

Synopsis

Inflammation is a complex set of immune responses that is critical for survival during injury or infection. It is also crucial for maintenance of tissue homeostasis under myriad conditions caused by cellular stress and tissue malfunction. A successful inflammatory response after the removal of noxious agents culminates into the resolution phase to promote tissue repair and induce adaptive immunity for efficient responses upon second encounter. Based on various immune factors involved and the duration of the processes, inflammation can be broadly classified into acute and chronic inflammation. Acute inflammation generally lasts from a few days to a week and involves activation of non-specific innate immune responses. Acute inflammation is best characterized during microbial infections and often ends with the eradication of infection. However, uncontrolled acute inflammatory responses can be detrimental, a classic example of which is sepsis. The persistence of stimuli or incomplete resolution of inflammation can lead to chronic inflammation, mediated by activation of adaptive immune cells. For example, failure to remove monosodium urate crystals leads to gout, a form of chronic inflammation-induced arthritis. Another example of chronic inflammation is rheumatoid arthritis, an autoimmune disorder affecting the joints in bones. Recent studies have shown the involvement of inflammation in a variety of modern human diseases such as obesity, diabetes and neurodegenerative disorders. Therefore, inflammatory responses are an important area of investigation that affects a wide variety of biological responses.

The process of inflammation is driven by a variety of mediators, of which the cytokine network occupies a key regulatory role. Most of the pathological inflammatory conditions are aggravated by pro-inflammatory cytokines like $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , IL-17 etc. Accordingly, the treatment of rheumatoid arthritis and inflammatory bowel disease patients with neutralizing antibodies or inhibitors to cytokines, e.g. $\text{TNF}\alpha$, IL-1 etc, is beneficial. Although, cytokine signalling is known to be involved in pathogenesis of variety of inflammatory disorders, the roles of these cytokines in regulation of homeostasis and inflammatory responses is still not clear. Other than that, studies on inflammatory mechanisms revealed the critical roles of signalling proteins involved in inflammation, e.g. p38 Mitogen Activated Protein Kinase (p38 MAPK), c-Jun N-Terminal Kinase (JNK), prostaglandins, mediators of nitrosative and oxidative stress etc. In order to obtain a better understanding of the inflammatory responses, this study aims to elucidate the roles of two pro-inflammatory mediators: Interferon-gamma ($\text{IFN}\gamma$) and Nitric Oxide Synthase 2 (NOS2). In the first part of this work, studies with $\text{IFN}\gamma$ were performed in an *in vitro* cellular model of macrophage activation. In the second part, the roles of NOS2 in an *in vivo* setting, i.e. *Salmonella* Typhimurium (*S. Typhimurium*) infection-induced model of sepsis, were investigated.

I) $\text{IFN}\gamma$, a type II Interferon, has pleiotropic roles in immunity, cancer biology, autoimmunity etc. $\text{IFN}\gamma$, a T helper (Th)-1 type effector cytokine, is a key activator of macrophages. It activates the macrophages towards the M1 phenotype, which are characterized by increased expression of pro-inflammatory cytokines like $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-12 and production of reactive nitrogen and oxygen intermediates. Concomitantly, $\text{IFN}\gamma$ upregulates Major Histocompatibility Complex (MHC) encoded class I (MHC-I) and class II (MHC-II) molecules for antigen presentation. Also, $\text{IFN}\gamma$ inhibits the differentiation of regulatory T cells and Th-2 type T cells. $\text{IFN}\gamma$ post binding to its receptor induces rapid responses via activation of the canonical Janus Kinase (JAK)-Signal Transducer and Activator 1 (STAT1) pathway. Additionally, $\text{IFN}\gamma$ can also activate various non-canonical pathways independent of STAT1 in a cell- and context-dependent manner. For example, $\text{IFN}\gamma$ mediated activation of p38 MAPK is involved in the regulation of autophagy, phagosome maturation and killing of intra-cellular pathogens. In the present study, the cross talk between $\text{IFN}\gamma$

