation efficiency of all three subunits was abolished significantly in the presence of NOS1 inhibitor TRIM. This data provided a link to the regulatory mechanism by which AP1 target genes are downregulated in the absence of NOS1. By preventing the nuclear translocation of Fos, Jun, and ATF2, the inflammatory genes regulated by AP1 fail to express in the nucleus.

Till now, our study suggested that NOS1 is an active molecule during early hours of LPS induced inflammatory signalling and it negatively regulates the AP1 nuclear translocation leading to perturbed inflammatory response. Nuclear fractions isolated from BMDM treated with LPS also exhibited elevated expression of Fos, Jun and ATF2 subunits. The cytoplasmic fractions at the same time was detected with a loss of subunits expression. Interestingly in accordance with the confocal data, the TRIM treated macrophages showed a reverse effect on subunit abundance. The nuclear fractions depicted low abundance of Fos, Jun and ATF2 compared to the cytoplasmic counterparts (Fig. 4.2.3.2 B). So far, the evidence indicated that AP1 nuclear translocation is facilitated by the presence of NOS1 which is abrogated with the inhibition of NOS1. This suggests a novel role of NOS1 in macrophage activation through its impact on AP1 transcription factor.

Inflammation is majorly associated with the release of cytokines that facilitate the battle against the invading pathogen. We next examined the expression of AP-1 mediated cytokine expression: IL12, IL23, INF-γ, MIP1-α, M-CSF and TNF-α in macrophages stimulated with LPS alone or along with TRIM. There was a significant increase in the cytokines expression in LPS induced macrophages which decreased with NOS1 inhibition (Fig. 4.2.4 A-F). This data is in accordance with the nuclear translocation studies on AP-1 subunits, connecting the concept that NOS1 inhibition leads to lower abundance of AP-1 transcription factor in the nucleus, which might be the reason for decreased expression of AP-1 mediated cytokines.

TIRAP-Jun interaction is a crucial determining factor for the severity of inflammatory response in macrophages. Therefore, we used docking studies to study the preferred orientation of the two proteins in 3 D space (Fig. 4.3.1.1 A-B). To validate the docked structure, molecular dynamics simulation was performed, and the most stable conformation was chosen for further studies. Our strategy was to inhibit the binding interface between TIRAP and Jun. For this, we used the Jun binding site in TIRAP structure as the docking site for potential drug candidates.

Small molecules or inhibitors have been widely used to check the interaction of important signalling molecules in order to prevent the onset of signalling molecule. In this study, we identified five repurposed drugs through virtual screening to inhibit the interaction of TIRAP and c-Jun (Fig. 4.3.1.2 A). Top five scoring drugs were tested in-vitro for their inhibitory potential on TIRAP-Jun interaction. Immunoprecipitation was performed with TIRAP and Jun in LPS triggered macrophages in the presence or absence of drugs. Strikingly, out of the five drugs, Gefitinib suppressed the interaction of TIRAP and Jun significantly compared to other drugs (Fig. 4.3.3 A). We next performed confocal analysis and confirmed the inhibitory potential of Gefitinib in macrophages (Fig. 4.3.3 C) Confocal data indicated a rapid interaction between TIRAP and c-Jun in BMDM as well as in lung sections of LPS injected mice. So far, our data suggested that Gefitinib potentially inhibits the TIRAP and Jun interaction. Docking studies on Gefitinib and TIRAP structure reveal the presence of residues in TIRAP that form conventional stable

hydrogen bonds and hydrophobic interactions with Gefitinib that imparts stability to the complex (Fig. 4.3.3 D).