dependent p38 MAPK activation and its effects on cellular responses of macrophages were investigated. The treatment of RAW 264.7, a mouse macrophage cell line, with the combination of IFN γ and p38 MAPK inhibitors (SB202190 or SB203580) led to the formation of giant intracellular vesicular structures (GIVS). Notably, treatment of RAW 264.7 with IFN γ or p38 MAPK inhibitors alone or the combination of IFN γ with ERK or JNK inhibitors did not lead to the generation of GIVS. The generation of GIVS, upon treatment with IFN γ and SB202190, was also observed in primary mouse peritoneal macrophages but to a lesser extent in other cells, e.g. L929 (a mouse fibroblast cell line) and was negligible in CT26 (a colon carcinoma cell line). Further investigations revealed that the genesis of these GIVS in RAW 264.7 cells was independent of IFN γ -induced nitrite production, autophagy and cell viability. Immunofluorescence microscopic analysis demonstrated that these GIVS were positive for the late endosomal and lysosomal membrane markers such as CD63, Lysosome Associated Membrane Protein (LAMP) 1/ LAMP2 respectively. Importantly, the generation of GIVS was blocked by chloroquine (an inhibitor of endosomal and lysosomal acidification) and Ly294002 (a PI-3K inhibitor). Kinetic studies using live cell imaging showed increased fusion but not fission of LAMP1 positive vesicles, resulting in the formation of GIVS upon treatment of RAW 264.7 cells with IFN γ and SB202190. Interestingly, the cells with large lysosomes showed normal phagocytosis and cell surface expression of MHC I and transferrin receptors (CD71), but lower cell surface expression of MHC II and LAMP1, upon IFN γ stimulation. Surprisingly, IFN γ -induced mammalian Target of Rapamycin (mTOR) activation is inhibited with SB20190 treatment with simultaneous increase in total amounts of transcription factor EB (TFEB), a master regulator of lysosome biogenesis. TFEB was majorly translocated to the nucleus and showed increased transcription of lysosomal biogenesis genes, *Naglu1* and *Neu1*, in RAW 264.7 cells treated with the combination of IFN γ and SB202190. Altogether, the study elucidated the possible roles of inhibition of p38 MAPK during IFN γ mediated regulation of lysosomal biogenesis and/or homeostasis. The implications of formation of enlarged lysosomes are discussed in the context of macrophage functions.

II) Sepsis is a syndrome with a considerable global burden in terms of morbidity and mortality. It is characterized as a life-threatening organ damage due to dysregulated host immune responses during infection. Enhanced innate immune responses and suppressed adaptive immunity together contribute to persistent organ damage and recurrent infections, which often contribute to impaired recovery and mortality of sepsis patients. The overwhelming inflammatory responses due to hyper-activated innate immune cells are a hallmark of sepsis. Therefore, molecules that attenuate inflammatory responses are potential therapeutic targets to treat sepsis. Nitric Oxide (NO) is a highly reactive molecule that has multiple functions in the host, ranging from cellular signaling and modulation of gene expression to innate antimicrobial responses. Although, the protective roles of NO in infectious diseases are well documented, its role in pathogenesis of sepsis is controversial. Based on this, the focus of the study was to investigate the role of NOS2 during *S. Typhimurium* infection-induced peritonitis (inflammation of the peritoneum) leading to sepsis in mice. A *S. Typhimurium* infection-induced sepsis model in mice was established, which was characterized by cytokine storm, neutrophil recruitment at the site of infection and organ damage leading to death of mice. The roles of NOS2 during *S. Typhimurium* infection-induced sepsis model in mice were elucidated using a mouse strain harbouring a genetic deletion in *Nos2*, the enzyme responsible for generation of NO in immune cells. Upon sepsis, *Nos2*^{-/-} mice had attenuated responses, such as induction of Reactive Oxygen Species (ROS), pro-inflammatory cytokines TNF α , IL-6 and IFN γ as well as reduced neutrophil recruitment into the peritoneal

cavity. However, responses such as induction of activation markers on neutrophils, infection-induced glucocorticoids in sera, phagocytic ability of recruited neutrophils and apoptosis of peritoneal cells were largely independent of NOS2-derived NO. Importantly, the dampened initial immune responses in *Nos2*^{-/-} mice correlated with increased microbial burden in peritoneal lavage, spleen and liver, which contributed to greater liver damage, cardiac dysfunction and reduced survival of *Nos2*^{-/-} mice upon sepsis. Therefore, NOS2 regulates the initial innate immune responses in this mouse model of *S. Typhimurium* infection-induced sepsis.

To further establish the precise roles of NO, studies with exogenous supplementation of DETA-NO, a NO donor, in *Nos2*^{-/-} mice were performed. DETA-NO supplementation 3 hours prior to infection significantly restored innate immune responses, including neutrophil recruitment, increase in serum IL-6 and CCL2 amounts etc. Administration of DETA-NO also led to significant reduction in microbial burden in peritoneal lavage, spleen and liver. Consequently, there was reduced organ damage and increased survival of *Nos2*^{-/-} mice upon sepsis. However, no beneficial effects of DETA-NO were observed in C57BL/6 (wild type) mice. The finding from this study demonstrates that NOS2-derived NO is required to promote beneficial host innate immune responses in this model of peritonitis induced sepsis. The important implications of these findings are discussed in the context of the complex roles of NO during sepsis.

Overall, the present study elucidated the uncharacterized roles of small molecule inhibitors of p38 MAPK in functional responses of macrophages to IFN γ and has demonstrated NOS2 to be a critical regulator of host innate immune responses during *S. Typhimurium* infection-induced sepsis.