We further determined in-vivo efficacy of Gefitinib in LPS injected mice. Mice administered with Gefitinib before LPS exhibited remarkable recovery from the endotoxin shock and showed higher survivability compared to the LPS injected mice (Fig 4.3.4 B). Lung morphology from the same group of mice suggests that Gefitinib protected mice from LPS induced lung injury (Fig. 4.3.4 C). Cytokines detected through real-time PCR were also detected in the lungs of affected mice and demonstrated results similar to the survival data. LPS group of mice represented the higher expression of inflammatory cytokines in contrast to the Gefitinib group of mice (Fig. 4.3.5 B-G). Importantly our observations showing a decrease in phospho c-Jun expression after gefitinib administration in LPS injected mice provide promising evidence for the molecular targets of gefitinib (Fig. 4.3.5 H). The data implies that Gefitinib is a potential anti-inflammatory drug and can be utilized for therapeutics of chronic inflammatory disorders such as sepsis. Here we report a unique mechanism utilized by Gefitinib to suppress the inflammatory outbursts in macrophages.

In addition to the above studies, we reported AP-1 dimerization pattern in determining the inflammatory response in macrophages. Our data signifies that NOS1 promotes the formation of Fos-Jun dimer over Jun-ATF2 dimer, and we observed a reverse effect in the presence of NOS1 inhibitor TRIM in macrophages stimulated with LPS (Fig. 4.4.1.1 A and D). Our results showed an abundance of Jun-ATF2 dimer with NOS1 inhibition. Previous literature has reported that Jun-ATF2 dimer leads to anti-inflammatory response owing to low efficiency to regulate the transcription of pro-inflammatory cytokines. Based on this, we further detected the anti-inflammatory cytokines in Raw macrophages and THP-1 macrophages. Strikingly, NOS1 inhibited macrophages exhibited higher expression of anti-inflammatory cytokines suggesting that abundance of Jun-ATF2 dimer of AP-1 might be the cause of anti-inflammatory response in macrophages.

Overall, this thesis summarizes the molecular mechanisms of NOS1 mediated inflammation and different molecules that participate in orchestrating the cascade.

6. Summary and Conclusion

Several lines of evidence have recorded the contribution of NOS2 derived nitric oxide in cellular toxicity during septic shock. However, the role of NOS1 has not been well elucidated in the context of sepsis-associated inflammatory responses. This study emphasizes the role of NOS1 in sepsis and the associated mechanism that leads to activation of inflammatory cascades in macrophages.

Here, we have studied the mechanism by which NOS1 controls the activity of AP1 transcription factor that ultimately control the transcriptional regulation of inflammatory cytokines thereby enhancing the inflammatory responses. Detailed investigation of the molecular mechanism underlying the TLR4 signaling pathway indicated that LPS induced NOS1 activation that further facilitated nuclear translocation of AP1 transcription factor. Therefore, with inhibition of NOS1, we observed lack of AP1 nuclear translocation. This negatively regulates the AP1 mediated cytokine gene expression. We next, examined the mechanism of AP1 mediated inflammatory response. In our study, we identified a novel interaction between inflammatory mediators TIRAP and c-Jun in LPS stimulated macrophages.

Targeted disruption of TIRAP and c-Jun through potential repurposed drug was the next objective of the thesis. An efficient strategy to balance the inflammatory lesions in chronic diseases is based on the identification of mediators that give rise to abrupt signaling. Using molecular modeling of TIRAP and c-Jun interaction and computer-assisted screening, Gefitinib, an EGFR (Epidermal growth factor receptor) inhibitor, was selected and its efficiency to impede the TIRAP-c-Jun interaction was experimentally validated. Gefitinib is a drug currently being used for certain breast, lung and other cancers. These findings illustrate that Gefitinib can be repurposed as an anti-inflammatory therapy for chronic diseases like sepsis. Together, this study provides a novel insight into the mechanism of TIRAP-mediated activation of c-Jun in TLR4 induced pathway. Furthermore, inhibition of TIRAP and c-Jun

interaction is a potential therapeutic strategy for the chronic inflammatory disorders. The work from this study offers further exploration of the molecular targets of inflammation that are well appreciated for designing novel drugs for chronic inflammatory diseases.

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